Determining the Effect of Isotretinoin on CYP2D6 and CYP3A4 Activity in Patients with Severe Acne

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Abstract

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Cytochrome P450 2D6 (CYP2D6) and 3A4 (CYP3A4) are critical enzymes involved in the metabolism of commonly used drugs. There are high inter-individual variabilities in CYP2D6 drug-metabolizing activities, majorly caused by genetic polymorphism and transcriptional regulation. Previous *in vitro* and preclinical *in vivo* studies have shown the CYP2D6 expression was regulated by small heterodimer partner (SHP) as a transcriptional repressor via interacting with hepatic nuclear factor (HNF) 4α. Investigating potential drug-drug interactions resulting from CYP2D6 downregulation *in vitro* and *in vivo* is necessary. Isotretinoin (13-*cis*-retinoic acid) is an FDA-approved drug used to treat severe, recalcitrant, nodulocystic acne and neuroblastoma. Previously 13-*cis*-retinoic acid, its isomer, all-*trans*-retinoic acid, and its metabolite 4-oxo-13-*cis*-retinoic acid were shown to downregulate CYP2D6 expression and induce SHP expression *in vitro,* but the effect was not observed in healthy males. CYP3A4 was shown to be induced after treatment with isotretinoin *in vitro* and *in vivo*. A larger participant size and mixed sexes were necessary to fully capture the role of retinoid impact on CYP2D6 and CYP3A4 regulation. In this study, thirty-three participants (22 females and 11 males) who

required isotretinoin treatment for acne were recruited. There were two study time points, including pre-isotretinoin control and after isotretinoin initiation for at least a week. Dextromethorphan plasma and urinary metabolic ratios were compared between two study time points to indicate changes in CYP2D6 and CYP3A4 activity after isotretinoin administration. No statistically significant difference was reported in CYP2D6 activity, while a weak induction in CYP3A4 activity was observed after isotretinoin administration. Future investigation on the mechanism of CYP2D6 regulation by retinoids in humans is necessary.

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Chapter 1:

Introduction

Abstract

Cytochrome P450 2D6 (CYP2D6) enzyme is predominately expressed in the liver and responsible for metabolizing approximately 25% of the commonly used drug. The *CYP2D6* gene is highly polymorphic and has non-, decreased-, or functional genetic variants with varied frequencies across different populations. CYP2D6 polymorphisms contribute to high inter-individual variability observed in CYP2D6 drug-metabolizing activity. CYP3A is primarily expressed in the liver and gut and is responsible for metabolizing approximately 50% of commonly used drugs. Dextromethorphan, a widely used antitussive agent, has been previously used for CYP2D6 phenotyping as well as a dual-probe substrate for CYP2D6 and CYP3A4 activity in clinical studies. Single time point plasma or cumulative urinary dextromethorphan metabolic ratios have been used as surrogate markers for CYP2D6 and CYP3A4 *in vivo* activity. Small heterodimer partner (SHP) - hepatic nuclear factor (HNF) 4α pathway has a critical role in CYP2D6 expression regulation demonstrated by previous *in vitro* and CYP2D6 humanized mice studies. There are potential drug-drug interactions between CYP2D6 substrates and drug that downregulates CYP2D6 transcriptional regulation. Previous studies suggested that 13-*cis*-retinoic acid, all-*trans*-retinoic acid, and 4-oxo-13-*cis*-retinoic acid have drug-drug interaction potentials with cytochrome P450 enzymes and/or transporters via diverse mechanisms. Isotretinoin (13-*cis*-retinoic acid), as an approved drug for severe acne and neuroblastoma treatment, has been investigated for its contribution to the regulation of CYP2D6 and CYP3A4

activity. Previously in human hepatocytes, 13-*cis*-retinoic acid, all-*trans*-retinoic acid, and 4-oxo-13-*cis*-retinoic acid have been shown to induce SHP and downregulate CYP2D6 mRNA expressions as well as induce CYP3A4 expressions. However, downregulation of CYP2D6 *in vitro* by retinoids did not translate in healthy male adults with isotretinoin administration, while CYP3A4 induction was observed in the participants likely via activation of pregnane X receptor (PXR).

1.1 CYP2D6 Pharmacogenomics and Expression Regulation

1.1.1 Background to Cytochrome P450 Enzymes and CYP2D6

Cytochrome P450 (CYP) enzymes constitute one of the enzyme superfamilies that participate in phase I drug metabolism of xenobiotics and endogenous compounds[1]. CYPs typically oxidize drugs into more hydrophilic compounds for excretion[1]. There are 57 putative *CYP* functional genes, along with 58 pseudogenes within 18 families, that are encoded in humans[2]. Among the 57 functional CYP enzymes, 12 belong to CYP1, CYP2, and CYP3 -families and are responsible for the metabolism of 70-80% of approved drugs and xenobiotics[3]. The expression and activity of CYP2A6, CYP2B6, CYP2C9, CYP2C19, and CYP2D6 are highly variable between individuals[4]. The variability in activity is mainly due to gene mutations, including copy number variants, insertions and deletions, and missense mutations[4]. The polymorphisms of *CYP* genes greatly affect the metabolism of the drugs that are substrates for those CYP enzymes and lead to variations in drug response and adverse effects between individuals[4].

Cytochrome P450 2D6 (CYP2D6) has been extensively studied. CYP2D6 is predominantly expressed in the liver, intestinal tissue, and brain, with the highest expression in the liver[5–7]. The typical substrates for CYP2D6 are mostly lipophilic bases and include analgesics, antidepressants, antihypertensives, anti-cancer agents, antipsychotics, antiarrhythmics, and antiemetics[5,8]. CYP2D6 has a high affinity and low capacity for many of its substrates and appears to be easily saturated at relatively low substrate concentrations[8]. Compared to other CYPs, CYP2D6 is mostly not inducible by environmental agents or common enzyme inducers, including steroids[9]. 3-methylcholanthrene, phenobarbital, and rifampin are well-known as

inducers of drug-metabolizing enzymes[10]. Yet, previous real-time-PCR (RT-PCR) data measuring CYP2D6 mRNA showed that these common xenobiotics do not induce CYP2D6 mRNA in human hepatocytes compared to controls[10–12]. However, some natural products such as valerian and *G. biloba* increased CYP2D6 activity in a dose-dependent manner in human hepatocytes[13]. Valerian and *G. biloba* increased CYP2D6 activity by approximately 141% and 143% at 1875 and 219 µg/mL concentrations, respectively, using dextromethorphan as probe substrate[13]. Allosteric activation of CYP2D6 enzyme by the compounds in the herbs was proposed as the mechanism of this observed increase[13]. CYP2D6 can be inhibited by many drugs leading to clinical drug-drug interactions that range from mild to fatal in severity[7]. The inhibitors include irreversible inhibitors such as cimetidine, paroxetine, and methamphetamine and reversible inhibitors such as olanzapine[7].

1.1.2 Overview of CYP2D6 Genetic Polymorphisms

Previous studies have shown that the *CYP2D6* gene is highly polymorphic and has many different variants that are functional, have reduced function, or are non-functional[14]. Most genetic variants are single nucleotide polymorphisms (SNPs), nucleotide insertions or deletions, and structural gene copy number variants, including multiplications, large deletions, and duplications[5]. Some non-functional *CYP2D7* and *CYP2D8* pseudogenes share high similarity with *CYP2D6* and add complexity to precise genotype determination for CYP2D6[5,15]. The frequency of CYP2D6 genetic variants has been shown to vary between different populations[16]. In the European population, the predominant *CYP2D6* alleles (71%) are the functional alleles, and the non-functional alleles account for 21% of the allele variability (mainly *CYP2D6*4*)[16]. In Asians, CYP2D6 functional alleles only represent ~50% of CYP2D6 alleles[16]. The reduced

function allele (*CYP2D6*10*) has a median of 41% frequency of *CYP2D6* alleles in Asians and Pacific Islanders, while a lower frequency is present in North, Central, and South America[16]. In Africans and African Americans, functional alleles also represent about 50% of the frequency of the *CYP2D6* alleles, and reduced function alleles account for 35% of the variability (mainly *CYP2D6**17)[16]. The common CYP2D6 variant allele frequencies across populations are shown in Table 1.1[14,17,18].

CYP2D6 polymorphisms contribute to the large inter-individual variability observed in the pharmacokinetics and disposition of CYP2D6 substrates[19]. Non- or decreased function alleles decrease the clearance and increase the exposure of the CYP2D6 substrate drugs, which can affect drug response and/or lead to adverse drug reactions[8]. The activity scores (AS) have been broadly used to translate CYP2D6 genotypes into phenotypes. Each allele has been assigned to values of 0, 0.5, and 1 to represent no, decreased, or normal function of CYP2D6 as shown in Figure 1.1, and the sum of the values is used as AS of CYP2D6 genotypes[20,21]. There are four phenotype groups that have been identified: poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM), and ultra-rapid metabolizers (UM)[22,23].The Clinical Pharmacogenetics Implementation Consortium (CPIC) method recommends that AS=0 is classified as PM, AS=0.5 as IM, AS=1 to 2 as EM, and AS>2 as UM[24–27]. Individuals who are homozygous for deficient CYP2D6 alleles are identified as poor metabolizers (PM) and have significantly reduced clearance of CYP2D6 substrate drugs[8]. Standard doses of CYP2D6 substrate drugs can result in several-fold higher drug exposure and lead to an increased risk of toxicity or adverse effects in individuals who are PMs[28,29]. Individuals with heterozygosity for one decreased-function allele and one non-functional allele are identified as intermediate

metabolizers (IM)[22]. Oral clearances of dextromethorphan and tolterodine have been shown to be 53- and 22-fold higher, respectively, in extensive metabolizers (EM) than in PMs[30,31].

Gene copy number variations in *CYP2D6* also lead to altered drug exposure[8]. Individuals with more than two copies of CYP2D6 functional alleles are identified as ultra-rapid metabolizers (UM) and have decreased drug concentrations of tramadol[32] and metoprolol observed[33]. Similarly, other studies have shown the codeine metabolite morphine (formed by CYP2D6) concentration increased 10- to 30-fold in the UMs and could potentially lead to toxicity from codeine intake[34]. Probe substrate drugs, including dextromethorphan, tramadol, and metoprolol, have been used for CYP2D6 phenotyping by measuring the metabolic ratios of these drugs[35–39]. The frequencies of the metabolic ratios for dextromethorphan as a CYP2D6 probe substrate drug have shown bimodal or trimodal distributions, which could reflect the CYP2D6 EM, IM, and PM groups[36,40].

1.1.3 Regulation of CYP2D6 Expression and Changes in CYP2D6 Activity During Pregnancy

The mechanisms of CYP2D6 transcriptional regulation have not been extensively described. Both hepatocyte nuclear factor (HNF) 4α and small heterodimer partner (SHP) mRNA expression were positively associated with CYP2D6 mRNA expression in liver tissues[41,42]. The mRNA level of CYP8B1, the target gene for the SHP-HNF4α regulatory pathway, was shown to be a good predictor of CYP2D6 mRNA expression and explains about 25% of the CYP2D6 expression variability[41]. Both HNF4α and SHP mRNA expression were positively associated

with CYP2D6 mRNA expression in liver tissues, suggesting that SHP-HNF4α pathway has a critical role in CYP2D6 expression regulation[41]. Transcription factors, including HNF4α and CCAAT/enhancer-binding protein (C/EBP) α, have been identified to be involved in altering the transcriptional regulation of basal CYP2D6 expression *in vitro*[43]. Knockdown of C/EBPα led to significantly decreased CYP2D6 expression in HepG2 cells[44]. Knockdown of HNF4α or deletion of liver HNF4α led to decreased expression of CYP2D6 in human hepatocytes and mice[45,46]. The overexpression of transcription factor SHP suppresses HNF4α mediated activation of CYP2D6 promoter in HepG2 cells, and knockdown of SHP expression led to significantly increased CYP2D6 expression in CYP2D6 transgenic mice[47,48]. Krüppel-like factor 9 (KLF9) enhanced HNF4α on activation of CYP2D6 promoter and weakly activated CYP2D6 promoter in HepG2 cells[47]. Nuclear receptor farnesoid X receptor (FXR) as a modulator of SHP (upregulation of SHP expression) might also affect the expression and activity of CYP2D6, and there are potential drug-drug interactions between FXR agonists and CYP2D6 substrates[43,49]. Bile acids might also have a potential role in altering CYP2D6 expression and leading to large inter-individual variability in CYP2D6 activity[43]. A positive and marginal correlation was observed between total bile acid concentrations in the liver and CYP2D6 mRNA expression[41].

