A validation study measuring the cytochrome P450 enzyme subfamily 3A4 activity in free-living adults

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Abstract

Introduction: The activity of cytochrome P450 enzyme subfamily, CYP3A4, is an important determinant of the activity of certain anti-cancer drugs used in lung cancer, but there is currently no assay of CYP3A4 enzyme activity available for routine clinical use. This thesis presents results of human testing and validation of a previously published CYP3A4 assay in serum and Dried Blood Spot analysis (DBS) format. Previous researchers developed this assay in healthy male non-smokers, who were not taking medications and had normal medical test results. A number of lifestyle, medical and genetic factors are known to modulate CYP3A4 activity and the current project validated the assay in free-living adults, both male and female. These individuals would be expected to have a wider range of CYP3A4 metabolic activity and give values which would be more representative of those found in patients with lung cancer. Testing for another CYP450 subfamily, CYP1A2, was initially included in these studies as a control, but this enzyme also has a minor role in metabolism of certain drugs used in lung cancer and testing for CYP1A2 was included in all assays presented.

Methods: An indirect test for activity of CYP3A4 and CYP1A2, was performed using test doses of Midazolam and caffeine respectively. Participants fasted for at least 8 hours prior to testing. After a baseline venous blood sample participants consumed the test drugs (100 mg caffeine and 2 mg of Midazolam). Peak concentrations of both doses occur around 60 minutes after ingestion, and a second blood test was taken at this time point. Liquid chromatography/mass spectroscopy (LCMS) was used to measure the serum concentrations of each test drug and its primary metabolite (Midazolam-OH/midazolam and paraxanthine/caffeine). After baseline correction, an index of enzyme activity was calculated using the ratio of metabolite/substrate concentrations at 60 minutes (Metabolic ratio). Medical and dietary factors can affect activity, and relevant information from each participant's medical and diet was captured. A scoring system (The "DiMQu" score) was created and correlation with measured metabolic ratio was performed. DBS testing was conducted to assess assay performance using known concentrations of substrates recovered from whole blood.

Results: The performance characteristics of the LCMS assay from serum confirm that the method works well. The assay linear range that was sufficient for the expected range of analyte concentrations. The coefficient of variation (CV) for repeated testing was checked at lower limit of detection and was <15% for all analytes which meets the FDA standard (LLOQ <20%). Additional reproducibility testing at different concentrations within the linear range of the assay showed CV was <7%. Assay accuracy was within 20% of the known value. Repeated testing of samples after 3 months showed intra-subject agreement (CVs <10%) confirming 3-month stability after storage at -80C. Exploratory correlational analyses suggested a trend for negative relationship between age and CYP3A4 metabolic ratio which is consistent with the literature. Additional analysis confirmed that females had greater CYP3A4 metabolic ratio than males which is also consistent with previous literature. However, there was no significant sex difference for the CYP1A2 metabolic ratio. There was a trend suggesting CYP1A2 metabolic ratio correlated with DiMQu score, however, this was not statistically significant. Similarly, the CYP3A4 metabolic ratio was not correlated with DiMQu score. Finally, further exploratory testing of confirmed that all analytes of interest were also detectable in pooled whole venous blood spotted onto filter paper (DBS).

Discussion: Even an indirect measure of the activity of CYP3A4 and CYP1A2 enzymes has the potential to provide useful information on likelihood of both side-effects of cancer treatments and in determining the safety of patient dietary or herbal treatment therapies. This assay could potentially enable health care professionals to detect changes in CYP3A4 and CYP1A2 activity over the course of treatment and anticipate changes in response to certain anti-cancer drugs over time. Based on the findings in this thesis, the LCMS assay performance is acceptable for further experimental use with patients and testing with free-living subjects suggests the limits of detection are appropriate. Whilst direct DBS testing for both CYP3A4 and CYP1A2 assays was not performed with the free-living subjects, testing using whole blood and addition of known concentrations of standards suggest this approach is also feasible using the assay developed. DBS has the advantage of being a less invasive form of testing and is promising for future studies with cancer patients.

Résumé

Introduction: L'activité de la sous-famille du cytochrome P450, CYP3A4, est un déterminant important de l'activité de certains médicaments anticancéreux utilisés dans le cancer du poumon, mais il n'existe actuellement aucun test qui vise à déterminer l'activité de l'enzyme CYP3A4 pour un usage clinique de routine. Cette thèse présente les résultats des tests sur les humains et de la validation d'un test CYP3A4 précédemment publié sous forme de sérum et analyse de prélèvements de sang séché (DBS). Des chercheurs précédents ont mis au point ce test chez des hommes non-fumeurs en bonne santé, qui ne prenaient pas de médicaments et dont les résultats aux tests médicaux étaient normaux. On sait qu'un certain nombre de facteurs liés au mode de vie, à la condition médicale et à la génétique modulent l'activité du CYP3A4 et le projet en cours l'a validé chez des adultes, hommes et femmes. On s'attendrait à ce que ces individus aient une plage d'activité métabolique plus large pour le CYP3A4 et donnent des valeurs qui seraient plus représentatives de celles trouvées chez les patients atteints d'un cancer du poumon. Le test d'une autre sous-famille du CYP450, le CYP1A2, a été initialement inclus dans ces études à titre de contrôle, mais cette enzyme joue également un rôle mineur dans le métabolisme de certains médicaments utilisés dans le cancer du poumon et le test du CYP1A2 a été inclus dans tous les tests présentés.

Méthode: L'activité enzymatique du CYP3A4 et du CYP1A2 a été mesurée à l'aide de doses de Midazolam et de caféine, respectivement. Les participants ont jeûné pendant au moins 8 heures avant le test de sang. Après un échantillon de sang veineux de base, les participants ont consommé les médicaments testés (100 mg de Caféine et 2 mg de Midazolam). Les concentrations maximales des deux doses sont atteintes environ 60 minutes après l'ingestion et un second test sanguin a été effectué à ce moment. La chromatographie en phase liquide / spectroscopie de masse (LCMS) a été utilisée pour mesurer les concentrations sériques de chaque médicament testé et de son métabolite principal (Midazolam-OH /Midazolam et Paraxanthine/Caféine). Après correction de la ligne de base, l'activité enzymatique a été calculée en utilisant le rapport des concentrations métabolite/substrat à 60

minutes (rapport métabolique). Des facteurs médicaux et diététiques peuvent affecter l'activité enzymatique. Les informations pertinentes provenant des antécédents médicaux et du régime alimentaire de chaque participant ont été capturées. Un système de notation (le score «DiMQu») a été créé et une corrélation avec le rapport métabolique mesuré a été réalisée. Des tests DBS ont été réalisés pour évaluer les performances des tests en utilisant des concentrations connues de substrats récupérés dans du sang veineux entier.

Résultats: Les caractéristiques de performance du dosage LCMS à partir de sérum confirment que la méthode fonctionne bien. La gamme linéaire du test était suffisante pour la plage attendue des concentrations en analyte. Le coefficient de variation (CV) pour les tests répétés a été vérifié à la limite inférieure de détection et était <15% pour tous les analytes répondant à la norme FDA (LLOQ <20%). Des tests de reproductibilité supplémentaires à différentes concentrations dans la plage linéaire du dosage ont montrés que le CV était <7%. La précision du test se situait à moins de 20% de la valeur connue. Des tests répétés sur des échantillons après 3 mois ont montré un accord intra-sujet (CV <10%) confirmant la stabilité à 3 mois après stockage à -80 °C. Des analyses exploratoires par corrélation suggèrent une tendance à la corrélation négative entre l'âge et le rapport métabolique du CYP3A4, ce qui est cohérent avec la littérature. Une analyse supplémentaire a confirmé que les femmes avaient une activité plus grande du CYP3A4 que les hommes, ce qui est également cohérent avec la littérature antérieure. Cependant, il n'y avait pas de différence de sexe significative pour le rapport métabolique du CYP1A2. Il y avait une tendance suggérant une corrélation entre le rapport métabolique du CYP1A2 et le score de DiMQu, mais ce n'était pas statistiquement significatif. De même, le rapport métabolique du CYP3A4 n'était pas corrélé avec le score DiMQu. Enfin, d'autres tests exploratoires ont confirmé que tous les analytes d'intérêt étaient également détectables dans du sang veineux entier, taché sur du papier filtre (DBS).

Discussion: Même une mesure indirecte de l'activité des enzymes CYP3A4 et CYP1A2 pourrait fournir des informations utiles sur la probabilité d'effets secondaires des traitements anticancéreux et sur la détermination de la sécurité des traitements à base de plantes ou de traitements diététiques du patient. Cet essai permettrait également aux professionnels de la santé de surveiller les changements d'activité du CYP3A4 et du CYP1A2 au cours du traitement et d'anticiper les changements dans la réponse à certains médicaments anticancéreux au fil du temps. Sur la base des résultats de cette thèse, les performances du test LCMS sont acceptables pour une utilisation clinique et les tests sur des sujets autonomes ont confirmé que les limites de détection étaient appropriées. Bien que le test direct de DBS pour les tests CYP3A4 et CYP1A2 n'ait pas été réalisé sur les sujets, les tests utilisant du sang entier et l'addition de concentrations connues d'étalons suggèrent que cette approche est également réalisable à l'aide du test développé. La méthode DBS a l'avantage d'être une forme de test moins invasive et est prometteuse pour de futures études sur des patients atteints de cancer.

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Noor Mady. December 2019

Contribution of Authors

Dr. Jagoe and Noor Mady designed the research project. Noor Mady conducted all the data collection, statistical analysis, literature review and the full writing of the thesis. The LC-MS/MS and DBS analysis was performed in Dr. Christoph Borchers laboratory at the Segal Cancer Centre, Proteomics Centre. The laboratory analysis protocol, performed by Constance Sobsey, is described in Appendix 1. Dr. Jagoe critically reviewed the thesis.

1 CHAPTER I: INTRODUCTION

1.1 The incidence and mortality of lung cancer

In Canada, cancer is the leading cause of death and mortality is estimated at 30%.[1,2] Alarmingly, it is estimated that 1 in 2 Canadians will develop cancer during their lifetime.[1] In 2017, there were over 200,000 new cases of cancer and nearly 90 000 deaths from cancer.[1] More specifically, lung, breast, colorectal and prostate are the most commonly diagnosed (excluding skin cancer) cancers in Canada and represent half of all new cancer cases.

Among the new cases, 14% of patients are diagnosed with lung cancer.[1] More males are diagnosed with lung cancer as compared to females. However, the rate of lung cancer diagnosis in females kept increasing until 2006 when it began to plateau. In Canada, lung cancer impacts males and females almost equally (see Table 1).

Along with increasing rates of cancer diagnosis, survival rates are also increasing. Over 60% of Canadians diagnosed with cancer have a life expectancy of 5 years or more.[1] However, this rate is much lower for lung cancer patients and is estimated at 17%. The 5-year survival rates are generally lower for small cell cancer patients as compared to non-small cell.[3]

Table 1. Diagnosis based on Lung Cancer Stage

	Males (%)	Females (%)
Lung and bronchus		
Stage I	17.8	23.7
Stage II	8.4	8.5
Stage III	20.0	19.4
Stage IV	52.0	47.1
Unknown	1.8	1.4

Note. Based on Canadian Cancer Society, Canadian Cancer Statistics: A 2018 special report.

1.1.1. Lung Cancer Treatments

Lung cancer is grouped as either Non-small cell lung cancer (NSCLC) or Small cell lung cancer and this classification is based on the cancer cell origin.[1] Non-small cell lung cancer and Small cell lung cancer may be differentiated based on histological appearances.

Lung cancer treatments include: surgery, radiation therapy, chemotherapy, chemoradiation, targeted therapy, immunotherapy and endobronchial therapies.[1,4] The best treatment choice depends on various factors, such as the histological and molecular type of lung cancer, tumour stage, overall health of the patient and lung function.

Surgery and radiation therapy are considered as localized treatments.[1,4] Surgery is commonly used for the treatment of Stage I and II NSCLC and the type of surgery depends on where the cancer is located in the patient's lung(s). Radiation therapy can be used for local control of tumours and it is also often combined with chemotherapy to treat NSCLC. Endobronchial therapies may also be an option to remove blockage in bronchi (e.g. stent placement).[1,4] Chemotherapies, targeted treatments and immunotherapies are considered to be primarily systemic therapies in lung cancer.[1] Immunotherapy is commonly used for advanced or metastatic NSCLC that is not responsive to chemotherapy or targeted treatments. This treatment option allows the body to potentially reactivate its immune response to the tumor. Targeted treatments and chemotherapy will be addressed in the following sections.

1.1.2. The Targeted Treatments for Lung Cancer

Targeted therapies are used in the treatment of non-small cell lung cancer (NSCLC) with specific mutations. The specific target therapy choice depends on the genetic mutations present in the cancer cells. Erlotinib and Gefitinib both target mutated epidermal growth factor receptors (EGFR).[5] By blocking EGFR, Erlotinib hinders cancer cell growth. In addition, Maemondo and colleagues (2010) found that compared to chemotherapy, Gefitinib first-line

treatment of patients with NSCLC tumors with EGFR gene mutations, resulted in better progression-free survival.[6] Another treatment example is Crizotinib, an anti-cancer drug that is an ALK and ROS1 inhibitor, found to be highly effective in patients carrying ALK fusion gene.[7]

1.1.3. Chemotherapy treatments

Chemotherapy is the most commonly used form of treatment in lung cancer as the majority of patients present with advanced disease.[1] Chemotherapy is commonly a combination of drugs and although it may be administered independently, it may also be used prior (neo-adjuvant) or post-surgery (adjuvant) for NSCLC.[1] Some chemotherapy medications, such as etoposide and paclitaxel, are used as second line treatments for lung cancer. Etoposide works by preventing re-ligation of DNA strands and promotes apoptosis of cancer cells.[10] Paclitaxel's mechanism of action involves targeting tubulin and blocking the progression of mitosis and promotion of apoptosis.[11]

In comparison to the targeted therapies, the traditional chemotherapy drugs stop cell division by inhibiting DNA transcription and/or protein synthesis. Chemotherapy drugs are less specific and are also cytotoxic: toxic to living cells. In contrast to chemotherapy, the newer targeted treatments are not directed against the cell machinery for DNA replication, they're targeted to specific receptors. Therefore, targeted therapies are considered to be more specific as well as cytotoxic and cytostatic, in other words, inhibiting cell growth and division.

1.2 CYP 450 Enzymes

Drugs are metabolized through various enzymes and primarily through the CYP 450 enzyme superfamily.[8] The CYP 450 superfamily is comprised of 18 families and includes over 50 enzymes, however only a small number of those enzymes are considered to metabolize 90% of drugs (CYP3A4/5 34%; CYP1A2 10%).[8] Hereditable and

environmental factors can impact the activity of these enzymes. These include genetic polymorphisms, sex, age, diet, medications and botanicals, for example: Chinese herbs. The resulting variation in CYP activity could create interindividual differences in anti-cancer drug response.

