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Leveraging the Tools and Techniques of Precision Medicine to Better Understand the Biological Underpinnings of Psychiatric Disorders and Psychotropic Treatment Effects

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Leveraging the Tools and Techniques of Precision Medicine to Better Understand the Biological Underpinnings of Psychiatric Disorders and Psychotropic Treatment Effects

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Chemistry

by

Lauren Catherine Seaman

2019

ABSTRACT OF THE DISSERTATION

Leveraging the Tools and Techniques of Precision Medicine
to Better Understand the Biological Underpinnings of
Psychiatric Disorders and Psychotropic Treatment Effects

by

Lauren Catherine Seaman
Doctor of Philosophy in Chemistry
University of California, Los Angeles, 2019
Professor William M. Gelbart, Chair
Professor Erika Lynn Nurmi, Co-Chair

The dawn of a new era of medicine has begun as clinicians and researchers shift their focus to more individual-centric diagnostic, treatment, and disease management strategies. Precision medicine is a multidisciplinary approach to human health care that takes into account a person's genetic makeup, behaviors, and environmental factors when evaluating pathophysiology, tailoring treatments, and designing novel therapeutic moieties. In this dissertation I break down the critical subfields of this discipline to explain and apply the emerging tools and techniques we now have at our disposal to better understand the underlying biology of complex human psychiatric disorders.

We begin with pharmacokinetics and pharmacodynamics, two branches of precision medicine that are involved directly with the temporal dynamics of pharmaceutical therapies and aid in disentanglement of how the body processes drugs versus how the drugs affect our bodies. I discuss detailed research across three separate drugs; risperidone, methamphetamine, and nicotine, integrating quantitative metabolic studies, genetic assessment, neuroimaging, and receptor analysis to clearly define inter-patient variability in risk and response.

Following this is work I accomplished in the realm of gene and environment interactions in young children experiencing anxiety disorders, which over time, led to what I hold as my largest contribution to the personalized medicine field; microbiome and host interactions. I am attempting to unlock a more direct, biological mechanism to something known as antipsychotic-induced weight gain (AIWG) through the examination of bile acids and the gut microbiome and their crosstalk and interplay with host physiology. Results are abundant throughout this document and each study presented here within brings a unique piece of the precision medicine puzzle to the table.

Pharmacokinetics, pharmacodynamics, genomic technology, gene-environment interactions, and the host-microbiome axis are the salient concepts in my toolbox of personalized medicine techniques that I believe can be leveraged in a variety of combinations to accomplish large goals in the medical and biotechnology fields. Whether it be through careful patient assessment with companion diagnostics, proper medication selection based on risk vs. reward value in harmony with an individual’s personal makeup, perseverance of high level disease progression and treatment monitoring, or even one day tailoring drug discovery to the highly specific receptors and biological pathways involved in these grievous diseases, it is clear precision medicine will pave the way for a better life for many people in the future.

The dissertation of Lauren Catherine Seaman is approved.

Erika Lynn Nurmi

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William M. Gelbart, Committee Chair

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2019

DEDICATION

It is my in my warmest regard that I dedicate this work to every single person that has crossed my path during my time in graduate school. Every soul has affected me in some way, and my life would not be what it is today without the support, love, kindness, challenges, pain, and beautiful happiness that people have shown me.

Specifically, I would like to thank my parents. If my Dad hadn't helped me save a dying tree sapling on the side of the road when I was just 3 years old, I'm not sure I would be here today, still striving to make our world a better place. If my Mother hadn't shown me what true strength and intelligence in a woman looks like, coupled with a penchant for books of all kind, I might not have made it to the point of writing this dissertation at all! Their unwavering love has propelled me to this point in my journey, and I am grateful every single day.

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Chapter 2 allowed me to work with wonderfully intelligent people in the fields of drug addiction, autism, and pharmacokinetics. I would like to thank Dr. Andy Dean for his extensive support and role as the project PI in the research with CYP2D6 and methamphetamine usage. Additionally, I would like to thank Dr. James McCracken as project PI for the Risperidone Pharmacokinetics research, as well as the RUPP Autism Network. Case study #2 is unpublished work currently, and all the authors are recognized in this chapter as such.

Chapter 3 presented the opportunity to collaborate with Dr. Arthur Brody, the lead PI on both PD case studies 1 and 2. Case study one is published in the journal Neuropsychopharmacology (doi: 10.1038/npp.2017.48.), which has granted copyright permissions for use in dissertations. Case study two is published in the journal Psychopharmacology (doi: 10.1007/s00213-018-5077-3), which also grants copyright permissions for use in dissertations. The remaining authors who helped with this work and contributed to these two publications are listed in the chapter.

Within chapter 4 I would like to acknowledge the guidance of Dr. Arthur body once more and Dr. Erika Nurmi as the two head researchers of the first case study. In the second case study I

received an enormous amount of support from Dr. Edythe London as she was the project head, and an expert on substance use disorder. I am grateful to the additional researchers who worked on these projects alongside me as well.

Delving deeper into how our genes and environment interact would not have been possible without the mentorship and ideas of Dr. Jessica Borelli who was the lead PI on both case studies in chapter 5 in which I helped with extensive genetic analysis. I would like to thank the additional authors of both of these works. GxE case study 1 was published in Behavioral Brain Research (doi: 10.1016/j.bbr.2016.07.035.) which allows for personal use distribution of copyrighted materials, which included dissertations. GxE case study 2 was published in Research in Development Disabilities (doi: 10.1016/j.ridd.2018.03.002) which has the same copyright permissions, allowing me to reproduce both of these works here.

Finally, in Chapter 6, I included two works that are my own personal accomplishments in terms of idea development and implementation. However, it is worth nothing that I would like to specifically express gratitude to Dr. Kym Faull for his expert guidance on the mass spectrometry method and Dr. Erika Nurmi for her support in the development of my final dissertation project. They taught me invaluable knowledge and helped propel my scientific career. Both works are still currently unpublished.

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Chapter 1

An Introduction and Overview of the World of Personalized Medicine

Personalized medicine—two simple words with a hidden universe of meaning, potential, and hope. It takes other names, such as precision medicine, stratified medicine, or theranostics, but in the end, all represent the same overarching goal; to tailor medical decisions, pharmaceutical interventions, and even long-term care and disease management to each unique individual. While this is not a new philosophy, as Hippocrates once said, “It is more important to know what sort of person has a disease than to know what sort of disease a person has”, the rapid advancement of diagnostic and informatic technologies has pushed the field back into the limelight of the medical world. These novel approaches revolve around understanding the molecular hierarchy of disease, specifically focusing broad scope genomics, and the development of the personalized medicine concept was first coined in this context but continues to expand to encompass many specific measures. In fact, over the last 10 years, the focus of the medical treatment world has shifted from “reaction” to “prevention” and can be clearly observed in the 870% increase of personalized medicine drugs, treatments and diagnostics products available in the market over this time period.[1]

This increased momentum is most dominant in the growing biotech field, and drug development pipelines are full of targeted treatments; 42% of all compounds have the potential to be personalized medicines.[2] While effective and tailored drug molecules are the pot of gold at the end of the rainbow, we must first understand how these substances address the issue of inter-patient variability. What better place to start than the most basic building blocks of life – our DNA? As every single human being differs to some extent in their genetic sequence, or

genome, it stands to reason that this avenue would be critical in evaluating as how we respond to treatment at both the population and individual levels.[3] Since successful completion of the Human Genome Project in 2003, which encompassed complete nucleotide base pair sequencing as well as phenotypic linking, more than 1,800 genes directly related to disease have been discovered, characterized and can be targeted in condition-specific clinical testing.[4] Researchers have capitalized on this improved understanding of population history, and over the years, we have built on this genetic technology. Modern advances allow for further examination of a patient's fundamental biology and regulatory genome variation through RNA expression and protein synthesis, which can lead to downstream mechanistic insights for treatment planning and drug development.[5]

Since these concepts can be applied to transformative health care and are based on the complex dynamics of systems biology, it is necessary to first evaluate simple nucleotide genetic mutations and how they may be connected to certain diseases. One of the most invaluable tools used in precision diagnostics is the genome-wide association study (GWAS). These studies are based on the millions of single nucleotide polymorphisms (SNPs) and other genetic variations that humans display and asks if the allele of interest in one of these variants is found more frequently than expected in individuals with a certain disease.[6] These broad scope assays are vital, as common and complex diseases are influenced by multiple genes, and the likelihood of developing certain ailments is one of the most powerful predictive measures. In addition, an individual's genetic makeup partially influences how well or poorly they respond to certain pharmaceutical interventions, and therefore has the potential to entirely alter their course of treatment.

Interlacing a person's genetics with their treatment response has evolved into the field known as pharmacogenomics (PG), which will be discussed in detail in the Genomic Technology of Chapter 3. Briefly, since an individual's genetic information can be utilized in this approach, it aims to combat the typical "one size fits all" medication prescribing and dosing methods. The lynchpins of PG are the genes directly involved with drug metabolism, notably cytochrome P450s[7], VKORC1[8], and TPMT,[9] and their allelic variations are linked closely to how an individual will respond to an active compound or drug metabolite. Enzymatic structural changes caused by mutations in these genes can make a drug more or less effective in different individuals, and even minute alterations can drastically impact the safety of a pharmaceutical. These genetic markers display clinically important consequences in the average population, and are an invaluable tool being leveraged for personalized medicine.

While these genomic markers are a critical foundation of the personalized medicine field, there are a number of factors that must be tested and considered when treating individuals with any type of therapy. Companion analyses and diagnostics are the remaining slices of the personalized medicine pie and help researchers and medical professionals understand the underlying biology of specific diseases and conditions. Here we must define a new term, "theranostics", which is the effort to develop more specific and individualized therapies for various ailments, and to combine diagnostic and therapeutic capabilities into a single agent.[10] Briefly, theranostics takes advantage of both particles (x-ray, free radical, nanotechnology) and imaging/devices (MRI, ultrasounds, fluorescence microscopy) to provide point-of-care therapy delivery while being able to examine and evaluate the effects of a drug in real time. This approach to treatment can engender better prognoses for many morbid diseases in a wide range of patient subpopulations.

Based on the desired target, this conglomerate of strategies can be varied immensely, but typically include identifying unique biomarkers related to the disease of interest. These "biomarkers", or biological markers, refer to objective indications of a medical state that are observed from outside the patient and can be measured accurately and reproducibly.[11] These non-subjective and quantitative phenotypes related to illness are one of the most sought-after aspects of personalized medicine research, as they can offer unambiguous data to demonstrate whether interventions are effective, ineffective, safe, or unsafe. When these biomarkers are used as outcomes in drug trials, they can be considered surrogate endpoints for clinically meaningful results.[12] Additionally, biomarkers have started to be incorporated in preventative care, for example, pre-screening for lung[13] and breast cancer[14] related gene mutations and the protein production gene linked to Alzheimer's disease.[15] In all cases, discerning biomarker data, either epidemiological, therapeutic, and/or pathophysiological, allows for the improvement of the drug development process, in addition to a deeper understanding of the biological hierarchy of many grievous diseases.

With this arsenal of tools in place, we can finally discuss the current state of the field of personalized medicine, and how it is contributing directly to interventions in the biomedical realm. The first and foremost sector impacted by these scientific advancements is that of drug development, and as I briefly mentioned above, this industry has seen tremendous growth with the addition of personalized medicine tactics. Because of the heterogenous nature of most diseases, utilizing genomic information to combat one-sized-fits all drug therapies has quickly become one of the most effective strategies of targeted drug design. This shift has resulted in a boom in the biotechnology or "biotech" industry that involves living systems or organisms in the development of products and devices.[16] And while biotech is used in numerous other sectors such as food production, agriculture, and fuel, it's prevalence in medicine and pharmaceuticals

allows for implementation of novel genomic data and techniques. Large pharmaceutical focused biotech companies such as Amgen and Genetech boast missions of “curing grievous diseases” instead of just treating the symptoms, and believe these cures exists inside of our own unique biological traits.

Diseases such as cancer, Alzheimer’s, asthma, diabetes, heart disease, and HIV are all heavy hitters when it comes to human mortality, and in conjunction with their enigmatic pathological mechanisms, they become prime targets in the personalized medicine field. Identifying genetic determinates of these common diseases through GWAS can provide valuable knowledge about the architecture, even though the vast majority of disease-associated loci have yet to be biologically explained. A vanguard example of this personalized medicine technique is that of cancer genomics. A total of 1,762,450 new cases of cancer, along with 606,880 unique deaths from cancer are expected to occur in 2019 in the United States alone.[17] However, these fatalities are down over 27% since their peak in 1991, thanks to early detection diagnostics and patient tailored treatments. Appropriately coined “oncogenomics”, high through-put sequencing methods are used to characterize genes,[18] identify genome hybridization,[19] detect copy number variation in oligonucleotides,[20] perform karyotyping,[21] and reveal chromosomal breakpoints (BAC-end sequencing).[22] These technologies allow for a significantly better understanding of cancer, particularly in the realm of tumor heterogeneity, and can improve prognosis for thousands of individuals that may not find success in the treatments typically applied to the general population.

Cancer certainly isn’t the only field to benefit from the proliferation of the “omics” aspect of precision medicine, which takes groups of biological molecules and chemicals that inform us about the structure, function, and dynamics of an organism and aims to collectively characterize

and quantify these systems. The suffix “-ome” describes a totality in biology, and large areas like genome, the proteome, and the metabolome encompass a plethora of knowledge that unless broken down can be overwhelming for researchers to utilize in personalized and targeted project development. We’ve already covered the importance of personal genomics, but it is worth mentioning that other biochemical branches such as epigenomics, which is concerned with chemical modification on DNA due to environmental factors,[23] proteomics, the large-scale analysis of proteins and their structures and functions,[24] and metabolomics, that systematically evaluates the unique chemical fingerprints of metabolites[25] are helping shape the personalized medicine landscape. The ability of classify derangements that underlie disease is the future of comprehensive diagnosis, progression monitoring, and new therapeutic discovery.

With these base biological foundations, the potential of the field can truly grow. So finally, to wrap up the current horizon of this exciting research, we can exemplify this by discussing artificial intelligence (AI) in precision medicine. A true paradigm shift is occurring with the advancement of AI, machine learning, and the development of neural networks.[26] Patient information, such as vitals, clinical symptoms, laboratory test results, personality phenotypes, etc., is being collected on a near constant basis, providing millions of data that have the potential to be employed by both physicians and researchers. One can imagine it would require significant computer power and detailed algorithms to not only organize this valuable commodity, but also to parse it into meaningful scientific connections. The innovation of biostatistical methods and AI programs can improve the quality of patient care, enable cost-effective strategies both in healthcare and drug development, increase overall efficiency in prescriptions and testing predictions, and reduce readmission and mortality rates.[27] So while it is hard to say with confidence that AI is the final frontier, it is clear that there would be no personalized medicine without these technological wonders.

Unfortunately, with any wonderful area of science, there are challenges and pitfalls that while difficult, can also inspire us to push further into our understanding and advance the field in many aspects. Of course, as PM is practiced more widely and continues to grow, the main difficulty comes when dealing with regulations, patient privacy and confidentiality, and intellectual property rights, which will all be discussed in more detail in the final chapter of this thesis. However briefly, the Food and Drug Administration (FDA) in the United States is responsible for overseeing all pharmaceutical and chemically based therapeutic modalities, and in order to incorporate aspects such a genetic and biomarker information in clinical use and drug development, regulatory science standards need to be updated.[28] This is of vital importance to ensure quality control, accuracy, precision, and reliability in our endeavors to create a better standard of care for many diseases. And perhaps the most critical issue with the widespread implementation of PM is the protection of the patients it hopes to aid. Due to the unique nature of individualized genetic information, this poses a significant challenge in both managing the psychological effects patients might experience, as well as what constitutes personal health information for confidentiality purposes. As the discipline continues to evolve, we are responsible for addressing these concerns and implementing adequate tools in order to accelerate the adoption of stratified medicine to further aspects and fields of generalized medicine.

By now, you have most likely surmised that personalized medicine is a truly interdisciplinary area of research, bounding a variety of scientific fields and technologies. This is why I chose to explore the potential of a variety of individual aspects of PM in order to expand my knowledge as a scientist beyond just that of simply organic chemistry. I have always been conscious of our impact, as both scientists and humans, on the planet and its environment, and what we will be

able to both accomplish and leave behind to improve the quality of life of our species. As I began to pursue my graduate studies here at UCLA, I considered how my science might have real-world impact, and I quickly realized that my background in organic chemistry would provide a formidable foundation for translational research. Rapidly this crystallized into the realization that I would need to acquire a better understanding of a variety of biological sciences, and so I sought out mentorship from many talented individuals in a myriad of departments in order to nurse my desire to paint a complete picture of targeted medicine and drug design. I found myself in the laboratory of Dr. Erika Nurmi, a brilliant psychiatrist with a passion for helping others and advancing the fundamental science we use to do so. Through 6 years of intensive research and training, I believe I have created a cohesive story of how personalized medicine can be utilized to understand, treat, and change how we approach a variety of diseases. Let me briefly walk you through what that entailed, and what this dissertation will aim to convey.

Chapter 2 will set the stage in the realm of pharmacokinetics (PK) – which is succinctly defined as the time course/movement of a drug throughout the body, examining aspects of absorption, distribution, metabolism, and excretion. I was able to participate in research of two separate drugs and their pharmacokinetic properties, methamphetamine and risperidone, specifically leveraging a unique cytochrome p450 enzyme genotype to better understand deleterious side effects, and how they might be attenuated. Chapter 3 examines the reverse side of the PK coin, pharmacodynamics (PD), where now we are concerned with how a drug affects the body, instead of how the body affects a drug. Here I learned about neurobiology, and the consequences of cigarette smoking and nicotine on neurochemical balances due to brain structure and function. While PK and PD are the bread and butter for not only personalized medicine, but also targeted drug design, it is also important to consider genomic technology and within that pharmacogenetics (PG), and I do so in Chapter 4. PG is the branch of pharmacology that is concerned with the effects of genetic variation on reactions to pharmaceuticals, and here

I was able to bring a huge component of personal genomics to the table. Continuing this theme, we come to Chapter 5, which is all about the work I accomplished in the realm of gene and environment interactions, which over time, led to what I hold as my largest contribution to the personalized medicine field – Chapter 6; microbiome and host interactions. Here I spent years attempting to unlock a more direct, biological mechanism to something known as antipsychotic-induced weight gain (AIWG) through the examination of bile acids and the gut microbiome and their crosstalk and interplay with host physiology.

My story ends on a hopeful note; a putative mechanism of how this unwanted side effect of AIWG, which also has strong ties to cardiometabolic syndrome, one of the most profound diseases of our era, works on a biochemical level, and how we can leverage this information to better inform therapeutic treatment of various psychiatric diseases with not only antipsychotics, but other psychotropic drugs as well. I am currently running a replication study of a promising preliminary finding in this area that was recently funded by the NICHD, and I cannot express how excited I am for the potential my work has to impact the lives of so many people that may suffer from similar conditions. Each chapter represents a piece of the puzzle for my own personalized medicine story, and each unique situation taught me invaluable science and skills that I will utilize as I pursue my career as a scientist and research in this paramount field. I invite you to buckle up, strap in, and enjoy the rollercoaster ride that is the following PhD dissertation.

Chapter 2

Pharmacokinetics

General Background

In my quest to better understand and contribute to the growing field of personalized medicine, I worked on a variety of scientific projects in specific categories of this overarching concept. The roots were established first in the field of pharmacokinetics, a branch of pharmacology which is simply the study of drug absorption, distribution, metabolism, and excretion.[29] Stemming from the ancient Greek *pharmakon* meaning “drug” and *kinetikos* meaning “moving, putting in motion”, pharmacokinetics (PK) employs a conglomerate of techniques to determine the fate of a pharmaceutical substance once given to a living organism, and aims to understand how the organism itself affects this active drug compound. In combination with pharmacodynamics, which will we discuss in a later chapter, PK helps inform and influence medicinal dosing levels to maximize therapeutic benefits while minimizing adverse side effects.

One can imagine that the human body is a complex system, and a drug must undergo multiple steps and transformations as it is being liberated, absorbed, distributed, metabolized, and/or excreted (LADME). Liberation refers to the process of the release of the biologically active compound from the pharmaceutical formulation. Absorption is simply how that substance enters blood circulation, and distribution is the subsequent distribution or dissemination throughout the various organs, tissues, and bodily fluids. Metabolism, which can be referred to as biotransformation or inactivation as well, occurs when the organism recognizes the drug as a foreign entity and begins the irreversible transformation of the parent compound into chemically and structurally different metabolites. Lastly, there is excretion, where any leftover original drug

and its metabolites are removed from the body through avenues such as urine, feces, or perspiration.[30]

All the above phases are individually susceptible to physio-chemical interactions between the active drug substance and the organism in question. Both compartmental and non-compartmental mathematical modelling for these connections is used to express unique equations for a milieu of pharmacokinetic properties. Parameters such as dose, clearance, bioavailability, elimination half-life, concentration, and fluctuation are just a few of the vital measurements that are typically completed during the PK branch of modern drug development. In fact, PK is so crucial to avoid a potential compound's attrition before or during clinical trials, the industry has focused countless efforts into improving the understanding of these drug characteristics, and candidate failure in this branch has dropped to only 5% in recent years.[31] While lack of safety or efficacy are the most common causes for a drug getting the axe, attempting to overcome poor bioavailability or uncover predictive kinetic behavior can dictate the overall success of a program[32], making PK one of the first lines of defense against development failure.

Because of the importance of PK analysis in both drug development and clinical applications, it is worth discussing the main avenues of measurement for this subfield. Having the ability to develop strong associations between drug concentrations and their pharmacological responses allows both doctors and personalized medicine researchers to apply PK principles to patients on a case-by-case basis. A drug's active effect in the body is often directly related to its concentration in various peripherals or tissue. This is potential proxy of the concentration at inaccessible direct receptor binding sites, a concept known as kinetic homogeneity; as the concentration of a compound increases in plasma, serum, urine, or saliva, the concentration in

most tissues will increase proportionally.[33] With this information, we can construct a concentration vs. time profile for any given pharmaceutical, and hypersensitive bioanalytical methods are necessary for this purpose.

The gold standard analytical technique used in PK is mass spectrometry. While I will be discussing mass spectrometry and related methods in great detail later in chapter 6, a brief overview will help paint the picture of why it is such a critical component to PK in development settings. There are three essential steps in this type of measurement: ionization, separation, and mass detection.[34] First, individual molecules in a small sample are broken apart, or ionized, in the ion source of the machine where they typically become cations through the loss of an electron. Because of this, the ions can be sorted and separated according to their atomic mass and charge state. Commonly, this is accomplished through column chromatography with either gas or liquid as a mobile phase and can be tailored to the specific molecular properties of the items of interest. Finally, as the ions pass to the detector of a machine, which measures the relative abundance of each species, and this data can be analyzed further to parse apart critical information about the compound originally injected. The high selectivity and sensitivity of mass spectrometry allows for detection of xenobiotics in complex bodily matrices such as those mentioned above at very low concentrations with reliability.

An Introduction to Cytochrome P450

With a strong understanding of how pharmacokinetics is critical to multiple aspects of pharmaceuticals, I began to explore the application of these principals to different areas of personalized medicine. As previously discussed, there are 5 major areas of focus in PK, represented by LADME. When I began my graduate work, I had the opportunity to explore the metabolism piece of pharmacokinetics in a couple of various ways, while learning techniques and concepts that would help drive the story of how personalized medicine can shape our future endeavors. It is necessary to flesh out the discussion of metabolism in order to better understand how the upcoming studies function in their entirety. Metabolism, in the broadest definition of the word, is the sum of all chemical reactions that happen intercellularly in living organisms. To give a sense of how complex this collective of pathways is, Figure 2-1 demonstrates the core metabolic functions of just ONE eukaryotic cell.

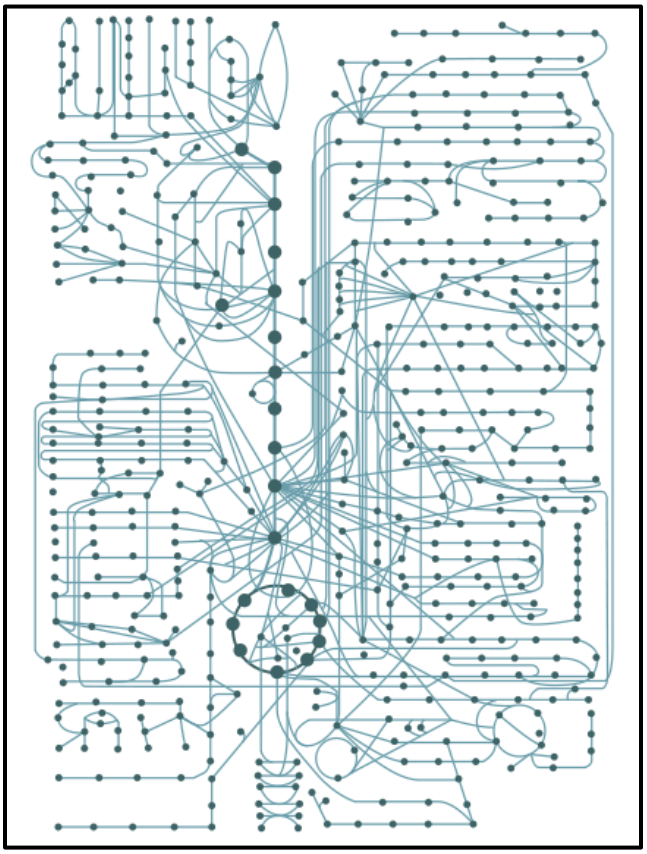

Figure 2-1. An artistic diagram of the core metabolic pathways in a eukaryotic cell, such as the cells that make up the human body. Each line is a reaction, and each circle is a reactant or product.

Image credit: "Metabolism diagram," by Zlir'a (public domain).

And in lieu of attempting to tackle explaining this entire miniature universe, I will focus on catabolic metabolism, as this relates to only the reactions involved in breaking down compounds, providing the strongest foundation for what happens to a drug once it enters the body. Xenobiotics, or chemical moieties found within an organism that are not naturally produced by that organism, are quickly targeted by catabolic processes, mainly located in the

liver. Many of these molecular transformations that occur within cells require multiple steps, energy, and enzymes to accomplish.[35] Ingested or injected drugs are either activated (or inactivated) by these hepatic enzymes to form metabolites, which are then conjugated and excreted in either bile or urine.[36] Commonly, this occurs in an enzyme complex called the microsomal biotransformation, of which the main component is a heme containing cytochrome monooxygenase, also known as cytochrome P450's. (CYPs)

CYP proteins/enzymes are predominantly oxidization catalysts and membrane associated in nature and add one oxygen atom to a variety of small and large molecule substrates. In humans, the CYP family accounts for 75% of total drug metabolism, transforming thousands of compounds that act as substrates in a variety of tissues throughout the body.[37] This is in part because the active site of a cytochrome P450 contains a heme-iron center that is tethered directly to the protein via cysteine-thiolate flanking residues[38], allowing for a wide array of reactions to undergo catalysis. These CYP enzymes are present in most tissues of the human body, and additionally play critical roles in the breakdown of endogenous chemicals. There are 57 human genes divided among 18 families of cytochrome p450 genes and the proteins they encode, making these loci prime functional targets for genetic evaluation and penetrance understanding.[39] In order to avoid lengthy explanation of these salient metabolic tools, the CYP families are listed in detail in Table 2-1.

Family	Members	Names
CYP1	3 subfamilies, 3 genes, 1 pseudogene	CYP1A1, CYP1A2, CYP1B1
CYP2	13 subfamilies, 16 genes, 16 pseudogenes	CYP2A6, CYP2A7, CYP2A13, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2F1, CYP2J2, CYP2R1, CYP2S1, CYP2U1, CYP2W1
CYP3	1 subfamily, 4 genes, 2 pseudogenes	CYP3A4, CYP3A5, CYP3A7, CYP3A43
CYP4	6 subfamilies, 12 genes, 10pseudogenes	CYP4A11, CYP4A22, CYP4B1, CYP4F2, CYP4F3, CYP4F8, CYP4F11, CYP4F12, CYP4F22, CYP4V2, CYP4X1, CYP4Z1
CYP5	1 subfamily, 1 gene	CYP5A1
CYP7	2 subfamilies, 2 genes	CYP7A1, CYP7B1
CYP8	2 subfamilies, 2 genes	CYP8A1, CYP8B1
CYP11	2 subfamilies, 3 genes	CYP11A1, CYP11B1, CYP11B2
CYP17	1 subfamily, 1 gene	CYP17A1
CYP19	1 subfamily, 1 gene	CYP19A1
CYP20	1 subfamily, 1 gene	CYP20A1
CYP21	2 subfamilies, 1 gene, 1 pseudogene	CYP21A2
CYP24	1 subfamily, 1 gene	CYP24A1
CYP26	3 subfamilies, 3 genes	CYP26A1, CYP26B1, CYP26C1
CYP27	3 subfamilies, 3 genes	CYP27A1, CYP27B1, CYP27C1
CYP39	1 subfamily, 1 gene	CYP39A1
CYP46	1 subfamily, 1 gene	CYP46A1
CYP51	1 subfamily, 1 gene, 3 pseudogenes	CYP51A1

Table 2-1. Humans have 57 genes and more than 59 pseudogenes divided among 18 families of cytochrome P450 genes and 43 subfamilies. This is a summary of the genes and of the proteins they encode. (http://drnelson.uthsc.edu/human.P450.table.html)

Pharmacokinetic Metabolism and *CYP2D6*

And while some CYPs metabolize very few, or sometimes just even one, substrates, both *CYP3A4* and *CYP2D6* account for over 34% and 25% of drug deactivation and bioactivation respectively.[40] These two enzymes can be both inhibited or induced by pharmaceuticals, resulting in clinically significant drug-drug interactions that may results in unanticipated adverse reactions, but also potentially beneficial therapeutic outcomes. Because of this, it is of critical importance to understand the genetic variability at these loci and how this variance functionally impacts individual's abilities to metabolize certain drugs; a strong

foundation of pharmacokinetics and pharmacogenetics as a whole. The research opportunities I encountered in my early years of graduate school allowed me to dive into the world of *CYP2D6* specifically, and it is important to understand the basic science and genetics behind perhaps the most well-characterized metabolic enzyme.

Serendipitously, *CYP2D6* is primarily expressed in the liver, which is the central hub for all drug processing and excretion in the human body, but also has a strong presence in areas of the central nervous system[41], allowing for pivotal drug-host interactions. Biochemically, *CYP2D6* act through the mechanism of hydroxylation, demethylation, and dealkylation of specific functional groups.[42] But what makes this enzyme intriguing is the fact that *CYP2D6* is characterized by a high inter-individual variability in catalytic activity mainly caused by genetic polymorphisms[43] and copy number variation.[44] Appropriately named, the *CYP2D6* allele is what mediates these deviations in phenotypes, and individuals possessing certain allelic variants will manifest normal, decreased, absent, or excessive *CYP2D6* function.[45] There are four distinct metabolizer classes, and their nomenclature, phenotypes and related genotypes can be seen in Table 2-2. These denominations will be used throughout the two case studies in this chapter.

The second year of my graduate research career, I had the opportunity to be a part of a project examining the effects of

Table 2-2. The four metabolizer status classifications for the CYP2D6 gene and related enzyme. Genotype at this locus and functionally related phenotype are also displayed for each variation. Enzymatic activity directly impacts drug metabolism rate.

Classification	Genotype	Phenotype
Ultrarapid Metabolizer (UM)	3 or more fully functioning genes (CNV)	Increased enzymatic activity
Extensive Metabolizer (EM)	2 wild type, fully function genes	Normal activity
Intermediate Metabolizer (IM)	1 wild type allele, 1 non-functioning/defective allele	Decreased activity
Poor Metabolizer (PM)	2 non-functioning alleles/1 or more deleted gene	No enzymatic activity

methamphetamine (MA) usage on neurodegeneration. *CYP2D6* is selectively responsible for the metabolism of MA, thus individuals with genetic variation and therefore altered metabolizer classes may experience differing responses to the drug as well as to the deleterious side effects. Detailed information and findings from this work can be seen in the upcoming PK case study #1. Additionally, I was also involved in a pharmacokinetic project aimed at autistic youth in my 3rd year as a graduate student. Here we were interested in whether or not *CYP2D6* genotype had an effect on these patient's response to a second-generation antipsychotic, risperidone, metabolized by this enzyme. This study also added *CYP3A4*/5 analysis into the mix to paint a more complete PK picture. More background and results of this research can be found in PK case study #2. Overall, I feel these two studies provided me with a robust background on pharmacokinetics, and allowed me to begin to truly apply these principles to my overarching goal of a better understanding of personalized medicine.

Case Study #1: *CYP2D6*, brain structure, and cognitive function in methamphetamine dependence*

A Dean, LC Seaman, ED London, EL Nurmi

**This work is currently unpublished.*

Background / Specific Research Aims

Methamphetamine (MA) use disorder is a highly prevalent public health problem, both nationally and worldwide. Cognitive dysfunction is a common sequela of MA use; however, individual susceptibility to cognitive impairment is highly variable and the mechanism of degeneration is largely unknown. Metabolism of MA is dependent on the cytochrome p450 2D6 (*CYP2D6*) enzyme, which is inactivated by common polymorphisms. Previous research has found that *CYP2D6* extensive metabolizers (EMs), with two wild-type or functional alleles, have higher rates of cognitive impairment than intermediate/poor metabolizers (IM/PMs) with at least one

non-functional allele. We hypothesized that this may result from the elevated exposure of EMs to toxic metabolic byproducts. To evaluate this hypothesis, we examined whether EMs, relative to IM/PMs, display other markers of neurotoxicity such as differences in cerebral gray matter volume.

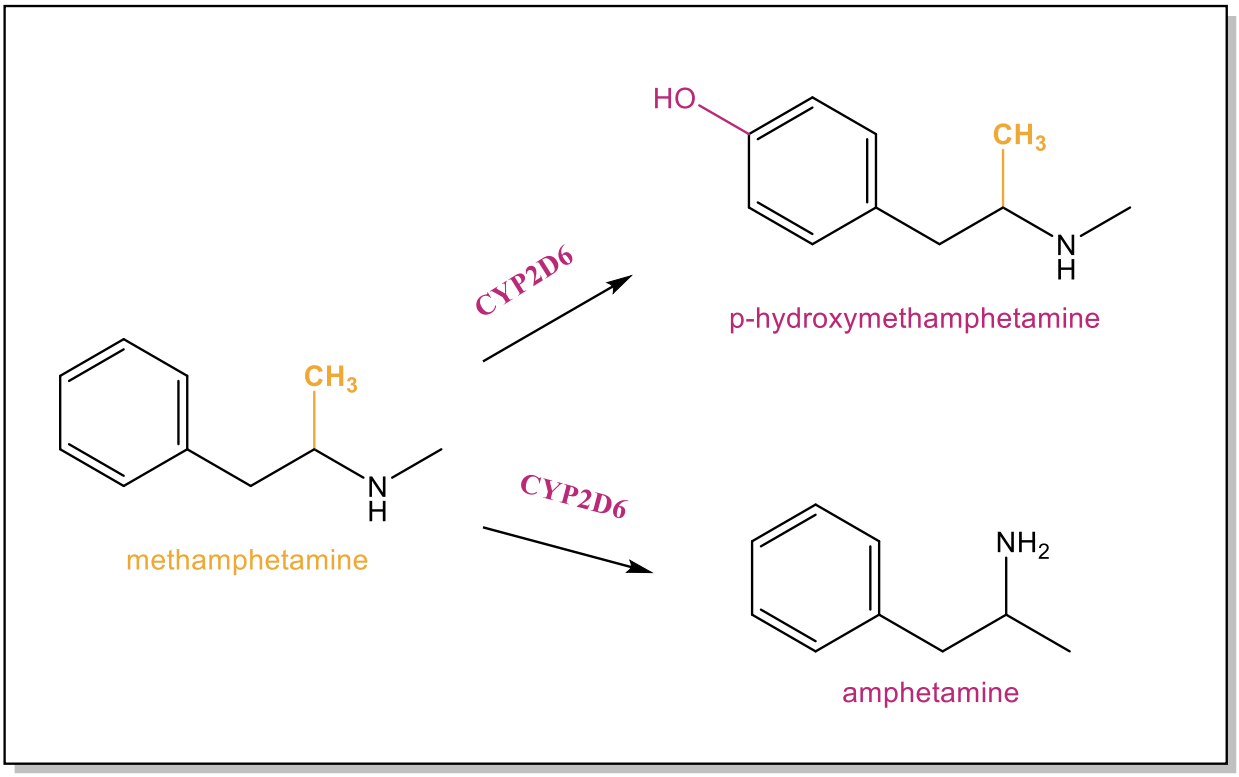

Figure 2-2. Methamphetamine is metabolized by the liver enzyme CP2D6. The drug can either undergo hydroxylation to form p-hydroxymethamphetamine or dealkylation to form amphetamine. These represent the two major metabolites.

This project sought to evaluate whether methamphetamine-dependent participants with different variants of the cytochrome P450 gene (cytochrome P450, family 2, subfamily D, polypeptide 6; *CYP2D6*) exhibit differential rates of cognitive impairment and abnormalities in brain structure. The *CYP2D6* gene codes for one of the primary enzymes responsible for methamphetamine metabolism. Depending on the functionality of inherited *CYP2D6* alleles, individuals vary in how readily they metabolize methamphetamine, ranging from poor (two non-functional alleles) to

extensive metabolism (two functional alleles). Previous research has found that *CYP2D6* extensive metabolizers have higher rates of cognitive impairment than intermediate/poor metabolizers.[46] The authors hypothesized that, because extensive metabolizers are exposed to a greater load of metabolic byproducts (e.g., amphetamine, 4-hydroxymethamphetamine) than intermediate/poor metabolizers, metabolic byproducts may be more neurotoxic than the parent methamphetamine compound. Our project sought to further evaluate this hypothesis by examining whether extensive metabolizers display other markers of neurotoxicity relative to intermediate/poor metabolizers; namely, abnormalities in cerebral gray matter volume and white matter integrity. At the time of the grant submission, we already had structural MRI and *CYP2D6* genotype on 57 methamphetamine-dependent subjects; with this R21, we sought to add 24 more methamphetamine-dependent subjects to this dataset to evaluate hypotheses. We hypothesized that: 1) methamphetamine-dependent participants who are extensive metabolizers (based on *CYP2D6* genotype) would have significantly lower cognitive scores than intermediate/poor metabolizers (but not significantly different estimates of premorbid IQ); and 2) relative to intermediate/poor metabolizers, extensive metabolizers would have alterations in gray matter volume and white matter integrity which have previously been hypothesized to be associated with neurotoxicity (i.e., reduced cortical gray matter and reduced fractional anisotropy in the frontal lobe).

General Methods

Subjects:

Otherwise healthy, adult (18-55 years old) methamphetamine-dependent individuals, n=86. Inclusion criteria: DSM-IV-TR MA dependence by Structured Clinical Interview. Must test positive for MA metabolite in urinalysis during first screen. Caucasian or Hispanic. Exclusion

criteria: History of Axis I psychiatric disorder other than MA dependence or nicotine dependence (due to high co-morbidity) or evidence of a serious neurological or medical condition.

Imaging:

Structural MRI was acquired on the testing day using a Siemens Sonata 1.5 tesla system with a standard 32-channel phased array head coil. A high-resolution whole-brain T1-weighted anatomical sequence (MP-RAGE) was collected for spatial normalization and voxel-based morphometry. Diffusion tensor imaging (DTI) data was obtained using 30 gradient directions at b=1000, 5 repeats of b=0 (no diffusion weighting), with 2.5x2.5x2.5 mm resolution. Scanning time took approximately 30 minutes.

Neuropsychological Assessment:

The neuropsychological battery shown in Table 3 was completed on the testing day after participants sustained 5 days of abstinence. This collection of tests was designed to tap multiple cognitive domains, with an emphasis on tests of executive function, memory and processing speed. Inclusion of normative data for performance comparison purposes. Assessment took approximately 2.5 hours to complete.

NEUROPSYCHOLOGICAL BATTERY	DOMAIN
COGNITIVE TESTS	
Wechsler Test of Adult Reading	Premorbid IQ estimation
Rey Auditory Verbal Learning Test	Verbal learning and memory
Brief Visuospatial Memory Test-Revised	Visual learning and memory
Continuous Performance Test – 2	Visual attention/inhibitory control
Stroop Test	Executive functioning/inhibitory control
Finger Tapping Test	Motor speed
Verbal Fluency	Language and executive functioning
Wisconsin Card Sorting Test	Executive functioning/concept formation
Trailmaking Test	Psychomotor speed and set-shifting
Victoria Symptom Validity Test	Effort/motivation
INVENTORIES	
Time/date last drug use	Recency of drug, cigarette & caffeine use
Alcohol breathalyzer	Exclude alcohol intoxication
Stanford Sleepiness Scale	Alertness
Beck Depression Inventory-II	Depressive symptoms
Amphetamine Cessation Symptom Assessment Scale	Meth withdrawal and craving
Positive and Negative Affect Schedule	Emotional States

Table 2-3. Neuropsychological battery of tests utilized in the reported MA study.

Genetic Testing:

Blood samples were collected, and genomic DNA was extracted. The two most common variants in Caucasian and Hispanic populations account for 94% of the genetic variation in *CYP2D6*, rs1065852 and rs3892097. TaqMan SNP genotyping assays were used to genotype these loci in all individuals with 93.69% sensitivity, >99% specificity, and >99% positive predict value.

Statistical Analyses:

No significant differences in age, sex, ethnicity, level of education, or smoking were observed between the EM and IM/PM groups. Differences in performance on cognitive measures were assessed by t-test (one-tailed) for equivalence of the means.

Studies & Results

During the period of this grant, we completed assessments on 29 methamphetamine-dependent participants. The participants received a 1.5 T structural MRI scan, provided a blood sample for *CYP2D6* genotyping and were administered a comprehensive neurocognitive battery. Combined with existing data from former participants, we now have *CYP2D6* genotype and structural MRI from 86 methamphetamine-dependent subjects (extensive metabolizers n = 60; intermediate/poor metabolizers n = 26), with smaller subsets having been administered various cognitive tests.

The extensive metabolizer and intermediate/poor metabolizer subjects are generally well-matched on demographic variables. The two groups do not differ with respect to age, gender, education, premorbid IQ (Shipley Vocabulary Test), or ethnicity (p's > .05). Further, the extensive and intermediate/poor metabolizers do not significantly differ on measures of methamphetamine and nicotine use, including years of heavy methamphetamine use, age of onset of methamphetamine use, days of methamphetamine used in the last 30 days, grams of methamphetamine used in the last week, cigarette smoker status (yes/no) or pack years of cigarette smoking (p's > .05).

Because of unavailable data in former participants, only a subset of the subjects have been administered cognitive tests, with the largest subsets having been administered the Stop Signal Test (SST; extensive metabolizers n = 47; intermediate/poor n = 23) and the Wisconsin Card

Sorting Test (WCST; extensive metabolizers n = 44; intermediate/poor n = 18). The extensive metabolizers had a significantly slower go reaction time on the SST than intermediate/poor metabolizers (t(68) = -2.016; p = .048); however, the two groups did not differ in stop signal reaction time (SSRT, p > .05). On the WCST, relative to intermediate/poor metabolizers, the extensive metabolizers had a higher percentage of perseverative errors (t(60) = 1.745; p = 0.017), shown in Figure 2-3. Other WCST indices did not significantly differ between the groups (p's > .10).

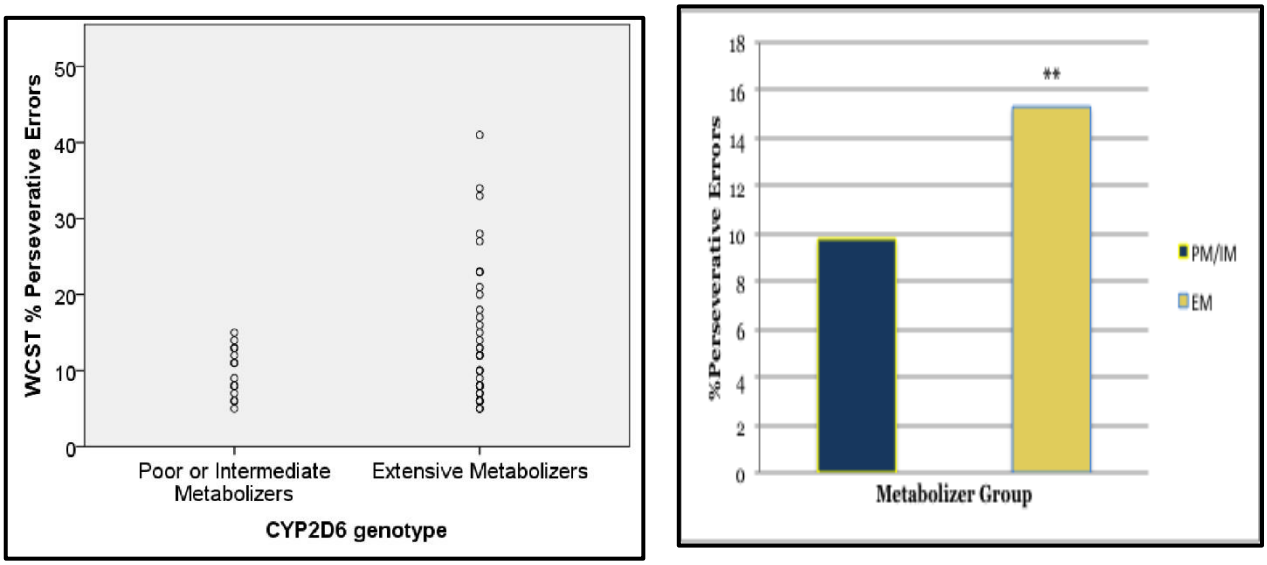

Figure 2-3. MA users who are CYP2D6 extensive (EM) vs. intermediate/poor (PM/IM) metabolizers show increased perseverative errors on the WCST.
Utilizing the Wisconsin Card Sorting Task (WCST) as a measure of cognitive ability, we observed a significant and marked increase of percentage of perseverative errors in the EM participants, almost double that seen in PM/IM individuals. (p=0.014, Cohen's d effect size = 1.4) This data supports our prediction of greater cognitive decline in EM individuals, due to the increase of toxic MA metabolism byproducts that are reaching the brain.

We next compared the extensive and intermediate/poor metabolizer groups on measures of cerebral gray matter volume. Images were preprocessed (segmented, modulated and

smoothed) using the voxel-based morphometry (VBM) toolbox in SPM8. Statistical analyses were conducted using FSL's Randomise (http://www.fmrib.ox.ac.uk/fsl/randomise, version 5.1) to implement voxelwise permutation-based nonparametric inference for ANCOVA with age and gender as covariates. Given the association of WCST performance with frontal lobe integrity in meta-analytic data[47], statistical models were applied to data in an explicit mask of gray matter in the frontal lobe based on an atlas rendered in Montreal Neurological Institute (MNI) 152 space (see Figure 2-4).[48, 49] Threshold-free cluster enhancement [50] was used to correct for multiple comparisons, and a statistical threshold of $p < 0.05$, corrected for the entire search volume, was applied to each of the resulting statistical maps.

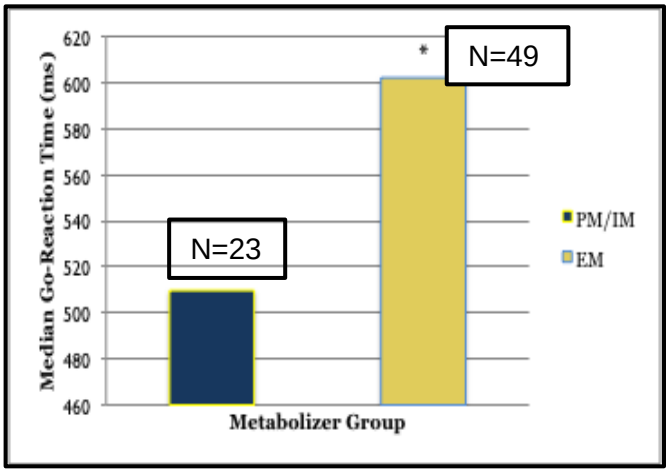

Figure 2-4. MA users who are extensive (EM) vs. poor/intermediate (PM/IM) metabolizers show an increased median reaction time on the of SST, as measured by go-reaction response. Consistent with our predictions, the significant increase in go-reaction time displayed by EM individuals on the Stop Signal Task (SST) ($p=0.036$, Cohen's d effect size = 2.0) reflects dysfunction in response inhibition and impulsive action. Speculatively, because the SST has a mix of stop and go trials, a delayed reaction time on a go trial would indicate a difficulty in adaptation and executive functioning, potentially due to neurotoxic damage in the prefrontal cortex.

Results revealed that, relative to intermediate/poor metabolizers, the extensive metabolizers had less gray matter volume in the right cingulate/paracingulate gyri (see Figure 2-5). In contrast, there were no regions in which the

extensive metabolizers had greater gray matter volume than the intermediate/poor metabolizers.

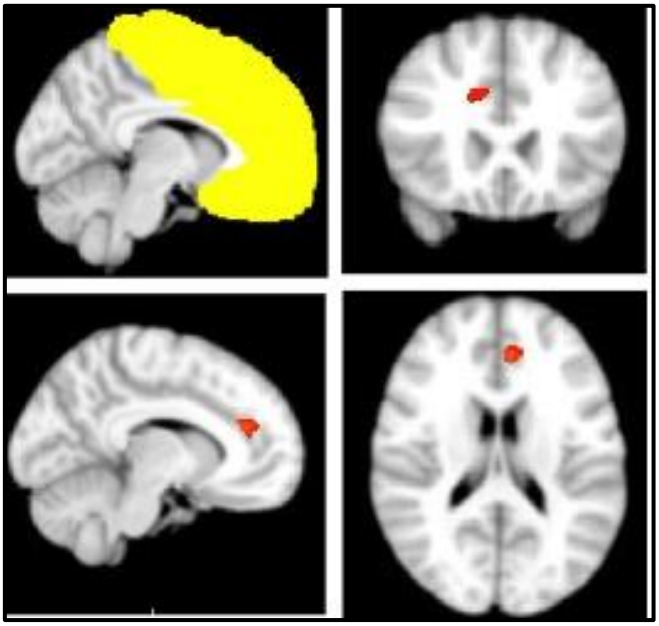

Figure 2-5. MA users who are extensive (EM) vs. poor/intermediate metabolizers (PM/IM) display decreased cerebral gray matter volume. EMs (n=57) exhibited reduced gray matter volume in the right anterior cingulate gyrus relative to IM/PMs (n=26) by threshhold free cluster enhancement ($p<0.05$, corrected) controlled for age and gender. Sagittal slice (in yellow) in the upper left corner shows the frontal lobe mask used as a region of interest. There were no brain regions where EM's displayed higher grey matter than PM/IM's. These findings support our hypothesis that the metabolized products of MA, amphetamine and 4-hydroxymethamphetamine, are more toxic to neuronal cells than the parent compound.

Lastly, group differences in estimates of white matter integrity (i.e., fractional anisotropy; FA) were evaluated using Diffusion Tensor Imaging (DTI) data processed and analyzed in FSL. Analyses were conducted using tract-based spatial statistics (TBSS) within a thresholded FA "skeleton" of white matter tracts. Voxel-wise statistics were conducted within the FA skeleton, using threshold-free cluster enhancement and permutation testing in FSL's Randomise. Age and gender were included as covariates of no interest.

At corrected thresholds, the extensive and intermediate/poor metabolizers did not exhibit differences in FA, regardless of contrast selection (i.e., extensive > intermediate/poor or intermediate/poor > extensive). At reduced statistical levels ($p < .15$, corrected), the most appreciable differences suggested that extensive metabolizers have greater FA than intermediate/poor metabolizers in regions of the corpus callosum, anterior commissure, and left

cerebral white matter (Figure 2-6). However, these differences were not observed at traditional levels of significance.

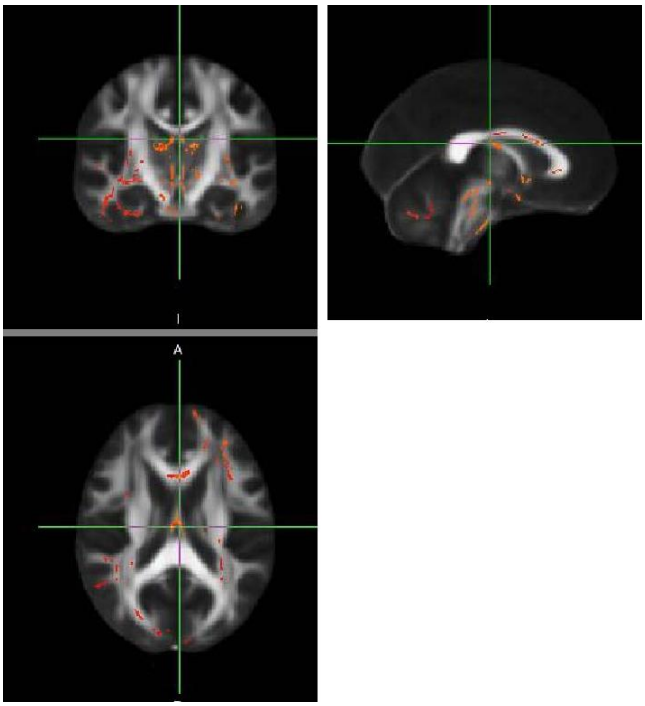

Figure 2-6. Observed changes in fractional anisotropy between extensive (EM) and intermediate/poor (IM/PM) metabolizers.
IM/PMs (n=23) tended to have lower fractional anisotropy (FA), a measure of white matter integrity, than EMs (n=39) at reduced statistical thresholds ($p<0.15$, corrected) in regions of the corpus callosum, anterior commissure, and cerebral white matter. While it might be contrary to initial hypotheses, when taken together with our results above, this finding suggests that FA scores may not be indicative of neurotoxicity and that low FA itself may not reflect neuronal damage or cognitive disability.

Significance & Conclusions

The present data provide some support for the hypothesis that, based on *CYP2D6* genotype, extensive metabolizers are more susceptible to methamphetamine-induced neurotoxic effects than intermediate/poor metabolizers. Specifically, relative to intermediate/poor metabolizers, extensive metabolizers exhibited worse performance on a test of executive function (Wisconsin Card Sorting Test perseverative errors, $p = 0.017$) and had less gray matter volume in the right cingulate/paracingulate gyri ($p<.05$, corrected). Since extensive metabolizers have a higher degree of exposure to the metabolic byproducts of methamphetamine (e.g., amphetamine and 4-hydroxymethamphetamine)[51] than intermediate/poor metabolizers (extensive metabolizers readily metabolize methamphetamine while intermediate/poor metabolizers are more likely to excrete methamphetamine unchanged), these data may suggest that the metabolic byproducts

are more toxic than the parent compound. This is consistent with *in vivo* studies showing that 4-hydroxymethamphetamine is more toxic to rat neuronal cells than methamphetamine. [52] Should these data be replicated, possible harm-reduction strategies could be explored to reduce the toxicity of methamphetamine for users who are engaged in the early stages of behavioral treatment (e.g., administration of *CYP2D6* inhibitors).

There were no significant differences between extensive and intermediate/poor metabolizers on a measure of white matter integrity (fractional anisotropy; FA). Nonsignificant trends in some regions were contrary to hypotheses; extensive metabolizers exhibited higher FA than intermediate/poor metabolizers. Although this may suggest that FA scores were not indicative of neurotoxicity, it should be noted that low FA may not be synonymous with neuronal damage. For example, cigarette smokers have been shown to have *higher* FA than nonsmokers in several white matter tracts.[53] Likewise, increased FA is associated with attention- deficit hyperactivity disorder (ADHD;)[54] and visuospatial deficits in Williams syndrome.[55] In HIV+ individuals, both decreased and increased FA have been found relative to healthy control subjects, depending on the specific brain region.[56] As such, current nonsignificant findings in FA may not indicate the absence of neurotoxicity.

Our data strongly support the hypothesis that *CYP2D6* extensive metabolizers are more vulnerable to methamphetamine-induced neurotoxic effects than those with impaired enzymatic activity, resulting from common gene polymorphisms. This could be due to the fact that MA metabolites are more toxic to the underlying neurocircuitry and neuronal cells than the parent compound, and future toxicity studies can now address this issue.

We were unable to test for gene deletions or duplications with our methods, which decreases the

sensitivity of our test, so that it is possible that a small number of IM/PM individuals may have been missed or misclassified. While our pilot study was not powered to support correction for multiple testing for 10 cognitive measures, effect sizes are large and warrant replication in a larger population.

