PERSONALIZATION OF IMMUNOSUPPRESSIVE MEDICATION FOR KIDNEY TRANSPLANT RECIPIENTS

by

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Personalization of Immunosuppressive Medication for Kidney Transplant Recipients

A Dissertation in three papers submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at George Mason University

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DEDICATION

I dedicate this dissertation to my parents Azita Roshan and Mohsen Nayebpour, and to my brother Mohammad Amin Nayebpour and to my late grandparents Hasan Roshan and Azarm Azimi. Above all, I dedicate this to Iran.

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Panel of Reactive Antibody ……. PRA
Donor Specific Antibodies ……..……DSA
Time in Therapeutic Range ……..…….TTR
Donor Derived Cell-free DNA…… dd-cfDNA
African-American ……..…….AA
Coefficient Variability …….CV
End Stage Renal Disease……...…….ESRD
prospective payments system …….PPS

ABSTRACT

PERSONALIZATION OF IMMUNOSUPPRESSIVE MEDICATION FOR KIDNEY TRANSPLANT RECIPIENTS

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This thesis presents three independent essays for the fulfillment of doctoral dissertation in Public Policy. The common theme in these essays is the practice of personalized medicine for kidney transplant recipients. The field of kidney transplantation is one of the costliest fields in the healthcare system and it is paid by the Federal government. Increasing the quality of transplant outcomes has been a major focus for the CMS, particularly for underserved populations such as African Americans who already face the worst transplant outcomes. Studies show that implementing personalized medicine practices increases the quality of care, reduces graft rejection and increases graft survival rates. Such results directly translate into reducing the cost of kidney care. In this manuscript I developed a personalized medicine model based on gut microbiome information and gene markers to optimize the administration of an immunosuppressive drug called Tacrolimus. This model proves to be superior than existing models in

predicting optimum required dose. In the next step I investigated the role of gut microbiome in kidney transplant outcomes and used the change in the relative abundance of bacterial genera as a tool for predicting graft rejection and graft failure. Finally, the existing policies of insurance coverage for personalized medicine for kidney disease were surveyed. I present an argument that expanding Medicare coverage to personalized medicine for kidney transplantation is essential. This 3-essay dissertation presents a package for extending our knowledge of personalized medicine in kidney disease and it offers possible tools for implementing such practices.

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Introduction and Policy Relevance

The field of kidney disease is one of the costliest fields in medicine. The complexities associated with End Stage Renal Disease (ESRD) led Congress in 1974 to expand Medicare coverage to treat ESRD patients. Since then, ESRD has been the only field of medicine covered by Medicare for patients less that 65 years old. This reality has made the field of ESRD a topic of interest among public health scholars. Except the topic of health insurance, no specialized medical field like ESRD has caused decades-long debates among policy analysts. All aspects of kidney transplantation and dialysis are under some level of control of the Federal government. Allocation of organs, prioritizing recipients, payment of dialysis and transplant surgery, post-transplant medication, and facilitating kidney donation are all affected by policies of the Federal government. For example Organ Procurement and Transplantation Network (OPTN) is a non-profit organization sponsored by the Federal government under the jurisdiction of Centers for Medicare & Medicaid Services (CMS) to provide a framework for organ allocation across the country. Furthermore, Medicare covers the cost of ESRD through payments of dialysis, transplant surgeries and medication. CMS's policies also affect kidney donation by establishing national and local programs for facilitating donation through limited financial incentives such as tax breaks, tuition credits, transportation and lodging cost,

childcare and post-donation care. The involvement of the Federal government in ESRD provides a unique opportunity for the field of kidney transplantation to use government resources to further improve quality of care for ESRD patients.

Particularly, kidney transplantation has faced several experimentations and changes in policies to lower the cost of care, expand equity and improve quality of care. The last of such changes in policy happened in 2014, knowns as the new Kidney Allocation System (KAS) in which the allocation process of kidney was substantially changed. The most recent major policy change was the law passed in 2020 by Congress to expand Medicare coverage of immunosuppressive medication for life, which used to be only 36 months post-transplant surgery. It is evident that CMS has shifted its focused away from fee-for-service paradigm and towards long-term outcome-oriented payment paradigms, such as the Prospective Payment System (PPS). Among ESRD patients, African Americans remain the least-served population. Transplant outcomes such as graft failure and waitlist relisting is highest among African Americans. At the same time African Americans are disproportionately present on the waitlist for kidney transplant compared to other racial groups based on their proportion of the population. It is known that many treatments in the field of kidney transplantation is tailored towards European decent patients. Among the most essential treatments is the dose selection of Tacrolimus (Tac), the leading immunosuppressive medication in the country. European decent patients require low dose of Tac while African Americans due to their genotype tend to require higher dose of Tac,

caused by the expression of the CYP3A5 gene. All transplant centers in the country initiate their Tac administration by low dose, and based on the need of patients the dose is adjusted. Studies unanimously show that starting Tac therapy with higher dose for African American patients leads to significantly improved transplant outcomes. But this practice rarely takes place. That is why personalized medicine has the potential of filling this gap. It is well established that adopting personalized medicine for dose selection leads to improved transplant outcomes, particularly for minorities. The number of studies and clinical trials to clinically prove this hypothesis is limited. Although they all point to the same direction, i.e., the promising conclusion that personalized dose selection practice is beneficial for all ESRD patients. That is why it is essential for the Federal government to fund more research on this topic in order to further expand quality of care for ESRD minorities. There has been no comprehensive attempt to summarize all aspects of personalized medicine for kidney disease to help kidney transplant centers and advocates for establish such practices in their centers. Furthermore, the field of personalized medicine has been introduced to a new dimension, i.e., the gut microbiome. Since the last decade there has been an immense amount of studies and focus on the role of gut microbiome on human health. In the field of kidney disease, there are limited number of such studies that investigate the role of gut microbiome on transplant outcomes.

This dissertation sheds light to the role of gut microbiome in personalized medicine for kidney transplantation and it provides original research to show how gut microbiome could be applied in personalized dose selection of Tacrolimus. The results of this dissertation could help hospitals, physicians, patient advocacy groups, insurance companies and pharmaceutical companies to further understand the role of gut microbiome in personalized medicine, but it can also provide evidence for policy makers to make informed decisions regarding the future direction of Medicare expansion in personalized medicine.

PAPER #1: The Role of Insurance in the Practice and Expansion of Personalized Medicine in Kidney Transplantation: A Public Policy Perspective

1.1 Introduction

1.1.1 Kidney Transplantation and Medicare

End Stage Renal Disease (ESRD) is the only medical condition which is covered by Medicare for patients under the age of 65. In 1972, congress enacted this legislation for qualified patients, titled Social Security Amendments of 1972; P.L. 92-603. This was the first ever legislation in the US to cover the needs of patients based on medical conditions rather than on age (Kirchhoff, 2018). Medicare spends about 7% of its annual budget on ESRD treatment, while ESRD patients account for 1% of the total patients under the Medicare program (Hart et al., 2019). Medicare benefits for ESRD patients include thrice-weekly dialysis treatment, kidney transplant, and post-transplant medication. Previously Medicare coverage lasted 36 months post-transplant, but in December 2020 the US Congress passed the Comprehensive Immunosuppressive Drug Coverage for Kidney Transplant Patients Act in which provides lifetime Medicare coverage for immunosuppressive drugs for kidney transplant recipients (Gill et al., 2021). Throughout the years, US Congress has enacted a number of modifications to Medicare benefits for

ESRD patients, such as the Medicare Improvements for Patients and Providers Act of 2008 (MIPPA) which imposed a bundled payment system for dialysis providers. The 21st Century Cures Act (CURES Act) in 2016 will allowed Medicare-eligible ESRD patients to enroll in Medicare Part C private managed care plans (Hart et al., 2019).

It is important to note that Medicare did not create a specific program for ESRD patients. It rather expanded the existing Medicare coverage to ESRD patients as opposed to creating a unique program for ESRD patients. This anecdote is important because fundamentally any argument that wishes Medicare to cover a certain group of patients needs to show how the expansion of Medicare will result in cost-effective outcomes for Medicare, and not only the targeted patients.

In this paper I will first introduce the concept of personalized medicine for kidney disease and then present an argument for having this practice covered by Medicare. In this chapter, personalized medicine will be limited to the pharmacogenomics, i.e., the role of genes in efficacy of drugs, and will not include microbiome therapies since there is little literature about their effect of kidney transplantation. Thus, for the remainder of chapter 1, pharmacogenomics and personalized medicine might be used interchangeably.

1.1.2 Personalized Medicine in Kidney Transplantation

Pharmacogenomics is the study of the impact of genetics on individual drug response. It aims to maximize therapeutic impact of drugs and minimize adverse drug reactions (Hefti

et al, 2016; Weinshiboum et al, 2017). The most useful definition of pharmacogenomics, which has been influenced by marketing campaigns, is "giving the right drug at the right dose to the right patient at the right time" (McLeod et. al., 2001). Pharmacogenomics as a concept is not new. Researchers have always been aware that therapeutic agents have significant heterogeneity in their efficacy and toxicity across different populations (McLeod, 2001). It took few decades to discover the genetic basis of this phenomenon and to apply it in medical treatments (ibid). Genetic factors determine 20 to 95 percent of drug response variability in human body (Belle, et. al., 2008). Among the existing drugs in the market, gene variations could interfere with the administration of 100-150 of all of the 1200 FDA approved drugs (Collins, 2016). Information about a patient's genetic constitution can help us select the proper drug dosage by knowing if a patient has a low or high metabolism. If a drug is known to be generally safe (low to no side effects) and therapeutic for the general public, the use of pharmacogenomics seems to be no longer necessary. This is, however, not the case if a drug is known to be risky for a specific population. If a drug is known to be toxic, and/or, extremely vital, it is important to know the gene-enzyme relationship. For example, the enzyme thiopurine-methyltransferase (determined by the TPMT gene) metabolizes azathioprine, an immunosuppressive drug used to treat Crohn's disease (Schwartz, 2004). If this drug is not properly metabolized, it can change into a toxic substance directly linked to higher risk of skin cancer and lymphoma. Therefore, TPMT activity is always monitored before

drug administration. Another example is the Cytochrome P450 gene which has a significant effect on metabolism of many drugs. Variations (polymorphisms) of the cytochrome P450 genes significantly affect the function of enzymes in human body. The effects of polymorphisms are observed in the breakdown of medications. Drugs can be metabolized quickly or slowly depending on the polymorphisms of this gene. For example, if a cytochrome P450 enzyme metabolizes a drug slowly, smaller dosage is needed because the drug stays active longer in human body. Higher dosage is needed if a drug is quickly metabolized and broken down quickly. According to NIH's Genetics Home Reference, Cytochrome P450 enzymes account for 70-80% percent of enzymes involved in drug metabolism (NIH, 2019). Knowing a patient's whole genome sequence (or targeted gene sequence) before initiating a treatment can significantly help physicians predict a patient's response to a drug.

Today, Pharmacogenomics resides under the umbrella of Personalized Medicine, i.e., the idea of tailoring health treatment to individuals, not populations. Personalized Medicine as a practice, and pharmacogenetics specifically, looks at the genetic constitution of patients as a critical element of treatment and prevention (Kalow, 2006). Advances in sequencing techniques and computational capabilities have made this field ready for wider application in medical treatment.

In the case of kidney transplantation, we focus on immunosuppressive medication. Immunosuppressive therapy is a critical part of kidney transplantation.

Immunosuppressive therapies have significantly improved graft survival rates and have reduced acute rejections (Provenzani et al, 2013). When a kidney is transplanted, a patient's body recognizes this organ as a foreign object and triggers the immune system, i.e., white blood cells will attack the grafted organ, causing a failure

Table-1: summary of drug-gene associations. (Thervet, et al., 2010)

Drug	Genotype (rs number)	Phenotype
Tacrolimus	*CYP3A5*3* (rs776746)	*3 homozygotes (CYP3A5 non-expressers) have higher dose-adjusted trough blood concentrations and lower dose requirements compared to *1 carriers; evaluated in a randomized control trial with no effect on 3 month outcomes
	ABCB1 3435C>T (rs1045642) 2677G>A/T (rs2032582) 1236C>T (rs1128503)	No clear effect on pharmacokinetics
	*CYP3A4*22* (rs35599367)	May explain additional variability in dose-adjusted blood concentration in combination with *CYP3A5*1/*3*
	*POR*28* (rs1057868)	Associated with lower dose-adjusted blood concentration but only in CYP3A5 expressers
	PPARA (rs4253728 and rs4823613)	Associated with dose adjusted blood concentration but results conflicting
Cyclosporine	*CYP3A5*3* (rs776746)	No clear effect on pharmacokinetics
	ABCB1 3435C>T (rs1045642) 2677G>A/T (rs2032582) 1236C>T (rs1128503)	No clear effect on pharmacokinetics
	*CYP3A4*22* (rs35599367)	Associated with higher dose-adjusted blood concentration
	*POR*28* (rs1057868)	Associated with lower dose-adjusted blood concentration in CYP3A5 non-expressers
Mycophenolate	*UGT1A9* (rs6714486) (rs17868320)	Associated with lower MPA exposure
Sirolimus	*CYP3A5*3* (rs776746)	Associated with higher dose-adjusted blood concentration
Everolimus	*CYP3A5*3* (rs776746)	No clear effect on pharmacokinetics

Therefore, immunosuppressive drugs reduce the offensive status of a patient's immune system and results in acceptance of the foreign organ.

Application of immunosuppressive drugs have always been complicated due to their narrow therapeutic index, i.e., overexposure leads to toxicity and underexposure increases the risk of acute rejection. Another element which adds to the complication is the vast pharmacokinetic inter-patient variability in immunosuppressive drugs (ibid). Therefore, transplant centers routinely monitor and adjust dosage of immunosuppressive drugs for their patients to reach therapeutic levels. The therapeutic range of Tacrolimus has not been clearly defined in the transplant literature and not all practitioners agree on a single optimum level. Some researchers suggest a range of 5-20, some 5-15, and others 8-10 ng/ml (Jusko et al., 1995; McMaster et at., 1995). Trial-and-error has been the most common practice to determine the best dosage (Provenzani et al, 2013). The best way to administer immunosuppressive drugs is still a matter of intense debate among physicians (Chinnadurai, 2021). Pharmacogenomics has already been applied to chronic kidney disease, dialysis and transplantation, but not as intensely and widely as other chronic diseases such as cardiovascular disease, Alzheimer disease, cancer, and asthma (Birdwell, 2015). Among all immunosuppressive drugs, cyclosporine and tacrolimus are the ones which have been heavily studied for their pharmacogenomics affects. For example, patients who do not express CYP3A5 need lower dosage of tacrolimus compared to patients who express CYP3A5, controlling for age. Studies show that CYP3A5 explains

39% of tacrolimus inter-individual variability (Elens, 2014). Other genes have been shown to affect tacrolimus absorption, such as ABCB1 polymorphism, but studies are conflicting in the level of its impact (ibid). Other variants include CYP3A4*22, POR*28, and PPARA (ibid). Table-1 shows a list of few critical genes which impact tacrolimus and other immunosuppressive drugs.

A fertile subject for research is whether adjusting dosage of tacrolimus based on the association of CYP3A5 with tacrolimus pharmacokinetics actually improves transplant outcomes or not. Little research has been done to address this question, however, especially in the randomized control trial setting to prove or disprove this hypothesis. According to Birdwell's survey, only one randomized control study has tested this hypothesis (Thervet, et al., 2010). This study shows that pharmacogenomics-guided dosage does indeed helped patients reach therapeutic levels by day 3, although no difference was observed in patient and graft survival, based on a 3 month follow up. More studies can improve our knowledge of this issue. Currently, transplant physicians select the initial dose of Tac based on a patient's Body Mass Index (BMI), usually 0.10-0.20 mg/kg per day then they adjust it on a daily basis by monitoring the trough concentration of Tac until it reaches 8-12 ng/ml for the first 3 months and 5-10 ng/ml for the 3-6 months post-transplant (Jusko et al., 1995). Although selecting Tac dose based on BMI is recommended by the US Food and Drug Administration (FDA), recent studies have shown that this approach is not effective, particularly in patients who are obese and

African Americans (Shih et al., 2014). It is believed that using a personalized dose selection tool at the start of immunosuppressive therapy could reduce the number of dose adjustments and Tac trough level fluctuations. Studies show that higher intra-patient Tac trough fluctuations (coefficient of variance greater than 40%) and lower time spent in therapeutic range (less than 40%) significantly increases the risk of graft loss, acute rejection, and poor kidney graft function (Davis et al., 2020; Taber et al., 2017). Lack of drug management and immunosuppressive medication modulation also results in sever infectious conditions for kidney transplant recipients (Shih et al., 2014). Achieving an early immunosuppressive therapeutic level is proven to significantly decrease the risk of acute rejection by 58% by day 2 after transplantation (Schiff et. al, 2007). The need for an individualized Tac dose adjustment plan for kidney transplant recipients is significant and utilization of a personalized plan will significantly improve transplant outcomes for all patients. Improved transplant outcomes such as lower rejection rates, shorter hospital stays, and higher organ utilization all translate into reducing the healthcare cost of post-transplant kidney care.

