

Metabolic Insights into Bariatric Surgical Diabetes Resolution

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This thesis is dedicated to:

My Parents, Patricia & Nelson

Sister, Renee

and Fiancée, Jiun Yi

For their eternal and unconditional love, understanding and support.

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Statement of Originality

To the best of my knowledge, this is to certify that the content of this thesis and its scientific conclusions which I have presented for consideration for my PhD degree is a result of my own work. Any work, ideas, contributions, and assistance I have received contributing to this work are fully acknowledged in accordance with college policy in the acknowledgements section and throughout the text. Any original publications from work produced while preparing this thesis has also been listed in the relevant section.

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Abstract

Background

Bariatric surgery is currently the most effective form of treatment for individuals with severe obesity and Type 2 Diabetes (T2D) and induces systems-wide metabolic changes. While effective, there is significant heterogeneity in the duration of T2D remission reported within the literature. The underlying mechanisms which contribute to improved glycaemic control after surgery have not yet been completely elucidated. Therefore, there is currently no effective or reliable method of predicting the probability of surgical resolution of T2D in the pre-operative setting. The constant evolution and technological improvements in the field of metabolomics have enabled scientists to explore systems biology with a different perspective and investigating metabolic mechanisms that contribute to the resolution of T2D after surgery provides an opportunity to investigate this pathology with a different viewpoint.

Methods

This thesis utilises predominantly Liquid Chromatography- Mass Spectrometry (LC-MS) as a metabolic profiling method. After pre-processing of the data, a combination of multivariate modelling, univariate statistics and correlation analysis was used as a method of exploratory analysis to explore the metabolic differences in a cohort of individuals with obesity and T2D. The effect of bariatric surgery on the metabolic profiles of these individuals are then evaluated, as well as signatures of T2D resolution. Methodological techniques which potentially simplify metabolic profiling studies are also adopted and evaluated with the clinician-scientist in mind. Finally, utilising random forest, a machine learning algorithm, I evaluated whether any of the metabolites profiled might have a role in the prediction of surgical T2D resolution.

Results

Amino acid (AA) and lipid signatures differentiating individuals with and without T2D were identified. Some of these metabolites were significantly correlated with glycated

haemoglobin (HbA_{1c}). Bariatric surgery in particularly Roux-En-Y Gastric bypass resulted in a reduction in some of the metabolites linked to glycaemic control. Novel serum AA ratio and lipid signatures of surgical T2D resolution were also identified.

Two targeted LC-MS methods of serum amino acid quantification were compared and found to be comparable. The use of total creatinine or osmolality as a normalisation factor for urinary tryptophan and kynurenine metabolites data acquired by LC-MS was found to improve multivariate modelling. 24-hour urine values of tryptophan and kynurenine metabolites with creatine used as a post-acquisition normalisation factor was found to strongly correlate with their respective corresponding serum values, thus indicating urine's suitability to be used as a surrogate biofluid.

Conclusion

The exploratory and discovery nature of this thesis has confirmed existing and identified several novel amino acid and lipid signatures linked to metabolic dysfunction in T2D and its association with T2D surgical resolution. Some of the findings also explain a subset of the beneficial effects of surgery for T2D and other obesity associated conditions. The results from these studies give clues to mechanistic drivers and can form the basis of future studies to establish causality.

Overview

Part I : Background, Thesis Overview And Exploratory Metabolomics



Chapter 1

Bariatric Surgery and
Surgical Diabetes
Resolution

Chapter 2

Metabolomics

Chapter 3

Study Overview and
Exploratory Untargeted
Analysis

Part II: Targeted Profiling of Metabolic Pathways



Chapter 4

Amino Acids

Chapter 5

The Tryptophan Pathway

Chapter 6

Serum Lipids

Part III: Clinical Applicability of Findings



Chapter 7

Current Predictors of
Surgical Diabetes
Resolution & Random
Forest Modelling

Chapter 8

Conclusion, Limitations
and Further Work

Presentations and Publications

Publications

- KTD Yeung, N Penney, L Whiley, H Ashrafian, M Lewis, S Purkayastha, A Darzi & E Holmes. The impact of bariatric surgery on serum tryptophan – kynurenine pathway metabolites. *Scientific Reports* 12, 294 (2022)

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- Kynurenic Acid – A Clue for Different Mechanisms of Action in Bariatric Surgery?

Imperial College London, Department of Surgery & Cancer Dec 2019 Annual Research Afternoon.

- The Role of Amino Acids in Type 2 Diabetes Resolution After Bariatric Surgery

25th International Federation for the Surgery of Obesity and Metabolic Disorders (IFSO) World Congress, 23-27 August 2022, Miami, USA.

- Serum Amino Acid Ratios as Proxies to Mechanism Contributing to Improved Metabolic Health after Bariatric Surgery
- Serum Lipidomic Signatures of Type 2 Diabetes and Early Surgical Diabetes Resolution

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Abbreviations

ADA	American Diabetes Association
ALT	Alanine Aminotransferase
AUROC	Area Under the Receiver Operating Characteristic curve
BCAA	Branched Chain Amino Acid
BCAT	Branch Chain Aminotransferase
BCKDH	Branched Chain Keto Acid Dehydrogenase
BMI	Body Mass Index
BPD-DS	Biliopancreatic Diversion with Duodenal Switch
BTR	Branch Chain Amino Acid to Tyrosine Ratio
CAWG	Chemical Analysis Working Group
CE	Cholesterol Ester
CER	Ceramide
CIT	Citrulline
CKD	Chronic Kidney Disease
COPD	Chronic Obstructive Pulmonary Disease
CVD	Cardiovascular Disease
DAG	Diacylglycerols
DCER	Dihydroceramides
DM	Diabetes Mellitus
ESI	Electron Spray Ionisation
FDR	False Discovery Rate
FFA	Free Fatty Acids
G ₃ P	Glycerol 3 Phosphate
GDM	Gestational Diabetes Mellitus
GGR	Glutamine to Glutamate Ratio
GLP ₁	Glucagon Like Peptide
GLUT ₄	Glucose Transported Type 4
GPR	G Protein Coupled Receptor
HCER	Hexosylceramide
HDL	High Density Lipoprotein
HMDB	Human Metabolome Database
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HPLC	High-Performance Liquid Chromatography
I ₃ AA	Indole-3-Acetic Acid
IDF	International Diabetes Federation

IDO	Indoleamine 2,3-Dioxygenase
IGB	intra-gastric balloon
IGT	Impaired Glucose Tolerance
IMS	Individualised Metabolic Surgery Score
KAT	Kynurenine Aminotransaminase
KMO	Kynurenine 3 Monooxygenase
KTR	Kynurenine to Tryptophan Ratio
KYN	Kynurenine
KYNA	Kynurenic Acid
KYNU	Kynureninase
LAGB	Laparoscopic Adjustable Gastric Band
LAT ₁	Large Neutral Amino Acid Transporter
LCAT	Lecithin-Cholesterol Acyltransferase
LCER	Lactosylceramide
LCMS	Liquid Chromatography Mass Spectrometry
LDL	Low Density Lipoprotein
LPC	Lyso-Phosphatidylcholines
LPCAT	Lysophosphatidylcholine Acyltransferase
LPE	Lysophosphatidylethanolamine
LPG	Lyso-Phosphatidylglycerol
LPI	Lyso-Phosphatidylinositol
LSTT	Leucine and Serine to Threonine and Tryptophan Ratio
MDT	Multi-Disciplinary Team
MGB	Mini or One Anastomosis Gastric Bypass
MS	Mass Spectrometry
MSI	Metabolomics Standard Initiative
mTOR	Rapamycin Molecular Target
NAD	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAFLD	Non-Alcoholic Fatty Liver Disease
NBSR	National Bariatric Surgery Registry
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NIDDM	Non-Insulin Dependent Diabetes Mellitus
NIST	National Institute of Standards and Technology
NMDA	N-Methyl-D-aspartate
NMN	Beta-nicotinamide mononucleotide
NMR	Nuclear Magnetic Resonance Spectroscopy
OAGB	One Anastomosis Gastric Bypass

OECD	Organisation for Economic Co-operation and Development
OPLS-DA	Orthogonal Partial Least Squares Discriminant Analysis
PAG	Phenylacetate glutamine
PC	Phosphatidylcholine
PCA	Principal Component Analysis
PDGF	Platelet Derived Growth Factor
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PLA ₂	Phospholipase A ₂
PP ₂ A	Protein Phosphatase Enzyme
PPAR	Peroxisome Proliferator Activated Receptors
PSAT	Phosphoserine Aminotransferase
PYY	Peptide YY
QC	Quality Control
QPRT	Quinolinate Phosphoribosyltransferase
RCT	Randomised Control Trial
RF	Random Forest
ROS	Reactive Oxygen Species
RP	Reverse Phase
RYGB	Roux-En-Y Gastric Bypass
SCFA	Short Chain Fatty Acid
SGLT ₁	Sodium Glucose Transport Protein-1
SHMT ₁	Serine Hydroxymethyltransferase 1
SHMT ₂	Serine Hydroxymethyltransferase 2
SM	Sphingomyelin
T ₂ D	Type 2 Diabetes
TAG	Triglycerides
TCA	Tricarboxylic Acid
TDO	Tryptophan 2,3-Dioxygenase
TLR-4	Toll-Like-Receptor 4
TMAO	Trimethylamine N-Oxide
TNF- α	Tumour Necrosis Factor Alpha
TPH	Tryptophan Hydroxylase
TRP	Tryptophan
UHPLC	Ultra-High-Performance Liquid Chromatography
VEGF	Vascular Endothelial Growth Factor
VLCD	Very Low-Calorie Diet
VLDL	Very Low-Density Lipoprotein
VPR	Valine to Phenylalanine Ratio

VSG	Vertical Sleeve Gastrectomy
WHO	World Health Organisation
XA	Xanthurenic Acid

Chapter 1: Bariatric surgery and surgical diabetes resolution

1.1 Obesity and Type 2 Diabetes

Global Pandemic - Obesity

Obesity is a condition where there is an excessive accumulation of body fat. It is a serious but preventable and reversible condition. Recognised by the World Health Organisation (WHO) as one of the greatest public health challenges of the 21st century (1), in 2021, obesity is a global pandemic that is now estimated to affect over 1.9 billion adults worldwide (1). Adults with a Body Mass Index (BMI) between 25-29.9 kg/m² are classified as overweight, 30-34.99 kg/m² are classified as living with obesity and above 35 kg/m² is considered to be severely obese. Within the United Kingdom, it is estimated that one in four adults are affected by this condition(2). Within England in 2018, 63% of adults were overweight or living with obesity (3). There is significant variability globally, with the United States reporting adult obesity rates of 40% compared to Japan and Korean where rates are less than 10%(4).