However, by demonstrating an association between *CYP2D6* genotype and cognitive decline and brain abnormalities, we propose an identifiable risk factor for cognitive dysfunction in MA dependence. Subsequently, this risk factor has the potential to be utilized by clinicians for both early intervention and individualized treatment planning, while also aiding in the development of harm reduction strategies (i.e., the use of *CYP2D6* inhibitors) to prevent cognitive damage related to MA relapse while individuals are in treatment.

Case Study #2: Genetic Determinants of Risperidone Pharmacokinetics in Children with Autism Spectrum Disorder (ASD): Relationship to Treatment Outcomes and Side Effects*

**This paper is currently submitted for publication.*

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INTRODUCTION

The antipsychotic risperidone (RSP) is safe and effective for the acute and intermediate-term treatment of severe and challenging behaviors such as aggression, self-injury, and extreme mood lability that are commonly associated with autism spectrum disorder.[57, 58] Individual treatment benefit and adverse events, such as antipsychotic-induced weight gain (AIWG), however, are highly variable. Identifying predictors of therapeutic response and adverse event risk could guide treatment selection and may reveal fundamental mechanisms for prevention or treatment.

Available data support the hypothesis that drug response and risk for some adverse events is heritable, determined in part by individual genetic differences. For AIWG, investigations using family study designs show heritability for this adverse effect may be as high as 60-80%.[59, 60] Both genome-wide and candidate gene studies have identified common genetic variants to function as predictors, but the amount of variance in AIWG explained by these findings remains modest. [61-63] The strongest published associations have implicated common variants of MC4R, leptin, and HTR2C as partial predictors of AIWG[64]. Attempts to identify genetic associations for other adverse events such as extrapyramidal and tardive dyskinesia (TD) have only been. [65, 66] While a genome-wide association study identified 5 potential genes impacting antipsychotic treatment response in schizophrenia,[67] established moderators of genetic response in ASD are lacking.[68]

A significant limitation in the vast majority of pharmacogenetic studies of antipsychotic treatment has been the failure to include drug pharmacokinetic (PK) information in the examination of genetic effects. This is especially relevant in studies of drug distribution, metabolism, excretion (DME), and transporter gene variants, where mutations may directly impact plasma and tissue drug concentrations. RSP, like most antipsychotics, undergoes extensive first-pass hepatic metabolism (hydroxylation) to 9-hydroxyrisperidone (9-OH-RSP, paliperidone). Hydroxylation is primarily catalyzed by cytochrome P450 2D6 (*CYP2D6*), with cytochrome P450 3A4/5 (*CYP3A4*/5) representing a secondary pathway of metabolism.[69] Interest in the possible effects of genetic variants of these drug metabolizing enzymes is heightened by the observations that they are both expressed in brain.[70] Although 9-OH-RSP was often presumed to possess equivalent pharmacodynamic properties to RSP, it is now recognized that RSP has substantially higher affinity for 5-HT2A and α1 and α2 adrenergic receptors.[71, 72] Therefore, differences in adverse events could be influenced by shifts in the ratio of RSP/9-OH-RSP, as well as the sum of plasma RSP and 9-OH-RSP (active moiety) concentrations. In addition, both RSP and 9-OH-

RSP are substrates and inhibitors of members of the adenosine-triphosphate-binding cassette (ABC) superfamily of transport proteins, including the multidrug resistance-1 protein P-glycoprotein (MDR1 or P-gp), encoded by the *ABCB1* gene,[73] and the breast cancer resistance protein (BCRP), encoded by the *ABCG2* gene,[74] both of which influence drug efflux across membranes in the intestines, blood-brain-barrier, and renal tubules. Genetic variants in *ABCB1* and *ABCG2* may be additional sources of variability in therapeutic and adverse effects of psychotropics such as RSP. Lastly, *NR1I2* encodes the nuclear receptor pregnane X (PXR) that is a transcriptional regulator of multiple genes involved in the absorption, distribution, metabolism, and extraction of drugs and other xenobiotics, including the cytochrome P450s and ABC drug transporters.[75, 76] *NR1I2* polymorphism rs7643645 (A>G) was correlated with a 2.8 fold lower risperidone total active moiety concentration when comparing GG to AA genotypes ($p = 0.031$).[77]

Multiple mutations in relevant drug metabolism enzymes (DME) and transporter genes are common across all human populations, and allele frequencies vary according to genetic background. *CYP2D6* is polymorphic, with over 100 functional variants identified. Absent *CYP2D6* metabolic capacity ("Poor Metabolizers" or PMs) occurs in 5-10% of Caucasians as a result of inheriting two nonfunctional alleles, with the alleles *CYP2D6**3, *4, *5, *6 representing 95% of responsible variants underlying PMs. Decreased *CYP2D6* function ("Intermediate Metabolizers" or IMs) can be observed in those individuals with one nonfunctional allele, or in individuals possessing other mutations which convey reduced enzyme activity (e.g., *CYP2D6**10 allele).[78] Gene deletion and duplication occurs in approximately 7% and 5% if the American population respectively.[44] Gene duplication (3 or more copies) of functional alleles results in ultrarapid metabolizer status (UMs). *CYP3A4*/5 is also polymorphic, but with fewer variants, although some 6% of Caucasians, 22% of Asians, and 26% of African Americans have been found to carry the *CYP3A4**20 variant, an insertion-mediated frameshift resulting in loss of enzyme activity. Other

relatively common *CYP3A4* variants which also reduce enzymatic activity include the *CYP3A4**1B and *3 alleles, found in 4-5% and 1% of Caucasians respectively. Similarly, the genes encoding drug transporters are known to have common variants. *ABCB1* and *ABCG2* have identified variants which influence expression and/or substrate affinity, and which can be found in as many as 20% of Caucasians. *NR1I2* variants are also common.

Studies examining the impact of differing *CYP2D6* and *CYP3A4*/5 genotypes on RSP pharmacokinetics have yielded mixed findings, but most extant studies are underpowered to detect genetic effects, or contain confounding factors such as smoking, wide age ranges, and varying exposure lengths. Extensive Metabolizer (EM, or wild type) subjects did not differ from PM subjects in the sum of plasma RSP and 9-OH-RSP concentrations (active moiety) in several reports.[79-84] Nevertheless, differences in RSP and 9-OH-RSP concentrations,[80] and higher ratios of RSP/9-OH-RSP in PMs[79-81, 83] have been found between *CYP2D6* PMs versus EMs, in the absence of differences in active moiety levels. However, some contrasting reports from larger samples have observed higher RSP and active moiety plasma concentrations in impaired metabolizers (heterozygous and homozygous for inactive *CYP2D6* alleles) versus EMs.[85-87] Using population PK analyses, one of the largest studies showed that *CYP2D6* allele groups linked to metabolizer phenotype classification predicted 52% of the variance of RSP concentrations, while the area under the curve (AUC) values for activity moiety concentrations were significantly (28%) higher for *CYP2D6* PMs versus combined EM/UMs and versus IMs. [87] None of these studies examined downstream beneficial or adverse clinical drug effects.

Few reports have examined effects of *CYP3A4*/5, ABC-family variants, and RSP pharmacokinetics to confidently establish PK or pharmacodynamic relationships. No *CYP3A4*/5 effects were observed for any RSP PK parameters in three studies.[79, 88, 89] However, one study

noted that carriers of the *CYP3A4* SNP rs35599367 A-allele to have a 30% reduction in 9-OH-RSP clearance,[87] and in another report, the *CYP3A5**3 allele conferred higher active moiety and 9-9-OH-RSP concentrations,[80] which remained significant even after correcting for 2D6 genotypes. For *ABCB1*, three studies have found significant effects of the *ABCB1* SNP rs1045642 (C3435T) on active moiety concentrations;[79, 84, 85] two of these studies also noted effects of additional *ABCB1* variants. In addition, a haplotype comprised of 3 SNPs (rs1128503, rs1045642, and rs2032583) in *ABCB1* was associated with significantly different 9-OH-RSP and active moiety concentrations; the haplotype predicted 11% of active moiety concentrations.[79] However, one report failed to find PK effects of *ABCB1* variants. None of these studies examined resultant clinical drug effects in relation to PK.

Studies testing associations between *CYP2D6*, 3A4/5, and *ABCB1* genotypes and clinical effects, such as response and weight gain, have yielded mixed results. Reports on *CYP2D6* have generally not found associations with clinical response.[82, 85, 90, 91] In a positive cohort study of 40 children with ASD, disruptive behaviors were rated as improved, no change, or worsened; UMs (n=2) showed no response or adverse effects, whereas PMs (n=2) experienced improvement and greater adverse effects.[92] One report of a sample of 116 outpatients treated with various antipsychotics (including 26 receiving RSP) for psychosis noted a greater proportion of responders were *CYP2D6* EMs versus non-responders (67% versus 46%, p = 0.023). I can't find this reference In a smaller study, EMs also showed greater improvement. I can't find this reference However, a monotherapy RSP study of adults with schizophrenia found *CYP2D6* PMs showed greater PANSS-Total score reduction (p = 0.029).[93] Two reports have identified associations between efficacy and *CYP3A4*/5 variants. In the Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) study, a marginal effect for clinical response was seen for *CYP3A5* genotypes in 175 subjects treated with RSP (p = 0.04).[94] A study in 130 patients with

schizophrenia receiving RSP monotherapy reported minor A-allele homozygotes of *CYP3A4**1G versus other genotypes experienced significantly less percent improvement in PANSS score (28% versus 42/43%, p = 0.021), however this result did not survive statistical correction.(Du et al., 2010) Drug transporter variants may be associated with the clinical efficacy of RSP. Greater reductions in behavior and sociability problems in children with autism treated with RSP were observed in *ABCB1* 1236T-allele (rs1128503) carriers (p = 0.002), although the magnitude of difference was small (4.7%).[90] Likewise, TT homozygotes at this SNP experienced greater improvement of psychotic symptoms in adults with schizophrenia.[95]

Adverse events and relationships to DME variants have also been reported. Lane et al (2006) found that *CYP2D6**10 EMs gained 1.14 and 0.80 kg less than those heterozygous and homozygous for *CYP2D6**10 respectively after 6 weeks of RSP exposure. Similarly, null alleles (*4) were also associated with weight gain in several other studies.[96, 97] In contrast, a study of 45 children with autism treated with RSP for up to 12 months found that both *CYP2D6* UMs and PMs showed smaller increases in waist circumference than EMs; PMs did not differ from EMs in BMI increases.[90] While other reports have noted associations between *CYP2D6* and *ABCB1* variants with adverse effects and intolerance of RSP,[85, 86, 98, 99] only one study has reported an association between *ABCB1* variants and RSP-associated weight gain only in females.[100] Several studies have found significant relationships between RSP PK (specifically active moiety concentrations) and adverse events of akathisia, tremor, and other “neurologic” symptoms.[87] A meta-analysis of 20 studies with varying antipsychotics found no association between TD and *CYP2D6*, however when analyses were re-examined using prospective studies only, homozygous and heterozygous individuals with *CYP2D6* reduced function alleles showed an increased odds ratio of 1.83 – 2.08 for TD versus EM patients, and mutant allele carriers also possessed increased risk for parkinsonian symptoms (OR = 1.64).[101] In a sample of 325 outpatients receiving chronic RSP,

CYP2D6 PMs were 3.1-fold more likely to experience moderate to severe adverse drug reactions[98] and discontinued RSP due to adverse events at a 6-fold greater rate than EMs. Similarly, variants in *ABCB1* have been associated with RSP-induced extrapyramidal symptoms.[85, 99] Finally, RSP-induced prolactin elevation in children was associated with number of functional *CYP2D6* alleles;[102] however, this result was not replicated by later reports.[90, 103-105]

Taken together, aside from some associations with adverse events such as EPS and possibly AIWG, the extant literature on the impact of *CYP2D6*, *CYP3A4*/5, and *ABCB1* mutations on risperidone PK, efficacy, and adverse events is inconclusive and incomplete, given the relative absence of integrated analyses of RSP PK with genetic and clinical outcomes. We are unaware of a single study of investigating DME genetics with RSP PK and clinical response in children, despite its widespread clinical use in pediatric psychiatry. Given the lack of consistent and integrated prior data, we sought to concurrently test the impact of common PK variants on plasma RSP levels and treatment outcomes in a sample of children receiving standard RSP monotherapy.

METHODS

Subjects

The research was conducted under the auspices of the National Institute of Mental Health (NIMH) RUPP Autism Network using two protocols approved by individual site Institutional Review Boards and by a NIMH Health Data Safety Monitoring Board.[106, 107] Written informed consent (and assent from the child, when capable) was obtained from a parent or guardian prior to enrollment. In the earlier study, youth (ages 5 to 17 years) meeting DSM-IV criteria for autistic disorder accompanied by severe irritability (aggression, tantrums, and/or self-injurious

behaviors) defined by a score of > 18 on the Aberrant Behavior Checklist (ABC) Irritability subscale were treated for 8 weeks with risperidone monotherapy or placebo.(McCracken et al., 2002) Only 10% of subjects had previously received (ineffective) antipsychotic treatment. Those subjects deemed non-responders who had been randomized first to placebo in the 8-week acute phase were offered open-label treated with risperidone according to an identical titration and assessment protocol. The Aman et al. study applied similar inclusion and exclusion criteria (except age range of 4 – 13 years) and all subjects received RSP monotherapy, titrated similarly to the 2002 RUPP study, with or without behavior management for up to 24 weeks. Of the 225 total outpatients enrolled from the two trials, weekly measures of weight, height, BMI, and genotype data were available for a combined 184 subjects from their initial 8-week acute exposure to RSP. Plasma samples for RSP and 9-OH-RSP drug levels were available from 120 subjects and were analyzed using combined liquid chromatography/mass spectrometry according to previously published protocols; sample loss was primarily due to inadequate samples and blood draw refusals.

Clinical response was assessed by changes in parent-reported behavior on the Aberrant Behavior Checklist-Community Version (ABC).(Aman, Singh, Stewart, & Field, 1985) The ABC is a widely-used outcome measure in clinical trials involving individuals with intellectual and developmental disabilities. It contains five subscales defined by factor analysis: Irritability, Hyperactivity, Stereotypic Behavior, Social Withdrawal, and Inappropriate Speech. The Irritability subscale was one of the primary endpoints for the two clinical trials. Weight and height were obtained weekly, and weight was transformed to standardized z-scores using anthropometric indices based on the 2000 CDC growth charts using the CDC SAS program, as previously described.[108] Adverse events were assessed using a parent-reported checklist of items, as well as by spontaneous report from parents to the study physicians.

Genotyping

Genomic DNA was extracted from whole blood using QiaAmp DNA Blood Mini Kits (Qiagen, Valencia, CA). Genotyping was performed using the TaqMan genotyping platform (Life Technologies, Grand Island, NY) with Qiagen Type-it Fast SNP Probe PCR Kit according to manufacturer's protocols. All markers were in Hardy-Weinberg Equilibrium, 10% of the dataset was genotyped in duplicate with perfect concordance, and allele frequencies were consistent with those reported by the HapMap Consortium. Genes chosen for analysis were those known to regulate risperidone metabolism (*CYP2D6*, *CYP3A4*/5, *NR1I2*) and influence absorption and disposition (*ABCB1*, *ABCG2*, *NR1I2*). The four most common functional SNPs in *CYP2D6* that are associated with >90% of IM and PM phenotypes in Caucasian and African American individuals were genotyped (rs1065852 [missense], rs3892097 [splice donor], rs16947 [missense], rs28371706 [missense]) and gene copy number variation (CNV) was determined using Taqman real-time PCR. Activity score was assigned by summing the number of null (0), partial (0.5), and wild-type (1) alleles plus or minus CNV duplication or deletion. For metabolizer status assignment, EMs had a score of 1.5-2, IMs 0.5-1, PMs 0 and UMs>2.[109] We did not test for rare polymorphisms/alleles, since these, if present at all, would have represented only a tiny percent of our sample. Similarly, common functional variants in *CYP3A4*/5 (rs2740574, rs55785340, rs4987161, and rs776746) and *NR1I2* (rs7643645, rs1523130, rs2472677) were selected and classified as EM, IM, or PM according to the existing literature;[110] (PharmGKB, https://www.pharmgkb.org/view/vips.jsp). Three known functional variants in *ABCB1* (C1236T/rs1128503, C3435T/rs1045642, rs4148740 and G2677T/rs2032582 by strong linkage disequilibrium (r2=0.8) with rs1128503) and 7 tag SNPs across the coding region of *ABCG2* were also included in the analyses (rs2725248, rs3114020, rs17731538, rs2231142, rs13137622, rs7681519, rs2725256).

Statistical Analysis

Because the distributions of RSP, 9-OH-RSP, and active moiety concentrations were skewed, those data were log-transformed to normalize distributions to meet the assumptions of the statistical model. ANOVA analysis was conducted with RSP PK concentrations as the dependent variable and genotype as a fixed factor. Potentially confounding covariates such as sex, age, dose, race and ethnicity were entered in the initial model; those identified as non-significant were thereafter dropped from the final model. Due to race effects, analyses were repeated within racial groups. Statistical significance was set as $p < 0.05$. Given the exploratory nature of the study, p values are reported as their uncorrected value; the effects of statistical correction for multiple tests per hypothesis are reported in the text. To examine change in treatment outcome over time, a repeated measures linear mixed model was employed (e.g., weighted z-score from baseline and ABC subscales). Genotype, visit (as a continuous variable), and interaction of genotype by visit were entered as predictors. Frequency of adverse events in genotype groups was examine by chi square analysis.

RESULTS

Risperidone PK: Demographic Influences

The RUPP and RUPP-PI groups were compared using the appropriate Chi-square, ANOVA, or t-test to ensure comparable samples. The samples were mostly comparable, with the exception of the mean younger age of the RUPP-PI sample (90.6 + 29.9 months versus 102.3 + 31.9 months, $p = 0.06$), and a slightly higher mean daily dose for the RUPP-PI study (2.1 + 0.6 mg/day versus 1.8 mg/day, $p = 0.019$), and, reflecting the above, the baseline mean body

weights and mean risperidone dose (mg/kg/day) were significantly different. The RUPP sample also contained a higher proportion of Hispanic subjects and a trend towards fewer Black subjects; separate analyses by ancestry are reported. All analyses included age, sex, ancestry, and dose as covariates. Interestingly, RSP plasma level was not correlated with age (r=0.029, p=0.75), sex (F=1.096, p=0.337) or dose (r=-0.053, p=0.564). As clinicians followed a protocol of flexible dosing, the latter may be explained by apparent implicitly detected CYP metabolizer phenotype. After covarying for baseline age and weight, both strong determinants of dose, those with at least one functional *CYP2D6* or *CYP3A4* enzyme received a mean dose of 0.076 mg/kg/day or 2.1 mg daily, while those with no functional *CYP2D6* or 3A4 were prescribed 0.069 mg/kg/day or 1.8 mg daily (p=0.047). Inference of impaired metabolizer status by prescribing clinicians has been similarly noted in a prior report.[111]

	RUPP (n=34)	**RUPP-PI (n=86)**	**Combined (n=120)**	**P-value**
Gender [n (%) male]	85.3 (%)	83.7 (%)	101 (84.2%)	χ^2=0.045, p=0.832
Baseline age [months]	102.3 ± 31.9	90.6 ± 29.9	93.9 ± 30.8	T=1.90, p=0.06
Baseline BMI	18.3 ± 5.0	17.7 ± 3.0	17.9 ± 3.8	T=0.89, p=0.37
Ethnicity [n (%)]				
White, non-Hispanic	21 (61.8%)	61 (70.9%)	82 (68.3%)	χ^2 = 0.95, p=0.33
Black, non-Hispanic	2 (5.9%)	14 (16.3%)	16 (13.3%)	
Native American	0	2 (2.3%)	2 (1.7%)	X^2 = 2.68, p=0.10
Asian or Pacific Islander	1 (2.9%)	4 (4.7%)	5 (4.2%)	
Hispanic	6 (17.6%)	4 (4.7%)	10 (8.3%)	
Black, Hispanic	0	1 (1.2%)	1 (0.8%)	
Other	4 (11.8%)	0	4 (3.3%)	X^2 = 5.38, p=0.02

Baseline weight [mean kgs]	34.0 ± 17.9	28.9 ± 10.9	30.7 ± 14.2	T=2.37, p=0.019
Final dose [mg/kg/d]	0.06 ± 0.03	0.08 ± 0.03	0.07 ± 0.03	T=-4.08, $p=6.7x10^{-5}$
Final dose [mg/d]	1.8 ± 0.6	2.1 ± 0.6	2.0 ± 0.6	T=-2.71, p=0.007
Risperidone [nmol/L]	27.3 ± 38.9	18.5 ± 37.7	21.0 ± 38.1	T=1.13, p=0.26
9-OH-risperidone [nmol/L]	42.5 ± 30.9	51.4 ± 49.4	48.9 ± 44.9	T=-0.98 p=0.24
Total Active Moiety [nmol/L]	69.8 ± 60.5	69.9 ± 81.4	69.8 ± 75.8	T=-0.008, p=0.99
Mean weight gain [kg]	2.8 ± 2.0	2.6 ± 1.5	2.7 ± 3.9	T=1.40, p=0.16

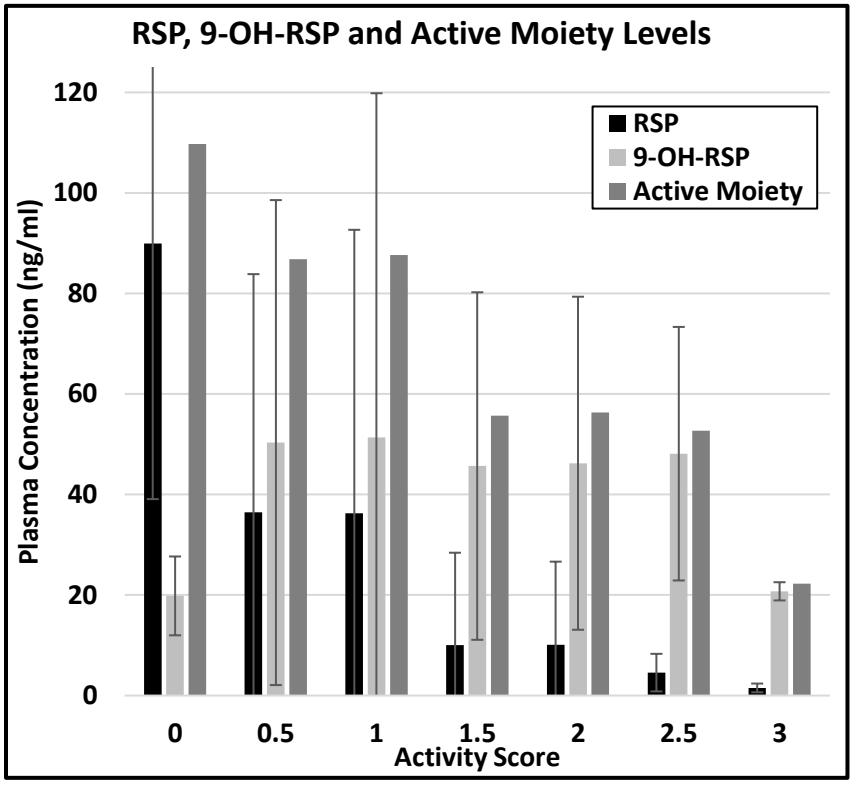
Table 2-4. Characteristics of the RUPP, RUPP-PI, and Combined RUPP Autism Risperidone Pharmacokinetic/Pharmacogenetic Samples

Risperidone PK: Pharmacogenetics

CYP2D6. Concentrations for RSP, 9-OH-RSP, and total active moiety (RSP + 9-OH-RSP), as well as the RSP/9-OH-RSP ratio are reported in Table 1 for the separate studies and the combined sample. Observed mean concentrations were comparable to those reported in the literature;[79, 83, 85] however, levels showed marked variability. *CYP2D6* activity score (AS)[109]was the strongest determinant of RSP levels (see Figure 2-7). Of the 119 subjects with valid *CYP2D6* genotypes, 24 (20%) had wildtype activity (AS = 2) at the *CYP2D6* locus, having two normal function alleles and 6 subjects (5%) had duplications resulting supernormal activity (AS > 2). Two (2%) subjects were poor metabolizers with no enzyme function (AS = 0). The majority of subjects (N=87, 73%) had some combination of reduced function or null alleles: 13 (11%) with AS = 0.5, 28 (24%) with AS = 1, and 46 (39%) with AS = 1.5 (see Figure 2-8). *CYP2D6* AS predicted RSP parent drug level ($p=7.71 \times 10^{-6}$) and the ratio of RSP/9-OH-RSP ($p=8.71 \times 10^{-9}$),

controlling for age, sex, ancestry, total daily dose, and plasma processing batch. In addition to total daily dose ($p=0.019$), parent drug (RSP) level ($p=8.13 \times 10^{-12}$) was the strongest predictor of 9-OH-RSP concentration. Given this observation, parent drug level was added to the model, revealing a significant effect of AS on 9-OH-RSP (3.01×10^{-4}).

Figure 2-7. CYP2D6 Activity Score Predicts RSP, 9-OH-RSP, and Active Moiety Levels.



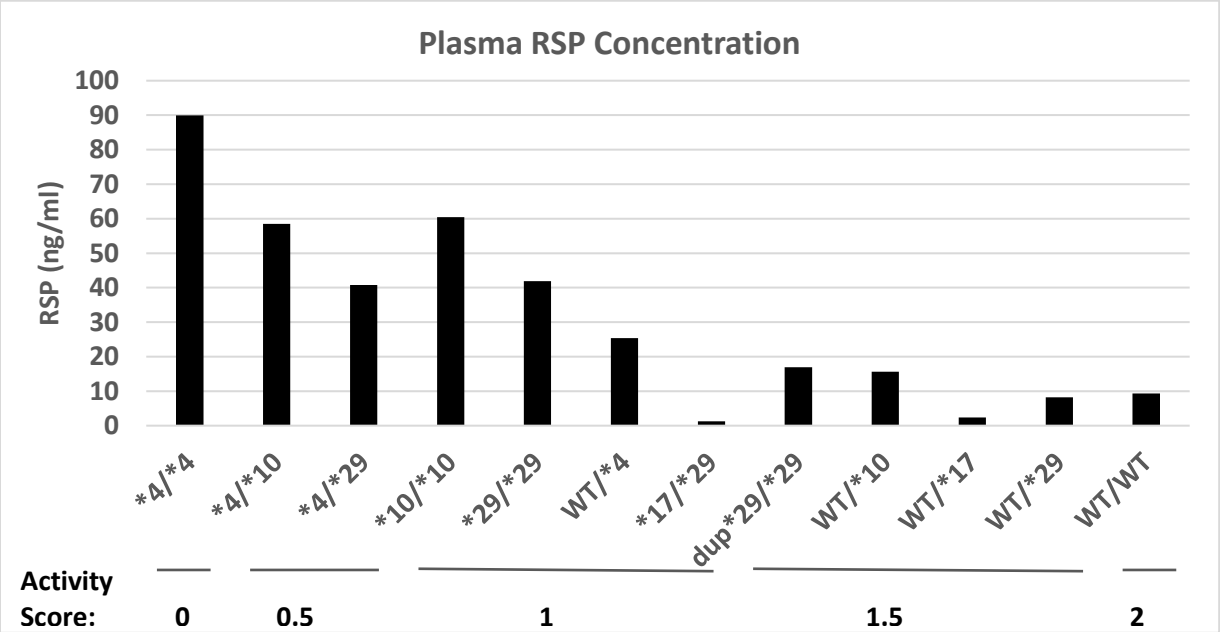


Figure 2-8. RSP Levels Correspond to CYP2D6 Genotype in Our Sample. Predicted Activity Score of each genotype is shown below the graph.

As noted in previous literature, *CYP2D6* *4 and *10 alleles resulted in the highest RSP levels. Consistent with prior reports suggesting substrate specificity, *17 does not appear to be a reduced function allele for RSP transport in our sample. In the case of two reduced function alleles, a duplication of one of the partial alleles was apparently able to compensate for impaired function with increased quantity, resulting in wildtype RSP levels. While classic UMs (AS > 2) showed only slightly reduced RSP levels compared to EMs, total active moiety was more than halved in individuals with an AS of 3. (see Figure 8).

CYP3A4/5. The multiple rare *CYP3A4* polymorphisms tested were invariant in our sample, and therefore *CYP3A4* did not contribute to RSP PK variability. At the *CYP3A5* locus, 25 subjects

(21%) possessed one allele conveying impaired function and 80 subjects (67%) possessed two reduced function alleles. After covarying for *CYP2D6* effects in the total sample, *CYP3A5* showed a marginal contribution to only RSP parent drug level (p = 0.02). Ancestry effects, discussed below, were prominent.

Other PK Genes. Carriers of the minor allele at *NR1I2* rs1523130 show higher levels of 9-OH-RSP (p=0.044) in a dominant pattern independent of *CYP2D6* or *CYP3A4* genotype. No other PK effects of *NR1I2* variants were observed. Transporter gene variants in *ABCB1* and *ABCG2* had no effect on plasma drug levels.

Risperidone PK: Ancestry Effects

The range of RSP level was much greater in Caucasians, with a range of 254 compared to 92 in non-Caucasians, likely reflecting the prevalence of *CYP2D6* inactivating polymorphisms. Given the large differences identified in minor allele frequencies between Caucasian and non-Caucasian populations, we performed separate exploratory analyses of the effects of *CYP2D6* and *CYP3A5* variation in these two groups. Since all Caucasians (N=84) in our sample carried impaired activity alleles at *CYP3A5*, not all phenotypes were represented (N EM = 0, IM = 14 and PM = 67), and *CYP3A5* variation did not account for variability within the Caucasian sample (p = 0.15). In Caucasians, RSP concentrations were strongly predicted by *CYP2D6* genotype alone (p = 1.1 x10-5). Conversely, in this underpowered sample of non-Caucasians (N=36), RSP concentration was determined predominantly by *CYP3A5* genotype (p = 0.005), with a marginal influence of *CYP2D6* status (p=0.035), and contrary to findings in Caucasians, a marginally significant effect of dose (p=0.004).

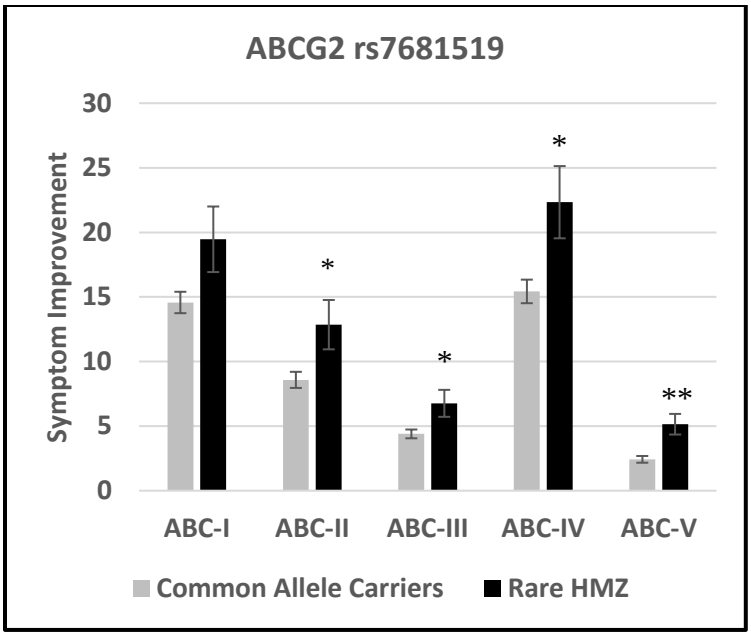
Risperidone PK: Clinical Outcomes

Pairwise correlational analyses were performed to assess the possible relationships between PK concentrations and clinical outcomes. Individual values for RSP, 9-OH-RSP, total active moiety, and for the RSP/9-OH-RSP ratio (all log-transformed) were examined against change from baseline to Week 8 for the five clinical efficacy ABC subscales. Of the total of 20 tests, ABC-I (irritability subscale) change was positively correlated with RSP ratio ($r=0.22$, $p=0.015$) and negatively correlated with 9-OH-RSP level ($r=-0.18$, $p=0.047$). ABC-III (stereotypy subscale) was also negatively correlated with 9-OH-RSP level ($r=-0.22$, $p=0.016$). None of the PK values were significantly correlated with RSP-associated weight gain (BMI Z-scores, all p values >0.3). From the Adverse Events Report Form, we examined relationships between RSP PK concentrations and the presence or absence of side effects. In 23 children with reported extrapyramidal symptoms (EPS), symptoms were positively associated with levels of 9-OH-RSP ($p=0.021$) and total active moiety ($p=0.014$). Sedation, which was commonly reported in 95 children, was positively associated with RSP Ratio ($p=0.047$). Drooling was positively associated with higher daily RSP doses (2.16 ± 0.59 mg/d vs. 1.92 ± 0.62 mg/day, $p=0.015$).

Risperidone Pharmacogenomics: Clinical Outcomes

Genotype groups at each locus were analyzed as possible predictors of clinical efficacy and adverse event outcomes. Neither *CYP2D6* nor *CYP3A4*/5 metabolizer status was significantly associated with changes on treatment response endpoints, as measured by the five ABC subscales, or side effects, including weight gain, sedation, EPS, tremor, and drooling. While not

significant, our data suggests that *CYP2D6* PMs may show both attenuated response and increased side effects; however, these analyses were severely underpowered given only 2 PMs in our sample. *CYP2D6* PMs showed half to one quarter the improvement in ABC-I, -II, and IV compared to IM/EMs (Cohen's D effect sizes = 0.8-0.9). Similarly, PMs had nearly twice the degree of weight gain as IM/EMs (BMI-Z score 1.1 verses 0.6 respectively, D = 0.8). Variant rs7681519 in the *ABCG2* transporter gene is the only genetic variant globally associated with dimensional ABC score. Homozygotes for the minor allele showed an enhanced response (ABC-I p = 0.07, D = 0.5; ABC-II p=0.04, D = 0.6; ABC-III p = 0.03, D = 0.6; ABC-IV p = 0.02, D = 0.6; ABC-V p = 0.001, D = 0.9). Consistent with the direction of effects discussed above, minor allele *ABCG2* homozygotes were also protected from AIWG (p=0.04). The C-allele at *NR1I2* rs1523130 was associated with enhanced ABC-I response (p=0.02, dominant pattern, D = 0.5). *ABCB1* rs4148740 minor alleles showed greater weight gain (p = 0.002, recessive pattern, D = 1.6). With regard to other adverse effects, all participants who reported tremor (14/14) were carriers of the C-allele at *NR1I2* rs1523130 (p=0.039) and 92% of those reporting EPS (p=0.017) and 86% of those reporting drooling (p=0.044) were common allele homozygotes at *ABCG2* rs2231142.



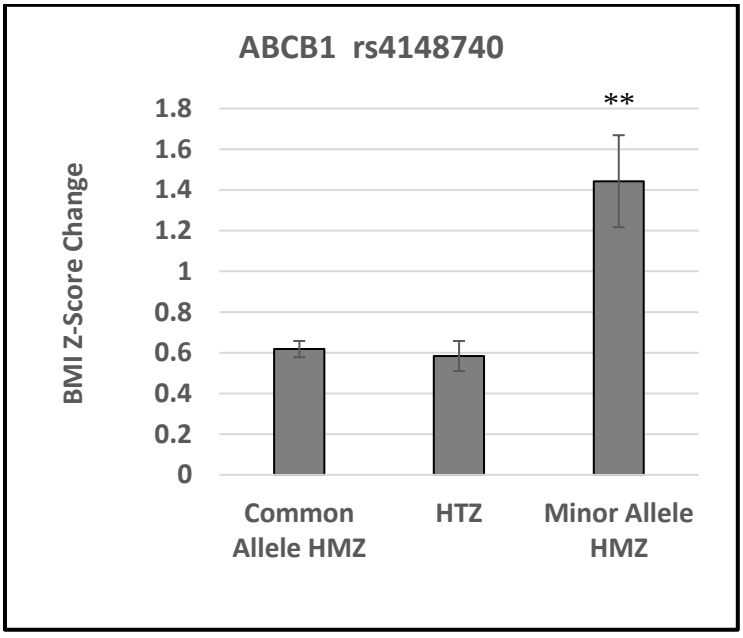


Figure 2-9. Transporter Variants are Associated with Enhanced ASD Treatment Response and Adverse Effect Profile.
Top. Minor allele homozygotes (Minor HMZ), compared to common allele homozygotes and heterozygotes (Common Allele Carriers) of *ABCG2* rs7681519 show greater improvement on all subscales of the Aberrant Behavior Checklist (ABC).
Bottom. *ABCB1* rs4148740 minor allele homozygotes show nearly 3-fold greater weight gain compared to those with common alleles. * $p<0.05$, ** $p<0.005$

DISCUSSION

This study represents one of the most comprehensive attempts to delineate the role of multiple DME and drug transporter genes on antipsychotic pharmacokinetics and clinical effects of treatment and is especially unique given our sample of children and adolescents. There are several main findings.

Plasma RSP levels showed only weak association with clinical outcomes. With the exception of marginal effects of 9-OH-RSP on irritability, stereotypy and EPS, no efficacy or side effect associations, including with weight gain, were evident. Speculatively, negative effects of 9-OH-RSP on outcome and adverse effects may imply that paliperidone may not achieve equal efficacy to RSP for ASD irritability and stereotypy and may be more likely to produce EPS. In line with these data, *NR1I2* rs1523130 was associated with both 9-OH-RSP levels and irritability. Sedation, on the other hand, was associated with RSP to 9-OH RSP ratio, suggesting the opposite relationship. BMI changes were independent of RSP plasma levels.

The paucity of association between RSP PK values and clinical outcomes we observed is surprising but in agreement with literature reports. Extant data has suggested possible relationships between drug level and the presence of neurologic symptoms. We speculate that, given the mechanism of action of RSP as an antagonist of dopamine 2 and serotonin 2A receptors, PK values, especially of active moiety, above a certain threshold are sufficient for inducing clinical change, and that a “dose-response” relationship between exposure and other clinical effects, positive or negative, may require much higher exposures to be detected.

Despite some contradictory findings in the literature, our data confirms the primary and significant role of *CYP2D6* in determining RSP concentrations, especially in Caucasians. The *CYP2D6* poor metabolizer genotype group displayed 702% higher RSP and 133% higher total

active moiety concentrations. Significant but more modest associations were also observed for the effects of *CYP3A5* genotypes on RSP and active moiety concentrations, which predominated over *CYP2D6* in non-Caucasian subjects in predicting drug levels. RSP metabolism appears to be handled by different CYP metabolic pathways across ancestry. As fully functional *CYP3A5* alleles are apparently rare in Caucasians but *CYP2D6* variation is common, the finding that *CYP2D6* variation is the primary driver of RSP variability is not surprising. Inactive copies of *CYP3A4* are common in populations of African ancestry, emphasizing the importance of compensation by *CYP3A5*. Since *CYP2D6* PM alleles are less common in non-Caucasian populations, *CYP2D6* contributes less to non-Caucasian variation. Larger and more diverse samples would be needed to further stratify effects according to ancestry. These findings are consistent with prior reports of the predominant role of *CYP2D6* genotype on RSP concentration[85-87] and contribution of *CYP3A5* variants to RSP and active moiety concentrations[80, 87] in adults, suggesting that conflicting studies may have been underpowered or influenced by population background differences, especially with regard to genetic ancestry.[79, 88, 89]

Contrary to a few other published reports, we found no impact of ABC-family gene variants on RSP pharmacokinetics. The absence of associations between variants in *ABCB1* with RSP PK endpoints is difficult to reconcile with the two reports finding effects of multiple *ABCB1* variants on 9-OH-RSP and active moiety concentrations,[79, 85] even though we genotyped at least one of the SNPs (rs1045642, C3435T) found previously to be predictive of PK differences. The fact that other groups have also been unable to identify RSP PK effects of *ABCB1* genotypes suggests that any true effects are relatively small.[87] It is conceivable that developmental differences may influence the relationship between these gene variants and RSP PK, given the pediatric age range of our sample.

Despite a strong effect of *CYP2D6* on plasma RSP, *CYP2D6* genotype did not predict outcome. Given the few relationships noted between RSP PK and clinical effects, this is not surprising. Genetic predictors of RSP response outcomes are undoubtedly far more complex than genetic determinants of plasma concentration, requiring sample sizes in the tens of thousands for unequivocal discovery. PMs in our study may have in fact suffered worse outcomes and adverse effects; however, our study was underpowered to statistically test this relationship. While neither of the metabolic CYP genes were related to outcomes, transporter proteins were nominally associated. One possible interpretation of this finding is that plasma concentration, which is largely related to CYP enzymes, is less important than brain exposure, which is dependent on transporter proteins, in determining outcomes. The consistent association of *NR1I2* and transporter variants across measures of efficacy and adverse effects in the absence of RSP PK associations might reflect their potential role in influencing brain exposure. The fact that the same genes and variants (*ABCG2* and *NR1I2*) were associated with therapeutic outcomes and adverse effects adds credence to these results.

ABCG2 intronic SNP rs7681519 and *NR1I2* 5'UTR SNP rs1523130 appear to tag regulatory DNA blocks, as evidenced by numerous promoter and enhancer marks, evidence of DNase protection and regulatory protein binding, and functional gene regulation based on eQTL databases (haploreg). Interestingly, the G-allele of *ABCG2* rs7681519 is in strong LD (r2=0.78, D'=0.99) with the C-allele of rs2725263, empirically shown to produce increased expression of *ABCG2*.[112] Based on this strong association, carriers of the G-allele of rs7681519 should have reduced brain exposure to RSP given the increased *ABCG2* efflux pump expression at the BBB. Similarly, the T-allele of *NR1I2* rs1523130 has also been previously shown to increase RSP clearance and in our sample was observed to predict reduced response and greater side

effects.[87] *ABCG2* rs2231142 is a missense variant resulting in an amino acid change from glutamine to lysine; common alleles have been previously associated with higher RSP levels and greater adverse effects,[113] which we also observed in our analysis. Linkage disequilibrium (LD) between SNPs within *ABCG2* is <0.4, reflecting independent associations. *ABCB1* rs4148740 is an intronic variant tagging enhancer marks, sites of protein binding and motif changes. Minor alleles, associated with increased AIWG in our sample and have previously been associated with remission of major depression and increased adverse effects of antidepressants transported by *ABCB1*, suggesting that it is a functional variant producing greater psychotropic exposure.[114-116]

These data add to extant knowledge of moderators of RSP pharmacokinetics in humans. Consistent with in vitro and in vivo studies, we confirmed the importance of *CYP2D6* and *CYP3A5* genetic variants in determining steady state concentrations of RSP and active moiety. Our sample was larger than the majority of similar reports and participants demonstrated excellent treatment compliance, were mostly treatment-naive, and were all non-smokers and medically healthy, which presumably aided our ability to discern important but subtle effects of gene variants on RSP pharmacokinetics and outcome.

Our study has several limitations. Although our sample is larger than the majority of similar studies, it still lacks sufficient power to test variants of low frequency or small effect, as well as interactions between multiple loci. As a sample of children with ASD, the clinical effects under observation may not extrapolate to the treatment of psychosis or other clinical conditions. Our RSP PK estimates are only based on single trough samples, which may be insufficient to fully capture the range of actual exposures as compared to repeated sampling studies. A large number of tests were performed (tests of 14 genetic variants, 4 (not independent) plasma

measures, 5 treatment outcomes and 5 adverse effects). Few of these findings would survive correction for multiple testing.

There have been considerable efforts to identify clinically significant moderators and predictors of benefits and adverse effects of RSP and other antipsychotics, which in theory could yield more personalized and effective management. However, the results of these efforts have been mostly disappointing. Moderator analyses of clinical benefit in the two RSP studies of children with ASD that formed the sample studied in this report only identified one significant moderator, baseline severity of irritability, out of 33 variables tested. Similar attempts to identify genetic predictors of antipsychotic response have not yet uncovered significant predictors of outcome, with the possible exception of studies of AIWG, where prior studies have noted replicated and robust associations with variants in MC4R and other genes involved in energy balance. In theory, genetic variants that directly impact the distribution and metabolism of the drug represent reasonable candidates to test for their impact on clinical outcomes. Although we demonstrated significant associations with RSP PK values and identified several nominal associations between gene variants and clinical outcomes, additional studies are needed to confirm these associations. More research on the mechanisms and magnitude of clinical effects of drug transporter gene variants is especially needed. Overall, the prediction of clinical effects of medications such as RSP are complex, and will require considerably more effort to achieve clinical application. These important efforts, however, may eventually help to identify patients who would benefit most from RSP therapy or are at highest risk for adverse outcomes.

Chapter 2 Wrap-Up

Overall, the completion of these two projections based heavily in pharmacokinetics not only taught me in-depth knowledge of LADME principles, but also helped me learn an enormous amount of the liver-based metabolism of pharmaceutical drugs. Specifically, cytochrome p450 enzymes are invaluable tools in our working knowledge of drugs and xenobiotic treatments for human diseases, and I believe that with this strong PK foundation at the base of my personalized medicine pyramid, I will truly be able to apply sound scientific reasoning to my future targeted strategies for biological mechanistic evaluation and drug design.

Chapter 3

Pharmacodynamics

General Introduction

As we saw in chapter 2, one of the most powerful tools we have in understanding pharmaceuticals is that of pharmacokinetics; because being able to identify the ways our own body will process and affect the drug is over half of the battle. The second piece to this puzzle comes in the form of a field known as pharmacodynamics (PD). I touched briefly on these principles in chapter 1, but we can consider PK and PD a sort of yin and yang relationship. Where PK is in the most basic sense how the body will affect a drug or drug like substance, PD is how that substance or its metabolites will interact with and affect the human body.

Pharmacodynamics stems from two Greek words: *pharmakon,* meaning drug, and *dynamikos,* representing force or power. Whether or not we are talking about pharmaceutical moieties or even drugs of abuse, people take these substances to achieve a desired biological, physiological, and/or pharmacological responses. Because of this, most drugs are developed based on the PD theory that the compound will interact with a native biological structure (i.e. a receptor, an enzyme, a transporter, etc.) and that this interaction manifests as a specific effect on the body.[117] The majority of drugs either mimic or inhibit normal biochemical processes or inhibit pathological processes of microbial organisms, and there are 7 main drug interactions that researchers consider when developing a novel drug.[118]

1. Stimulating action / receptor agonism
2. Depressing action / receptor agonism
3. Blocking / antagonizing action

4. Stabilizing action
5. Exchanging / replacing native substance action
6. Direct beneficial chemical reaction
7. Direct harmful chemical reaction

In general, PD endpoints and measures are essential for establishing the benefit-to-risk ratio for any therapeutic intervention. These systematically evaluate a drug's activity in the body using biomarkers and or clinical outcomes to quantify both efficacy and safety.[119] Pharmacodynamics places emphasis on dose-response relationships, which is the direct cause and effect of drug concentration and represents a large sphere of the temporal dynamics involved in this field. As we consider drugs as ligands for their biochemical receptors, it is also possible to measure the receptor binding and consequent effect, informing about the therapeutic window and duration of action of a constituent.[120]

Identifying and Implementing Pharmacodynamic Biomarkers

The bulk of pharmacodynamics is focused around the concept of biomarkers, which are most commonly endogenous macromolecules that can be measured in bodily fluids. These provide a more direct biological link to disease and clinical response, but pose significant challenges due to heterogeneity, varying levels of activity, and immunoreactivity.[121] However, they are vital in the bigger picture of personalized medicine, as they allow for earlier and more robust drug safety and efficacy measures as well as accurate patient stratification.[122] They propel our understanding of how drugs, both novel and already established, work on a cellular level, and in combination with targeted bioanalytics and PK/PD modelling, can ensure proper

pathophysiological validation of various human diseases and improve the way medicine approaches and treats these issues.

My graduate advisor, Dr. Erika Nurmi, worked closed with a researcher who was deeply interested in the effects that nicotine has on neuroinflammation, Dr. Arthur Brody. I had the opportunity in my 3rd and 4th years of graduate school to work closely with both of them on two separate but related pharmacodynamic projects focusing on cigarette smoking. We can consider nicotine the drug in these scenarios, as although it is derived from the tobacco plant, it is still a stimulant and potent parasympathomimetic alkaloid that acts as a receptor agonist as most nicotinic acetylcholine receptors.[123] It is the main psychoactive compound in cigarettes currently available on the market and has a high addiction liability in the general population, but this mechanism of action is still somewhat poorly understood.[124]

We leveraged a biomarker for neuroinflammation, the expression of translocator protein (TSPO), which is quantifiable through positron emission topography (PET) imaging to better understand nicotine's direct effects on the brain after individuals smoke a cigarette. In the first study, represented below as PD Case Study #1, we demonstrated that smokers have impaired inflammatory functioning compared with non-smokers and that the extent of this effect could be determined by an individuals genotype at the TSPO locus. To better understand how this mechanism operated on a more neurochemical level, the second study, PD Case Study #2, evaluated the effect of overnight smoking abstinence on this same TSPO biomarker, implementing dynamic imaging of both satiated and abstinent smokers for comparison. Results showed that chronic cigarette smoking was indicative of global impairment of microglial activation that can persist throughout the duration of a smoker's life. More background explanation, PD details, results, and discussion can be found directly below in each case study.

PD Case Study #1 : Effect of Cigarette Smoking on a Marker for Neuroinflammation: A [11C]DAA1106 Positron Emission Tomography Study *

**This study is currently published in Neuropsychopharmacology* [125]

Brody AL, Hubert R, Enoki R, Garcia LY, Mamoun MS, Okita K, London ED, Nurmi EL, Seaman LC, Mandelkern MA.

INTRODUCTION

Inflammation is a critical component of normal tissue repair and is fundamental to the body's defense against infection. [126] In the brain, microglia continuously scan the surrounding extracellular space[127] in order to respond swiftly to damage or infection by becoming activated and participating in neuroinflammation[128]. In this context, activated microglia participate in functions such as clearance of apoptotic cells and extracellular pathogens, removal of degenerating neurons and extracellular proteins, and cytokine/chemokine production.[128] When activated, microglial cellular morphology changes and the expression of the translocator protein (TSPO) 18 kDa is increased, thereby making the expression of TSPO a marker for neuroinflammation.

The radioligand N-(2,5-dimethoxybenzyl)-N-(5-fluoro-2-phenoxyphenyl) acetamide labeled with carbon-11 (abbreviated as [11C]DAA1106) has emerged as a reliable second-generation

radiotracer for labeling TSPO[129-131] with high affinity[132-135] for positron emission tomography (PET) scanning in vivo. Because [11C]DAA1106 and other newer radiotracers have higher affinity for TSPO than previously used radiotracers (eg, [11C]PK11195), they are more useful for quantifying PET data by having the sensitivity to account for genetic TSPO predispositions (discussed in more detail below)[136] and smaller changes in neuroinflammation.[135] TSPO was originally called the 'peripheral benzodiazepine receptor[131]' because it was identified by benzodiazepine binding but was renamed to acknowledge its many potential functions and location in the central nervous system (as well as in the periphery).[137] Specific binding of DAA1106 correlates with the presence of activated microglia identified by immunohistochemistry in situ[135] and immunohistochemistry combined with autoradiography in brain tissue.[138]

PET studies using [11C]DAA1106 and similar radiotracers have examined a range of conditions thought to be associated with neuroinflammation. This method was used recently to demonstrate increases in radiotracer binding in patients with Alzheimer's disease[139-144], Lewy body dementia[145], amyotrophic lateral sclerosis[146], stroke[147], and non-smokers with major depression[148], but not Parkinson's disease[149] or normal aging[150]. Increases in this marker have also been demonstrated in animal models of brain injury[134, 151-153] and stroke[154], along with subsequent normalization with time after a brain insult[152, 154, 155]. In contrast, a decrease in the marker for neuroinflammation was found with administration of propofol anesthesia (Hines et al, 2013).

Over the past 30+ years, a large body of research has addressed the effects of cigarette smoking on inflammation in the body[126]. A driving force behind this research is the known impairment of wound healing by smoking. Comprehensive literature reviews have

recommended preoperative and postoperative abstinence periods of >4 weeks in smokers undergoing surgical procedures[157, 158]. Though the mechanism by which smoking impairs wound healing has not been fully elucidated, cigarette smoke contains >250 toxins, many of which are known to affect healing[158], and studies of laboratory animals exposed to cigarette smoke have demonstrated significant alterations (both decreases and increases) in markers of neuroinflammation[159]. Reviews of this literature indicate that the inflammatory healing response is attenuated in smokers by reduced inflammatory cell chemotactic responsiveness, diminished migratory function, and increased oxidative stress.[160, 161]

In the absence of studies directly examining the effect of human cigarette smoking on neuroinflammation in vivo, we used PET scanning to determine whether cigarette smokers have altered binding of [11C]DAA1106, a marker for neuroinflammation, compared with non-smokers. We hypothesized that non-smoker vs smoker effects would occur globally throughout the brain, as prior research by our group[162-165] and others[166, 167] demonstrates widespread effects of smoking when studying systems (eg, the nicotinic cholinergic system) that are widely distributed. We also sought to examine the effect of menthol, as menthol cigarette smoking is common (~1/3 of US smokers) (SAMHSA, 2009) and menthol smokers have more difficulty quitting in standard treatment programs[168-170], elevated serum nicotine/cotinine/exhaled carbon monoxide (CO) levels (in some[171], but not all [172, 173], studies), and more severe upregulation of brain nicotinic acetylcholine receptors[165] when compared with non-menthol cigarette smokers. Therefore, we also hypothesized that effects of smoking on [11C]DAA1106 binding would be greater in menthol than in non-menthol smokers.

MATERIALS AND METHODS

Forty-five participants (30 smokers and 15 non-smokers) completed the study and had usable data. These participants underwent telephone and in-person screening, a bolus [11C]DAA1106 PET scanning session, blood draws during PET to determine TSPO affinity genotype and plasma nicotine (and metabolite) levels, and a structural magnetic resonance imaging (MRI) scan, as described below. An additional 6 participants underwent PET scanning but were excluded due to genotype (n=4, see below) or technical PET scanning issues (n=2) (Figure 11).

Participants were veterans who were recruited through Internet (eg, Craigslist) advertisements and posted flyers. Inclusion criteria were: (1) healthy adult (18–65 years) cigarette smokers (10–40 cigarettes per day) who met DSM-IV criteria[174] for Nicotine Dependence or non-smokers (<100 cigarettes lifetime and none within the past year), (2) smoking primarily (>80%) either menthol or non-menthol cigarettes (for the smoker group), (3) ability to read, write, and give voluntary informed consent, and (4) an exhaled CO ⩾ or <8 ppm (and urine cotinine ⩾ or <200 ng/ml) during the study screening visit to support smoking or non-smoking status, respectively. Exclusion criteria

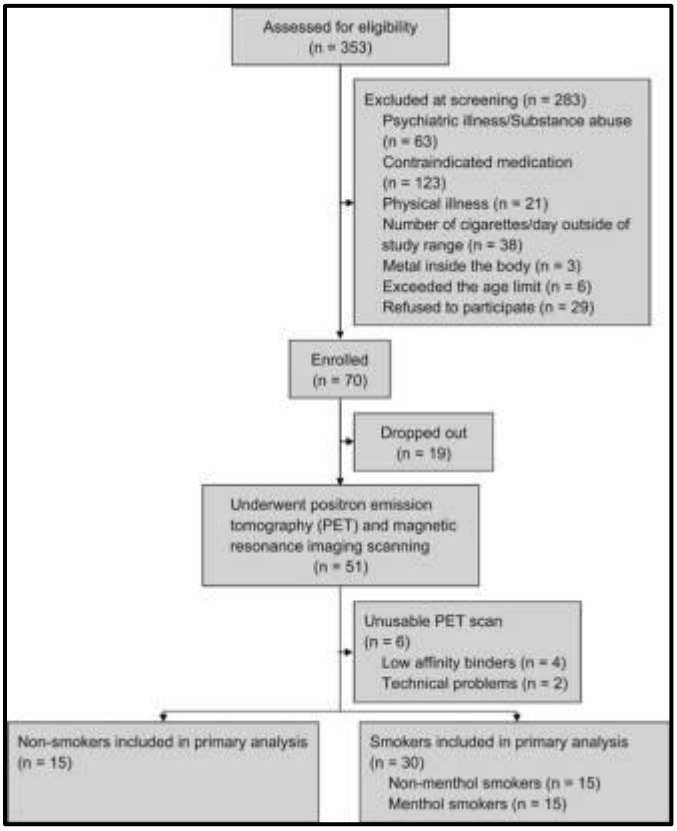


Figure 3-1. Flow diagram showing the number of potential and actual participants at each step of the study, including reasons for potential participants being screened out of participation.

were: (1) any Axis I diagnosis (including mood, anxiety, psychotic, and substance abuse disorders) within the past year, (2) any current medication or history of a medical condition that might affect the central nervous system at the time of scanning (eg, current treatment with a psychotropic medication or history of severe head trauma with loss of consciousness, epilepsy, or other neurological diseases), (3) regular use (>1 × /week) of anti-inflammatory medication, such as steroidal or non-steroidal anti-inflammatory medications (eg, corticosteroids, ibuprofen, naproxen, aspirin, or celecoxib (Celebrex)), (4) unstable cardiovascular disease, severe liver disease, or renal insufficiency, which might make tolerating study procedures difficult, or (5) pregnancy. Occasional drug/alcohol use not meeting criteria for abuse or dependence was not exclusionary, but participants were instructed to abstain from drug/alcohol use for at least 48 h prior to PET scanning.