Lastly, the impact of gut microbiome on immunosuppressive medication treatments has been of great interest for researchers. Gene sequencing of the gut microbiome has shed light on some previously unexplainable dynamics of immunosuppressive medication effectiveness. Xiao et. al (2018), Swanson (2015) and Ahmad et. al (2016) discuss the current understanding of microbiome influence on kidney transplant outcomes in their

papers. All investigations point to the fact that gut microbiota has a critical role in the development of human diseases and particularly in kidney disease (Xiao et. al; 2018). There are 1000 different types of microbiota in the human gut (Gin et. al; 2010) and the composition of the gut microbiome differs from person to person (Lynch et. al; 2016). The gut microbiome of humans is involved in important activities such as food digestion, regulating metabolism and the immune system, and promotion of angiogenesis, i.e., development of new blood vessels (Thaiss et. al, 2016; Neish, 2009; Manfredo et. al, 2018). Imbalance in the gut microbiome is associated with inflammatory bowel disease, obesity, diabetes, colorectal cancer, cardiovascular disease and nervous system disease (Xiao et. al; 2018). New studies have observed the critical role of gut microbiota on the immune system (Belkaid et. al, 2014). The change in gut microbiota diversity has effects on distant organs as well (ibid). Several studies have investigated the change of the microbiome composition after a kidney transplant operation (Lee et.al, 2014; Zaza et al., 2017). All studies have observed: 1) a decrease in the baseline predominant organisms, 2) a decrease of diversity and 3) emergence of new dominant bacterial population after transplantation (Xiao et. al; 2018). All studies conclude that the above changes lead to an increased risk of post-transplant infection (ibid). In the specific case of kidney transplant, change in gut microbiota composition has been associated with increased risk of graft failure in renal transplant by influencing the dosing of immunosuppressant drugs (Zaza et al., 2017). For example, Lee et al. investigated the role of human microbiome on

Tacrolimus dosage and concluded that *Faecalibacterium prausnitzii* abundance in the first week of transplantation is positively associated with higher future tacrolimus dosing at 1 month (Lee et al., 2014). Other studies have even observed the role of gut microbiome in the prognosis of kidney transplant outcomes (Ardalan et al., 2017; Ahmad et al., 2016; Fricke et al., 2014). Ardalan et al. report that gut dysbiosis causes accumulation of uremic toxins, systemic inflammation, and infection that influence the pathogenesis of acute kidney injury, chronic kidney disease, emergence of infection, changes in drug metabolism and graft rejection (Ardalan et al., 2017). Dysbiosis can be caused by immunosuppression and antimicrobial therapies, ischemia-reperfusion (I/R) injury, and dietary restrictions (ibid). Studies are still in their early stages and results are somehow mixed due to small sample sizes, but more studies are underway to explore microbiome's effect on kidney disease. What has not been done is the development of an algorithm to detect a personalized optimum medication dosage based on a person's pre-transplant gut microbiome composition.

1.2 Challenges in Personalized Medicine in Kidney Transplantation

There are two main challenges in adopting the practice of Personalized Medicine in Kidney Transplantation: a) whether applying genotype/microbiome-guided dose selection will result in better outcomes, b) the payment of practicing personalized dose selection. Regarding the first challenge, most researchers and practitioners believe that the

projection of studies point to the conclusion that personalized treatments do offer better transplant outcomes, even though the studies are still limited (Thervet, et al., 2010). Most results show strong evidence that personalized dose selection practices lead to shorter time to reach therapeutic levels, which in turn leads to better transplant outcomes (ibid). More clinical trials with different cohorts of patients can strengthens this argument. Particularly in the case of African American patients who historically experience poor transplant outcomes, the hypothesis of most studies is to expect better outcomes for this specific population by applying personalized dose selection (Taber et al., 2017).

The main challenge in applying personalized medicine in kidney transplant is, in fact, the payment. Hospitals and healthcare providers are the ones who perform this service, but reimbursement is not guaranteed. Specially in the case of patients who already face significant financial challenges, paying for more expenses is difficult to justify. Based on an extensive survey by Hresko et al. at Duke University Institute for Genome Sciences & Policy, the coverage of pharmacogenomics (PGx) tests significantly varies across different insurance companies. Medicare does in face cover such tests in a limited capacity, but most of the largest private insurance companies are the ones that cover the largest number of PGx tests. It has been evident that adoption of PGx tools have been extremely slow in the medical world, but more so has been the payment processes for such tools. The slow pace of adoption has been due to lack of knowledge among physicians, patients' interest, and lack of insurance coverage. What has been causing

hesitancy among insurance companies is the lack of evidence that PGx tests and practices lead to better outcomes (Hresko et al., 2012). Hresko surveyed the top 10 insurance companies in the country: Kaiser Foundation Group, Coventry Corporation Group, UnitedHealth Group, Independence Blue Cross Group, Aetna Group, Highmark Group, Humana Group, Wellpoint, HCSC Group, and Cigna Health Group. The results show that among all these companies, in total, 27 PGx tests are covered (table 2). It is important to note that these 27 PGx tests were covered for drugs which have mentioned on their label the impact of genetic variations. If such label is not on the drug, it is not covered by any insurance company. Most PGx tests are deemed investigational and not medically necessary. Aetna was proven to cover the largest number of PGx tests. Medicare also recognizes the coverage of PGx tests and it covers the cost of targeted gene testing for Warfarin response. Medicare covers the PGx testing of CYP2C9 or VKORC1 alleles to predict warfarin effectiveness only when Medicare beneficiaries are candidates for anticoagulation therapy and few other requirements (Hresko et al., 2012). In June 2020, the CMS Medicare issued a future Local Coverage Determination (LCD) for pharmacogenomics testing. This LCD described Medicare's intent to cover single gene, multi-gene panels to improve the safety of specific medications. The definition for coverage is stated as "*medically necessary, appropriate, and approved for use in the patient's condition and are known to have a gene(s)-drug interaction that has been demonstrated to be clinically actionable as defined by the FDA (PGx information*

required for safe drug administration) or Clinical Pharmacogenetic Implementation Consortium (CPIC) guidelines (category A and B)" (CMS, 2020). One important criterion for both Medicare and private companies is not only there needs to be evidence that a gene-drug interaction exists, but also whether knowing about the genotype will have any clinical utility. In several cases, knowing about a gene-drug interaction does not necessarily translate into clinical utility, thus, it will not be justified for reimbursement or coverage (CMS, 2020). In the PGx cases where insurance companies have covered, strong randomized clinical trials have been performed and shown the effectiveness of applying genotyping in drug treatment, either via dose selection or drug selection. It is interesting to note that the difference between coverage of PGx tests is also impacted by the type and number of randomized clinical trials that each insurance company decides to review (Hresko et al., 2012)

If the benefits of performing personalized dose selection could be advocated by all stakeholders, that will be the start of advocacy for Medicare coverage. Stakeholders are kidney transplant centers (i.e., hospitals), patient advocacy groups such as the American Kidney Fund (AKF), the National Kidney Foundation (NKF), the government agencies in charge of overseeing kidney transplant outcomes and allocation of organs such as Organ Procurement and Transplantation Network (OPTN) and United Network for Organ Sharing (UNOS) and notably Medicare who pays all the expenses, and physician advocacy groups such as American Organ Transplant Association and American Society

of Transplantation (AST) who have a vested interest in to improve the outcome of their treatments. There are numerous of such advocacy groups, all listed in a thorough repository on the NKF webpage. Each of these stakeholders possess different interests in advancing better outcomes in kidney transplantation. Two group of stakeholders that have the ultimate power of the purse and direct financial interests are hospitals and Medicare. In the next section we argue that how the interests of these parties could align and materialize the practice of personalized medicine in kidney transplant.

1.3 How Medicare covers ESRD

It is essential to understand the different parts of Medicare program in order to explore possible ways to expand it to prospective treatments. There are 4 distinct parts in Medicare programs:

1- Part A: Hospital insurance. This part covers the cost of inpatient services such as transplant surgery.

2- Part B: Supplementary medical insurance. This part covers physician services, hospital outpatient services, dialysis, medical equipment, preventative services and prescription drugs. Generally, patients pay a 20% coinsurance for part B insurance, but it may vary based on income level.

3- Part C: Medicare Advantage. Patients who wish to receive Medicare part A and B through a private insurance company can use Part C. The Federal Government

pays private health plans that participate in Part C. Additional services that are not covered under Part A of B will be provided to patients at additional premium such as dental, vision, fitness programs, and any service that can be customized to treat a specific condition.

4- Part D: outpatient prescription drugs. Patients who are enrolled in Part A and Part B may also enroll in Part D which provides outpatient prescription drugs via private insurance companies.

As of 2015, 59% of ESRD patients used Medicare as their primary payer, 8% had Medicare as secondary payer (covered by employer-sponsored insurance as primary), 14% were in Part C plans, and 19% had non-Medicare coverage, i.e., they were pre- or post-Medicare entitlement (patients in the waiting period for receiving dialysis or patient after 36 months of receiving a transplant) (Kirchhoff, 2018). In 2016, Medicare spent approximately $61,996 per ESRD patient, compared to $9,889 per non-ESRD patients [ibid]. Total Medicare spending on ESRD patients in 2016 was $35.4 billion, which equals to 7% of total Medicare spending. This proportion of expenditure on ESRD patients (i.e., 7%) has been stable since 2004 (Kirchhoff, 2018).

It is important to clearly lay out how does Medicare pay for ESRD services and what does it exactly cover. When ESRD benefits were first implemented by the US Congress in 1972, Medicare paid health care providers separate amounts for testing, supplies, drugs and treatments. This system was known as fee-for-service and it significantly improved

the conditions of ESRD patients. Although, the fee-for-service system proved to significantly increase the cost of kidney care regardless of providing better outcomes. In order to control costs, Congress required CMS to implement a prospective payment system (PPS) for dialysis services. With PPS, the annual amount of payment to a healthcare provider is established in advance at the beginning of the fiscal year, regardless of the actual volume of care provided to patients. It has been evident that since the implementation of PPS in 2011, the annual increase in Medicare fee-for-service spending has been modest, and the spending per-beneficiary has decreased (Kirchhoff, 2018). Congress has been satisfied with the PPS plan because the growth in Medicare spending has been due to the increase in covered lives and not due to higher costs (Hart et al., 2019). Although, this still does not reflect the quality of outcomes. In the case of kidney transplantation, Medicare provides reimbursement for medical services performed in a hospital such as surgery, medication, tests, and in-patient recovery. To be more specific, Medicare Part A covers the cost of performing surgery with all the associated treatments including laboratory tests. Medicare Part B covers the cost of immunosuppressive drugs. Patients are responsible to pay 20% of the Medicare-approved expenses unless they fall below the minimum income level and are covered by complimentary Medicaid coverage for the 20% co-payment.

Patients do not pay for any Medicare-approved laboratory tests (Kirchhoff, 2018). Laboratory tests are defined as "*medically necessary clinical diagnostic laboratory tests*"

ordered by a physician, which includes certain blood tests, urinalysis, tests on tissue specimens and screening tests [ibid]. Genetic test for pharmacogenomics purposes have not been covered by Medicare, but there are several arguments from numerous stakeholders to encourage reimbursement of such genetic tests (Hresko et al., 2012). In this paper, I will describe the current knowledge of personalized medicine in the field of kidney transplantation and its benefits and challenges in transplant outcomes. This paper will describe the framework of personalized medicine in kidney transplantation, then provide a policy perspective argument for Medicare to cover the cost of such practices in kidney transplantation.

1.4 An Argument for Medicare Payment for Personalized Medicine in Kidney Transplantation

Based on the studies cited earlier, there is no doubt about the impact of CYP3A5 on Tacrolimus effectiveness for kidney transplant patients. What is lacking is more randomized clinical trials (RTC) to show the clinical benefits of applying genotype-guided Tac dose selection. It will be prudent for CMS or other healthcare agencies like the NIH to provide more grants for research in order to conduct more RCTs. Since the existing studies point towards the efficacy of genotype-guided treatments, distributing grants is justified, because it will not be solely an investigation in the dark, but the existing path does encourage favorable outcomes for this investment. If the efficacy of

PGx testing for Tac can be established, cost-effectiveness can then be analyzed. Based on the small cost for targeted genotyping, circa $200 per sequencing and the associated overhead costs, the benefits strongly surpass the costs. If the benefits include one day shorter hospital stay (an amount equal to on average $6000-10,000 per day) and longer graft survival and fewer rejections, demonstrating cost-effectiveness will not be difficult. On the other hand, there needs to be advocacy from the non-government stakeholders to prepare the conditions in which Medicare can cover the costs of PGx testing for kidney disease. Notably, one of the most important criteria for PGs coverage is labeling. While it is important to have FDA's approval on drug-gene interactions, printing PGx information on the drug package seems to be the most important criterion based on Hreski's survey. Having this label on drug packaging is a necessary condition, but not sufficient. It is upon the drug manufacturers to print such information on the packaging. This would potentially also benefit drug manufacturers by having their drugs experience targeted dose selection for higher efficacy. Since the adoption of PGx testing by Aetna and Humana, there has been a vast amount of data regarding the utility of such coverage. This could be an incentive for Medicare since these private companies have been bearing the initial cost of investigation and evidence generation. The last challenge for adopting PGx as a routine practice in kidney transplantation, is the fact that patients might end up facing a co-payment cost, in the case of Medicare usually close to 20%. As noted before, for many patients who already face financial difficulties, this could be an extra burden.

Based on an opinion piece by Geruso et al. in the Harvard Business Review, they studied the impact of PGs testing on premiums or co-pays (Geruso et al., 2018). They reached the conclusion that the increase in premiums and co-pays are significantly low and compared to other medical expenses it is minuscule. They also argue that the benefits of PGx in the short and long run can have direct financial return for patients, notably shorter hospital stays and lower adverse drug reactions.

Due to the high cost of ESRD for Medicare, there has been a great emphasis on quality of care and outcomes. Short term rejection rate remains an important criterion for evaluating transplant centers, but Medicare has also shifted its attention towards long term survival rate (Kirchhoff, 2018). Kidney transplantation is more cost-effective than dialysis after 3.1 years, thus it is much more financially beneficial to improve graft survival rate beyond 5 years. Using PGx tests to improve dose selection at the very beginning of transplant surgery is a viable strategy to improve graft survival rate. The recent move from Congress to cover life-time immunosuppressive medication coverage noted the benefits of long-term care, e.g., the cost of returning to dialysis due to kidney failure is around eight times the cost of immunosuppressive medication. If all stakeholders use the momentum created in the US Congress and provide more evidence on the benefits of PGx testing for kidney transplantation, it is not farfetched to see personalized medicine as a routine practice in kidney transplantation.