The cause of obesity is due to a multitude of interlinked genetic, environmental and behaviour factors. However, the fundamental pathophysiological process is hypothesised to be a result of a prolonged imbalance between calorific consumption and expenditure. Surplus calories are stored as fat within the human body. In particular, excess fat which lines the internal organs, otherwise known as visceral fat, is strongly linked with metabolic disorders(5). Pro-inflammatory factors are then secreted by macrophages accumulated within this excess adipose tissue leading to a chronic low grade inflammatory state (6) which contributes to the development of several obesity related co-morbidities.

Over the last several decades, humans have had easy access and exposure to a variety of foods that are cheaper but increasingly energy dense and higher in fat, salt and sugar content. Modern habits and technology have also led to a general decline in physical activity

patterns and a more sedentary lifestyle contributing to the increasing prevalence of obesity. Historically and through evolution humans have needed to survive by conserving energy and have had to endure malnutrition leading to a genotype that tends to favour these circumstances. This evolutionary history results in significant difficulty when one actively attempts to reverse processes leading to obesity (7).

There is no single genetic mutation that can be directly attributed to the dramatic rise in the prevalence of obesity(8). However, several genetic risk variants linked to markers of obesity including BMI, weight circumference and satiety responsiveness have been identified (9, 10). The factors of genetic predisposition along with environmental influences, impaired physiological and metabolic states have all contributed to the growing rate of obesity.

If left untreated, those living with obesity have an increased risk of developing potentially serious obesity related comorbidities with a significant impact on both quality and expectancy of life(11). These conditions include and are not limited to type 2 diabetes (T2D), hypertension, hypercholesterolemia, dyslipidaemia, atherosclerosis, coronary heart disease, asthma, stroke, gastro-oesophageal reflux disease, many types of cancer, gallstones, osteoarthritis, obesity related infertility, polycystic ovarian syndrome, steatohepatitis, chronic kidney disease, dementia and depression(12).

As BMI increases, the risk of developing these related comorbidities also increase. There is an estimated reduction in life expectancy between five to twenty years depending on additional risk factors such as age, gender, race, severity of obesity and related comorbidities(13-15). In 2016, the WHO reported that there were more deaths globally attributed to individuals who were overweight or living with obesity rather than underweight(1). In the UK, excess adiposity is now responsible for more deaths in the middle and old-age groups than smoking(16).

Treatment of Obesity

Obesity is entirely preventable, and prevention should form the mainstay of population level government intervention and policies. Education, accessibility and affordability to healthier food choices and regular physical exercise should be promoted. Motivating and enabling individuals to make these choices regardless of socio-economic background is important in the fight against obesity. Examples of this include the movement to improve the nutrition level of school meals, the banning of junk food advertisements and the introduction of sugar tax policies (17).

However, for those already living with obesity, treatment is challenging. Complex alterations in underlying hormonal states and metabolism along with physical constraints hamper weight loss efforts and in fact, sometimes promotes weight gain leading to poor results at sustained weight loss attempts both via lifestyle and pharmacological interventions. In addition, further factors such as the role of the brain in energy balance is also yet to be fully understood (18). The poor success rate of traditional treatment methods aimed at reducing energy intake while increasing expenditure via physical exercise suggests the relationship between weight, exercise and other aetiological factors are complex and still poorly understood(8).

Currently, the management of obesity in the United Kingdom is structured into tiers. Tiers 1 and 2 are delivered at a primary care level and cover health promotion and lifestyle interventions. Tier 3 is specialist weight management delivered within the hospital setting often in the form of group sessions. This includes personalised diet advice, time restrictive diet, exercise programs and behaviour coaching. When participants are compliant and motivated, these programs have the potential to be effective(19). There are also pharmacological options available, some of which have shown early promise in clinical trials, but none have proven to yield successful and sustained long term results yet(20, 21). Finally, tier 4 service is surgical intervention for obesity and its associated co-morbidities, otherwise known as bariatric or metabolic surgery. This currently is the most successful treatment for severe obesity (22-24) but is not without risk.

Obesity & Type 2 Diabetes

Diabetes is a chronic disease characterised by persistent hyperglycaemia underlying pathological alterations to glucose and lipid metabolism. Insulin is a peptide hormone produced by the beta cells within the pancreas, it is secreted in response to blood glucose levels and regulates glucose homeostasis by promoting absorption of glucose from the blood. Systemic inflammation secondary to obesity causes an imbalance between the immune system and systemic metabolic functions. This in turn leads to dysregulation of glucose homeostatic mechanisms(25). Hyperglycaemia can be caused by the reduction of insulin mediated glucose uptake mechanisms in peripheral tissues (insulin resistance) and/or the failure of pancreatic insulin secretion (β -cell dysfunction)(26). Insulin resistance itself is a result of many pathophysiologic processes affecting insulin signalling, glucose transporter dysfunction, lipotoxicity, excess fatty acid, altered amino acid and lipid metabolism, oxidative stress, and systemic inflammation(27). Many cellular and molecular pathways linked to the above have been postulated as contributing to insulin resistance, but a unifying theory remains elusive.

There are three main types of diabetes, type 1 diabetes is where the body is not able to produce insulin due to the complete autoimmune destruction of beta cells in the pancreas. Type 2 diabetes(T2D) which is the most common type, it is a heterogenous classification, traditionally described as a disease of insulin resistance and β -cell dysfunction. Research is only just beginning to understand the large variations and further sub classifications linked to insulin resistance, deficiency or other factors within this diagnosis(28) including factors of genetic (29) and ethnic (30) predisposition. T2D is not exclusive to those with obesity as there are also cohorts of lean patients who also have T2D, linked to rapid β -cell failure(31). Finally, gestational diabetes occurs during pregnancy and usually resolves after delivery.

The cause of T2D is complex(32). Obesity, dietary patterns and T2D are profoundly linked(33-35): one in three people with T2D are overweight and one in ten are classified as living with obesity. Obesity leads to increased risk of T2D and both contribute exponentially to the risk of developing serious associated microvascular and macrovascular comorbidities and conditions affecting the heart, eyes, kidneys, blood vessels and nervous system (36).

T2D is a significant cause of blindness (37) and renal failure(38) globally. Chronic systemic inflammation secondary to obesity is thought to be an important mechanistic driver of T2D (39)

Interestingly, metabolic outcomes after bariatric surgery have also led to the idea that obesity and T2D are both separate and intertwined conditions(40). After surgery, most patients with T2D benefit from improved glycaemic control(41). However, in some, despite significant surgical alterations in anatomy such as after Roux-En-Y gastric bypass (RYGB) do not experience any improvement(42). There are also cohorts of patients living with T2D that do not have an elevated BMI(43). Such variations in outcomes currently cannot be fully explained by our current understanding and leads to the hypothesis that while obesity contributes to the T2D phenotype, other factors also impact this pathological phenotype.

In 2019, there were approximately 463 million adults living with T2D worldwide. This is not limited to higher income countries with 79% living in low- and middle-income countries. This number is predicted to rise to 700 million by 2045. During the same year, diabetes was attributed to 4.2million global deaths(44). Within the United Kingdom, 4.5 million people are currently living with T2D and the National Health Service (NHS) spends one tenth of its yearly budget treating T2D. 80% of this is associated with healthcare costs treating complications of diabetes(45) equating to a staggering £1 Million per hour. T2D and obesity place an enormous burden on global health systems. Halting the rise in diabetes and obesity was one of the nine voluntary global targets highlighted by the WHO in its global action plan for the prevention and control of noncommunicable diseases 2013-2020 report(46).

Currently, diagnostic criteria rely on laboratory-based measures of fasting blood glucose levels, calculated insulin sensitivity indexes as well as oral glucose tolerance tests and measurement of glycated haemoglobin (HbA_{1c}). These diagnostic tests detect T2D at a stage where pathological processes are already well established. The search for novel predictive metabolic markers that are both sensitive and specific to the T2D spectrum is urgently required to allow intervention and prevention at an earlier stage.