For the telephone screening, a thorough smoking history, including age of first cigarette, maximum smoking habit, menthol or non-menthol cigarette use, length and dates of abstinence periods, previous treatments used, and current smoking habit, was obtained. A brief medical, psychiatric, and substance use history was also obtained during the telephone screening. During a subsequent in-person visit, eligibility criteria were confirmed and general demographics, smoking history, and symptom ratings were obtained with screening questions from the SCID for DSM-IV, the Smoker's Profile Form, the Fagerström Test for Nicotine Dependence (FTND)[175, 176] (to assess severity of Nicotine Dependence), Shiffman–Jarvik Withdrawal Scale (SJWS)[177] (to measure craving and withdrawal), and Spielberger State Trait Anxiety Index (STAI)[178] and Beck Depression Inventory (BDI)[179] (to confirm the absence of potentially confounding psychiatric symptoms). A brief medical review of systems and chart review were also performed by a study physician (ALB or MSM), along with an exhaled CO measurement (Micro+ Smokerlyzer Breath CO Monitor; Bedfont Scientific, UK), urine cotinine

screen (The Accutest NicAlert; Jant Pharmacal, Encino, CA), breathalyzer (AlcoMatePro), urine toxicology screen (Test Country I-Cup Urine Toxicology Kit), and urine pregnancy test (Test Country Cassette Urine Pregnancy Test) to verify inclusion/exclusion criteria.

Participants meeting inclusion/exclusion criteria who wished to participate underwent a [11C]DAA1106 PET scanning session 1 week later, using a procedure similar to the one developed in previous studies.[144, 180, 181] At 1400 hours on the day of PET scanning, participants arrived at the VA Greater Los Angeles Healthcare System PET Center and underwent a brief clinical interview, breathalyzer, and urine cotinine, toxicology, and pregnancy screens, in order to verify continued meeting of inclusion/exclusion criteria (including confirmation of reports of drug abstinence at the time of scanning). From 1430 to 1445 hours, smokers smoked to satiety (2–3 cigarettes, favorite brand) in an outdoor area adjacent to the PET center. From 1445 to 1500 hours, participants were positioned on the PET scanner and a venous line was placed. At 1500 hours, participants received a bolus injection of 377 (±62) MBq of [11C]DAA1106 and underwent dynamic PET scanning of the brain for the next 90 min. PET scans were obtained using the Philips Gemini TruFlight PET Scanner (Koninklijke Philips Electronics N.V., Eindhoven, The Netherlands). [11C]DAA1106 was prepared by an established method (Wang et al, 2012). An investigational new drug (IND) approval from the Food and Drug Administration (IND 122041) was obtained to use the radiotracer [11C]DAA1106 for the study described here.

A 5-ml blood sample was drawn prior to the initiation of PET scanning for genotyping of each individual's TSPO affinity subtype (high [C/C], medium [C/T], or low [T/T]), because these affinity subtypes have been shown to affect radiotracer binding for all currently used radiotracers determining TSPO availability.[136, 182, 183] For this sample, venous blood was drawn via a port in the catheter placed for radiotracer injection. Genomic DNA was extracted from whole blood

using the QiaAmp DNA Blood Mini Kits (Qiagen, Valencia, CA) by study collaborators (EN and LS) and TSPO single-nucleotide polymorphism (rs6971) genotyping using the TaqMan Allelic Discrimination (Thermo Fisher Scientific, Canoga Park, CA) platform was performed in duplicate, according to the manufacturer's specified protocol. Quality control was ensured by perfect concordance of replicate samples, expected minor allele frequencies, and adherence to Hardy–Weinberg equilibrium. Only scans from participants with the high- or medium-affinity genotypes (known to be >90% of North Americans;[184]) were included in study analyses in order to avoid a potential confound. The exclusion of low-affinity binders from data analysis is standard practice in recent research in this field.[146, 149, 185, 186]

In addition, blood samples were drawn 10 and 60 min after the initiation of PET scanning for determination of plasma nicotine/cotinine levels. Afternoon plasma cotinine has been shown to be a good measure of nicotine exposure for the past 24 h[187]. Samples were centrifuged to obtain plasma, packed on dry ice, and shipped to the Clinical Pharmacology Laboratory at the University of California, San Francisco for assay by gas chromatography by Peyton Jacob and colleagues.

One week after the PET scanning session, an MRI scan of the brain was obtained on a 3.0-T scanner (Signa; GE Medical Systems, Milwaukee, WI) in order to aid in localization of regions on the PET scans. The MRI had the following specifications: three-dimensional Fourier-transform spoiled-gradient-recalled acquisition with TR=30 ms, TE=7 ms, 30-degree angle, 2 acquisitions, and 256 × 192 view matrix. The acquired volume was reconstructed as roughly 90 contiguous 1.5-mm thick transaxial slices.

As in previous research by our group[162, 188-190] , MRI/PET co-registration was performed using the Statistical Parametric Mapping software (FIL Methods Group, UK), and automated volumes of interest (VOIs) were determined on MRI using FSL tools for structural MRI. These automated VOIs were transferred from each participant's MRI to his/her co-registered PET scan and visually inspected using PMOD (PMOD Technologies, Zurich, Switzerland). The primary VOI was whole brain (including gray and white matter) for reasons cited in the Introduction section. However, as automated volumes are easily attained and regional differences are possible, VOIs were also determined for the amygdala, caudate, hippocampus, nucleus accumbens, putamen, and thalamus, similar to VOIs obtained in prior research (Takano et al, 2010; Yasuno et al, 2012).

In order to obtain a quantitative measurement of VOI binding to TSPO in the brain, standardized uptake values (SUVs) were calculated using the standard definition of SUV=mean tissue activity concentration (Bq/ml)/(injected dose (Bq)/body weight (g)). Mean tissue activity concentration from 20 to 40 min postinjection was used, based on time activity curves demonstrating stable activity during this time period. SUV was used as the primary outcome measure because it avoids invasive arterial blood sampling and has been shown to strongly correlate with total volume of distribution (Vt) values[191, 192], has good test–retest reproducibility, and has less intersubject variability than Vt for a similar radiotracer.

For statistical analysis of data, an analysis of variance (ANOVA) was performed, with whole-brain SUV as the measure of interest and both group (smokers vs non-smoker) and TSPO genotype (mixed or high affinity) as between-subject factors[141, 142]. To determine whether group differences were due to differences in particular brain regions, a multivariate ANOVA (MANOVA), using the smaller automated VOIs, was performed with the same structure as the

preceding ANOVA, followed by univariate ANOVAs for the individual VOIs. To quantify between-group differences, percentage of difference was calculated as: 100 × (SUVnon-smokers−SUVsmokers)/SUVnon-smokers. Based on prior research reporting greater brain exposure to cigarette smoke in menthol than in non-menthol cigarette smokers, we also performed an ANOVA for whole-brain SUV with the same structure as the above test, using non-smoker vs menthol vs non-menthol cigarette preference as a between-subject factor. As an exploratory analysis, linear analyses were performed for the smoker group, with whole-brain SUV value as the dependent variable and independent variables related to smoking, controlling

Variable	Non-smoker group (*n*=15)	Whole smoker group (*n*=30)	Non-menthol smoker subgroup (*n*=15)	Menthol smoker subgroup (*n*=15)
Age, years	47.6 (±13.8)	52.1 (±8.1)	49.9 (±8.4)	54.4 (±7.4)
Sex (% female)	26.7	20.0	20.0	20.0
Race/ethnicity (%)				
African American	26.7	46.7	33.3	60.0
Asian	26.7	10.0	6.7	13.3
Hispanic	26.7	13.3	13.3	13.3
White	20.0	30.0	46.7	13.3
Height (inches)	68.9 (±4.0)	68.2 (±4.0)	68.3 (±4.7)	68.2 (±3.5)
Weight (kg)	88.1 (±23.4)	84.0 (±16.2)	83.9 (±17.9)	84.1 (±14.9)
Cigarettes per day	0 (±0)	13.9 (±3.8)	13.5 (±3.8)	14.4 (±3.9)
Exhaled carbon monoxide (ppm)	1.6 (±0.6)	13.3 (±4.7)	13.0 (±4.2)	13.5 (±5.2)
Fagerström Test for Nicotine Dependence (FTND)	0 (±0)	4.0 (±2.3)	4.1 (±2.1)	3.9 (±2.4)
Beck Depression Inventory	1.0 (±1.3)	1.7 (±2.3)	1.3 (±1.8)	2.1 (±2.8)
State Trait Anxiety Inventory	58.5 (±15.0)	66.7 (±17.8)	68.3 (±18.1)	65.1 (±18.0)
Caffeine use (coffee cup equivalents/day)	1.1 (±1.3)	2.0 (±1.6)	2.0 (±1.7)	1.9 (±1.5)
Alcohol drinks per day	0.6 (±1.4)	1.0 (±2.1)	0.7 (±1.6)	1.3 (±2.5)

Marijuana cigarettes per week	0.0 (±0.0)	0.3 (±1.3)	0.5 (±1.8)	0.1 (±0.4)

for TSPO genotype. Statistical tests were performed using the statistical software program SPSS/PASW version 24 (SPSS, Chicago, IL).

RESULTS

Table 3-1. Baseline Demographics and Rating Scale Scores for the Non-Smoker and Smoker Groups All values are presented as means (±SD) or percentages. Using χ^2 tests for categorical variables and Student's *t*-tests for continuous variables, no between-group (or between-subgroup) tests were significant, other than differences in measures of smoking (cigarettes per day, exhaled carbon monoxide, and FTND scores) between the smoker groups/subgroups and the non-smoker group (all *P*-values<0.0005).

Study groups had no significant differences in age, sex, race/ethnicity, height, weight, depression/anxiety levels, or caffeine, alcohol, or marijuana use (Table 3-1). On average, the groups were middle-aged, mostly male, and had generally low levels of depression/anxiety and drug/alcohol use. No significant between-group differences were present for body weight or injected dose of radiotracer, which were used to calculate SUV.

Brain region	**SUV values—non-smokers (*n*=15)**	**SUV values—smokers (*n*=30)**	**SUV values—non-menthol smoker subgroup (*n*=15)**	**SUV values—menthol smoker subgroup (*n*=15)**
Whole brain	0.20 (±0.03)	0.17 (±0.04)	0.18 (±0.04)	0.16 (±0.02)
Accumbens				
R	0.20 (±0.03)	0.17 (±0.04)	0.17 (±0.04)	0.16 (±0.03)
L	0.21 (±0.03)	0.17 (±0.04)	0.17 (±0.04)	0.16 (±0.03)
Amygdala				
R	0.18 (±0.03)	0.15 (±0.04)	0.16 (±0.04)	0.13 (±0.02)

L	0.17 (±0.03)	0.15 (±0.04)	0.16 (±0.04)	0.14 (±0.02)
Caudate				
R	0.18 (±0.04)	0.14 (±0.03)	0.15 (±0.03)	0.14 (±0.02)
L	0.18 (±0.03)	0.15 (±0.03)	0.15 (±0.03)	0.14 (±0.02)
Hippocampus				
R	0.19 (±0.03)	0.16 (±0.03)	0.17 (±0.04)	0.15 (±0.02)
L	0.19 (±0.03)	0.15 (±0.03)	0.16 (±0.04)	0.14 (±0.02)
Putamen				
R	0.23 (±0.03)	0.19 (±0.05)	0.21 (±0.06)	0.18 (±0.03)
L	0.23 (±0.03)	0.19 (±0.04)	0.20 (±0.05)	0.18 (±0.03)
Thalamus				
R	0.22 (±0.04)	0.19 (±0.04)	0.20 (±0.04)	0.18 (±0.03)
L	0.22 (±0.03)	0.18 (±0.04)	0.19 (±0.04)	0.17 (±0.03)

Table 3-2. Standardized Uptake Values (SUVs) for the Whole Brain and Smaller Regions of Interest for Non-smokers and Smokers (and the Non-Menthol Smoker and Menthol Smoker Subgroups) Abbreviations: L=left; R=right. All values are mean±SD. All regions were analyzed using analysis of variance, with group (non-smoker vs smoker or non-smoker vs non-menthol smoker vs menthol smoker) and genotype as between-subject factors. All regions were significant for the non-smoker vs smoker comparison at the P≤0.05 level, except for the right and left accumbens, which approached significance (P-values=0.08 and 0.06, respectively). Similarly, all regions were significant for the three group comparisons (non-smoker vs non-menthol smoker vs menthol smoker) at the P≤0.05 level. The automated regions listed here were generated using the FSL toolkit.

PET data analysis comparing smokers and non-smokers revealed a significant effect of group for whole-brain SUV values (ANOVA, F=8.3; df=1,41; P=0.006), due to smokers having mean

16.8% lower values than non-smokers (Table 3-2). Consistent with this global finding, in the analysis of the smaller VOIs, a significant multivariate effect of group was found (MANOVA; F=2.8, df=12,30; P=0.01), with all VOIs having a significant (or trend-level) between-group effect on univariate analysis (Table 3-2), owing to smokers having lower SUV values than non-smokers (range 14.6–19.7%) in all VOIs studied.

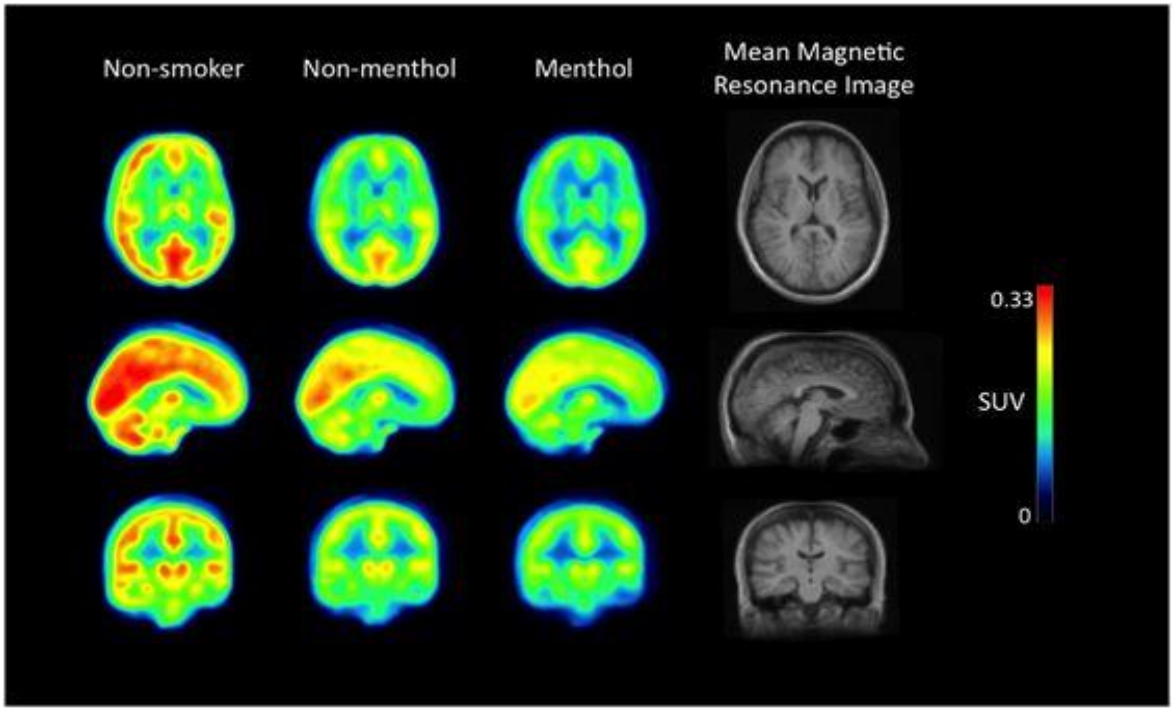


Figure 3-2. Mean positron emission tomography (PET) images from the study subgroups (non-smokers, non-menthol cigarette smokers, and menthol cigarette smokers) demonstrating higher [11C]DAA1106 standardized uptake values (SUVs) for non-smokers than the two smoker subgroups. The first three columns consist of mean SUV PET images (transaxial, sagittal, and coronal from top to bottom) for the three study groups/subgroups (n=15 each) and the far right column shows the group mean magnetic resonance image. For this figure representation of study results, the PET scans were spatially normalized into standard Montreal Neurological Institute (MNI) template space.

For the three-group comparison (non-smokers vs non-menthol cigarette smokers vs menthol cigarette smokers), the whole-brain SUV comparison was significant (ANOVA, F=6.1; df=2,39; P=0.005), owing to a range of values from non-smokers (highest) to non-menthol cigarette smokers (middle) to menthol cigarette smokers (lowest) (Table 3-2). In the multivariate analysis

of smaller VOIs, a significant effect of group was found (MANOVA; F=1.8, df=24,56; P=0.03), with all VOIs having a significant between-group effect, owing to the range (from high to low) of SUV values from smokers to non-menthol smokers to menthol smokers (Table 3-2). In comparing only the non-menthol with the menthol cigarette smokers, the whole-brain SUV comparison did not reach significance (ANOVA; F=3.6; df=1,26; P=0.07), and similar results were found for the smaller VOIs (ANOVAs; Ps=0.03–0.21), possibly owing to the smaller samples used for comparing the non-menthol with the menthol cigarette smoker subgroups.

In the exploratory analysis of smoking-related variables, a significant relationship was found between cigarettes per day and whole-brain SUV (F=6.3; P=0.02), indicating that higher levels of reported smoking were associated with lower levels of TSPO availability. Similarly, a significant relationship between the stimulation subscale scores of the SJWS and whole-brain SUV was also found (F=5.6; P=0.03), indicating that higher levels of withdrawal stimulation were associated with lower levels of TSPO availability. No significant associations were found for FTND scores, CO levels, plasma nicotine/cotinine levels, or other subscales on the SJWS.

DISCUSSION

Cigarette smokers have less [11C]DAA1106 binding than non-smokers throughout the brain, indicating less TSPO availability. Though several explanations for this finding are possible, a straightforward one is that smoking results in global impairment of microglial activation. This explanation is consistent with much prior research demonstrating that smokers have impaired inflammatory functioning in other parts of the body, which leads to compromised wound healing[126, 156]. Furthermore, the inverse correlation between [11C]DAA1106 binding and

participant reports of cigarette use per day indicates that the severity of impaired microglial activation may be related to the amount of current cigarette usage. Of note, the fact that study results were global (rather than regional) is also consistent with prior research demonstrating widespread effects of smoking on brain receptors.[162, 163, 165-167, 190] These global effects of smoking are in line with known properties of cigarette smoke, namely, that it rapidly enters the body and brain due to high permeability through lung, vasculature, and brain cells.[193] Taken together, study results may demonstrate a significant widespread brain abnormality in smokers in the satiated state.

The negative association between SUV values and cigarettes per day, but not plasma nicotine levels (or other measures of smoking behavior), may indicate that components of cigarette smoke other than nicotine are responsible for the low level of microglial activation found here. Laboratory studies support this theory, with several studies demonstrating that whole tobacco smoke administration results in greater alterations in inflammatory markers than nicotine alone.[194, 195] However, given the evidence that nicotine indeed impairs[196, 197] or attenuates[198] some inflammatory processes, and the relatively small sample of smokers studied here in the correlational analysis, the exact relationship between nicotine and neuroinflammation in human smokers remains to be confirmed.

Although impairment of neuroinflammation by smoking is a straightforward explanation of the study results, other explanations are possible, given the complex effects of cigarette smoking on the brain. Cigarette smoke contains thousands of constituents,[199] with hundreds having known toxic effects. [200, 201] It is possible that one or more of these constituents directly interfered with [11C]DAA1106 binding to TSPO, which would have resulted in the difference in binding between smokers in the satiated state and non-smokers found here. Additionally, acute smoking

is known to disrupt blood–brain barrier function,[202] which could have created differences in radiotracer binding for smokers and non-smokers for the PET time period of interest used here.

In addition to the overall difference between smokers and non-smokers, the menthol cigarette smoker subgroup had less [11C]DAA1106 binding than the non-menthol cigarette smoker subgroup. This finding is consistent with prior research by our group[165] showing greater upregulation of nicotinic acetylcholine receptors throughout almost all brain regions in menthol than in non-menthol cigarette smokers. Also, research by others demonstrates that menthol cigarette smoking is associated with more severe biological abnormalities in some[171], but not all,[172, 173] studies that have examined this issue. Therefore, as in prior research, the present finding may be due to greater brain exposure to cigarette smoke (leading to greater impairment of microglial activation) in menthol cigarette smokers, a direct effect of menthol flavoring, or some other mechanism.

The primary limitation of this study was the absence of arterial blood sampling such that total distribution volume (Vt) was not ascertained. Vt may control for the potential confounds of between-subject differences in radiotracer metabolism and binding to vascular endothelium and plasma protein.[149, 203, 204] Although Vt is a common outcome measure in PET studies examining TSPO in conditions other than tobacco dependence,[205-207] recent research demonstrates that the less invasive SUV measure tends to correlate well with Vt within individual PET studies[191, 208] and has high test–retest reliability[209]. Other similar studies have used pseudo-reference regions for PET data analysis[146, 205, 210-213] to minimize potential confounds, but this method would not have been appropriate here due to the hypothesized and confirmed effect of smoking throughout the brain. Additional limitations included a modest sample size and the fact that smokers were scanned in the satiated state, such that we did not determine whether results

were due to acute or chronic cigarette smoking. Future research could examine smokers in the abstinent state to determine the relationship between decreased [11C]DAA1106 binding and recency of smoking.

In summary, cigarette smokers in the satiated state have decreased TSPO availability, which is related to participants' current smoking level (higher levels of smoking were associated with less TSPO availability). This effect appeared to be greater for menthol than for non-menthol cigarette smokers. Future research could examine the time course of recovery of TSPO availability upon smoking cessation and the interplay between smoking, neuroinflammation, and the progression of diseases thought to be mediated by neuroinflammation.

PD Case Study #2: Effect of overnight smoking abstinence on a marker for microglial activation: a [11C]DAA1106 positron emission tomography study.*

**This study is currently published in Psychopharmacology.*[214]*

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INTRODUCTION

Microglia are the main innate immune cells in the central nervous system (CNS).[215] Under homeostatic conditions, they continuously monitor the surrounding environment for signs of infection or homeostasis-perturbing events.[216, 217] Microglia react to counteract such perturbations in order to protect neurons, which have a limited capacity to regenerate, leading to elevated levels of activated microglia in neurodegenerative diseases.[218, 219] In this context, activated microglia participate in various functions, such as clearance of apoptotic cells and extracellular pathogens, removal of degenerating neurons and extracellular proteins, and cytokine/chemokine production.[128] When activated, microglial cellular morphology changes and expression of the translocator protein (TSPO) 18 kDa is increased, thereby making expression of TSPO a marker for neuroinflammation.

Epidemiological studies have shown that cigarette smokers have a lower risk of neurodegenerative diseases than the general population,[220, 221] and much research has examined the mechanism by which smoking could diminish neuroinflammation, which contributes to neurodegenerative damage. Extensive pre-clinical work has shown that nicotine

and/or other agonists at nicotinic acetylcholine receptors (nAChRs) protect against neuronal cell damage[222] via binding to α7 nAChRs and inhibition of microglial activation.[220, 223-225]

The radioligand N-(2,5-dimethoxybenzyl)-N-(5-fluoro-2-phenoxyphenyl) acetamide labeled with carbon-11 ([11C]DAA1106) has emerged as a reliable second-generation radiotracer for labeling TSPO[129-131] with high affinity[132, 133, 135, 138] for positron emission tomography (PET) scanning in vivo. Specific binding of DAA1106 is reported to be greater than previously used ligands (e.g., PK11195) but less than at least one recently developed ligand (PBR28). [136] Specific binding of DAA1106 correlates with the presence of activated microglia identified by immunohistochemistry in situ[135] and immunohistochemistry combined with autoradiography in brain tissue.[138] [11C]DAA1106 was chosen for use here because of these favorable properties and previous experience by our group with this radiotracer.[125, 226]

Our group recently used [11C]DAA1106 with PET scanning to compare smokers who had smoked to satiety (~ 15 min prior to scanning) with nonsmoking controls.[125] The groups differed in whole brain standardized uptake values (SUVs) for the radiotracer, with smokers having 16.8% lower values than nonsmokers (and lower mean SUVs in menthol- than nonmenthol-cigarette smokers). Smokers also had lower SUVs (by 14.6–19.7%) in a range of smaller brain volumes of interest. In addition, whole-brain SUV was negatively correlated with participant-reported cigarettes per day. These study findings were consistent with much prior research demonstrating that smokers have less inflammatory functioning than nonsmokers.

For the study presented here, we sought to determine if the reduction in [11C]DAA1106 SUV (the marker for neuroinflammation) found in satiated smokers was still present in early (overnight) smoking abstinence. In order to study smokers in a state with potentially significant

differences from nonsmoking controls, overnight (> 12 h) abstinence from smoking was chosen as the time point of interest because nicotine would be expected to be recently eliminated from the body at that time (plasma half-life of ~ 2 h;[124] and withdrawal symptoms (e.g., urge to smoke and anxiety/irritability) would be expected to be elevated.[227, 228] As in our prior study, we hypothesized that smoker vs. nonsmoker effects would occur globally, based on prior research demonstrating that cigarette smoke is rapidly absorbed[229] and results in saturation (or near saturation) of nicotinic acetylcholine receptors throughout the brain.[162, 163, 190, 230] We also sought to examine the effect of menthol, because menthol smoking is common (~ 1/3 of US smokers) (SAMHSA 2009), menthol smokers have more trouble quitting in standard treatment programs than nonmenthol-cigarette smokers,[157, 168, 169] menthol smoking has been found to lead to elevated serum nicotine/cotinine and exhaled carbon monoxide levels[171] (though not all studies agree on this point;[231, 232] and menthol smokers had lower mean [11C]DAA1106 SUVs than nonmenthol smokers in our prior study of smokers in satiety.[125]

METHOD

Forty participants (22 cigarette smokers and 18 nonsmokers) completed the study and had usable data. These participants underwent telephone and in-person screening, overnight smoking abstinence prior to the PET session (for the smoker group), a bolus [11C]DAA1106 PET scanning session, a blood draw to determine TSPO affinity genotype, and a structural magnetic resonance imaging (MRI) scan, as described below. Four additional participants were enrolled, but were excluded because they were homozygous for the low affinity TSPO genotype. All participants provided written informed consent on forms approved by the Institutional Review Boards of either the VA San Diego Healthcare System or VA Greater Los

Angeles Healthcare System. A subset of the nonsmoker group in the analysis presented here consisted of participants who were included in our previous report (n = 13).

Participants were recruited through posted flyers and the Internet (e.g., Craigslist). Inclusion criteria were healthy adult (18 to 65 years old) daily cigarette smokers (range 2–25 cigarettes/day and evidence of tobacco dependence on the Fagerström test for nicotine dependence (FTND)) or nonsmokers (< 100 cigarettes lifetime and none within the past year), smoking primarily (> 80%) either menthol or nonmenthol cigarettes (for the smoker group), ability to give voluntary informed consent, and an exhaled CO > or < 8 ppm (and urine cotinine > or < 200 ng/mL) during the study screening visit to support smoking or nonsmoking status, respectively. Exclusion criteria were any psychiatric diagnosis (including mood, anxiety, psychotic, and substance abuse disorders other than tobacco use disorder) within the past 6 months; any current medication or history of a medical condition that might affect the central nervous system at the time of scanning (e.g., current treatment with a psychotropic medication, or history of severe head trauma with loss of consciousness, epilepsy, or other neurological diseases); regular use (> 1×/week) of anti-inflammatory medication, such as steroidal or nonsteroidal anti-inflammatory medications (e.g., corticosteroids, ibuprofen, naproxen, aspirin, or celecoxib (Celebrex®)); unstable cardiovascular disease, severe liver disease, or renal insufficiency, which might make tolerating study procedures difficult; or pregnancy. Occasional drug/alcohol use not meeting criteria for abuse or dependence was not exclusionary, but participants were instructed to abstain from drug/alcohol use for at least 48 h prior to PET scanning.

For the telephone screening, a thorough smoking history, including age of first cigarette, maximum smoking habit, menthol- or nonmenthol-cigarette use, length and dates of abstinence

periods, previous treatments used, and current smoking habit, was obtained. A brief medical, psychiatric, and substance use history was also obtained. During a subsequent in-person visit, eligibility criteria were confirmed and general demographics, smoking history, and symptom ratings were obtained with the FTND[175, 176] (to assess severity of nicotine dependence), Spielberger State Trait Anxiety Index (STAI)[178] , and Beck Depression Inventory (BDI)[179] (to confirm the absence of potentially confounding psychiatric symptoms) for all participants, and Smoker's Profile Form and Shiffman–Jarvik Withdrawal Scale (SJWS)[177] (to measure craving and withdrawal) for participants scanned at UCSD. A brief medical review of systems and chart review were also performed by a study physician, along with an exhaled carbon monoxide (CO) measurement (Micro+ Smokerlyzer Breath CO Monitor; Bedfont Scientific, Ltd., UK), urine cotinine screen (The Accutest® NicAlert™; Jant Pharmacal Corp., Encino, CA), breathalyzer (AlcoMatePro), urine toxicology screen (Test Country I-Cup Urine Toxicology Kit), and urine pregnancy test (Test Country Cassette Urine Pregnancy Test), in order to determine if participants met inclusion/exclusion criteria.

Participants who met inclusion/exclusion criteria and wished to participate underwent a [11C]DAA1106 PET scanning session 1 week later at either the University of California at San Diego (UCSD) Center for Molecular Imaging (n = 15; 10 overnight abstinent smokers and 5 nonsmokers) or VA Greater Los Angeles Healthcare System (VAGLAHS) PET Center (n = 25; 12 overnight abstinent smokers and 13 nonsmokers), using a procedure similar to the one developed in previous studies.[143, 144, 180, 181] Smokers were instructed to abstain from cigarettes and other nicotine-containing products from prior to midnight on the night before PET scanning. The PET session was initiated in the afternoon with participants undergoing a brief clinical interview, breathalyzer, exhaled CO level, and urine toxicology and pregnancy screens, in order to verify continued meeting of inclusion/exclusion criteria (including drug abstinence at the time

of scanning). An exhaled CO of < 6 ppm was considered consistent with overnight abstinence. Following these procedures, participants were positioned on the PET scanning bed and a venous line was placed. They then received a bolus injection of 350 (± 53) MBq of [11C]DAA1106 and underwent dynamic PET scanning of the brain for the next 90 min. PET scans were obtained using either an ECAT HR+ PET scanner (CTI PET systems, Knoxville, TN) (UCSD) or a Philips Gemini TruFlight PET Scanner (Koninklijke Philips Electronics N.V., Eindhoven, the Netherlands) (VAGLAHS). [11C]DAA1106 was prepared by an established method.[233] All scans consisted of eighteen 5-min frames. For the UCSD scanner, a 20-min transmission scan from a Ge-68 rod source was performed for attenuation correction following the dynamic scan. The reconstruction used a manufacturer's OSEM algorithm with 4 iterations and 16 subsets. For the VAGLAHS scanner, CT scanning was performed for attenuation correction following the dynamic scan, and the manufacturer's RAMLA algorithm was used for image reconstruction. Investigational new drug (IND) approvals from the Food and Drug Administration (INDs 133984 (UCSD) and 122041 (VAGLAHS)) were obtained to use the radiotracer [11C]DAA1106 for the studies described here.

A 5-ml blood sample was drawn prior to the initiation of PET scanning for genotyping of each individual's TSPO affinity subtype (high (C/C), medium (C/T), or low (T/T)), because these affinity subtypes affect radiotracer binding of all currently used radiotracers determining TSPO availability.[136, 182, 183] Genomic DNA was extracted from whole blood using QiaAmp DNA Blood Mini Kits (Qiagen, Valencia, CA) (by E.N. and L.S.) and TSPO single-nucleotide polymorphism (rs6971) genotyping using the TaqMan Allelic Discrimination (Thermo Fisher Scientific, Canoga Park, CA) platform was performed in duplicate, according to the manufacturer's specified protocol. Quality control was ensured by perfect concordance of replicate samples, expected minor allele frequencies, and adherence to Hardy–Weinberg equilibrium. Only scans from

participants with the high- or medium- affinity genotypes, present in > 90% of North Americans,[184] were included in study analyses, in order to avoid a potential confound. The exclusion of homozygous low-affinity binders from data analysis is standard practice in recent research in this field.[146, 149, 185, 186]

Within 1–2 weeks of PET scanning, an MRI scan of the brain was obtained, in order to facilitate localization of regions on the PET scans. At UCSD, high-resolution T1-weighted 3D MRIs were obtained on a Siemens 3 T Skyra scanner (Erlangen, Germany), with the following specifications: TR = 2300 ms, TE = 3 ms, 9° angle, 2 acquisitions, and 160 × 256 × 256 matrix. Participants who received PET scans at VAGLAHS had MRI scans with the following specifications: 3 T GE Medical Systems Signa scanner (Milwaukee, WI) three-dimensional Fourier-transform (3DFT) spoiled-gradient-recalled acquisition with TR = 30 ms, TE = 7 ms, 30° angle, 2 acquisitions, and 256 × 192 view matrix.

As in previous research by our group, MRI/PET co-registration was performed using Statistical Parametric Mapping software (FIL Methods Group, UK), and automated volumes of interest (VOIs) were determined on MRI using FSL tools for structural MRI. These automated VOIs were transferred from each participant's MRI to his/her co-registered PET scan and visually inspected using PMOD (PMOD Technologies Ltd., Zurich, Switzerland). The primary VOI was whole brain (including gray and white matter) for reasons noted above. However, since automated volumes are easily attained and regional differences are possible, VOIs were also determined for the amygdala, caudate, hippocampus, nucleus accumbens, putamen, and thalamus, similar to VOIs obtained in prior research.[144, 181]

In order to obtain semi-quantitative measurements of radiotracer binding to TSPO in brain, SUVs were calculated using the standard definition of SUV = mean tissue activity concentration (Bq/mL)/(injected dose (Bq)/body weight (g)). Mean tissue activity from 20 to 40 min post-injection was used, based on time-activity curves from our previous study demonstrating stable activity during this time period. SUV was used as the primary outcome measure because it avoids invasive arterial blood sampling.

For the statistical analysis of data, demographic and rating scale variables were compared between groups using Student t tests for continuous variables and Chi-square tests for categorical variables, in order to determine if groups were similar on these variables. For the central study analysis, an analysis of covariance (ANCOVA) was performed, with whole-brain SUV as the measure of interest and both group (overnight abstinent smoker vs. nonsmoker) and TSPO genotype (heterozygous or homozygous for the high affinity allele) as between-subject factors.[142, 148] Research site (UCSD vs. VAGLAHS) was included in the model as a nuisance covariate, in order to control for possible systematic differences in SUVs related to the different PET scanners or other differences in methodology at the two sites (which were somewhat, but not significantly, different). To determine if group differences were due to regional effects, a multivariate ANCOVA (MANCOVA), using the smaller automated VOIs, was performed with the same structure as the preceding ANCOVA, followed by univariate ANCOVAs for the individual VOIs. The use of an overall MANCOVA for determination of main effect of group (nonsmoker vs. overnight abstinent smoker) controls for multiple comparisons. For the smaller VOIs, means of left and right SUVs were used. For descriptive purposes, percent difference between study groups for both sites was calculated as: 100 * (SUVnonsmokers − SUVsmokers) / SUVnonsmokers. Based on prior research reporting greater brain exposure to cigarette smoke in menthol- than nonmenthol-cigarette smokers, we also

performed an ANCOVA for whole brain SUV with the same structure as the above test, using nonsmoker vs. menthol- vs. nonmenthol-cigarette preference as a between-subject factor, followed by the same analyses for smaller automated VOIs as for the preceding analysis. For the exploration of associations between PET data and smoking-related behavioral symptoms, correlations were determined for the smoker group between whole brain SUV and smoking-related ratings (for smokers with completed rating scales), while controlling for genotype. For exploratory associations between SUVs and demographic variables, correlations or tests for associations with the same structure as in the preceding analyses were performed for age, sex, and race/ethnicity for the whole study sample. Statistical tests were performed using the statistical software program SPSS version 24 (SPSS Inc., Chicago, IL).

RESULTS

The nonsmoker and overnight abstinent smoker groups did not differ significantly in age ($P = 0.24$), sex ($P = 0.71$), race/ethnicity ($P = 0.50$), height ($P = 0.18$), weight ($P = 0.25$), depression ($P = 0.16$) or anxiety ($P = 0.94$) levels, or caffeine ($P = 0.23$), alcohol ($P = 0.27$), or marijuana ($P = 0.29$) use (Table 3-3). On average, participants were middle-aged, mostly male, and had generally low levels of depression/anxiety and drug/alcohol use. On the scan day, participants had low levels of exhaled CO (1.9 ± 0.8 ppm for the nonsmoker group and 3.3 ± 1.4 ppm for smokers), and smokers had a mean of 17.9 ± 2.3 h abstinence at the time of radiotracer injection (due to overnight abstinence and availability of PET scanning for research at our institutions in the mid-to-late afternoon).

Variable	Nonsmoker group (*n* = 18)	Whole smoker group (*n* = 22)	Nonmenthol-smoker subgroup (*n* = 16)	Menthol-smoker subgroup (*n* = 6)
Age	46.9 (± 13.4)	41.9 (± 13.2)	41.5 (± 12.6)	43.0 (± 16.7)
Sex (% female)	22.2	27.3	25.0	33.3
Race/ethnicity (%)				
African American	22.2	36.4	37.5	33.3
Asian	22.2	9.1	12.5	0
Hispanic	22.2	13.6	12.5	16.7
White	33.3	40.1	37.5	50.0
TSPO genotype (% high affinity)	83.3	77.2	87.5	50.0
Height (in.)	69.7 (± 4.2)	68.0 (± 3.4)	68.5 (± 3.5)	66.6 (± 2.6)
Weight (kg)	89.8 (± 23.8)	82.3 (± 16.5)	86.8 (± 15.8)	70.1 (± 12.1)
Cigarettes/day	0 (± 0)	11.2 (± 6.7)	11.1 (± 6.7)	11.7 (± 7.3)
Exhaled carbon monoxide (ppm) at screening visit	1.7 (± 0.7)	12.7 (± 9.0)	11.6 (± 8.7)	15.7 (± 9.6)
Fagerström Test for Nicotine Dependence (FTND)	0 (± 0)	4.3 (± 2.9)	4.6 (± 3.0)	3.5 (± 2.6)
Beck Depression Inventory	1.1 (± 1.4)	2.1 (± 2.9)	2.3 (± 2.8)	1.8 (± 3.5)
Trait Anxiety Inventory	30.9 (± 9.1)	31.2 (± 11.6)	31.4 (± 12.0)	30.4 (± 11.8)

Variable	Nonsmoker group (*n* = 18)	Whole smoker group (*n* = 22)	Nonmenthol-smoker subgroup (*n* = 16)	Menthol-smoker subgroup (*n* = 6)
Caffeine use (coffee cup equivalents/ day)	0.9 (± 1.0)	1.3 (± 1.0)	1.2 (± 1.0)	1.6 (± 1.0)
Alcohol drinks/week	0.2 (± 0.5)	1.0 (± 1.7)	1.2 (± 1.9)	0.6 (± 0.8)
Marijuana cigarettes/week	0.1 (± 0.5)	0.5 (± 1.6)	0.7 (± 1.8)	0.2 (± 0.6)

Table 3-3. Baseline demographics and rating scale scores for the nonsmoker and overnight abstinent smoker groups. All values are presented as means (± standard deviation) or percentages. Using χ^2 tests for categorical variables and Student *t* tests for continuous variables, no between-group (nonsmoker vs. smoker) tests were significant, other than differences in measures of smoking (cigarettes per day, exhaled carbon monoxide, and FTND scores; all *P* values < 0.0005)

PET data analysis comparing overnight abstinent smokers and nonsmokers revealed a significant effect of group for whole brain SUVs (ANCOVA, P = 0.004), due to the overnight abstinent smokers having lower values than nonsmokers. Mean whole brain SUVs were 0.87 for nonsmokers and 0.73 for overnight abstinent smokers at UCSD and 1.00 for nonsmokers and 0.83 for overnight abstinent smokers at VAGLAHS (15.5% lower at UCSD and 17.0% lower at VAGLAHS). Consistent with these global findings, a significant multivariate effect of group was found (MANCOVA, P = 0.002) for the smaller VOIs, with all VOIs having a significant between-group effect on univariate analysis (range of P values < 0.0005 to 0.026), due to overnight-abstinent smokers having lower SUV values than nonsmokers (range 6.8 to 29.5%) in all VOIs studied.

For the three-group comparison (nonsmokers vs. nonmenthol-cigarette smokers vs. menthol-cigarette smokers), the whole brain SUV comparison was significant (ANCOVA, P = 0.001), due to nonsmokers having the highest values, followed by nonmenthol-cigarette smokers, and then menthol-cigarette smokers. In the multivariate analysis of smaller VOIs, a significant effect of group was found (MANCOVA, P < 0.0005), with all VOIs having a significant between-group effect, due to the range (from high to low) of SUV values from smokers to nonmenthol smokers to menthol smokers. In comparing only the nonmenthol- with the menthol-cigarette smokers, the whole brain SUV comparison was significant (ANCOVA, P = 0.02), and similar results were found for the smaller VOIs (ANOVAs; P = 0.02 to 0.07), possibly less highly significant than the overall analysis due to the smaller samples used for comparing the nonmenthol- with the menthol-cigarette smoker subgroups. In comparing results from the two study sites, ratios of smoker to non-smoker SUVs were similar (Table 3-4).

Region	**Ratio of mean SUVs between all overnight abstinent smokers and nonsmokers by site**		**Ratio of mean SUVs between overnight abstinent nonmenthol smokers and nonsmokers by site**		**Ratio of mean SUVs between overnight abstinent menthol smokers and nonsmokers by site**	
	UCSD	**VAGLAHS**	**UCSD**	**VAGLAHS**	**UCSD**	**VAGLAHS**
Whole brain	0.84	0.83	0.92	0.85	0.73	0.71
Accumbens	0.73	0.80	0.80	0.83	0.63	0.69
Amygdala	0.87	0.80	0.95	0.82	0.75	0.69
Caudate	0.85	0.85	0.93	0.88	0.73	0.71
Hippocampus	0.89	0.79	0.97	0.81	0.77	0.70
Putamen	0.73	0.78	0.80	0.80	0.63	0.66

Region	Ratio of mean SUVs between all overnight abstinent smokers and nonsmokers by site		Ratio of mean SUVs between overnight abstinent nonmenthol smokers and nonsmokers by site		Ratio of mean SUVs between overnight abstinent menthol smokers and nonsmokers by site	
	UCSD	VAGLAHS	UCSD	VAGLAHS	UCSD	VAGLAHS
Thalamus	0.70	0.79	0.76	0.80	0.60	0.71

Table 3-4. Ratios of mean standardized uptake values (SUVs) between overnight abstinent smokers and nonsmokers for the two study sites. Ratios of mean standardized uptake values (SUVs) for whole brain and smaller volumes of interest (VOIs) between overnight abstinent smokers and nonsmokers for the two study sites, demonstrating that results from the two sites were consistent with one another. All values were less than 1, indicating lower [11C]DAA1106 binding on positron emission tomography scans for overnight abstinent smokers (and subgroups of smokers based on cigarette type) than nonsmokers for all VOIs and both study sites

UCSD, University of California at San Diego; VAGLAHS, VA Greater Los Angeles Healthcare System

In the exploratory analysis of smoking-related behavioral variables, a significant inverse relationship was found between depth of inhalation of cigarettes (as rated by participants on the smoker's profile form) and whole brain SUV (correlation = −0.74, P = 0.02), suggesting that higher levels of cigarette smoke exposure were associated with lower levels of TSPO availability (Figure 3-3). No significant associations were found for cigarettes per day, FTND, SJWS scores, age, sex, or race/ethnicity.

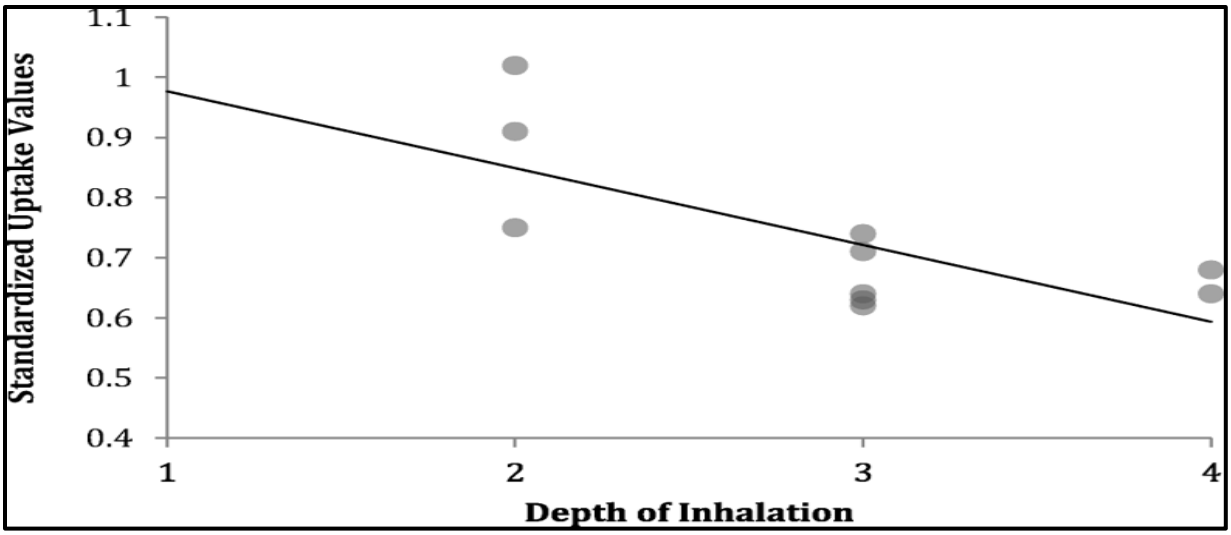


Figure 3-3. Scatterplot showing inverse correlation (− 0.74, P = 0.02) between whole brain standardized uptake values and smokers' ratings of depth of inhalation (for study smokers who filled out this questionnaire). The correlation value controlled for translocator protein genotype (high vs. medium affinity).

DISCUSSION

Cigarette smokers who underwent overnight abstinence had less [11C]DAA1106 binding than nonsmokers throughout the brain, indicating less TSPO availability (and less neuroinflammatory function). Furthermore, menthol-cigarette smokers had lower levels of [11C]DAA1106 binding than nonmenthol-cigarette smokers. These findings are remarkably similar to those of our previous study of smokers in the satiated state, and indicate that chronic cigarette smoking leads to reduced TSPO availability found in satiety and persisting into early (overnight) abstinence. In addition, the global (rather than localized) effect found here is consistent with prior research demonstrating global effects of smoking on other molecules (such as nAChRs) that are located throughout the brain.[28, 163, 165-167, 189, 190]

A straightforward explanation for the central study finding is that chronic cigarette smoking leads to global impairment of microglial activation in early abstinence. This explanation is consistent with much prior research demonstrating that smokers have impaired inflammatory functioning in other parts of the body, an effect that leads to compromised wound healing and lasts for weeks.[126, 156-158] It is also consistent with basic science research demonstrating that microglial cells express nAChRs[225] and that pre-treatment with nicotine (or acetylcholine) inhibits microglial activation. [225, 234, 235] Similarly, in an animal study of autoimmune encephalomyelitis, nicotine administration was found to decrease microglial activation,[198] although other condensate of cigarette smoke was found to increase it. In addition, the inverse correlation in this study between [11C]DAA1106 binding and participant self-reports of depth of inhalation indicates that severity of impaired microglial activation may be related to the amount of exposure to cigarette smoke (since depth of inhalation is known to affect absorbed constituents of tobacco smoke).[124]

Other explanations for study findings are possible. For example, cigarette smoking has been shown to reduce numbers of resident microglial cells,[236] which would presumably lead to less radiotracer binding. Cigarette smokers are also known to have higher metabolism of some medications than nonsmokers,[223] which (if applicable to [11C]DAA1106) would be expected to lessen radiotracer uptake and binding. In addition, cigarette smoking may result in inflammation in parts of the body other than brain,[237] which could lead to sequestration of the radiotracer and less binding in brain.

In addition to the overall difference between smokers and nonsmokers, the menthol cigarette smoker subgroup had less [11C]DAA1106 binding than the nonmenthol-cigarette smoker subgroup. This finding is consistent with prior research by our group showing greater up-

regulation of nicotinic acetylcholine receptors throughout almost all brain regions in menthol- than nonmenthol-cigarette smokers, along with a similar finding for TSPO availability in our prior study with [11C]DAA1106 PET and smokers in satiety. Also, research by others demonstrates that menthol-cigarette smoking is associated with more severe biological abnormalities in some,[171] but not all,[172, 173] studies that have examined this issue. Therefore, as in prior research, the present finding may be due to greater brain exposure to cigarette smoke (leading to greater impairment of microglial activation) in menthol-cigarette smokers, a direct effect of menthol flavoring or some other mechanism.

The primary limitation of this study is the absence of arterial blood sampling, precluding the determination of total distribution volume (Vt), which is the gold standard outcome measure for this type of research. Vt may control for the potential confounds of between-subject differences in radiotracer metabolism and binding to vascular endothelium and plasma protein[149, 203, 204]. While Vt is a common outcome measure in PET studies examining TSPO in conditions other than tobacco dependence,[205-207] the SUV measure was used because it is less invasive than methods that include arterial blood sampling and arterial sampling was not feasible. Other similar studies have used pseudo-reference regions for PET data analysis[146, 205, 210-213] to minimize potential confounds, but this method would not have been appropriate here due to the hypothesized and confirmed effect of smoking throughout the brain. Additional limitations included scanning at two sites with different model scanners (though results were similar at the two sites), a modest sample size, the absence of measurement of brain nAChR levels, and the fact that smokers were scanned in early abstinence, such that we did not determine whether TSPO availability normalizes with prolonged abstinence. Associations between TSPO availability and brain nAChR availability as determined with PET or single photon emission computed tomography imaging (as we and others have done in prior research) or evaluation of

normalization of TSPO availability with prolonged abstinence could be addressed in future research.

In summary, cigarette smokers in the overnight abstinent state have lower TSPO availability than nonsmoking controls, similar to our recent finding in satiated smokers. The effect was global and greater for menthol- than nonmenthol-cigarette smokers. Future research could build upon this and our previous[125] initial studies and directly compare TSPO availability in smokers in satiety with those undergoing short-term and prolonged abstinence. This approach could provide direct information about the time course of normalization of available TSPO levels in smokers who maintain abstinence. Additional future studies could focus on the interplay between smoking, neuroinflammation, and the progression of diseases thought to be mediated by neuroinflammation.

Chapter 3 Wrap-Up

The ability to identify clinically relevant biomarkers and quantify them in a meaningful manner is one of the most critical skills I have learned as a scientist in the last six years. The application for this field of pharmacodynamic research is endless, and can easily be combined with thorough pharmacokinetics, detailed pharmacogenetics, and eventually host and environmental interactions in order to paint an entire picture for a specific pharmaceutical candidate or treatment. When I had the chance to design my own research study later in my career, I proposed a biomarker forward hypothesis in hopes of solving an enigmatic drug side effect problem, which will be discussed in depth in chapter 6. Being exposed to neurobiology, genetics, imaging, and drugs of abuse over the course of the two salient case studies above

pushed me to learn more about fields that I was uncomfortable in calling my own, and I began to be able to implement a truly multidisciplinary approach to my own research.

Chapter 4

Genomic Technology

General Introduction to Genomic Technology in Precision Medicine

Finally, there is the third piece of the pharmacology triforce: genetics. Because of the heavy presence of genomic technology (GT) in precision medicine, this branch of research is one of the most critical to understanding how we can impact clinical care for unique individuals as opposed to perpetuating a “one-size-fits-all” model. And while chapter 1 scratched the surface on a variety of these genetic or pharmacogenetic concepts, there are complex inner workings worth discussing and applying.

By now, we understand the word pharmacogenetics has Greek roots, stemming from *pharmakon* (drug) and *genesis,* regarding origin, which was first adapted to the biological word for genetics in 1860. There are an estimated 30,000-40,000 genes in the human genome, both protein-coding and non-coding, representing a huge untapped market for medical and disease innovation.[238] This is why a core component of personalized medicine is based on utilizing an individual’s genetic profile to curate the best therapeutic choices by facilitating predictions about whether that person will experience benefits or suffer serious side effects. There has been a distinct shift away from empirical and average-based medicine since the mid 2000’s as the technology and processing of genetic data continues to improve exponentially. In pharmacogenetics, human genomic information is used to study individual responses to pharmaceutical treatments, and connections are made between specific drugs, gene variants,

and physical response. When fabricating these ties, researchers utilize two main areas of genetic profiling: SNP genotyping for candidate genes or genome-wide association studies.

SNP Genotyping Methods

Single nucleotide polymorphisms, or SNPs for short, represent the bulk of our knowledge in common genetic variation among people. Each SNP is a difference in a single DNA building block, the nucleotide, for example, replacing cytosine (C) with a thymine (T) in a certain stretch of DNA sequence.[239] These changes occur normally throughout a person's DNA, and there are roughly 4-5 million SNPs in an individual's genome. While the majority of SNPs tend to exist in non-coding regions, when they manifest within a gene or in a regulatory region, they may play a more direct role in disease by affecting the gene's function. As just one SNP can have a direct biological effect, the process of selecting a "candidate" gene to study direct disease hierarchy is a popular method in pharmacogenetics. Candidate gene studies rely on a set of determinates based on *a priori* hypothesis about the role of a selected gene, a group of pathway related genes, or a phenotype.[240] However, overall the scientific community's experience with these types of studies has been disappointing due to the large variance contribution to disease and genetic heterogeneity seen within and between populations. This is not to say this method of genotyping does not have merit; there are many functionally defined genetic loci that are used as biomarkers for direct pharmacokinetic and pharmacodynamic parameters as well as known genes encoding receptors and transcription factors directly involved in disease etiology.

GWAS Technology

The boom of genetic technology is allowing researchers to branch off from candidate gene studies. Rapid, affordable, and accurate genomic analyses such as next-generation sequencing and whole genome/exome sequencing harbor the potential to provide profound amounts of data

for millions of variants at a time. Stemming from these are genome-wide association studies (GWAS), which are a relatively new way for scientists to identify genes involved in human disease. Instead of only examining one or two SNPs in a candidate gene, this technique leverage millions of SNPs at a time. As most cate[241]gorical diseases are complex in nature, biostatistical methods can be employed to parse the significant associations and help pinpoint variations that occur more frequently in people with these ailments, indicating potential knowledge about genes that may contribute to a person's risk of developing a certain disease. GWAS allows us to capture the cumulative effects of many variants with small penetrance, but that still change the manifestation of a disease.[242] Multifactorial and grievous illnesses, such as Parkinson's Disease,[243] Type 2 Diabetes[244] and Schizophrenia[245] have all benefited in the last year from in-depth GWAS research. And while we still have much to uncover about how human disease works on the level of our macromolecules such as DNA and RNA, there has been incredibly progress that has allowed for new pharmacogenetic diagnostic tests to be implemented in clinical care and directly impact treatment outcomes.

I was extremely lucky to be able to partake in pharmacogenetic and genomic pathway analysis research that leveraged both SNP genotyping as well as GWAS techniques. They were focused in an area of growing concern for psychiatry: addiction. Substance Use Disorder (SUD) is a highly prevalent and debilitating public health issue, both nationally and worldwide, affecting more than 600 million people.[246] This can include alcohol, nicotine, cannabis, opioids, stimulants, and various recreational drugs. As the disorder persists, physical and mental sequelae are common and often result in profound functional impairment. Despite the clear importance to public and individual health, therapies are few and only partially effective, in part due to the rudimentary current understanding of the underlying genetic and biological underpinnings of addiction. The significant heritability of SUD suggests a moderate to strong

genetic basis, but the failure to identify specific risk alleles indicates that the underlying mechanisms are multifactorial and heterogeneous.[247] The limited utility of traditional therapy has led the field of complex genetics to explore dimensional measures of functional neurocognitive phenotypes as more proximal to brain biology. Below, GT case study #1 is a candidate gene study of the influence of genetic variation in cholinergic receptors, nicotine's direct target in the brain, on striatal dopamine release after smoking a nicotine containing cigarette and subsequently confer addiction risk. GT case study #2 applied advanced genetic techniques such as GWAS and polygenic risk scores to better understand the stark difference in the impulsivity trait as seen in addicts versus the general population and how it might be related to their susceptibility to developing a substance related disorder. Both are excellent examples of how pharmacogenetics can be an invaluable tool moving forward in psychiatric diagnoses and individualized treatments.