Table-2: Coverage Policies for Pharmacogenomics Tests by Insurer

Test	Drug Indication	Insurer					Tech Assessments		FDA Approvals	
		Aetna	Indep-endence BCBS	Cigna	Humana	United Health	BCBS TEC	EGAPP	FDA-cleared test	Revised Drug Label with PGx Info
Apo E	Lipid lowering medications	No [1]	-	No [2]	-	-	-	--	No	Yes
BRAF	Cetuximab, pantimumab	-	No [3]	-	-	-	-	--	Yes	No
Caris TargetNOW Molecular Profiling	Inform cancer therapy	No [1]	-	-	No [4]	-	-	--	No	N/A
CYP2C19	Clopidogrel	Yes [1]	Yes [5]	No [6]	No [4]	-	-	--	Yes	Yes
CYP2C19	Proton Pump Inhibitors	No [1]	No [7]	-	No [4]	-		--	Yes	Yes
CYP2C9/ VKORC1	Warfarin	No [1]	No [8]	No [6]	No [4]	-	-	--	Yes	Yes
CYP2D6	Tamoxifen	No [1]	No [9]	No [6]	No [4]	-	No [10]	--	Yes	Yes
CYP2D6	Tetrabenezine	Yes [1]	-	-	Yes [4]	-	-	--	Yes	Yes
CYP2D6	Donepezil	No [1]	-	-	-	-	-	--	Yes	No
CYP2C9	Proton pump inhibitors	-	-	-	No [4]	-	-	Insufficient evidence to recommend for or against use [11]	No	Yes
CYP450 (not specified/multiple)	SSRIs	No [1]	-	No [6]	No [4]	-	-	Insufficient evidence to recommend for or against use [11]	N/A	N/A
Dihydropyramidine Dehydrogenase (DPYD)	5-Fluorouracil	No [1]	-	-	No [4]	-	No [12]	--	No	Yes
EGFR	Erlotinib	Yes [1]	No [13]	-	Yes [4]	-	Yes [14]	--	No	No
ERCC1	Cisplatin, carboplatin, oxaloplatin	-	-	-	No [4]	-	-	--	No	No

Test	Drug Indication	Insurer					Tech Assessments		FDA Approvals	
		Aetna	Indep-endence BCBS	Cigna	Humana	United Health	BCBS TEC	EGAPP	FDA-cleared test	Revised Drug Label with PGx Info
HLA-B*1502	Carbamazepine	Yes (in Asian patients) [1]	-	-	Yes (in Asian patients) [4]	-	-	--	No	Yes
HLA-B*5701	Abacavir	Yes [1]	-	Yes [6]	Yes [4]	-	-	--	No	Yes
IL28B	Interferon therapy for Hepatitis C	No [1]	-	-	-	-	-	--	No	Yes (Peg-interferon α2B, Teleprivir, Boceprivir)
KIF6	Statin		No [15]					--	No	N/A
KRAS	Erlotinib	Yes [1]	No [16]	-	No [4]	-	-	--	Yes	No
MGMT Methylation	Temozolomide (Temodar)	-	-	-	No [4]	-	-	--	No	No
MTHFR	Antifolate chemotherapy	No [1]	-	-	-	-	-	Insufficient evidence for or against [11]	Yes	No
rs3798220	Aspirin	No [1]	-	-	-	-	-	--	No	No
TPMT	Mercaptopurine, azathiopurine	Yes [1]	Yes [17]	Yes [6]	Yes [4]	-	-	--	No	Yes
Thymidylate Synthase	5-Fluorouracil	No [1]	-	-	-	-	No [18]	--	No	No
Urovysion	Follow-up treatment for bladder cancer	Yes [1]	-	-	-	--	--	--	Yes	N/A
UTG1A1	Irinotecan	No [1]	-	No [19]	No [4]	-	-	--	Yes	Yes
Whole Genome/Whole Exome/Genome-wide Association study	Pharmacogenetics (not specified)	-	-	-	No [4]	-	-	--	No	N/A

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PAPER #2: The Role of Gut Microbiome in Personalized Optimum Dose Selection of Tacrolimus for Kidney Transplant Recipients

Abstract

This paper introduces a new personalized dose selection model for Tacrolimus based on gene markers and gut microbiome profile. Previous studies have explored strong associations between Tacrolimus dosing after kidney transplant operation and the abundance of certain species of bacteria in the gut. There have been no attempts to translate the knowledge of gut microbiome into clinical utility. By recruiting 10 kidney transplant patients from the George Washington University Hospital, we collected the first database containing pre- and post-transplant gut microbiome data paired with targeted gene sequencing of CYP3A5 for the purpose of developing the first gene/microbiome-guided Tacrolimus dose selection model. The pre-transplant relative abundance of *Phocaeicola* in days≤10 was negatively associated with optimal Tac dose and the increase in relative abundance of *Bacteroides* after transplantation in 11≤days≤90 was positively associated with optimum Tac dose with $p<0.01$. The bias and precision of our model were significantly improved compared to those of the current state-of-the-art optimum Tac dose model, i.e., Jacobson et al. ($p=0.05$ one-sided Wilcoxon signed-rank test). Further

validation of this model by an independent cohort of patients is required. Conducting clinical trials to investigate weather using this model translates into improved outcomes will determine the utility of this model.

2.1 Introduction

2.1.1 Tacrolimus

Tacrolimus (Tac) is the most widely used post-transplant maintenance immunosuppression regimen in the United States [1]. Tac has a narrow therapeutic window, i.e., low exposure leads to graft rejection and high exposure leads to toxicity [2]. That is why Tac trough levels are routinely monitored to be in the therapeutic range by continuously adjusting the dose. Therapeutic levels are usually defined as 8-12 ng/ml for the first 3 months and 6-10 ng/ml for months 3-6 post-transplant, although each transplant center follows its own unique protocol [3]. Reaching the therapeutic level by dose adjustment is particularly difficult due to the wide inter-individual variability of Tac. This variability is presumably caused by the expression of cytochrome gene P4503A5 (CYP3A5), which affects Tac's pharmacokinetics [3]. It is known that the expression of CYP3A5*1 allele is associated with high metabolization of Tac, hence higher dose of Tac is needed to reach therapeutic levels [4]. Studies show that African

Americans are generally high expressors of CYP3A5 and need higher dose of Tac to reach therapeutic levels compared to European Americans [2]. Currently transplant centers do not practice genotype-guided dose selection. A trial-and-error approach with routine Tac through level monitoring is practiced instead [3]. Some centers apply a rule of thumb approach by selecting the initial Tac dose as 0.10-0.30 mg/kg per day in two divided doses [5]. Although selecting Tac dose based on bodyweight is recommended by the US Food and Drug Administration (FDA), recent studies have shown that this approach is not effective, particularly in patients who are obese [5, 6].

Studies have shown that a lack of drug management for immunosuppressive medication results in sever post-transplant complications such as infections, delayed graft functions and acute rejections for kidney transplant recipients [7, 8]. Having a strategy to select optimal initial Tac dose is critical because achieving an early immunosuppressive therapeutic level is proven to significantly decrease the risk of acute rejection [7]. Additionally, a personalized Tac dose selection strategy is critical in reducing intrapatient Tac trough fluctuations and increasing time in therapeutic range. Studies show that higher intrapatient Tac trough fluctuations (coefficient of Variance greater than 40%) and lower time spent in therapeutic range (less than 40%) significantly increases the risk of graft loss [9, 10].

Lack of an optimal Tac dose selection strategy has encouraged researchers to adopt more advanced approaches for initial Tac dose selection, particularly through genotyping [2].

Such personalized approaches are proven to be significantly effective in reaching Tac therapeutic levels in fewer days and with less dose adjustments [2]. Among the personalized Tac dose selection models, Jakobson et al. from the of University of Minnesota have developed the most seminal model, supported by the National Institute of Allergy and Infectious Disease (NIAID). This genotype-guided dose selection model incorporates few clinical and center-specific factors in order to make it practical and easy to use. This model has been validated by the same team in a retrospective comparison study on 795 patients to predict initial Tac trough levels and optimum Tac (Prograf) dose. The personalized model of Jakobson was significantly superior to the basic clearance approach for the first 6 months post-transplant with low bias and high precision [3]. Equation 1 shows the Jakobson model for prediction of Tac apparent clearance (CL/F) in the first 6 months post-transplant. Equation 2 shows the total daily dose requirement (TDD) based on the predicted Tac clearance and the desired goal for trough concentration.

Equation (1)

$CL/F\ (l\ h^{-1}) =$

$38.4 \times [(0.86, \text{if days 6-10}) \text{ or } (0.71, \text{if days 11-180})]$
$\times [(1.69, \text{if CYP3A5*1/*3 genotype}) \text{ or } (2.00, \text{if CYP3A5*1/*1 genotype})]$
$\times (0.7, \text{if receiving a transplant at a steroid sparing center})$
$\times [(\text{age in years}/50)^{-0.4}]$
$\times (0.94, \text{if CCB is present})$

CCB=calcium channel blocker
CL/F= Tac apparent clearance

Equation (2)

$TTD_{Jacobson}$ (mg) =

$[CL/F(I\ h^{-1}) \times Tac\ trough\ goal\ (ng\ ml^{-1}) \times 24\ h]/1000$

TDD= total daily dose

The example presented by Jakobson et al. is the following: in order to prospectively select the optimal Tac dose for a 50-year-old patient with 85 kg on day 3 post-transplant with a Tac trough level goal of 10 ng/ml and a genotype of CYP3A5*1/*1 in a CCB and steroid using center, we first calculate the CL/F and then TDD.

$CL/F\ (l\ h^{-1}) = 38.4 \times (2\ for\ CYP3A5*1/*1\ genotype) \times [(50/50)^{-0.4}] \times (0.94\ for\ CCB\ use) = 72.2$

$TDD\ (mg) = [72.2 \times 10\ ng/ml \times 25\ h]/1000 = 17.5$

The optimal daily Tac dose for this patient is 17.5 mg or 8.5-8 mg twice daily. If we use the FDA's weight-based dosing method (0.1 mg/kg/day) the predicted dose would be equal to 8.5 mg per day, which would significantly under-dose the patient. Jakobson's model has not been tested in a prospective trial yet.

2.1.2. Gut Microbiome and Kidney Transplant

One element that has been absent from personalized Tac dose selection models is the human gut microbiome. There are more than 100 trillion microbial cells in the human gut which significantly influence the host immune system and overall health [17]. Even the

smallest change in the diversity and composition of the human gut microbiome could affect the host health [18]. It has been established that kidney transplantation leads to microbiome dysbiosis caused by the administration of immunosuppressive and antibiotic drugs [12, 13]. Microbiome dysbiosis is associated with several post-transplant complications such as increased risk of infections, diarrhea and graft failure [12, 13]. Examples of bacterial taxa that have proven association with kidney transplant outcomes are as follows, *Janthinobacterium*, *Clostridia*, *Bacilli*, *Lactobacillales* which are associated with spontaneous tolerance; numerous genera in *Lachnospiraceae* which are negatively associated with serum creatinine and blood urea nitrogen (BUN); Butyrate-producing bacteria are associated with less development of respiratory viral infections and *Lactobacillus plantarum* which is associated with reduced risk of clostridium difficile (C. Diff) infection incidence [14]. It is known that at least 30% of all non-antibiotic drugs alter the abundance of at least one bacterial strain [18]. Lower gut bacterial diversity has been associated with reduced immune functioning and metabolic syndrome [18]. Recent studies have explored the possibility of a significant connection between human gut microbiome and the metabolism of drugs, particularly drugs which impact the immune system such as Tac [15, 16, 17]. No direct causal relationship has been proven in any study regarding microbiome dysbiosis and drug metabolism and transplant outcomes. What makes this relationship difficult to study is the complex and bidirectional dynamic between gut microbiome, immunosuppressive medication, and

antibiotics, i.e., initial Tac and antibiotic administration causes microbiome dysbiosis, and in return, microbiome dysbiosis affects the immune system via a variety of pathways and manipulates dose adjustments of immunosuppressive medication and antibiotic efficacy [13].

2.1.3 Gut Microbiome and Tacrolimus

Post-transplant dysbiosis of the gut microbiome caused by the administration of antibiotics and immunosuppressants can result in the abundance of certain bacterial communities that can metabolize immunosuppressants into less potent metabolites [14]. For example, Lee et al. discovered a significant association between *Faecalibacterium prausnitzii*, which is one of the most abundant bacterial species in the gut microbiome, and Tac dose in the first month after kidney transplantation [15]. Patients with higher abundance of *Faecalibacterium prausnitzii* in the first week of transplantation demanded higher dose of Tac at 1 month [15]. Lee et al. opines that Tac absorption and/or metabolism may be affected by the colonic mucosa since a healthy colonic mucosa requires butyrate from bacterial sources such as *Faecalibacterium prausnitzii*. An additional study reached the conclusion that *Faecalibacterium prausnitzii* can metabolize Tac into a less potent novel metabolite M1 (9-hydroxy-tacrolimus), which is 15-fold less potent than Tac in inhibiting the proliferation of activated T cells [16].

Studies have unanimously reached the conclusion that if drug-microbiome interactions could be predicted for every patient, it will facilitate the selection of optimal treatments and dose which leads to fewer dose adjustments and adverse reactions [18]. Although many studies have explored drug-microbiome interactions, there is a major lack of translation into clinical use [18]. A framework that can incorporate clinical information, gene markers and microbiome profile of a given patient into a personalized dose selection practice does not exist. Such personalized practices must be clinically relevant, cost-effective, fast and non-invasive to be accepted [18]. This paper is an attempt to incorporate gut microbiome profile in a personalized Tac dose selection model. This will be based on the genotype-guided Tac dose selection model of Jacobson et al. and it will improve the overall performance of that model.

2.2 Methods

2.2.1 Population

Seventeen kidney transplant patients from The George Washington University Hospital (GWUH) were consented and enrolled in this study. IRB was approved by the GWUH Office of Human Research (#NCR191914) and George Mason University IRB

#1906208-1. Summary of clinical and demographic information is presented in table 1. Exclusion criteria were pediatric, pregnant, and simultaneous kidney-pancreas recipients. All patients underwent induction therapy based on test results such as Panel of Reactive Antibodies (PRA) and Donor Specific Antibodies (DSA). DSA is a measure to predict antibody-mediated rejection. PRA is a measure to determine the potential level of sensitization of a given patient towards the pool of kidney donors, as a result of prior exposure to external HLA antigens during blood transfusions, pregnancies, or previous organ transplantations. If a patient possesses a high PRA, he/she will undergo a desensitization process to reduce the risk of graft rejection. Patients who were highly sensitized received Velcade (Bortezomib) preoperatively. All patients received two doses of Thymoglobulin, while sensitized patients received three doses. Following Thymoglobulin induction, all patients received Simulect. Induction therapies are pre-medicated with methylprednisolone, acetaminophen, and diphenhydramine. Induction therapy is the process of administering immunosuppressive therapy at the time of kidney transplantation to reduce the risk of allograft rejection. After the transplant surgery, all patients started with 4 mg of Tac. On a case-by-case basis, some patients were subject to antibiotics (Atovaquone) and stimulant laxatives.

2.2.2 Sample Collection and Sequencing

For each patient a series of sample collection activities took place. 3mL of blood was drawn for gene sequencing at the time of routine clinic visit. Blood was stored in ZYMO DNA/RNA Shield Blood Collection tubes which ensure sample stability during storage/transport at ambient temperatures without the need for refrigeration or specialized equipment. The nucleic acids (DNA & RNA) in samples are preserved at ambient temperature (DNA >1 year, RNA up to 1 month). Targeted gene sequencing was performed by Illumina Next Generation sequencer by *The Sequencing Center* lab in Fort Collins, CO. The list of targeted genes is available in appendix A. We focused on the expression of the gene CYP3A5. Because it is well established that the cytochrome P4503A5 (CYP3A5)*1 allele singlehandedly has the most significant impact on metabolization of Tac [3]. The cytochrome P4503A5 has 25 allelic expressions (from *1 to *9) but only the alleles *1, *3, *6 and *7 are frequently found in the population. Therefore, the existence of single nucleotide polymorphisms CYP3A5*3(rs776746, g.6986A>G), CYP3A5*6 (rs10264272, g. 14690 G>A) and CYP3A5*7 (rs41303343, g.27131-27132insT) were analyzed. Subjects who did not express CYP3A5*3, *6 or *7 alleles were categorized as CYP3A5*1/*1 genotype and those who expressed one CYP3A5*3, *6 or *7 allele were categorized as CYP3A5*1/*3, *1/*6 or *1/*7 genotype, respectively.

Two stool samples were collected per patient for gut microbiome sequencing. The first stool sample was collected 1 week before transplant and the second stool sample was collected 1-2 months post-transplant. Collection was performed by subjects by using a paper feces catcher and a coring brush which eliminated the risk of contamination. Stool samples were stored inside ZYMO DNA/RNA Shield Fecal Collection Tubes–DX. These tubes preserve the bacterial communities in ambient temperature (DNA>2 years, RNA>1 month). Whole DNA of bacteria was sequenced using shotgun metagenomics to the strain level by *CosmosID* lab in Germantown, MD (https://www.cosmosid.com). For each patient, only the bacterial taxa with relative abundance more than 1% were included in the analysis, which is the common practice in gut microbiome studies to exclude irrelevant taxa [15]. Appendix C shows the 76 remaining genera. Shannon diversity index for both pre- and post-transplant stool sample was calculated, and the difference was analyzed. Shannon diversity index is a popular metric in biology for measuring the number of species living in a sample (richness) and their relative abundance (evenness). The number of unique species that exist in an environment represents the richness of that environment. But that is not enough to measure the diversity of a given environment; because if one species dominates the whole environment it will not be a diverse environment. That is why in addition to richness, Shannon diversity considers the relative abundance of the species to measure the evenness of the environment as well.

