Study of the Impact of Isotretinoin Administration on CYP3A4 Activity Using 6β-Hydroxylation of Cortisol and Cortisone as Endogenous Biomarkers

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Abstract

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Isotretinoin is an FDA approved treatment for severe cystic acne and neuroblastoma and has been deployed in several non-FDA approved treatments of various cancers.[1-5] Cortisol (COL) and Cortisone (CON) are metabolized to their 6β-hydroxy metabolites primarily by CYP3A4.[6] Cortisol and 6β-hydroxycortisol (6β-OHCOL) are converted by 11β-HSD type 2 to cortisone and 6β-hydroxycortisone (6β-OHCON) respectively. Inversely, cortisone and 6β- hydroxycortisone are converted to cortisol and 6β-hydroxycortisol by 11β-HSD type 1.[6,7] Previous in vitro work has suggested that CYP3A4 in the liver is the main enzyme responsible for the 6β-hydroxylation of cortisol and cortisone making cortisol metabolism an attractive choice as an endogenous biomarker of CYP3A4 activity.[6] This research investigated the effect of isotretinoin on CYP3A4 activity in severe acne patients using the 6β-hydroxylations of cortisol and cortisone as endogenous biomarkers. The plasma concentrations and renal clearance of cortisol, cortisone, 6β-hydroxycortisol, and 6β-hydroxycortisone are reported, along with the urinary and plasma metabolic ratios, and the apparent formation clearances of 6β- hydroxycortisol, and 6β-

hydroxycortisone. The results show that there was an increase in the plasma concentrations of cortisol and 6β-hydroxycortisol suggesting that isotretinoin is impacting cortisol production. Furthermore, a decrease in 6β-hydroxycortisol renal clearance was found which supports isotretinoin impacting the interconversion of cortisol and cortisone in the kidney. Surprisingly, we saw a decrease in the formation clearance of 6β-hydroxycortisone which could imply CYP3A4 inhibition. Lastly, there was no change in plasma or urinary metabolic ratios which supports CYP3A4 not being induced by isotretinoin.

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List of Abbreviations

COL	Cortisol
CON	Cortisone
6β-OHCOL	6β-hydroxycortisol
6β-OHCON	6β-hydroxycortisone
CYP3A4	cytochrome P450 family 3 subfamily A member 4
CYP3A5	cytochrome P450 family 3 subfamily A member 5
CYP3A7	cytochrome P450 family 3 subfamily A member 7
CYP3A43	cytochrome P450 family 3 subfamily A member 43
CYP2D6	cytochrome P450 family 2 subfamily D member 6
PXR	pregnane X receptor
RXR	retinoid X receptor
13cisRA	13-cis-retinoic acid, isotretinoin
atRA	all-trans-retinoic acid
CL	clearance
CL_f	formation clearance
11β-HSD	11β-hydroxysteroid dehydrogenase
DX	dextrorphan
DM	dextromethorphan
SNPs	single nucleotide polymorphisms
LC-MS	liquid chromatography mass spectrometry
QC	quality control
KPi	potassium phosphate buffer
TEM	ion source temperature
CUR	curtain gas
GS1	nebulizing gas
GS2	drying gas
CAD	collision activated dissociation gas
IS	ion-spray voltage
EP	entrance potential
DP	declustering potential
CE	collision energy
PAR	peak area ratio
CV	coefficient of variance
LOD	limit of detection
LOQ	limit of quantification
S/N	signal to noise ratio
GM	geometric mean
GMR	geometric mean ratio
CI 90%	90% confidence interval
DME	drug metabolizing enzyme
DDI	drug-drug interaction
DDF	drug-food interaction
AUC	area under the plasma concentration-time curve
ADH	alcohol dehydrogenase

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Dedication

To my family, Yvonne, Rémy, and Selena for all of the love and support throughout the years. Without them this would not be possible.

Chapter 1. Introduction

1.1 General biochemistry of CYP3A and its role in drug metabolism

Cytochrome P450 family 3 subfamily A (CYP3A) is a human gene locus that includes a group of hemoprotein monooxygenases that can catalyze reactions involving drug metabolism and steroid synthesis.[7] The CYP3A subfamily metabolizes more drugs than any other group of enzymes in the CYP superfamily.[25] The subfamily has four members: CYP3A4, CYP3A5, CYP3A7, and CYP3A43. CYP3A5 is a polymorphic isoform and is functional only in humans that carry the CYP3A5*1 allele which is most common among Sub-Equatorial African populations.[28,29] The CYP3A5*3 allele results in lack of CYP3A5 expression, it is expressed in high frequency in Asian populations, and is almost fixed in European populations.[28,29] CYP3A7 is expressed in fetal livers. As the fetus matures the levels of CYP3A7 decrease through pediatric age and into adult stages.[30] With the exception of the testis, CYP3A43 is not expressed in human tissues at significant levels and is about 0.1% of CYP3A4 expression level in the liver.[31]

CYP3A4 is localized to the endoplasmic reticulum of hepatocytes and enterocytes. CYP3A4 is the most abundant CYP3A isoform (85.4%) in the liver and small intestines.[8,9,32,33,34,35] It is responsible for the phase I metabolism of around 50% of currently used medications including, but not limited to: immunosuppressants like tacrolimus, chemotherapeutics including tamoxifen, statins like atorvastatin, and corticosteroids including cortisol and cortisone.[5-19,20-24,26,27] Hepatic CYP3A4 has been shown to have an *in vivo* half-life of 70 to 140 hours.[10]

Although there is little genetic variability in CYP3A4 amongst healthy populations, the enzyme activity has high interindividual variability (20-100-fold) *in vivo* and *in vitro*. However,

this is mostly due to homeostatic regulatory mechanisms and is likely not attributed to genetic polymorphisms.[9,10,15,16,36-38] Additionally, it has been shown that females have around two-fold higher tissue concentrations of CYP3A4 and metabolize CYP3A4 substrates faster than men.[9,17,39] Lastly, CYP3A4 is expressed in very low levels in the fetus and reaches adult levels after the first year of life.[30,40]

1.2 CYP3A4 drug interactions and regulation of expression

Drug metabolizing enzymes (DMEs) can be induced, reversibly inhibited, and irreversibly inhibited by other drugs, foods, and supplements. This effect on DMEs can lead to a change in how a second drug impacts the body, either increasing or decreasing effectiveness. This reaction between two or more drugs is known as a drug-drug interaction (DDI) and when it is between a drug and food it is known as a drug-food interaction (DFI). These drug interactions are a major concern when taking medications. To address this for prescription medications the potential drug-drug interactions of comedications need to be considered by a pharmacist before prescriptions are filled for a drug. Unfortunately, for other products like food, drinks, and supplements no such gatekeeper exists. Therefore, use of these products without the doctor's knowledge can cause therapeutic failure or drug toxicity due to unmanaged or unknown DDIs.

CYP3A4 can be inhibited by a number of drugs including, but not limited to, azole antifungals (ketoconazole, fluconazole, itraconazole), indinavir, ritonavir, clarithromycin and nefazodone.[10,11,41] One natural product, grapefruit, has also been shown to potently inhibit intestinal CYP3A4 with an effect similar to erythromycin, with both grapefruit juice and felodipine increasing felodipine levels around three-fold.[12,13,42] On average grapefruit increases the felodipine area under the plasma concentration-time curve (AUC) by 240%.[43] CYP3A4 is

also inducible by many different drugs, such as carbamazepine and rifampin.[5,14,19] Due to the large number of drugs CYP3A4 metabolizes and the possibility that drugs inhibit or induce its activity, it is important to identify potential drug-drug interactions with this enzyme.[6,21-24]

Induction of CYP3A4 transcription occurs when a xenobiotic binds to the ligand-binding pocket of pregnane X receptor (PXR), this complex then translocates to the nucleus where it binds to a response element on the CYP3A4 promoter as a heterodimer with retinoid X receptor (RXR).[14,19,43-45] Previous *in vitro* and *in vivo* studies have shown that retinoids including isotretinoin can induce CYP3A4.[5,46-49] For example, treatment of human hepatocytes with isotretinoin led to an increase in CYP3A4 mRNA expression.[5,50] Similarly, human hepatocytes showed an increase in PXR activity when treated with isotretinoin using a PXR reporter gene assay.[5,50] This work supports the hypothesis that isotretinoin induces CYP3A4 via the activation of PXR.

