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Exploring the potential application of glucuronides as a prognostic biomarker for disease

Volume 1: Research thesis

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Copyright statement

Abstract

The detoxification pathway in the liver is the vital pathway for detoxifying xenobiotics and endogenous molecules in all vertebrates. The majority of molecules require three phases for detoxification, phase I preparation, phase II conjugation and phase III excretion. Research to date demonstrates how human gene polymorphisms and lifestyle factors may alter the fate of the molecule. Additionally, research to date indicates that genotyping can be useful for personalised medicine before administering a drug, the information is used to inform the patient which drug can be or cannot be used for treatment. This is to prevent adverse toxicological reactions during treatment of a disease. Therefore, monitoring the fate of glucuronides in relation to diseases, should have invaluable potential in detection of relevant diseases at earlier stages. The glucuronide species may reflect disease causation influences by genetics and non-genetic factors. For instance, in disease influenced by lifestyle factors, the glucuronides may include glucuronides that have been produced from xenobiotic molecules that have been through glucuronidation. Since research have not addressed the relation of the fate of glucuronide species and diseases. It is important to clarify the concept for disease prognostics based on glucuronide species in their excretion. Therefore, the aim of the research is to explore the potential application of glucuronides for disease prognosis and disease, by revealing the relationship between glucuronides and disease. The research presented in the thesis demonstrate that glucuronide species are potentially associated with diseases. For instance, to date, in prostate cancer three glucuronides are associated with the disease: androsterone glucuronide (ADT-g), androstane-3α, 17β-diol glucuronide (3α-diol-G), and androstane-3a, 17b-diol-17 glucuronide (3α-diol-17G), when prostate cancer is influenced by gene variants. In type 2 diabetes, influenced by lifestyle, three glucuronides are associated with the disease: 2-phenylethanol glucuronide, deoxycholic acid 3-glucuronide, and androstanediol glucuronide.

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Statement of declaration

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or processional qualification except as specified.

Abbreviations

2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine	PhIP
2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine	EDDP
3,4-Methylenedioxymethamphetamine	MDMA
Acetaminophen	APAP
ADT-glucuronide	ADT-G
Alpha	α
Alternative splicing	AS
Alzheimer's disease	AD
Amino groups	$-NH_2$
Androstanediol glucuronide	A-diol-g
Androstane-3α,l7β-diol-3-glucuronide	3α-diol-3G
Anthocyanins	ACs
ATP binding cassette transporter	ABC Transporter
Blood brain barrier	BBB
Beta	β
Base pair	bp
Breast cancer resistance protein	BCRP
Butylated hydroxyanisole	BHA
Butylated hydroxytoluene	BHT
Hydroxy-tert-butyl	BHT-OH
Carboxylesterase	CE
Carboxylic acid groups	-COOH
Colorectal cancer	CRC
Cyclooxygenase-1	COX-1
Cyclooxygenase-2	COX-2
Cytochrome p450	CYP450
DNA methyltransferases	DNMTs
Enzyme-linked immunosorbent assay	ELISA
Escherichia coli	*E. coli*

Estrone-3-glucuronide	E1G
Folinic acid, fluorouracil and irinotecan	FOLFIRI
Gas Chromatography	GC
Glucuronide transporter	GusB
Glutathione S-transferases	GSTs
Glutathione	GSH
High resolution mass spectrometry	HRMS
High-performance liquid chromatography	HPLC
Hydroxyl group	-OH
Irinotecan	CPT-11
Liquid Chromatography tandem mass spectrometry	LC-MS/MS
Messenger RNA	mRNA
Michaelis constant	K_m
MicroRNAs	miRs
Microsomal ethanol oxidising system	MEOS
Multi drug resistance	MDR
N-acetyl-p-benzoquinone imine	NAPQI
N-acetyltransferases	NATs
Norketamine	NK
Oestrogen response element	ERE
Organic anion transporter	OAT
Parkinson’s disease	PD
Personalised medicine	PM
Phenylketonuria	PKU
Rate of reaction	V_{max}
Reactive oxygen species	ROS
Sulfotransferases	SULTs
Type 2 Diabetes Mellitus	T2DM
Ubiquitin	Ub
Ultra-performance liquid chromatography	UPLC

Ultra-high performance liquid chromatography-High resolution mass spectrometry	UPLC-HRMS
Urine diphosphate-glucuronosyltransferase	UGT
Urine-diphosphate glucuronic acid	UDP-GA
β-Glucuronidase	GusA

Table of gene variants

Gene	Gene variant	Gene variant and condition
CYP1A2	*CYP1A2*1C*, rs71581941, and rs2470890	*CYP1A2*1C*: lung cancer. rs71581941: breast cancer. rs2470890: breast cancer.
CYP3A4	*CYP3A4*20* (rs67666821), *CYP3A4*1b*	*CYP3A4*20*: unclear, possess increased and decreased expression. *CYP3A4*1b*: breast cancer but association is unclear.
CYP3A5	*CYP3A5*3*	Prostate cancer
CYP2B6	*CYP2B6*6*	Poor metaboliser of methadone
CYP2D6	*CYP2D6*4* (rs3892097)	Poor metaboliser of methadone
CYP2E1	*CYP2E1*5B*	Alcohol dependence, association is unclear.
UGT1A1	*UGT1A1*28*	Gilbert's syndrome; Colorectal cancer
UGT2B15	*UGT2B15*2*	Prostate cancer; Breast cancer
UGT2B10	rs61750900 and rs2942857	Lung cancer.
UGT2B17	UGT2B17 deletion	Prostate cancer; Breast cancer.
ABCA1	rs2230806	Coronary heart disease.
ABCB1	rs1128503, rs2032582, and rs1045642	rs1128503: Alters methadone concentrations for opioid withdrawal. rs2032582: opioid dependence. rs1045642: colorectal cancer, opioid dependence, and cocaine addiction.
ABCG1	rs72552713	Alcohol induced gout.
ABCG2	rs3109823	Influences myelosuppression when irinotecan is administered.
OATP1B1	rs71581941	Rotor syndrome.

Chapter 1: Introduction

1.1 Glucuronides as a biomarker for disease

Biomarkers are molecules that can be used as a measurable indicator to determine the presence of a disease or biomarkers can be used to identify disease severity (Law et al. 1995; Rodriguez et al. 1997; Colomer et al. 2000). The criteria that makes a good biomarker include: the biomarker must be present in peripheral blood and or/bodily fluids (such as blood, urine or salvia) (Takahashi et al. 2000; Wurst et al. 2000; Warankulsuriya et al. 2000), easily detectable for quantification (An et al. 2000), robust (Leonardi et al. 2001), associated to a clinical condition (Bhatavdekar et al. 2000), and cost effective (Henriksson et al. 2010). Therefore, in order for glucuronides to be a good biomarker, glucuronide molecules need to fit the criteria.

The application of glucuronides as a biomarker for diagnostics and prognostics, glucuronide molecules must meet the specified criteria to be considered as a good biomarker for disease. Thus, research to date demonstrates that glucuronides can be detected in peripheral blood (Wang et al. 2004), urine (Lutz et al. 2006), and saliva (Qi et al. 2002). Furthermore, glucuronides can easily be quantified (Annesley and Clayton 2005). Research to date does not reveal if glucuronide molecules are robust as a disease biomarker. Lastly, glucuronides could be associated to clinical conditions (Lévesque et al. 2014; Johnson et al. 2016; Zhao et al. 2017) and refer to Aim and Objectives. Research to date does discuss or evaluate the cost-effectiveness for glucuronides as a biomarker. According to the research to date, this demonstrates that glucuronides could be a good biomarker for disease diagnostics and prognostics. However, further research is required to explore the cost-effectiveness and the robustness of glucuronides as a biomarker for disease.

1.2 Overview of detoxification pathways

The human detoxification pathway is the vital pathway in the liver for the detoxification of xenobiotics and endogenous molecules (Bosma et al. 1994; Pacifici et al. 1997; Stillwell et al. 1997; Dietrich et al. 2004). The molecules which can be detoxified include xenobiotics pollutants Turteltaub et al. (1990); Bhattacharyya et al. (1995); Caporaso et al. (2001), medicinal drugs (Lee et al.

1996; Turan et al. 2001; Yamamoto et al. 2008; Tomasi et al. 2010), illicit drugs (Vitcheva and Mitcheva 2007; Meyer et al. 2008; Bunten et al. 2010), food additives (Yu et al. 1997; Dreiseitel et al. 2008; Mozrzymas et al. 2017), and endogenous molecules (Schmid et al. 1957; Hong et al. 2004; Park et al. 2006). These pathway serves to reduce the toxicity effects of the molecules by changing the structure of the molecule by the addition of glucuronic acid to a molecule, changing the charge of the molecule, becoming water soluble. However, some molecules do not undergo three phases, the three phases have been discussed below. For the detoxification of molecules (van der Deure et al. 2008; van de Steeg et al. 2012; Kotsampasakou et al. 2015), organic anion transporters (OATs), transports transport molecules into the cell for detoxification such as OATP1B1 transporting bilirubin into the liver. An overview discussion of OATs has been presented in Appendix 4 Section A4 1.0. Below discusses a generalised explanation of the detoxification pathway.

Phase I is mediated by cytochrome P450s (CYP450s), known as the "preparation" phase in the detoxification pathway, preparing molecules by different biochemical reactions (Slaughter and Edwards 1995; Huan et al. 1998; Meyer et al. 2001), oxidations, reductions or hydrolysis. These reactions expose or introduce a functional group: hydroxyls (-OH) (Figure 1.1), thiol (sulfhydryl) (-SH), amino groups (-NH2) or carboxylic acids (-COOH) (Williams 1967; Williams 1972; Schwartzman et al. 1987; Boon et al. 1997; McKinney et al. 2004; Butler et al. 2013; Ashrap et al. 2017; Sang et al. 2018), for phase II conjugation (Sun et al. 2012;; Alkharfy et al. 2013; Zhu et al. 2015), in most cases. Irinotecan for example (Figure 1.1), the inactive metabolites does not require glucuronidation. CYP450s contain a heme group which aids the binding of molecules to the protein active site, classifying CYP450s as heme-thiolate proteins (Ohno et al. 2000; Auclair et al. 2001). Essentially, phase I is the preparation of molecules exposing the functional groups for phase II conjugation via glucuronidation by Urine Diphosphate (UDP)-glucuronosyltransferase (UGT) and other phase II conjugation reactions (Figure 1.2) (Bosma et al. 1995; Sparks et al. 2004; Huber et al. 2009).

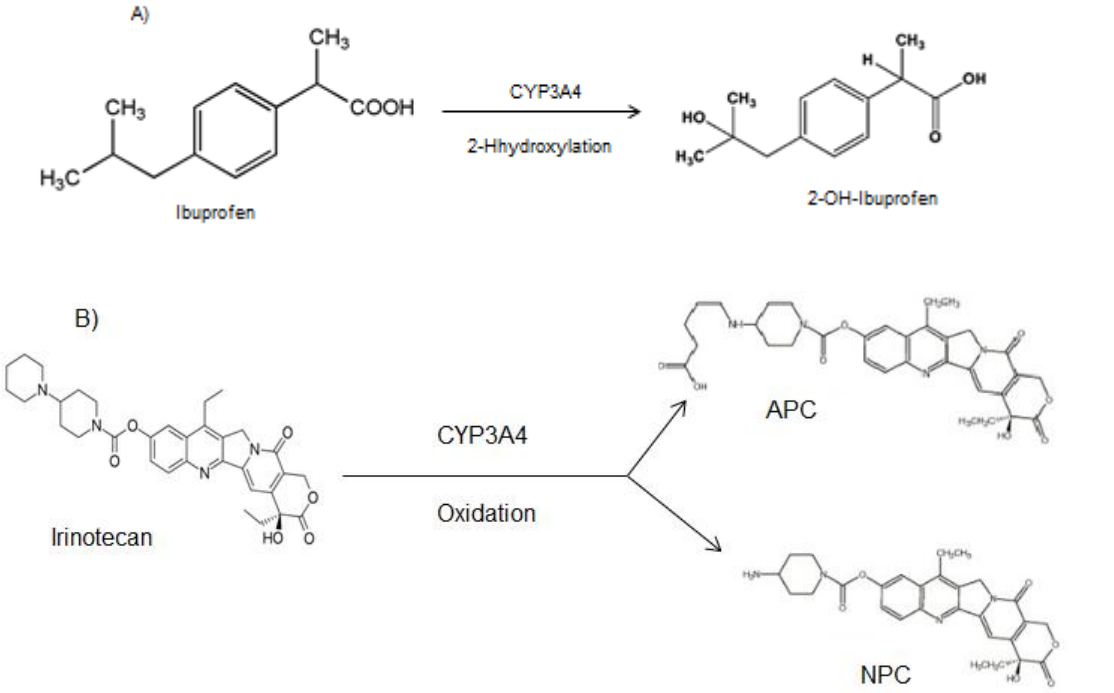


Figure 1.1: Examples of phase I detoxification. A) Ibuprofen is hydroxylated by CYP3A4 to produce the metabolite 2-OH-ibuprofein (Chang et al. 2008). **B)** Irinotecan is oxidised by CYP3A4 which synthesises two inactive metabolites, APC and NPC.

Phase II is the conjugation phase which detoxifies a majority of molecules, by conjugating molecules to the functional group for detoxification. Conjugation may include: sulphation that attaches sulfhydryl (thiol) (-SH) groups (Sharer et al. 1995), glucuronidation (attachment of glucuronate, UDP-GA), (Iyer et al. 1998), acetylation that attaches acetyl groups (CH_3CO) (Windmill et al. 2000), and glutathione conjugation which conjugates glutathione (GSH) molecules (Buratti et al. 2015). Glucuronidation is the major phase II conjugation process due to the unlimited availability of glucose (discussed further in Section 8.0). In glucuronidation, UGTs conjugate urine diphosphate glucuronate (glucuronic acid, UDP-GA) to the introduced or exposed functional group on the molecule, synthesising a glucuronide (Figure 1.2). Molecules become detoxified by glucuronidation due to the addition of glucuronate to a hydrophobic molecule, increasing water solubility and the polarity of a molecule (Kang et al. 1995; Trapnell et al. 1998; Silva et al. 2003).

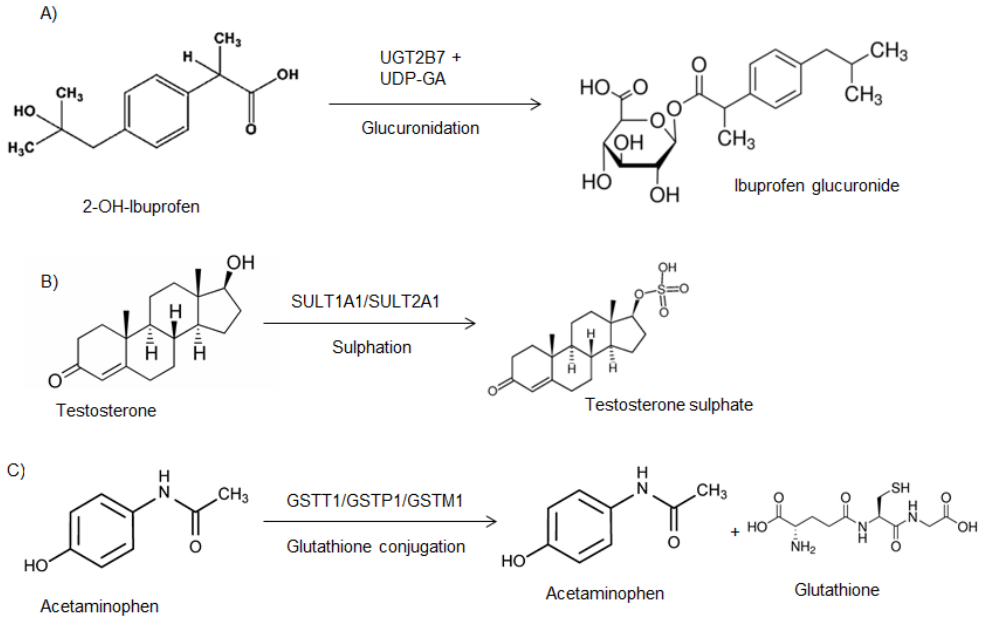


Figure 1.2: Examples of phase II conjugation reactions. A) 2-OH-Iburprofen is conjugated to UDP-GA for glucuronidation by UGT2B7 (Sakaguchi et al, 2004). **B)** Testosterone can be sulphated in phase II detoxification by SULT1A1 and SULT2A1 (Schulze et al. 2013). **C)** Glutathione conjugation of acetaminophen by GSTT1, GSTP1 or GSTM1 for phase II detoxification, by which glutathione is conjugated to acetaminophen.

Phase III known as the “excretion” phase, requiring transporters for the elimination of phase I and II products. These transporters are mainly ATP binding cassette (ABC) transporters, for example multidrug resistance transporters such as MDR1 (Radosevich et al. 1989; Valverde et al. 1992; Kim et al. 1996). ABC transporters situated on cell membranes, efflux phase I and II metabolites from the cell (Wang et al. 2008; Pohl et al. 2011; Song et al. 2014; Crow et al. 2017; Aguiar et al. 2019). Once out of cells phase I and II products, enter the bile duct and into the small intestines or the urinary tract.

ABC transporters use active transport, requiring hydrolysis of ATP to ADP for molecule transportation (George and Jones 2013; Karasik et al. 2017). ABC transporters are expressed on the blood brain barrier (BBB), on the apical surface of a cell membrane (Carl et al. 2010; Bakhsheshian et al. 2013; Chapy et al. 2015; Liu et al. 2018). The presence of ABC transporters on the BBB, prevents endogenous and xenobiotic molecules from entering the brain and induce a toxicological effect. (Shumacher et al. 2012; Disdier et al. 2015; DisMøllgård et al. 2017). Additionally, ABC transporters are expressed on the intestinal barrier, on the apical membrane surface (van den Bosch et al. 2007;

Ogasawra et al. 2010; Perdomo et al. 2013; Kis et al. 2016; Sawangrat et al. 2018). Transporters expressed on the intestinal barrier, efflux phase I and II metabolites into the bile or urine for elimination. The molecular mechanism for molecule transport are discussed below.

ABC transporters, use a mechanism of active transport, facilitated by ATP hydrolysis, using nucleotide-binding domains 1 and 2 (Hou et al. 2001; Berger et al. 2005; Scott-Ward et al. 2007; Kloch et al. 2010; Nagao et al. 2012; Corradi et al. 2015; Urbatsch et al. 2018). These research articles postulated NBD1 is utilised for ATP hydrolysis whilst, NBD2 is associated with the opening of the transporter. Additionally, Figure 1.3 presents a theoretical model of the ATP switch model (proposed by Higgins and Linton 2004). It is not yet clear whether ATP hydrolysis and opening of the transporter occurs synergistically or alternatively.

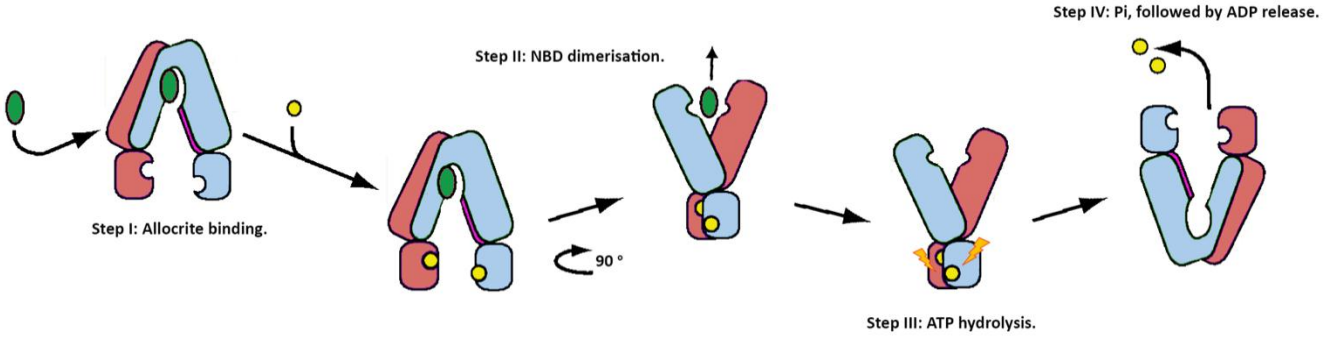


Figure 1.3: ATP switch model of ABC transporters. **Step I**) molecule (green oval) enters the transporter. **Step II)** the transporter closes and the NBDs dimerise, with ATP (yellow circles), binding to the nucleotide binding domains. After closure, the transporter rotates to release the molecule. **Step III)** ATP is hydrolysed to ADP. **Step IV)** ADP is released from the NBDs (Higgins and Linton 2004).

Furthermore, ABC transporters are subjected to phosphorylation by protein kinase A (PKA) or protein kinase C (PKC). PKAs and PKCs phosphorylate the ABCA and ABCB family of transporters (See et al. 2002; Roosbeek et al. 2004; Nandi et al. 2008), and the ABCC family of transporters (Chambers et al. 1990; Chambers et al. 1992; Chambers et al. 1994; Sachs et al. 1996). Thus if a transporter is phosphorylated by PKC in a disease, an accumulation of toxic molecules may occur, allowing the disease to develop. Alternatively, increased expression of a ABC transporter could be a contributing factor in chemo-resistance, such as the overexpression of MRP4 as discussed in Section 3.1.4.

All potential phosphorylation sites have been presented in Appendix 4 Table 1. Collectively, phosphorylation by PKA induced increased expression of the transporters. Conversely, phosphorylation by PKC induces internalisation, reducing transport activity.

Organic anion transporters (OATs) excrete anionic molecules from the body. OATs are predominantly expressed within the renal tubes of the kidney (Eraly et al. 2005) and the liver (Rost et al. 2005). Eraly et al. (2005) reported that in *OAT1* knock-out mice, the transport of para-aminohippuric acid (PAH) was reduced. In a later study, Bulacio et al. (2015), reported that when cisplatin (chemotherapy drug for cancer), that is known to induce acute kidney injury (AKI), increasing *OAT5* expression. Furthermore, when N-acetylcysteine is co-administered with cisplatin, the expression of *OAT5* returns back to basal expression. Therefore, this clearly demonstrates that OATs also transport molecules for elimination from the body.

1.2.1 Glucuronide as carbon source for *Escherichia coli*

E. coli residing in the large intestine and urinary tract scavenge glucuronides as a carbon-based nutrient source (Liang et al. 2005). The *gus* operon (Jefferson et al. 1986; Liang et al. 2005), expresses glucuronidase by the gene known as *gusA* (*uidA*), which cleaves glucuronides into glycones (nutrients) and aglycones (toxic molecules), the latter that is excreted. In the *gus* operon system there is also a *gusR* gene coding for the repressor protein. GusR suppresses transcription when glucuronides are not present. In the presence of glucuronides (Figure 1.4), GusB transports glucuronides across membranes from outside of the cell and into the cytoplasm. There is evidence to show that GusC enhances glucuronide transport into *E. coli*, but detailed molecular mechanisms remains obscure (Liang et al. 2005). However, the specific molecular function of GusC remains unclear but, it is thought that GusC is a porin-like protein. Typically, porin proteins allow the diffusion of molecules

across the outer membrane (Nakae 1976). Further studies are required to elucidate the function of GusC.

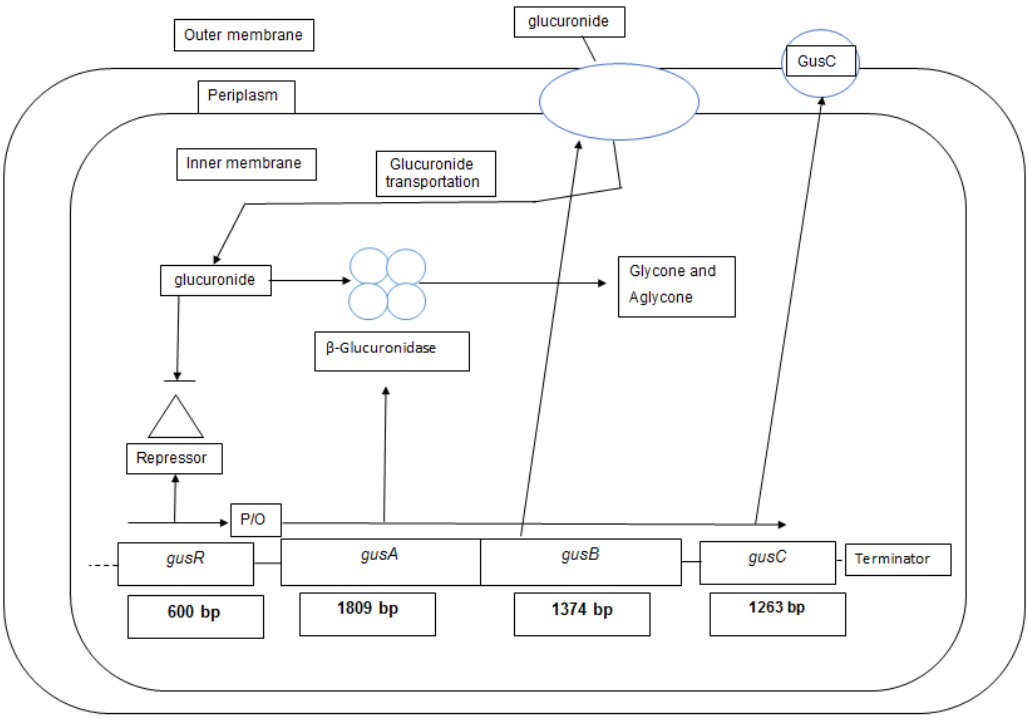


Figure 1.4: The *gus* operon system in *E. coli*. Adopted from the research of (Jefferson et al. 1986; Liang et al. 2005). If the glucuronides produced in a disease do not reflect abnormalities, the aglycone component of glucuronides could be used for disease diagnostics.

Aglycones released from *E. coli* are either eliminated from the human body or they enter the enterohepatic circulation (Figure 1.5). In the enterohepatic circulatory system, aglycones are “reabsorbed” back into the bloodstream by passive diffusion, through the large intestine. Once in the bloodstream, an aglycone enters back into the liver (Samuel et al. 1968; Kent et al. 1972; Walsh and Levien 1975; Inoue et al. 1979; Blumenthal et al. 1980). Once aglycones enter the liver, they are detoxified via glucuronidation, again by the enterohepatic circulation system (Figure 1.5). Enterohepatic circulation occurs if the aglycone part remains in the body.

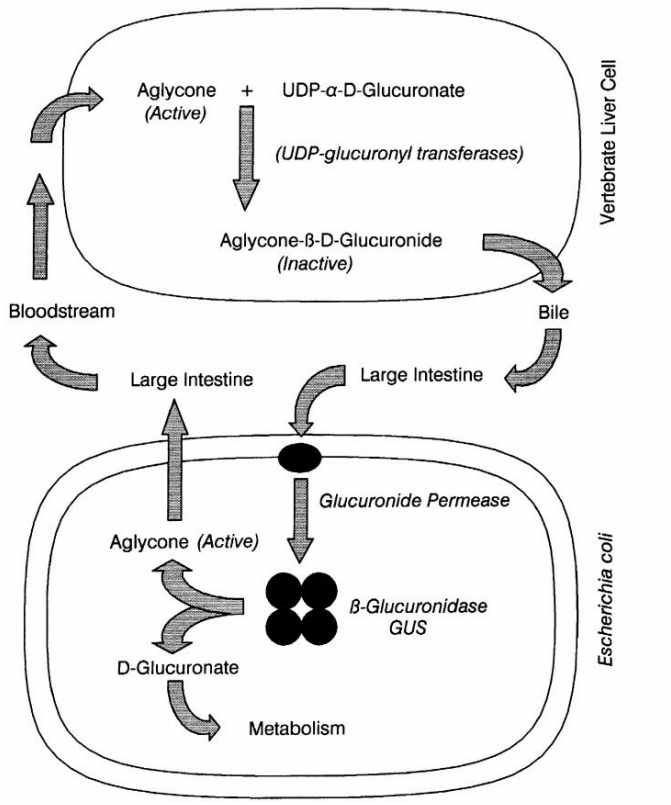


Figure 1.5: Enterohepatic circulation (Adopted from: Wilson et al. 1992, p. 9). Used with the permission from Elsevier. Copyright © 1992 ACADEMIC PRESS, INC. Published by Elsevier Inc. All rights reserved.

1.3 Influences of genetic variation on glucuronide production

The three phases of the detoxification pathway can be affected by genetic variation which can result in abnormalities and alterations, in the detoxification of molecules by different molecular mechanisms (Spencer et al. 2006; Balin and Cascalho 2010), as discussed below. From here on in, for Section 1.3 and 1.4, they will discuss how genetic variation influence molecule detoxification and likewise for epigenetic regulation.

Genetic variation accounts for the differences between individuals. Genetic variation within a population is influenced by three factors: mutation (Lai et al. 2019), gene flow (Botigué et al. 2013) and genetic shuffling (Star and Spencer 2013). In human somatic cells, the rate of mutation has been reported to be approximately 10^{-10} per base pair (bp), per cellular generation (Balin and Cascalho 2010; Gurskaya et al. 2012). Whilst in germline cells, the rate of mutation has been reported to be approximately 1.2×10^{-8} (Milholland et al. 2017). In diseases, the rate of mutation increases. For instance, in cancers, the

rate of mutation occurs for every 2,200 to 2,800 bps (Hodis et al. 2013; Jayaraman et al. 2014; Hao et al. 2016). However, DNA can be repaired by several repair mechanisms. In germline cells, DNA can be repaired by any of the following: nucleotide excision repair (NER) (Jansen et al. 2001), base excision repair (BER) (Olsen et al. 2001), mismatch repair (MMR) (Velasco et al. 2004) or, DNA double strand break repair (DSBR) (Allard and Colaiácovo 2010). In somatic cells non-homologous end joining (NHEJ) (Felgentreff et al. 2014), occurs for DNA repair.

1.3.1 Gene flow and genetic shuffling

Gene flow alters gene frequencies within a population via vertical gene transfer, transferring genes from parent to offspring (Lee et al. 2015). Vertical gene flow can be influenced by genetic shuffling and may also alter the integrity of a gene. The difference of gene flow compared to genetic shuffling is that gene flow, is associated with the movements of a gene from one generation to another (Lee et al. 2015). Genetic shuffling leads to the production of different traits (Spencer et al. 2006), as discussed below.

Genetic shuffling is caused by DNA recombination, leading to genetic mutation or potential newly formed genes. DNA recombination can be interpreted as an exchange of DNA strands which synthesise new DNA sequences (Spencer et al. 2006; Vanhooff et al. 2009; Qi et al. 2015). This is often observed at regions where DNA has been broken, a chance for re-joining to another DNA sequence. DNA recombination can therefore, strongly influence genetic variations. Thus it is logical to believe that these genetic events could also induce alterations in the glucuronidation of molecules.

1.3.2 Alternative splicing

Genetic variations can be caused by alternative splicing (AS) (Li et al. 2016) and sometimes known as exon skipping. In exon skipping, an exon skipped or spliced out of the pre-mRNA transcript (Gurskaya et al. 2012; Cui et al. 2017). AS patterns could disrupt the frameshift of the coding region, promoting protein isoforms and introducing gene polymorphism or premature stop codons. Exon

skipping alone can influence a range of conditions for example: cystic fibrosis (Pagani et al. 2000; Pagani et al. 2003; Pagani et al. 2005; Aznarez et al. 2007) and neuromuscular diseases (Incitti et al. 2010; Dick et al. 2013; Barthelme et al. 2015). Exon skipping can also be observed in other conditions such as cancer (Kahles et al. 2018; Wang et al. 2019). Therefore, in diseases such as cancers, AS could be a target for therapeutic applications for disease treatment.

Genetic variation can be tested currently through two common methods, gene mapping (Fledel-Alon et al. 2013) and DNA sequencing (Choudhary et al. 2017). These methods determine all potential variants that may be present, driving personalised medicine (PM). PM has more beneficial effects to a specific patient compared to standard treatment methods (Horgan et al. 2014; Minsker et al. 2016). This is because PM is tailored to a specific patient, thereby the treatments provided to a patient, is more effective.

AS patterns have be observed in the detoxification pathway, within phase II glucuronidation. Rouleau et al. (2013) reported two *UGT1A* isoforms, i1s and i2s (truncated). The study reported that the i2s negatively regulates i1s via protein-protein interactions. Rouleau et al. (2013) reported that the i2s variant had less sensitivity towards drug-induced cellular death. The study identified that the production of reactive oxygen species (ROS) were reduced and increased expression of superoxide dismutase 1. Later, Labriet et al (2018) reported “intronisation” (the conversion of exons to introns) of exon 1-2 and exon skipping of exons 4-6 of *UGT2B10* which are human specific. It is believed that these AS patterns on xenobiotics and endogenous molecule glucuronidation could be altered but further research is required. Reduced function of the glucuronosyltransferase genes such as the *UGT2B*, may lead to certain chronic diseases such as having the *UGT2B17* gene deleted from the genome (See Section 5.2.3). Therefore, AS could be a subject for understanding the relationship between disease and glucuronide production.

1.4 Epigenetics

Epigenetics defines molecular processes which regulates gene expression, by acting upon DNA, without causing a mutation to a DNA sequence of a gene (Waddington 2012). There four common epigenetic regulatory processes have been discussed below: DNA methylation, histone modifications, ubiquitination and microRNAs.

1.4.1 DNA methylation

DNA methylation is a regulatory cellular process affecting gene expression (Panning and Jaenisch 1996; Danam et al. 1999; Fuks et al 2002). DNA methylation regulates gene expression through “silencing” the gene. Gene “silencing” happens by the attachment of methyl groups (CH_3) to cytosine and guanine rich regions (CpG islands) in a gene promoter. DNA methylation is catalysed by DNA methyltransferases (DNMTs). *DNMT1* is expressed on chromosome 19p3.2, *DNMT2* is located at chromosome 5 and DNMT3A is located at 2p23.3 whilst, *DNMT3A* is located at 2p23.2 and *DMNT3B* is located at chromosome 20q11.21, in humans. Gene silencing occurs since RNA polymerase cannot recognise methyl groups attached by a DNMT. Consequently, transcription is suppressed (Palacios et al. 2010; Hu et al. 2013; Bechis et al. 2015; Hong et al. 2017), thereby gene silencing occurs. DNA methylation is also a heritable trait, for at least three generations.

*UGT1A1*28* is an extensively studied variant of *UGT1A1* due to the association of Gilbert’s syndrome (a condition where an individual have increased unconjugated bilirubin concentrations, Section 6.1.2) and bilirubin glucuronidation. *UGT1A1*28* has reduced expression due to an insertion within the gene promotor. Epigenetic regulation of the *UGT1A1**28 has been reported by Bélanger et al. (2010), they reported that methylation of *UGT1A1* within the 5’-flanking region at CpG -4 region negatively regulates *UGT1A1* expression (Figure 1.6). A later study by Yasar et al. (2013), confirms this and reported that the transcription factor response element (USF) (-4 region) of *UGT1A1*28* is methylated unlike the wild-type UGT1A1. Therefore, the methylation of the USF

element may also be accounted for the reduced expression of *UGT1A1*28* and reduced

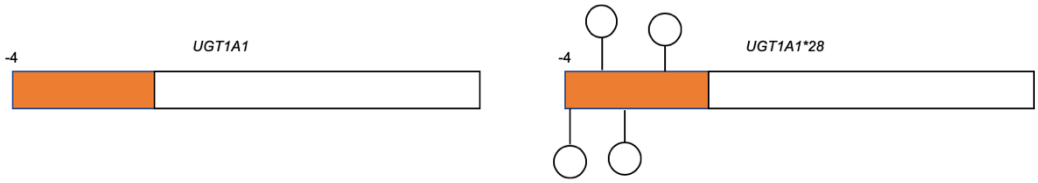


Figure 1.6: Differences between of the USF region of *UGT1A1* and *UGT1A1*28*. The orange region presents the -4 USF region of the *UGT1A1* gene. The markers on *UGT1A1*28* represent the methylation markers on the USF region.

1.4.2 Histone acetylation and phosphorylation

Histones are positively charged proteins that are rich with the histidine amino acid (Hildebrand et al. 1977; Paponov et al. 1980; Righetti et al. 1983). The function of histones allows the condensation of DNA to chromatin. Histones are subjected to epigenetic regulation by acetylation and phosphorylation, discussed below.

Histone acetylation is the addition of acetyl groups ($COCH_3$) onto lysine residues at histone 3 (H3) and 4 (H4) (Figure 1.7). The addition of acetyl groups neutralises the positive charge, relaxing H3 and H4, enhancing gene transcription (Kato et al. 2007; Chen et al. 2010; Dagdemir et al. 2013; Verdone et al. 2015; Nicolas et al. 2018). Furthermore, histone phosphorylation contributes to cell division for chromosome condensation and segregation (Wei et al. 1999; Ota et al. 2002; Huang et al. 2006; Wilkins et al. 2013; Kruitwagen et al. 2015). In addition there are other mechanisms such as DNA reparation which stimulates histone phosphorylation.