GT Case Study #1 : Cholinergic Genetic Variation Moderates Smoking-Induced Striatal Dopamine Release*

This work is currently unpublished.

L.C. Seaman, K.S. Mallya, K. Ta, J.L. Chartie, J.T. McCracken, A.L. Brody, E.L. Nurmi

Introduction

Smoking is the leading preventable cause of mortality in the United States, formally established as the cause of 21 diseases encompassing cancer, cardiovascular diseases, and respiratory diseases.[248] These smoking-related diseases account for an excess of 480,000 deaths each year in the United States alone. While there are currently several FDA-approved pharmacological treatments for smoking cessation, these approaches have limited efficacy, and even with the best treatments, nicotine abstinence rates at 1 year are only 23%.[249] Given the high heritability (0.75)[250] of nicotine dependence, genetic variability of nicotine receptors may explain the significant interindividual variability in clinically relevant smoking-related phenotypes including differences in the rewarding properties of smoking and response to smoking cessation treatments.

The rewarding effects of nicotine, the addictive compound in tobacco smoke[251, 252], are due to activation of the mesolimbic dopamine tract in the brain.[253] Dopaminergic neurons within this tract project their axons from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and release dopamine in response to nicotine binding to nicotinic acetylcholine receptors (nAChRs).[254, 255] This well-known reward pathway has been implicated in nicotine dependence and many other drugs of abuse.[256] Variations in smoking-induced dopamine release within this

pathway may underlie differences in individual sensitivity to the reinforcing properties of smoking[257] and other clinically important smoking-related phenotypes.

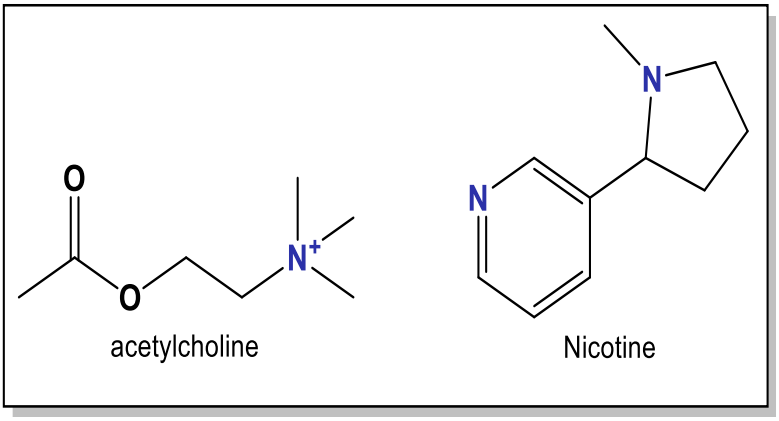


Figure 4-1. The chemical structure of the neurotransmitter acetylcholine and the exogenous stimulant nicotine. Both activate the nicotinic acetylcholine receptors in the human brain.

nAChRs are transmembrane homopentameric or heteropentameric non-selective cation channels composed of subunits encoded by nine α-subunits and three β-subunit genes.[258] The various nAChR combinations differ in localization as well as their pharmacologic and kinetic properties.[258] Within the brain, the two main types or nAChRs are the α7 homo-oligomer and the α4β2 hetero-oligomer.[258] To date, the majority of research investigating the link between nicotine dependence and specific nAChR subunits has focused primarily on the α4β2 subunit based on its high affinity for nicotine, widespread distribution in the brain, and the success of varenicline, a partial α4β2 agonist, as a smoking cessation therapy.[249] Another well-studied genetic risk region for heaviness of smoking, defined by cigarettes smoked per day (CPD), is the chromosome 15 cluster of *CHRNA3, CHRNA5,* and *CHRNA4* which showed significant association with CPD in 3 genome-wide association studies.[259] While these regions have yielded promising effects, recent research has shown the importance of other nACHR genes in nicotine-dependence phenotypes including CHRNB4, *CHRNA7*, *CHRNA6*, and *CHRNB3* loci.[260] Of specific interest is *CHRNA7*, which has been implicated by a strong body of work to be

involved in nicotine reward, dependence, and withdrawal phenotypes in both animals and humans.[261-263]

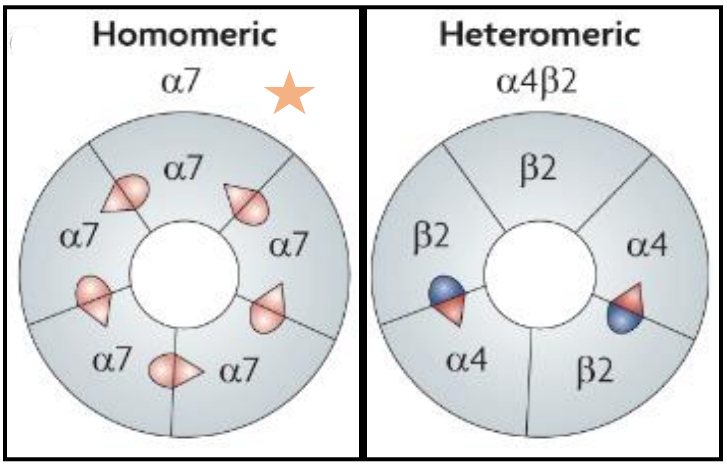


Figure 4-2. Structure of the most common nAChRs, $(\alpha7)_5$ and $(\alpha4)_2(\beta2)_3$. nAChRs are homomeric or heteromeric pentamers of α and β subunits including α2-α10 and β2-β4.

α7 subunits may also play a role in short-term nicotine reward through a modulatory effect on β2 activation.[264] Within the VTA, single recordings of dopaminergic neurons demonstrate two types of neuronal firing rhythms in vivo which occur spontaneously and in response to nicotine: a slow, regular single-spike firing and bursting mode.[265, 266] When α7 -/- mice were injected with IV nicotine there was a large and rapid increase in the firing rate of DA neurons that was short-lasting and followed by a plateau at a slightly lower firing rate.[264] These findings suggest that α7 normally masks an inhibitory effect of GABAergic neurons in wild-type mice, and taken together, this data also suggests that β2 nAChRs may be responsible for switching neurons from a resting to excited state, with α7 nAChRs finely tuning this state after β2 nAChR activation. In this way, both α7 and β2 may be involved in the neurobiochemical events underlying nicotine reinforcement

To briefly highlight the importance of human studies on *CHRNA7*, several groups have shown an association between *CHRNA7* in nicotine dependence, smoking risk, and smoking cessation. In a sample of 710 African Americans, *CHRNA7* and *CHRNA10* both showed a modest

association with nicotine dependence risk.[267] Similarly, in a study of 501 female Israeli students (ages 20-30) a nominally significant ($p<0.05$) genotypic association was shown in SNPs in *CHRNA7* (rs1909884), *CHRNA9* (rs4861065), and *CHRNB3* (rs9298629) with nicotine dependence.[267] In yet another sample of 177 patients with schizophrenia an association was shown between the homozygous 113 bp allele in *CHRNA7* and smoking risk ($p=0.015$).[268] *CHRNA7* also appears to play a role in smoking cessation. In a randomized, double-blind, placebo-controlled study comparing the efficacy of varenicline and buproprion (n=1175), rs6494212 in *CHRNA7* was associated with continuous abstinence from nicotine ($p=0.0038$) (weeks 9-12).[269]

In our previous work we demonstrated a relationship between smoking-induced dopamine release in the VTA/NAc pathway and functional genetic variants within the dopamine system. In a study of 45 tobacco-dependent smokers, smoking-induced dopamine release was measured using positron emission tomography (PET) screening in patients who either smoked a cigarette or did not smoke a cigarette while screening. Smoking-induced dopamine release in the VTA/NAc was measured using the radiotracer raclopride, a competitive D2 dopamine receptor antagonist, labeled with radioactive carbon (11C), which revealed an association between gene variants in the dopamine transporter variable number tandem repeat (VNTR), D2 receptor Taq A1/A2, D4 receptor VNTR, and catechol-O-methyltransferase Val158Met polymorphisms and smoking-induced dopamine release.[269] While these findings suggest that genetic variation in the DA system plays a salient role in smoking related behaviors, variation in DA dynamics in cigarette smokers remains only partially explained.

Given recent evidence for the possible role of other nAChR genes in nicotine dependence, we tested common genetic variation in 6 nAChR genes (*CHRNA3, CHRNA4, CHRNA5, CHRNA7,*

CHRNB2, CHRNB4) as additional potential moderators of smoking-induced DA release. We hypothesized that more direct moderators of DA signaling in the striatum would include genetic polymorphisms in the nAChRs, as they are both the downstream targets of synaptic DA as well as the proximal site of nicotine action in the brain. Utilizing PET imaging with the same 11C raclopride radiotracer as described above, we were able to visualize in humans how smoking a tobacco cigarette directly impacts dopamine release in our pathways of interest and how this subsequently is related to, or affected by functional genetic variations in the corresponding nAChRs. Our research and findings are described here within.

METHODS

One hundred and two otherwise healthy adult smokers who met DSM-IV criteria for nicotine dependence completed the study. Subjects were adults (age 21-65 years) initially screened during a telephone interview in which medical, psychiatric, and substance abuse histories were obtained. Subjects who passed this screening were then assessed in person using screening questions from the Structured Clinical Interview for DSM IV. [First MB, JBW: Structured Clinical Interview for DSM-IV Axis I Disorders…] Subjects with a history of any axis I disorder other than nicotine dependence were excluded. Subjects were also excluded if they had a history of medications that might affect the central nervous system (e.g. current treatment with a beta-blocker or analgesic medication, history of head trauma with loss of consciousness, history of epilepsy). Subjects who did not meet the criteria for abuse or dependence but were occasional users of alcohol, caffeine or other drugs were allowed to participate but were instructed to abstain from these substances for 24 hours before scanning. Subjects who experienced caffeine withdrawal symptoms or consumed more than two cups of coffee per day (200 to 300 mg/day caffeine) were also excluded. Pregnant women were excluded due to potential risk to

the fetus of radiation exposure. After a complete description of the study, written informed consent was obtained.

During the initial visit, additional screening data were obtained including smoking history data, current smoking level, years smoked, brand of cigarette smoked, and quit periods. Fagerstrom Test for Nicotine Dependence (FTND)[175], the Beck Depression Inventory[176], the Spielberger State-Trait Anxiety Inventory[270], and the Shiffman-Jarvik Withdrawal Scale were included in the psychological assessment battery. An exhaled carbon monoxide (CO) level was obtained using the MicroSmokerlyzer (Bedfont Scientific Ltd, Kent, England) at the initial visit to verify smoking status (subjects were considered to be active smokers if a CO level of >8 ppm was obtained).

Study Overview

On a single afternoon visit participants were interviewed with standardized questions; completed rating scales related to cigarette usage, mood, and personality; had blood drawn for genetic testing; and underwent [11C]raclopride PET scanning and either smoked a cigarette or did not smoke during the scan. On a separate morning, patients underwent structural MRI within 1 week of PET scanning.

PET Scanning

Subjects underwent a pre- and post-cigarette smoking single [11C]raclopride bolus-plus-continuous-infusion PET session[190] and structural magnetic resonance imaging (MRI) to aid in interpretation of the PET scans. Subjects abstained from smoking for 3 hours prior to scanning and scanned <1 minute after smoking a cigarette.

Genetic Testing

Whole blood samples were collected and genomic DNA was extracted using the Gentra Puregene Blood Kit according to the manufacturer's protocol (Qiagen, Valencia, Calif.).

Genotyping was performed using the Thermo Fisher TaqMan genotyping platform (Thermo Fisher Scientific, Waltham, MA, USA) with Qiagen Type-it Fast SNP Probe PCR Kit (Qiagen, USA) according to manufacturer's protocols. Tag SNPs (tSNPs) were selected using Broad Tagger software, capturing all common variability >5% in most and >10% in all cases at an r2>0.8 in most and 0.6 in all cases. For quality control purposes, a subset of data was run in duplicate, demonstrating perfect concordance. All markers were in Hardy-Weinberg Equilibrium and minor allele frequencies (MAF) were similar to those previously published by the HapMap Consortium.

Statistical Analysis

Univariate analysis of variance (ANOVA) was performed with the percentage change in ventral caudate/nucleus accumbens binding potential as the dependent variable and the marker genotype as a between-subject factor with ethnicity and age as covariates. Because sex was not a significant predictor of binding potential, it was removed from the model.

In post-hoc analysis, a combined risk measure of two significant variants (*CHRNA7* rs12915695 and *CHRNA4* rs2236196) was constructed to determine whether each conferred independent, additive effects. Genotype at each locus was given a score of 0 or 1, with 1 denoting the presence of at least one allele associated with enhanced binding potential. Scores were summed across the two loci and each subject received an overall risk score from 0 to 2. A one-way ANOVA was applied as above.

Genetic variants and imaging data were also tested for correlation with behavioral measures of craving, dependence (FTND), number of cigarettes per day, number of years smoking, and exhaled CO levels.

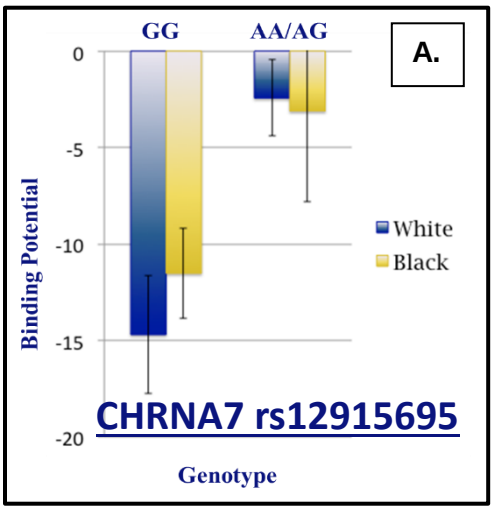
RESULTS

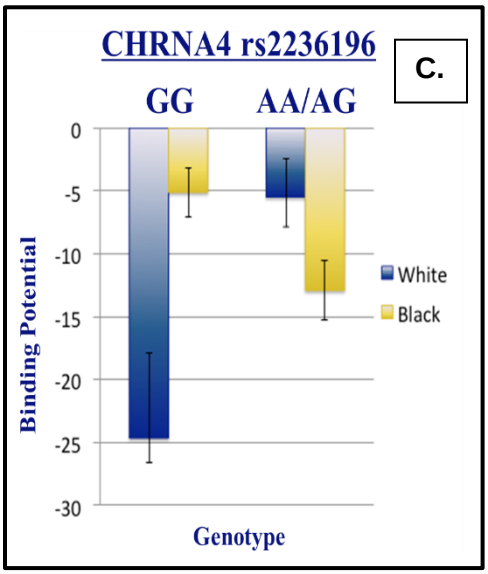
Table one displays the results that map the various nAChR subunit genetic variants and their role in nicotine-induced dopamine release. Two independent gene variants were associated with percentage change in VTA/NAc binding potential (Figure 18). Prominently, homozygotes for the common allele (GG) at an intro 2 polymorphism in *CHRNA7* (rs12915695) demonstrated a greater than three times reduction in radiotracer binding potential (BP), corresponding to a significant increase in smoking-induced dopamine release, compared to minor A-allele carriers

Gene	SNP ID	Gene Region	HM MAF	Our MAF	p-value	Previous Association With Related Phenotype	PubMed ID
CHRNA3	rs578776	3'UTR	0.17	0.21	N.S.	heavy smoking, nicotine dependence, risk lung CA	18618000, 19029397, 20886544, 19259974, 18978787
CHRNA4	rs755203	promoter	0.41	0.49	0.001		
	rs2236196	3'UTR	0.36	0.33	N.S.		
	rs1044396	intronic	0.45	0.36	N.S.	nicotine dependence in men	19290018, 15154117, 19429020, 19211801, 17613539, 18534558, 19693267
CHRNA5	rs16969968	exon 4	0.39	0.40	N.S.	heavy smoking, risk nicotine dependence, risk lung CA	19029397, 18618000, 19259974, 17135278, 18385676, 18385739, 18978790, 20418888, 19951401
CHRNA7	rs12915695	intron 2	0.19	0.22	0.001	risk nicotine dependence	19307444
	rs8028396	exon 2	0.39	0.34	N.S.		
	rs6494212	intronic	0.25	0.33	N.S.		
	rs7170028	intronic	0.49	0.46	N.S.	risk nicotine dependence	19307444, 16314871
	rs2651417	intronic	0.36	0.35	N.S.		
	rs7179008	intronic	0.19	0.23	N.S.		
	rs883473	intronic	0.42	0.32	N.S.		
CHRNB2	rs9427092	promoter	0.16	0.15	N.S.		
	rs4845652	3'	0.13	0.11	N.S.		
	rs2072659	3'UTR	0.10	0.09	N.S.		
CHRNB4	rs12443170	intronic	0.09	0.10	N.S.		
	rs11072768	intronic	0.18	0.19	N.S.	heavy smoking	19029397

Table 4-1. Results mapping nAChR subunit polymorphisms and their published role in nicotine-induced dopamine release.

(-10.8% vs. -3.0% respectively, p=0.001, d=0.97). This finding survived multiple comparison corrections (p=0.009, Caucasian). PET brain images from this set of subjects confirm a larger reduction in raclopride binding in the striatum (resulting from greater dopamine release) after smoking a cigarette. (Figure 18) It is important to note that raclopride BP in both genotype groups was even at baseline. Additionally, a variant in the 3' UTR





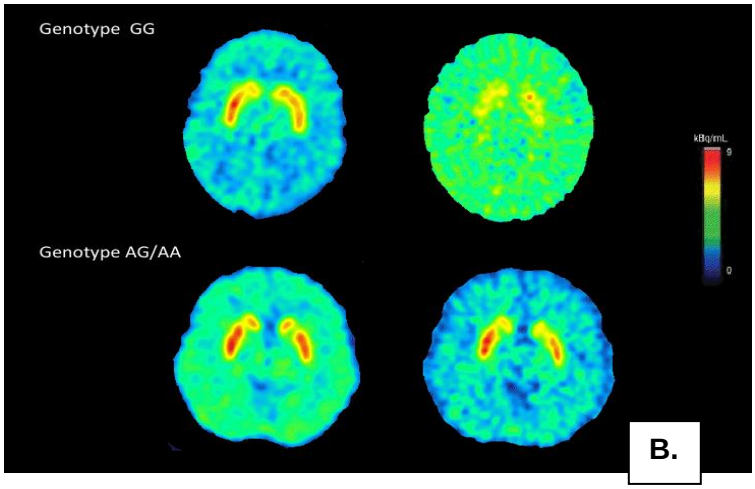


Figure 4-3. NAChRs are Associated with Smoking-Induced DA Release

A. CHRNA7 Genotype Predicts Smoking-Induced Striatal DA Release. Homozygotes for the common allele (GG) at an intron 2 polymorphism (rs12915695) showed greater than 3X reduction in radiotracer binding potential after smoking a cigarette, indicating significantly greater DA release, compared to minor A-allele carriers (-10.8% vs. -3.0% respectively, p=0.001, d=0.97), surviving multiple comparison correction (p=0.009 in caucasians).

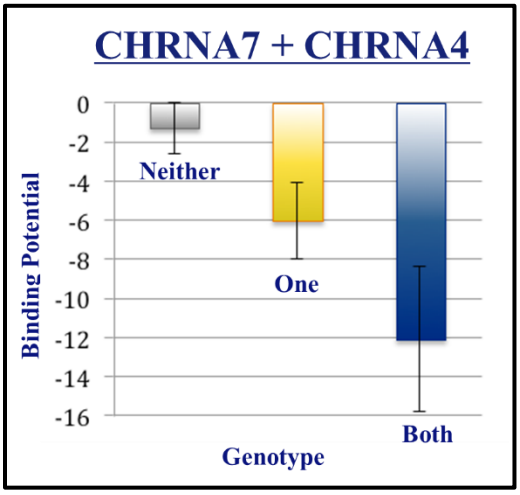
B. CHRNA7 Genotype Differences in Striatal Dopamine Release after Smoking. PET brain images are shown with highest levels of raclopride binding in red and lowest in blue. A larger reduction in raclopride binding (resulting from greater dopamine release) after smoking a cigarette is evident in the striatum (red) of GG homozygotes (top) vs. A-allele carriers (bottom).

C. CHRNA4 Genotype Predicts Smoking-Induced Striatal DA Release. A complex race by genotype interaction was observed at a variant in the 3' UTR of the alpha 4 subunit (CHRNA4, rs2236196) where significant reduction in radiotracer binding was shown for Caucasian GG homozygotes, but African American carriers of the A-allele (interaction p=0.001, d=2.17).

of the CHRNA4 subunit (rs2236196) showed a complex race by genotype interaction. In Caucasians, a significant reduction in radiotracer binding was shown for common allele GG homozygotes whereas African American carriers of the minor A-allele showed a greater reduction in radiotracer binding (p=0.001, d=2.17).

In order to test for independent or additive effects between these two variants we calculated a composite score for each subject by assigning one point for the presence of each allele associated with reduced BP. This model and concurrent analysis demonstrated that each "risk" allele additively contributes to greater dopamine release in a dose-dependent manner; subjects with 0 alleles displayed little to no change in BP, subjects with one allele showed an intermediate reduction in BP, and subjects with two alleles showed close to double the reduction in BP as individuals with just one contributing allele, and therefore the greatest nicotine-induced dopamine release (p=0.007). (Figure 4-4)

Figure 4-4. CHRNA7 and CHRNA4 Composite Score Predicts Dopamine Release in Caucasians.
As shown previously in the Caucasian subset, homozygotes for the common allele (GG) at an CHRNA7 polymorphism (rs12915695) and carriers of the minor allele (G) at a CHRNA4 polymorphism (rs2236196) show significant reduction in radiotracer binding potential, corresponding to a much greater dopamine release than their counterparts. Composite analysis demonstrates that each risk allele additively contributes to greater dopamine release (p=0.007). Individuals with both variants display the greatest release, those with neither have almost no release, and those with only one are intermediate.

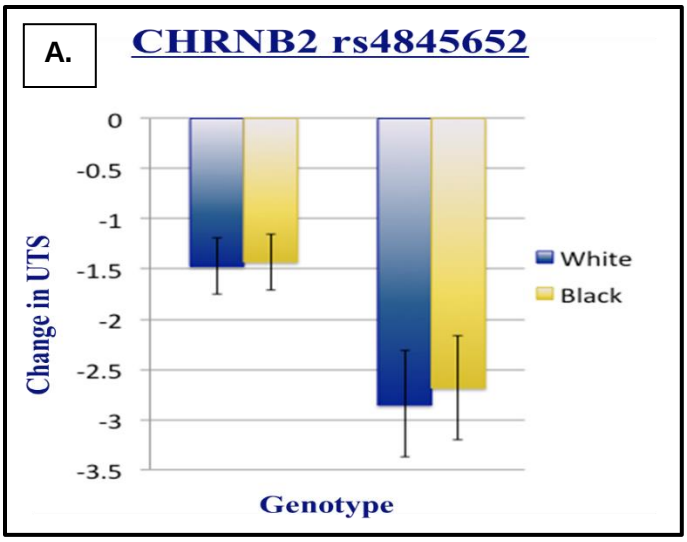


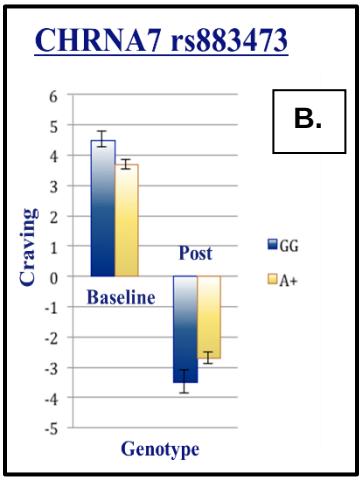
Auxillary cholinergic receptor variants were discovered to have significant effects in other smoking related endophenotypes. An individual's genotype at a polymorphism just distal (3') to the CHRNB2 gene (rs4845652) was able to predict a change in urge to smoke

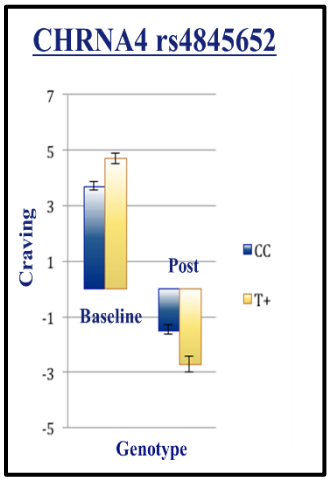
(UTS). Minor T-allele carriers of this polymorphism displayed close to twice a greater reduction in nicotine craving after smoking a tobacco cigarette than homozygotes for the common allele (CC) as measured by the change in UTS ($p=0.003$). (Figure 4-5)

Craving, as measured by self-report, was found to be affected by two nAChR variants, one at the *CHRNA7* gene locus as well as the same variant as above, located on *CHRNB2* (rs8834373 and rs4845652 respectively.) GG homozygotes at *CHRNA7* rs8834373 and T-allele carriers at CHRNB2 rs4845652 have greater baseline nicotine craving before ($p<0.01$ & $p<0.001$) and greater reduction of craving after ($p<0.05$ & $p<0.001$) smoking a cigarette as compared to A-allele carriers and CC homozygotes, respectively. (Figure 4-5)

Finally, an individual's genotype from a single variant located in the promoter region of the *CHRNA4* gene (rs755203) showed correlations with both the number of years said individual had smoked cigarettes as well as their score on the Fagerstrom Test for Nicotine Dependence (FTND). Homozygotes for the common allele (CC) at this SNP has a significantly longer smoking history in years (22.3 ±1.6 vs. 17.6±1.69, $p<0.05$) and greater FTND scores, indicating a greater dependence for nicotine from cigarettes, (6.5 ±0.3 vs. 5.5 ±0.3) than T-carriers ($p<0.01$). (Figure 4-5)







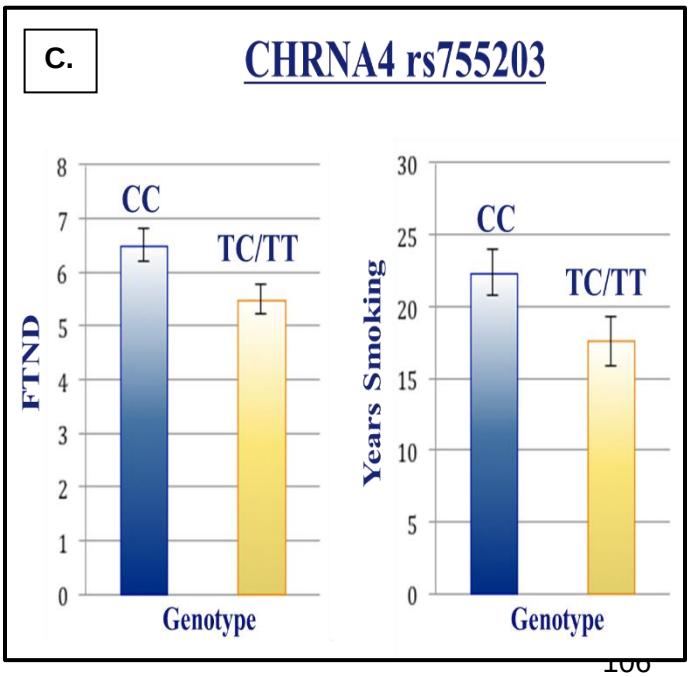


Figure 4-5. NAChRs are Associated with Smoking Endophenotypes and Measures of Nicotine Dependence.

A. **CHRNB2 Genotype Predicts Change in Urge to Smoke (UTS).** Minor T-allele carriers at a polymorphism (rs4845652) just distal (3') to the CHRNB2 gene displayed close to 2X greater reduction in nicotine craving after smoking a cigarette than homozygotes for the common allele (CC), as measured by the change in urge to smoke, (p=0.003). Both Caucasians and African Americans show the same direction of effects.

B. **Variation in Subunits CHRNA7 and CHRNA4 is Associated with Craving.** GG homozygotes at CHRNA7 rs8834373 and T-carriers at CHRNB2 rs4845652 have greater baseline craving before ($p<0.01$ & $p<0.001$) and greater reduction after ($p<0.05$ & $p<0.001$) smoking a cigarette as compared to A-carriers and CC homozygotes, respectively.

C. **CHRNA4 Genotypes that Predict DA-Release are Associated with Measures of Nicotine Dependence.** CC homozygotes at rs755203 show greater DA release and had a longer smoking history (22.3 ±1.6 vs. 17.6±1.69, $p<0.05$) and greater Fagerstrom Test for Nicotine Dependence (FTND) scores, indicating a greater dependence for nicotine from ciagrettes, (6.5 ±0.3 vs. 5.5 ±0.3) than T-carriers ($p<0.01$).

DISCUSSION

Overall, in a broad picture, these data suggest that structural and/or functional variation in midbrain nAChRs contributes to differential smoking-induced striatal dopamine release. Most notably, smokers with a homozygous common allele genotype (GG) at *CHRNA7* locus rs12915695) consistently and significantly demonstrated close to triple the reduction in BP for 11C raclopride, when measured with PET, than their A-allele carrying counterparts. As the radiotracer raclopride is a selective antagonist for dopaminergic D2 receptors in the striatum, DA will competitively bind in the same locations. When the brain is flooded with nicotine from cigarette smoke, and subsequent high dopamine release for GG individuals, the radiotracer will essentially be forced out of these receptor spots, causing large decreases in binding potential. On the other hand, it appears that individuals carrying an A allele (either homozygous AA or heterozygous AG) have a stunted reaction to nicotine in the same neurological regions, and less dopamine is released into the VTA/NAc. One can speculate, based on the salient role of dopamine in reward[271, 272], that the majority of the population might be at risk for positive biological feedback from cigarette smoking.

Interestingly, we observed a complex race by genotype interaction at *CHRNA4* rs2236196 in regards to smoking-induced dopamine release. Caucasian smokers with a GG genotype and African Americans with either an AA or AG genotype experienced much greater striatal dopamine release after smoking a cigarette. While a finding such as this may be difficult to interpret accurately, it is critical to take note of. Pharmacotherapies for smoking cessation are commonly a “one size fits all” category of treatment. Interventions such as nicotine gum, the nicotine patch, bupropion, and more recently varenicline have wildly varying degrees of success, and perhaps this CHRNA4 finding can begin to help explain why this might occur.

Factors such as age, sex, race, and ethnicity may significantly alter the efficacy of treatment for an individual attempting to quit smoking in part because of their level of nicotine-induced dopamine release. Future work needs to be done to attempt to disentangle the many diversities of an individual, their genetic makeup, and their pharmacological responses to classic smoking cessation treatments. Additionally, it is our hope that findings such as this will engender the movement to more individualized treatments for nicotine dependence and perhaps inform small molecule drug targets in the specific nicotinic receptor subtypes.

Our genetic findings at both nicotinic α4 and α7 cholinergic receptor subunits are consistent with animal and in vitro studies that have previously implicated these subunit types in DA-mediated reinforcement associated with cigarette smoking. However, it is worth discussing that past animal studies have revealed conflicting evidence for the role of *CHRNA7* in nicotine dependence and withdrawal. A study by Laviolette and van der Kooy (2003) showed that following direct intra-VTA administration of methyllycaconitine (MLA), a nAChR antagonist considered to be relatively selective for α 7 pentameric nAChRs, a decrease was seen in the conditioned rewarding effects of nicotine in rats.[263] In a similar investigation, Markou and Paterson (2001)[273] demonstrated that MLA reduced intravenous nicotine self-administration, also in rats, suggesting that α7 nAChRs do in fact play a role in nicotine reinforcement. In contrast, a study by Walters, et. al[274] found that treatment with the α4 α2 antagonist dihydro-beta-erythroidine (DH, E) blocked nicotine conditioned place preference while the α7 antagonist MLA had no effect on this behavior, insinuating that the α2 subunit, but not the α7 subunit, is a critical component in nicotine reward systems. It will be vital to clearly define accepted nAChR subunit effects in smoking for future research before beginning to move studies of selective agonists and antagonists to a human population.

Putative effects on nicotine dependence and nicotine craving were observed in nAChR variants that did not correlate with smoking-induced striatal dopamine release, but were nonetheless consistent with previous research findings, indicating their relevance in future smoking-related investigations. Our data at *CHRNA7* rs883473 implicating GG homozygotes as experiencing higher baseline cigarette craving and more robust satisfaction from consequently smoking a cigarette than A-allele carrying individuals are supported by minimal, but strong findings at this same locus related to smoking cessation[275] as well as severity of nicotine dependence.[276]

Of additional interest is the SNP located on the promoter region of CHRNA4 (rs755203) where we observed that smokers with a CC common allele genotype had spent significantly more years smoking as well as had displayed greater FTND test scores, indicating elevated nicotine dependence. A study in 2013 communicated that this SNP has a significant connection to the pathophysiology of nicotine dependence as well as the related phenotype being a critical component in the level of verbal memory and executive functioning in schizophrenia patients.[277] Taken together, we can surmise that this variant is not only consequential in identifying and addressing nicotine dependence, but also an important factor to examine in other psychiatric ailments, as many mood disorders include very high co-morbidities of smoking.

As *CHRNB2* is one of the most studied nicotinic receptor genes, it is reassuring that we also found significant results at a specific locus in this region (rs4845652). Individuals carrying the minor T-allele reported significantly elevated baseline nicotine craving and then subsequently less craving after smoking one cigarette of their choice. While this finding can be interpreted in a few different ways, a unique perspective is to consider the implications for the bulk of the smoker population – or the individuals with a common CC genotype. These people are still reporting high craving, but are in turn experiencing much less satisfaction from smoking their

cigarette than carriers of the T variation. We can look at this in two differing ways; one is that these individuals might be less at risk for developing nicotine dependence, as cigarettes are not as rewarding to them. However, it could manifest itself as these smokers engage in smoking even more tobacco cigarettes than their counterparts to achieve the same level of relief from craving. The latter, and more concerning view point, is supported by recent findings that *CHRNB2* rs4845652 T-allele carriers may be associated with lower levels of nicotine dependence.[278] We believe further examination of this locus is necessary to expand upon how this finding can be utilized for components such as early intervention and treating planning.

An important limitation of the current study is the small sample size. We were only able to successfully image and genetically test 102 individual smokers for this work. Promisingly, Cohen's D effect sizes at both variants are significant and large, and each variant confers unique, additive effects. We believe this warrants larger exploration in upcoming research studies of smoking populations that may include neuroimaging.

GT Case Study #2: Polygenic Contributions to Decision-Making on a Laboratory Test of Reward-Based Risk-Taking*

Nurmi EL, Laughlin CP, Seaman LC, Kohno M, Hellemann GS, Palmer A, DeWit H, London ED

This work is currently unpublished.

INTRODUCTION

The ability to make decisions in uncertain conditions that involve the balance between risk and reward is fundamental to personal development as well as survival. Heightened risk-taking behavior, however, has been linked to neuropsychiatric disorders, such as Attention Deficit-Hyperactive Disorder (ADHD), addictions, and mood disorders[279-284]. For these reasons, the biological bases of risk-taking behavior, including their neural underpinnings[285, 286] and genetic architecture[287, 288] have been a subject of recent interest. A twin study that estimated the contributions of genes and environment across seven risk taking domains revealed additive genetic but individually unique environmental influences; heritability estimates from a meta-analyses of twin studies ranged from 29% in financial risk taking to 55% in safety[288]. In a twin study using the Iowa Gambling Task, which evaluates reward-based decision-making in the presence of risk, a latent "decision-making" factor was identified, explaining between 20%-46% of the variance across task administration timepoints[289]. A genome-wide association study (GWAS) on risk preferences, focusing on risk aversion, used a sample of 10,455 adults, and failed to identify single-nucleotide polymorphisms (SNPs) reaching genomewide significance[290]. The results suggested that risk aversion is a complex trait that is highly polygenic, likely to be driven by many genetic variants, each with a small effect size.

In this study, we evaluated the propensity for risk-taking, using the Balloon Analog Risk Task (BART), [284] a laboratory test of reward-based, risky decision-making, in two relatively large, independent samples. Using a youth-adapted variant of the BART, a candidate gene study of 223 youths (average age 11.3 years) indicated that females, but not males, who are carriers of the *COMT* [158]Met allele had higher risk-taking propensity compared to Val homozygotes. [287] A version of the BART adapted for administration to rats was used in a study of inbred strains, indicating a moderately heritable pattern of risk-taking behavior in rats, with about 55% of the variance in risk-taking behavior attributable to heritable factors, and data consistent with a polygenic model.[291]

In summary, risky decision-making can profoundly influence well-being, and has been associated with several psychiatric disorders. Genetic factors appear to contribute to risk taking, but the underlying genetic architecture remains unknown. *The goals of the present study were to develop and test a polygenic model to estimate the variability in risky decision-making that is explained by common genetic variation. Furthermore, we sought to apply our polygenic model to ADHD and bipolar samples available in the public domain, to estimate shared genetic risk for and risky decision making in psychiatric disorders characterized by risk-taking behavior. In our final analyses, we will similarly test shared genetic variation between risky decision making and substance use disorder and smoking cessation.*

METHODS

Participants:

Data for this study came from two projects. One was the Consortium for Neuropsychiatric Phenomics (the CNP project), a study performed at the Semel Institute of the University of California Los Angeles, to examine the underlying genetic and neural factors, such as memory,

and their link to three target neuropsychiatric illnesses, schizophrenia, bipolar disorder, and attention-deficit hyperactivity disorder. The other project was the Genetics of Impulsivity, performed at the University of Georgia and the University of Chicago, in Georgia and Illinois, respectively (the GIP project).

CNP Sample[292]

Subjects, ages 21-50, were recruited by community advertisements in the Los Angeles area and were only included if they identified as either “Caucasian, not of Hispanic or Latino descent,” or “Hispanic or Latino, of any race,” as per NIH racial and ethnic minority group guidelines. Exclusion criteria included neurological disease, history of head injury with loss of consciousness, use of psychoactive medications, substance dependence within the past six months, and a positive drug screen on the day of testing. Diagnoses for all individuals were verified using structured clinical interviews (SCID-IV;[174]). Included subjects underwent a neuropsychological battery and submitted blood samples for genotyping. All subjects gave written informed consent in line with the procedure approved by the Institutional Review Board at UCLA. In total, 1,600 healthy individuals, 58 patients with schizophrenia, patients with bipolar disorder, and participant-hyperactivity disorder were evaluated for this study. However, due to substantial population stratification, only Caucasian healthy individuals were included in the polygenic score generation (*N* = 837;).

GIP Sample[293, 294]

934 Caucasian ancestry participants 18-30 years of age were recruited from Athens, GA and Chicago, IL. Subjects with recent psychiatric treatment for psychiatric illness or substance use were excluded. Participants completed assessments individually in a behavioral laboratory. DNA

was collected via a saliva sample for DNA collection in an Oragene DNA kit (DNA Genotek Inc., Kanata, ON, Canada).

Balloon Analog Risk Task. The BART is a computerized behavioral measure of risky decision-making.[284] Virtual balloons are presented on a computer screen, one balloon per trial, and the participant can "pump" the balloons up by pressing a response key, virtually inflating the balloons. Each pump produces a set increase in an amount of money (e.g., 5 cents per pump) or points earned on that trial. However, after a certain number of pumps, determined probabilistically, the balloon explodes and no money or points are earned on that trial. The participant must decide when to "cash out" of a given trial, by pressing a response key, to retain earnings in a cumulative bank. The objective is for the participant to earn as much money or points as possible across the task. Versions of the BART vary with respect to the number of trials/balloons used, as well as the probability of explosions [e.g., some tasks have used balloons with a single probability of explosion,[282] while others have used different-colored balloons with different probabilities of explosion. [284, 295] The primary dependent variable of the task is the mean or total number of pumps on trials in which the balloon did not explode; these have been termed "'adjusted pumps"'. Adjusted pumps are preferred to absolute pumps because explosions artificially restrict the range of pumping behavior for evidence of the bias associated with absolute pumps see [296].

Genetic Analyses. Genotyping was performed using the Omni Illumina 500,000 SNP chip. For all genotype data, markers were excluded for quality control if they had less than a 95% genotyping rate, a minor allele frequency less than 1%, deviated significantly from Hardy Weinberg equilibrium ($p < 10^{-6}$), or were identified as having non-random genotyping failure ($p < 10^{-10}$). Individuals were excluded for missing genotypic data (<2% genotypes), missing phenotypic data, or deviation from expected autosomal heterozygosity ($F_{het} < .2$). GWAS was performed on each of the CNP (OMNI only) and GIP datasets as follows. Principal component analysis (PCA) was

performed within study as well as joint with the 1000 Genomes (1KG) ancestry informative markers for use in QC and modeling efforts. Partial correlations ere used to control for variability in population structure. Plink[297] was used to perform two linear regressions with Mean Adjusted Pumps as the dependent variable of interest and Total Adjusted Pumps as (expected) negative control, supplying sex, age and the first five PCA dimensions as covariates (Mean Adjusted Pumps ~ sex + age + D1:5, Total Adjusted Pumps ~ sex + age + D1:5). Each set of summary statistics was clumped and, along with the paired genotypes from its complement study, used to create polygenic scores for each individual in the target sample.[298] These scores were then compared using a partial correlation analysis that controlled for the same covariates in the target dataset as in the source's GWAS. Outlier detection and removal was conducted on the polygenic scores via Cook's distance to remove any egregiously anomalous scores. Imputation to 1KG Phase 3 was also performed on each dataset and the same methodology was applied. A MEGA analysis GWAS was performed on the merged raw genotypes of the CNP and GIP datasets. After stand QC measures (see methods above), PLINK, as used to perform a linear regression, was run using the following model (Mean Adjusted Pumps ~ gt + sex + age + dataset_code + D1:D5). A quantile-quantile (Q-Q) plot of observed vs. expected p-values and Manhattan plot of the linear regression results was performed in R. PRS where then derived and the best MEGA PRS was then tested against the public ADHD and bipolar disorder datasets using PRS methods above.

RESULTS

When a PRS for risky decision-making is constructed based on BART performance in the CNP sample, this PRS predicts BART performance in the GIP sample (r^2=0.11, p=0.00094) and remains significant when corrected for multiple comparisons made in empirically determining the optimal p-value threshold for SNP inclusion in the model (FDRp=0.0057). Likewise, when the

PRS is derived from the GIP dataset and applied to the CNP sample, an identical correlation is observed at a similar level of significance (CNP r2=0.11, p=0.0012, FDRp=0.011).

When 135 Caucasian individuals with psychiatric disorders impacting risk-taking behavior, bipolar disorder, ADHD, and schizophrenia, are added to the CNP sample, significance is improved, suggested that risk-taking is a continuous trait across healthy individuals and those diagnosed with these disorders. An ANOVA of group membership (healthy, schizophrenia, bipolar, and ADHD) was significant ($p<0.05$) and followed an expected pattern where groups characterized by prominent impulsivity (ADHD and bipolar disorder) showed an increased mean adjusted pumps compared to healthy individuals, whereas mean adjusted pumps in the schizophrenia group were decreased (p=0.01).

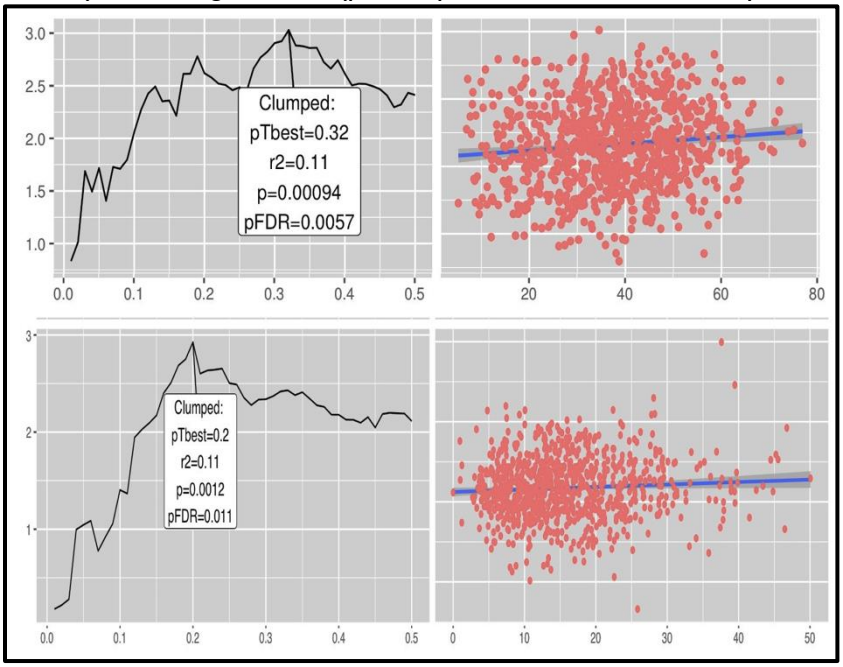


Figure 4-6. Risky Decision Making as Measured by BART Performance is a Heritable, Polygenic Trait.

Top Panel: PRS for BART performance derived in the CNP sample predicts BART performance in GIP participants. Bottom Panel: PRS for BART performance derived in the GIP sample predicts BART performance in the

The MEGA analysis linear regression identified two variants rs12023073 and rs6891903 that were approaching genome-wide significance. Both SNPs (rs6891903 and rs12023073) are intronic and

map within the catenin delta 2 *(CTNND2)* and immunoglobulin superfamily member 21 *(IGSF21)*

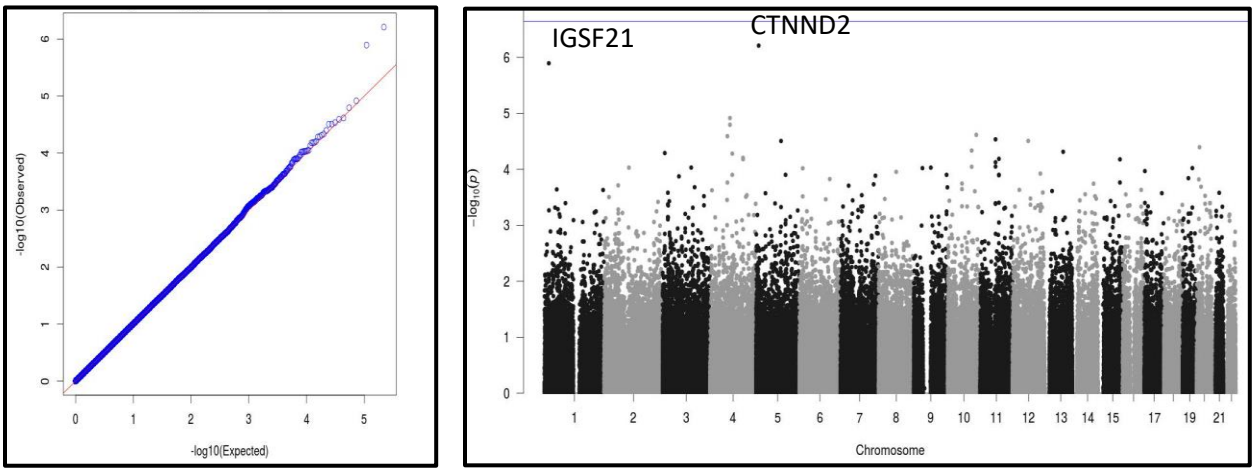


Figure 4-7. Mega-analysis GWAS Results for BART Performance in the Combined CNP and GIP sample. Left Panel: Q-Q Plot show excellent correction for population effects and two SNPs with lower than expected p-value. Right Panel: Manhattan plot showing the location of two SNPs with suggestive lower than expected p-values. Blue line represents genome-wide significance.

genes. The Q-Q plot demonstrates that the principle components correct for any ancestry effects.

An exploratory analysis applying the PRS derived from the MEGA analysis to large samples from the Psychiatric Genomics Consortium public datasets, we observed a significant association with the bipolar disorder phenotype (best p=0.00066, pFDR=0.018). but only a marginal association with the ADHD phenotype (best p=0.048, pFDR=0.28).

DISCUSSION

In line with the few previous rodent and human studies, our findings confirm a heritable, polygenic component to risky decision-making explaining 11% of the variance in BART performance. The bidirectionality of similar effect size and significance level is compelling. The PRS model performs best when additional individuals with psychiatric diagnoses impacting risk-taking behavior are included. These data are in line with prior studies demonstrating that both individuals with ADHD and bipolar disorder demonstrate increased impulsivity scores on behavioral tasks and more conservative BART performance is seen in individuals with schizophrenia.[299] The improvement in PRS performance when psychiatric samples are included provides support for the conceptualization of a spectrum of risky decision-making throughout the population with more

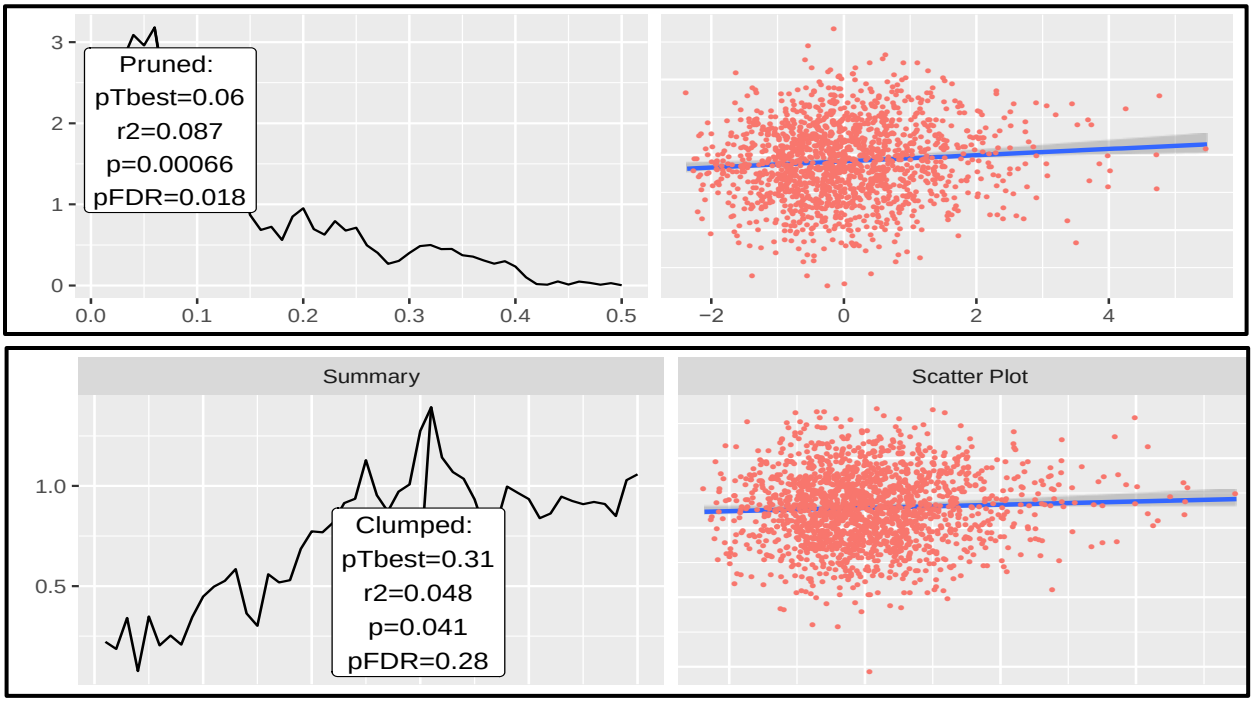


Figure 4-8. An exploratory analysis applying the PRS derived from the MEGA analysis to large samples from the Psychiatric Genomics Consortium public datasets, we observed a significant association with the bipolar disorder phenotype (top panel) (best p=0.00066, pFDR=0.018). but only a marginal association with the ADHD phenotype (bottom panel) (best p=0.048, pFDR=0.28).

extreme phenotypes in individuals with mental illness.

The MEGA analysis provided additional power and identified two intronic SNPs that approach genomewide significance, rs12023073 and rs6891903. The genes containing these SNPs are both good biological candidates for risk-taking and relevant phenotypic associations have been published. CTNND2 (Catenin delta 2) is known to be involved in neuronal development, specifically maintenance of dendritic spines and synapse.[300] CTNND2 is among the genes affected by CNVs found in ADHD and, along with other cell adhesion genes, GWAS-associated SNPs in addiction. An intronic *CTNND2* variant (rs11133644) was recently associated with social conformity.[301] *CTNND2* is often deleted in Cri-du-Chat syndrome, which commonly manifests in hyperactivity and impulsivity phenotypes. Associations with impulsivity and excitement seeking have been observed with SNPs in family member CTNNA2 in a Native American sample[302] and a meta-analysis of GWAS respectively.[303] A population level GWAS using the UK Biobank identified a sex-specific (males) genome-wide intronic SNP (rs140089781) in *CTNNA2* to be associated with alcohol consumption.[304] High drug use has been shown to associate with SNPs in family member CTNNA3.

The second most significant association from the MEGA analysis was rs12023073, which is an intronic variant in *IGSF21* (immunoglobulin superfamily member 21). This gene encodes a protein that is part of the immunoglobulin super family that multiple human tissue analysis has identified that it is most highly expressed in the brain and is believed to play an integral role in inhibitory synaptic development. Tanabe et al. (2017) through an unbiased expression screen and proteomics in mice identified postsynaptic IgSF21 to interact with presynaptic neurexin2α.[305] *Igsf21* knockout mice from that safe study were found to have a number of phenotypic abnormalities including: impaired inhibitory presynaptic organization, diminished GABA-mediated

synaptic transmission in hippocampal CA1 neurons, and deficits in sensory gating.

This study represents the first genome-wide assessment of heritability of risky decision-making based on BART performance. It benefits from comparing similar objective behavioral measurements in moderately sized, healthy, genetically homogenous samples. The ability to include a smaller number of subjects with psychiatric diagnoses assessed with the same protocol extends the implications of the results. The main limitation of this study is limited power. Given the highly complex phenotypes being evaluated, it is possible that a much larger sample could definitively identify genes associated with risky decision making at the genomewide significant level and that an association with ADHD diagnosis could be demonstrated. Additionally, a replication sample to confirm the MEGA PRS would be useful to further validate the findings.

In conclusion, we demonstrate for the first time that polygenic scores derived from a genome-wide association study of a risk-taking phenotype successfully predict the same phenotype in an independent sample. We found that 11% of the variance in performance on the BART was captured by common genetic variation, consistent with the idea that risk-taking behavior is a polygenic trait. A MEGA-analysis GWAS combining the two samples, while underpowered, produced suggestive association at two functionally relevant genes. A PRS derived from this MEGA-analysis successfully predicted categorical bipolar diagnosis in a large public sample. A marginal trend was seen when applied to an ADHD population.

Chapter 4 Wrap-Up

With the advent and continuous growth of the genomic revolution, it becomes possible to study individual genetic variation throughout the whole genome on an unprecedented scale. The study

of pharmacogenetics and genetics as a whole embody all of these new technologies, and while I have only been able to scratch the surface during my time in graduate school, I know both the wet-lab, such as various forms of genotyping, and biostatistical, such as high-throughput genomic data pruning, techniques I was able to acquire will be indispensable in my future; I will be able to understand how human diseases operates on the fundamental biological level and derive meaningful knowledge from human genetics.

Chapter 5

Gene x Environment Interactions

General Introduction to Gene-Environment Interactions

After traversing the pertinent fields of pharmacology, the more subtle details of personalized medicine begin to take shape in the form of the applied combinations of these principles. While the underlying biochemistry of how our bodies function in response to pharmaceuticals is both fascinating and paramount, it is important to zoom out and examine how the world around us can both affect and change this delicate homeostasis. Because we just covered how critical human genetics is to keeping the machine that is precision medicine well-oiled in Chapter 4, I will first discuss how the environment can interact with our genes.