Multiple clinical studies have indicated an increase in CYP2D6 activity during pregnancy. The mean oral clearance of metoprolol was higher in 22-26 weeks and 34-38 weeks of pregnancy compared to ≥3 months postpartum[50]. The metabolic ratio of dextromethorphan/dextrorphan (DM/DX) was used as the probe of CYP2D6 activity. Plasma DM/DX ratio decreased by 53% after 2 hours of oral dosing of dextromethorphan in homozygous and heterozygous CYP2D6 extensive metabolizers during pregnancy, suggesting an increased CYP2D6 activity was

observed during pregnancy[51]. Urinary DM/DX ratio decreased by 26-48% in a 0–24-hour cumulative urine in CYP2D6 extensive metabolizers during pregnancy compared to postpartum[52]. Similarly, in another study 0-4-hour urine DX/DM MR increased by 43% in CYP2D6 extensive metabolizers during pregnancy compared to postpartum[53]. Evaluation of other CYP2D6 probe substrates, including clonidine and paroxetine, also showed increased clearance during pregnancy[54,55]. However, the increase in CYP2D6 activity during pregnancy was not observed in CYP2D6 PMs or IMs[51,55]. Similarly, decreased steady-state plasma concentrations of paroxetine were only observed in CYP2D6 extensive metabolizers while not in CYP2D6 IMs or PMs[55]. One potential cause could be the transcriptional induction of CYP2D6 during pregnancy[43]. Concurrent changes of multiple transcription factors, such as decreased SHP or increased KLF9 expression, have potential roles in activating CYP2D6 promoter and altering HNF4α regulation *in vitro* during pregnancy[43]. Several *in vitro* and *in vivo* studies indicated female hormones (estradiol and progesterone) and the use of oral contraceptives do not significantly alter the CYP2D6 expression and activity during pregnancy[9,56,57]. Vitamin A supplement was also not observed to change CYP2D6 activity during pregnancy[53]. However, a positive correlation was reported between *all-trans*-retinoic acid concentration and increased CYP2D6 activity during pregnancy and postpartum[53]. More studies are necessary to elucidate the mechanisms of CYP2D6 transcriptional regulation and potential mechanisms that lead to increased CYP2D6 activity during pregnancy.

1.1.4 Clinical Drug-Drug Interactions with CYP2D6 Substrates

Drug-drug interactions (DDI) have been observed between CYP2D6 substrates such as dextromethorphan, metoprolol, and others, with inhibitors such as paroxetine, quinidine,

terbinafine and perhexiline[58]. These interactions were different between CYP2D6 EMs and PMs[58]. Pre-treatment with quinidine significantly suppressed dextrorphan formation clearance and increased plasma concentration of dextromethorphan in CYP2D6 EMs[59]. Similarly, treatment with fluoxetine also impaired propafenone metabolism by CYP2D6 with decreased oral clearance and increased plasma AUC and C_{max} of R- and S- propafenone in CYP2D6 EMs[60]. Paroxetine treatment also increased atomoxetine (CYP2D6 substrate) AUC in CYP2D6 EMs[61]. Terbinafine and quinidine, two CYP2D6 inhibitors, increased the metabolic ratio of DM/DX in CYP2D6 EMs but not in PMs[62,63]. Coadministration of metoprolol with CYP2D6 inhibitors (diphenhydramine or dronedarone) impaired the CYP2D6-mediated metoprolol metabolism and increased the AUC of metoprolol in CYP2D6 EMs but not in PMs[64–67]. Increased metoprolol exposure led to clinically significant negative chronotropic and inotropic effects[64–67]. Diphenhydramine and propafenone inhibited the CYP2D6-mediated metabolism of venlafaxine and mexiletine significantly in CYP2D6 EMs but not in PMs[68,69]. Coadministration of CYP2D6 inhibitors, including paroxetine, amiodarone, and cimetidine with tramadol, showed a significantly decreased formation of the active metabolite of tramadol, (+) O-desmethyl tramadol[70]. Greater reductions in (+) O-desmethyl tramadol concentrations were observed in EMs compared to IMs[70]. CYP2D6 inhibitors can cause clinically relevant DDIs with CYP2D6 substrates, especially those with a narrow therapeutic window[66]. These studies show that CYP2D6 substrate clearances can be inhibited, leading to clinically relevant effects that are often more pronounced in the CYP2D6 EMs than IMs or PMs[58].

1.2. Dextromethorphan: A Dual-Probe Substrate for CYP2D6 and CYP3A4 Activity

1.2.1 Dextromethorphan Pharmacokinetics

Dextromethorphan (DM) has been widely used as an antitussive agent[71,72]. Dextromethorphan is metabolized via O-demethylation to dextrorphan (DX) primarily by CYP2D6 and via N-demethylation to 3-methoxymorphinan (3MM) primarily by CYP3A. Dextrorphan and 3-methoxymorphinan are further metabolized to 3-hydroxymorphinan (3HM) by CYP3A4 and CYP2D6, respectively[72] (Figure 2.1). Dextrorphan also undergoes glucuronidation to dextrorphan-O-glucuronide primarily by UGT 2B isoforms[73].

The pharmacokinetic parameters of dextromethorphan and its metabolites are significantly different in CYP2D6 EMs and PMs, as observed in previous clinical studies[59,74]. Dextromethorphan undergoes substantial hepatic first-pass metabolism leading to very low bioavailability of 1-2% for CYP2D6 EMs, while much higher bioavailability of 80% is observed in PMs[74]. Plasma C_{max} and AUC of dextromethorphan are significantly greater in PMs compared to EMs[74]. The median half-life of dextromethorphan is about 2.4 hours for EMs and 19.1 hours for PMs[74]. The dextromethorphan fraction metabolized by CYP2D6 to dextrorphan was calculated to be 0.98 in EMs[75]. The median half-life of dextrorphan has been reported to be about 1.4 hours for EMs and 10.1 hours for PMs[74]. Dextrorphan is primarily eliminated through glucuronidation to dextrorphan-O-glucuronide and the formation of 3-hydroxymorphinan (3HM) based on *in vitro* data[73]. The secondary metabolite, 3-hydroxymorphinan (3HM), also has different half-lives in EMs (median of 2 hours) and PMs (median of 20 hours), but its kinetics are formation rate-limited explaining this difference[74]. The 3-methoxymorphinan (3MM) shows

elimination rate-limited kinetics in EMs[76]. In the presence of 2% BSA, the formation clearance of dextrorphan-O-glucuronide was observed to be twelve-fold more efficient than the formation clearance of 3HM in human liver microsomes[73]. Dextrorphan-O-glucuronide is then eliminated renally by glomerular filtration and active secretion[77]. In addition to the renal excretion, dextromethorphan and its metabolites are also eliminated by biliary excretion and the enterohepatic cycle, which might lead to their long half-lives in PMs[59].

1.2.2 Dextromethorphan as an *in vivo* Probe Substrate for CYP2D6 Activity

Metabolic ratios, including metabolite to parent AUC ratios, plasma concentration ratios, and urinary amount ratios, have been used for determining changes in P450 enzyme activities in drug-drug interaction and phenotyping studies[78]. The increasing metabolite to parent ratio generally suggests an induction of that metabolic enzyme pathway, while decreasing metabolite to parent ratio indicates an inhibition of the metabolic enzyme that is forming the metabolite[73]. The metabolic ratio depends on the formation clearance and elimination clearance of the metabolite. Using simulations, Lutz and Isoherranen, 2012[73] demonstrated that induction or inhibition of the elimination pathway of the metabolite would decrease or increase the metabolic ratio (AUC_m/AUC_p) of the primary measurement potentially misleading in the interpretation of the data. It is necessary to fully understand the elimination pathways of the metabolite and the specific metabolizing enzymes contributing to the metabolite clearance for better interpretation of the changes in the metabolic ratio[73].

The metabolic ratios (MR) of dextromethorphan/dextrorphan (DM/DX) have been used for CYP2D6 phenotyping to distinguish CYP2D6 PMs and EMs in human populations[79]. Moreover, plasma, urine, and saliva metabolic ratios of DM/DX all have been assessed and used as indicators of CYP2D6 metabolic activity in clinical studies[80–83]. Single time point (1, 1.5, 2, 3, 4, 5, 6, 8 hours) plasma metabolic ratios of DM/DX have been assessed as an alternative of using the plasma AUC or 0–24-hour cumulative urine[82]. Significant correlations were found between the single-time point measurement for all the time points (1, 1.5, 2, 3, 4, 5, 6, 8 hours) and the two gold standard ways of measuring CYP2D6 activity (AUC extrapolated to infinity and 0-24-hour urine) ($r>0.8$; $p<0.05$) in CYP2D6 EMs after a 15 mg oral dose of DM[82]. The single-time point measurements for plasma DM/DX MRs at 2, 3, 4, and 5 hours for the single-dose study were significantly correlated with the corresponding multiple-dosing studies[82]. The single-time point measurement at 2-, 3-, 4-, and 5-hour plasma DM/DX MRs were suggested as measures of CYP2D6 activity with good validation, relatively easy lab measurement, and no side effects[82].

Cumulative urine collections (0-2, 4, 6, 8, 12 hours) for urine metabolic ratios of DM/DX also showed significant correlations with 0-24 hours cumulative urine metabolic ratios in CYP2D6 EMs[82]. Urine DM/DX metabolic ratios were found to be constant in 0-4, 0-6, and 0-8-hour urine samples[84], and DM metabolites (Dextrorphan) seem to maintain relative consistency after 4 hours in children who were 3-8 years old and CYP2D6 EMs[85]. There was a trend of higher intra-individual variability (CV%) in DM/DX metabolic ratios for shorter urine collection[86]. The %CV was 58% for 0-4 hours, 57% for 0-8 hours, and 44% for 0-24 hours in CYP2D6 EMs[86]. Even though 4-hour urine collection appears to be an acceptable way to characterize DM/DX metabolic ratios[85], a longer urine collection is preferred to reduce the intra-individual variability

for the metabolic ratio. A strong correlation was found between 3-hour plasma and 4-, 8-, and 24-hour urine metabolic ratio of DM/DX[80,87,88]. However, plasma DM/DX metabolic ratio was suggested to be more advantageous for measuring CYP2D6 activity *in vivo* with less variability compared to urinary metabolic ratios (ranging from 0.01-2.53 in plasma versus 0.0007-4.252 in urine)[39,81]. Similarly, another study reported a weak correlation between urinary DM/DX MRs and DM oral clearance ($r=0.24$; $p=0.04$), while stronger correlations were observed between DM oral clearance and 3-hour, 4-hour, 6-hour, and 8-hour plasma DM/DX metabolic ratios with correlation coefficients (r) all greater than 0.5 and p-values all smaller than 0.004[89].

1.2.3 Using Dextromethorphan as a Dual-Probe for Assessment of Human CYP2D6 and CYP3A4 Activity *in vivo*

CYP3A enzymes are primarily expressed in the gut and liver and are responsible for metabolizing more than 50% of the drugs that are metabolized by CYP enzymes[90]. *CYP3A* family includes *CYP3A4*, *CYP3A5*, *CYP3A7*, and *CYP3A43* genes. CYP3A expression has been shown to be sex and tissue-specific, and women have higher CYP3A mRNA and protein expression in the liver than men[91,92]. CYP3A4 has the highest CYP3A protein content in the liver and contributes to the majority of CYP3A activity in the liver with CYP3A5, CYP3A7, and CYP3A43 being minor in most individuals[3]. CYP3A7 is predominantly expressed in the fetal liver with persistent expression after birth in neonates and can metabolize retinoic acid and other endogenous compounds[93]. The variants *CYP3A5*3*, **6,* and **7* are associated with the loss of protein function of CYP3A5[94]. *CYP3A5*3* is predominantly found in Caucasians, and *CYP3A5*6* and *CYP3A5*7* are primarily detected in African Americans [94,95]. These genetic variations contribute to the interracial variability in CYP3A5 activity and pharmacokinetics of

CYP3A5 substrates[94,95]. Other rare variants, including *CYP3A5*2, *8*, and **9*, have unknown functional significance[96]. According to the Clinical Pharmacogenetics Implementation Consortium (CPIC) guidance, CYP3A5 phenotypes are assigned based on the combined allelic status of the diplotypes (Table 1.2)[96]. CYP3A5 extensive metabolizers have two functional alleles (*CYP3A5*1/*1*)[96]. CYP3A5 intermediate metabolizers carry one functional and one non-functional allele (*CYP3A5 *1/*3, *1/*6, *1/*7*)[96]. CYP3A5 poor metabolizers carry two non-functional alleles (*CYP3A5*3/*3, *3/*6, *3/*7, *6/*7, *6/*6, *7/*7)* and are CYP3A5 non-expressers[96]. CYP3A43 has been shown to participate in cancer development, and its function is specific depending on different tissues[97].

There is large inter-individual variability observed in CYP3A activity[98]. CYP3A4, the most abundant CYP3A isoform expressed in the gut and liver, has a greater than 50-fold variation in hepatic expression and greater than 20-fold variation in enzymatic activity *in vivo* among individuals[99]. Unlike CYP2D6, CYP3A4 is highly inducible by xenobiotics, including rifampicin, dexamethasone, and paclitaxel through the activation of nuclear receptor pregnane X receptor (PXR) or constitutive androstane receptor (CAR)[100]. *CYP3A4* is not as polymorphic as *CYP2D6.* CYP3A4 phenotypes are variable and have unimodal distribution[3]. *CYP3A4*22* has been shown to account for 7% of the variability in mRNA levels in the liver samples, and an intronic variant rs4646450 accounts for 3-5% of expression and CYP3A4 hepatic activity and variability in human liver[101]. Using a multivariate analysis, Klein et al.[101] reported ~8-9% variability in atorvastatin hydroxylase activity due to two linked peroxisome proliferator-activated receptor alpha (PPARα) SNPs, and ~33% of the variability was explained by genetic and nongenetic factors.