1.3 General properties of Isotretinoin and its relationship to CYP3A4

Isotretinoin, the generic drug name for 13-*cis*-retinoic acid (13*cis*RA) is a small molecule (300.4 g/mol) prescription medication previously sold under the brand name Accutane®. The first FDA approved isotretinoin containing product was approved in 1982.[51] Isotretinoin is available in 10, 20, 30, and 40 mg soft gelatin capsules taken orally to treat severe recalcitrant acne.[1-4] Isotretinoin has several non-FDA approved uses in the treatment of various cancers like thyroid and lung cancer.[3,4] Isotretinoin's mechanism of action is not known but it treats acne by inhibiting sebaceous gland function and keratinization and has been shown to reduce sebaceous gland size and sebum production.[4] It is in a class of drugs known as retinoids, which are a group of compounds derived from vitamin A (retinol). More specifically, isotretinoin is a first

generation retinoid, meaning it is a naturally occurring metabolite of vitamin A.[52] In the human body, isotretinoin is reversibly cis-trans isomerized to its geometric isomer *all-trans*-tretinoin also known as *all-trans*-retinoic acid (*at*RA).[1,2,5,53,54] In healthy human livers endogenous 13*cis*RA is present at lower average concentrations (8.3 pmol/g) than retinol (14.1 nmol/g) and *at*RA (42.6 pmol/g).[55] This holds true also for individuals with steatosis and nonalcoholic steatohepatitis.[55] Conversely, in human serum endogenous 13*cis*RA was found to circulate at slightly higher concentrations than *at*RA in both fed and fasted individuals.[56] In a study of participants receiving 13*cis*RA at 0.5 mg/kg/day for three months, the average measured isotretinoin concentration in serum was 483 nM.[57]

In humans 13*cis*RA is metabolized through 4-hydroxylation by CYP26s and other CYP isoforms including CYP3A4 and CYP2C8 to 4-hydroxy-13*cis*RA which is then oxidized by CYP26s and alcohol dehydrogenases (ADHs) to the main metabolite 4-oxo-13*cis*RA which can also reversibly cis-trans isomerize to 4-oxo-*at*RA.[2,4,5,55,58,59] Additionally, isotretinoin can reversibly isomerize to *at*RA which is then 4-hydroxylated also by CYP26s to 4-hydroxy-*at*RA and is subsequently oxidized also by CYP26s and ADHs to 4-oxo-*at*RA which can isomerize to 4-oxo-13*cis*RA.[4,55,60] This partial metabolic pathway of isotretinoin is shown below in Figure 1

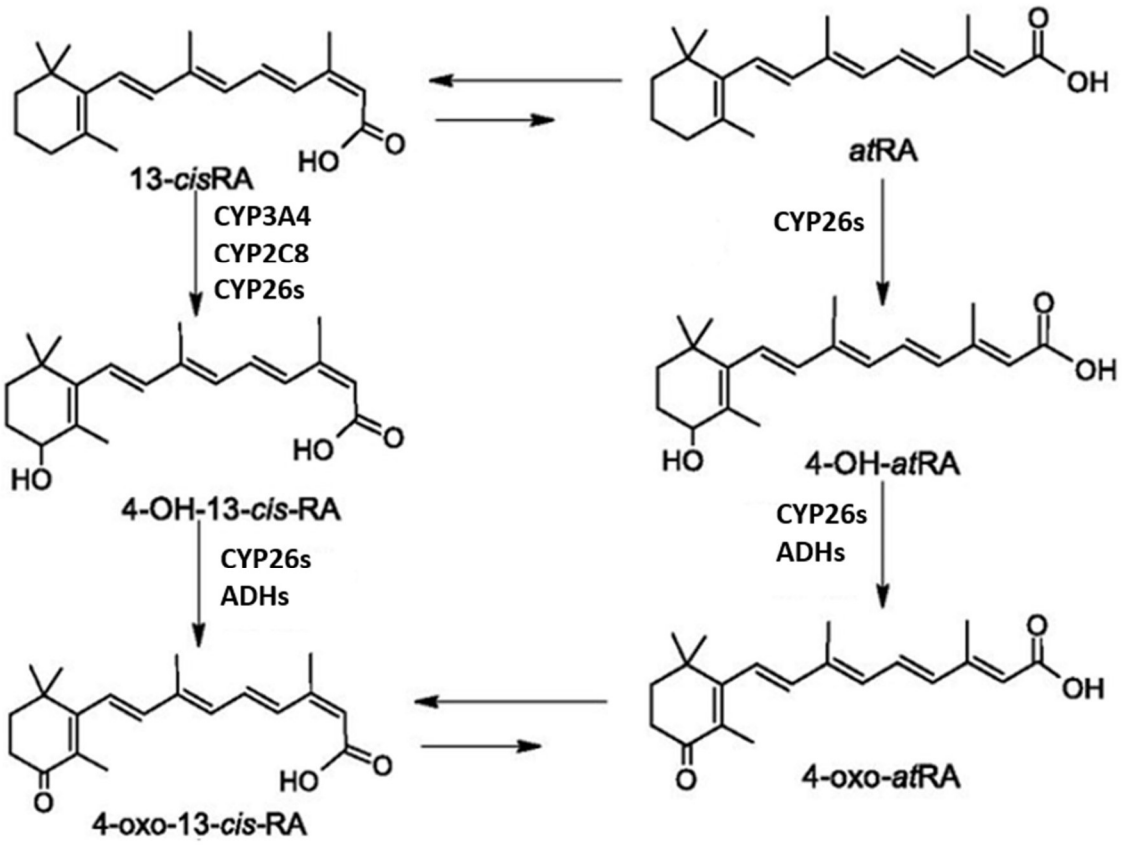

Figure 1. 13*cis*RA metabolic pathway shown with chemical structures and known metabolizing enzymes.[4,55]

Isotretinoin has an average half-life of around 20 hours in either fed or fasted conditions and it is more than 99.9% bound to plasma proteins, mainly albumin.[1] The lack of an intravenous dosage form of isotretinoin means its disposition cannot be well characterized.[60] Isotretinoin blood concentrations after oral dosing follow a linear two-compartment model with lag time before the onset of first-order absorption.[60] Due to isotretinoin's high lipophilicity it is absorbed best with a high fat meal.[1] Following a 40 mg oral dose, subjects given a high fat meal had a higher maximum concentration and larger AUC than fasted subjects.[61] Maximum isotretinoin concentration ranged from 74 to 511 ng/mL at 1 – 4 hours after a 100 mg oral dose.[62] Following the same 100 mg oral dose, the amount excreted unchanged in the feces is 53 – 74% of the oral

dose, which likely reflects a combination of biliary excretion and unabsorbed drug.[62] After an 80 mg oral dose, pediatric neuroblastoma patients had an average apparent oral clearance (CL) of 15.9 L/hr.[63]

Previous studies have shown that isotretinoin has the potential to cause drug-drug interactions by way of a number of DMEs.[3,5] In human hepatocytes, 13*cis*RA treatment decreased the mRNA expression of OATP1B1, CYP1A2, CYP2C9, and CYP2D6 while it increased the expression of CYP2B6 and CYP3A4 mRNA.[3,5,50] More specifically and relevant to this work, clinical studies have suggested that 13*cis*RA may be an inducer of CYP3A4.[3] Isotretinoin regulates CYPs through gene expression via binding to RAR or PXR.[3,5]

1.4 General properties of cortisone, cortisol, & their 6β-hydroxy metabolites

Cortisone is a naturally occurring inactive pregnene (21-carbon) glucocorticosteroid hormone.[64,65] Therapeutically cortisone is used as a prodrug for the treatment of adrenal insufficiency and as an anti-inflammatory drug.[65] Cortisol is an active pregnene glucocorticosteroid and also commonly used therapeutically as a treatment for inflammation and adrenal insufficiency.[64,66] In the liver, cortisone is converted to the active cortisol, by 11β-hydroxysteroid dehydrogenase (11β-HSD) type 1.[6,20] In addition, cortisol can be converted back to inactive cortisone by 11β-HSD type 2 in the kidney.[6,20] Cortisol and cortisone are irreversibly metabolized to 6β-hydroxycortisol and 6β-hydroxycortisone, primarily by CYP3A4.[6] Increased cortisol in the blood leads to increasing blood pressure and glucose levels and also suppresses the immune system.[67,68] The mechanism of action for cortisol is through the binding of glucocorticoid receptors in the cytoplasm, this complex then translocates to the nucleus and impacts gene expression that elicits downstream effects like inhibiting inflammatory

transcription factors including phospholipase A2 and NF-kappa B, while promoting anti-inflammatory transcription factors.[68-70]