Individual differences exist in the metabolic activity of anticancer drugs by CYP3A enzymes and as a result, patients have different pharmacokinetic profiles.[8] Since the hepatic detoxification enzyme pathways (CYP450) play a vital role in drug metabolism, their activity levels are relevant in determining ideal drug doses in cancer treatment. Additionally, CYP 1A1/2, CYP2D6 and CYP3A4 are dominant enzyme pathways for common systemic therapies used in the treatment of lung cancer.[9]

1.2.1 CYP 3A4 function and relevance

The CYP3A family consists of four genes: CYP3A4, CYP3A5, CYP3A7 and CYP3A3. Among the many existing enzymes, CYP3A4 is considered to be the most important drug metabolizing one.[12] The highest expression of CYP3A4 enzymes is in the liver and intestine. Almost 50% of all drugs are metabolized by the CYP3A4 enzyme and substrate drugs can be found in most therapeutic drug classes.[8, 13, 14, 15] It is responsible for processing several therapies for lung cancer, such as gefitinib, erlotinib and taxanes (widely used in chemotherapies).[12] They are also known to be involved in the metabolism of hormones such as testosterone and oestrogen.[15] Commonly used substrates for the CYP3A4 enzyme are midazolam, erythromycin, cortisol and testosterone.[15]

The CYP3A4 enzyme can be inhibited and induced by various drugs and/or substances. For example, inhibition of CYP3A4 can occur from antifungal agents, macrolide antibiotics, erythromycin, some antidepressant such as fluoxetine, some antiarrhythmic medication such as amiodarone, blood pressure drugs like verapamil, liquorice, and grapefruit juice.[13, 15] The activity of this enzyme may also be induced by several agents such as antibiotic

rifampicin, anticonvulsants including carbamazepine, pulmonary artery hypertension mediation bosentan, HIV medication efavirenz, St. John's wort, and topiramate for epilepsy and migraine treatment.[13,15] The net induction/inhibition of an enzymatic driven metabolism depends on several variables including the significance of the metabolic pathway involved with respect to the total clearance, the amount of the of a substrate consumed (e.g. concentration of Midazolam) and whether that substrate is an inducer or an inhibitor.[13] Drug-drug interactions, as a result of these conditions, may result in reduced plasma levels at standard doses. For example, when given together, rifampicin, phenytoin or carbamazepine may significantly decrease plasma concentrations for a range of substrates metabolized through CYP3A4 enzymes.[15] The opposite scenario is possible with a potent inhibitor of CYP3A4 and a drug dependent on CYP3A4 enzyme metabolism. In this case, it is possible for the substrate drug concentration to increase 10-20 times in plasma levels and cause possible toxicity.[15] For example, simvastatin is primarily metabolized through CYP3A4 and its levels may be greatly increased with the addition of a strong inhibitor such as verapamil.

Previously mentioned targeted treatments, Erlotinib and Gefitinib, are metabolized through the CYP3A4 liver enzymes.[8,6] Chemotherapy medications, such as etoposide and paclitaxel, are also metabolised through the CYP3A4 enzymes.[10, 11]

1.2.2 CYP1A2 function and relevance

The CYP1A2 enzyme belongs to the CYP1A family along with CYP1A1, CYP1B1 and CYP1D1P. CYP1A2, along with others, is classified under significant metabolic enzymes responsible for both biotransformation of chemicals and for the metabolic activation of pre-carcinogens.[15] Similarly to CYP3A4, it is primarily expressed in the liver. Along with CYP1A1, the CYP1A2 enzyme is responsible some of the metabolism of common lung cancer systemic therapies like Erlotinib.[9]

Various drugs and other chemicals may inhibit the CYP1A2 enzyme such as the antibiotics norfloxacin and ciprofloxacin, antidepressants paroxetine and fluvoxamine and blood pressure medication verapamil.[13] This enzyme is inducible by various substances including polycyclic aromatic hydrocarbons in tobacco, caffeine, vegetables (cabbage, broccoli), modafinil used in narcolepsy and omeprazole for gastroesophageal reflux disease.[9,13] By inducing CYP1A2, the drug efficacy will decrease.[15] This is due to the increase in enzyme activity which will cause a decline in concentration levels.

1.3 Influential factors on metabolic activity

1.3.1 Genetic polymorphisms

Genetic polymorphisms in the CYP enzyme family can lead to increased and/or reduced activity levels, or differences in the inducibility of CYP enzymes when exposed to substrates.[14] In some cases, genetic polymorphisms cause reduced enzyme activity levels which may result in less metabolic clearance.[14] As described below, several polymorphisms exist for the CYP3A4 enzyme but their clinical relevance is difficult to assess as these polymorphisms are only present at very low frequencies (0-5%) in all populations studied.[16] In contrast, there are some cases of higher frequency polymorphisms for the CYP1A2 enzymes, and, some may be clinically relevant.[16, 17]

Over 30 single nucleotide polymorphisms exist in CYP3A4 enzymes, however, they are low in frequency.[14] The Farmacogene Variation Consortium provides summary information on relevant studies with regards to CYP3A4 activity.[18] The main polymorphisms for CYP3A4 are the following: decreased (CYP3A4*8, CYP3A4*11, CYP3A4*13, CYP3A4*17, CYP3A4*18A, CYP3A4*22), increased (CYP3A4*18A) or no (CYP3A4*20) enzyme activity, based on in vitro and/or in vivo data. In another study which considered CYP3A4 allele frequency, using data from 56 945 individuals from five major populations, researchers concluded that among several other

important CYP450 enzymes, CYP3A4 showed the lowest cumulative variant allele frequency.[16] In the same study, when compared to other main CYP450 enzymes based on activity, the majority of CYP3A4 enzyme allele variants had mostly normal enzyme activity and none had increased enzyme activity. The CYP3A4 enzyme also had the lowest percentage of alleles associated with decreased activity compared to the other enzymes: 5.1% in Europeans, 0.3% in Africans, 2.0% in East Asian, 0.7% in south Asian and 2.7% in Admixed American populations. Admixed individuals are those who come from two or more genetically distinct backgrounds.[19] The authors concluded that perhaps there is strong selection pressure to conserve CYP3A4 and limit SNPs (single nucleotide polymorphisms) with greatly increased or decreased activity.[16]

The first allele shown to be inactive was the CYP3A4*20, with very low frequencies of 0.06% in white, 0.26% in black and 0.22% in Chinese.[20] Researchers have found the CYP3A4 *22 polymorphism to be associated with reduced CYP3A4 activity and better response to simvastatin, a lipid-lowering medication.[21] In that study, individuals with the CYP3A4*22 polymorphism showed lower total cholesterol and LDL cholesterol as compared to controls. This reduction in CYP3A4 activity, is associated with better response to the medication as the substrate is not being processed as rapidly into the inactive metabolite. CYP3A4*18, common in East Asians, is known to be a rapid metabolizer of sex steroids and was found to be associated with low levels of bone mineral in Korean women, a risk factor for osteoporosis.[16, 22] In conclusion, ethnic differences exist within frequency of CYP3A4 genetic polymorphisms and some studies have begun to shed light on the potential clinical relevance of the CYP3A4 SNPS. However, their low frequencies suggest that impact of SNP variation is minor at the population level and other factors influencing CYP3A4 activity should also be considered.

CYP1A2 polymorphisms vary more than CYP3A4 in activity and inducibility, however, (with one exception CYP1A2*F) they too are generally low in frequency. The inducibility of an enzyme refers to its increased rate of

biosynthesis due to the presence of its substrate. Based on the Farmacogene Variation Consortium, the following data summarizes polymorphisms for the CYP1A2 enzyme: decreased activity(CYP1A2*1C, CYP1A2*1K, CYP1A2*3, CYP1A2*4, CYP1A2*7, CYP1A2*8, CYP1A2*11, CYP1A2*15, CYP1A2*16), higher inducibility (CYP1A2*1F), no activity (CYP1A2*6), based on in vitro and/or in vivo data.[18] The CYP1A2*1F polymorphism has the greatest allele frequencies: 65.5% in Europeans, 43.9% in Africans, 58.8% in East Asians, 46.6% in South Asians and 73.3% in Admixed Americans.[16] The CYP1A2*1F polymorphism has been associated with a high inducibility of CYP1A2 in smokers.[9] Polycyclic aromatic hydrocarbons in tobacco smoke have been shown to induce CYP1A2 enzyme, which has been shown to metabolize Erlotinib, as mentioned above, is a targeted systemic therapy used to treat some patients with lung cancer.[9] This induction may accelerate catabolism and lower plasma concentrations. In the major study conducted by Zhou and colleagues (2017), the next most common polymorphism was *1K which is associated with decreased enzyme activity, and is detected in 2.4% in Europeans, 6.1% in Africans, 0.2% in East Asians, 6.9% in South Asians and 2.5% in Admixed Americans. All other CYP1A2 polymorphism studied in that sample were below 1.4% allele frequency, the prevalence of the majority of these less than 1 in 4500.[16] Thus, CYP1A2 enzymes do vary more genetically as compared to CYP3A4. Although more research is needed, it is plausible that lung cancer patients who are also current smokers, that have the CYP1A2*1F polymorphism (highly inducible), and are undergoing targeted treatments (i.e. Erlotinib) may not respond optimally at standard doses.

In summary, genetic polymorphisms do provide some important information that is relevant to our understanding of enzyme activity. However, it appears that genetic polymorphisms alone do not explain the majority of interindividual variability in enzyme activity or drug metabolism.[23]

1.3.2 Sex

Several studies using human liver microsomes and drug clearance studies have shown that females exhibit greater CYP3A enzyme family activity and greater CYP3A4 specific enzyme activity when compared to males.[24,25,26] Furthermore, when midazolam (a CYP3A4 substrate) was administrated intravenously to 100 healthy participants, females had higher clearance compared to males.[27] However, some authors have questioned the relevance of the measured differences in CYP3A4 activity. For example, in a study on healthy volunteers, researchers concluded that females had higher CYP3A4 enzyme activity, as measured by conversion of midazolam into its metabolite, but no differences in concentration of midazolam were found in females compared to males.[28] Similarly, in a meta-analysis of studies comparing oral versus intravenous midazolam, females showed greater clearance of midazolam, but again no statistically significant differences in concentrations of midazolam between males and females was found.[29] In contrast, there is evidence indicating that males have increased CYP1A2 enzyme activity compared to females. [24,25,26] Thus, there is some evidence for sex-related differences in CYP3A4 and CYP1A2 enzyme activity which may impact drug metabolism but this does not necessarily lead to differences in circulating drug levels, presumably because of other compensatory mechanisms.

1.3.3 Age

Older age is associated with reduced liver size, blood flow and less drug clearance through CYP450 enzyme pathways.[30,31,32] In addition to relevant sex differences mentioned previously, researchers who administered IV midazolam to 100 healthy subjects found that age is inversely correlated to CYP3A activity and midazolam clearance was reduced as age increased.[27] Some have reported that unlike CYP3A, CYP1A2 enzyme is not significantly impacted by age.[33] However, in this latter study there was no testing for SNPs for CYP1A2 or other factors which may have confounded the results.

1.3.4 Obesity

There is little direct evidence looking at the impact of obesity on enzyme activity as it relates to drug metabolism. However, in a study with obese and diabetic individuals, CYP3A4 enzyme activity was found to be decreased.[34] It has been reported that CYP3A4 substrates, alprazolam, cyclosporine and midazolam showed lower drug clearance in obese individuals and lower clearance of most other CYP3A4 substrates is common in obesity.[35] In contrast, looking at CYP1A2 activity, mice fed with a high fat diet had reduced CYP1A2 expression.[36] However, no significant differences in caffeine clearance were found among obese and non-obese non-smokers.[37] These results were the same after weight loss. Furthermore, in a study of children comparing obese and non-obese children, using caffeine as a substrate to assess CYP1A2 enzyme activity, researchers concluded that no statistically significant difference existed in the metabolic ratios of their participants.[38]

1.3.5 Diet/Nutrition

Different foods and beverages can inhibit or induce CYP450 enzymes.[13,39,40] A summary of common products consumed are included in our sample Dietary Questionnaire (see Table 2).

Table 2. Summary of Dietary Questionnaire and Enzyme Activity

	CYP1A2		CYP3A4	
Item	Inducer	Inhibitor	Inducer	Inhibitor
Coffee	✓			
Tea	✓			
Red Wine*		✓		✓
Beer*		✓		✓
Spirits/Liquor*		✓		✓
Carbonated Soda	✓			
Grapefruit / Seville oranges				✓
Cruciferous Vegetables	✓			
Charbroiled, barbequed, or pan-fried meat	✓			
Jufeng Grape Juice	✓			
Indian food (curcuma)*		✓		✓
Liquorice Candy				
Ground black pepper				✓
Garlic			✓	

Note. Coffee and tea includes decaffeinated options. Meats include: chicken, beef, pork, sausage, lamb and turkey. Cruciferous vegetables include: cauliflower, broccoli, cabbage, bok choy, brussel sprouts, turnips, radish. * indicates minor inhibitor or inducer

Low intakes of protein, certain minerals and vitamins intake can alter CYP450 enzyme activity.[41] Vitamin D, which is primarily catabolized through CYP3A4 enzymes is frequently low in patients with cancer.[42] Specifically, almost 80% lung cancer patients have subnormal 25-hydroxyvitamin D concentrations and about 20% have undiagnosed deficient levels <25nmol/L.[43] CYP3A4 is inducible by Vitamin D and a study showed humans consuming oral vitamin D supplements had increased break down of Atorvastatin.[44,42] Other researchers have demonstrated that seasonal sunlight exposure-related changes in Vitamin D are also correlated with CYP3A4 intestinal levels.[45] In a study using Midazolam as a substrate, 9 healthy individuals received single doses of intravenous midazolam in different nutritional states.[46] Some had fasted overnight, others for 36 hours and a third

group received a high fat diet. These researchers found increase Midazolam clearance with short-term fasting, however, having a high fat diet didn't impact the clearance results.[46] Short term fasting also decreased clearance of Hydroxymidazolam. This may be potentially clinically relevant for patients that experience severe weight-loss or cachexia as is common in advanced cancer.[46] In a study on vitamin C deficiency, researchers found decreased overall CYP proteins (and specifically CYP2B1/2B2) in liver microsomes of rats with this deficiency.[47]

1.3.6 Botanicals

Traditional Chinese herbs, which are often used by patients to manage symptoms, may also alter the activity of these enzymes.[13] Tetrahydropalmatine (tet) is commonly found in traditional Chinese medicine and known to inhibit CYP3A enzymes. In a sample of beagle dogs, researchers found that the compound tet increased accumulation of midazolam, a common CYP3A4 enzyme substrate.[48] However, in that study, tet didn't significantly impact levels of caffeine (CYP1A2 substrate) or metoprolol (CYP 2D6 substrate). In addition to tet, researchers have studied other purified herbal compounds (neferine and berberine) commonly found in traditional Chinese medicine: in vitro. All of these compounds, namely tet, neferine and berberine, significantly inhibited the CYP3A4 enzyme.[49] In another study of 42 healthy volunteers, 4-week green tea catechin consumption led to a mild decrease in CYP3A4 enzyme activity and no significant effect on CYP1A2 activity.[50] A study also found that CYP3A4 enzyme activity is inhibited by cannabis, and this has been shown to affect clearance of drugs such as Ketamine.[51] Therefore, having a better understanding of the role botanicals play when taken in conjunction with other drugs, may be useful in cancer treatment.