Multiple probe substrates, including erythromycin, midazolam, verapamil, and endogenous biomarkers (4β-Hydroxycholesterol, 6β-hydroxycortisol, 6β-hydroxycortisone) have been used to evaluate CYP3A activity changes *in vivo*[102–104]. Even though intravenously administered midazolam provides a reasonable estimate of hepatic CYP3A activity, it quickly causes pharmacological effects, including sedation or amnestic effects, which limits its use as an *in vivo* probe substrate[105]. Oral administration of midazolam captures both intestinal and hepatic CYP3A activity; however, its use is complicated due to the requirement of multiple blood samples for determining midazolam clearance[106]. Intravenous dosing of verapamil could cause a reduction of blood pressure and prolonged atrioventricular conduction[107]. The disadvantages of using an erythromycin breath test (ERMBT) include radiolabeled ^{14}C and intravenous administration[105]. The ratio of 6β-hydroxycortisol to free cortisol (6βFF/FF) was also used as an endogenous marker for hepatic CYP3A activity and phenotyping[108–110]. It is a non-invasive *in vivo* measurement of CYP3A activity; however, it is susceptible to high within-day, inter-individual, and intra-individual variability[109,110].

Dextromethorphan has been suggested and assessed as an indicator of CYP3A activity simultaneously while measuring CYP2D6 metabolic activity[102,111]. Dextromethorphan is easy to administer and does not require intensive blood sampling if only cumulative urine collections are needed. Both dextromethorphan/3-methoxymorphinan (DM/3MM) and dextrorphan/3-hydroxymorphinan (DX/3HM) have previously been used as *in vivo* CYP3A activity markers. The cumulative urinary dextromethorphan/3-methoxymorphinan (DM/3MM) metabolic ratio was found to be strongly correlated with oral and intravenous verapamil clearances, and the plasma ratio of $AUC_{norverapamil}/AUC_{verapamil}$ after coadministration of verapamil and

dextromethorphan orally[102]. The dextrorphan/3-hydroxymorphinan (DX/3HM) metabolic ratio has also been proposed to reflect CYP3A activity and has been used when 3-methoxymorphinan circulating concentrations were not detected in 2-hour plasma samples[51]. Urinary 3HM/DX MRs were statistically significantly correlated with midazolam oral clearance (r=0.45)[106].

1.3 Brief Overview of Retinoic Acids and their Roles in Enzyme Expression Regulation

1.3.1 Brief Overview of Retinoid Metabolic Pathway

Retinoids are endogenous compounds structurally similar to vitamin A (retinol) (Figure 1.2) and responsible for maintaining many physiological processes, including cell growth, vision, reproduction, healthy skin, immune response, and gene transcriptional regulation[112,113]. Vitamin A (retinol) is obtained via dietary retinyl esters and β-carotene[113]. The storage and metabolism of retinoids in the form of retinyl esters in the liver stellate cells[114,115]. Retinyl esters are hydrolyzed to retinol and oxidized to the endogenous bioactive form of vitamin A[115]. *All-trans* retinoic acid (*at*RA) is the active metabolite of retinol[116]. *At*RA and its isomer 13-*cis* retinoic acid (13-*cis*RA) can interconvert in humans[117]. For example, 13-*cis*RA rapidly isomerized to *at*RA when incubated with conceptual cytosol, suggesting that embryonic isomerase partially catalyzes this conversion[117]. A previous study showed that isomerization between *at*RA and 13-*cis*RA is partly mediated by glutathione S-transferases (GSTs)[118]. GST Pi and Mu classes were shown to have higher isomerase activities. Hence these GSTs are expected to have high catalytic activity of 13-*cis*RA formation in tissues with high concentrations of the isoenzymes[118]. *At*RA and 13-*cis*RA are metabolized to downstream metabolites 4-oxo-*at*RA and 4-oxo-13-c*is*RA sequentially via 4-

hydroxylation (Figure 1.2)[119–122]. It is assumed that retinoids administered as drugs or as dietary supplements will follow similar metabolic pathways.

1.3.2 Isotretinoin as a Drug: Pharmacology and Pharmacokinetics

Isotretinoin (13-*cis*RA) is commonly used as therapy for severe, recalcitrant nodular acne and for the treatment of neuroblastoma[123,124]. Isotretinoin has been studied for head, neck, thyroid, and lung cancers and also has been investigated for male infertility[125–128]. Recent studies reported that systemic isotretinoin treatment positively affects semen parameters, including concentration[128], volume, total sperm count, progressive motility, and vitality while deteriorating sperm morphology[129].

Acne is a common condition for adolescents and young adults[130]. The therapeutic oral dosing of isotretinoin for treating severe acne is 0.5-1.0 mg/kg/day[131]. Adverse effects, including cheilitis and xerosis, often will increase with a higher dose[131]. Other side effects include dry skin, dry mucous membranes, nosebleeds, and increased total cholesterol concentrations and serum triglycerides[132]. A significant side effect of isotretinoin is mood changes, depression and suicidality. Overall 26 out of 247 participants (10.5%) diagnosed with acne vulgaris reported mood changes, most commonly depression, after isotretinoin administration[133]. It is necessary to monitor for mood changes in patients who need isotretinoin for therapeutic uses, especially with pre-existing mood disorders[133].

Isotretinoin exposure during pregnancy was estimated to result in 18-22% spontaneous abortion, and of survived births, 20-30% were estimated to develop craniofacial defects, cardiac and central nervous system abnormalities, thymic hypoplasia, and limb reduction or duplication, referred to as Accutane embryopathy[134]. The potential cardiovascular risk of isotretinoin and its derivatives was reported recently[135]. A healthy 18-year-old adolescent was reported with heart failure due to dilated cardiomyopathy and renal infarction that appeared after 5-months of isotretinoin treatment[135]. Yet, the overall significance of these side effects requires further study. Isotretinoin-treatment may also cause ulcerative colitis (UC) during the first six months after isotretinoin treatment[136].

Isotretinoin is highly lipophilic and extensively bound to plasma albumin (f_u=0.0004)[137,138]. It has relatively low bioavailability (~25%) following oral dosing, and taking isotretinoin with food increases the bioavailability by 1.5 to 2- fold[138,139]. Approximately 20-30% of the isotretinoin dose isomerizes to *at*RA[140]. After a single dose of 80mg oral isotretinoin, the harmonic means of distribution and elimination half-lives of 13-*cis*RA were 1.3 and 17.4 hours, respectively[140]. Peak plasma concentrations of 4-oxo-isotretinoin (4-oxo-13-*cis*RA), the primary active circulating metabolite of isotretinoin, were reported to be approximately one-half of those of isotretinoin 6 to 16 hours after isotretinoin dosing[140]. The mean AUC ratio of metabolite (4-oxo-isotretinoin) to parent (isotretinoin) following the single dose was 2.5 (n=15; all males)[140]. The harmonic means of the apparent elimination half-lives for isotretinoin and 4-oxo-isotretinoin were 15.8 and 29.2 hours calculated after the last dose in a multiple-dosing study, suggesting the metabolite 4-oxo-isotretinoin is elimination rate limited[141]. The metabolite 4-oxo-isotretinoin appeared to accumulate in plasma, and the steady-state concentrations were two to five-fold higher than those

of the parent drug isotretinoin[141]. High inter-individual variability of plasma concentrations of isotretinoin have been observed after isotretinoin administration in previous studies, and some patients potentially have sub-optimal drug exposures[142].

1.3.3 Regulation of Drug Metabolizing Enzyme Expression by Retinoic Acids

Isotretinoin (13-*cis*RA) has been shown to induce gene regulation through binding to retinoic acid receptors (RAR)[143,144]. *At*RA has a higher affinity to RARs and directly activates RAR (α, β, and γ) and retinoid X receptor (RXR α, β, and γ), which then bind to retinoic acid response elements (RARE) on the target gene promoter regions, causing cellular effects and regulating biological processes[143,145]. RAR signaling has significant roles in regulating cell differentiation and maintaining molecular functions during embryogenesis and adult life[144,146]. The changes in cellular concentrations of retinoic acid tightly regulate the activation and signaling via RARs[147–149]. Previously, *at*RA was reported to reduce CYP7A1 mRNA expressions in primary human hepatocytes and HepG2 cells, likely via activation of farnesoid X receptor (FXR)/RXR signaling[150]. Another study indicated that *at*RA appeared to activate the c-Jun N-terminal kinase (JNK)/ERK pathway and the downstream protein AP-1, which additionally represses the HNF4-α activation on the promoter of CYP7A1[151]. *At*RA appeared to selectively regulate specific FXR targets, including stimulating small heterodimer partner (SHP), fibroblast growth factor 19 (FGF19), and organic solute transporter α (OST α) mRNA levels[150]. A synergistic effect was observed between *at*RA and chenodeoxycholic acid (CDCA) in the human I-BABP and BSEP genes minimal promoters[150]. *At*RA and CDA caused a mild reduction in CYP27A1 and NTCP mRNA levels in primary human hepatocytes[150]. In *at*RA-treated human hepatocytes, SHP expression was increased[150].

Drug-drug interactions (DDI) for retinoic acid have been widely studied *in vitro* and, to a lesser extent, *in vivo*. Isotretinoin was shown to induce SHP mRNA expression and decrease CYP2D6 mRNA expression in human hepatocytes, likely via the repression of HNF4α activity[137]. SHP expression was induced, and CYP2D6 expression was also downregulated after *at*RA treatment in hepatocytes and CYP2D6 humanized mice[48,137]. The metabolite of isotretinoin, 4-oxo-13-*cis*RA, was also shown to induce the mRNA expression of SHP and decrease the expression of CYP2D6 in human hepatocytes[137]. Isotretinoin resulted in a weak induction in CYP3A4 expression *in vitro* and *in vivo,* potentially mediated by Pregnane X Receptor (PXR) activation[137]. Isotretinoin and *at*RA appeared to inhibit hepatic transporters organic anion-transporting polypeptide 1B1 (OATP 1B1) and 1B3 (OATP1B3) *in vitro*[152]. Further studies have shown that isotretinoin, *at*RA, and 4-oxo-13-*cis*RA downregulate OATP1B1, CYP1A2, and CYP2C9 mRNA expression and increase CYP2B6 mRNA expression dose-dependently in human hepatocytes[153]. After dosing *at*RA for 14 days, CYP2E1 and N-acetyltransferase enzymes were induced in patients with prostate cancer (only in fast acetylators) by 83% and 29%, respectively[154]. These studies provide evidence that *at*RA, isotretinoin, and its circulating metabolite 4-oxo-13-*cis*RA all have drug-drug interaction potential with cytochrome P450 enzymes and/or transporters via diverse mechanisms.

Table 1.1 Allele Frequencies[a] of Common *CYP2D6* Variant Alleles Across Populations[b].

***CYP2D6* Allele**	**European**	**African**	**Hispanic/Latino**	**Asian**	**Predicted Function[c]**
*2	14.81	16.11	16.48	9.53	Normal
*3	1.57	0.35	0.86	N/A[d]	Decreased
*4	13.80	3.63	9.55	0.51	None
*5	3.08	5.67	2.96	6.24	None
*6	1.06	0.26	0.55	N/A	None
*9	2.54	0.53	1.46	N/A	Decreased
*10	1.58	3.83	1.65	12.88	Decreased
*17	0.16	15.71	1.72	0.036	Decreased
*29	0.07	8.40	1.24	N/A	Decreased
*41	9.83	2.40	5.72	4.02	Decreased

[a]Allele frequencies are shown as percentages (%)

[b]All values are from the TOPMed studies[17]

[c]Predicted functions are from Clinical Pharmacogenetics Implementation Consortium guideline (CPIC)[155]

[d]N/A indicates no frequency value is given from the reference TOPMed studies.

Table 1.2 Assignment of CYP3A5 Phenotypes Based on Genotyped Diplotypes.

CYP3A5 Phenotypes	CYP3A5 Diplotypes
Extensive metabolizer (CYP3A5 expresser: carry two functional alleles)	**1/*1*
Intermediate metabolizer (CYP3A5 expresser: carry one functional and one non-functional allele)	**1/*3, *1/*6, *1/*7*
Poor metabolizer (CYP3A5 non-expresser: carry two functional alleles)	**3/*3, *3/*6, *3/*7, *6/*7, *6/*6, *7/*7*

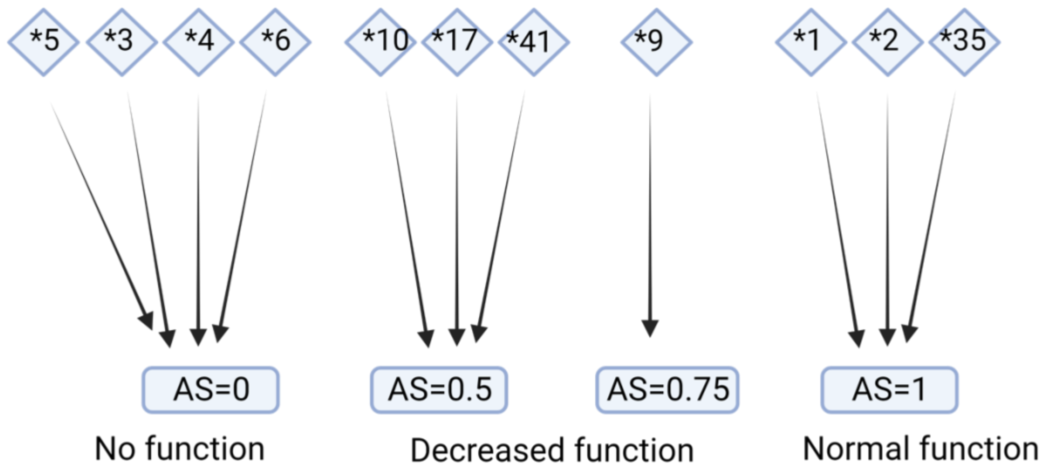

Figure 1.1 Assignment of CYP2D6 Activity Scores (AS) Based on CYP2D6 Genotype. Blue diamonds represent the *CYP2D6* variant alleles present, and blue ovals represent the AS assigned to the corresponding alleles. The allele types, including normal, decreased, and no function, are indicated. The sum of the AS for all alleles in diplotypes is used as the *CYP2D6* AS for each subject *CYP2D6* genotype.