Not surprisingly, we refer to this equilibrium as gene-environment interaction, and it is important for improving both accuracy and precision in the assessment of both influences, as one cannot usually exist without the other. Gene-environment interaction or GxE as I will refer to it throughout this work, is defined as “a different effect of an environmental exposure on disease risk in persons with different genotypes” or alternatively, “a different effect of a genotype on a disease risk in persons with different environmental exposures.”[306] A simply way to approach this is to consider an environmental risk factor as a “high-risk genotype” and can be things such as physical exposure (e.g. radiation, extreme temperature), chemical (e.g. polycyclic aromatic hydrocarbons, free radicals), biological (e.g. viruses), a behavior pattern (e.g. late age at first pregnancy), or a life event (e.g. job loss, car accident, injury).[307] Research assume the variables to be dichotomous in these scenarios in order to facilitate ease and clarity of measurement and

discussion. Subtle differences in genetic variation cause people to respond in distinct and differing ways when exposed to the same environmental agent. Using statistical methods and thorough phenotyping assessments, researchers can classify predictions about disease risk in individual patients, stratifying a large amount of unique health information.

Testing Gene-Environment Interactions

In order to test these associations, individuals must be classified by the presence or absence of both the exposure and the high-risk genotype. Exposures are relatively facile to uncover and quantify, while the genetic component can be more of an obstacle. The most common strategies to evaluate GxE involve identifying a susceptibility gene, implementing ecogenetic or candidate genes, measuring a linked genetic marker, or evaluating positive vs. negative family history.[306] Overall, the identification of subsets of populations with high disease risks due to particular combinations of environmental stressors and genetic mutations with allow development of more targeted screening, interventions, preventative strategies, as well as improved maintenance of everyday health.[308]

There is a pocket in this field of research that focuses on the effects of early childhood development. Studies are indicating that gene-environment interactions during this period of development may have long lasting and penetrating effects on health that do not manifest until adulthood. One can imagine “risk” factors such as those encountered during the beginning of our lives would have resounding implications in psychological health and well-being. In fact, researchers have seen that the behaviors of rat moms towards their newborn pups changes the lifelong responses of those offspring to stress. The parental behavior patterns change the activity of genes in the pup’s brains, specifically genes that are involved in the response to

stress hormones such as cortisol.[309] Considering there is a myriad of adult psychiatric disorders that operate through complex mechanisms that are not entirely understood, GxE interactions represent a valuable pathway for studying long term effects of childhood life events.

Applying Gene-Environment Interactions to Psychiatric Research

Through a long-standing collaboration with Dr. Jessica Borelli, I had a golden opportunity to incorporate my growing database of genomic science into her work with childhood attachment patterns and future psychiatric disorder risk and susceptibility. I present two case studies that revolved around GxE interactions in a diverse community of school-aged children. The first, GxE Case Study #1, we examined moderating effects of a SNP in the FKBP5 gene, which encodes for FK506 binding protein 5 and plays a role in immunoregulation and basic cellular processes[310] on the links between parenting intensity (overcontrol) and child attachment. We assessed whether this genetic variation moderates the links between maternal and child attachment, and subsequently children's emotional regulation. Detailed results can be seen below. Secondly, GxE Case Study #2, we expanded upon what is known about children's genetic and environmental risk for anxiety by examining the unique and interactive effects of mother-child language style matching and a specific OPRM1 polymorphism on children's separation anxiety disorder symptoms. OPRM1 is a μ-opioid receptor encoding gene, and these neuronal receptors can mediate acute changes in neuronal excitability via suppression of presynaptic release of GABA.[311] Findings from this study provide support for a differential susceptibility model of childhood separation anxiety and are detailed below.

GxE Case Study #1: Interactive effects of attachment and FKBP5 genotype on school-aged children's emotion regulation and depressive symptoms. *

***This work is currently published in Behavioral Brain Research*[312]

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1. Introduction

A large body of research demonstrates that child attachment insecurity and genetic factors are each associated with elevated risk for emotion dysregulation and psychopathology.[313, 314] However, few studies have examined associations between these two factors [6] and even fewer have evaluated their interaction in predicting child outcomes. Using a developmental psychopathology framework,[315] in a middle childhood community sample, we examine interactions of parenting and genes in predicting child attachment security, as well as attachment security (both children's and mothers') and genes in predicting children's emotion regulation and depressive symptoms.

1.1. Emotion regulation and depressive symptomatology

Emotional reactions are multilayered experiences comprised of psychological, physiological, and behavioral components.[316] Emotion regulation (ER) refers to the conscious or unconscious processes by which individuals modulate their emotions; some ER strategies result in worsened distress and poor psychological outcomes. Emotion suppression – a conscious process of pushing negative emotions away – is, ironically, associated with increases in negative emotion.[317, 318] Rumination, another maladaptive ER strategy,[319] involves a passive and

repetitive focus on the origins, meaning, or future implications of negative emotion;[320] it is a well-established risk factor for childhood depression.[321]

Because multiple systems are involved in emotions and their regulation, multi-method assessment of ER is essential.[322] Whereas traitlike ER strategies such as suppression and rumination are typically assessed via self-report, momentary emotional changes inaccessible through self-report can be measured physiologically.[323] Respiratory sinus arrhythmia (RSA), which indexes parasympathetic vagal influences on the heart,[324] is one such measure. Although the meaning of task-related RSA changes has been debated, generally speaking, greater decreases in RSA signify an adaptive response, namely, recruitment of additional resources to adequately cope with stress on ER capacity.[324-327] Indeed, greater task-related decreases in children's RSA are associated with fewer behavior problems, greater social competence, and more adaptive ER strategies,[326, 328, 329] but see [330], for conflicting effects).

1.2. Attachment insecurity, emotion dysregulation, and depressive symptoms

One of the core tenets of Bowlby's attachment theory is that attachment and ER are inextricably intertwined.[331, 332] Attachment security develops through interactions with attachment figures (typically caregivers) who fulfill two central functions, serving as a secure base from which the child can explore (i.e., promoting the child's autonomy) and serving as a safe haven to which the child can return in times of need (i.e., providing comfort); Caregivers who consistently fulfill these functions promote the child's confidence that his/her emotional needs can be expressed and resolved within close relationships.[333] In contrast, children exposed to caregiving that fails to provide comfort (e.g., rejecting the child for showing emotion) develop attachment avoidance,[334] which involves learning to deactivate their emotional responses and adopting a strategy of self-reliance,[335] responses that are phenotypically similar to emotion suppression.[336] Similarly,

children whose parents are unable to promote children's autonomy (e.g., by engaging in intrusive or overcontrolling parenting), or who respond inconsistently to children's attachment needs, develop anxious attachment.[337] Indeed, parental overinvolvement, a construct theoretically related to overcontrol, is associated with attachment insecurity in school-aged children.[338] Like other forms of parental insensitivity,[339] overcontrol may teach children that their environment is chaotic and unpredictable.[340] Children parented this way adopt hyperactivating ER strategies[336] such as rumination that prolong and intensify negative emotions in order to increase the likelihood of eliciting a caregiver response.[341]

The theoretical claim that attachment insecurity is associated with suboptimal ER throughout the lifespan is now supported by a large body of research (see [342] for a review). Even in middle childhood, until recently a relatively understudied developmental phase in the attachment literature,[343] links between attachment and ER abound: Attachment insecurity is associated with greater parent-reported child emotion dysregulation[343] and with physiological indices of emotion dysregulation, including cortisol reactivity,[344] startle magnitude,[345] event-related potentials,[346] galvanic skin response,[347] and cardiovascular reactivity.[348] Although less research has examined links between parental attachment security and children's ER (see [349]), it is thought that attachment transmits across generations,[350] and it follows that parental attachment insecurity should also be associated with impaired child ER.

In theory, children's insecure attachment also predisposes them to affective pathology such as depression because children's lack of emotional security results in emotional (e.g., poor ER;[351] and cognitive (a sense of loss and uncontrollability) risk factors for depression.[352] Consistent with these predictions, associations between attachment insecurity and depression have been documented at various stages of development, including middle childhood.[353] Research

expanding our understanding of factors that contribute to the development of depression in middle childhood is particularly important given the dramatic rise in depression prevalence in adolescence.[320]

1.3. FKBP5, emotion dysregulation, and depressive symptoms

In addition to environmental risk factors, children's ER and risk for depressive symptomatology may also be influenced by their genes. The hypothalamic-pituitary-adrenal (HPA) axis links the nervous and endocrine systems in the regulation of the human stress response. Hypothalamic corticotropin-releasing hormone, the main activator of the HPA axis, regulates the release of adrenocorticotropic hormone from the pituitary, in turn triggering adrenal secretion of cortisol, a glucocorticoid and key stress hormone.[354] Cortisol, through its action at glucocorticoid receptors, has widespread effects throughout the body, diverting resources to cope with acute stressors. The stress response is limited by cortisol feedback on the hypothalamus. This negative feedback loop is regulated by the glucocorticoid receptor co-chaperone, FKBP5, which modulates glucocorticoid receptor sensitivity [30]. Functional polymorphisms in the FKBP5 gene, however, alter FKBP5 expression and result in downstream glucocorticoid receptor resistance and dysregulation of the neuroendocrine stress-response.

The two most studied polymorphisms, rs3800373 and rs1360780, map to the 3′ untranslated region and intron 2 respectively. Since they are in near-perfect linkage disequilibrium ($r2 > 0.9$), genetic effects cannot be independently differentiated; however, functional effects of the rs3800373-rs1360780 haplotype may be conferred by the rs1360780 variant, which maps to a glucocorticoid response element in intron 2 and results in a hyperactive allele,[310].[355] Minor alleles at these loci (rs3800373 C-allele, rs1360780 T-allele) present at a frequency of 20–40% across racial groups, result in poor regulation of the stress response system following exposure

to stress.[310] Not surprisingly, these FKBP5 minor alleles have been associated with mental illnesses such as major depression, bipolar disorder and post-traumatic stress disorder.[310, 356] Additionally, environment by gene effects have been repeatedly observed, with FKBP5 minor alleles conferring risk for harmful psychological effects specifically in the context of childhood trauma exposure.[357] During childhood, FKBP5 minor alleles also predispose individuals to stress-mediated epigenetic changes that further dysregulate the FKBP5 negative feedback loop, thus moderating the long-term impact of environmental stressors.[358] Given that emotion dysregulation and depression have been associated with both attachment insecurity and genetic vulnerability as well as the fact that some research suggests an association between attachment insecurity itself and genetic variation,[359] a comprehensive assessment must examine both factors simultaneously. Designs involving both predictors are able to determine if one is a more proximal correlate of the outcomes of interest than the other, and they are also able to explore the impact of their interaction. Two dominant theoretical perspectives regarding gene-environment interactions in predicting psychological outcomes are the diathesis-stress and the differential susceptibility models. The diathesis-stress model[360] suggests that minor alleles will be associated with negative outcomes in the presence of negative parenting and/or attachment insecurity. In contrast, the differential susceptibility theory posits that genetic factors are markers of plasticity, or openness to environment inputs[361, 362]– children with genetic sensitivity may demonstrate worse outcomes in the context of negative environmental circumstances, but enjoy more optimal outcomes than expected in favorable environments. Evidence for the differential susceptibility theory continues to mount, as scholars reinterpret data from prior studies previously conceptualized as evidence for diathesis and conduct new studies to test the model.[363]

Studies show that interactions of parenting behaviors and monoaminergic gene variants predict attachment security, ER, and depressive symptoms.[347, 359, 364-367] HPA axis candidate genes have also been studied for genetic influences on these phenotypes, including mineralocorticoid, glucocorticoid and oxytocin receptors.[368, 369] The most replicated genetic findings occur at the FKBP5 locus. FKBP5 minor alleles interacted with chronic low family support in predicting child mental health status in 255 children with comorbid depression and disruptive behaviors.[370] The interaction of FKBP5 gene variants and adverse life events predicted first episode depression onset in a large-scale 10-year prospective community study of adolescents and young adults[371] and symptoms of anxiety, depression, anger, and dissociation in adolescents.[372] Similarly, FKBP5 variants demonstrated significant interactions with mild to moderate life events in predicting anxiety and depression in preschoolers.[373] Finally, consistent with the differential susceptibility model, post-institutionalized girls with FKBP5 minor alleles exhibited more depressive symptoms at higher levels of peer victimization, but fewer depressive symptoms at lower levels of victimization,[374] suggesting that gene variants confer openness to experience rather than simple risk for negative outcomes.[375] Thus, based on the reliability of previous findings in independent samples, well-described impact of functional variation, and clearly demonstrated interaction with environmental stressors, we examine genetic influences of the FKBP5 risk haplotype (tagged by the rs3800373 variant) on attachment, ER, and depressive symptoms.

1.4. Current investigation

Using a diverse, community-based sample of school-aged children, we build upon prior work and examine interactive associations between attachment and genes to predict child ER outcomes. Using multimodal measurement of ER and incorporating assessments representing both mother and child perspectives, we evaluate four hypotheses.

First, we examine whether parenting behavior (operationalized as parental overcontrol), moderated by genes (FKBP5 genotype), predicts quality of children's attachment (Hypothesis 1). Consistent with the differential susceptibility hypothesis,[361] we predict that the greatest variability in child attachment will occur among children with FKBP5 minor alleles; among these children, low parental overcontrol will be associated with the highest attachment security and high parental overcontrol with the lowest attachment security.

Next, we examine whether children's attachment security, which we propose represents the internalization of parenting behavior, moderated by children's genes (FKBP5 genotype), predicts ER as measured by three indices – stressor-related RSA, emotion suppression, and rumination (Hypothesis 2) – and depressive symptoms (Hypothesis 3). We predict that in children with FKBP5 minor alleles, high attachment insecurity will be associated with the greatest degree of emotion dysregulation/depression and low attachment insecurity with the least.

Finally, we investigate whether maternal attachment, moderated by child genes, predicts the same three indicators of emotion dysregulation and depression. We expect that among children with minor alleles only, maternal attachment insecurity will be associated with greater emotion dysregulation and depression (Hypothesis 4).

2. Method

2.1. Participants

School-aged children (N = 106, 49% girls) and their mothers (see Table 5-1 for sample descriptors) were recruited from the community using various methods, including online

postings and flyers. In order to participate, mothers had to be proficient in English and have at least one child between the ages of 9 and 12. The sample was diverse socioeconomically (48% reported an annual income of less than $60,000), racially (63% Caucasian, 22% African American, 10% Asian, and 5% other racial category or mixed race), and ethnically (22% Hispanic, 78% non-Hispanic).

	Child Gender				t(98)
	Female		Male		
	Mean	SD	Mean	SD	
Child Age	10.35	1.19	10.22	1.03	0.57
Mother Age	40.10	7.17	39.10	7.10	0.70
Maternal Attachment Anxiety[a]	2.30	1.08	2.33	1.18	−0.13
Maternal Attachment Avoidance[a]	2.35	1.08	2.55	1.22	−0.86
Maternal Overcontrol	7.90	4.21	7.98	4.73	−0.10
Child RSA Reactivity	−0.02	0.98	0.03	1.02	−0.24
Child Attachment Security[a]	3.24	0.54	3.05	0.49	1.84
Child Trait Rumination	10.40	6.84	10.32	6.57	0.06
Child Emotion Suppression[a]	2.30	0.76	2.70	0.86	−2.46*
Child Depressive Symptoms[a]	0.19	0.22	0.24	0.19	−1.16

Table 5-1. T-tests between Key Study Variables.

a - Values represent item-level mean scores.

* - p < .05.

2.2. Procedure

The study was approved by the Institutional Review Board. Upon arriving at the laboratory, mothers and their children provided informed consent and assent. Then, in separate rooms, mothers and children completed self-report measures of attachment. Children also reported on their emotion suppression, rumination, and depressive symptoms. Mothers self-reported their use of overcontrol. Finally, children participated in the performance challenge task (PCT) while their cardiovascular reactivity was monitored.

2.3. Measures

2.3.1. Maternal attachment style

The Experiences in Close Relationships-Revised scale (ECR-R;[376]) is a self-report measure assessing individual differences with respect to attachment-related anxiety (e.g., I often worry my partner doesn't really love me) and avoidance (e.g., I prefer not to show a partner how I feel deep down) as they occur in the context of adult romantic relationships. Each subscale consists of 18 items rated on 7-point scales, with higher values signifying greater insecurity. Item-level scores were averaged to yield attachment avoidance and anxiety scores. The ECR-R's reliability and validity have been extensively demonstrated.[373] In this study, $\alpha_{anxiety} = 0.92$ and $\alpha_{avoidance} = 0.94$.

2.3.2. Maternal overcontrol

Mothers completed the USC Parental Overcontrol Scale (USC-POS;), a 10-item questionnaire designed to measure parental overcontrol (e.g., I expect my child to tell me everything that happens when he/she is away from home) among parents of school-aged children. Parents rate each item on a 5-point scale (from 0, Not at all descriptive, to 4, Extremely descriptive); item scores were summed to create a total score. The USC-POS's validity has been demonstrated in two prior studies (see [368, 377]). Discriminant validity was shown in this sample by a nonsignificant association between USC-POS scores and social desirability as measured by the Balanced Inventory of Desirable Responding questionnaire, $r = -0.13$, ns. Cronbach's alpha was comparable to reports in prior studies, $\alpha = 0.64$.

2.3.3. Child attachment security

Children completed the Security Scale, a 15-item questionnaire presented in the form of self-esteem questionnaire (e.g., Some kids find it easier to trust their mom but other kids are not sure if they can trust their mom). After selecting which group of children they are most like, respondents indicate how true that statement is for them (sort of true or really true). Responses

to the items are averaged to create a mean score, with higher scores suggesting higher security (range: 1–4). The measure has strong reliability and validity.[378] In this study, Cronbach's alpha was 0.88. Note that the Kerns Security Scale does not differentiate between anxiety and avoidance, two dimensions of insecurity.

2.3.4. Child genetic plasticity

Saliva was collected and DNA isolated using DNA Genotek's (Ontario, Canada) Oragene Saliva Collection Kit (OG-500). FKBP5 variant rs3800373 was genotyped using Life Technologies' (Carlsbad, CA) TaqMan platform according to the manufacturer's protocol. To ensure quality control, a subset of the sample was genotyped in duplicate with perfect concordance. Minor allele frequencies were similar to expected published values and Hardy-Weinberg Equilibrium was satisfied. In line with existing literature, the minor C-allele was considered the risk/plasticity allele, with CC homozygotes at greatest risk/plasticity. Since the minor allele is present at similar frequencies across racial groups and race was not a significant predictor of the dependent variables, race was not expected to be a confound. There was insufficient saliva to allow for genotyping of n = 5 children and n = 2 children had undetermined genotypes; therefore we report findings on n = 99 children.

2.3.5. Child emotion suppression

The Emotion Regulation Questionnaire for Children and Adolescents (ERQ-CA;[379] is a 10-item measure probing responses to happiness, sadness, and anger. The items are grouped into two subscales: emotion suppression and cognitive reappraisal. The current investigation utilized the emotion suppression subscale, comprised of 4 items that describe conscious forms of masking experienced emotion (e.g. When I am feeling happy, I am careful not to show it). Children respond using a 5-point scale (1 = strongly disagree, to 5 = strongly agree). Internal consistency

and convergent validity of the measure have been reported.[379] Cronbach's alpha for the emotion suppression subscale was 0.74 for this sample.

2.3.6. Child rumination

Children completed the Children's Response Style Questionnaire (CRSQ;), comprised of 25 items that assess rumination in response to feeling sad. There are three subscales: ruminative responses, distracting responses, and problem-solving. We utilized the total score of the 13-item ruminative responses scale; items prompt children to describe how often they employ a passive, self-focused response to sad mood (e.g., think about how alone you feel). Children respond on a 0 (almost never) to 3 (almost always) scale, with higher scores indicating greater rumination. The ruminative responses subscale has good psychometric properties, including high internal consistency and test-retest reliability.[380] Cronbach's alpha for the ruminative response subscale was 0.84 in this sample.

2.3.7. Child depression symptoms

Children completed the Children's Depression Inventory (CDI;[381]), a 27-item measure of depressive symptomatology. The CDI prompts children to choose the statement from a series of three statements that best describes how they felt over the past two weeks (e.g., "I am sad once in a while," "I am sad many times," or "I am sad all the time"). These responses are converted to a 0–2 scale; a mean score across 27 items was computed, with higher scores indicating more depressive symptoms. The CDI has good test-retest reliability, internal consistency, and validity.[381] Cronbach's alpha in this sample was 0.84.

2.3.8. Performance challenge task

Children completed the Performance Challenge Task (PCT;[382]). Mothers, who were told their children should complete the task independently, sat in a chair placed a few feet behind the child and observed. The task consisted of solving six geometric puzzles adapted from the Block Design task in the Wechsler Intelligence Scale for Children, 3rd edition. For each puzzle, the screen displayed a reduced-size picture of the completed puzzle, and ten pieces that could be moved into an empty frame. Children were instructed to move the pieces to complete the 9-piece puzzle. Puzzles were made impossible by only including eight of the nine correct pieces, along with two extraneous pieces. Each puzzle was displayed to the child for 50 s, after which a cartoon frown face appeared, indicating the child's failure to solve the puzzle. For the next 10 s, a progress bar appeared, showing the child's progress in comparison to other imaginary children, who supposedly got 5 out of 6 puzzles correct.

Children sat approximately 20 inches in front of a computer monitor on which stimuli were presented and used a computer mouse to move the puzzle pieces. The screen was connected to an experimenter-controlled computer located in an adjacent room. Experimenters used the computer to present experimental stimuli using E-Prime 2.0 software (Psychology Software Tools, Pittsburgh, PA), which sent recording signals to the physiological data collection program, BioLab (Mindware Technologies, Gahanna, OH).

2.3.9. Child cardiovascular reactivity

Prior to the PCT, research assistants connected children to HRV equipment and tested the signals to make sure the electrodes were functioning well. Disposable Mindware 1.5-inch foam electrodes with 7% chloride wet gel and touchproof snap leads were connected to a BioNex 8-slot chassis equipped with an impedance cardiograph (Mindware Technologies, Gahanna, OH). HRV data were collected using BioLab 2.5 acquisition software and later edited for peak errors

and noise due to movement using the BioLab HRV 2.0 application (Mindware Technologies, Gahanna, OH).

Children were instructed to remain as still as possible. Children first completed a 5-min resting baseline during which they watched a nature video while their physiological activity was recorded. During the PCT, a 60-s segment of physiological data was recorded for each of the six unsolvable puzzles. For this study, we calculated mean scores across the five-minute baseline nature video (baseline RSA) and across the six unsolvable puzzles for each child (stressor-related RSA). RSA data from n = 7 children were lost due to equipment malfunction. To assess RSA reactivity, we created a standardized residual score (stressor-related RSA controlling for baseline RSA), which we used as the dependent variable in hypothesis testing.

2.4. Data analytic plan

Hypotheses were tested using hierarchical linear regressions, controlling for demographic variables that were significantly associated with dependent variables. Due to the strong association between rumination and depression,[380] we initially included rumination in the regression predicting depressive symptoms; the pattern of effects was unchanged, therefore we present the results of the more parsimonious analyses not including rumination. Since the AA (N = 47) and AC (N = 38) genotype groups produced identical patterns across all analyses, we determined that the C-allele was acting in a recessive manner and therefore combined A-allele carriers (AA and AC) into a single group that was compared with CC homozygotes (N = 14).

In order to adopt a conservative data analytic strategy that minimizes the number of analyses conducted to avoid type I error inflation, we tested all attachment × gene interaction effects simultaneously in each of the models, and subsequently performed post-hoc analyses of

interactions that emerged as statistically significant in these more complex omnibus models. Initial analyses revealed that attachment × gene interactions were not statistically significant for avoidance; therefore, in the models presented here, we included two interaction terms (child attachment security × FKBP5 and maternal attachment anxiety × FKBP5). As is customary in attachment research (e.g.,[383]), we controlled for maternal attachment avoidance when attachment anxiety was a predictor variable.

We used the PROCESS macro for SPSS[384] to test moderation by FKBP5 of associations between child and mother attachment and outcome variables. The PROCESS macro tests significance by creating 95% bias-corrected confidence intervals using 1000 bootstrapped samples. In the moderation models, the independent variable is the environmental factor (mother overcontrol, child attachment, mother attachment) and the moderator is the level of genetic risk/plasticity, treated as a dichotomous variable (AA and AC = lower risk/plasticity; CC = higher risk/plasticity). Dependent variables include child attachment security, measures of ER, and depression. For each individual hypothesis, a significance threshold of $p < 0.05$ was assumed.

After conducting analyses, we followed the guidelines proposed by Roisman et al.[385] for evaluating whether statistically significant moderation effects fit diathesis-stress or differential susceptibility models . Following their recommendations, we examined the simple slopes of the association between X and Y among children with low risk/plasticity (AA or AC) and high risk/plasticity (CC) alleles. For all significant moderation effects, we then determined the percentage of cases that fell past the crossover point, termed the proportion affected (PA) index. The crossover point in an interaction is the first value of X at which the regression lines cross. Roisman et al. recommend that when 16% or more cases fall beyond the crossover point,

the interaction can be interpreted as providing evidence of differential susceptibility, but when between 2 and 16% of cases fall beyond this point, the conclusion is less clear.

3. Results

Compared to girls, boys reported greater use of expressive suppression to regulate emotion, t(98) = −2.46, p = 0.016, but no other gender differences emerged (see Table 10). As shown in Table 5-2, child age was negatively associated with emotion suppression. Both maternal attachment anxiety and avoidance were positively associated with maternal overcontrol, and maternal attachment anxiety was positively correlated with child depressive symptoms. Maternal overcontrol was negatively associated with child attachment security and positively with child depressive symptoms. Child attachment security was inversely associated with children's emotion suppression and children's depressive symptoms. FKBP5 minor alleles were correlated with lower stressor RSA and positively associated with children's depressive symptoms, but were not significantly associated with any of the environmental variables. A repeated-measures t-test suggested that for the sample as a whole, RSA decreased from baseline to PCT, t(90) = 4.79, p = 0.0001.

Variable	1	2	3	4	5	6	7	8	9	10	11
1. Child Age	–										
2. Mother Age	0.37**	–									
3. Maternal Attachment Anxiety	−0.03	−0.07	–								
4. Maternal Attachment Avoidance	−0.01	−0.10	0.51**	–							
5. Maternal Overcontrol	−0.04	−0.20	0.37**	0.44**	–						
6. Child Attachment Security	0.12	0.12	−0.23	−0.17	−0.33**						
7. *FKBP5*	−0.08	0.16	−0.11	0.15	0.09	−0.06	–				
8. Child RSA Reactivity	−0.003	−0.22*	0.06	0.002	0.11	−0.12	−0.21*	–			
9. Child Expressive Suppression	−0.20*	−0.15	0.01	0.09	0.13	−0.26**	−0.01	0.16	–		
10. Child Trait Rumination	0.01	0.09	0.19	−0.07	0.10	−0.10	−0.01	0.02	0.14	–	
11. Child Depressive Symptoms	−0.06	0.08	0.22*	0.10	0.22*	−0.36**	0.78**	−0.11	0.06	−0.15	–

Table 5-2. Zero-order Correlations between Key Study Variables.

* - p < 0.05.

** - p < 0.01

3.1. Predictors of child attachment security

Beyond the variance explained by the covariates and the main effects of maternal overcontrol and FKBP5 genotype alone, $R2 = 0.16$, $p = 0.009$, the interaction between maternal overcontrol and FKBP5 genotype did not significantly add to the prediction of child attachment security, $\Delta R2 = 0.01$, $p = 0.50$ (i.e., the simple slopes were not significantly different from each other and a model with only one slope would be more parsimonious). However, we describe the simple slopes as descriptive statistics. The negative association between maternal overcontrol and child attachment security was statistically significant when children were heterozygous for the FKBP5 minor allele (AC), $b = -0.04$, $p = 0.001$, or homozygous for risk/plasticity (CC), $b = -0.05$, $p = 0.01$, but not when children had low genetic risk/plasticity (AA), $b = -0.03$, $p = 0.05$.

3.2. Predictors of children's emotion regulation and depressive symptoms

Beyond the variance explained by the covariates and the main effects of child attachment security, maternal attachment avoidance, and FKBP5 genotype alone, $R2 = 0.15$, $p = 0.03$, the two interactions of child and maternal attachment with FKBP5 together yielded no significant improvement in the prediction of RSA reactivity, $\Delta R2 = 0.06$, $p = 0.07$ (see Table 5-3). When decomposing this combined effect into the two separate interaction effects – the interaction between child attachment and FKBP5 by itself significantly improved prediction, $\Delta R2 = 0.04$, $p = 0.048$, whereas that between maternal attachment anxiety and FKBP5 did not, $\Delta R2 = 0.005$, $p = 0.50$. Fig. 5-1 depicts the 2-way interaction effect. For the significant interaction effect, child attachment security was inversely associated with RSA among children with high risk/plasticity (CC), $b = -1.21$, $p = 0.01$, but not among children with low risk/plasticity (AA or AC), $b = -0.10$, $p = 0.64$. According to the PA index, 100% of cases fell beyond the crossover point (2.22 on attachment security), providing evidence of minor alleles acting exclusively as a protective factor whose impact is enhanced when attachment security is high.

Table 5-3. FKBP5 Genotype × Child Attachment Predicting Child RSA Reactivity, Emotion Suppression, and Child Rumination. Child gender was only significantly associated with emotion suppression; therefore we included it as a covariate only in the analysis in which emotion suppression was a dependent variable. PROCESS generates unstandardized b values, which are reported here.

t - $p < 0.10$.

* - $p < 0.05$.

a - Standardized residual of mean RSA during insolvable puzzles controlling for baseline RSA.

	Child RSA Reactivity[a]				Child Emotion Suppression				Child Rumination			
	β	*b*	SE	95% CI	*β*	*b*	SE	95% CI	*β*	*b*	SE	95% CI
Step 1 R^2		0.15*				0.17*				0.16*		
Constant		−2.02	2.48	[−6.97, 2.93]		−1.07	2.10	[−5.27, 3.12]		−12.64	16.10	[−44.62, 19.34]
Child Gender[b]	–	–	–	–	0.20	−0.29	0.17	[−0.05, 0.62]	–	–	–	–
Child Attachment	−0.14	1.09	0.69	[−0.30, 2.47]	−0.23	0.93	0.57	[−0.19, 2.07]	−0.27	8.24	4.44	[−0.58, 17.06]
Mat Anxiety	0.01	−0.24	0.33	[−0.91, 0.43]	−0.05	0.01	0.29	[−0.55, 0.41]	−0.07	−0.08	2.17	[−4.40, 4.24]
Mat Avoidance	0.01	0.08	0.11	[−0.15, 0.30]	0.06	0.10	0.09	[−0.07, 0.27]	0.08	−1.12	0.66t	[−2.09, −0.20]
FKBP5	−0.28	2.19*	0.57	[−1.86, 6.23]	−0.01	3.69*	1.76	[0.18, 7.18]	−0.01	20.29	13.34	[−6.23, 46.80]
Step 2 ΔR^2		0.06t				0.06 t				0.06*		
Ch Attach x *FKBP5*	−1.40*	−1.13*	0.56	[−2.25, −0.07]	−1.61*	−1.14*	0.47	[−2.09, −0.20]	−1.69*	−7.69*	2.68	[−15.01, −0.37]
Mat Anxiety x *FKBP5*	0.31	0.19	0.28	[−0.37, 0.74]	−0.15	−0.08	0.24	[−0.56, 0.41]	−0.50	1.66	1.84	[−2.01, 5.34]

Beyond the variance explained by the covariates, including child gender, and the main effects, R2 = 0.17, p = 0.018, the two interactions between attachment and FKBP5 did not significantly contribute to the prediction of emotion suppression, ΔR2 = 0.06, p = 0.057 (see Table 5-3). When decomposing this combined effect into the separate interaction effects, the interaction

between child attachment and FKBP5 significantly added to the prediction, ΔR2 = 0.06, p = 0.018, whereas the interaction between maternal attachment anxiety and FKBP5 did not, ΔR2 = 0.009, p = 0.75. For the significant interaction, child attachment security was inversely associated with emotion suppression among children with high risk/plasticity (CC), b = −1.19, p = 0.004, but not among children with low risk/plasticity (AA or AC), b = −0.22, p = 0.18 (see Fig. 5-1). The PA index showed that 25.53% of cases fell beyond the crossover point (a value of 3.14 on attachment security), providing strong evidence for differential susceptibility.

Beyond the variance explained by the covariates and the main effects, R2 = 0.16, p = 0.015, the combined interactions between attachment and FKBP5 significantly added to the prediction of rumination, ΔR2 = 0.06, p = 0.041 (see Table 12). Decomposing this joint effect showed that the interaction between child attachment and FKBP5 was significant, ΔR2 = 0.04, p = 0.039, whereas that between maternal attachment anxiety and FKBP5 was not, ΔR2 = 0.008, p = 0.37. Child attachment security was inversely associated with rumination among children with high risk/plasticity (CC), b = −9.96, p = 0.004, but not among children with low risk/plasticity (AA or AC), b = 0.15, p = 0.92, (see Fig. 5-2). The PA index revealed that 22.34% of cases fell beyond the crossover point (3.14 on attachment security), providing evidence of differential susceptibility.

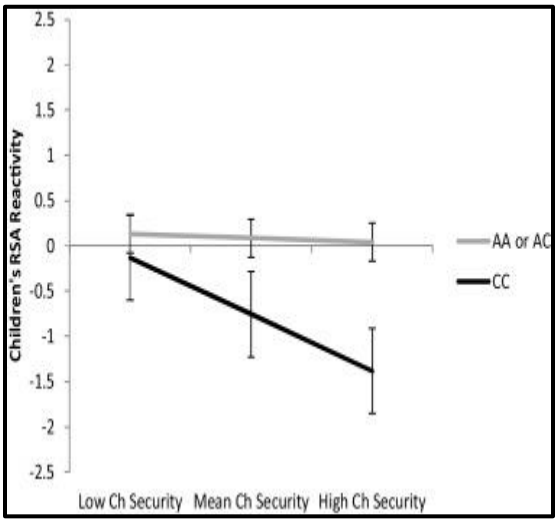

Figure 5-1. Attachment × FKBP5 rs3800373 predicting child RSA reactivity. The C-allele of FKBP5 rs3800373 is believed to confer sensitivity to environmental stress.

Beyond the variance explained by the covariates and the main effects, R2 = 0.30, p =0.00001, the interactions between attachment and FKBP5 genotype jointly added to the prediction of depressive symptoms, ΔR2 = 0.12, p = 0.001 (see Table 5-4). Decomposing this joint effect showed that the interaction between child attachment and FKBP5 added significantly to the prediction, ΔR2 = 0.06, p = 0.005, whereas that between maternal attachment anxiety and FKBP5 did not, ΔR2 = 0.03, p = 0.07. Attachment security was more strongly inversely associated with depressive symptoms among children with high risk/plasticity (CC), b = −0.43, p = 0.0001, as compared to those with low risk/plasticity (AA or AC), b = −0.09, p =0.03 (see Fig. 5-3). According to the PA index, 15.95% of cases fell beyond the crossover point (3.65 on child attachment security), providing tentative evidence for differential susceptibility.

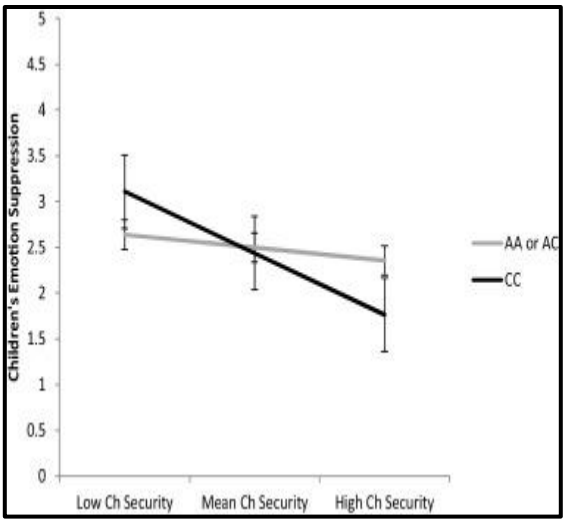

Figure 5-2. Attachment × FKBP5 rs3800373 predicting children's emotion suppression, controlling for child gender and maternal attachment avoidance.

We interpret the trend-level effect for the interaction between maternal attachment anxiety and FKBP5 – of note is that when analyzed separately from the first interaction effect (i.e., without

	Child Depressive Symptoms[a]			
	β	*b*	SE	95% CI
Step 1 R^2		0.30***		
Constant		−0.39	0.46	[−1.31, 0.52]
Child Attachment Security	−0.31	−0.22	0.12	[−0.03, 0.48]
Maternal Attachment Anxiety	−0.19	−0.07	0.06	[−0.21, 0.05]
Maternal Attachment Avoidance	−0.09	−0.01	0.02	[−0.04, 0.04]
FKBP5	0.18	0.79*	0.38	[0.03, 1.55]
Step 2 $\triangle R^2$		0.12***		
Child Attachment Security × *FKBP5*	−1.72	−0.30**	0.11	[−0.51, −0.09]
Maternal Attachment Anxiety × *FKBP5*	0.73	0.09[t]	0.05	[−0.01, 0.20]

Table 5-4. FKBP5 Genotype × Maternal Attachment Anxiety Predicting Children's Depressive Symptoms.

t - p = 0.06.

a - Pattern of effects remains the same when controlling for children's rumination. PROCESS generates unstandardized b values, which are reported here.

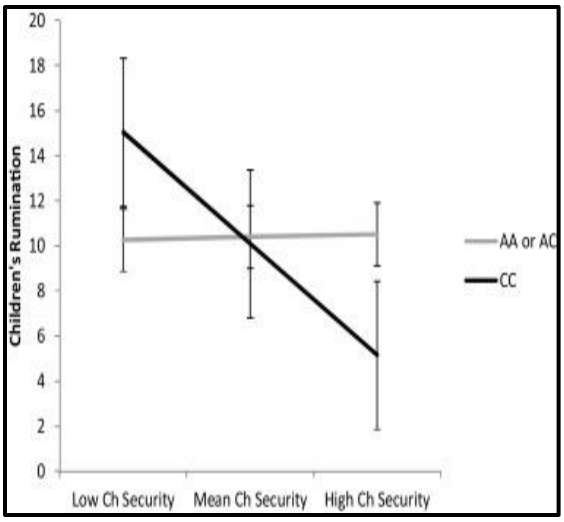

Figure 5-4. Attachment × FKBP5 rs3800373 predicting children's rumination, controlling for maternal attachment avoidance.

controlling for the first interaction effect), the finding is statistically significant, p = 0.01. Maternal attachment anxiety was positively associated with depressive symptoms among children with high risk/plasticity (CC), b = 0.13, p = 0.01, but not among those with low risk/plasticity (AA or AC), b = 0.03, p = 0.25 (see Fig. 5-5). The PA index revealed that 7.21% of cases fell beyond the crossover point (1.64 on maternal attachment anxiety), providing weak evidence for differential susceptibility.

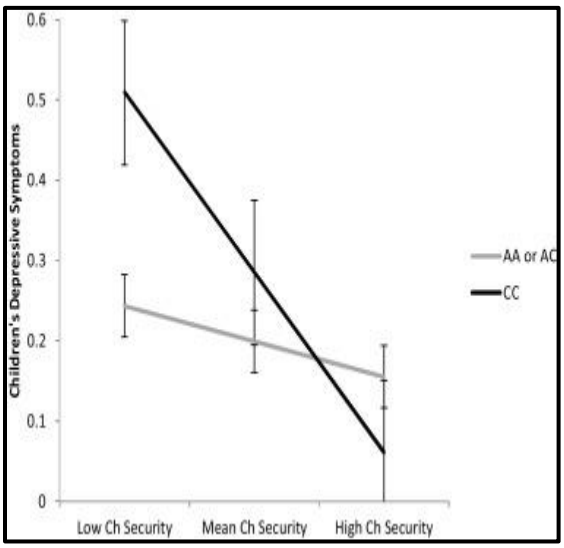

Figure 5-5. Attachment × FKBP5 rs3800373 predicting children's depressive symptoms, controlling for maternal attachment avoidance. The pattern remains unchanged when also controlling for rumination.

It is important to note that in computing the PA indices, we examined single interactions in isolation (e.g., child attachment security × FKBP5).

We sought to advance the state of knowledge pertaining to the interactive associations of attachment and genetic risk with children's ER and depressive symptoms. Our findings are supportive of the notion that relationship factors and children's genotype interact in predicting child outcomes, and can be understood within a differential susceptibility framework.

Importantly, in our sample, FKBP5 was not significantly associated with any of the variables representing environmental factors, suggesting the absence of gene-environment correlations. In combination with our other findings, these non-significant associations suggest that while minor alleles may make children vulnerable to the effects of maternal attachment, children with these alleles do not have mothers with greater attachment insecurity.

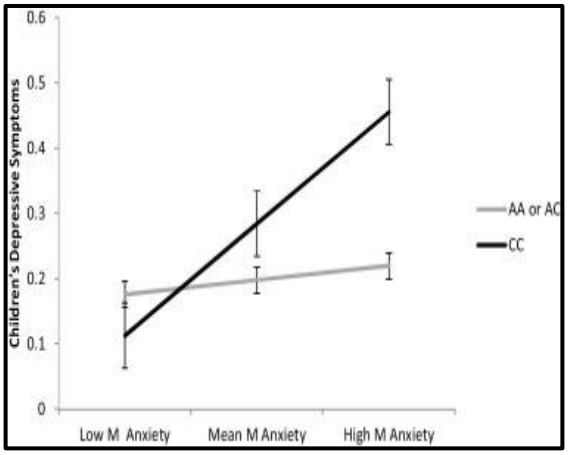

Figure 5-6. Attachment × FKBP5 rs3800373 predicting children's depressive symptoms, controlling for maternal attachment avoidance and child attachment security. The pattern remains unchanged when also controlling for rumination. M = maternal attachment.

4.1. Predictors of child attachment security

Although parental overcontrol was negatively associated with child attachment security, the FKBP5 genotype was not. Further, although the effect of the interaction between overcontrol and FKBP5 genotype on attachment security was not itself statistically significant, we noted that higher overcontrol predicted lower attachment security and lower overcontrol predicted higher attachment security among children with minor alleles. Prior work reports a link between parental over involvement and attachment insecurity,[338] but here we found that this link only holds for children with minor alleles. We present this pattern of effects to facilitate future examination of these associations with larger samples.

4.2. Children's attachment security as a predictor

Although we identified few main effects between attachment or FKBP5 and children's ER, the interaction between these two factors predicted all outcomes. With respect to state-like reactivity, we found that among children with the CC genotype, children's attachment security was associated with a greater decrease from baseline RSA, suggesting readiness to respond to the stressor. In contrast, there was no association between attachment security and RSA reactivity in children with the AC or AA genotypes. This pattern, where genetic susceptibility is associated with more optimal functioning in positive environments, is consistent with a differential susceptibility framework. Importantly, however, the meaning of task-related decreases in RSA has been a topic of debate. Some scholars argue that decreases in RSA in response to a task, referred to as RSA withdrawal, represent readiness to engage the autonomic nervous system in mounting a response to a stressor.[386] This may be true for community samples, for which decreases in RSA are more likely to suggest adaptive readiness. In support of this assertion, task-related decreases in RSA are associated with fewer externalizing problems, more prosocial behavior, and less sadness.[328] Others argue that greater decreases in RSA in response to a stressful task indicate that the stimulus poses a greater regulatory challenge to the individual than can be easily handled. Indeed, associations between RSA decreases and rumination, internalizing and behavioral problems, and poorer executive functioning have been reported (e.g., [330]). Given these competing perspectives, our interpretation of this finding – that attachment security is associated with greater adaptive decreases in RSA among CC children – is tentative.

With respect to the traitlike measures of ER (suppression, rumination), children's attachment was associated with ER for children homozygous for the minor C-allele, but not among children

with AA or AC alleles. Both rumination and suppression are considered maladaptive forms of ER – rumination can be conceptualized as a hyperactivating form of ER and suppression, a deactivating one. Our results show that children with CC genotypes had less optimal ER when security was low, but more optimal ER when security was high, as compared to children without minor alleles, providing evidence for differential susceptibility.[385]

With respect to depressive symptoms, there was a negative association between children's attachment security and depressive symptoms only for CC children, with modest evidence for differential susceptibility. However, because those with high child attachment security reported very low depressive symptoms regardless of genotype, trend-level protective effects seen for children with FKBP5 minor alleles and positive environments may be limited in significance by floor effects (i.e., pathology rating scales cannot capture improvement beyond the absence of symptoms).

When considering these four interaction effects of child attachment security and child genotype on ER and depression, the findings provide strong support for differential susceptibility: Minor alleles predict more maladaptive ER/depression when children's attachment security is low and more optimal ER/depression when attachment security is high. That is, minor alleles may confer sensitivity to the influence of the environment rather than exclusively conferring risk.

4.3. Maternal relationship variables as predictors

In comparison to the dependence of the associations between child attachment and children's ER on genetic plasticity, associations between maternal attachment and ER did not depend on FKBP5 genotype, nor were there main effects of maternal attachment on children's ER reactivity or strategies. Although contrary to theoretically-based predictions, the lack of significant effects

is unsurprising in that few studies have reported main effects between parent attachment and child ER (but see[387], for an example of main effects of interview-based maternal attachment), and to our knowledge no studies have examined these links using parents' self-reported attachment style. Further, in the present study, parents reported on attachment in the context of romantic relationships. Although emerging research shows that attachment security measured in the context of romantic relationships is associated with parenting behavior and emotion[388], we would not expect parents' romantic attachment security to be as strongly linked to children's ER strategies as caregiving behavior.

Maternal attachment anxiety interacted with children's FKBP5 genotype in predicting children's depressive symptoms; when maternal attachment anxiety is high, children with minor alleles report higher depressive symptoms, whereas when anxiety is low, children with minor alleles report lower depressive symptoms. Importantly, the nature of these associations varies as a function of children's attachment security; children with CC genotypes and low security have the strongest positive association between maternal attachment anxiety and children's depressive symptoms. Overall, with respect to depressive symptoms, minor alleles may confer openness to both the negative and positive influence of the caregiving environment (in this case, maternal attachment anxiety).

Maternal avoidance did not interact with children's genotype to predict outcomes. There are at least two possible explanations for this pattern, one conceptual and the other, methodological. Conceptually, parents higher in attachment anxiety may parent in a way that heightens the child's attachment needs, for example, by catastrophizing when the child is afraid, which in turn, could intensify and prolong negative emotional experiences. Elevated reactivity may then enhance children's risk for affective forms of pathology, such as depression and anxiety.[389] If

parents do not heighten children's attachment needs, children may be better at regulating their emotion in the face of ordinary stressors. High avoidance parents may treat children in a way that results in children suppressing emotion, which may more likely result in externalizing rather than internalizing problems.[390]

From a methodological perspective, the absence of interactive associations between maternal attachment avoidance and child genotypes with child-reported reactivity and depression could also be explained by reporter biases.[335] Self-reported attachment is only weakly associated with attachment representations as derived from interviews,[391] and avoidance in particular may be the dimension least likely to correspond across measurement modalities. For these reasons, it is impossible to ascertain whether the absence of interaction effects with avoidance reflects a true lack of association, or whether interview-based, but not self-reported, assessments of avoidance would yield effects.

4.4. Implications

In the present study, multiple interactive associations between attachment and FKBP5 in the prediction of ER and depression provide evidence for differential susceptibility.[361, 362] Children with this genotype had the most optimal psychological profiles (lower emotion suppression and rumination, greater RSA response) in positive caregiving contexts (i.e., when they reported high attachment security) and the most maladaptive outcomes (greater emotion suppression and rumination, higher depression) in negative caregiving contexts (i.e., low child attachment security or high maternal attachment anxiety).

These effects lead us to wonder about the developmental period of plasticity of children with FKBP5 minor alleles – is their plasticity time-limited or are they open to the influence of their

environments throughout their lives? In future studies it will be interesting to examine parenting by gene interactions, measuring parenting constructs at different points in the child's development. This would enable researchers to assess whether FKBP5 minor alleles confer plasticity during a sensitive or critical period, or whether children carrying minor alleles have greater plasticity throughout development.

If replicated and extended using longitudinal designs, these findings could have implications for understanding which children are most at risk for emotion dysregulation and depressive symptomatology. One of the central predictions of attachment theory is that insecure attachment confers risk for emotion dysregulation[351] and internalizing pathology,[353] and these claims are supported in middle childhood for both dysregulation[346] and internalizing pathology.[353] Similarly, FKBP5 minor alleles have been linked to emotion dysregulation and depression in prior studies.[310, 356] In contrast, our findings suggest that associations between attachment security in the mother-child dyad and self-reported and physiological emotion reactivity and with depressive symptoms, depend on FKBP5 genotype sensitivity. These findings imply that in future work researchers should evaluate the contributions of both attachment and FKBP5 genotype, individually and interactively, to child outcomes.

If replicated, these findings could have implications for intervention as well. If we assume that a causal mechanism underlies the findings, then it follows that for children with the CC genotype, attachment insecurity has the strongest impact on ER and depression. Translating these findings into the realm of prevention/intervention, then, it might make sense to identify CC children with histories of attachment-related adversity (e.g., youth in foster care) for treatment, or to target parents carrying minor alleles for engagement in parenting programs grounded in attachment theory during pregnancy. Alternatively, infants could be screened for FKBP5

genotype; the parents of infants with the CC genotype could then receive parenting interventions. Further, depending on the developmental specificity of plasticity, minor allele homozygotes may also be more receptive to the impact of interventions, making the potential gain from interventions targeted towards these populations greater.

4.5. Limitations and strengths

It is important to contextualize this study's findings in light of its limitations. First, the interpretation of genetic findings is limited by the relatively small sample size (N = 106), which constrains our ability to draw conclusions regarding effects that were non-significant.[369] In order to reduce the likelihood of inflation of Type I error due to multiple testing, we conducted joint analyses of both interaction effects with each outcome measure. However, this analytic approach may have resulted in inflated Type II errors. Many of the interactions involving maternal attachment anxiety and FKBP5 were statistically significant when examined in isolation, but were not significant after controlling for child attachment and its interaction with FKBP5. Accordingly, our findings warrant replication in larger independent samples with greater power to detect effects. Second, given the limited statistical power, we only considered one strong candidate gene variant in this study; however, many variants are likely to interact with one another and with environmental factors to contribute to stress phenotypes. Similarly, a larger study would allow researchers to include measures of potential confounding factors that cannot be ruled out here.

In addition, this study was cross-sectional, limiting to some extent the conclusions we can draw. It is impossible to know if the measures of overcontrol and maternal and child attachment security solely reflect current conditions (i.e., current overcontrol), or instead reflect variability in these constructs that emerged when the child was younger. Answers to these questions would

provide information regarding the developmental timing of the influence of parent-child relationship quality and child genotypes on child outcomes. Further, the correlational nature of the study makes it challenging to infer the direction of effects among the environmental variables and outcome measures (e.g., does attachment insecurity cause depression or does depression cause attachment insecurity?) as well as to rule out the influence of unmeasured confounding factors.

There are also limitations with respect to measurement. Our measure of children's attachment security does not differentiate between attachment anxiety and avoidance, two dimensions of attachment insecurity. Therefore, we were unable to examine whether anxiety and avoidance were associated with our measures of emotion hyperactivation (rumination) and deactivation (suppression), respectively. In recent years, investigators have worked to develop the first self-report attachment measures for school-aged children that differentiate between these dimensions;[392] using this measure in future research may help generate and test more targeted hypotheses. Further, we used a self-report measure of child attachment security and three of the four dependent variables in the analyses with child attachment as a predictor were also measured with child self-report. Shared method variance may have contributed to these effects. On the other hand, these effects were entirely consistent with the findings using an objective, physiological measure as the outcome.

Finally, in this study we assessed mothers' self-reported attachment anxiety and avoidance in romantic relationships. Although to our knowledge no self-report measures of attachment-based caregiving systems exist, in future studies it would be interesting to examine children's genotypes as moderators of the links between behavioral measures of parenting sensitivity or

parental reflective functioning, two constructs thought to promote secure attachment in children, in predicting child outcomes.

This investigation also has significant strengths – our use of a multimethod approach to assessing ER-related constructs enhances confidence in our findings. Further, incorporating both parent- and child-reports of attachment and parenting provides a multilayered assessment of the parent-child relational context. Few studies examine the interrelations between factors across genetic, relational, subjective, physiological, and clinical levels of analysis − as such, our study has the potential to speak to the complexity of intersecting patterns of risk in the prediction of internalizing psychopathology.

4.6. Conclusion

In sum, using a cross-sectional design involving a community sample, we found that children with high attachment insecurity and homozygous FKBP5 minor alleles manifested the greatest signs of maladaptive ER and depressive symptoms, whereas children with high attachment security and minor alleles showed the most positive ER outcomes. These findings provide preliminary evidence for the genetic sensitivity or plasticity of children with FKBP5 minor alleles − when immersed in a positive caregiving environment, they show the best outcomes, whereas in a negative caregiving context, they manifest the greatest risk.

GxE Case Study #2: Interaction between the Opioid Receptor OPRM1 Gene and Mother-Child Language Style Matching Prospectively Predicts Children's Separation Anxiety Disorder Symptoms. *

**This work is currently published in Research in Development Disabilities*

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1. Introduction

While experiencing distress in response to separation from caregivers is typical and adaptive for young children, separation distress normatively decreases between the ages of three and five.[393] However, four to five percent of children take longer to outgrow these developmentally-typical worries, developing separation anxiety disorder (SAD).[393, 394] For children aged nine to twelve, SAD commonly manifests as excessive distress when separating from caregivers. Once apart, children with SAD experience sadness, withdrawal, and poor concentration. Adolescents with SAD report greater somatic complaints and school refusal.[393] Importantly, SAD in children is a risk factor for future maladjustment; participants' retrospective reports of childhood SAD predict both current and future adult psychopathology, including anxiety, panic, and depressive disorders.[395, 396] Children with SAD who are treated for school refusal may return to school, but many continue to experience social and emotional difficulties in adulthood, suggesting that the psychological effects of the disorder extend far beyond presenting symptoms.[397] Given the strong links between early SAD diagnosis and later pathology, identifying factors that confer risk for SAD is important for developing prevention and intervention efforts. Further, consistent with a developmental psychopathology framework, examining prospective predictors of SAD symptoms within community samples is necessary in the identification of risk factors.

2.2. *Predictors of separation anxiety*

Various genetic and environmental factors have been suggested as candidate risk factors for the development of SAD symptoms, both individually and in interaction with one another. Two prevailing theories exist regarding interactions between genetic and environmental influences on development. According to the diathesis stress hypothesis, possession of certain "risky" alleles is thought to increase vulnerability to pathology when combined with life stress (e.g. MAOA gene polymorphism;[398, 399] this hypothesis holds that in the absence of the risky alleles or the life stressor, individuals will not be as likely to exhibit pathology. Thus, negative environmental factors may serve as a catalyst for the development of psychopathology among individuals who possess the diathesis allele.

An alternative theory of gene by environment interactions is the differential susceptibility hypothesis. It holds that some genes can affect individuals' sensitivity to all environmental influences, both negative and positive.[400] Belsky (2005) argues that it may be evolutionarily beneficial for siblings to differ in their degree of susceptibility to environmental influence.[361] If children experience maladaptive parenting, those with lower plasticity will be less affected by it, but, if they experience supportive parenting, those with higher plasticity will maximally reap the benefits of parental support. In support of this theory, there is evidence for a number of genetic factors promoting differential susceptibility, rather that rendering individuals vulnerable to risk, such as the serotonin transporter polymorphism 5HTTLPR[401, 402] and dopamine receptor gene D4 (DRD4;[402]). In the current study, we will examine the interaction between genetic and environmental influences on children's SAD symptoms. We aim to distinguish whether the OPRM1 polymorphism A118G confers greater SAD symptoms in combination with an adverse environmental context (diathesis stress), or whether children with the allele have greater SAD

symptoms in a negative context, but fewer symptoms than those without the allele in a positive context (differential susceptibility).