In humans, cortisol levels have been shown to change on a diurnal cycle with concentrations in the saliva and serum being higher in the morning than the afternoon.[71] In healthy adults, the average total plasma concentration of cortisol is 20 μg/dL with an average of 1 μg/dL being free cortisol.[72] Treatment with oral hydrocortisone has been shown to achieve physiologically equivalent cortisol plasma concentrations.[73] The main protein for transporting glucocorticoids in the blood is corticosteroid-binding globulin (CBG) with 80-90% of circulating glucocorticoids bound to CBG.[74,75] Additionally, around 5-15% of glucocorticoids bind non-specifically to albumin with the remaining 5-10% circulating free as active hormones.[74,75] Cortisol concentration has also been shown to increase with stress and can exceed the 28 μg/dL binding capacity of CBG.[72,76,77] In healthy adult men, the cortisol secretion rate is between 15 and 20 mg per day with women's rates being slightly lower.[72] The partial metabolic pathways of cortisol and cortisone are shown below in figure 2.

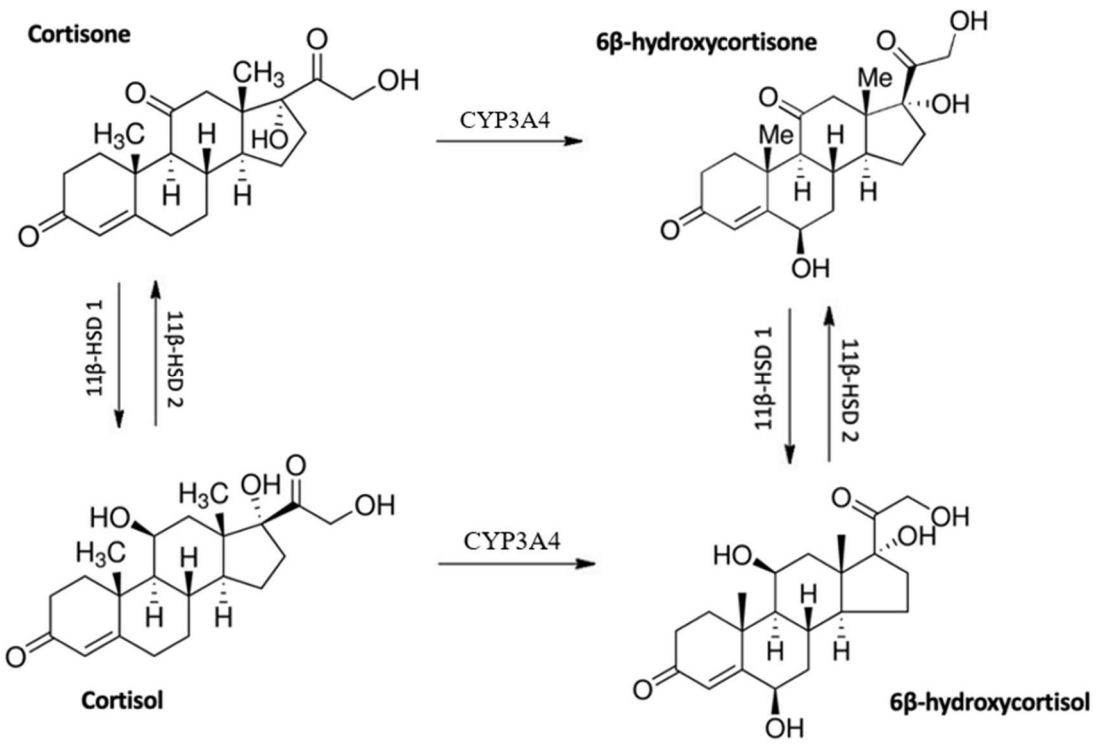

Figure 2. Cortisone and cortisol metabolic pathway shown with chemical structures and known metabolizing enzymes.

1.5 Endogenous biomarkers of CYP3A4 activity

Traditionally, CYP3A4 activity *in vivo* was assessed using a probe substrate drug with the classic example being midazolam. However, the FDA maintains a database of recommended probe substrates that include alfentanil, felodipine, and tacrolimus among others.[24,41] More recently, the use of plasma and urinary metabolic ratios of endogenous compounds as biomarkers of CYP3A4 activity have been investigated, including 11β-testosterone:testosterone and 6β-hydroxyandrostenedione:androstenedione, among others.[24] Currently the preferred endogenous biomarker for CYP3A4 evaluation is oxidation of cholesterol to 4β-hydroxycholesterol.[78] By using an endogenous biomarker of enzyme activity, the need for dosing with probe drugs could be eliminated in some circumstances such as, individualized treatment, early risk assessment, chronic conditions impacting CYP3A4 activity, and where dosing with a substrate probe is not

possible.[22,24,78] Furthermore, endogenous biomarkers are not appropriate for comprehensive drug-drug interaction studies as they only account for hepatic CYP3A4 metabolism, whereas probe drugs like midazolam with their oral and intravenous formulations can characterize both hepatic and intestinal CYP3A4 metabolism.[78]

Previous *in vitro* studies have shown that CYP3A4 in the liver is the primary enzyme responsible for the 6β-hydroxylation of cortisol and cortisone.[6,79] Based on the *in vitro* studies the $f_{m,CYP3A4}$ of cortisol and cortisone in the 6β-hydroxylation was around 60%.[6] Because CYP3A4 is the main contributor to 6β-hydroxylation of cortisol and cortisone, these metabolites make good targets for endogenous markers of CYP3A4 activity. In the past, the 6β-hydroxycortisol-to-cortisol ratio has been utilized to detect CYP3A4 induction with rifampin and CYP3A4 inhibition by clarithromycin.[6,80-84] Several methods have been used to characterize CYP3A4 activity using the 6β-hydroxy metabolites: urinary ratio of 6β-hydroxycortisol:cortisol, urinary ratio of 6β-hydroxycortisone:cortisone, plasma ratio of 6β-hydroxycortisol:cortisol, plasma ratio of 6β-hydroxycortisone:cortisone, formation clearance (CL_f) of 6β-hydroxycortisol, CL_f of 6β-hydroxycortisone, and the combined CL_f of 6β-hydroxycortisol and 6β-hydroxycortisone.[5,6,21-24] However, there can be many confounding factors that lead to variability in these measurements such as hepatic CYP3A4 induction, increased cortisol synthesis, the interconversion of cortisol and 6β-hydroxycortisol to and from cortisone and 6β-hydroxycortisone, and the fact that 6β-hydroxylation only accounts for less than 10% of cortisol and cortisone elimination.[5]

1.6 Goals, Aims, & Hypothesis

Previous work has suggested that isotretinoin treatment induces CYP3A4 *in vitro*.[3,5] Furthermore, clinical studies suggested that this *in vitro* induction translates to an *in vivo* induction of CYP3A4.[3,5] Additionally, the 6β-hydroxylation of cortisol and cortisone have been explored as endogenous biomarkers of CYP3A4 activity.[5,6,21-24,80-84] The aim of this work was to determine if treatment with 13*cis*RA impacted CYP3A4 activity by comparing the metabolic ratios and formation clearance of cortisol, cortisone and their 6β-hydroxy metabolites before and after isotretinoin dosing. Our hypothesis is that treatment with isotretinoin will lead to an induction of CYP3A4. To test this, a clinical study was conducted in 33 participants with severe acne during which they received an average daily dose of isotretinoin at 1 mg/kg/day for an average of 66 days.[85] Urine and blood were collected before and during treatment with 13*cis*RA and analyzed for the concentrations of cortisol, cortisone, and their 6β-hydroxy metabolites in order to characterize the metabolic ratios and formation clearances.