Treatment of T2D

Current treatment of T2D is aimed at achieving tight glycaemic control to prevent T2D related complications with modification of risk factors and is traditionally achieved through long term medications and lifestyle modifications. While this reduces the risk of microvascular complications, it does not reduce the risk of cardiovascular events, stroke, diabetes neuropathy or renal failure(47-49). So, factors beyond persistent hyperglycaemia from T2D and the obesity phenotype such as chronic inflammation and metabolic dysfunction must in theory be contributory to the development of these complications.

Current National Institute for Health and Care Excellence (NICE) guidelines classify treatment into diet, lifestyle and drug interventions (50). It is suggested that a target HbA_{1c} should be set between 48mmol/mol to 53mmol/mol depending on medication choices. Those with an HbA_{1c} of 58mmol/mol or above are considered to have poorly controlled disease. Drug therapy is tailored according to the level of glycaemic control, medication allergies, intolerances, and relevant past medical history. Medications are prescribed in a stepwise protocolised manner titrated to response and examples of medications used include metformin, dipeptidyl peptidase-4 inhibitor, pioglitazone, sulfonylurea, sodium-glucose cotransporter 2 inhibitor and glucagon-like peptide 1 mimetic(50). Insulin therapy is reserved for those with difficulty controlling HbA_{1c} despite multi-drug therapy and non-pharmacological modifications. Despite a wide plethora of medications made available and various non-pharmaceutical methods to help achieve these aims, only approximately 10% of patients ever achieve glycaemic and metabolic control in a sustained manner(51).

It is well documented that in those who are living with obesity, weight loss prevents T2D in most cases and in those already living with T2D, weight loss leads to an improvement in glycaemic control. While there are some successful examples (52), sustained weight loss remains a significant challenge in these circumstances due to a variety of physical, metabolic and psychological reasons. Unfortunately, some medications used for optimising glycaemic control unintentionally causes weight gain. In those who succeed with low calorie diets and lifestyle modifications, weight loss is rarely persistent beyond one to five years(53). This has led to a search for alternative forms or targets of therapy with the gastro-

intestinal tract emerging as one prime candidate with recent level one evidence showing the superiority of bariatric surgery over stand-alone medical therapy in the treatment of T2D in those with obesity(54, 55).

1.2 Bariatric / Metabolic Surgery

1.2.1 Indication and selection for Surgery

The current National Institute for Health and Care Excellence (NICE) criteria for bariatric surgery under the NHS are as follows(56) in *table 1-1*:

• BMI > 40 kg/m² • BMI between 35-40 kg/m² with obesity related co-morbidities such as type 2 diabetes • The patient has tried all appropriate non-surgical measures to achieve or maintain adequate weight loss • The patient has been or will receive Tier 3 intensive obesity management services • Fitness for General Anaesthesia • Willingness to engage in long term follow up • No Contra-indications for Bariatric Surgery
There are lower thresholds in two cohorts of patients: • Consider surgery for patients with severe T2 Diabetes who have a BMI of 30 or above • In patients with Asian family origins, the BMI threshold can be reduced by a further 2.5 kg/m²

TABLE 1-1 NICE CRITERIA FOR BARIATRIC SURGERY

If the patient wishes to be considered for surgery and fulfils the above criteria, then a referral by the general practitioner to a bariatric surgical centre is warranted. A thorough assessment by individual members of the multi-disciplinary team (MDT) is essential. Members of the MDT include the bariatric nurse specialist, bariatric physician, bariatric psychiatrist or psychologist, specialist dietitian, anaesthetist, and the surgeon.

Patients hoping for a 'quick fix' do not necessarily have the correct motivation or engagement potential and often do not achieve satisfactory results thus pre-operative counselling and patient selection is extremely important. Individuals that require further support, behavioural coaching or counselling should be identified and an MDT consensus should be reached before proceeding with surgery. It is essential to recognise that bariatric

surgery is a part of a wider process that is associated with improved weight and metabolic outcomes.

Several common lifestyle and social habits also need to be considered. Smoking is a significant risk factor for developing both immediate and longer term post-operative complications in bariatric surgery. It is strongly recommended smoking cessation occurs before surgery and some centres consider this to be an absolute contra-indication. Those who drink alcohol need to be counselled that intestinal absorption rates increase due to short gut transit after RYGB increasing the effects of alcohol but also leads to blood levels that would be illegal for driving. Women of childbearing age should also be counselled to use appropriate contraception for 18 months after surgery due to the dramatic catabolic effects of surgery on the patient's body, nutrition and metabolic state.

1.2.2 Types of Bariatric Surgery

The two main operations performed both globally and within the UK are vertical sleeve gastrectomy (VSG) and Roux-en-Y gastric bypass (RYGB)(57, 58), with VSG becoming increasingly the most popular procedure as it is a relatively simpler procedure with lower rate of complications(59). The vast majority of these procedures are performed laparoscopically and 80% of patients are discharged by the third day after surgery(58).

Historically the procedures were classified into those that are “restrictive” signifying restriction of food intake and “malabsorptive” indicating a reduction in nutritional absorption. In fact, the mechanisms of action are far more complex than this and are multifactorial involving a wide range of both weight dependent and weight independent factors.

Vertical sleeve gastrectomy (VSG) was traditionally described as a more “restrictive” procedure: a large portion of the stomach is resected using laparoscopic staplers along the greater curve beginning from approximately 3-5cm from the pylorus to the lateral edge of the gastro-oesophageal junction, forming a new tube-like stomach approximately 80-100ml in size, see figure 1-1.

Roux-En-Y gastric bypass (RYGB) was a procedure that is traditionally described as having both “restrictive” and “malabsorptive” mechanisms; involves the creation of a 15-30ml egg sized gastric pouch which is anastomosed with the distal jejunum. Food that is consumed ‘bypasses’ the excluded stomach remnant, duodenum and proximal jejunum and this is known as the roux limb. Biliary and pancreatic digestive excretions travel down the divided proximal jejunum known as the BP limb. It is then anastomosed in a Y configuration with the roux limb where it joins to form the common channel, see *figure 1-1*.

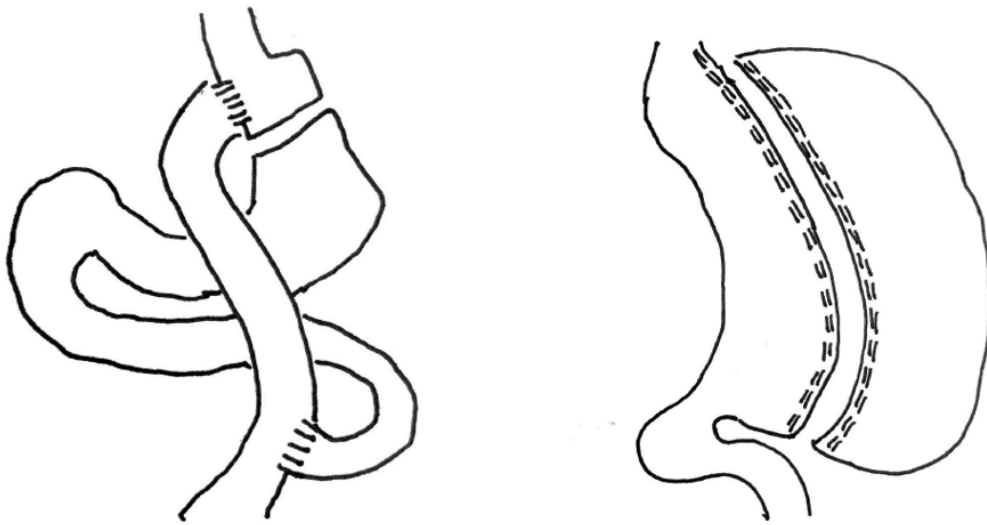


FIGURE 1-1 ILLUSTRATION OF ROUX-EN-Y GASTRIC BYPASS (LEFT) AND VERTICAL SLEEVE GASTRECTOMY (RIGHT)

Other “restrictive” procedures include the intra-gastric balloon (IGB) where a soft and expandable balloon is fitted endoscopically in the stomach often as a temporary or first stage procedure to provide volumetric restriction within the stomach leading to a degree of weight loss by limiting food intake. The laparoscopic adjustable gastric band (LAGB) is placed laparoscopically just below the gastro-oesophageal junction which is connected to a subcutaneous port via tubing. The band is adjusted by injecting fluid into the system and limits the amount of food a patient can tolerate. This procedure has high variability in success and has become less common due to a lifetime risk of complications and the eventual need for the band to be surgically removed and requiring subsequent revisional bariatric procedures(60).

The vertical banded gastroplasty is no longer commonly performed and biliopancreatic diversion with duodenal switch (BPD-DS) has not gained popularity globally due to a higher rate of morbidity and mortality along with severe long time nutritional deficiencies.

There are also other newer procedures which have gained significant attention and popularity. The mini or one anastomosis gastric bypass (MGB/OAGB) takes the principals of RYGB, where a longer gastric pouch is created, and a loop of small bowel is anastomosed distally to create a loop. This procedure has shown promise for its weight loss profile and resolution of obesity related co-morbidities.

1.2.3 *Risks of Bariatric Surgery*

Bariatric surgery has been shown to be safe. The latest UK National Bariatric Surgery Registry (NBSR) Report 2020 found that bariatric surgery was associated with a hospital mortality rate of 0.04% and complication rate of 2.4%(58). A recent meta-analysis of 3.6million patients who underwent bariatric surgery between January 2014 and July 2020 reported an overall pooled mortality rate of 0.08%(61). These rates are no different when compared to most routine general surgical and orthopaedic procedures and is predominantly due to significant improvement in minimally invasive surgical techniques and anaesthesia expertise(62). However, despite compelling evidence of safety, there are still significant misconceptions related to the safety of bariatric surgery due to commonly associated co-morbidities and perceived anaesthetic and surgical risk associated with obesity. When comparing the most commonly performed bariatric procedures, RYGB was found to have a higher rate of adverse events(63), complications(64), readmission (65) and future re-operation(66). Some common bariatric surgery complications are displayed in *table 1-2*.