2.2.2. *Environmental predictors of separation anxiety*

Due to the highly relational nature of SAD symptoms, interpersonal factors have been hypothesized to predict risk for SAD.[332] For instance, both attachment insecurity and harsh parenting predict greater separation anxiety in school-aged children.[403-406]

The manner in which individuals impact one another's behaviors and emotions, referred to as co-regulation, may be another interpersonal factor involved in SAD development. Co-regulation between parents and infants is considered to be highly important for the emergence of children's emotional self-regulation.[407, 408] In co-regulated interactions, parents respond in a contingent and matched way to their infants' emotional displays. Seeing their internal states mirrored by their parents provides infants with a visual representation of their internal states and conveys the powerful message that their caregivers understand their emotions and are available to help them regulate their emotions.[409] Over time, a consistent pattern of co-regulated interactions teaches children how to regulate their emotions independently, while fostering a sense of security that their caregivers are there for them in times of distress.[409, 410]

Co-regulation can be measured in terms of the patterning or matching of facial expressions (Fogel, 1994), language, vocal tonality, or physiology. Greater matching is linked with more optimal functioning, such as stronger parent-infant bonds,[411] secure infant attachment,[412] increased vagal tone in response to an emotional stressor,[413] lower externalizing symptoms,[414] increased emotion regulation in early childhood,[415, 416] higher obedience to parental instructions,[417] and lower negative emotionality.[418] The majority of studies on co-regulation have

explored this construct during infancy, when co-regulation may predominantly occur through facial affect matching. However, in early and middle childhood, language becomes an increasingly important tool for communication and, thus, may be involved in the dyadic co-regulation process. Like affect matching, higher linguistic matching within a parent-child dyad may reflect greater parental sensitivity and co-regulation.[348, 419-421]

A novel method of assessing dyadic co-regulation that has emerged in the literature is language style matching (LSM;[422, 423]), a metric quantifying the similarity of function word use between individuals.[422] Function words express grammatical relationships in a sentence and lack clear lexical meaning (e.g., the, and, of, have, could). Variability in the use of function words, which comprise more than half of colloquial speech,[424] is not affected by the content of the speech,[422] and individuals tend to be unaware of their own and others' function word use.[425] Thus, comparing the use of function words allows us to examine matching that the participants themselves are unaware of and that is unrelated to the topic of their speech.

The results of extant studies reveal that higher LSM is associated with more positive relational outcomes. In speed-dating conversations, LSM predicted mutual romantic interest, even when controlling for perceived similarity, and higher LSM in couples' instant messages predicted greater relationship stability after three months.[423] Additionally, bloggers with illnesses reported feeling more social support when LSM was greater between comments on their blog posts and the posts themselves.[426] Thus, early evidence hints that LSM is a valuable indicator of relationship quality.

Prior work also suggests that LSM is a noninvasive measure of the dyadic matching involved in parent-child co-regulation; scholars argue that dyads who have a history of behavioral matching

may also have more closely aligned speech patterns.[427] Although research on LSM is in its infancy, preliminary work suggests that higher LSM between parents and children is associated with greater child attachment security, lower children's galvanic skin response in anticipation of a reunion with their parents, and less self-reported negative emotion in response to a stressful task.[348, 427] These findings have lead scholars to contend that LSM may tap into a history of parent-child co-regulation that has become ingrained linguistically.

Given the influence of co-regulation on parent-child relationship quality and emotional development, it seems plausible that the absence of co-regulation could elicit the emergence of separation anxiety. Indeed, LSM is associated with factors that have been found to influence separation anxiety, such as attachment quality and physiological dysregulation,[348, 427] but has never been directly examined for its association with separation anxiety. Prior research on SAD etiology has focused on individual-level variables (e.g., attachment security, parenting behavior), rather than on measures of dyadic-level processes. Since LSM reflects the level of matching between parent and child, it may constitute a more robust predictor of risk for relationship-focused psychopathology, allowing us to explore the influence of parent-child interaction on separation anxiety in a novel manner.

The links between LSM and SAD symptoms may be especially important to investigate in middle childhood, an understudied developmental stage.[343] As it is developmentally appropriate for children in this age range to experience separations from their parents and engage in greater autonomy-seeking, symptoms of SAD may indicate a lack of security in the parent-child relationship and a failure to express developmentally-expected autonomy striving. Children exhibiting maladaptive responses to hypothetical separation from parents have been found to

have poorer academic performance and lower self-esteem, indicating that symptoms of SAD in this age range may be cause for clinical concern.[428]

1.1.2. Genetic predictors of separation anxiety

Genetic factors may also increase risk for SAD symptoms. Forty-seven percent of children with SAD in one sample had mothers with an anxiety disorder and a meta-analysis of twin studies on SAD estimated moderate heritability (43%), suggesting the important role of shared genetic risk.[429-431]

Research regarding the impact of specific genes on SAD is limited,[432] although one study found an association between the duplication of gene GTF2I and increased SAD symptoms, possibly through its role in downregulating calcium entry into cells, which could affect downstream signaling pathways.[433] Other genes have been associated with generalized anxiety in human and animal studies, such as the rs2254298 polymorphism in the oxytocin receptor gene (OXTR;)[434] and the 5HTTLPR polymorphism in the serotonin transporter gene (SLC6A4[435]).

One genetic factor that has not been previously examined for its association with children's separation anxiety is the gene encoding the μ opioid receptor (OPRM1), which is involved in the HPA axis, the brain's reward and stress response systems, as well as in physical and social pain processing via the anterior insula and dorsal anterior cingulate cortex.[436-439] OPRM1 has been extensively researched,[439] and a functional, nonsynonymous single nucleotide polymorphism in the first exon of this gene, called A118G or rs1799971, has been linked to psychological outcomes.[440-443]

The presence of the minor G-allele, in about 15–30% of those with European ancestry, 40–50% of those with Asian ancestry, and 1–3% of Latinos and African Americans,[444-446] results in a non-conservative amino acid substitution from asparagine to aspartic acid,[447] altering gene expression, ligand sensitivity and signal transduction.[439, 448] Because the μ opioid receptor system inhibits the HPA axis, individuals with the minor allele may have altered HPA axis functioning, impacting their stress response.[437, 439, 449] G-allele carriers were found to have higher resting cortisol and a blunted cortisol response to a psychosocial stressor.[439, 450] A recent study also showed an association between G-allele possession and neuroticism, the tendency to experience negative emotions in stressful situations.[451]

Further, gene by environment interactions have been observed for OPRM1 in both human and rodent models. Genetically altered mice lacking the Oprm1 gene show abnormal maternal separation and attachment,[452] an effect reversed by enhanced maternal care.[453] Although candidate gene studies in humans suggest that the A118G polymorphism interacts with environmental factors to affect stress reactivity, there are varied findings on the nature of this interaction. Among psychiatric patients, A-allele homozygotes had greater susceptibility to the effects of maternal caregiving on their levels of fearful attachment, while those with the minor allele had similarly high levels of fearful attachment regardless of caregiving.[443] However, studies involving other populations have found that G-allele carriers were more sensitive to stress.[440] For example, one study conducted with African American adolescents found that individuals with the minor allele and high life stress had greater depression symptoms than A-allele homozygotes.[442] While these studies are limited in scope and complexity, they preliminarily suggest that children's susceptibility to environmental risk factors may be influenced by their OPRM1 genotype, but are varied in terms of their support for the differential susceptibility or diathesis stress hypothesis. The majority of evidence among community

samples indicates that the OPRM1 minor allele is associated with increased stress sensitivity, so it may be particularly difficult for children with this genotype to regulate distress. When combined with maladaptive parent-child relationship quality, minor allele carriers may be at greater risk for relationship-focused psychopathology, such as SAD; however, it is unclear if possession of the minor allele may be associated with lower SAD symptoms among dyads with high co-regulation.

1.2. Current study

Here we provide the first exploration of the interaction between parent-child LSM and OPRM1 to predict children's concurrent and future SAD symptoms. We test the following four hypotheses: First, based on findings on the link between parent-child relationship quality and SAD, we predict that LSM will be negatively associated with concurrent and future SAD symptoms.[403-406]

Second, we hypothesize that the OPRM1 118G allele will be associated with greater concurrent and future SAD symptoms, in line with previous findings linking the allele with greater stress reactivity.[439-441, 450, 451]

Third, we predict that these two factors will interact such that low LSM will have a stronger association with SAD symptoms among children who possess the 118G allele. This reflects previous findings on interactions between the A118G polymorphism and environmental factors.[440-443, 453] Importantly, current research does indicate whether the role of the polymorphism follows the diathesis stress or differential susceptibility hypothesis for psychopathology, so we are unable to make any predictions in that respect.

Our fourth hypothesis concerns the potentially unique and important role of LSM as a measure of parent-child interaction quality. While previous research has examined the effects of intra-individual level variables on separation anxiety, our study aims to utilize LSM to capture the influence of dyadic matching above and beyond the contribution of individual-level characteristics of the members of the dyad. We expect that this measure will provide additional nuance in our understanding of the development of SAD and the importance of dyadic factors. Specifically, since LSM has previously been associated with attachment security (Borelli et al., 2016), we will control for mothers' and children's self-reported attachment in our analyses to study the role of their interactions independently of each dyad member's perception of their relationship. We predict that the effects of LSM, both separately and in conjunction with genetic risk, will remain after controlling for the attachment styles of the mother and child.

2. Method

2.1. Participants

Participants were recruited from Southern California via online postings, flyers displayed in community gathering sites (e.g., coffee shops), and word-of-mouth to participate in a study of child development. Forty-five children aged between nine and twelve (49% females, M_{age} = 10.37 years, SD_{age} = 1.09) and their mothers (M_{age} = 40.24, SD_{age} = 6.70) took part in this longitudinal study, which was an extension of a larger study. The sample was diverse in terms of race (53.3% Caucasian, 17.8% African American, 8.9% Asian American), ethnicity (17.8% Hispanic), and family income (median: $61,000–$80,000 annually, with 17.8% reporting under $40,000). Mothers needed to be proficient in English to be eligible for participation and all children were fluent in English.

2.2. Procedure

Prior to data collection, the study was approved by the Institutional Review Board. The Time 1 assessment (T1) was conducted in a laboratory. After providing consent (mothers) and assent (children), children reported their symptoms of SAD and provided a saliva sample to a trained research assistant, later used to determine OPRM1 genotype. The children then completed a standardized Performance Challenge Task (PCT) in which they attempted to complete a series of six unsolvable puzzles on a computer while their mothers observed. The puzzles in this standardized task were adapted from the Block Design task in the Wechsler Intelligence Scale for Children-III [454] and were a slightly modified version of a task used in a previous study.[348] In each puzzle, children had 50 s to recreate an image of a completed puzzle by dragging red and white square tiles into an empty nine-piece puzzle frame. Unbeknownst to the participants, some of the tiles needed to solve the puzzle were missing (children are given ten tiles, of which two are incorrect). For ten seconds after each of the six puzzles, children were presented with a progress bar indicating that other children who had taken part in the study got five of the six puzzles correct, while the child had not completed any correctly. During this task, the mother's chair was placed six feet behind the child and the mother was asked not to help her child unless necessary. Following this task, mothers and children were taken into separate rooms and interviewed about their own and the other's experiences during the PCT. These interviews, described below, were used to compute the LSM score of the dyad.

At the outset of the data collection, we only anticipated collecting data from families at a single time point. However, two years later (Time 2 [T2] assessment; range: 2 years, 3 months to 3 years, 5 months), we obtained additional funding to conduct an online assessment of the youth in the study. At this time, we re-contacted T1 participants via phone and email. Due to the fact that we had not planned for a longitudinal follow-up, we had not made any attempts to maintain

relationships with the participants in the study, and therefore were only able to successfully reenroll 42% of the original T1 sample. Importantly, the participants who did and did not participate in the T2 data collection did not significantly differ in T1 measures of SAD symptoms, LSM, OPRM1, age, gender, or attachment security (child or mother). However, those who completed the follow-up T2 data collection reported significantly higher household income at T1 than those who did not, $t(45) = -2.56$, $p = 0.01$. Consequently, we controlled for income in further analyses. In the T2 data collection, youth again reported on their SAD symptoms though an at-home online survey.

2.3. Measures

2.3.1. Language style matching

Following the PCT, the child and mother independently completed semi-structured interviews about their experiences during the stressor task. At the start of the interview, mother and child were shown the same two-minute video clip of the child working on the fifth and sixth impossible puzzles. This visual prompt was designed to reinstate their emotions at the end of the puzzle task. The mother and child were asked four parallel questions regarding their own and the other person's thoughts and feelings during the puzzle task. Participants' responses to the questions in the interview formed the corpus for the LSM calculation (mean child word count = 365, SD = 245; mean mother word count = 894, SD = 458).

The interviews were transcribed verbatim, and research assistants were trained to manually review and edit transcripts according to the procedures outlined by Pennebaker, Francis, and Booth (2001). The edits allowed the participants' speech to be analyzed by the Linguistic Inquiry and Word Count system (LIWC[455]). For example, editors removed interviewer speech from

transcripts so that it was not included in measures of participant speech and flagged “filler” words and “nonfluencies” (e.g., “um,” “like”) to ensure that the program would not mistake those words for meaningful word production. The LIWC program calculates the percentages of words in the text sample that fall within certain categories. Nine function-word categories are used in calculating LSM: auxiliary verbs (e.g., might), articles (e.g., the), common adverbs (e.g., naturally), personal pronouns (e.g., you), indefinite pronouns (e.g., someone), prepositions and relative pronouns (e.g., of, which), negations (e.g., not), conjunctions (e.g., and), and quantifiers (e.g., most).[422]

To produce the LSM metric, we followed the procedures of Gonzales et al. (2010), a method used in a number of other studies.[423, 426, 427, 456, 457] We first calculated the proportions of words in each function word category out of the total number of words in the responses and took the absolute difference of the proportions within each dyad (i.e., |Mother − Child|). Next, we divided this value by the sum of the dyad’s proportions of that function word category (i.e., (|Mother − Child|)/(Mother + Child)). This provides the difference between mother and child word usage adjusted for combined word usage in that category. Finally, we subtracted this value from 1 in order to determine the level of similarity in the frequency of certain types of words in the dyad’s speech. The equation is shown below:

$$LSM_{wordcategory} = 1 - [(|Mother - Child|)/(Mother + Child)]$$

To illustrate the computation of this metric, we provide an example for one function word category: The auxiliary verb category generated by LIWC provides the proportion of auxiliary verbs used by a speaker out of the total number of words uttered by the speaker (e.g., 457 auxiliary verbs/10,000 words = 0.0457 for mother; 80 auxiliary verbs/6000 words = 0.0133 for her child). Using the proportional values generated by LIWC (Maux = 0.0457; Caux = 0.0133),

we computed the absolute value of the difference between mother and child auxiliary word usage as a proportion of the total auxiliary word usage:
(|Maux − Caux|)/(Maux + Caux) = (|0.0457 − 0.0133|)/(0.0457 + 0.0133) = 0.5520. We then subtracted this value from 1, yielding LSMaux = 0.4480 for this particular dyad.

We performed the same calculation for each of the nine function word categories for each dyad. Then, we averaged the nine individual word category LSM calculations to look at matching across all function words, as validated by of Gonzales et al. (2010). This creates an overall LSM score for each mother-child dyad.

LSMTotal = (LSMwordcategory1 + LSMwordcategory2 + … + LSMwordcategory)/(9)

Overall, the algorithm used to compute LSM is thought to measure the degree of linguistic matching within a dyad independently from the content of their speech. LSM scores range between 0 and 1, with higher scores signifying greater verbal matching (Gonzales et al., 2010). We provide narrative responses from dyads with high and low LSM scores in Table 5-5i98.

	High LSM		***Low LSM***	
	Child	**Mother**	**Child**	**Mother**
Questions about child's thoughts	*I was thinking about um, like, like how I was supposed to do the puzzles, because once, once I got it right, well not, well I ha-had a triangle, but I was flipping it inside the thing*	*Um initially he was probably excited and happy. After he got the first one wrong he's like, "What oh okay okay I did something wrong I gotta refocus."*	*I was thinking that I was really gonna accomplish it. I was thinking I did really well and that I almost had it and I almost finished the whole thing.*	*He probably was thinking about finishing in the allotted time also. I'm sure he probably figured by looking at it he knew exactly what to do, but once he started, you know, and then having to turn the pieces around it changed his outlook.*

Questions about child's feelings	*I was curious about how to do it. I was wondering if when I put the block – put it down to see if it that actually was right.*	*Um he probably felt um excited in the beginningand then after probably frustrated. Yeah.*	*I felt really good and I enjoyed that. I did pretty good and I'm proud of myself.*	*Um I think he knew that he didn't do good, you know, because he didn't get any of them right. But it wasn't like a devastating thing. It was more of an excitement to try to get the next one finished.*
Questions about mother's thoughts	*She might have been thinking that – she might have been thinking about the correct answer in her head, but she couldn't really just say it out loud. I think she was thinking uh— thinking about how I did.*	*Um, initially I was thinking um that he would get them all right because I know that he's really good at kind of stuff. Um and then after a while I just figured um … the point was for him to get them wrong.*	*Maybe she was thinking about how good I was doing it and how she was helping me. She might have thought I was doing pretty good and she might have been proud of me.*	*That I hoped he would make it in the allotted time um and that it wasn't too hard for him. He did good for his first time seeing those types of puzzles. So now I think he will probably want to do more puzzles like that.*
Questions about mother's feelings	*I mean, a little disappointed. I kept on getting them wrong. Maybe, is this really how he's – is this really how my son is doing? 'Cause I was getting them wrong.*	*Um…I felt…I don't know I guess content or happy or yeah. I like watching him do you know things you know that may be a challenge so.*	*She might have thought that I was doing really good and she tried to help me really hard. I think she felt really good that I almost had it and I had one more puzzle piece to complete.*	*It felt good. He was interested in doing it, you know, so I always like just to watch him do things. I love it, I love it. I mean that it was nice, it went by well, and it was great.*

Table 5-5. Narrative Examples of High and Low LSM Interviews.

2.3.2. OPRM1

Trained research assistants collected saliva samples immediately following consent procedures. We isolated DNA using DNA Genotek's (Ontario, Canada) Oragene Saliva Collection Kit (OG-500). The OPRM1 genotype was then determined using Life Technologies' (Carlsbad, CA) TaqMan platform, following the manufacturer's protocol. To ensure quality, we genotyped a subset of the sample a second time, which yielded perfect concordance with the original results. The minor allele frequency in our sample was similar to that reported in previous studies and

satisfied Hardy-Weinberg Equilibrium. There was insufficient saliva to allow for the genotyping of one sample out of the 45 who participated in the T2 data collection, so we report on 44 children.

2.3.3. Separation anxiety

Children reported their anxiety symptoms in the Multidimensional Anxiety Scale for Children at both time points (MASC[458]). The scale has 39 items that are divided into four subscales: Physical Symptoms, Social Anxiety, Harm Avoidance, and Separation/Panic. Children rated how much each item applied to them (e.g., from the Separation/Panic subscale: “I try to stay near my mom and dad”) on a 4-point Likert-type scale from 0 (“never true about me”) to 3 (“often true about me”). The MASC is a reliable and valid measure of anxiety symptoms in community samples.[459] The 9-item Separation/Panic subscale was used to assess SAD symptoms. It has been found to significantly predict the presence and severity of SAD among children aged 7–17.[460, 461] Internal consistency for the Separation/Panic scale in this sample was 0.78 at T1 and 0.80 at T2.

2.3.4. Dyad-level covariates

To assess attachment, mothers completed the Experiences in Close Relationships Scale Revised at T1 (ECR-R[376]), a 36-item measure consisting of an attachment anxiety scale (e.g., I often worry my partner doesn’t really love me; $\alpha = 0.92$) and an avoidance scale (e.g., I prefer not to show a partner how I feel deep down; $\alpha = 0.94$). Children completed the 15-item Kerns Security Scale at T1[378]; e.g., Some kids find it easy to trust their mom but other kids are not sure if they can trust their mom; $\alpha = 0.88$). We included these variables as controls in our analyses. In this way, we were able to test whether our dyadic-level matching variable (LSM) predicted SAD symptoms above and beyond the contributions of mother and child attachment.

2.4. Data analytic plan

After examining the distributions of our key variables, we determined whether demographic factors (age, race/ethnicity, gender, income) should be included as covariates in subsequent hypothesis testing through t-tests and zero order correlations. To aid the interpretation of our data, we also performed a t-test to examine differences in separation anxiety levels at the two time points.

To test our hypotheses, we first conducted four linear regressions to examine the main effects of LSM and OPRM1 genotype on T1 and T2 SAD symptoms. OPRM1 was analyzed as a dichotomous variable, with 1 indicating that the child possessed at least one G allele. We controlled for T1 separation anxiety when examining T2 separation anxiety scores. In all four regressions, we controlled for child age, gender, and family income.

We next analyzed the effectiveness of the interaction between LSM, and OPRM1 genotype, measured at T1, in predicting T1 and T2 SAD symptoms, using Hayes' publicly available, free statistical PROCESS macro software.[462] PROCESS operates by using 1000 bootstrapped samples to estimate 95% bias-corrected confidence intervals for the interaction coefficient and, in moderation analysis, provides estimates of simple slopes for the conditional effects. We performed four moderation analyses, testing both LSM and OPRM1 as moderators of the relation between the other independent variable and T1 and T2 separation anxiety. We controlled for child age and gender, family income, and the main effects of LSM and OPRM1 in all four moderations. When predicting T2 separation anxiety, we controlled for T1 symptoms. We also controlled for maternal and child attachment to isolate the effects of dyadic linguistic matching on SAD symptoms from individual-level factors.

After conducting analyses, we followed guidelines proposed by Roisman et al. for evaluating whether significant moderation effects fit diathesis-stress or differential susceptibility models.[385] This entails examining the simple slopes of the association between X and Y among children with low risk/plasticity (AA) and high risk/plasticity (AG and GG) alleles. For significant moderation effects, we determined the percentage of cases that fell past the crossover point, termed the proportion affected (PA) index. The crossover point in an interaction is the first value of X at which the regression lines cross. According to Roisman et al. (2012), when 16% or more cases fall beyond the crossover point, the interaction can be interpreted as providing evidence of differential susceptibility, but when between 2 and 16% of cases fall beyond this point, the conclusion is less clear.

3. Results

Prior to hypothesis testing, we examined the distributions of our study variables and their associations with demographic factors. The results of initial analyses revealed that all study variables were normally distributed. Twenty-four percent of the children possessed at least one G-allele. An independent samples t-test indicated that girls endorsed significantly more symptoms of SAD than boys at T1, $t(44) = 2.36$, $p = 0.02$, but no other gender differences were observed. Zero-order correlations revealed that younger children had greater SAD symptom severity at both time points (Table 5-6). On average, children reported significantly greater SAD symptoms at T2, $t(44) = -9.55$, $p = 0.001$.

Table 5-6. Zero-Order Correlations Between Key Study Variables.
Note: Ma= Maternal, Anx = Anxiety, Avoid = Avoidance, C = Child, Sep = Separation.
*p < 0.05, ***p < 0.0001.

	Mean (SD)	LSM	OPRM	M Anx	M Avoid	C Security	T1 Sep Anx	T2 Sep Anx
Age	10.29 (1.11)	−0.02	0.02	0.01	0.19	0.03	−0.34*	−0.32*
LSM	0.82 (0.16)	–	0.10	0.07	0.12	0.07	0.06	0.25
OPRM1	–		–	0.02	−0.02	−0.08	−0.07	0.08
M Anx	2.08 (0.98)			–	0.51***	−0.18	0.18	0.01
M Avoid	2.23 (1.03)				–	−0.17	−0.09	−0.01
C Security	3.24 (0.51)					–	0.21	0.24
T1 Sep Anx	1.07 (0.56)						–	0.49***
T2 Sep Anx	1.86 (0.49)							–

3.1. Hypothesis testing

Through regression analyses, we explored the main and interactive effects of LSM and OPRM1 genotype in the prediction of T1 and T2 SAD, including simple slopes analyses to gain further information on significant interactions. As shown in Table 5-6, the LSM x OPRM1 interaction was not a significant contributor to the model predicting T1 SAD symptoms after controlling for child age child gender, income, mother and child attachment, and the main effects of LSM and OPRM1 on T1 SAD symptoms.

However, a parallel regression analysis (see Table 5-7) revealed that the LSM x OPRM1 interaction was a significant contributor to the model predicting T2 SAD symptoms with a large

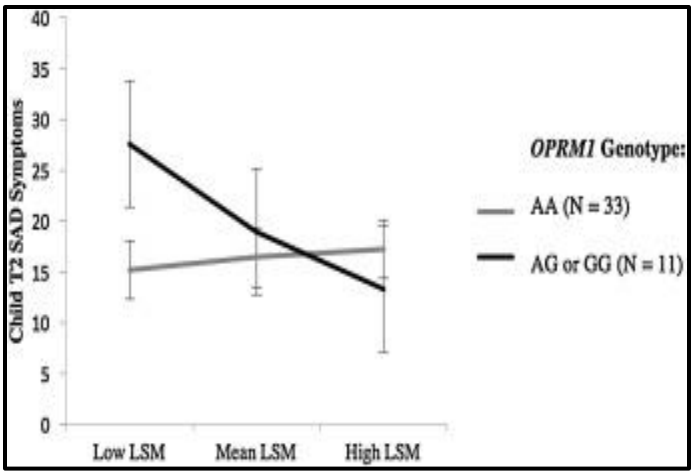
effect size after controlling for T1 SAD symptoms, child age, child gender, income, mother and child attachment, and the main effects of LSM on T2 SAD symptoms.

A simple slopes analysis revealed that among homozygous AA allele children, LSM was positively associated with T2 SAD symptoms after controlling for T1 SAD symptoms, b = 6.73, p = 0.03. However, among children who possessed at least one G allele, LSM was negatively associated with T2 SAD symptoms, controlling for T1 SAD symptoms, b = −52.38, p = 0.004 (see Fig. 5-7). The PA index revealed that 18.18% of the cases fell beyond the crossover point (.90 on LSM), providing evidence for differential susceptibility.

Predictors	T1C SAD Symptoms				T2C SAD Symptoms			
	ΔR^2	*b*	*SE*	*p*	ΔR^2	*b*	*SE*	*p*
				0.036				0.0023
C Age		−0.19	0.07	0.01		−0.58	0.55	0.30
C Gender		−0.30	0.16	0.07		0.63	1.19	0.60
Family Income		−0.03	0.05	0.49		0.17	0.32	0.59
M Attachment Avoidance		−0.06	0.08	0.45		0.36	0.60	0.55
M Attachment Anxiety		0.16	0.09	0.09		−0.81	0.67	0.24
C Attachment Security		0.36	0.16	0.03		0.83	1.19	0.49
T1C SAD symptoms		–	–	–		3.50	1.14	0.004
C *OPRM1* genotype		−0.40	2.09	0.85		51.00	14.72	0.002
LSM		−0.11	0.36	0.76		6.73	2.94	0.03
LSM x *OPRM1*	0.0007	0.48	2.44	0.85	0.17	−59.11	17.31	0.002

Table 5-7. Results of Regression Analyses Examining OPRM1 as a Moderator of the Relationship Between LSM and Child SAD Symptoms.
Note: C = child

Figure 5-7. OPRM1 genotype moderates the association between mother-child LSM and T2 separation anxiety symptoms, controlling for T1 separation anxiety symptoms.



Reversing the independent and moderator variables revealed that among children in dyads with high LSM, $b = -3.94$, $p = 0.05$, and mean level LSM, $b = 2.50$, $p = 0.06$, OPRM1 minor allele possession was not associated with T2 SAD symptoms, although the slopes approached significance. Among children with low LSM, those with minor alleles had significantly higher T2 SAD symptoms than A-allele homozygotes, $b = 0.63$ $p = 0.002$.

4. Discussion

This study examined the independent and interactive roles of OPRM1 genotype and mother-child LSM, a form of behavioral matching measured at T1, in the prediction of children's self-reported T1 and T2 SAD symptoms. Both LSM and OPRM1 had significant main effects on T2, but not T1 SAD symptoms. In addition, as hypothesized, the interaction between LSM and OPRM1 genotype predicted T2 SAD symptoms, controlling for T1 SAD symptoms. Specifically, the G-allele was significantly associated with greater T2 SAD symptoms among low LSM dyads only. Approaching significance, the G-allele was also associated with higher separation anxiety in dyads with medium levels of LSM, but with reduced SAD symptoms among high LSM dyads. Our findings are consistent with previous research linking the OPRM1 minor allele with greater sensitivity to environmental effects.[440-442] They are also consistent with findings on the negative association between LSM and factors that play a role in separation anxiety, including physiological stress reactivity and attachment insecurity.[348, 403-406, 427]

Moreover, our findings support a differential susceptibility model of the relation between OPRM1 genotype and T2 SAD symptoms. Possession of the OPRM1 minor G allele is non-significantly associated with fewer SAD symptoms when LSM is high and significantly associated with greater SAD symptoms when LSM is low; for AA homozygotes, LSM and SAD symptoms are significantly positively related with a much smaller coefficient. The lack of a significant difference between the genotypes for the high LSM group may be due to the limited statistical power from our small sample size. It is possible that, with more power, we would have found significant evidence that the G-allele is associated with reduced SAD symptoms among children who experience greater behavioral matching. The PA analysis confirms the differential susceptibility interpretation, suggesting that possession of the minor allele confers greater plasticity or openness to environmental influence. This is a novel finding, as the roles of the A118G polymorphism and LSM in human SAD have not yet been examined. However, our conclusions are tentative given the small sample size, resulting in limited statistical and external validity. In a study among psychiatric patients, the AA homozygous allele conferred greater plasticity to the effect of maternal caregiving on levels of fearful attachment, suggesting that the role of the polymorphism may differ in clinical populations.[443] Further research with larger samples is required to reach more definite conclusions.

Given that the gene by environment effect held after controlling for maternal and child attachment, we propose that LSM is linked to T2 SAD symptoms beyond the effects of individual attachment style because it is an indicator of ongoing co-regulation between dyad members. Increased co-regulation among dyads with higher LSM may be related to children's greater emotion regulation and security in their relationships. For children who are particularly sensitive to stress, which the minor genotype seems to engender, co-regulation may be

especially crucial for them to learn how to deal with their negative emotions. When the parent-child relationship is characterized by low LSM, indicating low co-regulation, the child may not develop the coping skills needed to independently handle separation from parents or other daily stressors.

On the other hand, some of our findings did not support our hypotheses. Although the interaction between OPRM1 genotype and LSM predicted future SAD symptoms, the variables were not associated with concurrent symptoms. Given that LSM is theorized to reflect a dyad's long-term history of co-regulation, we would expect LSM to be associated with both concurrent and future SAD symptoms. Similarly, we would expect the role of OPRM1 to remain stable with time. Future study could investigate whether the factors affecting SAD development vary with age. Secondly, among AA homozygotes, LSM was positively associated with T2 SAD symptoms. However, the slope for this effect is substantially smaller than that for G allele carriers and the SAD scores fall within a lower range. Thus, homozygous children with higher LSM may be more willing to be open about their fears, resulting in higher, but still relatively low SAD symptoms.

A further unexpected finding was that, contrary to previous research indicating that separation anxiety decreases with age,[393] the mean level of SAD symptoms was higher at T2, when the children were aged twelve to fifteen, than at T1, when the children were aged nine to twelve. One possible explanation is that the T2 data collection period occurred around the time that most children start middle school, a transitional period has been associated with greater psychological distress.[463, 464] Increased independence and academic and social demands may act as additional stressors that heighten children's levels of separation anxiety. Additionally, the T2 data collection occurred during the six months following the 2016 U.S. Presidential election,

which spurred increases in anxiety particularly among Latino/a populations.[465] The increase in T2 SAD symptoms could reflect a general increase in anxiety among vulnerable populations within the United States (about half of our sample was non-White). Children with the OPRM1 minor allele may be especially sensitive to these stressors (transition to middle school or the election), particularly if they have not developed effective emotion coping skills through co-regulation. Further research could investigate this possibility by examining how children's current stressors interact with genetic and relational factors.

4.1. Strengths and limitations

This study is characterized by a number of strengths. The longitudinal design allowed us to examine the prospective predictive effects of LSM and OPRM1 in a racially and ethnically diverse sample. Further, LSM is an unobtrusive metric of behavioral matching that allows us to examine subtle variation in the synchrony of dyadic relationships. It is worth noting that this matching occurred even though we interviewed mothers and children separately, suggesting that they were carrying a co-regulatory mental representation of their relationship. Additionally, the fact that the gene by environment effect occurred independently of mother and child self-reported attachment highlights the importance of investigating dyadic interactions beyond each individual's perception of the relationship.

There are also important limitations to the current design. Participants elected to take part in the study in response to our recruitment methods, introducing possible sampling bias. Those willing to participate in a study on parent-child relationships may systematically differ from the general population, such as by having greater interest in the topic, and our study did not include fathers. Additionally, there was variability in the time interval between T1 and T2 for participants

because the T1 data collection took several months to complete, affecting the amount of time between data collections for different participants.

This is a small candidate gene by environment study that requires replication in larger samples to provide definitive results. Our hypotheses test only one genetic and one environmental factor in a complex system that leads to anxiety symptoms. Although we have proposed some possible explanations, the mechanism through which LSM and the OPRM1 minor allele affect separation anxiety remains unclear. Additionally, even though we employed a longitudinal design, we cannot test for causal relations between our variables. In particular, because the environmental factor that we are measuring cannot be separated from the genetic contributions of the participants, we are not examining a true gene by environment interaction. That is, variability in LSM may itself be related in part to other genetic factors of the dyad, rather than representing a purely environmental influence. Further, since we only assessed SAD symptoms in this study, it is also possible that children with the minor OPRM1 allele and high LSM are at heightened risk for less relational forms of anxiety-related problems. In future studies, it will be important to explore whether the proposed protective effects of LSM apply to multiple forms of anxiety or just to separation anxiety. Finally, with respect to our sample, although the first step in a developmental psychopathology study involves exploring correlates of risk in community samples, it would be advantageous to study these constructs within a clinical sample.

5. Conclusion

Children who carry the minor 118G allele of the OPRM1 gene experience more symptoms of SAD than those who are AA homozygous in the context of low parent-child dyadic behavioral matching (LSM), but they experience fewer SAD symptoms in high LSM contexts. Since SAD is

most common in children from low-income backgrounds[393] who face multiple forms of adversity, future studies should assess the relative roles of LSM and OPRM1 in the context of additional risks. Greater knowledge of the factors that contribute to the development of SAD can inform both treatment and preventative measures. If the effects identified in the current study are replicated, this could point toward the importance of testing whether interventions designed to strengthen the parent-child relationship reduce the risk of developing SAD symptoms in children who carry the minor OPRM1 allele.

Chapter 5 Wrap-Up

Obtaining a deeper understanding of how the environment affects the lives of people every day has extreme value in personalized medicine. In the above studies I specifically contributed the genetic component of the gene-environment interactions, but through this process I was able to learn a significant amount about a large portion of environmental effects and their implications in human health and disease. It is often overlooked that the world around us can have such a profound impact on our daily lives, our physical health, and even more importantly, our mental health. By leveraging this knowledge, we can integrate it and develop and apply genome-based strategies for the early detection, diagnosis, and treatment of psychiatric disease. It aids us in predicating disease rates and provides a formidable foundation for well-information recommendations of therapeutic strategies as the medical community shifts their focus to more stratified approaches. Additionally, public health can be significantly impacted as we uncover more about these unique GxE interactions, as policy-makers could design more “user-friendly” living conditions, allowing for a delay or even prevention of many diseases.

Chapter 6

Microbiota and Host Interactions

General Introduction

We have slowly inched our way through the trenches of the multitude of personalized medicine tools and techniques to finally reach this point. My final 2 chapters will not only attempt to leverage the most salient components I've covered but will also present a unique and novel research project that I was able to develop utilizing the in-depth knowledge I have acquired through a diverse graduate school career. First, let's get started by discussing the general basis for this area of research – host biological interactions.

The term host might spur a small amount of negative connotation, sometimes associated with a parasitic relationship, but in biology it simply means an organism that harbors a guest, whether it is parasitic, mutualistic, or symbiotic.[466] A biological interaction is the effect that a pair of organisms have on each other. And although it is typically forgotten, humans play host to the large collective bacterial population in our bodies, and I will be specifically focusing on those found in the gut, appropriately named the gut microbiome. These human-bacterium interactions are long-term in nature, strongly influencing the evolution of our species as a whole and are therefore considered symbiotic and mutualistic.

Understanding the Gut Microbiome

In humans, the gut microbiota has the largest number of bacteria and the highest number of unique species compared to other areas of the body; a complex and dynamic ecosystem of approximately 200 bacterial species exists in the gastrointestinal tract.[467] It is important to

differentiate between the gut microbiota and the gut microbiome, even though these terms are typically interchangeable. The microbiota / flora are the actual organisms while the microbiome is their collective of genes, which number roughly over 2 million, and is vastly greater than the human genome itself.[468] Due largely to rapid advancements in analytical techniques in microbiology, molecular genetics, and bioinformatics, the true diversity of these microorganisms is quickly being revealed and its contributions to homeostasis in both health and pathogenesis are continuously emerging. This field of study is one of the most active and exciting in both biology and medicine and stands to be one of the greatest contributors to precision health care to date.

The gut microbiota have four general roles in the human body that are imperative to regular biological function. First, there is the clear metabolic role; bacteria aid in salvaging calories,[469] producing short-chain fatty acids,[470] synthesize vitamin K and folic acid,[471] as well as participate in certain drug metabolic pathways. Next, the gut bacterium have the exclusive ability to deconjugate bile acids through the production of an enzyme known as bile salt hydrolase.[472]Third, interestingly, these bacteria play a major role in the prevention of host colonization by foreign pathogens. (28856738) And lastly, the gut microbiome stimulates immunologic effects through avenues such as immonuglobulin A production, promotion of anti-inflammatory cytokines, and induction of regulatory T cells.[473] We can see that this diverse microbial community has an extensive repertoire and is a key factor in shaping the human biochemical profile, and therefore has a direct impact on our health. This has stimulated an exciting amount of research in the past 5-10 years, and scientists are striving to identify the functionality of many microorganisms and their relationship with implicit human biology, signaling, and pathways and how the composition of this unique body can influence disease pathology.

With over 1000 identified species of bacteria, it can be difficult to classify distinct enterotypes of the human gut microbiota, however the improvement of genomic technologies such as 16S ribosomal RNA sequencing and shotgun metagenomics has allowed for a detailed look at the composition of diversity between individuals.[474] A general consensus of the phylum level composition in the human gut is emerging and is focused around the *Firmicutes* and *Bacteroidetes* classifications to represent the dominant bulk of variable bacteria.[475] A variety of populations, whether it be children vs. adults, healthy individuals vs. disease states, or obese vs. lean persons, have quantifiable and distinct differences in gut microbiota compositions, pointing to a more direct biological link to certain human archetypes. But in order to elucidate these connections to disease we must also understand how the bacteria communicate to the host directly.

Microbiota and Bile Acid Crosstalk

As mentioned above, one of the primary functions of the gut microbiota is to influence the chemical structure of a major class of endogenous signaling molecules, the bile acids. These species are examples of trans-genomic metabolites arising from the interactive metabolism between the host genome and the gut microbiome.[476] Outlined in Figure 30, bile acids (BA) are synthesized in the liver from the nutrient cholesterol. The two primary BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA) undergo amidation and subsequent conjugation by either glycine or taurine, and therefore present downstream as molecules that are fully ionized at physiological pH.[477] This enhances the amphipathic and detergent properties which facilities lipid digestion and absorption. Only a small fraction (1-5%) of all BAs escape reabsorption into the distal ileum and enterohepatic circulation, however these are the few that enter the colon and are subjected to the transformative powers of the gut microbiota. It is here that a

bidirectional relationship exists, whereas unique bacterial enzymes can structurally and functionally modify the BAs, but the bile acids can also exert antimicrobial selection pressures on the community of bacteria. It is this host-based relationship that builds the blocks for a potential feedback mechanism that can control bacterial populations while also influencing hormonal signaling, lipid and glucose homeostasis, and disease perturbations and warrants further explanation in a multitude of fields, but specifically in those revolving around weight-gain and metabolic syndrome.

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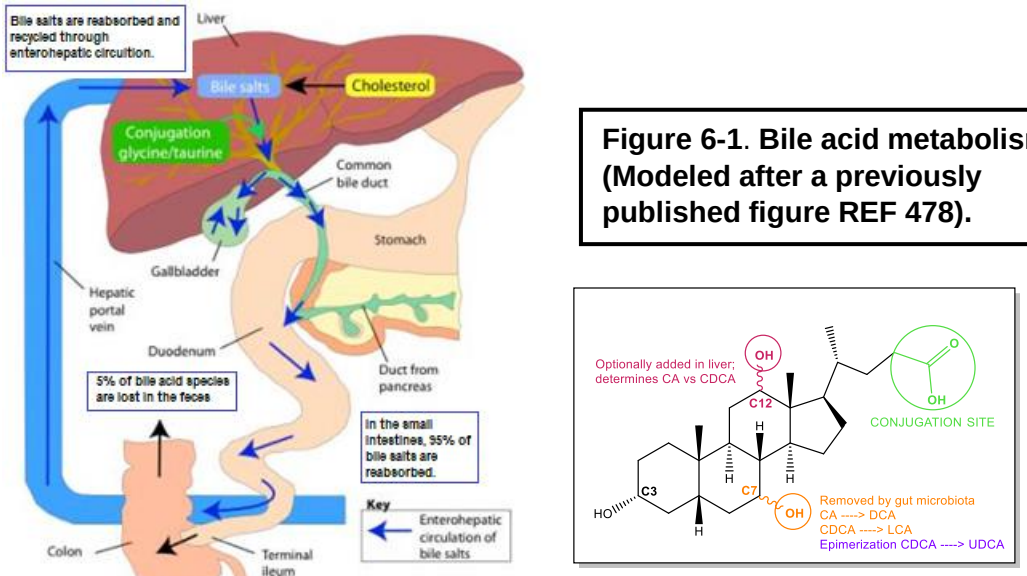


Figure 6-1. Bile acid metabolism (Modeled after a previously published figure REF 478).

The Gut Microbiota, Bile Acids, and Psychotropic-Induced Weight Gain

After extensive research into both the fields of the gut microbiome and bile acids, I took the ball and ran with it. I had spent many years deep in the thralls of psychiatric medicine and therapeutics, however one question always lingered; why do a large percentage of individuals experience such adverse and weight related side effects on a variety of psychotropic medicines? In my third year, I had a serendipitous opportunity to delve into the world of bariatric surgery in the same sense, and I learned a large amount about how both salient biological

components can play a significant role in weight-loss. I became fervent in my quest to better understand how human metabolism is controlled on a biochemical level, and because of Dr. Nurmi's previous work in the genetics of antipsychotic-induced weight gain, we were able to sit down and brainstorm a completely novel idea – elucidating a more direct, biological mechanism of this deleterious side effect. You can read more about how antipsychotic induced-weight gain (AIWG) works in the case study presented in this chapter below. I had to work from the ground up, and I spent a large amount of time developing a novel method for detecting human bile acids in plasma utilizing high performance liquid chromatograph tandem mass spectrometry. I truly felt that this was one of the crowning chemistry jewels of my chemistry PhD, and the entire method is presented here. Being cognizant of the backbones of precision medicine is rewarding as a scientist but being able to implement them in a way that has the potential to change people's lives is rewarding to my soul. In both this chapter and the next, chapter 7, I would like to share the preliminary results and continuing project I have developed during the last 2 years of my graduate career that brings a new viewpoint to the enigma that is weight gain following pharmaceutical treatment.

Original Method: Fit-For-Purpose HPLC-MS/MS Targeted Detection and quantitation of 11 Bile Acids and Their Biological Precursor, 7-alpha-hydroxycholest-4-en-3-one*

***** *This work is currently unpublished*

Lauren C Seaman, Hüseyin Kayadibi, Kym F Faull, Erika L Nurmi

Introduction

Classically viewed as the simple “dish-detergent” molecules of the digestive system, bile acids (BAs) are quickly gaining recognition as critical metabolic signaling compounds, hormone regulators, and disease state clinical biomarkers. BAs are the major component of bile, and are synthesized from cholesterol through a hepatic cytochrome p450 7A1-mediated enzymatic pathway[479]. They are powerful dietary lipid emulsifiers, allowing for solubilization and subsequent digestion of fats and oils through a mixed-micellular surfactant process.[480] Bile acids regulate their own synthesis, utilizing a negative feed-back loop that spans across the liver and the gut[481], where they can additionally act as antimicrobial agents.[482] Interestingly, recent research has begun to uncover the role of BAs as salient biological signaling molecules, acting as hormonal ligands for nuclear receptors such as the farnesoid X receptor (FXR), pregnane X receptor (PXR), and cell-surface G-protein coupled receptors (GPCRs),[483] which have all been implicated in glucose and lipid homeostasis in humans[484-486] . Stemming from this, BAs are now increasingly being identified as markers for hepatic disease states[487], chronic inflammation[488], and cancer[489, 490], making them a desirable, measurable target for many fields of work.

Bile acids obtain their robust structural diversity from cholesterol catabolism and amino acid conjugation in the liver, as well as enzymatic transformations by bacteria found in the gut[491]. Additionally, bile acids can become charged and associate with alkali metal cations such as Na^+ or K^+ to form bile salts, which can act as electron acceptors in intestinal homeostasis.[492] Importantly, they lend themselves to cross-talk between the human (or host) and gut microbial metabolism[493]. While the overall bile acid pool may contain close to 150 unique species[494], generally speaking this collection is composed of two primary BAs (cholic acid [CA] and chenodeoxycholic acid [CDCA] in humans, and CA and β-muricholic acid [BMCA] in rodents which are synthesized in hepatocytes, as well as 4 major secondary BAs (deoxycholic acid [DCA], lithocholic acid [LCA], ursodeoxycholic acid [UDCA], and hyocholic acid [HCA])[495]. These secondary bile acid species are produced through structural modifications of primary BAs in the small intestines and colon following interaction with present microbial populations. Various enzymes produced by these bacterial species have the capabilities to deconjugate, hydroxylate, dehydroxylate, and epimerize[491, 496-498] both primary and secondary BAs altering their chemical properties and binding affinities, indicating microbial importance in host enterohepatic circulation. Moreover, each of the individual BAs has the potential to further become conjugated at their terminal acetyl junction with either glycine or taurine[499], yielding an overall wide range of molecular variants. This provides an interesting analytical challenge for both sensitive and selective measurement of BAs.

While this challenge has been attempted by numerous researchers over the past decade, many of the published analytical methods suffer various shortcomings based on specific measurement needs. Liquid chromatography tandem mass spectrometry (LC-MS) has been frequently utilized for the separation and concurrent detection of BAs in both human and animal biological samples. However, successful methods often contain a plethora of pitfalls – making application

of these techniques to broad research studies, both clinical and laboratory based, an arduous task. Involved and tedious sample preparation[500], long analytical cycles[500, 501], lack of complete chromatographic separation[502], a requirement for expensive equipment or reagents[494, 503-505], and negative ion selectivity[494, 506, 507] seem to permeate the bulk of currently available BA measurement methods. It can be frustrating to attempt to successfully reproduce an analytical method with very narrow parameters. In fact, many clinical measurements of bile acids are accomplished through a blanket enzymatic assay that can only measure relative bile acid pool size[508, 509] completely circumventing the potential for these compounds as biomarkers in many metabolic and non-metabolic studies alike. This was the driving motivation for the method development described herein.

High performance liquid chromatography coupled with mass spectrometry (HPLC-MS) is the analytical foundation of this method, allowing for coverage of the diverse range of bile acid hydrophobicity[510] as well as a multitude of isobaric species. Given the range in chemical structures, a one-size-fits all approach is not the optimal way to present a method, which is why various MS parameters, sample preparations and chromatographic options were tested and presented as viable and reproducible options. Facile biological sample preparation for both human and rodent plasma was combined with a medium throughput analytical cycle (45 min) with both positive and negative ion ESI mode detectability options. Utilization of reversed-phased C_{18} separation via column chromatography provided excellent coverage of the main bile acid pool constituents, as well as 7-alpha-hydroxy-4-cholesten-3-one (C4) (a predictive and rate limiting marker for bile acid synthesis),[511] while still effectively separating isobaric conjugates and species. Multiple reaction monitoring mode was implemented to identify and track unique fragments of the BAs of interest, validating accurate measurement of 11 unique compounds in a lipid-rich biological matrix. Additionally, classically troublesome samples, such as pediatric,

disease state, and older (>10 years' storage) plasma collections were successfully measured. We feel the HPLC-MS parameters and options presented within provide an excellent starting point for a wide array of research goals involving BAs, and hopefully present others with the ability to incorporate these important molecules in more and growing studies.

Materials and Methods

Chemical Materials

All organic solvents used in biological sample preparation and mobile phases were prepared with LC-MS grade solvents and chemicals obtained from Sigma-Aldrich (St. Louis, MO). This included methanol, acetonitrile, formic acid, triethylamine, and ammonium acetate. Nine bile acid standards (cholic acid, taurocholic acid, glycocholic acid, chenodeoxycholic acid, taurochenodeoxycholic acid, deoxycholic acid, taurodeoxycholic acid, ursodeoxycholic acid, and lithocholic acid) were also purchased from Sigma-Aldrich (St. Louis, MO). One rodent-dominant bile acid (β-muricholic acid) was purchased from Cayman Chemical (Ann Arbor, MI). Additionally, one deuterated internal standard (chenodeoxycholic acid-2,2,4,4-d_4 [CDCA-D_4]), which was chosen due to its predominance in the human bile acid pool[512] and shared molecular weight with two other unique BAs, and 7-alpha-hydroxy-4-cholesten-3-one, were also procured from Sigma-Aldrich (St. Louis, MO). The structures of all the BA species measured can be seen in Figure 6-2.

Collection of Human Plasma

Samples were collected from pediatric patients with autism spectrum disorder, pediatric and adult healthy controls for use in the bile acid profiling method application. Blood was initially taken into Vacutainer blood collection tubes (Becton, Dickinson and Company, Franklin Lakes,

NJ) which were treated with 7-10 mg of K_2EDTA to prevent erythrocyte clotting. Venous blood samples were stored immediately at 4^{o}C until further processing could occur – within 1 week. All specimens were centrifuged at *2000xg*, 4^{o}C for 10 minutes in a Beckman Coulter Allegra 6R (Beckman Coulter, Indianapolis, IN) to separate red blood cells from what is deemed “blood plasma”. This resulting plasma sample was aliquoted into 500 µL fractions for long term storage at -80^{o}C. The oldest of the plasma samples used in the following measurements dated back to 1999, representing 18 years of -80^{o}C storage with viable detection of bile acids, while the newest plasma sample was collected 1 week before LC-MS analysis, indicating a robust method application timeframe.

Plasma Sample Preparation

Prior to profiling analysis, samples were thawed at 4oC, and 100 µL aliquots were transferred to 1.5 mL Eppendorf microcentrifuge tubes (Eppendorf, Hauppauge, NY), spiked with the internal standard (CDCA-D4, in a final concentration of 5 pmol/µL [5 uM] (5 µL addition of 20 pmol/µL in methanol). For a liquid-liquid based extraction, 300 µL of 100% methanol was added (1:3 v/v), before 1 min of high speed vortexting, followed by a 15-minute incubation at room temperature (RT). Following centrifugation (16,000xg, 5 min, RT) the resulting supernatant was transferred to a clean microcentrifuge tube. This supernatant was dried

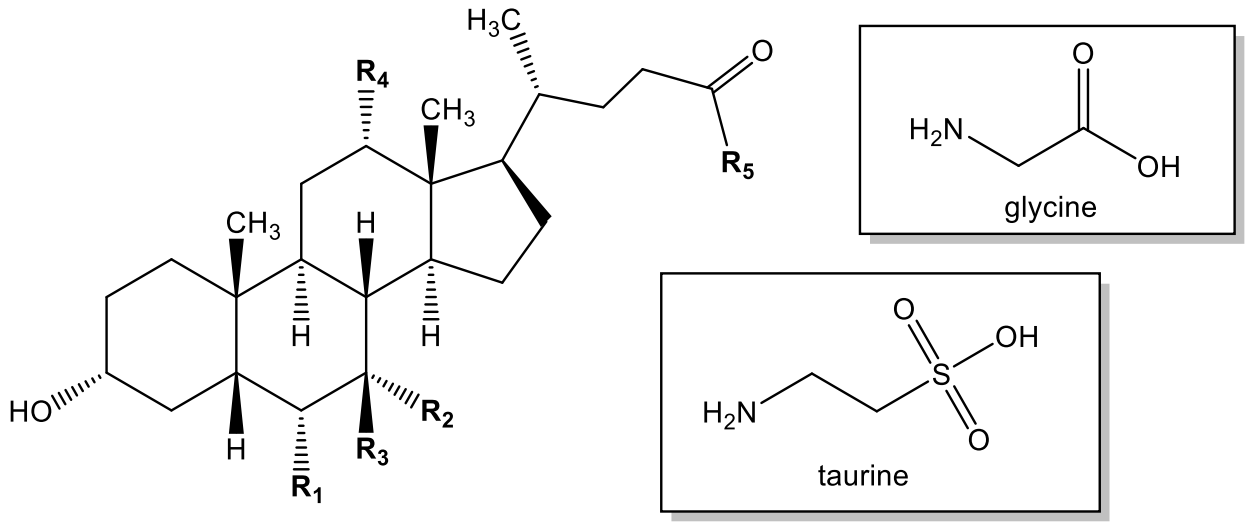
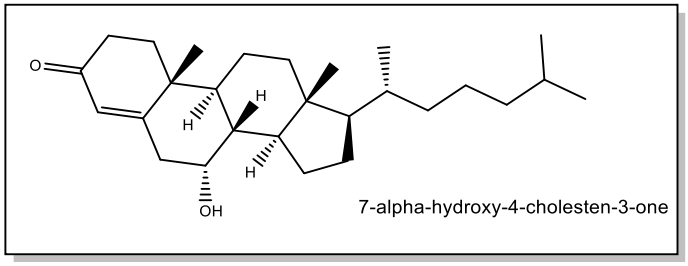


Figure 6-2. Structure variations and substitutions of the 11 bile acid species measured with their corresponding abbreviations. Tauro-conjugates have the amino acid taurine connected by the N terminus at the R_5 position. Glyco-conjugates have glycine connected by the N terminus at the R_5 position. Underneath the table is the structure of the 12th species evaluated, the bile acid synthesis precursor: 7-alpha-hydroxy-4-cholesten-3one.



down in a vacuum centrifuge (Savant SpeedVac Concentrator, ThermoFisher, Waltham, MA) for 2 hours, and the pellet was re-dissolved in 20 µL of 60/40% MeOH/H2O. A final round of high speed vortexing for 30 seconds, accompanied by centrifugation (16,000xg, 5 min, RT), provided a clear supernatant which was transferred to 300 µL polypropylene HPLC vials (American Chromatography Supplies, Vineland, NJ) for subsequent analytical examination.

Compound	R_1 (α)	R_2 (α)	R_3 (β)	R_4 (α)	R_5

Cholic acid *(CA)*	H	OH	H	OH	OH
Chenodeoxycholic acid *(CDCA)*	H	OH	H	H	OH
Deoxycholic acid *(DCA)*	H	H	H	OH	OH
Lithocholic acid *(LCA)*	H	H	H	H	OH
Ursodeoxycholic acid *(UDCA)*	H	H	OH	H	OH
Taurocholic acid *(TCA)*	H	OH	H	OH	$NHCH_2CH_2SO_3H$
Taurochenodeoxycholc acid *(TCDCA)*	H	OH	H	H	$NHCH_2CH_2SO_3H$
Taurodeoxycholic acid *(TDCA)*	H	H	H	OH	$NHCH_2CH_2SO_3H$
Glycocholic acid *(GCA)*	H	OH	H	OH	$NHCH_2COOH$
Glycochenodeoxycholic acid *(GCDCA)*	H	OH	H	H	$NHCH_2COOH$
β-Muricholic acid *(BMCA)*	OH ($R_1\beta$)	H	OH	H	OH

HPLC-MS Analysis – Positive & Negative ESI Conditions

Analysis was performed by an Agilent 1260 Infinity high performance liquid chromatograph (HPLC) (Agilent, Santa Clara, CA) coupled to an Agilent 6460 triple quadrupole mass spectrometer equipped with an electrospray ionization source operating in either the negative ion mode (ESI-) or the positive ion mode (ESI+) for targeted detection options. While the conditions described below produced the most sensitive and reliable BA detection results, additional chromatographic conditions were tested, and they are summarized in the Table 6-

1.

Ion Mode	Column	Mobile Phase A	Mobile Phase B	Gradient Length	Separation of BAs
Positive	Kinetex C18 1.7 uM, 150 x 2.1 mm	10 mM Ammonium Acetate in H2O	100% MeOH	70 mins	9 out of 11
Positive	Luna Omega polar C18 1.6 uM 100 x 2.1 mm	0.1% Formic Acid in H2O	100% MeOH	60 mins	7 out of 11
Negative	Kinetex C18 2.6 uM, 150 x 2.1 mm	10 mM Ammonium Acetate in H2O	100% MeOH	62 mins	6 out of 11
Negative	Hypercarb Porous Graphite Carbon 3 uM, 100 x 1 mm	0.1% Triethylamine in water	0.1% Triethylamine in ACN	62 mins	9 out of 11

Table 6-1. Compilation of four additional chromatographic conditions tested for bile acid detection sensitivity and separation.