1.3.7 Medications and Nutritional Supplements

Drug-drug interactions may also produce relevant clinical outcomes within the context of cancer treatment. For example, Atorvastatin and Verapamil are inhibitors of CYP3A4, while Dexamethasone is an inducer.[13] In another study, cannabidiol was shown to greatly inhibit CYP3A family enzyme activity (CYP3A4 and CYP3A5).[52] A summary of commonly used drugs and their primary role in relation to CYP enzymes (see Table 3).[13,53]

Table 3. Summary of effects of Medications/Nutritional Supplements on Enzyme Activity

		CYP1A2		CYP3A4	
	Role	Inducer	Inhibitor	Inducer	Inhibitor
Cimetidine	Heart burn				✓
Clarithromycin	Antibiotic				✓
Erythromycin	Antibiotic				✓
Desogestrel*	Birth Control				✓
Fluoxetine*	Antidepressant		✓		✓
Norfloxacin	Antibiotic		✓		
Verapamil (*CYP1A2 only)	Blood pressure		✓		✓
Dexamethasone	Anti-inflammatory and immunosuppressant			✓	
Rifampicin	Antibiotic			✓	
Carbamazepine	Anticonvulsant			✓	
Bosentan	Pulmonary artery hypertension mediation			✓	
Topiramate	Epilepsy and migraine			✓	
Lansoprazole*	Acid reflux/ulcers	✓			
Nicotine		✓			
Omeprazole	Gastroesophageal reflux disease	✓			

Note.* indicates minor inhibitor or inducer

1.3.8. Inflammation and drug metabolism

Studies in cancer patients have shown that a there is a correlation between existing inflammatory response and reduced expression of CYP3A4 as well as other genes related to drug metabolism.[54] It is known that the CYP3A4 enzyme is a common route of metabolism of several anti-cancer medications. Importantly, inflammation is known

to alter CYP3A4 gene expression in such a way that leads to subsequent increases in IL-6 levels.[55] IL-6 levels are elevated in several inflammatory diseases.[55] In another study, increased C-reactive protein levels, a marker of inflammation, were strongly associated with impaired CYP3A4 activity in cancer patients.[56] Markers of systemic inflammation are strongly associated with poor prognosis in lung cancer and one potential contributing mechanism for this observation is that cancer-related systemic inflammation modulates metabolism of key anti-cancer drugs, through reducing their clearance and resulting in increased levels of toxicity.[55,57,58,59]

1.3.9. Summary of influential factors

The various factors mentioned above may have a potential impact on CYP3A4 and CYP1A2 activity. These causes, both hereditable, disease related or environmental, may lead to the induction or inhibition of the enzymes involved.[8] If this enzyme activity is increased, this may result in a reduction in effect of certain drugs used for cancer treatment due to increased metabolism and elimination. In contrast, inhibition of specific elimination pathways can also lead to toxicity[14] and, for example, inhibition of CYP3A4 enzymes function by consumption Atorvastatin or a range of other drugs may increase the risk of side-effects.[14] More importantly, given the large number and diverse nature of factors which can modulate CYP3A4 and CYP1A2 activity, it is logical and desirable to establish a clinically applicable test to assess CYP3A4 and CYP1A2 activity directly.

1.4 Assays

1.4.1 Enzyme Assays

Direct approaches to assessing enzyme activity involve calculating the number of moles of substrate converted per unit time; also known as velocity values.[60] The measure of velocity is derived from the initial part of the reaction which approximates a linear shape. As the substrates deplete, the reaction reduces in speed and ceases. To conduct a direct enzymatic assay, there are various considerations to assess. The enzymatic assay needs to be near the

optimal pH for the respective enzyme and optimized for temperature.[60] Other factors such as enzyme concentration should be low, at catalytic amounts, but enough for the reaction to proceed. Buffers should correspond to the optimal pH for the enzyme of choice and all assay components should be compatible as they may influence the ionic strength and the pH.[60] These assays need to undergo a process of validation to confirm the robustness of the results and to assess variability.[61]

The kinetic analysis behind traditional enzyme assays may involve using plasma and then the addition of the substrate of interest. By measuring metabolites at various time intervals during the assay, it is possible to obtain an "area under the curve" assessment. This analysis is used to calculate the amount of enzyme present in the system, based on its activity in plasma. This type of assessment is used for example when researchers measure alanine transaminase (a representative enzyme of global liver function and a marker of potential liver disease) in routine blood tests, or pancreatic disease related enzymes such as lipase and amylase or other enzymes in plasma to detect clinical abnormalities.[62]

In contrast, phenotyping of CYP450 enzymes, often involves an indirect approach to measure enzyme activity. This type of assay is still highly useful in research and clinical settings as it offers rapid information on drug metabolism and is a useful surrogate for direct measurement of enzymatic activity.[63] Enzyme activity is inferred from the ratio of concentrations of metabolite to that of a known substrate.[64] Midazolam is metabolized to OH-midazolam by CYP3A4 and therefore, it can be used as a probe to asses CYP3A4 activity. Midazolam clearance is one method of phenotyping CYP3A4 activity and a previous study has shown that a correlation exists between CYP3A hepatic concentration and Midazolam clearance, using human liver samples.[65] The same researchers found a correlation between Midazolam clearance and a single 30-minute ratio of the plasma concentrations of Midazolam and its metabolite. This 30-minute ratio of plasma OH-Midazolam/Midazolam, correlated significantly

with hepatic CYP3A content (pmol/mg S-13 protein), which was measured in liver biopsy tissue of liver donors and recipients, 1-4 hr after the Midazolam dose.

A similar approach has been used for CYP1A2, using caffeine as substrate. Previously, caffeine urinary metabolic ratios where used for CYP1A2 activity phenotyping, however, they were found to be poorly reproducible.[63] This was exemplified through a study that compared several popular urinary caffeine ratios (i.e. [5-acetylamino-6-amino-3-methyluracil + 1-methyluric acid+ 1-methylxanthine]/ 1,7-dimethyluric acid) used to measured CYP1A2 activity in research and the resulting weak correlations between these ratios showed that they were not measuring the same variables.[66,63] These researchers proposed that caffeine's complex metabolism along with many factors impacting renal excretion, such as ethnicity-specific variations in renal transport, meant that the results obtained through these urinary ratios were not exact.[66] As a result, phenotyping through the use of serum metabolic ratios gained popularity and is now widely used to assess the resulting enzymatic effect of drug therapy or a condition. This is commonly achieved through cocktail studies (see Table 4).

Phenotyping approaches are useful, however, they present with their own challenges such as determining the time of analysis, whether to complete a time-course study or a single-time point assessment. Time-course analysis refers to when researchers take measures through an enzyme assay and develop an area under the curve. The phenotyping approach utilized in this study to infer enzyme activity, using a single time point measurement of the concentrations of Midazolam and its metabolite, has been previously employed by other researchers.[67,68,64] A study used single-time point phenotypic assessment of CYP3A4 using Midazolam (1-hr post) in Chinese males and found it is predictive of oral Midazolam clearance.[69] This time point is based on the peak metabolism of the chosen substrates at 60-minutes based on previous literature.[70,71] Others have also proposed phenotyping serum ratios for Midazolam at one hour after dose to infer activity.[64] Representative concentration-time profiles for Midazolam and

caffeine, chosen probes for the enzymes of interest CYP3A4 and CYP1A2 respectively, may be found in a previously validated study.[70] In addition, based on previous time-course analysis completed on CYP3A4 and CYP1A2 activity using Midazolam and caffeine as probes, peak concentrations occur at around 1 hour post-ingestion. Thus for a single time point measurement, the 1-hour time point is the easiest to reliably detect the substrate and metabolite concentrations and likely also the optimal point to distinguish differences in MR between individuals.[70,71]

1.4.2 Selected Approach

To distinguish between traditional "direct" kinetic enzyme assessment techniques and the proposed validated indirect assessment of enzyme activity, the following points need to be considered. The goal was to measure the functional activity of the enzymes of interest, which should be occurring in the liver (and intestine) using a relatively non-invasive method. It would be difficult and too invasive to obtain liver samples of patients to detect enzyme activity. Although the indirect test adopted has limitations, part of the mandate of this project was to establish a test which was both feasible to perform rapidly in a clinic and acceptable to patients. As a result, the preferred course of action was to infer enzyme activity indirectly, from a MR of circulating product/substrate concentrations at a single time point.

Time-course analysis is more robust than single time measures. In this research project, a time-course approach was considered but a single-time point analysis, at 60-minutes post drug administration was preferred. From a clinical standpoint, the single-time point assay was deemed more feasible for use with the intended eventual target population (i.e. advanced lung cancer patients). In addition, the assay under study was previously validated by Bosilkovska and colleagues (2014) where multiple blood tests were performed to measure multiple different substrate/products and thus a time-course analysis for each substrate was possible. The published time-

concentration curves show that by 8 hours CYP1A2 had reduced to below 600ng/mL and CYP3A4 concentrations had reached 0ng/mL.

1.4.3 Previous Assay Techniques

There have been various experimental techniques used to analyze CYP enzymes. Some approaches include fluorescence, nuclear magnetic and electron paramagnetic resonance spectroscopy, X-ray crystallography etc., and techniques may involve both soluble and membrane-bound forms of CYP3A4.[72] Traditionally, high-performance liquid chromatography (HPLC) was the commonly used to quantify CYP enzyme activity. The HPLC system is focused on the quantification of the amount of metabolite present.[73] However, it has been established that this process is costly and inefficient as it involves a lengthy run for each sample and procedure to extract the proteins, it requires large assay volumes and enzyme concentrations needed to obtain the limit of detection.[73] Mass spectroscopy using high throughput allows for better sensitivity and specificity.[74] Most researchers have now opted for faster assays, including: fluorescence assay, luminescence assay and mass spectroscopy assay, that allow for the better assessment of several samples in the matter of seconds.

The fluorescence and luminescence-based assays are both fast and cheap and minimize the amount of reagent used.[73] However, in addition the luminescence is flexible with the types of tissues used. Arguably, the best option, although more costly, is the mass spectroscopy-based assay, as it is highly sensitive and accurate.[73] Furthermore, its use protects from potential disadvantages that come with the other assays. For example, in the fluorescence assay, there may be possible interference with compounds that exhibit fluorescence properties that affect the measurement of metabolite. Due to the lower concentrations of substrates used in order to achieve a better signal-to-noise ratio reading, the luminescence assay tends to give a lower activity reading than the fluorescence assay method.[73]

Liquid chromatography-mass spectroscopy (LC-MS) method was employed in this project. LC-MS couples the compound analysis capacity of mass spectroscopy with the physical separation abilities of liquid chromatography. LC-MS can be used with the cocktail methods described below to determine the activity of several enzymes at the same time.[73]

1.4.4 Multi-drug cocktails

Given that the purpose of this study was to look at enzyme activity through liquid chromatography-mass spectrometry, it is important to consider previously used methods and approaches as well. A number of groups have developed mass spectroscopy and having explored the idea of a drug cocktail, to assay a range of CYP enzymes.[70,71,76,77,78,79,80] Different cocktails have different doses of substrate drugs, and in those study designs, subjects have blood tests at different time points depending on the peak time of metabolism of the CYP enzyme of interest (see Table 4). LC-MS has been used to study human liver microsomes using several substrates.[75] This method is highly efficient and accurate in its quantification approach of metabolites.

Table 4. Summary of Several Previously Tested Multi-drug Cocktails

Author, Year	Participants	CYP Enzyme(s)	Substrate(s)	Technique
Bosilkovska and al., 2014*	N= 10, healthy	CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A, P-glycoprotein	Caffeine, Bupropion, Flurbiprifen, Omeprazole, Dextromethorphan, MidazolamCaffeine, Bupropion, Flurbiprifen, Omeprazole, Dextromethorphan, Midazolam FEX	LC-MS Time-course analysis.
Bosilkovska and al., 2016*	N= 30, healthy	CYP 1A2, CYP 2B6, CYP 2C9, CYP 2C19, CYP2D6, CYP3A	Caffeine, Bupropion, Flurbiprofen, Omeprazole, Dextromethorphan, Midazolam	HPLC-MS/MS (MS/MS: tandem mass spectrometry method) Time-course analysis.
Kakuda et al., 2014	N= 14, healthy	CYP1A2, CYP3A, CYP2C9, CYP2C19, CYP2D6,	Midazolam, Dextromethorphan, Caffeine, Omeprazole, Warfarin, Vitamin K	LC-MS Time-course analysis.
Blakey et al., 2004	N=12, healthy	CYP1A2, CYP2C9, CYP2D6, CYP 2E1, CYP3A4	Caffeine, Tolbutamide, Debrisoquine, Chlorzoxazone Midazolam	HPLC separation followed by tandem mass spectrometry. Time course analysis for Midazolam (CYP3A4 activity) and single time point analysis at 6h30 min for caffeine (CYP1A2 activity).
Ghassabian et al., 2009	N=11, Schizophrenic patients	CYP 1A2, CYP 2C19, CYP 2C9, CYP 2D6, CYP 3A4	Caffeine, Omeprazole, Losartan, Dextromethorphan, Midazolam	LC-MS Time-course analysis.
Derungs et al.,2016	N=16 Healthy	CYP1A2 CYP2B6 CYP2C9,CYP2C19, CYP2D6, CYP3A4	Caffeine, Efavirenz, Losartan, Omeprazole, Metoprolol, Midazolam	Reversed phase HPLC tandem mass spectrometry Time-course analysis.

Note. * Indicates that the study also included the dried blood spotting format of blood analysis. Time-course analysis refers to performing blood testing on the same participants at several time intervals post-probe ingestion to develop an AUC.

Through these multi-drug cocktail studies, some researchers concluded interesting clinically relevant findings. For example, some determined that etravirine, a HIV drug, induces CYP3A but inhibits CYP2C9, CYP2C19 and P-glycoprotein.[77] This study was conducted with 14 healthy subjects (ages: 18-55), normal BMI and medical exam results and mostly non-smokers.[77] Other researchers assessed 12 healthy, Caucasian, non-smoking volunteers between the ages of 22-48 years old in a multi-drug cocktail and confirmed that there were no drug interactions with co-administration.[78] In this study researchers evaluated potential interactions of coadministration of drugs in their cocktail indirectly using measured blood pressure and glucose. No significant evidence for metabolic interactions was seen. An assay using LC-MS was validated in a pilot study of 11 schizophrenic patients by quantifying a cocktail of substrates and their metabolites to phenotype CYPs 1A2, 2C19, 2C9, 2D6 and 3A4 enzymes.[79] Analytes of interest were separated in a run completed within minutes and patients had no adverse effects.[79]

To avoid potential effects from added (non-drug) ingredients when using commercial drug preparations for drug cocktails, researchers developed a method, using spray dried di-calcium phosphate particle as a drug carrier of multiple enzyme substrates, that can be used by any multi-cocktail approach.[81] These researchers were able to validate these mini-tablets using the Basel cocktail in healthy volunteers using LC-MS.[81] They believe this approach can simplify protocols, increase participant adherence.[81]

In summary, multi-drug cocktails provide a potentially useful tool for studying toxicity and drug-drug interactions to address relevant clinical treatments as well as drug development. In these studies, researchers have tested different combinations of cocktails and techniques of analysis.

Some researchers have also examined the DBS format for these drug assays. Instead of quantifying the analytes of interest using serum or plasma from a venous blood sample, a simple finger prick is used to extract a few drops of whole blood which are immediately dried onto sterile filter paper. Bosilkovska and colleagues (2016) used the DBS approach with the Geneva cocktail and looked for evidence of drug interactions between components of the cocktail of test substrates (which included caffeine and midazolam). The authors confirmed that the Geneva cocktail substrates do not cause mutual interactions. This was achieved through developing pharmacokinetic profiles of the substrates and their metabolites and then assessing AUC metabolite/AUC substrate administered either alone or as a Geneva cocktail.[76] Using the DBS technique, other researchers employed liquid chromatography with high-resolution mass spectrometry assay technology to develop a systemic toxicological assessment of blood samples at low concentrations.[82] Thus the DBS method is promising for future studies with cancer patients, because it is less invasive as a way of obtaining blood samples. In addition, the lack of interactions between components of the multidrug cocktails used for CYP enzyme testing, suggests the assays are unlikely to be affected by other drugs which patients may be taking for medical reasons.

1.4.5 Limitations of previous research

In a study by Bosilkovska and colleagues (2014), all participants were non-smokers, not taking medications and they all had normal results on their electrocardiogram, physical and liver function tests.[70,71] Similarly, in the previously cited studies, participants often presented with no serious medical conditions, with healthy BMI, younger age groups, and non-smoking.

Some highlighted issues in these studies include delays between the time participants ingest the drug and the blood testing and the use of excipients which are commonly found in commercial formulas.[81] These may potentially impact the activity of the CYP enzymes and thus bias the results. Some researchers justified their choices of

participants (male, Caucasian, healthy, non-smoking) to limit impact of ethnicity and sex on outcomes.[81] However, this approach reduces the generalizability of study findings to the overall population. Most importantly, it is still unclear whether of the published assays would work well with typical cancer patients. It seems likely that, with these highly selected groups, the results may not be representative of the range of different metabolic ratios seen in typical cancer patients. As an intermediate step before attempting to use this assay for studies of patients with lung cancer the current study recruited medically stable free-living adults with no restrictions on age and of both sexes.