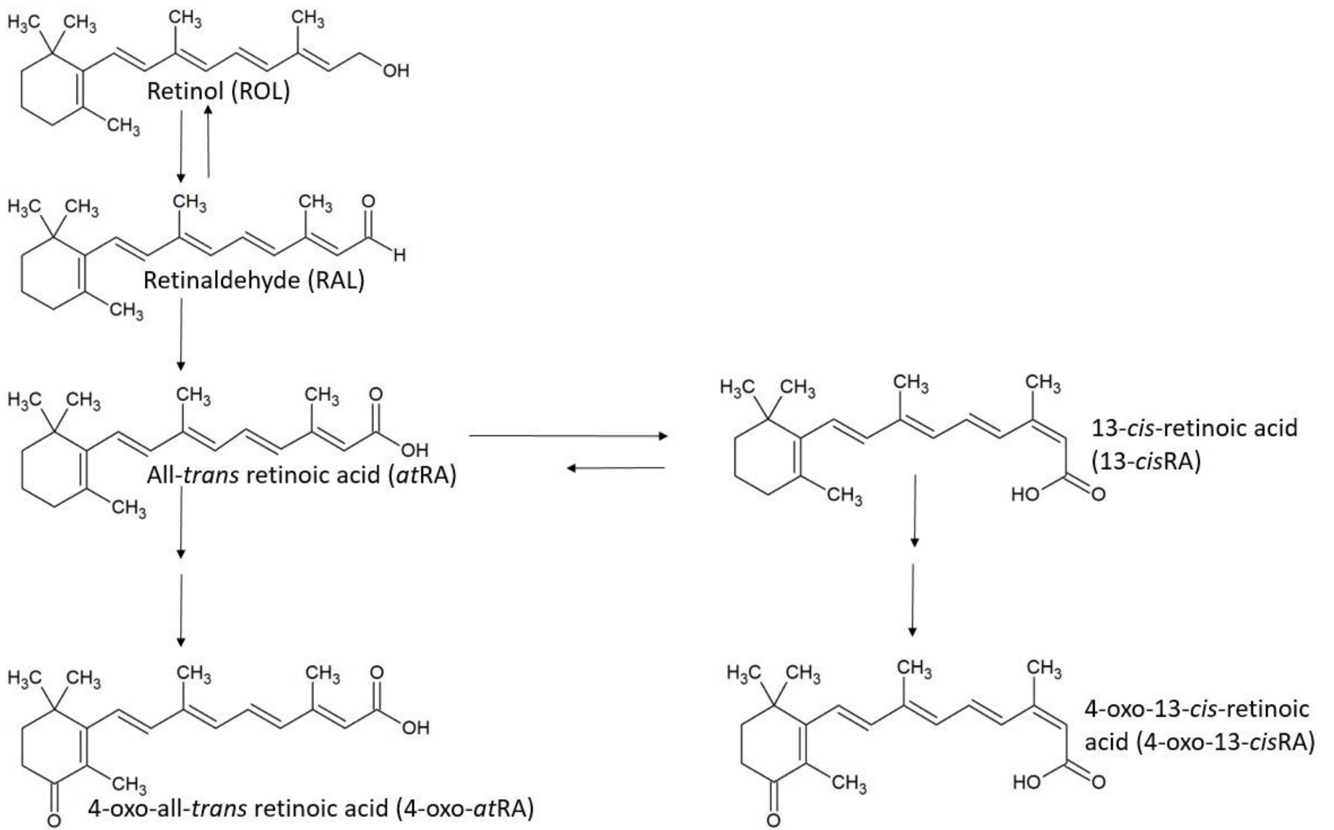

Figure 1.2 Retinoid Metabolic Scheme.

Chapter 2:

Effect of Isotretinoin on CYP2D6 and CYP3A4 Activity in Patients with Severe Acne

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Abstract

Previously, retinoids have decreased CYP2D6 mRNA expression *in vitro* and induce CYP3A4 *in vitro* and *in vivo* in humans. This study aimed to determine whether isotretinoin (13-*cis*-retinoic acid) administration changes CYP2D6 and CYP3A4 activities in patients with severe acne. A total of 33 patients (22 females and 11 males, 23.2 ± 5.7 years old) who were anticipated to receive isotretinoin for therapeutic reasons were enrolled. All participants were genotyped for CYP2D6 and CYP3A5. Their CYP2D6 activity scores (AS) ranged from 0 to 3. All participants received dextromethorphan (DM) 30 mg orally as a dual-probe substrate of CYP2D6 and CYP3A4 activity at two study time points: pre-isotretinoin treatment and with concomitant isotretinoin for at least one week. The concentrations of isotretinoin, DM and their metabolites were measured in 2-hour post-dose plasma samples and in cumulative 0–4-hour urine collections using liquid chromatography-mass spectrometry. In CYP2D6 extensive metabolizers, urinary dextrorphan (DX)/DM metabolic ratio (MR) (CYP2D6 activity marker) trended lower with isotretinoin administration compared to pre-isotretinoin (geometric mean ratio (GMR) = 0.784).

The urinary 3-hydroxymorphinan (3HM)/DX MR (CYP3A4 activity marker) was increased (GMR = 1.181) following isotretinoin administration compared to pre-isotretinoin. The urinary DX-O-glucuronide/DX MR (proposed UGT2B marker) was increased (GMR = 1.216) with isotretinoin administration compared to pre-isotretinoin. Administration of isotretinoin did not alter CYP2D6 activity, suggesting that the predicted downregulation of CYP2D6 based on *in vitro* data does not translate to humans. A weak induction of CYP3A4 activity by isotretinoin was observed. The data also suggest dextrorphan glucuronidation is increased following isotretinoin administration.

2.1 Introduction

Cytochrome P450 2D6 (CYP2D6) is a critical enzyme responsible for the clearance of approximately 20-25% of commonly used drugs, although it only accounts for 2-4% of total liver CYP content[4,5,156,157]. The disposition of CYP2D6 substrate drugs shows high inter-individual variability due to genetic polymorphisms in CYP2D6[158]. In addition to genetic variability in intrinsic CYP2D6 activity, CYP2D6-mediated clearance varies due to differences in CYP2D6 protein expression[159]. A positive correlation exists between CYP2D6 copy number and CYP2D6 mRNA levels in human liver tissue[41]. CYP2D6 mRNA expression in the liver also varies between individuals, even within the same genotype group, suggesting transcriptional regulation of CYP2D6[42,160]. The mechanisms of CYP2D6 transcriptional regulation have not been well defined. Previous studies have shown that CYP2D6 is generally not inducible by common xenobiotic inducers[9,10]. In human hepatocytes and CYP2D6 humanized mice, CYP2D6 activity is regulated by hepatocyte nuclear factor (HNF) 4α[45,46]. Small heterodimer partner (SHP) acts as a transcriptional repressor for CYP2D6 expression via interacting with HNF4α and repressing CYP2D6 transcription[41,48].

Isotretinoin (13-*cis*-retinoic acid, 13-*cis*RA) is approved by EMA and FDA for treatment of severe, recalcitrant, nodulocystic acne[124]. Isotretinoin, its isomer all-*trans*-retinoic acid (*at*RA), and its active metabolite 4-oxo-13-*cis*-retinoic acid (4-oxo-13-*cis*RA) increase SHP mRNA expression and decrease CYP2D6 mRNA expression in human hepatocytes[137]. However, in mice, treatment with 13-*cis*RA and *at*RA had no impact on Shp mRNA expression, while 4-oxo-13*cis*RA increased the mRNA expression of Shp and mouse Cyp2d isoform[137]. In contrast, in CYP2D6 humanized mice, treatment with *at*RA induced SHP and downregulated CYP2D6

expression[48]. The increase in SHP mRNA expression likely resulted in reduced CYP2D6 expression via repression of HNF4α activity[48]. Based on human hepatocyte studies, a 35-77% decrease in CYP2D6 expression was predicted with combined effects of 13-*cis*RA, *at*RA, and 4-oxo-13-*cis*RA after isotretinoin 20 mg twice daily dosing for twenty weeks[137]. However, no decrease in CYP2D6 activity was observed in healthy male CYP2D6 extensive metabolizers (EM) (n=8) receiving isotretinoin 40 mg twice daily dosing for two weeks[137]. Dextromethorphan was used as the CYP2D6 probe substrate. The dextrorphan/dextromethorphan (DX/DM) AUC ratios and mean formation clearance of DX were instead increased 1.25- and 1.29-fold, respectively, indicating CYP2D6 was potentially weakly induced *in vivo*[137]. This study only recruited healthy adult males and may not have fully captured the role of retinoids in regulating CYP2D6 activity[137]. For example, females may have different CYP2D6 regulation as Cyp2d22 wild-type female mice have higher Cyp2d22 hepatic mRNA expression at the estrous phase of the estrous cycle compared to male mice and ovariectomy decreased Cyp2d22 mRNA expression in female mice[161]. Previous studies have reported that DM/DX urinary metabolic ratios were significantly lower in healthy female CYP2D6 EMs compared to males[162,163].

Retinoids have also been shown to be *in vitro* inducers of CYP3A4 via activation of pregnane X receptor (PXR)[137] and/or constitutive androstane receptor (CAR)[164]. Previously, isotretinoin administration resulted in a weak CYP3A4 induction in humans *in vivo* based on the formation clearance of 3-methoxymorphinan (3MM) from DM and 6βOH-cortisol formation as an endogenous hepatic CYP3A4 biomarker[137]. However, this finding requires further confirmation. Our study aimed to test whether isotretinoin regulates CYP2D6 and CYP3A4 activity in male and female patients with severe acne using DM as a dual-probe substrate *in vivo*.

2.2 Methods

Study Participants

The study was registered on clinicaltrials.gov (NCT03076021) and approved by the University of Washington Investigational Review Board. All participants provided written informed consent, and parents provided consent for participants younger than 18 years old. The study was designed to have 80% power to detect a 25% change in DX/DM metabolic ratio with an α=0.05. A total of 33 patients (22 females and 11 males) with severe acne that were expected to be treated with isotretinoin were recruited for the study. The participants were ≥12 years old and not pregnant. Participants were excluded from the study based on the following exclusion criteria: body weight < 80 lbs or BMI ≥ 30 kg/m^2; history of allergic or adverse reactions to isotretinoin, vitamin A, or dextromethorphan; pregnancy; inability to follow isotretinoin risk evaluation and mitigation strategy (iPLEDGE) program requirements; had chronic or persistent cough accompanying asthma, smoking or COPD; had a productive cough, fever, known kidney disease, known liver disease, diabetes, mental illness requiring treatment; taking medications or supplements known to interact with dextromethorphan or CYP2D6 activity or taking drugs known to increase the risk of adverse effects from dextromethorphan within 14 days.

Study Design and Drug Administration

Each participant received dextromethorphan 30 mg orally on each of the two study days. The first study day was the control day, which occurred prior to the administration of isotretinoin. Participants then took an average of 1.01 ± 0.20 mg/kg/day of isotretinoin per clinical care for at least a week, with a median of 66 days of treatment (range 10-194 days), before study day 2. On

both study days, blood samples were collected (into foil wrapped EDTA tubes and immediately placed on wet ice) 2 hours post-dextromethorphan administration and approximately 1 hour after the start of a non-standardized meal. Plasma was separated by centrifugation (10 min at 3,000 g) and stored at –80° C until analysis. Laboratory lights were covered with UV fluorescent light filter sleeves. Plasma was used for quantification of dextromethorphan (DM), dextrorphan (DX), 3-hydroxymorphinan (3HM), 3-methoxymorphinan (3MM), dextrorphan-O-glucuronide (DX-O-glucuronide), all-*trans*-retinoic acid (*at*RA), 13-*cis*-retinoic acid (13-*cis*RA), 4-oxo-13-*cis*-retinoic acid (4-oxo-13-*cis*RA), and retinol (ROH) concentrations. Cumulative urine was collected 0-4 hours post-dextromethorphan administration on both study days. The urine was kept refrigerated until the collection was complete, then all urine was combined, volume measured, and aliquots stored at –80° C until analysis. Urine samples were used to quantify DM, DX, 3HM, 3MM, and DX-O-glucuronide.