The chromatographic methods were reverse-phase in nature, utilizing a long alkyl chain stationary phase (C18) paired with differing mobile phase systems for each positive and negative ion modes to facilitate both dampening of adduct effects, elution of widely varying hydrophobicity levels, as well as separation of isobaric

BA species. For ESI+, a Cadenza CD-C18 column (3.0 µm, 250 x 2 mm, Imtakt, Portland, OR) was selected for use due to its high steric selectivity, as CDCA and UDCA only differ by an inversion at a single stereocenter. The column was heated (55°C) for the entire gradient program, allowing for consistent and reliable retention times as well as avoidance of lipid content buildup on the column matrix that might have been leftover in the samples from earlier plasma preparation.[513] The aqueous mobile phase solvent A consisted of a volumetric preparation of 1 L of ultrapure water with 1 mL of neat formic acid (100/0.1 v/v). Organic mobile phase solvent B was comprised of a volumetric preparation of 1 L of acetonitrile with 1 mL of neat formic acid (100/0.1 v/v).

Time (min)	***%A***	***%B***	***Flow Rate (µL/min)***	***Pressure***
0	99	1	200	400
3.5	99	1	200	400
38	0	100	250	400
40	0	100	250	400
42	99	1	250	400
45	99	1	250	400

Table 6-2. Positive Ion ESI HPLC gradient conditions. Solvent A consisted of 100% / 0.1% ultrapure water to neat formic acid. Solvent B consisted of 100% / 0.1% acetonitrile to neat formic acid. Pressure represents maximum instrument threshold. The column utilized with this gradient was a Cadenza CD-C18 reverse phase (3.0 µm, 250 x 2 mm).

The gradient separation is described in Table 17. The injection volume of prepared biological samples was 8 µL. To minimize injector carry-over, a complete gradient wash cycle with a strong solvent (methanol) was performed sporadically throughout sample analysis as "blanks".

Time (min)	*%A*	*%B*	*Flow Rate (µL/min)*	*Pressure*
0	99	1	70	400
2	99	1	70	400
7	50	50	70	400
11	25	75	70	400
32	0	100	70	400
35	0	100	70	400
37	99	1	70	400
45	99	1	70	400

Table 6-3. Negative Ion ESI HPLC gradient conditions. Solvent A consisted of 100% / 0.1% ultrapure water to triethylamine. Solvent B consisted of 50%/37.5%/12.5%/0.1% water to acetonitrile to methanol to triethylamine. Pressure represents maximum instrument threshold. The column utilized with this gradient was a Kinetex EVO C18 reverse phase column (1.7 µm, 150 x 2.1 mm), requiring a decreased flow rate.

For ESI-, a Kinetex EVO C18 column (1.7 µm, 150 x 2.1 mm) (Phenomenex, Torrance, CA) was selected for use for base stable capabilities along with high selectivity due to small particle size. The column was again heated to a constant temperature of 55^{o}C for the entire duration of the chromatographic run. The aqueous mobile phase solvent A consisted of a volumetric preparation of 1 L of ultrapure water with 1 mL of triethylamine (TEA) (100/0.1 v/v) as a higher pH was observed to improve chromatographic separation of isobaric BA species. The organic mobile phase solvent B was prepared as a volumetric solution of 500 mL of ultrapure water, 375 mL of acetonitrile with 125 mL of methanol and an addition of 1 mL of TEA (50/37.5/12.5/0.1 v/v/v/v). The gradient separation is detailed in Table 6-3. The injection volume of patient samples was 8 µL. To minimize injector needle contamination, a complete wash cycle with a high polarity solvent (methanol) was performed occasionally throughout sample analysis as “blanks”.

MS/MS Conditions & Optimization

As mentioned above, the highest sensitivity results were obtained with an Agilent 6460 series triple quadrupole mass spectrometer (QQQ). However, both positive and negative ESI methods were replicated and confirmed on an LCQ DecaXP Plus mass spectrometer (ThermoFisher, Waltham, MA). Mass spectrometry parameters for the QQQ were as follows: capillary voltage was set to 4000 V, gas flow was adjusted to 8 L/min, the gas temperature was 300 ^{o}C, the nebulizer pressure was set at 15 psi, and the delta electron multiplier voltage (EMV) was set to +200/-0 for ESI+ and +0/-200 for ESI-. All the 12 BA species in this assessment yielded characteristic fragments when subjected to collision-induced dissociation and were therefore assayed using multiple reaction monitoring (MRM). The noted transitions for each of the 12 standards and 1 deuterated internal standard are provided in Table 6-4 (ESI+) and Table 4 (ESI-). Source parameters of fragmentor voltage and collision energy were optimized for each bile acid transition by direct flow injection at the HPLC flow rate and appropriate solvent composition (HPLC MS/MS conditions listed above). These optimizations were automatically performed using Agilent MassHunter Optimization software, and tested in duplicate before finalizing the values, and are also included in Table 6-5 & 6-6.

Method Validation

The finalized method presented above was further tested according to certain parameters set forth by the FDA for bioanalytical method validation.[514] Linearity was evaluated over a concentration range of 20 pM to 2 mM with a 5-point calibration curve. Samples designated as quality controls were prepared for each of the 12 species in MeOH/H2O (60/40 v/v) and utilized for both linearity assessment and matrix effect testing; QC1 (2mM) QC2 (200nM)

QC3 (20nM) and QC4 (2nM) QC5 (200pM). The limit of detection (LOD) was determined with a signal-to-noise (S/N) ratio greater than 3. Additionally, the lower limit of quantification (LLOQ) was determined with a S/N ratio greater than 5. Evaluation of the matrix effect from plasma biological fluids allowed for an assessment of reliability and selectivity of the proposed HPLC MS/MS method. This was accomplished by comparing the integrated peak area of the deuterated standard (CDCA-D4) in both spiked solvent (60% methanol) and in spiked plasma samples of fixed volumes. Potential for injector or column carry-over was tested by direct comparison of blank solvent samples versus QC1 and QC5.

Table 6-4. Positive Ion MRM transition monitoring species, product ions, and instrument source parameters. Bile acid acronyms can be found in Appendix 1. The majority of (M+H) ions were undetectable, or existed only at minimal levels, and dominating charged species consistently experience the loss of 1 or 2 water molecules.

Compound	***Molecular Weight (g/mol)***	***Monitoring Species***	***Precursor Ion***	***Product Ion***	***Fragmentor (V)***	***Collision Energy (V)***
TCA	515.7	$(M+H)-H_2O$	498	462	75	12
TCDCA	499.7	$(M+H)-2H_2O$	464	125	125	44
TDCA	499.7	(M+H)	500	464	105	8
GCA	465.6	(M+H)	466	412	95	12
GCDCA	449.6	$(M+H)-2H_2O$	432	414	100	15
CA	408.6	$(M+H)-2H_2O$	373	355	95	12
CDCA	392.6	$(M+H)-2H_2O$	357	357	90	0
DCA	392.6	$(M+H)-2H_2O$	357	357	90	0
LCA	376.6	$(M+H)-H_2O$	359	177	75	16
UDCA	392.6	$(M+H)-2H_2O$	357	357	90	0
BMCA	408.6	(M+H)+17	426	355	100	10
C4	400.7	(M+H)	401	177	135	20
CDCA-D4	396.6	$(M+H)-2H_2O$	361	261	132	23

Percent error of accuracy and precision of the described method were analyzed by measuring QC1, QC3, and QC5 on 3 intra-day (analyzed on the same day) replicates as well as an inter-day (measured on 3 separate days) replicate. Finally, the recovery of the deuterated standard (CDCA-D4) was evaluated. Control plasma was spiked with CDCA-D4 before sample preparation, and additionally in a sample after preparation was complete. Recovery was calculated as: area under the peak of the pre-spiked sample divided by the area under the peak of the post-spiked sample. This was repeated in 3 replicates of fixed volume and concentration.

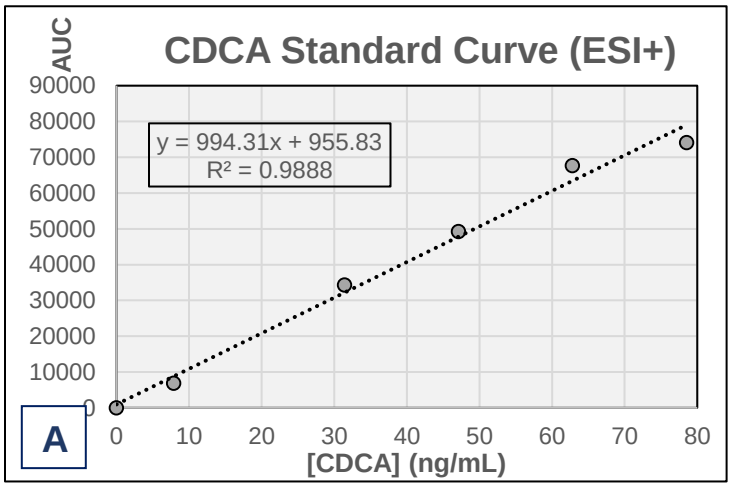
Table 6-5. Negative Ion MRM transition monitoring species, product ions, and instrument source parameters. Bile acid acronyms can be found in Appendix 1. βMCA was undetectable in negative ESI. Product ions for glycine and taurine conjugates are classic fragments of these charged amino acids and consistent across species.

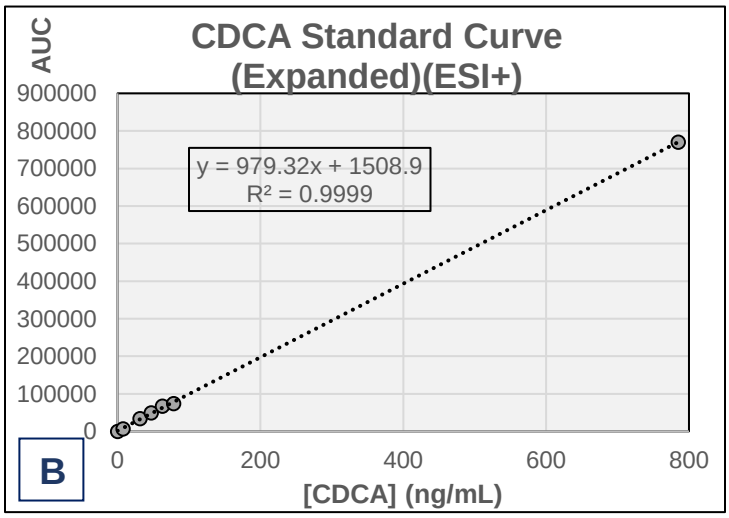
Compound	***Molecular Weight (g/mol)***	***Monitoring Species***	***Precursor Ion***	***Product Ion***	***Fragmentor (V)***	***Collision Energy (V)***
TCA	515.7	(M-H)	514	80	270	75
TCDCA	499.7	(M-H)	498	80	280	80
TDCA	499.7	(M-H)	498	80	270	70
GCA	465.6	(M-H)	464	74	220	45
GCDCA	449.6	(M-H)	448	74	200	45
CA	408.6	(M-H)	407	289	290	40
CDCA	392.6	(M-H)	391	391	240	0
DCA	392.6	(M-H)	391	391	240	0
LCA	376.6	(M-H)	375	369	190	11
UDCA	392.6	(M-H)	391	391	240	0
BMCA	408.6	(M-H)+46	452	406	110	5
C4	400.7	(M-H)	399	177	155	18
CDCA-D4	396.6	(M-H)+46	441	359	142	35

Data Processing and Statistical Analysis

Raw data files were interpreted using MassHunter qualitative analysis software (Agilent). Integration of bile acid chromatographic peaks was accomplished both by automatic integration as well as manual integration in the case of the more difficult to detect BA signals (LCA, GCA).

In order to determine bile acid concentration in patient and control plasma samples, a standard curve of the previously measured QC samples was included with each sample batch, also eliminating the majority of errors that may occur due to batch effects. As the concentrations of each QC sample is known, linear regression was utilized to determine unknown sample concentrations by graphing integrative peak area vs. sample concentration. An example of the linear standard curve for all BAs can be seen in Figure 6-3. Dilution factors were taken into account to arrive at the final concentration of each BA in plasma.





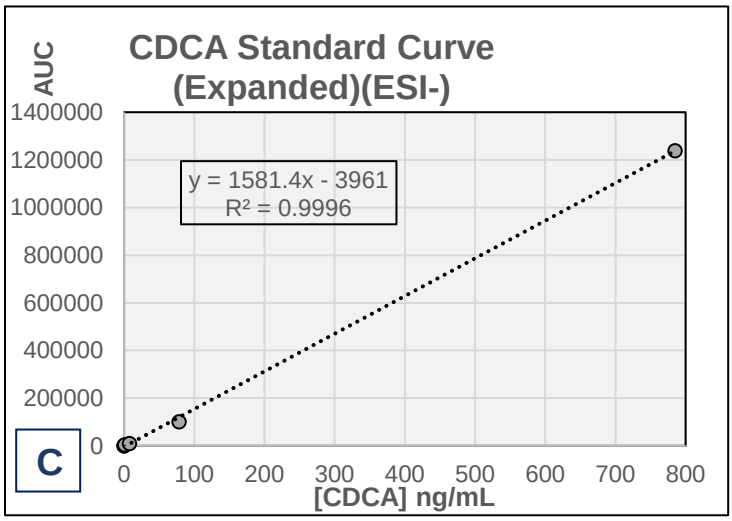


Figure 6-3

A. A representative BA calibration curve in positive mode detection; graph shows chenodeoxycholic acid (CDCA) which is the most dominant primary BA in humans. Six points in the lower range were examined, 0 nM, 2 nM (0.785 ng/mL), 20 nM (7.85 ng/mL), 80 nM (31.4 ng/mL), 120 nM (47.1 ng/mL), and 160 nM (62.8 ng/mL). This curve was created for all 12 species measured in the method and utilized for calculations of LOD and LLOQ. Linearity was high, with an R2 of 0.99. The standard curves were utilized in final concentration calculations of human samples.

B. A representative BA calibration curve in positive mode detection; graph shows chenodeoxycholic acid (CDCA) which is the most dominant primary BA in humans. Eight points over a large range were examined, 0 nM, 2 nM (0.785 ng/mL), 20 nM (7.85 ng/mL), 80 nM (31.4 ng/mL), 120 nM (47.1 ng/mL), 160 nM (62.8 ng/mL), 200 nM (78.5 ng/mL), and 2 uM (785 ng/mL). This was also generated for all 12 BA species, to confirm accuracy and precision at higher concentrations, and that the ULOQ was not passed.

C. A representative BA calibration curve in negative mode detection; graph shows chenodeoxycholic acid (CDCA) which is the most dominant primary BA in humans. Five points over a large range were examined, 0 nM, 2 nM (0.785 ng/mL), 20 nM (7.85 ng/mL, 200 nM (78.5 ng/mL), and 2 uM (785 ng/mL). This was also generated for all 12 BA species, and higher concentrations were more accurate in the negative ion mode. Linearity was high at an R2 of 0.99, similar to that of ESI+.

Results and Discussion

Chromatographic Optimization and Application

In order to produce and maintain adequate chromatographic separation and overall peak shape for all BA species in positive ion detection mode, it was necessary to lower the pH of the mobile phases. Both 10 mM ammonium acetate and ~24 mM formic acid (or 0.1% v/v) solutions were tested in aqueous (water) and organic (acetonitrile or methanol) with the presented MRM method. While both were sufficient to achieve sharp peaks and stable retention times, it was found that the presence of ammonium acetate caused both predominant ammonium adducts in electrospray as well as bile salt build up in the column due to the high percentage of organic phase needed to move the compounds off. Formic acid addition did not produce formate adducts while still lowering the pH of both mobile phases to between 2-4 and positively impacted the sensitivity of the assay. These findings correlating pH and BA retention are consistent with previously published work.[494, 515, 516]

In contrast, we found it was necessary to increase the pH during negative ion detection of BA species. This was mainly due to the fact that utilizing ammonium acetate or formic acid in a buffer would tend to protonate one of the various hydroxyl sites on the unconjugated bile acids and allow for easier coupling with acetate and formate adducts while hindering the ability to ionize the steroidal backbone efficiently. We found through the addition of 0.1% triethylamine (TEA) in both mobile phases, the pH increased sufficiently to allow for proper ion formation and unique fragmentation. This constituent also allowed for a greater magnitude of sensitivity as well as improved resolution for the isobaric BA species. This effect also has been reported in previous literature describing superior BA separation methods with high-pH anion exchange

chromatography.[517] Partial addition of the stronger eluent methanol in the acetonitrile organic phase also helped to prevent bile salt formation in the column and machine.

The sensitivity of both systems was found to be sufficient for the detection of selected BA species in normal human and rodent plasma despite a large concentration range in biological fluids. Although the majority of the bile acids observed in both healthy controls and disease sample analysis can be distinguished by the targeted method below, there are a multitude of both unidentified as well as additional secondary and tertiary species that could potentially act as differential markers of certain diseases. This warrants further translational exploration.

Targeted MS/MS Analysis of 12 Bile Acid Species with Positive ESI

Overall, 11 unique bile acid species and one bile acid precursor were targeted in a single HPLC-MS/MS method operating in the positive ion mode of detection. Utilization of the above optimized chromatographic conditions for ESI+ allowed for detection of all compounds in biological samples with a high level of separation. While MRM was implemented due to increased sensitivity and unique fragmentation options, chromatography and the

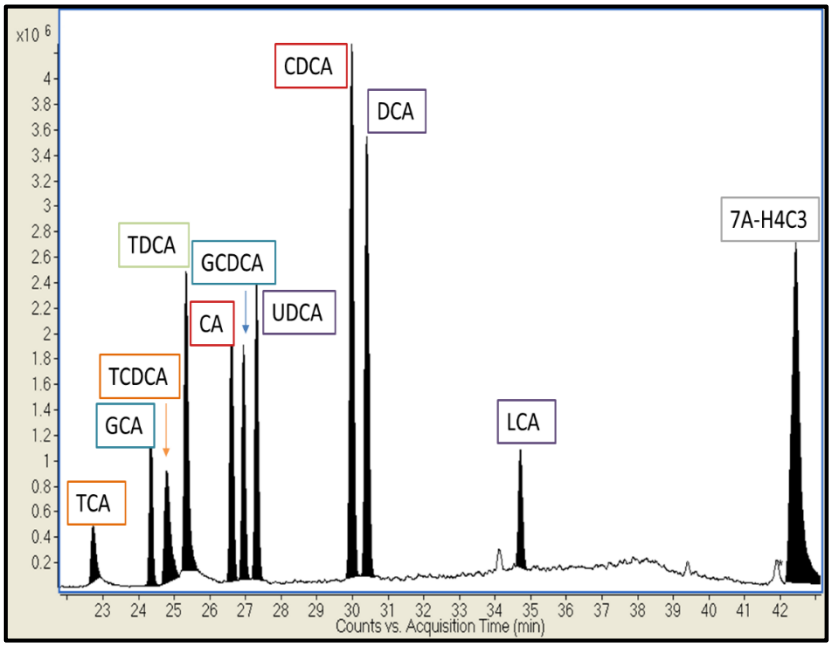


Figure 6-4. Extracted ion chromatogram (EIC) of the 10 human specific bile acids and their precursor (7α-H4C3 or C4) measured in one mixture, separated by the described HPLC method and positive ESI. All species give unique retention times with quantitative peaks, including isobaric compounds, during a 45-minute gradient. As the column utilized is C18 reverse phased based, it can be concluded that 7α-H4C3 is the strongest retainer and most likely displays the highest non-polar character.

length of the method alone was sufficient to separate even isobaric BA species. (Figure 6-4) Due to conjugation with either taurine or glycine which significantly alters the thermodynamic properties of a BA, we observed a decreased retention time for these species across the board, allowing for a facile distinction between conjugated and unconjugated variants. It is worth noting that although conjugated BA species do not comprise the majority of bile that is reabsorbed through the portal vein due to bile salt hydrolase activity in the ileum[518], the amino acid addition has been shown to change the physiological activity of these molecules[519] and is relevant in clinical applications.

Combining superior LC separation with optimized MRM transitions offered significant sensitivity for the bile acids examined. Coupling this with consistent reproducibility allows us to confidently report positive-ion monitoring as a feasible approach for biological analysis of these compounds.

Targeted MS/MS Analysis of 12 Bile Acid Species with Negative ESI

Additionally, the same 11 BA species and BA precursor were targeted in a single HPLC-MS/MS method operating in the negative ion mode of detection. As described above, differing chromatographic conditions were used for ESI- and adequately allowed for detection of all compounds in biological matrixes (specifically EDTA treated blood plasma). Alternating the variant of C18 particle column used in this polarity allowed for complete LC separation of

these molecules and produced unique retention times to that of positive ion detection (Figure 6-5). MRM ions, fragmentations, and parameters for this method are listed in Table 6-5.

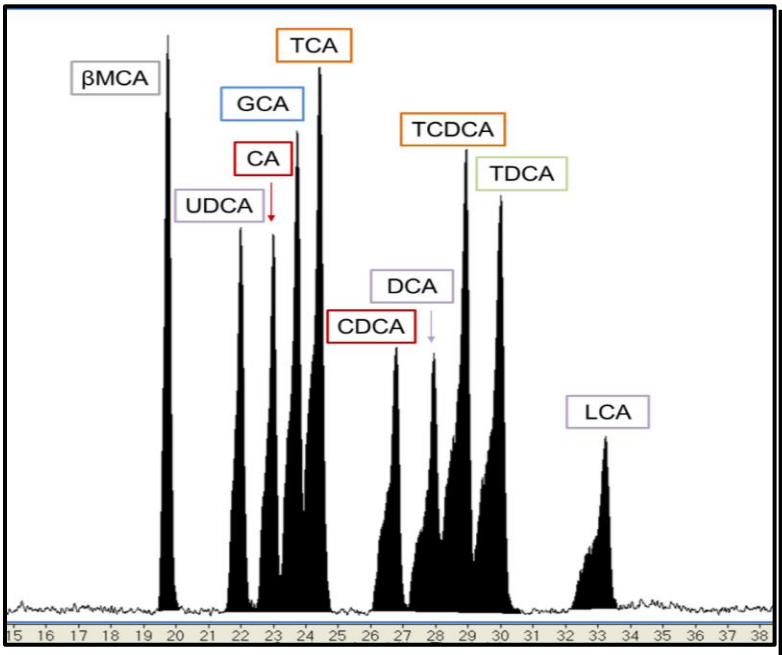


Figure 6-5. Extracted ion chromatogram (EIC) of 10 bile acids measured in one mixture, separated by the described HPLC method and negative ESI. All species give unique retention times with quantitative peaks, including isobaric compounds, during a 45-minute gradient. As the column utilized is C18 reverse phased based, it can be concluded that β-MCA (a rodent dominant BA) is the most polar bile acid measured, likely due to its unique stereochemistry at C-6. It can be seen that negative ESI detection produced slightly broader peaks than ESI+ with less defined retention times but is still suitable for sensitive measurement.

Comparison of Positive Ion vs. Negative Ion MS Detection

It is worth discussing the main differences between ESI+ and ESI- detection of bile acids, as both methods were sufficient for identification and quantification of all targeted species. The multitude of univariate mass spectrometry methods for the detection of bile acids was previously discussed in the introduction, and we are unaware of a report that discusses both applicable methods for immediate use in research endeavors.

The most notable variance between positive and negative modes of detection is the bile acid ions and adducts that are formed and subsequently monitored. Comparing Figures, one can clearly see that the predominant fragment species in each mode are quite unique, and differ from the classically observed (M+H) and (M-H) peaks. While no significant difference in

sensitivity based on peak integration was seen between these differing transition fragments, ESI+ tended to produce more robust signals and lower detectable ranges for all BA species; ESI- excelled at detecting toxic secondary bile acid species (i.e. LCA and DCA) that are not as dominant in peripheral blood.

We were able to confidently confirm that BAs are susceptible to changes in pH, altering the ionization and detection patterns based on mobile phase selection. There are significant differences in this application for either ESI+ or ESI-. Greatest sensitivity was achieved with lower pH (acidic spiked) mobile phases in positive detection MS, while the opposite was true for negative detection, and a higher than neutral pH produced improved resolution. These differences can partially be explained by the steroidal backbone of the examined species, where hydroxyl substitutions greatly vary both the lipophilicity and hydrophilicty of BAs. This variation is ideal for specific method development that may include media or additional species that thrive in a particular pH, allowing for complete optimization.

Method Validation

To ensure the maximum signal detection for each bile acid species measured, optimization of MRM ion transitions was completed with Agilent MassHunter software. It was found that although isobaric variants CDCA, DCA, and UDCA produced unique fragments, a higher sensitivity was achieved when monitoring a loss of the same two water fragments with no collision energy, where separation was achieved through chromatography and retention time verification. Similar methodology was also used for internal standard (IS) monitoring, as it was a deuterated form of CDCA. This ensured specific and reproducible results across both method applications. According to FDA guidelines, the HPLC-MS/MS method was validated

for accuracy and precision on intra- and inter-day injections, as well as linearity in both detection ion modes.

In positive ESI, the limit of detection (LOD) for the 12 compounds detected ranged between 1.9-3.1 ng/mL for primary BAs (CA,CDCA), 3.6-11.6 ng/mL for secondary BAs (DCA,LCA,UDCA,BMCA), 0.73-5.6 ng/mL for conjugated BAs (GCA,GCDCA,TCA,TCDCA,TDCA) and was 15.4 ng/mL for C4,final concentration. ; in negative ESI the LOD's ranged between 22.4-27.4 ng/mL for primary BAs,17.6-27.3 ng/mL for secondary, 12.3-24.0 ng/mL for conjugated BAs and was 26.8 ng/mL for C4. Linearity was explored over a broad range of concentrations as discussed above; 20 pM to 2 uM, due to the fact that there are various concentrations of BA present in bile, blood, urine, and feces, and although molarity concentrations can be utilized for reporting standard measurements, we found that individual BAs were better represented in final data using ng/mL, particularly for clinical applications. Tables 6-6 and 6-7 show the accuracy and precision of both intra- and inter-day measurements of the 11 BAs and 1 precursor targeted. As the limit of detection in measurement was approached, there was a marked decrease in accuracy for all species. However, negative ESI was superior in combating this variance, and further exploration of this effect would be warranted. While the range of extraction recovery of all analytes was averaged out to be 70.1%, the lowest percentage contributors are due to the more toxic, secondary BA species (particularly LCA) as the human body is efficient in removing these from the bloodstream before reabsorption and can experience stronger matrix effects. [520]

Table 6-6. Intra-day (same day in triplicate, n=3) and inter-day (3 separate days in triplicate, n=9) measured means (±standard deviation), accuracy, and precision for each of the 12 BA species measured in positive ion mode. The spiked concentrations are shown in ng/mL and vary based on the molecular weight of the compound, as original QC samples were prepared in molarities; 20 nM, 200 nM, 20 uM are the respective concentrations. Ng/mL concentrations were calculated using the calibration curve. Precision >35% for inter-day measurements is ideal, indicating acceptable machine variability and robust method application. It is worth noting that overall, samples closer to the LOD (20nM set) had lower accuracies across the board. This could be improved by fine tuning methods at lower concentrations. Both TCDCA and C4 were not detectable at the 20 nM concentration during this experiment, as this fell close to if not below the LOD. ESI+ provided the greater sensitivity and precision.

Sample (+)	Spiked (ng/mL)	Measured (mean ± SD)	Accuracy (%)	Precision (RSD%)
CA				
Intra-day	8.17	10.8±0.7	112.9	6.9
	81.7	78.8±0.6	96.5	0.8
	817	817.3±5.9	100.0	0.7
Inter-day	8.17	14.0±2.1	171.0	15.4
	81.7	75.3±2.1	92.2	2.8
	817	817.6±0.2	100.1	0.0
CDCA				
Intra-day	7.85	11.6±2.1	117.7	18.4
	78.5	74.3±0.7	94.7	0.9
	785	785.4±21.2	100.0	2.7
Inter-day	7.85	13.1±1.7	167.0	12.8
	78.5	72.7±2.2	92.6	3.0
	785	785.5±13.3	100.1	1.7
DCA				
Intra-day	7.85	8.2±5.0	103.9	61
	78.5	78.2±1.2	99.6	1.5
	785	785.0±6.9	100.0	0.9
Inter-day	7.85	12.8±4.6	163.3	36.0
	78.5	73.0±4.5	93.0	6.2
	785	785.5±6.4	100.1	0.8
LCA				
Intra-day	7.53	5.7±0.0	76.1	0.0
	75.3	77.3±5.6	102.6	7.3
	753	752.8±5.4	100.0	0.7
Inter-day	7.53	13.4±5.4	178.1	40.4
	75.3	68.8±8.3	91.4	12.1
	753	753.6±4.7	100.1	0.6
UDCA				

Intra-day	7.85	9.4±0.3	120.0	3.2
	78.5	76.1±0.6	96.9	3.4
	785	785.2±10.2	100.0	12.7
Inter-day	7.85	12.7±3.5	162.3	27.8
	78.5	73.1±3.9	93.1	5.3
	785	785.5±6.9	100.1	0.9
TCA				
Intra-day	10.3	17.3±0.2	168.0	4.5
	103	95.3±2.5	92.5	2.6
	1030	1030.7±20.6	100.1	2.9
Inter-day	10.3	-	-	-
	103	93.6±3.6	90.9	3.9
	1030	1030.9±13.2	100.1	1.3
TCDCA				
Intra-day	9.97	-	-	-
	99.7	96.4±3.3	96.9	3.4
	997	997.3±127.0	100.0	12.7
Inter-day	9.97	12.0±8.6	120.0	71.7
	99.7	97.5±11.4	97.8	11.7
	997	997.2±72.6	100.0	7.3
TDCA				
Intra-day	9.97	11.8±0.7	118.5	6.0
	99.7	96.7±3.9	97.0	4.0
	997	997.2±6.1	100.0	0.6
Inter-day	9.97	14.4±2.7	144.3	18.5
	99.7	94.8±3.8	95.1	4.0
	997	997.4±19.0	100.0	1.9
GCA				
Intra-day	9.31	11.2±0.5	120.6	4.3
	93.1	89.9±0.6	96.6	0.7
	931	931.2	100.0	0.5
Inter-day	9.31	13.0±1.6	140.1	11.9
	93.1	89.0±1.4	95.6	1.6
	931	931.4±	100.0	1.3
GCDCA				
Intra-day	8.99	11.0±0.3	122.4	2.9
	89.9	86.6±0.6	96.3	0.7
	899	899.2±6.7	100.0	0.7
Inter-day	8.99	11.6±0.8	129.0	6.8
	89.9	87.0±0.6	96.8	0.7
	899	899.3±9.9	100.0	1.1
BMCA				

Intra-day	8.17	9.8±0.7	119.4	7.4
	81.7	84.0±2.1	102.8	16.3
	817	817.2±7.0	100.0	0.9
Inter-day	8.17	10.2±3.0	125.1	29.8
	81.7	79.4±3.8	97.2	4.8
	817	817.2±5.9	100.0	0.7
C4				
Intra-day	8.01	-	-	-
	80.1	76.2±10.4	95.2	13.7
	801	803.2±47.3	100.3	5.9
Inter-day	8.01	-	-	-
	80.1	66.9±15.7	83.6	15.7
	801	802.2±52.2	100.1	6.5

Table 6-7. Intra-day (same day in triplicate, n=3) and inter-day (3 separate days in triplicate, n=9) measured means (±standard deviation), accuracy, and precision for each of the 12 BA species measured in negative ion mode. The spiked concentrations are shown in ng/mL and vary based on the molecular weight of the compound, as original QC samples were prepared in molarities; 20 nM, 200 nM, 20 uM are the respective concentrations. Ng/mL concentrations were calculated using the calibration curve. Precision >35% for inter-day measurements is ideal, indicating acceptable machine variability and robust method application. It is worth noting that overall, samples closer to the LOD (20nM set) had lower accuracies across the board. This could be improved by fine tuning methods at lower concentrations. Both TCDCA and TDCA were not seen at the lowest concentration (20nM). However, C4 still had stable measurements in this range, showing that negative ESI is preferable to measure this bile acid precursor, likely due to its unique chemical structure that differs from the typical steroid backbone of the other 11 bile acids. ESI- had slightly lower inter-day precision than ESI+ overall.

Sample (-)	Spiked (ng/mL)	Measured (mean ± SD)	Accuracy (%)	Precision (RSD%)
CA				
Intra-day	8.17	8.8±0.1	108.1	0.8
	81.7	81.0±5.1	99.1	6.3
	817	817.1±6.5	100.0	0.8
Inter-day	8.17	5.0±1.5	61.1	30.6
	81.7	85.2±5.9	104.3	7.0
	817	816.7±5.0	100.0	0.6
CDCA				
Intra-day	7.85	12.0±1.9	125.7	16.2
	78.5	73.9±1.7	94.2	2.3
	785	785.4±3.6	100.1	0.5

Inter-day	7.85	9.7±6.4	124.2	65.4
	78.5	76.4±14.3	97.3	18.7
	785	785.2±38.4	100.0	4.9
DCA				
Intra-day	7.85	11.3±4.4	143.8	38.6
	78.5	74.7±0.2	95.2	0.2
	785	785.3±18.6	100.0	2.4
Inter-day	7.85	8.5±6.8	108.3	79.4
	78.5	77.8±14.6	99.1	18.7
	785	785.1±46.7	100.0	5.9
LCA				
Intra-day	7.53	13.3±3.9	134.0	29.0
	75.3	68.9±4.6	91.5	6.7
	753	753.6±2.4	100.1	0.3
Inter-day	7.53	8.9±4.2	117.5	47.5
	75.3	73.8±5.1	98.1	6.9
	753	753.1±2.4	100.0	0.3
UDCA				
Intra-day	7.85	11.3±1.6	89.3	14.0
	78.5	74.7±3.1	95.2	4.2
	785	785.3±8.7	100.0	1.1
Inter-day	7.85	8.3±3.0	106.3	35.9
	78.5	78.0±13.5	99.3	17.3
	785	785.0±7.6	100.0	1.0
TCA				
Intra-day	10.3	18.7±0.5	143.9	2.4
	103	123.5±8.1	95.0	6.6
	1030	1030.7±12.3	100.1	1.2
Inter-day	10.3	22.5±4.4	173.4	19.4
	103	119.2±9.0	91.7	7.6
	1030	1031.2±8.5	100.1	0.8
TCDCA				
Intra-day	9.97	10.7±1.0	107.0	9.7
	99.7	99.2±4.5	99.2	4.5
	997	1000.1±13.7	100.0	1.4
Inter-day	9.97	-	-	-
	99.7	110.4±4.3	110.4	3.9
	997	999.1±19.8	99.9	2.0
TDCA				
Intra-day	9.97	10.8±0.7	108.0	6.5
	99.7	98.8±9.6	99.1	9.8
	997	997.1±18.5	100.0	1.9

Inter-day	9.97		-	-	-
	99.7		112.0±8.0	112.3	7.1
	997		995.9±18.1	99.9	1.8
GCA					
Intra-day	9.31		8.2±1.5	88.5	18.3
	93.1		94.3±5.8	101.3	6.2
	931		930.9±4.2	100.0	0.5
Inter-day	9.31		5.7±2.9	61.8	50.6
	93.1		97.0±5.7	104.2	5.9
	931		930.6±9.8	100.0	1.1
GCDCA					
Intra-day	8.99		9.7±0.3	108.2	2.7
	89.9		89.1±5.3	99.1	6.0
	899		899.1±7.2	100.0	0.8
Inter-day	8.99		6.7±2.6	74.6	38.7
	89.9		92.4±4.6	102.8	5.0
	899		898.8±7.9	100.0	0.9
BMCA					
Intra-day	8.17		10.0±0.5	117.6	5.4
	81.7		79.6±0.5	97.5	0.7
	817		817.2±8.8	100.0	1.1
Inter-day	8.17		7.4±2.3	90.2	30.6
	81.7		82.6±2.1	101.1	2.5
	817		816.9±6.5	100.0	0.8
C4					
Intra-day	8.01		8.5±0.1	108.1	0.8
	80.1		77.8±5.0	99.1	6.3
	801		785.1±6.3	100.0	0.8
Inter-day	8.01	10.7±4.8	136.7	44.9	
	80.1	75.3±24.8	96.0	32.9	
	801	785.3±9.5	100.0	1.2	

Finally, to confirm the scientific application of this analytical method, plasma samples from a pediatric autistic population (n=14) that had been taking the antipsychotic medication Risperidone (RSP) [521] were processed and measured according to the ESI+ parameters described above. These samples represent a unique challenge for method validation, as many of them were over 15 years old at the time of measurement, having been stored long

term at -80°C. It is also important to quantitate BAs in child populations such as these, as current data is limited, but has the potential to represent critical clinical indications early in life. The children in the study underwent an 8-week time course of RSP, at which point their final fasting BA concentrations were measured. The average concentrations of the 5 main bile acids that exist in plasma can be seen in Table 6-8. Interestingly, overall significant changes were observed in BA concentrations before and after taking this medication. This alone demonstrates the clinical importance of better understanding enterohepatic circulation patterns and contributors through targeted analytical methods.

Table 6-8. Fasting plasma BA concentrations of pediatric patients (aged 4-17 years old) following 8 weeks of risperidone pharmaceutical treatment, displaying the average, median, minimum, and maximum concentrations for each of the 5 main BA species occurring in humans as a test for method application. Values are represented in ng/mL, and sample preparation allowed for detection below the limit of quantitation from liquid concentration before injection.

n = 28, Following Treatment *All values in ng/mL*				
Bile Acid	**Average**	**Median**	**Min**	**Max**
CA	16.81	13.5	1.86	51.09
CDCA	25.91	18.23	5.04	65.22
DCA	39.94	35.6	6.99	78.79
UDCA	42.92	18.79	6.2	150.52
TCA	8.81	4.62	1.35	47.02

Conclusion

Increasing awareness and importance of bile acids as signaling molecules in both basic and translational research spurs a need for reliable and facile quantitative methods. BA diversity and challenging chemical properties make fit-for-purpose mass spectrometry the most straightforward approach to many related quantitative and qualitative measurements. Targeted detection through reversed-phase HPLC coupled with MS allows for sensitive and reliable determination of unconjugated, glycine, and taurine conjugated BAs in human and rodent plasma fluids in either positive or negative ion modes.

Measurement of these species, along with the indicative precursor 7a-hydroxycholest-4-en-3-one can generate detailed profiles that have the potential to be vital components of clinical testing for disease states including metabolic syndrome, inflammation, psychiatric disorders, and liver problems. We believe having a wide array of equipment and procedural choices when developing a targeted bile acid MS/MS detection workflow will be invaluable for translational and clinical research alike. It is the hope that this understudied field of BAs and their related biological and pathological mechanisms will become more accessible to many laboratories.

MxH Case Study #1: A Putative Development Pathway; Microbiome – Bile Acid Cross Talk in the Biological Mechanism of Psychotropic-Induced Weight Gain*

***This work is currently unpublished.*

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Introduction

Antipsychotic (AP) medications are critical for the stabilization of psychotic, mood, and behavioral disorders.[522] APs are prescribed to 1-2% of adults (~7M)[523] and 0.8-1.2% of youth (~1M).[524, 525] Prescribing rates have been steadily rising for decades (increasing 750% in the first decade of the millennium),[526] and is greatest in the demographic groups most vulnerable to adverse effects—the underprivileged, elderly and very young.[527-530] While effective, antipsychotics carry serious risks—most commonly, weight gain and cardiometabolic abnormalities, which affect up to 60% of patients[531] and present a major obstacle to long-term treatment.[532-534] An increased susceptibility to and magnitude of AIWG has been repeatedly documented in children.[533, 535, 536] Within a period of only 11 weeks, youth starting risperidone (RSP) may gain up to 3 kg. Associated abnormalities such as central obesity, insulin resistance, dyslipidemia, and systemic inflammation[537-539] reach sufficient magnitude in childhood to trigger and accelerate atherosclerosis.[537, 539-543] Cardiovascular disease accounts for the largest proportion of premature deaths in the severely mentally ill, whose life expectancy is dwarfed by

that of the general population by as much as 20 years—a gap that has increased over recent decades, paralleling antipsychotic prescribing.[544, 545]

Little is known about the mechanism of AIWG and its subsequent influence on metabolic syndrome (MS). Differential risk across the drug class is not explained by simple receptor binding profiles at contributing sites such as the muscarinic system, but other mechanistic links are lacking. A few supported risk genes explain only a small percentage of the risk.[62, 546, 547] Novel, testable hypotheses are sorely needed that examine how these APs and other similar psychotropic medications interact with our underlying biology. We asked ourselves where a logical place to begin the search for fundamental links to AIWG might be and began to uncover relevant research in opposite situations – bariatric surgery patients who were losing significant amounts of weight.

While it has long been suggested that the various benefits of bariatric surgery are simply a result of mechanical restriction of the stomach and malabsorption of nutrients caused by intestinal changes, contemporary studies are beginning to uncover more contributing factors. Based on alterations in the flow and anatomic routing of ingested nutrients following surgery, hypotheses have emerged implicating bile acids as key molecules in this avenue of weight-loss.[548] Classically viewed as the simple “dish-detergent” molecules of the digestive system, bile acids (BAs) are quickly gaining recognition as critical metabolic signaling compounds, hormone regulators, and disease state clinical biomarkers. BAs are the major component of bile and are synthesized from cholesterol through a hepatic cytochrome p450 7A1 (CYP7A1)-mediated enzymatic pathway.[479] They are powerful dietary lipid emulsifiers, allowing for solubilization and subsequent digestion of fats and oils through a mixed-micellular surfactant process.[480]

BAs have been found to regulate lipid, glucose and cellular energy homeostasis by acting as signaling molecules at nuclear hormone receptors in the liver and intestines known as farnesoid X (FXR).[549] Systemic levels of BAs are significantly elevated in patients following both major bariatric procedures, Roux-en-Y gastric bypass and sleeve gastrectomy, suggesting a critical role for these molecules in the mechanism of weight regulation. Interestingly, in a study of human BA profiles, total plasma BA concentrations were higher in both obese and diabetic subjects than in healthy controls[550], indicating there are complex interactions between the host and these regulatory compounds that can change how they affect metabolic states. This can potentially be delineated by the differing agonist and antagonist properties of various primary and secondary BAs at FXR. Primary (1^{o}) BAs (cholic acid [CA] and chenodeoxycholic acid [CDCA]) are potent agonists for FXR, while the secondary (2^{o}) BA ursodeoxycholic acid (UDCA) is an FXR antagonist.

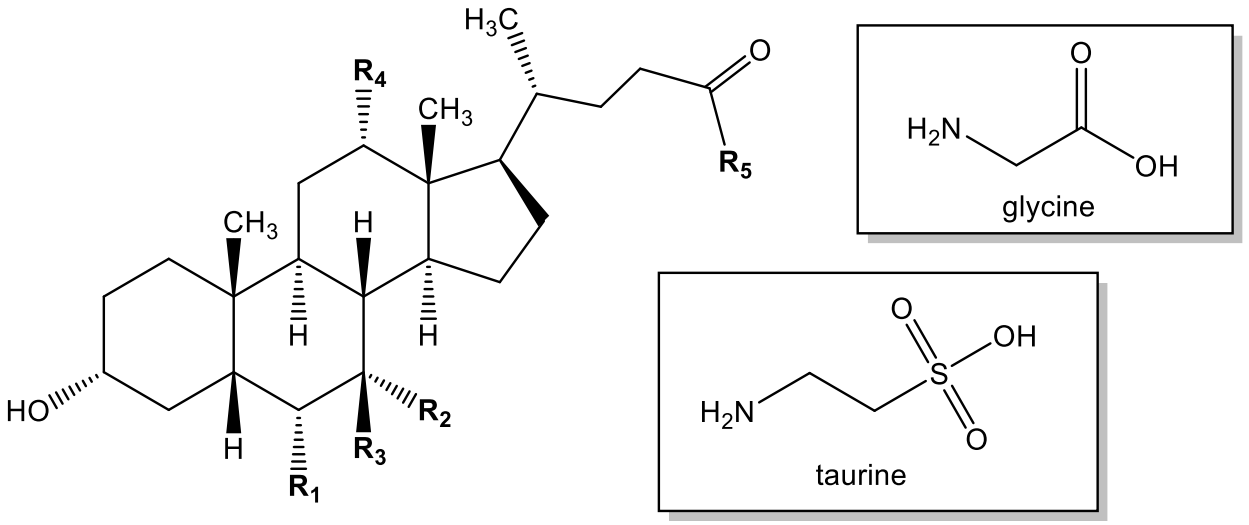
As the binding potential for BAs changes in accordance to their relative lipophilicity and hydrophilicity, this study aims to measure the concentrations of a set of primary and secondary BAs in a specific population that has undergone treatment with an antipsychotic medication utilizing high performance liquid chromatograph tandem mass spectrometry (HPLC-MS), and test whether or not the relative makeup of a person's bile acid pool can provide us with significant biological clues to explain how weight-gain following treatment occurs on a molecular level. A better understanding of this complex mechanism will provide the foundation for targeted treatment and drug development strategies for pharmaceutical-induced and, potentially, other-cause weight gain and metabolic dysfunction.

Materials and Methods

Collection of Human Plasma & Vital Metrics

Samples were collected from pediatric patients with autism spectrum disorder, n=30, after initiating and sustaining a second-generation antipsychotic Risperidone (RSP) treatment for an 8-week trial, fasting at two timepoints: baseline and after 2 months on medication. Blood was initially taken into Vacutainer blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) which were treated with 7-10 mg of K2EDTA to prevent erythrocyte clotting. Venous blood samples were stored immediately at 4oC until further processing could occur – within 1 week. All specimens were centrifuged at 2000xg, 4^{o}C for 10 minutes in a Beckman Coulter Allegra 6R (Beckman Coulter, Indianapolis, IN) to separate red blood cells from what is deemed “blood plasma”. This resulting plasma sample was aliquoted into 500 µL fractions for long term storage at -80^{o}C.

All participants underwent baseline clinical evaluation by the study psychiatrist including general health, diet, and body measurements (height, weight, body mass index [BMI]. At both visits, repeated body measurements, medication dose and adherence since the prior session, any new treatments received (with special focus on antibiotics), and the occurrence and reason for any protocol deviations were recorded.



Compound	R_1 (α)	R_2 (α)	R_3 (β)	R_4 (α)	R_5	Type of BA
Cholic acid *(CA)*	H	OH	H	OH	OH	1º
Chenodeoxycholic acid *(CDCA)*	H	OH	H	H	OH	1º
Deoxycholic acid *(DCA)*	H	H	H	OH	OH	2º
Lithocholic acid *(LCA)*	H	H	H	H	OH	2º
Ursodeoxycholic acid *(UDCA)*	H	H	OH	H	OH	2º
Taurocholic acid *(TCA)*	H	OH	H	OH	$NHCH_2CH_2SO_3H$	2º

Figure 6-6. Structure variations and substitutions of the 6 bile acid species measured with their corresponding abbreviations. For bile acids as a whole, tauro-conjugates have the amino acid taurine connected by the N terminus at the R_5 position. Glyco-conjugates have glycine connected by the N terminus at the R_5 position.

Chemical Materials for Mass Spectrometry Analysis

All organic solvents used in biological sample preparation and mobile phases were prepared with LC-MS grade solvents and chemicals obtained from Sigma-Aldrich (St. Louis, MO). This included methanol, acetonitrile, formic acid, triethylamine, and ammonium acetate. 6 bile acid standards (cholic acid, taurocholic acid, chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid, and lithocholic acid) were also purchased from Sigma-Aldrich (St. Louis, MO). Additionally, one deuterated internal standard (chenodeoxycholic acid-2,2,4,4-d4 [CDCA-D4]), which was chosen due to its predominance in the human bile acid pool[512] and shared molecular weight with two other unique BAs, was also procured from Sigma-Aldrich (St. Louis, MO). The structures of all the BA species measured can be seen in Figure 6-6.

Plasma Sample Preparation

Prior to BA profiling analysis, samples were thawed at 4oC, and 100 µL aliquots were transferred to 1.5 mL Eppendorf microcentrifuge tubes (Eppendorf, Hauppauge, NY), spiked with an internal standard (CDCA-D4, in a final concentration of 5 pmol/µL [5 uM] (5 µL addition of 20 pmol/µL in methanol). For a liquid-liquid based extraction, 300 µL of 100% methanol was added (1:3 v/v), before 1 min of high speed vortexting, followed by a 15-minute incubation at room temperature (RT). Following centrifugation (16,000xg, 5 min, RT) the resulting supernatant was transferred to a clean microcentrifuge tube. This supernatant was dried down in a vacuum centrifuge (Savant SpeedVac Concentrator, ThermoFisher, Waltham, MA) for 2 hours, and the pellet was re-dissolved in 20 µL of 60/40% MeOH/H2O. A final round of high speed vortexing for 30 seconds, accompanied by centrifugation (16,000xg, 5 min, RT), provided a clear supernatant which was transferred to 300 µL polypropylene

HPLC vials (American Chromatography Supplies, Vineland, NJ) for subsequent analytical examination.

HPLC-MS Analysis – Positive ESI Conditions

Analysis was performed by an Agilent 1260 Infinity high performance liquid chromatograph (HPLC) (Agilent, Santa Clara, CA) coupled to an Agilent 6460 triple quadrupole mass spectrometer equipped with an electrospray ionization source operating in the positive ion mode (ESI+) for targeted detection options. The conditions described below were developed by our lab for a personalized method and produced the most sensitive and reliable BA detection results in-house.

The chromatographic method was reverse-phase in nature, utilizing a long alkyl chain stationary phase (C18) paired with a mobile phase system to facilitate both dampening of adduct effects, elution of widely varying hydrophobicity levels, as well as separation of isobaric BA species. For ESI+, a Cadenza CD-C18 column (3.0 µm, 250 x 2 mm, Imtakt, Portland, OR) was selected for use due to its high steric selectivity, as CDCA and UDCA only differ by an inversion at a single stereocenter. The column was heated (55^{o}C) for the entire gradient program, allowing for consistent and reliable retention times as well as avoidance of lipid content buildup on the column matrix that might have been leftover in the samples from earlier plasma preparation.[513] The aqueous mobile phase solvent A consisted of a volumetric preparation of 1 L of ultrapure water with 1 mL of neat formic acid (100/0.1 v/v). Organic mobile phase solvent B was comprised of a volumetric preparation of 1 L of acetonitrile with 1 mL of neat formic acid (100/0.1 v/v). The injection volume of prepared biological samples was 8 µL. To minimize injector carry-over, a complete gradient wash cycle with a strong solvent (methanol) was performed sporadically throughout sample analysis as “blanks”.

All the 6 BA species in this assessment yielded characteristic fragments when subjected to collision-induced dissociation and were therefore assayed using multiple reaction monitoring (MRM). The finalized method presented above was further tested according to certain parameters set forth by the FDA for bioanalytical method validation for properties such as linearity, limit of detection, limit of quantitation, precision, and accuracy.[514]

Data Processing and Statistical Analysis

Raw data files were interpreted using MassHunter qualitative analysis software (Agilent). Integration of bile acid chromatographic peaks was accomplished both by automatic integration as well as manual integration in the case of the more difficult to detect BA signals (LCA).

In order to determine bile acid concentration in patient and control plasma samples, a standard curve of the previously measured QC samples was included with each sample batch, also eliminating the majority of errors that may occur due to batch effects. As the concentrations of each QC sample is known, linear regression was utilized to determine unknown sample concentrations by graphing integrative peak area vs. sample concentration. Dilution factors were taken into account to arrive at the final concentration of each BA in plasma.

Results

First and foremost, it is important to quantitate BAs in child populations such as the ones described above, as current data is limited, but has the potential to represent critical clinical indications early in life, particularly in the case of monitoring and attenuating psychotropic side effects. The children in the study initiated and sustained an 8-week time course of RSP, at which point their final fasting BA concentrations were measured. The average concentrations of the 5 main bile acid that exist in plasma (LCA, a toxic secondary BA species, was too low of a concentration to reliably detect across samples) can be seen in Table 6-8.

In this pilot dataset of 30 children from the RUPP RSP trial, we also observed marked changes in the total BA pool and diversity, with predominant increases in 1° BAs. Primary BAs were significantly more pronounced in those with high versus low AIWG (p=0.01, Fig. 35A). This pattern was replicated in 4 subjects started on RSP with moderate weight gain recruited at Baylor College of Medicine, a collaborator's site, with a 1° BA increase of 960 nmol/L, similar to 1,374 nmol/L in the RUPP sample.

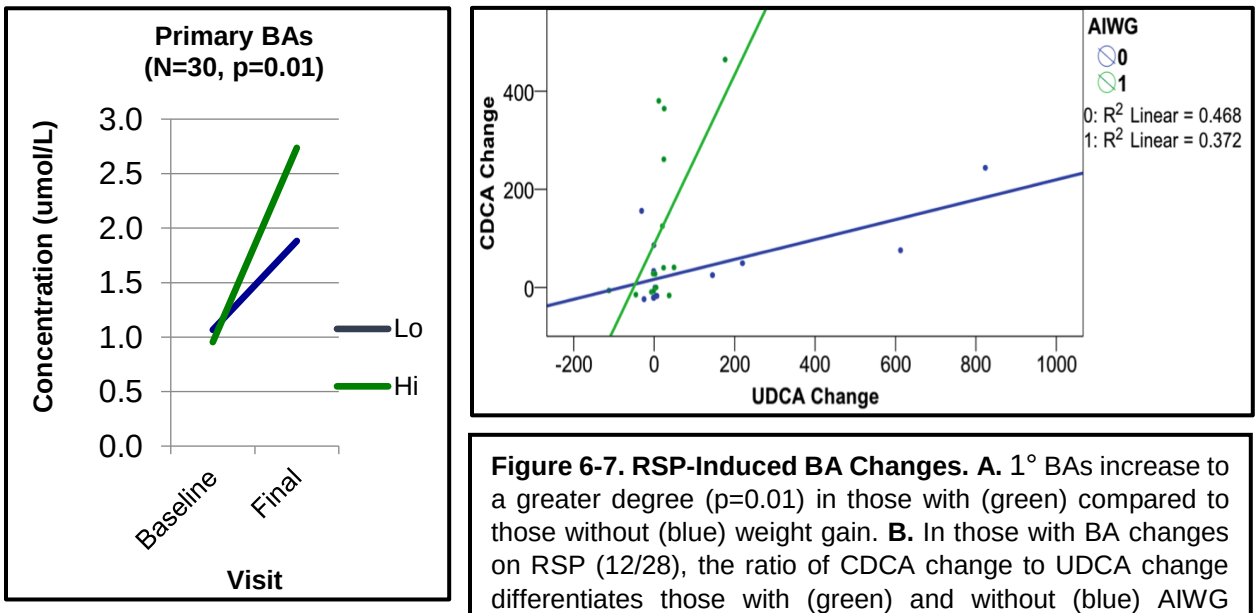


Figure 6-7. RSP-Induced BA Changes. A. 1° BAs increase to a greater degree (p=0.01) in those with (green) compared to those without (blue) weight gain. **B.** In those with BA changes on RSP (12/28), the ratio of CDCA change to UDCA change differentiates those with (green) and without (blue) AIWG (p=0.001). All 28 subjects shown. Units are nmol/L.

Both CDCA and UDCA measures at baseline and 8 weeks were available for 28/30 RUPP participants. 12 of these 28 displayed a significant change (from baseline to 8 weeks) in either CDCA or UDCA (>0.5 SD). In this subset showing BA change with RSP, the ratio of the change in CDCA to the change in UDCA differentiated those with AIWG (ΔCDCA/ΔUDCA>1) from those without (ΔCDCA/ΔUDCA<1). This algorithm correctly predicts AIWG status in 11/12 (92%) participants whose ratios change (p=0.001, Fig. 35B), with a sensitivity of 100% and specificity of 86%. Sex, age, race/ethnicity, and RSP dose/plasma level were tested but did not contribute to BA differences. Test-Retest reliability of CDCA, UDCA, and 1° BA repeated measurements in the same individual (N=2) were precise within 4 nmol/L.

Discussion

While previous studies have described AIWG and resulting metabolic syndrome (MS), and even searched for genes conferring risk, mechanistic approaches are lacking. As far as we are aware, this examination of peripheral bile acids in pediatric patients taking psychotropic medications is the first to demonstrate that significant changes are occurring in the BA pool following treatment. There are multiple factors that make these findings potentially so significant. Within the last few decades, it has been discovered that bile acids and bile salts have the ability to activate specific nuclear receptors, expressed in liver and GI tract cells, such as farnesoid X receptor (FXR) and G protein coupled receptor TGR5.11 Binding and subsequent activation of these receptors alter the expression of multiple genes that encode protein or enzyme products involved in the regulation of glucose, fatty acids, metabolism, bile acid synthesis, metabolism, and even energy metabolism.[486, 551, 552] In more recent years, it has also been shown that bile acids can stimulate the expression of endocrine L-cells[553]

which are implicated in secretion of satiety hormones peptide YY (PYY) and glycogen-like protein 1 (GLP-1). This provides elucidative connections for BA quantification and the biological mechanism of AIWG/MS.