1.4.6 Study Rationale

Given the highly selected nature of the subjects and controlled conditions used in most prior studies using this LCMS assay it is important to verify that the assay parameters (e.g. limits of detection with the test substrate doses used) are still valid for a broader population. To address this concern, this study deliberately focussed on testing of free-living adults, both males and females, including a wide range of ages and some subjects with some chronic illness or taking medication. These factors would be expected to lead to more variation in CYP3A4 and CYP1A2 activity and thus a greater range of values for substrate and metabolite concentrations than were seen in prior studies of young healthy males on controlled diets. The objective of this thesis was to use the previously published methodology and establish if the assay could measure substrate and metabolite concentrations accurately using the low oral test doses of substrates used in prior studies.

Although CYP3A4 activity is important in the metabolism of certain anti-cancer drugs used in patients with lung cancer, there is currently no assay of CYP3A4 enzyme activity available for routine clinical use. By establishing this indirect assay of CYP3A4 activity, it is hoped that this will prove feasible to use in clinical studies in patients with lung cancer and facilitate further studies to establish whether this assay has clinical utility. If successful, this sort of approach may eventually help inform clinicians about drug safety and dosing of anti-cancer drugs. For

example, if a patient presents with high levels of CYP3A4 enzyme activity at the beginning of their cancer treatment and these levels are reduced later, we would expect drugs metabolised through the CYP3A4 pathway, potentially including some anti-cancer drugs, to be present in their system longer. Whilst this may be beneficial for anti-cancer effect if concentrations were limiting, it may increase the risk of drug-related side effects. Another example is a patient that doesn't respond to the treatment as expected because of occult induction of CYP3A4 enzyme activity levels. In this situation, higher CYP3A4 activity would result in more rapid metabolism of the active drug and reduced anti-cancer effect in that individual. Additionally, CYP1A2 was originally included as an additional control to determine whether changes in CYP3A4 enzyme activity were generalizable to other CYP450 family or more specific to the CYP3A subfamily. However, with emerging information about the role of CYP1A2 in the metabolism of some systemic therapies in lung cancer, the results for CYP1A2 are included with the intention that the assay will include testing of both enzymes.

2 CHAPTER 2: RESEARCH QUESTION, OBJECTIVES, HYPOTHESIS

2.1 Aims

1. To confirm that the assay under study meets the necessary standards of performance by determining lower and upper limits of detection, reproducibility, linearity and accuracy with known concentrations of analytes.
2. To confirm that the results obtained for the free-living subjects are within the limits of detection established for the assay and determine the 3-month stability of assay results for all subjects on repeat testing.
3. To assess the relation between CYP3A4 and CYP1A2 enzyme activity (measured through MR), and key factors reported to be modulate activity in the literature.
4. To assess the relation between measured enzyme activity and DiMQu score developed for this project.
5. To assess assay performance using known concentrations of substrates recovered from whole blood using the Dried Blood Spot format.

2.2 Objective

The primary objective of this study is to develop and test the methodology for a clinical measure of CYP3A4 and CYP1A2 enzyme activity, based on metabolism of a small oral dose of midazolam and caffeine respectively, using a blood sample in healthy participants.

2.3 Research Question

Is the present assay suitable for measuring levels of CYP3A4 and CYP1A2 enzyme activity reliably in free-living healthy participants?

2.4 Primary and Secondary Outcomes

The primary outcomes of the present study are the concentration ratios of Hydroxymidazolam to Midazolam and paraxanthine to caffeine at 1-hour after dosing in serum.

The secondary outcomes of the present study are whether DBS testing has the necessary sensitivity to quantify all analytes of interest.

2.5 Hypothesis

It is predicted that the LC-MS assay described, using the doses of substrates chosen, can accurately measure serum substrate and metabolite concentrations in free-living adults to evaluate individual CYP3A4 and CYP1A2 activity.

3 CHAPTER III: METHODS AND PROCEDURES

This study has been approved by the Jewish General Hospital Ethics board (CODIM-MBM-16-235).

3.1 Study Design and Population

The study design employed is a single-arm pilot study. A total of ten adult participants (aged 18 years or older) with no acute illness or no new treatment were recruited. Midazolam was used as a CYP3A4 substrate to assess enzymatic activity in this study. Previous researchers have tested assays for several sub-families of the cytochrome P450 enzymes by using a test dose of a known substrate, namely midazolam, measuring levels of the drug and the metabolite at a standardized time interval post-dose using mass spectrometry.[70,71] Therefore, the CYP3A4 enzyme activity was measured by observing the time for which a small dose of a specific drug is metabolized in the body. A simultaneous assay of the CYP1A2 enzyme was also performed using a different substrate, caffeine, with similar pharmacokinetics.

3.2 Testing Procedure

The consent form was given to the participants to sign (see Appendix 2). Participants were asked to fast overnight (minimum 8 hours) prior to testing. Upon arrival, their heights and weights were measured. A blood sample was taken from the arm and they immediately consumed 100 mg caffeine and 2 mg of midazolam, which are test substrates of CYP1A2 and CYP3A4 respectively. The participants completed a medical questionnaire, a simple dietary questionnaire and a list of any medications or complementary health treatments they were consuming (see appendix 3) and had height and weight measurements. After an hour, when peak concentration occurs, a second blood test was taken.

3.3 DiMQu Scoring Method

Participants were asked to complete a dietary and medical history questionnaire (see appendix 3). The items in the dietary questionnaire were scored based on their known inducing or inhibiting properties for CYP3A4 or CYP1A2 enzymes (see Tables 2 and 8). In order to arrive at a global assessment of likely impact of both diet and medicines on CYP3A4 and CYP1A2 activity for each individual, a DiMQu (Diet and Medication Questionnaire) score for each enzyme was calculated. The score took into account the strength of reported induction or inhibition of any drugs consumed. In addition, the score for both inducing and inhibiting effects for each enzyme also incorporated the frequency of consumption of the dietary products (see Table 5). Items that were rarely used or used on a monthly basis received a score of 0, items that were used on a weekly basis received a score of 1 and items used daily a score of 2. The sum of inhibiting and inducing items was calculated for each individual for each enzyme. As above, the final DiMQu score for each enzyme was calculated by subtracting the total inhibiting score from the total induction score. For example, for CYP3A4, if the subject consumed one strongly inducing drug (score 2) and one inducing food item 4 times a week (score 1) and daily consumption of an inhibiting food (score 2), their net DiMQu score for CYP3A4 was (2+1)-2= 1.

Table 5. Scoring of Dietary, Herbal and Medical Substance Use

Level	Score	Frequency
Low	0	never or rarely
		1-3 per month
Medium	1	1-3 per week
		4-6 per week
High	2	1-2 times a day
		3 or more times a day

3.4 Sample Analysis

The full laboratory analysis protocol, performed by Constance Sobsey, is described in Appendix 1. The LC-MS/MS analysis was performed in Dr. Christoph Borchers laboratory at the Segal Cancer Centre, Proteomics Centre.

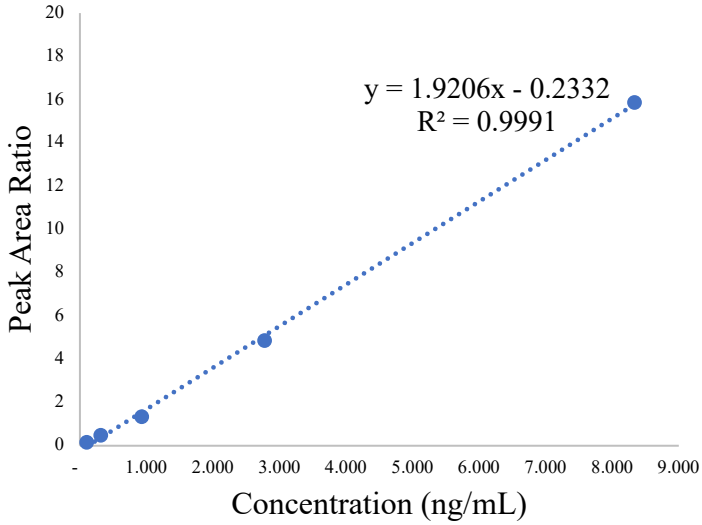
Standards. Stock standards were diluted in 60% methanol and added to commercial pooled charcoal-stripped serum to establish standard curves. Calibration curves were prepared using standards for all 4 analytes of interest (Midazolam/1-OH-Midazolam, Caffeine/Paraxanthine). Isotope-labeled versions of each compound [Midazolam-D_4 maleate, α-Hydroxymidazolam-D_4, Caffeine-(trimethyl-d9) and 1,7-Dimethylxanthine-(dimethyl-d6)] were used as internal standards.

Sample preparation. Patient serum samples (50uL aliquots) were thawed on ice and internal standards with a known concentration were added to each sample. Proteins were precipitated with acetonitrile and centrifugation. The supernatants were diluted 1:1 in water before injection into the mass spectrometer.

Quantitation. Concentration values were obtained by a multiple reaction monitoring mass spectrometry with internal standards. The use of individual internal standards for each analyte allows us to accurately measure the precise concentration value. The peak area ratio of each analyte and its internal standard are used to generate a standard curve (see figure 1) that can be used to extrapolate the concentrations of each analyte in patient samples. The concentration calculations were performed in Skyline software. The concentrations are then used to make a ratio for each pair of metabolites (see figure 2). The metabolic ratios were calculated by dividing the concentrations of Hydroxymidazolam by Midazolam and the concentrations of paraxanthine by caffeine.

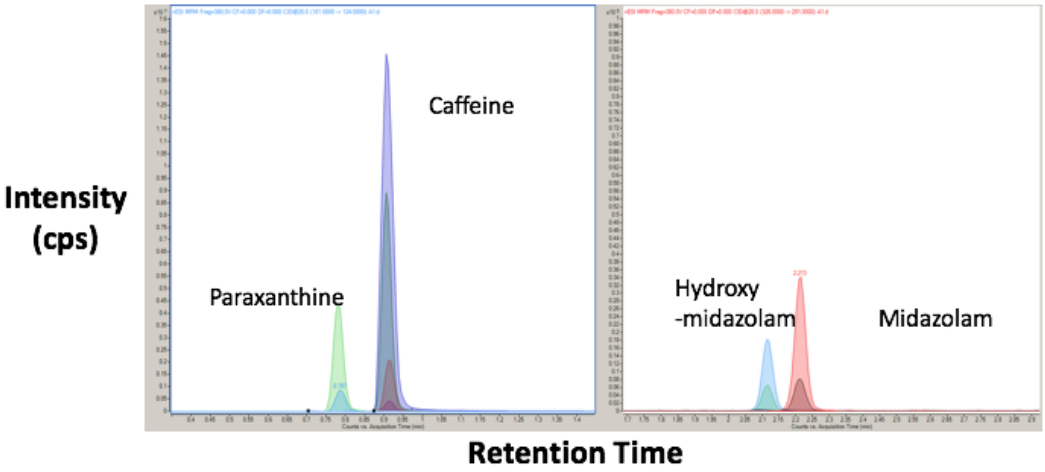
Dried Blood Spot. There were four levels of labeled standards (1x, 5x, 25x, 50x) were that were added into whole blood and then spotted onto the center of HemaSpot cards (see Appendix 1). HemaSpot cards are made up of an 8-petal shape that allows the blood to disperse evenly from the center out onto all the petals. The extracted sample was injected incrementally as 2 µL, 10 µL and 20 µL. The petal shape enabled extraction of the analytes in a low volume of methanol, therefore requiring less volume and less dilution.

Figure 1. Sample Standard Curve of Hydroxymidazolam and its Internal Standard



Note. Peak area ratio is calculated for each analyte and its internal standard and used to generate a standard curve to extrapolate the concentrations of each analyte.

Figure 2. Metabolic Ratios



Note. The Metabolic ratio is calculated by dividing the concentrations of for each pair of metabolites, i.e. Hydroxymidazolam (blue) by Midazolam (pink). The smaller peaks within each larger peak represent the internal standard for each analyte.

4 CHAPTER IV: RESULTS

4.1 Descriptive Statistics

A total of 10 participants (mean age 38 ± 11.79; *n*= 5 males) took part in this study, the majority of whom were either staff or graduate students at the Jewish General Hospital (see tables 6 and 7). Among the participants, three individuals had chronic illnesses. Additionally, there were no smokers in our sample. In total, 4 individuals reported complementary natural health products use and 6 were taking medications (see Table 8).

Table 6. Descriptive Statistics

	Mean	**SD**
Age	38	11.8
Height (cm)	171.4	11.5
Weight (kg)	68.2	10.1
BMI	23.2	1.7
DiMQu* Total Score		
CYP3A4	-2.8	
CYP1A2	6.0	

*Note. * DiMQu = Diet and Medication Questionnaire aggregate score for exposure to inducing and inhibiting factors. A positive score = net inducing*

Table 7. Descriptive Statistics

	N
Females/Males	5/5
Chronic Illness	3
Smokers	0
Complementary Natural Health Products Use	4
Current Medication use	6

Table 8. Study Subjects' Medications/Nutritional Supplements and predicted CYP Pathway effects

	CYP1A2		CYP3A4	
	Inducer	Inhibitor	Inducer	Inhibitor
Modafinil	✓		✓	
Curcumin*		✓		✓
Omega 3 Fatty Acid		✓		
Melatonin		✓		
Loratidine			✓	
Oregano Oil				✓
Teva-venlafaxine XR				✓

*Note.** indicates minor inhibitor or inducer

4.2 Assay Performance

The published assay was implemented, optimized, and validated in Dr. Borchers lab prior to the testing of patient samples. This included experiments to determine each assay's linear range, assess the recovery of analytes during extraction, and to confirm the specificity of the assay.

The performance characteristics of the LCMS assay from serum are summarized in table 9 and confirm that the method works well and can be used for clinical studies. The assay linear range that was sufficient for the expected range of analyte concentrations.[70,71] The coefficient of variation (CV) for repeated testing was checked at lower limit of detection and was <15% for all analytes which meets the FDA standard (LLOQ <20%).[83] The coefficient of variation at the lower limit of quantification (LLOQ) determines the assay's precision. The quality control points (low/medium/high) all fell within the criteria (known concentration ± 15%). These quality control points are samples with known concentrations that are independent of the standard curve. The use of charcoal stripped serum allowed a blank sample containing no caffeine and paraxanthine (which are commonly seen in blood samples, even

from fasting individuals). This allowed for the use of a control, therefore, when the analyte is not present there is no signal.

In order to verify the performance of the assay on the day of testing, we applied the following validation and quality control criteria. Each standard curve included at least 6 points within the linear range and demonstrated high linearity (R^2>0.985). The signal for each analyte in the blank charcoal-stripped serum was less than 20% of the signal at the lower limit of quantification. Also, the response for both analytes and internal standards in the double blank solvent was lower than 20% of the signal at the lower limit of quantification, showing that no carry-over occurred during analysis. The transition ratios were consistent between labeled and unlabeled standards, showing that the samples were consistent with calibrators and quality controls and did not contain unexpected interferences.

4.2.1. Accuracy

With respect to the accuracy of concentration measurements, the measured concentration of calibration standards based on the line of best fit, was accurate within +/-20% for each standard within the linear range for each analyte. Quality control samples (high/medium/low), consisting of charcoal-stripped serum spiked with known quantities of each analyte, were included with each batch and the measured concentration in these samples was also accurate within +/-20% of the known value.

4.2.2. Reproducibility

The reproducibility of the assay was further demonstrated since each of the 3 sets of technical replicates was analyzed during the assay validation process. For each analyte, known concentrations of the standard were added into the serum at 3 levels (low, medium, high). For each level, three identical samples were extracted and analyzed in parallel. The resulting reproducibility testing at different concentrations within the linear range of the assay

showed CV was <7%. These performance indicators ensure the validity of the measured concentrations for this study.