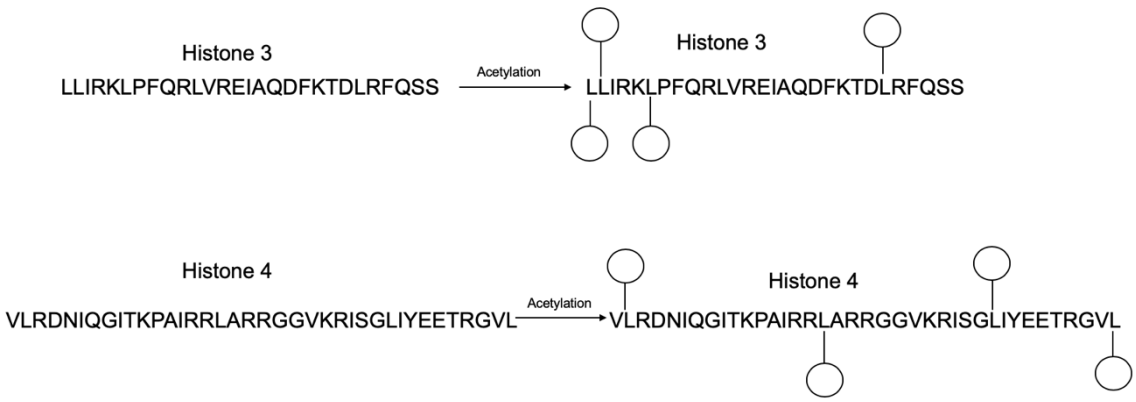


Figure 1.7: Lysine acetylation on histone 3 and histone 4. Partial protein sequences of histone 3 and histone 4 protein have been used. The markers at the lysine (L) residues present acetylation markers. Histone 3 UNIPROT ID: P68431. Histone 4 UNIPROT ID: P62805.

However, to date there is not any research which discusses histone acetylation and phase II glucuronidation. Clearly, further research is needed to reveal whether histone acetylation and phosphorylation the role in influencing *UGT* expression and in turn, the effects on molecule glucuronidation.

1.4.3 Ubiquitination

Ubiquitination is associated with “marking” proteins for degradation (Nguyen et al 2013; Maurer et al. 2016). Proteins marked for degradation have ubiquitin protein chains attached onto lysine residues, acting as a marker for a proteasome (Fletcher et al. 2015; Wu et al. 2018). The marked protein for degradation is recognised by a protease and subsequently degraded. A common example is p53 degradation by mouse double minute 2 homolog protein (MDM2), an E3 ubiquitin protein (Pichiorri et a. 2010; Sparks et al. 2014; Zhu et al. 2018). Thus, the degradation of p53 could be also be a potential disease causing factor for cancer.

The effects of ubiquitination on glucuronosyltransferases have not been explored. This does not necessarily imply that ubiquitination does not affect glucuronosyltransferase proteins. Therefore, it is important that this concept is explored to understand the potential impacts ubiquitination has on molecule detoxification and disease.

1.4.4 microRNA

microRNAs (miRNAs) represent another epigenetic factor, regulating gene expression. miRNAs are non-coding RNAs which are approximately 22 nucleotides long (Ebhardt et al. 2010; Fang et al. 2013; Rolle et al. 2016). miRs regulate gene expression by targeting messenger (m)RNA transcripts for degradation causing gene "silencing" (Zeng et al. 2003; Wu et al. 2006; Hausser et al. 2009; Helfer et al. 2012; Antic et al. 2015; Freimer and Blelloch 2018). mRNA degradation occurs in two ways, the first involves the binding of miRNAs to the 3' untranslated region (UTR) (A/U rich regions of the 3' UTR). Alternatively, the polyadenylation region on mRNA transcripts can be removed (Wu et al. 2006; Beilharz et al. 2009). miRs regulate the expression of different *UGT*s by binding to different regions of a UGT which has been reported in a number of studies (Table 1.1), that the miRs regulate gene expression.

Table 1.1: Some reported miRNAs which regulate some *UGT* expression

miR	*UGT* gene	Predicted binding position if reported	miR effect on gene expression if reported	Reference
miR-21–3,	*UGT1A1*	Not reported	Reduced expression	(Papageorgiou and Court 2013)
miR-103b				
miR-141–3p				
miR-200a-3p				
miR-376b-3p				
miR-103b	*UGT1A3*	+656 to +677	Suppression	
miR-141-3p		+52 to +76		
miR-22a-3p		+54 to +76		
miR-135a-5p	*UGT2B4*	202 to 224	Decrease	(Wijayakumara et al. 2017)
miR-410-3p		213 to 235	Significantly reduced expression	
miR-3664-3p	*UGT2B7*	77 to 99	Significantly reduced expression	
miR-216b-5p	*UGT2B10*	Not reported	Reduced	(Dluzen et al. 2016)
miR-331-5p	*UGT2B15*	Not reported	Significantly reduced expression	(Wijayakumara et al. 2018)
miR-376c	*UGT2B17*	3'-UTR	Reduced expression	(Wijayakumara et al. 2015)

The evidence presented in Table 1.1 shows that miRNAs negatively regulate *UGT* expression. Therefore, the negative regulation in *UGT* expression could contribute to the accumulation in the number of toxic molecules within the human body. Consequently, the accumulation of toxic molecules could lead to

the development of diseases. Additionally, this leads to the suggestion that in despite of gene variants that act as a predisposition for diseases, gene variants may not necessarily be the sole cause of disease.

1.4.5 Epigenetics and disease

The previous sections, Section 1.3.1 to 1.3.4 discusses different epigenetic regulatory mechanisms for gene expression. In prostate cancers for example research has reported hypermethylation patterns of *GSTP1* with hypermethylation (Prior et al. 2010; Chen et al. 2013; Martignano et al. 2016; O'Reilly et al. 2019). Thus, the expression of *GSTP1* is non-existent and consequently the suppression of *GTSP1* could contribute to the accumulation of toxic molecules and metabolites. The accumulation of toxic molecules in the prostate could therefore contribute to the severity of prostate cancer. Ubiquitination in prostate cancers has also been reported (Guan et al. 2008; Chang et al. 2012; Wang et al. 2016; Ma et al. 2018). Tumour necrosis factor (TNF)-receptor associated factor 4 (TRAF4) has E3 ligase functional mechanisms (Kim et al. 2017; Diao et al. 2018), degrading proteins that have been marked for degradation. Singh et al. (2018) reported that in prostate cancer TRAF4 degrades Tropomyosin receptor kinase A (TrkA), by which TrkA regulates prostate cancer metastasis. The ubiquitination of TrkA by TRAF4 promotes prostate cancer metastasis. However, histone modification by acetylation and phosphorylation has not been reported thus far. miRNAs also influence prostate cancer and severity. One particular miR, miR-141 has been associated with prostate cancer. Richardson et al. (2019) reported that when miR-141 is highly expressed, biochemical recurrence of prostate cancer happens. Furthermore, the expression of miR-145 is thought to contribute to the aggressiveness of prostate cancer. It is recommended that further studies should be conducted to elucidate whether gene expression in prostate cancer is altered by miR-141 and miR-145.

1.5 Rationale

The detoxification pathway is important for human health. The detoxification of molecules may vary in different individuals influenced by ethnicity (Premawardhena et al. 2003; et al. 2004; Westlind-Johansson et al. 2006; Zhou et al. 2009; Santos et al. 2014; Court et al. 2017). Both genetic and non-genetic factors could affect the detoxification of molecules leading to diseases. Thus, the presence of a disease may correspond to abnormalities in altered glucuronide production which could be used to reveal a glucuronide spectrum. The different glucuronide spectrum could therefore be used for disease prognosis and diagnosis. Therefore, finding or clarifying this association of the altered glucuronide spectrum with certain disease is of interest of this research.

1.6 Aim and Objectives

The aim of this research was to clarify the association of glucuronides for disease prognosis.

To ensure the aim was achieved the following objectives were outlined:

1. Evaluate how genetics affect the detoxification pathway
2. Evaluate lifestyle factors which may affect the detoxification pathway
3. Evaluation of published gene polymorphic frequencies on relevant genes from different human ethnicities, using bioinformatic tools, databases, and published peer-reviewed research.
4. Reveal potential glucuronide spectrums reflecting objectives 1 and 2.
5. Postulate the potential of clinical applications of the research.

1.7 Thesis structure

To ensure the aim and objectives are successfully achieved multiple chapters have been presented in this thesis. Below provides an outline of the different chapters within the thesis.

Chapter 1: Introduction.

Chapter 2: The Metabolic Fate of Different Medicinal Drugs.

Chapter 3: The Metabolic Fate of Different Illicit Drugs.

Chapter 4: The Metabolic Fate of Different Food Additives.

Chapter 5: The Metabolic Fate of Different Endogenous Molecules.

Chapter 6: The Metabolomic Fate of Different Xenobiotics.

Chapter 7: The Potential Application of Glucuronides for Disease Prognostics.

Chapter 8: Conclusion.

Chapter 2: Methods

2.1 Method for databases

The method undertaken involved evaluating data in research to date. Journal databases were used to obtain relevant literature: Google Scholar, Web of Science, PubMed and Scopus (Table 2.1). Bioinformatic databases and tools including: OMIM, NCBI, gnomAD and Exome Aggregation Consortium (ExAC) (Table 2.1) were used to access allele frequencies for different human ethnicities and research to date was used.

Table 2.1: Databases used for the research

Database	Database URL	Reference (if possible)
Google Scholar	https://scholar.google.com/	Not applicable
Web of Science	https://apps.webofknowledge.com/WOS_GeneralSearch_input.do?product=WOS&search_mode=GeneralSearch&SID=D3ZluaMLfytEi3HsYdu&preferencesSaved=	Not applicable
PubMed	https://pubmed.ncbi.nlm.nih.gov/	Not applicable
NCBI	https://www.ncbi.nlm.nih.gov/	Not applicable
OMIM	https://www.omim.org/	(Amberger et al. 2015; Amberger et al. 2019)
gnomAD	https://gnomad.broadinstitute.org/	(Karczewski et al. 2019)
Exome Aggregation Consortium (ExAC)	Database no longer available: refer to: https://gnomad.broadinstitute.org/	ExAC:(Lek et al. 2016) gnomAD:(Karczewski et al. 2019)
Ensembl	https://www.ensembl.org/index.html	(Hunt et al. 2018)
ClinVar	https://www.ncbi.nlm.nih.gov/clinvar/	(Landrum and Kattman 2018)

Online Mendelian Inheritance in Man (OMIM) is a database which report genes and their function and gene variants associated to a disease (Amberger et al. 2019). OMIM was used for one particular disease, phenylketonuria (PKU) (OMIM%21600) discussed in Chapter 4. This was to gain a further understanding of how the disease is caused at the molecular level, since aspartame is metabolised by phenylalanine hydroxylase (Fusetti et al. 1998). From the information provided by OMIM in relation to PKU, a further search was taken using google for the discussion of aspartame and the relation to disease as discussed in Section 4.1. The use of Web of Science, PubMed and google scholar is discussed in Section 2.3. Additionally, OMIM was used to explore allele frequencies of the different variants discussed in this research.

gnomAD and ExAC are bioinformatic databases that report allele frequencies of gene variants, homozygous and heterozygous, within a specific population or ethnic group (Lek et al. 2016; Karczewski et al. 2019). Both gnomAD and ExAC, along with published peer-reviewed research on human population genetics was used to demonstrate which ethnicities, had the variant as evident in Chapters 3 to 7. This is because one ethnicity will be at a higher risk of a disease compared to another population or ethnicity. For instance, prostate cancer occurs more frequently in black men compared to white men and Asian men, in England alone (Lloyd et al 2015). Thus, treatment could be prioritised for individuals who are at a higher risk of a disease, compared to another individual.

Ensembl is a bioinformatic database that provides such as information on a gene and reporting variant(s) of a gene population genetics of a gene variant and the association to disease (Hunt et al. 2018). Additionally, Ensembl provides a genome browser which can be used to analyse a particular area of the human genome (Hunt et al. 2018). Ensembl was used to explore population genetics of a gene variant and to explore an association of a variant to a disease, using a variant ID (rs#) or the gene variant annotation such as *CYP2D6*4* .

ClinVar is an archive bioinformatic database which contains gene variants and the relationship of a gene variant (intergenic, exon or a gene from mitochondria to a disease (Landrum and Kattman 2018). ClinVar was used to help associate gene variants discussed in this research, to a disease along published peer-reviewed research using PubMed and google scholar.

2.2 Workflow of bioinformatic and genetic analysis

Below presents a workflow diagram of the process undertaken. An explanation of each database and the purpose why each database has been used is provided in Section 2.1.

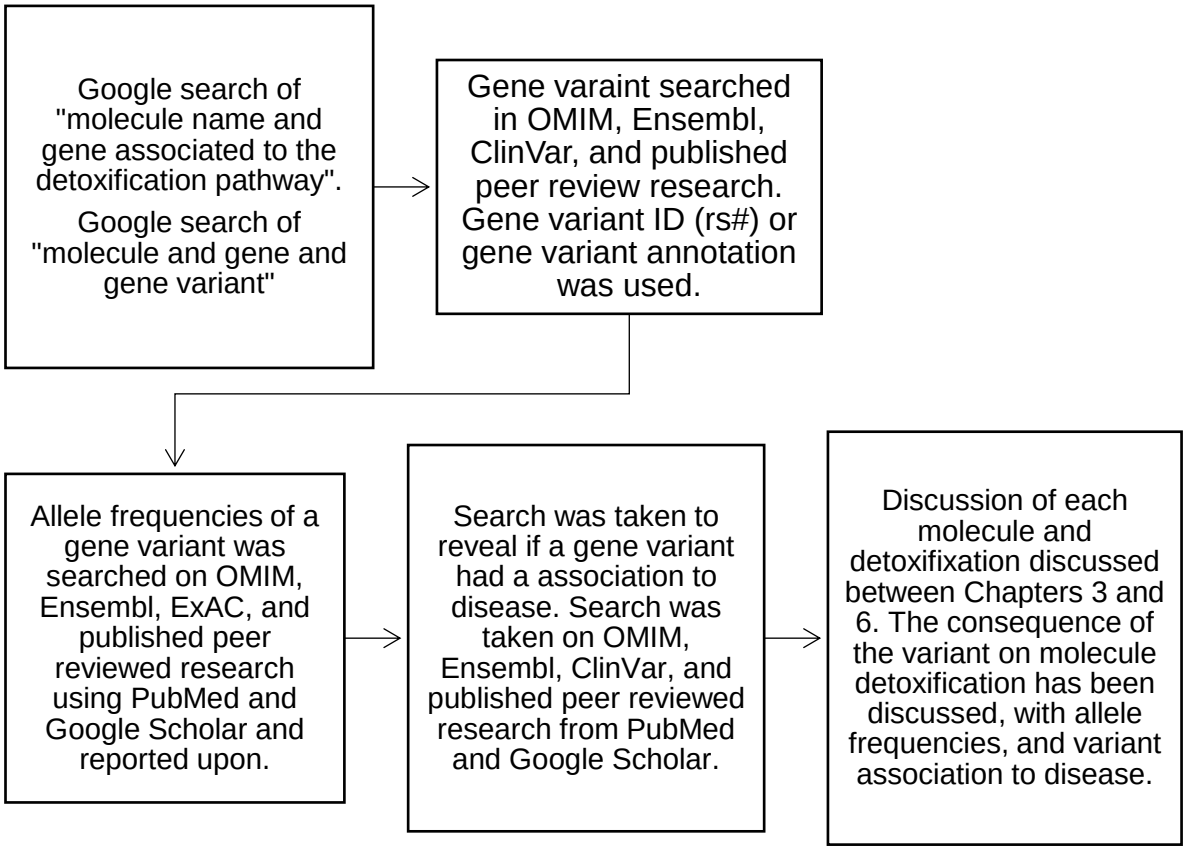


Figure 2.1: Workflow of the bioinformatics method undertaken.

2.3 Method for Chapter 8

To collect the results that have been presented in Chapter 8, three journal databases were used, Google Scholar, Web of Science and PubMed. Using the three journal article databases, search terms were applied to retrieve all the papers for analysis, which may report glucuronides associated to a disease (type 2 diabetes mellitus, colorectal cancer, prostate cancer, breast cancer, liver cancer, and Parkinson's disease). Figure 2.1 presents a flow diagram of the process undertaken for Chapter 8. Once all the papers were collected, all the papers were analysed using inclusion and exclusion criteria (Table 2.2 and Figure 2.2), in accordance to the PRISMA recommended guidelines for systematic reviews (Moher et al 2012). The inclusion and exclusion criteria have been designed to retrieve journal articles that have reported glucuronides associated to a disease.

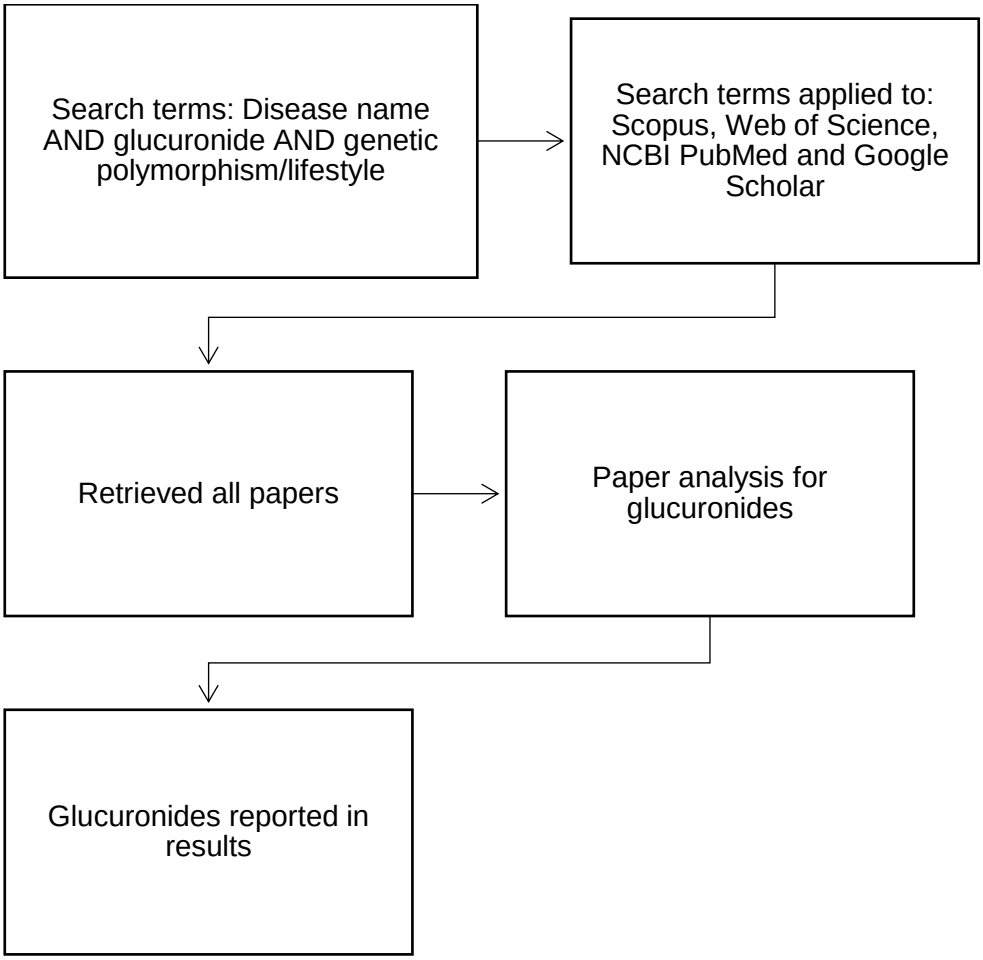


Figure 2.2: Workflow of the method used for Chapter 8.

Table 2.2: Inclusion and exclusion criteria for journal article analysis

Part	Description	Inclusion criteria	Exclusion criteria
1	Journal title analysis	Keywords (at least one): glucuronides, disease, gene polymorphisms or lifestyle related factors, human sample.	Duplicate studies by title Not relevant No keyword(s) mentioned
2	Abstract analysis	Glucuronide(s) detected/biomarker(s)/metabolites Human sample analysis Disease influenced by gene polymorphism(s) or lifestyle factor(s).	Retracted papers Samples which do not derive from human Glucuronides not reported Disease not specified No abstract provided
3	Full paper analysis	Analysis using human samples Glucuronides associated to a disease Glucuronide detection from individuals who have a disease caused by both gene polymorphism(s) and lifestyle factor(s).	Abstracts provided only (full text not available) Reviews (literature and other systematic reviews). Letters to the editor Conference papers Papers that are not available in English Glucuronides which are reported that are not associated to a disease. Glucuronides that do not come from human

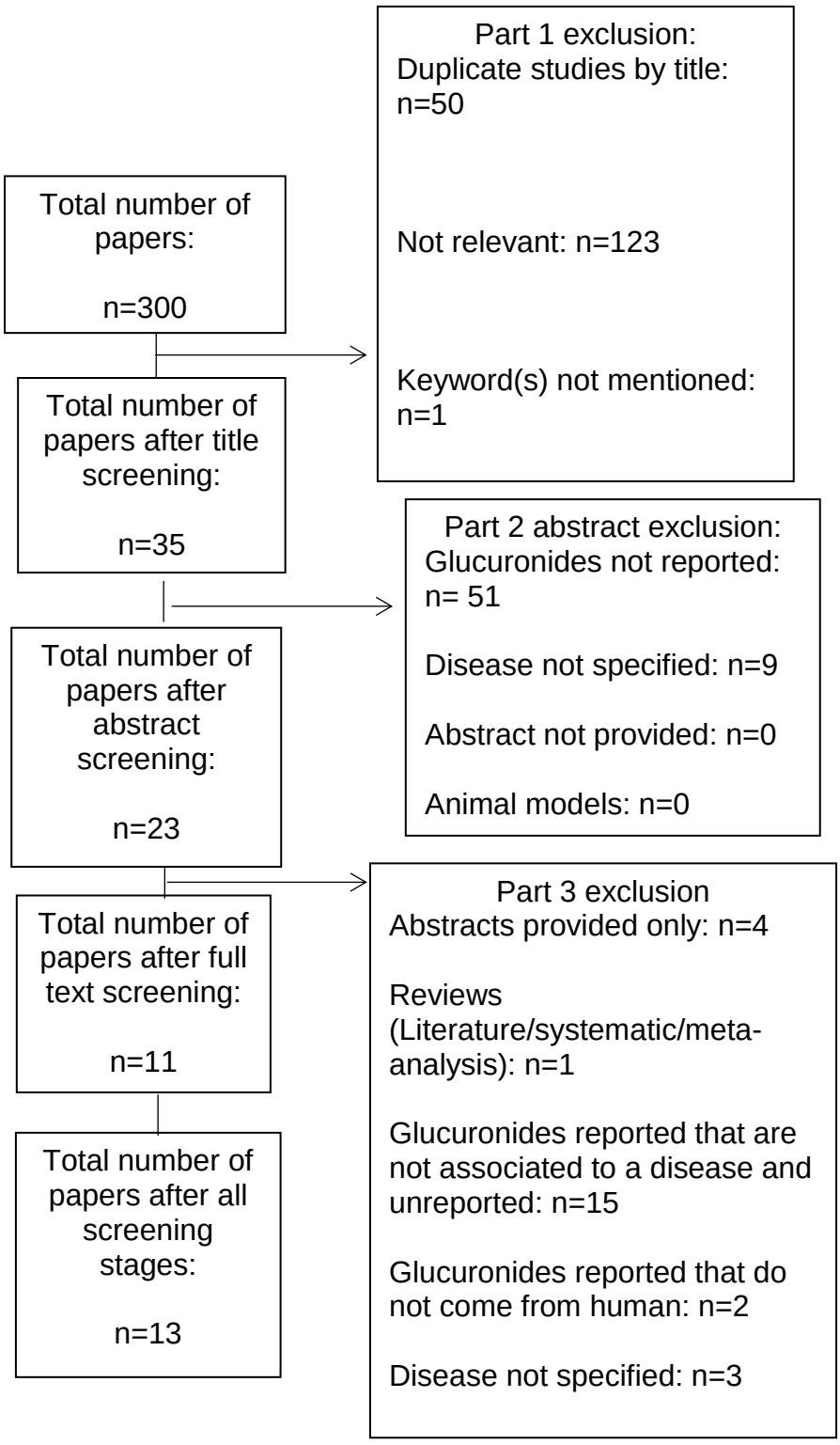


Figure 2.3: Flow diagram for literature analysis

Chapter 3: The Metabolic Fate of Different Medicinal Drugs

Medicinal drugs are perceived to elicit therapeutic effects to treat or manage a disease. However, there is misconceptions in the views of medicinal drugs. This is because each individual responds to a drug due to different efficiencies of drug detoxification, which can be linked to linked to the genetic makeup of an individual. However, current practices of prescribing and administering drugs does not consider the genetics of an individual and therefore, should be considered before prescribing a drug to a patient (Zineh et al. 2004; Frueh et al. 2008). Thus, an individual may present severe adverse toxicological reactions to a drug that has been prescribed for treatment, since the standard method is a "one fits all approach". Therefore, the application of personalised medicine should be considered as a standard practice for prescribing medicinal drugs. Personalised medicine is designed to tailor a drug or treatment to a patient based on the genetics of a patient (Younossi et al. 2013; Guglielmo et al. 2018). Thereby, personalised medicine could prevent the risks of adverse toxicological reaction caused by a prescribed drug, potentially achieving a higher success rate for disease treatment. Below discusses the implications of how genetic variation can alter the detoxification of medicinal drugs.

3.1 Irinotecan

3.1.1 Irinotecan overview

Irinotecan (CPT-11) (Figure 3.1) is a chemotherapeutic-based drug for the treatment of colorectal cancers (CRCs) (Morise et al. 2014) and small cell lung cancers (SCLCs) (Kondo et al. 2018). CPT-11 inhibits topoisomerase I by a binding mechanism preventing DNA ligation, reparation and replication (Fayad et al. 2009; Tamura et al. 2012; Meisenberg et al. 2016). This promotes the induction of the apoptotic pathway, triggering cellular death to cancerous cells (Motwani et al. 2001; McDermott et al. 2005; Haug et al. 2008).

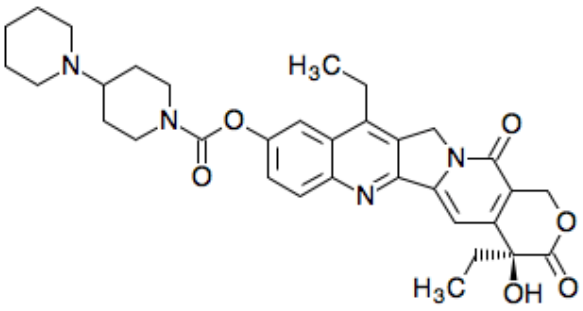


Figure 3.1: Molecular structure of irinotecan

In CRC's CPT-11 is administered as the first drug for treatment if the CRC metastasises. CPT-11 is usually co-administered with cetuxumab or fluoruracil (5-FU) and folinic acid (FOLFIRI) (Figure 3.2), as a drug combination therapy (Uemura and Yamada 2006; Ruzzo et al. 2008; Huang et al. 2012; Pham et al. 2015; Liang et al. 2016). The FOLFIRI drug regime treatment aims to reduce the size of CRCs. Liang et al. (2016) reported that before treatment the metastases size (mm) of 218.71 ± 38.57 and after treatment the metastases size has been reduced with a size of 63.09 ± 12.69. Therefore, the FOLFIRI treatment is effective on CRCs. Despite this evidence, chemotherapy-drug based treatment may be rendered ineffective when ABC transporters are over expressed.

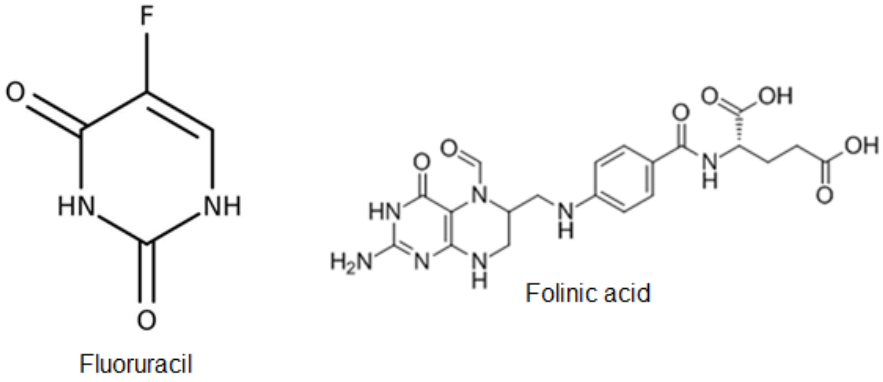


Figure 3.2: Molecular structure of fluoruracil and folinic acid

CPT-11, unlike its use for CRCs, is the second choice of treatment for SCLCs. SCLC's account for approximately 15% of all cancers of the lung. Kondo et al. (2018) concluded that CPT-11 achieved a high disease control rate (DCR) and increased survival time in patients. Despite the small sample size (n=30) of this study, earlier research conducted on 321 patients with SCLC, who were treated

with CPT-11, reported a low dosage (60 mg) was effective with minor toxicological effects (Morise et al. 2014). This suggests that high drug dosage may not reflect a higher efficacy.

3.1.2 Irinotecan processing by cytochrome p450

In phase I CPT-11 is a substrate for CYP3A4. CYP3A4 prepares CPT-11 for phase II by a oxidative for phase II glucuronidation (Santos et al. 2000; Mathijssen et al. 2004). The oxidative reaction synthesises two inactive metabolites, 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin (APC) and 7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecin (NPC) (Figure 3.3).

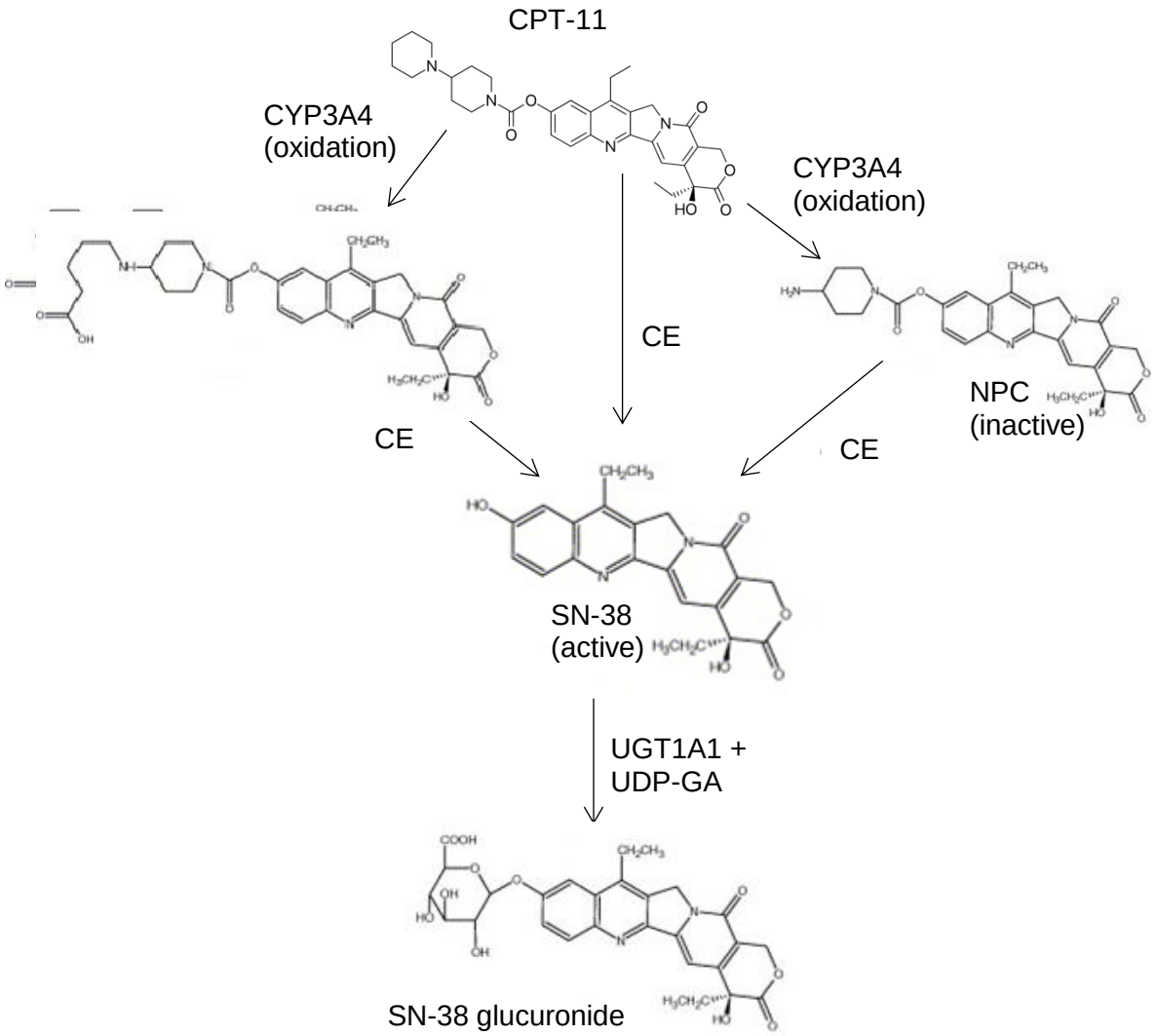


Figure 3.3: CPT-11 detoxification pathway. When CYP3A4 reacts with CPT-11, two inactive metabolites are produced, APC and NPC. APC and NPC could then go through a esterification reaction catalysed by carboxylesterase (CE). Alternatively, CPT-11 can be synthesised to SN-38by an esterification reaction CE. SN-38 can then be glucuronidated by UGT1A1.

The oxidative reaction catalysed by CYP3A4 protein produces the inactive metabolite APC. APC can be synthesised to SN-38, by an esterification reaction that is catalysed by carboxylesterase (CE) (Guichard et al. 1998; Wu et al. 2002; Sanghani et al. 2004; de Jong et al. 2006; Han et al. 2009; van der Bol et al. 2011; Marangon et al. 2015; Satoh et al. 2018), (Figure 3.3). NPC is also synthesised to SN-38 by CE (Figure 3.3). Alternatively, CPT-11 can be synthesised directly to SN-38 by an esterification reaction by CE, or a hydrolysis reaction by CYP3A4. Subsequently, SN-38 is conjugated to UDP-glucuronate

(glucuronic acid, UDP-GA) for phase II detoxification, synthesising SN-38 glucuronide.

SN-38, like the parent molecule CPT-11, inhibits topoisomerase I as discussed in Section 3.1.1. In CRCs the *CYP3A4*20* polymorphism is expressed, with a reduced function (Apellániz-Ruiz et al. 2015; Gómez-Bravo et al. 2018). The reduced function is a result of protein truncation (Westlind-Johansson et al. 2006; Zhou et al. 2011; Apellániz-Ruiz et al. 2015). Rierra et al. (2018) demonstrated that *CYP3A4*20* protein function could be increased. Thereby, this may indicate that gene expression can vary in different human ethnicities. Alternatively, there could be a lack of research associating the expression patterns of *CYP3A4*20*.

The *CYP3A4*20* (rs67666821) polymorphism is more frequently observed in the Spanish population (Table 3.1). It has been hypothesised that the founder effect is the main cause for the polymorphism (Apellániz-Ruiz et al. 2015). The founder effect explains that the loss of genetic variation, is a result of the formation of a new population from a small number of individuals who came from a larger population (Mayr 1942; Robertson 1952; Mayr 1959; Mckusick et al. 1964). Thereby, the founder effect can introduce characteristics that may have not existed in the previous larger population.

Table 3.1: Allele distribution frequency of the *CYP3A4*20* allele in different human populations.

Population	Country or region (if specified)	Homozygous (if specified)	Heterozygous (if specified)	% of population affected	Reference
European	NS*	NS*	NS*	0	(Zhou et al. 2017)
	Germany	NS*	0	<0.1	(Westlind-Johansson et al. 2006)
	Sweden	0	NS*	<0.1	(Karczewski et al. 2019)
	Italy	NS*	0	NS*	(Apellániz-Ruiz et al. 2014)
	Spain	NS*	24	1.6	(Apellániz-Ruiz et al. 2014)
	Argentina	NS*	0	0	Apellániz-Ruiz et al. 2014)
Africans	NS*	NS*	NS*	<0.1	(Zhou et al. 2017)
	Libya	NS*	0	0	(Apellániz-Ruiz et al. 2014)
	Algeria	NS*	0	0	(Apellániz-Ruiz et al. 2014)
	African American	NS*	1	0	(Apellániz-Ruiz et al. 2014)
	NS*	0	NS*	<0.1	(Karczewski et al. 2019)
Asian	East Asian	NS*	NS*	<0.1	(Zhou et al. 2017)
	South Asian	NS*	NS*	0	(Zhou et al. 2017)

**NS: Not specified*

3.1.3 Phase II detoxification of irinotecan

CPT-11 requires phase II conjugation for detoxification. CPT-11 is conjugated to UDP-glucuronate (UDP-GA, glucuronic acid) by UGT1A1 and UGT1A6. UGT1A1 is the predominant UGT1A for CPT-11 glucuronidation (Ando et al. 1998; Iyer et al. 2002; Marcuello et al. 2004; Alkharfy et al. 2013; Jannin et al. 2017; Shirasu et al. 2019). The glucuronidation of CPT-11 by UGT1A1 is altered by a polymorphism associated which influences toxicological reactions.

