INVESTIGATING THE UTILITY OF METABOLOMICS AS A TOOL FOR PREDICTING GRAFT OUTCOMES IN KIDNEY TRANSPLANT RECIPIENTS

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ABSTRACT

Transplantation has greatly improved the lives of people with end-stage kidney failure, increasing life expectancy by an average of 10 years. However, threats to extended transplant survival continue to pose legitimate concern. Furthermore, the demand for healthy viable organs greatly exceeds supply and ensuring maximal longevity is of utmost importance. Early allograft kidney injury may negatively impact long-term outcomes. Similarly, the emergence of chronic rejection presents a major obstacle for prolonged graft survival and signifies a fundamental failure to achieve stable immune adaptation.

Metabolomics focuses on the global measurement of small molecules and is a promising tool in the setting of kidney transplantation. Metabolites reflect ongoing bodily changes occurring at multiple levels —molecule, cell, tissue, organ— and offer a unique perspective that may improve our understanding of the intricate processes involved in graft injury and rejection. This thesis examines metabolite concentrations in the serum before transplantation, and how they may influence immediate and long-term transplant outcomes.

To begin, we measured the levels of one individual metabolite (oxythiamine) prior to kidney transplant surgery and tested for association with 1) signs of functional thiamine deficiency early post-transplant; and 2) level of uremia (dialysis adequacy) pre-transplant. Afterwards, we investigated if there are characterizable differences in the pre-transplant serum metabolome of kidney transplant recipients, and whether those differences are associated with chronic rejection outcomes.

In the first study, we found that oxythiamine levels are associated with dialysis adequacy at transplant. Patients treated with peritoneal dialysis, who have no residual kidney function and low dialysis adequacy, are particularly vulnerable to manifesting high oxythiamine levels. This

subset of patients may be at an increased risk for developing acute thiamine deficiency in the early post-transplant period. In the second study, we were able to demonstrate the presence in serum of innate metabolomic differences between patients, which were associated with chronic rejection outcomes, suggesting that, even before transplantation, the metabolite environment may be an important factor involved in the predisposition of alloimmune differentiation towards a rejection response.

LAY SUMMARY

Transplantation is the prevailing treatment for people with end-stage kidney failure. However, some people experience adverse reactions immediately after surgery, which can harm organ longevity. Furthermore, we know that some people will inevitably develop chronic forms of rejection, which also limits kidney transplant survival. Metabolomics is an analytical chemistry technique used to test for hundreds of small molecules (called metabolites) in the blood. Metabolites reflect ongoing bodily processes and may provide valuable insights about which patients are more likely to develop adverse responses. It may also help identify why that risk is higher and strategies to reduce that risk.

In this research project, we investigated 1) whether high levels of one particular metabolite are linked to serious deteriorations after kidney transplant surgery that closely resembles thiamine deficiency, and 2) whether distinct metabolite patterns are present before transplantation that can predict who is more likely to develop chronic rejection years later.

PREFACE

The work comprising this thesis was conducted across three distinct locations: BC Children's Hospital Research Institute (Vancouver, BC, Canada), Vancouver General Hospital (Vancouver, BC, Canada), and The Metabolomic Innovation Centre (Edmonton, AB, Canada). The study entitled "Investigating oxythiamine levels in children undergoing kidney transplantation and the risk of immediate post-operative metabolic and hemodynamic decompensation" was approved by the University of British Columbia's Research Ethics Board (H18-02704). Furthermore, the study entitled "Investigating serum immunometabolomic profiles associated with kidney transplant alloimmune outcomes" was approved by the University of British Columbia's Research Ethics Board (H19-01908).

Chapter 2 is a retrospective study which included a clinical chart review and serum metabolite analysis. A version of this chapter has been submitted for publication consideration. The contributors to this work are Or Golan, Roger Dyer, Dr. Graham Sinclair, and Dr. Tom Blydt-Hansen. All authors contributed to the study conception and design. Ethics submission, material preparation, data collection, and statistical analysis were performed by Or Golan under the supervision of Dr. Blydt-Hansen. Sample analysis was performed by Roger Dyer. The first draft of the manuscript was written by Or Golan, and all authors revised subsequent versions of the manuscript. All authors read and approved the final manuscript.

Chapter 3 is a retrospective, nested case-control study which included a clinical chart review and serum metabolite analysis. A version of this chapter is currently being prepared for manuscript submission. The contributors to this work are Or Golan, Dr. Karen Sherwood, Dr. Atul Sharma, Dr. David Wishart, and Dr. Tom Blydt-Hansen. Study conception and design, ethics submission, material preparation, data collection, preliminary statistical analysis, and

written composition were performed by Or Golan under the supervision of Dr. Blydt-Hansen. The principal investigator of this project was Dr. Blydt-Hansen, and he was extensively involved in study conception and design. Dr. Karen Sherwood was involved with and supervised serum sample collection from the BC Transplant Biolibrary (BCTB). Dr. Atul Sharma was the senior statistician and developed the three prediction models. Sample analysis was performed by The Metabolomic Innovation Centre (TMIC; Dr. David Wishart).

My supervisor, Dr. Tom Blydt-Hansen, along with my committee members, Dr. Graham Sinclair and Dr. Paul Keown, provided vital feedback on the interpretation of all results and throughout this written document.

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LIST OF ABBREVIATIONS

CKD – chronic kidney disease

GFR – glomerular filtration rate

ESRD – end-stage renal disease

HD – hemodialysis

PD – peritoneal dialysis

eGFR – estimated glomerular filtration rate

HLA – human leukocyte antigen

DSA – donor-specific antibodies

dnDSA – *de novo* donor-specific antibodies

CNI – calcineurin inhibitor

AR – acute rejection

CR – chronic rejection

TCMR – T cell-mediated rejection

APC – antigen presenting cell

TNF – tumor necrosis factor

ABMR – antibody-mediated rejection

ADCC – antibody-dependent cell-mediated cytotoxicity

GC-MS – gas chromatography-mass spectrometry

LC-MS – liquid chromatography-mass spectrometry

FDR – false discovery rate

PPCA – probabilistic principal component analysis

TPP – thiamine pyrophosphate

OTPP – oxythiamine pyrophosphate

GSH – glutathione

ROS – reactive oxygen species

GSSG – glutathione disulfide

HPLC-MS – high-performance liquid chromatography tandem mass spectrometry

MRM – multiple-reaction monitoring mode

PROMIS – Patient Records and Outcome Management Information System

SD – standard deviation

IQR – interquartile range

Tregs – regulatory T cells

FOXP3 – forkhead box protein 3

AHR – aryl hydrocarbon receptor

IEM – inborn errors of metabolism

BCTB – British Columbia Transplant Biolibrary

TMIC – The Metabolomics Innovation Centre

DG – diglycerides

TG – triglycerides

PC – phosphatidylcholines

LPC – lysophosphatidylcholines

SM – sphingomyelins

Cer – ceramides

CE – cholesteryl esters

UHPLC – ultra-high-performance liquid chromatography

LOD – limit of detection

CoV – coefficient of variation

PCA – principal component analysis

PLS – partial least squares

PCR – principal component regression

LDA – linear discriminant analysis

LR – logistic regression

AUC – area under the curve

RMSEP – root mean square error prediction

ROC – receiver operator characteristic

CI – confidence interval

LOOCV – leave-one-out cross validation

VIF – variance inflation factors

VIP – variable importance scores

MMF – mycophenolate mofetil

TAC – tacrolimus

CSA – cyclosporine A

TCR – T cell receptor

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Chapter 1:

Introduction

1.1 Chronic Kidney Disease

As a medical term, chronic kidney disease (CKD) encapsulates disorders which affect kidney structure and, consequently, function (1). CKD presentation is varied and dependent on disease etiology, pathology, and severity; however, ultimately, all patients experience deterioration of renal function. The decline in kidney function can occur rapidly (within months), but more often disease progression is prolonged (spanning several years)(2). To be diagnosed with CKD, a patient must display signs of kidney damage (i.e., albuminuria) or decreased kidney function (i.e., glomerular filtration rate (GFR) <60 mL/min per 1.73 m^2) for three or more months. CKD is further classified into five stages based on the severity of GFR deterioration (3).

The gradual decrease of GFR, characteristic of CKD, can result in various complications: increased risk of cardiovascular disease, acute kidney injury, infection, cognitive decline, and impaired physical function (4-8). Some pharmacological treatments, and even certain lifestyle changes, can slow down the deterioration of renal function (9). However, there are no effective therapies that target the primary disease process, and, in most cases, progression of CKD is irreversible (2).

The prevalence of CKD increases for older adults, partly due to the increased prevalence of other co-existing conditions: diabetes, obesity, hypertension, and cardiovascular disease (10). In fact, hypertension and diabetes are the primary causes of CKD, with the latter accounting for an estimated 30-50% of all cases (11). Moreover, adults can be diagnosed with CKD after infections or exposure to drugs and toxins because such experiences can result in glomerular and tubulointerstitial diseases; these etiologies are more prevalent in developing countries (1). For a

small portion of the CKD patient population, disease etiology is genetic. Congenital abnormalities of the kidney or urinary tract lead to disease diagnosis at birth or in early childhood. Other genetic causes, such as autosomal dominant polycystic kidney disease, usually manifest in adulthood (12). Familial history of kidney disease and race/ethnicity are additional genetic determinants that can contribute to increased disease risk. Epigenetic factors are also increasingly recognized as being influential in susceptibility to CKD (13).

Estimates of CKD incidence are difficult to determine due to the differences among countries in the rates of underlying disease and government-sponsored treatment, but yearly worldwide CKD incidence may be as high as 200 cases per million people. In the United States, the incidence is closer to 400 cases per million people (1). The United States has a proportionally higher incidence of disease in racial and ethnic minorities, likely due to a combination of genetic and environmental risk factors, social determinants of health, and continued healthcare inequality (14). For similar reasons, racial and ethnic minorities in Canada are also at an increased risk for both CKD diagnosis and progression (15).

Determining disease prevalence of early stage CKD is not any easier, as estimates are prone to biases caused by the limitations in the methods used to measure GFR and identify kidney damage. In fact, prevalence of CKD is highly dependent on which GFR estimation equation is used (16). In the United States, disease prevalence is estimated at 1,800 cases per million people (1). Between 2013-2016, the percent prevalence in the general adult population was 14.8%, but steeply rises to 32.2% in adults over 60 years old (17). Prevalence estimates in other countries are subject to slight variation, but the overall trends are similar (16).

For the pediatric population, disease prevalence in the United States for 2018 was 2.7 cases per thousand children. Interestingly, CKD shows a bimodal age distribution with young

children (aged <4) and adolescents (aged 18-21) more likely to be diagnosed. Like their adult counterparts, the pediatric CKD population has an increased presence of concomitant conditions (e.g. diabetes, hypertension, and cardiovascular diseases). However, their overall frequencies were much lower when compared with the adult CKD population (17).

Diagnosis with CKD poses a substantial burden, and, for 2012, the WHO estimated that the disease accounted for 1.5% of deaths worldwide. Individuals with CKD are at an increased risk for morbidity as well as cardiovascular and all-cause mortality. Furthermore, these individuals are more likely to die prematurely than to experience kidney failure (11).

An advanced stage of CKD, known as end-stage renal disease (ESRD), occurs when native kidney function can no longer sustain life, i.e., the presence of kidney failure. To be diagnosed with kidney failure, a patient must either have a GFR of less than 15 mL/min per 1.73 m^2 or require treatment with dialysis or transplantation. Unfortunately, kidney failure is an inevitable outcome of progressive CKD. The only suitable treatment options for a patient with ESRD are renal replacement therapy, kidney transplantation, or palliative (non-dialytic) care (1, 2, 11).

1.2 End-Stage Renal Disease

In Canada, the prevalence of ESRD is estimated at 1,200 cases per million people, and, in 2018, 40,289 Canadians –exclusive of Quebec—were living with kidney failure (18, 19). Most of these individuals (approximately 58%) undergo treatment with dialysis, spending an average of 3.8 years on renal replacement therapy before kidney transplantation (20). Dialysis patients have a five year survival rate that is anywhere from 13% to 60% lower than the general age-matched population (21). The demand for a new kidney continues to outweigh availability; in

2018, 40% of Canadians on the transplant waiting list received a new kidney, and 2% died waiting (18, 20).

An overwhelming majority of patients who receive treatment for ESRD are citizens of affluent countries and have access to universal healthcare (22). Because of the substantial costs associated with renal replacement therapies, poorer countries cannot accommodate treatment for as many people. Developing countries, such as China and India, with an increasingly elderly population are projected to have a disproportionate increase in the number of ESRD cases, a trend which will augment if the increase in prevalence of hypertension and diabetes continues (19).

The primary etiologies of ESRD differ between adults and children, and, for the former, the most frequent cause is diabetic nephropathy. Additionally, ESRD can be attributed to hypertension, autosomal dominant polycystic kidney, and chronic glomerulosclerosis. In children, common causes of ESRD include focal segmental glomerulosclerosis, renal dysplasia, obstructive uropathy, and reflux nephropathy (23).

Kidney transplantation is the preferred treatment for ESRD, as transplant recipients have reduced mortality, cardiovascular events, and better self-reported quality of life when compared with dialysis patients (24). However, transplant surgery is limited by the scarcity of donor organs.

1.3 Renal Replacement Therapies

The primary objective of all renal replacement therapies are to substitute the functions of a normal kidney: expulsion of nitrogenous wastes, electrolyte concentration homeostasis, and regulation of extracellular volume. There are two general subcategories of renal replacement therapy, hemodialysis (HD) and peritoneal dialysis (PD). HD uses an external machine as a

semipermeable membrane (the dialyser) to allow ion exchange between the patient's blood and the dialysate. Through diffusion, blood biochemistry can be altered toward that of the dialysate. Compared with HD, PD is a much simpler technique that uses the peritoneal membrane as a semipermeable membrane and circumvents the need for an external machine (2).

Both dialysis modalities can be administered as intermittent or continuous therapies. Comparisons between intermittent and continuous therapies are made by measuring urea clearance, a surrogate marker of the total therapeutic dosage administered, and differs from assessment of native renal function, which uses creatinine clearance (25). Clinical decisions between the use of peritoneal or hemo-based renal replacement therapy involve several considerations: patient-dependent factors, treatment goals, location of treatment, and healthcare resources (25). In 2004, more than 1.3 million people worldwide were receiving dialysis treatment with 89% using hemo-based modalities (26). Contrastingly, PD appears to be the more prevalent treatment type in Asian countries, likely attributable to its relative ease and cost efficiency (27-29). Home dialysis therapy, such as PD or home HD, are favourable because these treatment options are dramatically less disruptive to patients' lives and have comparable clinical outcomes to in-centre dialysis (30). However, across the world, the majority of ESRD patients continue to be treated with in-hospital HD (31).

The survival rate of dialysis patients in Japan is far superior than other countries. Patients who began dialysis treatment between 2004 to 2008 had an unadjusted 5-year survival rate of 60%, 41%, or 39% in Japan, Europe, and the United States, respectively (17, 32, 33). Across Europe and North America, Caucasians have the shortest (adjusted) dialysis survival rate (17, 34, 35). The international disparities in dialysis survival are not completely understood, but can be partially explained by variations in patient characteristics as well as in the national prevalence of

cardiovascular and all-cause mortality (36, 37). To some extent, these variations are also attributed to modifiable differences in clinical practice (31).

Chronic HD patients are prone to high mortality rates in the first few months following treatment initiation (38-40). Early referral to a specialist is associated with better outcomes in the introductory dialysis period, including improved patient preparedness, experience, and survival. Starting dialysis earlier, i.e., in patients with better kidney function, has not resulted in demonstrable benefits (41-43). When a patient experiences irretrievable and permanent loss of renal function, long-term dialysis treatment ensues; this occurrence is referred to as the initiation of maintenance dialysis. When clinicians make decisions about the start of maintenance dialysis, considering patient preparedness in addition to estimated GFR (eGFR) may reduce needlessly early treatment and improve patient outcomes (44).

For ESRD patients treated with renal replacement therapy, both adequate nutrition and sufficient dialysis are needed to reduce morbidity and mortality (2). If residual renal function declines to zero, PD inadequacy can become an issue. Inadequacy may also result from long-term PD treatment because of diminished peritoneal membrane efficiency in transporting waste products, fluid, and electrolytes (2).

Dialysis efficiency is characterized by adequate biochemical change and depends on blood flow, dialysate flow, and the dialysis membrane surface area. Contrastingly, dialysis efficacy can be evaluated by calculating urea kinetics, which requires measurements of the residual renal urea clearance, the rate of increase of urea concentration between dialysis sessions, and the reduction in urea concentration during dialysis. Kt/V (where K is the dialyser clearance, t is the duration of dialysis in minutes, and V is the urea distribution volume estimated as total body water) is a clinical metric used as an assessment tool for dialysis adequacy (2). Clinical

guidelines, published by the National Kidney Foundation, recommend a Kt/V of 1.2 per session as the minimum threshold required for well-nourished adult patients hemo-dialysed three times per week. For peritoneal dialysis in adults, a minimum weekly Kt/V of 1.7 (and 1.8 for children) is considered adequate (45). As a tool for quantifying dialysis adequacy, Kt/V is by far the most researched, but whether it provides an accurate depiction of dialysis effectiveness remains unclear (46, 47). Because of operational differences, the immediate clearance for intermittent therapy is much higher than continuous therapy, but the efficacy of continuous therapy tends to be superior because of extended treatment duration (25). Improvements in Kt/V can be achieved through increases in dialyzer size or flow rate, or by prolonging the dialysis session; however, the existence of a dose-response relationship between Kt/V and patient outcomes appears unlikely (2, 31, 48).

In addition to dialysis efficiency, dialysis session duration is also a probable contributor to HD outcomes. As an intermittent treatment, HD is not a perfect replacement of innate kidney function, and multiple strategies are implemented to prolong treatment either by session duration, frequency, or a combination of both. Most clinicians believe longer treatment time provides benefits beyond Kt/V, such as improved clearance of so called middle molecules (uremic toxins larger than urea) and removal of fluid volume with concurrent reduction in hemodynamic instability (31). However, these perceived benefits lack conclusive evidence (49). When compared with shorter sessions, prolonged dialysis sessions are correlated with longer patient survival and better volume management, blood pressure levels, and phosphorus levels (50-52). These trends have led to increased session lengths for patients receiving in-hospital dialysis treatment in high income countries (53). Individuals undergoing thrice weekly dialysis are at an

increased risk of complications and mortality because of the weekly extended gap (a two-day interval) between sessions (54, 55).

Dialysis is a life-saving treatment for patients with kidney failure, but remains expensive, intrusive, and physiologically limited —many uremic toxins are not sufficiently cleared. Clinical focus should be aimed toward improvements in experience through increased access to kidney transplantation, patient education on dialysis modalities, rehabilitation of malnourished patients, and aligning clinical decision making with patient and familial desires (31).

In contrast, transplantation completely compensates for innate kidney function and is the current gold-standard for treatment of kidney failure. Unfortunately, organ availability is severely restricted, and, as a result, millions of ESRD patients worldwide must commence with dialysis treatment. Although renal replacement therapy can successfully clear the majority of solutes, there are several treatment limitations. Dialysis therapy requires a substantial volume of fluid to function effectively, which involves tethering to stationary fluid and power sources. Renal replacement therapy does not account for the other responsibilities of the kidney, such as the intricate modulation of solute concentration as well as metabolic and endocrine functions (25). Advancements in artificial kidney research are attempting to address these pressing issues, but feasible devices are years away from fruition (56).

1.4 Renal Transplantation

1.4.1 General epidemiology

Ultimately, a kidney transplant is the best treatment option for patients with ESRD. Kidney transplantation is the superior type of renal replacement therapy as patients with a successful transplant have far better survival than patients undergoing dialysis (57). When compared to patients who remained on renal replacement therapies, those transplanted with a

deceased donor organ achieved additional survival of between 6 months and more than three years, irrespective of the presence of comorbidities at the time of transplantation (58). Another large national study, which utilized data from the United States Renal Data System, demonstrated that the average increase in life expectancy of transplant recipients was 9.8 years when compared to their dialysis equivalents. The magnitude of increased life expectancy decreases in older recipients and recipients with preexisting comorbid conditions (59).

The major caveat of transplantation is that the demand for healthy viable organs greatly exceeds the supply. Therefore, transplant research is essential to ensure donor organs are utilized as optimally as possible because of their value and scarcity. Kidney transplantation allows patients to regain a sense of normalcy as it imposes the fewest restrictions on daily life. However, they are still burdened by the effects of being an immunocompromised population and the importance of medication adherence (2).

Access to and treatment with kidney transplantation is highly varied between countries. For example, in 2013, transplantation rates in some Asian and Eastern European countries were less than 10%, but greater than 50% in Nordic countries. Interestingly, the countries with the highest proportion of renal transplants have some of the lowest incidence rates of ESRD, which suggests that efforts to abate CKD progression may have additional benefits, i.e., result in a higher proportion of patients who receive kidney transplants (31).

The optimal time for a pre-emptive transplant is a contentious issue, as clinicians must contemplate the benefits of dialysis avoidance against the detriments of early exposure to chronic immunosuppression. Until recently, studies in the pediatric population have not demonstrated substantive benefits of pre-emptive transplantation; nonetheless, a recent nationwide study of pediatric patients with ESRD demonstrated that it does appear to confer a survival advantage,

especially when compared to children who have been on dialysis for more than one year. Children treated with dialysis for more than one year had a 52% higher risk of graft failure in the first five years post-transplant than those who underwent transplantation pre-emptively. Furthermore, patients on dialysis for more than 18 months had an 89% higher risk of death, regardless of donor type (60).

Immunosuppressive medication is compulsory for all transplants to avert graft rejection. The only exception are transplants performed with a donor that is a perfect genetic match, i.e., an identical twin. Patients must continue with immunosuppressive treatment for as long as the allograft remains in place (2). Indeed, short-term graft survival has improved immensely in the past 25 years with increasingly sophisticated assessment of donor-recipient compatibility and immunosuppressive regimens as well as improvements in surgical techniques; however, long-term allograft survival has failed to follow suit. The estimated half-life of a kidney graft is 9 years with a deceased donor, and slightly increases to 12 years with a living donor (61). As significant improvements in long-term outcomes remain elusive, new and innovative strategies are warranted.

1.4.2 Factors known to affect transplant success

Donor-recipient HLA matching

The Human Leukocyte Antigen (HLA) complex is an important determinant of transplantation outcome, specifically the degree of HLA matching between donor and recipient. Overall, better HLA matching reduces the risk of rejection thereby improving allograft survival (62). The reason being mismatched (foreign) donor HLA antigens elicit an intensive alloimmune response, and the epitopes of donor HLA antigens are primary targets.

HLA typing for the A, B, and DR loci is widespread as mismatches against these antigens have well-known detrimental effects on graft outcome (2). For example, mismatches at the DR locus increase the risk of early acute rejection (63). The clinical relevance of the DQ locus has gained recent attention (64, 65).

Sensitive cross-matching techniques are used to screen patients before surgery for the existence of donor-specific antibodies (DSA), which can result from previous blood transfusions, pregnancy or miscarriage, or previous transplants. Highly sensitized patients can undergo antibody removal and immunosuppressive treatment before transplantation; however, this cohort has an increased risk of infection and developing chronic antibody-mediated rejection (66).

Because of the resulting anti-HLA sensitization, HLA mismatches severely hinder the success of subsequent transplants. This is of concern, specifically for younger patients, as they will almost certainly require re-transplantation during their lifespan; therefore, maximizing donor-recipient histocompatibility matching is especially critical for this subset of patients (67).

Another, more recent strategy, involves assessment of donor-recipient compatibility at the structural level using epitope data. Eplets are polymorphic amino acid residues (i.e., functional epitopes), and the amount of donor-recipient eplet mismatch can be calculated by computer programs. The prevailing thought is that mismatched functional epitopes provide better prognostic value for future development of anti-donor HLA antibodies than standard HLA loci typing (68). Epitope matching is a more sensitive method of identifying compatible donors for sensitized patients (69). Eplet matching for HLA-DR, -DQ, and -DP loci may soon be integrated into routine clinical practice with the use of computer programs like HLAMatchmaker. Epitope matching is a novel approach that can potentially improve graft outcomes by reducing the incidence of *de novo* DSA (dnDSA) development (70).