Table 9. Performance Characteristics of LC-MS Assay from Serum

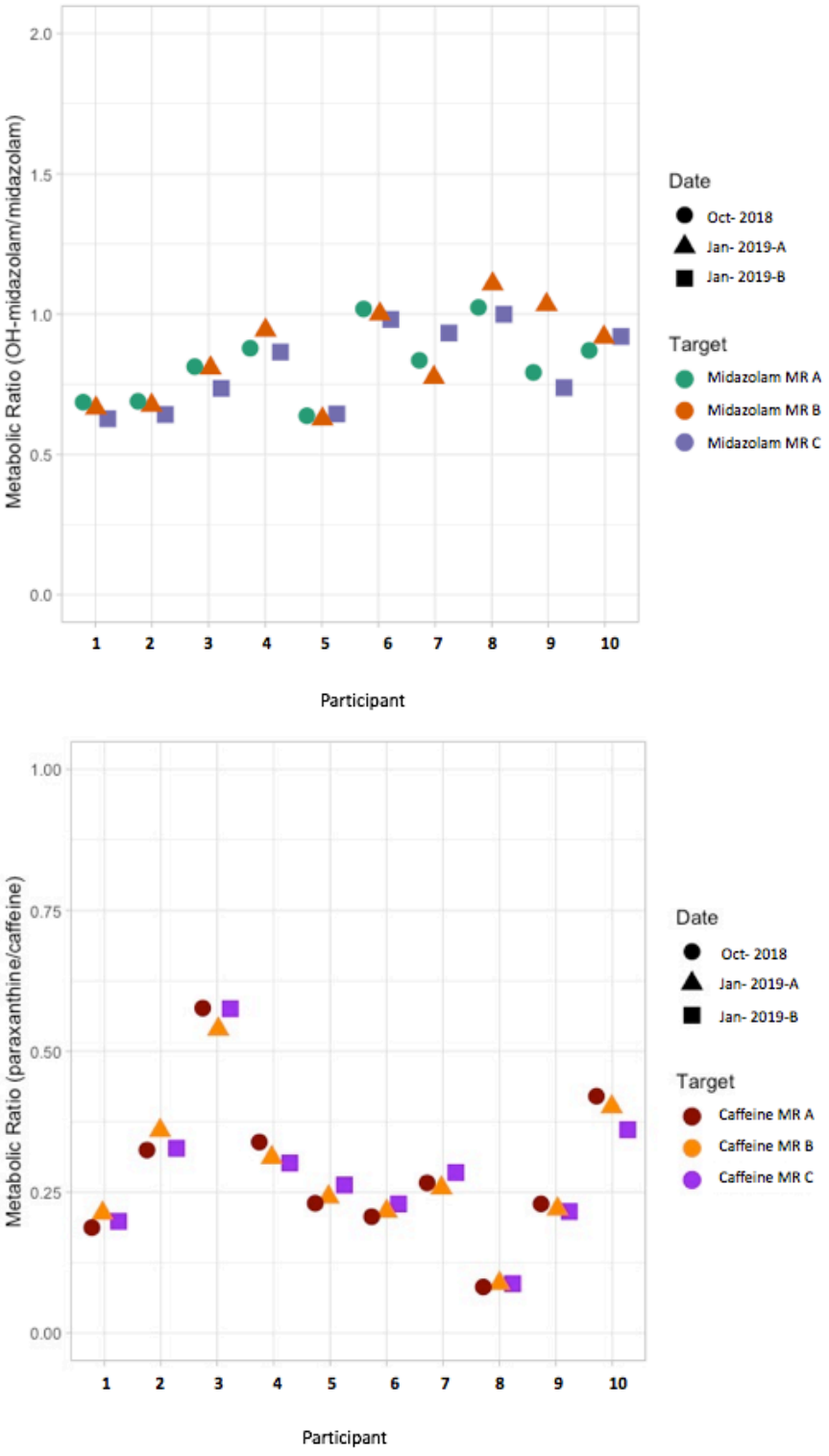
Analyte	Desired Range (ng/mL)	Achieved Linear Range (ng/mL)	Number of points in curve	Number of QC passed	Linearity (R^2)	CV at LLOQ (%)
Caffeine	25-5000	20-5000	7	3/3	0.9914	<15
Paraxanthine	25-5000	10-2500	7	3/3	0.9942	<15
Midazolam	0.1-100	0.10-24.4	6	3/3	0.9990	<15
Hydroxymidazolam	0.2-200	0.10-24.4	6	3/3	0.9950	<15

Note. Average coefficient of variation (CV) is of 3 replicates prepared in parallel at each level included in linear range. Quality Control (QC) is set at high, medium and low.

4.2.3. Stability

Due to the necessity of sample storage, their stability over time needed to be assessed in order to ensure that there is no change in the metabolites. Coefficients of variation were calculated for reproducibility by determining whether experimental repetitions produce consistent results. A lower coefficient of variation represents a reproducible result within a run. Inter-day stability is used to assess whether the concentration of metabolites the samples have changed in the overtime. Samples analyzed on different days (October versus January, see Appendix 1 for protocol) showed agreement in the values generated from analysis (CVs <6%) confirming 3-month stability after storage at -80C. The stability, assessed over three time points, are depicted in figure 3.

Figure 3. Stability of Midazolam and Hydroxymidazolam and Caffeine and Paraxanthine



4.2.4. Raw Data

The raw data is depicted in tables (10 and 11). Baseline testing showed no contamination with midazolam but high caffeine and paraxanthine concentrations for subjects 3,4,10.

Table 10. CYP1A2 Enzyme Substrate and Analyte Concentrations

Participant	Baseline Caffeine	Peak (60 mins) Caffeine	Baseline Corrected Caffeine	Baseline Parax	Peak (60 mins) Parax	Baseline Corrected Parax	Metabolic Ratio: Corrected Parax: Caffeine
1	316.7	2246.3	1929.7	273.7	446.6	172.9	0.09
2	487.3	2887.5	2400.3	732.0	971.8	239.7	0.10
3	3761.4	6263.4	2502.0	3297.2	3526.5	229.3	0.09
4	1747.4	5704.5	3957.1	1537.5	1809.3	271.7	0.07
5	336.6	2752.2	2415.6	463.1	674.5	211.4	0.09
6	212.6	2656.5	2443.9	341.2	576.7	235.5	0.10
7	352.6	3704.4	3351.9	633.9	996.6	362.7	0.11
8	111.6	2881.9	2770.3	81.0	246.4	165.4	0.06
9	201.1	3346.0	3144.9	318.1	750.3	432.3	0.14
10	898.4	3547.0	2648.7	1293.7	1393.0	99.3	0.04

Note. Parax refers to paraxanthine.

Table 11. CYP3A4 Enzyme Substrate and Analyte Concentrations

Participant	Baseline Midazolam	Peak (60 mins) Midazolam	Baseline OH-Midazolam	Peak (60 mins) OH-Midazolam	Metabolic Ratio OH-Midazolam: Midazolam
1	0.06	7.38	0.03	4.86	0.66
2	0.06	7.02	0.04	4.70	0.67
3	0.06	11.40	0.03	8.95	0.78
4	0.06	7.34	0.04	6.57	0.89
5	0.06	6.48	0.03	4.15	0.64
6	0.06	4.59	0.04	4.60	1.00
7	0.06	7.03	0.03	5.93	0.84
8	0.06	8.65	0.03	9.01	1.04
9	0.06	4.39	0.03	3.97	0.90
10	0.06	7.59	0.03	6.85	0.90

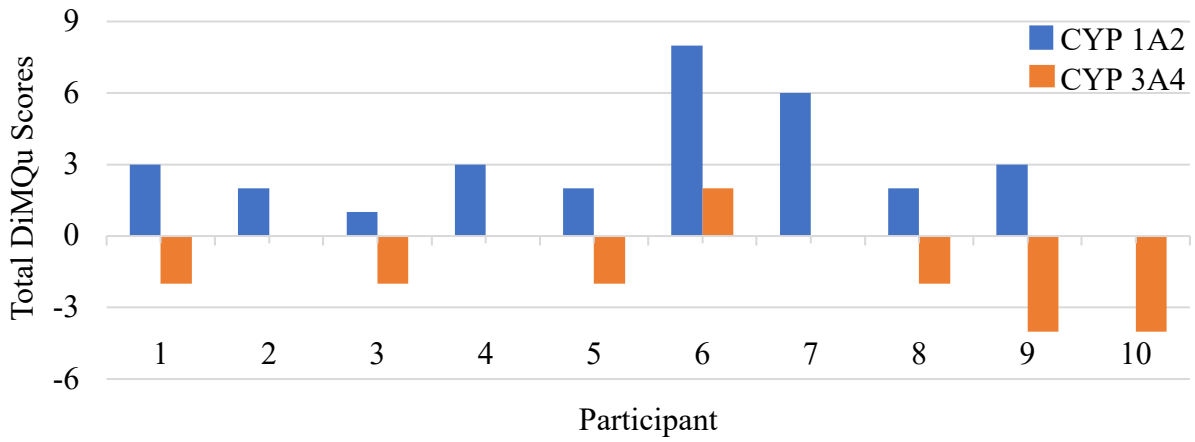
Note. All baseline values for Midazolam and OH-Midazolam are below the lower limit of quantitation (<LLOQ).

4.3 Exploratory Analysis between enzyme activity and key factors

4.3.1. DiMQu Scores

The participants total DiMQu raw scores are depicted in Figure 4. Generally, scores for the CYP1A2 enzyme predicted a net inducing effect of diet and medications consumed (principally caffeine-containing products) for 9/10 participants, whereas the same analysis indicated either no predicted effect or a modest net inhibiting effect of diet and medication use on CYP3A4 enzyme activity.

Figure 4. DiMQu Scores



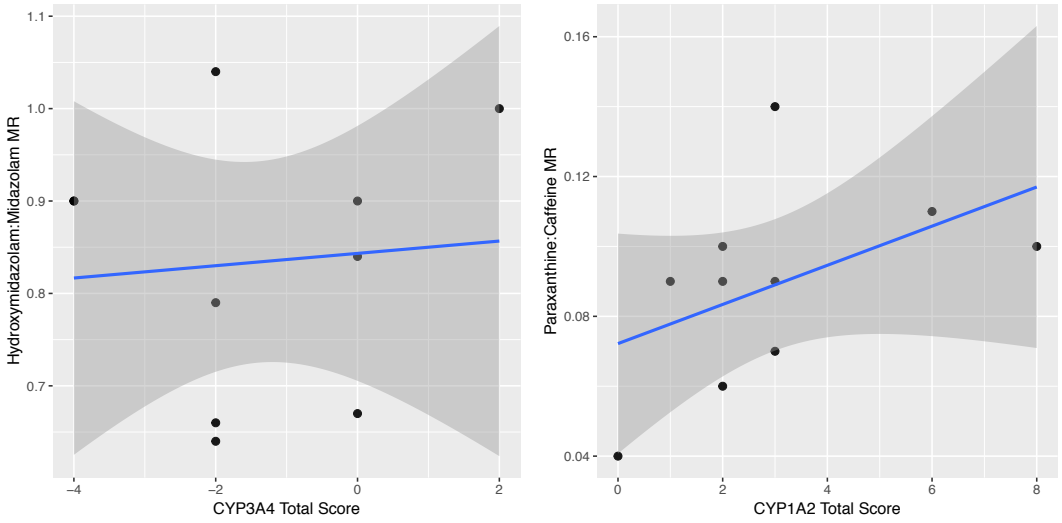
Note. A positive DiMQu score is more inducing, a negative score is more inhibiting. No bar indicates the score is 0.

4.3.2. DiMQu Scores and Enzyme Activity

There was no correlation between activity (MR) for CYP3A4 and the corresponding DiMQu score for CYP3A4 (*rho*=0.064, *p*=0.86; see Figure 5). However, there was a trend towards correlation between activity of CYP1A2 and corresponding DiMQu score (*rho*=0.61, *p*=0.06; see Figure 5b).

Figures 5. Scatterplots of Metabolic Ratios and DiMQu Scores

5a) 5b)

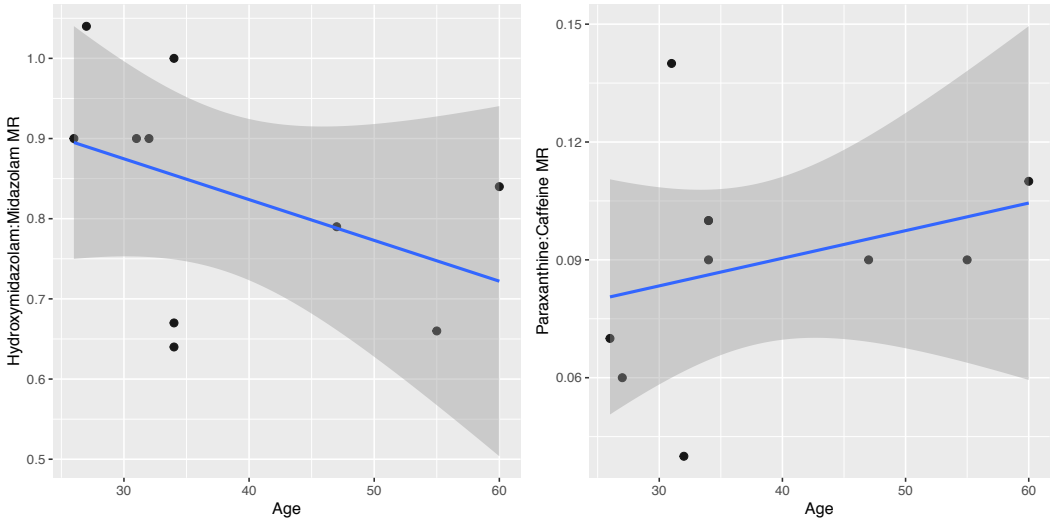


4.3.3. CYP3A4 Enzyme Activity and Age

Neither CYP3A4 nor CYP1A2 activity were significantly correlated with age, though as expected from the literature there was a trend for reduced activity with age for CYP3A4 (*rho*=-0.60, *p*=0.06; see Figure 6) but not CYP1A2 (*rho*=0.42, *p*=0.23; see Figure 6b).

Figure 6. Scatterplots of CYP3A4, CYP1A2 enzyme Metabolic Ratios and Age

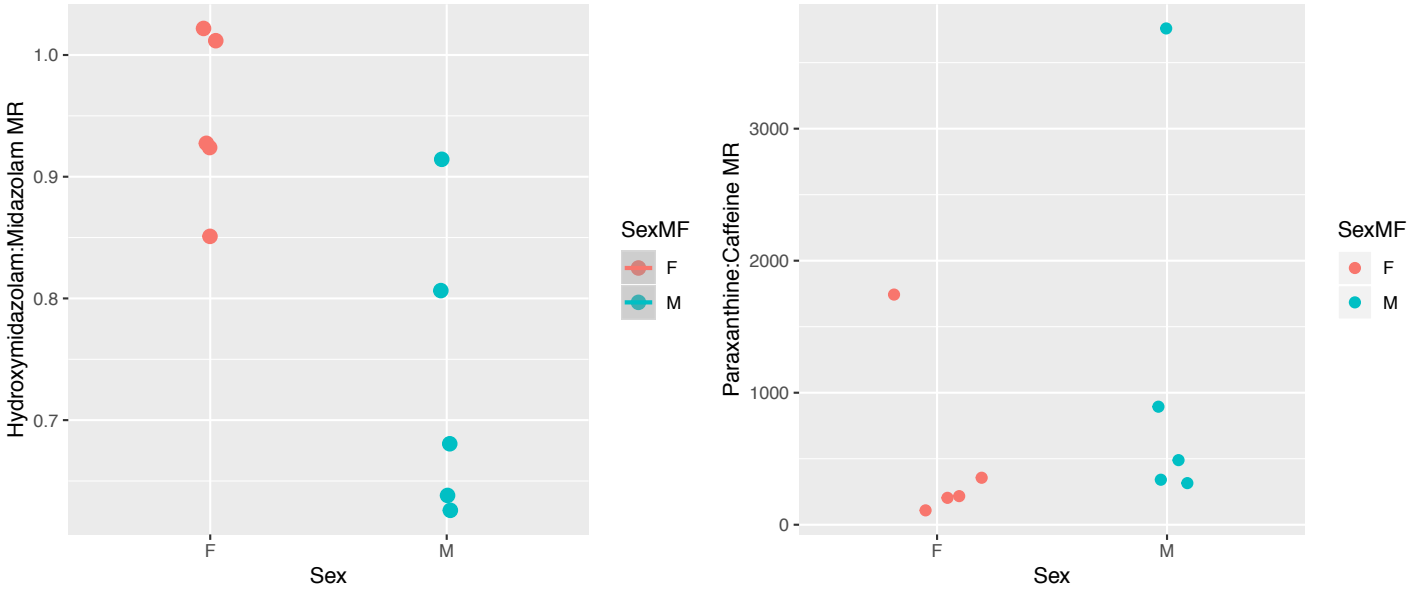
6a) 6b)



4.4 Sex and Metabolic Ratio

Mean CYP3A4 activity was higher in females than males (t=3.31, p=0.01; see Figure 7) which is consistent with the literature but there was no difference in activity between males and females for CYP1A2 (t=0.78, p=0.46 ; see Figure 7).