Chapter 2: Experimental Procedures and Materials and Methods

2.1. Clinical Study Design

This study was conducted at the University of Washington, approved by the University of Washington Institutional Review Board, and registered with clinicaltrials.gov (NCT03076021). All participants and/or the legal guardians of underage participants gave written informed consent. The study was designed to detect if dosing with isotretinoin impacted cytochrome P450 2D6 (CYP2D6) activity by measuring and comparing the metabolic ratio of dextrorphan (DX) to dextromethorphan (DM) before and during an isotretinoin dosing regimen.[32] Thirty-three participants were enrolled. The study demographics were as follows: 22 females and 11 males aged 16 to 45 with an average age of 24. Of the 33 participants, 25 were Caucasian, 7 were Asian, and 1 was African American.

Study day one was done prior to the participants beginning isotretinoin treatment and was used as a control. After the first study day participants took isotretinoin at an average of 1.01 ± 0.20 mg/kg/day for 10 – 104 days with an average duration of treatment of 66 days. On both study days, participants arrived at the clinic where they received a 30 mg dose of DM orally and blood samples were drawn at two hours post-DM dosing. Blood was collected into light protected EDTA tubes on ice and plasma was separated by centrifugation for 10 minutes at 3,000 g and stored at -80° C. Urine was collected over four hours following DM dosing. Plasma and urine were stored at -80° C and cortisol, cortisone, and their 6β-hydroxy metabolites were quantified from the samples together with the retinoids.[86] Only the metabolic ratio for 6β-hydroxycortisol to cortisol was calculated due to the lack of quantifiable levels of 6βOH-cortisone in plasma.

All participants also provided DNA via a buccal swab for genotyping of CYP2D6 and CYP3A5 single nucleotide polymorphisms (SNPs).[85] Genomic DNA was extracted from buccal swabs using a Qiagen© QIAmp DNA Mini Kit (Germantown, MD). CYP3A5 genotyping assays were done on an Applied Biosystems™ StepOnePlus Real-Time PCR System using a Taqman® (Waltham, MA) allelic discrimination assay.[85,86] The allele, SNP, and assay IDs can be found below in Table 1.

Table 1. CYP3A5 alleles, SNP IDs, & Assay IDs

Allele	SNP ID	Assay ID
*3	rs776746	C_26201809_30
*6	rs10264272	C_30203950_10
*7	rs41303343	C_32287188_10

2.2 Chemicals & Reagents

Cortisone, cortisol, and 6β-hydroxycortisol were purchased from Millipore Sigma (St. Louis, MO) and 6β-hydroxycortisone was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Deuterium labelled internal standards of cortisone-2,2,4,6,6,9,12,12-d_8 and cortisol-9,11,12,12-d_4 as 100 µg/mL solution in methanol were purchased from Millipore Sigma (St. Louis, MO). Liquid Chromatography Mass spectrometry (LC-MS) (Optima) grade methanol, acetonitrile, water, and formic acid were purchased from Thermo Fisher Scientific (Waltham, MA). Monobasic and dibasic potassium phosphate were purchased from Millipore Sigma (St. Louis, MO). Ultra-Low Hormone and Steroids Mass Spect Gold Human Serum (blank serum) was purchased from Golden West Diagnostics (Temecula, CA).

2.3 Preparation of calibrators, quality controls, urine, and plasma samples

For urine analysis, calibration standards and quality control (QC) samples for measurement of cortisol, cortisone, 6β-hydroxycortisol, and 6β-hydroxycortisone were prepared in 100 mM potassium phosphate buffer (KPi), pH 7.4 to mimic human urine. The calibration curve included 10 concentrations listed in Table 2. Quality control (QC) samples were prepared at three concentrations listed in Table 2. To prepare calibrator and QC samples 25X working stocks containing all four analytes were made in methanol for the calibration curve and for the QCs. Then, calibrators and QCs were prepared by adding 20 µL of 25X calibrator stock solution to 470 µL of KPi in a 1.7 mL microcentrifuge tube. This 490 µL calibrator or QC sample or 490 µL of urine were used for sample analysis. For sample preparation a solution including both labeled internal standards was made in methanol to 1.36 µM. To prepare urine samples, calibrators, and QCs for analysis, 10 µL of the solution containing labeled internal standards was added to each sample. All samples, QCs, and calibrators were then vigorously vortexed for 10 seconds, followed by the addition of 750 µL of acetonitrile and another 10 seconds of vigorous vortexing. All samples, QCs, and calibrators were then centrifuged at 15,000 g for 15 minutes and 250 µL of the supernatant was removed to a 96 well plate for LC-MS/MS analysis.

Table 2. Calibration curve & quality control concentrations (nM) for quantification of urine

Concentration in Urine (nM)				
	Cortisol	6βOH-Cortisol	Cortisone	6βOH-Cortisone
Calibrator	nM	nM	nM	nM
s0	0	0	0	0
s1	0.69	0.66	0.69	0.67
s2	2.8	2.6	2.8	2.7
s3	14	13	14	13
s4	28	26	28	27
s5	70	67	70	67
s6	138	132	139	133
s7	180	172	181	173
s8	235	225	236	227
s9	276	264	277	266
LQC	2.8	2.6	2.8	2.7
MQC	70	67	70	67
HQC	180	172	181	173

For plasma analysis, calibration standards and quality control (QC) samples for measurement of cortisol, cortisone, 6β-hydroxycortisol, and 6β-hydroxycortisone were prepared in the mass spect gold blank serum which was determined to be free of interfering endogenous cortisol and its metabolites. The concentrations used for the calibration curves and quality control samples are listed for each analyte in Table 3. Cortisol-d_4 was used as an internal standard. The calibrators and QCs were prepared as described for the urine samples. First a 25X working solution was made in methanol and then this working solution was diluted into blank serum to the final concentrations in Table 3.

Table 3. Calibration curve & quality control concentrations (nM) for quantification of plasma

Concentration in Serum (nM)				
	Cortisol	6βOH-Cortisol	Cortisone	6βOH-Cortisone
Calibrator	nM	nM	nM	nM
s0	0	0	0	0
s1	25	0.25	15	0.25
s2	75	0.50	20	0.50
s3	150	1	30	1
s4	250	3	50	3
s5	400	4	75	4
s6	600	5	100	5
s7	800	7	150	7
s8	900	10	200	10
LQC	100	0.75	25	0.75
MQC1	350	1	30	1
MQC2	500	2	40	2
HQC	750	6	60	6

For the calibration curves, quality controls, and plasma samples, 60 µL of the samples from curves, QCs, and human plasma were added to a 96-deep well PCR plate. For the plasma quantification the labeled internal standard was added directly to the organic precipitation solution (acetonitrile) to a final concentration of 0.68 µM by adding 250 µL of 27.2 µM cortisol-d_4 to 10 mL of acetonitrile. Then, using a multichannel pipette 90 µL of acetonitrile containing 0.68 µM cortisol-d_4 in methanol were added and the samples mixed thoroughly by pipetting. All the samples were then centrifuged at 4° C for 45 minutes at 3,000 g, the supernatant was transferred to a new PCR plate and centrifuged for a second time at 4° C for 30 minutes at 3,000 g. For analysis, 75 µL of supernatant was removed to a new 96-well LC-MS/MS plate for analysis.

2.4 HPLC-MS/MS

The HPLC-MS/MS method for analyzing urine samples was adapted from previous work[1,2] with the addition of deuterium labeled cortisone-d_8 and cortisol-d_4 internal standards. For urine, the samples were analyzed using an Agilent 1290 Infinity LC System (Santa Clara, CA) coupled to a Sciex 5500 QTRAP mass spectrometer (Framingham, MA). The analytes were separated using a Hypersil GOLD^{TM} (1.9 μm, 100 × 2.1 mm^2) column and a 2.1 mm ID filter cartridge (0.2 μm) both from Thermo Fisher Scientific (Waltham, MA). A gradient elution of seven minutes was used with water and 0.1% formic acid (A) and acetonitrile and 0.1% formic acid (B) at a 0.4 mL/min flow rate. Starting conditions were 10% B for 30 seconds, increased to 90% B over the next three minutes, kept at 90% for the next 1.5 minutes, before decreasing back to 10% B in the next 10 seconds, then re-equilibrating at 10% B for one minute and 50 seconds. Negative ion electrospray ionization was used for analyte detection. As described previously,[5,6] the following transitions were monitored: cortisol *m/z* 407.062 > 331.0, cortisone *m/z* 405.061 > 329.3, 6β-hydroxycortisol *m/z* 423.056 > 347.1, 6β-hydroxycortisone *m/z* 421.035 > 345.2, cortisol-d_4 *m/z* 411.062 > 335.0, and cortisone-d_8 *m/z* 413.061 > 337.3. Other MS parameters were the same for all analytes and internal standards and were as follows: ion source temperature (TEM) at 500, curtain gas (CUR) at 10, nebulizing gas (GS1) at 80, drying gas (GS2) at 60, collision activated dissociation gas (CAD) at -2, ion-spray voltage (IS) at -4500, and entrance potential (EP) at -10. The declustering potential (DP) for cortisone, cortisone-d_8, and both 6β-hydroxy metabolites was at -45, while for cortisol and cortisol-d_4 the (DP) was at -30. The collision energy (CE) for cortisol, 6β-hydroxycortisol, and cortisol-d_4 was at -26, and for cortisone, 6β-hydroxycortisone, and cortisone-d_8 the CE was at -22.