2.2.3 Clinical data

Clinical data was collected from electronic health records in Cerner and deidentified per protocol. Among which, Tac dose and Tac trough levels were the most critical entries. All Tac dose entries (total daily dose in Mg) was collected continuously from the day of transplant surgery until day 90 post-transplant. *Envarsus* was the dominant immunosuppressant drug in this transplant center. Tac doses were routinely adjusted by transplant physicians to reach therapeutic levels. Tac trough levels were measured by the George Washington University Hospital Lab. Trough levels of the first day of kidney transplant and trough levels measured in the afternoon were excluded to reflect true and stable concentration levels. Four time frames were defined for the analysis, i.e., 2≤days≤5 ,6≤days≤10, 11≤days≤30, 31≤days≤90. For each time frame, **observed optimal Tac dose** was defined as the average administrated dose of Tac in that time frame, which corresponded to the occurrence of stable therapeutic Tac levels (8-12 ng/ml). Our model was developed based on the observed optimal Tac doses in order to predict future optimal Tac doses based on gut microbiome taxa and Jacobson's initial total daily dose. A future study shall validate this model based on a testing set.

2.2.4 Statistical Analysis

Relevant bacterial taxa were selected by plotting their pre-transplant relative abundance and the change in their relative abundance against observed optimal Tac dose for each subject. Pearson correlation coefficient was calculated for bivariate analysis in all levels of species, genus, and family. Taxa of genus level was ultimately selected for analysis because in the species level there were a significant number of unknown species. Significant bacterial genera were selected for possible inclusion in the model with p-value<0.05, graphs 1 to 4 in appendix B. We used pre-transplant relative abundance of bacteria for predicting optimal Tac dose for days 2-10 since the pre-transplant microbiome profile is the closest to that time frame. For predicting the optimal Tac level for days 11-90, we used the change in relative abundance of bacteria, since pre- and post-transplant relative abundances showed no correlation with observed optimal tac dose. Ordinary Least Square (OLS) linear regression was used to add the relevant microbiome variables to the Jacobson model, previously presented in equations 1 and 2. The total daily dose derived from the Jacobson model for the i^{th} subject is named $TDD\text{-}Jacob_i$. The observed optimal Tac dose for the i^{th} subject ($Dose_{pred,i}$) is the dependent variable of our model. The results of the final models were compared to the Jacobson model via bias and precision. Prediction error for i^{th} patient (PE_i) was defined as the difference between predicted and observed dose, shown in equation 3. We followed the definitions of

Jacobson et al. for bias and precision [3]. Bias was defined as the median prediction error and precision as the median absolute prediction error, shown in equation 3.

Equation (3)

$PE_i = Dose_{pred,i} - Dose_{obs,i}$.

Bias = median (PE_i)

Precision = median ($|PE_i|$)

PE_i = prediction error for i^{th} patient.

$Dose_{pred,i}$ = predicted optimal Tac dose for i^{th} patient.

$Dose_{obs,i}$ = observed optimal Tac dose for i^{th} patient.

To compare the outcome of our model against Jacobson's model we evaluated relative predictive performance in all the four time frames. One-sided Wilcoxon signed-rank test was used on the paired prediction errors for relative bias and on paired absolute prediction errors for relative precision [25]. Null hypothesis was set as bias and precision of the two models are similar against the alternative hypothesis that bias and precision of our model is less than Jacobson's. A p-value<0.01 was evidence that the models do not have similar predictive power and our model is superior at predicting optimal Tac dose in each time frame.

2.3 Results

All patients possessed the CYP3A5*1/*1 allele which made them high expressors of CYP3A5. This controls for the effect of gene markers on Tac dosing which has been absent in all previous gut microbiome studies. Various models were examined to reach the most relevant, simple, and efficient models. Table 1 shows the results of the OLS models for predicting optimal Tac dose in the four time frames. The variable **Phocaeicola** represents the pre-transplant relative abundance of *Phocaeicola* in the form of percentage. The variable **ΔBacteroides** represents the change in relative abundance of *Bacteroides* from pre-transplant to post-transplant in the form of percentage. Each column represents on OLS regression results. The rows are independent variables. The value of TDD(Jacobson) is calculated separately based on equation 2. The adjusted R-squared value for all models were above 60%. The last 4 rows of table-1 compares the paired bias and precision of each OLS prediction of optimal Tac dose vs. those of Jacobson. The overall significance of each regression (F-Statistic) is less than 0.01, which rejects the hypothesis that all regression coefficients are equal to zero. The n for each of the regressions was 17, with 2 independent variables in each regression, and the outcomes of the regression (F-statistic and Adj-R2) justified the validity of using the OLS model.

Table 3: OLS Regression Results for Optimal Tac Dose

	Dependent Variable			
Independent Variables:	**Optimal Dose, 2≤days≤5**	**Optimal Dose, 6≤days≤10**	**Optimal Dose, 11≤days≤30**	**Optimal Dose, 31≤days≤90**
TDD-Jacob$_{2\text{-}5days}$	1.11 (0.98)			
TDD-Jacob$_{6\text{-}10days}$		1.76 (1.56)		
TDD-Jacob$_{11\text{-}180days}$			3.32 (2.86)	2.07 (2.89)
Pre-tx abundance of Phocaeicola	-74.63** (22.15)	-105.39** (20.42)		
ΔRelative abundance of Bacteroides			46.17** (12.77)	49.08** (12.92)
Constant	4.74	5.02	-16.06	-7.57
Prob>F	0.01	0.009	0.007	0.008
Adj.R^2	0.64	0.66	0.68	0.67
Bias	0.49	0.55	-0.11	-1.47
Precision	1.49	2.44	2.56	3.58
Jacobson's Bias	-2.83	-4.95	-3.18	-0.85
Jacobson's Precision	2.99	4.95	3.66	2.58

**p-value<0.01

For predicting optimal Tac dose in days≤10, the pre-transplant abundance of *Phocaeicola* was the most significant variable with p<0.01. For predicting optimal Tac dose in

days≥11, the change in the relative abundance of *Bacteroides* was the most significant variable with $p<0.01$. The bias and precision of our model were significantly less than those of Jacobson et al., with $p<0.05$. for all time frames in one-sided Wilcoxon signed-rank test. The graphs below show how our model improves the predictive power of Jacobson's model by having less prediction errors. As evident in figure 5 to 8, blue dots (predicted values) are much closer to the red diamonds (observed values) compared to the hallow green dots (Jacobson's predictions).

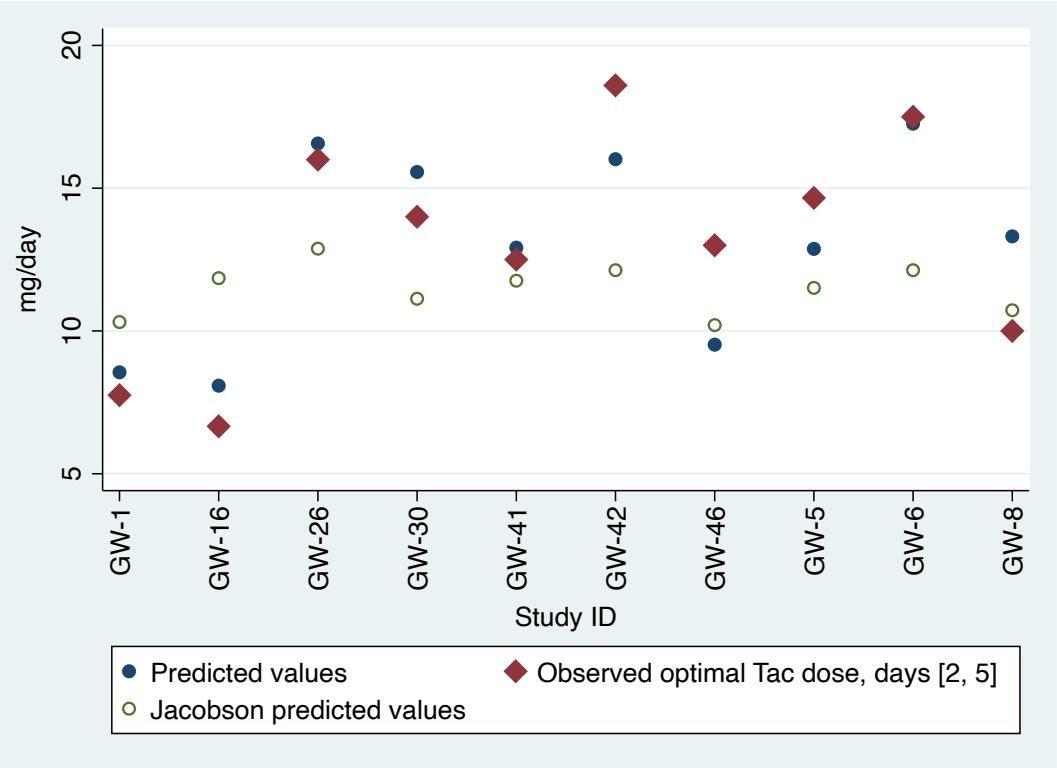

Figure 1-a: Predicted values vs. observed values for days 2-5

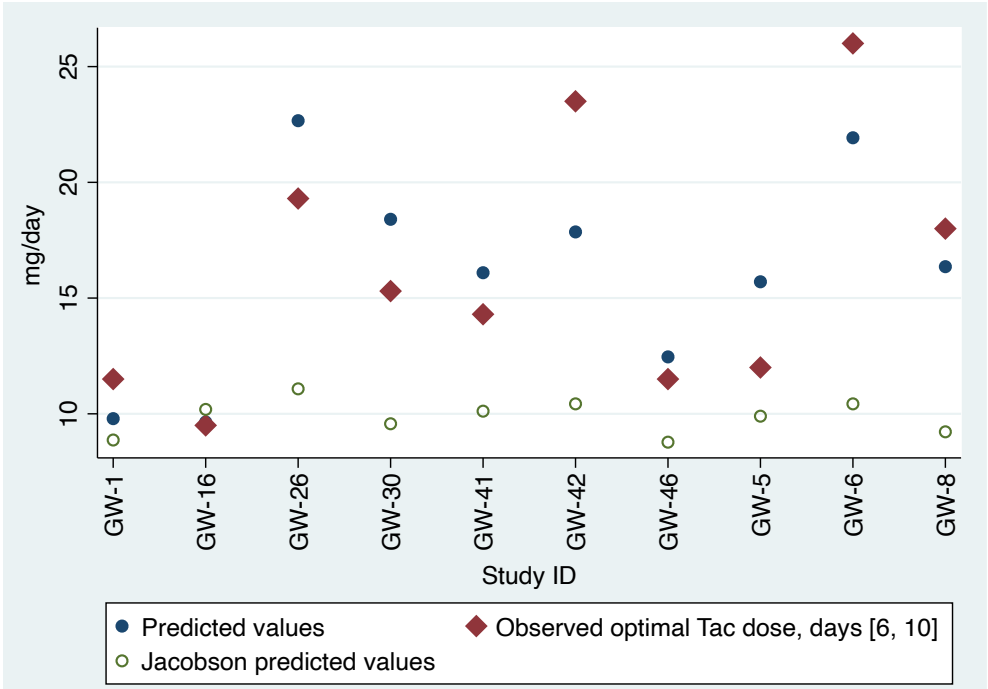

Figure 1-b: Predicted values vs. observed values for days 6-10

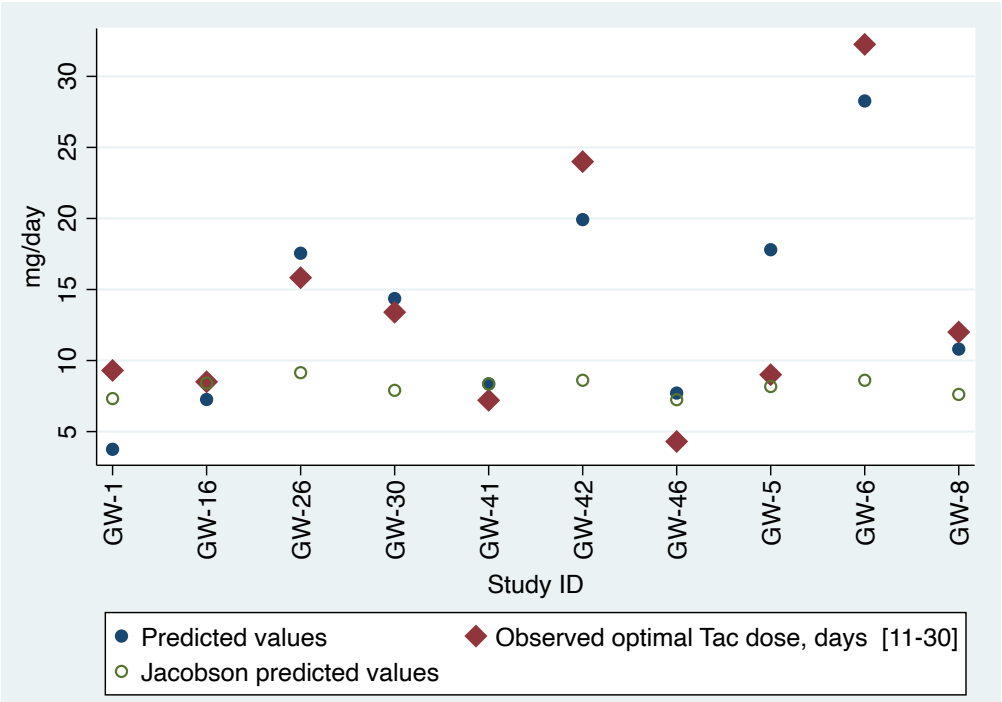

Figure 1-c: Predicted values vs. observed values for days 11-30

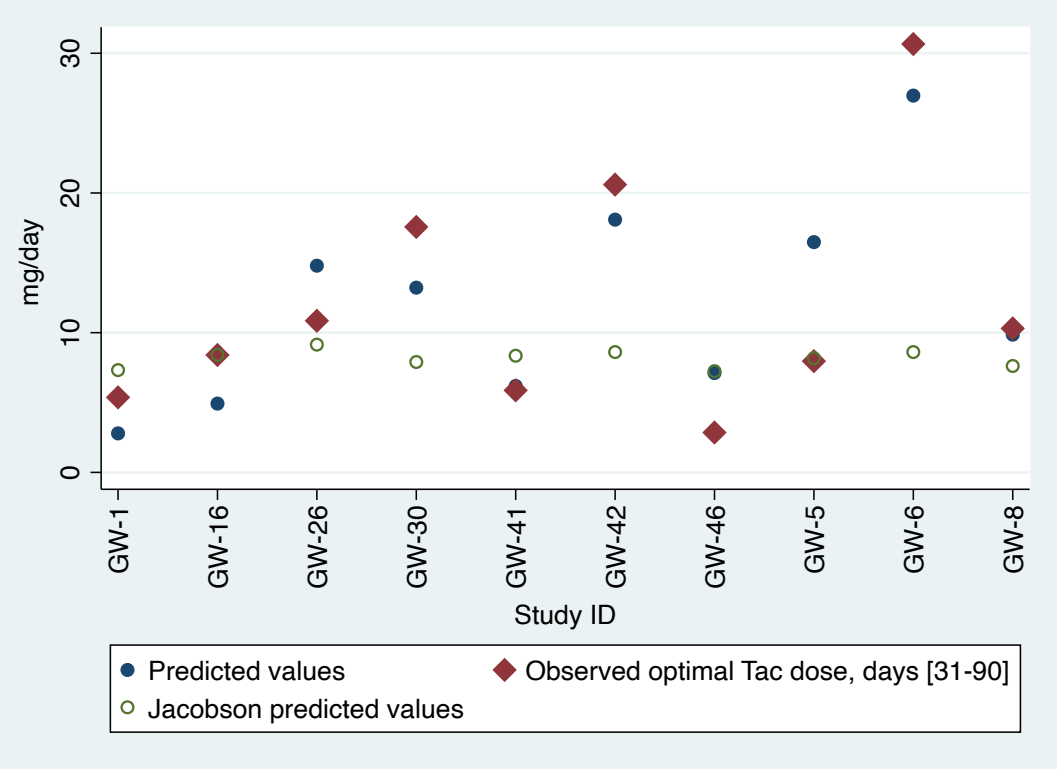

Figure 1-d: Predicted values vs. observed values for days 31-90

Applying the results of our models on the four time frames, we propose the following personalized optimal dose selection model for Tacrolimus.