Early	Late (>30 days post-operative)
General	
○ Intra-abdominal Bleeding ○ Wound Infection ○ Venous Thromboembolism ○ Bowel Obstruction ○ Pneumonia	○ Incisional Hernia ○ Dumping Syndrome ○ Anaemia ○ B12 Deficiency ○ Micronutrient Deficiencies ○ Wernicke's Encephalopathy ○ Gallstone Disease ○ Depression ○ Bowel Obstruction ○ Weight Regain
RYGB Specific	
○ Anastamotic Leak ○ Internal Hernia	○ Internal Hernia ○ Marginal Ulcer ○ Anastamotic Stricture ○ Gallstone Disease
VSG Specific	
○ Staple Link Leak	○ Gastro-oesophageal Reflux ○ Sleeve Stricture

TABLE 1-2 COMMON COMPLICATIONS ASSOCIATED WITH BARIATRIC SURGERY

Despite the above, in appropriate cases, the benefits associated with bariatric surgery far outweigh the risks. Bariatric surgery not only leads to sustained weight loss, but an improvement is also seen in many associated co-morbidities. The post-surgical modulation of lipid and glucose metabolism and metabolic profiles as well as changes conferred to the gut microbiome and host co-metabolism are some of the mechanisms which are thought to contribute these beneficial effects(67-70).

Longer-term data is still required, although several large studies have reported significant long-term results. The Swedish Obese Subjects (SOS) study has reported the longest follow up cohort to date. Over 4000 study and 1000 reference patients were followed up for an average of 24 years, those who underwent surgery was found to have a 3 year longer life expectancy compared to those in the control group who received standard obesity care. However, when compared to the general population, life expectancy was still shorter by 5.5 years(71). Multiple large scale observational studies have found patients who undergo

bariatric surgery to have an associated lower all-cause mortality and obesity related comorbidities when compared to matched controls(72-77). So, one must weigh up the risk of complications against the consequences of not effectively treating obesity.

1.2.4 *The History of Surgical Type 2 Diabetes Resolution*

Surgery and T2D are two terms that are not conventionally paired together. However, the landscape of diabetes treatment is rapidly evolving. Bariatric surgery was initially intended for weight loss, but it has now been shown to be very successful in the treatment of T2D and other obesity related comorbidities in those living with obesity. These effects have led to the term ‘metabolic’ surgery.

Looking back, the effects of gastro-intestinal manipulation resulting in improvement in diabetes was first reported in 1925(78) in a case following gastro-jejunostomy. In 1955, an improvement in glycaemic control was noted in patients who had diabetes mellitus and who underwent Bilioth II subtotal gastrectomy for gastric cancer or ulcer disease(79).

In 1987, the first metabolic surgery case series was published by Pories et al. 86 out of 88 patients with severe obesity and diabetes who had undergone the Greenville gastric bypass was reported to become euglycemic four months following surgery. Beneficial effects on hypertension and mental health were also noted(80).

Following on, in 1992 *Pories et al* published a paper titled “is type II diabetes mellitus (NIDDM) a surgical disease?”. Expanding from their first publication, this paper reported a cohort of 515 patients with severe obesity who underwent the Greenville gastric bypass, 55.9% had diabetes prior to surgery and only 5.8% remained following surgery. The authors proposed that Non-Insulin Dependent Diabetes Mellitus (NIDDM) in patients with obesity could be treated by the gastric bypass and suggested that patients with severe NIDDM who were not overweight should also perhaps be considered for surgery. This perhaps was the original landmark publication of ‘metabolic’ surgery. The study also reported a 10% staple line failure rate between the partitioned stomach pouches, and on the basis of this the procedure was modified to divide the stomach which is more in keeping with the modern gold standard Roux-en-Y gastric bypass (RYGB).

In 1995, Pories et al then published “Who would have thought it? An operation proves to be the most effective therapy for adult-onset Diabetes Mellitus”. This seminal paper reported

long term cohort data including 608 patients spanning over 14years reported a mortality and morbidity rate of 1.5% and 8.5% respectively. Surgery led to sustained weight loss and 82.9% of patients with NIDDM and 98.7% with glucose impairment remained euglycaemic(81). Improvements in hypertension, sleep apnoea, cardiorespiratory failure, arthritis and infertility were also reported. The authors conclude that “no other therapy has produced such durable and complete control of diabetes mellitus”.

Since then, a significant number of randomised controlled trials have shown the superiority of metabolic surgery over intense medical therapy in the treatment of T2D and observational data has also shown bariatric surgery to be successful in reducing rates of micro and macrovascular complications(70, 82, 83). Some of these studies reporting T2D remission rates are summarised below in *table 1-3*.

Author	Year	Treatment Groups	Participant Numbers	Time (months)	T2D Remission Rates
Mingrone et al. (55)	2021	RYGB v BPD v MT	20/20/20	120	25% v 59% v 5%
Purnell et al. (84)	2020	RYGB v LAGB	2256/827 T2D	84	57% v 22.5%
Ikramuddin et al. (85)	2018	MT & RYGB v MT	60/60	60	55% v 14%
Simonson et al. (86)	2018	RYGB v MT	19/19	36	42% v 0%
Schauer et al. (87)	2017	RYGB v VSG v MT	50/50/50	60	29% v 23% v 5%
Wentworth et al. (88)	2017	RYGB v MT	22/23	60	23% v 9%
Corurcoulas et al. (89)	2015	MT v (RYGB or LAGB) & MT	21/20/20	36	0% v 40% v 29%

TABLE 1-3 LONG TERM TYPE 2 DIABETES RESOLUTION RATES PUBLISHED IN CLINICAL TRIALS.

RYGB= ROUX-EN-Y GASTRIC BYPASS, VSG= VERTICAL SLEEVE GASTRECTOMY, BPD= BILIOPANCREATIC DIVERSION, LAGB= LAPAROSCOPIC ADJUSTABLE GASTRIC BAND, MT= MEDICAL THERAPY

The 2nd Diabetes Surgery Summit (DSS-II) held in 2016 was a joint multidisciplinary group meeting composed of 48 voting delegates representative of multiple international medical societies. A consensus statement stated that there is now sufficient level one clinical and mechanistic evidence to recommend metabolic surgery for those with T2D and BMI over 40kg/m² and to be considered in those with BMI 30-39.9kg/m² and poorly controlled T2D despite optimal lifestyle and medical therapy (90). This has since been adopted by many countries. Within the United Kingdom, this recommendation has been included into the NICE guidelines for bariatric surgery but it is yet to be incorporated in the managing blood glucose in T2D for adults guidelines yet(50).

A recent large systematic review and meta-analysis published in the lancet included 174,772 participants reported 6.1 years increase in life expectancy in individuals living with obesity who underwent bariatric surgery when compared to matched individuals who underwent non-surgical management for obesity. The benefit of bariatric surgery on all-cause mortality was particularly pronounced in individuals with T2D where life expectancy was 9.3 years longer compared to 5.1 years in those without T2D(91).

Uptake of bariatric surgery has not increased in an expected manner despite high level clinical evidence. Part of the reason may be because currently guidelines and selection criteria for bariatric surgery are still based on Body Mass Index (BMI) alone. T2D and other metabolic measures such as hypertension and obstructive sleep apnoea is still seen only as secondary outcomes. Despite evidence that bariatric surgery in those with obesity and T2D lead to metabolic benefits and reduced overall healthcare costs(92). While there is significant heterogeneity in the definition of T2D remission reported in literature(93), the guidelines and evidence recommending surgery for those with obesity and T2D are not widely known, encouraged or shared amongst clinicians and surgeons (94). We must also refer to the principal of treatment of T2D, which is to reduce risk of end organ damage. T2D remission after bariatric surgery has been shown to reduce risk of incident microvascular disease and even in cases to later relapse, a 'T2D free' period instigated by surgery has beneficial effects on long term end organ damage risk(95).

It was also found that another significant barrier for access to bariatric surgery is the limited knowledge about the safety and effectiveness of surgery in both patients and referring practitioners(96).

The beneficial effects of surgery on T2D have also yet to be translated to the non-obese population. Metabolic surgery for those with uncontrolled T2D and a lower BMI of <35 kg/m² is reported to be safe and has the potential to be effective in the treatment of T2D but further evaluation is required. Similar T2D remission rates have been reported in meta-analysis comparing cohorts above and below a baseline BMI of 35(97). However, longer-term data and randomised control studies are required to fully establish its effects and the mechanisms for which this is achieved in this demographic (98-100).

However, as long-term data becomes available, we are now also seeing that up to one third of patients don't achieve sustained T2D resolution (97, 101-104). Whilst there are some prediction scores published in the current literature that estimate a likelihood of T2D resolution (42), there is still no reliable method to predict or identify patient's diabetes resolution or potential risk of relapse at the pre-operative time point (105). There is also little research into the other fraction of patients who don't achieve either initial or sustained T2D resolution after surgery.