Plasma samples were analyzed using an AB Sciex 6500 QTRAP system (Framingham, MA) coupled to an Agilent 1290 Infinity II LC System (Santa Clara, CA). The column, filter, gradient, and solvents used were the same as for the urine analysis described above, with the only difference being that a mobile phase flow rate of 0.5 mL/min was used. The MS/MS detection was done using negative ion electrospray ionization as described for urine samples except for curtain gas which was set to 35 for all analytes. The chromatograms were integrated using Sciex Analyst® software version 1.6.3 (Framingham, MA).

2.5 Method Validation

The method was validated in accordance with the FDA Guidelines for Industry Bioanalytical Method Validation.[87] For validation of urine analysis method, the acceptance criteria for the calibration curves were defined as greater than 75% of nonzero concentrations with less than 15% error from the nominal concentrations, 20% error was accepted at the limit of quantification (LOQ). Calibration curves for each analyte were prepared in duplicate, weighted 1/x, and fit linearly to the data using Analyst. Blank KPi with and without the internal standards was analyzed for any interference with the analytes of interest. The accuracy and precision were determined on three separate days. Intraday variability was determined by the coefficient of variance (CV) of five replicates of each QC on the same day. Inter-day variability was calculated by the CV of 3 replicates of each QC on three separate days. Intra- and Inter-day variability can be found in Table 4. The limit of detection (LOD) was defined for each analyte as the lowest concentration with a reproducible signal to noise ratio (S/N) > 3. For the urine, the LOD was established as 0.59 nM for cortisol, 0.56 nM for 6β-hydroxycortisol, 0.59 nM for cortisone, and 0.57 nM for 6β-hydroxycortisone. The LOQ was defined for each analyte as the lowest

concentration with a S/N > 10 and with a CV% determined as <20%. The LOQ for urine was established as 0.69 nM for cortisol, 0.66 nM for 6β-hydroxycortisol, 0.69 nM for cortisone, and 0.67 nM for 6β-hydroxycortisone.

Table 4. Accuracy and precision for all analytes in human urine.

Urine	LQC		MQC		HQC	
	% error	%CV	% error	%CV	% error	%CV
Cortisol-Intraday	-1.1	6.3	-6.3	2.8	-1.3	3.2
Cortisol-Interday	-3.4	6.2	-6.9	3.3	-4.1	4.8
6β-OH-COL-Intraday	-9.2	2.2	-7.7	2.8	-1.3	2.0
6β-OH-COL-Interday	-10.8	2.4	-6.8	3.0	-1.4	5.7
Cortisone-Intraday	-14.6	2.7	-13.2	3.9	-9.4	5.2
Cortisone-Interday	-14.9	5.4	-11.5	3.6	-8.1	4.6
6β-OH-CON-Intraday	-13.4	3.4	-9.0	3.3	-9.4	3.1
6β-OH-CON-Interday	-9.6	5.8	-7.4	4.2	-6.0	6.4

For validation of the plasma analysis method, calibration curves were prepared in duplicate on three separate days as described previously. Quality controls (QCs) were prepared in five replicates in the same fashion as the calibration curves. Cortisol was quantified using the peak area ratio (PAR) of cortisol *m/z* 407.062 > 331.0 and cortisol-d_4 *m/z* 411.062 > 335.0, 6β-hydroxycortisol was quantified using the PAR of 6β-hydroxycortisol *m/z* 423.056 > 347.1 and cortisol-d_4 *m/z* 411.062 > 335.0, cortisone was quantified using the PAR of cortisone *m/z* 405.061 > 329.3 and cortisol-d_4 *m/z* 411.062 > 335.0, and 6β-hydroxycortisone was quantified using the PAR of 6β-hydroxycortisone *m/z* 421.035 > 345.2 and cortisol-d_4 *m/z* 411.062 > 335.0. All calibration curves were weighted 1/x and fitted linearly to the data. The same acceptance criteria for urine calibration curves were used as the plasma calibration curves. Blank serum with and without the internal standard was analyzed for any interference with the analytes of interest. The methods accuracy and precision were determined on three separate days. Intra-day

variability was determined by the CV of five replicates of each QC on the same day. Inter-day variability was calculated by the CV of 3 replicates of each QC on three separate days. Intra- and inter-day variability can be found in Table 5. The LOD was defined for each analyte as the lowest concentration with a reproducible signal to noise ratio (S/N) > 3. Cortisol LOD was at 15 nM, 6β-hydroxycortisol at 0.15 nM, cortisone at 5 nM, and 6β-hydroxycortisone at 0.15 nM. The LOQ was defined for each analyte as the lowest concentration with a S/N > 10 and a CV% <20%. The LOQ for cortisol was 25 nM, 6β-hydroxycortisol was 0.25 nM, cortisone was 15 nM, and 6β-hydroxycortisone was 0.25 nM.

Table 5. Accuracy and precision for all analytes in human plasma.

Plasma	LQC		MQC1		MQC2		HQC	
	% error	%CV	% error	%CV	% error	%CV	% error	%CV
Cortisol-Intraday	-4.3	2.8	3.8	1.9	16.4	1.7	-1.5	3.1
Cortisol-Interday	-4.4	2.7	3.5	2.8	16.0	1.9	-0.9	3.2
6β-OH-COL-Intraday	-9.4	3.8	-13.6	6.5	-3.1	2.2	-10.0	3.6
6β-OH-COL-Interday	-10.7	4.0	-14.8	5.6	-5.6	3.9	-7.7	4.7
Cortisone-Intraday	-13.5	4.3	-11.1	4.6	-6.5	2.3	-7.3	4.1
Cortisone-Interday	-14.2	4.8	-10.3	3.3	-6.4	2.7	-4.7	4.1
6β-OH-CON-Intraday	13.2	7.4	-5.3	5.8	2.4	3.2	-11.0	4.4
6β-OH-CON-Interday	14.9	9.0	-3.1	9.6	3.6	2.8	-7.1	6.0

2.6 Pharmacokinetics and Statistical Analysis

The geometric mean (GM) and 90% confidence interval (CI) are reported for the concentrations of cortisone, cortisol, their 6β-hydroxy metabolites, the ratios of the parent compounds to their metabolites, and the ratios of treatment to control. Wilcoxon matched-pairs signed rank tests were used to compare the plasma concentrations, plasma and urinary metabolic ratios, formation clearances, and renal clearances of participants to determine if isotretinoin

treatment impacted cortisol, cortisone, or their metabolites disposition. Kruskal-Wallis tests were used to compare formation clearances between CYP3A5 genotypes.

Formation clearance of 6β-hydroxy cortisol ($CL_{f(6\beta OHX)}$) was used as a measure of CYP3A4 activity. The $CLf_{(6\beta OHX)}$) was calculated using equation 1,[6] where the plasma concentration measured 2 hours post dextromethorphan administration was used as a surrogate for cortisol and cortisone AUC, A is the amount of the 6β-hydroxy metabolite and [X] is the plasma concentration of cortisol or cortisone, at the 2-hour blood draw; and time is 2 hours. Descriptive statistics, outlier, Wilcoxon, and Kruskal-Wallis tests were calculated using GraphPad Prism 10.2.2 (La Jolla, CA).

$$CL_{[(6\{30HX)} = \frac{A(6\{30\_)}{[X]\times time}$$ Equation 1.