Figure 7. Sex and Metabolic Ratio



4.5 Dried Blood Sampling (DBS) Assay Performance

The DBS testing, using pooled whole blood showed that all our analytes of interest can successfully extracted from HemaSpot cards and measured using the same LCMS assay. Despite using this different technique, the results show that all analytes would have been detected within the linear range for the 10 participants in the study (see Table 12).

Table 12. Dried Blood Spot Method Compared to Serum Analysis

Analyte	Desired Range (ng/mL)	Achieved Serum Linear Range (ng/mL)	Subject Serum Concentration Range (ng/mL)	Pooled Whole Blood DBS Range (ng/mL)
Caffeine	25-5000	20-5000	110-6300	80-4000
Paraxanthine	25-5000	10-2500	80-3600	40-2000
Midazolam	0.1-100	0.10-24.4	4-12	0.4-20
OH-Midazolam	0.2-200	0.10-24.4	3-10	0.4-20

Note. DBS was assessed in capillary blood. LloQ can be further reduced through optimization of the extraction procedure and using more petals.

5 CHAPTER V: DISCUSSION

5.1 Key findings

This thesis was aimed at initial feasibility testing of an indirect assay for CYP3A4 and CYP1A2 activity in preparation for use in future studies focussed on patients with lung cancer. The results show the assay performance is acceptable for clinical use and testing with free-living subjects confirmed the limits of detection are appropriate. The higher CYP3A4 activity in females versus males was consistent with the literature.[24,25,26] Additionally, the trend towards inverse correlation with age and CYP3A4 enzyme activity has also been previously reported and is a further indirect validation of the assay results.[30,31,32] There was no inverse correlation between CYP1A2 enzyme activity and age and the no sex difference in CYP1A2 enzyme activity, but there was a strong trend towards increased CYP1A2 activity and exposure to dietary factors (mainly caffeine-containing products) which would be expected to induce CYP1A2 activity.

As discussed above multiple factors (dietary, genetic, drug etc.,), are known to influence CYP3A4 and CYP1A2 activity. The objective of the development of the DiMQu score was to determine whether there was any correlation between consumption of certain dietary components and drugs that are reported in the literature and known to inhibit or activate the CYP enzymes under study, and measured MR for the specified enzyme. The DiMQu score as constructed does not and cannot capture all the possible factors which influence the CYP enzyme activity and the correlation analysis with MR was an exploratory exercise only. Whilst the questionnaire could be expanded and optimised in future studies, it seems highly unlikely that this approach will provide a reliable predictor of CYP3A4 and CYP1A2 enzyme activity. Thus, it is still believed that some form of measurement of CYP enzyme activity is the only way to determine activity robustly.

Direct DBS testing for both CYP3A4 and CYP1A2 assays was not performed with the free-living subjects. However, laboratory testing using whole blood and addition of known concentrations of standards suggest this

approach is also feasible using the assay developed. Our results with DBS are consistent with previous research which suggest that DBS can substitute for serum testing and yields similar results.[70,71,76]

5.2 Study limitations

The small size (10 subjects) of the current study is clearly an important limitation. In addition, though the protocol for recruitment of subjects was deliberately designed to recruit a wide range of subjects, the mean age, smoking habits and number and type of co-morbidities is not truly representative of the wider population of patients with lung cancer who are older with more comorbidities and greater proportion of current or ex-smokers. As a result, because all of these factors may influence the activity of CYP3A4 and/or CYP1A2, it is likely that the range of enzyme activities encountered in any future clinical studies with lung cancer patients will be greater than those seen in the current study. Nevertheless, the limits or detection and assay linear response range for samples obtained from subjects in the current study was easily achieved, making it relatively straightforward to detect substrate and metabolite levels in all subjects. Thus, it is anticipated that the assay can be easily adapted to extend the linear range and limits of detection for future studies in the lung cancer population.

As detailed above, the assay being studied in this thesis gives an indirect global index of enzyme activity. CYP3A4 enzyme is concentrated in the liver and intestine, and oral test substrate (Midazolam) is a subject to metabolism by both.[63] It is possible that use of intravenous Midazolam, to isolate the activity of hepatic CYP3A only, may be more appropriate in some circumstances, but given that several of the anti-cancer drugs of interest in lung cancer (e.g. Gefitinib) are also orally administered the total activity of intestinal and liver CYP3A4 is still of major interest. Furthermore, the potential practical difficulties with administering the test dose (establishing i.v. access, sedation) make it far less feasible and practical for wider use in the lung cancer population.

After taking the test drugs, a single time-point sampling approach was chosen to capture peak concentrations of substrate and metabolite at about 1 hour. The literature suggests that this is a reasonable approach and MR values correlate with AUC methods and drug clearance.[63,64,65,70,71] In addition, studies have shown that changes in MR at single time point change appropriately when individuals also ingest known enzyme inducers or inhibitors. [64,67,68,69] However, we did not do a formal precision assessment of the MR at different time points in the same individual to determine how much the MR varied with time of blood sampling around the intended 1-hour time point. This is potentially important as a. time to peak concentration will vary between 1-2 hours in different individuals b. sample timing may vary for the same individual if they are tested on different occasions. Knowing the precision of the MR will help determine whether differences between individuals, or in the same individual over time, are real or due to physiological/technical variation.

Caffeine was chosen as the test substrate for CYP1A2 as it is commonly used for this purpose experimentally and peak concentrations of its metabolite occurs around the same time after ingestion as that for Midazolam which facilitates measurement of MR for both Midazolam and Caffeine using a single blood test. However, the use of caffeine presents some disadvantages. Caffeine is so widely used and consumed it is very commonly found as a contaminant in clinical samples including in pooled serum used to dilute standards used to construct the standard curves. To address the latter problem charcoal-stripped serum was used as diluent with good effect. Some individuals (see Table 10, subjects 3,4,10) had a relatively high level of caffeine and paraxanthine in the baseline serum samples making it difficult to measure the caffeine/paraxanthine peak concentrations attributable to the test caffeine dose administered. Given that the decay half-life for caffeine is in the order of 5-6 hrs and that in the current study, participants were only asked to fast for a minimum of eight hours prior to testing, it is likely that the protocol did not allow long enough to eliminate previously consumed caffeine and paraxanthine. This is particularly important in those with habitual high caffeine consumption and slower metabolisers of caffeine. One

indicator is the relative concentration of paraxanthine, which is cleared more slowly than caffeine, and can be present in concentrations which exceed those of caffeine many hours after consumption (e.g. Table 10, subject 10). It is also possible that some subjects did not adhere to the instructions to fast prior to testing and consumed caffeine in the hours just prior to testing leading to very high baseline levels of caffeine and paraxanthine (e.g. possibly Table 10, subjects 3,4). In developing the protocol, we were aware of the potential for presence of low levels of caffeine and paraxanthine at baseline but by reference to the concentration curves in the literature (Bosilkovska et al., 2014) we anticipated baseline concentrations of caffeine <600 and peak concentrations of > 1500 giving an expected ratio of peak to background of around 3 allowing for reliable measurement of the effect of the test dose of caffeine. Whilst this was true for the majority (7/10) of the subjects there is certainly a strong case for adjusting the protocol to include an extended period of caffeine abstinence (e.g. 36 hrs) and perhaps more specific education of subjects about potential other sources of caffeine (e.g. green tea), which would be easily incorporated without making it too onerous for patients to perform the assay correctly.

Baseline correction of peak caffeine and paraxanthine concentrations was performed using a simple baseline subtraction. By reference to the prior literature (Bosilkovska et al 2014) the decay curves of both caffeine and paraxanthine are relatively flat (i.e. only slow decline per hour) 8 hours after consumption of a test dose of caffeine, thus the simple baseline subtraction approach is a reasonable approximation. It could be argued that this method is too simplistic and may over-estimate the contribution of baseline caffeine to the caffeine peak at 60 minutes after consumption of the test dose. However, when no baseline correction for caffeine and paraxanthine was performed the correlation between DiMQu score and CYP1A2 MR (see Figure 5b) was reduced, so the simple baseline subtraction approach was retained. Future studies could include repeated measures over time to track the clearance of caffeine and paraxanthine, perhaps using DBS testing. This would enable precision assessment around the peak concentration, as mentioned above, but would also give more data on the rate of change in caffeine and

paraxanthine levels many hours after consumption of caffeine which in turn could be used to adjust the baseline correction of peak concentrations.

Midazolam was used as the substrate for CYP3A4 in this study. Whilst this test substrate performed well experimentally with no reports of sedation or other side effects, it did pose some administrative problems as Midazolam is a controlled substance, and in Canada it is also only available in liquid format intended for intravenous use. Unfortunately, there were long delays obtaining the necessary approvals and paperwork from both Health Canada for use off-label in this study, and from research pharmacy at the hospital as they needed to order, store and dispense the Midazolam on the day of use. The Midazolam used was dispensed in a syringe and has a bitter taste which was masked with co-administration of fruit juice. A pill formulation of Midazolam suitable for use with this assay is available in the US but the quantity needed for this study was not sufficient for the minimum order required for shipping.

Genetic testing of our participants for variant SNPs for CYP3A4 and CYP1A2 was not performed. It is likely that this would have been most relevant for the CYP1A2 enzyme as, unlike CYP3A4, polymorphisms (e.g. *1F) are present at high percentages in the population. Markers of inflammation were also not measured in this study, however this was unlikely to be relevant to the population in this study who were clinically stable. In addition, we did not measure vitamin D levels to determine whether subjects had potentially important deficiency that may have impacted enzyme activity. Participants in the study were asked about their alcohol consumption and smoking habits, however, we did not ask specifically about cannabis, a potential CYP3A4 inhibitor. Since recreational cannabis use is now legalized in Canada and medical cannabis is already popular among cancer patients, this is another important factor to consider with potential impact on CYP3A4 enzyme activity and drug metabolism. Whilst obtaining full data on all these factors would have been desirable, the lack of this data only serves to underline how difficult it is to capture all the factors that have a potential to modulate CYP3A4 and CYP1A2

activity and determine their net effect on enzyme activity. This in turn highlights the potential usefulness of more direct tests of enzyme activity such as the assay being tested in the current project.

In one study using the Basel cocktail, the authors noted that they were unable to detect the expected strong induction of CYP3A4 by rifampicin. Glucoronidation of OH-midazolam is a secondary step in metabolism of midazolam (by UDP glucuronosyl transferases) and the authors tested the effect of this step on recovered OH-midazolam concentrations by treating their samples with glucuronidase before measuring OH-midazolam concentrations. In their study, this change in protocol, led to an increase in recovery of OH-midazolam and altered final MR as well as revealing the induction of CYP3A4 activity by rifampicin.[80] As a result, the authors suggest that because there is a secondary glucuronidation of the primary hydroxymidazolam product, an assay which does not include glucuronidase treatment will underestimate the change in the MR. Experiments are currently underway in our research group to evaluate whether this additional step in the assay has any major impact on recovered OH-midazolam concentrations.

6 CHAPTER VI: FUTURE DIRECTIONS

6.1 Conclusions and future directions

In the absence of routine measurement of the levels of anti-cancer drugs, oncologists have little direct information to guide them in adjusting doses to obtain optimum outcomes while minimizing serious side-effects. Monitoring changes in both CYP3A4 and CYP1A2 activity which are principal metabolisers of several key anti-cancer drugs used in lung cancer, could provide additional, potentially useful, information to help promote safe, effective use of certain anti-cancer drugs. Equally importantly, an assay of CYP3A4 activity would also be very helpful in monitoring the safety of dietary changes or herbal treatments that patients may want to use in conjunction with their regular oncological treatment.

Many drugs are metabolized through more than one pathway and changes in one metabolic pathway may result in reciprocal changes in another.[14] For example, when reviewing drugs that are primarily metabolized through the CYP3A4 enzyme pathway, it's important to consider that there may be potential changes in other parallel metabolic pathways e.g. CYP1A2, for the same substrate.[14] Since there may be several routes of metabolism for a given drug, caution is needed in making any assumption about possible direct correlation between drug concentrations and the activity of e.g. CYP3A4 or other enzyme. Thus, further studies are needed to confirm whether changes in CYP3A4 and CYP1A2 enzyme activity measured using the assay presented in this thesis, are associated with changes in concentrations or activity of specific target drugs of interest.

One simple future study would be to further validate this assay in patients with lung cancer and establish more clearly the likely range of substrate and metabolite concentrations that are encountered and to perform the precision assessment with repeated testing of the same individual over time. In addition, further testing of the DBS approach is warranted. Venous blood sampling is more invasive, expensive, and time-consuming than DBS and it seems likely that simple finger-prick blood testing would increase participant enrollment in future studies. Furthermore,

DBS testing, including the HemaSpot cartridges used in this study, are amenable to home testing. Thus, participants may be able to complete more extended time course experiments at home using the DBS approach.

In addition to validation and technical development to increase convenience of testing in lung cancer patients, future studies should also focus on the clinical relevance of the results. If CYP3A4 and CYP1A2 are involved in metabolism of anti-cancer drugs, further studies are needed to establish what level of enzyme activity is likely to result in clinically-relevant changes in drug activity. Ideally this could be pursued using direct measurement of concentrations of a given anticancer drug e.g. Erlotinib, and parallel studies of CYP3A4/CYP1A2 activity levels in the same patient. However, assays for most anti-cancer drugs are not available routinely. Nevertheless, similar studies could be performed using other (non-oncological) medications known to be metabolised via one of these pathways where direct assays are available. Another approach might be to look for clinical markers of anticancer effect e.g. tumour response to a given drug, and measured CYP3A4/CYP1A2 activity. Where higher enzyme activity might be expected to be associated with reduced anti-cancer effect. Finally, and perhaps more feasible, would be a study of easily measured and rapidly occurring toxicity such as skin changes (acne-like skin eruptions) associated with EGFR-inhibitor use in some patients and their CYP3A4/CYP1A2 activity. In this study the hypothesis would be that higher enzyme activity would be associated with lower skin toxicity.

Another way in which the CYP3A4 and CYP1A2 assays could be helpful in management of patients with lung cancer is to monitor changes in enzyme activity over time. Specifically, it would be of interest to evaluate whether changes in enzyme activity are associated with variations in systemic inflammation. In addition, this assay could be used to monitor the potential impact of various complementary health approaches. For example, Traditional Chinese herbs and other botanicals are commonly used by patients to reduce symptoms, but their impact on anticancer drug metabolism is usually unknown making it difficult for both oncologists and patients to know how best to use these treatments. In a study performed at the Peter Brojde Lung Cancer Centre recently, 15 stage 4

NSCLC patients were studied whilst taking a 6-week course of a standardised Chinese herb formula. The results showed that this formula was apparently safe (using standard blood tests including coagulation and liver function testing) with only a small minority reporting mild adverse effects, but as a group patients reported improved markers of quality of life.[84] However, even in this carefully performed study, the researchers were not able to determine if the Chinese herbs used had other subclinical change on liver function, including CYP3A4 or CYP1A2 activity. Thus, parallel testing with the CYP3A4/CYP1A2 assay presented here, would be a valuable additional approach to identify potential impacts of complementary health approaches, medical or dietary changes which may occur during the course of anti-cancer treatment.