This leads to a vacuum of knowledge. How does bariatric surgery lead to improved T2D outcomes? It is of clinical importance that clinicians can identify those most likely to benefit from surgical intervention to be able to provide personalised medical advice. Understanding of the precise mechanisms leading to surgical resolution of T2D is vital in this but also presents a new angle and opportunity for us to increase our understanding of how obesity is associated with T2D pathophysiology. Identifying how and where these powerful mechanisms achieve improved glycaemic control will allow clinicians to identify patients with underlying metabolic disturbances most likely to be corrected by surgical intervention. A deeper mechanistic understanding also has the potential to aid the discovery of other targeted non-surgical interventions.

1.2.5 Roux-En-Y Gastric Bypass vs Sleeve Gastrectomy for T2D Resolution

When comparing RYGB to VSG, cohort and meta-analysis of observational studies found that RYGB led to higher rates of T2D remission when compared to VSG(63, 106-108) but recent randomised control trials(102, 109, 110) and several meta-analyses (41, 111-113) comparing RYGB and VSG found no significant difference in long term T2D remission, homeostatic model assessment of insulin resistance (HOMA-IR) or improved glucose tolerance between the two procedures. Another meta-analysis also found rates of T2D remission between the two operations did not correlate with percent of excess weight loss(114). The above suggests that while both are effective in achieving T2D remission, the mechanisms for which this is achieved are numerous, varied and not completely elucidated. Some of these mechanisms are discussed below.

1.2.6 Mechanisms of Action

In some patients with T2D, within hours or days after surgery the need for medication and insulin to achieve glycaemic control dramatically decreases. While weight loss is associated with improved insulin sensitivity, this does not occur as rapidly suggesting that there are additional non-weight dependant mechanisms contributing to this overall effect(115). Achieving a threshold of 20% total weight loss has been reported to be strongly associated with initial T2D resolution, however, beyond this threshold the likelihood of T2D remained the same (116) which insinuates mechanisms beyond weight loss.

The precise mechanisms leading to the anti-diabetes effect of surgery are yet to be fully elucidated. These effects seem to be achieved via a combination of improving the utilisation of insulin secreted and decreasing insulin resistance(117). Some mechanistic studies suggest resolution of T2D after surgery is solely due to weight loss(118), other observational studies have also found greater post-operative weight loss to be associated with T2D remission(119). While weight is an important contributory factor, by reducing beta-cell stress(120) and systemic inflammation(121) most now also agree that many other overarching mechanisms contribute to this beneficial effect.

A modest 5-10% weight loss has been reported to be associated with significant improvements in metabolic parameters such as reduction of hypertension and circulating triglycerides. *Aminian et al* reported a cohort of patients with obesity and T2D who achieved <25% weight loss after bariatric surgery but 65% still displayed improved glycaemic control (122). Another well-constructed study found weight loss, weight regain and index weight and BMI to be non-statistically different when comparing those with and without T2D remission after surgery(123).

There are many hypotheses reported within the current literature with regulation of gut hormones such as secretin, glucagon, ghrelin, peptide YY (PYY) and Glucagon-Like Peptide (GLP-1), changes in circulating amino acids, bile acids (BA), modulation of pro-inflammatory cytokines and adipokines and the alteration of gut microbiome composition implicated. Some of these effects are found to be independent of weight(124, 125). The

weight independent effects of RYGB leading to metabolic benefits have been described as the BRAVE effects- Bile flow alteration, Reduction in gastric size, Anatomical gut rearrangement and altered flow of nutrients, Vagal manipulation and Enteric gut hormone modulation(126).

Calorific restriction in the immediate days after surgery or due to an intentional very low calorie diet (VLCD) resulted in equally improved insulin sensitivity and Beta cell function suggesting that this is a major weight independent contributor to the early reduction of glycaemic load after surgery(127). However, VLCD induced weight loss was found to decrease levels of PYY, Cholecystokinin (CCK), GLP-1 and insulin but increase level of ghrelin and most significantly, hunger levels. one year later. These hormones changes remain distant from baseline up to one year later, illustrating the compensatory hormonal changes that render sustained weight loss so difficult(128). Surgery leads to the opposite, and the hormonal effects causes early satiety along with a change in feeding behaviours contributing to sustained weight loss(129).

The foregut hypothesis suggests that exclusion of the proximal intestine diminishes an unidentified anti-incretin response that leads to improved insulin sensitivity(130). Supporting this, in vivo experiments where the duodenum and jejunum have been surgically bypassed in T2D rodent models have led to improved glucose tolerance and blood glucose levels (125, 131). However, a recent randomised control trial investigating this hypothesis using an endoscopically implanted endoluminal duodenal-jejunal bypass liner found it not to be superior for glycaemic control in T2D when compared to intensive medical therapy alone(132). A meta-analysis of experimental surgeries designed to test the foregut hypothesis in humans compared to conventional operations found no extra value in foregut interventions in achieving diabetes remission(133).

The hindgut hypothesis stipulates that the increased speed and delivery of undigested nutrients to the distal intestine and colon leads to enhanced secretion of distal gut peptides such as GLP-1 which improves glycaemic control. This theory is supported where RYGB has been shown to increase release of GLP-1 and PYY when compared to VSG. VSG led to a more

significant reduction in ghrelin, but both led to comparable satiety and weight loss(134).Further illustrating that there are many mechanisms that confer the beneficial effects of bariatric surgery. What is less clear is the physiological significance of increased GLP-1 secretion. RYGB was found to still reduce weight and improve glucose homeostasis in rodent models deficient of GLP-1 receptors(135). In humans, GLP-1 rise after RYGB was not associated with an improvement in fasting glucose(136) but suppression of gut hormones including GLP-1 modified brain reward response to food(137). So, the verdict on GLP-1's significance and precise role in post-operative glucose homeostasis is still widely debated(138) and yet to be fully determined.

Bile acids are ligands of the G-protein-coupled receptor (TGR5) and the nuclear farnesoid X receptor (FXR) and have vital roles in modulating inflammation(139) and a collection of effects known as the 'SLIMMER' effects (140). This includes modulation of Satiety, Lipid & cholesterol metabolism, Incretins and glucose homeostasis, energy Metabolism, gut Microbiota and Endoplasmic Reticulum stress. BAs stimulate the secretion of GLP-1 and PYY from L-cells found mostly in the jejunum and colon via TGR5. Activation of intestinal FXR also leads to an autoregulation cycle of BA production(140). In vivo experiments have found that the gut restricted FXR agonist reduces diet induced weight gain, systemic inflammation, insulin resistance and hepatic gluconeogenesis(141, 142). Furthermore, VSG in FXR (143) or TGR5(144) knocked out mice led to a significant reduction in the beneficial effects of surgery on body weight and glucose tolerance signifying a substantial contributory role for these pathways to the benefits of bariatric surgery.

In human clinical studies, a significant increase in serum BA levels after RYGB were only noted a few months to years after surgery(145) while this correlated to body weight and incretin effects, BA do not seem to mediate GLP1 and PYY response in the early post-operative phase(146). The precise mechanisms for this delayed response are again not fully elucidated. Nevertheless, current literature demonstrates a complex relationship between BA, FXR, TGR5 with energy metabolism and glucose homeostasis(147). *Pattou et al.* found that the absence of bile within the alimentary limb after RYGB caused a blunting in active glucose uptake by the sodium-glucose cotransporters within the intestine. This was effect

was reversed in experiments by the addition of sodium or bile further demonstrating another effect of bile diversion on intestinal glucose absorption(148).

Gut remodelling induced by surgery may also play a part in the metabolic improvements. It has been demonstrated in rodent models that remodelling after RYGB caused a change in energy demands and upregulation of glucose transporters leading to an increase in gut glucose metabolism and disposal(149). L-cells have also been found to increase in number after RYGB suggesting a possible mechanism for the exaggerated gut hormone changes described above (150, 151).

Even in those with clinical T2D remission and significant weight loss after surgery, Beta cell function which is assessed by the administration of intravenous glucose seems to either remain impaired or show minimal improvement(152-154). This may explain why some are more susceptible to T2D relapse(155). Interestingly, Beta cell function was found to increase 5.8-fold after RYGB compared to VSG or IMT(156) and when challenged with oral glucose, beta cell function improved suggesting an GI derived effect or insulin response which is critical to beta cell function post-operatively(152) and even in rarer cases of hypoglycaemia after RYGB(157).

Finally, the gut microbiome are the microorganisms and their collective genomes that reside within the gut. The gut microbiome can influence phenotypic expression (158) and abnormalities related to the microbiome have been linked to many disease states including obesity and T2D, thereby representing another innovative target for intervention(159-163). Patients with T2D can be identified based on the gut microbiome alone using metagenome wide association techniques(162).

In obesity, lower gut bacterial richness has been shown to be related to increased insulin resistance(164). Early gut microbiome profiling studies reported an increase in Firmicutes/Bacteroidetes ratio (160, 165) to be associated with obesity and T2D(160) however, the latest literature suggest that butyrate producing gut bacteria are lacking in those with T2D and an increase in the abundance of firmicutes are positively correlated with pro-inflammatory markers such as Interferon (IFN)- γ and IL-6(166).

Experimental faecal transplant in humans from lean donors to patients with metabolic syndrome resulted in increased insulin sensitivity, suggesting a degree of causality(167). Colonisation of germ-free mice with faecal microbiota obtained from young or old aged mice led to an influence on the relative abundances of lipid and fatty acid content within brain and liver tissues(168). Colonisation by old aged mice microbiota led to increased monounsaturated fatty acids and decreased total cholesterol and polyunsaturated fatty acids within brain tissue. The relative abundance of common lipids found within brain cortex tissue including phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE) and sphingomyelin (SM) were also reduced in those transplanted with old aged mice donors. Similar changes were observed within liver tissues (168).