Chapter 3. Results

3.1 CYP3A5 Genotypes

CYP3A5*1 is the wildtype functional allele. Participants were genotyped for three non-functional SNPs of CYP3A5; CYP3A5*3, CYP3A5*6, and CYP3A5*7. Participants were then characterized as poor, intermediate, or extensive CYP3A5 metabolizers based on the presence of zero, one, or two functional alleles, respectively.[32,33,85] One participant was an extensive CYP3A5 metabolizer with the CYP3A5*1/*1 genotype. Six participants were intermediate CYP3A5 metabolizers with the genotype CYP3A5*1/*3. Twenty-six participants had the CYP3A5*3/*3 genotype and were classified as poor CYP3A5 metabolizers.

3.2 Plasma Concentrations and Urinary and Plasma Metabolic Ratios

The concentrations of cortisol, 6βOH-cortisol and cortisone in plasma are reported as the GM and 90% CI in Table 6. The individual participants' concentrations are shown in figure 3. The plasma concentrations of cortisol and 6βOH-cortisol were significantly increased with isotretinoin treatment. However, the metabolite-to-parent ratio of 6βOH-cortisol/cortisol was unchanged, meaning they increased at a similar rate. The increase in 6βOH-cortisol can be attributed, at least in part, to an increase of cortisol according to equation 2.

$$C_{ss,metabolite} = \frac{C_{ss,parent} \times CL_f}{CL_{metabolite}} \quad \text{Equation 2.}$$

Table 6. Plasma concentrations (nM) reported as geometric means & 90% confidence intervals for before (control) and during (treatment) dosing with 13*cis*RA.

Analyte	n	Day 1 Control	Day 2 Treatment	Treatments/Control	P-Value
Cortisol	32	248 (219 - 280)	309 (270 - 353)	1.25 (1.10 - 1.41)	0.0041
6β-OHCOL	30	1.20 (1.07 - 1.34)	1.43 (1.25 - 1.63)	1.19 (1.04 - 1.36)	0.0053
Cortisone	32	31 (28 - 35)	35 (31 - 40)	1.12 (1.01 - 1.25)	0.066

Cortisol: 1 outlier removed. 6β-OH-cortisol: 2 outliers & 1 unquantifiable sample removed. Cortisone: 1 unquantifiable sample removed. Only 1 participant had quantifiable levels of 6βOH-cortisone in plasma.

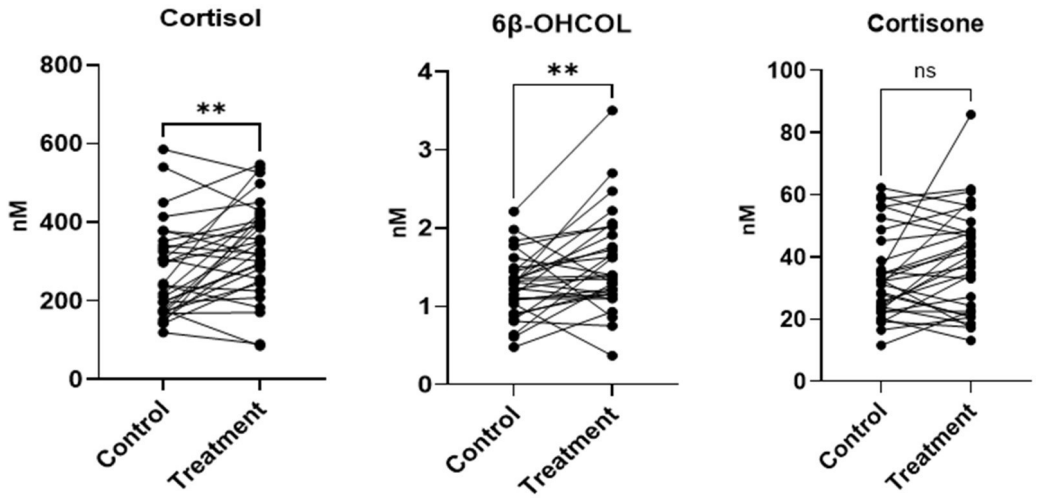

Figure 3. Individual plasma concentrations of cortisol, cortisone and 6βOH-cortisol, on day 1 (Control) and day 2 (Treatment). ns: $P > 0.05$, **: $P \leq 0.01$.

The plasma metabolic ratios (MR) for 6β-OH-cortisol to cortisol are reported as the GM and 90% CI in Table 7. The change (treatment/control) in MR is reported as the GMR and 90% CI in Table 7. The individual participants' plasma metabolic ratios are shown in figure 4. The plasma MR for 6β-OH-cortisone to cortisone was not calculated as only one subject had quantifiable levels of plasma 6β-OH-cortisone on both study days. Based on the analysis there was no significant change in plasma 6β-OH-cortisol to cortisol ratio due to isotretinoin treatment.

Table 7. Plasma metabolic ratios reported as geometric means & 90% confidence intervals for before (control) and during (treatment) dosing with 13*cis*RA.

Analyte	n	Day 1 Control	Day 2 Treatment	Treatments/Control	P-Value
6β-OHCOL:COL	31	0.0050 (0.0044 - 0.0056)	0.0047 (0.0042 - 0.0054)	0.93 (0.83 - 1.03)	0.26

1 outlier & 1 unquantifiable subject removed.

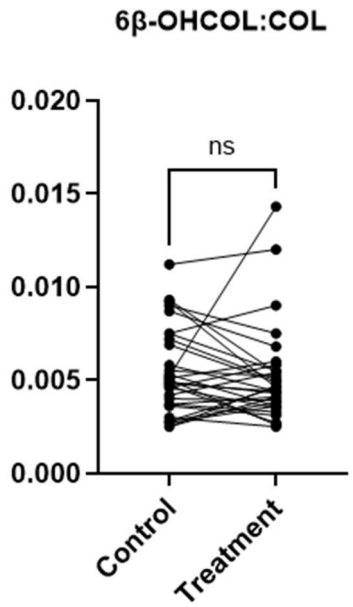

Figure 4. Individual plasma metabolic ratios of 6β-hydroxycortisol to cortisol. ns: P > 0.05.

The urinary metabolic ratios (MR) for 6β-OH-cortisol to cortisol and 6β-OH-cortisone to cortisone are reported as the GM and 90% CI in Table 8. The change (treatment/control) in MRs is reported as the GMR and 90% CI in Table 8. The individual participants' urinary metabolic ratios are shown in Figure 5. The individual metabolite's urinary ratios did not change.

Table 8. Urinary metabolic ratios reported as geometric means & 90% confidence intervals for before (control) and during (treatment) dosing with 13*cis*RA.

Analyte	n	Day 1 Control	Day 2 Treatment	Treatments/Control	P-Value
6β-OHCOL:COL	32	0.33 (0.28 - 0.41)	0.35 (0.29 - 0.43)	1.06 (0.85 - 1.32)	0.57
6β-OHCON:CON	24	0.16 (0.13 - 0.19)	0.16 (0.16 - 0.19)	0.97 (0.76 - 1.24)	0.57

6βOHCOL:COL: 1 outlier removed. 6βOHCON:CON: 1 outlier and 8 unquantifiable samples removed. Sum: 8 unquantifiable samples removed

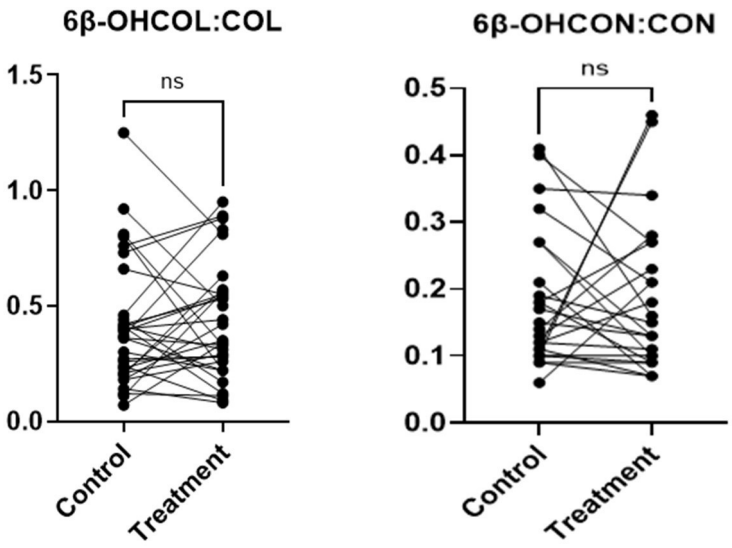

Figure 5. Individual urinary metabolic ratios of 6β-hydroxycortisol to cortisol and 6β-hydroxycortisone to cortisone ns: P > 0.05.