*UGT1A1*28* alters the fate of CPT-11 molecules by contributing to the accumulation of unconjugated CPT-11 within the liver. Consequently, this

causes neutropenia and severe diarrhoea, a common adverse effect in individuals who have the *UGT1A1*28* polymorphism. *UGT1A1*28* has a two base pair (bp) insertion (thymine (T) and adenine (A) ($(TA)_7$) within the TATA box in the promotor region of the gene. The wildtype (WT) *UGT1A1* promotor region has a sequence of $(TA)_6$ (Ando et al. 1998; Peters et al. 2003; Liu et al. 2008; Zhao et al. 2013; de Souza 2017). Therefore, before administering medicinal drugs, patients should be screened for polymorphisms of *UGT1A*. Human allelic distributions of *UGT1A1*28* have been provided in Table 3.2.

Table 3.2: Human allele distribution of *UGT1A1*28*.

Population	Country or ethnicity	Number of individuals from the population	Number of homozygous (% affected)	Number of heterozygous (% affected)	P-value reported in the study	Reference
Europe	UK	5 9	3 (5)	26 (44)	NS*	(Premawardhena et al. 2003)
	Iceland	69	7 (10.1)	33 (47.8)	NS*	(Premawardhena et al. 2003)
	Germany	343	39 (17.9)	80 (36.7)	NS*	(Borucki et al. 2009)
	Italy	98	16 (16.3)	NS*	<0.001	(Biondi et al. 1999)
	Greek Cypriots	47	4 (4.85)	19 (40.4)	NS*	(Premawardhena et al. 2003)
Asia	Chinese	50	1 (2)	11 (22)	NS*	(Premawardhena et al. 2003)
	Japanese	8	1 (13)	2 (25)	NS*	(Liu et al. 2007)
	Filipino	37	0	9 (24)	NS*	(Liu et al. 2007)
American	Caucasian	56	7 (13)	22 (39)	NS*	(Liu et al. 2007)

**NS: Not specified*

UGT1A6 is the second UGT1A that can glucuronidate CPT-11. But, UGT1A6 is not as extensively studied as UGT1A1 (Hanioka et al. 2001; Carlini et al. 2005; Mross et al. 2007; Bellemare et al. 2010; Stingl et al. 2014; Wang et al. 2017). However, no polymorphism of *UGT1A6* has yet been associated with altered fate of CPT-11.

3.1.4 Irinotecan transportation for elimination

CPT-11 is a substrate for multiple transporters: *ABCC4* (multidrug resistance protein 4 (MRP4, MOATB)) (Norris et al. 2005), *ABCG2* (breast cancer resistance protein, BCRP) (Zhang et al. 2008) and *ABCB1* (MDR1) (Iyer et al. 2002). *ABCC4* is predominantly expressed in the prostate (Ho et al. 2008) and colon (Chen et al. 2017). *ABCG2* is also expressed in the colon (Candeil et al. 2004), duodenum (Gutmann et al. 2005), small intestines (Enokizono et al. 2007), and seminal vesicles (Thompson et al. 2008). This demonstrates that the expression of transporters is not limited to one location.

MDR1 is the predominant transporter for CPT-11 and SN-38 but, there are other ABC transporters for both molecules (Lalloo et al. 2004) In colorectal cancers, *ABCB1* is over-expressed (Bessho et al. 2006; Riganti et al. 2009; Stein et al. 2012; Takács et al. 2015; Liu et al. 2017). Over-expression of *ABCB1* impedes cancer treatment, causing chemo-resistance towards drug based therapies. This will therefore, promote CRC to develop and metastasise. Therefore, ABC transporter inhibitors could be used to reduce *ABCB1* over-expression. Furthermore, in colorectal cancers, rs1045642 has been linked to reduced expression (Hoffmeyer et al. 2000; Brant et al. 2003; Shahwan et al. 2007; Drain et al. 2009; Campa et al. 2012; Marna et al. 2015; Jin and Song 2017). Decreased expression may influence an accumulation in toxic molecules which could contribute to further disease progression.

Similarly to MDR1, MRP4 is also over-expressed in CRC (Holla et al. 2008; Cheung et al. 2015). The overexpression of *ABCC4* also renders drug-based chemotherapy ineffective (Gradilone et al. 2008; Lai et al. 2012; Zhang et al. 2014). The over-expression of *ABCC4* has been observed in cancers such as: neuroblastomas (Norris et al. 2005), colorectal cancer (Holla et al. 2008), prostate cancer (Ho et al. 2008) lung cancer (Maeng et al. 2014) and osteosarcomas (He et al. 2015). Alternatives to drug-based cancer treatments could be used to minimise the risk of chemo-resistance in the presence of over-expressed ABC transporters.

A variant of the *ABCG2* gene is associated with promoting further adverse reactions towards CPT-11. rs3109823 has been reported to influence

myelosuppression (Cha et al. 2009; Wang et al. 2011; Moriya et al. 2014; Bircsak et al. 2018). Myelosuppression decreases bone marrow activity and as a result, reducing the production of red blood cell (RBC) and platelets (Warkentin et al. 2014; Taylor et al. 2017). Before prescribing CPT-11, it is recommended that patients should be screened for rs3109823 and *UGT1A1*28*.

3.1.5 Irinotecan and disease

With an altered detoxification pattern of CPT-11, the application of CPT-11 for CRC treatment is ineffective (Bessho et al. 2006; Riganti et al. 2009;Lai et al. 2012; Zhang et al. 2014). This is due to that in the presence of *UGT1A1*28* glucuronidation of CPT-11 and SN-38 is significantly reduced. Consequently, CPT-11 and SN-38 accumulate in the liver inducing adverse effects (Section 2.1.3). Furthermore, with the presence of rs3109823, a variant affecting *ABCG2*, influences further adverse effects (Section 2.1.4). Therefore, alternatives of CPT-11 for treatment programmes could be considered. Furthermore, genotyping a patient for the variants is recommended (Campa et al. 2012; de Souza et al. 2017; Sharma et al. 2019), to avoid adverse toxicological reactions towards a drug. Thus, the application of personalised medicine for the treatment of CRC should be considered.

3.2 Acetaminophen

3.2.1 Acetaminophen overview

Acetaminophen (APAP, paracetamol), is an analgesic drug used to treat acute to moderate pain (Chou et al. 2007; Toms et al. 2008). The mechanism of action of APAP is to inhibit prostaglandin (PGs) synthesis (Rahme et al. 2002; Högestätt et al. 2005). PGs are synthesised by cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (Figure 3.4), to promote inflammation at the site of injury or infection. APAP is a weak inhibitor of COX-1 and COX-2 but, APAP does not inhibit COX-1 and COX-2 in peripheral tissues (Rahme et al. 2002; Graham et al. 2005; Hinz et al. 2008; Griesser et al. 2010). However, APAP is

not classified as a nonsteroidal anti-inflammatory drug (NSAID) because APAP does not relieve inflammation.

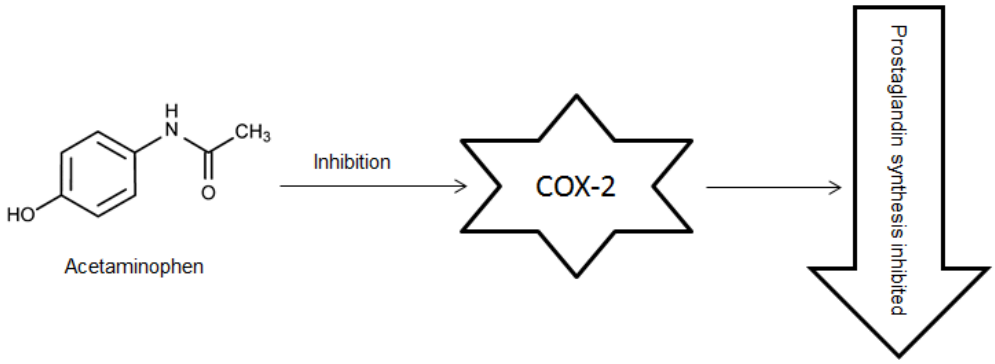


Figure 3.4: Diagram of acetaminophen inhibiting prostaglandin synthesis. The mechanism for inhibition of COX-2 remains unclear.

3.2.2 Acetaminophen detoxification by cytochrome p450

When APAP is present in the liver, *CYP3A4* gene expression is stimulated, coding for CYP3A4 protein. CYP3A4 bioactivates APAP by an oxidative reaction (Figure 3.5). However, CYP3A4 activity can be suppressed by APAP in a dose-dependent relationship (Feierman et al. 2002; Zhang et al. 2004; Kim et al. 2009; Ben-Shacher et al. 2012; Raza and John 2015; Limonciel et al. 2018). Thus, excess accumulation of APAP can lead to drug-induced liver failure.

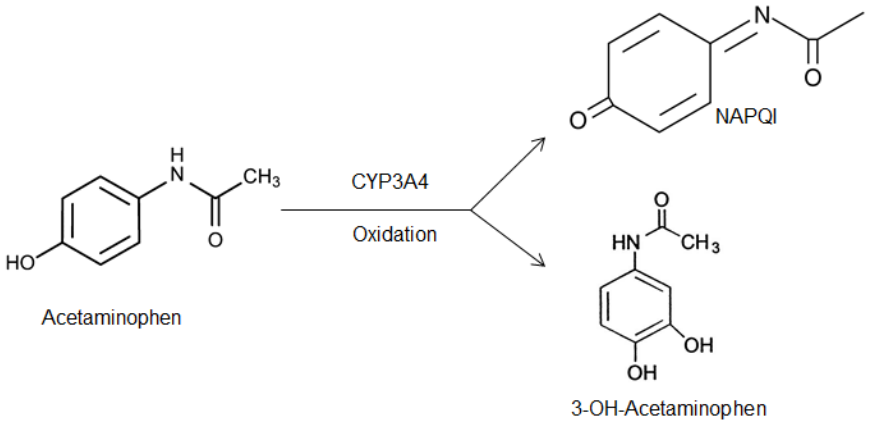


Figure 3.5: Phase I detoxification of acetaminophen by CYP3A4. NAPQI is the active metabolite produced from the oxidation reaction which can induce toxicological effects. 3-OH-Acetaminophen is an inactive metabolite produced from the reaction.

Other CYP450s can prepare APAP for phase II, CYP2E1 and CYP1A2. CYP2E1 and CYP1A2 were the first two reported CYP450s to react with APAP. CYP2E1 and CYP1A2 hydroxylate APAP, synthesising a toxic metabolite, N-

acetyl-p-benzoquinone imine (NAPQI or NAPBQI) (Zaher et al. 1998; Sumioka et al. 2001; Guo et al. 2004; Dai et al. 2006). The presence of NAPQI in the liver can promote adverse toxicological effects including liver damage (Fong et al. 2010; Stutchfield et al. 2015; Rubin et al. 2018).

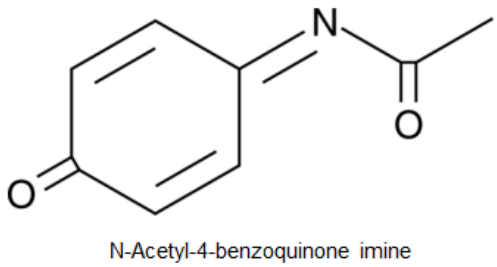


Figure 3.6: Molecular structure of acetyl-p-benzoquinone imine.

Table 3.3: Human allele distribution of *CYP2E1*5B*

Population		N*		CYP2E1			P-value	References
				Genotype frequency (%)				
				*1A/*1A	*1A/*B	*5B/*5B		
				(GG)	(GC)	(CC)		
Asian								
Iranian		200		97.0	3.0	0.0		(Shahriary et al. 2012)
Taiwanese		231		58.0	35.1	6.9	<0.001	(Wang et al. 1999)
Indian		350		98.0	2.0	0.0	0.31	(Ruwali et al. 2009)
Han Chinese		166		80.6	NS*	40	<0.001	(Wang et al. 2009)
Japanese		NS*		NS*	NS*	60	<0.001	(Krishnakumar et al. 2011)
European								
French		172		91.6	4.7	0.0	0.29	(Bouchardy et al. 2000)
British		155		96.8	3.2	0.0	0.88	(Yang et al. 2001)
German		297		94.9	4.4	0.7	0.11	(Neuhaus et al. 2004)
Poland		316		94.4	5.6	0.0	0.08	(Gajecka et al. 2008)
Italian		245		93.4	6.2	0.4	0.03	(Boccia et al. 2008)
American								
Brazilian		212		88.2	11.8	0.0	<0.001	(Marques et al. 2006)
American		399		96.2	3.8	0.0	0.6	(Liu et al. 2001)
Chilean		148		71.0	27.0	2.0	<0.001	(Quiñones et al. 2001)
Mexican Mestizos		239		72.0	23.8	4.2	<0.001	(Gordillo-Bastidas et al. 2010)
Mexican Huichols		99		21.2	54.5	24.2	<0.001	(Gordillo-Bastidas et al. 2010)
Turkish		206		96.1	3.9	0.0	0.54	(Ulusoy et al. 2007)

**N: population size; P-value presents significance difference. P-values are the values reported in the studies.*

Interestingly, there is a potential epigenetic mechanism that has been postulated to protect the liver from APAP induced liver damage, using miR-122 (Chowdhary et al. 2017), discussed below. Typically, miR-122 is associated with decreasing cholesterol and fatty acids (Esau et al. 2006; Ghosh et al. 2010; Wang et al. 2013; Raitoharju et al. 2016; Sendi et al. 2018). The general

function of miRs has been discussed in Section 1.3.4 and presented in Figure 3.7.

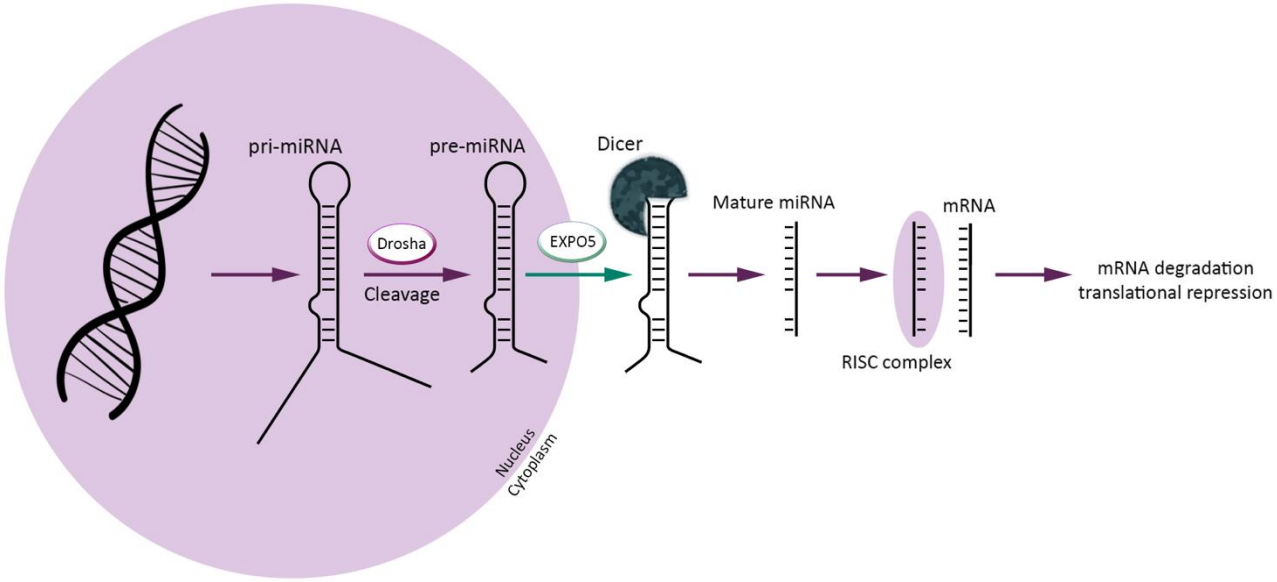


Figure 3.7: miRNA synthesis and mRNA degradation pathway. miRNA synthesis occurs by a small series of cascade events. miRNA is first synthesised through transcription by RNA polymerase II, producing a primary (pri)-miRNA transcript with hairpin structure (Lee et al. 2004; Gatfield et al. 2009; Nagpal et al. 2013; Nakamachi, et al. 2016). Subsequently, DROSHA proteins (type III ribonuclease), cleave the pri-miRNA hairpin structure to yield pre-miRNA (Ma et al. 2013; Johanson et al. 2015; Kim et al. 2017). From the nucleus of the cell, exportin (EXPO5) transports the pre-miRNA to a DICER complex in the cytoplasm, which cleaves pre-miRNA to release the mature miRNA for mRNA degradation.

In APAP induced liver toxicity, miR-122 expression is altered but, further elucidation is required into the function of miR-122 and protecting the liver from APAP induced damage (Chowdhary et al. 2017). Chowdhary et al. (2017) reported that if miR-122 expression is decreased, it is correlated with decreased expression of two transcription factors, HNF4α and HNF6. These transcription factors may regulate APAP detoxification by increasing *CYP2E1* and *CYP1A2* expression. Additionally, miR-122 was found to regulate *CYP1A2* expression through a pathway involving transcriptional repressor CTCF, aryl hydrocarbon receptor (AHR) (increased expression), and the mediator of RNA polymerase II transcription subunit 1 (MED1, transcription regulator) (increased expression) (Chowdhary et al. 2017). The specific molecular mechanism(s) were not discussed and therefore, the molecular mechanism(s) of the pathway should be explored further.

3.2.3 Acetaminophen phase II detoxification

APAP and NAPQI can be detoxified by glucuronidation or GSH conjugation (Chen et al. 2003; Srikanth et al. 2005). If APAP and NAPQI are detoxified by glutathione (GSH) conjugation, one of the three GSTs could conjugate GSH molecules to APAP or NAPQI: GSTT1, GSTP1 and GSTM1 (Huber et al. 2009; Roušar et al. 2012; Raza et al. 2015; Kim et al, 2018). The conjugation reaction synthesises APAP-GSH and NAPQI-GSH metabolites (Figure 3.8). However, discussed in Section 3.2.4, excess APAP and NAPQI can induce liver necrosis and liver failure, due to depleted concentrations of GSH in the liver.

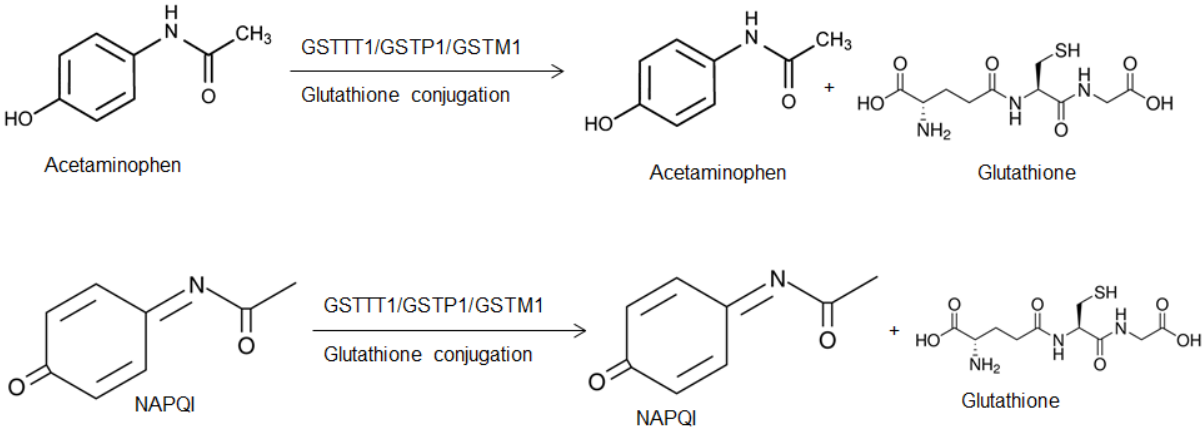


Figure 3.8: Phase II detoxification of acetaminophen and NAPQI. The metabolites produced at the end of the conjugation has glutathione conjugated to each of the molecules.

Glutathione S-Transferase Theta 1 (GSTT1) is one of the three GSTs capable of detoxifying APAP molecules and NAPQI metabolites. A gene deletion of *GSTT1* influences susceptibility towards APAP inducted liver toxicity (Lucena et al. 2008; Buchard et al. 2012). The consequence of the gene variant contributes to APAP induced liver hepatotoxicity since GSH is not conjugated to APAP for detoxification. However, there are two other GSH conjugation enzymes for NAPQI and APAP GSH conjugation. Thus far, other research is needed to explore whether there are other *GSTT1* variants which may influence APAP induced hepatotoxicity.

A variant of GSTP1, GSTP1 Ile/Ile, is thought to influence the risk of asthma (Romieu et al. 2006; Imode et al. 2008). In a review by Subrahmanyam et al.

(2018), the authors commented on a 1.8 times increased risk of asthma occurrence when infants are exposed to APAP, with the GSTP1 Ile/Ile variant. In other research it has been demonstrated that individuals with the Ile/Ile genotype are at a higher risk of asthma from pollutants (Schultz et al. 2010; Hwang et al. 2013; Al-Arifa and Jahan 2016). This suggests that the function of *GSTP1* with the Ile/Ile genotype is reduced.

Glutathione S-transferase Mu 1(*GSTM1*) is a third GST, conjugating GSH to APAP and NAPQI. Alike to *GSTT1*, *GSTM1* can also be deleted causing a potential predisposition to APAP induced liver failure (Lucena et al. 2008; Buchard et al. 2012). Allele frequency of *GSTM1* deletion was not identifiable. Clearly, further research is needed to find an association between potential *GSTM1* variants and APAP induced hepatotoxicity.

APAP is also conjugated to UDP-GA for detoxification by UGT1A protein. Both UGT1A1 and UGT1A6 can glucuronidate APAP. This has been reported in some studies (Mutlib et al 2006; Osabe et al. 2008; Liu et al. 2011; Giamarellos-Bourboulis et al. 2014; Mehboob et al. 2017). However, if an individual with Gilbert's syndrome self-administers APAP, glucuronidation of APAP is reduced (Esteban et al. 1999; Ito et al. 2002; Rauchschwalbe et al. 2004). Individuals with Gilbert's syndrome are unlikely to detoxify APAP effectively. As a result, those with Gilbert's syndrome are likely to develop liver damage.

3.2.4 Phase III transportation of acetaminophen

ABCC4 codes for multidrug resistance protein 4 (MRP4) whilst, *ABCC12* encodes for cystic fibrosis transmembrane regulator (CFTR) and multidrug resistance protein 9 (MRP9). A limited number of studies have reported induced expression of *ABCC4* and *ABCC12* induced by APAP (Zamek-Gliszczynski et al. 2006; Aleksunes et al. 2008; Khan et al. 2011; Schäfer et al. 2013; Lima et al. 2015). Which therefore draws the conclusion that MRP4 and MRP9 transports APAP for excretion from the cell. But, increased concentrations of APAP leading to APAP induced liver damage and failure, leads to the up-regulation of *ABCC4*. This is reported in some murine studies (Aleksunes et al.

2006; Campion et al. 2008) and a human liver sample study (Barnes et al. 2007). The increased *ABCC4* expression may also account for an increase in other potential toxic molecules that could be associated with APAP induced liver damage and failure.

Therefore, to date the only known pathological effect of which APAP induces is liver failure and damage (Fong et al. 2010; Stutchfield et al. 2015; Rubin et al. 2018). This is because APAP and the toxic metabolite, NAPQI, depletes GSH molecules in the liver. Consequently, NAPQI interacts with molecules in the liver (Mitchell et al 1973; Miner and Kissinger 1979; Dybing et al. 1983; Moore et al. 1985; Farber et al. 1988; Trenti et al. 1992), inducing liver necrosis and failure. There are also other contributing factors that could influence APAP induced hepatotoxicity such as gene variants that code for glutathione conjugating which yet, needs to be explored.

3.3 Hydrocortisone

3.3.1 Hydrocortisone overview

Cortisol is a glucocorticoid that can be used for medicinal application , referred to as hydrocortisone, that is commonly used as creams (Figure 3.9). The medicinal applications of hydrocortisone is to treat a number of skin conditions including: eczema (Korting et al. 1995) and dermatitis (Jorrizo et al. 1995). The mechanisms of action of hydrocortisone involves the binding of hydrocortisone molecules binding to glucocorticoid receptors (Ayrout et al. 2017), which leads to the formation of a receptor-ligand complex. This complex subsequently translocates to the cell nucleus and binds to a glucocorticoid response element (GRE) of the targeted gene(s) to regulate expression (Rainville et al. 2017). Additionally, hydrocortisone possess anti-inflammatory properties via the suppression of COX-1 and COX-2 preventing the synthesis of PGs (Kirkby et al. 2012; Dong et al. 2018). Thus, this demonstrates that hydrocortisone has a number of influences on molecular processes. Alike to other medicinal drugs discussed in this Chapter, hydrocortisone is also detoxified.

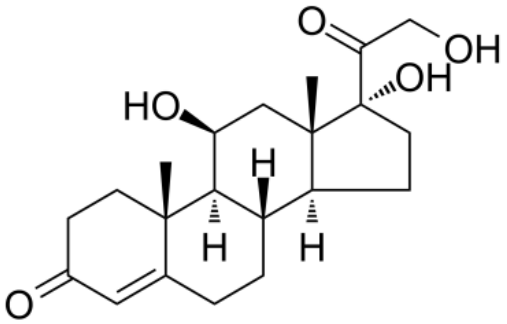


Figure 3.9: Molecular structure of Hydrocortisone.

Other applications of hydrocortisone include hormone therapy treatment for diseases. For instance, in Addison's disease cortisol production is reduced due to dysfunction of the adrenal glands (Kyriazopoulou et al.1984; Candrina and Giutina 1987). Thus, hydrocortisone is used to restore cortisol concentrations in a patient with Addison's disease (Baxter et al. 2013; Murray et al. 2016). Therefore, not only hydrocortisone can be used for dermatological conditions and allergies but, it can also be used for hormone replacement therapy for a disease.

3.3.2 Hydrocortisone and phase I and phase III detoxification

Hydrocortisone is a substrate of CYP3A4 in phase I of the detoxification pathway. The expression of *CYP3A4* is influenced by the concentration of hydrocortisone. The encoded protein by *CYP3A4* hydroxylates hydrocortisone to synthesise 6β-hydroxycortisol (Kuzikov et al. 2019), Figure 3.10. Once 6β-hydroxycortisol has been synthesised, the metabolite is subsequently effluxed out from the cell by MDR1 (encoded by *ABCB1*) (Kyle et al. 2018) and then eliminated from the body. Thus, to date it is not elucidated that 6β-hydroxycortisol requires phase II conjugation.

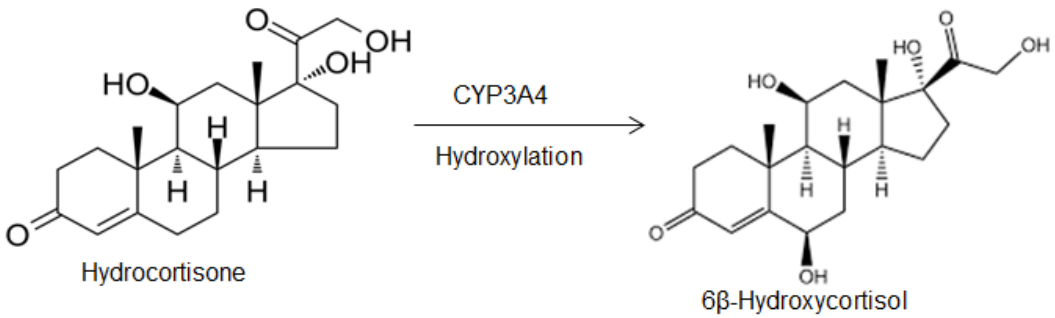


Figure 3.10: Phase I detoxification of hydrocortisone. CYP3A4 detoxifies hydrocortisone by a hydroxylation reaction.

3.3.3 The undesired effects of hydrocortisone

In despite of the medicinal applications of hydrocortisone there are considerations that should be taken into account. However the use of hydrocortisones could promote adverse effects including indirect effects of disease causation such as Type 2 Diabetes Mellitus (T2DM) (Kamba et al. 2016; Sue and Milanes 2019). As discussed in Section 2.3.1, hydrocortisone increase cortisol concentrations. Increased cortisol concentrations can lead to increased blood glucose concentrations (Vila et al. 2010; Kamba et al. 2016; Lakhani et al. 2017). Therefore, hydrocortisone acts as an indirect cause for the development of T2DM. Furthermore, individuals with T2DM should avoid the use of hydrocortisone since, hydrocortisone could increase the severity of T2DM by promoting a further increase in blood glucose concentrations.

Furthermore, it should be recognised that T2DM can also be influenced by a individual's lifestyle choice. It is evident that individuals who consume a high fat and glucose containing diet, accompanied with the lack of exercise is a contributing factor (Lindström et al. 2006; Nisar et al. 2009; Ganiyu et al. 2013; Cassidy et al. 2016; Barreira et al. 2018). Furthermore, T2DM can also influenced by genetics. In a study conducted by Eny et al. (2008), reported that a variant in *GLUT2*, Thr110Ile, has influential roles contributing to an increased consumption of a higher glucose intake. Furthermore, in a genome-wide DNA methylation study, multiple genes have been reported to have methylation including *CYP7A1* (Guo et al. 2019). The *CYP7A1* gene encodes for the CYP7A1 protein which is important for the metabolism of cholesterol (Pullinger et al. 2002; Chen et al. 2005; Gälman et al. 2008). The methylation of *CYP3A7*

could prevent the metabolism of cholesterol and therefore, cholesterol could accumulate in the human body contributing to obesity and in turn, T2DM. In despite of the presence of a gene variant and epigenetic regulatory mechanism(s), the variant and epigenetic mechanism(s) may not be the sole cause of the disease. Therefore, the prescribing of hydrocortisone to a patient should be done with caution. This is because if patients who are prescribed hydrocortisone with undiagnosed T2DM as a result of lifestyle or genetic influences, the severity of T2DM could be increased. Furthermore, T2DM is currently determined by determining blood glucose concentrations but associating glucuronides to T2DM could also be used. Thus far, only two research papers have reported glucuronides associated with T2DM (Appendix 2 and see Table 8.3).

3.4 Clotrimazole

3.4.1 Clotrimazole overview and cytochrome p450

Clotrimazole (Figure 3.11) is another dermatological cream based treatment for fungal based infections including: thrush (Reddy et al. 2017), ringworm (Trivedi et al. 2017) and athlete's foot (Abed and Hussein 2017). The mechanisms of action of clotrimazole suppresses the synthesis of ergosterol within fungal membranes, preventing fungal cell wall synthesis (Taylor et al. 1983; Haller 1985). The suppression of ergosterol synthesis is influenced by a dose dependent manner and thus, prevents the growth of a pathogenic fungus.

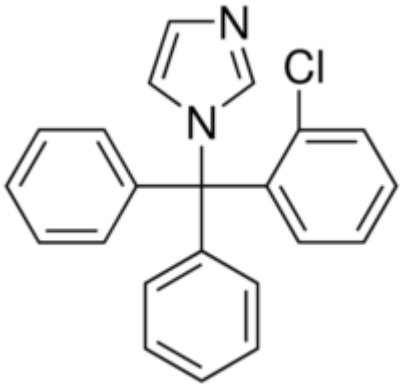


Figure 3.11: Molecular structure of clotrimazole.

To date, studies have reported that clotrimazole is able to suppress: *CYP2E1* (Zanger et al. 2000), *CYP1A2* (Zhang et al. 2002), *CYP2D6* (Zhang et al. 2002) and *CYP3A4* (Monostory et al. 2004). Conversely, clotrimazole has been reported to induce *CYP2B6* expression (Faucette et al. 2004). Therefore, *in vivo*, *the* suppression of the named CYP450s may allow drug-drug interactions to occur which could potentially be toxic to an individual who use clotrimazole and other medicinal drugs. However, the molecular interaction for phase I detoxification of clotrimazole by CYP2B6 is unclear.

3.4.2 Clotrimazole and phase II and phase III

Alongside with interfering with phase I detoxification, clotrimazole can also interfere with phase II conjugation of other molecules. In one study, Das et al. (1986), reported clotrimazole can interfere with glucuronidation and sulphation of polycyclic aromatic hydrocarbons (PAHs), using primary neonatal BALBlc mouse keratinocyte cell cultures. However, thus far, there is not further research exploring the effects of clotrimazole and phase II conjugation.

In humans an association of clotrimazole and ABC transporters are yet to be revealed. However, in a study using *Saccharomyces cerevisiae* revealed clotrimazole is a substrate of Pdr5p (Knorre et al. 2016). Pdr5p is a nucleotide triphosphate (NTP)-dependent transporter which relies on NTP for the efflux of molecules from the cell. Additionally, the Pdr5p transporter is studied for antifungal resistance (Reis de Sá et al. 2014). Clearly, further research is required to elucidate whether clotrimazole is transported in humans or whether, clotrimazole interferes with the transport of other molecules.

3.4.3 Clotrimazole and disease

Clotrimazole and other azole based anti-fungal treatment not only has antifungal properties, but they can induce toxicological reactions (Khoza et al. 2017; Bühler et al. 2019). Clotrimazole is thought to induce hepatotoxicity and clotrimazole induced hepatotoxicity. This can be tested by determining liver and blood plasma concentrations of aspartate aminotransferase (AST), and alanine aminotransferase (ALT) reported in a murine study (Khoza et al. 2017) and a

human case report study (Bühler et al. 2019). However, further research is recommend. However, monitoring a patient on a drug should be considered to monitor and identify potential changes in molecular biomarkers, which could indicate toxicity and other adverse effects.

3.4 Conclusion

In despite of the perception of medicinal drugs and their medicinal properties, medicinal drugs can influence other complications such as Type 2 Diabetes Mellitus by hydrocortisone. Therefore, it is recommended that patients with type 2 diabetes mellitus, should not use hydrocortisone and caution must be taken if a patient has undiagnosed type 2 diabetes mellitus. Additionally, medicinal drugs also change the fate of a molecule during detoxification. In despite of there has not been a disease associated with clotrimazole but, the anti-fungal treatment has capabilities of inducing hepatotoxicity. Similarly, acetaminophen depletes glutathione molecules in the liver, which leads to hepatotoxicity and this may lead to acetaminophen related deaths depending upon the severity of the damage in the liver caused by acetaminophen. The toxicity of irinotecan is influenced by a number of variants namely, *UGT1A1*28* (associated with neutropenia and severe diarrhoea) and *ABCG2* rs3109823 influences myelosuppression.

Chapter 4: The Metabolic Fate of Different Illicit Drugs

Unlike medicinal drugs, illicit drugs are used for recreational purposes, by which a illicit drug acts upon the central nervous system (CNS), altering the state of mind of the illicit drug user. Illicit drugs such as cocaine promotes brain stimulation by enhancing dopamine production by binding to dopamine receptors (Navarro et al. 2013; Dobbs et al. 2016; Wang et al. 2019), which can therefore lead to enhanced senses such as hallucinations and altered behavioural patterns. Furthermore, the use of heroin can lead to mental health disorders including: anxiety (Applebaum et al. 2010), depression (Cornford et al. 2012), bipolar (Maremmani et al. 2012), and schizophrenia (Chiappelli et al. 2017). This clearly demonstrates there are multiple adverse effects associated with illicit drugs. However, gene variants within the detoxification pathway can alter the fate of the drug (Bunten et al. 2010; Levran et al. 2013). The purpose of this chapter is to discuss the metabolomic fate of different illicit drugs.

4.1 Methadone

4.1.1 Methadone and phase I detoxification

Methadone, a narcotic, is an illicit synthetic opiate (Figure 4.1) but methadone has also been used for medicinal applications (Lossignol et al. 2013; Rasmussen et al. 2015; Mercadante and Bruera 2018). Methadone is used in treatment programmes to withdraw individuals from illicit opioid addictions (Amato et al. 2003; Ilgen et al. 2006). Additionally, methadone can be used as an alternative to morphine for pain treatment (Shelton et al. 2008; Verthein et al. 2014). Both methadone and morphine bind to the mu (μ)-opioid receptor (OPRM1), to elicit therapeutic effects. Methadone is administered as a racemic mixture consisting of (R-) and (S-) enantiomers (Bunten et al. 2011; Chen et al. 2014; Peng et al. 2018). However, the use of methadone can also lead to addiction and thus, methadone can be abused which may lead to methadone related fatalities.

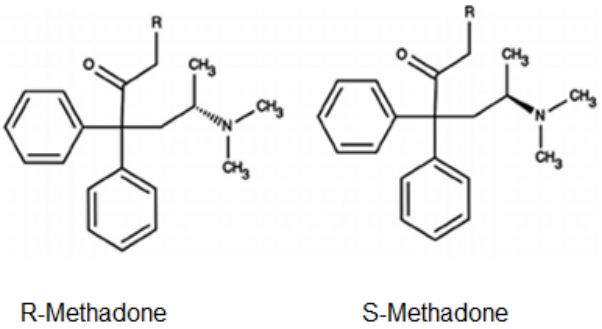


Figure 4.1: Molecular structure of methadone. The left presents the (R)-enantiomer whilst the right presents the (S)-enantiomer of methadone.