CYP2D6 and CYP3A5 Genotyping

Buccal swabs were collected for genomic DNA extraction from participants using Qiagen QIAamp DNA Mini Kit (Germantown, Maryland). CYP2D6 and CYP3A5 assays were performed on a StepOnePlus instrument following the recommended protocols from the manufacturer (Applied Biosystems, Waltham, Massachusetts). *CYP2D6* copy number was determined and samples were genotyped for nine core single nucleotide polymorphisms (SNPs) including *CYP2D6*2, *3, *4, *6, *9, *10, *17, *35, and *41* using Taqman allelic discrimination assays (Invitrogen). *CYP2D6* genotype was determined and were translated into activity scores (AS) and expected phenotypes using previously published genotyping strategy and methods[20,21,165]. The CYP2D6 activity scores were calculated by the summation of values that

were assigned to each allele. CYP2D6 metabolizer phenotypes were assigned with the corresponding AS values: poor metabolizer (AS=0), intermediate metabolizer (AS=0.5), extensive metabolizer (AS=1-2), and ultrarapid metabolizer (AS>2). To determine if the changes in CYP3A activity are different among CYP3A5 expressors and non-expressors after isotretinoin administration, samples were also genotyped for three non-functional SNPs of CYP3A5, including *CYP3A5*3* (rs776746), *CYP3A5*6* (rs10264272), and *CYP3A5*7* (rs41303343) using TaqMan allelic discrimination assays. Combined CYP3A5 allelic status for each of these three SNPs (a *CYP3A5*3/*6/*7* metabolic composite) was used for characterizing extensive, intermediate, and poor metabolizers of CYP3A5 as described previously[96,166]. The wild-type functional allele is *CYP3A5*1.* CYP3A5 extensive metabolizers were identified with two functional alleles (*CYP3A5*1/*1*). CYP3A5 intermediate metabolizers were identified with one functional allele (*CYP3A5*1/*3, *1/*6*, or **1/*7*). CYP3A5 poor metabolizers were identified with two non-functional alleles (*CYP3A5*3/*3, *3/*6, *3/*7, *6/*7, *6/*6*, or **7/*7*).

Quantification of Retinoids, Dextromethorphan and Metabolites in Plasma and Urine

All-*trans* retinol and *at*RA were purchased from Millipore Sigma (Burlington, Massachusetts). The 13-*cis*RA was purchased from Toronto Research Chemicals (Toronto, Canada). The *at*RA-d_5 and 13-*cis*RA-d_5 were purchased from Cayman Chemical (Ann Arbor, Michigan), and retinol-d_6 was purchased from Cambridge Isotopes Laboratories (Tewksbury, Massachusetts). The 4-oxo-13-*cis*RA and 4-oxo-13-*cis*RA-d_3 were purchased from Santa Cruz Biotechnology (Dallas, Texas). Mass-spectrometry grade formic acid, water, methanol, and acetonitrile were purchased from Fisher Scientific (Pittsburgh, Pennsylvania). Dextromethorphan, dextrorphan, 3-

hydroxymorphinan, 3-methoxymorphinan, dextromethorphan-d_3, and dextrorphan-d_3 were purchased from Sigma Aldrich (St. Louis, Missouri).

Plasma retinoids were analyzed and quantified using atmospheric pressure chemical ionization (APCI) positive ion mode on AB Sciex 6500 QTRAP mass spectrometer coupled with Agilent 1290 Infinity II LC using previously validated methods[167–169]. Plasma dextromethorphan (DM) and its metabolites (DX, 3MM, and 3HM) were quantified as previously described[21]. To quantify urine DM, DX, 3MM, and 3HM, urine samples along with calibration curves and quality controls were diluted 1:10 with water and protein precipitated with an equal amount of ice-cold acetonitrile containing internal standards of DM-d_3 and DX-d_3. To quantify dextrorphan-O-glucuronide both in plasma and urine, samples were diluted by 1:1000 with water and protein precipitated with an equal amount of ice-cold acetonitrile containing internal standards of DX-d_3. The processed samples were analyzed using AB Sciex 6500 QTRAP mass spectrometer coupled with Agilent 1290 Infinity II LC and equipped with a Kinetex EVO C18 column (2.1×100 mm, 2.6 µm). The acquisition method was as previously described[21,137] with minor modifications. MS/MS transitions monitored for the analytes were: DM, m/z 272>215; DX, m/z 258>157; DX-O-glucuronide, m/z 434>258; 3HM, m/z 244>157; 3MM, m/z 258>171; DM-d_3, m/z 275>215; DX-d_3, m/z 261>157. The lower limits of quantification (LLOQ) for DM, DX, DX-O-glucuronide, 3HM, and 3MM were 1, 1, 90, 3, 0.33 nM in plasma and 1, 150, 9000, 45, 6 nM in urine.

The LC-MS/MS data were quantified using MultiQuant 3.0.3. All analyte concentrations were calculated by peak area ratios of the analytes to internal standards. The calibration curves for each analyte were fitted by linear regression and weighed by 1/x, with the exception of DX-O-glucuronide, which was weighted by $1/x^2$. FDA Bioanalytical Guidance was used to refer to bioanalytical acceptance criteria[170].

Data and Statistical Analysis

The participants' demographics are expressed as mean and standard deviation (SD). Retinoid concentrations in plasma are reported as geometric means and 90% confidence intervals. The geometric mean ratios (GMR) and their 90% confidence intervals (90% CI) were used to compare the changes in retinoid concentrations after isotretinoin treatment (study day 2) to endogenous concentrations (study day 1). GMR of treatment/control is the geometric mean of each participant's study day 2 retinoid concentration over the study day 1 retinoid concentration.

DX/DM and DX-O-glucuronide/DX metabolic ratios were calculated by dividing the molar concentration (plasma) or molar quantity (urine) of the metabolite by that of the parent. DX plus DX-O-glucuronide/DM urinary metabolic ratio was calculated by dividing the sum of the molar quantity of metabolites by that of the parent. The 3MM/DM and 3HM/DX metabolic ratios in urine were calculated by molar quantity of metabolite by that of parent. Urine samples with concentrations above or within 5% of the lower limit of quantification were accepted, and concentrations lower than that were excluded from data analysis. The geometric mean ratio (GMR) and its 90% confidence interval (90% CI) were used to compare plasma or urinary

metabolic ratios between study day 1 (pre-treatment control) and study day 2 (after treatment with isotretinoin for at least a week). GMR of treatment/control metabolic ratio is the geometric mean of each participant's study day 2 metabolic ratio over study day 1 metabolic ratio. The 90% CI of the GMR for the metabolic ratio after isotretinoin treatment over pre-treatment control was compared to the bioequivalence range of 0.8 and 1.25 according to FDA Guidance of Clinical DDI Studies[171]. If the 90% CI does not include 1, there is an indication of a potential DDI. A non-parametric Mann-Whitney test was performed to evaluate sex differences in CYP2D6 activity pre-isotretinoin and after isotretinoin treatment among CYP2D6 EMs. The non-parametric Mann-Whitney test was also used to assess the differences in urinary 3HM/DX metabolic ratios between *CYP3A5* genotypes pre-isotretinoin and after isotretinoin treatment. A p-value < 0.05 was considered statistically significant. Statistical analyses were all conducted on GraphPad Prism 9.5.1 (GraphPad Software, Inc., La Jolla, California).

To evaluate the relationship between plasma retinoid concentrations (*at*RA, 13-*cis*RA, and 4-oxo-13-*cis*RA) and urine DX/DM, 3MM/DM, and 3HM/DX metabolic ratios in CYP2D6 EMs, a linear mixed-effect regression analysis was conducted by treating two repeated measurements (pre-treatment control and post-isotretinoin treatment) from each participant as a cluster. To conduct the analyses, both plasma retinoid concentrations and urine metabolic ratios were log-transformed. The mixed effect modeling was only applied to the intercept, allowing the baseline urinary metabolic ratios to differ across participants, while the slope estimate (i.e., the relationship between urinary metabolic ratios and plasma retinoid concentrations) was derived using fixed effect only as the population means. All analyses were performed using the NLME package in R Studio (R version 4.1.2) (R Studio, Boston, Massachusetts)[172].

2.3 Results

Participant Demographics

Thirty-six participants were enrolled and genotyped, and thirty-three (22 females and 11 males; 25 White, 7 Asian, and 1 Black) completed the study. The mean (± SD) age of the participants was 23.2 ± 5.7 years old, with a weight of 64.6 ± 15.4 kg and height of 168.5 ± 10.9 cm. Two participants had a CYP2D6 activity score (AS) of 0, one had an AS of 0.5, five had an AS of 1.0, five had an AS of 1.5, 17 had an AS of 2.0, one had an AS of 2.0 - 2.5, and two had an AS of 3.0. Twenty-six participants were CYP3A5 poor metabolizers with *CYP3A5 *3/*3* genotype. Six participants were CYP3A5 intermediate metabolizers with *CYP3A5 *1/*3* genotype. One participant was a CYP3A5 extensive metabolizer with *CYP3A5 *1/*1* genotype.

Retinoid Concentrations

The geometric mean and 90% CI of retinoid concentrations on both study days 1 and 2 are reported in Table 2.1. Administration of isotretinoin increased plasma retinoid (except retinol) concentrations on study day 2 (2-hour post-dose) compared to study day 1 (endogenous concentrations). The geometric mean of the steady-state concentration values for *at*RA, 13-*cis*RA, and 4-oxo-13-*cis*RA measured on study day 2 (2-hour post-dose) were 41 nM, 1,085 nM, and 2,385 nM, respectively. The 13-*cis*RA and 4-oxo-13-*cis*RA concentrations increased 397- and 356-fold on study day 2 compared to study day 1. The *at*RA concentrations increased 15-fold on study day 2 compared to study day 1. Retinol concentrations decreased by 20% on study day 2 compared to study day 1 (GMR = 0.8 treatment/control).

Dextromethorphan Metabolic Ratios

The 2-hour post-dose plasma and 0–4-hour post-dose cumulative urine metabolic ratios of DX/DM were used as *in vivo* markers of CYP2D6 activity (Figure 2.1). The 0–4-hour post-dose cumulative urine metabolic ratio 3HM/DX was used as an *in vivo* marker for CYP3A4 activity. The geometric mean and 90% CI of plasma and urine metabolic ratios on both study days 1 and 2, as well as the GMR of treatment/control with 90% CI, are shown in Table 2.2.

Both the urinary DX/DM and DX plus DX-O-glucuronide/DM metabolic ratios in CYP2D6 extensive metabolizers (AS = 1–2) trended lower with isotretinoin treatment (GMR = 0.784 and 0.911 treatment/control), but the 90% CI of the GMRs included one suggesting that the observed changes were not statistically significant (Table 2.2; Figure 2.2 b, c). For participants who were CYP2D6 extensive metabolizers (AS = 1–2), no statistically significant differences were observed between study day 1 and 2 plasma DX/DM metabolic ratios (GMR 1.014 treatment/control; 90% CI of the GMR included 1) (Table 2.2; Figure 2.2 a). The impact of the administration of isotretinoin on plasma and urine DX/DM ratio was also analyzed in individual AS sub-groups (Figure 2.2 d, e; Figure 2.5). The DX/DM urinary metabolic ratio in AS=2 group was statistically significantly lower on study day 2 than study day 1 (GMR 0.580 treatment/control; 90% CI: 0.412-0.816) (Figure 2.2 e) while no statistically significant differences were observed in DX/DM plasma metabolic ratio in the AS=2 group (GMR 0.856 treatment/control; 90% CI included 1) (Figure 2.2 d). No statistically significant differences in DX/DM 0-4-hour urinary metabolic ratio between female and male participants were observed on either study day in CYP2D6 EMs (p-value = 0.2 and 0.8) (Figure 2.6).

The urinary 3MM/DM metabolic ratio was not statistically significantly different after isotretinoin treatment compared to control (GMR 0.652 treatment/control; 90% CI included 1) (Table 2.2; Figure 2.3 a). Urinary 3HM/DX metabolic ratio increased after isotretinoin treatment compared to control (GMR 1.181 treatment/control; 90% CI 1.034 – 1.349) (Table 2.2; Figure 2.3 b). There was no statistically significant difference in urinary 3HM/DX metabolic ratio between CYP3A5 expressers (*CYP3A5 *1/*1* and **1/*3* genotypes) and CYP3A5 nonexpressers (*CYP3A5 *3/*3* genotype) on either study day (p-value = 0.2 and 0.4) (Figure 2.7 a, b). Additionally, there was no statistically significant difference observed in urinary 3HM/DX metabolic ratio after isotretinoin treatment (study day 2 3HM/DX MR to study day 1 3HM/DX MR ratio) between CYP3A5 expressers and non-expressers (p-value= 0.5) (Figure 2.7 c).

The urinary DX-O-glucuronide/DX metabolic ratio increased after isotretinoin treatment compared to control (GMR 1.216 treatment/control; 90% CI 1.063 – 1.391) (Table 2.2; Figure 2.4 b). Conversely, plasma DX-O-glucuronide/DX ratio was unchanged between the two study days (GMR 1.017 treatment/control; 90% CI included 1) (Table 2.2; Figure 2.4 a).