Equation (4)

TDD (mg/day) =

$TDD_{Jacobson}$ × [(1.11, if 2≤days≤5) or (1.76, if 6≤days≤10) or (3.32, if 11≤days≤30) or (2.07, if 31≤days≤90)
- (74.63 × pre-tx relative abundance of *Phocaeicola*, if 2≤days≤6)
- (105.39 × pre-tx relative abundance of *Phocaeicola*, if 6≤days≤10)
+ (46.17 × change in relative abundance of *Bacteroides*, if 11≤days≤30)
+ (49.08 × change in relative abundance of *Bacteroides*, if 31≤days≤90)
+ [(4.74, if 2≤days≤6) or (5.02, if 6≤days≤10)]
- [(16.06, if 11≤days≤30) or (7.57, if 31≤days≤90)]

TDD = total daily dose (mg/day)

$TDD_{Jacobson}$ = predicted total daily dose by Jacobson model defined by equation 2.

goal of 10 ng/ml and a genotype of CYP3A5*1/*1 in a CCB and steroid using center, and a pre-transplant relative abundance of %6 *Phocaeicola*. To calculate the predicted optimal Tac dose, we first find $TDD_{Jacobson}$ for this case, which is 17.5 mg. Afterwards we follow the streps of equation 4.

TDD = 17.5 × (1.11, if 2≤days≤5) – (74.63×0.06, *Phocaeicola* if 2≤days≤5) + (4.74, if 2≤days≤5) = 19.7 mg/day. This example shows that Jacobson's model is underdosing the patient, and it needs to be increased by 2.2 mg/day.

2.4 Discussion

Using the above example, our model does not prescribe a definite 19.7 mg/per Tac dose for the given patient on day 3, it rather presents a guideline to the physician to aim for 19.7 mg and plan dose adjustments towards this value. Our model has shown to be more accurate than Jacobson in predicting optimal Tac doses by having a significantly smaller bias and precision error. It is, however, critical to note that the use of bacterial abundance as a predictor does not imply a causal relationship between Tac metabolization and bacterial genera. Based on our analysis, *Phocaeicola* and *Bacteroides* are significantly associated with optimal Tac dose levels, *Phocaeicola* for the first 10 days and *Bacteroides* for days 11-90. This association is in line with previous research on the effects of *Phocaeicola* and *Bacteroides* on health outcomes. Various species of the genera *Phocaeicola* and *Bacteroides* are among the most abundant species in the human gut, about 30% of the human gut microbiota, and they play a critical role in the balance of the colonic ecosystem [20]. They are known to regulate the degradation of complex heteropolysaccharides to small chain fatty acids and the synthesis of vitamins and bioactive compounds [19]. Bacteroides particularly has been subject to significant attention, and it has been considered as the next generation probiotics candidate due to its involvement in host health [20]. *Bacteroides* usually dominates the human gut microbiome by 20-50% of the total genera [20]. Lee et al. have previously studied the role of *Bacteroides* in kidney transplant outcomes. Lower abundance of *Bacteroides* post-

transplant was associated with diarrhea and acute rejection [21]. This is particularly of interest since our results indicated a positive correlation between the increase in the abundance of *Bacteroides* post-transplant and higher optimal Tac dose in the days 11-90 post-transplant which might potentially have decreased the risk of rejection. A skin transplant study on mice showed that fecal transplant of high-dose Tac-treated mice which contains high abundance of *Bacteroides* into low-dose Tac-treated mice will increase the allograft survival rate [22].

In an in-vitro study by Guo et al., they reached the same conclusion as Lee et al. regarding *F. prausnizii* which produces less potent metabolites, thereby requiring higher doses of Tac. But they also found that Bacteroidales, an order of bacteria which includes Bacteroides genus, produce inactive metabolites as well, which correlates with demanding higher Tac dose for efficacy [23]. The aforementioned studies support our choice of using *Bacteroides* as a predictor for optimal Tac dose.

A point of weakness of our study is that it has been limited to a small sample size (n=10) while we plan to increase the size to 50 within 12 months. It also requires validation by a cohort of new patients in order to test the model.

Funding disclosure: the cost of sample collection, sequencing and staff were jointly covered by CareDx and Virginia BioAnalytics LLC.

Appendix A: list of targeted genes

CYP3A5
CYP3A4
CYP3A7
CYP2C19
FMO3
C6
ABCB1
HSD11B1
NR1I2
IL10
IL12A
LEP
POR
HUS1
UGT1A9
HPRT1
UGT1A9
SLCO1B1
NFATC1
ABCC2

Appendix B: Demographic Information

Characteristics	***Measure at time of transplant (N=17)***
Age (Mean±SD)	58.9±11.01
BMI (Mean±SD)	33.35±3.78
Male (n, %)	6 (60%)
African American (n, %)	6 (60%)
Live donor (n, %)	6 (60%)
Previous Transplant (n, %)	2 (20%)
Dialysis (n, %)	8 (80%)
Cold Ischemic time hours (Mean/Median)	9.24/1.91
CPRA (Mean±SD)	45.36±43.14
History of hypertension (n, %)	10 (100%)
Diabetes (n, %)	4 (40%)
Hepatitis C (n, %)	2 (20%)
High risk CMV (n, %)	5 (50%)
High risk EBV (n, %)	0 (0%)
Creatinine (Mean±SD)	6.45±4.69

Appendix C: Correlation Between Optimal Tac Dose and Relative Abundance of Bacterial Genera

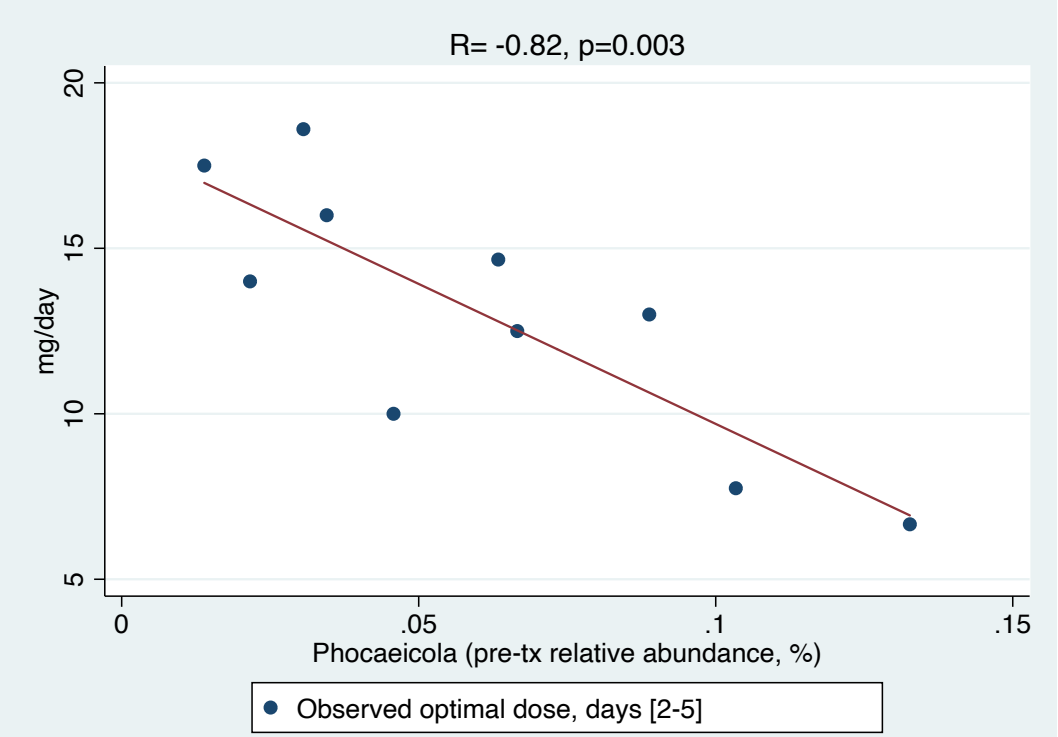

Figure c-1: Association between Phocaeicola and observed optimal Tac dose in days 2-5

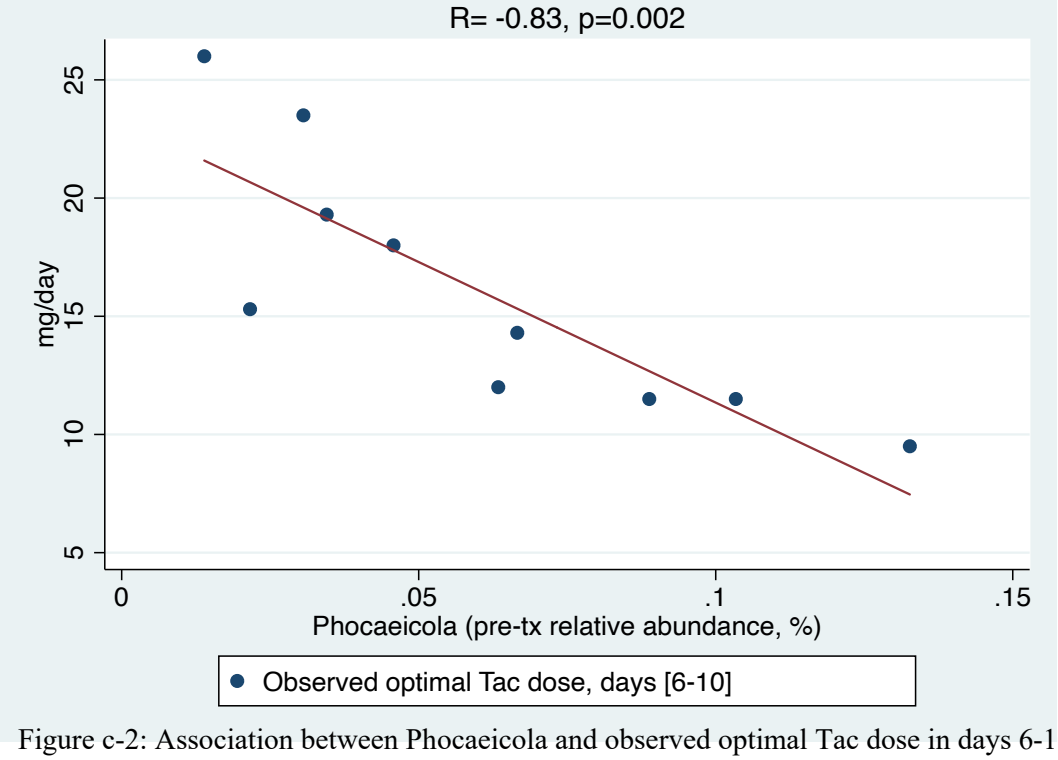

Figure c-2: Association between Phocaeicola and observed optimal Tac dose in days 6-10

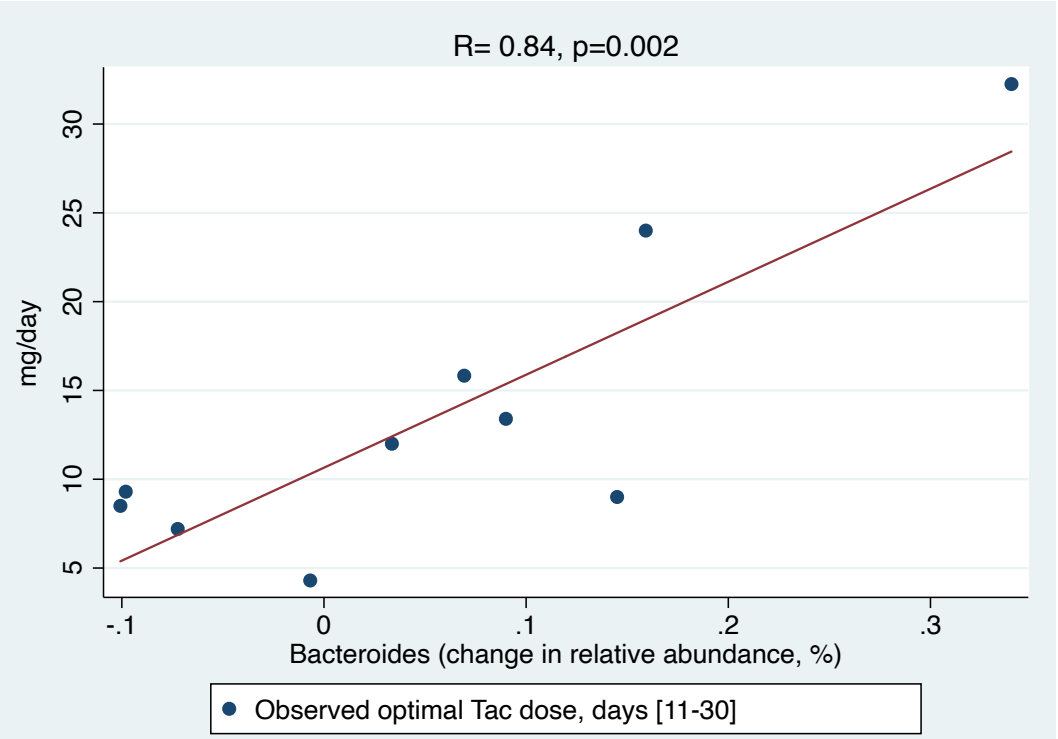

Figure c-3: Association between Bacteroides and observed optimal Tac dose in days 11-30

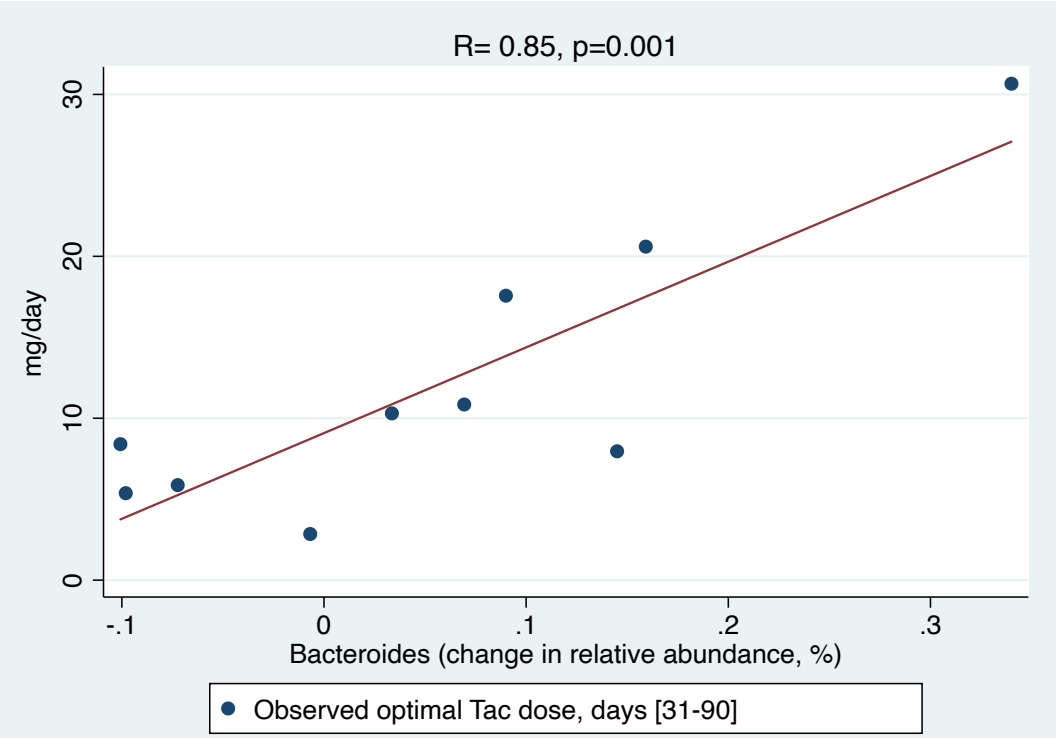

Figure c-4: Association between Bacteroides and observed optimal Tac dose in days 31-90

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PAPER #3: The Role of Gut Microbiome in Personalized Probiotic Regimens and Prediction of Outcomes for Kidney Transplant Recipients, controlling for CYP3A5

Abstract

The gut microbiome and its impact on human health has been the subject of interest among researchers. Diversity and composition of gut microbiome significantly changes after kidney transplantation. Prior studies show that gut microbiome dysbiosis has significant effects on transplant outcomes such as spontaneous tolerance, acute rejection, serum creatinine and blood urea nitrogen, infections and diarrhea. In this paper, we investigate which genera of bacteria exhibit the significant changes in relative abundance after kidney transplantation among high expressers of CYP3A5 gene, and controlling for age, race and gender by recruiting 10 kidney transplant patients. Association between gut microbiome dysbiosis and Tacrolimus trough level fluctuation and AlloSure Donor Derived Cell-free DNA were analyzed. Shannon diversity decreased after the transplant operation for all subjects. The average relative abundance of *Alistipes* decreased by 4% with p=0.01 across all subjects and the average relative abundance of *Bacteroides* increased by 8% with p=0.03 for African Americans. Every 0.1% increase in relative abundance of *Faecalibacterium* in African Americans after transplant surgery was associated with 20%

increase in Tac CV till 60 days post-transplant (p=0.01). After transplant surgery, every 1% increase in the relative abundance of *Lachnospiraceae* was associated with 1.08 units drop in AlloSure dd-cfDNA levels controlling for patient age (p=0.05).