Donor kidney

Kidney transplantation can occur with either a cadaveric or living organ donor. In most countries, patients who have suffered irreversible brain damaged (deemed as 'brainstem death') are eligible for organ donation. Organ shortage is a widespread problem worldwide, and the number of people requiring new organs continues to rise; therefore, countries are increasingly allowing organ retrieval after cardiac death. Donation after cardiac death has comparable results to organ donation after brain death (2). Another increasingly accepted practice is donation from expanded criteria donors.

Transplantation with an organ derived from a living donor is highly favourable; it minimizes the risk of ischemic injury and often results in better long-term graft outcomes (66). Living donation may be feasible if a related or unrelated person volunteers as a donor. Unrelated living donors are acceptable providing there is no use of coercion.

The quality of the donor organ should be taken into account as it affects the ability to respond to stressors as well as organ longevity. When the donor receives intensive care, instances of acute tubular damage and inflammation can occur, which correlate with detrimental events such as ischemic acute renal failure and delayed graft function (68). The latter has been implicated as a substantial risk factor for graft loss (71). Niemann and colleagues demonstrated that the occurrence of delayed graft function can be diminished by subjecting the deceased donor organ targeted mild hypothermia prior to transplantation (72). Peri-transplant stress associated with deceased donor organs can be greatly reduced with optimum post-operative care, pulsatile ex-vivo machine perfusion, rapid organ transfer, and prompt implantation (66, 73).

Long-term graft outcomes are unequivocally impacted by the quality of the donor organ, primarily donor age. Older donors may have pre-existing kidney lesions, which cannot be

reversed and negatively affect the response to stressors (2). Approximately 3% of transplantation surgeries result in primary nonfunction (74).

Immunosuppression

All transplanted patients, apart from those receiving an organ from an HLA identical donor, must undergo treatment with immunosuppression medication to mitigate graft rejection. The risk of rejection is highest in the first three months post-surgery (2). The conventional immunosuppressive regimen encompasses a calcineurin inhibitor (CNI), an antiproliferative agent, and corticosteroids (75). There are two readily used CNIs: tacrolimus and cyclosporine. The use of tacrolimus is more extensive, and it's generally regarded as the superior immunosuppressant (75). However, various factors affect CNI selection, and, under certain circumstances, the use of cyclosporine is more appropriate. Each CNI is associated with a distinct set of side effects and both present efficacy issues —particularly apparent when examining the frequency of dnDSA development (68).

All patients undergo peri-transplant induction immunosuppression therapy (either with an interleukin 2 blocker or anti-thymocyte globulin) to prevent early acute rejection (76). Post-transplantation, immunosuppressive doses are initially high but are eventually tapered to a lower maintenance dose, usually within the first year.

1.4.3 Graft outcomes

Transplantation triggers a defensive response from the recipient's immune system in reaction to the detection of foreign donor antigens. This alloimmune response is inevitable, and remains the primary obstacle for graft survival. If it is left unchecked, the resulting rejection leads to graft destruction. However, graft loss can be mediated by both immunological and non-immunological mechanisms. Rejection can be subcategorized temporally as hyperacute (occurs

within minutes), acute (occurs within days to weeks), late acute (occurs after three months), or chronic (occurs after months or years) or by pathophysiological criterion such as cellular, vascular, or humoral (77). Rejection diagnoses often combine both terminology groupings (e.g. chronic antibody-mediated rejection). Rejection can be further classified by additional characteristics such as severity (with use of Banff criterion)(78), treatment receptiveness, and the presence or absence of symptomatic renal dysfunction (77).

The donor kidney is exposed to immunological stressors even before transplantation, through the systemic effects of donor brain death or perioperative ischemia-reperfusion injury. A consequence of ischemia-reperfusion is increased HLA antigen expression in the graft. HLA up-regulation leads to the release of chemokines, proinflammatory cytokines, and adhesion molecules which, in turn, intensify the recipient immune response and cellular graft infiltration, increasing the risk of subsequent rejection (79, 80).

The effect of immune-mediated graft injury can be influenced by multiple factors such as severity, timing, persistence, and histologic pathology (66). These variables influence therapeutic responses.

1.4.4 Rejection types

Acute rejection

Acute rejection (AR), characterized by a decline in renal function, usually develops in the first few months post-transplant in anywhere from 10 to 30 percent of patients (2). AR is clinically confirmed with a renal biopsy, as rejection varies in type (cellular, vascular, or humoral) and severity (81). Both arms of the immune system (innate and adaptive) are involved in early AR. When AR is detected by the presence of histological changes on surveillance biopsy, but there are no accompanying clinical signs or symptoms, it is termed subclinical AR.

Rejection origin can be defined as either cellular (T-cell mediated) or humoral (antibody-mediated); however, the cause of AR can be mixed (i.e., both cellular and humoral in origin)(81).

Because late AR often manifests as a severe rejection episode, it can prove difficult to reverse, and leaves the patient at an increased risk of subsequent graft failure. Late AR is characterized by active inflammation and tubulointerstitial damage, and may also be accompanied by the presence of graft-directed antibodies (82). It can develop as a result of an aggressive alloimmune response to the graft, for example, in patients who are not immunologically well-matched. Patients with attenuated immunosuppressive regimens (either due to cancer, prior severe infection, or non-adherence) are also at risk (77).

There are several factors associated with AR events that may negatively influence long-term graft outcomes: more than one rejection episode within the first 3 months after transplantation, vascular or humoral rejection, delayed graft function (defined by return to dialysis in the first week post-transplant), and failure to return to nadir creatinine levels (<130 μmol/L)(2).

Chronic rejection

Chronic rejection (CR) represents a form of unabated immune injury to the graft and is likely a result of insufficient immunosuppression, leading to uncontrolled anti-graft lymphocytes or antibodies. The characteristic feature of CR is a gradual decline in renal function. It can be accompanied by interstitial-infiltrating T cells and macrophages or T cell invasion of the renal parenchyma (77). Contrastingly, chronic antibody-mediated rejection is caused by pre-existing DSA or newly formed (after transplantation) DSA and is often coincided by characteristic deposits on the capillary endothelium (83).

T cell-mediated rejection (TCMR)

The treatment, management, and prevention of T cell-mediated rejection (TCMR) has improved tremendously over the past two decades and translated to sustained improvement in short-term graft survival. Cellular rejection most commonly manifests as a form of AR. Mechanistically, T cell-mediated injury begins when donor antigens appear on antigen presenting cells (APCs) and are presented to recipient T lymphocytes (77).

Donor antigens from immature graft-derived dendritic cells mature into APCs and are present within the graft environment, but also home to lymphoid organs where they activate recipient T cells (84). Activated T cells then differentiate into distinct subtypes, proceed to the allograft, and mediate the destruction of the transplanted organ. T lymphocytes can sense alloantigens both directly (via donor APCs) and indirectly (via recipient APCs) (85). Eventually donor APCs will disappear, and the indirect pathway becomes the primary source of long-term cell-mediated injury to the allograft (86).

Through the use of adhesion molecules, T cells tether the endothelium and, in a process called diapedesis, cross the peritubular capillaries to enter the allograft (79). T cells mediate graft injury three-fold: direct contact with tubular epithelial cells, the effects of cytokines released into the local milieu, and activation of inflammatory or vascular endothelial cells. Cytotoxic T cells release perforin and granzymes (A and B) which mediate apoptosis in targeted graft cells (87). Conversely, T helper cells mediate graft injury by secretion of tumor necrosis factor (TNF) α and β, causing apoptotic cell death (88).

When T cells invade renal tubule cells and proliferate within the interstitial space, it causes tubulitis —a typical histologic feature of an AR episode. These T lymphocytes secrete inflammatory cytokines which elicit chemokine production from tubular epithelial cells.

Chemokines then perpetrate a positive feedback loop by attracting more T cells to the allograft (89). Injured tubular cells can undergo epithelial-mesenchymal transition and cause interstitial fibrosis (90). Tubular epithelial cell death and basement membrane rupture can result in tubular atrophy and graft dysfunction (91).

Corticosteroid administration is the standard initial treatment for an episode of acute cellular rejection (81). However, cellular rejection can be recurrent or steroid-resistant. These rejection types respond better to treatment with polyclonal or monoclonal anti-T cell antibodies. Moreover, increases to immunosuppression dosage after a bout of acute cellular rejection may help prevent future rejection episodes (81).

Antibody-mediated rejection (ABMR)

Humoral rejection most commonly manifests as a result of antibodies directed against HLA molecules. Discovering the destructive potential of anti-HLA alloantibodies and their intrinsic link to antibody-mediated rejection (ABMR) has revolutionized the field of transplant medicine. The past decade has yielded scientific breakthroughs that have shifted our fundamental understanding of chronically failing allografts. Donor-specific anti-HLA antibodies have become increasingly accepted as the primary mediators of the chronic deterioration of grafts (92). This notion completely opposes the historical dogma that primarily attributed such allograft losses to CNI toxicity or chronic allograft nephropathy. In addition, rejection has been traditionally considered as a process predominantly mediated by T-cells –a rejection type in which clinical treatment and management has improved tremendously. Now, we recognize that current immunosuppressive protocols inadequately regulate humoral immunity, resulting in renal dysfunction and subsequent graft loss (93-95).

The development of ABMR occurs early and continuously, cycling between intervals of injury and repair. This process eventually results in the gradual deterioration of renal function and leads to graft loss. ABMR is identified by the presence of histological changes caused by circulating DSA and diagnosed by renal biopsy (81). DSAs can be found prior to surgery or emerge *de novo* post-transplantation, and not all DSAs are equally pathogenic. For example, dnDSA are predominately directed against class II HLA molecules, and class II DSAs are regarded as more harmful than class I (96). The incidence of dnDSA has been estimated to range anywhere from 10-35% in the renal transplant population (96-99). In a recent clinical trial, just under 40% of DSA-positive patients were also diagnosed with biopsy-proven ABMR (97). The presence of circulating dnDSA can be detected before full-blown rejection is diagnosed (100-102). Therefore, some postulate dnDSA represents a mechanism of sustained graft injury (98).

Circulating DSAs can bind to HLA antigens, or other targets on the graft endothelium, and initiate graft injury. The level of DSA pathogenicity may also be influenced by the heavy chain isotype. Complement activating DSAs lead to the rapid initiation of the classical pathway, and injury resulting from this process usually leads to swift allograft loss (103). Endothelial cell binding DSAs induce cell proliferation or antibody-dependent cell-mediated cytotoxicity (ADCC), processes which result in chronic graft injury that mechanistically occurs via natural killer cells (104, 105). The lesions that result from DSA-activated pathways can cause permanent damage that eventually compromises graft function (92).

ABMR is considered to be a dynamic and continuous process of injury and repair. It should be regarded as a term that encompasses a collection of diseases with varying pathologies, which are often accompanied by the detection of circulating DSA (92). Peritubular capillary

deposition of C4d —an inactive by-product of the classical complement pathway— functions as a universal marker of active ABMR (106, 107).

With the advent of modern therapeutics, there are multiple interventions that can be used to successfully reverse episodes of AR. However, none of these address the root cause, and antibody-secreting plasma cells can still be found in the spleen and bone marrow of patients, i.e., DSAs may remain detectable in circulation (108). These DSAs can lead to a slow-progressing form of antibody-mediated injury indicated by signs such as persistent glomerulitis as well as peritubular capillary inflammation and C4d deposition. Moreover, individuals who develop dnDSAs often have a more indolent form of antibody-mediated graft injury that occurs without AR episodes, sometimes termed subclinical or indolent ABMR (109, 110).

A surveillance biopsy study conducted over the first post-transplantation year revealed substantial and multidirectional variations in humoral immunity as evidenced by oscillating DSA, Cd4 deposition, and glomerulitis scores (111). The data presented by studies such as this one support a continuum-based theory for explaining the natural progression of chronic ABMR (112). Therefore, graft injury resulting from a singular episode of ABMR is improbable; instead, it appears to manifest in a dynamic manner, most likely in the initial post-transplant period and continues at fluctuating intensities thereafter (92).

The mainstay of ABMR treatment concentrates on reducing antibodies and inhibiting complement activation. For antibody reduction, current therapies are plasma exchange with low-dose IVIG, high-dose IVIG, and rituximab (113). High-dose IVIG is also used in clinical practice for complement inhibition (114, 115).

Vascular rejection

Vascular rejection is characterized by the following histological features: vascular infiltrating mononuclear cells, endothelial cell apoptosis, and the production of matrix proteins and collagens by fibroblasts. Natural killer cells, anti-MHC antibodies, and interferon-γ are all involved in vessel invasion (77). Usually, vascular rejection is T-cell mediated, as macrophages and T cells (both CD4 and CD8) invade the endothelium via activated adhesion molecules (ICAM-1 and VCAM). Adhesion molecules are activated by chemokine signalling cascades (116). However, a distinctive subtype of vascular rejection that is antibody-mediated has also been described (117).

Vascular rejection is often severe and resistant to therapy with glucocorticoids; consequently, standard clinical guidelines recommend treatment with potent anti-lymphocyte antibody therapy (e.g. antithymocyte globulin)(75, 81).

1.4.5 Immunosuppression-related

CNI toxicity

The invention of CNIs revolutionized the field of transplantation by significantly improving one-year graft survival rates. CNIs are now the cornerstone of transplant immunosuppression; unfortunately, they are also nephrotoxic (118).

With continued medical and pharmacological advancement, clinicians are much better at identifying and treating rejection earlier. However, long-term graft loss rates have remained largely unchanged for the past two decades, and late graft injury as a result of CNI toxicity is one suspected culprit. The presence of characteristic toxicity lesions in renal arteries and tubules support this notion (119). Along with evidence from CNI avoidance, dose reduction, and early/late withdrawal studies which demonstrate improved renal structure and function (120-

122). Patients treated with CNIs for non-renal solid organ transplant or autoimmune disease also experience renal damage and sometimes progress to renal failure (123).

Patients with deteriorating graft function from suspected CNI nephrotoxicity, who are at low immunological risk, are recommended to withdraw treatment with CNIs and continue maintenance immunosuppression with an antiproliferative agent (usually mycophenolate mofetil) and corticosteroids (124). Approaches like CNI dose reduction or substitution can be implemented for high immunological risk patients experiencing signs of CNI toxicity, alongside vigilant rejection monitoring (125).

Non-adherence

Medication non-compliance represents a major obstacle in transplant medicine as an estimated 23-50% of recipients exhibit non-adherent behaviours such as intermittent omission of medication (accidental or deliberate), self-induced dose reductions, avoidance of specific medication, or total therapeutic termination (126, 127). Poor compliance substantially increases the risk of AR and graft loss, with some estimates suggesting it accounts for 36% of all graft loss (126). High intraindividual variance in medication trough level, which suggests the presence of non-adherent behaviours, reliably predicts the subsequent development of dnDSA and graft loss (128). As a patient progresses further from transplantation (i.e., with increasing time post-transplant), the likelihood of mediation nonadherence increases (129).

Individualized strategies which focus specifically on attenuating the medication burden (i.e., pill volume) and eliciting positive behavioural changes may assist with improvement of compliance levels (130). Educational interventions, such as improving patients' health literacy levels or counselling sessions on adherence to immunosuppressive medications, may be beneficial (130). Other interventions that could improve adherence rates and, by extension, graft

outcomes include psychosocial support, personalized care planning, and self-monitoring tools (68).

1.4.6 Recurrent glomerular disease

After kidney transplantation, diseases with glomerular pathophysiology (including diabetes) can reoccur and result in subsequent graft loss. Diagnostic indicators of disease reoccurrence include proteinuria and hematuria. Recurrent glomerular disease has a 10-year incidence rate of 8.4%, which represents the third most frequent cause of late graft rejection, succeeding CR and death with a functioning graft (131).

1.4.7 Death with a functioning graft

Unfortunately, death with a functioning graft is a possible outcome after transplantation and the risk increases with transplant duration (132). Mortality amongst kidney recipients is usually attributed to cancer or cardiovascular events. The risk of cardiovascular-related mortality is highest in the first three months post-transplant surgery and decreases thereafter (133). The incidence of cancer is two- to three-fold higher than the general population, and clinical outcomes are often worse (134). Cancer diagnosis is intrinsically tied to chronic immunosuppression; both the length and strength of immunosuppression increases cancer risk. As an immunocompromised population, these patients often succumb to cancers that are associated with viral infections (134).

1.5 Metabolomics

As a discipline, metabolomics focuses on the global measurement of small molecules that comprise the metabolome —the collection of metabolites found in a living cell or organism— and offers the advantages of utilizing high-throughput techniques. In the past two decades, metabolomics has garnered increasing recognition in organ transplantation research for its

potential to progress the use of personalized medicine and as an appealingly quick and non-invasive tool. As another ‘omics tool, it provides information complementary to other systems biology techniques such as genomics and proteomics (135).

With advancements in analytical chemistry techniques, it is now possible to measure hundreds or thousands of small-molecule metabolites at once (136, 137). Some of these metabolites are already integrated into standard clinical monitoring, but the benefit rests in the large amount of lesser-known metabolites that can also be quantified (136, 138). The ability to measure many metabolites at once provides a more comprehensive depiction on the state of the underlying physiological processes (135). Metabolomics has the potential to detect early changes in cellular signaling with a high degree of sensitivity and specificity. It is an attractive tool in renal transplantation because new advancements could allow for clinical monitoring of drug effects and possible graft perturbations before the presence of histological or pathological evidence, and enable individualized immunosuppression therapy (139). However, metabolomics has not yet made these aspirations feasible.

Common analytical chemistry techniques used in metabolomics include GC-MS (gas chromatography–mass spectrometry), LC-MS (liquid chromatography–mass spectrometry) or NMR spectroscopy, with measurements usually performed on biofluids, as opposed to tissues (135). The rationalization being that a biofluid produced by or surrounding an organ will provide high-caliber information about its physiological state (135). Cells communicate with biofluid by direct and indirect (via extracellular fluid) mechanisms. Cellular products, such as metabolites, can enter various biofluids via membrane diffusion or vesicle-mediated transport, and, as a part of cell-death processes, cells release their contents into biofluids. Therefore, the justification is that, to a certain extent, biofluids can represent the biochemical changes of cells and organs.

These changes, if mechanistically sound, may provide prognostic value and become a utilizable biomarker (139).

Blood is a particularly attractive biofluid because metabolite perturbations are easily detected; however, these changes are not easily traceable to one specific cause or organ. Blood is an ambiguous biofluid and likely more reflective of systemic physiology. Small molecules can travel freely via the circulatory system. A continued challenge is the difficulty of rationalizing the source and cause of these metabolite signals, and parsing out the consequential from the inconsequential changes (135).

Metabolites reflect changes occurring at multiple levels, molecule, cell, tissue, and organ, and are closely intertwined with genomic, proteomic, immune, and environmental systems. Therefore, to further advancements on graft injury and rejection, or risk-stratification, the field will require multidisciplinary input and cooperation. Nonetheless, metabolomics is an exciting and promising tool in renal transplantation. It offers a unique perspective that may improve our understanding of the intricate processes involved in allograft injury and rejection (135).

Graft injury and subsequent rejection is a highly complex and multidimensional process; therefore, determining cause-effect associations between chemical markers and disease can be difficult. Furthermore, a newly established biomarker can only be of value if it can feasibly integrate into clinical practice. Current heavily relied upon clinical markers, such as creatinine, are sub-optimal because changes are only detected with advance kidney injury. This is problematic as interventions may be less effective or permanent injury may have already taken place (140). Molecular signatures confer more information than measurement of a single parameter as the latter will never be able to capture the complete intricacies involved in kidney function (139).

Translational transplant research is predominately focused on the development of new strategies that improve long-term allograft survival (66); ‘omics technologies are highly attractive and create new opportunities (141). In renal transplantation, research has previously focused on metabolomics as a tool for biomarker development as a prognostic indicator of donor organ injury, post-transplantation function, renal dysfunction, AR and subclinical rejection (142-146).

The development of disease can be categorically separated by genetic predisposition, a biochemical stage, and a symptomatic stage (147, 148). Current diagnostic tools used in post-transplant management include clinical indicators (e.g. creatinine) and histological assessment in the form of invasive renal biopsies. Both tools detect disease occurrence in the symptomatic stage when the injury is too progressive and no longer fully reversible. Molecular markers derived from metabolic patterns can potentially identify changes in the biochemical phase where pathological consequences are not yet permanent (147, 148). Therefore, diagnostic strategies based on metabolite panels may result in significant advancements in the areas of preventative and personalized transplant medicine.

‘Omics technologies can accommodate both non-targeted and targeted analyses. A non-targeted approach can be advantageous because it captures unfiltered and nonbiased data; however, the specific chemical compounds that constitute the recorded signal are often unknown (139). Contrastingly, a targeted approach incorporates multi-analyte assays that measure known compounds, and is advantageous because the results can be better interpreted (139). However, this approach may be inherently biased because only select compounds are measured.

There are various other known factors that can introduce bias into metabolomic studies: timing of sample collection, sample collection procedure, sample processing, stability and

storage, extraction procedures, dilution of sample, analytical method used (LC-MS versus GC-MS), assay sensitivity, reliable range of response, etc. (149). One common consideration in metabolomics is how to address the variable to observation ratio, often the number of metabolites greatly exceeds observations. A substantial portion of the data generated does not directly relate to the disease process and will not convey valuable information. The large variation in the informativeness of each metabolite tends to make metabolomic data quite noisy (139). In general, metabolites tend to be more complex and heterogeneous than genes and proteins. As a result, classical statistical approaches are insufficient. The statistical methods in metabolomic studies usually utilize algorithms developed for data reduction and filtering, controlling false discovery rate, and high-dimensional modelling (150, 151).

Metabolomic studies frequently use multivariate classifiers to explore the likelihood of differentiating disease states by their metabolite profiles. Data collected for such studies are highly dimensional and correlated, making traditional sample size calculations difficult to perform. Ideally, pilot data are generated to provide conclusive sample size calculations. Specifically, the data are subject to the same analysis plan proposed for the formal study, but supplemented with bootstrapping methods. Nevertheless, preliminary data are often unavailable.

Recently, a simulation study with targeted false discovery rates (FDR) has proposed estimates for adequate sample sizes in metabolomics: if the FDR=0.05 and 300 metabolites are analyzed with the assumption that 20% of analytes will differ ($p < 0.05$) between groups, a study will require a minimum of 15 samples per group (152). However, it is important to consider this caveat: the suggestion was developed under a specific analysis model, probabilistic principal component analysis (PPCA). As a general guideline, experienced investigators often cite that 25-30 samples are required in each phenotypic class to develop a robust multivariate classifier.

Historically, and in the context of renal transplantation research, metabolomics has not received as much attention as genomics or proteomics, which might be attributed to the complexity of metabolomic assays (139). The metabolome includes molecules with a wide range of physicochemical profiles; analyte analysis often requires the combination of multiple assays (149, 153). For successful implementation into routine clinical care multiple criteria must be considered: realistic sample collection and handling procedures, practical and validated bioanalytical methods, and general health economics (154).

Single-compound surrogate markers are unable to sufficiently monitor a complex organ such as the kidney. However, non-targeted profiling cannot be feasibly translated into the clinic because of the requirement for complex analyses and software tools (139). Ideally, combinatorial biomarker patterns, consisting of 5-15 individual parameters, will provide more comprehensive information, and be superior both in specificity and sensitivity.

1.6 Hypothesis and Objectives

In both studies, we utilize metabolomic techniques to investigate the viability of identifying patients pre-transplant who may be at a higher risk of later experiencing adverse graft-related events.

In Chapter 2, the oxythiamine study, we hypothesized that surgical stress and ischemia-reperfusion injury may precipitate functional thiamine deficiency with oxythiamine toxicity post-operatively in children.