Further faecal transplant experiments using the gut microbiota from Roux-en-Y gastric bypass (RYGB) treated mice to transfuse non-operated germfree mice, lead to significant weight-loss and decreased fat mass compared to the transfer of gut microbiota from mice who have undergone sham surgery(169). Similar effects have been demonstrated using stools from human patients who have undergone RYGB(68).

The most prominently reported gut microbiome changes after bariatric surgery are a decrease in abundance of Firmicutes and relative increase in abundance of Bacteroidetes and proteobacteria(170). Some of these microbiota changes are reported to be correlated those that achieve post-operative T2D remission(171, 172). However, despite an exponential number of publications related to this topic, not all studies have been able to replicate the same changes(173) throwing caution in the interpretation of potential causality. The precise mechanisms by which the microbiome influences health or disease are not entirely established, but multiple mechanisms have been implicated including reduced energy harvest of non-digestible food types, modified gut permeability leading to decreased systemic inflammation, altered bile acid metabolism and carbohydrate fermentation products such as short-chain fatty acids (SCFAs) acting as signalling molecules to increase

satiety(163, 174-176). Some have reported certain bacterial taxa to be associated with an increased likelihood of T2D resolution after surgery(171). While not the focus of this thesis, further research is still needed to further examine the cause, effect and therapeutic potential of the gut microbiome in obesity and T2D.

Bariatric surgery also reduces obesity induced systemic inflammation and is said to be one of the mechanisms contributing to glucose homeostasis. This will be further examined throughout this thesis. Beyond the GI Tract, there is also evidence to suggest there are brain regulated glucoregulatory systems connecting the central nervous system to pancreatic islet, liver and gut(177).

In summary, bariatric surgery leads to a whole host of interwoven systemic changes than confer weight loss, T2D remission and additional metabolic benefits of which some of the mechanisms are described above. However, despite extensive research, there has yet to be any significant progress in translating these findings to the bedside clinical environment. Further research is required to elucidate the molecular mechanisms by which this is achieved. The use of metabolomics may yield further molecular and metabolic insights and ultimately, a deeper understanding of the T2D pathogenesis and surgery induced resolution may allow for a personalised precision-based medicine approach. It could enable us to identify novel targets for intervention, enhance current treatments, and assist clinicians in the risk stratification and prediction of metabolic response to improve the selection and counselling of potential surgical candidates.

Chapter 2: Metabolomics

2.1 Metabolomics

2.1.1 *Metabolomics*

Metabolomics(178), also referred to as metabonomics(179) or metabolic profiling was first described in 1998. It refers to the comprehensive and dynamic quantification and analysis of biological end product metabolites found within tissue or fluid samples. These metabolites can originate from the host (endogenous), be produced by symbiotic or infectious microbes that live within the host (microbial) or be absorbed (exogenous) from a wide variety of ingested materials such as food and medications. The metabolome refers to the total profile and concentrations of these metabolites and is typically measured by analytical chemistry methods such as nuclear magnetic resonance spectroscopy (NMR) or mass spectrometry (MS). Longitudinal or cross-sectional metabolic profiling provides an opportunity to study molecular level systemic metabolic differences related to states of health, disease and response to intervention(179). This can be applied to both individual and population-based level studies(180). Metabolomics can be applied to fields of studies such as microbiology, food science, toxicology, or environmental science but perhaps most the exciting, is its potential to enhance clinical medical practice. Metabolomic studies can have a role in biomarker discovery, enhancement of current diagnostics, disease screening, population level risk stratification or aid in the development of precision based therapeutic options. Commonly analysed bio samples include blood serum or plasma, urine, faecal extracts, tissue biopsies or extracts and exhaled breath compounds (181-183).

Individual metabolic profiles are widely variable and are a result of complex interactions(184) between the host's environment, lifestyle, diet, gut microbiome and the host's genome (genomics), all of which contribute to the phenotypic expression of both healthy and disease states. Metabolomic data generated are extensive in nature and are closely associated with other '-omics' studies of the transcriptome (transcriptomics), proteome (proteomics) and metagenome (metagenomics). While no single metric can illustrate the full biological complexity of health and disease, an integrated multi-omics systems biological approach, using multivariate chemometric and computational statistical

techniques to integrate and interpret the data acquired can be used to generate or to test hypotheses. This bridges the genotype to phenotype gap and can enhance our understanding of health, disease, and the effect of therapeutic interventions.

When applied to large scale longitudinal epidemiological studies, data generated from metabolic profiling can be used to identify dysregulated metabolic pathways and link potentially novel biomarkers associated with disease risk and thus has the potential to supersede or augment current conventional clinical markers of disease. When combined with gene wide association studies, it allows a deeper understanding of the influence and interactions between genes of risk and factors leading to the expression of a diseased phenotype. Taking a step further, studying the effect of intervention can also further enhance our understanding of pathophysiology. Ultimately this may allow for subsequent individual risk stratification(185) and treatment in an era moving towards precision based, personalised medicine.

The first metabolome-wide association study (MWAS) identified and demonstrated association in selected urinary biomarkers and elevated blood pressure in individuals (184). Since then, the number of metabolomic studies have increased exponentially over the last decade and have investigated diseases such as T2D(186, 187), obesity(188), cancers(189-193), cardiovascular disease(194), inflammatory bowel disease(195, 196), kidney disease(197), Alzheimer's disease (198), hepatic disease(196, 199) and have also recently been used to define pathological metabolic signatures of SARS-Cov-2 infection(200). Studies mapping metabolic associations between closely related diseases such as T2D and obesity have also been performed(201-203) leading to deeper mechanistic pathophysiological insights.

2.1.2 Modalities of Metabolomic Studies

The metabolome is complex and contains a significant number of metabolites at different concentrations, each with their own biochemical characteristics. There have been many analytical techniques and methods developed and applied to profile molecular signatures of complex biological samples but the two most widely used spectroscopic methods of metabolic profiling are Nuclear Magnetic Resonance spectroscopy (NMR) and Mass Spectrometry (MS), which is usually prefaced by a method for separating molecules such as gas or liquid phase chromatography. Both NMR and MS measure a wide range of metabolites with different properties, and it is important to recognise that these techniques are complementary to each other, due to the vast range of physiochemical properties of metabolites found in various biofluids, both methods are required for comprehensive analysis. The main differences between these two platforms are listed in *table 2-1*. Modalities and protocols are chosen depending on the different classes of organic compounds being measured. The majority of data presented within this thesis is acquired using mass spectrometric methods so this will be explored in more detail.

	Nuclear Magnetic Resonance	Mass Spectrometry
Reproducibility	Extremely high	Less reproducible and more likely to yield variability due to technical factors
Speed	Relatively rapid (Typically, 4 minutes per sample)	Slower than NMR (When coupled with chromatography, typically >10 minutes per sample)
Sensitivity	Lower than MS	Highly sensitive providing accurate mass, isotopic distribution and structural information. Also, superior in detecting smaller fragment ions for metabolite identification and secondary pathway metabolites. However, the target molecule must ionise, otherwise sensitivity will be poor.
Selectivity	Generally used for non-targeted analysis	Suitable for both targeted and non-targeted analysis
Samples	Samples are not destroyed during analysis	Small volumes required for analysis, but MS is a destructive method, samples used for analysis are consumed.
Measurement of Metabolites	All hydrogen containing metabolites within detection limits are measured simultaneously	Lower detection limits but different separation chromatographic or electrophoresis methods and ionisation modes are required to measure different groups of metabolites
Pre-analysis preparation	Often do not require or minimal preparation required	Requires sample preparation, derivatisation or extraction dependant on analysis protocol. Machine and chromatography columns also need conditioning.
Quantification	Inherently Quantitative (i.e., double the signal equates to double the concentration)	Requires validated quantitative methods using internal standards & calibration curves for quantification due to ionisation effect

TABLE 2-1 MAJOR DIFFERENCES BETWEEN NUCLEAR MAGNETIC RESONANCE VS MASS SPECTROMETRY

2.1.3 Nuclear Magnetic Resonance Spectroscopy

The NMR phenomenon was first described by Purcell et al(204) and Bloch et al(205). NMR combines the technology of superconducting magnets and pulse sequences. In brief, the molecular structures contained within an analyte can be determined by observing the interaction of nuclear spins under the influence of a powerful magnetic field.

Organic chemistry NMR analysis relies on the abundance of nuclei which are spin-active such as ^1H , ^{13}C and ^{31}P found in many organic compounds. For the purpose of metabolomic profiling, ^1H is by far the most measured nucleus. For nuclei that have a non-zero spin and are electrically charged (see Introduction to NMR Spectroscopy, *Abraham et al* (206) for further explanation). When a sample is subjected to an external magnetic field, the hydrogen nuclei adopt a spin state by absorbing this energy. As the spin rate returns to the base level, this energy is re-emitted, and the frequency of this energy is measured. This is the NMR signal. Although all ^1H nuclei within a chemical are exposed to the same external magnetic field, the exact impact of that field depends on the chemical environment of the proton nucleus such that protons that are more heavily shielded by electron clouds will experience a slightly lower overall magnetic field. These differences cause different chemical shifts representing protons in different chemical environments (e.g., CH_2 vs CH_3).

The intensity of NMR signal is proportional to the concentration of nuclei in the receiver coil so NMR spectroscopy can be used to quantify many metabolites simultaneously. It also does not need the application of pre analytical separation techniques, and the sample does not get destroyed. Different compounds are identified using their characteristic chemical shift and multiplicity of the signal.