3.1 Introduction

3.1.1 The Gut Microbiome

There are more than 100 trillion microbial cells (mostly bacteria) in the human gut, which significantly influence the host immune system, metabolism and overall health [17]. To compare the scale of microbiome collective genomes to human genome, the former encodes more than three million genes, actively producing thousands of metabolites, while the latter consists of 23000 genes [25]. Here we will use the term *gut microbiome* as the collective genomes of the bacteria in human gut, and *gut microbiota* as the community of bacteria itself although they are usually used interchangeably in the literature. Studies have shown that even the smallest change in the diversity and composition of the humane gut microbiota could affect the host health [18]. This is due to the involvement of gut microbiota in the fermentation of non-digestible substrates that regulate the production of short chain fatty acids such as acetate, propionate, and butyrate to modulate biological responses of host gastrointestinal health [25, 26]. When the gut

microbiota is in *symbiosis*, it can provide the host with various metabolic capabilities that promotes overall health. It is established that *dysbiosis* in the gut microbiota is associated with the pathogenesis of intestinal and extra-intestinal disorders [17]. A species-rich gut ecosystem is more likely to be robust against environmental interventions such as surgery and medication [25]. Diversity is particularly important because, in the absence of certain species due to intervention, other functionally related species can compensate for the function of absent species [25]. In this paper we focus on bacterial communities. It is known that the factors influencing gut microbiome variety among humans are age, sex, ethnicity, genetics, and environmental factors such as diet, geography, and medication [17].

3.1.2 Gut Microbiota in Kidney Transplant

Both human and animal studies show that kidney transplantation leads to microbiome dysbiosis in the gut [27]. This is mainly caused by the administration of immunosuppressive and antibiotic drugs [12, 13]. Microbiome dysbiosis is associated with several post-transplant complications, such as increased risk of infections, diarrhea, interstitial fibrosis, reduced tolerance, modification of immunosuppressant levels in blood and graft failure [12, 13, 27]. Examples of bacterial taxa that have proven association with kidney transplant outcomes are as follows, *Janthinobacterium*, *Clostridia*, *Bacilli*, *Lactobacillales* are associated with spontaneous tolerance; lower abundance of

Bacteroidetes at the phylum level is associated with acute rejection; numerous genera in *Lachnospiraceae* are negatively associated with serum creatinine and blood urea nitrogen; *Lactobacillus plantarum* is associated with reduced risk of clostridium difficile infection, and reduction in genera *Ruminococcus*, *Dorea*, and *Coprococcus* are associated with incidents of diarrhea [14, 27]. Lower gut bacterial diversity has been associated with reduced immune functioning and metabolic syndrome [18]. No direct causal relationship has been proven in any study regarding microbiome dysbiosis and transplant outcomes. What makes this relationship difficult to study is the complex and bidirectional dynamics between gut microbiome, immunosuppressive medication, and antibiotics, i.e., initial immunosuppressive and antibiotic administration causes microbiome dysbiosis, and in turn, microbiome dysbiosis affects the immune system via a variety of pathways and manipulates dose adjustments of immunosuppressive medication and antibiotic efficacy [13]. The same relationship exists for the gut microbiota composition and graft rejection or infections, i.e., microbial dysbiosis could facilitate rejection or infections, which in turn, rejection or infection cause more microbial dysbiosis. Salvadori et al. have summarized all the relevant findings of the role of microbiota in kidney transplant [27]. In this pilot study, we will look at the role of gut microbiota in prediction of post kidney transplant outcomes and measures to personalize nutritional treatments. We use time in Tacrolimus blood level fluctuation and AlloSure Donor Derived Cell-free DNA (dd-cfDNA) as proxies for transplant outcome. Prier studies have established that Tacrolimus

blood level fluctuation is strongly associated with graft rejection and failure, and high levels of dd-cfDNA is also associated with risk of active rejection [28, 38]. Therefore, these measures will be used as proxies for graft rejection or failure.

3.1.3 Tacrolimus Blood Level Fluctuation and Time in Therapeutic Range

Tacrolimus (Tac) is the most widely used post-transplant maintenance immunosuppression regimen in the United States [1]. Tac has a narrow therapeutic window, i.e., low exposure leads to graft rejection and high exposure leads to toxicity [2]. Tac trough levels are routinely monitored to be in the therapeutic range by adjusting Tac dose. Therapeutic levels are usually defined as 8-12 ng/ml for the first 3 months and 6-10 ng/ml for months 3-6 post-transplant, although each transplant center follows its own unique protocol [3]. Reaching the therapeutic level by dose adjustment is particularly difficult due to the wide inter-individual variability of Tac. This variability is presumably caused by the expression of cytochrome gene P4503A5 (CYP3A5) which affects Tac's pharmacokinetics [3]. It is known that the expression of CYP3A5*1 allele is associated with high metabolization of Tac, hence higher dose of Tac is needed to reach therapeutic levels [4]. Prior studies show that African Americans (AA) are generally high expressors of CYP3A5 and thus need higher dose of Tac to reach therapeutic levels compared to European Americans [2]. Prior studies also show that Tac blood level fluctuation is strongly associated with acute rejection and poor kidney graft function [28]. Park et al.

show that Coefficient Variability (CV) of Tac trough levels above 33.7% in the 1st year post-transplant is significantly associated with allograft loss [29]. Coefficient Variability (CV) is a statistical measure for calculating fluctuation which defined as the ratio of the standard deviation to the mean. The higher the CV, the greater the level of fluctuation, expressed as a percentage.

Rozen-Zvi et al. additionally found that the combination of high Tac trough level CV and exposure to low Tac levels (<5 ng/mL) is a significant predictor of high-risk patients in early post-transplant period [30]. Other studies show that intra-patient Tac variability is higher among African-American kidney patients, and it is a significant risk factor for deleterious outcomes, i.e., 10% increase in Tac coefficient of variability (CV) increases the risk of acute rejection by 20% and risk of graft loss by 30%. A high Tac CV of >40% is a significant predictor for disparities in African American patients compared to white patients [31]. Additionally, Davis et al. found that high Tac CV (>44.2%) combined with low Time in Therapeutic Range (TTR<40%) during the first year of post-transplant, significantly increased the risk of graft loss [9].

In this paper, we hypothesize that the change in abundance of certain gut microbiome bacterial genera are associated with high Tac fluctuation and low TTR, which proposes the idea that the abundance or the lack of those genera could be associated with graft rejection. We know that post-transplant dysbiosis of the gut microbiome can promote an increase in the abundance of certain bacterial communities that can metabolize

immunosuppressants into less potent metabolites [14]. For example, Lee et al. discovered a significant association between *Faecalibacterium prausnitzii* which is one of the most abundant bacterial species in the gut microbiome and higher levels of Tac dose in the first month post-transplant [15]. Patients with higher abundance of this species in the first week of transplantation demanded higher dose of Tac at 1 month [15]. Lee et al. believe that Tac absorption and/or metabolism may be affected by the colonic mucosa and we know that a healthy colonic mucosa requires butyrate from bacterial sources such as *Faecalibacterium prausnitzii*. Another study reached the conclusion that *Faecalibacterium prausnitzii* metabolizes Tac into a less potent novel metabolite M1 (9-hydroxy-tacrolimus) which is 15-fold less potent than Tac in inhibiting the proliferation of activated T cells [16]. We will show new associates between the gut microbiome and Tac blood level fluctuations and TTR.

3.1.4 AlloSure Donor Derived Cell-free DNA

The AlloSure test (manufacture name) is a clinical-grade, targeted, next generation sequencing assay that measures single-nucleotide polymorphisms to accurately quantify donor-derived cell-free DNA (dd-cfDNA) in renal transplant recipients without separate genotyping of either the donor or the recipient. The AlloSure test is intended to assess the probability of allograft rejection in kidney transplant recipients with clinical suspicion of rejection. The AlloSure result is the percent of donor-derived cell-free DNA in the total

cell-free DNA present in kidney transplant recipients. dd-cfDNA level greater than 1% indicate a probability of active rejection (antibody-mediated rejection or T cell-mediated rejection) [38]. dd-cfDNA levels 1% and below reflect absence of active rejection. In this study we will examine the association between the change in abundance of gut microbiome bacterial communities and dd-cfDNA levels. The AlloSure test is a novel predictive test that has been used in a few transplant center including the George Washington University Hospital and it has proven to be significantly accurate in predicting graft rejection. Since the AlloSure test data for all of our subjects were readily available we planned to execute the first ever association study between dd-cfDNA and gut microbiome.

3.2 Methods

3.2.1 Population

TWELVE kidney transplant patients from The George Washington University Hospital were consented and enrolled in this study. IRB was approved by the GWUH Office of Human Research (#NCR191914) and George Mason University IRB #1906208-1. Summary of clinical and demographic information is presented in Table 1. Exclusion criteria were pediatric, pregnant, and simultaneous kidney-pancreas recipients.

All patients underwent induction therapy based on test results such as Panel of Reactive Antibodies (PRA) and Donor Specific Antibodies (DSA). DSA is a measure to predict antibody-mediated rejection. PRA is a measure to determine the potential level of sensitization of a given patient towards the pool of kidney donors, as a result of prior exposure to external HLA antigens during blood transfusions, pregnancies, or previous organ transplantations. If a patient possesses a high PRA, he/she will undergo a desensitization process to reduce the risk of graft rejection. Patients who were highly sensitized received Velcade (Bortezomib) preoperatively. All patients received two doses of Thymoglobulin, while sensitized patients received three doses. Following Thymoglobulin induction, all patients received Simulect. Induction therapies are pre-medicated with methylprednisolone, acetaminophen, and diphenhydramine. Induction therapy is the process of administering immunosuppressive therapy at the time of kidney transplantation to reduce the risk of allograft rejection. After the transplant surgery, all patients started with 4 mg of Tac. On a case-by-case basis, some patients were subject to antibiotics (Atovaquone) and stimulant laxatives.

3.2.2 Sample Collection and Sequencing

For each patient, a series of sample collection activities took place. Three mL of blood was drawn for gene sequencing at the time of routine clinic visit. Blood was stored in ZYMO DNA/RNA Shield Blood Collection tubes which ensure sample stability during

storage/transport at ambient temperatures without the need for refrigeration or specialized equipment. The nucleic acids (DNA & RNA) in samples are preserved at ambient temperature (DNA >1 year, RNA up to 1 month). Targeted gene sequencing was performed by Illumina Next Generation sequencer by *The Sequencing Center* lab in Fort Collins, CO. The list of targeted genes is available in appendix A. The existence of single nucleotide polymorphisms CYP3A5*3(rs776746, g.6986A>G), CYP3A5*6 (rs10264272, g. 14690 G>A) and CYP3A5*7 (rs41303343, g.27131-27132insT) was analyzed. Subjects who did not express CYP3A5*3, *6 or *7 alleles were categorized as CYP3A5*1/*1 genotype and those who expressed one CYP3A5*3, *6 or *7 allele were categorized as CYP3A5*1/*3, *1/*6 or *1/*7 genotype, respectively.

All patients in this study possessed the CYP3A5*1/*1 allele which made them high expressors of CYP3A5. This controls for the effect of CYP3A5 on Tac metabolism which has been absent in all previous gut microbiome studies.

Two stool samples were collected per patient for gut microbiome sequencing. The first stool sample was collected 1 week before transplant and the second stool sample was collected 4-8 weeks post-transplant. Collection was performed by subjects by using a paper feces catcher and a coring brush which eliminated the risk of contamination. Stool samples were stored inside ZYMO DNA/RNA Shield Fecal Collection Tubes–DX. These tubes preserve the bacterial communities in ambient temperature (DNA>2 years, RNA>1 month). Whole DNA of bacteria was sequenced using shotgun metagenomics to the

strain level by *CosmosID* lab in Germantown, MD. For each patient only the bacterial taxa with relative abundance more than 1% were included in the analysis, which is the common practice in gut microbiome [15]. Shannon diversity index for both pre- and post-transplant stool sample was calculated, and the difference was analyzed. Shannon diversity index is a popular metric in biology for measuring the number of species living in a sample (richness) and their relative abundance (evenness). For each subject Jaccard beta diversity index was calculated to compare the pre- and post-transplant composition of the gut microbiome. This index, which is presented as a percentage, reflects the level of dissimilarity between the two stool samples.

3.2.3 Clinical data

Clinical data was collected from electronic health records in Cerner and deidentified per protocol. Among which, Tac dose and Tac trough levels were the most critical entries. All Tac dose entries (total daily dose in mg) were collected continuously from the day of transplant surgery till day 90 post-transplant. *Envarsus* was the dominant immunosuppressant drug in this transplant center. Tac doses were routinely adjusted by transplant physicians to reach therapeutic levels, 10 ng/ml for *Envarsus* users and 200 ng/ml for *Cyclosporin* users. Tac trough levels were measured by the George Washington University Hospital Lab. For each patient, the Coefficient of Variability (CV) of Tac trough levels was calculated for the first 30 days and 90 days post-transplant. CV is

measured as (SD/mean)×100%. Measure of AlloSure Donor Derived Cell-free DNA was provided by CareDx as a routine test in the George Washington University Hospital at 1, 2, and 3 months post-transplant. For patients who had multiple AlloSure measures in each month, the median was used.

3.2.4 Statistical Analysis

Change in alpha diversity (Shannon index) was analyzed for all samples. Shannon diversity index is calculated as below:

Shannon Diversity = $\sum[(Pi) * Ln(Pi)]$

Where Pi= proportion of total sample represented by species i, divided by the number of individuals of species i by total number of species.

The analysis was performed separately for African Americans and non-African Americans in order to explore the effect of race on diversity. T-tests on pre- and post-transplant paired samples were performed to compare the change in alpha diversity using the significance level defined at p=0.05. In order to see which bacterial genera experienced significant change in their abundance, we analyzed the difference between pre- and post-transplant relative abundance of all genera by t-tests for paired samples using the significance level defined at p=0.05. Species level analysis was not possible due to too many unknown species. This phenomenon occurs at the time of sequencing. The existence of species can be detected, but mano of them will not be necessarily assigned to

a certain known species. All analysis were also performed on AA and non-AA subjects separately. After selecting the bacterial genera which experienced the most significant level of change, we used a random effects regression model to control for interpatient variability, along with race, age and gender. Since for each subject we have two values for gut microbiome relative abundance, i.e., pre- and post-transplant, we can set our data in a panel format. The Random Effects regression model is used to estimate the effect of individual-level factors that vary between subjects. In our case, the time of producing and collecting samples and different diets contribute to the random effect.

The model is set as below:

$Y_{it} = \alpha + \beta X_{it} + u_i + \varepsilon_{it}$

Where i= 1 to 12 (number of subjects); t= 1 (for pre-transplant) and 2 (for post-transplant); Y_{it} is the dependent variable representing the relative abundance of Alistipes in subject i for period t; ε_{it} is the residual as a whole where the residual is a combination of cross section and time series; u_i is the individual residual which is the random characteristic of unit observation the i-th and remains at all times; α is constant; β is the coefficient for each independent variable; X is the set of independent variables defined as below:

1/ Post-Transplant: a dummy variable which equals to 1 when the observation is for post-transplant.

2/ AA: a dummy variable which equals to 1 when the subject is African-American.

3/ Age: a continuous real number representing subjects' age.

4/ Male: a dummy variable which equals to 1 when the subject is male.

In the next step, Tac CV of the first 30 and 90 days post-transplant was plotted against the change in relative abundance of all genera to detect any possible association. Pearson correlation coefficient was calculated and those with $p<0.05$ were selected. Same analysis was performed on dd-cfDNA to detect any possible association between dd-cfDNA and change in abundance of genera. After selection of the relevant genera, Ordinary Least Square model was used to control for the effects of age and gender. Since all subjects were high expressers of CYP3A4 we did not include a control variable for that.

3.3 Results

3.3.1 Microbiome Diversity

Figure 1-a and 1-b show the gut microbiome composition of all subjects in genus level, categorized by pre- and post-transplant. Genus with abundance less than 0.5% were excluded (studies usually exclude at less than 1%, but for extra level of investigation we excluded at 0.5%). Figure 1-c shows the aggregate microbiome composition by pre- and post-transplant cohorts.