Correlation between plasma retinoid concentrations and urinary dextromethorphan metabolic ratios

Based on linear mixed-effect regression analysis, there were no significant correlations between changes in log plasma retinoid concentrations (*at*RA, 13-*cis*RA, and 4-oxo-13-*cis*RA) and log urinary DX/DM MR (p-value = 0.2, 0.3, and 0.3, respectively), 3MM/DM MR (p-value = 0.4,

0.5, and 0.4, respectively), and 3HM/DX MR (p-value = 0.5, 0.3, and 0.3, respectively) after isotretinoin treatment in CYP2D6 EMs (Figure 2.8).

2.4 Discussion

Our study assessed the effect of isotretinoin on CYP2D6, CYP3A4, and UGT2B activities in patients with severe acne. Our results show no statistically significant downregulation of CYP2D6 activity in participants treated with isotretinoin compared to pre-treatment control using 2-hour plasma, 0-4-hour urinary DX/DM, and urinary DX plus DX-O-glucuronide/DM metabolic ratios as CYP2D6 activity markers although a deceasing trend was observed in the 0-4-hour urinary DX/DM MR after isotretinoin treatment (GMR 0.784 treatment/control) in CYP2D6 EMs. A weak induction of CYP3A4 activity after isotretinoin administration was observed in our study using a 0-4-hour urinary 3HM/DX metabolic ratio. No previous findings have shown induction of UGT2B expression by retinoids; however, the 0-4-hour urinary DX-O-glucuronide/DX metabolic ratio increased after isotretinoin administration, preliminarily suggesting an increased UGT2B activity *in vivo*.

Previously, a concentration-dependent induction of SHP mRNA and downregulation of CYP2D6 mRNA were observed in human hepatocytes after retinoid (*at*RA, 13-*cis*RA, and 4-oxo-13-*cis*RA) treatment and *in vivo* CYP2D6 downregulation was predicted due to the HNF4α-SHP regulation mechanism[137]. However, in male mice, following *at*RA, 13-*cis*RA, and 4-oxo-13-*cis*RA treatment no correlation between Shp induction and mouse Cyp2d downregulation was observed[137]. Similarly, no decrease in CYP2D6 activity following 13*cis*RA treatment was

observed in humans *in vivo*[137]. Our findings are consistent with this previous data in humans and suggest that the CYP2D6 downregulation by retinoids *in vitro* does not translate into CYP2D6 gene regulation by retinoids *in vivo* in humans. This lack of translation was suggested to be due to the lack of endogenous regulators such as bile acids, stellate cells and Kupffer cell-derived factors in *in vitro* hepatocyte systems and their potential role in SHP regulation[137]. In support of this, treatment of rat primary hepatocytes with bile acids was shown to interfere with the activation of Ntcp gene expression by retinoids mediated through Fxr activation and Shp induction[173]. In addition, estrogens and bile acids have been suggested to contribute to the regulation of CYP2D6 expression in CYP2D6 humanized mice[174,175]. Incorporating these necessary endogenous regulators into the *in vitro* systems may be necessary to predict the clinical DDI profile of isotretinoin.

Previously, a study by Stevison et al.[137] demonstrated a weak increase in DX/DM AUC ratio (GMR 1.25 treatment/control) and increased mean dextrorphan (oral) formation clearance from 1,840 L/hour to 2,369 L/hour with isotretinoin, suggesting that 13-*cis*RA is a CYP2D6 inducer. In contrast, no statistically significant changes in CYP2D6 activity after isotretinoin administration were observed in the current study. The differences in the participant population and study design between Stevison et al.[137] and our study might explain some differences in the observed CYP2D6 activity changes after isotretinoin administration. For instance, the current study enrolled a more diverse participant (patient) group than the prior investigation. The CYP2D6 genotype inclusion criteria between our study and Stevison et al.[137] study was different. Our study had a more diverse CYP2D6 genotype population than Stevison et al.[137] which excluded all *CYP2D6 *3* and *CYP2D6*4* participants. The effect of isotretinoin on CYP2D6

activity in different CYP2D6 genotype groups might be different. In our study, the CYP2D6 AS 2 participants showed a significant decrease in 0-4-hour urinary DX/DM metabolic ratio after isotretinoin treatment suggesting downregulation of CYP2D6 activity (Figure 2.2 e). This was not observed in participants with CYP2D6 AS of 1 or 1.5 participants (Figure 2.5). Stevison et al.[137] study recruited eight healthy males. In contrast, our study had a predominantly female, larger group of patients with severe acne. However, these differences in study populations did not impact the outcomes although some studies have reported that DM/DX (0-8-hour[162]; 0-10-hour[163]) urinary metabolic ratios were significantly lower in healthy, Caucasian, female CYP2D6 EMs compared to male CYP2D6 EMs. We found no significant differences in DX/DM 0-4-hour urinary metabolic ratio between females and males who are CYP2D6 EMs possibly due to the smaller number of participants. This result is similar to a previous study reporting no significant difference in DM/DX 0-24-hour urinary metabolic ratios between female and male healthy volunteers who were CYP2D6 EMs [176]. Stevison et al.[137] study measured full plasma AUC of dextromethorphan and dextrorphan, and the calculated magnitudes of change in DX/DM AUC ratio and dextrorphan formation clearance were 1.25- and 1.29-fold higher, respectively, after isotretinoin administration. The single time point 2-hour plasma metabolic ratio or 0–4-hour cumulative urinary metabolic ratio might not be sensitive enough to detect this weak induction in CYP2D6 activity. The interpretation of the single time point metabolic ratio might also be confounded by changes in the elimination rate of metabolite[73]. For example, induction of dextrorphan glucuronidation might decrease the metabolic ratio of DX/DM we observed. However, the trend towards decreased ratio was still observed when 0-4-hour urinary DX plus DX-O-glucuronide/DM MR was analyzed in CYP2D6 EMs. This observation suggests the

increased glucuronidation (Table 2.2, Figure 2.4 b) had a minor effect on the urinary DX/DM MR.

Previous studies have suggested that retinoids induce CYP3A4 via PXR activation *in vivo* and *in vitro*[137]. Our results are consistent with a weak induction of CYP3A4 activity with isotretinoin treatment based on the 0-4-hour urinary 3HM/DX MR (GMR 1.18 treatment/control; n=29) (Figure 2.3 b). However, no statistically significant changes were observed in the 0-4-hour urinary 3MM/DM MR (n=12), likely due to the smaller number of available data (Figure 2.3 a). Most of the participants had 3MM urinary concentrations below the limit of quantification and therefore were not included in the data analysis in this study. Hence 3HM/DX MR was used as a CYP3A4 *in vivo* activity indicator as previously described[51]. Yet, a limitation of this measure is that 3HM/DX metabolic ratio is also affected by the changes in the formation clearance and elimination of DX. The elimination pathway of DX (Figure 2.1) includes glucuronidation and metabolism to 3HM[73]. Based on larger DX-O-glucuronide *in vitro* formation clearance compared to 3HM, the major elimination pathway of DX would be glucuronidation[73].

An interesting finding in our study is the significant increase in urinary DX-O-glucuronide/DX metabolic ratio following isotretinoin treatment (GMR 1.216 treatment/control) (Table 2.2; Figure 2.4 b). A previous study showed that UGT2B isoforms, including UGT2B4, UGT2B7, UGT2B15, and UGT2B17, are primarily responsible for the glucuronidation of dextrorphan to dextrorphan-O-glucuronide suggesting the change in urinary ratio may be due to induction of these UGTs[73]. UGT1A1 was found to be induced by about 2-fold after treatment with retinoids

(30 µM *at*RA, 13-*cis*RA, or 4-oxo-13-*cis*RA) in human hepatocytes[153]. Another *in vitro* study indicated that vitamin A (100 µM) competitively inhibits UGT1A1, UGT2B4, UGT2B7, and UGT2B15 activity[177]. Retinoids including *at*RA and its metabolite, 4-hydroxy-retinoic acid (4-OH-RA), have been shown to be substrates of UGT2B7[120]. However, induction of UGT2B activities by retinoids *in vitro* or *in vivo* has not been previously shown. A significant limitation of this work is that the metabolic ratio of DX-O-glucuronide/DX has not been established or evaluated as a UGT2B *in vivo* marker before. Hence, whether the observed increase in DX-O-glucuronide/DX metabolic ratio after isotretinoin treatment indicates induction of UGT2B isoforms is inconclusive. Future *in vitro* studies would be helpful in understanding and predicting the mechanisms of retinoids' impact on UGT2B isoform expression and activities *in vivo.*

2.5 Conclusions

The *in vitro* study demonstrating the downregulation of CYP2D6 activity by isotretinoin does not translate into significant *in vivo* effects in humans. However, this study demonstrated a weak induction in CYP3A4 activity after isotretinoin administration *in vivo*, likely via a PXR-mediated pathway as previously reported. Future studies using other CYP3A4 probe substrates are necessary to determine if the CYP3A4 induction by retinoids *in vivo* is reproducible. This study has shown an increased dextrorphan-O-glucuronide to dextrorphan metabolic ratio after isotretinoin treatment and reported a preliminary observation of increased apparent UGT2B activity with retinoids. Future work is needed to demonstrate how isotretinoin treatment impacts the activity of UGT2B isoforms *in vitro* and *in vivo*.

Table 2.1 Retinoid Concentrations Measured on Both Study Days. Retinoid concentrations measured prior to treatment on Study Day 1 (Control) and with concurrent isotretinoin treatment on Study Day 2 (Treatment) for all participants (n=33).

Retinoids	**Study Day 1 Control**[1]	**Study Day 2 Treatment**[1]	**Treatment/Control GMR**[2] **(90%CI)**
Retinol (µM)	1.5 (1.4 – 1.6)	1.2 (1.1 – 1.3)	0.8 (0.7 – 0.9)
[a]*at*RA (nM)	2.8 (2.5 – 3.0)	41.1 (34.1 – 49.6)	14.9 (12.5 – 17.7)
[b]13-*cis*RA (nM)	2.7 (2.3 – 3.2)	1085 (904.1 – 1301)	397.3 (311.4 – 506.8)
[c]4-oxo-13-*cis*RA (nM)	6.7 (5.2 – 8.7)	2385 (1912 – 2974)	356.2 (254.7 – 498.3)

Retinoid concentrations were measured 2 hours post dose of Dextromethorphan and approximately 1 hour after a non-standardized meal.

[1]Study days 1 and 2 data were reported as geometric mean (90% CI).

[2]Treatment/Control geometric mean ratio with 90% CI were reported

[a]*at*RA: all-*trans*-retinoic acid

[b]13*cis*RA: 13-*cis*-retinoic acid

[c]4-oxo-13*cis*RA: 4-oxo-13-*cis*-retinoic acid

Table 2.2 Dextromethorphan Metabolic Ratios on Both Study Days. Dextrorphan (DX) with or without dextrorphan-O-glucuronide to dextromethorphan (DM), dextrorphan-O-glucuronide to dextrorphan (DX) plasma and urinary metabolic ratios (MR), 3-methoxymorphinan (3MM) to dextromethorphan (DM), 3-hydroxymorphinan (3HM) to dextrorphan (DX) urinary metabolic ratios (MR) for pre-treatment Study Day 1 (Control) and with concurrent isotretinoin treatment Study Day 2 (Treatment).

	n of Participants	Study Day 1 Control[1]	Study Day 2 Treatment[1]	Treatment/Control GMR[2](90%CI)
DX/DM Plasma MR	27[a]	3.0 (2.2 – 4.3)	3.1 (2.3 – 4.1)	1.014 (0.802 – 1.282)
DX/DM Urinary MR	24[b]	16.0 (10.2 – 25.0)	12.5 (8.9 – 17.6)	0.784 (0.553 – 1.111)
(DX + DX-O-glucuronide)/DM Urinary MR	21[c]	540.5 (329.9 – 885.7)	492.2 (324.4 – 746.8)	0.911 (0.591 – 1.404)
DX-O-glucuronide/DX Plasma MR	32[d]	62.5 (54.6 – 71.6)	63.5 (53.7 – 75.2)	1.017 (0.896 – 1.153)
DX-O-glucuronide/DX Urinary MR	25[e]	30.9 (26.2 – 36.6)	37.6 (31.9 – 44.3)	1.216 (1.063 – 1.391)
3MM/DM Urinary MR	12[f]	0.11 (0.074 – 0.16)	0.071 (0.049 – 0.103)	0.652 (0.404 – 1.055)
3HM/DX Urinary MR	29[g]	0.27 (0.24, 0.31)	0.32 (0.28, 0.38)	1.181 (1.034 – 1.349)

[1]Study days 1 and 2 data were reported as geometric mean (90% CI).

[2]Treatment/Control geometric mean ratio with 90% CI were reported

[a]Only participants who were extensive metabolizers of CYP2D6 (activity scores 1-2) were included.

[b]Only participants who were extensive metabolizers of CYP2D6 (activity scores 1-2) were included, three participants had concentrations below limit of quantification and were excluded from the analysis.

[c]Only participants who were extensive metabolizers of CYP2D6 (activity scores 1-2) were included, six participants had concentrations below limit of quantification and were excluded from the analysis.

[d]One participant had concentrations below limit of quantification was excluded.

[e]Eight participants had concentrations below limit of quantification were excluded.

[f]Twenty-one participants who had concentrations below limit of quantification were excluded.

[g]Four participants who had concentrations below limit of quantification were excluded.