3.3 Formation Clearance

Formation Clearance of the individual 6βOH-metabolites was used as an *in vivo* biomarker of CYP3A4 activity. Equation 1 was used to calculate $CL_{f,}$ and values are reported as GM and 90% CI in Table 9. The impact of isotretinoin treatment on CL_f is reported as the GMR and 90% CI in Table 9. Individual CL_f values are shown in Figure 6. Surprisingly, the formation clearances of both metabolites (1 significantly) were decreased with isotretinoin administration suggesting that CYP3A4 activity may actually be inhibited.

Table 9. Formation clearance (mL/hr) reported as geometric means & 90% confidence intervals for 6β-OH-metabolites before (control) and during (treatment) dosing with 13*cis*RA.

Analyte	n	Day 1 Control	Day 2 Treatment	Treatments/Control	P-Value
6β-OHCOL	31	1.20 (0.86 - 1.68	0.65 (0.45 - 0.93)	0.54 (0.38 - 0.76)	0.051
6β-OHCON	29	4.48 (2.89 - 6.96)	0.47 (0.32 - 0.70)	0.11 (0.07 - 0.15)	< 0.0001

6β-OHCOL: 2 outliers removed. 6β-OHCON: 3 outliers and 1 unquantifiable removed. Sum: 3 outliers and 1 unquantifiable removed.

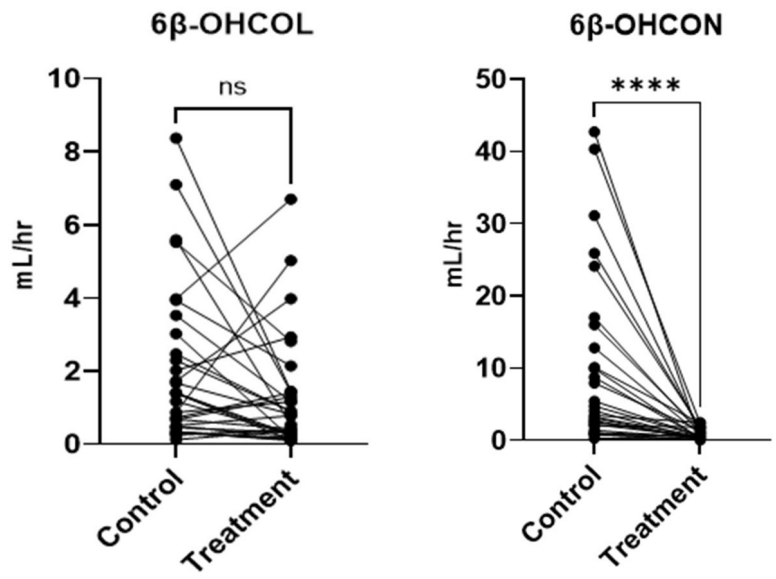

Figure 6. Individual formation clearance (mL/hr) before (control) and with (treatment) dosing with 13*cis*RA. ns: $P > 0.05$, ****: $P \leq 0.0001$.

To determine if CYP3A5 metabolic status impacted baseline formation clearance, the $CL_{f,control}$ was compared between the three CYP3A5 metabolic categories based on genotype. There was no statistical difference in the three groups suggesting that CYP3A5 metabolic status and genotype do not impact baseline formation clearance. The descriptive statistics are in Table 10 and the individual CL_f values shown in Figure 7.

Table 10. $CL_{f,control}$ reported as geometric means & 90% confidence intervals for 6β-OH-metabolites by CYP3A5 metabolism status.

Analyte	Extensive (1)	Intermediate (8)	Minimal (25)
6β-OHCOL	0.87	1.64 (0.43 - 6.18)	1.06 (0.75 - 1.5)
6β-OHCON	15.41	10.22 (2.71 - 38.50)	7.56 (5.42 - 10.54)

6β-OHCOL: 1 outlier removed. 6β-OHCOL: 3 outliers removed. Combined: 3 outliers removed.

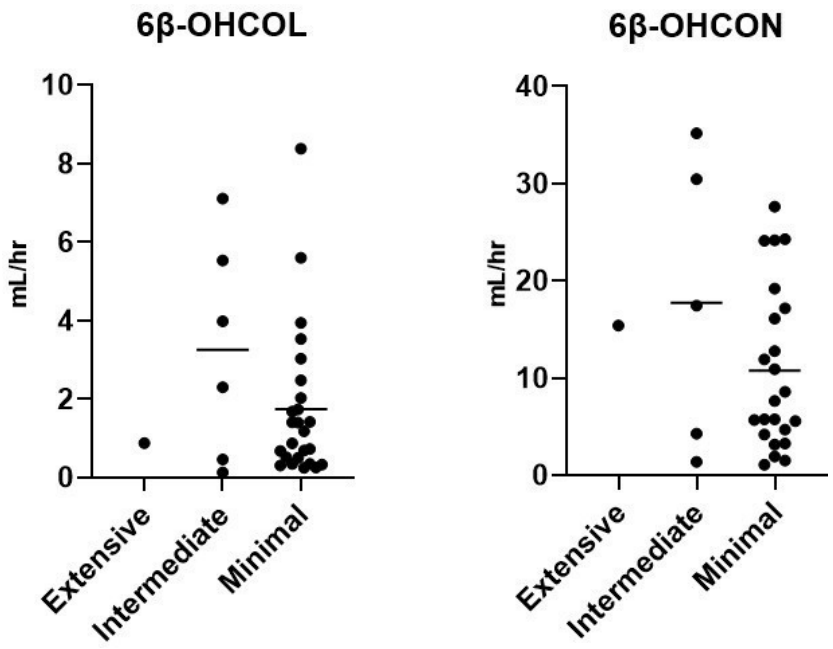

Figure 7. Individual formation clearances with means on Day 1 (control) by CYP3A5 metabolizer status (based on genotype).

Additionally, the ratios of $CL_{f,treatment}$:$CL_{f,control}$ were compared between CYP3A5 metabolic categories to determine if isotretinoin treatment impacted CL_f differently by genotype. Although there was a trend towards differences in the CL_f of the three CYP3A5 metabolic categories, none were statistically significant. This suggests that isotretinoin treatment did not impact CL_f in a genotype dependent manner. The descriptive statistics are in Table 11 and the individual CL_f values are shown in Figure 8.

Table 11. Ratio of $CL_{f,treatment}$:$CL_{f,control}$ reported as geometric means & 90% confidence intervals for 6β-OH-metabolites by CYP3A5 metabolism status.

Analyte	Extensive (1)	Intermediate (8)	Minimal (25)
6β-OHCOL	1.34	0.73 (0.32 - 1.68)	0.46 (0.31 - 0.69)
6β-OHCON	1.68	0.62 (0.23 - 1.67)	0.42 (0.28 - 0.65)

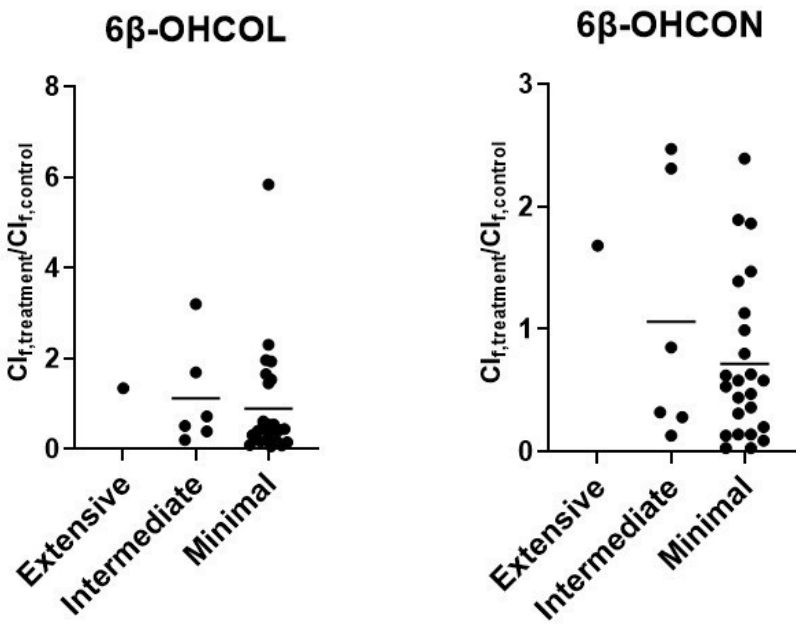

Figure 8. Individual formation clearance ratios with means by CYP3A5 metabolizer status (based on genotype).