We can strengthen these tenuous links by examining a similarly growing field of study – the gut microbiome. Recent evidence has fueled a growing enthusiasm for the role of the collective bacterial population in the human gastrointestinal tract in host metabolism and energy harvest,[554] glucose homeostasis,[491] and pharmaceutical breakdown.[555] Preclinical studies of the antipsychotics olanzapine (OLZ) and RSP link AIWG with obesogenic microbiome changes in mice, including an increased Firmicutes to Bacteroidetes (F/B) phyla ratio.[556-559] Moreover, preclinical studies have also demonstrated that gut bacteria are necessary for AIWG. In the only translational study published to date, our team confirmed that AIWG is associated with microbiome changes in children.[560]

The relationship between the microbiome and BA pool is bidirectional. The microbiome regulates BA synthesis and reabsorption and shapes the BA pool.[561] Both gram positive and negative bacteria produce intracellular enzymes such as bile salt hydrolase and hydroxysteroid dehydrogenase (HSDH) that can deconjugate taurine and glycine adduct bile salts[562] as well as hydroxylate 1° BAs to form 2° and 3° forms.[563] This deconjugation and epimerization results in an increase in BA hydrophilicity, affecting membrane permeability and efficiency of lipid solubilization. While almost all BAs are natural ligands for the hepatic FXR receptor, binding affinity and activity are substantially altered by BA modifications.[564] For example, the 1° BA CDCA is hepatically synthesized and excreted into the intestinal lumen in bile. Gut bacteria then convert CDCA to UDCA, which has opposing effects on downstream FXR signaling.[565-569]

Since FXR signaling is promoted by 1° BAs (CDCA and CA) but inhibited by the 2° BA UDCA,

the microbiome could influence metabolism by shifting the balance between these BA species. Conversely, BAs alter intestinal bacterial composition by moderating oxidative and pH stress in the small bowel and chelating vital cellular ions, thus promoting or inhibiting the growth of certain species.[476] Direct antimicrobial effects result from detergent-like disruption of bacterial membranes[561] and indirect effects are mediated by FXR activation, triggering upregulation of genes involved in epithelial mucosal defense.[482]

We hypothesize that a ΔCDCA/ΔUDCA<1 could occur when epimerase+ bacteria are unavailable to convert CDCA to UDCA, resulting in excess CDCA and AIWG. Therefore, we believe future experiments should examine whether relative abundance of a key bacterial species (B. fragilis) that can catalyze the first step in epimerization was affected by RSP exposure. In Figure 6-8, we have proposed a potential active biological mechanism of AIWG.

The data presented here, while preliminary, provide the foundation for a potentially targetable and biological based model of AIWG. Further research is needed to replicate findings in larger samples, clarify links between microbiome and BA, and determine whether associations are causal or simply an epiphenomenon or downstream effect. Some weaknesses of the current student include the small sample size of participants, and while power was sufficient to generate statistically meaningful results, future studies that aim to affix the microbiome and bile acids would benefit from at least double the sample population (n=60+) to help delineate downstream affects.

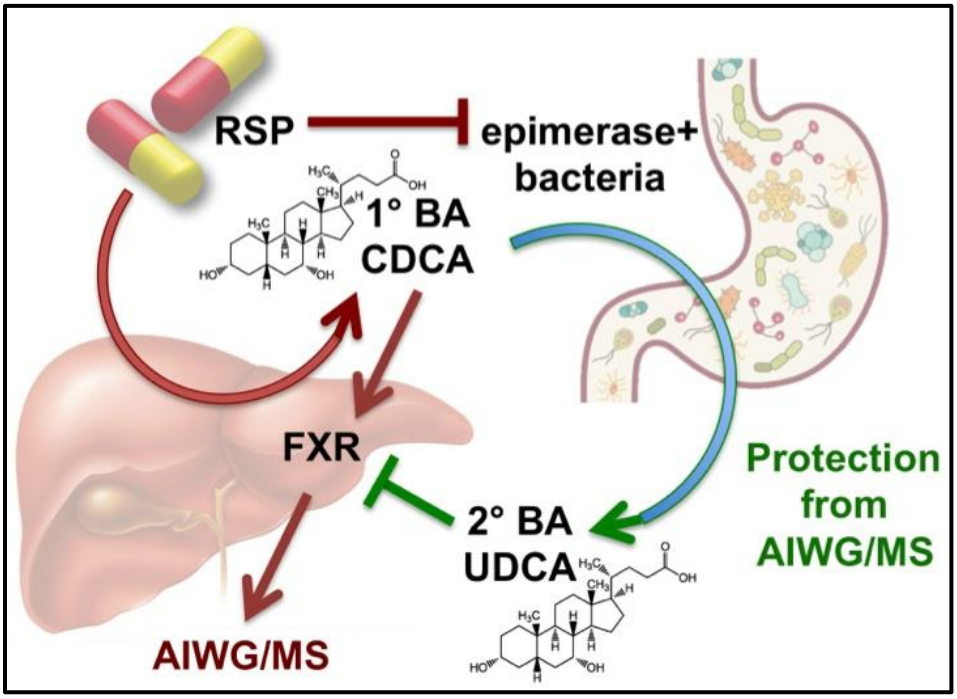


Figure 6-8. Proposed Model of AIWG/MS. RSP impacts BA and gut microbial composition, favoring more obesogenic profiles. These pathways promote FXR signaling, negatively impacting lipid and glucose homeostasis, adiposity, and BMI. A protective mechanism whereby CDCA is converted to UDCA by epimerase+ gut bacteria counteracts this pathway; UDCA blocks FXR signaling, thus opposing AIWG/MS. The balance between these two pathways dictates metabolic response to RSP.

It is also worth noting that addition of more bile acid species in the analytical quantification would be desirable for continuing work. This study was only able to reliable detect and measure 5 major human BAs, however there are close to 150 unique compounds in human bile.[494] Conversely, as more compounds are added to a MS analysis, it could potentially result in issues with multiple testing, spurious associations, and the need for a larger population size. These secondary bile acid species are produced through structural modifications of primary BAs in the small intestines and colon following interaction with present microbial populations. Various enzymes produced by these bacterial species have the capabilities to deconjugate, hydroxylate, dehydroxylate, and epimerize[491, 496] both primary and secondary BAs altering their chemical properties and binding affinities, indicating

microbial importance in host enterohepatic circulation and overall metabolic homeostasis, and quantifying additional variants has the potential to yield even more insightful biological connections to AIWG. Our team has already begun to add 10 additional BA species to the bioanalytical method described above for future studies.

Finally, in order to disentangle direct BA involvement with AIWG/MS, downstream biomarkers for FXR activation would be an ideal target to assay in later work on this topic. FXR signaling results in secretion of gut-derived FGF19 and subsequent suppression of precursors of hepatic BA synthesis (7α-hydroxycholesterol and 7α-hydroxy-4- cholesten-3-one [C4]). Both of these factors can be measured successfully in blood to provide additional insight on the direction of the proposed mechanism.

Conclusion

Antipsychotics have revolutionized treatment of several severe medical conditions; therefore, their use is necessary despite serious side effects. We have shown that RSP treatment results in unfavorable bile acid changes which are significantly linked with AIWG in pediatric populations. This drug exposure resulted in BA pool alterations that can differentiate those who experience AIWG from those without and has the prospective to guide clinical care moving forward. Additionally, due to an evolving understanding of the gut microbiome's involvement with host metabolism, we have proposed an extended hypothesis and theoretical model for bile acid-microbiome cross talk based on chemistry and function. Further research is needed to replicate findings in larger samples, clarify links between microbiome and BA, and determine whether associations are causal or simply an epiphenomenon or downstream effect. Ultimately, the identification of an easily measurable biomarker of AIWG risk could provide clinical guidance. If proven, highly actionable treatment targets are clear, and potential treatments are immediately available.

Chapter 6 Wrap-Up

Chapter 7 will directly continue from the conclusions of the above case study, wrapping up my dissertation as a whole, and presenting the current, larger replication project to validate my preliminary findings.

Chapter 7

Future Directions, Conclusions, and Final Thoughts

Future Directions

Based on the encouraging and intriguing preliminary results just discussed in Chapter 6, and with the help and leadership of my PI, Dr. Nurmi, we decided to write and submit a R21 research proposal grant to the NIH to explore our putative biological pathway of antipsychotic-induced weight gain further. Below you fill find this document, that was just recently accepted and funded by the National Institute of Child Health and Human Development (NICHD). It is ongoing, and I will be continuing to serve as a research assistant on this grant and perform the pertinent bile acid measurements and statistical and quantitative analysis as we accumulate enough participants. I am beyond excited at the prospect this study holds to inform the world of adverse treatment side effects following psychotropic medication, which I will discuss further in the conclusions following.

Current Study and Future Directions : The role of bile acid-microbiome cross-talk in psychotropic-induced weight gain and cardiometabolic dysfunction*

***This is a submitted and funded R21 grant proposal.

I. SIGNIFICANCE.

A. Antipsychotic-Induced Weight Gain (AIWG) & Metabolic Syndrome.

Antipsychotic (AP) medications are critical for the stabilization of psychotic, mood, and behavioral disorders.[522] APs are prescribed to 1-2% of adults (~7M)[523] and 0.8-1.2% of youth (~1M).[524, 525] Prescribing rates have been steadily rising for decades (increasing 750% in the first decade of the millennium),[526] and is greatest in the demographic groups most vulnerable to adverse effects—the underprivileged, elderly and very young.[527-530] While effective, antipsychotics carry serious risks—most commonly, weight gain and cardiometabolic abnormalities, which affect up to 60% of patients[531] and present a major obstacle to long-term treatment.[532-534] An increased susceptibility to and magnitude of AIWG has been repeatedly documented in children.[533, 535, 536] Within a period of only 11 weeks, youth starting risperidone (RSP) may gain up to 3 kg. Associated abnormalities such as central obesity, insulin resistance, dyslipidemia, and systemic inflammation[537-539] reach sufficient magnitude in childhood to trigger and accelerate atherosclerosis.[537, 539-543] Cardiovascular disease accounts for the largest proportion of premature deaths in the severely mentally ill, whose life expectancy is dwarfed by that of the general population by as much as 20 years—a gap that has increased over recent decades, paralleling antipsychotic prescribing.[544, 545]

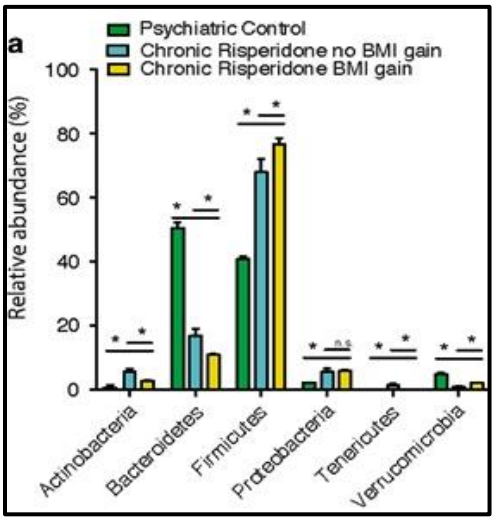
Despite this increase, very little is known about the mechanism of AIWG/MS. Differential risk across the drug class is not explained by simple receptor binding profiles, but other biological mechanistic links are lacking. A few supported risk genes explain only a small percentage of the risk.[62, 546, 547] Novel, testable hypotheses are sorely needed.

B. The Gut Microbiome & AIWG.

Recent evidence has fueled a growing enthusiasm for the role of the collective bacterial population in the human gastrointestinal tract in host metabolism and energy harvest,[570] glucose homeostasis,[571] and pharmaceutical breakdown.[572] Preclinical studies of the antipsychotics olanzapine (OLZ) and RSP link AIWG with obesogenic microbiota changes in mice, including an increased Firmicutes to Bacteroidetes (F/B) phyla ratio.[573-576] Moreover, preclinical studies have also demonstrated that gut bacteria are *necessary* for AIWG.[573-576] In fact, AIWG is absent in germ-free mice but can be induced by microbiome transplant.[573] Similar F/B changes, increased adiposity, and inflammation were reported in OLZ-treated rats and could be prevented by co-administration of an antibiotic cocktail that effectively sterilized the gut.[575].[574] Similarly, mice receiving RSP developed AIWG, mediated by decreased energy expenditure and transferrable by fecal transplant.[576]

In the only translational study published to date, our team confirmed that AIWG is associated with microbiome changes in RSP-treated children.[577] In line with preclinical studies, RSP treatment was associated , cross-sectionally, with elevated F/B ratio and a host of differences in the metabolic potential of the gut microbiota (Fig. 7-1a).[578] Concurrently, a small longitudinal study of children enrolled within days (mean=3.2, s.d.=5.2) of starting RSP substantiated these findings. Within 1-3 months of RSP initiation, the F/B ratio had begun to increase, appearing to

plateau by 5-6 months (Fig. 7-1b). Importantly, the F/B ratio was positively correlated with the magnitude of AIWG (Fig. 7-1c).



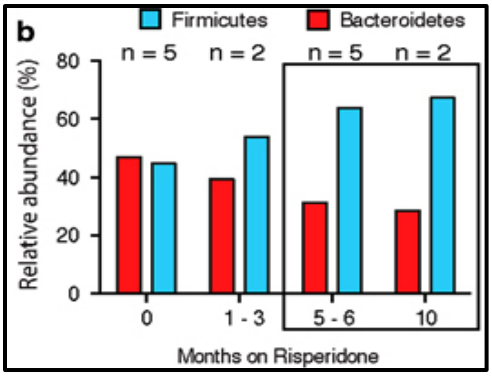
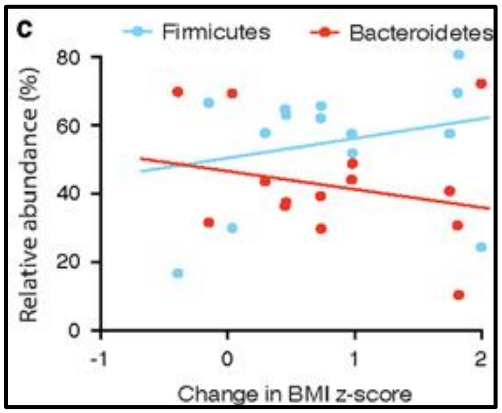


Figure 7-1a. Cross-Sectional RSP-Related Microbiome Changes. Phyla-level differences between controls (green) and chronic RSP–treated subjects with AIWG (yellow) and without (blue) reveal phyla changes similar to those seen in obesity (increased *F/B* ratio).**7-1b. Prospective RSP-Associated F/B Increase.** By 1-3 mo., relative abundance of *F* (blue) *vs. B* (red) diverges, with maximum increase in F/B ratio at 5-6 mo. **7-1c. F/B ratio predicts AIWG.** BMI (z-score) change is positively correlated with *F* (blue) to *B* (red) relative abundance.



C. Bile Acids Regulate Energy Balance.

While BAs were traditionally assigned the simple role of lipid surfactants aiding in fat absorption, they are now considered steroid hormones with a regulatory role in energy balance via tropism for specific hepatic, intestinal, and adipose tissue nuclear receptors.[551, 553, 579] Primary (1°) BAs, chenodeoxycholic acid (CDCA) and cholic acid (CA), are synthesized from cholesterol in the liver.[580] 1° BAs are potent agonists for the liver nuclear receptor farnesoid X (FXR), involved in lipid and glucose homeostasis, while the secondary (2°) BA ursodeoxycholic acid (UDCA) acts as an FXR antagonist.[565-569] In a study of human BA profiles, total plasma BA concentrations were higher in both obese and diabetic subjects than in healthy controls.[550] Specifically, the 1° BAs

CDCA and CA were associated with insulin resistance[550] and CDCA was positively correlated with BMI. Rodent and human data suggest that oral CDCA treatment, once used to dissolve gallstones, results in unfavorable lipid changes (increased total cholesterol/LDL[581] and decreased HDL).[582] Conversely, the secondary BA UDCA has been shown to promote positive lipid changes and reduce inflammation.[583-586] UDCA also reduces intestinal absorption of more toxic 1° BAs.[587] Furthermore, BA sequestrants, which disrupt BA enterohepatic circulation, lower plasma glucose and long-term glucose regulation marker HbA1c in hyperlipidemia clinical trials.[588]

The relationship between the microbiome and BA pool is bidirectional. The microbiome regulates BA synthesis and reabsorption and shapes the BA pool.[561] Both gram positive and negative bacteria produce intracellular enzymes such as bile salt hydrolase and hydroxysteroid dehydrogenase (HSDH) that can deconjugate taurine and glycine adduct bile salts[562] as well as hydroxylate 1° BAs to form 2° and 3° forms.[563] This deconjugation and epimerization results in an increase in BA hydrophilicity, affecting membrane permeability and efficiency of lipid solubilization. While almost all BAs are natural ligands for the hepatic FXR receptor, binding affinity and activity are substantially altered by BA modifications.[564] For example, the 1° BA CDCA is hepatically synthesized and excreted into the intestinal lumen in bile. Gut bacteria then convert CDCA to UDCA, which has opposing effects on downstream FXR signaling.[565-569] Rodent studies highlight the importance of signaling through the FXR receptor as a mechanism for microbiome effects on energy balance. Data from germ-free and antibiotic treated mice suggest that the microbiome promotes diet-induced obesity through FXR signaling,[589, 590] and FXR deficiency reduces weight and improves glucose homeostasis in mouse models of obesity.[591] Following bariatric surgery in rodents, hepatic FXR expression is decreased.[554] However, beneficial metabolic effects can be achieved by reducing or increasing signaling in a context-specific manner. Since FXR signaling is promoted by 1° BAs (CDCA and CA) but inhibited by the 2° BA

UDCA, the microbiome could influence metabolism by shifting the balance between these BA species.

Conversely, BAs alter intestinal bacterial composition by moderating oxidative and pH stress in the small bowel and chelating vital cellular ions, thus promoting or inhibiting the growth of certain species.[476] Direct antimicrobial effects result from detergent-like disruption of bacterial membranes[561] and indirect effects are mediated by FXR activation, triggering upregulation of genes involved in epithelial mucosal defense.[482]

D. Preliminary Data:

In a pilot dataset of 30 children from the RUPP RSP trial who had initiated and sustained RSP treatment for an 8 week trial,[107, 521, 592] we observed marked changes in the total BA pool and diversity, with predominant increases in 1° BAs. 1° BAs were significantly more pronounced in those with high versus low AIWG (p=0.01, Fig. 6-7a). This pattern was replicated in 4 subjects started on RSP with moderate weight gain recruited by Dr. Calarge, co-investigator,[577] with a 1° BA increase of 960 nmol/L, comparable to 1,374 nmol/L in the RUPP sample. Both CDCA and UDCA measures at baseline and 8 weeks were available for 28/30 RUPP participants. 12 of these 28 displayed a significant change (from baseline to 8 weeks) in either CDCA or UDCA (>.5 SD). Of note, the ratio of the change in CDCA to the change in UDCA differentiated those with AIWG (ΔCDCA/ΔUDCA>1) from those without (ΔCDCA/ΔUDCA<1). This pattern correctly predicts AIWG status in 11/12 (92%) participants whose ratios change (p=0.001, Fig. 6-7b), with a sensitivity of 100% and specificity of 86%. Sex, age, race/ethnicity, and RSP dose/plasma level did not contribute to BA differences. Test-Retest reliability of CDCA, UDCA, and 1° BA repeated measurements in the same individual (N=2) were precise within 4 nmol/L.

We hypothesized that a ΔCDCA/ΔUDCA<1 could occur when epimerase+ bacteria are unavailable to convert CDCA to UDCA, resulting in excess CDCA and AIWG. Therefore, using data from the prospectively followed RSP-treated children enrolled by Dr. Calarge,[577] we examined whether relative abundance of a key bacterial species *(B. fragilis)* that can catalyze the first step in epimerization was affected by RSP exposure. As predicted, the relative abundance of *B. fragilis* decreased over 200-fold after 1-3 months of RSP exposure.

In order to explore whether these effects are specific to AIWG, we tested a small pilot sample of children taking SSRIs. Ten youth were selected, 5 with moderate (>1 Z-score) SSRI-induced weight gain (SIWG) and 5 without. Remarkably, the trends were in the same direction, with individuals with greater weight gain showing greater elevation in 1° BA and ΔCDCA/ΔUDCA>1. However, given the small sample size, the differences were not significant. . Using this ratio, SIWG status can be correctly predicted in 6/8 (75%) of individuals with BA change on an SSRI. In summary, our preliminary data suggests that alterations to the BA balance may contribute to AIWG, a link that to our knowledge has not been previously investigated. We have shown that RSP-exposed children exhibit substantial changes in their BA pool, with opposite patterns of 1° vs. 2° BAs seen in youth at risk versus protected from AIWG. It is possible that antipsychotic drugs may impact BA synthesis and metabolism directly, as both first and second generation antipsychotics concentrate in bile,[573] slow enterohepatic BA circulation, and can trigger hepatic cholestasis, resulting in a reduced flow and clearance of BAs and toxic accumulation of 1° BAs.[593, 594] However, based on previous data supporting an essential role for the microbiome in AIWG and the fact that the key regulator of UDCA levels is bacterial conversion from CDCA, we propose to test a model of AIWG based on altered BA-microbiome interaction.

E. Scientific Premise.

Integrating the prior literature reviewed above and our own pilot data, we hypothesize that (Aim 1A) RSP treatment increases 1° BAs and that resistance to these changes is conferred by the ability of epimerase+ gut microbiota to convert 1° (CDCA) to the 2° (UDCA) BA, which opposes the downstream effects of 1° BAs at the FXR receptor. We further propose that those who experience significant AIWG can be differentiated from those who are protected based on RSP-induced changes in: (Aim 1A) CDCA/UDCA>1, (Aim 1B) increased FXR activity and (Aim 2A) loss of epimerase+ bacterial species in the gut, reflecting an impaired ability to convert CDCA to UDCA to compensate for increasing CDCA. While preclinical studies from three independent groups, in both mice and rats exposed to two different antipsychotics, report consistent effects on the microbiome, translation from rodent to human is inherently problematic. Our data examining RSP-induced microbiome changes in humans, although unique, are largely based on a cross-sectional study and very small longitudinal sample. Time course of changes is not well defined. BAs are gaining support as a hormonal signal involved in energy balance; however, our pilot data, while in agreement with our theoretical model based on BA chemistry and function, is also based on a small sample.

These data, while preliminary, provide the foundation for a compelling model of AIWG (Fig. 37). Further research is needed to replicate findings in larger samples, clarify links between microbiome and BA, and determine whether associations are causal or simply an epiphenomenon or downstream effect. Understanding the role BAs play in AIWG will serve several functions. It will lead to the identification of an easily measurable biomarker of AIWG risk could provide clinical guidance. If proven, highly actionable treatment targets are clear, and potential treatments are immediately available. UDCA is available orally as a gallstone treatment and epimerase+ bacteria are a chief component of probiotics. Developing approaches that may be relevant include drugs directly impacting FXR signaling and prebiotic strategies

used to shape microbial populations. The mechanisms involved in AIWG/MS may be relevant to other drug classes and will likely inform our overarching concept of normal and dysregulated metabolic balance.

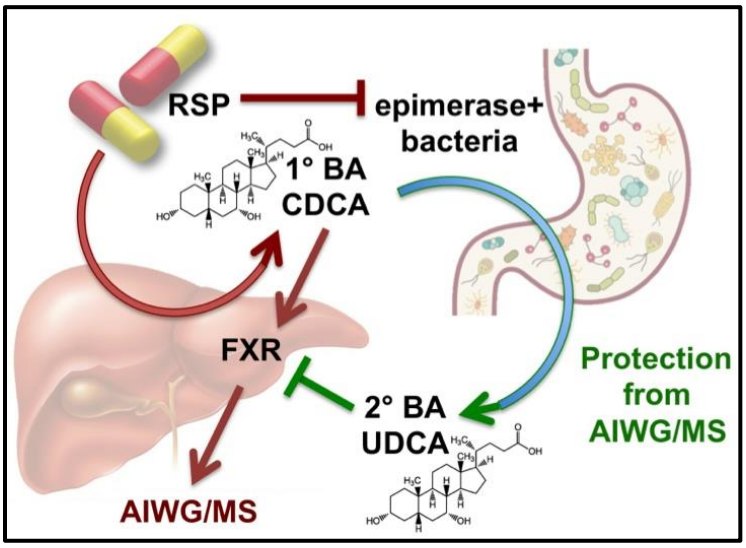


Figure 7-2. Proposed Model of AIWG/MS. RSP impacts BA and gut microbial composition, favoring more obesogenic profiles. These pathways promote FXR signaling, negatively impacting lipid and glucose homeostasis, adiposity, and BMI. A protective mechanism whereby CDCA is converted to UDCA by epimerase+ gut bacteria counteracts this pathway; UDCA blocks FXR signaling, thus opposing AIWG/MS. The balance between these two pathways dictates metabolic response to RSP.

II. INNOVATION.

While previous studies have described AIWG/MS, and even searched for genes conferring risk, mechanistic approaches are lacking. Our team's prior work represents the only published examination of the role of the microbiome in AIWG in humans. We developed and will implement our own BA measurement assay, the details of which we will publish. An appreciation of the role of BAs as signaling molecules is just beginning to emerge. This project would be the first to include BA signaling pathways in the study of AIWG and the first to integrate BA and microbiome changes. Uniting preclinical and clinical data and integrating microbiome and BA findings, we propose to test a novel potential mechanism contributing to AIWG. This model has compelling hooks to actionable treatment targets and aligns with the goals of precision medicine.

III. APPROACH: METHODS

STUDY OVERVIEW	**2. Collect (baseline, 1-, 2-, & 6-mo f/u):**	**3. Analyze:**	**4. Expected Results:**
1. Recruit: children starting RSP as part of naturalistic treatment for any diagnosis UCLA: 20 participants/Y BCM: 10 participants/Y	blood (fasting), stool, diet tracking, height, weight, waist circumference, skinfold body fat, med changes (including RSP dose, antibiotics, other meds)	***Blood:*** BAs, lipids, glc ***Stool:*** Microbiome, epimerase activity, BAs	**Aim 1A:** 1° BAs will increase & AIWG will be predicted by changes in CDCA/UDCA **Aim 1B:** Downstream markers of FXR signaling will increase, moreso in AIWG+. **Aim 2A:** Epimerase+ bacterial species will decrease in AIWG+ vs. AIWG- **Aim 2B:** Explore time course

Study Design and Procedures.

60 unmedicated youth initiating RSP treatment within clinical programs at UCLA and BCM will be recruited, screened, and consented for participation in order to provide 52 subjects with complete data (additional 15% to account for attrition). UCLA will recruit 20 subjects and BCM 10 subjects during each study year. Subjects will be paid $50 at each of 4 collection visits and $30 for providing written dietary information on 5 non-visit weeks.

Assessments:

All participants will undergo baseline clinical evaluation by the study psychiatrist including general health, and diet and anthropometric measurements (height, weight, blood pressure, body mass index [BMI], waist circumference, skinfold body fat) will be taken following standard procedures. The Pubertal Developmental Scale (PDS), a psychometrically sound child self-report yielding a continuous pubertal change score and a five-level categorical classification of pre- to post-pubertal, will be administered.[595] At each visit, repeated body measurements, medication dose and adherence since the prior session, any new treatments received (especially antibiotics), and the occurrence and reason for any protocol deviations will be recorded. Fasting

blood and stool samples will be collected prior to initiation of RSP treatment and at 1-, 2- and 6-months on RSP.

Several factors support the feasibility of our recruitment target. UCLA and BCM are leading regional centers for the evaluation and treatment of youngsters with pediatric mental illness. The UCLA Child Psychiatry Division has served as home to numerous funded clinical studies and draws referrals from a catchment of over 16 million. The clinical operations (inpatient, partial, outpatient hospital services) are housed in close proximity to the research sites. At both sites, RSP is the antipsychotic most commonly prescribed to children and teens given the superior evidence base for clinical treatment of psychosis, mood stabilization, aggression, impulsivity, tics, OCD, and other developmental disorders. Recruiting will be facilitated by announcements at faculty meetings and fellow didactics; emails to faculty and fellows; and outreach to social work, nursing and support staff. Study staff will be available continuously on call (during business hours Mon. through Fri. when clinics operate and recruitment will occur) to meet with potential participants following their treatment visit. We have successfully completed recruitment for multiple funded pediatric trials, supported by a vibrant community outreach/referral network including UCLA-affiliated community clinics, primary care physicians, mental health providers, schools, churches, community organizations and centers, and an active and expanding web and social media presence. Of note, the ethnic minority participation rate for our similar studies is ~40%.

AIM 1: Methods*.* Whole blood will be collected in EDTA-treated Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 4C at 2000 rpm for 10 minutes within 24 hours of collection to separate plasma. Plasma will be stored at -80C until processing. BAs will be measured in plasma samples using a solid-phase extraction method. We will measure the principal BAs found in humans, including 1°, 2°, and conjugated BAs:[579, 596] Specifically, we will

measure CA, CDCA, UDCA, deoxycholic acid (DCA), and lithocholic acid (LCA) and their taurine (T) and glycine (G) conjugates (TCA, TCDCA, TDCA, TUDCA, TLCA, GCDCA, GDCA, GCA, GLCA), iso-epimers (isoLCA, isoDCA) and precursors (7α-hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one [C4]). A Cadenza CD-C18 high efficiency column (Imtak, Portland, OR) that is specifically designed with unique ligand density provides excellent steric selectivity for chromatographic separation. Separated BAs will be analyzed with an Agilent 6460 Triple Quadrupole LC/Mass Spectrometer with Standard ESI Source (Agilent Technologies, Santa Clara, CA) in the positive-ion mode. Quantitative data will be analyzed in the multiple reaction monitoring mode by using optimal parameters for each individual BA. In our pilot studies, the lower limit of quantification was 0.5 nmol/L for all BAs. Downstream indicators of FXR signaling and measures of inflammation (cytokine panel) will be quantified by commercially available ELISA assays (ThermoFisher). FXR signaling results in secretion of gut-derived FGF19 and subsequent suppression of prescursors of hepatic BA synthesis (7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one [C4]).

<u>AIM 1A.</u> To measure changes to BA composition longitudinally during 8 weeks of RSP treatment and compare BA pool composition in those with and without AIWG.

Hypothesis: 1° BAs will increase with RSP exposure, with greater increases in those with AIWG. In those with RSP-induced BA changes, ΔCDCA/ΔUDCA ratio>1 will identify participants who gain clinically significant weight compared to ΔCDCA/ΔUDCA ratio<1 in those protected from weight gain.

Analytic Plan 1A: Levels of 1° BAs will be calculated at individual time points and analyzed using a repeated measures mixed linear model. Covariates such as dose, age, sex, and

race/ethnicity will be included in the model. Sex-specific effects will be considered. Change in CDCA and UDCA will be calculated at individual time points. Participants with significant changes in either measure (>.5 SD) will be analyzed for ΔCDCA/ΔUDCA ratio and ratio>1 will be used to predict AIWG status. Actual versus predicted assignments will be compared using a Chi Square test. Sex-specific effects will be considered.

Power 1A: The effect size for increases in 1° BA's determined in the pilot study is large (d=.74). The proposed sample size (n=52), with 3 within subject measurement points and 2 groups and assuming a within-subject autocorrelation of r=.50), provides sufficient power (81%) to detect changes of this magnitude. The proposed UDCA/CDCA ratio rule is only applicable to participants that show significant changes in those values in response to antipsychotics. Based on the preliminary data, ~50% of the sample is expected to change. In our pilot, the proportion of participants with ratio>1 who have AIWG is 100%, while those with ratio <1 who have AIWG is only 14%. The minimum sample size to detect a difference in proportions of this magnitude with sufficient power (80%) is n=14, requiring a total sample size of n=28 to be recruited to account for the 50% of the participants without BA changes. As these estimated proportions are based on small samples, this is likely to underestimate the required sample size, but as the sample size available (n=52) is nearly twice the estimate, we are confident that this aim is sufficiently powered. Additional analyses of the baseline microbiome of participants resistant to changes in CDCA and UDCA will be explored to assess potential protective factors for future study.

AIM 1B. To bolster the evidence for the proposed mechanism, we will compare downstream markers of FXR signaling in those with and without AIWG.

Hypothesis: Downstream indicators of BA-induced FXR signaling will reveal suppression of BA synthesis and increased secreted FGF19 reflecting increased FXR activity overall. We expect greater FXR signaling in those with versus those without AIWG.

Analytic Plan 1A: Blood levels of these markers at the three visits will be analyzed using a repeated measures mixed linear model. AIWG+ or – group status will be added to the model as a between subject variable to test for differential effects. Covariates such as dose, age, sex, and race/ethnicity will be included in the model. Sex-specific effects will be considered. Power 1B: Given the large effects seen in BA increase (d=.74) and existing literature showing that CDCA produces large effects on downstream markers of FXR activity in contrast to UDCA,[597, 598] we anticipate that downstream markers will be altered by a greater magnitude than BAs themselves (see Power 1A), and thus the study is powered to detect these effects.

Alternative Approach: If pilot findings do not replicate, FXR signaling indicators may provide clues to an alternative mechanism. Additionally, the relationship of other BAs with AIWG can be explored in this larger, better-powered study.

AIM 2: Methods. Stool samples will be collected prior to initiation of RSP treatment and at 1-, 2- and 6-months on RSP. Samples will be freshly collected and transported to the Nurmi laboratory where they will be aliquoted and frozen within 30 minutes. If patients are unable to provide stool samples at the time of their visit, collection kits will be sent home with parents. Samples provided in a home setting will be placed on provided dry ice and picked up within 24 hours in a frozen state. All stabilized samples will be stored at -80C until DNA extraction. Extracted DNA will be subjected to multiplex paired-end whole genome sequencing on an Illumina HiSeq instrument (Illumina, San Diego, CA) in the UCLA microbiome core. Computational analysis will be performed using Qiime 2 to identify OTUs and relative abundances and to perform diversity

analyses. The CDCA to UDCA conversion requires two steps that can be formed by distinct but co-located bacteria. The first step is performed by species expressing 7α-hydroxysteroid deyhydrogenase (HSDH) that can form oxo derivatives (ketones) of CDCA, including *Escherichia coli, Bacteroides fragilis* and *Pseudomonas* species. The second step is performed by species expressing 7β-HSDH that reduces ketones to epimerized hydroxyls, including *Collinsella aerofaciens* and *Ruminococcus gnavus.* Bacteria that contain both α– and β-HSDHs include *Clostridium limosum,* and *Clostridium baratii.* The specific enzyme sequences are available for these species. The low level of homology (~50-80%) between species will allow us to differentiate which epimerizing bacteria are present through their enzymatic sequence signature. A further confirmatory functional assay will measure epimerase activity by culture of stool samples, followed by addition of CDCA to culture medium and LCMS measurement of UDCA and LCA.[507, 579, 599, 600] Fresh stool samples collected can be used for both analyses.

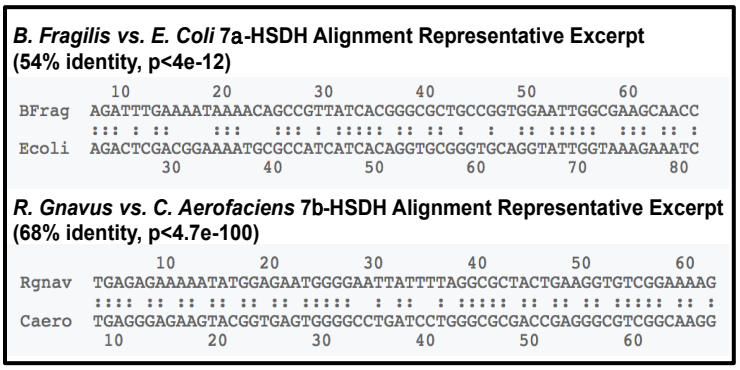


Figure 7-3. Representative Genetic sequence of *B. Fragilis* and *R. Gnavus.* Both of these bacterial species produce the specific 7-βHSDH enzyme needed to epimerize CDCA to UDCA.

<u>AIM 2A.</u> To measure longitudinal changes in the gut microbiota during 8 weeks of RSP treatment and compare relative abundance of epimerase+ bacterial species in those with and without AIWG.

Hypothesis: Epimerase+ bacterial species will be depleted in those with vs. without AIWG.

Analytic Plan 2A: The change in relative abundance of epimerase+ bacteria will be assessed based on both OTUs and enzyme activity using a repeated measures linear mixed model. Covariates such as dose, age, sex, and race/ethnicity will be included in the model. Sex-specific effects will be considered.

Power 2A: The proposed sample size (n=52, ~26 expected in each group [AIWG+ and AIWG-], with 3 within subject measurement points and 2 groups) provides sufficient power (81%) to detect differences in the relative abundance of epimerase+ bacteria of the same magnitude as observed in the pilot data (d=.71 difference at study endpoint under the assumption that both groups are equivalent at study beginning and a within-subject autocorrelation of r=.50).

Alternative Approach: If pilot findings do not replicate, other microbiome-AIWG relationships can be explored in this larger, better-powered study.

<u>Exploratory Aim 2B.</u> To measure the time course of gut microbiota and BA composition changes during RSP exposure to help clarify the mechanism of AIWG and facilitate the identification of early biomarkers.

Given that the earliest data previously collected for BA and microbiome changes were 8 weeks from baseline, the mid-point and 6-month follow-up assessments will help resolve the order and timing of BA and microbiome peak changes, as well as changes relative to one another. Since a longer time period will likely result in greater rates of attrition, loss to follow-up, RSP discontinuation, concomitant drug addition, and antibiotic prescription, we expect a much smaller of subjects to provide useable data for a 6-month follow-up, hence this timepoint will be observational and analyses will be exploratory.

Rigor and Reproducibility:

Scientific Premise of Proposed Research (see Significance section).

Rigorous Experimental Design.

Our methods have been validated by our own prior work and independent published data. Multiple approaches have been enlisted to protect against bias, including independent, blinded data analysis and sound statistical methods. In order to facilitate transparency, we have described in detail our experimental protocol and other methods. Similarly, we will share the study design details, analytic methods, and trial data with the scientific community.

Consideration of Sex and Other Relevant Biological Variables.

Given that major indications for antipsychotic treatment in youth have a male bias, extra efforts will be made to recruit female subjects to ensure representation, especially in light of previous data indicating that female rodents are at greater risk of microbiome and metabolic effects. Inclusion of females will enable us to robustly test for sex effects. In our pilot studies, sex, age, race/ethnicity, and RSP dose/plasma level were not significant contributors to our models.

Authentication of Key Biological and/or Chemical Resources. Our methodological approaches have been selected with attention to prior validation and shared acceptance by the field. We will ensure transparency in data sharing and continue to apply new methods to our data as they emerge to improve interpretation.

Timeline	Preaward	Y1	Y2 (mo 1-8)	Y2 (mo 9-11)	Y2 (mo 12)
UCLA	**Update IRB, consents, announcements**	**Recruiting & Collection** N (2/mo) =24	**Recruiting & Collection** N (2/mo) =16	**Sample Processing, Data Analysis, Interpretation**	**Manuscript preparation, data sharing**
BCM		N (1/mo) =12	N=1/mo) =8		

Overall Dissertation Conclusions

Precision medicine is an emerging approach for disease treatment and prevention that heavily weighs individual variability in genes, environment, and lifestyle for each person. It is in stark contrast to a one-size-fits-all approach in which both doctors and drug developers focus on empirical data and what works best for the “average” person in the general population. The importance of these techniques is so high, in 2016, President Barack Obama backed an initiative to fund precision medicine and accelerate this type of technology into everyday life. Personalized medicine has the potential to tailor therapy and inform targeted drug design for the best therapeutic responses and highest safety margins to overall ensure better patient care. Earlier diagnoses, informative risk assessments, and optimal treatments are pieces of human healthcare that should not be overlooked, and have the utmost potential to improve the quality of life for many people.

By leveraging the tools and techniques I described throughout this dissertation, I feel I have had the opportunity to truly explore the potential that precision medicine has to change the landscape of scientific research and pharmaceutical manufacturing. I would like to take this opportunity to briefly summarize the salient findings I have presented, and touch on how I feel they all fit snugly together in the puzzle that is psychiatric health.

We began our journey in the field of pharmacokinetics (PK), where I was able to learn a vast amount of both didactic knowledge and hands-on techniques for evaluating how drugs are processed by the human body and what this could mean for downstream effects and diagnostics. PK Case Study #1 was entitled *CYP2D6*, brain structure, and cognitive function in methamphetamine dependence and utilized functional genotyping at a pertinent metabolic

enzyme to assess whether or not MA addicts were more susceptible to neurodegredation based on variation at this locus'. Our data strongly support the hypothesis that *CYP2D6* extensive metabolizers are more vulnerable to methamphetamine-induced neurotoxic effects than those with impaired enzymatic activity, resulting from common gene polymorphisms. This could be due to the fact that MA metabolites are more toxic to the underlying neurocircuitry and neuronal cells than the parent compound, and future toxicity studies can now address this issue. A clearly defined genetic risk factor such as this has the potential to be utilized by clinicians for both early intervention and individualized treatment planning, while also aiding in the development of harm reduction strategies, a lynchpin of personalized medicine.

PK Case Study #2, Genetic Determinants of Risperidone Pharmacokinetics in Children with Autism Spectrum Disorder (ASD): Relationship to Treatment Outcomes and Side Effects, was my first exposure to the deleterious pharmaceutical side effect that is antipsychotic-induced weight gain. I was unaware back then of the implications, but this project is what truly piqued my interested in regard to defining pathways and mechanisms that are more closely related to our underlying biology, and through our examinations of the same liver based enzymatic genotype, we were able to understand how drug metabolites can also play a critical role in individual patient outcomes. The *CYP2D6* poor metabolizer genotype group displayed 702% higher risperidone (RSP) and 133% higher total active moiety concentrations and RSP metabolism appears to be handled by different CYP metabolic pathways across ancestry. These data add to extant knowledge of moderators of RSP pharmacokinetics in humans, and while the entire picture is still somewhat poorly understood, this study may eventually help to identify patients who would benefit most from RSP therapy or are at highest risk for adverse outcomes.

From these promising roots in pharmacokinetics, I was able to make a smooth transition to the yin of this yang, pharmacodynamics (PD). Focusing efforts on how already known xenobiotic substances affect the human body is of great importance when considering new modality conception and synthesis further down the line. The comprehension I gained during my research in this field changed how I approach solving more complex enigmas related to a variety of human disease, and truly helped me learn a large amount of invaluable neurobiology. PD Case Study #1 was the Effect of Cigarette Smoking on a Marker for Neuroinflammation: A [11C]DAA1106 Positron Emission Tomography Study, a paper I was able to publish with fantastic collaborators and experts in the field. When activated, microglia increase the expression of translocator protein (TSPO) 18 kDa, thereby making the TSPO expression a marker for neuroinflammation, and we examined this diagnostic effect directly through radiochemistry in combination with genetics in a high-risk population. Cigarette smokers have less [11C]DAA1106 binding than non-smokers throughout the brain, indicating less TSPO availability, and though several explanations for this finding are possible, a straightforward one is that smoking results in global impairment of microglial activation which could lead to compromised wound healing throughout the body. As nicotine dependence is an immense and wide-spread public health issue, future research could examine the time course of recovery of TSPO availability upon smoking cessation and the interplay between smoking, neuroinflammation, and the progression of substance use disorder as well as other diseases thought to be mediated by neuroinflammation.

And some of this future research came with a related pharmacodynamic study, seen in PD Case Study #2: Effect of overnight smoking abstinence on a marker for microglial activation: a [11C]DAA1106 positron emission tomography study. Again utilizing Using positron emission

tomography scanning, our group recently demonstrated that smokers in the satiated state had 16.8% less binding of the radiotracer [11C]DAA1106 (a radioligand for TSPO) in the brain than nonsmokers. These results in overnight abstinent smokers are similar to those in satiated smokers, indicating that chronic cigarette smoking leads to global impairment of microglial activation which persists into early abstinence. Both studies were significant in the fact that they brought me the opportunity to discover more about theranostic platforms such as brain imaging, and how these can be implemented in cutting edge research to make informed choices of receptors in both investigatory science as well as drug delivery mechanisms.

In order to lay the final brick in my formidable foundation of precision medicine repertoire, I had to dive deep into the world of genomic technology. At the end of the day, the true backbone of personalized medicine lies in DNA based understanding, applications, and manipulations. I was fortunate enough to participate in both major forms of human genetic evaluations currently used in medicine – candidate gene and genome-wide studies. GT Case Study #1 was titled Cholinergic Genetic Variation Moderates Smoking-Induced Striatal Dopamine Release and focused on a subset of nicotinic acetylcholine receptor encoding candidate genes to inform direct dopamine (DA) release variation in nicotine consumption. Findings at nicotinic α4 and α7 cholinergic receptor subunits are consistent with animal and in vitro studies implicating these subunits in DA-mediated reinforcement associated with smoking. Correlation of these variants with behavioral endophenotypes suggests substantial effects on dependence and craving. Overall, a comprehensive understanding of genetic moderators of nicotine reward and dependence risk may facilitate the development and individualization of successful treatment strategies.

GT Case Study #2: Polygenic Contributions to Decision-Making on a Laboratory Test of Reward-Based Risk-Taking was one of the more critical projects that I had to the chance to develop and participate in due to its usage of genome-wide association studies and polygenic risk scores, two of the most pertinent genomic approaches. Heightened risk-taking behavior has been linked to neuropsychiatric disorders, such as Attention Deficit-Hyperactive Disorder (ADHD), addictions, and mood disorders, and our findings confirm a heritable, polygenic component to risky decision-making explaining 11% of the variance in balloon analog risk task (BART) performance. We demonstrated for the first time that polygenic scores derived from a genome-wide association study of a risk-taking phenotype successfully predict the same phenotype in an independent sample and revealed shared genetic underpinnings with categorical bipolar diagnosis. This is a succinct and effective biomarker, one of the true hallmarks of precision medicine. Having the confidence and grasp on the genomic technology involved in disentangling biomarkers such as this will be indispensable in my future career.

As I moved forward and began to apply these principles to environmental factors, the story of my research began to take an entirely new shape. We do not live in a vacuum, and to not consider how profoundly the world around us affects our health on a cellular level would be foolish. Due to Dr. Nurmi's strong connections with a top researcher in the field of childhood attachment, I was able to work on understanding how our genes and the environment interact to engender various disease states as well as treatment outcomes. The first endeavor, GxE Case Study #1: Interactive effects of attachment and FKBP5 genotype on school-aged children's emotion regulation and depressive symptoms, was a wonderful way to expose me to the intricate workings of gene-environment interactions. Research demonstrates that child attachment insecurity and genetic factors are each associated with elevated risk for emotion dysregulation and psychopathology but had not been considered in tandem. Higher levels of

overcontrol predicted lower child attachment security only in FKBP5 minor allele carriers, and these findings can be conceptualized in a differential susceptibility framework, where the FKBP5 minor allele confers either risk or resilience, depending on the parenting environment.

Following up with the same encouraging team, I worked on GxE Case Study #2: Interaction between the Opioid Receptor OPRM1 Gene and Mother-Child Language Style Matching Prospectively Predicts Children's Separation Anxiety Disorder Symptoms. Psychiatric disorders tend to be particularly distressing in children and adolescents, and this is especially true for anxiety-based disorders, yet they remain largely underexplored in this population. There is also considerable evidence of genetic risk for anxiety, including possession of the OPRM1 minor allele, 118G, and the interaction between mother-child language style matching and OPRM1 genotype significantly predicted separation anxiety disorder (SAD) symptoms beyond the main effects of the two variables in our study. Greater knowledge of the factors that contribute to the development of SAD can inform both treatment and preventative measures, and this could point toward the importance of testing whether interventions designed to strengthen the parent-child relationship reduce the risk of developing SAD symptoms in children who carry the minor OPRM1 allele. As precision medicine pushes the agenda of implementing standard patient genotyping, studies such as these provide cardinal information on how these new and strange health data can be utilized and tailored based on each person’s unique situation.

And finally, we come to the apogee of my doctoral research career. Through serendipitous events that I will discuss briefly in my final thoughts following this section, I came to discover the fascinating world that is that surrounding the human gut microbiome. Over the last 10 years, scientists have begun to unearth the almost limitless potential the gut microbiota and microbiome bring to elucidating human disease pathophysiology. I knew immediately when I

first began reading about this symbiotic presence contained within all of us that I wanted to incorporate it in my PhD project; so I set out to accomplish just that. The final chapter of my story highlights the blood, sweat, and tears that I poured into my work on bile acid and gut microbiome crosstalk. I stumbled into the world of bioanalytics ignorant and bright eyed, but quickly realized the true potential it has to inform us about biochemical moieties that could influence disease and adverse drug side effects. My original method: Fit-For-Purpose HPLC-MS/MS Targeted Detection and quantitation of 11 Bile Acids and Their Biological Precursor, 7-alpha-hydroxycholest-4-en-3-one was a labor of love, and it plays a pivotal role in my proposed research study. Classically, bile acids (BA) are defined as our body's simple detergent molecules. They are produced in our liver from the catabolism of cholesterol and stored in the gallbladder. Due to their steroidal backbone, they can act as hormones and have significant regulatory effects in host energy metabolism. They accomplish this through binding at various nuclear receptors in the gut and liver, namely FXR (farnesoid X receptor) and PXR (pregnagane X receptor) but also G-protein coupled receptors (such as TGR5). These have serious downstream impact on glucose and lipid homeostasis, as well as pathway functions in disease states such as cirrhosis and cancer tumorogenesis. I identified an unmet need in the mass spec quantification of these compounds in human matrices and was able to develop a novel method for their robust detection. I am still adding additional bile acids to this method as I write this, and it has the potential for even more growth in the future!

The real dessert came when I had the opportunity to apply this bioanalytic method to actual patient samples in my microbiota-host interaction Case Study #1: A Putative Development Pathway; Microbiome – Bile Acid Cross Talk in the Biological Mechanism of Psychotropic-Induced Weight Gain. I assembled a top tier collaborative research team to aid me in my quest to better understand the biochemical circuitry and pathways involved in the complex weight gain

and metabolic syndrome that plagues the majority of patients taking psychotropic medications. Differential risk across the drug class is not explained by simple receptor binding profiles at contributing sites such as the muscarinic system, but other mechanistic links are lacking. We observed marked changes in the total BA pool and diversity, with predominant increases in 1° BAs. Primary BAs were significantly more pronounced in those with high versus low antipsychotic-induced weight gain (AIWG), and in this subset showing BA change with antipsychotic treatment, the ratio of the change in CDCA to the change in UDCA differentiated those with AIWG (ΔCDCA/ΔUDCA>1) from those without (ΔCDCA/ΔUDCA<1). Because of these strong biomarker-based findings, but the paucity of understanding on *why* this might be, we began to pull in previous data from collaborators in the same populations involving the gut microbiota composition. We crafted an expositive mechanism of psychotropic-induced weight gain leverage both of these salient factors, and with the help of the incredible scientists around me, I drafted a replication project grant proposal to link bile acid changes to gut microbiota shifts in individuals experience the deleterious effects of weight gain following antipsychotic medication treatment, which is currently in progress and explained in the future directions above this blurb. Encouragingly, a recent paper published in *Nature Medicine* reveals that metformin, a medication commonly used to treat antipsychotic-induced weight gain, acts through the microbiome-bile acid signaling pathway that we identified in our study, further validating our hypothesis.[601, 602] This opens the door to very personalized and patient centered treatment approaches, and even hopefully one day, informed and specific biological targets for future drug design in these areas, as I believe we have brought yet another piece of the AIWG puzzle to the table, and hopefully clinical implementation might not be far away.

My Final Thoughts

I believe I had a truly unique and inspiring graduate school experience. There was a defining moment in my undergraduate career when it hit me that chemistry was my calling. I had been a psychology major for three years, intending to continue on to medical school to become a psychiatrist. Pursuing the chemistry requirements for this goal led me to discover my love and talent for this versatile and fascinating subject. As my chemistry professors recognized my aptitude and excitement, they urged me to switch my major. The decision to alter my path crystallized when I realized that using chemistry to design new therapeutics was the way I could best contribute my talents towards healing and medicine.

As I pursued my studies here at UCLA, completing chemistry graduate courses and teaching undergraduate chemistry labs, I considered how my science might have real-world impact. However, I was met with an unfortunate situation; the year I matriculated, there were an abundance of students in the organic chemistry discipline. Combined with a tepid funding environment, most of the labs I was initially interested in being a part of were full or not taking more students due to this lack of resources. I struggle for close to a year to find somewhere that I might belong in the scientific community and was beginning to feel hopeless when I stumbled across the laboratory of Dr. Erika Nurmi. I realized that my background in organic chemistry would provide a solid foundation for translational research. The many opportunities to integrate my chemistry background with translational projects in psychiatry and clinical medicine drew me to her unique style of research, as she is a neuroscience PhD but also an actively practicing psychiatrist. To me, she has always been Wonder Woman. Not only does she attempt to do it all in terms of teaching, guiding new students, treating patients, running clinics, provoking novel research, and challenging the status quo of women in science, but she also made time to be my

mentor in this crazy journey. She helped me form the beautiful story that I presented to you today, and I could not be prouder of what I've accomplished under her nourishing wings.

As my research has progressed, I have come to appreciate the need for biologically based, personalized treatments for a myriad of debilitating disorders. Specifically, in the field of psychiatry, targeted drug therapies are lacking, and those pharmaceuticals currently in use show wide variability in efficacy across individuals. I have found my niche in a personalized medicine field known as pharmacomicrobiomics, which aims to capture the gut microbiome's interaction with human ingested xenobiotics and pharmaceuticals. My flagship dissertation project presented in chapters 6 and 7 embodies my passion about this subject, as I have strong personal ties to metabolic syndrome. My entire life I have struggled with my weight; I was obese before I even knew what the word medically meant. When I moved to California to attend graduate school, I left everything I had ever known behind on the East coast, and that negatively impacted my health even more. I reached almost 400 pounds my second year of education here at UCLA, and beyond anything else, I knew I NEVER wanted anyone else to ever feel the way that I felt in that moment. I spent sleepless nights and countless hours researching the gut microbiota and how they might influence obesity, weight-gain, weight-loss, and associated diseases. While my own journey swept me through losing over 230 pounds over the next 3 years, I couldn't not confer this knowledge to the research community. Even if it only helped one person in the future, I knew it was vital that I incorporate the human microbiota and microbiome into a meaningful scientific study.

My personal dissertation project and culmination of the tools and techniques I previously examined begins to elucidate a direct biological link to the unexplored partners of the gut

microbiome and bile acids. This work specifically aims to confirm the link between microbiome changes and antipsychotic-induced weight gain, to further define concrete biological mechanisms underlying this serious adverse event, and to ideally present clinically relevant biomarkers for the advancement of future research and treatment. I believe that my unique training and skillset offer a unique perspective to the pharmacomicrobiomics and precision medicine disciplines. Through my initial undergraduate and graduate education, I have developed the organic and analytical chemistry skills for a future in targeted drug design; however, the second phase of my graduate training provides me the necessary understanding of brain and gut biology that will inform my choice of drug targets. Insights about the underpinnings of complex pharmaceutical responses revealed by mechanistic and genetic studies, such as those I have begun to examine, will provide rich future opportunities to unite my inner chemist and biologist in the development of novel therapeutics to improve overall health and quality of life.

While my choice to incorporate learning a whole new field into my training has made this trajectory lengthy and challenging, my project has already delivered publishable results that have generated great enthusiasm in my field. I plan to complete the confirmatory analyses that will solidify this project even as I move away to the next phase of my scientific career. It will also pave the way for this endeavor, as I integrate my interests in clinical biomarkers, psychopathology, and effective, individualized treatments. My work has the potential to solve a problem that has perplexed the field for many years, will have clear clinical implications and will highlight viable treatment strategies for implementation work. After completion of my doctorate, I hope to pursue a scientific position in the pharmaceutical industry where I can leverage the knowledge and skills I have acquired during my graduate training to design, develop, and deliver precision therapeutics for psychiatric disease.

I hope to not only become a shining member of the biotechnology industry with my unique approach to solving scientific perplexities that I garnered from the precision medicine tool box I put together over the last six years, but I also strive to bring passion and compassion into my work. This planet is a beautiful place, and life is a wonderful experience. We all deserve the best care, the most happiness, and the brightest of futures. I want to provide that for people that may have lost hope along the way. I want to transform the way we approach the treatment of grievous diseases. I want to change the world. And while it may sound like I'm auditioning for Miss America; I am beyond content to instead be receiving the title of Dr. Lauren Seaman. Thank you for your time and for being a part of my story.

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