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6.3 Appendices

6.3.1 Appendix 1: Lab Protocols

Jagoe Assay Development Project **31-Oct-2018**

Context

- Previously observed deterioration in chromatography performance, so revised LC method to ensure proper retention when there was higher organic (18 µL injection).
- However, after subsequent run of QCs and calibration curve in buffer, sensitivity still appeared to be worse than previous runs. On closer examination, this was primarily the case for the calibration curve and appeared to be due to poor chromatography.
- Samples were remade from original standards and diluted in glass vials (since there was a concern that the 96-well plate was causing adsorption of the standards from buffer (suggested by better sensitivity in serum than in buffer). The remade samples showed the exact same profile as the previous batch – poor chromatography for the standards, which appears to limit sensitivity.
- After trying to remake solvents, etc., it was ultimately determined that this may be due to different pH between the samples and the standards prepared in buffer (as observed on pH test strips). As a result, it was decided to prepare the standards in serum to improve retention.

Purpose

1. Prepare and analyze calibration curve standards in charcoal-stripped to confirm that samples prepared in serum now match the sensitivity (A-F), linearity (R2> 0.995), and accuracy (QCs +/- 20%) before spiking QCs and analysing patient samples.
2. Prepare aliquots of spiked serum to use as an ongoing QC.
3. Analyze patient samples

Linear range & IS concentrations

	Cal low	Cal high	IS concentration
Caffeine	20	4500	1000
Paraxanthine	20	1800	400
Midazolam	0.25	25	2
OH-Midazolam	0.25	25	2
(all values in ng/mL)			

Calibration standards & spiked-serum QCs for Geneva Cocktail Assay

		Equivalent [ ] in serum, ng/mL										
		A	B	C	D	E	F	G		QC – High	QC - Med	QC-Low
Caff		5000	2000	800	320	128	51.20	20.48		2500	500	100
Parax		2500	1000	400	160	64	25.60	10.24		1250	250	50
Mida		25	10	4	1.60	0.64	0.26	0.10		12.5	2.5	0.5
OH-Mida		25	10	4	1.60	0.64	0.26	0.10		12.5	2.5	0.5

Experiment 1 Sample List

Sample Type	Samples	Sample	Spiking	IS	Notes
Standards A-G in buffer	7 x Stds in **serum**	45 uL serum	5 uL stds (10x conc)	5 uL IS	
Serum blank (charcoal stripped)	2 x Plasma	50 uL serum	-	5 µL IS	
QCs in charcoal-stripped serum	3 x QCs in plasma	45 uL serum	5 uL stds (10x conc)	5 uL IS	

Protocol for Sample Preparation of QC Samples (Experiment 1)

		Volume (uL)	Organic	Total (uL)	Final Organic
Aliquot serum to Eppendorf lo-bind 1.5 mL tube		45	*0%*	45	*0%*
Add unlabeled standards mix, vortex	+	5	*60%*	50	*6%*
Add IS mix, vortex	+	5	*60%*	55	*11%*
Add acetonitrile to precipitate proteins	+	195	*100%*	250	*80%*
Vortex, Centrifuge 10,000 rpm for 3 minutes					
Remove 50 uL supernatant					
Transfer to MS vial		50	*80%*	50	*80%*
Dilute 1:1 with water	+	50	*0*	100	*40%*

Experiment 2 Sample List

Sample Type	Samples	Sample	Spiking	IS	Notes
Patient Serum	N=20 x 3 replicates as shown below	50 µL serum	--	5 µL IS	As below
Serum blank	3 (prepare 1 in each replicate run)	50 µL serum		5 µL IS	SB-01, etc
QCs	3 (per replicate run)	As prepared in previous experiment			QC-L1, QC-M1, etc

Participant ID	Before – rep 1	After – rep 1		Before – rep 2	After – rep 2		Before – rep 3	After – rep 3	
04NIMQEJ	A-01	K-01		A-02	K-02		A-03	K-03	
HMKE4WLY	B-01	L-01		B-02	L-02		B-03	L-03	
FSZN2KS0	C-01	M-01		C-02	M-02		C-03	M-03	
5PO7P014	D-01	N-01		D-02	N-02		D-03	N-03	
OCKNEYEQ	E-01	O-01		E-02	O-02		E-03	O-03	
JB73636T	F-01	P-01		F-02	P-02		F-03	P-03	
J4OGFV77	G-01	Q-01		G-02	Q-02		G-03	Q-03	
8Z9UMG7U	H-01	R-01		H-02	R-02		H-03	R-03	
9BDV3X3V	I-01	S-01		I-02	S-02		I-03	S-03	
W6Q7KF83	J-01	T-01		J-02	T-02		J-03	T-03	

Protocol for Sample Preparation of Experimental Samples (Experiment 2)

		Volume (uL)	Organic	Total (uL)	Final Organic
Aliquot serum to Eppendorf lo-bind 1.5 mL tube		50	*0%*	50	*0%*
Add IS mix, vortex	+	5	*60%*	55	*6%*
Add acetonitrile to precipitate proteins	+	195	*100%*	250	*79%*
Vortex, Centrifuge 10,000 rpm for 3 minutes					
Remove 50 uL supernatant					
Transfer to MS vial		50	*79%*	50	*79%*
Dilute 1:1 with water	+	50	*0*	100	*40%*

MassHunter>Methods>Connie>Geneva Cocktail>_____.m

LC Settings

Parameter	Settings
System	Agilent 1290 Infinity system
Column	Zorbax Eclipse plus RRHD C18, 2.1x15mm, 1.8um (Column labeled "A")
Guard Column	none
Column temp. (°C)	50°C +/- 0.8°C
Mobile Phase A	H_2O + 0.1% formic acid
Mobile Phase B	Acetonitrile + 0.1% formic acid
Flow Rate (ml/min)	0.6
Run Time (min)	5.2
Injection Volume (µl)	Default Injection volume in method: 5 uL Perform 2 injections of each sample: 2 uL for caffeine, paraxanthine; 18 uL for midazolam, OH-midazolam
Sample dispensing	0.0 mm needle draw position (Vial/Well bottom sensing on); 10 uL/min draw speed, 20 uL/min eject speed; 3 sec equilibration time, flush with 5.0 times injection volume
Needle Wash	Flush Port - 10 sec
AS Temperature (ºC)	4
Max Pressure	1000 bar

LC Gradient

Step	Time (min)	% Mobile Phase B
Applying sample to column	0.00-0.50	2% → 20%
Pre-elution	0.50-1.00	2% → 20%
Elution	1.00-3.00	20 →25%
Post-elution	3.00-3.50	25 →98%
Wash	3.50-5.00	98 →100%
Reset	5.00-5.3	100 → 2%
Equilibration	5.3-5.8	2%
Post time	+0.2 min	2%

MS Method

Analyte	Transition	CE
Hydroxymidazolam (IS)	346/328	22
Hydroxymidazolam (IS)	346/203	31
Hydroxymidazolam	342/324	22
Hydroxymidazolam	342/203	31
Midazolam (IS)	330/295	28
Midazolam (IS)	330/253	40
Midazolam	326/291	28
Midazolam	326/249	40

Analyte	Transition	CE
Caffeine (IS)	204/144	21
Caffeine (IS)	204/116	27
Caffeine	195/138	21
Caffeine	195/110	27
Paraxanthine (IS)	187/127	20
Paraxanthine	181/124	20

MS Settings

Parameter	Settings
System	Agilent 6495 MS/MS
Ion Source	AJS ESI
Method	MRM-MS
Delta EMV (+)	400
Source gas temp	150 C
Gas Flow	15 l/min
Nebulizer	30 psi
Sheath Gas Temp	250 C
Sheath Gas Flow	11 l/min
Polarity	positive

Capillary	3500V + / 3000 V neg
Nozzle voltage	300 V pos / 1500 V neg
Resolution (MS1 and MS2)	Unit Res
Fragmentor	380
Cell Accelerator Voltage	5
Cycle time	3.04 cycles/s, 329 ms/cycle (dwell 20/transition)
Time filtering	Peak width 0.03 min

Validation Criteria for Geneva Cocktail Assay at JGH

Double Blank	• 1 replicate per run, injected 2x	• Response <20% of LLoQ per analyte
Blank with IS	• 1 replicate per run, injected 2x	• Response <20% of LLoQ per analyte
Ionization Effects	• All specimens and calibrators/QCs	• IS peak area variation 80-120%
Transition Ratio	• Specimens vs calibrators/QCs	• Samples consistent with calibrators/ QCs
Calibration Curve	• Minimum of 6 levels bracketing curves	• R2>0.995

Jagoe Assay Development Project **03-Jan-2019**

Context

- Agilent 6495 has been in use for a major project since November and now have the opportunity to run final replicates for Jagoe project. Initial test of standards extracted from serum (samples from Oct 31, 2018) showed that the retention time is sufficiently stable that no methods changes are necessary. The column performance does not appear to have changed. The standards in buffer still generate poor peak shape. May be useful to replace the column eventually.

Purpose

1. Prepare and analyze calibration curve standards in charcoal-stripped to confirm that samples prepared in serum now match the sensitivity (A-F), linearity (R2> 0.995), and accuracy (QCs +/- 20%)
2. Prepare aliquots of spiked serum to use as an ongoing QC.
3. Analyze patient samples

Linear range & IS concentrations

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		Equivalent [ ] in serum, ng/mL										
		A	B	C	D	E	F	G		QC – High	QC - Med	QC- Low
Caff		5000	2000	800	320	128	51.20	20.48		2500	500	100
Parax		2500	1000	400	160	64	25.60	10.24		1250	250	50
Mida		25	10	4	1.60	0.64	0.26	0.10		12.5	2.5	0.5
OH-Mida		25	10	4	1.60	0.64	0.26	0.10		12.5	2.5	0.5

Experiment 1 Sample List

Sample Type	Samples	Sample	Spiking	IS	Notes
Standards A-G in buffer	7 x Stds in **serum**	45 uL serum	5 uL stds (10x conc)	5 uL IS	
Serum blank (charcoal stripped)	2 x Plasma	50 uL serum	-	5 µL IS	
QCs in charcoal-stripped serum	3 x QCs in plasma	45 uL serum	5 uL stds (10x conc)	5 uL IS	

Protocol for Sample Preparation of QC Samples (Experiment 1)

		Volume (uL)	Organic	Total (uL)	Final Organic
Aliquot serum to Eppendorf lo-bind 1.5 mL tube		45	*0%*	45	*0%*
Add unlabeled standards mix, vortex	+	5	*60%*	50	*6%*
Add IS mix, vortex	+	5	*60%*	55	*11%*
Add acetonitrile to precipitate proteins	+	195	*100%*	250	*80%*
Vortex, Centrifuge 10,000 rpm for 3 minutes					
Remove 50 uL supernatant					
Transfer to MS vial		50	*80%*	50	*80%*
Dilute 1:1 with water	+	50	*0*	100	*40%*

Experiment 2 Sample List

Sample Type	Samples	Sample	Spiking	IS	Notes
Patient Serum	N=20 x 3 replicates as shown below	50 µL serum	--	5 µL IS	As below
Serum blank	3 (prepare 1 in each replicate run)	50 µL serum		5 µL IS	SB-01, etc
QCs	3 (per replicate run)	As prepared in previous experiment			QC-L1, QC-M1, etc

Participant ID	Before – rep 1	After – rep 1		Before – rep 2	After – rep 2		Before – rep 3	After – rep 3	
04NIMQEJ	A-01	K-01		A-02	K-02		A-03	K-03	
HMKE4WLY	B-01	L-01		B-02	L-02		B-03	L-03	
FSZN2KS0	C-01	M-01		C-02	M-02		C-03	M-03	
5PO7P014	D-01	N-01		D-02	N-02		D-03	N-03	
OCKNEYEQ	E-01	O-01		E-02	O-02		E-03	O-03	
JB73636T	F-01	P-01		F-02	P-02		F-03	P-03	
J4OGFV77	G-01	Q-01		G-02	Q-02		G-03	Q-03	
8Z9UMG7U	H-01	R-01		H-02	R-02		H-03	R-03	
9BDV3X3V	I-01	S-01		I-02	S-02		I-03	S-03	
W6Q7KF83	J-01	T-01		J-02	T-02		J-03	T-03	

Protocol for Sample Preparation of Experimental Samples (Experiment 2)

		Volume (uL)	Organic	Total (uL)	Final Organic
Aliquot serum to Eppendorf lo-bind 1.5 mL tube		50	*0%*	50	*0%*
Add IS mix, vortex	+	5	*60%*	55	*6%*
Add acetonitrile to precipitate proteins	+	195	*100%*	250	*79%*
Vortex, Centrifuge 10,000 rpm for 3 minutes					
Remove 50 uL supernatant					
Transfer to MS vial		50	*79%*	50	*79%*
Dilute 1:1 with water	+	50	*0*	100	*40%*

MassHunter>Methods>Connie>Geneva Cocktail>_____.m

LC Settings

Parameter	Settings
System	Agilent 1290 Infinity system
Column	Zorbax Eclipse plus RRHD C18, 2.1x15mm, 1.8um (Column labeled "A")
Guard Column	none
Column temp. (°C)	50°C +/- 0.8°C
Mobile Phase A	H_2O + 0.1% formic acid
Mobile Phase B	Acetonitrile + 0.1% formic acid
Flow Rate (ml/min)	0.6
Run Time (min)	5.2
Injection Volume (µl)	Default Injection volume in method: 5 uL Perform 2 injections of each sample: 2 uL for caffeine, paraxanthine; 18 uL for midazolam, OH-midazolam
Sample dispensing	0.0 mm needle draw position (Vial/Well bottom sensing on); 10 uL/min draw speed, 20 uL/min eject speed; 3 sec equilibration time, flush with 5.0 times injection volume
Needle Wash	Flush Port - 10 sec
AS Temperature (°C)	4
Max Pressure	1000 bar

LC Gradient

Step	Time (min)	% Mobile Phase B
Applying sample to column	0.00-0.50	2% → 20%
Pre-elution	0.50-1.00	2% → 20%
Elution	1.00-3.00	20 →25%
Post-elution	3.00-3.50	25 →98%
Wash	3.50-5.00	98 →100%
Reset	5.00-5.3	100 → 2%
Equilibration	5.3-5.8	2%
Post time	+0.2 min	2%

MS Method

Analyte	Transition	CE
Hydroxymidazolam (IS)	346/328	22
Hydroxymidazolam (IS)	346/203	31
Hydroxymidazolam	342/324	22
Hydroxymidazolam	342/203	31
Midazolam (IS)	330/295	28
Midazolam (IS)	330/253	40
Midazolam	326/291	28
Midazolam	326/249	40

Analyte	Transition	CE
Caffeine (IS)	204/144	21
Caffeine (IS)	204/116	27
Caffeine	195/138	21
Caffeine	195/110	27
Paraxanthine (IS)	187/127	20
Paraxanthine	181/124	20

MS Settings

Parameter	Settings
System	Agilent 6495 MS/MS
Ion Source	AJS ESI
Method	MRM-MS
Delta EMV (+)	400
Source gas temp	150 C
Gas Flow	15 l/min
Nebulizer	30 psi
Sheath Gas Temp	250 C
Sheath Gas Flow	11 l/min
Polarity	positive