3.4 Renal Clearance

The renal clearance for each analyte was calculated and reported as the GM and 90% CI while the change from day 1 to day 2 is given as the GMR and 90% CI both in Table 12. Individual renal clearance values for each analyte are shown in Figure 9. The renal clearance of 6β-OHCOL decreased significantly with isotretinoin treatment.

Table 12. Renal clearance (mL/min) reported as geometric means & 90% confidence intervals for all analytes before (control) and during (treatment) dosing with 13*cis*RA.

Analyte	n	Day 1 Control	Day 2 Treatment	Treatments/Control	P-Value
Cortisol	30	1.50 (0.98 - 2.29)	0.81 (0.53 - 1.23)	0.47 (0.32 - 0.70)	0.073
6β-OHCOL	30	112 (82 - 152)	57(40 - 81)	0.51 (0.36 - 0.72)	0.0049
Cortisone	31	32 (22 - 48)	16 (10 - 25)	0.44 (0.30 - 0.65)	0.11

Cortisol: 3 outliers removed. 6βOHCOL: 2 outliers and 1 unquantifiable removed. Cortisone: 1 outlier and 1 unquantifiable removed. 6βOHCON: only 1 participant with quantifiable plasma concentrations.

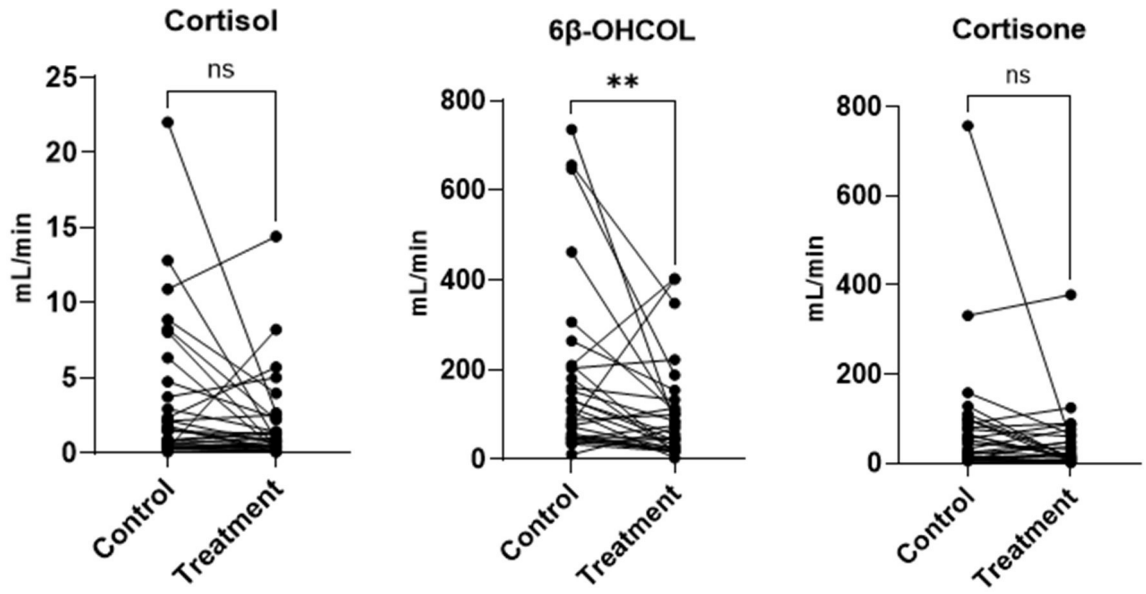

Figure 9. Individual renal clearance (mL/min) of all analytes before (control) and with (treatment) dosing with 13*cis*RA. ns: $P > 0.05$, **: $P \leq 0.01$.

Chapter 4: Summary and Conclusions

4.1 Discussion

If the hypothesis that isotretinoin treatment induces CYP3A4 activity was correct we would expect an increase in plasma and urinary metabolic ratios, and increase in formation clearances, and no change in the renal clearances. We see a significant increase in the plasma concentrations of cortisol and 6β-OHCOL but no change in their metabolic ratios, suggesting that isotretinoin is having a direct impact on cortisol production. The finding that there was no change in plasma or urinary metabolic ratios does not support the induction of CYP3A4, but the metabolic ratio may be unchanged if both formation and renal clearance change to the same degree.

The formation clearance of 6β-OHCON decreased while there was no significant change for 6β-OHCOL. If this effect was due to CYP3A4 inhibition/downregulation it would be expected that both metabolites would be affected. The more likely explanation is that isotretinoin is impacting the interconversion of cortisol to cortisone in the kidneys which impacts reabsorption and thus renal clearance. and urinary excretion of the metabolites. The change in the renal clearance of 6β-OHCOL agrees with the idea that there is an effect on conversion in the kidney.

These findings do not agree with previous studies that suggested isotretinoin may be an inducer of CYP3A4.[5,85] In fact, the decrease in the formation clearance of 6β-hydroxycortisol would imply CYP3A4 inhibition. However, there could be multiple reasons for the difference. Some of these previous findings were measured using mRNA levels and or the formation clearance of 3-methoxymorphinan from dextromethorphan. First, these studies may have had a different sample collection time from our study or even a varied collection time in their own.

Considering the large fluctuations in cortisol and cortisone levels throughout the day, differing collection times could lead to large differences in measured values. Second, the levels of mRNA do not always accurately portray the activity of an enzyme.[88] Finally, the use of a probe drug like dextromethorphan may have unknown impacts on other elimination pathways of cortisol and cortisone, for example, the 11β-hydroxysteroid dehydrogenases. Without controlling for these confounding factors, the ability to use the 6β-hydroxylation of cortisol and cortisone to assess CYP3A4 activity with any accuracy becomes difficult.

It is important to note that the urinary metabolic ratios may be altered by changes in renal clearance due to either an increase in renal blood flow or the renal extraction ratio. Indeed, 6β-hydroxy-cortisol renal clearance was significantly altered by isotretinoin potentially explaining the difference between urinary and plasma measures, these findings do not support isotretinoin treatment inducing CYP3A4 activity.

4.2 Conclusions

These results do not support the induction of CYP3A4 by isotretinoin treatment. However, it is important to note a number of confounding factors. First, unlike previous work, our study included genotyping for CYP3A5 (1 extensive, 6 intermediate, and 26 poor metabolizers), which allowed us to determine that there was no statistical difference in the formation clearance between CYP3A5 metabolizers and non-metabolizers. Thus, this study mainly reflects CYP3A4 metabolism. Secondly, previous work has included healthy males while this study was a majority female (22 females and 11 males) and all participants had severe acne. Compared to previous data[73], the participants in this study had elevated average levels of cortisol and slightly increased average levels of cortisone. However, it is important to note that the

concentrations of both hormones vary widely throughout the day so differences in measurement time can have a large impact on results. Lastly, this study did not investigate changes to 11β-hydroxysteroid dehydrogenases type 1 and 2, or any other elimination pathways for cortisol or cortisone. Meaning that isotretinoin may be having an unknown effect on the interconversion of cortisol to and from cortisone or 6β-hydroxycortisol to and from 6β-hydroxycortisone or some other elimination pathway.

In conclusion, the overall results of this research do not support the hypothesis that isotretinoin treatment induces CYP3A4 activity using the 6β-hydroxylation of cortisol and cortisone as endogenous biomarkers. It does, however, suggest that isotretinoin has an effect on the production of cortisol and the conversion of cortisol to cortisone in the kidney and may in fact inhibit CYP3A4. These effects warrant further specific study. To further determine if severe acne itself could be confounding the data, an option would be a study in acne patients using a different CYP3A4 probe drug like midazolam as the results from biomarkers and dextromethorphan metabolic ratios are inconclusive. Furthermore, investigation into the disposition of 11β-HSD type 1 and 2 is warranted to elucidate isotretinoin treatments impact, if any, on the interconversion of cortisol to and from cortisone or 6β-hydroxycortisol to and from 6β-hydroxycortisone. With that, it would make sense to also investigate the other elimination pathways of cortisol, cortisone, and their 6β-hydroxy-metabolites.

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