Aim 1: Determine the levels of serum oxythiamine and thiamine in pediatric patients with pre-kidney transplant samples and the association with clinical signs of functional thiamine deficiency immediately post-transplant.

Aim 2: Test the association of oxythiamine levels with dialysis adequacy or kidney function in the same patients pre-transplant.

In Chapter 3, the adult metabolomics study, we hypothesized that differences in the host metabolome at transplantation directly influence early alloimmune differentiation and are associated with post-transplant alloimmune outcomes in transplant recipients.

Aim: To determine whether we can characterize differences in the pre-transplant serum metabolome (metabotypes) of transplant recipients associated with chronic rejection.

Chapter 2:

Investigating oxythiamine levels in children undergoing kidney transplantation and the risk of immediate post-operative metabolic and hemodynamic decompensation

2.1 Introduction

In CKD, waste products of metabolism accumulate as renal clearance declines. When waste products reach harmful concentrations with progression to end-stage kidney failure, they are considered as uremic toxins. Uremic toxins are defined by their ability to negatively interfere with biological functions, a common example being antimetabolites. Antimetabolites are molecules that interfere with normal cellular metabolic processes, usually by binding to metabolic enzymes (155).

Oxythiamine is one such antimetabolite that antagonizes the coenzyme thiamine (156). Thiamine is a water-soluble B vitamin important in normal cellular metabolism. Usually, thiamine is pyrophosphorylated into thiamine pyrophosphate (TPP) for use at many points in intermediary metabolism, including the pentose phosphate pathway, branched chain amino acid metabolism, glycolysis, and the citric acid cycle (157, 158). In uremia, oxythiamine binds to TPP-dependent enzymes in its pyrophosphorylated form as oxythiamine pyrophosphate (OTPP) (156, 159). Oxythiamine decreases the retention of thiamine in tissues and the activity of TPP-dependent enzymes, along with increasing the rate of TPP dephosphorylation (159-165). Coupled with evidence that CKD is associated with a multi-organ decrease in thiamine transporters, the pre-transplant metabolic environment may predispose a patient to functional thiamine deficiency (165-168). When precipitated by an acute metabolic stress, functional thiamine deficiency can manifest clinically as Beriberi syndrome, a state of cardiovascular

collapse and metabolic acidosis (169). The negative effects of oxythiamine accumulation can be reversed in the short term by administration of high-dose thiamine to outcompete OTPP (159).

Thiamine is essential for cellular regeneration of not only ATP, but also glutathione (GSH), the latter functioning as a necessary anti-oxidant. A compulsory aspect of transplantation is reperfusion, which allows for tissue reoxygenation and return to aerobic respiration. A consequence of reperfusion is the rapid generation of reactive oxygen species (ROS) in ischemic tissues. Under normal conditions, mitochondria produce superoxide as a by-product of oxidative phosphorylation, which will eventually be reduced to water and simultaneously oxidizes GSH to glutathione disulfide (GSSG). Requirements for regeneration of ATP and GSH from ADP and GSSG respectively are particularly important during reperfusion to protect against harmful events, such as acute cell swelling and production of ROS (170).

Thiamine deficiency can lead to two different clinical syndromes: beriberi and Wernicke-Korsakoff. Beriberi can be further classified into either “dry” or “wet”, which are distinguished by neurologic (dry beriberi) or cardiac (wet beriberi) symptoms. Additionally, there is a rapidly progressing form of cardiac beriberi termed Shoshin beriberi (169). High cardiac output failure, oliguria, edema, hyperglycemia, and lactic acidosis are the standard characterizations of thiamine deficiency. These clinical manifestations are mainly the result of an inability to utilize the Krebs cycle (171).

Our group previously reported a case of Shoshin Beriberi syndrome, with onset in the immediate post-transplant period and presenting with severe lactic acidosis, hyperglycemia, and cardiovascular instability typical of thiamine deficiency. It was rapidly reversed after a single pharmacologic dose of intravenous thiamine (172). Similar episodes have been reported in adult transplant recipients (173, 174). Kidney transplant surgery and ischemia-reperfusion injury are

major stressors that may be expected to trigger Beriberi syndrome in susceptible patients. Uremic oxythiamine toxicity was suspected, but to date has never been confirmed in such cases. Oxythiamine levels are further expected to vary with the severity of uremia. Understanding the risk factors for elevated oxythiamine levels pre-transplant could permit anticipation of risk for Beriberi syndrome and the opportunity for early intervention with thiamine.

Therefore, we hypothesized that surgical stress and ischemia/reperfusion injury may precipitate functional thiamine deficiency with oxythiamine toxicity post-operatively. The aims of the study were as follows: (1) To determine the levels of serum oxythiamine and thiamine in patients with pre-kidney transplant samples and the association with clinical signs of functional thiamine deficiency immediately post-transplant; (2) Test the association of oxythiamine levels with dialysis adequacy or kidney function in the same patients pre-transplant.

2.2 Methods

2.2.1 Patient Population

All pediatric patients who underwent kidney transplantation at a single tertiary pediatric transplant center between January 2013–September 2018 were eligible for inclusion. These patients also received chronic kidney disease care pre-transplant at the same institution. Patients were excluded if they did not have pre-transplant serum samples available for testing or peri-operative laboratory and clinical data available for analysis. The study was approved by the institutional review board at the University of British Columbia (H18-02704; H16-01140).

2.2.2 Serum Samples

Serum samples are routinely collected each month while on the transplant waiting list. In cases where residual sample exceeded what was clinically required for future testing, the Immunology Laboratory provided a serum aliquot for metabolite analysis. For each patient, the

sample closest to the time of transplant was selected for testing. In a subset of patients who had available post-surgery samples (between 1–12 months), a follow-up sample was retrieved to determine post-transplant oxythiamine and thiamine concentrations, following the normalization of kidney function.

2.2.3 High-performance Liquid Chromatography Tandem Mass Spectrometry (HPLC-MS)

The ACQUITY UPLC H-Class System and Xevo TQ-S (Waters, Mississauga, ON, Canada) instruments were used for all HPLC-MS analyses with a hydrophilic interaction chromatography (HILIC) approach operating in a positive-ion, multiple-reaction monitoring mode (MRM). The analytical column was a 1.7μm particle size, 2.1mm x 50 mm BEH HILIC with guard column (Waters, Mississauga, ON, Canada) and the following mobile phases: (A) 96% acetonitrile (EMD/Millipore, Burlington, MA, USA)/4% H2O with a final concentration of 10mM ammonium formate, and (B) 10 mM ammonium formate in deionized water (Thermo Fisher Scientific, Burnaby, BC, Canada). Both A and B contained 0.05% formic acid (Millipore Sigma, Oakville, ON, Canada). All solvents were LC/MS grade. Gradient chromatography was employed at a flow rate of 0.3ml/min with initial conditions of 85% A and 15% B held for two minutes after injection followed by a gradient to 75% A and 25% B over two minutes and held for two minutes, before returning to starting conditions for column stabilization. Column temperature was maintained at 30°C throughout. Detection was normalized to a ^{13}C-thiamine internal standard (Millipore Sigma, Oakville, ON, Canada). To deduce analyte amounts, a linear standard curve was constructed (from 0.05 nmol/L to 50 nmol/L) with a 0.05 nmol/L limit of quantitation. Serum samples, previously stored at -80°C, were thawed on ice and a 20 μl aliquot was combined with 10 μl of internal standard and 120 μl of acetonitrile. The solution was vortexed and then centrifuged at 14,000 RCF for ten minutes, the resulting supernatant was

injected into the column. The retention times for thiamine and oxythiamine were 4.6 minutes and 4.0 minutes, respectively, with complete baseline separation between the two compounds. Conditions for each compound's MRM are listed in **Table 1**.

2.2.4 Data Collection

The kidney transplant charts and laboratory record of included patients were reviewed retrospectively to extract pertinent information (demographic, clinical, and laboratory data). Data sources included documents available in the multi-organ transplant patient charts and clinical databases: the solid organ transplant clinical database (REDCap), the Provincial Renal Agency's information system (Patient Records and Outcome Management Information System; PROMIS), and BC Children's Hospital clinical health information system (PowerChart). Twenty-four-hour lactate and glucose levels is a time-averaged mean value based on the repeated measurements of each substance collected over the first day after transplantation. The effectiveness of dialysis was quantified using Kt/V (where K is the dialyzer clearance, t is the duration of dialysis, and V is the urea distribution volume), which is a metric commonly used to measure the adequacy of both hemodialysis and peritoneal dialysis treatment (45).

2.2.5 Statistical Analysis

All statistical analyses were completed in R and results with a $p < 0.05$ were considered statistically significant. Parametric variables are presented as mean (standard deviation (SD)) and nonparametric variables are presented as median (interquartile range (IQR)). Descriptive statistics were used to describe oxythiamine and thiamine levels. A Wilcox signed rank test was conducted to confirm the return of oxythiamine to normal levels post-transplant. Oxythiamine levels were tested separately for association with severity of lactic acidosis and hyperglycemia in the first 24 hours post-transplant using the Pearson correlation coefficient. The Kolmogorov-

Smirnov test was performed to assess the normality of the sample distribution. A Kruskal-Wallis one-way analysis of variance was performed to compare oxythiamine levels partitioned by different dialysis modalities (HD, PD, and no dialysis (CKD)), followed by a post-hoc analysis using Dunn's test with p-values adjusted using the Benjamini-Hochberg method. Patients with a weekly Kt/V of < 2 were classified as being poorly dialyzed (45). A Mann-Whitney U test was used to compare oxythiamine levels partitioned by dialysis adequacy ($Kt/V \geq 2$ or < 2). A sensitivity analysis was performed, excluding two high oxythiamine outliers.

2.3 Results

A total of 48 patients were eligible for study inclusion. One patient was excluded from the analysis for being an extreme outlier. Their post-transplant oxythiamine was 25 nM, over a 12-fold increase from pre-transplant levels, giving indication of a possible sample or instrument error. Of the remaining 47 patients, 43 had pre-transplant serum samples and 37 had follow-up post-transplant serum samples available for testing. Relevant baseline cohort characteristics are reported in **Table 2**. The majority of patients were male (68%) with ages ranging from 1 to 19 years (mean 11.5 years). Most patients were on dialysis at the time of transplant (66%), with about twice as many patients on PD than HD. Donor type was approximately equally distributed across our study cohort.

2.3.1 Thiamine and oxythiamine levels before and after transplantation

Serum oxythiamine levels were not normally distributed amongst our patient cohort ($p < 0.001$, Kolmogorov-Smirnov normality test). For the 37 patients who had both pre- and post-transplant serum samples available for testing, there was a 60% reduction in median oxythiamine levels from 0.35 nM (IQR 0.24, 0.58) to 0.14 nM (IQR 0.06, 0.24) pre- and post-transplant, respectively ($p < 0.001$, CI 0.11–0.36, Wilcoxon Signed Rank Test) (**Figure 1**).

2.3.2 Oxythiamine and relation to key symptoms of functional thiamine deficiency

There was a strong positive association between 24-hour glucose and 24-hour lactate levels (Pearson's $r = 0.70$, $p < 0.001$). Serum oxythiamine was associated with 24-hour lactate levels (Pearson's $r = 0.38$, $p = 0.02$), but not as closely associated with 24-hour glucose levels (Pearson's $r = 0.30$, $p > 0.05$) (**Figure 2**). There were two distinct outliers with high pre-transplant serum oxythiamine levels (2.03 nM and 2.24 nM), one of which presented post-transplant with clinically manifest Shoshin beriberi syndrome (2.03 nM). The other did not manifest any clinical adverse effect. A sensitivity analysis excluding the two high oxythiamine levels indicates that the association with both 24-hour lactate and 24-glucose is driven principally by these outliers (Pearson's $r = -0.13$, $p = 0.4$ and Pearson's $r = 0.09$, $p = 0.5$; 24-hour lactate and 24-hour glucose, respectively).

2.3.3 Serum oxythiamine differs by dialysis modality

Median oxythiamine levels differed by modality, measuring 0.67 nM (IQR 0.31, 0.74), 0.34 nM (IQR 0.28, 0.56), and 0.25 nM (IQR 0.17, 0.38) for PD, HD and CKD, respectively ($p = 0.05$, Kruskal-Wallis Rank Sum Test). Following a post-hoc test, a difference was identified in pre-transplant serum oxythiamine levels between the PD and CKD groups ($p < 0.05$, Dunn's Test for Multiple Comparisons) (**Figure 4**).

2.3.4 Serum oxythiamine is associated with dialysis adequacy

The effectiveness of dialysis was quantified using Kt/V. Patients with a $Kt/V < 2$ were considered poorly dialyzed (45). Serum oxythiamine levels were negatively associated with dialysis adequacy (Pearson's $r = -0.44$, $p = 0.02$). Median oxythiamine levels were higher in patients with poor dialysis adequacy (0.92 nM (IQR 0.51, 1.01) vs. 0.40 nM (IQR 0.24, 0.51) with $Kt/V \geq 2$; $p < 0.01$, Mann-Whitney U test) (**Figure 4**). A sensitivity analysis excluding the

two high outliers remained significant for dialysis adequacy ($p = 0.03$, Mann-Whitney U test). Nine of the ten patients with poor dialysis adequacy were on PD, including both outliers.

2.4 Discussion

In this study, we were able to confirm that the patient in our pediatric cohort who developed Shoshin beriberi syndrome post-surgery had markedly high serum oxythiamine —a 5-fold increase in comparison to median oxythiamine levels— in the immediate pre-transplant period (172). Serum oxythiamine levels were correlated with patients' lactate levels 24 hours after transplant, but patients with more modest elevation did not manifest overt clinical signs of functional thiamine deficiency. Patients on peritoneal dialysis were at an increased risk of having higher oxythiamine levels, as were patients who had low dialysis adequacy. High oxythiamine levels appear, therefore, to be more prevalent in patients where uremic toxins are not being cleared as effectively. In our cohort, those were predominately patients on peritoneal dialysis.

All patients undergoing renal transplantation are supplemented with a multivitamin that includes 5 mg of thiamine, suggesting the patient who developed Shoshin beriberi syndrome had functional thiamine deficiency with oxythiamine toxicity. To the best of our knowledge, this is the first report to confirm oxythiamine toxicity in a child. Similar episodes of presumed functional thiamine deficiency have been reported in adult transplant recipients (173, 174). Functional thiamine deficiency has been well-documented in ESRD patients and occurs mechanistically through the inhibition of transketolase activity by uremic toxins (167, 175). Zhang et al. have demonstrated that oxythiamine is directly responsible for transketolase activity inhibition (159). Oxythiamine is thought to be primarily of dietary origin, resulting from acidic thiamine-rich foodstuffs cooked at high temperature; however, an altered gut microbiome in ESRD patients may also be associated with abnormal oxythiamine production (165).

Of the two patients with markedly high oxythiamine levels, only one of them manifested clinical thiamine deficiency; therefore, high oxythiamine levels alone may not be sufficient to precipitate functional thiamine deficiency. After excluding these two cases, we were not able to identify a "forme frutste" of oxythiamine toxicity at lower levels of oxythiamine elevation. That included identifying milder fluctuations in lactic acidosis and hyperglycemia in the immediate peri-operative period that were not strongly associated with the level of oxythiamine pre-transplant. Thiamine transporters may be downregulated in CKD, which has been previously demonstrated in animal models (167). This downregulation would result in impaired vitamin absorption and, theoretically, would subject this population to an increased susceptibility for oxythiamine toxicity. However, if thiamine absorption is indeed diminished, there would likely be interindividual variability and patients may display vastly different levels of impaired thiamine absorption. If sufficient levels of the vitamin are still present, thiamine may be able to outcompete OTPP and prevent the manifestation of functional thiamine deficiency. In addition, the level of surgical stress a patient endures may differ and also impact bodily thiamine supply. Critically ill patients who underwent substantial physical stress, such as a major operation, were found to have depleted thiamine levels, but the crude difference in B vitamin levels pre- and post-surgery varied widely between individuals (176). Interestingly, apart from thiamine, body composition may also be an important predictor of post-operative lactate levels (177).

We identified dialysis adequacy as being strongly associated with oxythiamine levels, which aligns with the premise that some ESRD patients are at higher risk for oxythiamine toxicity related to the level of dialysis clearance. Patients on PD were more likely to have low adequacy, which included both patients with the highest serum oxythiamine levels. This raises the concern that children on PD may be more at risk for oxythiamine toxicity. These findings are

in contrast to adult dialysis studies, which identified 15-fold increased oxythiamine levels in adult HD patients compared with normal, and 4-fold in adult PD patients (159). These differences may be related to the choice of modalities for PD delivery in children compared to adults, with use of continuous cycler for PD and more frequent and longer runs for hemodialysis. The smaller number of cases with each modality precludes a more in-depth analysis of this finding.

Early allograft kidney injury and hemodynamic instability imperil patient safety, extends hospitalization and need for critical care resources, and may negatively impact long-term allograft outcome (178-180). Functional thiamine deficiency associated with oxythiamine toxicity has only been recently recognized, but is readily amendable to treatment. High-risk patients may be identified by the risk factors outlined: those with malnutrition, sub-optimal dialysis adequacy, and children who are undergoing peritoneal dialysis. Clinical decompensation from functional thiamine deficiency should be considered preventable, since thiamine is a water-soluble vitamin and generally safe to administer. Patients at high risk could be safely treated with high-dose thiamine prior to surgery to prevent serious morbidity post-transplant (172, 173).

We recognize some limitations posed by this study. Serum samples were used, based on their availability retrospectively for analysis, whereas prior analysis in an adult cohort of ESRD patients uses plasma samples (159). Internal validity of these measurements was confirmed, however, by demonstrating significant decline in serum oxythiamine post-operatively in our cohort. Ideally, we would have measured thiamine levels in tandem with oxythiamine. However, thiamine is measured by its active form thiamine diphosphate and extracted from erythrocytes, requiring whole blood samples that were not available for analysis. Unfortunately, this limited our ability to determine on the role of thiamine supplementation in our pediatric cohort. Lastly, it

is important to acknowledge that our sample size was limited and presenting with Shoshin beriberi syndrome following transplant surgery is a rare phenomenon (169, 172-174). Nonetheless, this case series is the first to examine in depth the link between oxythiamine toxicity and perioperative risk in pediatric transplant recipients and its relationship with dialysis adequacy.

Thiamine sufficiency is difficult to determine, and clinical testing is not currently a part of standard care. However, as previously mentioned, thiamine supplementation is already integrated into standard care for kidney transplant patients. Oxythiamine is not a clinically available test. Although this study is the biggest to date in children, it does not provide conclusive results. A future multi-centre study which identifies patients with high oxythiamine levels and randomizes them to treatment with thiamine at the time of transplantation is required. In the interim, clinicians should consider pre-emptive treatment for patients who begin to manifest early signs of toxicity post-transplant or prophylactic treatment for patients who, as identified by this study, could be at higher risk.

In conclusion, we found that oxythiamine levels are associated with dialysis adequacy at transplant. Pediatric patients on PD with no residual kidney function and low dialysis adequacy are particularly vulnerable to manifesting high oxythiamine levels. These patients may be at an increased risk for developing acute Shoshin beriberi syndrome in the early post-transplant period. High dose thiamine supplementation is deserving of consideration as a preventative measure for functional thiamine deficiency with oxythiamine toxicity, which may be precipitated by the stress endured during transplant surgery and subsequent ischemia-reperfusion injury.

2.5 Tables & Figures

Table 1. MRM conditions by analyte

Analyte	MRM (m/z)	Product Ion (m/z)	Cone Voltage (V)	Collision Energy (V)
^{13}C-thiamine	267.9	121.8	25	16
thiamine	264.9	121.9	25	16
oxythiamine	266	122.9	32	19

Table 2. Baseline characteristics of pediatric cohort at transplantation (**n=47**)	
Sex	
Male	32 (68)
Age at transplant (years)	
Mean (±SD)	11.5 ± 5.75
Etiology	
Non-glomerular disease	32 (68)
Donor Type	
Living donor	24 (51)
Modality Type	
Hemodialysis	10 (21)
Peritoneal Dialysis	21 (45)
No Dialysis	16 (34)
Dialysis Length (days)	
Mean (±SD)	689 ± 417
First transplant	44 (94)
Cold-ischemia time (minutes)	
Mean (±SD)	300 ± 258
Warm-ischemia time (minutes)	
Mean (±SD)	29 ± 13

Variables are expressed as crude numbers (percentages), unless otherwise noted. BMI, body mass index

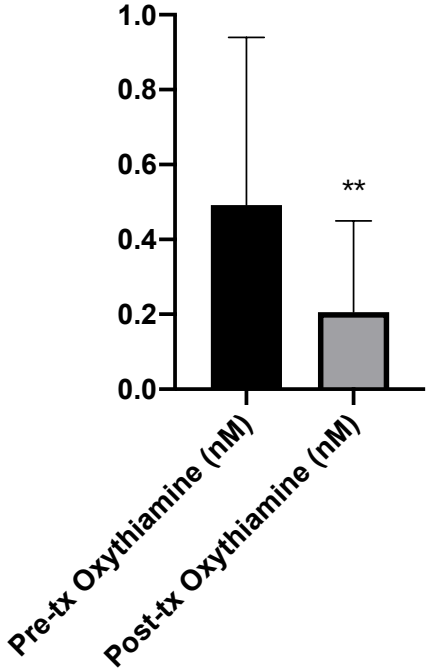


Figure. 1 Comparison of serum oxythiamine levels in patients pre- and post-transplant. A total of 37 patients had both samples available for testing. There was a 60% reduction in median oxythiamine levels from 0.35 nM (IQR 0.24, 0.58) to 0.14 nM (IQR 0.06, 0.24) pre- and post-transplant, respectively ($p < 0.001$, CI 0.11–0.36, Wilcoxon Signed Rank Test).

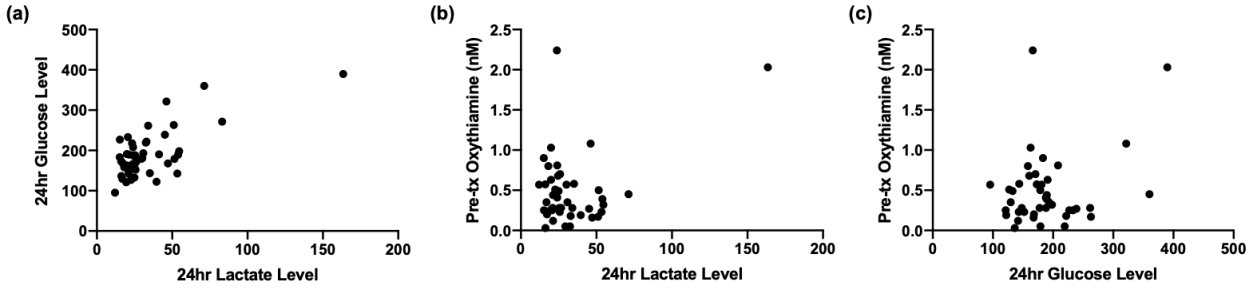


Figure. 2 Correlation analysis with clinical indicators of functional thiamine deficiency (lactic acidosis and hyperglycemia) in pediatric kidney transplant patients. **(a)** Twenty-four-hour lactate and glucose levels were strongly associated (Pearson's $r = 0.70$, $p < 0.001$); **(b)** Serum oxythiamine was associated with 24-hour lactate levels (Pearson's $r = 0.38$, $p = 0.02$); **(c)** Oxythiamine levels were not as closely associated with 24-hour glucose levels (Pearson's $r = 0.30$, $p = 0.05$).