Capillary	3500V + / 3000 V neg
Nozzle voltage	300 V pos / 1500 V neg
Resolution (MS1 and MS2)	Unit Res
Fragmentor	380
Cell Accelerator Voltage	5
Cycle time	3.04 cycles/s, 329 ms/cycle (dwell 20/transition)
Time filtering	Peak width 0.03 min

Validation Criteria for Geneva Cocktail Assay at JGH

Double Blank	• 1 replicate per run, injected 2x	• Response <20% of LLoQ per analyte
Blank with IS	• 1 replicate per run, injected 2x	• Response <20% of LLoQ per analyte
Ionization Effects	• All specimens and calibrators/QCs	• IS peak area variation 80-120%

Transition Ratio	• Specimens vs calibrators/QCs	• Samples consistent with calibrators/ QCs
Calibration Curve	• Minimum of 6 levels bracketing curves	• R2>0.995

Jagoe Assay Development Project **06-Feb-2019**

Purpose

- Purpose is to try to translate existing method to DBS. Test sensitivity in small volumes.
- Use a limited calibration curve but spot different volumes onto cards (5 µL, 9.2 µL, 20 µL)

Materials

- Stds & IS
- 5 x whatman cards, 5x hemaspot
- Charcoal-stripped plasma (since this is all that is available at the moment)

Calibration standards

		Equivalent [ ] in serum, ng/mL				IS Concentration
		A	B	C	D	
Caff		5000	2500	500	100	1000
Parax		2500	1250	250	50	400
Mida		25	12.5	2.5	0.5	2
OH-Mida		25	12.5	2.5	0.5	2

- Prepare from Standards Mix of 31 Oct 2018, in 60% MeOH
    a. Dilute mix - 10x to Std A
    b. Dilute Std A - 2x to Std B
    c. Dilute Std B - 5 x to C
    d. Dilute Std C - 5x to D
- Use IS of Jan 3, 2019; Dilute 2.5uL of IS per 100 µL of MeOH (e.g., 25 µL IS + 975 µL MeOH)

Prepare DBS samples:

- Prepare standards A-D in buffer according to table showing standards preparation (10x final serum concentration)
- Aliquot 25 µL per Eppendorf lo bind vial (3 x A, B, C, D, blank), dry down in speedvac
- To each vial, add 250 uL of charcoal stripped plasma or whole blood, vortex and immediately spot for each sample:
    a. 1 x whatman card, 5 µL, 5 µL, 9.2 µL, 9.2 µL, 20 µL to each of the 5 positions
    b. 1 x spot on card, 80 µL total per card
    c. Excess spiked plasma may be stored at -20C
- Follow card instructions for drying and storage, etc.
    a. E.g., The DBS collection cards were bent so that the back of the card was not in contact with any surface to prevent loss of blood that soaked through the filter paper. DBS samples were allowed to dry at room temperature for at least 1 h and were then packed in a sealable plastic bag containing desiccant until analysis (no later than 15 days postspotting).

Extract DBS samples:

- Start by testing 1 x sample from highest standard to see if detectability is adequate. Start with testing 9.2 µL spots
    a. Whatman cards: punch entire spot with 6mm punch
    b. HemaSpot: tear one leaf (equivalent to 9.2 µL)
- Fold paper into the bottom of Eppendorf vials
- Add MeOH (100 µl) with IS mix (2.5 µL/100 µL), seal & vortex
- Dilute supernatant 10:1 with H2O in MS vials before analysis

LC-MRM-MS/MS

MassHunter>Methods>Connie>Geneva Cocktail>_____.m

LC Settings

Parameter	Settings
System	Agilent 1290 Infinity system
Column	Zorbax Eclipse plus RRHD C18, 2.1x15mm, 1.8um (Column labeled "A")
Guard Column	none
Column temp. (°C)	50°C +/- 0.8°C
Mobile Phase A	H_2O + 0.1% formic acid
Mobile Phase B	Acetonitrile + 0.1% formic acid
Flow Rate (ml/min)	0.6
Run Time (min)	5.2
Injection Volume (µl)	Default Injection volume in method: 5 uL Perform 2 injections of each sample: 2 uL for caffeine, paraxanthine; 18 uL for midazolam, OH-midazolam
Sample dispensing	0.0 mm needle draw position (Vial/Well bottom sensing on); 10 uL/min draw speed, 20 uL/min eject speed; 3 sec equilibration time, flush with 5.0 times injection volume
Needle Wash	Flush Port - 10 sec
AS Temperature (°C)	4
Max Pressure	1000 bar

LC Gradient

Step	Time (min)	% Mobile Phase B
Applying sample to column	0.00-0.50	2% → 20%
Pre-elution	0.50-1.00	2% → 20%
Elution	1.00-3.00	20 →25%
Post-elution	3.00-3.50	25 →98%
Wash	3.50-5.00	98 →100%
Reset	5.00-5.3	100 → 2%
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Hydroxymidazolam	342/203	31
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Midazolam (IS)	330/253	40
Midazolam	326/291	28
Midazolam	326/249	40

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Caffeine (IS)	204/116	27
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Sheath Gas Temp	250 C
Sheath Gas Flow	11 l/min
Polarity	positive

Capillary	3500V + / 3000 V neg
Nozzle voltage	300 V pos / 1500 V neg
Resolution (MS1 and MS2)	Unit Res
Fragmentor	380
Cell Accelerator Voltage	5
Cycle time	3.04 cycles/s, 329 ms/cycle (dwell 20/transition)
Time filtering	Peak width 0.03 min

6.3.2 Appendix 2: Consent Form

Jewish General Hospital
Peter Brojde Lung Cancer Centre
Dr. Thomas Jagoe

Consent form
Measuring CYP3A4 activity: A validation study in free-living adults

Introduction
You are invited to participate in a clinical research study to evaluate a method to measure the activity of a specific type of liver enzymes. You have the right to know about the purpose and procedures that are to be used in this research study, and to be informed about the potential benefits, risks, compensation, and discomfort of this research.

This document will explain the purpose and procedures of this research study, and will also set out any potential benefits and any potential risks or discomfort that you should know about before you decide if you will participate in this research. It is important that you read the following information and ask as many questions as you need to. You do not have to take part in this study if you do not want to. If you decide you do not want to participate this will not affect your normal treatment or care in any way.

1. Purpose of study:
Many patients with lung cancer are now treated with newer anti-cancer drugs that are metabolized by a specific type of liver enzyme called the CYP3A4 enzymes. These enzymes are part of normal metabolism in the body but have an important role in deactivating some drugs that are now used for treating lung cancer. Many other substances including certain foods, medications and even complementary medicines such as herbal therapies can change the activity of the CYP3A4 enzymes. If the CYP3A4 enzyme activity changes substantially while a patient with lung cancer is on treatment this may reduce the effectiveness of their cancer treatment. There are no tests of CYP3A enzyme activity available currently and we want to develop one to help predict when a patient may need to have their cancer treatment changed. We want to prove that this method works well in people without lung cancer, before proceeding to do the same testing on patients with lung cancer.

The best way to measure CYP3A4 enzyme activity is to check how rapidly a small dose of a specific drug is metabolized in the body. The test drug is taken by mouth and blood levels of the drug are checked about 1-hour after taking the drug. If this is successful we hope that in future patients can complete this test at home and not have to come into hospital.

The activity of another closely-related member of the same enzyme family (the CYP1A2 enzyme) will be measured on the same blood samples. The CYP1A2 enzyme activity test is included to help us determine whether any changes in CYP enzyme activity are generalized or specific for the CYP3A4 family alone.

2. Procedures:

- You will be asked to eat nothing and drink only water for at least 8 hours prior to coming for the test between 0800 -1000. It is especially important that you do not take any caffeinated drinks or any usual medication including sleeping pills for 8hrs prior to the test.
- When you arrive, your height and weight will be measured (without shoes and wearing light indoor clothes only) as your overall body size may alter metabolism of the test drugs.
- You will then have a blood sample taken from the arm (5mls = approx. 1 teaspoon).
- Immediately after this you will be asked to take two tablets: 100 mg caffeine (to test the CYP1A2 enzymes) and 2 mg of midalozam (to test the CYP3A4 enzymes).
- You will then be asked to complete a medical questionnaire, a simple dietary questionnaire and a list of any medications and complementary health treatments that you are using.
- One hour after you took the two test drugs you will be asked to provide the second venous blood sample.

Any side-effects from these procedures, such as drowsiness caused by the test drug midazolam, are expected to be very minimal at the low dose being used, and will usually have peaked by the time you finish the tests. However, **you are advised not to drive for a further 5 hours after the second blood test just to be safe**.

3. Risks, Discomforts and Side-Effects:

There is nothing to suggest that there are any additional expected risks involved in taking the study drug, nor with the method of blood withdrawal that are performed. The oral formulation of midazolam is not available in Canada and so we will use a liquid form which has is made for intravenous use. There are no known safety concerns and the intravenous preparation has been widely used for oral sedation in children. The midazolam liquid can taste bitter, so we will give you some sweet-tasting fruit juice at the same time as the midazolam to counteract the bitter taste.

There will be the usual minor discomfort of the venous blood sampling. The test dose of caffeine is about the same as in a cup of filter coffee. When given directly into the vein midazolam can make people feel sleepy, but the oral dose used in this study is 5 or 10 times lower than the equivalent intravenous dose and is not usually associated with any sedative effect. All precautions will be taken to avoid the occurrence of unexpected adverse reactions and participants will be closely monitored. With all medicines or procedures, there may be risks that we do not know about.

4. Benefits:

There are no medical benefits to your participation in this study. However, information learned from this research may lead to better treatment in the future for people with lung cancer by helping oncologists decide on the correct dose of their cancer treatments drugs.

5. Alternative to Study Participation:
You do not have to participate in this study if you do not want to.

6. Voluntary participation/withdrawal:
Your participation in this study is voluntary, and even if you decide to participate in the study now you can withdraw from the study at any time. You do not have to give a specific reason for withdrawing from the study and your future medical care and your patient-doctor or patient-therapist relationship will not be affected in any way. In addition, your participation in the study may be stopped by the study team, without your consent if:

- you are unable or unwilling to follow the study procedures set out above
- the study doctor feels it is in your best interests to withdraw you from the study.

It is important that you inform your study doctor of any change in your health or if you experience any side effects whether or not you think it is relevant to this study or related to the test drugs used in the study.

7. New Information Learned
If new information is learned that could affect your choice to continue in the study, you will be told about this information and given an opportunity to decide if you want to continue your participation in the study.

8. Confidentiality:
During your participation in this study, the study doctor and their team will collect and record information about you in a study file. They will only collect information required to meet the scientific goals of the study.

The study file may include information from your medical records concerning your past and present state of health, your lifestyle, as well as the results of the tests, exams and procedures that you will undergo during this research project. Your research file does also contain other information, such as your name, sex and date of birth.

The blood samples will be sent to the 6th floor Segal Cancer Centre in lab of Dr Christoph Borchers and conserved for maximum two years for the exclusive objectives of this study and then destroyed.

All information collected during the research project will remain confidential to the extent provided by law. You will only be identified by a code number. The key to the code linking your name to your study file will be kept by the doctor in charge of this research study [Pulmonary Oncology, PBLCC].

To ensure your safety, a copy of this information and consent form will be placed in your medical chart. As a result, any person or company whom you give access to your

medical chart will have access to this information. The study data will be stored for at least 10 years by the doctor in charge of this study.

The data may be published or shared during scientific meetings; however, it will not be possible to identify you.

You have the right to consult your study file in order to verify the information gathered, and to have it corrected if necessary. However, in order to protect the scientific integrity of the research project, assessing certain information before the project is ended may require that you be withdrawn from the study.

If you withdraw (or are withdrawn) from the study, any information collected up to the point of withdrawal for the purpose of this research may still be used in order to protect the scientific integrity of the study.

9. Costs and compensation:
You will not be paid for your participation in this study. There will be no costs to you for participating in this study. The drugs and research procedures will be provided to you free of charge.

10. Compensation in case of injury:
Should you suffer harm of any kind following the administration of any procedure related to the research study, you will receive the appropriate care and services as required by your state of health.

By agreeing to participate in this research study, you do not give up any of your legal rights nor discharging the doctor in charge of this research study, the sponsor or the institution, of their civil and professional responsibilities.

11. Contact information or questions:
If you have any questions about the research now or later, if you notice anything unusual, or if you think you have had a research-related injury, you should call Dr Thomas Jagoe at phone number 514-340-8222 ext. 2734.

For any questions concerning your rights as a person taking part in this study or if you have comments or wish to file a complaint you can communicate with the CIUSSS West-Central Montreal Health's Local Commissioner of Complaints & Quality of Services, Rosemary Steinberg, at (514) 340-8222 ext. 5833.

STATEMENT OF CONSENT

Measuring CYP3A4 activity: A validation study in healthy subjects

I have reviewed the information and consent form. Both the research study and the information and consent form were explained to me. My questions were answered, and I was given sufficient time to make a decision. After reflection, I consent to participate in this research study in accordance with the conditions stated above.
I authorize the research study team to have access to my medical record for the purposes of this study. I do not give up any of my legal rights by signing this consent form.

I agree to participate in this study.

Name of Participant	Signature	Date

Person Obtaining Consent:
I have explained the research project and the terms of this consent form to the research participant and have answered all of their questions.

Name of Person Obtaining Consent	Signature	Date

Commitment of the Principal Investigator:

I certify that this information and consent form were explained to the research participant, and that the questions the participant had were answered. I undertake, together with the research team, to respect what was agreed upon in the information and consent form, and to give a signed and dated copy of this form to the research participant.

Name of Principal Investigator	Signature	Date

The Medical/Biomedical (MBM) Research Ethics Committee of the Centre intégré universitaire de santé et de services sociaux of West Central Montreal Health (CIUSSS WCMH) has approved and is responsible for the continuing ethical oversight of the study.

6.3.3 Appendix 3: Dietary/Medical History Questionnaire

QUESTIONNAIRE

		Insert check mark in the boxes which best represent your intake					
Food/Drink	**Serving Size**	**Never or Rarely**	**1-3 per month**	**1-3 per week**	**4-6 per week**	**1-2 times a day**	**3 or more times a day**
Coffee, regular	1 cup (250 mL)						
Coffee, decaffeinated	1 cup (250 mL)						
Tea, regular	1 cup (250 mL)						
Tea, decaffeinated	1 cup (250 mL)						
Carbonated Soda: e.g. Coke, Pepsi, 7UP, Sprite, Root Beer, Dr. Pepper	1 can (355 mL)						
Cruciferous Vegetables: e.g. Cauliflower, Broccoli, Cabbage, Bok Choy, Brussel Sprouts, Turnips, Radish	1 cup of vegetables						
Charbroiled, barbequed, or pan-fried meat: e.g. Chicken, Beef, Pork, Sausage, Lamb, Turkey	1 portion (3oz = about size of your palm)						
Red Wine	1 glass (5oz/ 150ml)						
Beer	1 bottle/can(12oz /355 ml)						
Spirits/Liquor	1 shot (1.5 oz/ 44ml)						
Garlic cloves	1 serving						
Grapefruit / Seville oranges	1 medium-sized orange						
Jufeng Grape Juice	1 cup (250 mL)						
Liquorice Candy	1 medium-sized pack of candy						
Indian food	1 serving						
Ground black pepper (for seasoning/taste in a meal)	-						

1

Smoking

Do you smoke (please circle one)? (Y / N)

How many cigarettes do you smoke in a day?

_____

Medical History:
Please list any current chronic medical problems or conditions that you have. If none, please write 'none.'

Complementary Natural Health Products:
Please list any complementary or natural health products that you take (example: curcumin, herbal products, vitamins, supplements, spices, etc.).

Name of Product	Amount of Product Taken	How Often Is It Taken

Current Medications:
Please list any medications you are currently taking, whether prescription or over the counter (Please remember to include any hormonal treatments, birth control, etc.).

Name of Medication	Dose of Medication	How Often Is It Taken?	How Do You Take it?

Time of first blood test: _____

Time of second blood test: _____

Height: _____

Weight: _____