2.1.4 *Mass Spectrometry*

Modern MS techniques were developed in the early 1900s by Arthur Jeffrey Dempster and Francis William Aston who were both physicists. Through time, discoveries and technological advancements related to MS have led to the award of several Nobel prizes such as the 2002 Nobel Prize in Chemistry awarded to John Fenn for the development of (ESI) electron spray ionisation which was used in the generation of data presented in this thesis.

MS can be coupled with separation techniques such as (LC) liquid or (GC) gas chromatography. This allows analytes to be separated in gaseous or liquid columns before being introduced chronologically into the mass spectrometer for analysis, which is particularly useful in complex samples such as biofluids. Ultra-high-performance LC (UHPLC) which refers to the pressure capacity within the column is currently the most utilised form of separation for metabolomic studies. LC-MS is versatile with a variety of separation techniques compatible with a wide range of mass analysers.

The LC process involves samples injected into a solvent mixture (mobile phase), which is then transferred into the chromatography column (stationary phase). These phases are either polar or nonpolar and elucidation of molecules depend on their polar interactions. Reversed phase (RP) chromatography is commonly used due to its stability for semi-polar and non-polar metabolites found in biological fluids. It has a polar mobile phase with non-polar stationary phase. This facilitates further differentiation; non-polar metabolites favour the non-polar stationary phase and consequently travel down the column at a slower rate than polar metabolites which favour the polar mobile phase. In order to further separate molecules for analysis, applied methods include gradient elution by gradually altering the ratio of aqueous : organic solvent in the stationary phase(207).

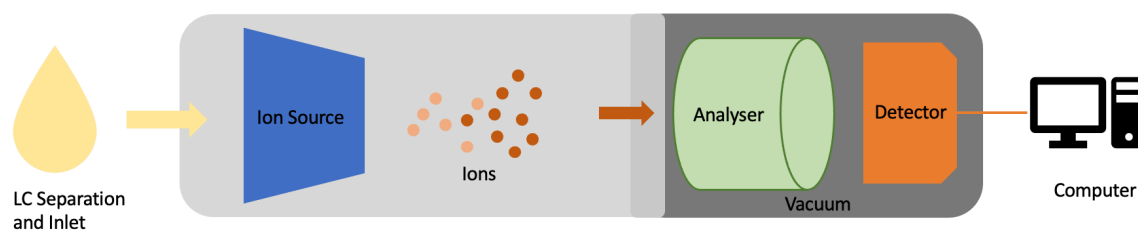


FIGURE 2-1 MASS SPECTROMETRY SCHEMATIC

The basic concept of an MS instrument is divided into three parts (*Figure 2-1*). The first is after separation via chromatography, or infusion, samples are ionised to gaseous ions at the ionisation source. The ions are then separated using magnetic or electric fields in the analyser under a vacuum. Electric fields are preferred due to their practicality compared to large heavy magnets. The generated fields operate in a continuous or cyclic manner and the respective ions will have different trajectories and separate according to their mass-to-charge ratio (m/z). Dependant on the physical properties of each sample, different ionisation methods and pressures may be utilised during different phases along with positive or negative detection modes to enhance detection(208). Finally, the detector system detects the intensities and charge of separated ions. This information is then used to produce a three-dimensional spectrum containing a chromatogram (retention time), mass to charge ratio and signal intensity dimension.

Data presented within this thesis were acquired using quadrupole- time-of-flight (Q-TOF) MS where a combination of quadrupole (four parallel rods) and time of flight mass analyses are used in tandem. The time-of-flight mass analyser provides high resolution and accurate mass data useful in discovery metabolomics experiments, whilst the quadrupole allows for ion pre-selection which can be used for generating specific ion fragmentation patterns critical for metabolite identification.

2.2 Metabolic Workflow and Metabolite Identification

2.2.1 Metabolomic Workflow

A metabolomic workflow can be designed to study the metabolite specific effect of disease or intervention within a study population. Profiling can either be untargeted which aims to profile and characterise all metabolites within a biological sample; or targeted where pre-selected metabolites that belong to the same family or related to a metabolic pathway or disease process are measured and quantified against known markers and standards using pre-determined protocols.

Untargeted analysis focuses the entire metabolic ‘fingerprint’ and is a hypothesis generating method with the potential to yield novel insights or aid biomarker discovery into pathology and disease processes. Targeted analysis offers precise quantification and is used in studies designed to evaluate specific biological pathways and to address specific hypothesis. Both approaches should be used in a complementary fashion in metabolomics pipelines. Study designs often either compare subjects to healthy controls or study the dynamic metabolic changes related to disease or intervention.

The analytic workflow can be divided into stages and is illustrated below in figure 2-2. Once the study has been designed and the criteria for selection have been established, detailed clinical information should be collected with the biological samples. Biological samples belonging to a study must be treated identically. This includes the pre-processing methodology of samples such as centrifugation of whole blood to serum, to the storage container, storage conditions and freeze and thaw cycles. Most samples are immediately frozen at -80°C but depending on the analysis protocol, further processing of samples may be required at the time of analysis.

In the data acquisition phase, samples are typically processed according to standardised operating protocols, which are dependent on the analysis platform. Pre-acquisition normalisation is performed if required. Regardless of methodology but particularly in instances of mass spectrometry, it is ideal to acquire data all in one batch to keep experimental conditions as identical as possible to minimise effects such as drift and batch

effects. Samples are analysed in random run orders to avoid the introduction of unintended or unknown bias that could affect interpretation. Quality control (QC) samples made of from pooling samples obtained from a series of study participants are also typically included in the run order to condition the platform and used as a reference for intra-study reproducibility.

Raw spectral data are pre-processed and converted into manageable data formats such as a .csv file using analytical packages or software. This process is described in further detail later. Post-acquisition normalisation of data for biofluids with variable concentration such as urine is also often utilised to allow for meaningful comparison. If targeted profiling methodology has been utilised, then metabolite concentrations are calculated against known measured standards using calibration curves. With untargeted profiling, multivariate and univariate analysis can be used to identify features of interest. These features can then be related to metabolites in a subsequent process known as metabolite identification, a time consuming and difficult process also described in detail later in this chapter.

Biological evaluation or bioinformatic analysis can be performed with many methods such as pathway mapping or enrichment analysis, correlation network analysis and multi-omics integration. Finally, in the ideal setting, any significant findings will need to be clinically validated on larger population study cohorts.

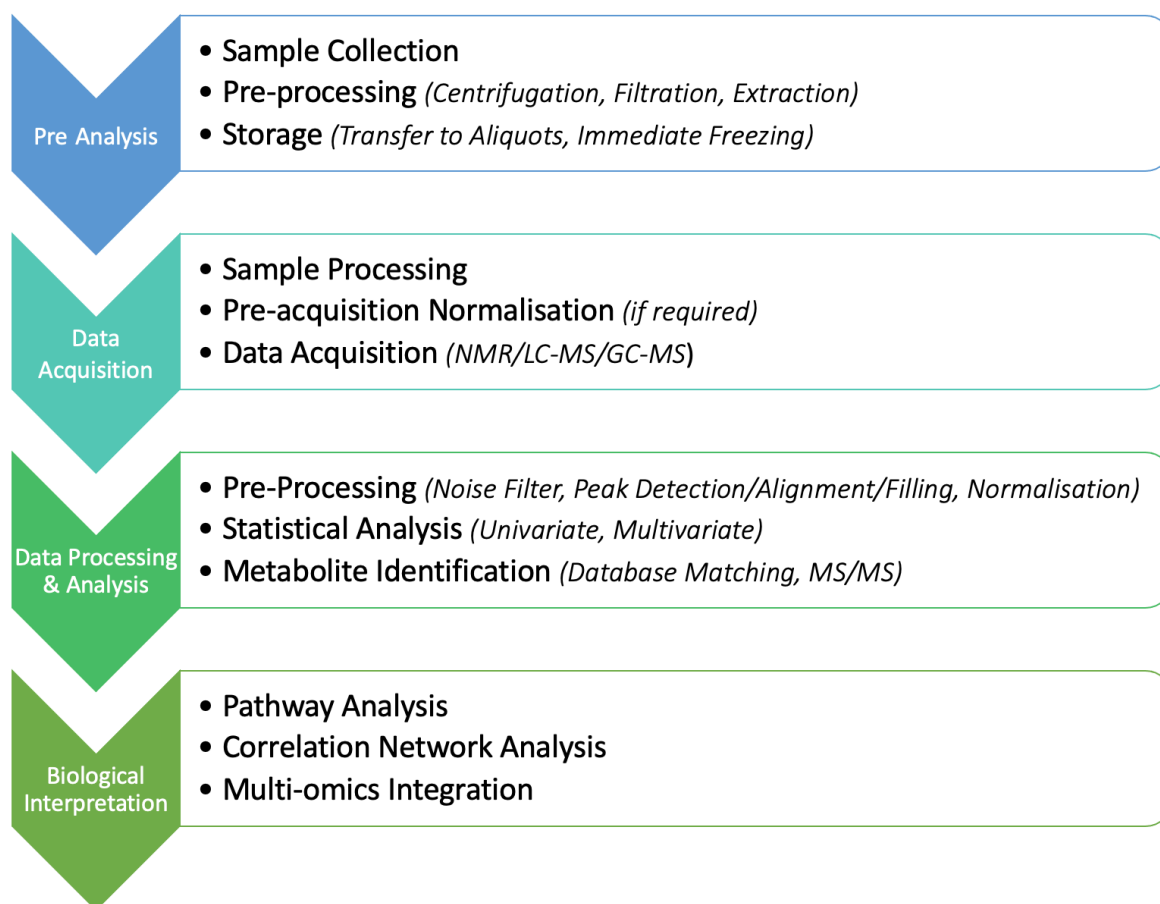


FIGURE 2-2 METABOLOMICS WORKFLOW

2.2.2 Metabolite ID Bottleneck

Metabolite identification (MetID) of features of interest is fundamental in the biological interpretation of raw untargeted metabolomic data, however this is not straightforward and is considered to be a major bottleneck in metabolomics. Selected features of interest rarely directly present as the parent metabolite. Ionisation effects such as adduct formation and in-source fragmentation can occur presenting several mass-to-charge ratios with the same retention time all resulting from the same metabolite. Co-elution where multiple metabolites with the same retention time are detected simultaneously can also occur, all of which serve to make identification of chemicals from standard databases difficult.