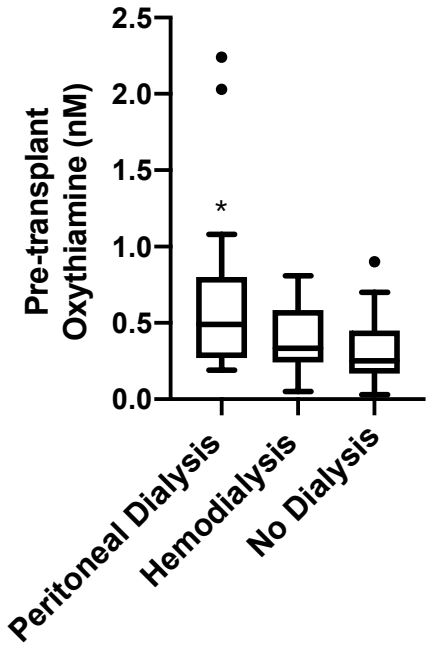


Figure. 3 Levels of pre-transplant serum oxythiamine differed when partitioned by dialysis modality. Median oxythiamine levels measured 0.67nM (IQR 0.31, 0.74), 0.34nM (IQR 0.28, 0.56), and 0.25nM (IQR 0.17, 0.38) for PD, HD, and no dialysis, respectively (Kruskal-Wallis Rank Sum Test, $p < 0.05$). Following a post-hoc Dunn's Test for Multiple Comparisons, a difference ($p < 0.05$) was found between the PD group and the group that did not undergo dialysis treatment.

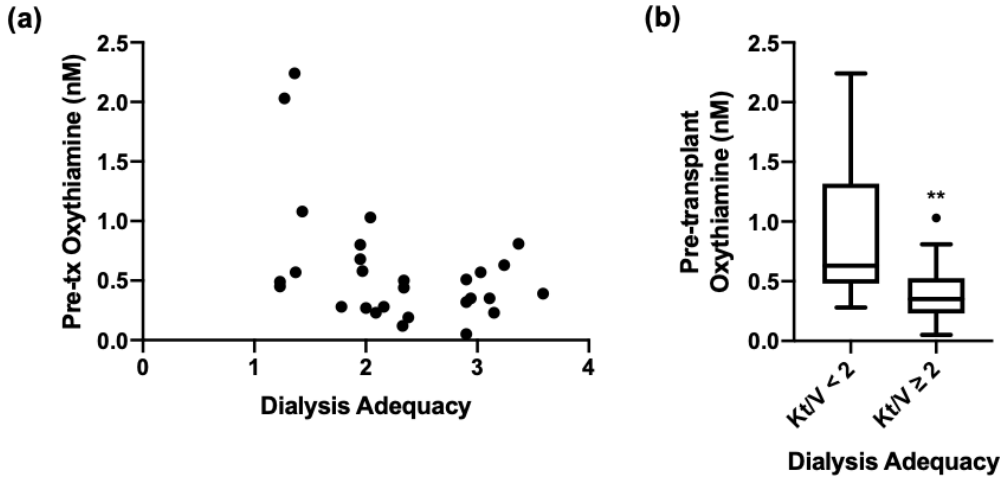


Figure. 4 Levels of pre-transplant serum oxythiamine differed by dialysis adequacy. Adequacy was measured using Kt/V. Patients with a Kt/V of below 2 were considered to have poor dialysis adequacy. **(a)** Serum oxythiamine was negatively associated with dialysis adequacy (Pearson's $r = -0.44$, $p = 0.02$); **(b)** Median oxythiamine levels were higher in patients with poor dialysis adequacy (0.92nM (IQR 0.51, 1.01) vs. 0.40nM (IQR 0.24, 0.51) with $Kt/V \geq 2$; Mann-Whitney U Test, $p < 0.001$).

Chapter 3:

Investigating serum immunometabolomic profiles associated with kidney transplant alloimmune outcomes

3.1 Introduction

Kidney transplantation is the most effective treatment for people with end-stage kidney disease. After transplant, the overriding preoccupation of transplant recipients and their caregivers is the risk of rejection and subsequent graft failure. Survival of the new organ hinges on achieving a state of adapted alloimmune quiescence with strict adherence to a lifelong immunosuppressive regimen. A careful balance must be achieved between preventing rejection and avoiding adverse consequences of excess immunosuppression, including the risk of infection, malignancy, and drug-specific toxicities (181). Despite immunosuppression, the emergence of CR in some cases represents fundamentally a failure to achieve stable immune adaptation and is heralded by the development of *de novo* donor-specific HLA antibodies (182). The extent of HLA mismatching, instability of immunosuppression exposure and early rejection are known to play critical roles, but we lack a clear understanding of the different individual patient factors that predisposes some but not others to CR. The challenge is that currently, we lack a reliable method to predict individual rejection risk, and to permit patient-specific tailoring of immune suppression or rejection monitoring for to those at highest risk.

We hypothesized that differences in the host metabolic environment (also known as the metabolome) at transplantation directly influence early alloimmune differentiation and are associated with post-transplant alloimmune outcomes in kidney transplant recipients. The aim of this study was to determine whether we can characterize differences in the pre-transplant serum metabolome (metabotypes) of transplant recipients associated with CR.

What is well known, but poorly understood, is that factors related to host metabolism at the time of transplant play a critical role to influence allograft outcome. This includes the associations of both prolonged uremia (183, 184) and malnutrition (185) to adversely affect graft survival. While uremia and malnutrition result in many diverse derangements of host metabolism, very specific changes may also be important. This includes higher levels of free fatty acids pre-transplant (186) and non-esterified fatty acids early post-transplant (187) that have been associated with substantially improved graft survival. From a genetic and developmental perspective, children are at higher risk for developing dnDSA than adults (182, 188), as are females (189), which suggests innate differences in alloimmune responsiveness. These many gross and subtle differences in metabolism highlight the pivotal role of the host environment at the time of kidney engraftment in predisposing the early alloimmune response and T cell differentiation toward either adaptation or inflammation.

Optimal host alloimmune adaptation requires both suppression of effector responses and induction of regulatory immune cell phenotypes that together maintain alloimmune quiescence. The latter is a dynamic process that is heralded by the expansion of regulatory T cells (Tregs) (190, 191). Tregs are important in the resolution of immune activation, and are a suppressive subset of CD4+ T cells with characteristic expression of interleukin 2, receptor alpha chain CD25, and transcription factor forkhead box protein 3 (FOXP3) (192). The relative balance between regulatory and effector T cell responses within the allograft is implicated as a marker of graft outcome (193-195). Increased Tregs in circulation (196, 197) and the persistence of allograft-infiltrating peripheral Tregs (198) are associated with better long-term graft outcomes.

The ways in which the host metabolic environment influences early T cell differentiation is now better understood. Tregs become functionally specialized in relation to their environment

and the local milieu of metabolites is an important modulator of their phenotype and function (199, 200). Tregs preferentially utilize fatty acid oxidation (201), in contrast to effector T cells (e.g. Th17), which rely heavily on glycolysis (202, 203). While glycolysis inhibition favours Treg differentiation (204), lipid metabolism and substrate availability are considered important factors regulating T cell fate decisions (186, 187, 201, 203-205). Vitamin A and D levels influence the differentiation of peripheral Tregs (206, 207) (208, 209) and Treg stabilization during inflammation (202). Dietary metabolites that activate the aryl hydrocarbon receptor (AHR) can tilt T cells to either Th17 or Treg differentiation in a ligand-dependent manner (210-212). Metabolites such as kynurenine, indole-3-carbanole and 3,3'-diindolylmethane (via AHR) promote production of Tregs and inhibition of Th17 development (213, 214). The impact of the host metabolome on graft outcome are therefore likely mediated via their influences on early alloantigen-specific T cell differentiation events, predisposing the outcome toward either Treg or effector responses.

The field of metabolomics seeks to understand how patterns of metabolite levels relative to one another (known as metabotypes) can provide a complete phenotypic representation of different states of health or disease (215, 216). The metabotype effectively summarizes the integration of genetic, epigenetic and environmental influences on physiological processes, in a given tissue. Metabolite measurements can be incorporated within existing clinical assays and therefore have great potential as a tool for patient risk-stratification and monitoring of therapeutic responses. Metabolomic methods have long been used for diagnosing inborn errors of metabolism (IEM) using samples derived from biospecimens such as urine, blood, or cerebrospinal fluid (217). These techniques have also been applied to diagnosis of heart failure (218), staging of CKD (219, 220) and detection of AR (221, 222). Immunometabolomics is an

emerging field focused more specifically on understanding metabotypes associated with immune cell differentiation and responses. In the setting of transplantation, as with other immune processes, it is expected that the host metabolome will influence the early alloimmune response after transplantation. This information is expected to provide robust risk prediction for early and late alloimmune risk, but has not yet been studied comprehensively.

Herein, we applied targeted metabolomic techniques to develop a comprehensive characterization of the serum metabolome of kidney transplant recipients, and elucidate patterns of change associated with risk of CR.

3.2 Methods

Our study cohort comprised of adult patients who underwent kidney transplantation surgery at a single tertiary transplant centre between January 2008–March 2012. We used a nested case-control study design with samples sourced retrospectively from the British Columbia Transplant Biolibrary (BCTB). The purpose of the study was to use metabolomic techniques to analyze baseline serum samples from these patients and to assess the predictive value for subsequent graft loss.

Study subjects had pre-transplant serum samples collected as part of the BCTB: a transplant biobank program at the clinical immunology lab in Vancouver General Hospital. The BCTB, established in 2008 as part of a national Genome Canada study, contains sequential serum samples from more than 600 renal transplant recipients, the majority of which have stored pre-transplant (i.e., baseline) serum samples available. Complete electronic medical records were accessible through PROMIS —a centralized provincial-wide registry and clinical information system regulated by the British Columbia Renal Agency. Individuals were invited to participate in the biobank because they were preparing to or had already received a kidney transplant. Each

patient was asked to provide approximately 4 extra tubes of venous blood (i.e., 15 ml or 3 teaspoons). Research samples (for biobanking) were collected simultaneous to blood samples required for mandatory clinical testing. Samples were collected either prior to the time of transplantation or when the patient was seen in-hospital for a post-transplant clinic visit. Individuals included in the biobank were over the age of 18, receiving a kidney transplant from a living or deceased donor, and able to provide informed consent.

Our study was approved by the institutional review board at the University of British Columbia (H19-01908). The study was classified as minimal risk because samples were retrospectively derived from the BCTB, and, at the time of initial sample retrieval, all biobank participants provided informed consent, which encompassed the use of their biological samples for future transplant-related research.

3.2.1 Sample Selection

Patients were excluded from study inclusion if they lacked a baseline sample, were HLA identical, or had less than one year of allograft survival. Cases were defined as graft loss attributable to CR, including active cellular, antibody, or mixed types of rejection. Controls were defined as patients free from CR and with stable allograft function (inclusive of those who died with a functioning graft). Stable allograft function was identified by either a minimum graft survival of eight years or stable post-transplant eGFR, i.e., a near-constant ($1 > x > -1$) eGFR slope. Samples were matched 2:1 (control: case) under the following hierarchy: follow-up time, immunosuppression regimen, induction immunosuppression, age, donor type, and sex.

3.2.2 Direct Flow Injection and Liquid Chromatography Tandem Mass Spectrometry

All samples were sent to The Metabolomics Innovation Centre (TMIC) for targeted quantitative metabolomics testing. Serum samples were analyzed using a combination of direct

injection mass spectrometry (MxP500 Kit) and reverse phase liquid chromatography tandem mass spectrometry (LC/MS-MS). The MxP500 Kit is a commercially available assay, purchased from BIOCRATES Life Sciences AG (Austria). When combined with the ABI 5500 Q-Trap (Applied Biosystems/MDS Sciex) mass spectrometer, the MxP500 Kit can be used for the targeted identification and quantification of 630 distinct endogenous metabolites: amino acids, acylcarnitines, biogenic amines, bile acids, organic acids, steroids, diglycerides (DGs), triglycerides (TGs), phosphatidylcholines (PCs), lysophosphatidylcholines (LPCs), sphingomyelins (SMs), ceramides (Cers), cholesteryl esters (CEs), and sugars. The method used combines the derivatization and extraction of analytes with selective mass-spectrometric detection using MRM. Isotope-labeled internal standards were used for metabolite quantification. The kit included a 96 deep-well plate with a filter plate attached, sealing tape, and the reagents as well as the solvents required for assay preparation. The first 14 wells were loaded as follows: one blank, three zero samples, seven standards, and three quality control samples (provided with each Kit). Serum samples were analyzed with adherence to the protocol outlined in the user manual. In short, samples were thawed on ice, vortexed, and then centrifuged at 13,000 g. For each sample, 10 µL was loaded onto the centre of the filter paper on the upper 96-well plate and dried with a stream of nitrogen. As a derivatization step, 20 µL of a 5% solution of phenyl-isothiocyanate was added and samples were subsequently incubated. With the use of an evaporator, filter spots were dried once more. Then, 300 µL of methanol containing 5 mM ammonium acetate was added to extract the metabolites. Metabolite extracts were collected by centrifugation into the lower 96-well plate and diluted with Kit MS running solvent. Mass spectrometric analysis was performed on a API5500 Qtrap® tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) equipped with

a solvent delivery system. The mass spectrometer received the samples via liquid chromatography followed by direct injection. The Agilent 1290 UHPLC and AB SCIEX QTrap 5500 instruments were used for all HPLC-MS analyses. The analytical column was provided with the purchased MxP500 Kit. The mobile phases were as follows: (A) 2,000 mL water and 4 mL formic acid and (B) 2,000 mL acetonitrile and 4 mL formic acid. All solvents were LC/MS grade. Gradient chromatography was employed at a flow rate of 0.8ml/min with initial conditions of 100% solvent A held for 15 seconds. Further details on the elution gradients are provided in **Table 1.** Column oven temperature was maintained at 50°C throughout. A System Suitability Test was completed to check the UHPLC-MS system performance before Kit preparation. The Biocrates MetIQ software regulated the entire assay workflow inclusive of sample registration, automated calculation of metabolite concentrations, and data export into other analysis programs. A targeted profiling scheme was used to quantitatively screen for known small molecule metabolites using MRM, neutral loss, and precursor ion scans.

3.2.3 Volcano Plot

Metabolite concentrations derived from LC-MS were visualised using a volcano plot to explore the relationship between cases and controls. To deal with measurements below the limit of detection (LOD), they were replaced by LOD/√(2) using Microsoft Excel. The LOD was defined as three times the background within the zero samples (phosphate-buffered saline). Data were assigned to a sample type (“case” or “control”). The resulting data were imported into the R software environment for statistical computing. Data columns with near-constant metabolite concentrations (coefficient of variation (CoV) < 0.46) were excluded from further analysis. Data were log2 transformed and a volcano plot was constructed as an initial exploratory analysis to screen for candidate metabolites in subsequent classifier development. Student’s t-test was used

to test for statistical significance. Metabolites with an absolute fold-change >1 and p-value <0.05, without Bonferroni adjustment, were labeled to identify potential high-impact metabolites: metabolites with fold-change >1 marked by orange and metabolites with p-value <0.05 marked by red.

3.2.4 Analysis 1 – Developing a Classifier Using All Measured Metabolites

The objective of the analysis was to fit principal components analysis (PCA) and partial least squares (PLS) models to baseline blood metabolomic data and assess the predictive value for subsequent graft loss.

PCA is a data reduction tool commonly used for exploratory data analysis. PCA uses an unsupervised learning approach for prediction models. Dimension reduction with PCA can be used to parse out pertinent information (summarized by a handful of components) from noise. However, due to its unsupervised nature, it does not use information from the dependent variable (y) to compute PCA loadings or scores, i.e., PCA identifies patterns based on maximizing variation without considering pre-determined phenotypic differences among samples (223, 224). Therefore, it may be more difficult to identify important patterns using this approach. However, if an association is found, PCA results are less prone to over-fitting.

Principal component regression (PCR) is a combination of PCA and multiple linear regression. The linear combinations used in PCR are the principal component scores of the x-variables; however, the number of latent variables are optimized to maximize the capacity to predict the y-variable (224).

Similar to PCR, PLS combines data reduction with multiple linear regression; however, it uses a supervised learning approach in defining loadings on latent factors. That is, in PLS, latent variables are identified by having maximum covariance between scores in x-space and the

outcome of interest. Therefore, PLS components account for relation to the y-variable, and the resulting model predicts a property, y, using information from both the dependent and independent variables (224).

A classifier is developed by using information from x-variables with known phenotypic differences (grouping characteristics). The ultimate goal is to use the classifier as a method to reliably predict these grouping characteristics in new observations (samples). There are several different approaches to classifier development, including various linear regression methods, linear discriminant analysis (LDA), and logistic regression (LR), all of which focus on maximizing the separability among known categories. For high-dimensional datasets, data reduction techniques, such as PCA or PLS, can summarize information contained in the x-variables by intermediate latent variables, which are subsequently used in regression, LDA, or LR (224).

A model's ability to fit the given data is directly dependent on complexity. A highly complicated model can fit almost any data; however, an overfitted model lacks generalizability, and its ability to accurately predict new cases is diminished. For regression models, higher complexity can result from increasing either the number of variables or the number of components. In the context of PCA or PLS, there are multiple methods to estimate optimal model complexity (i.e., number of components): cross-validated area under the curve (AUC), Q^2 statistic (essentially the cross-validated R^2), root mean square error prediction (RMSEP)(224).

Although, PCR and PLS can handle several hundred regressor variables, there are arguments against using all available variables, mainly because a larger number can lead to problems with overfitting, predictive performance, and interpretation (225, 226). There are multiple strategies for variable selection. For all, one applies algorithms for variable selection to

identify the subset of candidates potentially worthy of further evaluation. An additional strategy for choosing potential regressor variables is to identify candidates which have a univariate association with the y-variable ("bivariate screening"). However, variables with a weak correlation may still be important if they can describe the variation of y not captured by other regressor variables. With no general rule for the best method of variable selection, selection needs to be an iterative process (224).

Here, we start by developing PCR and PLS models using all measured metabolites, i.e., using all possible x-variables. Hereafter, this approach will be referred to as the all metabolite model.

In each serum sample, 630 distinct metabolite concentrations were measured. Data columns with near-constant metabolite concentrations (CoV < 0.46) were excluded from further analysis. Metabolite data were $\log_{10}$ transformed to account for a right-skewed distribution commonly seen with laboratory measurements, which typically have a floor or minimum value. Samples were randomly split into separate training and test datasets with a distribution of 75% training set to 25% test set. Stratified random sampling was employed to ensure equal distribution of cases and controls among each group. Receiver Operator Characteristic (ROC) plots were used to illustrate test characteristics. The AUC was calculated and reported with the corresponding 95% confidence intervals (CI). All PCR/PLS analyses were conducted in the R software environment for statistical computing using the pls package (227).

Developing the Predictive Model Using Principle Component Regression (PCR) and Projection on Latent Structure Discriminant Analysis (PLS-DA)

Best practice is to use different samples for calibration (training) and test (prediction) because, even with the use of statistical tools to avoid overfitting (e.g. Q^2 statistic), the model

prediction performance may be too optimistic. Results from the external validation (either by a test set or leave-one-out cross validation (LOOCV)) are what indicate the discrimination performance of the model (classifier) on data that were explicitly excluded from the training procedure. Two methods were used to develop the model (224):

Training/test Split

The data was split into training and test sets to obtain an estimation of the predictive performance for new cases. This method hinges on having enough samples (of both cases and controls) to provide adequate statistical power in each group, and therefore is more reliable with larger sample sizes. As a general rule of thumb, to develop a robust multivariate classifier, ~25-30 samples are required in each phenotypic class. First, the optimum complexity of the model was estimated, i.e., the optimal number of PCR or PLS components was identified through cross validation (*vide infra*). Then, the model was constructed from the training dataset using the predetermined number of optimal components, and the fitted model was applied to the test set. Discriminant scores were calculated for each sample, and then a ROC curve was used to illustrate the overall sensitivity and specificity.

Leave-one-out-cross-validation (LOOCV)

LOOCV provides a reasonable initial estimate of model performance on test data, and this validation strategy can be used to create models from a relatively small number of samples. LOOCV was used to split the data into training and validation sets where observations are withheld one-at-a-time to test the classifier trained on the remaining observations. First, the optimum complexity of the model was estimated, i.e., the optimal number of PCR or PLS components. For PCR, the optimal number of components was determined using the Q^2 statistic, which measures the goodness of fit for predicting case vs. control status on a 0-1 scale. For PLS,

the optimal number of latent variables was determined using the cross-validated AUC and further confirmed with the Q^2 statistic and RMSEP. Then, the model was constructed from the training set using the predetermined number of optimal components, and the fitted model was applied to the corresponding validation set. Using either regression in PCR or discriminant analysis in PLS-DA, a single discriminant score was calculated for each sample, and then a ROC curve was used to summarize overall sensitivity and specificity.

3.2.5 Analysis 2 – Logistic Regression Model with Candidate Predictors

The objective of the analysis was to identify candidate predictors (from the metabolite data) based on univariate AUC criteria, develop a LR model using the these candidate predictors, and assess the predictive value of the model for subsequent graft loss.

Predictive Modelling with Logistic Regression

Inclusion of too many variables can compromise the performance of a regression model (228). Nevertheless, for this analysis, the goal was prediction accuracy. In a predictive model, accuracy supersedes parsimony as long as problems with collinearity and overfitting are avoided (228). Potential collinearity issues can be identified through variance inflation factors (VIFs). Generally, VIFs with an upper bound of 5-10 are acceptable (228). Overfitting can also compromise the generalizability of the predictive model, but can be averted by validation on external data either through cross-validation or withheld test data.

There are many procedures available to identify candidate predictors: backward selection, forward selection, stepwise selection, best subsets, and bivariate screening. Bivariate screening evaluates individual candidate predictors in single-predictor models, and candidates that meet predetermined criteria are identified for subsequent inclusion in regression models (229). Here,

we use bivariate screening to identify candidate predictors for a LR model and include all predictors that meet selection criterion in the final model.

Training/test Split

There was a concern that the total number samples may not be enough to provide adequate statistical power for a training/test split; therefore, the subsequent classifier training and test performance was performed as a proof of concept.

Samples were randomly split into separate training and test datasets for independent calibration and validation of the LR model. The sample distribution was 65% training set to 35% test set. Stratified random sampling was employed to ensure equal distribution of cases and controls among each group. As a first step, ROC curves were used to examine the relationship between individual metabolite concentration and sample phenotypes (case vs. control). The ROC curve plots sensitivity (true positive rate) versus 1-specificity (false positive rate) as the threshold is varied across the range of sample concentrations. In this analysis, the AUC served as a measure of overall discrimination, where the diagonal line with AUC = 0.5 represents chance agreement. As a result, failure of the 95% confidence interval to cross 0.5 represents statistical significance at $p = 0.05$. For this reason, candidate predictors were identified through bivariate screening, and metabolites with a lower AUC 95% CI > 0.51 were included in the LR model. Beta coefficients and odds ratios (e^b) were calculated for each regressor variable. The severity of multicollinearity was quantified by calculating the VIF of each predictor. The fitted model was then applied to training and test data, and ROC plots used to illustrate test characteristics for model predictions.

Fitting the Full Dataset

The model was also trained on the full dataset. As a first step, ROC curves were used to examine the relationship between individual metabolite concentration and sample phenotypes (case vs. control). As discussed above, the AUC served as a measure of overall discrimination for this analysis. Candidate predictors were identified through bivariate screening, and metabolites with a lower AUC 95% CI > 0.5 were included in the LR model. Beta coefficients and odds ratios (e^b) were calculated for each regressor variable. The severity of multicollinearity was quantified by calculating the VIF of each predictor. ROC plots were used to illustrate test characteristics for model predictions, and LOOCV was performed to assess the external validity of the fitted model.

3.2.6 Analysis 3 – Developing a Classifier Using the Top 60 Metabolites

The objective of the analysis was to fit PCA and PLS models to baseline blood metabolomic data and assess the predictive value for subsequent graft loss.

Here, we revisit PCR and PLS strategies and develop a model using the top 60 measured metabolites (ranked by univariate AUCs). Somewhere between the top 75-90 metabolites, the predicative signal was lost, as evidenced by a loss of significance in the cross-validation step. Therefore, the number 60 represents the top 10% of all measured metabolites, whilst remaining below the threshold where the signal dissipates. Hereafter, this approach will be referred to as the top 60 metabolite model.