The (R)-enantiomer of methadone is responsible for eliciting therapeutic effects and conversely, the (S)-enantiomer is toxic (Lamont et al. 2006; Eap et al. 2007). The toxicity of the (S)-enantiomer (Eap et al. 2007; Lin et al. 2009), is due to inhibition of sodium/potassium (Na^+/K^+) pumps. The inhibition of Na^+/K^+ pumps causes prolonged QT intervals of a heartbeat. Consequently, *Torsades de pointes* (polymorphic ventricular tachycardia) occurs, demonstrated by rapid and irregular QRS complexes on an electrocardiogram (ECG) (Lamont et al. 2006; Viskin et al. 2010; Price et al. 2013; Nicotera et al. 2016). Thereby, individuals who have *Torsades de pointes* may have spontaneous ventricular fibrillation. Thus, CYP2B6 has a greater affinity towards the (S)-enantiomer.

CYP2B6 demethylates the (S)-enantiomer producing an inactive metabolite (Wang et al. 2010; Gadel et al. 2013; Zahari et al. 2016) (Figure 4.2). The inactive metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) is subsequently excreted in the urine (Larson and Richards 2009; Leimans et al. 2012; Holm and Linnet 2015; Musile et al. 2018). The fate of the (R)-enantiomer is not clear. Substrate affinities towards methadone of the non-polymorphic CYP2B6 are presented in Figure 4.3. The K_m represents substrate concentration when the velocity of a reaction is equal to a half the maximum velocity (V_{max}).

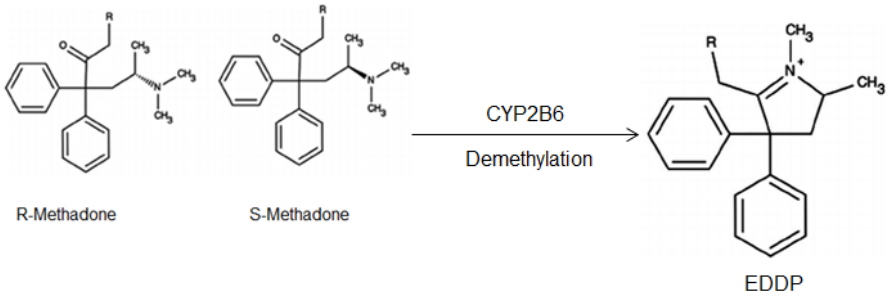


Figure 4.2: Demethylation of methadone by CYP2B6.

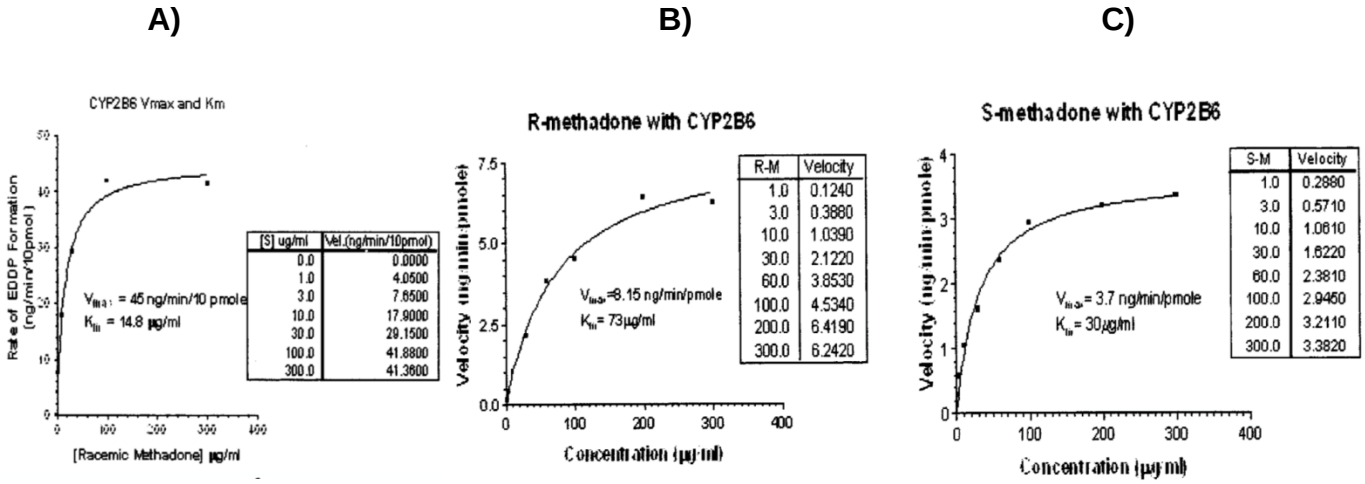


Figure 4.3: K_m and V_{max} of methadone in the presence of CYP2B6 (Adopted from: Gerber et al 2004). **A)** K_m and V_{max} of racemic methadone, K_m = 14.8 µg/ml, V_{max} = 45 ng/min/10 p mol. **B)** R-methadone, K_m = 73 µg/ml and V_{max} = 8.15 ng/min/p mol. **C)** S-methadone, K_m = 30 µg/ml and V_{max} = 3.7 ng/min/p mol. Used with the permission from Wiley publishers.

A polymorphism of the *CYP2B6* gene, *CYP2B6*6,* alters the fate of methadone due to reduced gene expression. As a result, the (S-) enantiomer of methadone is not detoxified effectively, classifying individuals with the polymorphism as poor metabolisers, as reported in some post-mortem analysis studies (Bunten et al. 2010; Bunten et al. 2011[a]; Bunten et al. 2011[b]; Gadel et al, 2013; Kharasch et al. 2016). The kinetic parameters of CYP2B6*6 also show protein function is reduced. The (R-) and (S-) enantiomers have an equilibrium dissociation (K_s) of 97 ± 7 and 40 ± 7, respectively. The V_{max} of (R-) and (S-) enantiomers have been reported to be 5.7 ± 0.3 and 3.5 ± 0.3, respectively. Conversely, the wild-type (WT) has a K_s of 75 ± 18 for the (R-) enantiomer and 25 ± 5 for the (S-) enantiomer. The V_{max} of (R-) enantiomer and (S-) enantiomer

are 20.8 ± 0.6 and 14.6 ± 1.0, respectively (Gadel et al. 2013). Thus, demonstrating kinetic parameters of CYP2B6*6 is reduced. Furthermore, the polymorphism is prevalent in the African and Caucasian population (Table 4.1).

Table 4.1: *CYP2B6*6* human allele frequencies in ethnic populations

Country or Ethnic background	Total number of people reported to have the variant	% reported that has not specified Homozygous or Heterozygous	P-value reported in the study	Reference
Japanese	884	17.6	0.77	(Jacob et al. 2004)
Koreans	88	15.9	P<0.01	(Klein et al. 2005)
Han Chinese	386	18.4	NS*	(Guan et al. 2004)
Mongolians	200	21.0	NS*	(Davaalkham et al. 2009)
African	418	28	P=0.05	(Peko et al. 2019)
Caucasian	270	28.2	NS*	(Jacob et al. 2004)
Europeans	NS*	3.4	NS*	(Zhou et al. 2017)

NS Not Specified*

In addition to CYP2B6, another CYP450 detoxifies methadone, CYP2D6. A transition mutation within *CYP2D6*, G1934A, introduces a premature stop codon causing protein truncation by a splicing defect (Gough et al. 1990; Saxena et al. 994; McLellan et al. 1997; Duzhak et al. 2000), causing a non-functional variant. This defect produces the *CYP2D6*4* (rs3892097) variant. Alike to the *CYP2B6*6* variant, individuals carrying this variant cannot detoxify methadone. Therefore, methadone related deaths may occur. *CYP2D6*4* affects approximately 20% of the European population (Table 4.2). Therefore, both *CYP2B6*6* and *CYP2D6*4* should be screened in post-mortem analysis to rule out methadone overdose.

Table 4.2: Human allele distribution of *CYP2D6*4*

Region	Country or ethnicity	% of homozygous (number of people)	% of heterozygous (number of people)	% of population affected if homozygous or heterozygous not specified	P-value reported in the study	Reference
	Brazilian	NS*	NS*	9.4 (191)	NS*	(Friedrich et al. 2014)
Europe	Germany	NS*	NS*	20.7 (589)	NS*	(Saches et al. 1999)
	Greek	3.18 (9)	78 (27)	Not applicable	NS*	(Arvanitidis et al. 2007)
	United Kingdom	NS*	NS*	24.2 (NS*)	NS*	(Daly 2015)
Asia	Han Chinese	NS*	NS*	1 (NS*)	NS*	(Zhou et al. 2009)
	Pakistan	4.5 (8)	12 (21)	NS*	NS*	(Anwarullah et al. 2017)
America	African-American	NS*	NS*	0.3 (NS*)	NS*	(Del Tredici et al. 2018)
	Hispanic	NS*	NS*	0.3 (NS*)	NS*	
	Caucasian	NS*	NS*	19.6 (NS*)	NS*	

**NS: Not Specified*

4.1.2 Methadone and phase II conjugation

To date, it is not elucidated in research whether methadone and the methadone metabolite EDDP can be conjugated to UDP-glucuronate (UDP-glucuronic acid, UDP-GA) for glucuronidation (Goldberger et al. 1998). But, methadone is able to suppress glucuronidation activities of UGT2B4 and UGT2B7. Raungrut et al. (2010) reported that in humans, methadone suppressed glucuronidation activity of UGT2B4. In a similar study by Gelston et al (2012), they conducted a study which explored the detoxification of codeine and buprenorphine in patients. The study reported that methadone suppressed glucuronidation activity of UGT2B4 and UGT2B7 for codeine and buprenorphine in humans. Therefore, if there is a non-functional variant of either *CYP2B6* or *CYP2D6*, methadone molecules could accumulate and suppress glucuronidation activities of UGT2B4 and UGT2B7. The consequent suppression of glucuronidation activities may allow other toxic molecules including methadone to accumulate and as a result, an individual may have toxicological reactions due to the presence or toxic molecules or a fatality may occur.

4.1.3 Methadone and phase III

In phase III of the detoxification pathway methadone is transported by the P-gp ABC transporter for elimination from the body via urination (George et al. 2000; Preston et al. 2003). *ABCB1* is expressed in various locations around the body including: brain, liver, kidneys, small intestine and colon. Multiple studies have reported that methadone is a substrate for MDR1 (Bouër et al. 1999; Wang et al. 2004; Ortega et al. 2007; Tournier et al. 2010; Hung et al. 2013; Mouly et al. 2015; Gibbs et al. 2018). However, methadone transport can be affected by other medicinal drugs such as quinidine, due to the suppression of MDR1 which increases the concentration of methadone molecules in the blood (Funao et al. 2003; Rautio et al. 2006; Pires et al. 2009).

Three polymorphisms have been highly studied associated with *ABCB1*. Three main polymorphisms affect *ABCB1* expression: rs1128503 (synonymous mutation, C1236T), rs2032582 (Ala893Thr, missense mutation, exon 21) and rs1045642 (C3435T, synonymous mutation). These three ABCB1 variants alter the fate of methadone.

4.1.3.1 rs1128503

rs1128503 (T1236C) is a synonymous mutation that is thought to have an influence on opioid dependence susceptibility (OMIM %610064). This is because individuals who have the rs1128503 polymorphism require an increased dose of methadone for heroin withdrawal. Individuals with the TT homozygous genotype require up to five times more methadone (150 mg/day) compared to individuals who have the CT heterozygous genotype and the non-variant (Levran et al. 2008; Levran et al. 2013). Therefore, rs1128503 may act as a predisposition to methadone related fatalities. Allele distributions have been presented in Table 4.3.

Table 4.3: Human allele distribution of rs1128503.

Region	Country or ethnicity	Number of individuals	% of homozygous	% of heterozygous	% if homozygous or heterozygous not specified	P-value reported in the study	Reference
Asia	Han Chinese	185	35.14	47.03	Not applicable	>0.05	(Yan et al. 2017)
	Indian	87	NS*	NS*	33	NS*	(Chowbay et al. 2013)
	Japan	48	60.4	39.6	Not applicable	0.393	(Komoto et al. 2006)
	Jordan	339	NS*	NS*	0.35	NS*	(Al-Diab et al. 2015)
Europe	Macedonia	107	21.5	44.9	Not applicable	NS*	(Naumovska et al. 2014)
	Finland	3377	NS*	NS*	5.1	NS*	(Lek et al. 2016)
	Germany	188	48.9	13.3	Not applicable	NS*	(Hoffmeyer et al. 2000)
Africa	Morocco	110	3(2)**	42 (55)**	Not applicable	NS*	(Senhaji et al. 2015)

NS: Not specified*
*(n)**: presents the individuals who have the polymorphism with Chron's disease*

4.1.3.2 rs2032582

rs2032582 is another variant that alters the activity of *ABCB1*. Two mutations can arise from rs2032582: G2667T (Ser893Ala) and G2667A (Ser893Thr). Ala893Thr has been observed to reduce transport activity (Rebecchi et al. 2009; van Assema et al. 2012; Fehér et al. 2014; Madadi et al. 2016). But it is not apparent how Ala893Ser alters transport activity. Both allele distributions have been presented in Table 4.4 (A and B).

Table 4.4 (A): Allele frequency distribution of ABCB1 rs2032582. Distribution of Ser893Ala (Adopted from: Karczewski et al. 2019).

Population	Allele Count	Allele Number	Number of Homozygotes	Allele Frequency
African	22863	24940	10496	0.9167
Ashkenazi Jewish	6469	10356	2018	0.6247
Other	3964	7192	1136	0.5512
Latino	19358	35316	5325	0.5481
European (non-Finnish)	70437	128632	19278	0.5476
East Asian	9492	19922	2278	0.4765
European (Finnish)	11752	25008	2775	0.4699
South Asian	10692	30604	1968	0.3494
Female	72854	129082	21641	0.5644
Male	82173	152888	23633	0.5375
Total	155027	281970	45274	0.5498

Table 4.4 (B): Allele frequency distribution of ABCB1 rs2032582. Distribution of Ser893Thr (Adopted from: Lek et al. 2016)

Population	Allele Count	Allele Number	Number of Homozygotes	Allele Frequency
South Asian	717	16508	18	0.04343
Other	27	908	1	0.02974
Latino	654	11462	15	0.05706
European (Non-Finnish)	1623	66622	24	0.02436
European (Finnish)	227	6598	3	0.0344
East Asian	1203	8638	91	0.1393
African	47	10398	0	0.00452
Total	4498	121134	152	0.03713

4.1.3.3 rs1045642

rs1045642 is a synonymous nucleotide mutation (C3435T). In a similar manner to rs1128503 and rs2032582, rs1045642 has been reported to contribute to opioid dependence, influencing methadone concentrations for opioid and heroin

withdrawal if the polymorphism is homozygous (Levran et al. 2013). Thus, implying the function of rs1045642 is increased. The molecular function of rs1045642 should be explored to reveal whether transport activity is affected. Table 3.5 presents the allele distributions of rs1045642.

Table 4.5: Human allele distribution of *ABCB1* rs1045642

Population	Country or ethnicity	Number of individuals	% of homozygous	% of heterozygous	Reference
Europe	UK (Caucasian)	190	24	48	(Ameyaw et al. 2001)
	Germany (Caucasian)	188	28	48	(Hoffmeyer et al. 2000)
	Portugal	92**	56 (35)***	73 (46)***	(Santos et al. 2014)
	Finland	9692	38.60	NS*	(Karczewski et al. 2019)
	European-American	152**	19 (32.58) ***	26 (44.8)***	(Pang et al. 2014)
Asia	Saudi Arabia	96	26	38	(Ameyaw et al. 2001)
	China	132	26	42	(Ameyaw et al. 2001)
	Han Chinese	185	40	44.32	(Yan et al. 2017)
Africa	Ghana	206	0.0	3.4	(Ameyaw et al. 2001)
	Kenya	80	4	26	(Ameyaw et al. 2001)
	Sudan	96	26	38	(Ameyaw et al. 2001)
	African-American	99**	13 (13.1)***	39 (39.4)***	(Santos et al. 2011)

NS: Not specified*
***: Number of patients in study*
****: Patients who have been identified with the polymorphism. (n) = % of those with the polymorphism.*

Furthermore in phase III, other drugs are able to alter the fate of methadone (Kharasch et al. 2004). Quinidine is a medicinal drug, (Figure 4.4), that is synthesised to aid in the treatment for heart arrhythmias (Zakrzewska-Koperska et al. 2018) and atrial fibrillation (Mazzanti et al. 2019). Quinidine is a substrate of MDR1, by which quinidine suppresses transport activity of MDR1 (Funao et al. 2003; Rautio et al. 2006; Pires et al. 2009; Syvänen et al. 2012; Bui et al. 2015; Morsy et al. 2018). Thus, when quinidine is taken by a methadone user, plasma concentrations of methadone increase (Kharasch et al. 2004; Kharasch et al. 2009; Mercer and Coop 2011; Meissner et al. 2014; Feng et al. 2017),

which could contribute to methadone related deaths. Therefore, in post-mortems that are associated with methadone related deaths, quinidine and quinidine metabolites should be screened.

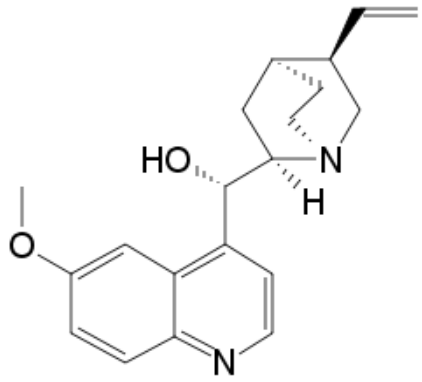


Figure 4.4: Molecular structure of quinidine.

4.2 Cocaine

4.2.1 Cocaine overview and phase I detoxification

Cocaine is a recreational drug (Figure 4.5), which stimulates the central nervous system (CNS) and inhibits the reuptake of serotonin, noradrenaline and dopamine (Tomasi et al. 2010; Kosten et al. 2013; Smith et al. 2015; Siciliano et al. 2018). This is one of the molecular characteristics of cocaine addiction. Consequently, prolonged exposure and use of cocaine can lead to psychotic reactions (auditory and visual hallucinations and paranoia) (Mitchell and Vierkant 1991; Cuomo et al. 1994; Bartlett et al. 1997). In phase I detoxification, cocaine is detoxified by CYP3A4 (Pasanen et al. 1995; De Rienzo et al. 2000; Ramírez et al. 2004; Vitcheva and Mitcheva 2007; Gallelli et al. 2017), by a demethylation reaction. Alternatively, cocaine can be detoxified by a esterification reaction by carboxylesterase (CE) (Figure 4.6).

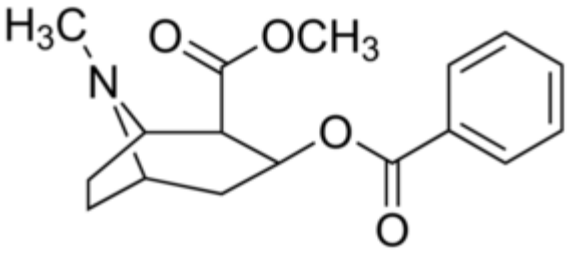


Figure 4.5 Molecular structure of cocaine.

If cocaine is detoxified by esterification, the major metabolite benzoylecgonine (inactive metabolite), is produced. Alternatively, if cocaine is demethylated by CYP3A4, the minor active metabolite norcocaine is synthesised (Kloss et al. 1983; Plessinger and Woods 1990; Pellinen et al. 1994; Smirnow and Logan 1996; Arinç and Bozcaarmutlu 2003; Vitcheva and Mitcheva 2007), (Figure 4.6). Norcocaine is an active toxic metabolite which can elicit toxicological reactions in the liver leading to liver necrosis and failure (Thompson et al. 1979; Kloss et al. 1982).

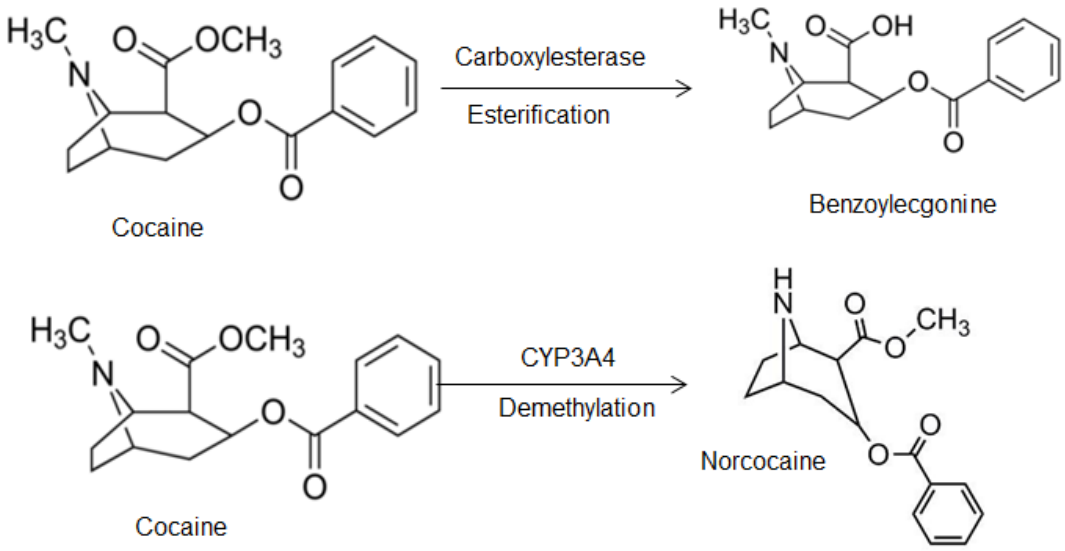


Figure 4.6: Chemical reaction processing of cocaine. The esterification reaction is the major reaction for cocaine producing benzoylecgonine. Whilst the demethylation reaction by CYP3A4 is the minor reaction.

4.2.2 Cocaine and phase II conjugation

After phase I preparation, like methadone, cocaine does not require glucuronidation. This may be due to the resultant major metabolite benzoylecgonine of the esterification reaction by carboxylesterase, being inactive and non-toxic. Yao et al. (2013) characterised cocaine molecule metabolites and glucuronides in murines that are not named. Thus, demonstrating that cocaine could be conjugated to UDP-GA but requires further exploration. Furthermore, research to date has not revealed whether norcocaine is glucuronidated.

4.2.3 Cocaine and phase III transport

Cocaine is also thought to be a substrate of MDR1 however, this is based on limited knowledge (Raje et al. 2003; Tournier et al. 2010; Isaza et al. 2013; Salvatore et al. 2015). Additionally, MDR1 is thought to have an influence in cocaine addiction (Candiotti et al. 2013; Salavatore et al. 2015), but evidently further research is required. One variant of MDR1 is thought to influence cocaine addiction, rs1045642, due to reduced gene expression (Candiotti et al. 2013; Sychev et al. 2018). Reduced expression may account for an accumulation in cocaine molecules in the brain, inhibiting dopamine receptors. As a result, dopamine production is reduced which could contribute to addiction (Candiotti et al. 2013; Sychev et al. 2018).

Cocaine is also a substrate of a proton-antiporter flux transporter system in the blood brain barrier (BBB) (Andrè et al. 2009; Chapy et al. 2014; Chapy et al. 2015; Auvity et al. 2017). However, the molecular mechanisms of the transport system are not clear. Clearly, a further elucidation on the molecular mechanisms of the transport system is recommended.

4.3 Ketamine

4.3.1 Ketamine and cytochrome p450

Ketamine (Figure 4.7) is used as an illicit drug (recreational) and a medicinal drug (anaesthetic) (Akeju et al. 2016; Carspecken et al. 2018). Like other illicit

drugs discussed above, ketamine acts upon the CNS (Garcia et al. 2008; Kittelberger et al. 2012; Tang et al. 2015; Glasgow et al. 2018). The effects of ketamine include hallucinations (Hunt et al. 2006), loss of coordination (Hunt et al. 2006) and nausea (Heinz et al. 2006). Long-term exposure of ketamine can lead to cognitive impairment (Morgan et al. 2006; Nikiforuk et al. 2010; Liang et al. 2013). When ketamine is administered, *CYP3A4*, *CYP2B6* and *CYP2C9* are induced. CYP3A4 has been shown to be the major CYP450 for phase I preparation by demethylation (Figure 4.7). CYP2B6 and CYP2C9 have been shown to have less activity towards ketamine (Hijazi and Boulieu 2002; Negrusz et al. 2005; Legrand et al. 2008; Mössner et al. 2011; Li et al. 2013; Lin et al. 2015; Zheng et al. 2017). The demethylation of ketamine by CYP3A4 synthesises norketamine (NK).

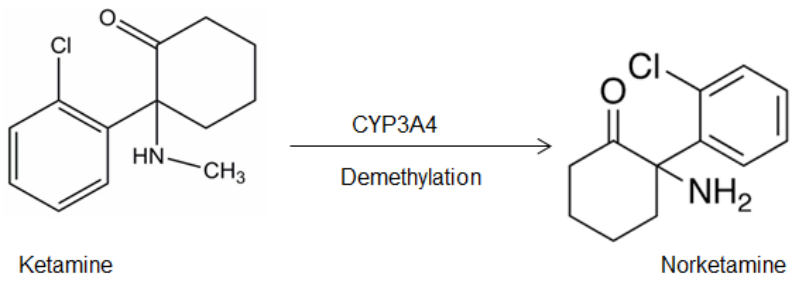


Figure 4.7: Phase I detoxification of ketamine.

NK is an active metabolite that inhibits the N-methyl-D-aspartate receptor (NMDA receptor) (Ebert et al. 1997; Graven-Nielsen et al. 2000). NMDA receptors are important for synaptic plasticity and memory. If NK binds to these receptors, receptor function is suppressed (Ebart et al. 1997; Liu et al. 2001; Negrusz et al. 2005; Stone et al. 2008; Olofsen et al. 2012; Sałat et al. 2015). Consequently, memory impairment may occur, influenced by the amount and frequency of ketamine use.

Additionally, NK could be converted to 6-OH-norketamine (6-OH-NK) by CYP2B6 by hydroxylation (Figure4.8), and subsequently removed from the body. NK hydroxylation is also affected by *CYP2B6*6* (Li et al. 2013; Li et al. 2015), due to reduced function of *CYP2B6*6*. Therefore, polymorphism

screening in post-mortem analysis could detect polymorphism contribution to death.

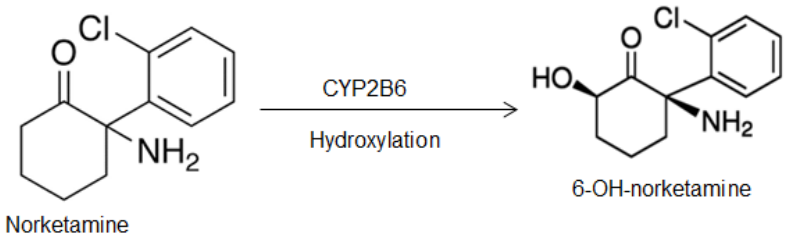


Figure 4.8: The conversion of norketamine to 6-OH-norketamine

4.3.2 The fate of ketamine in Phase II and phase III metabolism

Ketamine molecules do not go through glucuronidation. It is not currently known whether glucuronidation of norketamine occurs. A metabolite of ketamine, 3-OH-norketamine (3-OH-NK) has been observed to undergo glucuronidation (Turfus et al. 2009). Therefore, *in vivo* studies are recommended to reveal whether 3-OH-NK is conjugated to UDP-GA for glucuronidation.

In phase III ketamine has been associated with two ABC transporters, ABCB1 and ABCG2 (Ganguly et al. 2018). Thus, further elucidation is necessary to explore the association of *ABCB1* and *ABCG2.* Ketamine may act as an inhibitor or inducer for the two transporters.

4.4 3,4-methylenedioxymethamphetamine

4.4.1 3,4-methylenedioxymethamphetamine and cytochrome P450

3,4-Methylenedioxymethamphetamine (MDMA) is another illicit drug (Figure 4.9) with stimulatory effects, leading to increased production of serotonin (Klomp et al. 2012), dopamine (Hondebrink et al. 2012) and noradrenaline (Hysek et al. 2012). When MDMA is taken, enhanced visual hallucinations (Lamers et al. 2003) and increased emotions and empathy to other individuals can occur (Curran et al. 2004). Long term exposure to MDMA may lead to

psychosis, hypertension, seizures and hyperthermia. Like cocaine, MDMA is demethylated by CYP3A4. Additionally, CYP2D6 has also been shown to demethylate MDMA (Meyer et al. 2008; de la Torre et al. 2012; Hysek et al. 2014; Steur et al. 2016; Rodgers et al. 2018). These reactions produce a number of metabolites: 3,4-methylenedioxyamphetamine (MDA) from detoxification by CYP3A4 (Figure 4.9), 3,4-dihydroxymethamphetamine (HHMA, toxic) and 4-hydroxy-3-methoxymethamphetamine (HMMA, inactive), from CYP2D6 (Abraham et al. 2009; Antolino-Lobo et al. 2011; Desrosiers et al. 2013; Steuer et al. 2016), Figure 4.9. These are the known metabolites produced in phase I detoxification of MDMA.

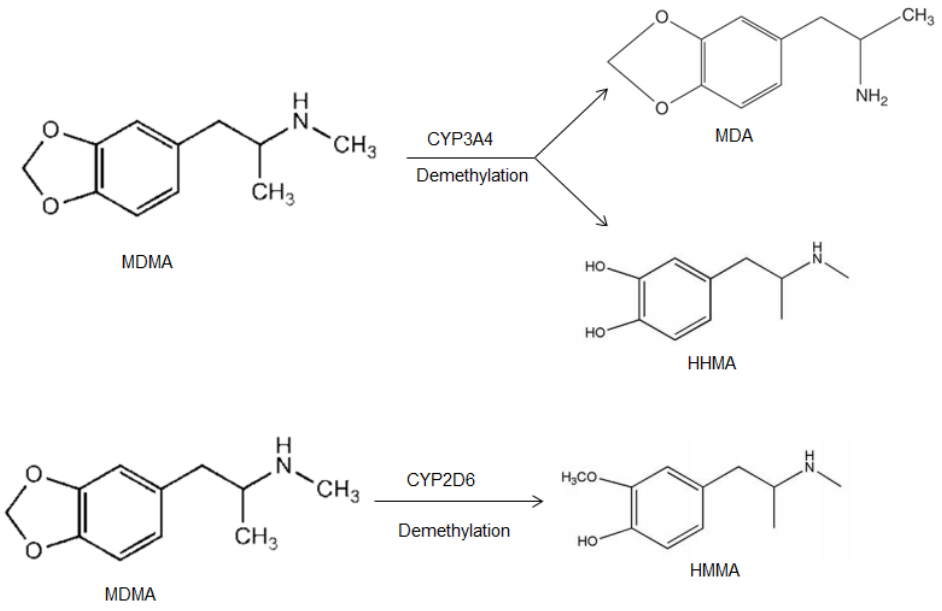


Figure 4.9: Phase I detoxification of 3,4-Methylenedioxymethamphetamine.

*CYP2D6*4* is also associated in altering the fate of MDMA, in a similar manner to methadone. A post-mortem analysis study was conducted, involving MDMA related deaths. The study reported that *CYP2D6*4* may also influence MDMA related deaths (Gilhooly et al. 2002), as a result of reduced expression of the *CYP2D6*4* variant. Therefore, screening for the polymorphism should occur during post-mortems where MDMA is implicated and thought to be the cause of death.

4.4.2 3,4-methylenedioxymethamphetamine phase II and phase III metabolism

MDMA molecules are not conjugated to UDP-GA for glucuronidation. However, the MDMA metabolite HMMA goes through glucuronidation. Glucuronidation of HMMA increases the solubility and reduces toxicity of the molecule. The increase in solubility is one of the general effects of glucuronidation (Silva et al. 2003; van Dorp et al. 2009; Zhang et al. 2014; Taylor et al. 2017). HMMA glucuronidation is thought to occur by UGT2B15 (Shoda et al. 2009; Schwaninger et al. 2011 [a]) (Figure 4.10), but further research is recommended. Alternatively, HMMA can be sulphated by SULT1A3 and SULT1E3 (Schwaninger et al. 2011 [b]). Both SULT1A3 and SULT1E3 synthesise HMMA-sulphate which can subsequently be excreted.

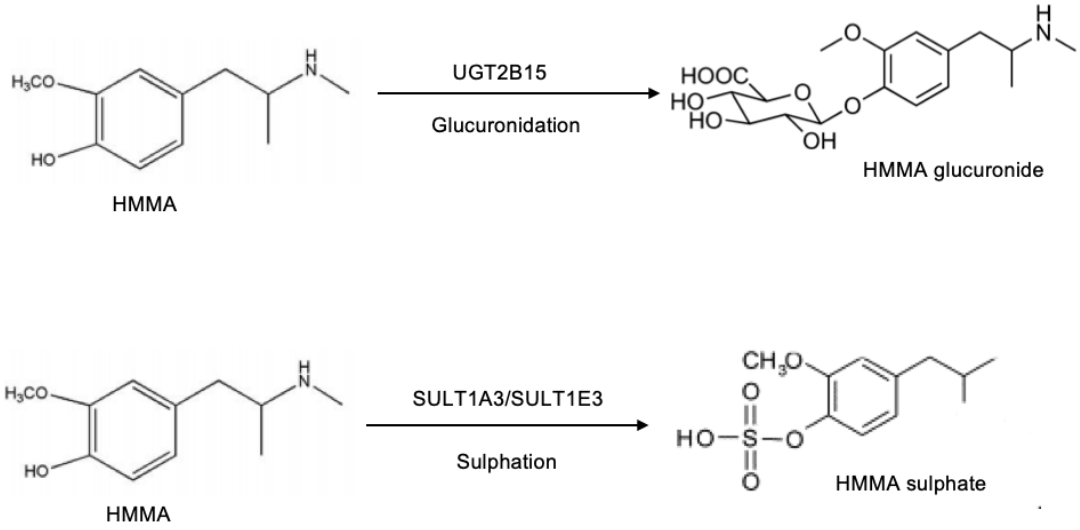


Figure 4.10 Phase II detoxification by conjugation of HMMA.

MDMA is thought to be a substrate for ABCB1 (Ketabi-Kiyanvash et al. 2003; Tournier et al. 2010). Thus far, it is clear that there is very limited knowledge on MDMA and phase III transportation. Therefore, it is recommended that further studies should explore other ABC transporters that may transport MDMA. Additionally, exploring how variants of a ABC transporter alter the fate of MDMA in phase III.

4.5 Conclusion

Illicit drugs are taken for recreational uses altering the behaviour of an individual by acting upon the central nervous system and receptors within the brain. But the detoxification of the drug molecules are influenced by gene variants, which could contribute to the severity of the adverse effects within an illicit drug user. Furthermore, the presence of non-functional variants of CYP2B6 and CYP2D6 can lead to drug related deaths. Thus, in treatment programmes such as heroin withdrawal using methadone, a patient should be screened for non-functional variants which could pose a risk to the patient.

Chapter 5: The Metabolomic Fate of Different Food additives

Food additives are used in foods to maintain food quality, enhance the taste and appearance of food (Wang 2000; Prat-Larquemin et al. 2000; Es-Safi et al. 2000). However, the use of food additives can promote toxic effects to an individual and thus precautions are required before the consumption of food. Below discusses the implications of gene variants on different food additives.

5.1 Aspartame

5.1.1 Aspartame overview and phase I detoxification

Aspartame is an artificial non-saccharide sweetener synthesised from phenylalanine (Phe, F) and aspartic acid (Asp, D) (Figure 5.1), that is used as an alternative to sugar (Blackburn et al. 1997; Wang and Schroeder 2000; Ilbäck et al. 2003). The acceptable daily intake (ADI) of aspartame is approximately 40 mg/kg of body-weight (bw)/day. However, aspartame is highly toxic to individuals with phenylketonuria (PKU) (OMIM #261600). PKU is a result of a mutation in phenylalanine hydroxylase (Gámez et al. 2000; Muntau et al. 2002; Hennermann et al. 2005; Burton et al. 2007; Hoeksma et al. 2009; Sanayama et al. 2011; Rossi et al. 2014; Bell et al. 2017), causing an excess toxic accumulation of phenylalanine. The consumption of aspartame by individuals with PKU can result in seizures and cognitive impairment. However, PKU can be managed by a low phenylalanine diet.

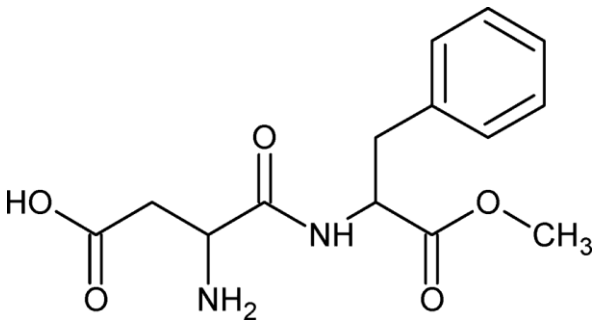


Figure 5.1: Molecular structure of aspartame.