Among all patients (n=12), Shannon diversity decreased after the transplant surgery, but the change was not statistically significant with significance defined at p=0.05 (figure 1-d). However, Shannon diversity was reduced 0.7 units for non-AA subjects after transplantation (n=4 paired observations). This change was significant with p=0.001 in a T-test for paired samples. No significant change was observed among African Americans with n=8 paired observations (figure 1-e). The average Beta diversity among all subjects was 54.36% (SD=17.4%, min=29, max=88) which means on average the post-transplant gut microbiome composition was 54.36% different from the pre-transplant gut microbiome composition. There was no significant difference among AAs and non-AAs.

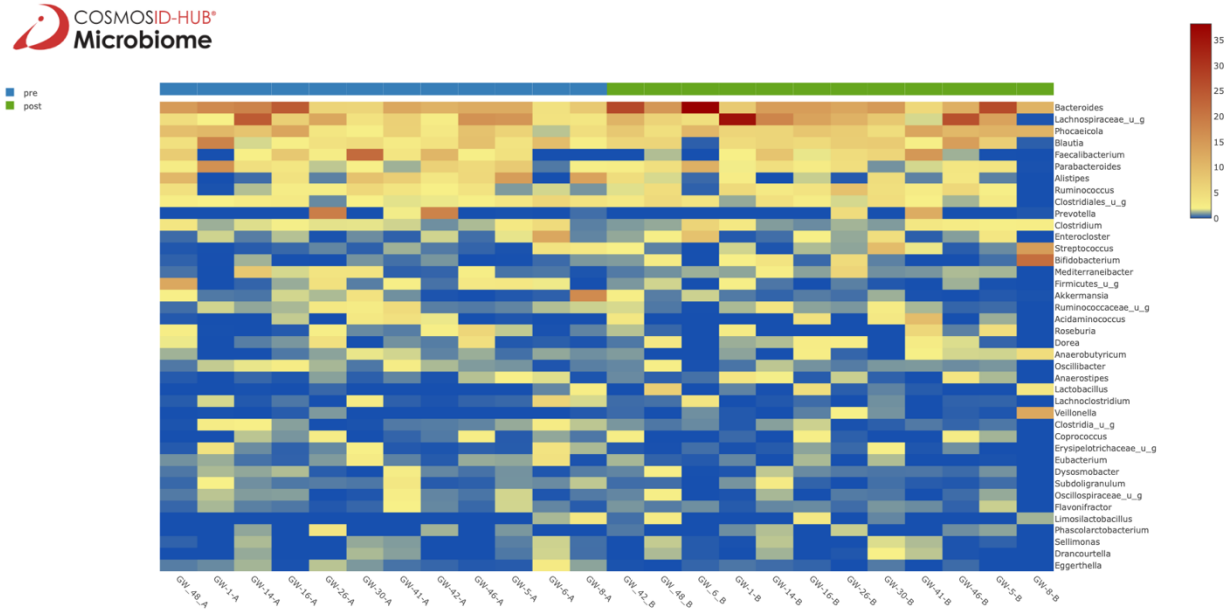

Figure 2-a: Gut Microbiome Composition for pre- and post- kidney transplant

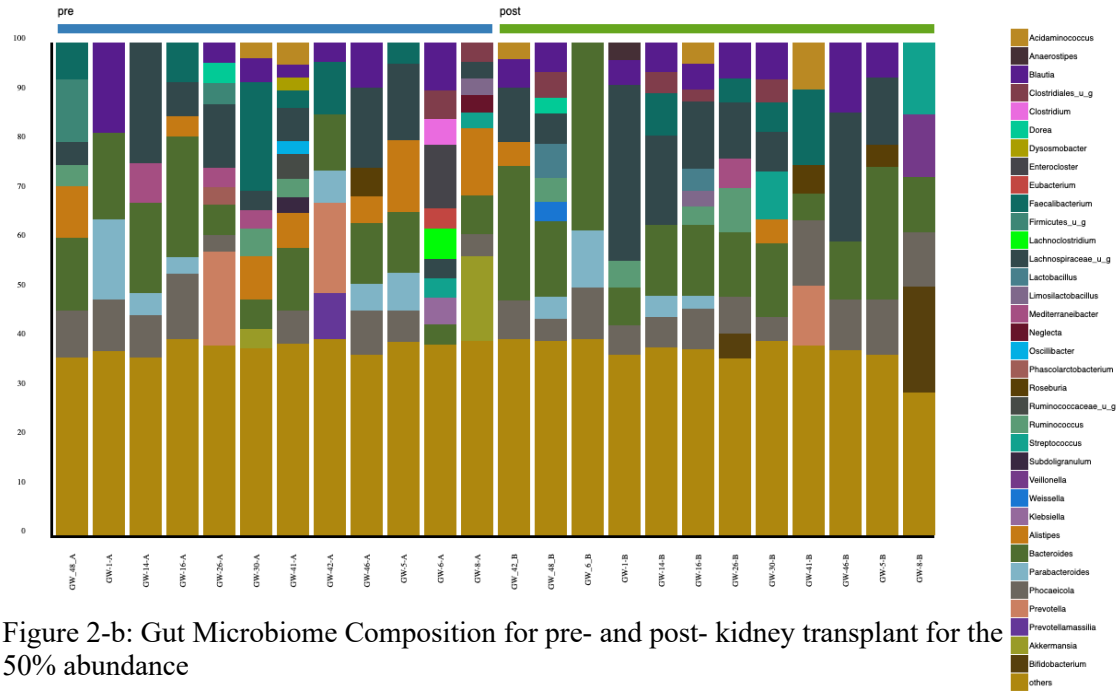

Figure 2-b: Gut Microbiome Composition for pre- and post- kidney transplant for the 50% abundance

Fig. 2-c

Figure 2-c: Gut Microbiome Composition for pre- and post- kidney transplant aggregate cohorts

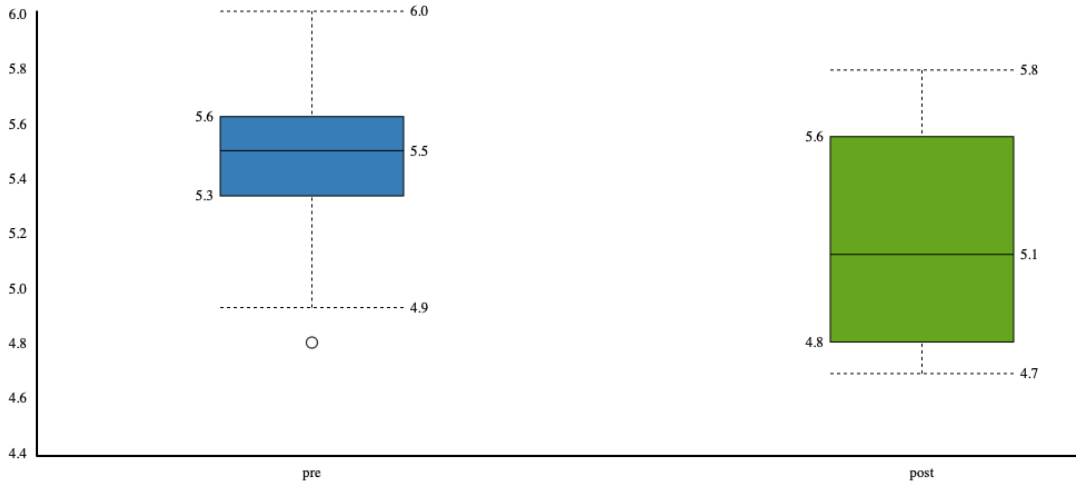

Figure 3: Shannon Diversity for pre- and post-transplant samples

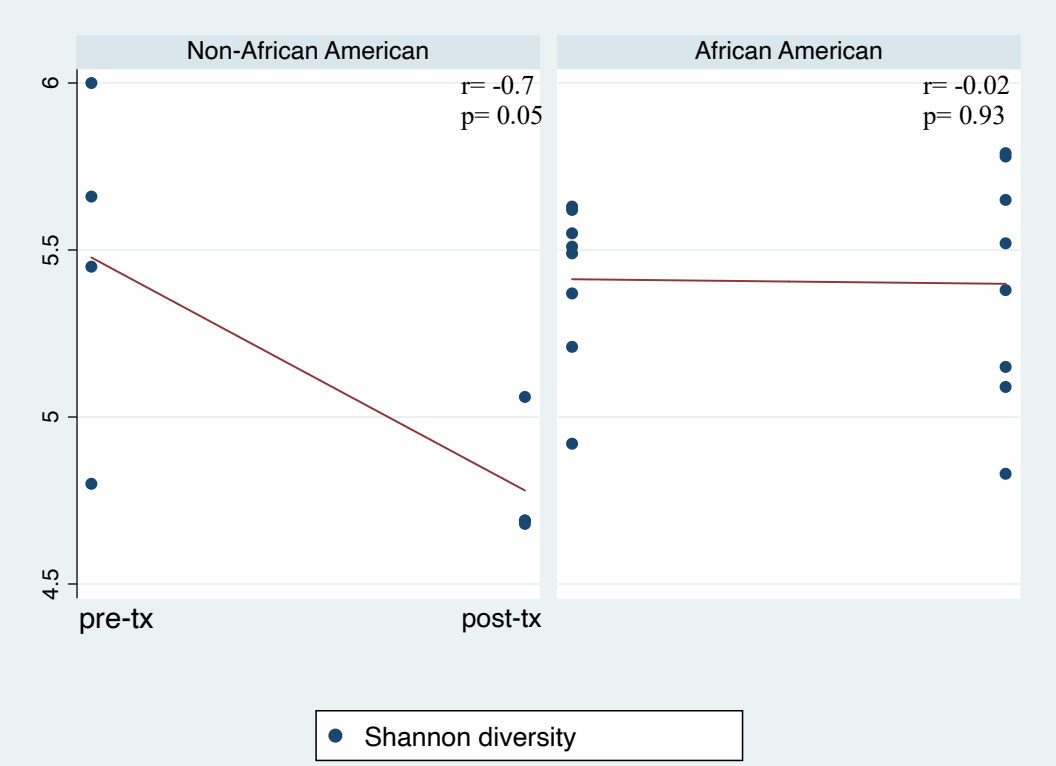

Figure 4: Change in Shannon Diversity for all patients and AA patients

3.3.2 Altered Bacterial Genera

Alistipes and *Eggerthella* were significantly altered among all subjects. T-test for paired samples showed that after transplant operation, relative abundance of *Alistipes* decreased

on average 4% with p=0.01, and relative abundance of *Eggerthella* decreased on average 0.6% with p<0.01. figure 2-a shows the change in relative abundance of *Alistipes* and *Eggerthella* with correlation coefficient of r=-0.46 and p=0.02 for *Alistipes*. The random-effect regression model controlling for age, race and gender confirmed the significant drop in *Alistipes* (coef=-0.04, p<0.005), represented in table 2. We forgo the analysis of *Eggerthella* since the change in relative abundance of this genus was less than 1% and not clinically relevant.

Among African American subjects, *Bacteroides*, *Clostridium*, and *Flavonifractor* were significantly altered after transplant surgery. Figure 2-b shows the change in relative abundance of *Bacteroides* with r=0.46 and p=0.02. After transplant operation, relative abundance of *Bacteroides* increased on average 8% for AAs. T-test for paired samples showed that this change was significant with p=0.03. We forgo the analysis of *Clostridium* and *Flavonifractor* since the change in their relative abundance was less than 1% and not clinically relevant. The random-effect regression model controlling for age and gender confirmed the significant change in *Bacteroides* (coef=0.08, p<0.04) for AAs, represented in table 2.

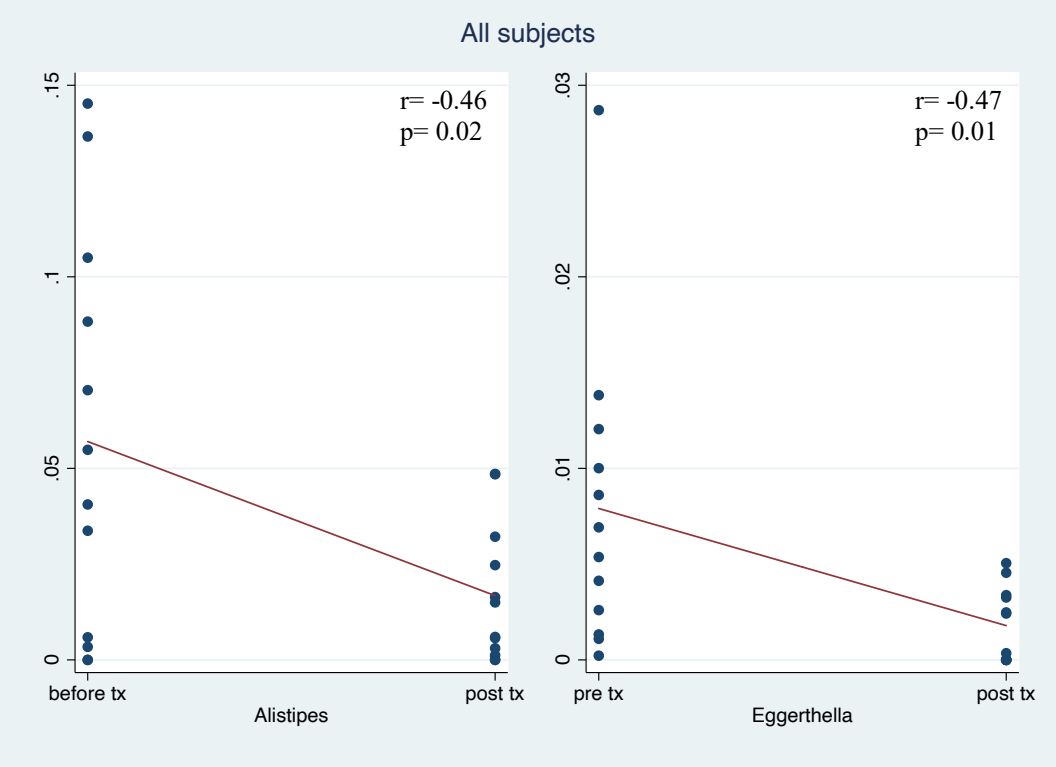

Figure 5: Change in the relative abundance of Alistipes and Eggerthella in all subjects

Table 4: Random-Effects Regression Results for Change in Relative Abundance of Bacterial Genera

	All Subjects (n=34, panel data)	African Americans (n=26, panel data)
	Dependent var	**Dependent var**
Independent var	*Relative Abundance of Alistipes*	*Relative Abundance of Bacteroides*
After Tx	-0.04**	0.08*
	(0.01)	(0.04)
AA	0.03	-
	(0.02)	
Age	0.002*	-0.0007
	(0.0009)	(0.002)
Male	-0.03*	-0.04
	(0.01)	(0.05)
Constant	-0.069	0.19
	(0.063)	(0.16)
Prob>chi2	0.004	0.19
Overall R^2	0.44	0.28

** p<0.01 *p<0.05

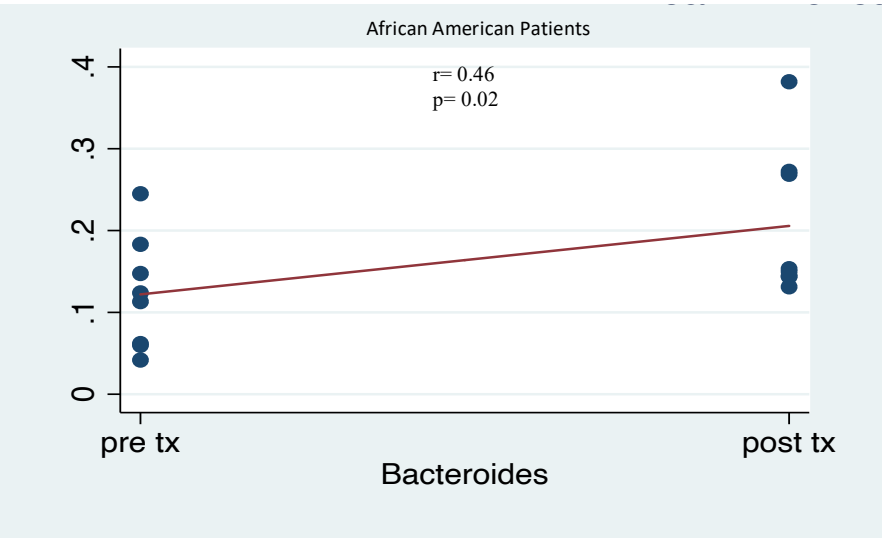

Figure 6: Change in relative abundance of Bacteroides among AA subjects

3.3.3 Tacrolimus Fluctuation

Among African Americans, the change in relative abundance of *Faecalibacterium* was associated with Tac CV within 30 and 60 days after transplant with Pearson correlation coefficient r=0.67 (p=0.06) and r=0.52 (p=0.18) respectively, shown in figure 3. After controlling for age and gender, OLS regression shows coef=222.23 (p=0.009) within 30 days and coef=214.77 (p=0.008) within 60 days post-transplant, i.e., every 0.1% increase in relative abundance of *Faecalibacterium* in AAs after transplant surgery is associated with ~20% increase in Tac CV till 60 days post-transplant. Table 3 reflects the results. TTR did not correlate with any bacterial genera or species.