First, metabolite data were $\log_{10}$ transformed to account for a right-skewed distribution and scaled by SD to ensure inter-comparability. Two approaches were used to develop the model: training/test split and full dataset/LOOCV. Both methods were previously described in

detail in the ‘all metabolite model’ section. All PCR/PLS analyses were conducted in the R software environment for statistical computing using the pls package (227).

Developing the Predictive Model Using Principle Component Regression (PCR) and Projection on Latent Structure Discriminant Analysis (PLS-DA)

Training/test Split

Samples were randomly split into separate training and test datasets for independent calibration and validation of the model. The sample distribution was 65% training set to 35% test set. Stratified random sampling was employed to ensure equal distribution of cases and controls among each group. As a first step, ROC curves were used to examine the relationship between individual metabolite concentration and sample phenotypes (case vs. control) in the training dataset. The AUC served as a measure of overall discrimination and candidate selection. In this analysis, the metabolites of the top 60 –representing 10% of total measured metabolites – univariate AUCs were identified and selected as variables in the subsequent PLS model. After the PLS model was created, it was validated in the independent test dataset.

Variable importance scores (VIP) were calculated as the weighted sum of absolute regression coefficients and quantifies the contribution of individual metabolites to the reduction in overall sum of squares in the optimal model. VIPs were then compared with the ones derived from the model trained on the full dataset.

Full dataset/LOOCV

The model was also trained on the full dataset. As a first step, ROC curves were used to examine the relationship between individual metabolite concentration and sample phenotypes (case vs. control), and the AUC served as a measure of overall discrimination and candidate selection. In this analysis, the metabolites of the top 60 –representing 10% of total measured

metabolites – univariate AUCs were identified and selected as predictors in the subsequent PCR/PLS models. After the PCR and PLS models were created, they were validated by LOOCV. The VIPs were calculated, and the correlation between VIPs and univariate AUCs was measured using Spearman's rank correlation rho and Kendall's rank correlation tau.

Moreover, the feasibility of a minimalist model was explored to determine which metabolites were essential to capture the differences between cases and controls. Initially, a minimalist VIP model was developed using the top 15 metabolites. However, to parse the signal further, the process was repeated with the top 13 metabolites and top 10 metabolites.

3.3 Results

3.3.1 Sample Selection

Forty-two patients with graft loss were identified. Thirty-seven individuals met case criteria and were included in the final study population: three patients experienced premature graft failure due to primary non-function (n=1) or recurrent disease (n=2), and two patients were not found in PROMIS.

Three hundred and sixty-five patients with ongoing graft survival were identified. Eighty-three individuals were included as controls in the final study population. Controls required a follow-up time equivalent to or longer than the corresponding case. As a result, patients with less than 922 days of follow-up (lowest follow-up time amongst cases) were excluded (n=6). Then, controls were matched by immunosuppression exposure at four weeks post-transplant. Three distinct immunosuppression groups were identified: (1) mycophenolate mofetil (MMF) and tacrolimus (TAC); (2) MMF, TAC, and prednisone; (3) cyclosporine A (CSA). Next, controls were matched by induction immunosuppression (basiliximab or antithymocyte globulin). Once controls had equivalent (or more) follow-up time and identical immunosuppression exposure to

their respective case, they were matched as closely as possible by the remaining criteria with the following priority rank: age (± ten years), donor type (living versus deceased), sex. If no suitable control met the ten year age criteria, the suitable control closest in age was chosen.

After controls were matched 2:1 based on the procedure described above (n=74), an additional nine controls with the longest follow-up time (three from each immunosuppression regimen group) were chosen. All 'additional controls' with a follow-up time of less than eight years had eGFR stability assessed to ensure stable graft function, as defined above (**Figure 1**).

Therefore, 120 patients were included in the total study population. Relevant baseline and demographic characteristics for the cohort are reported in **Table 2**. Overall, patients were more likely to be male (63%), and age at transplant ranged from 22 to 72 years. Most individuals were on dialysis at the time of transplant (84%), with the majority receiving hemodialysis treatment (73%). The average follow-up duration for the study population was 8.7 years. Baseline characteristics were approximately equally distributed across case and control groups.

3.3.2 Volcano Plot

A total of 630 metabolites were measured in the serum. Three hundred and seventy-two metabolites were retained after filtering and omitting columns with negligible variance (CoV <0.46). Variation in each metabolite was identified by fold-change of all the cases with respect to all the controls. No metabolites had an absolute fold-change >1. Six metabolites were significantly different ($p < 0.05$, Student's t-test) between the case and control groups; cases had an increased concentration of p-Cresol-SO4 and a decreased concentration of the other five significant metabolites (alpha-AAA (amino adipic acid), diacylglycerol (16:0_16:1), glucosylceramide (d18:1/18:0), triacylglycerol (17:1_38.6), and triacylglycerol (18:3_38:5)(**Figure 2**).

3.3.3 Analysis 1 – Developing a Classifier Using All Measured Metabolites

In total, there were 120 serum samples included in the analysis. Of the 630 metabolites measured, 372 metabolites met the CoV criteria and were included in the subsequent analysis. The training data included 54 controls and 24 cases, and the test data included 29 controls and 13 cases. The training/test split suffers from small case numbers in both subsets, and there was a particular concern that the test dataset may be too small to assess out-of-sample (external) performance of the classifier.

Principle Component Regression (PCR)

For PCR using the full dataset (n=120), there was no evidence of phenotype separation in three dimensions (**Figure 3a**). A single component model was identified as the optimal number of latent variables. There was no difference between the mean discriminant scores in the case versus control groups (Welch's t-test, p=0.5). The corresponding ROC curve had a non-significant AUC of 0.54 (95% CI 0.43-0.65)(**Figure 3b**).

Projection on Latent Structure Discriminant Analysis (PLS-DA)

Training/test Split

In the training dataset (n=78), the optimal PLS model had one latent factor. There was a difference between the mean discriminant scores in the case versus control groups (Welch's t-test, p=0.03) on training data. The corresponding ROC curve had an AUC of 0.66 (95% CI 0.52-0.79)(**Figure 4a**). For external validation, the model was tested in an independent test set (n=42). There was no difference between the mean discriminant scores in the case versus control groups (Welch's t-test, p=0.9), and the ROC plot had a non-significant AUC of 0.52 (95% CI 0.31-0.73) (**Figure 4b**).

Fitting the Full Dataset (validation with LOOCV)

For PLS using the full dataset (n=120), there appeared to be some evidence of separation in three dimensions (**Figure 5a**), and the cross-validated AUC indicated the optimal model had three latent factors. However, the Q^2 statistic indicated the optimal model complexity had one latent variable. Ultimately, to avoid overfitting, the model was developed in accordance with the Q^2 statistic (optimal complexity = 1 latent factor). There was again a difference between the mean discriminant scores in the case versus control groups (Welch's t-test, p=0.01). The corresponding ROC curve had an AUC of 0.64 (95% CI 0.54-0.75)(**Figure 5b**). For validation purposes, LOOCV was performed and demonstrated no difference between the mean discriminant scores in the case versus control groups (Welch's t-test, p=1). The corresponding ROC plot had a non-significant AUC of 0.51 (95% CI 0.39-0.62)(**Figure 5c**).

Interpretation

There was no evidence of signal in the unsupervised PCR model or in the supervised PLS model. For PLS, the model created using the training dataset failed validation in the external test dataset. Additionally, the weak signal in the full dataset was spurious (could not be confirmed by LOOCV) and appeared attributable to overfitting.

3.3.4 Analysis 2 – Logistic Regression Model with Candidate Predictors

Training/test Split

The training data included 54 controls and 24 cases, and the test data included 29 controls and 13 cases.

In the univariate ROC analysis, which examined the relationship between individual metabolite concentration and sample phenotype, eight metabolites met the screening criterion (lower AUC 95% CI > 0.51)(**Table 3**). Then, these eight metabolites were used as the candidate

predictors for the LR model, and each variable had a VIF of less than four (**Table 3**). The corresponding AUC for the model was 0.81 (95% CI 0.71-0.91)(**Figure 6a**). The external validation in the test dataset had a significant AUC of 0.68 (95% CI 0.50-0.86)(**Figure 6b**).

Fitting the Full Dataset

There were 120 serum samples included in the analysis. In the univariate ROC analysis, which examined the relationship between metabolite concentration and sample phenotype, six metabolites were identified as having statistically significant AUCs (lower AUC 95% CI > 0.5) (**Table 4**). Then, these six metabolites were used as candidate predictors for the LR model, and each variable had a VIF of less than three (**Table 5**). The corresponding AUC for the model was 0.73 (95% CI 0.63-0.83)(**Figure 7a**), and validation with LOOCV resulted in a significant AUC of 0.69 (95% CI 0.58-0.79)(**Figure 7b**).

Interpretation

The targeted candidate LR model was based on univariate AUCs and was confirmed in the training/test split. When the model was developed and applied to the full dataset, it was successfully cross validated with LOOCV. Additionally, the model was more robust when applied to the full dataset, which was anticipated due to the small sample size in the training/test split.

3.3.5 Analysis 3 – Developing a Classifier Using the Top 60 Metabolites

Principle Component Regression (PCR)

The top 60 metabolites were identified in the full dataset (n=120) and used to train the PCR model (**Table 6**). There appeared to be some evidence of phenotype separation in three dimensions (**Figure 8a**). A three component model was identified as the optimal model complexity. There was a difference between the mean discriminant scores in the case versus

control groups (Welch's t-test, p=0.002). The corresponding ROC curve had a significant AUC of 0.69 (95% CI 0.58-0.79)(**Figure 8b**). The model was validated with LOOCV, and there was no difference between the mean discriminant scores in the case versus control groups (Welch's t-test, p=0.06). However, the corresponding ROC plot had a significant AUC of 0.63 (95% CI 0.52-0.74)(**Figure 8c**).

Projection on Latent Structure Discriminant Analysis (PLS-DA)

Training/test Split

Usually 25-30 cases are required to develop a robust multivariate classifier; thus, there was a concern that the subsequent training/test split may be underpowered. The training data included 50 controls and 22 cases, and the test data included 33 controls and 15 cases.

The top 60 metabolites were identified from the training dataset (n=72) and used to train the PLS model (**Table 7**). There was evidence of phenotype separation in three dimensions (**Figure 9a**). The optimal model complexity included five latent factors. There was a difference between the mean discriminant scores in the case versus control groups (Welch's t-test, p <0.001), and the ROC curve had an AUC of 0.98 (95% CI 0.96-1)(**Figure 9b**). The model was externally validated in the independent test set (n=48), and there was a difference between the mean discriminant scores in the case versus control groups (Welch's t-test, p=0.02). The corresponding ROC plot had a significant AUC of 0.70 (95% CI 0.54-0.86) (**Figure 9c**). The top 20 VIPs from the training dataset are presented in **Table 8**.

Fitting the Full Dataset

The top 60 metabolites were identified in the full dataset (n=120) and used to train the PLS model (**Table 9**). Although the order in which AUCs were ranked differed slightly, the top 60 AUCs in the training dataset and full dataset were identical, i.e., the same univariate

associations were identified. There was again evidence of phenotype separation in three dimensions (**Figure 10a**); however, the optimal model complexity had four latent variables. For the optimal model, there was a difference between the mean discriminant scores in the case versus control groups (Welch's t-test, $p < 0.001$). The corresponding ROC curve had an AUC of 0.95 (95% CI 0.92-0.99) (**Figure 10b**). The model was validated with LOOCV, and there was difference between the mean discriminant scores in the case versus control groups (Welch's t-test, $p < 0.001$). The ROC curve had a significant AUC of 0.75 (95% CI 0.65-0.84)(**Figure 10c**).

The correlation between VIPs and univariate AUCs was weak (Spearman's rho= 0.24, p=0.07; Kendall's tau=0.15, p=0.1)(**Figure 11**). The top 20 VIPs from the full dataset are presented in **Table 10**. Of the top 20 VIPs, eight metabolites were also found in the top 20 univariate AUCs. In contrast, 15 of the top 20 VIPs were also included in the top 20 VIPs from the training dataset.

Next, to assess the effectiveness of a minimalist VIP model, the optimal model was re-fitted with the top 15 metabolites ranked by VIP (**Table 10**). There was a difference between the mean discriminant scores in the cases versus control groups (Welch's t-test, $p < 0.001$), and the ROC curve had an AUC of 0.90 (95% CI= 0.84-0.96). The model was validated with LOOCV, and there was a difference between the mean discriminant scores in the case versus control groups (Welch's t-test, $p < 0.001$). The corresponding ROC curve had a significant AUC of 0.82 (95% CI 0.74-0.90). The discriminant scores in the top 15 metabolite model were highly correlated with the full (60 metabolite) model (Pearson's r=0.87, $p < 0.001$).

To determine the minimum number of metabolites required to capture the signal of interest, the optimal model was once again re-fitted, using exclusively the top 13 metabolites ranked by VIP. There was a difference between the mean discriminant scores in the cases versus

control groups (Welch's t-test, p <0.001), and the ROC curve had an AUC of 0.88 (95% CI= 0.82-0.95). The model was validated with LOOCV, and there was difference between the mean discriminant scores in the case versus control groups (Welch's t-test, p <0.001). The corresponding ROC curve had a significant AUC of 0.80 (95% CI 0.71-0.88). The analysis was repeated once more for the top 10 metabolites ranked by VIP, and the results of all minimalist models are summarized in **Table 11**.

The average measurement of each metabolite in the top 20 VIP ranking for both case and control groups are summarized in **Table 12**.

Interpretation

There was evidence of a signal in both the unsupervised PCR model and the supervised PLS model. The PCR model was validated by LOOCV and, because the outcome was not used to train the classifier, bolstered the results of the subsequent PLS analysis. Despite overfitting in the training dataset, the PLS model was validated in the independent test dataset, providing further confirmation for the presence of a signal. Furthermore, when the model was fitted to the full dataset, there was once again evidence of a clear signal, confirmed by LOOCV. The 15 metabolite "minimalist" model, which was determined by VIPs, sufficiently reproduced the full signal. In the top 13 and top 10 metabolite minimalist models, there was also evidence of a signal, albeit progressively weaker. Therefore, it appears that the majority of the signal was contained in the top 15 metabolites.

3.4 Discussion

In this study, we were able to demonstrate the presence in serum of innate metabolomic differences between patients, which were associated with CR outcomes. These findings suggest

that, even before transplantation, the metabolite environment may be an important factor involved in the predisposition of alloimmune differentiation towards a rejection response.

In the first analysis, PCR and PLS methods were used to develop the 'all metabolite' model. All measured metabolites with substantial variance (372 metabolites) were used as variables. There was no evidence of signal in the unsupervised PCR model or in the supervised PLS model. The inability to ascertain any meaningful signal when incorporating all 372 eligible variables is likely reflective of random noise. Noise can result from two main sources: error attributed to measurement tools and random error attributed to data collection/preparation processes.

In the second analysis, LR was used to develop a predictive model. The most impactful candidate predictors were identified with bivariate screening of univariate AUCs. The two models we developed included either 6 or 8 of the most impactful metabolites as variables. There was evidence of a predictive signal in the model created with the training data as well as the full dataset, and both models were successfully validated. A drawback of LR is the need to restrict the number of variables included in the model. Therefore, although the analysis yielded conclusive results, it only incorporated information from a handful of metabolites.

In the third analysis, PCR and PLS methods were used once more to develop the predictive model. However, only the top 60 metabolites (determined by univariate AUCs) were included as variables. There was evidence of a signal in both the unsupervised PCR model and the supervised PLS model, and both statistical modelling methods were successfully validated. In particular, the PLS analysis in the full dataset provided the most robust results because the model was trained on all available samples (n=120) with a statistical method that also considers pre-determined phenotypic differences among samples. The majority of the signal was determined to

originate from the top 15 metabolites, identified by VIP. The analysis yielded more conclusive results than the LR because it incorporated more information from the metabolite data. Thus, this final analysis will be the focus of the following discussion.

Historically, metabolomics has not received as much consideration in kidney transplantation research as other fields in 'omics research: existing transplant research networks investigate genetic, epigenetic, and proteomic determinants of rejection phenotypes (230-233). The metabolome is arguably more complex than both the genome and the proteome because it reflects an open environment constantly in flux and can be altered by the microbiome (139).

Lipids were consistently identified as important contributors to the predictive model. In fact, over half of the top 20 VIP metabolites were lipid-related molecules, originating from one of the glycerolipids, glycerophospholipids, or sphingolipids classes; however, there were no appreciable trends within lipid classes (234). Of the 630 total metabolites measured, 523 were lipid-related. Therefore, the VIP metabolite trend may reflect an over representation of lipids tested in the original metabolite kit. Furthermore, as demonstrated in the volcano plot analysis, none of the metabolites had an absolute fold change greater than two –indicative of either a doubling or halving in concentration. Thus, the metabolite changes described between the two groups reflect subtle concentration differences.

Lipid metabolites are well recognized as immunomodulating molecules (235). For example, dietary lipids may have regulatory effects on lymphocyte proliferation, cytokine production, and natural killer cell activity (236-239). These lipids are suspected to exert their immunomodulatory effects predominantly by altering plasma membrane composition (240, 241).

Glycerolipids comprise a large proportion of total plasma lipids, and the predominant molecules we identified were triglycerides and diglycerides. Dyslipidemia is a known risk factor

of chronic graft rejection, and evidence suggests that dyslipidemia can reduced the availability of cyclosporine, diminishing the drug's immunosuppressive effect (242). No studies have investigated the validity of this claim in tacrolimus, another, more widespread, immunosuppressive medication. However, given that both drugs are CNIs, have similar mechanisms of action, and are lipophilic, an equivalent interaction between lipid levels and reduced tacrolimus efficacy warrants legitimate scientific plausibility. In addition, increased fat consumption, and presumably the subsequent increased lipid level in the bloodstream, decreases tacrolimus bioavailability (243). Future studies are required to further elucidate the impact of dyslipidemia on the immunosuppressive action of tacrolimus.

Free fatty acids and their eicosanoid counterparts are known modulators of inflammation and the immune response (244). In transplantation, these metabolites have been implicated in AR (245-247). Baker and colleagues demonstrated that the pre-transplant level of arachidonic acid was an independent marker of long-term graft survival (186). However, our study was unable to corroborate these findings; neither arachidonic acid nor measured eicosanoids were predictive of long-term graft outcomes. Transplantation era is a distinguishable, and potentially meaningful, difference between the two studies. In Baker et al. patients were transplanted between 1991-1997 and treated with cyclosporine. Contrastingly, in our study, patients were transplanted between 2008-2012 and predominantly treated with tacrolimus (a more effective CNI).

In the plasma membrane, local signaling and trafficking is regulated by the dynamic interplay between proteins, glycerophospholipids, sphingolipids, and sterol lipids —structural components of the lipid raft. The lipid variation, structure, and composition of the raft appear to be influential in its overall function (248). Lipid rafts have gained increasing recognition as modulators of T cell receptor (TCR) signaling activity through their influence on the

composition of the immunological synapse. The immunological synapse refers to the membrane structure formed in the gap between T cells and APCs; it facilitates numerous events which proceed to prime the immune response (249). Lipid raft composition, specifically the level of glycosphingolipids, is correlated with the activation of T cells. Accumulating evidence suggests that alterations to the contents of the lipid raft can modulate TCR activity and affect Th_{17} differentiation whereby lower levels of glycosphingolipids attenuate Th_{17} differentiation and cytokine production (250, 251). The overall trend in our data supported these results, as two of the three identified sphingolipids in the top 20 VIP metabolites were decreased in the control group. Novel immunotherapies which alter the lipid composition of the plasma membrane by targeting lipid metabolism continue to garner scientific attention (252).

Emerging evidence suggests the presence of innate differences in immune cell composition between transplant recipients before individuals receive their new organ (253-258). Of particular interest are the differences in T cell subpopulations. Two subtypes of T cells with important clinical significance in transplantation are T helper cells and Tregs. T helper cells (Th_1, Th_2, Th_{17}) are a subtype of CD4 T cells responsible for releasing pro-inflammatory cytokines into the cellular environment, which ultimately serves to amplify inflammation. Elevated levels of pro-inflammatory cytokines are a hallmark of rejection (259, 260). Contrastingly, Tregs function to supress the inflammatory response, predominately through the release of pro-regulatory cytokines. Treg levels, as well as other characteristic markers, correlate with graft function; patients with graft stability have a more robust pro-regulatory response (261-264). Despite exerting opposite effects, Tregs and Th_{17} cells derive from the same precursor. The balance between these two opposing cell types has become a recent focus of investigation, as the outcome of this competitive antagonism may influence graft survival (265-268). The propensity

for different immunological phenotypes could be a consequence of differences observed in lipid membrane composition, primarily in the context of Th_{17} differentiation, and merit further exploration.

Differences in the level of circulating plasma membrane lipids have already been demonstrated in patients with CKD, and the composition of the blood lipidome was correlated with adverse outcomes (disease progression or death) (269, 270). The altered plasma lipidome may be attributable to inflammatory processes, and LPCs have been negatively associated with C-reactive protein —a common marker of inflammation (269, 271, 272). Patients with CKD have increased systemic inflammation resulting from the uremic state. The degree of inflammation varies depending on the underlying disease state (e.g. autoimmune) and the quality of dialysis (273). However, in our study, the one LPC identified in the top 20 VIP metabolites demonstrated the opposite trend. Nonetheless, other lipid species may also be altered by inflammation.

Because individuals with CKD exhibit differences in circulating plasma membrane proteins, it is not unreasonable to postulate the existence of innate variability in the plasma membrane composition of T cells as well. Variability may partially help explain the clear presence of intrinsic differences between patients and their predisposition toward a robust immune response. However, characterizing the complex relationship between membrane lipids and T cells as well as the corresponding functional significance is still very much in its infancy. Further research is required to elucidate the underlying mechanisms and fully illustrate any potential implications.

In conclusion, the metabolomic environment surrounding T cell activation is increasingly recognized as an element which influences T cell differentiation and subsequent immune

response in a highly intricate and sophisticated manner. To our knowledge, this is the first ever comprehensive characterization of the serum metabolome pre-kidney transplantation used to create a predictive algorithm that estimates risk for developing chronic rejection. Our study provides evidence of the informative value of the pre-transplant metabolome in the clinical setting. Research which validates the predictive model in other adult kidney transplant recipient populations and assesses the interventional potential is needed.