The association of with CYP450s to PKU remains unclear. In despite of this, CYP2E1 and CYP3A2 have been associated with increased expression in murine models, in the presence of phenylalanine (Labra-Ruiz et al. 2007; Nosti-Palacios et al. 2014; Onaolapoet al. 2016). Conversely, in individuals with PKU,

CYP2E1 expression has been postulated to be reduced (Mozrzymas et al. 2017). Thus, further elucidation of the association of PKU and *CYP2E1* expression in humans are recommended.

5.1.2 Aspartame and phase II and phase III detoxification

Aspartame does not require glucuronidation. Since aspartame is catabolised to phenylalanine by phenylalanine hydroxylase, it is not necessary for aspartame to go through the detoxification pathway. The phenylalanine produced is used as a molecule to synthesise the neurotransmitters dopamine (Mosnik et al. 1997) and serotonin (Downing et al. 1997). Therefore aspartame does not require phase II detoxification.

Upon aspartame metabolism to phenylalanine, L-type amino acid transporters (LATs) transport phenylalanine into the brain (Uchino et al. 2002; Møller et al. 2005; Omidi et al. 2008). LATs are solute carrier (SLC) transporters, requiring a sodium (Na^+) concentration gradient to transport amino acids (Hidalgo and Borchardt 1990; Munck and Munck 1994; Boado et al. 1999). Additionally, LATs transport other amino acids including: leucine (Uchino et al. 2002), isoleucine (Babu et al. 2003), and threonine (Fernández et al. 2003). Phenylalanine can be used as a precursor molecule for neurotransmitter synthesis, such as dopamine.

However, in PKU an accumulation of phenylalanine is toxic, which can lead to seizures (Harding et al. 2004; Martynyuk et al. 2007). Furthermore, the presence of increased phenylalanine can reduce transport of other amino acids such as tryptophan (Pietz et al. 1999; Matalon et al. 2006; Mazzola et al. 2012; Sanayama et al. 2014). Tryptophan is also a precursor molecule for dopamine and serotonin synthesis. In PKU, dopamine and serotonin synthesis is reduced in the presence of increased concentrations of phenylalanine (Figure 5.2), which can lead to seizures.

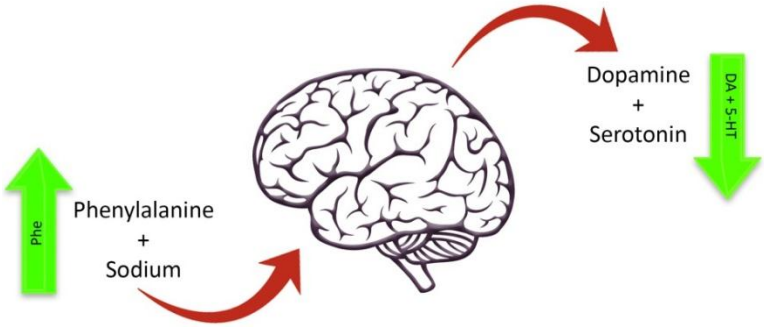


Figure 5.2: Phenylalanine transport consequence in phenylketonuria. In phenylketonuria, increased concentrations of phenylalanine (Phe) are transported into the brain by LAT1. Consequently, dopamine (DA) and serotonin (5-HT) synthesis is reduced. As a result, this can contribute to the onset of seizures.

Phenylalanine is a substrate of the broad neutral (^{0}) amino acid transporter 1 (B0AT1), coded by *SLC6A19* (Bröer et al. 2011; Belanger et al. 208). Like to other solute carrier transporters they can be expressed in the kidneys and the intestines, using a Na^+ concentration gradient (Kleta et al. 2004; Pinho et al. 2007; Fairweather et al. 2009; Bröer et al. 2011; Jiang et al. 2015; Javed et al. 2018). Phenylalanine transported from the kidneys is eliminated from the body by urination.

5.2 Anthocyanins

5.2.1 The properties of anthocyanins and phase I detoxification

Anthocyanins (ACs) (Figure 5.3) are pigments producing red to dark red-purple colourings in plants (Khoo et al. 2017). The aglycone of ACs, anthocyanidins, can be used as food colouring extracts assigned with an E number. E163 (food colouring additive) denotes the use of anthrocyanidin (Tennant and Klingberg 2016). The recommended ADI for ACs as a food additive is approximately 2.5 mg/kg bw/day in adults and 0.7 to 1.9 mg/kg bw/day for children.

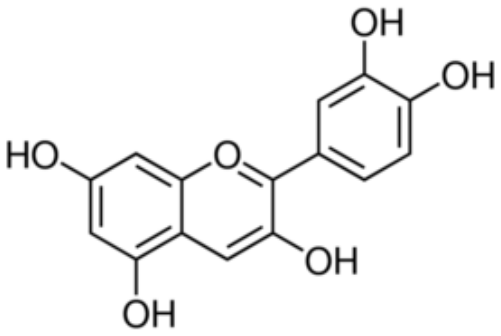


Figure 5.3: Molecular structure of an anthocyanin. Presents the molecular structure of cyanidin, a type of anthocyanin.

In vitro studies reported that ACs to have anti-cancerous properties. Zhao et al. (2014) reported AC-containing extracts from chokeberries, grapes and bilberries inhibited HCT-116 (human colon carcinoma) cancer cell line growth. A later *in vitro* study Bowen-Forbes et al. (2010), used chokeberries along with other fruits including *R. acuminatus*, *R. racemosus, R. jamaicensis and R. idaeus*, and several cancer cell lines; MCF-7 (breast), SF-268 (CNS), NCI-H460 (lung), HCT-116 (colon) and AGS (gastric) to determine AC effects on cancer. The study reported ACs from *R. acuminatus* shown the highest inhibition of cancer cell growth. Thi and Hwang (2018) tested AC properties on a SK-Hep1 (human hepatic adenocarcinoma) cell line, demonstrating the inhibition of cancer cell formation by ACs from chokeberries in a dose dependent manner. Speculatively, the addition of berries to the diet of cancer patients could supress cancer cell growth, thereby reducing tumour size.

Additionally, ACs possesses antioxidant properties (Xia et al. 2006; Mauray et al. 2010). Reactive oxygen species (ROS) including peroxides (H_2O_2) (Kallur et al. 2000), hydroxyls (-OH) (Ding et al. 2000) or singlet oxygen ($^1O_2$) (Karmat et al. 2000), can be produced by different pathways and reactions (St-Pierre et al. 2006; Cedarbaum 2010; Quinlan et al. 2013; Hu et al. 2016; Zhenyukh et al. 2018). Significantly high concentrations of ROS can lead to diseases such as prostate cancer (Bostwick et al. 2000) and cardiac disease (Tsutsui et al. 2001). ACs show more specificity to –OH radicals, due to the short life span (Ou et al. 2012; Huang et al. 2016). Furthermore, ACs could stimulate gastro-intestinal digestion, demonstrated by *in vitro* studies. ACs from Mulberries and purple

fleshed potatoes, were shown to promote gastro-intestinal digestion (Liang et al. 2011; Kubow et al. 2017). Thus, this demonstrates the benefits of berry and vegetable consumption.

ACs has been reported to possess anti-inflammatory properties, acting in a similar manner to nonsteroidal anti-inflammatory drugs (NSAIDs) (Taverniti et al. 2014; Kuntz et al. 2015). NSAIDs suppress COX-1 and COX-2 (Griffin et al. 2013; Carron et al. 2016), suppressing PG synthesis. Conversely, NSAIDs do not inhibit Nuclear–Factor B (NF-κB) due to limited drug efficacy (Viegas et al. 2011; Orlando et al. 2014; Nivanappa et al. 2016), whereas ACs have been shown to target NF-κB, reducing expression (Taverniti et al. 2014; Kuntz et al. 2015). Furthemore, a 2008 study demonstrated a mild inhibitory effect of ACs on *CYP3A4* expression and reported by two other studies (Dreiseitel et al. 2008; Srovnalova et al. 2014; Malhi et al. 2015). Whether other CYP450 expression is affected by ACs is not clear, leading to the need of further research.

5.2.2 Phase II and phase III detoxification of anthocyanins

Consumed ACs are able to enter glucuronidation (Wu et al. 2002; Felgines et al. 2003; Kay et al. 2004). Consumption of ACs below the ADI has a low probability of toxic effects. It has been demonstrated that ACs may go through glucuronidation in some studies (Milbury et al. 2007; Woodward et al. 2011; de Ferrars et al. 2014; Mueller et al. 2017), Figure 5.4. The specific UGT for AC glucuronidation are not yet known, thus requiring further research.

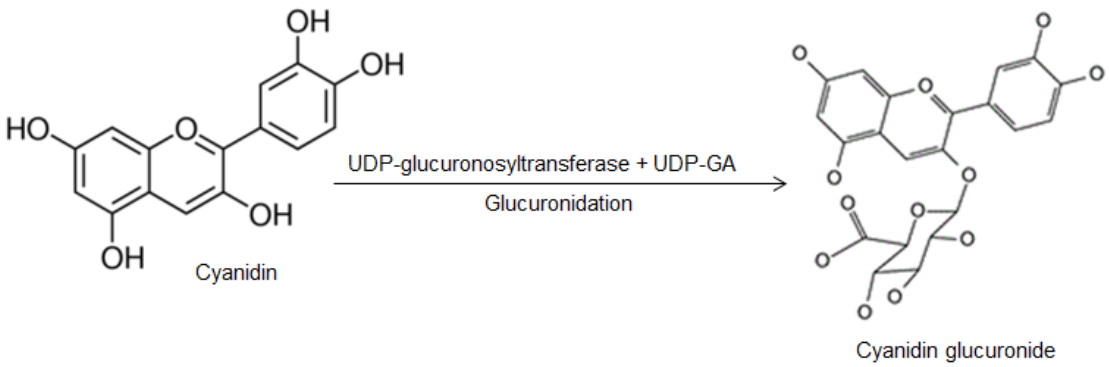


Figure 5.4: Phase II glucuronidation of cyanidin.

Organic anion polypeptide transporters (OATPs) are expressed on the BBB (Gao et al. 1999), liver (Kullak-Ublick et al. 2001) and intestine (Kobayashi et al. 2003). Anthocyanins are substrates of OATP1B1 and OATP1B3 (Riha et al. 2015) Anthocyanins have been reported by Riha et al. (2015) to increase OATP1B1 mRNA synthesis by approximately 60%. Conversely, OATP1B1 mRNA synthesis is reduced by 25% approximately. This demonstrates that anthocyanins are potential inhibitors for OATP1B3 and induce OATP1B1 mRNA production. Thus, further research is necessary to determine effects of these induced alterations on other molecules.

5.3 Butylated hydroxyanisole and butylated hydroxytoluene

5.3.1 Butylated hydroxyanisole and butylated hydroxytoluene and cytochrome p450

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Figure 5.5) are used as food preservatives because of their antioxidant activities (Adamson et al. 1977; Ito et al. 1983; Ito et al. 1986; Goodman et al. 1990; Whysner et al. 1994; Yu et al. 1997). The ADI recommended for consumption is 1.0 mg/kg bw/day for BHA, and 0-0.125 mg/kg bw/day for BHT. Potentially, consuming BHA and BHT may lead to toxic effects studies (Williams et al. 1991; Grillo and Dulout 1995; Grillo and Dulout 1995).

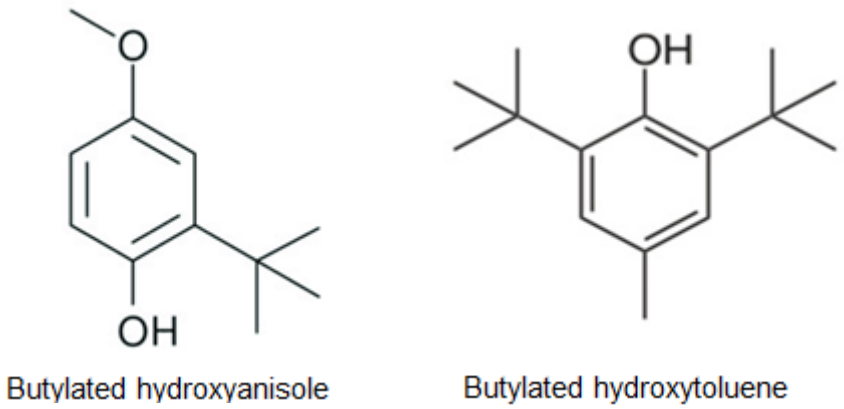


Figure 5.5: The molecular structure of butylated hydroxyanisole and butylated hydroxytoluene.

The potential toxicological effects of BHA and BHT have been explored in multiple animal and cell line studies (Williams et al. 1991; Grillo and Dulout 1995; Grillo and Dulout 1995). Consumption of BHT over the recommended ADI may produce toxicological effects, including tumorigeneses (Williams et al. 1991), genotoxic effects (chromosomal and DNA damage) (Grillo and Dulout 1995), and heart rate suppression (Kaplan et al. 2003). Speculatively, if BHT is consumed over the ADI, these observed effects could occur in humans, contributing to the potential development of disease.

The *CYP2J2* gene is expressed in various tissues including the heart (Wu et al. 1996), lungs (Zeldin et al. 1996), liver (Wu et al. 1996) and intestine (Zeldin et al. 1997). CYP2J2 primarily oxidises arachidonic acids (polyunsaturated acids found in phospholipids), but CYP2J2 can also detoxify BHT by an oxidative or hydroxylation reaction (Figure 5.6) (Yu et al. 1997; Thum and Borlak 2008; Lee and Murray 2010; Evangelista et al. 2013; McDougle et al. 2017). If BHT is detoxified by a hydroxylation reaction, hydroxy-tert-butyl (BHT-OH) is synthesised. Additionally, BHA has been identified to induce *CYP2J2* expression (Lee and Murray 2010). However, it is not extensively studied. This could be due to the ADI of BHA being higher than BHT and thus, requiring excess consumption for potential toxic effects to occur.

Two main metabolites of BHT have been extensively studied, BHT-OH (Figure 5.6) and BHT-QM (Figure 5.7). Both metabolites have the capabilities of inducing toxicological effects (DNA damage by cleavage), demonstrated in a number of studies (Nagai et al. 1993; Lewis et al. 1996; Oikawa et al. 1998). This genotoxic toxic effect induces cellular inflammation and subsequently, promotes the onset of mutagenesis and potential tumorigenesis.

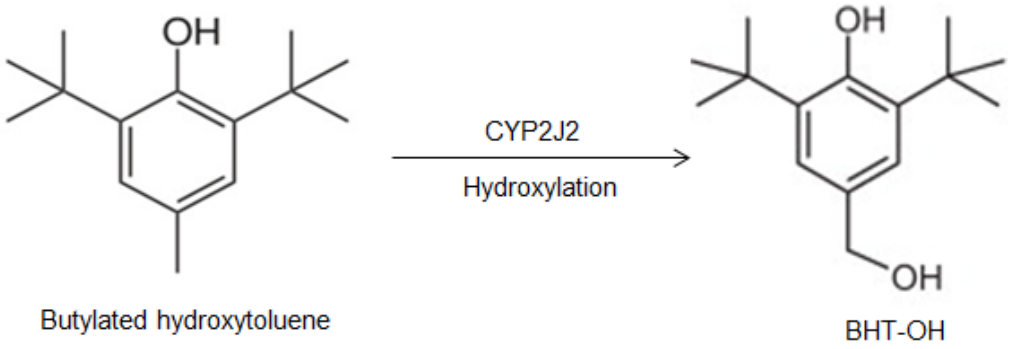


Figure 5.6: Phase I detoxification of butylated hydroxytoluene.

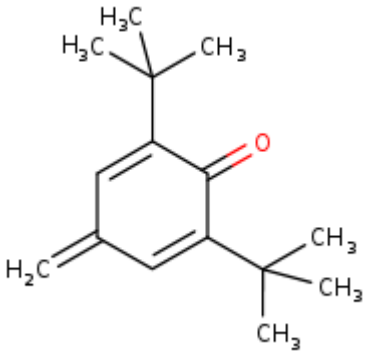


Figure 5.7: Molecular structure of BHT-QM (Adopted from: U.S. National Library of Medicine 2019).

5.3.2 Butylated hydroxyanisole and butylated hydroxytoluene and phase II metabolism

Both BHT and BHA can be conjugated to UDP-glucuronate (UDP-glucuronic acid, UDP-GA) for detoxification. Alike to anthocyanins, the specific glucuronidation enzyme is not known. Additionally, BHT has been reported to reduce glucuronidation of APAP (Boindogurong et al. 2006; Tamura 2011), whilst BHA has been demonstrated to increase APAP glucuronidation (Hazelton et al. 1985; Gregus and Klaassen 1985). Evidently, further research is necessary to understand the role of BHT and BHA in glucuronidation of other xenobiotics, excluding APAP. Further exploration of the effects of BHA and BHT on glucuronidation of endogenous molecules is necessary.

5.3.3 Butylated hydroxyanisole and butylated hydroxytoluene and phase III metabolism

BHA and BHT are not substrates of any ABC transporters. BHA has been revealed to be a substrate of OATP1A1 and OATP2B1 (Cheng et al. 2005). OATP1As are coded by solute carrier family 22 member 6 (*SLC22A6*). OATP1As are expressed in the adult kidneys on the basolateral surface of the proximal tubes and on the choroid plexus in the brain (Lopez-Nieto et al. 1997; Pritchard et al. 1999; Ohtsuki et al. 2002). *OATP2B1* is expressed within the liver (Kullak-Ublick et al. 200) and the intestines (Kobayashi et al. 2003). The transportation of BHT and BHA into the cell for phase I detoxification and phase II conjugation requires a Na^+ gradient. A phase III transporter for detoxification has not been unearthed in the research available.

Nrf2 is a basic leucine zipper (bZIP) protein transcription factor, regulating the expression of antioxidant protein coding genes such as *PRDX6* (Chowdhury et al. 2009) and *SOD1* (Woods et al. 2009). The presence of BHA and BHT stimulate ROS production by causing oxidative stress. In turn, the presence of ROS activates Nrf2 (Yuan et al. 2006; Jin et al. 2008; Lee and Murray 2010; Abdullah et al. 2012; Luo et al. 2015; Zheng et al. 2019). The activation of Nrf2 reduces *OATP1A1* mRNA production and conversely increases *OATP2B1* mRNA production (Cheng et al. 2005; Reisman et al. 2009; Aleksunes et al. 2012). Thus, substrates of OATP1P1 transporters may have an altered fate. But, substrates of OATP1P1 which are also substrates of OATP2B1 may still be transported. Therefore, further study is recommended to explore the impacts of BHT and BHA on the transportation of molecules.

5.4 Conclusion

Clearly, the consumption of food additives can promote toxicological effects. If aspartame is consumed by a person with phenylketonuria, seizures and other complications may arise. Phenylketonuria is a result of a genetic mutation that reduces enzymatic activity of phenylalanine hydroxylase causing an accumulation of phenylalanine within the human body. Furthermore, the full effects of anthocyanins and the impacts anthocyanins have on the detoxification pathway remain for elucidation. Conversely, butylated hydroxyanisole and butylated hydroxytoluene could be genotoxic leading to the potential of disease development along with suppressing glucuronidation activity of other molecules but, further exploration is needed. Thus, the consumption of food additives should be reduced as much as possible to avoid potential toxicological effects.

Chapter 6: The Metabolomic Fate of Different Endogenous Molecules

Endogenous molecules could be synthesised from degradation of other molecules such as bilirubin (Lad et al. 2004; Jansen et al. 2010) (Section 6.1), hormones (Lin et al. 2006; Tsutsui et al. 2008) (Section 6.2 and 6.3) or neurotransmitters. However, abnormal concentrations of endogenous molecules can influence the development of abnormal physiological conditions, leading to disease development (Huang et al. 2013). Thus, this chapter discusses the detoxification of endogenous molecules and how alterations to genes within the detoxification can contribute to the onset of disease.

6.1 Bilirubin

6.1.1 Bilirubin synthesis and cytochrome p450

Bilirubin, an insoluble molecule, is the end product of heme degradation (Lad et al. 2004; Jansen et al. 2010). Bilirubin synthesis requires two reactions (Figure 6.1). Heme is firstly oxidised by heme oxygenase (HO), synthesising biliverdin (Lad et al. 2004; Reed et al. 2010; Lin et al. 2013; Lee et al. 2017). Subsequently, biliverdin is reduced to bilirubin by NADPH-dependent biliverdin reductase (BVR) (Jansen et al. 2010; Subhanova et al. 2013; van Dijk et al. 2017). CYP1A1 or CYP1A2 is able to oxidise bilirubin as a degradation process, producing bilirubin oxidation products (Figure 6.2). Alternatively bilirubin can go through glucuronidation (Figure 6.3).

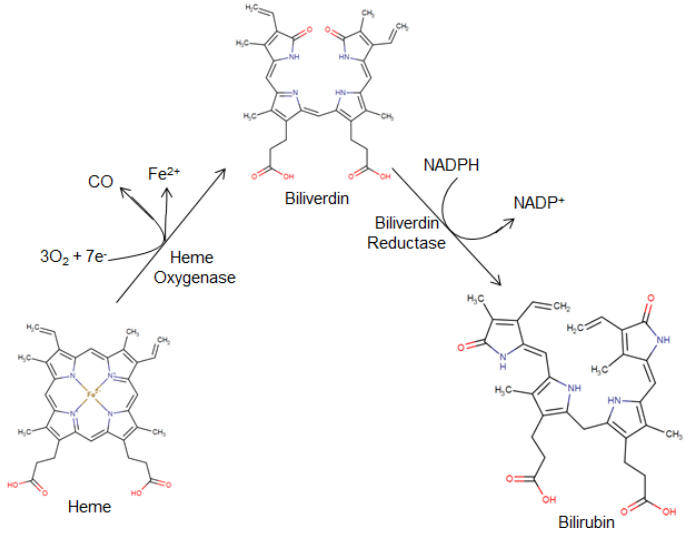


Figure 6.1: Enzymatic pathway for bilirubin synthesis. Bilirubin production occurs from heme degradation. Heme is first oxidised to biliverdin by heme oxygenase. Sequentially, biliverdin reductase reduces biliverdin to bilirubin. Heme HMBD ID: HMDB0003178. Biliverdin HMBD ID: HMDB0001008. Bilirubin HMBD ID: HMDB0000054.

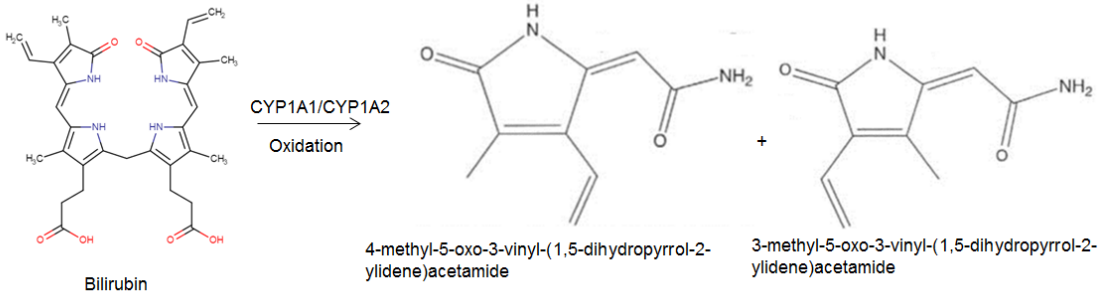


Figure 6.2: Oxidation of bilirubin to bilirubin oxidised products. The oxidisation reaction produces 4-methyl-5-oxo-3-vinyl-(1,5-dihydropyrrol-2-ylidene)acetamide and 3-methyl-5-oxo-3-vinyl-(1,5-dihydropyrrol-2-ylidene)acteamide. The oxidised molecular structures are adopted from (Wurster et al. 2008). Bilirubin HMBD ID: HMDB0000054.

6.1.2 Bilirubin and phase II glucuronidation

Bilirubin is also glucuronidated and detoxified by the process of glucuronidation, via the conjugation of urine diphosphate-glucuronic acid (UDP-GA) to the -COOH functional group by UGT1A1 (Figure 6.3). Bilirubin detoxification is affected by the *UGT1A1*28* polymorphism, a genetic predisposition for Gilbert's syndrome (Bosma et al. 1995; Ando et al. 1998). Gilbert's syndrome is characterised by an accumulation of unconjugated bilirubin in the liver, leading to jaundice. Under normal physiological conditions, bilirubin concentrations can be up to 1mg/dL. However, individuals with Gilbert's syndrome may have

unconjugated bilirubin concentrations between 1mg/dL and 6mg/dL (Huang et al. 2013; Flores – Villalba et al. 2016). However, Gilbert's syndrome should not be confused with Rotor syndrome (Section 6.1.3).

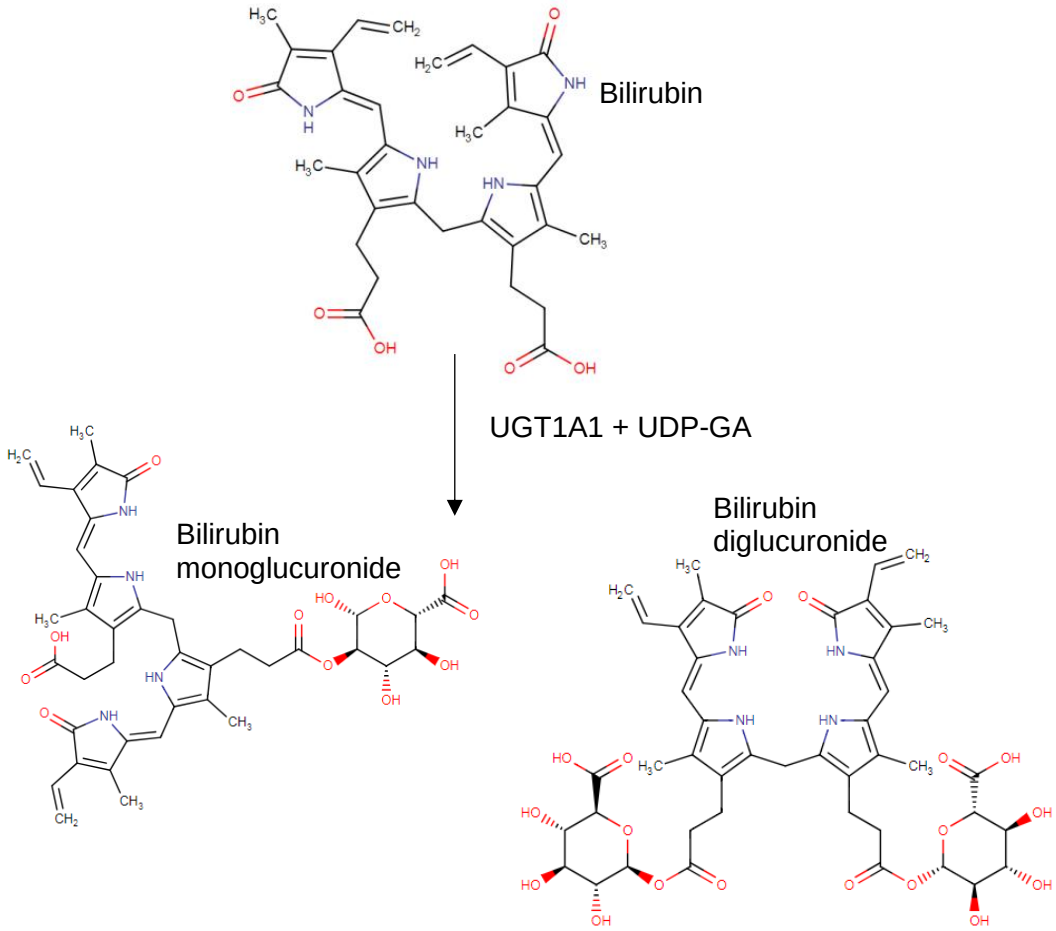


Figure 6.3: Bilirubin glucuronidation by UGT1A1. Once bilirubin is synthesised from heme degradation, bilirubin is subsequently glucuronidated. Bilirubin glucuronidation can either produce a monoglucuronide or a diglucuronide. Bilirubin HMDB: HMDB0000054; Bilirubin monoglucuronide HMDB accession number: HMDB0010332; Bilirubin diglucuronide HMDB accession number: HMDB0003325. Molecules in red present functional groups.

UGT1A6 is a second glucuronidation enzyme for the detoxification of bilirubin. However, the detoxification of bilirubin in the presence of UGT1A16 is 1/10 slower compared to UGT1A1 (Ciotti et al. 1998). Thus, *UGT1A6* may act as a compensator if *UGT1A1* is defective.

6.1.3 Bilirubin and phase III transport

OATP1B1 can transport endogenous molecules such as bilirubin (Passamonti et al. 2005; He et al. 2008; van de Steeg et al. 2012). Bilirubin is transported into liver cells for detoxification. Upon bilirubin glucuronide synthesis (Figure 6.4) MRP1 transports bilirubin glucuronides from the cell for subsequent elimination. Additionally, bilirubin is also a substrate for OAT1B3 (König et al. 2000; Hirano et al. 2004; Yamaguchi et al. 2006; Kindla et al. 2011; Zimmerman et al. 2013; Thomson et al. 2016; Alam et al. 2018). Mutations that affect bilirubin transport acts as a predisposition for diseases.

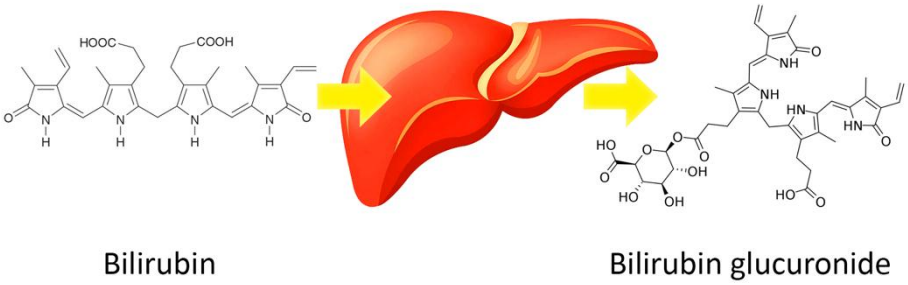


Figure 6.4: Bilirubin detoxification pathway. Bilirubin molecules are transported into liver cells by OAT1B1 or OAT1B3. In the cell CYP1A1 or CYP1A2 biotransform bilirubin for glucuronidation by UGT1A1. Upon bilirubin glucuronide synthesis, MRP1 excretes bilirubin glucuronides from the cell for subsequent elimination.

One mutation of the *OATP1B1* alters the fate of bilirubin. rs71581941 introduces a premature stop codon at amino acid position 580, a arginine to a stop codon (Arg580Ter). The consequence of this is a truncation of the transporter, producing a half TMD of TMD11 and TMD12, reducing the transport of bilirubin (Kim et al. 2007; van de Steeg et al. 2012; Kagawa et al. 2015; Yang et al. 2017). This mutation induces Rotor syndrome or Rotor type hyperbilirubinemia (OMIM #237450), causing jaundice. Rotor syndrome should not be confused with Gilbert's syndrome. In Rotor syndrome the molecule coproporphyrin (decomposition of porphyrin molecules), is detectable in urine. Additionally, measuring the concentrations of both unconjugated bilirubin and bilirubin glucuronide should also be considered to rule out Gilbert's syndrome.

The prevalence and allelic distribution of rs71581941 has is disproportionately associated with the Asian population (Table 6.1). A literature search was undertaken on allelic distributions. However, only one research article was found which primarily focusing on white Canadians (n=41), and reporting 1.4 % of Canadians and 0.8% of white Europeans with the variant (Boivin et al. 2010). An alternative search was conducted using two bioinformatics allele frequency databases on gnomAD and ExAC. ExAC was the only database which provided allele distribution, presented in Table 6.1.

Table 6.1: Human allele distribution of OATP1B1 rs71581941 (Adopted from: Lek et al. 2016)

Population	Allele Count	Allele Number	Number of Homozygotes	Allele Frequency
East Asian	41	8646	1	0.004742
Other	3	902	0	0.003326
Latino	32	11404	0	0.002806
European (Non-Finnish)	92	66476	0	0.001384
European (Finnish)	9	6574	1	0.001369
African	10	10326	0	0.0009684
South Asian	11	16480	0	0.0006675
Total	198	120808	2	0.001639

Additionally, a mutation in *OATP1B3* also contributes to the development of Rotor syndrome. The mutation in *OATP1B3* introduces a change at the amino acid position 253, changing arginine to a stop codon (Arg253Ter). The mutation causes an accumulation of bilirubin, which can also contribute to the development of Rotor syndrome (van de Steeg et al. 2012). The allelic frequency distribution has not been provided, possibly due to a lack of

knowledge to date on the association between Arg253Ter and Rotor syndrome, revealing a need for further research.

MRP1 is not limited to transporting xenobiotics, medicinal and illicit drugs, but MRP1 can transport endogenous molecules such as bilirubin glucuronides (Figure 6.4). Bilirubin glucuronide transportation by MRP1 has been evidenced (Corich et al. 2009; Gazzin et al. 2011; Bigo et al. 2014; Wang et al. 2017). Bilirubin is a substrate of other ABC transporters *ABCC2* (MRP2) (Toh et al. 1999; Fouassier et al. 2002) and *ABCC3* (MR3) (Bodó et al. 2003; Zelcar et al. 2006). MRP2 and MRP3 could be compensator transporters if MRP1 is defective.

6.2 Testosterone

6.2.1 Testosterone synthesis

Testosterone (Figure 6.5) is an androgen, synthesised in the Leydig cells (Mendelson et al. 1975; Chung and Allison 1979). Testosterone is synthesised from cholesterol, and requires several intermediate molecules, presented in Figure 6.5.

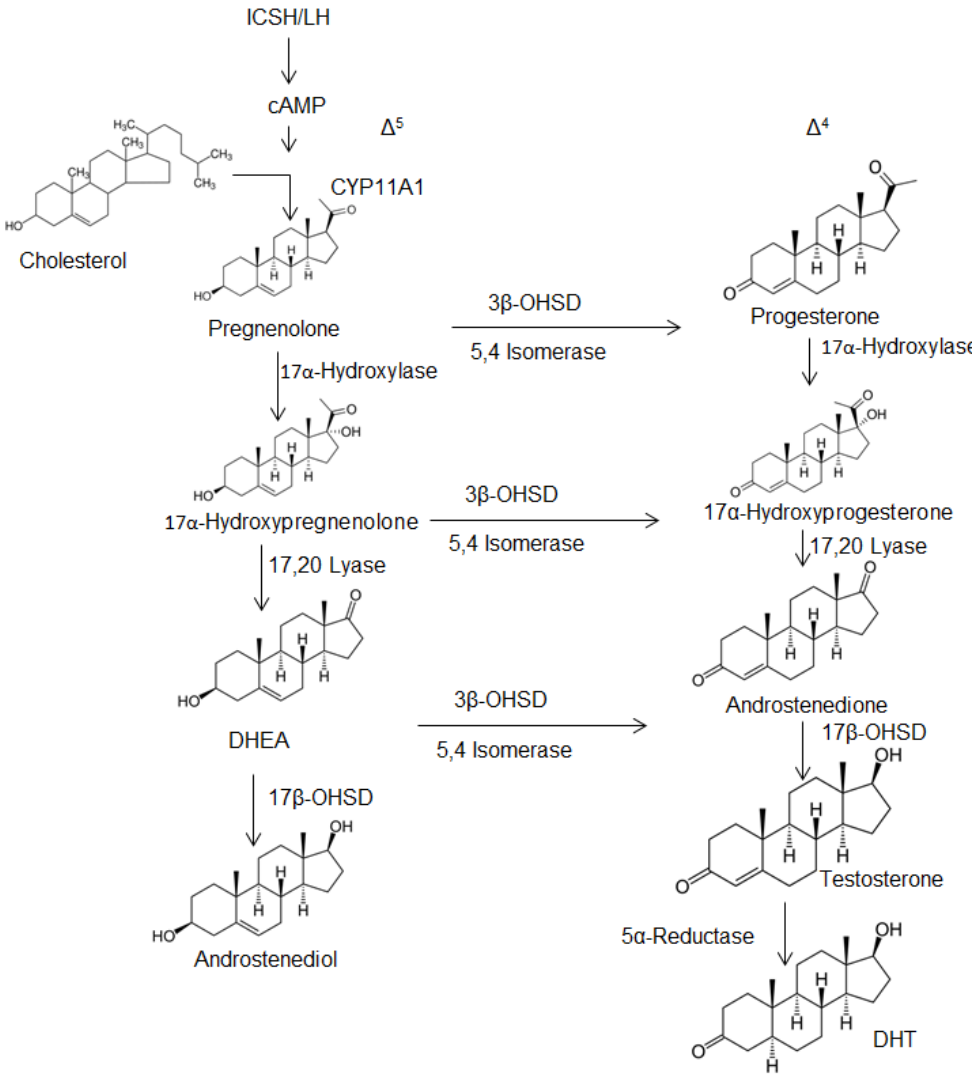


Figure 6.5: Testosterone synthesis pathway. In the Δ^5 pathway, cholesterol is cleaved to pregnenolone by CYP11A1 and converted to androstendiol by other biochemical reactions. Alternatively, Pregnenolone, 17α-hydroxypregnenolone or DHEA can be converted to progesterone, 17α-hydroxyprogesterone or adrenstendione, respectively within the Δ^4 pathway and synthesised to testosterone and DHT.