Table 5: OLS Regression for Association Between Tac CV and Faecalibacterium

Independent Var:	**African American** (n=14) Dependent Var: ***Tac CV 30 days***	**African American** (n=14) Dependent Var: ***Tac CV 60 days***
ΔFaecalibacterium	222.25** (46.11)	214.77** (44.28)
Age	1.39* (0.41)	1.58** (0.40)
Male	6.52 (4.52)	12.71* (4.34)
Constant	-12.12 (21.56)	-30.55 (20.70)
Prob>F	0.03	0.02
Adj.R^2	0.75	0.78

** p<0.01 *p<0.05

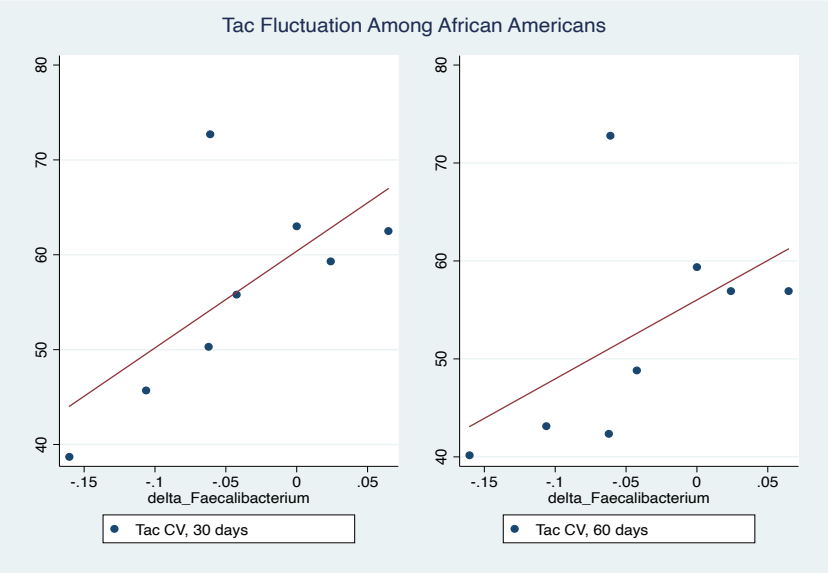

Figure 7: Association between change in abundance of Faecalibacterium and Tac CV

3.3.4 AlloSure Donor Derived Cell-free DNA

dd-cfDNA levels in 30 days, 60 days and 90 days were significantly correlated with the change in relative abundance of a genus belonging to the *Lachnospiraceae* family. Figure 4 shows the relationship with the associated Pearson correlation coefficients, r=-0.61 p=0.07 for dd-cfDNA levels in 30 days, r=-0.64 p=0.06 for 60 days, r=-0.77 p=0.01 for 90 days. OLS regression shows that after transplant surgery, every 1% increase in the relative abundance of a genus belonging to *Lachnospiraceae* is associated with 1.08 units drop in AlloSure dd-cfDNA levels (p=0.05) controlling for patient age.

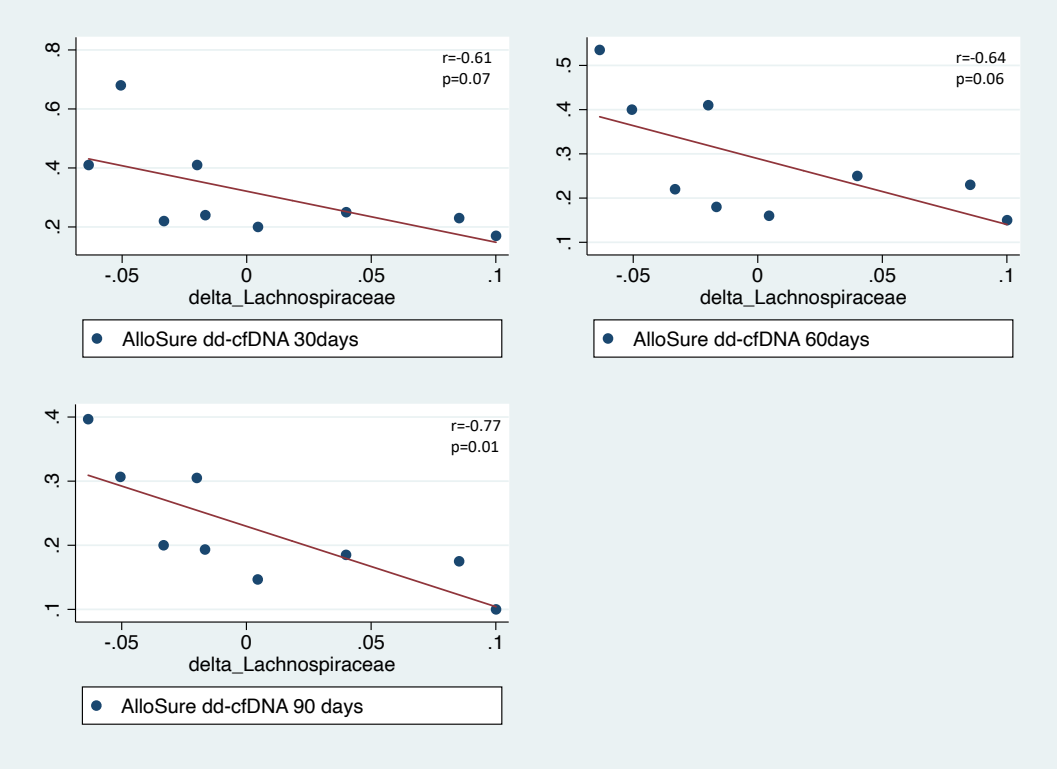

Figure 8: Association between Lachnospiraceae and dd-dfDNA

Table 6: Summary of Paper# 2 results

Measure	All Subjects	African Americans
Selected Bacterial genera with abundance>0.5%	76 genera, shown in appendix D	-
Shannon Diversity pre-Tx	5.5	5.4
Shannon diversity post-tx	5.1	5.38
Beta Diversity	54.36%	55.01%
CYP3A5	All high expressers	All high expressers
Altered Bacterial Genera	Alistipes (reduced by 4% controlling for race, age, gender with p=0.01)	Bacteroides (increased by 8% controlling for age, gender with p=0.04)
Tac CV	-	Every 0.1% increase in relative abundance of *Faecalibacterium* after tx is associated with ~20% increase in Tac CV till 60 days post-tx, controlling for age, gender (p<0.01)
ddcf-DNA	every 1% increase in the relative abundance of *Lachnospiraceae* is associated with 1.08 units drop in dd-cfDNA levels (p=0.05) controlling for patient age.	-

3.4 Discussion

Our analysis showed that alpha diversity decreased for all subjects, but more significantly for non-AA subjects. As mentioned earlier, change in diversity of gut microbiome has been associated to deleterious transplant outcomes. In order to remedy the change in diversity of gut microbiota, applying probiotic treatments to increase the abundance of *Alistipes* and *Eggerthella* may be able ameliorate this change of diversity for all patients after transplant operation. Using an intervention to restore the balance of bacterial communities in the gut has been practiced before, but in limited cases using probiotic medication or fecal transplant [31]. In the field of kidney disease, restoring the gut microbiome balance via probiotics have shown missed results, although no direct association to lower graft rejection or lower graft failure has been witnessed [31, 32]. *Alistipes* are anaerobic bacteria found mainly in the healthy human gastrointestinal tract microbiota and they have proven protective effects against diseases such as liver fibrosis, colitis, cancer immunotherapy, and cardiovascular disease [33]. In a mice skin transplant study, Mclntosh et al. found that the abundance of *Alistipes* was associated with prolonged graft survival. They concluded that *Alistipes* had a therapeutic role in skin transplant and administration of probiotics or fecal microbiome transplantation (FMT) are beneficial in transplant cases [34]. In a study by Hyunjeong et al. with 46 kidney transplant subjects, the role of gut microbiome in acute rejection was studied [35]. They realized at 3 months post-transplant, *Alistipes* was decreased in the acute rejection group

(p=0.0001, Wilcoxon rank-sum test). Linear discriminant analysis effect size (LEfSe) method showed that *Alistipes* was also significantly lower in the acute rejection group before kidney transplant. Our findings suggest the plausibility of administering probiotics or FMTs to increase the abundance of *Alistipes* in kidney transplant patients before and/or after transplant. It is known that the presence of Alistipes has been correlated with the promotion of healthy phenotypes, for example its protective roles in colitis, autism spectrum disorder, and various liver and cardiovascular fibrotic disorders [33]. Alistipes is a relatively new genus, and it has been the focus of recent clinical studies. Our analysis demonstrated that male subjects experienced even less abundance of *Alistipes* in their gut before and after transplant, thus needing an extra amount of *Alistipes* after transplant compared to other patients.

For African Americans in particular, the abundance of *Bacteroides* increased after transplant, but it might be beneficial not to alter this change via probiotic treatments because the increase of *Bacteroides* has been associated with favorable outcomes. *Bacteroides* has been subject to significant attention in transplant research, and it has been considered as the next generation probiotics candidate due to its involvement in host health [20]. *Bacteroides* usually dominates the human gut microbiome by 20-50% of the total genera and they play a critical role in the balance of the colonic ecosystem [20]. They are known to regulate the degradation of complex heteropolysaccharides to small chain fatty acids and the synthesis of vitamins and bioactive compounds [19]. Lee et al.

discovered that lower post-transplant abundance of *Bacteroides* was associated with diarrhea and acute rejection [21]. A skin transplant study on mice showed that fecal transplant of high-dose Tac-treated mice which contained high abundance of *Bacteroides* into low-dose Tac-treated mice will increase the allograft survival rate [22].

Our analysis showed that we can use the change in microbiome abundance as a signal for transplant outcomes, using proxies such as Tac CV and dd-cfDNA. Change in certain bacterial abundance after transplant could be a proxy for the level of Tac fluctuation (CV) and AlloSure Donor Derived Cell-free DNA. Higher levels of Tac CV (>40%) and higher levels of dd-cfDNA (>1%) are proven to be associated with poor outcomes and rejection. We believe that among African Americans, increase in abundance of *Faecalibacterium* could be a sign of high Tac fluctuation in 30 and 60 days post-transplant. This is in line with the seminal study of Lee et al. in which they discovered that higher abundance of *Faecalibacterium prausnitzii* which belongs to the *Faecalibacterium* genus in the first week of kidney transplant is associated with higher required Tac dose at 1 month [15]. *Faecalibacterium prausnitzii* metabolizes Tac into a less potent novel metabolite. Our analysis shows that *Faecalibacterium* is not only associated with higher required Tac dose, but also higher Tac fluctuation (CV) post kidney transplant. It is plausible to conduct a clinical trial to reduce the abundance of *Faecalibacterium* after kidney transplant for African American patients to see if it improves their Tac absorption.

Decrease in abundance of a genus belonging to the *Lachnospiraceae* family could indicate an increase in dd-cfDNA in all patients. Patients with higher dd-cfDNA lost significant amount of *Lachnospiraceae*. Abundance of *Lachnospiraceae* is associated with decreased lethality from graft-versus-host disease in allogenic blood/marrow transplantation and higher survival rate by its anti-inflammatory effect through induction of regulatory T cells [36]. Jenq et al. reached this conclusion by collecting fecal samples of 64 patients 12 days after bone marrow transplantation. They found that increased amount of genus *Blautia* which belongs to the *Lachnospiraceae* family was associated with reduced graft-versus-host disease lethality. Abundance of *Blautia* is known to be reduced by administration of antibiotics that inhibit anaerobic bacteria. The genera *Blautia* and *Roseburia* of the *Lachnospiraceae* family are the most active genera in the control of gut inflammatory processes, atherosclerosis, and maturation of the immune system [37]. It is known that patients with higher abundance of *Blautia* and *Roseburia* face minimal renal dysfunction with $p<0.05$ in a linear mixed effects regression model and FDR<5% [37]. We believe reduction in *Lachnospiraceae* family could indicate a probability of active rejection. A clinical trial study would be able to confirm this hypothesis and investigate whether increasing the abundance of *Lachnospiraceae* after kidney transplant could lead to lower levels of dd-cfDNA and higher graft survival rate.

3.5 Conclusion

Our study has been limited to a small sample size (n=17), although planning to increase the size to 50 within 12 months. All of our findings require validation by a cohort of new patients in a clinical trial. We propose conducting two interventions:

a) administering probiotics or FMTs to increase the abundance of *Alistipes* and *Lachnospiraceae* in a cohort of kidney transplant patients before and/or after kidney transplant and analyzing the associated graft survival rate for this cohort compared to a control group;

b) administering probiotics or FMTs to reduce the abundance of *Faecalibacterium* in a cohort of African American kidney transplant patients after transplant and analyzing the associated graft survival rate for this cohort compared to a control group.

As a result of these trials, we can also examine whether monitoring the abundance of *Lachnospiraceae* and *Faecalibacterium* could be a proxy for monitoring dd-cfDNA and Tac fluctuation, respectively.

Funding disclosure: the cost of sample collection, sequencing and staff were jointly covered by CareDx and Virginia BioAnalytics LLC.

Appendix A: list of targeted genes

CYP3A5
CYP3A4
CYP3A7
CYP2C19
FMO3
C6
ABCB1
HSD11B1
NR1I2
IL10
IL12A
LEP
POR
HUS1
UGT1A9
HPRT1
UGT1A9
SLCO1B1
NFATC1
ABCC2

Appendix B: Patient Characteristics

Characteristics	***Measure at time of transplant (N=17)***
Age (Mean±SD)	57.58 (11.67)
BMI (Mean±SD)	33.45 (3.69)
Male (n, %)	8 (66.6)
African American (n, %)	8 (66.6)
Live donor (n, %)	7 (58.3)
Previous Transplant (n, %)	3 (27.2)
Dialysis (n, %)	9 (81.8)
Cold Ischemic time hours (Mean, Median)	11, 2
CPRA (Mean±SD)	50.32 (45.16)
History of hypertension (n, %)	1 (9.1)
Diabetes (n, %)	4 (36.3)
Hepatitis C (n, %)	2 (16.6)
High risk CMV (n, %)	5 (45.4)
High risk EBV (n, %)	0 (0)
Creatinine (Mean±SD)	4.47 (3.6)

Appendix C

Bacteroides
Phocaeicola
Lachnospiraceae_u_g
Clostridiales_u_g
Blautia
Parabacteroides
Ruminococcus
Clostridium
Faecalibacterium
Alistipes
Ruminococcaceae_u_g
Enterocloster
Mediterraneibacter
Anaerobutyricum
Dorea
Firmicutes_u_g
Roseburia
Streptococcus
Acidaminococcus
Anaerostipes
Coprococcus
Eubacterium
Akkermansia
Oscillibacter
Drancourtella
Sellimonas
Bifidobacterium
Clostridia_u_g
Dialister
Dysosmobacter
Oscillospiraceae_u_g
Lachnoclostridium
Erysipelotrichaceae_u_g
Limosilactobacillus
Phascolarctobacterium
Prevotella
Burkholderiales_u_g
Flavonifractor
Eggerthella
Faecalimonas
Lactobacillus
Subdoligranulum
Sutterella
Longicatena
Megasphaera
Parasutterella
Porphyromonas
Actinomyces
Gemmiger
Oliverpabstia
Lacticaseibacillus
Neglecta
Ruthenibacterium
Veillonella
Weissella
Pseudoruminococcus
Anaerotruncus
Bariatricus
Catenibacterium
Desulfovibrio
Enterobacter
Enterococcus
Escherichia
Amedibacillus
Lachnospira
Evtepia
Klebsiella
Ligilactobacillus
Lawsonibacter
Longibaculum
Odoribacter
Paraprevotella
Pediococcus
Clostridioides
Prevotellamassilia
Tyzze

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