3.5 Tables & Figures

Table 1. UHPLC Gradient Part 1& 2

Part 1			
Time (min)	**Flow (mL/min)**	**A (%)**	**B (%)**
0.00	0.8	100	0
0.25	0.8	100	0
1.50	0.8	88	12
2.70	0.8	82.5	17.5
4.00	0.8	50	50
4.50	0.8	0	100
4.70	1.0	0	100
5.00	1.0	0	100
5.10	1.0	100	0
5.80	0.8	100	0
Part 2			
Time (min)	**Flow (mL/min)**	**A (%)**	**B (%)**
0.00	0.8	100	0
0.25	0.8	100	0
1.50	0.8	75	25
2.70	0.8	50	50
4.00	0.8	25	75
4.50	0.8	0	100
4.70	1.0	0	100
5.00	1.0	0	100
5.10	1.0	100	0
5.80	0.8	100	0

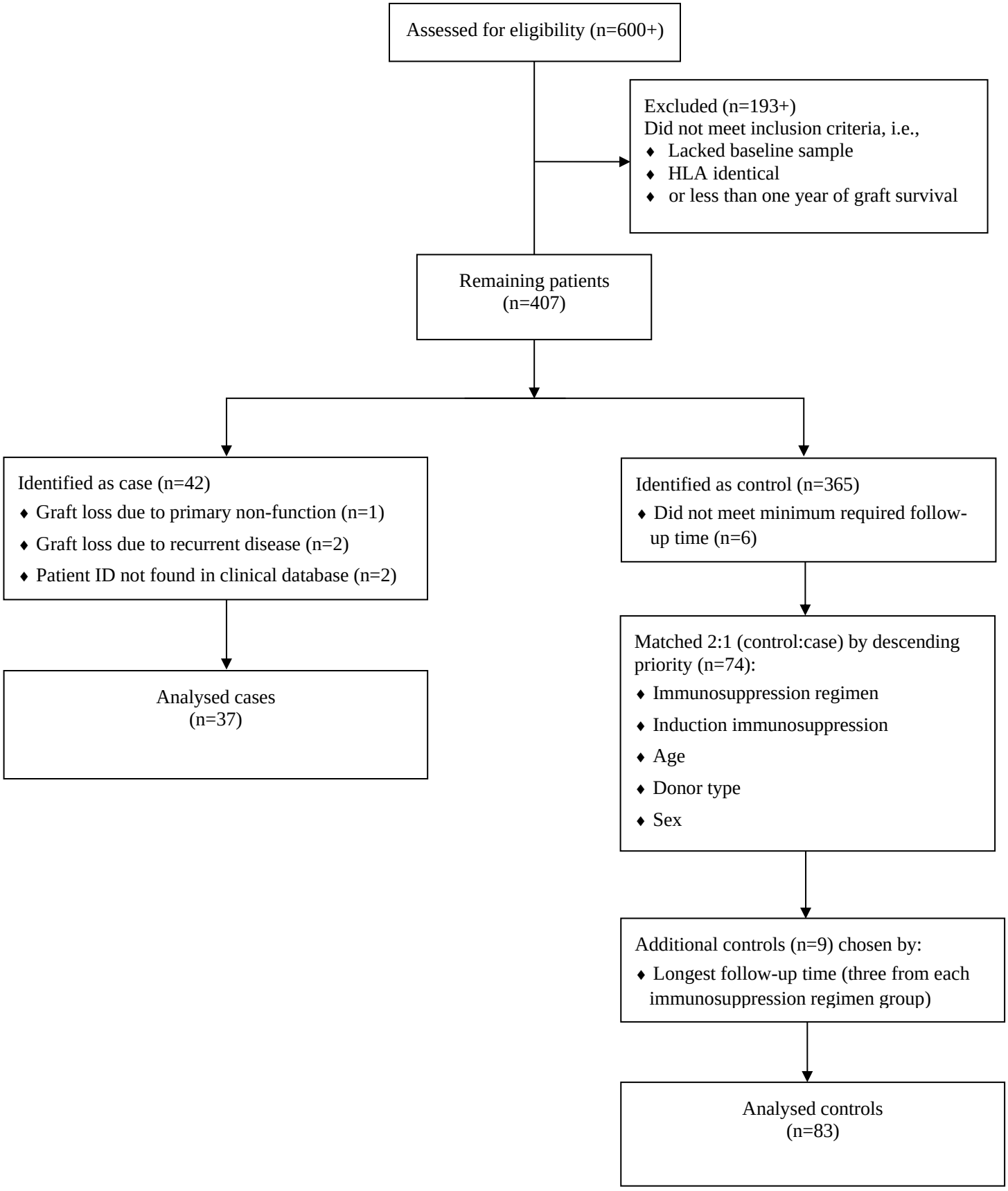
UHPLC, ultra-high-performance liquid chromatography

Table 2. Baseline characteristics of adult cohort at transplantation

	Study Cohort (n=120)	**Cases** (n=37)	**Control** (n=83)
Male sex	75 (63)	23 (62)	52 (63)
Age at transplant (years)	49 ± 13	47 ± 14	49 ± 13
Etiology			
Congenital/hereditary renal disease	14 (12)	5 (13)	9 (11)
Renal vascular disease	13 (11)	4 (11)	9 (11)
Glomerulonephritis	53 (44)	18 (49)	35 (42)
Diabetes	20 (17)	6 (16)	14 (17)
Nephropathy, drug induced	3 (2)	1 (3)	2 (2)
Other	5 (4)	0	5 (6)
Unknown etiology	12 (10)	3 (8)	9 (11)
Race			
Indigenous	10 (8)	2 (5)	8 (10)
Asian	9 (8)	1 (3)	8 (10)
Black	3 (2)	2 (5)	1 (1)
White	79 (66)	29 (78)	50 (60)
Indian sub-continent	11 (9)	1 (3)	10 (12)
Pacific Islander	6 (5)	1 (3)	5 (6)
Other/Multiracial	2 (1)	1(3)	1 (1)
Living donor	51 (43)	18 (49)	33 (40)
Modality type			
Hemodialysis	73 (61)	25 (68)	48 (59)
Peritoneal dialysis	27 (23)	7 (19)	20 (24)
No dialysis (pre-emptive)	19 (16)	5 (14)	14 (17)
Unknown	1	0	1
Dialysis length (days; n=100)	1461 ± 928	1337 ± 909	1518 ± 938
First transplant	116 (97)	36 (97)	80 (96)
Cold-ischemia time (minutes)	456 ± 300	473 ± 328	449 ± 289
Warm-ischemia time (minutes)	30 ± 18	30 ± 24	30 ± 15
Follow-up duration (days)	3163 ± 608	2913 ± 738	3274 ± 506

Values are expressed as n (%) or mean ± SD.

Figure 1. Sample selection and classification



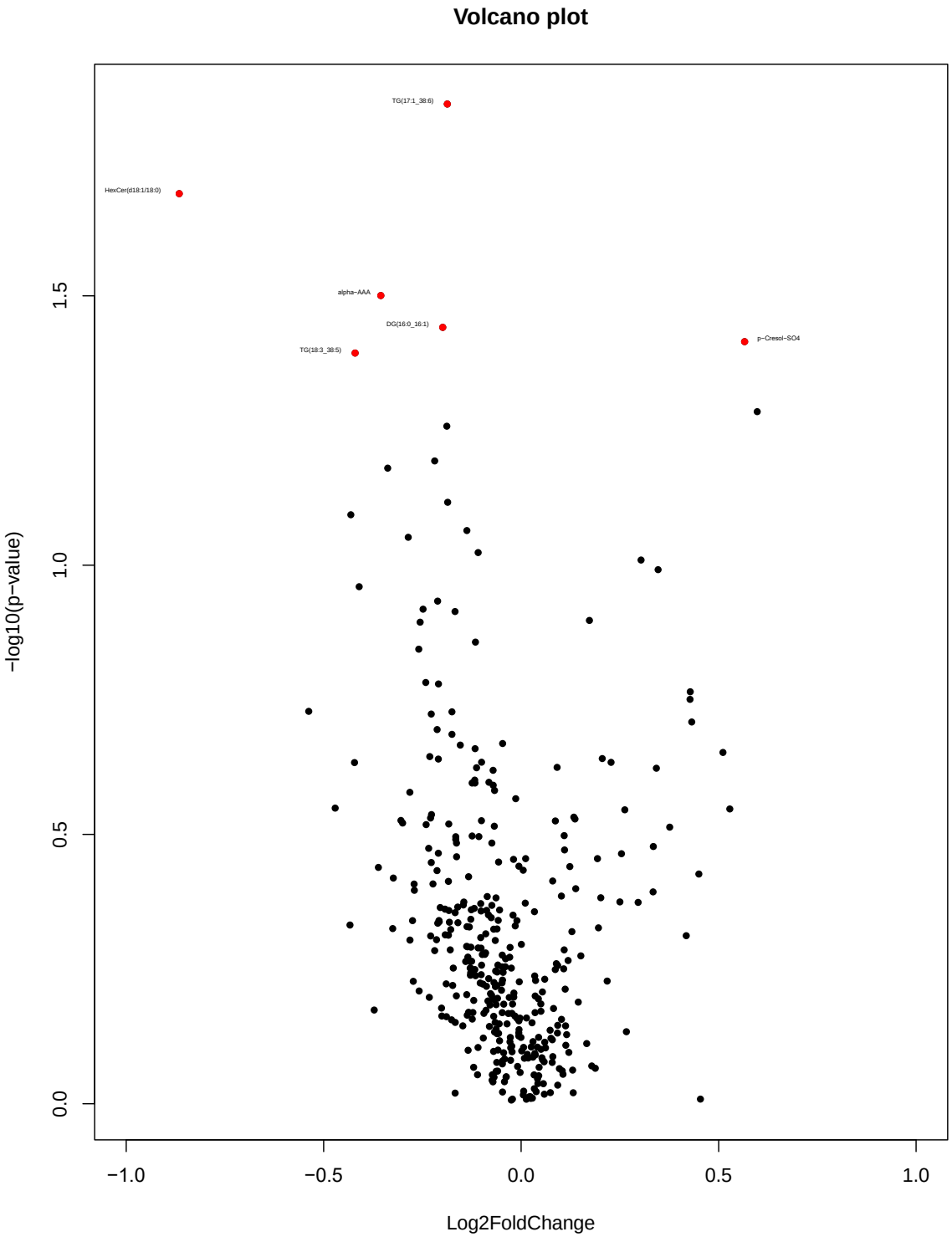


Figure 2. Volcano plot of measured metabolites. The horizontal access represents fold-change (case:control) of the log2 transformed metabolite data. The vertical access represents the -log10 transformed p-value, calculated using Student's t-Test. Red dots represent metabolites with p-value <0.05. Black dots represent metabolites without significant differences. No metabolites had an absolute fold-change >1.

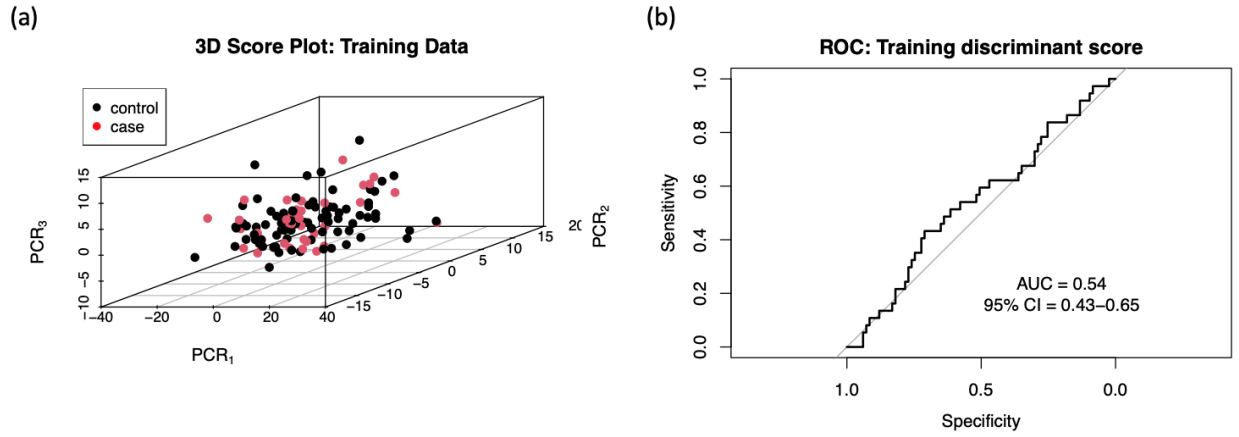


Figure 3. The PCR model trained on the full dataset (n=120): (a) three dimensional score plot; (b) the optimal model had a non-significant AUC of 0.54 (95% CI 0.43-0.65).

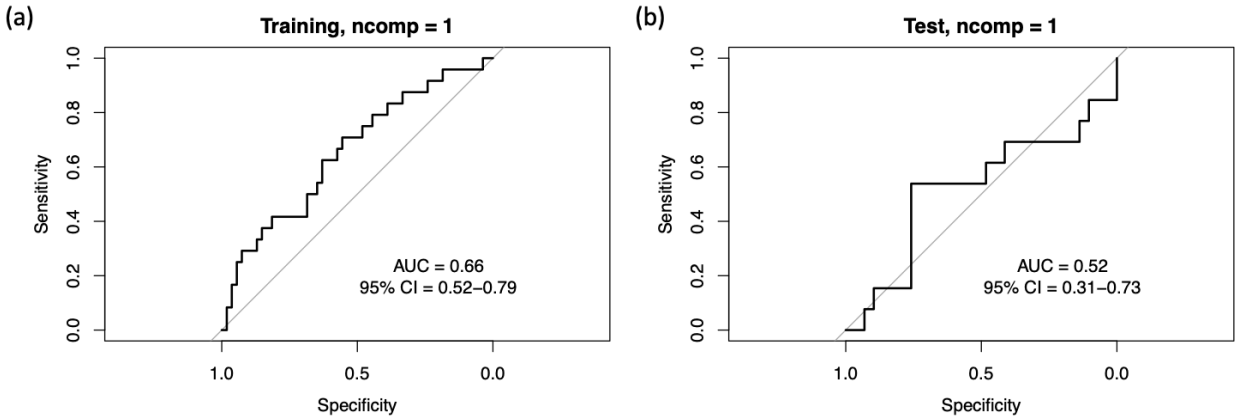


Figure 4. The PLS model for the training/test split: (a) the optimal model fit to the training dataset (n=78, 1 latent factor), had a significant AUC of 0.66 (95% CI 0.52-0.79), (b) but failed external validation in the test dataset (n=42).

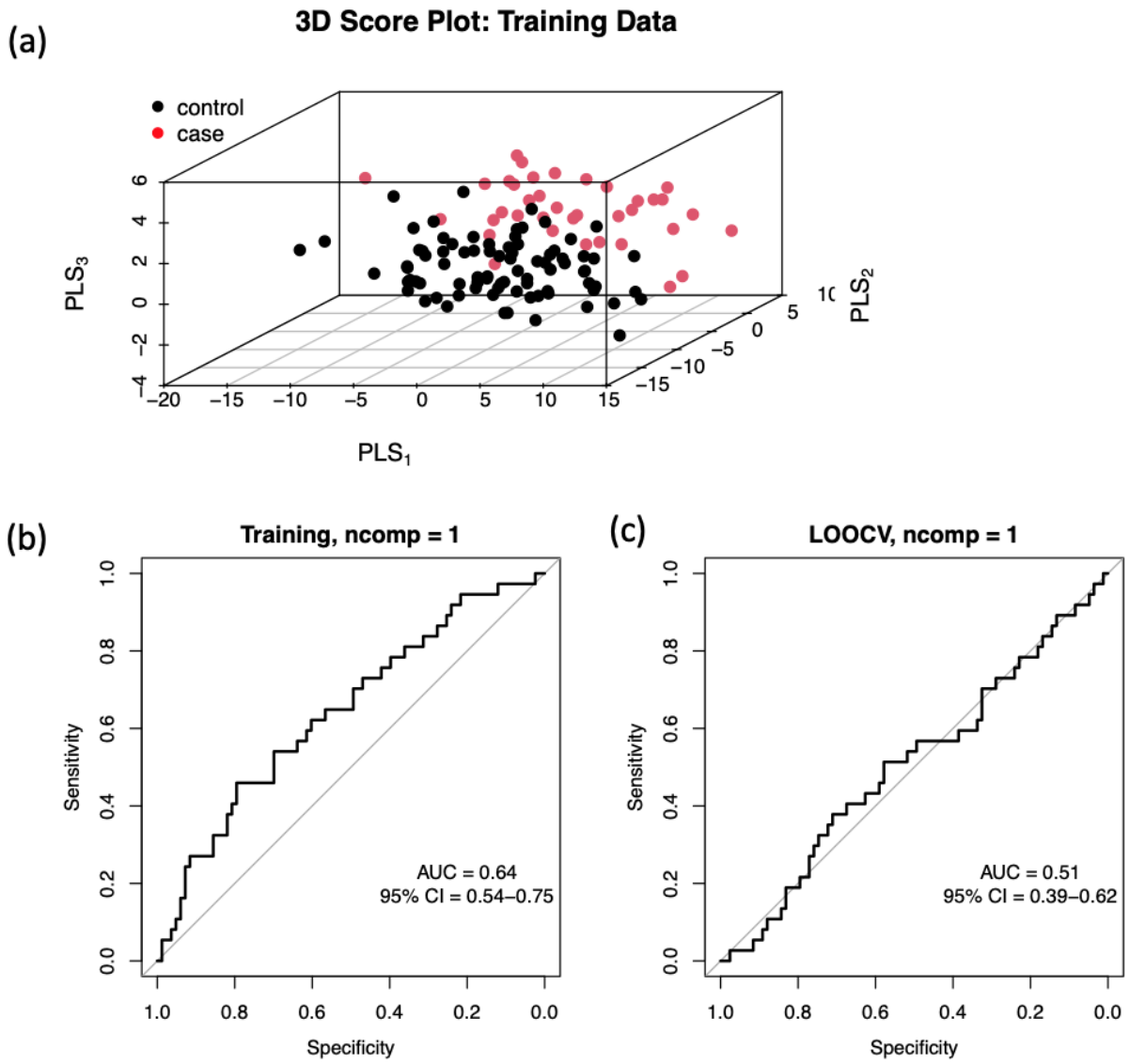


Figure 5. The PLS model trained on the full dataset (n=120): (a) three dimensional score plot; (b) the model had a significant AUC of 0.64 (95% CI 0.54-0.75), (c) but failed validation by LOOCV.

Table 3. Top 20 AUCs for univariate regression using the training data set

Metabolite	AUC	95% CI	
		Lower limit	Upper limit
C5.DC..C6.OH	0.682	0.549	0.816
PAG	0.660	0.519	0.800
His	0.659	0.534	0.785
TG.18.3_35.2	0.657	0.523	0.790
TMAO	0.656	0.529	0.783
PC.ae.C42.3	0.656	0.533	0.780
Creatinine	0.654	0.522	0.787
Putrescine	0.652	0.509	0.796
PC.ae.C40.2	0.647	0.505	0.788
Anserine	0.646	0.501	0.790
Asn	0.642	0.508	0.776
CE.20.1	0.640	0.511	0.768
C18.2	0.639	0.489	0.789
Cit	0.638	0.507	0.769
PC.ae.C42.2	0.637	0.504	0.771
HexCer.d18.1.18.0	0.630	0.493	0.766
PC.aa.C32.3	0.627	0.495	0.760
SM.C18.1	0.627	0.489	0.766
TG.17.0_34.3	0.627	0.495	0.760
SDMA	0.625	0.494	0.755

Metabolites with a lower limit CI > 0.51 where included as candidate predictors in the logistic regression model. AUC, area under the curve; CI, confidence interval

Table 4. Logistic regression analysis for the training/test split

Predictor	VIF	β	SE β	Z value	P-value
C5.DC..C6.OH	1.903	0.8202	4.0633	0.20	0.840
PAG	2.208	1.9816	1.2680	1.56	0.118
His	1.152	7.9666	3.7625	2.12	0.034
TG.18.3_35.2	1.111	-1.0306	0.7391	-1.39	0.163
TMAO	1.895	0.0309	1.1291	0.03	0.978
PC.ae.C42.3	1.059	-3.1554	3.3214	-0.95	0.342
Creatinine	3.200	-0.0214	2.6682	-0.01	0.994
CE.20.1	1.203	-1.5859	0.8837	-1.79	0.073

VIF, variable inflation factor; SE, standard error

Table 5. Top 20 AUCs for univariate regression using the full dataset

Metabolite	AUC	95% CI	
		Lower limit	Upper limit
TMAO	0.627	0.523	0.730
p.Cresol.SO4	0.626	0.519	0.733
C18.2	0.623	0.510	0.737
PC.aa.C38.1	0.619	0.507	0.731
HexCer.d18.1.18.0	0.618	0.510	0.727
C5.DC..C6.OH	0.610	0.499	0.721
BABA	0.609	0.496	0.722
TG.17.0_34.3	0.608	0.501	0.716
TG.18.3_38.5	0.603	0.496	0.709
Putrescine	0.601	0.487	0.715
lysoPC.a.C20.3	0.599	0.486	0.712
alpha.AAA	0.595	0.489	0.700
Hex2Cer.d18.1.22.0	0.589	0.480	0.697
Asn	0.588	0.476	0.700
DG.18.2_20.4	0.583	0.483	0.684
TG.16.0_38.1	0.583	0.476	0.691
TG.18.3_35.2	0.583	0.471	0.694
TG.17.1_36.3	0.579	0.460	0.699
C8	0.577	0.452	0.702
DG.18.0_20.0	0.577	0.472	0.683

Metabolites with a lower limit CI > 0.50 where included as candidate predictors in the logistic regression model. AUC, area under the curve; CI, confidence interval

Table 6. Logistic regression analysis for the full dataset

Predictor	VIF	β	SE β	Z value	P-value
TMAO	1.203	-0.00204	0.00350	-0.58	0.559
p.Cresol.SO4	1.201	0.00527	0.00281	1.87	0.061
C18.2	1.059	32.55358	13.90817	2.34	0.019
PC.aa.C38.1	1.159	-1.62842	0.81143	-2.01	0.045
HexCer.d18.1.18.0	1.059	-5.33897	3.50335	-1.52	0.128
TG.17.0_34.3	1.083	-1.20798	0.69484	-1.74	0.082

VIF, variable inflation factor; SE, standard error

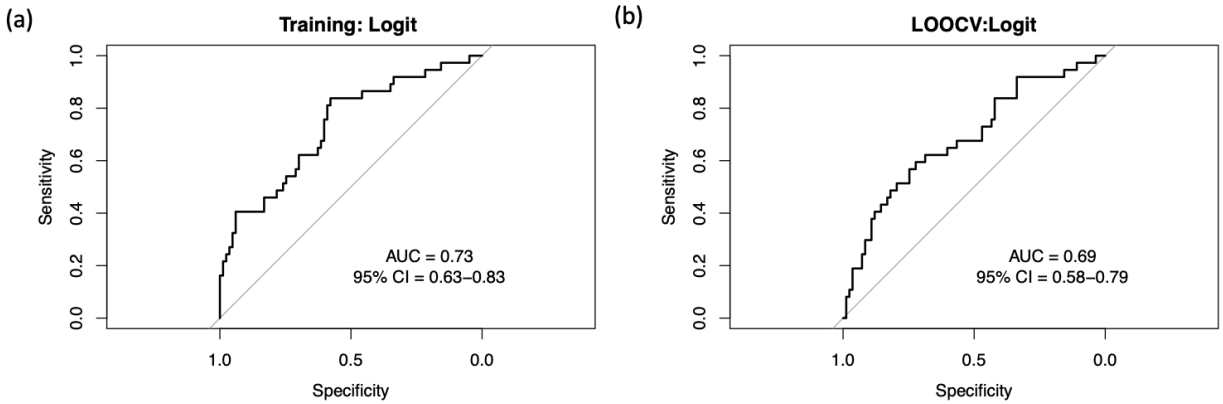


Figure 6. ROC curves for the logistic regression model in the training/test split: (a) the model in the training dataset (n= 78) had a significant AUC of 0.81 (95% CI 0.71-0.91); (b) the model was confirmed in the test dataset (n=42) with a significant AUC of 0.68 (95% CI 0.50-0.86).

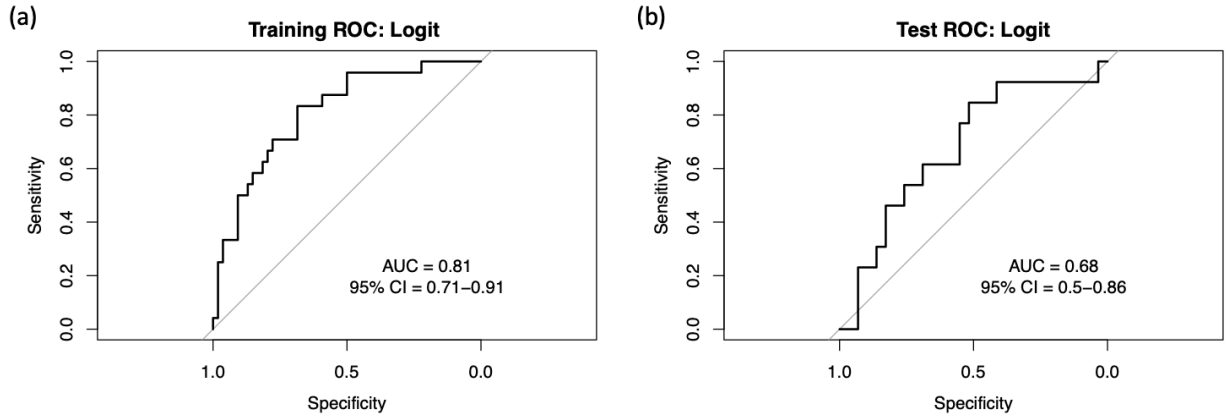


Figure 7. ROC curves for the logistic regression model fitted to the full dataset (n=120): (a) the model had a significant AUC of 0.73 (95% CI 0.63-0.83); (b) model confirmation with LOOCV had an AUC of 0.69 (95% CI 0.58-0.79).