6.2.2 Testosterone and cytochrome P450

Following synthesis, testosterone is secreted from the leydig cells into the peripheral circulatory system. Testosterone secretion is stimulated by the pituitary gland in response to gonadotropin-releasing hormone (GnRH) (Mongkonpunya et al. 1975; Happ et al. 1978). CYP3A4 hydroxylates testosterone, producing the metabolite 6-β-hydroxy testosterone (6β-OH-testosterone) (Figure 6.6) (Lin et al. 2006; Sivertsson et al. 2010; Patil et al. 2014; El-Sayed et al. 2016). Additionally, CYP3A5 has been reported to hydroxylate testosterone (Maezawa et al. 2010; Maguire et al. 2012; Badavi et

al. 2015; Smith et al. 2018). CYP3A4 is the major CYP450 for testosterone hydroxylation and CYP3A5 could act as a compensator for CYP3A4.

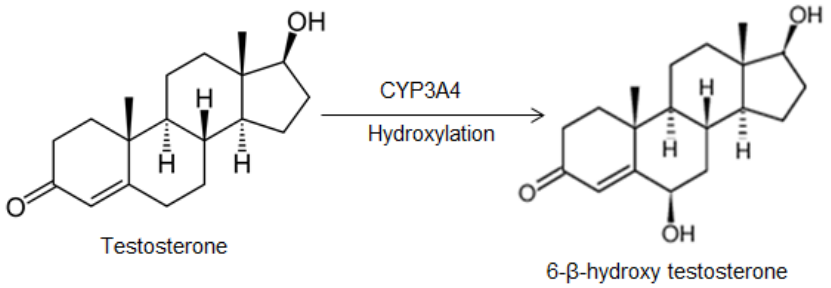


Figure 6.6: Phase I detoxification of testosterone.

A *CYP3A5* polymorphism (*CYP3A5*3*) affects phase I preparation of testosterone. *CYP3A5*3* is a result of a nucleotide mutation, G22893A, generating a cryptic splice site forming exon 3B. Consequently, the generation of exon 3B introduces a premature stop codon after amino acid position 102 (Kuehl et al. 2001; Fröhlich et al. 2004; Canonico et al. 2008). The premature stop codon produces a non-functional variant, causing protein truncation. Therefore, phase I preparation by *CYP3A5*3* promotes an accumulation of testosterone that has not been hydroxylated for phase II conjugation. Sequentially, the accumulated testosterone may promote abnormal cellular growth leading to tumour development, leading to prostate cancer (Stanbrough et al. 2006; Pierorazio et al. 2010).

Early diagnosis of prostate cancer increases survival. Generally, prostate cancer affects middle aged men and older however, younger men can also be affected. The risk of developing prostate cancer is approximately 2 to 4 times higher in black men (Jones and Chinegwundoh 2014; Machiori et al. 2016). Conversely, white men have a lower risk of developing prostate cancer. The reason(s) behind this remain unclear.

6.2.3 Testosterone and phase II

Testosterone is another molecule that requires glucuronidation, catalysed by UGT2B17 (Ekström et al. 2012; Zhu et al. 2015; Martín-Escudero et al. 2019), Figure 6.7. Testosterone glucuronidation prevents an excess accumulation of active testosterone. There are other UGTs which have the capabilities of testosterone glucuronidation such as UGT2A7 (Sten et al. 2009; Jenkinson et al. 2012; Wang et al. 2014; Basit et al. 2018). Alternatively, UGT2B15 can glucuronidate testosterone. Thus, UGT2B15 and UGT2A7 may act as compensators for UGT2B17.

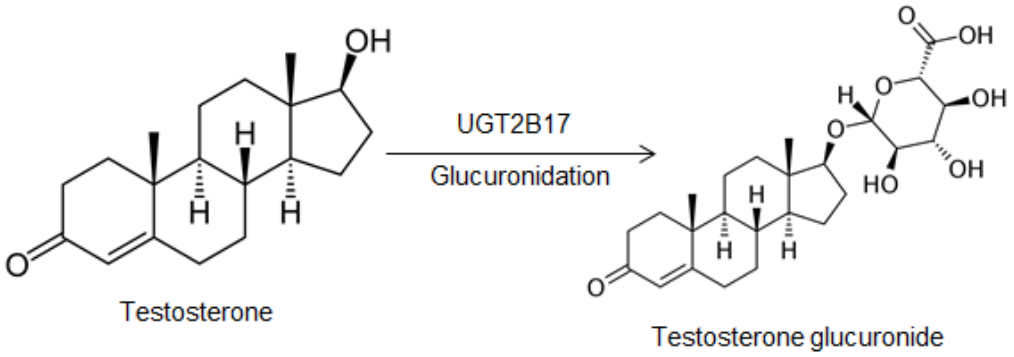


Figure 6.7: Phase II detoxification of testosterone

The *UGT2B17* gene can be deleted, causing problems with glucuronidation of testosterone. If the gene deletion is heterozygous, there could be reduced glucuronidation activity towards testosterone. Studies have reported that *UGT2B17* deletion is a potential genetic predisposition for prostate cancer (Gallagher et al. 2007; Bai et al. 2010; Kpoghomou et al. 2013; Urashima et al. 2015). However, as discussed above there are potential gene compensators which may reduce the risk of prostate cancer (Sten et al. 2009; Jenkinson et al. 2012).

*UGT2B15*2* (rs1902023) polymorphic variant (Asp85Tyr; exon 1), upregulates gene expression. Upregulated expression of *UGT2B15*2* may also act as a predisposition for prostate cancer (Ahern et al. 2011; Strahm et al. 2013; Romero-Lorca et al. 2015). This is due to increased glucuronidation activity, reducing the amount of biologically active testosterone. Thus, reduced presence of testosterone may contribute to the development of prostate cancer. To date,

four different studies have associated glucuronides with prostate cancer associated with genetics (Appendix 2 and Table 8.5) and only one study has reported a glucuronide associated with prostate cancer and lifestyle (Section 8.3.4.2).

In all prostate cancer a phase II coding gene for a glutathione conjugating enzyme, *GSTP1*, is hypermethylated (Millar et al. 1999; Song et al. 2002; Rosenbaum et al. 2005). Multiple studies have reported that the *GSTP1* gene is hypermethylated (Song et al. 2002; Enokida et al. 2005; Bryzgunova et al. 2008; Delgardo-Cruzata et al. 2012; Zhang et al. 2015; Hendriks et al. 2018). Clearly, this is one of the most extensively studied epigenetic mechanism of prostate cancer. Thus, the methylated *GSTP1* could also be a potential biomarker for the identification of prostate cancer. Furthermore, the methylation of *GSTP1* prevents gene expression for GSTP1 protein and therefore, toxic molecules could accumulate within the prostate which may contribute to the development and severity of prostate cancer.

Alternatively, testosterone can be sulphated which also aids in the elimination of testosterone from the body. Testosterone can be sulphated by SULT1A1 (Sparks et al. 2004) and SULT2A1 (Schulze et al. 2013), as an alternative phase II conjugation process. Upon the synthesis of testosterone sulphate metabolites, the inactive sulphate metabolite are subsequently eliminated via urination (Gonzalo-Lumbreras et al. 2003; Saudan et al. 2006; Piper et al. 2010).

5.2.4 Testosterone and Phase III transport

Testosterone in phase III is secreted from the testes, and prevented from penetrating the blood-testis barrier (BTB) by two transporters, MRP1 and MRP4 (Qian et al. 2001; Bart et al. 2004; Sivils et al. 2010; Dankers et al. 2013; Morgan et al. 2015). MRP1 and MRP4 actively transport testosterone from the leydig cells after synthesis. However, the link between these transporters and testicular cancer is not clear. Altered expression of *ABCC1* (MRP1) and *ABCC4* (MRP4) is present in prostate cancer (Sullivan et al. 2000; Cai et al. 2006).

In prostate cancer *ABCC1* and *ABCC4* are highly expressed (Zalcberg et al. 2000; Sullivan et al. 2000; Cai et al. 2006). The increased expression impedes drug-based cancer treatment. It is unclear whether over-expression is influenced by a mutation, or whether it is a natural molecular characteristic in prostate cancer. Nevertheless, studies have reported that p53 is able to regulate *ABCC1* (Thottassery et al. 1997; Sullivan et al. 2000), by binding to the promotor region of the gene. This implies that expression of *ABCC1* alters expression which could potentially contribute to *ABCC1* expression. Therefore, contributing to drug-based chemo-resistance.

6.3 Oestrogen

6.3.1 Oestrogen synthesis

Oestrogen is the primary sex hormone in females (Bowen et al. 2011). Oestrogen is an umbrella term for three specific hormones: oestradiol (Figure 6.8) and oestrone (Figure 6.8) and oestriol (Figure 6.9). Oestrone and oestradiol have functional roles in the reproductive menstrual cycle, with oestrone influencing the onset of the cycle (Sipavičiené et al. 2015), and oestradiol regulating ovulation (Wang et al. 2018). Oestriol has been observed to keep the uterus in a quiescent (resting) state to prevent early labour from occurring (Cohen 1985). Oestrogen is synthesised from cholesterol cleaved by CYP11A1 (CYP450scc), producing pregnenolone (Figure 6.8) (Tsutsui et al. 2008; Mast et al. 2011; Fan and Papadopoulos 2013). Once pregnenolone is synthesised, several intermediate molecules can be produced by different biochemical reactions until oestrogen is synthesised (Figure 6.8).

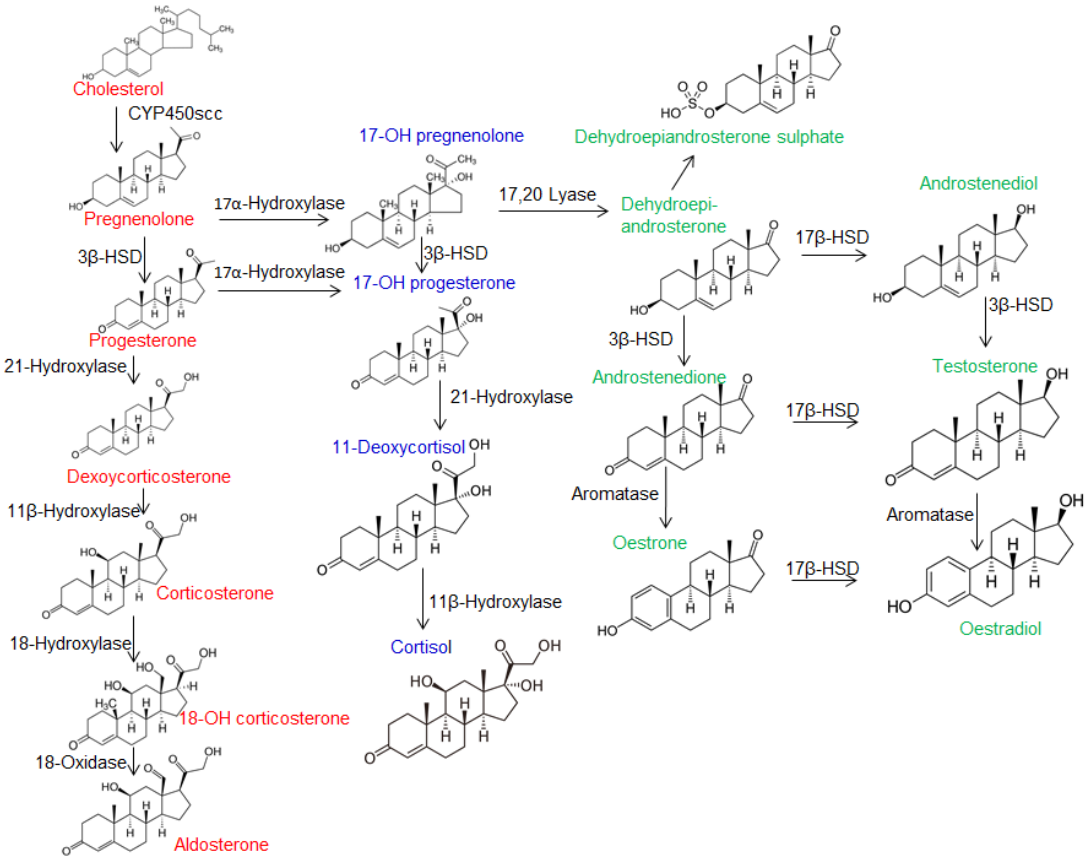


Figure 6.8: Oestrogen synthesis pathway. Hormones in red present mineralocorticoids. Hormones in blue present glucocorticoids. Hormones in green present the sex hormones.

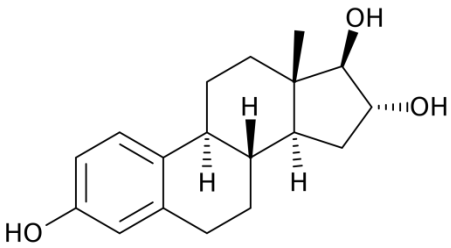


Figure 6.9: Molecular structure of oestriol.

6.3.2 Oestrogen and cytochrome P450

To ensure there is not an accumulation in active oestrogen, oestrogen is hydroxylated, by CYP1A2 (Hong et al. 2004) and CYP3A4 (Cheng et al. 2001), Figure 6.10. The discussion will be based on oestradiol as the primary oestrogen hormone. The kinetic parameters for CYP1A2 in the presence of oestradiol have been reported with a K_m of 58 ± 4 µM and a V_{max} of 0.7 ± 0.2 min^{-1}. Whilst, CYP3A4 has kinetic parameters where K_m has been reported to be 75 ± 18 µM and a V_{max} of 0.4 ± 0.2 min^{-1} (Yamazaki et al. 1998). This

demonstrates that CYP1A2 has a higher affinity for oestradiol. However, the rate of reaction (V_{max}) is higher for CYP3A4 compared to CYP1A2. In this case CYP3A4 may act as the compensator.

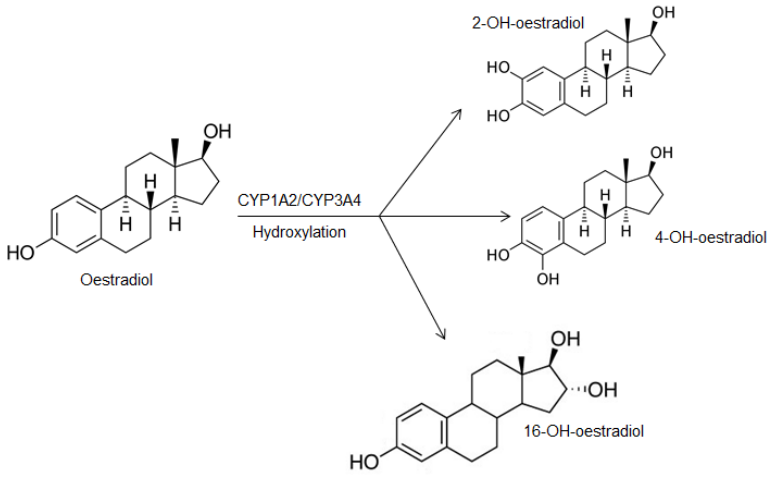


Figure 6.10: Phase I hydroxylation of oestradiol.

A molecular characteristic of breast cancer is abnormal oestrogen concentrations (Brinton et al. 1986; Colditz et al. 1990; Törnberg and Carstensen 1994). Similarly to testosterone, an accumulation of oestrogen leads to promotion of cellular growth. Alternatively, decreased oestrogen prevents cellular growth. In both situations cellular mutations and tumorigeneses could occur to compensate for the local environment. Additionally, a polymorphic CYP1A2 may influence the amount of oestrogen present (T1545C, rs71581941). rs2470890 has been associated with breast cancer in some studies (Anderson et al. 2012; Vukovic et al. 2016; Bai et al. 2017). However, the molecular function of the polymorphism is not known.

The *CYP3A4*1b* polymorphism has been observed in breast cancer. However, it is unclear whether *CYP3A4*1b* influences breast cancer. A number of studies reported a lack of functional association of *CYP3A4*1b* with breast cancer development (Spurdle et al. 2002; Chu et al. 2007; Fernández-Santander et al. 2013; Abdullah et al. 2016; Veiga et al. 2018). Thus, further research is required for a further elucidation into the expression of *CYP3A4*1b* and breast cancer.

6.3.3 Oestrogen and phase II conjugation

Similarly to testosterone, oestrogen can be glucuronidated by UGT2B17 (Hirata et al. 2010) or UGT2B15 (Hu et al. 2016), Figure 6.11. However, the *UGT2B15*2* polymorphism also affects oestrogen glucuronidation. The effect of *UGT2B15*2* on the glucuronidation of oestrogen is discussed below.

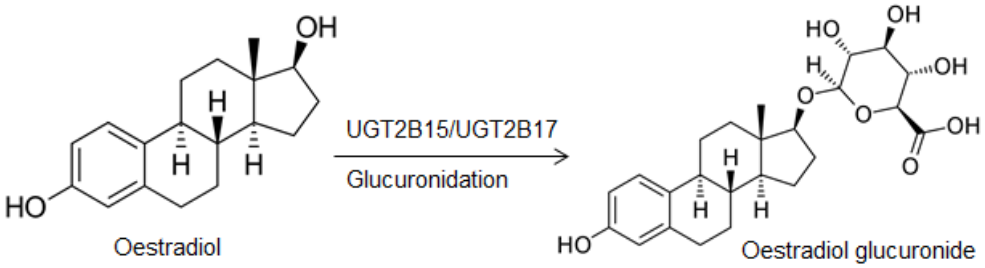


Figure 6.11: Phase II conjugation of oestradiol.

In addition to the association with prostate cancer, *UGT2B15*2* may be implicated in breast cancer. This has been reported to be due to decreased function (Park et al. 2006; De Smith et al. 2008; Sun et al. 2012; Divakaran et al. 2014). Thereby, reduced glucuronidation activity of oestrogen may leave oestrogen concentrations to accumulate which could contribute to the development of breast cancer. The allele frequencies of *UGT2B15*2* has been presented in Table 6.2.

Table 6.2: Human allele frequency distribution of *UGT2B15*2*

Population	Subject (n)	Allele frequency *2(D85Y)	*4(K523T)	Reference
Japanese	117	0.41	NS*	(Okugi et al. 2006)
	200	ND*	0.79	(Toide et al. 2002)
	77	0.49	NS*	(Riedy et al. 2000)
Chinese	78	0.42	NS*	(Riedy et al. 2000)
Caucasian	273	0.55	NS*	(Riedy et al. 2000)
	202	0.55	NS*	(Lampe et al. 2000)
	100	0.53	NS*	(Ménard et al. 2009)
	54	0.56	0.40	(Court et al. 2004)
	178	0.58	NS*	(Hajdinjak et al. 2004)
African	188	0.39	NS*	(Riedy et al. 2000)
African-Americans	41	0.44	NS*	(Court et al. 2017)
Hispanic	187	0.37	NS*	(Riedy et al. 2000)
Korean	50	0.44	0.89	(Hwang et al. 2014)

NS: Not specified*

A deletion variant polymorphism of *UGT2B17* is also associated with a predisposition for breast cancer (Park et al. 2006; Eskandari-Nasab et al. 2012). Several studies have reported on the effects of this polymorphism (Park et al. 2006; Eskandari-Nasab et al. 2012; Urashima et al. 2015; Packard et al. 2018). The *UGT2B17* gene deletion may causes an accumulation of oestrogen, promoting cellular growth in the breast tissue (Ménard et al. 2009). Sequentially, this promotion of cellular growth can contribute to the development of breast cancer. Thus, three studies have reported three glucuronides that could be produced in breast cancer (Appendix 2 and Table 8.6). Research to date on breast cancer has not elucidated potential glucuronides and lifestyle influences for breast cancer.

Similarly, in breast cancers, *GSTP1*, is also methylated (Lasabova et al. 2010). The methylation of GSTP1 has also been confirmed in later studies (Miyake et al. 2013 Fang et al. 2015; Kostovska et al. 2018). Evidently, the methylation of *GSTP1* could also be a potential biomarker for breast cancers along with screening for the potential glucuronide that are may be associated with breast cancer.

Oestrogen can be sulphated for detoxification as an alternative to glucuronidation (Sparks et al. 2004; Furimsky et al. 2007). Thus, upon the detoxification via sulfation, oestrogen sulphate metabolites are eliminated from the body. Thereby, sulphation is an alternative phase II detoxification process for oestrogen.

6.3.4 Oestrogen and phase III transportation

ABCG2 codes for the BCRP transporter component molecules, producing a half-transporter. A half-transporter is comprised of six transmembrane domains (TMDs) (Xu et al. 2004; Tusnády et al. 2006) (Figure 6.12). As a half transporter, BCRP is non-functional and requires dimerisation to occur for functionality (Kalinina et al. 2009; Lee and Levin 2015). Homodimerisation is the bonding of two identical protein chains (Kalinina et al. 2009; Rodríguez and Gutiérrez-de-Terán 2012; Liu et al. 2015; Guo et al. 2017; Maio et al. 2019), differing from the attachment of two different protein chains in heterodimerisation (Lee and Levin 2015; Cai et al. 2017; Cao et al. 2019). The BCRP transporter has been recognised as a homodimer transporter (Doyle et al. 1998; Kage et al. 2001; Henriksen et al. 2005; Leimanis and Georges 2007; Shigeta et al. 2010; Ogura et al. 2015). The functional dimer BCRP transporter, contrary to its name, is not only expressed within the breast tissue.

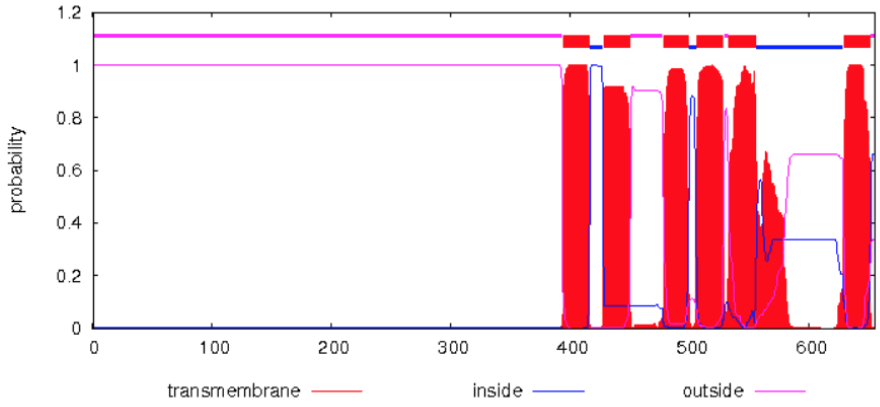


Figure 6.12: Predicted BCRP transmembrane topological structure. Predicted number of transmembrane domains using TMHMM v. 2.0 (TMHMM 2019), Accession number: AAC97367.1. Blue line presents TMDs within the cell, pink line presents TMDs outside of the cell.

BCRP transporters, in addition to their expression in the breast, are also expressed within the gastrointestinal tract (Gutmann et al. 2005), placenta (Wang et al. 2006) and the lungs (Nickel et al. 2017). The BCRP transporter is associated with oestrogen due to the oestrogen response element (ERE) found within *BCRP* (Ee et al. 2004; Lowery et al. 2009). The ERE comprises a palindromic sequence, putative (Pu)GGTCA, within the gene promoter region, induced by oestradiol. The presence of oestradiol promotes mRNA production (Ee et al. 2004; Wang et al. 2006; Day et al. 2009; Wu et al. 2012; Qian et al. 2015; Maddox et al. 2018). Receptors such as oestrogen receptor alpha and beta (ERα and ERβ), can bind to the ERE, to promote *BCRP* expression induced by oestrogen molecules. Therefore, increased oestrogen molecules, promotes an up-regulation in *BCRP* expression and consequently follows onto drug-based chemo-resistance.

6.4 Conclusion

Endogenous molecules are synthesised within the body from molecule degradation to the synthesis of hormones and neurotransmitters. Alterations to the concentrations of endogenous molecules can lead to the development of disease that is influenced by gene variants. Gilbert's syndrome is a result of increased bilirubin concentrations due to the *UGT1A1*28* variant which cannot glucuronidate bilirubin. Thus, jaundice can be observed and furthermore, individuals with *UGT1A1*28* could also be at a higher risk of hepatotoxicity if acetaminophen is consumed. Altered testosterone concentration may contribute to the development of prostate cancer. This is because the variant *UGT2B15*2* has increased gene expression and therefore, reducing testosterone concentrations, which could lead to the development of prostate cancer. Alternatively, the *UGT2B17* deletion genotype, increases testosterone concentration. Thereby, the increased concentrations promotes abnormal cellular growth leading to the potential development of prostate cancer. Alike to oestrogen altered concentrations can also be pathogenic. The same variants, *UGT2B15*2* and *UGT2B17* deletion genotype, can be observed in breast cancers. Therefore, clearly patients with a disease such as cancers should be screened for other polymorphisms alongside the known disease causing variants to determine whether a patient is at risk of a disease.

Chapter 7: The Metabolomic Fate of Different Xenobiotics

Xenobiotics are foreign molecules that are not naturally produced within the human body (Croom et al. 2012) Xenobiotics can come from a diverse range of sources such as the consumption of overcooked meats, smoking and pollution. All of which are detoxified via the detoxification pathway. As discussed in previous chapters and the fate of molecules in the presence of gene variants, the fate of xenobiotics are also influenced by gene variants as discussed in this chapter.

7.1 PhIPs

7.1.1 Introduction to PhIPs and phase I detoxification of PhIPs

Xenobiotics are molecules from sources outside of the human body. These molecules can be associated with cancer development, such as 2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) (Figure 7.1). PhIPs is a type of heterocyclic amine (HCA) molecule, produced from overcooked meats and fish (Tang et al. 2007; Reartes et al. 2012; Tang et al. 2013).

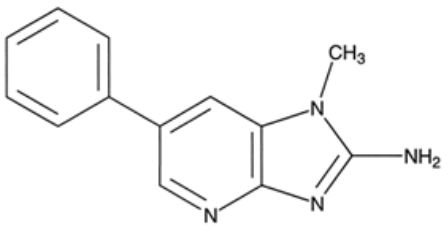


Figure 7.1: The molecular structure of PhIP

PhIP influences cancer development by exerting carcinogenic effects in the colon and mammary glands. Multiple studies reported induction of cellular proliferation at low doses of PhIPs (Zhu et al. 2005; Gooderham et al. 2007). This is through the stimulation of the MAP/ERK phosphorylation signal transduction pathway (Zhu et al. 2005; Gooderham et al. 2007). Additionally, PhIP was observed to inhibit the G_1 phase of the cell cycle preventing mitosis (Zhu et al. 2005; Gooderham et al. 2007), leading to potential tumorigenic mutations. Whereas a later study reported that the presence of PhIP molecules can increase the number of cells within G_1 phase of the cell cycle (Mimmler et al. 2017). Thus suggesting that PhIPs stimulate cellular division which could

also lead to cellular mutations and potential disease development. Other carcinogenic effects include the formation of PhIP DNA adducts (Turteltaub et al. 1993; Ghoshal et al. 1995; Josyula et al. 1998). DNA adducts are formed by the binding of a DNA segment to a carcinogenic molecule. This is due to the bioactivation by CYP1A1 or CYP1A2 by a hydroxylation reaction, synthesising an active metabolite, N-hydroxy-PhIP (N-OH-PhIP) (Huber et al. 1997; Galijatovic et al. 2001; Malfatti et al. 2005), Figure 7.2. The formation of DNA adducts leads to the activation of p53.

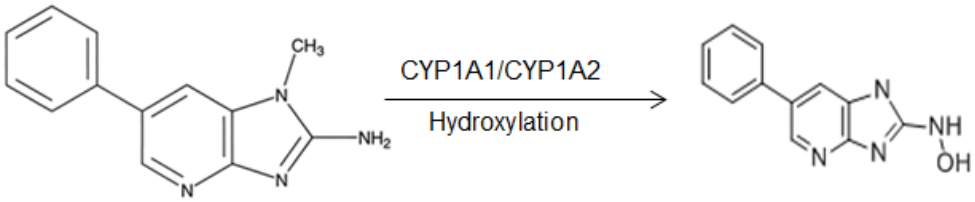


Figure 7.2: Phase I detoxification of 2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine. This phase I reaction produces the metabolite N-OH-PhIP.

p53 protein tumour suppressor coined as the “guardian of the genome”, is stimulated by cellular damage and oxidative stress. p53 activation induces the apoptotic pathway, preventing cellular mutations and development of cancerous cells. However, p53 is highly susceptible for degradation, therefore the vulnerability of p53 could be a factor in disease development (Ozeki et al. 2011; Zhang et al. 2014; de Stephanis et al. 2018). p53 degradation occurs through a cascade of events (Figure 7.3) involving attachment of mouse double minute 2 homolog (MDM2, E3 ubiquitin protein ligase). E3 ligase binds to p53 at the target recognising domain, enabling an E2 conjugating enzyme to binds to the E2 interacting domain of E3. Subsequently, E2 attaches ubiquitin (Ub) molecules to p53. As polyUb chains act as a marker for degradation by proteasomes, p53 is subsequently degraded by a proteasome.

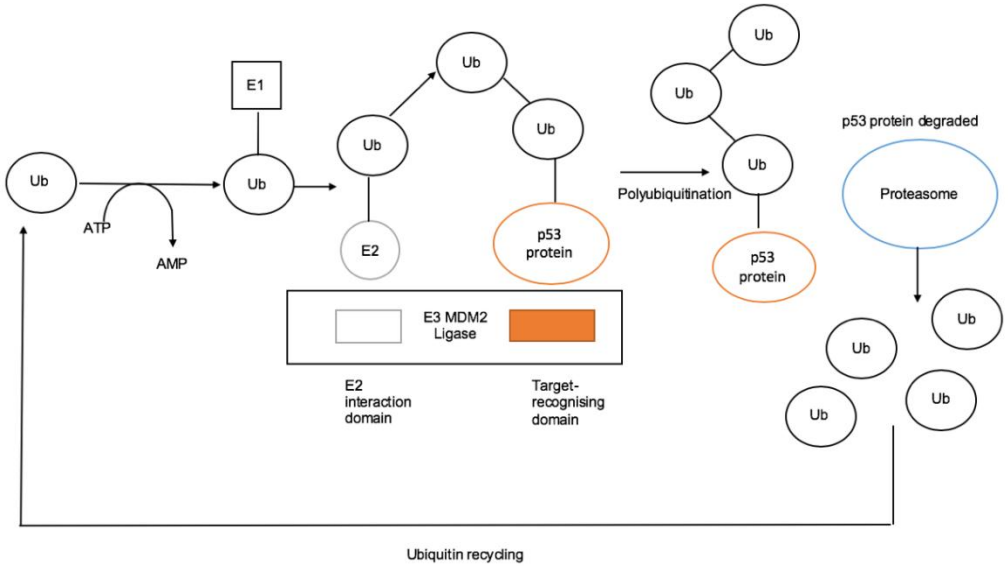


Figure 7.3: The pathway for p53 degradation. A cascade of events involving ubiquitinin (Ub), Ub activatior protein E1, congugating enzyme E2 (grey circle), and MDM2 ligase E3 (black rectangle) acting upon p53 (orange circle). E1 forms a ubiquitin-thioester (Lee and Schindelin 2008; Xu et al. 2010) leading to transfer of the thioester-linked Ub to a cysteine residue in E2. E3 MDM2 ligase binds p53 at the target recognising domain (orange rectangle), enabling E2 binding to the interaction domain (grey rectangle). E2 conjugates Ub to lysine residues in p53 for degradation (David et al. 2010; Cohen et al. 2013). Subsequently, p53 is degraded by a proteosome (blue oval) and free Ub is formed, and recycled.

DNA adducts could be used as a marker for cancer, due to PhIP accumulation leading to dose-dependent adduct development and potential influences in cancer development. Thus, the number of DNA adducts could be measured based upon the amount of meat consumed (Tang et al. 2007; Singh et al. 2010; Tang et al. 2013; Hemeryck et al. 2016; Durmus et al. 2019). Concluding this, individuals who consume large amounts of meat could be at a higher risk of developing prostate and colon cancer, compared to individuals who may consume only small amounts of meat (Aykan 2015; Saliba et al. 2019).

7.1.2 Phase II conjugation of PhIPs

After phase I preparation synthesising the metabolite N-OH-PhIP, subsequent glucuronidation by UGT1A10 occurs (Figure 7.4) (Malfatti and Felton 2001; Xie et al. 2003). PhIP glucuronidation by UGT1A10 has also been confirmed in later studies (Malfatti et al. 2005; Girard et al. 2008; Tang et al. 2013; Rogers et al. 2016; Lin et al. 2018), which demonstrate UGT1A10 is the major UGT1A for glucuronidation. Additionally, UGT1A1 can glucuronidate N-OH-PhIP

metabolites for detoxification. The *UGT1A1*28* polymorphism also alters the fate of N-OH-PhIP, due to reduced gene expression, promoting an accumulation of PhIP adducts (Girard et al. 2005). PhIP adducts are carcinogenic and therefore promote tumorigenesis (Girard et al. 2005; Cotterchio et al. 2008; Tang et al. 2013; Xiao et al. 2016). Thus, individuals with the *UGT1A1*28* could be at a higher risk of cancer. But, there are other UGTs able to glucuronidate N-OH-PhIP. These other UGTs include UGT1A9 (Yueh et al. 2001), UGT1A6 (Malfatti and Felton 2001) and UGT1A4 (Dellinger et al. 2007). Clearly, *UGT1A9*, *A6* and *A4* may act as compensators for both *UGT1A10* and *UGT1A1*28*.

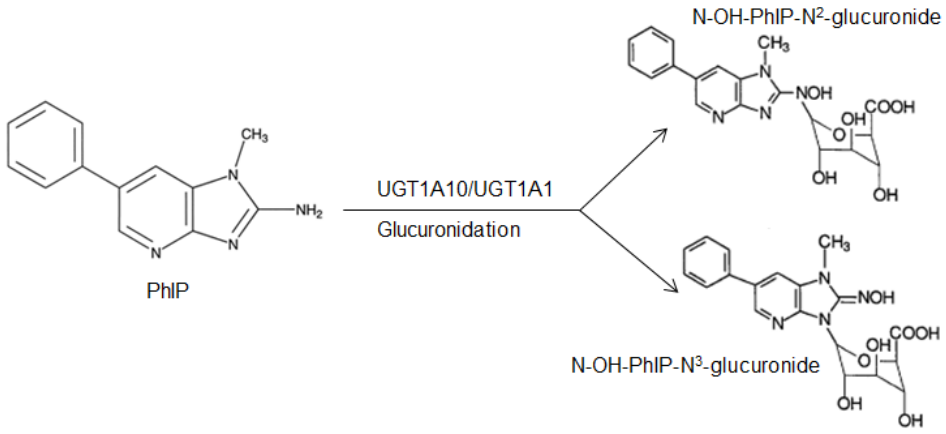


Figure 7.4: Phase II conjugation of a PhIP molecule. N-OH-PhIP glucuronide structures are adopted from (Stillwell et al. 2002).

7.1.3 Phase III transport of PhIPs

BCRP has capabilities of transporting xenobiotics, such as PhIPs, and medicinal drugs into the bile or urine for elimination (Dietrich et al. 2004; Cerveny et al. 2006; Enokizono et al. 2008; Graber-Maier et al. 2010; Vlamming et al. 2014; Shi et al. 2017). BCRP transporters prevent the carcinogenic effects exerted by PhIP by transporting PhIP molecules and metabolites into the bile or urine for elimination. To conclude this, the consumption of overcooked meats can lead to the formation of PhIP-DNA adducts within the colorectal region of the human body. The presence of the adducts within the colorectal region can be mutagenic contributing to CRC development.

7.2 Ethanol

7.2.1 Ethanol overview and phase I detoxification

Ethanol (Figure 7.5), commonly known as alcohol, is consumed for a variety of purposes. The consumption of alcohol often causes a common side effect, veisalgias (“hangovers”). This is influenced by different factors such as how much alcohol is consumed and ethnicity (Wetherill and Fromme 2009; Prat and Adan 2011; Penning et al. 2012). For instance, approximately 50% of the Asian population cannot consume alcohol, due to a missense mutation in the *ALDH* gene which codes for *ALDH2*2* (E487K) (Goedde et al. 1992).

Upon the consumption of alcohol ethanol molecules induces *CYP2E1* expression (Wolf et al. 2007; Cedarbaum 2011; Jin et al. 2013). Ethanol preparation in phase I of the detoxification pathway is the minor pathway for ethanol detoxification in the microsomal ethanol oxidising system (MEOS) system (Figure 7.5). Respectively, CYP2E1 oxidises ethanol in the presence of NADH, to acetaldehyde (Zhong et al. 2014; Xu et al. 2017). After subsequent synthesis of acetaldehyde, NAD+-dependent aldehyde dehydrogenase (ALDH2) oxidises acetaldehyde to acetone (Suprun et al. 2006; Quintanilla et al. 2007; Jelski et al. 2011; Choi et al. 2015). Acetone is subsequently excreted once the molecule has been synthesised.