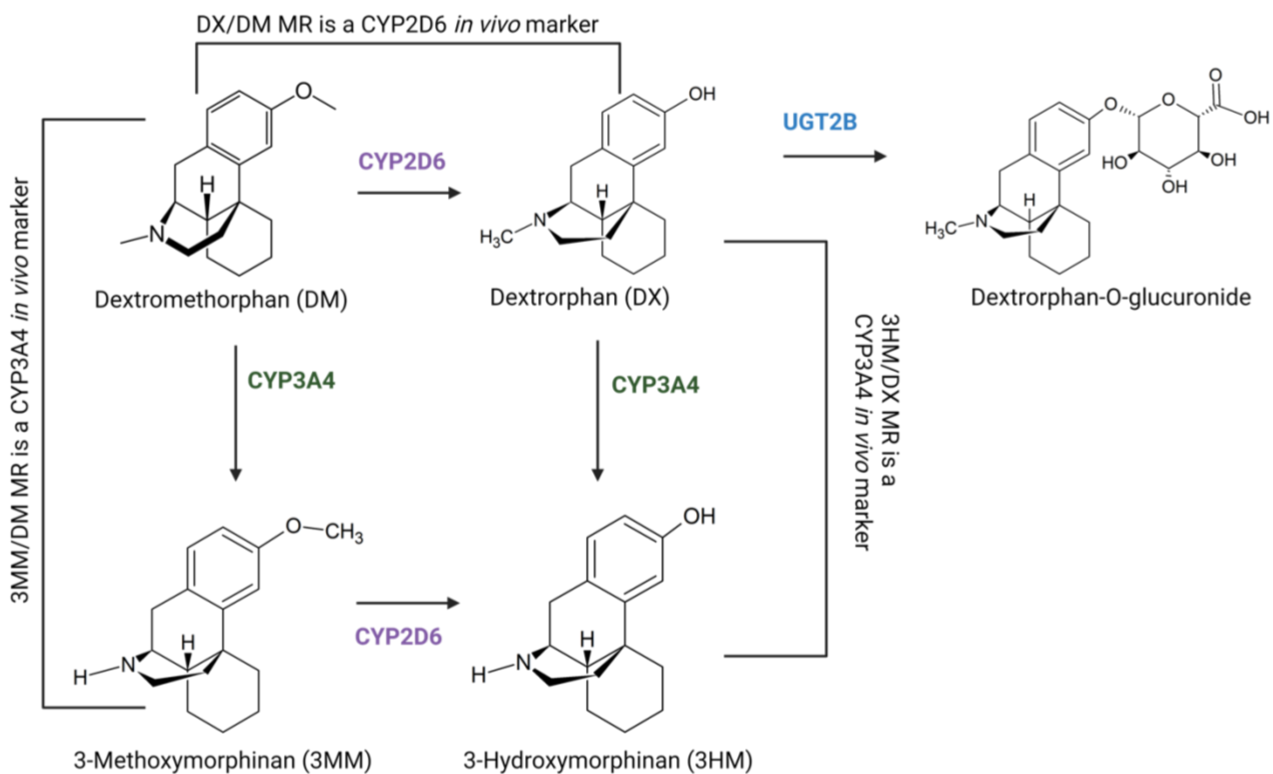

Figure 2.1 Dextromethorphan Metabolic Pathway. Surrogate markers for CYP2D6 (DX/DM metabolic ratio) and CYP3A4 (3MM/DM and 3HM/DX metabolic ratios) are shown in the scheme.

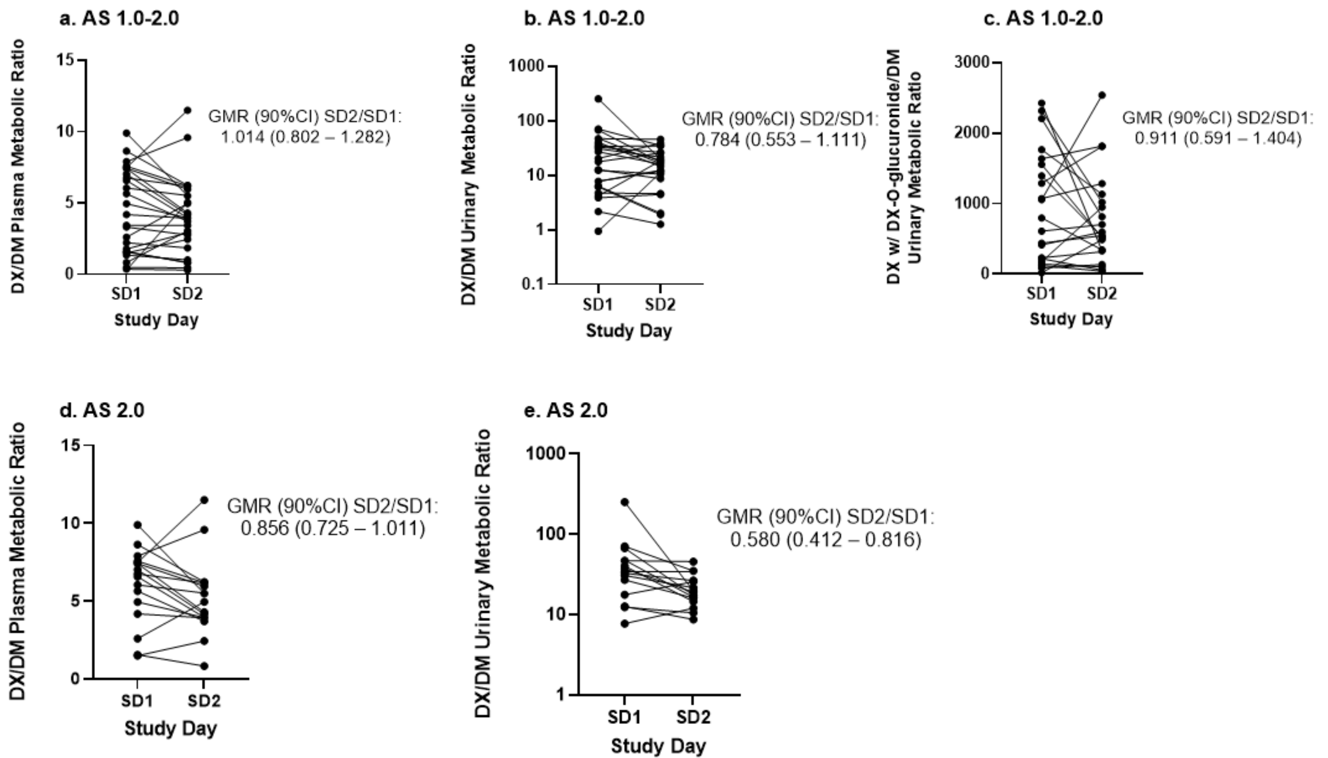

Figure 2.2 Plasma and Urinary Dextrorphan/Dextromethorphan Metabolic Ratios in Participants with CYP2D6 AS = 1-2 and 2. Dextrorphan/dextromethorphan (DX/DM) metabolic ratio in 2-hour plasma samples (n=27) (a) and 0-4-hour urine samples (n=24) (b), dextrorphan plus dextrorphan-O-glucuronide/dextromethorphan in 0-4-hour urine samples (n=21) (c) for pre-treatment on study day 1 and with concurrent isotretinoin treatment on study day 2 in participants who are extensive metabolizers with CYP2D6 activity scores 1-2. Dextrorphan/dextromethorphan (DX/DM) 2-hour plasma (n=17) (d) and 0-4-hour urinary metabolic ratios (n=15) (e) for pre-treatment on study day 1 and with concurrent isotretinoin treatment on study day 2 with CYP2D6 activity score = 2. The insets list the geometric mean ratios (90% confidence interval).

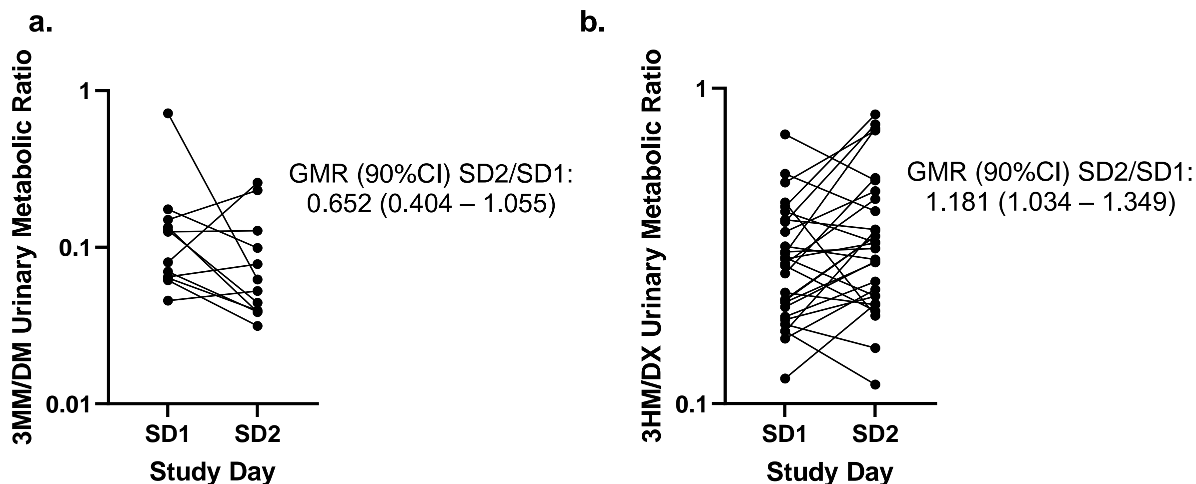

Figure 2.3 Urinary 3-Methoxymorphinan/Dextromethorphan and 3-Hydroxymorphinan/Dextrorphan Metabolic Ratios. 3-methoxymorphinan/dextromethorphan (3MM/DM) (n=12) (a) and 3-hydroxymorphinan/dextrorphan (n=29) (3HM/DX) (b) metabolic ratio in 0-4-hour urine samples for pre-treatment on study day 1 and with concurrent isotretinoin treatment on study day 2. The insets list the geometric mean ratios (90% confidence interval).

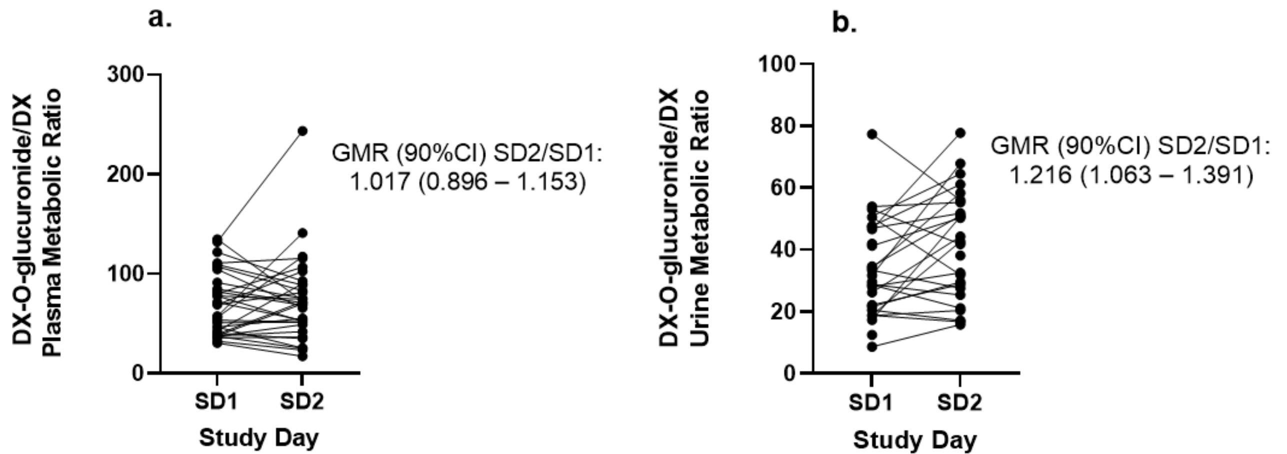

Figure 2.4 Plasma and Urinary Dextrorphan-O-Glucuronide/Dextrorphan Metabolic Ratios. Dextrorphan-O-glucuronide/dextrorphan (DX-O-glucuronide/DX) metabolic ratio in 2-hour plasma (n=32) (a) and 0-4-hour urine samples (n=25) (b) for pre-treatment on study day 1 and with concurrent isotretinoin treatment on study day 2. The insets list the geometric mean ratios (90% confidence interval).