Table 7. Top 60 AUCs in the full dataset

Metabolite	**AUC**	**95% CI**	
		Lower limit	Upper limit
TMAO	0.627	0.523	0.73
p.Cresol.SO4	0.626	0.519	0.733
C18.2	0.623	0.51	0.737
PC.aa.C38.1	0.619	0.507	0.731
HexCer.d18.1.18.0.	0.618	0.51	0.727
C5.DC..C6.OH.	0.610	0.499	0.721
BABA	0.609	0.496	0.722
TG.17.0_34.3.	0.608	0.501	0.716
TG.18.3_38.5.	0.603	0.496	0.709
Putrescine	0.601	0.487	0.715
lysoPC.a.C20.3	0.599	0.486	0.712
alpha.AAA	0.595	0.489	0.7
Hex2Cer.d18.1.22.0.	0.589	0.48	0.697
Asn	0.588	0.476	0.7
DG.18.2_20.4.	0.583	0.483	0.684
TG.16.0_38.1.	0.583	0.476	0.691
TG.18.3_35.2.	0.583	0.471	0.694
TG.17.1_36.3.	0.579	0.46	0.699
C8	0.577	0.452	0.702
DG.18.0_20.0.	0.577	0.472	0.683
C16.2	0.576	0.475	0.677
Trp	0.576	0.465	0.686
GCDCA	0.576	0.463	0.689
CE.20.3.	0.576	0.464	0.688
PC.ae.C44.3	0.576	0.459	0.694
TG.18.0_34.3.	0.575	0.465	0.684
DG.16.0_16.1.	0.574	0.473	0.676
DG.18.1_18.3.	0.573	0.457	0.689
TG.17.1_34.1.	0.573	0.46	0.686
Spermidine	0.572	0.477	0.666
lysoPC.a.C20.4	0.572	0.457	0.686

Metabolite	**AUC**	**95% CI**	
		Lower limit	Upper limit
SM.C24.0	0.572	0.463	0.682
TG.16.0_36.6.	0.572	0.463	0.682
TG.17.0_34.2.	0.572	0.463	0.682
C3.DC..C4.OH.	0.571	0.473	0.668
Orn	0.571	0.463	0.678
HipAcid	0.571	0.456	0.686
TG.16.1_34.3.	0.57	0.45	0.691
Hex2Cer.d18.1.24.0.	0.569	0.461	0.677
TG.16.0_35.2.	0.569	0.456	0.681
TG.16.0_33.2.	0.568	0.458	0.678
TG.16.0_35.3.	0.568	0.452	0.685
TG.22.6_34.3.	0.568	0.455	0.68
Cer.d18.2.23.0.	0.567	0.458	0.675
TG.17.1_32.1.	0.567	0.457	0.676
TG.18.2_35.3.	0.567	0.453	0.681
TG.20.4_34.2.	0.567	0.453	0.682
lysoPC.a.C16.0	0.566	0.452	0.68
PC.ae.C38.2	0.566	0.457	0.676
TG.18.1_35.2.	0.566	0.451	0.682
GLCAS	0.565	0.452	0.678
TCDCA	0.565	0.452	0.678
Cer.d18.2.16.0.	0.565	0.451	0.679
DHA	0.565	0.448	0.682
lysoPC.a.C18.1	0.565	0.453	0.677
PC.ae.C40.2	0.565	0.448	0.682
TG.17.0_34.1.	0.565	0.456	0.673
TG.20.5_34.1.	0.565	0.458	0.673
PC.aa.C28.1	0.564	0.452	0.677
PC.ae.C36.1	0.564	0.45	0.678

AUC, area under the curve; CI, confidence interval

Table 8. Top 60 AUCs in the training dataset

Metabolite	**AUC**	**95% CI**	
		Lower limit	Upper limit
BABA	0.704	0.564	0.845
C3.DC..C4.OH.	0.67	0.543	0.796
C18.2	0.66	0.52	0.8
Putrescine	0.653	0.512	0.795
C16.2	0.642	0.508	0.777
Hex2Cer.d18.1.24.0.	0.64	0.501	0.778
TG.18.3_38.5.	0.605	0.463	0.747
C5.DC..C6.OH.	0.597	0.451	0.744
Trp	0.597	0.46	0.734
PC.ae.C44.3	0.593	0.428	0.759
TG.17.1_32.1.	0.593	0.448	0.738
PC.ae.C38.2	0.592	0.447	0.736
alpha.AAA	0.591	0.452	0.731
SM.C24.0	0.59	0.444	0.735
HipAcid	0.589	0.434	0.743
PC.ae.C40.2	0.582	0.434	0.729
Cer.d18.2.16.0.	0.58	0.435	0.726
GCDCA	0.574	0.424	0.723
TG.16.0_38.1.	0.567	0.427	0.708
TMAO	0.566	0.43	0.702
Cer.d18.2.23.0.	0.565	0.422	0.709
DG.18.2_20.4.	0.564	0.432	0.696
GLCAS	0.562	0.409	0.714
C8	0.559	0.39	0.727
Hex2Cer.d18.1.22.0.	0.558	0.418	0.698
TG.17.0_34.3.	0.557	0.412	0.701
TG.20.5_34.1.	0.556	0.413	0.7
PC.ae.C36.1	0.555	0.408	0.702
TG.17.1_34.1.	0.555	0.408	0.703
TCDCA	0.553	0.407	0.698
DG.16.0_16.1.	0.552	0.417	0.687

Metabolite	**AUC**	**95% CI**	
		Lower limit	Upper limit
lysoPC.a.C20.3	0.55	0.406	0.694
TG.17.0_34.1.	0.549	0.403	0.694
TG.18.3_35.2.	0.547	0.393	0.7
TG.16.0_35.2.	0.546	0.397	0.696
Asn	0.545	0.394	0.695
TG.16.0_33.2.	0.541	0.392	0.69
TG.18.1_35.2.	0.541	0.384	0.698
PC.aa.C28.1	0.539	0.386	0.691
PC.aa.C38.1	0.539	0.38	0.698
TG.20.4_34.2.	0.537	0.383	0.69
lysoPC.a.C16.0	0.535	0.386	0.684
Spermidine	0.533	0.411	0.654
lysoPC.a.C20.4	0.533	0.386	0.68
TG.17.1_36.3.	0.533	0.373	0.693
TG.18.0_34.3.	0.531	0.386	0.677
CE.20.3.	0.53	0.385	0.676
p.Cresol.SO4	0.526	0.383	0.67
TG.18.2_35.3.	0.524	0.37	0.678
TG.17.0_34.2.	0.523	0.374	0.671
TG.16.0_36.6.	0.522	0.372	0.672
DG.18.1_18.3.	0.515	0.357	0.673
lysoPC.a.C18.1	0.509	0.369	0.649
HexCer.d18.1.18.0.	0.505	0.359	0.652
TG.16.1_34.3.	0.504	0.344	0.664
TG.22.6_34.3.	0.504	0.348	0.659
DHA	0.498	0.349	0.648
Orn	0.495	0.357	0.634
TG.16.0_35.3.	0.482	0.324	0.641
DG.18.0_20.0.	0.474	0.331	0.616

AUC, area under the curve; CI, confidence interval

Table 9. The top 20 metabolites ranked by VIPs from the training dataset

Metabolite	Ordered Scores	Scaled Scores
C3.DC..C4.OH.	0.0385	100.0
BABA	0.0297	77.1
C16.2	0.0274	71.1
C18.2	0.0231	60.1
TG.18.3_38.5.	0.0222	57.8
PC.ae.C38.2	0.0219	56.9
DG.18.2_20.4.	0.0217	56.4
C8	0.0215	55.9
Hex2Cer.d18.1.24.0.	0.0208	54.1
PC.ae.C40.2	0.0207	53.8
Hex2Cer.d18.1.22.0.	0.0175	45.6
Putrescine	0.0167	43.4
alpha.AAA	0.0167	43.3
DG.16.0_16.1.	0.0166	43.1
HipAcid	0.0157	40.8
SM.C24.0	0.0150	39.1
Spermidine	0.0144	37.4
Cer.d18.2.16.0.	0.0142	36.8
PC.aa.C28.1	0.0140	36.5
PC.ae.C44.3	0.0133	34.4

VIP, variable importance score

Table 10. The top 20 metabolites ranked by VIPs from the full dataset

Metabolite	Ordered Scores	Scaled Scores
DG.18.2_20.4	0.0276	100.0
HexCer.d18.1.18.0.	0.0258	93.5
C18.2	0.0244	88.7
PC.aa.C38.1	0.0242	87.8
Hex2Cer.d18.1.22.0	0.0227	82.4
C8	0.0215	78.2
PC.aa.C28.1	0.0200	72.8
CE.20.3.	0.0192	69.8
Hex2Cer.d18.1.24.0.	0.0185	67.3
Spermidine	0.0181	65.8
PC.ae.C38.2	0.0180	65.4
C16.2	0.0177	64.2
TG.18.3_38.5.	0.0176	63.8
C3.DC..C4.OH.	0.0171	62.1
PC.ae.C40.2	0.0164	59.4
p.Cresol.SO4	0.0157	57.1
BABA	0.0156	56.5
DG.16.0_16.1	0.0156	56.4
HipAcid	0.0153	55.6
lysoPC.a.C18.1	0.0149	54.2

VIP, variable importance score

Table 11. Minimalist VIP models

Minimalist Models	Training AUC (95% CI)	LOOCV AUC (95% CI)
Top 15 VIPs	0.90 (0.84-0.96)	0.82 (0.74-0.90)
Top 13 VIPs	0.88 (0.82-0.95)	0.80 (0.71-0.88)
Top 10 VIPs	0.82 (0.73-0.90)	0.70 (0.60-0.80)

AUC, area under the curve; CI, confidence interval; LOOCV, leave-one-out cross validation

Table 12. Top 20 VIP metabolite measurements stratified by group

Metabolites	Class	Controls	Cases
C3-DC (C4-OH)	Acylcarnitines	0.093 ± 0.034	0.113 ± 0.073
C8	Acylcarnitines	0.288 ± 0.078	0.278 ± 0.104
C16:2	Acylcarnitines	0.028 ± 0.004	0.029 ± 0.007
C18:2	Acylcarnitines	0.050 ± 0.014	0.058 ± 0.020
BABA	Amino acid-related	0.167 ± 0.103	0.199 ± 0.113
Spermidine	Biogenic Amines	0.196 ± 0.048	0.214 ± 0.117
HipAcid	Carboxylic Acids	203.993 ± 197.804	243.901 ± 189.295
CE(20:3)	Cholesterol Esters	23.912 ± 9.274	26.495 ± 11.303
p-Cresol-SO4	Cresols	135.743 ± 86.297	168.873 ± 79.991
DG(16:0_16:1)	Diacylglycerols	0.574 ± 0.313	0.484 ± 0.185
DG(18:2_20:4)	Diacylglycerols	0.290 ± 0.224	0.359 ± 0.283
lysoPC a C18:1	Glycerophospholipids	18.915 ± 6.093	20.379 ± 6.712
PC aa C28:1	Glycerophospholipids	2.727 ± 0.854	2.962 ± 0.957
PC aa C38:1	Glycerophospholipids	0.724 ± 0.334	0.610 ± 0.303
PC ae C38:2	Glycerophospholipids	1.598 ± 0.446	1.511 ± 0.351
PC ae C40:2	Glycerophospholipids	1.580 ± 0.396	1.518 ± 0.444
Hex2Cer(d18:1/22:0)	Glycosylceramides (Glycosphingolipid)	0.132 ± 0.078	0.160 ± 0.072
Hex2Cer(d18:1/24:0)	Glycosylceramides (Glycosphingolipid)	0.120 ± 0.068	0.137 ± 0.059
HexCer(d18:1/18:0)	Glycosylceramides (Glycosphingolipid)	0.100 ± 0.063	0.073 ± 0.064
TG(18:3_38:5)	Triacylglycerols	0.588 ± 0.393	0.442 ± 0.291

Values are expressed as mean ± SD

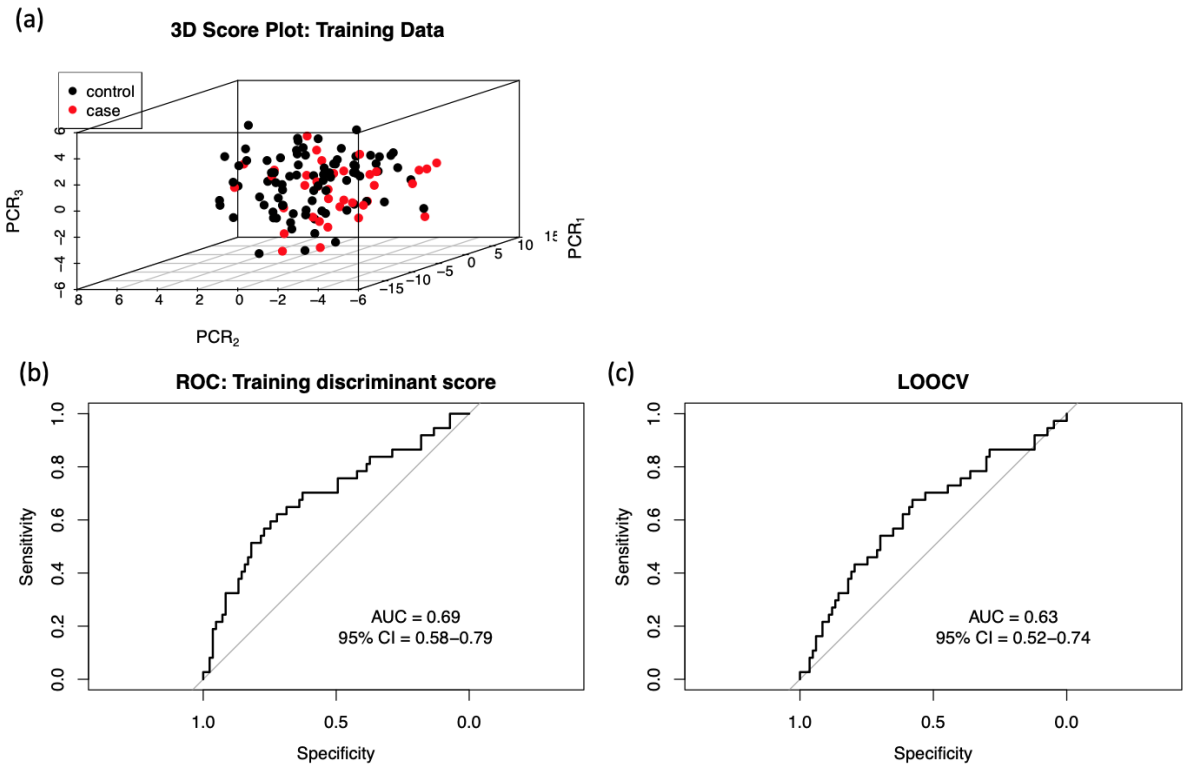


Figure 8. The PCR model trained on the full dataset (n=120): (a) three dimensional score plot; (b) the optimal model (3 components) had a significant AUC of 0.69 (95% CI 0.58-0.79), (c) and was confirmed by LOOCV, which had an AUC of 0.63 (95% CI 0.52-0.74)

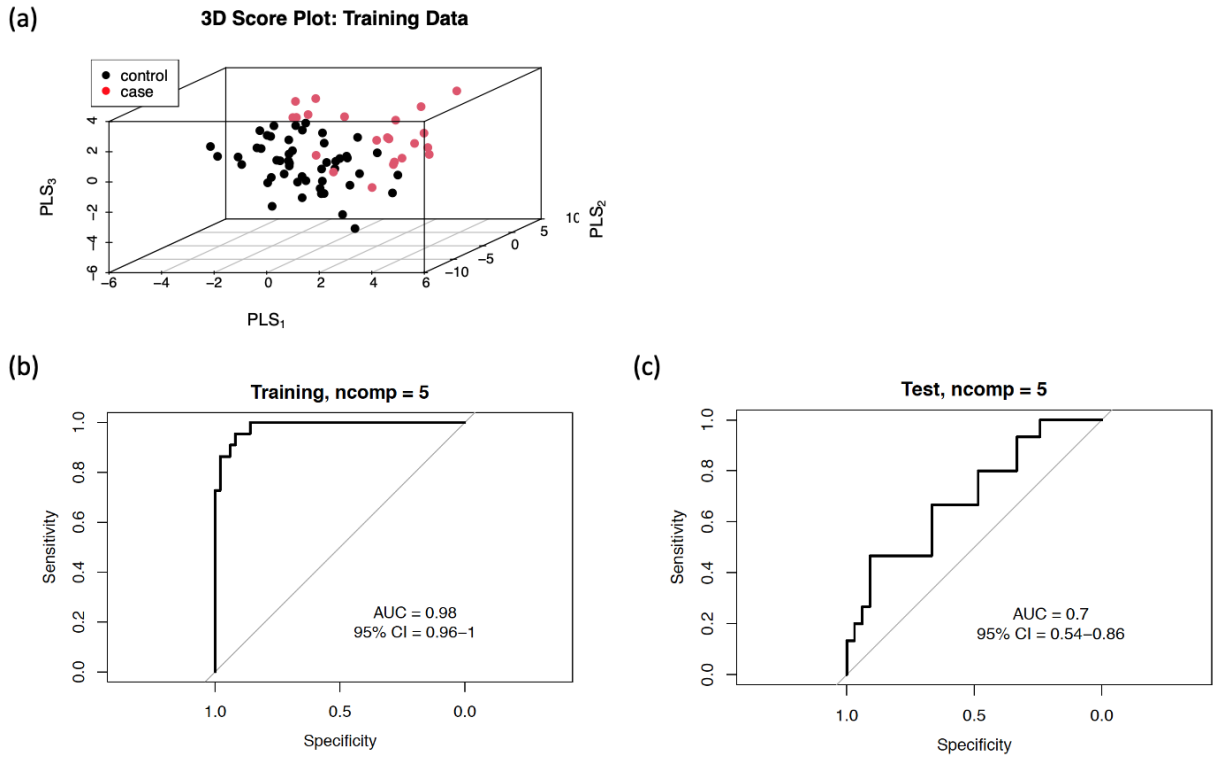


Figure 9. The PLS model for the training/test split: (a) three dimensional score plot; (b) the optimal model fit to the training dataset (n=72, 5 latent factors) had a significant AUC of 0.98 (95% CI 0.96-1); (c) the model was externally validated in the test dataset (n=48), which had an AUC of 0.70 (95% CI 0.54-0.86)

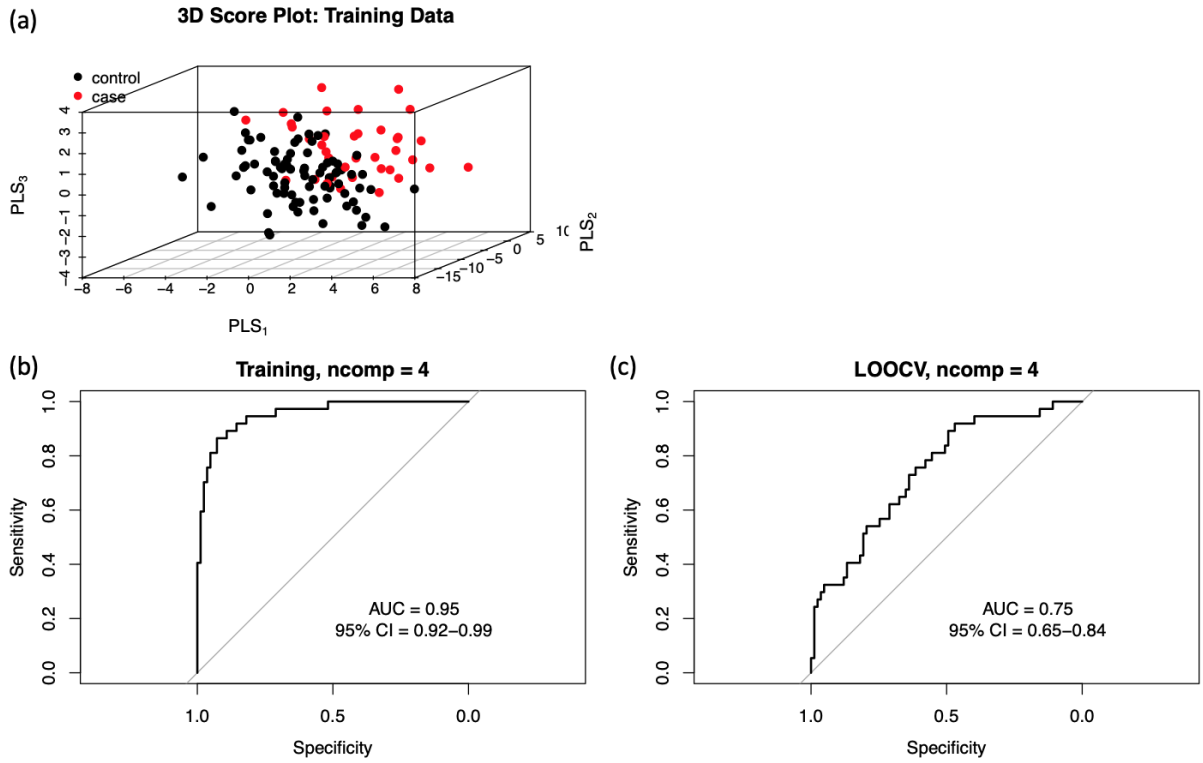


Figure 10. The PLS model trained on the full dataset (n=120): (a) three dimensional score plot; (b) the model had a significant AUC of 0.95 (95% CI 0.92-0.99), (c) and was confirmed with LOOCV, which had an AUC of 0.75 (95% CI 0.65-0.84)

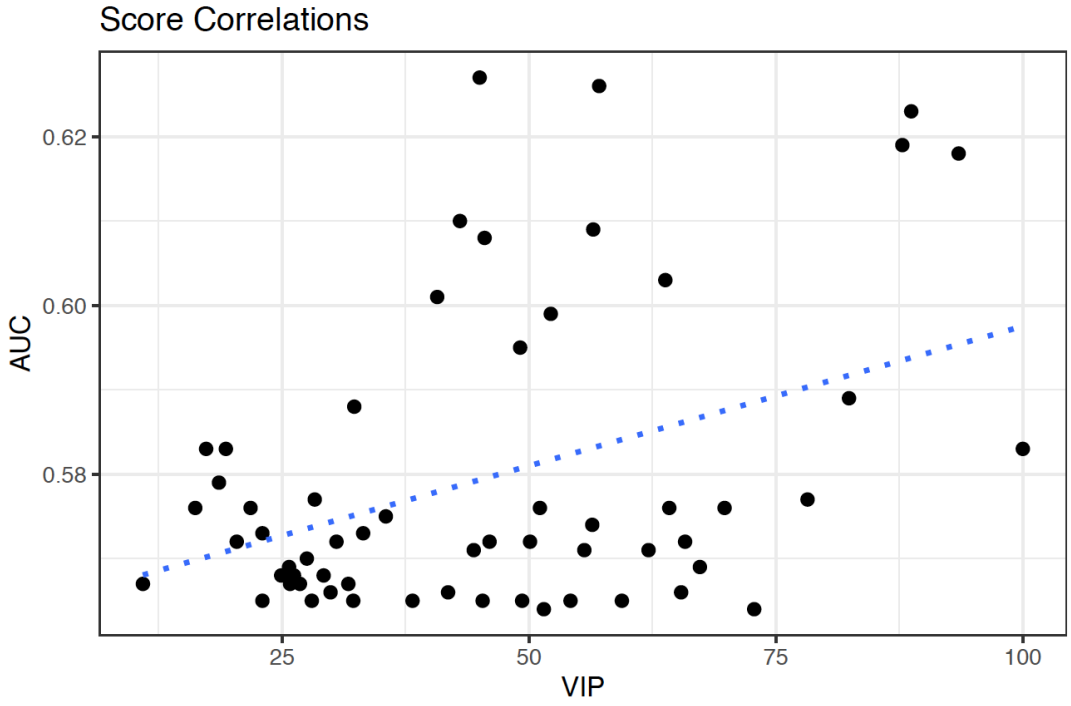


Figure 11. In the PLS model fitted to the full dataset, the correlation between top 20 VIPs and top 20 univariate AUCs was weak: Spearman's rho= 0.24, p=0.07; Kendall's tau=0.15, p=0.