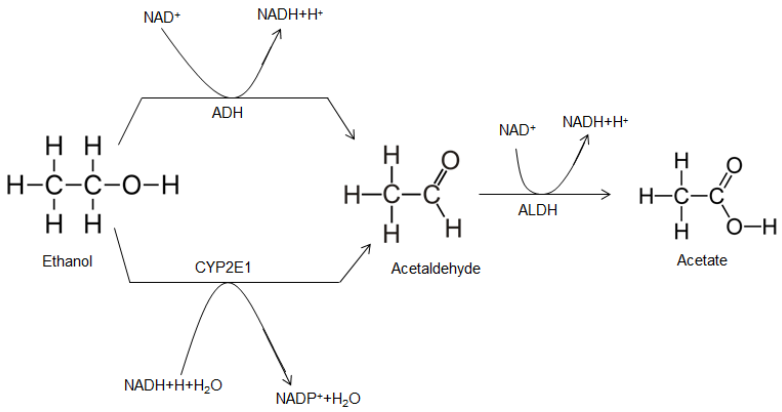


Figure 7.5: Microsomal ethanol oxidising system. In the cytoplasm ADH is constitutively expressed for the detoxification of ethanol to acetaldehyde. Alternatively, in the liver microsomes, CYP2E1 could be induced to synthesise acetaldehyde from ethanol. Acetaldehyde is then synthesised to acetate by ALDH.

A variant of *CYP2E1* has been reported to cause a mutation in the 5' flanking region. This is nucleotide mutation C1055T (rs2031920) (Hayashi et al.1991; Watanabe et al.1994). The variant is thought to contribute to alcohol dependence along with *ALDH2*. But, the mechanisms of *CYP2E1*5B* and *ALDH2* is not clear (Kim et al. 2010; Webb et al. 2010). Therefore, further investigation is necessary to understand how both genes contribute to alcohol dependence. Nevertheless, the *CYP2E1*5B*, was found to be associated with the Asian populations (Tang et al. 2010; Shahriary et al. 2012; Guaoua et al. 2014). The allele distributions are presented in Table 3.3.

7.2.2 Phase II detoxification of ethanol

The MEOS system is the primary detoxification pathway for ethanol. Ethanol can also go through glucuronidation (Helander et al. 2009; Reisfield et al. 2011; Saabi et al. 2013), the minor pathway for ethanol detoxification. Glucuronidation of ethanol synthesises ethyl glucuronides, catalysed predominantly by UGT1A1 and UGT2B7 (Figure 7.6) (Foti and Fisher 2005; Halter et al. 2007; Høiseth et al. 2010; Lostia et al. 2013; Stachel and Skopp 2016; Małkowska et al. 2018). Thereby, ethanol can be detoxified by two different processes: the MEOS system in Figure 7.5 or glucuronidation.

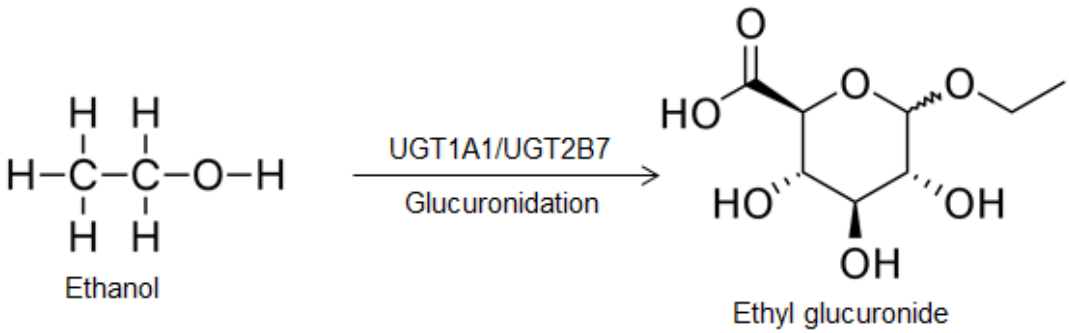


Figure 7.6: Phase II glucuronidation of ethanol.

Ethanol can interfere with APAP detoxification, particularly in individuals who abuse alcohol, by promoting liver damage. In addition to glucuronidation, ethanol molecules can be detoxified via GSH conjugation, depleting GSH molecules in the liver (Zhao and Slattery 2002; Vogt and Richie 2007; Otis and

Guidot 2010; Chitty et al. 2013; Chen et al. 2017). Due to this, individuals who take APAP are potentially at higher risk of liver failure. The reduction in GSH molecules allow APAP to interact with molecules in the liver, inducing liver failure. Thereby, impairing the detoxification of other molecules.

7.2.3 Phase III transportation of ethanol

In phase III, ethanol molecules are substrates for ABCA1 transporters, increases the transportation of cholesterol molecules (e Silva et al. 2000). Cholesterol itself has several vital roles including normal development of the brain (Hanaka et al. 2000; Guizetti et al. 2007). In the presence of alcohol abuse, offspring of an alcoholic user are likely to produce offspring with foetal alcohol syndrome (FAS) due to cholesterol transport interference from alcohol consumption (van der Gaag et al. 2001; Beulens et al. 2004; Guizzetti et al. 2007; Lesená et al. 2010; Zhou et al. 2014). ABCG1 also transports cholesterol however, the full impacts of alcohol on cholesterol transport by ABCG1 requires elucidation (Zhou et al. 2014).

A mutation in *ABCG1* can promote the onset of gout (Phipps-Green et al. 2010; Li et al. 2015; Duong et al. 2019). Gout is caused by excess uric acid (Villa et al. 1958; McCarty and Hollander 1961; Scott et al. 1964). The excess uric acid causes crystals to form and accumulate within the joints, presenting as lumps (Gaber et al. 2013; Onuma et al. 2014). The mutation contributing to alcohol induced gout is rs72552713. rs72552713 introduces a premature codon at Q216 (Gln126Ter, Q126X) and has been reported to increase uric acid concentrations (Ichida et al. 2012; Matsuo et al. 2016; Stiburkova et al. 2019). Additionally, rs72552713 is found to be disproportionately associated with the eastern Asian population (Table 7.1) as discussed in studies (Matsuo et al. 2009; Hamajimi et al. 2011; Matsuo et al. 2014 Matsuo; et al. 2016; Nakamura et al. 2018). The association of alcohol to gout and the non-functional ABCG1 could act as a contributing factor for alcohol induced gout.

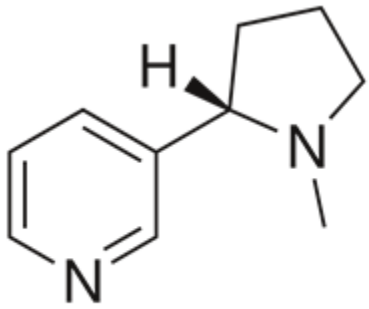
Table 7.1: Allele frequency distribution of rs72552713 (Adopted from: Karczewski et al. 2019).

Population	Allele Count	Allele Number	Number of Homozygotes	Allele Frequency
East Asian	97	19912	0	0.004871
Other	3	7172	0	0.0004183
Latino	1	35276	0	0.00002835
African	0	24938	0	0.000
Ashkenazi Jewish	0	10350	0	0.000
European (Finnish)	0	25042	0	0.000
European (non-Finnish)	0	128514	0	0.000
South Asian	0	30504	0	0.000

7.3 Nicotine

7.3.1 Nicotine and cytochrome p450

Cigarette smoking is considered a common practice (Gonzalez-Quintela et al. 2008). However, there are negative consequences associated with smoking. The carcinogenic effects of nicotine (Figure 7.7) remain unclear, *in vitro* studies demonstrate genotoxic effects (Ginzkey et al. 2013; Ginzkey et al. 2014a; Ginzkey et al. 2014b). However, smoking can lead to tumour development. A 2018 study compared the carcinogenic effects of vaporised nicotine from electronic (e) cigarettes to tobacco smoking, concluding that nicotine from e-cigarettes is less carcinogenic, but still has potential for carcinogenic effects (Stephens et al. 2018). The presence of nicotine stimulate the activation of *CYP1A1* and *CYP1A2* for phase I preparation (Anttila et al. 2001; Mohammed and Shervington 2008; Hukkanen et al. 2011; Ezzeldin et al. 2017). However, a *CYP1A2* polymorphism affects the detoxification process of nicotine (Sachse et al. 2003; Katoh et al. 2010), discussed below.



Nicotine

Figure 7.7: The molecular structure of nicotine.

*CYP1A2*1C* is a result of the nucleotide mutation G3858A, but the functional properties are not clear. It is thought that the polymorphism causes loss of function (LOF) (Nakajima et al. 1999). Conversely, later studies demonstrate that in individuals who smoke, who have the *CYP1A2*1C* polymorphism, the detoxification of nicotine is increased (Sachse et al. 2003; Katoh et al. 2010; Khono et al. 2013; Xiong and Li 2017). Thus, smokers who have the *CYP1A2*1C* polymorphism may have a reduced chance of cancer development due to the increased expression for nicotine detoxification. The discrepancies in the function of Allele frequency studies report that *CYP1A2*1C* is associated with the Asian population (Table 7.2).

Table 7.2: Frequency distribution of *CYP1A2*1C*

Region or Ethnic background	Country	Number of people (if specified)	% of Homozygous	% of Heterozygous	% if homozygous or heterozygous not specified	P-value reported in the study	Reference
Asia	China	NS*	NS*	NS*	22	NS*	(Xiong and Li 2017)
	Japan	NS*	NS*	NS*	23	NS*	
	Korea	186	NS*	NS*	29.6	NS*	(Myrand et al. 2008)
Africa	Egypt	212	93	7	Not applicable	P=0.21	(Bilgen et al. 2008)
Europe	Turkey	110	96	4	Not applicable	NS*	(Bilgen et al. 2008)
	Finland	152	NS*	NS*	1	NS*	(Karczewski 2019)
	Greece	10 (from 150 people)	6	1	Not applicable	P<0.05	(Gentile et al. 2018)

NS: Not specified*

7.3.2 Nicotine and Phase II and phase III

Nicotine is detoxified in phase II by glucuronidation, catalysed by UGT2B10 or UGT2B17. UGT2B10 and UGT2B17 glucuronidation activity towards nicotine has been observed in various studies (Lazarus et al. 2005; Chen et al. 2007; Chen et al. 2010; Murphy et al. 2014; Ware et al. 2016) (Figure 7.8). Two polymorphisms of *UGT2B10* have been reported to alter the fate of nicotine: rs61750900 and rs2942857.

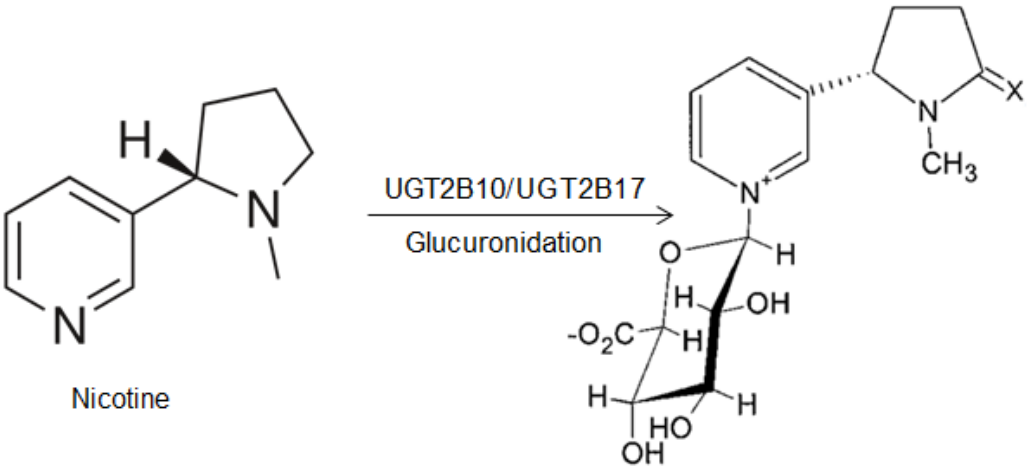


Figure 7.8: Phase II conjugation of nicotine for detoxification. The molecular structure of nicotine has been adopted from (Ghosheh and Hawes 2002).

6.3.2.1 rs61750900

rs61750900 (*UGT2B10*2*) is a missense mutation causing the amino acid change Asp67Tyr. The polymorphism is thought to act as a predisposition for lung cancer because rs61750900 is non-functional and therefore nicotine glucuronidation does not occur (Berg et al. 2010; Murphy et al. 2014; Taghavi et al. 2017). Therefore, the presence of unconjugated nicotine in the urine can be observed (Matsumoto et al. 2013; Wassenaar et al. 2015). Subsequently, long-term exposure in affected individuals may influence lung cancer onset (Sheppard et al. 2000; Chu et al. 2004; Le Marchand et al. 2008). Unconjugated nicotine in the urine may signify predisposition to lung cancer, however further research to determine the significance is needed. The allele distribution of rs61750900 has been presented in Table 7.3.

Table 7.3: Human allele frequency distribution of rs61750900

Region	Country or ethnicity	Number of people	% of homozygous	% of heterozygous	%if homozygous or heterozygous not specified	P-value reported in the study	Reference
Europe	Finland	1451	7.7	NS*	NS*	NS*	(Karczewski et al. 2019)
Asia	Japanese-Americans	674	NS*	NS*	3	NS*	(Murphy et al. 2014)
America	European-American	188	NS*	NS*	9.6	NS*	(Bloom et al. 2013)
	African-American	128	NS*	NS*	4.3	P=0.01	(Wassenaar et al. 2015)
	Native Hawaiians	311	NS*	NS*	4.7	NS*	(Murphy et al. 2014)
NS*	Caucasian	107	2	18	Not applicable	NS*	(Chen et al. 2010)
Ns*	Latinos	453	NS*	NS*	7.3	NS*	(Murphy et al. 2014)

NS: Not specified*

7.3.3.2 rs2942857

rs2942857 is another SNP which affects the glucuronidation activity of *UGT2B10* (Murphy et al. 2014). rs2942857 causes a splice variant, reducing glucuronidation activity. The splice variant causes an alteration between intron 2 and exon 3, disrupting the splicing acceptor site (Patel et al. 2015; Chen et al. 2016; Labriet et al. 2018). The variant is thought to predominantly affect those of African-American descent (Patel et al. 2015), potentially disposing the carrier to lung cancer (Murphy et al. 2014; Patel et al. 2015; Murphy et al. 2018), because of reduced glucuronidation activity. A search was undertaken on EVS for rs2942857, however it was unavailable. Additionally, rs2942857 was searched for on gnomAD and ExAC databases for allele distributions and no results were found. Evidently, further work is necessary to understand which ethnic backgrounds are affected by the polymorphism. Additionally, further studies are necessary to reveal the relationship between rs2942857 and lung cancer.

In smokers, the presence of nicotine suppresses both *ABCA1* and *ABCG2* expression, particularly in individuals with coronary artery disease (CAD) and coronary heart disease (CHD) (Song et al. 2015). Thereby increasing the

chance of pulmonary emphysema. The presence of nicotine reduces cholesterol transport by ABCA1 and ABCG2 and leads to an accumulation in cholesterol foam cell formation (Bochem et al. 2013; Song et al. 2015; Tao et al. 2017). Consequently, foam cells can develop to plaques, blocking arteries. As a result, this acts as a predisposition to the development of CAD and CHD. Additionally, several inflammatory markers related to smoking can be observed such as: fibrinogen (Dahl et al. 2001), tumour necrosis factor alpha (TNF-α) (Bolton et al. 2004), cytokines (Wojcik et al. 2008) and C-reactive proteins (Şahin and Aslan 2018). All of which can be influenced by mutation in *ABCA1* acting as a predisposition for CAD and CHD development.

ABCA1 rs2230806 is a genetic predisposition for CHD, causing an Arg219Lys (R219K) mutation (Villard et al. 2013; Cyrus et al. 2016; Wang et al. 2019). CHD is associated with an accumulation in low-density lipoprotein (LDL) cholesterol. The accumulation of LDL cholesterol suggests rs2230806 reduces function. Studies have reported that rs2230806 influences the severity of CHD (Cenarro et al. 2003; Kyriakou et al. 2007; Doosti et al. 2010; Zargar et al. 2013; Ghaznavi et al. 2018). Furthermore, it has been postulated that rs2230806 in the Asian population may serve as a protective mechanism from high-density lipoprotein (HDL) cholesterol, but not in Caucasians (Liu et al. 2015). This suggests that gene polymorphisms may function differently in different geographical and ethnic backgrounds. Table 7.4 presents allelic distribution for rs2230806.

Table 7.4: Human allelic distribution of rs2230806 (Adopted from: Lek et al. 2016).

Population	Allele Count	Allele Number	Number of Homozygotes	Allele Frequency
African	6512	10402	2077	0.626
East Asian	3846	8628	887	0.4458
South Asian	6078	16510	1120	0.3681
Other	306	908	55	0.337
Latino	3738	11564	594	0.3232
European (Non-Finnish)	18130	66734	2481	0.2717
European (Finnish)	1431	6614	143	0.2164
Total	40041	121360	7357	0.3299

7.4 Polycyclic aromatic hydrocarbons

7.4.1 Polycyclic aromatic hydrocarbons and cytochrome p450

Polycyclic aromatic hydrocarbons (PAHs) (Figure 7.9) are organic pollutant molecules which come from a number of environmental sources such as: grilled food (Lee et al. 2016), the burning of organic matter (Samburova et al. 2016), and smoking (Wang et al. 2016). If PAHs enter the body CYP450s bioactivate PAH molecules, by which the active toxic PAH metabolites able to elicit toxicological effects. For instance, the presence of pyrene molecules in the liver stimulate the activation of *CYP1A1* (Pandey et al. 2006), *CYP1B1* (Šulc et al. 2016) and *CYP3A4* (Juschyshyn et al. 2005). If pyrene molecules are hydroxylated by CYP3A4 (Figure 7.9), hydroxypyrene metabolites are synthesised (Jushchyshyn et al. 2005; Ciarrocca et al. 2014).

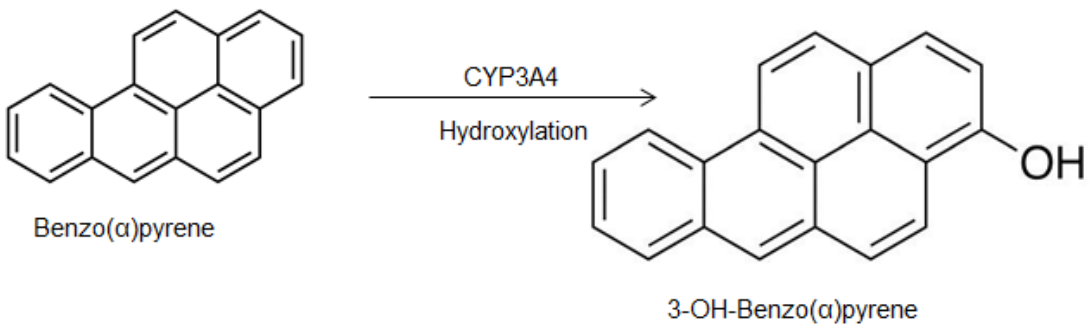


Figure 7.9: The bioactivation of benzo(α)pyrene by CYP3A4.

7.4.2 Polycyclic aromatic hydrocarbons and phase II and phase III

After subsequent synthesis of hydroxypyrene molecules, the molecules enter phase II. In phase II conjugation, hydroxypyrenes are conjugated to UDP-glucuroniate (UDP- glucuronic acid, UDP-GA) for detoxification by UGT1A6 (Figure 7.10) (Elovaara et al. 2007) and potentially UGT1A1 (Abnet et al.2007). Further research is recommended to validate whether pyrene is glucuronidated by UGT1A1. Furthermore, it would be interesting to reveal if pyrene molecules suppress glucuronidation activity towards other molecules.

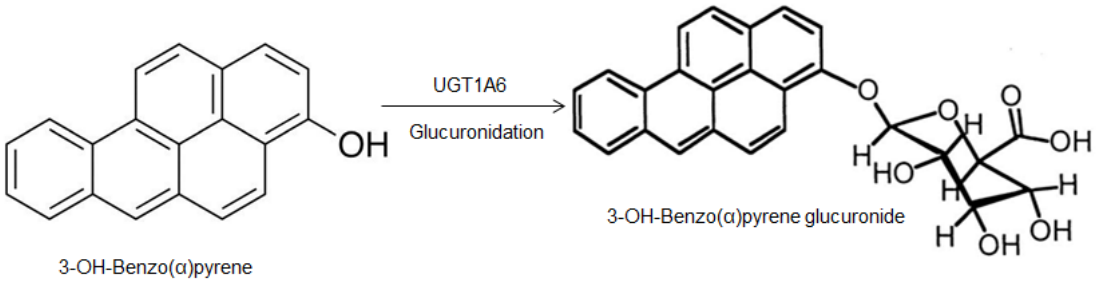


Figure 7.10: Phase II conjugation of 3-OH-benzo(α)pyrene. The molecular strucutre of 3-OH-Benzo(α)pyrene glucuronide has been adopted from (James et al. 2001).

It is thought to be believed that hydroxypyrene glucuronides are transported by a ABC transporter but, there is a lack of evidence. Järvinen et al. (2017) reported that hydroxypyrene glucuronides can be transported by MRP4. Thus further research is recommended to validate the finding. Additionally, other research could explore if hydroxypyrene glucuronides are transported by any other ABC transporters.

7.4.3 Polycyclic aromatic hydrocarbons and disease

As discussed in Section 2.4.3, clotrimazole can suppress glucuronidation activity of hydroxypyrene for detoxification. The toxicological effects of pyrene molecules in humans remains for elucidation (Drukteinis et al. 2005; Sharma et al. 2008; Tarantini et al. 2011), only presenting *in vitro* data. But, murine model studies have revealed the toxicological effects of benzo[α]pyrene (BaP)

molecules on murines. A study conducted by Uno et al. (2004), reported in *CYP1A1*$^{-/-}$ mice, the presence of BaP molecules could produce BaP-DNA adducts alongside with bone marrow depression. Therefore, in individuals with reduced *CYP1A1* expression BaP-DNA adducts could be formed which may lead to the development of disease. A later study conducted by Ribière et al. (2016) reported that if PAHs enter the body orally in the murine models used, intestinal inflammation is developed. Furthermore the faecal microbiota was also altered for instance, *Bacteroidales* species have been reported to be decreased. Therefore, if BaPs are orally entered into the body, inflammation and alterations to gut, mucous, and microbiota which could lead to other potential complications.

7.5 Conclusion

Pollution evidently poses a risk to human health, potentially more so in individuals with reduced gene expression that affects the detoxification pathway. The consumption of overcooked meats including fish, can lead to the development of PhIP-DNA adducts particularly in individuals with the *UGT1A1*28* variant. Subsequently, the presence of the adducts can induce mutagenic effects which can contribute to colorectal cancer development, influenced by diet. The association of nicotine and disease is unclear and requires elucidation but, it is thought that nicotine my induce carcinogenic effects. Polycyclic aromatic hydrocarbons can also influence disease development. For instance, people who work with burning organic materials have an increased risk of developing lung cancer compared to those who do not burn organic matter (Bootdee and Chantara 2014; Juntarawijit and Juntarawijit 2017). The accumulation of polycyclic aromatic hydrocarbons in the lungs as a result of inhalation, polycyclic aromatic hydrocarbons could lead to DNA damage, influencing the development of lung cancer.

Chapter 8: The Potential Application of Glucuronides for Disease Prognostics

8.0 Introduction

Glucose is a versatile molecule that is utilised by different molecular pathways (Figure 8.1), particularly for the synthesis of uridine-diphosphate (UDP)-glucuronate (glucuronic acid, UDP-GA). UDP-GA synthesis occurs via the uronic acid pathway (Figure 8.1), in the liver. Upon synthesis of UDPG-GA, the molecule is subsequently conjugated to xenobiotics and endogenous molecules (Esienberg et al. 1955; Horecker and Hiatt 1958; Oya 1961; Inoue et al. 1969), producing glucuronides. Even if there is a disruption in glucose concentrations in the blood, glucose is still available. In hypoglycaemic conditions glucose is still produced from glycogen to glucose (glycogenolysis) (Mutel et al. 2011; Kuhre et al. 2015; Pedersen et al. 2018) (Figure 8.1), upon the stimulation of glucagon secreted by the pancreas. Conversely, in hyperglycaemic condition glucose is present in large concentrations (Rahmoune et al. 2005; Seo et al. 2008; Pirola et al. 2011; Alam et al. 2014; Rahn et al. 2018), which can be synthesised to UDP-GA. Evidently, glucuronidation is the major conjugation process for phase II conjugation (Woelflingseder et al. 2019). Abnormalities in glucuronide specie production could lead to the development of glucuronide spectrums under abnormal conditions. Thus, the glucuronide spectrum may therefore, reflect all glucuronides produced associated with an abnormality in the detoxification pathway and disease. Thus, the glucuronide spectrum could be used in the application of disease diagnostics and prognostics.

The application of glucuronide spectrums for disease diagnostics and prognostics could distinguish between disease causation (genetics or lifestyle factors). Additionally, the spectrum could be used to monitor disease severity by determining the production of glucuronide species and concentrations overtime. This is due to if disease severity increases, the production of glucuronide species may correlate to disease severity. Therefore, the aim of this review is to explore the concept of using glucuronide species to reveal a glucuronide spectrum for disease prognostics.

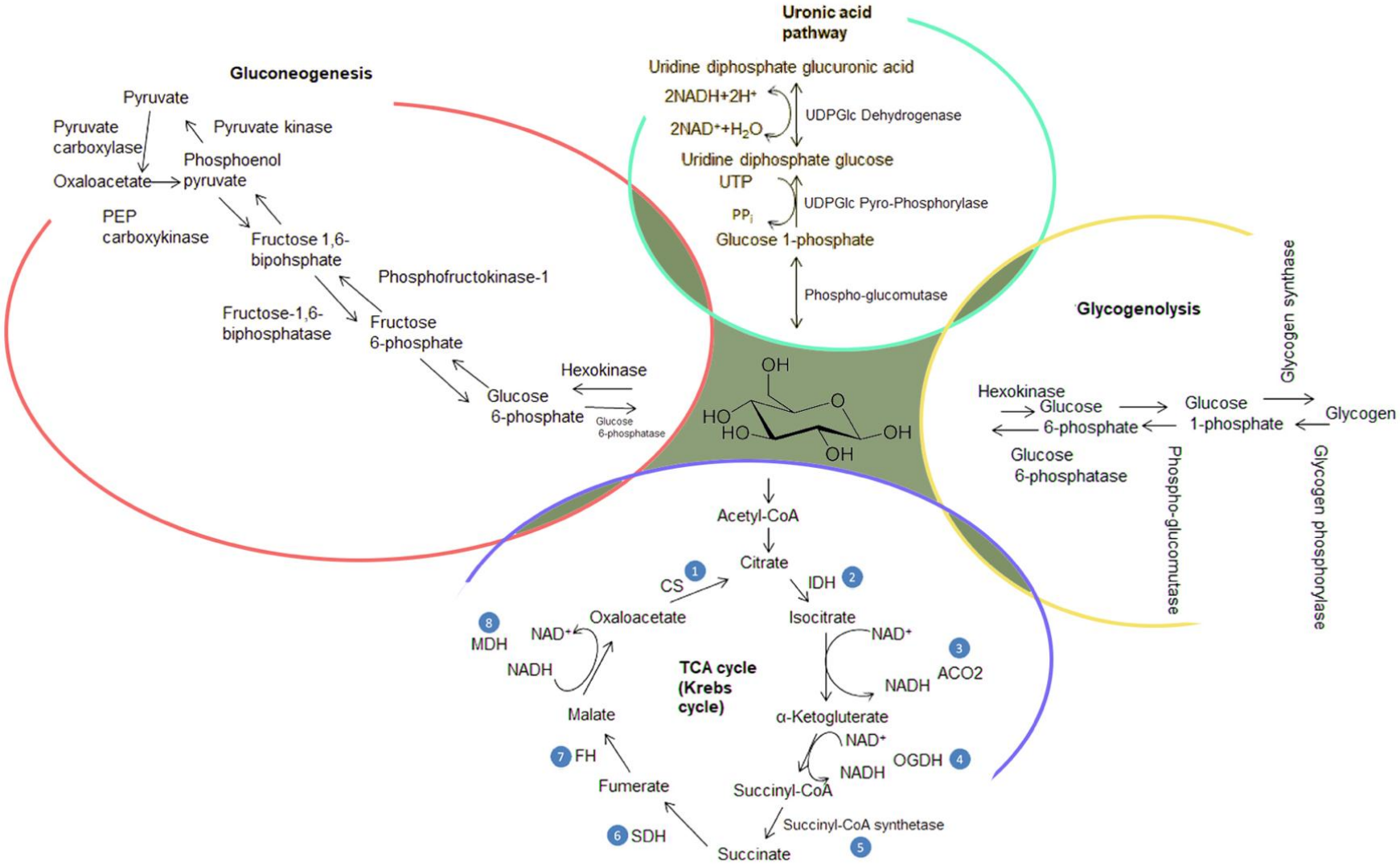


Figure 8.1: Glucose utilisation by different molecular pathways. Arrows pointing away from glucose shows glucose utilisation by a molecular pathway. Arrows pointing to glucose show glucose synthesis by a molecular pathway. In the Krebs cycle (citrate cycle) glucose can be synthesised and used as the starting molecule for the cycle. Gluconeogenesis converts pyruvate to glucose. Glycolysis produces pyruvate from glucose. The uronic acid pathway uses glucose to synthesise UDP-glucuronic acid. Glycogenolysis converts glycogen to glucose.

8.1.1 Colorectal cancer

The application of glucuronide spectrums to colorectal cancer (CRC) could be used to determine disease prognostics. Glucuronidation enzymes are not only expressed in the liver but, they are also expressed in the intestinal-colorectal region (Table 8.1). Thus, the expression patterns of the different uridine-diphosphate glucuronosyltransferases (*UGTs*) may be altered. For instance, in CRC, *UGT2B15* and *UGT2B17* are down regulated (Guo et al. 2017; Shi et al. 2018), as opposed to *UGT1A1* and *UGT1A10* which are up-regulated (Bélanger et al. 2010; Wang et al. 2013). The differences in gene expression, may produce different glucuronide species which could be used to construct a glucuronide spectrum.

Table 8.1: Expression patterns of UGTs in the intestinal-colorectal region

UGT	Expression location	Reference
UGT1A1	Small intestine	(Fujiwara et al. 2012)
UGT1A4	Duodenum	(Strassburg et al. 2000)
UGT1A5	Small intestine and Colon	(Finel et al. 2005)
UGT1A6	Large and small intestine	(Chen et al. 2005)
UGT1A8	Colon	(Mojarrabi et al. 1998)
UGT1A10	Duodenum	(Strassburg et al. 2000)
UGT2B15	Small intestine Colon	(Strassburg et al. 2000) (Sun et al. 2011)
UGT2B17	Small intestine Colon	(Strassburg et al. 2000) (Ohno and Nakajin 2008)

CRC can also develop from lifestyle factor such as dietary choices (Zhu et al. 2013; Chen et al. 2015; Schwingshackl et al. 2017). Individuals who consume a large amount of meats are more at risk of developing CRC (Malfatti et al. 2006; Fede et al. 2009; Gu et al. 2011; Chiavarini et al. 2017). For instance, research has revealed that individuals who consume large amounts are more than likely to have an increased number of PhIP adducts present in the large and small intestine (Nicken et al. 2010; Jamin et al. 2013; Nicken et al. 2016; Durmus et al. 2019). This evidently demonstrates that PhIP adducts can be used as a predictor biomarker for CRCs. In relation to glucuronidation, the amount of PhIP and PhIP glucuronides present reflects the amount of meat consumed (Malfatti et al. 2006; Fede et al. 2009). Thus, PhIPs and PhIP glucuronides could be used as predictor biomarkers in CRCs to determine the amount of meats

consumed. The presence of PhIPs and PhIP glucuronides and other molecules may cause increases UGT expression and therefore, increased glucuronide production occurs. Subsequently, increasing *gusA* expression in the gus operon system in *Escherichia coli* (*E. coli*).

The microbiome in the colorectal region is affected by the presence of CRC (Bronowski et al. 2008). This is because studies have reported that *E. coli* B2 phylotype strains, colonising around the cancer (Bronowski et al. 2008; Martinez-Medina et al. 2009; Prorok-Hamon et al. 2013; Mannion et al. 2016). Additionally, *gusA* expression is overexpressed in CRC (Gloux et al. 2010; Roberts et al. 2013; Gloux and Mondoloni 2016; Chen et al. 2017), due to the up-regulation of UGTs such as *UGT1A5* (Wang et al. 2013) and *UGT1A7* (Chen et al. 2019). The presence of glucuronides produced by up-regulated *UGT* expression induces an up-regulation in *gusA* expression coding for GusA (β-glucuronidase) to cleave glucuronides. Therefore, the application of a glucuronide spectrum could be used for CRC diagnostics. Furthermore, the glucuronide spectrum could be used to monitor disease severity overtime, based on the glucuronide species and concentrations produced.

8.1.2 Type 2 Diabetes mellitus

In Type 2 Diabetes Mellitus (T2DM) increased glucose concentrations is present varying from 5.6 to 6.9mmol/L for prediabetes (intermediate hyperglycaemia, IH) and 7mmol/L for T2DM. The increased concentrations of glucose can therefore be utilised by the uronic acid pathway (Figure 8.1) for UDP-GA synthesis (Eisenberg et al. 1958; Winegrad and Shaw 1964), for phase II glucuronidation. Due to the increased presence glucose concentrations an array of glucuronide species could be produced that are linked with T2DM.

In phase II the expression of several *UGT*s have been reported to be altered. *UGT2B7* has been reported to have reduced expression in the presence of mycophenolic acid (Dostalek et al. 2011). Additionally, the expression of *UGT2B1* expression has been reported to be reduced. In one study, *UGT2B1* mRNA was measured (Xie et al. 2013) and another study used *Ruellia tuberosa*

L. extracts to determine expression activity (Chang et al. 2018). Conversely, the expression of *UGT1A1*, *UGT1A6* and *UGT1A7* is increased (Xie et al. 2013). Therefore, there could be a diverse range of glucuronide species that could be used to construct a glucuronide spectrum due to the increased *UGT1A* expression and increased concentrations of glucose.

There are other complications that have been associated with T2DM leading to the development of other diseases and conditions. For instance, CRC (Levi et al. 2002), diabetic neuropathy (Sharma et al. 2002) and cardiovascular disease (Gæde et al. 2003). Individuals with T2DM have a decreased risk of prostate cancer as a result of reduced insulin production. Insulin may promote prostate cancer development (Hammarsten et al. 2010; Janghorbani et al. 2012; Tseng 2014; Valentino et al. 2017; Kachhawa et al. 2018), by regulating CYP2E1 expression. However, prostate cancer may still occur and could be determined by measuring prostate specific antigen (PSA) concentrations (Catalona et al. 2000; Thompson et al. 2004; Ulmert et al. 2008). Therefore, the use of glucuronide spectrums could be used to monitor disease severity by revealing whether glucuronides that are produced reflect disease severity. Since determining blood glucose concentrations only reveal the presence of IH or T2DM only.

8.1.3 Parkinson's disease

CYP2D6 encoded by *CYP2D6* is a cytochrome p450 (CYP450) which belongs to phase I of the detoxification pathway. CYP2D6 is not only involved in phase I detoxification of xenobiotics but, it is involved with synthesising dopamine (DA) (Hiroi et al. 1998). The common pathway for dopamine synthesis utilises phenylalanine as a precursor molecule. Alternatively, DA can be synthesised from tyramine by a hydroxylation reaction catalysed by CYP2D6, reported in a limited number of studies (Figure 8.2) (Hiroi et al. 1998; Zhu et al. 2005; Candiotti et al. 2009; Bromek et al. 2011). However, the synthesis of dopamine can be influenced by a variant of *CYP2D6*, as discussed below.

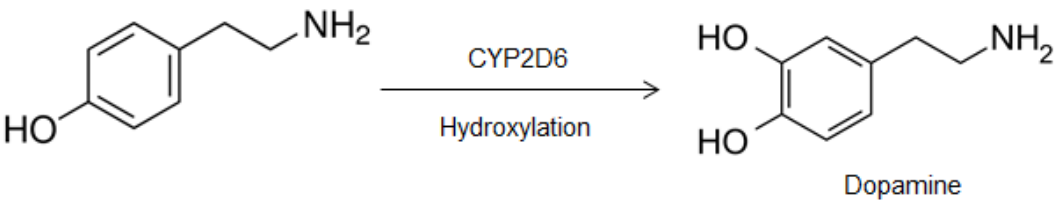


Figure 8.2: Hydroxylation of tyramine to dopamine

*CYP2D6*4* is a polymorphic variant of *CYP2D6* with a reduced function as discussed (Section 4.1.1) and commonly associated with the European population (Table 4.2). One of the molecular characteristics in Parkinson's disease (PD) is reduced dopamine production, due to the degradation of dopaminergic neurones (Cilla et al. 2010; Gröger et al. 2014; Kawashima et al. 2018). However, it appears that an elucidation is not made between reduced dopamine and *CYP2D6*4* in PD. In despite of *CYP2D6*4* has been associated with PD (Harhangi et al. 2001; Santt et al. 2004; Singh et al. 2008; Lu et al. 2013; Aslam et al. 2017). Therefore, further work is recommended to explore whether there is an association between *CYP2D6*4* and DA synthesis in PD. Furthermore, the association of glucuronidation activity in PD is unclear. However, Landolfi et al. (2017) explored glucuronidation of bisphenol A in PD and concluded, glucuronidation activity is reduced. Thus, further exploration is required to understand how phase II is affected in PD.