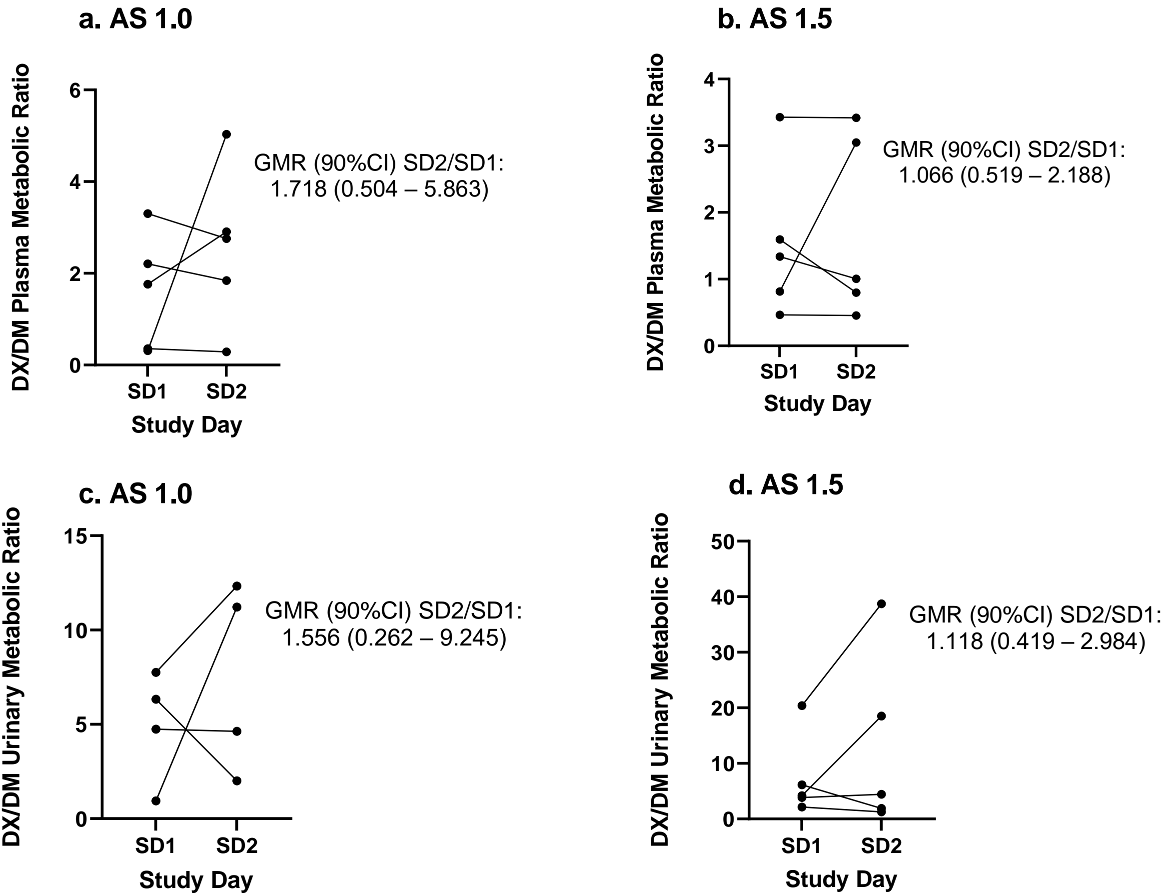

Figure 2.5 Plasma and Urinary Dextrorphan/Dextromethorphan Metabolic Ratios in Participants with CYP2D6 AS = 1 and 1.5. Dextrorphan/dextromethorphan (DX/DM) 2-hour plasma (n=5) (a) and 0-4-hour urinary metabolic ratios (n=4) (c) for pre-treatment on study day 1 and with concurrent isotretinoin treatment on study day 2 with CYP2D6 activity score = 1. Dextrorphan/dextromethorphan (DX/DM) 2-hour plasma (n=5) (b) and 0-4-hour urinary metabolic ratios (n=5) (d) for pre-treatment on study day 1 and with concurrent isotretinoin treatment on study day 2 with CYP2D6 activity score = 1.5. The insets list the geometric mean ratios (90% confidence interval).

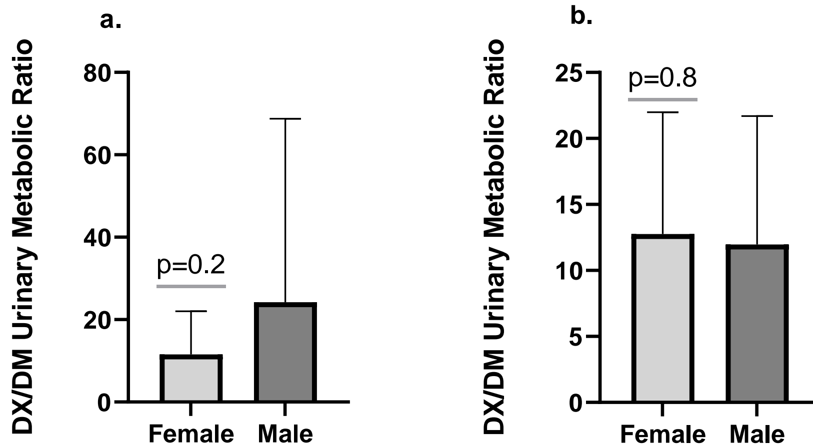

Figure 2.6 Urinary Dextrorphan/Dextromethorphan Metabolic Ratio in Female and Male CYP2D6 EMs. Dextrorphan/dextromethorphan (DX/DM) metabolic ratio in 0-4-hour urine samples for pre-treatment on study day 1 (16 females and 9 males) (a) and with concurrent isotretinoin treatment on study day 2 (16 females and 10 males) (b) between females and males who are extensive metabolizers with CYP2D6 activity scores = 1-2. The bar represents geometric mean and 95% confidence interval of DX/DM urinary metabolic ratio. Mann Whitney test was applied to compare DX/DM urinary metabolic ratio between females and males on either study day. P-value < 0.05 was considered statistically significant.

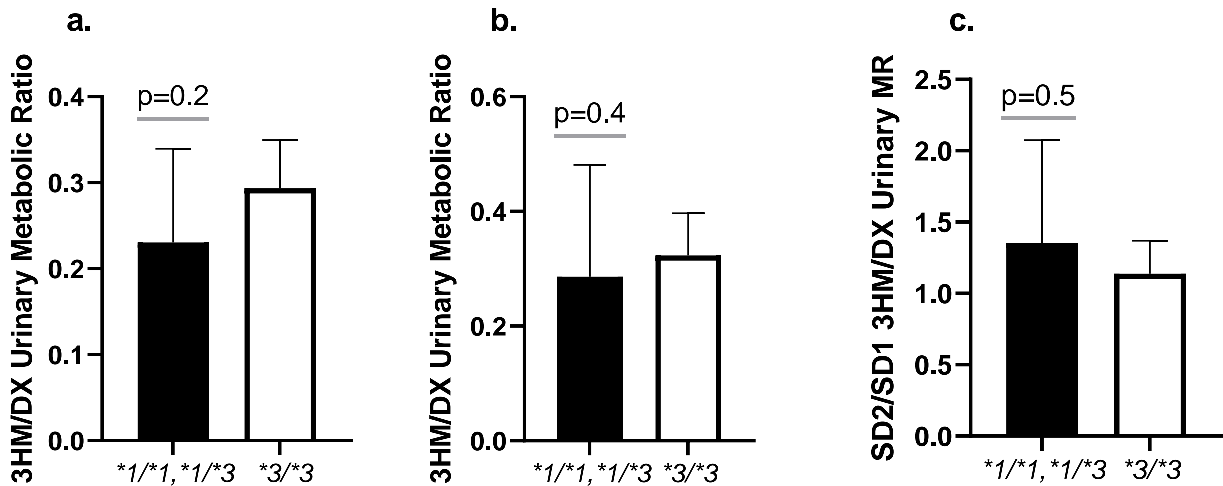

Figure 2.7 Urinary 3-Hydroxymorphinan/Dextrorphan Metabolic Ratio in Participants with Different CYP3A5 Genotypes. 3-hydroxymorphinan/dextrorphan (3HM/DX) metabolic ratio in 0-4-hour urine samples for pre-treatment on study day 1 (CYP3A5 expressers and nonexpressers: n=7 and 23, respectively) (a) and with concurrent isotretinoin treatment on study day 2 (CYP3A5 expressers and nonexpressers: n=6 and 25, respectively) (b) between CYP3A5 expressers (*CYP3A5* genotype: **1/*1* and **1/*3*) and non-expressers (*CYP3A5* genotype: **3/*3*). Changes of 3-hydroxymorphinan/dextrorphan (3HM/DX) with isotretinoin treatment between CYP3A5 expressers (n=6) and non-expressers (n=23) (c). The bar represents geometric mean and 95% confidence interval of 3HM/DX urinary metabolic ratio or ratio of study day 2 to study day 1 3HM/DX urinary ratio. Mann Whitney test was applied to compare 3HM/DX urinary metabolic ratio on study day 1, study day 2 and study day 2 to study 1 3HM/DX MR ratio between CYP3A5 expressers and non-expressers. P-value < 0.05 was considered statistically significant.

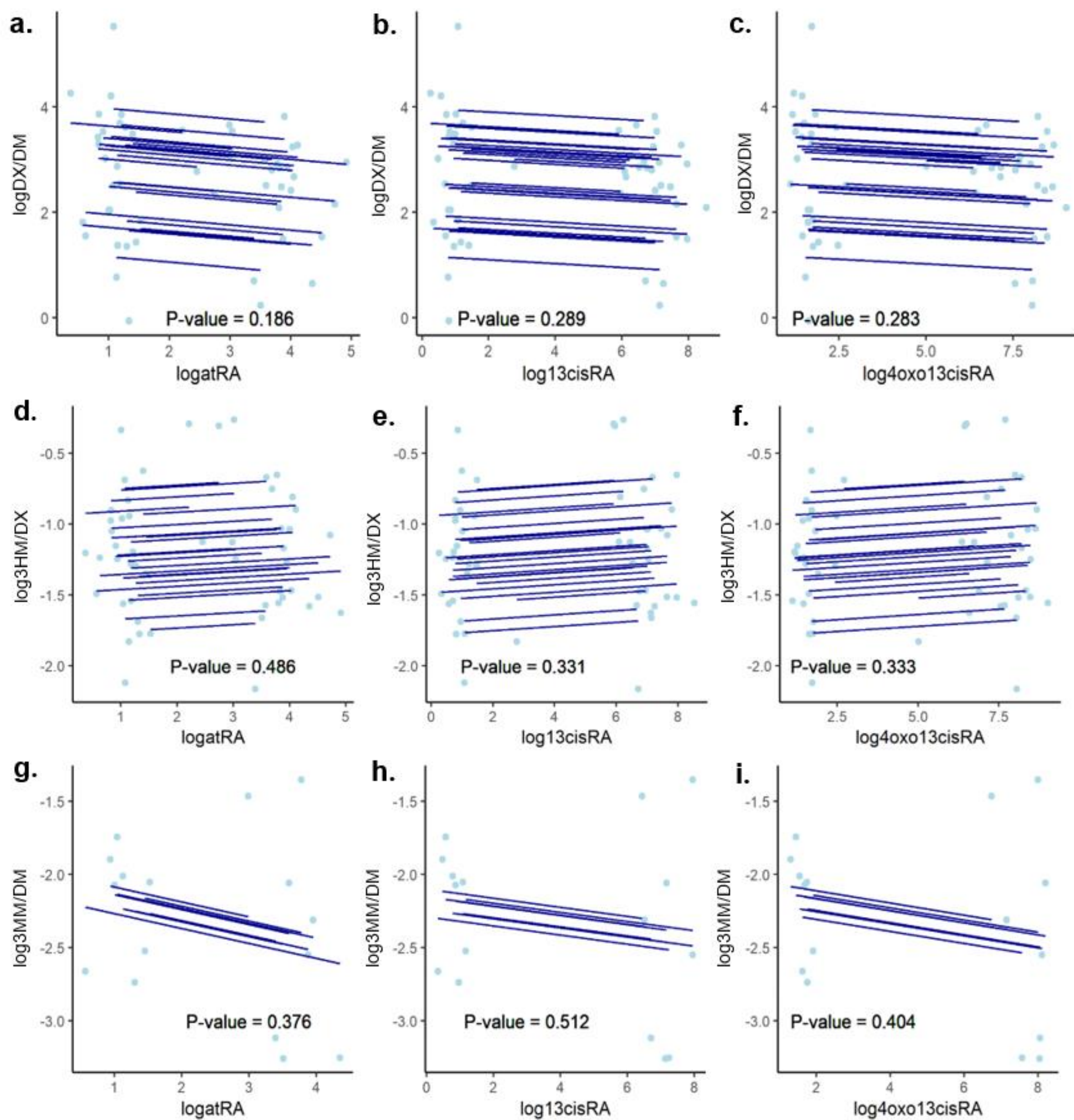

Figure 2.4 Correlation Between Plasma Retinoid Concentrations and Urinary Dextromethorphan Metabolic Ratios. Linear mixed-effect model of log transformed urinary dextrorphan/dextromethorphan (DX/DM) (a) - (c), 3-hydroxymorphinan/dextrorphan (3HM/DX)

(d) - (f), 3-methoxymorphinan/dextromethorphan (3MM/DM) (g) - (i) metabolic ratios versus plasma *at*RA, 13-*cis*RA, and 4-oxo-13-*cis*RA concentrations (nM). The light blue dots represent the two repeated measurements from 24 participants in (a) – (f) (3 participants were excluded from the analysis since the urinary concentrations were below the limit of quantification on one of the study days), 8 participants in (g) - (i) (18 participants were excluded from the analysis since the urinary concentrations were below the limit of quantification on at least one of the study days; 1 participant was excluded as an outlier). The dark blue lines represent the prediction for each participant from the linear mixed-effect model. P-value < 0.05 was considered statistically significant for the regression coefficient.

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