Chapter 4:
Conclusion

4.1 Summary

In the oxythiamine study, the patient who developed Shoshin beriberi syndrome after transplant surgery had serum oxythiamine that was 5-fold higher than median levels. Oxythiamine levels were moderately correlated with patients' lactate in the 24 hours after surgery; however, those with more subtle lactic acidosis did not present signs of functional thiamine deficiency. Patients treated with PD prior to transplantation were at an increased risk to have elevated oxythiamine levels, in addition to patients with poor dialysis adequacy. We concluded that patients may be more susceptible to elevated serum oxythiamine when renal clearance is poor and uremic toxins are more likely to persist. Increased serum oxythiamine may contribute to the formation of a metabolic environment where functional thiamine deficiency is more likely to occur. In our study cohort, PD patients were at highest risk.

In the adult metabolomics study, we confirmed the presence of innate metabolomic differences between kidney recipients, even before transplantation, which correlate with the development of CR. A novel finding that suggests the metabolome provides valuable information about the differences that predispose patients toward a detrimental alloimmune response which results in subsequent graft rejection. We concluded that these differences are present before surgery, and may be useful in risk-stratification and as a method for personalized immune monitoring.

4.2 Future directions

We have demonstrated that immunometabolomic profiling can offer important insights into the role that host metabolism plays in predisposing the risk for CR.

Clinical variables, such as the level of HLA-mismatch and donor-specific data (e.g. age and race), can also influence transplant outcomes. The above variables were not control-matched in our study. Therefore, to develop a robust prediction algorithm for allograft survival-risk, an important next step is the integration of these clinical features with the metabolomic profiling. Equally important is to characterize the pre-transplant metabolome in the pediatric population, determine if it correlates with long-term rejection outcomes as well, and compare/contrast to the metabolome we have described in the adult population. We must also improve our understanding on how these metabolomic profiles relate to the alloimmune response. For example, whether the metabotype is associated with established urinary chemokine biomarkers, such as CXCL10, or directly relates to tolerance-promoting Treg responses, are questions that mandate a more detailed exploration.

As extensions of the adult metabolomics study, these additional investigations will provide foundational preliminary data to design and power a definitive analysis to understand how pre-transplant differences in metabolism may prime alloimmune responses post-transplant. This approach is highly complementary to ongoing efforts to develop risk-stratification based on extent of HLA epitope mismatch, by elaborating on host metabolic factors that predispose toward more adapted immune responses. We expect to generate important hypotheses regarding metabolic influence on activated T cell differentiation that will be foundational for future research into mechanism and intervention.

Baseline metabolic states may promote preferential tolerogenic alloimmune responses, providing a potential marker to identify individuals at risk, and are also attractive targets for interventions aimed at reducing rejection rates. A better understanding of the metabolome will enable tailoring of immune suppression and monitoring to the patient's individual risk of

rejection. This will provide the basis for further scientific investigation and development of clinical applications to improve long-term outcomes after kidney transplantation.

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Appendix

List of Metabolites: MxP® Quant 500 Kit

Alkaloids (1)			
Trigonelline	Trigonelline		

Amine Oxides (1)			
TMAO	Trimethylamine N-oxide		

Amino Acids (20)			
Ala	Alanine	Leu	Leucine
Arg	Arginine	Lys	Lysine
Asn	Asparagine	Met	Methionine
Asp	Aspartate	Phe	Phenylalanine
Cys	Cysteine	Pro	Proline
Glu	Glutamate	Ser	Serine
Gln	Glutamine	Thr	Threonine
Gly	Glycine	Trp	Tryptophan
His	Histidine	Tyr	Tyrosine
Ile	Isoleucine	Val	Valine

Amino Acid Related (30)			
alpha-AAA	alpha-Aminoadipic acid	c4-OH-Pro	cis-4-Hydroxyproline
AABA	alpha-Aminobutyric acid	t4-OH-Pro	trans-4-Hydroxyproline
Ac-Orn	Acetylornithine	Kynurenine	Kynurenine
ADMA	Asymmetric dimethylarginine	Met-SO	Methionine sulfoxide
Anserine	Anserine	1-Met-His	1-Methylhistidine
5-AVA	5-Aminovaleric acid	3-Met-His	3-Methylhistidine
BABA	beta-Aminobutyric acid	Nitro-Tyr	Nitrotyrosine
Betaine	Betaine	Orn	Ornithine
Carnosine	Carnosine	PAG	Phenylacetylglycine
Cit	Citrulline	PheAlaBetaine	Phenylalanine betaine
Creatinine	Creatinine	ProBetaine	Proline betaine
Cystine	Cystine	Sarcosine	Sarcosine
DOPA	Dihydroxyphenylalanine	SDMA	Symmetric dimethylarginine
HArg	Homoarginine	Taurine	Taurine
HCys	Homocysteine	TrpBetaine	Tryptophan betaine

Bile Acids (14)			
CA	Cholic acid	GLCAS	Glycolithocholic acid sulfate
CDCA	Chenodeoxycholic acid	GUDCA	Glycoursodeoxycholic acid
DCA	Deoxycholic acid	TCA	Taurocholic acid
GCA	Glycocholic acid	TCDCA	Taurochenodeoxycholic acid
GDCA	Glycodeoxycholic acid	TDCA	Taurodeoxycholic acid
GCDCA	Glycochenodeoxycholic acid	TLCA	Taurolithocholic acid
GLCA	Glycolithocholic acid	TMCA	Tauromurocholic acid

Biogenic Amines (9)			
beta-Ala	beta-Alanine	Putrescine	Putrescine
GABA	gamma-Aminobutyric acid	Serotonin	Serotonin
Dopamine	Dopamine	Spermidine	Spermidine
Histamine	Histamine	Spermine	Spermine
PEA	Phenylethylamine		

Carbohydrates and Related (1)			
H1	Hexoses (including glucose)		

Carboxylic Acids (7)			
AconAcid	Aconitic acid	OH-GlutAcid	3-Hydroxyglutaric acid
DiCA(12:0)	Dodecanedioic acid	Lac	Lactic acid
DiCA(14:0)	Tetradecanedioic acid	Suc	Succinic acid
HipAcid	Hippuric acid		

Cresols (1)			
p-Cresol-SO4	p-Cresol sulfate		

Fatty Acids (12)			
AA	Arachidonic acid	FA(18:0)	Stearic acid
DHA	Docosahexaenoid acid	FA(18:1)	Octadecenoic acid
EPA	Eicosapentaenoic acid	FA(18:2)	Octadecadienoic acid
FA(12:0)	Lauric acid	FA(20:1)	Eicosenoic acid
FA(14:0)	Myristic acid	FA(20:2)	Eicosadienoic acid
FA(16:0)	Palmitic acid	FA(20:3)	Eicosatrienoic acid

Hormones and Related (4)			
AbsAcid	Abscisic Acid	Cortisone	Cortisone
Cortisol	Cortisol	DHEAS	Dehydroepiandrosterone sulfate

Indoles and Derivatives (4)			
Indole	Indole	3-IPA	3-Indolepropionic acid
3-IAA	3-Indoleacetic acid	Ind-SO4	Indoxyl sulfate

Nucleobases and Related (2)			
Hypoxanthine	Hypoxanthine	Xanthine	Xanthine

Vitamins and Cofactors (1)			
Choline	Choline		

Acylcarnitines (40)			
C0	Carnitine	C10:1	Decenoylcarnitine
C2	Acetylcarnitine	C10:2	Decadienoylcarnitine
C3	Propionoylcarnitine	C12	Dodecanoylcarnitine
C3-DC (C4-OH)	Malonylcarnitine (Hydroxybutyrylcarnitine)	C12-DC	Dodecanedioylcarnitine
C3-OH	Hydroxypropionylcarnitine	C12:1	Dodecenoylcarnitine
C3:1	Propenoylcarnitine	C14	Tetradecanoylcarnitine
C4	Butyrylcarnitine	C14:1	Tetradecenoylcarnitine
C4:1	Butenylcarnitine	C14:1-OH	Hydroxytetradecenoylcarnitine
C5	Valerylcarnitine	C14:2	Tetradecadienoylcarnitine
C5-DC (C6-OH)	Glutarylcarnitine (Hydroxyhexanoylcarnitine)	C14:2-OH	Hydroxytetradecadienoylcarnitine
C5-M-DC	Methylglutarylcarnitine	C16	Hexadecanoylcarnitine
C5-OH (C3-DC-M)	Hydroxyvalerylcarnitine (Methylmalonylcarnitine)	C16-OH	Hydroxyhexadecanoylcarnitine
C5:1	Tiglylcarnitine	C16:1	Hexadecenoylcarnitine
C5:1-DC	Glutaconylcarnitine	C16:1-OH	Hydroxyhexadecenoylcarnitine
C6 (C4:1-DC)	Hexanoylcarnitine (Fumarylcarnitine)	C16:2	Hexadecadienoylcarnitine
C6:1	Hexenoylcarnitine	C16:2-OH	Hydroxyhexadecadienoylcarnitine
C7-DC	Pimeloylcarnitine	C18	Octadecanoylcarnitine
C8	Octanoylcarnitine	C18:1	Octadecenoylcarnitine
C9	Nonaylcarnitine	C18:1-OH	Hydroxyoctadecenoylcarnitine
C10	Decanoylcarnitine	C18:2	Octadecadienylcarnitine

Lysophosphatidylcholines (14)			
lysoPC a C14:0	lysoPC a C18:0	lysoPC a C20:4	lysoPC a C28:0
lysoPC a C16:0	lysoPC a C18:1	lysoPC a C24:0	lysoPC a C28:1
lysoPC a C16:1	lysoPC a C18:2	lysoPC a C26:0	
lysoPC a C17:0	lysoPC a C20:3	lysoPC a C26:1	

Phosphatidylcholines (76)			
PC aa C24:0	PC aa C36:3	PC aa C42:0	PC ae C36:1
PC aa C26:0	PC aa C36:4	PC aa C42:1	PC ae C36:2
PC aa C28:1	PC aa C36:5	PC aa C42:2	PC ae C36:3
PC aa C30:0	PC aa C36:6	PC aa C42:4	PC ae C36:4
PC aa C30:2	PC aa C38:0	PC aa C42:5	PC ae C36:5
PC aa C32:0	PC aa C38:1	PC aa C42:6	PC ae C38:0
PC aa C32:1	PC aa C38:3	PC ae C30:0	PC ae C38:1
PC aa C32:2	PC aa C38:4	PC ae C30:1	PC ae C38:2
PC aa C32:3	PC aa C38:5	PC ae C30:2	PC ae C38:3
PC aa C34:1	PC aa C38:6	PC ae C32:1	PC ae C38:4
PC aa C34:2	PC aa C40:1	PC ae C32:2	PC ae C38:5
PC aa C34:3	PC aa C40:2	PC ae C34:0	PC ae C38:6
PC aa C34:4	PC aa C40:3	PC ae C34:1	PC ae C40:1
PC aa C36:0	PC aa C40:4	PC ae C34:2	PC ae C40:2
PC aa C36:1	PC aa C40:5	PC ae C34:3	PC ae C40:3
PC aa C36:2	PC aa C40:6	PC ae C36:0	PC ae C40:4
PC ae C40:5	PC ae C42:1	PC ae C42:4	PC ae C44:4
PC ae C40:6	PC ae C42:2	PC ae C42:5	PC ae C44:5
PC ae C42:0	PC ae C42:3	PC ae C44:3	PC ae C44:6

Sphingomyelins (15)			
SM (OH) C14:1	SM C18:0	SM (OH) C22:2	SM (OH) C24:1
SM C16:0	SM C18:1	SM C22:3	SM C26:0
SM C16:1	SM C20:2	SM C24:0	SM C26:1
SM (OH) C16:1	SM (OH) C22:1	SM C24:1	

Ceramides (28)			
Cer(d16:1/18:0)	Cer(d18:1/18:0(OH))	Cer(d18:1/24:0)	Cer(d18:2/18:0)
Cer(d16:1/20:0)	Cer(d18:1/18:0)	Cer(d18:1/24:1)	Cer(d18:2/18:1)
Cer(d16:1/22:0)	Cer(d18:1/18:1)	Cer(d18:1/25:0)	Cer(d18:2/20:0)
Cer(d16:1/23:0)	Cer(d18:1/20:0(OH))	Cer(d18:1/26:0)	Cer(d18:2/22:0)
Cer(d16:1/24:0)	Cer(d18:1/20:0)	Cer(d18:1/26:1)	Cer(d18:2/23:0)
Cer(d18:1/14:0)	Cer(d18:1/22:0)	Cer(d18:2/14:0)	Cer(d18:2/24:0)
Cer(d18:1/16:0)	Cer(d18:1/23:0)	Cer(d18:2/16:0)	Cer(d18:2/24:1)

Dihydroceramides (8)			
Cer(d18:0/18:0(OH))	Cer(d18:0/20:0)	Cer(d18:0/24:0)	Cer(d18:0/26:1(OH))
Cer(d18:0/18:0)	Cer(d18:0/22:0)	Cer(d18:0/24:1)	Cer(d18:0/26:1)

Hexosylceramides (19)			
HexCer(d16:1/22:0)	HexCer(d18:1/18:1)	HexCer(d18:1/24:1)	HexCer(d18:2/20:0)
HexCer(d16:1/24:0)	HexCer(d18:1/20:0)	HexCer(d18:1/26:0)	HexCer(d18:2/22:0)
HexCer(d18:1/14:0)	HexCer(d18:1/22:0)	HexCer(d18:1/26:1)	HexCer(d18:2/23:0)
HexCer(d18:1/16:0)	HexCer(d18:1/23:0)	HexCer(d18:2/16:0)	HexCer(d18:2/24:0)
HexCer(d18:1/18:0)	HexCer(d18:1/24:0)	HexCer(d18:2/18:0)	

Dihexosylceramides (9)			
Hex2Cer(d18:1/14:0)	Hex2Cer(d18:1/20:0)	Hex2Cer(d18:1/24:1)	
Hex2Cer(d18:1/16:0)	Hex2Cer(d18:1/22:0)	Hex2Cer(d18:1/26:0)	
Hex2Cer(d18:1/18:0)	Hex2Cer(d18:1/24:0)	Hex2Cer(d18:1/26:1)	

Trihexosylceramides (6)			
Hex3Cer(d18:1/16:0)	Hex3Cer(d18:1/24:1)	Hex3Cer(d18:1_20:0)	
Hex3Cer(d18:1/18:0)	Hex3Cer(d18:1/26:1)	Hex3Cer(d18:1_22:0)	

Cholesteryl Esters (22)			
CE(14:0)	CE(17:0)	CE(20:0)	CE(22:1)
CE(14:1)	CE(17:1)	CE(20:1)	CE(22:2)
CE(15:0)	CE(18:0)	CE(20:3)	CE(22:5)
CE(15:1)	CE(18:1)	CE(20:4)	CE(22:6)
CE(16:0)	CE(18:2)	CE(20:5)	
CE(16:1)	CE(18:3)	CE(22:0)	

Diglycerides (44)			
DG(14:0_14:0)	DG(16:0_20:3)	DG(18:1_18:2)	DG(18:2_18:3)
DG(14:0_18:1)	DG(16:0_20:4)	DG(18:1_18:3)	DG(18:2_18:4)
DG(14:0_18:2)	DG(16:1_18:0)	DG(18:1_18:4)	DG(18:2_20:0)
DG(14:0_20:0)	DG(16:1_18:1)	DG(18:1_20:0)	DG(18:2_20:4)
DG(14:1_18:1)	DG(16:1_18:2)	DG(18:1_20:1)	DG(18:3_18:3)
DG(14:1_20:2)	DG(16:1_20:0)	DG(18:1_20:2)	DG(18:3_20:2)
DG(16:0_16:0)	DG(17:0_17:1)	DG(18:1_20:3)	DG(21:0_22:6)
DG(16:0_16:1)	DG(17:0_18:1)	DG(18:1_20:4)	DG(22:1_22:2)
DG(16:0_18:1)	DG(18:0_20:0)	DG(18:1_22:5)	DG-O(14:0_18:2)
DG(16:0_18:2)	DG(18:0_20:4)	DG(18:1_22:6)	DG-O(16:0_18:1)
DG(16:0_20:0)	DG(18:1_18:1)	DG(18:2_18:2)	DG-O(16:0_20:4)

Triglycerides (242)			
TG(14:0_32:2)	TG(16:0_36:3)	TG(16:1_38:5)	TG(18:0_36:1)
TG(14:0_34:0)	TG(16:0_36:4)	TG(17:0_32:1)	TG(18:0_36:2)
TG(14:0_34:1)	TG(16:0_36:5)	TG(17:0_34:1)	TG(18:0_36:3)
TG(14:0_34:2)	TG(16:0_36:6)	TG(17:0_34:2)	TG(18:0_36:4)
TG(14:0_34:3)	TG(16:0_37:3)	TG(17:0_34:3)	TG(18:0_36:5)
TG(14:0_35:1)	TG(16:0_38:1)	TG(17:0_36:3)	TG(18:0_38:6)
TG(14:0_35:2)	TG(16:0_38:2)	TG(17:0_36:4)	TG(18:0_38:7)
TG(14:0_36:1)	TG(16:0_38:3)	TG(17:1_32:1)	TG(18:1_26:0)
TG(14:0_36:2)	TG(16:0_38:4)	TG(17:1_34:1)	TG(18:1_28:1)
TG(14:0_36:3)	TG(16:0_38:5)	TG(17:1_34:2)	TG(18:1_30:0)
TG(14:0_36:4)	TG(16:0_38:6)	TG(17:1_34:3)	TG(18:1_30:1)
TG(14:0_38:4)	TG(16:0_38:7)	TG(17:1_36:3)	TG(18:1_30:2)
TG(14:0_38:5)	TG(16:0_40:6)	TG(17:1_36:4)	TG(18:1_31:0)
TG(14:0_39:3)	TG(16:0_40:7)	TG(17:1_36:5)	TG(18:1_32:0)
TG(16:0_28:1)	TG(16:0_40:8)	TG(17:1_38:5)	TG(18:1_32:1)
TG(16:0_28:2)	TG(16:1_28:0)	TG(17:1_38:6)	TG(18:1_32:2)
TG(16:0_30:2)	TG(16:1_30:1)	TG(17:1_38:7)	TG(18:1_32:3)
TG(16:0_32:0)	TG(16:1_32:0)	TG(17:2_34:2)	TG(18:1_33:0)
TG(16:0_32:1)	TG(16:1_32:1)	TG(17:2_34:3)	TG(18:1_33:1)
TG(16:0_32:2)	TG(16:1_32:2)	TG(17:2_36:2)	TG(18:1_33:2)
TG(16:0_32:3)	TG(16:1_33:1)	TG(17:2_36:3)	TG(18:1_33:3)
TG(16:0_33:1)	TG(16:1_34:0)	TG(17:2_36:4)	TG(18:1_34:1)
TG(16:0_33:2)	TG(16:1_34:1)	TG(17:2_38:5)	TG(18:1_34:2)
TG(16:0_34:0)	TG(16:1_34:2)	TG(17:2_38:6)	TG(18:1_34:3)
TG(16:0_34:1)	TG(16:1_34:3)	TG(17:2_38:7)	TG(18:1_34:4)
TG(16:0_34:2)	TG(16:1_36:1)	TG(18:0_30:0)	TG(18:1_35:2)
TG(16:0_34:3)	TG(16:1_36:2)	TG(18:0_30:1)	TG(18:1_35:3)
TG(16:0_34:4)	TG(16:1_36:3)	TG(18:0_32:0)	TG(18:1_36:0)
TG(16:0_35:1)	TG(16:1_36:4)	TG(18:0_32:1)	TG(18:1_36:1)
TG(16:0_35:2)	TG(16:1_36:5)	TG(18:0_32:2)	TG(18:1_36:2)
TG(16:0_35:3)	TG(16:1_38:3)	TG(18:0_34:2)	TG(18:1_36:3)
TG(16:0_36:2)	TG(16:1_38:4)	TG(18:0_34:3)	TG(18:1_36:4)

Triglycerides (242)			
TG(18:1_36:5)	TG(18:2_38:4)	TG(20:1_34:1)	TG(20:4_35:3)
TG(18:1_36:6)	TG(18:2_38:5)	TG(20:1_34:2)	TG(20:4_36:2)
TG(18:1_38:5)	TG(18:2_38:6)	TG(20:1_34:3)	TG(20:4_36:3)
TG(18:1_38:6)	TG(18:3_30:0)	TG(20:2_32:0)	TG(20:4_36:4)
TG(18:1_38:7)	TG(18:3_32:0)	TG(20:2_32:1)	TG(20:4_36:5)
TG(18:2_28:0)	TG(18:3_32:1)	TG(20:2_34:1)	TG(20:5_34:0)
TG(18:2_30:0)	TG(18:3_33:2)	TG(20:2_34:2)	TG(20:5_34:1)
TG(18:2_30:1)	TG(18:3_34:0)	TG(20:2_34:3)	TG(20:5_34:2)
TG(18:2_31:0)	TG(18:3_34:1)	TG(20:2_34:4)	TG(20:5_36:2)
TG(18:2_32:0)	TG(18:3_34:2)	TG(20:2_36:5)	TG(20:5_36:3)
TG(18:2_32:1)	TG(18:3_34:3)	TG(20:3_32:0)	TG(22:0_32:4)
TG(18:2_32:2)	TG(18:3_35:2)	TG(20:3_32:1)	TG(22:1_32:5)
TG(18:2_33:0)	TG(18:3_36:1)	TG(20:3_32:2)	TG(22:2_32:4)
TG(18:2_33:1)	TG(18:3_36:2)	TG(20:3_34:0)	TG(22:3_30:2)
TG(18:2_33:2)	TG(18:3_36:3)	TG(20:3_34:1)	TG(22:4_32:0)
TG(18:2_34:0)	TG(18:3_36:4)	TG(20:3_34:2)	TG(22:4_32:2)
TG(18:2_34:1)	TG(18:3_38:5)	TG(20:3_34:3)	TG(22:4_34:2)
TG(18:2_34:2)	TG(18:3_38:6)	TG(20:3_36:3)	TG(22:5_32:0)
TG(18:2_34:3)	TG(20:0_32:3)	TG(20:3_36:4)	TG(22:5_32:1)
TG(18:2_34:4)	TG(20:0_32:4)	TG(20:3_36:5)	TG(22:5_34:1)
TG(18:2_35:1)	TG(20:0_34:1)	TG(20:4_30:0)	TG(22:5_34:2)
TG(18:2_35:2)	TG(20:1_24:3)	TG(20:4_32:0)	TG(22:5_34:3)
TG(18:2_35:3)	TG(20:1_26:1)	TG(20:4_32:1)	TG(22:6_32:0)
TG(18:2_36:0)	TG(20:1_30:1)	TG(20:4_32:2)	TG(22:6_32:1)
TG(18:2_36:1)	TG(20:1_31:0)	TG(20:4_33:2)	TG(22:6_34:1)
TG(18:2_36:2)	TG(20:1_32:1)	TG(20:4_34:0)	TG(22:6_34:2)
TG(18:2_36:3)	TG(20:1_32:2)	TG(20:4_34:1)	TG(22:6_34:3)
TG(18:2_36:4)	TG(20:1_32:3)	TG(20:4_34:2)	
TG(18:2_36:5)	TG(20:1_34:0)	TG(20:4_34:3)	