There are genetic tests which screen for a number of gene polymorphisms that can determine the onset of PD. Genetic tests can screen for the following genes and variants including : *LRRK2* (G2019S) (Illarioshkin et al. 2007), *PARK7* (*DJ1*; early onset, Leu166Pro) (van Duijn et al. 2001), *PINK1* (G411S) (Narendra et al. 2010), *PRKN* (*PRK2*) (exon deletion between 3 to 7, early onset) (Wu et al. 2005), and *SNCA* (G51D) (Kiely et al. 2013). Clearly, these criteria for genetic testing can complicate prognosis of PD. Instead, if there are glucuronide spectrums and glucuronide abnormalities, the spectrum could be used in the prognosis and diagnosis of PD. This is because if one of these gene variants is present, it does not necessarily mean an individual may develop PD.

8.1.4 Breast cancer

The aid of a glucuronide spectrum for breast cancer prognostics would be beneficial. This is because females who carry *BRCA1* and *BRCA2* are at risk of breast cancer however, they may not necessarily develop breast cancer (Ayub et al. 2013). Therefore, the application of a glucuronide spectrum can be used to determine the presence of the cancer. Another molecular characteristic of breast cancer is increased oestrogen concentrations (Schairer et al. 2000; Kaaks et al. 2005). Thus, there is a possibility that oestrogen glucuronides are produced, along with other glucuronides. However, increased oestrogen can be influenced by other factors such as the contraceptive pill.

The contraceptive pill is administered to prevent ovulation, which may contains oestrogen. Thus, when an individual uses the contraceptive pill, oestrogen concentrations increase (Lehtovirta et al. 1974; Briggs and Briggs 1977; Elliot et al. 1980). Even though there could be genetic predispositions, the predispositions may not necessarily cause breast cancer (Wiseman 2010). However, disruptions to the balance of oestrogen concentrations, increases the risk of breast cancer. Kumle et al. (2002) conducted a population study with 100,000 women from Norway and 96,0000 from Sweden. The study reported that women on the study who used contraceptive pills had a 30% increased chance of breast cancer. A later study, Morch et al (2017), conducted a cohort study (1.79 million participants) and concluded that the use of the contraceptive pill increases the risk of breast cancer, compared to women who do not use the contraceptive pill. However, it appears there is not any evidence to contradict that contraception pills influence breast cancer development. Thus, lifestyle factors also contribute to the development of breast cancer.

UGT2B15 encoding for the UGT21B5 protein is one of the phase II glucuronidation enzymes of the detoxification pathway. UGT2B15 has been reported to glucuronidate endogenous molecules such as oestrogen (Harrington et al. 2006) and testosterone (Strahm et al. 2013). In breast cancers, *UGT2B15* expression is up-regulated which may be due to increased oestrogen (Harrington et al. 2006; Hu and Mackenzie 2009; Sun et al. 2012; Romero-

Lorca et al. 2015). Therefore, the presence of oestrogen glucuronides could be detected in patient samples with breast cancer, along with other potential glucuronides associated with breast cancer.

8.1.5 Prostate cancer

Prostate cancer is another hormone driven cancer that is influenced by testosterone concentrations (Koo and Shim 2010; San Francisco et al. 2014; Claps et al. 2018). Testosterone influences the expression of *UGT2B15* (Strahm et al 2013) and *UGT2B17* (Basit et al. 2018). Increased testosterone up-regulates *UGT2B15* and *UGT2B17* expression and thereby, testosterone glucuronides are likely to be produced in prostate cancer, along with other possible glucuronides. Additionally, prostate cancer also alters phase I of the detoxification pathway.

CYP450 expression in prostate cancers has been observed to be altered, particularly CYP3A5. CYP3A5 has affinity to testosterone to hydroxylate testosterone for phase II glucuronidation (Yamori et al. 2004; Leskelä et al. 2007). In prostate cancer, the expression of CYP3A5 has been reported to be absent (Leskelä et al. 2007), down-regulated (Moilanen et al. 2007) or overexpressed (Jiang et al. 2015). These conflicting findings may account for the explanation that *CYP3A5* expression may vary depending upon the severity of the cancer. Additionally, the concentrations of testosterone can also influence *CYP3A5* expression. Therefore, this may contribute to glucuronide differences contributing to a glucuronide spectrum.

ABC transporter expression is altered, causing an alteration in the fate of a molecule. ABCB1 is one of the ABC transporter that has gained attention, due to the overexpression of the *ABCB1* gene (Reed et al. 2010; Zhu et al. 2013; Lombard et al. 2017; Nanayakkara et al. 2018). The overexpression of *ABCB1* consequently leads to the excretion of medicinal drugs used for cancer treatment causing resistance (Krech et al. 2012; Vallo et al. 2015; Liao et al. 2019), allowing the cancer to progress. *ABCC4* is another type of transporter

that can be detected in prostate cancer. However, the expression of *ABCC4* in prostate cancer is reduced (Peraldo-Neia et al. 2011; Montani et al. 2013; Li et al. 2017; Orellana-Serradell et al. 2019). Testosterone is a substrate of the ABCC4 transporter and due to the reduced expression of the *ABCC4* gene, testosterone accumulates in the prostate. Subsequently, the accumulation of testosterone can promote prostate cancer development. Thereby, testosterone glucuronides are likely to be present.

Currently, prostate cancer is diagnosed by measuring PSA concentrations, taking biopsies and MRI scans. However, MRI scans may not detect the cancer and a biopsy sample may not contain the cancer or multiple biopsies are required. The issue with determining PSA concentrations is that PSA can also be detected in prostatitis (Azab et al. 2012; Lokant and Naz 2014; Li et al. 2017; Lee et al. 2019). Clearly, there are difficulties in current prostate cancer tests and the application of a glucuronide could be used alongside current practices to determine the presence of the cancer.

8.1.6 Liver cancer

The liver is the predominant location for molecule detoxification (Girad et al. 2005; Mueller et al. 2010; Bartl et al. 2015; Hu et al. 2018). A disease that affects the liver could alter the detoxification process of molecules. The implications of liver cancer on the detoxification pathway provide conflicting reports. Ye et al. (2014) reported glucuronidation activity is reduced in liver cancers whilst, a later study reported glucuronidation catalysed by *UGT1A9* is increased (Ge et al. 2019). In other phases such as phase I, multiple *CYP450*s are down-regulated: *CYP1A2*, *CYP2A6*, *CYP2C8*, *CYP2C9*, *CYP2E1* and *CYP3A4* (Ren et al. 2018). The down-regulation of *CYP2C8* is thought to influence survival time of liver cancer after a hepatectomy (Wang et al. 2018). Further research is recommended to elucidate the connection between the expression of *CYP2C8* and survival time (Liu et al. 2019). Additionally, glucuronidation in phase II is altered. Since glucuronidation occurs in the liver potentially, there could be an array of glucuronides produced that may reflect liver cancer. Since the liver is the predominant location for molecule

detoxification, an array of glucuronides could be produced that reflects liver cancer.

Currently, liver cancers can be diagnosed by one of two ways, ultrasound scans (Cottone et al. 1983) or determining alpha-fetoprotein (AFP) concentrations (Chen et al. 1984). AFP protein is produced by the *AFP* gene and highly produced in the foetal liver. In the human adults, normal AFP concentrations is <10 ng/mL (Jeon et al. 2017). The issue with ultrasound is that the cancer may not be revealed from the scan. Determining AFP concentrations is an issue because AFP has been found to be associated with other cancers such as testicular (Paffenholz et al. 2018) and ovarian (Aldrink et al. 2018). Therefore, the application of glucuronide spectrums to determining the presence of liver cancer can be used alongside other diagnostic practices for this disease.

8.2 Results

A total number of 300 papers were collected from Scopus (n=62), Web of Science (n=48), NCBI PubMed (n=123) and Google Scholar (manual search) (n=67). After reviewing the papers for glucuronides associated to a disease (refer to Section 2.3), a total of three papers were eligible for colorectal cancer. Two papers were found reporting glucuronides associated to Type 2 Diabetes Mellitus (Table 8.3). Six papers were found to report glucuronides associated with prostate cancer. Four papers reported glucuronides associated with breast cancer (Table 8.5). Only two papers revealed glucuronides associated to colorectal cancer and genetics (Table 8.2) However, there were not any papers reporting an association of glucuronides for liver cancer and Parkinson's disease.

8.2.1 Colorectal cancer

8.2.1.1 Colorectal cancer, lifestyle and glucuronides

In the one study that was eligible after screening for CRC causation by lifestyle only one glucuronide was detectable, ethyl glucuronide. The study explored metabolites associated with a diet and the metabolite diet-disease relationship. To identify metabolites, the study used serum samples from 502 participants

using two different screening approaches; ultra-high-performance liquid-phase chromatography with tandem mass spectrometry (UPLC/MS) and gas chromatography–mass spectrometry (GC/MS) (Guertin et al. 2014). The ethyl glucuronide only shows that alcohol consumption may contribute to the cause of CRC development but, may not be a glucuronide specifically associated to CRC. Alternatively, ethyl glucuronide was detected in the patients who may have consumed alcohol.

8.2.1.2 Colorectal cancer, genetics and glucuronides

Once the inclusion and exclusion criteria were followed two papers reported glucuronides associated with colorectal cancer. The results are presented in Table 8.2.

Table 8.2: Glucuronides reported associated with colorectal cancer and genetics

Glucuronide	Human sample used	Glucuronide method detection	Polymorphism if specified	P-value reported by the study	Reference
5α-androstane-3α, 17β-diol glucuronide (3αdiol G)	Serum	Direct double-antibody radioimmunoassay	*MMP2* (rs837531, rs1816595, rs12924764, rs11643163, rs7187242)	0.91	(Meyer et al. 2012)
			CD14 (rs778583, rs778584, rs7721577, rs2569188, rs2569193, rs12517200, rs3822356, rs753279, rs1583005)	0.80	
			IK (rs2569188, rs2569193, rs12517200, rs3822356, rs753279, rs1583005, rs2286394)	0.80	
			PRG2 (rs10400305, rs3741089, rs490358, rs10792094, rs555097, rs3851114, rs3741085, rs10792095)	0.92	
SN-38 glucuronide	Venous blood	High-performance liquid chromatography	*UGT1A1*28*	P=0.022	(Hazama et al. 2010)

8.2.2 Type 2 Diabetes Mellitus

After examining the two papers related to T2DM causation by lifestyle all glucuronides have been reported (Table 8.3). This potential spectrum only presents three detectable glucuronides associated with T2DM and lifestyle factors. However, there could be more glucuronides which remains to be detected and added to the potential glucuronide spectrum.

Table 8.3: Glucuronides reported that are associated with T2DM caused by lifestyle factors.

Glucuronide name	Human sample used	Glucuronide detection method	P-value reported by the study	Study
2-phenylethanol glucuronide	Serum	UPLC/MS	Not Reported	(Zhao et al. 2017)
Deoxycholic acid 3-glucuronide	Serum	UPLC/MS	Not reported	(Zhao et al. 2017)
Androstanediol glucuronide (detected in males)	Serum	Roche Elecsys 2010 Immunoassay Analyser	P<0.05	(Li et al. 2017)

In one of the two studies referenced in Table 8.3 the ethnicity has been reported from the participants involved in the study. Whereas, the other study did not report ethnicity of the participants. Li et al. (2017) reported the participants in the study the ethnicities to be: African-American, Hispanic, Non-Hispanic and Mexican. However, the authors did not associate glucuronide production to a particular ethnicity. Thus, this suggests that androstanediol glucuronide (ADT-G) is present in all ethnicities. Additionally, the number of people reported for each ethnicity is not reported in the study. Conversely, Zhao et al. (2017) did not report any ethnicities in the study. Therefore, further work is recommended to associate whether glucuronides produced are the same in different ethnicities in the presence of T2DM or whether, there could be a variation in glucuronide production in different ethnicities.

8.2.3 Parkinson ’s disease

After following the protocol outlined in Section 2.3, using the inclusion and exclusion criteria, no eligible studies has been identified. Therefore, further work is necessary in the form of metabolomic studies to identify potential glucuronides associated with PD.

8.2.4 Prostate cancer

8.2.4.1 Prostate cancer, genetics and glucuronides

In Table 8.4 below presents all potential glucuronides that could be detected to aid in the diagnosis of prostate cancer, caused by genetics. This potential spectrum could be used alongside with other diagnosis methods to confirm the presence of prostate cancer. Multiple studies reported in Table 8.4 report the same three glucuronides androsterone glucuronide (ADT-glucuronide), androstane-3α,l7β-diol-3-glucuronide (3α-diol-3G) and androstane-3α,17β-diol-17-glucuronide (3α-diol-17G) for prostate cancer. Therefore, this validates that these three glucuronides are associated to prostate cancer.

Table 8.4: A potential glucuronide spectrum for prostate cancer influenced by genetics

Glucuronide name	Human sample used	Method of detection	Polymorphism if specified	P-value reported by the study	Reference
Androstane-3α,17β-Diol-Glucuronide	Prostate biopsy sample and serum sample	Enzyme Immunoassay (EIA)	*UGT2B15* (D85Y)	<0.001	(Grant et al. 2013)
Androsterone glucuronide and 3α-diol-3G	Plasma and tissue	Gas chromatography and Liquid chromatography-tandem MS	*SRD5A1* (rs518673, rs166050, rs518673, rs166050, rs518673 rs166050) *SRD5A2* (rs12470143, rs2208532, rs4952197, rs523349 and rs676033)	Not specified	(Lévesque et al. 2014)
ADT-glucuronide (ADT-G) 3α-diol-3G 3α-diol-17G	Plasma	Gas chromatography-mass spectrometry or liquid chromatography-tandem mass spectrometry	*UGT2B17* null deletion genotype	Not specified 0.0005 0.018	(Nadeau et al. 2011)
5α-androstane-3α,17β-diol glucuronide	Blood	Radioimmunoassay	*SRD5A2* (V89L)	P=0.002	(Hsing et al. 2001 [b])

Unfortunately, none of the studies, excluding one of the studies did not associate glucuronide production and ethnicity. Hsing et al. (2001), reported 5α-androstane-3α,17β-diol glucuronide in an Asian population. Thus, this suggests that glucuronide production may not vary between different ethnicities. However, multi-ethnic studies are necessary to elucidate the possibility of any variation in glucuronide production in a disease within different ethnicities.

8.2.4.2 Prostate cancer, lifestyle and glucuronides

The one study dated 2009 that matched all inclusion criteria for this systematic review, used blood samples and took into consideration participant lifestyle

(height, weight, smoking, physical activity and diet, including alcohol). Androstanediol glucuronide (A-diol-g) measured by a radio-immunoassay (RIA). The study concluded that smoking does not alter glucuronide concentrations ($P<0.0001$), physical activity did not alter glucuronide concentrations and alcohol consumption was not reported and the effect it had on A-diol-g production (Suzuki et al. 2009). Therefore, it is evident that further and up to date research is necessary to elucidate whether there are other glucuronides associated with prostate cancer and lifestyle. Alternatively, whether there are other potential lifestyle factors such as nightshift work alert glucuronide concentrations.

8.2.5 Breast cancer, genetics and glucuronides

The two applicable studies presented in Table 7.5 after analysing, only two glucuronides have been found to be produced in breast cancers. However, there could be other glucuronides associated to breast cancer which could be detected.

Table 8.5: Glucuronides associated with breast cancer and genetics

Glucuronide name	Human sample used	Method of detection	Polymorphism if specified	P-value reported by the study	Reference
Estrone-3-Glucuronide (E1G)	Plasma and Urine	RIA	CYP3A rs10273424	Not specified for plasma $P=1x10^{-5}$	(Johnson et al. 2012)
Estrone-3-Glucuronide	Urine	Enzyme-linked immunosorbent assay (ELISA)	CYP3A7*1C	Not reported	(Johnson et al. 2016)
Tamoxifen-N-Glucuronide (TAM-N-G)	Venous blood	HPLC	*UGT1A4* (rs6755571, rs2011425) and *UGT2B7* (rs7439366)	Not reported	(Romero-Lorca et al. 2015)
4-OH tamoxifen-O-Glucuronide (TAM-O-G)				Not reported	

After a further analysis of the papers, a search was taken into each of the studies referenced in Table 8.5, to explore whether ethnicities have been reported. Johnson et al. (2012), had patients of white ethnicity from the United Kingdom. However, Johnson et al. (2016) did not report any ethnicities. Romero-Lorca et al. (2015) did not report ethnicities. Therefore, providing evidence that multi-ethnic studies are recommended which elucidates whether glucuronide species produced are different within ethnic backgrounds.

8.2.6 Liver cancer and glucuronides

Unfortunately, after screening there were not any glucuronides that have been associated with liver cancer influenced by genetics or lifestyle factors. It is known that the liver functions to detoxify the body from endogenous (Bosma et al. 1995; Ando et al. 1998; Hong et al. 2004; Lin et al. 2006; Hirata et al. 2010; Ekström et al. 2012) and xenobiotic molecules (Iyer et al. 2002; Ortega et al. 2007; Larson and Richards 2009; Buchard et al. 2012; Raza et al. 2015; Kuzikov et al. 2019). Therefore, there could be an alteration in glucuronide production due to the presence of cancer. However, there could be difficulties in identifying potential glucuronides associated with liver cancer. This could potentially be due to the different types of liver cancer and thereby, metabolic/biomarker detection studies should be conducted to reveal any

potential glucuronides produced associated with different types of liver cancer (*see Discussion Section 8.3.5*).

8.3 Discussion and conclusion

The glucuronide spectrum consists of different glucuronide species that are produced from glucuronidation in phase II of the detoxification pathway. Abnormalities in the detoxification pathway may alter the production of glucuronide species which can be used to construct glucuronide spectrums. In previous work, genotyping was used to identify polymorphisms associated to a disease (Zwemmer et al. 2004; Hsiao et al. 2008; Jung et al. 2012; Jakobsen et al. 2014; Takeuchi et al. 2017), which is insufficient to determine a disease. This is because the presence of a variant may not be the cause of disease. Alternatively, determining a drug which an individual may or may not use (Bunten et al. 2011; Gadel et al. 2013; Kharasch et al. 2015; Burgess et al. 2017). This is because even though a gene variant is present the disease may not be present. Thus, by having a glucuronide spectrum can confirm the presence of a disease and could be used to monitor disease overtime.

The aim of this review was to construct a glucuronide spectrum using glucuronides that have been reported in published research that are disease associated. The aim of this review has been achieved. Glucuronide spectrums have been successfully constructed that reflects all known glucuronides associated to a disease. The glucuronide spectrums that reflect glucuronides produced in the presence of genetic variants with genetics could be used alongside genetic tests to determine gene variants and disease. This is because these results show glucuronide synthesis associated to a disease, with the presence of gene polymorphisms and lifestyle (see Results section).

8.3.1 Type 2 Diabetes Mellitus

Both 2-phenylethanol glucuronide and deoxycholic acid 3-glucuronide are naturally occurring glucuronides which are synthesised from 2-phenylethanol (Dong et al. 2015) and deoxycholic acid (Perreault et al. 2018), respectively. Both glucuronides are found to be predominantly synthesised in the presence of

T2DM (Sun et al. 2014; Zhao et al. 2015; Zhao et al. 2017). Therefore, this suggests that molecular pathways such as the phenylethylamine pathway and the Ehrlich pathway (2-phenylethanol synthesis) and deoxycholic acid synthesis pathway could be upregulated (Zhao et al. 2017). Alternatively, the UGTs which glucuronidate deoxycholic acid and 2-phenylethanol could be upregulated.

Androstanediol (ADT) is a metabolite of dihydrotestosterone (DHT), which is a neurosteroid with anticonvulsant activities. This is because androstanediol can bind to GABA receptors in the brain (Frye et al. 1996; Imamura and Prasad 1998; Frye et al. 2001; Reddy 2004; Kaminski et al. 2006; Reddy and Jian 2010; Krishnan et al. 2012; Shahrzad and Nasser 2015; Chuang et al. 2019). DHT is an intermediate molecule for testosterone synthesis and since ADT is a metabolite of DHT low concentrations of ADT-G is expected in T2DM (Joyce et al. 2016). 2-phenylethanol glucuronide was also detected in one of the excluded studies which used male Wistar rats, modelling T2DM. Sample collected were urine and blood samples which were analysed by UPLC/MS (Sun et al 2014).

Furthermore, glucuronides have been reported that are associated to T2DM and lifestyle factors in a murine study. In a excluded study that did not meet the inclusion criteria. Sun et al. (2014) reported that 2-phenyethanol glucuronide (P=0.02) is produced alongside with 6-hydroxy-5-methoxyindole glucuronide (P=0.03), in rats that were fed a high fat diet. Therefore, this contributes to the validation that 2-phenyethanol glucuronide is associated with T2DM. Furthermore once glucuronide spectrums for disease in humans have been revealed experimentally, glucuronide spectrums could be applied to disease diagnostics for animals.

8.3.2 Colorectal cancer

3αdiol G is a type of androstanediol glucuronide that can be detected in human samples (Morimoto et al. 1981; Moghissi et al. 1984; Lobo et al. 1987). Androstanediol glucuronide has also been detected in T2DM but, the specific type of androstanediol glucuronide detected in Li et al. (2017) was not discussed. Therefore, for disease diagnosis clarity and the exact glucuronide

name should be reported. However, there could as well be other glucuronides which may have not been reported but, yet could be detectable. This is because androstandediol is an umbrella term for the following specific androstanediol molecules: 3β-Etiocholanediol (5β-androstane-3β,17β-diol; etiocholane-3β,17β-diol), 3α-Etiocholanediol (5β-androstane-3α,17β-diol; etiocholane-3α,17β-diol), 3α-Androstanediol (5α-androstane-3α,17β-diol) and 3β-Androstanediol (5α-androstane-3β,17β-diol). Therefore, these other molecules could also be potential biomarkers for CRC. Additionally, SN-38 glucuronide has been associated with colorectal cancer and glucuronides because even though it is a drug, it is still glucuronidated in the presence of *UGT1A1*28* (Table 8.3).

8.3.3 Prostate cancer

In the present review, several glucuronides have been reported that are associated with prostate cancer influenced by genetics (Table 7.5). ADT-G derives from androsterone by which ADT is a metabolite of dihydrotestosterone. (DHT) (Pirog and Collins 1999; Bloch et al. 2006). 3α-diol-3G has also been found to be glucuronides associated with prostate cancer and genetics, which has been detected in multiple studies in Table 7.5, showing validation and evidence that 3α-diol-3G is associated with prostate cancer. Androstenadiols are also metabolites of DHT (Moghissi et al. 1984; Toscano and Horton 1987). Currently, ADT-glucuronide, 3α-diol-3G, 3α-diol-17G and 5α-androstane-3α,17β-diol glucuronide are the only known glucuronides that are associated with prostate cancer. But, there could be other glucuronides which are associated with prostate cancer but have not yet been detected. Thus, the detection of the known glucuronides in this case suggests that hormone concentrations are increased. Hsing et al. (2001) reported 3α-diol-17G in an Asian population, but the other studies did not report ethnicities. Therefore, multi-ethnic studies are recommended to reveal glucuronide spectrums for a disease in different ethnicities. This is because, the glucuronide species produced may have a variation in different ethnicities and thus, there could be a variation in the spectrum in different ethnicities.

8.3.4 Breast cancer

Oestrone-3-glucuronide (E1G) is synthesised from oestrone, a type of oestrogen (Strassburg et al. 2002; O'Connor et al. 2003; Thibaudeau et al. 2006). E1G is the only known glucuronide that is associated with breast cancer however, there could be other associated glucuronides which may be associated with breast cancer. Oestrone has functional roles in influencing the onset of the reproductive menstrual cycle alongside with oestradiol (Sipavičiené et al. 2015). In breast cancers, the concentrations of oestrone have been reported to increase in premenopausal woman (Miyoshi et al. 2003; Adly et al. 2006; Woolcott et al. 2010; Falk et al. 2013; Endogenous Hormones and Breast Cancer Collaborative Group 2015; Goedert et al. 2018), hence E1G has been detected. However, oestradiol glucuronides were not detected, despite of oestradiol concentrations have been reported to be increased in postmenopausal women, increasing the risk of breast cancer (Holmberg et al. 2001; Saito et al. 2004; Tamimi et al. 2007; Potter et al. 2009; Walker et al. 2011; Shoemaker et al. 2014; Sampson et al. 2017). Therefore, further metabolic/metabolite biomarker studies are recommended, to show other potential metabolites associated with breast cancer.

Tamoxifen is a hormone-therapy based drug used for the treatment of breast cancer (Frasor et al. 2006; Cajol et al. 2010; Cuzick et al. 2014; Neven et al. 2018). The mechanism of tamoxifen is by binding to oestrogen receptors (ERs) inducing conformational changes (Gorodeski et al. 1992; Webb et al. 1995; Shiau et al. 1998). The binding of tamoxifen to oestrogen receptors prevents further cancer development, preventing oestrogen from binding to oestrogen receptors. Tamoxifen has been demonstrated to undergo glucuronidation by UGT1A4 (Sun et al. 2004) and UGT2B17 (Romer-Lorca et al 2015). In the presence of gene variants, tamoxifen can still be glucuronidated as demonstrated in the results in Table 7.6.

8.3.5 Liver cancer

After examining all collected papers (n=87), there were not any papers that revealed glucuronides associated with liver cancer for both genetic and lifestyle.

The liver is responsible for the detoxification of xenobiotics and endogenous molecules. This is due to that the research available has not discussed glucuronide synthesis and liver cancer. This may be due to the fact that liver cancer can appear as primary liver cancer including: hepatocellular carcinoma (HCC) (Naugler et al. 2007), cholanigocarcinoma (bile duct cancer) (Inaba et al. 2007), liver angiosarcoma (Averbukh et al. 2018) and hepatoblastoma (Pham et al. 2015) or secondary liver cancer caused by other cancers that have metastasised such as colorectal cancer metastasis to the liver (Fong et al. 1999; Choti et al. 2002; Gupta et al. 2005; Zeng et al. 2008; Halama et al. 2011; Landreau et al. 2014; Moro et al. 2018). Exploring glucuronides from liver cancers could not only be used to determine the presence of a type of liver cancer but, the glucuronide species to construct a glucuronide spectrum, may be able to determine the difference between primary and secondary liver cancer.

8.36 Parkinson's disease

After reviewing all papers for PD (n=16), there were not any papers of glucuronides reported that are linked with the disease. Therefore, further research in the metabolomics of PD to establish all potential metabolites including glucuronides that are associated with PD. In despite of *CYP2D6*4* has been found to have a link with PD as discussed in Section 7.1.3 Once the metabolites have been revealed from metabolomic studies, then potential glucuronide spectrums for PD could be revealed.

8.4 Conclusion

The aim of the review was to explore the potential application of glucuronides for disease prognostics. The results presented in this review demonstrate that there are glucuronide spectrums associated with diseases. In colorectal cancer, three glucuronides have been associated with genetic predispositions of colorectal cancer (Table 8.3): 5α-androstane-3α, 17β-diol glucuronide and SN-38 glucuronide. In Type 2 Diabetes Mellitus, three glucuronides have been found to have an association with diet. In prostate cancer three glucuronides were reported associated with multiple polymorphisms, UGT2B15 (D85Y),

UGT2B15 null deletion genotype, *SRD5A1* and *SRD5A2* (see Table 8.4 for polymorphisms). Androsterone glucuronide is the only glucuronide that has been associated with prostate cancer and lifestyle factors. Breast cancer influenced by genetics has three glucuronides associated to the disease estrone-3-glucuronide (*CYP3A* rs10273424 and *CYP3A7*1C*) , tamoxifen-n-glucuronide (UGT1A4: rs6755571 and rs2011425), and 4-OH tamoxifen-O-glucuronide (UGT2B7: rs7439366). However, the link between glucuronide production and ethnicity is not fully elucidated. Therefore, multi-ethnic studies are recommended for future work.

8.5 Strengths and limitations

The strength of this review reveals that there are glucuronide spectrums diseases (*see Results section*), based on available published research which matched the inclusion criteria implemented in this review. Additionally, the concept of a glucuronide spectrum has not been discussed in previous research. To date, this is the first systematic review that has explored the concept of glucuronide spectrums associated to disease for prognosis. But, there are limitations that should be considered.

The limitations to this review is that the concentrations of glucuronides have not been discussed in the ellegible studies in relation to a disease. Furthermore, the ellegible studies used in the review did not discuss or compare glucuronide concentrations in patients diagnosed with a disease and healthy controls. Additionally, it is evident that the glucuronides reported were not linked to a particular ethnicity. Therefore, ethnic studies and glucuronide concentrations are recommended, glucuronide concentrations may vary in a disease amongst different ethnicities.

Chapter 9: Conclusion

9.1 Summary of the thesis

The aim of the thesis was to explore the potential application of glucuronides for disease prognosis. The aim of this chapter has been successfully achieved as it is evident within the results section of Chapter 8, with the revealing of different potential glucuronide spectrums, associated with a number of different diseases. Several Chapters have been produced to discuss the implications of gene variants influencing the fate of different molecules when they are detoxified within the liver to achieve the implemented objectives of the research. Respectively, objectives 1 and 2 was to evaluate genetic and lifestyle factors that may affect the detoxification pathway, which has been achieved. Objective 3 was to evaluate different human gene allele frequencies that is reported Chapters 3 to 7. Objective 4 was to reveal potential glucuronide spectrums reflecting objectives 1 and 2, which has been achieved in the results section of Chapter 8. Objective 5 was to postulate the potential clinical applications of the research has been discussed in Section 9.2 (see below).

Chapter 3 discussed the implications of gene variants on the detoxification on medicinal drugs. In despite of medicinal drugs having a role for their therapeutic effects, medicinal drugs can cause adverse effects and interfere with the detoxification other molecules. For instance, clotrimazole is used for the anti-fungal properties against dermatological conditions such as thrush. However, clotrimazole interferes with the glucuronidation of polycyclic aromatic hydrocarbons (PAHs) (Das et al. 1986). Clotrimazole can also induce hepatotoxicity which could also disrupt the detoxification of other molecules and lead to the progression of other diseases such as cancers. However, further research is recommended to reveal how clotrimazole influences the detoxification of medicinal drugs and endogenous molecules.

Chapter 4 discussed the detoxification of illicit drugs and how they are affected by genetic variation. For one example, In despite of methadone being used in opioid withdrawal programmes for the medicinal properties, methadone is an illicit drug. The use of methadone can also lead to addiction and prolonged use of methadone can lead to methadone related deaths. Methadone related deaths

can be influenced by two gene variants, *CYP2B6*6* (reduced expression) (Gadel et al. 2013) and *CYP2D6*4* (non-functional) (Gough et al. 1990) Both variants could also contribute to methadone related deaths as a result of increased methadone molecules. Furthermore, drug-drug based interactions may also influence methadone related deaths, for example quinidine and methadone. Quinidine alters the fate of methadone by suppressing MDR1 transport activity of methadone. Thus, individuals who take quinidine along with methadone, shows increased methadone concentrations and as a result this could also contribute to methadone related death (Kharasch et al. 2004; Kharasch et al. 2008).

Food additives are used to improve the quality, taste, appearance, and shelf life. Before food additives are applied to food, they are rigorously tested and declared whether an additive is safe by the European Food Safety Authority (EFSA). However, in despite of testing food additives, they still pose as a risk to human health. For instance, as discussed in Section 4.1, individuals with phenylketonuria (PKU) have a mutated phenylalanine hydroxylase (Gámez et al. 2000; Muntau et al. 2002). Thus, when phenylalanine concentrations are increased, phenylalanine is toxic. Increased phenylalanine can lead to seizures and other cognitive impairments (Martynyuk et al. 2007; Karimzadeh et al. 2012; Danafar and Hamidi 2015), due to the consumption of aspartame containing foods.

Endogenous molecules as discussed in Chapter 6, are naturally produced within the body (Mendelson et al. 1975; Chung and Allison 1979; Bowen et al. 2011). However altered concentrations of endogenous molecules can be harmful. In hormone driven cancers such as prostate cancer, the cancer can be influenced by increased hormone concentrations (Stanbrough et al. 2006; Coward et al. 2009; Muller et al. 2012), by which abnormal concentrations induce abnormal cellular growth. Furthermore, mutations within the detoxification pathways such as the *UGT2B17* deletion, increases testosterone concentrations. Furthermore, in other diseases such as Gilbert's syndrome, increased bilirubin concentrations are present that leads to jaundice due to the *UGT1A1*28* variant with reduced expression (Ando et al. 1998; Persico et al.

2001). Additionally, individuals who have Gilbert's syndrome cannot detoxify other molecules effectively such as PhIPs leading to a potential onset of colorectal cancer (CRC) (Girard et al. 2005; Chang et al. 2007).

Xenobiotics, discussed in Chapter 7, can also influence the onset of disease through different molecular interactions. For instance, as discussed in Section 6.1, PhIPs have the ability to bind to DNA forming adducts (Ghoshal et al. 1995; Dingley et al. 1999), that are carcinogenic contributing to colorectal cancer development. Xenobiotics can also interfere with the detoxification of other molecules. Ethanol can also be detoxified via glutathione (GSH) detoxification (Otis and Guidot 2010; Chitty et al. 2013; Chen et al. 2017), likewise for acetaminophen (APAP). Thus, while ethanol depletes GSH molecules in the liver, APAP is able to bind to molecules within the liver to promote hepatotoxicity.

The main strength from the thesis is that the aim has been successfully achieved by revealing different glucuronide spectrums for a number of disease (Section 7.3). The newly revealed glucuronide spectrums also reveal whether a disease is influenced by genetics or lifestyle factors. Furthermore, if a disease is influenced by genetic factors, the glucuronide spectrum could help to identify what gene variants are present that influenced a disease as evident in Tables 7.3, 7.5, and 7.6. Therefore, the application of glucuronides spectrums could be used for disease prognostics and diagnostics in the future.

The major limitation to the thesis is that there is not experimental work, validating the theoretical results presented in the results section of Chapter 7. Without the theoretical results that show whether glucuronide spectrums existed or not, it appeared inappropriate to conduct laboratory work until theoretical results have been reported. However, the experimental work can be done for future work to validate the theoretical results as discussed in Section 8.2 below.

9.2 Future directions

To develop the research further, the next step is to undertake laboratory work to validate the theoretical findings. The laboratory work will use human biological samples (blood and urine) from diagnosed patients, with a disease discussed in Chapter 7, who provide informed consent following the Data Protection Act 2018. Once the samples have been received the blood samples will be centrifuged to obtain the different components of blood and stored in the fridge until analysis. Urine samples will also be stored in the fridge until analysis or frozen. Furthermore, healthy controls will also be invited to provide blood and urine samples. Sample analysis will include screening for glucuronide spectrums by matching patient and healthy control based on age, gender (Kutner et al. 2000; Vaidya et al. 2003; Frankfort et al. 2006; Baum et al. 2009; Chiu et al. 2011; Booth et al. 2014; Yeh et al. 2016; Karlin et al. 2018; Wang et al. 2019), and ethnicity. This is to reveal whether there are variations within a glucuronide spectrum between different ethnicities and between healthy people and people who have been diagnosed with a disease. The screening method to reveal glucuronide spectrums will use the UPLC-HRMS system. This is because UPLC-HRMS is a common method for metabolomics studies (Nordström et al. 2006; Zelena et al. 2009; Syzmańska et al. 2011; Sun et al. 2014; Li et al. 2016; Schoeman et al. 2018; Zhao et al. 2019). Once a glucuronide spectrum has been determined, the next step would be to determine the concentrations of each glucuronide specie. The purpose of determining glucuronide concentrations is to elucidate if a specific glucuronide is produced in increased or decreased concentrations. Once, experimental data has been gained which reveals glucuronide spectrums (validating the results in the thesis and experimental repeats have been performed, validating the initial experimental findings) and concentrations are determined then the application of glucuronide spectrums could be used alongside current practices for disease prognostics.

9.3 Concluding remark

Glucuronides are molecules that are produced in the liver by the detoxification pathway of different molecules. However, the detoxification pathway can be affected by genetics and lifestyle factors, altering the fate of a molecule. The aim of the thesis was to explore potential glucuronide spectra for different diseases caused by genetics and lifestyle factors. The aim of the thesis has been successfully achieved with revealing different glucuronide spectra for different diseases. Thus, the next step from this research is to conduct experimental work to reveal different glucuronide spectrums and to determine the concentrations of each glucuronide specie.