To facilitate identification, tandem MS (MS/MS) is a mass spectrometry technique employed to further differentiate large ions with a similar m/z by means of fragmentation.

When exiting the first analyser, the quadrupole in the case of a Q-TOF instrument with MS/MS capabilities can select specific ions to be intentionally fragmented by applying voltage in a collision cell. Several different energy levels are typically used to increase the fragmentation pattern to yield product ions. Detection of these fragments by a second mass analyser gives further structural information to aid the identification of the parent ion. This is particularly useful in identifying separate ions with very similar m/z ratios and fragment patterns can provide a 'fingerprint' for database matching or comparison with a known chemical standard.

With the current workflow, researchers first manually search and compare spectra (m/z , RT and MS/MS fragmentation patterns) obtained from their own experimental to those found in online metabolomic database libraries. Examples of this include METLIN⁽²⁰⁹⁾, human metabolome database (HMDB)⁽²¹⁰⁾, mzCloud⁽²¹¹⁾ or XCMS online⁽²¹²⁾. Care must be taken to evaluate matches both for chemical and biological relevance. Once a potential match is identified, further experimental work is required to confirm the metabolite's identity.

MetID is extremely challenging as it is time consuming manual process which requires significant manpower and has large cost implications. The chemical analysis working group (CAWG) metabolomics standard initiative (MSI) defined four minimum standard levels for publications reporting metabolite identification(213). This includes- Level 1: Identified compounds. Level 2: Putatively annotated compounds. Level 3: Putatively characterised compound classes. Level 4: Unknown compounds. Gold standard level 1 identification of metabolites requires a minimum of two or more orthogonal data sets relative to chemical standards to be compared to experimental data measured in the same experimental conditions. In practical terms, pure chemical standards need to be purchased, then 'spiked' into the sample matrix and processed through the same experimental conditions as the original biofluid sample to confirm the identification of an unknown signal. Identification using data obtained from literature or external laboratories are classified as level 2 annotations.

In a typical untargeted mass spectrometry experiment, <2% of peaks are ever identified(214). Currently <10% of the entire known human metabolome has commercially available standards, reference materials and experimentally collected NMR or MS spectra(210) deposited in online libraries. While online databases continue to grow, newer computational programs have been designed to compute and predict fragmentation patterns and retention times. Such 'in silico' spectra have enabled the rapid rate of metabolomic database expansion which would otherwise not be possible via experimentation. Nevertheless, the need for cheaper and widely available reference materials are urgently required(215).

The collection, processing, subsequent analysis and interpretation of a huge amount of untargeted metabolomic data is challenging and attempting level 1 identification for every single peak is not practical. With a significant number of unknown signals, current metabolic datasets libraries are inadequate for comprehensive biological evaluation. Furthermore, in urine, metabolites are bio-transformed by processes such as glucuronidation or sulfation prior to being excreted.

2.3 Statistical Methods, Principal and Techniques

2.3.1 *Raw Spectral Data Pre-processing*

Pre-processing of spectral data acquired from MS experiments has several purposes. XCMS (XCMS, Scripps Institute, CA, USA) is the most commonly used data processing software for LCMS data(216) and is available as an online platform or R package. Intensity m/z peaks are detected, binned and reported along with retention times (RT). RT drift, which is a common phenomenon seen with LCMS instrument variation, can be adjusted using peak alignment. Peak groups that are not included in every sample from the initial peak grouping and alignment protocols are retrospectively analysed and reprocessed from raw data using peak filling, whereby peak intensity is generated from integration of a m/z ratio range at a specific retention time. Data are also filtered to reduce statistical 'noise' and to remove false peaks. The resulting data can then be normalised if required using a variety of methods to adjust for experimental confounding factors that may affect the interpretation of data. Normalisation of LCMS data acquired from urine samples are explored in further detail in a later chapter but for example, dilution of urine samples leads to MS signal sensitivity loss due to sample residue build up with the source of from detector loss over time. The final output from XCMS is a table where the intensity of features found within the samples are displayed along with its RT. This output is now suitable for statistical analysis.

2.3.2 *Multivariate Statistical Analysis*

It is common for metabolomic datasets to be large and often have more variables than samples. In particular, untargeted metabolic profiling can yield thousands of unidentified features as one metabolite can generate several adducts and fragments and biological samples contain hundreds to thousands of compounds. Isotopic differences are detected, and chemically related metabolites lead to a higher number of significant inter-metabolite correlations. The analysis is performed using various computational methods to reduce the data size and select features relevant to the scientific hypothesis. The overall aim of multivariate statistical modelling is to identify clusters related to biochemical patterns or identify features of separation between groups related to disease or intervention.

Commonly, data are first explored using principal component analysis (PCA). PCA is unsupervised, meaning analysis is performed without any labels or class information provided to the model calculation. PCA asks the question “are there any patterns or sample clusters/groups that exist within this data?”. The unsupervised nature of PCA means that any resulting clustering is based on the metabolite data alone i.e., it identifies inherent patterns based on the biochemical similarity of samples. PCA is used in metabolomics as a first pass quality control screening method to visualise any possible run order effects, by ensuring pooled quality control clustering and to identify any outliers resulting from sub-standard sample preparation or data acquisition (e.g., mis-injection). In some cases where extreme differences in study groups occur, PCA can also be used to identify metabolite patterns that distinguish between the study groups as well as identify those that occur within subgroups.

Following on, supervised modelling where class information is provided is used to ask the question “Are there any systematic patterns in the data which are different between defined sample groups?” The most commonly used method for LCMS data is orthogonal partial least squares discriminant analysis (OPLS-DA), which is a powerful method for identifying features that define the separation between two predefined groups. Inbuilt orthogonal correction removes confounding differences such as age and gender which are not related to separation of labelled classes (188) and can be visualised on an orthogonal plain.

Previously, partial Least Squares (PLS) regression was another commonly used multivariate regression method in analytical chemistry. Orthogonal projects to latent structures (OPLS) regression was first introduced in 2002(217) and OPLS-DA was subsequently introduced as an improvement for differentiating between classes(218). OPLS-DA models should be cross validated by methods such a k-fold; leave one out strategy to prevent over fitting, a scenario where visual differentiation or correlation with a class or response variable is achieved but in reality, the result is non-reproducible model is produced. The model statistics: R^2X goodness of fit and Q^2Y predictive ability of the model are used to evaluate the models produced. Variable importance of projection (VIP) scores and plots display the importance of each variable contributing to class separation within the model. A VIP score of over one is significant to the model. Finally, features identified through multivariate modelling need to be identified before further evaluation using univariate tests and fold change analysis.

2.3.3 *Challenges in bench to bedside clinical translation*

The potential promise of metabolomic studies in improving modern healthcare is substantial. However, challenges remain at every stage of the metabolomics workflow hindering the clinical translation from bench to bedside(219).

The metabolome is diverse in nature and contains many groups of metabolites such as amino acids, lipids, organic acids. These metabolites are variable in concentration, and not all are present in certain biofluids or tissues. This leads to two key issues, first the amount of data generated from metabolomic studies is vast in nature, Secondly, real time dynamic measurement of the entire metabolome is still not yet feasible with current methodology. Thus, we are not able to fully harness the potential of data collected so comprehensive analysis and interpretation of the complex human metabolome is still challenging. Current processes for metabolite identification are also extremely time consuming and expensive.

Nevertheless, technology will continue to evolve. The incorporation of machine learning and artificial intelligence algorithms may accelerate this progress. Ultimately, better characterisation and understanding of disease will result in a better understanding of therapy and aid scientists in seeking therapeutic targets. Risk stratification based on novel biomarkers may guide more efficient earlier and large-scale preventative interventions. All of the above requires significant collaboration between biologists, analytical chemists, medical clinicians, statisticians and computer engineers (220).

Chapter 3: Study Overview and Exploratory Untargeted Analysis

3.1 Thesis and Study Overview

3.1.1 Background

This thesis represents the journey I have taken over the course of the last four years. Throughout the text, I will illustrate the skills and knowledge I acquired during this time, incorporating basic science and clinical components into the chapters. I was offered the opportunity to investigate a cohort of patients who had already been enrolled into a prospective observation study initiated by my predecessor and supervisor Mr Nicholas Penney (NP). Details of the study's ethical approval are detailed in *appendix I* and GDPR transparency wording document is listed in *appendix II*.

Recruitment was performed by NP, all study participants were referred to the Imperial Weight Centre under current NICE guidelines (56) and were due to undergo bariatric surgery at Imperial College NHS Trust (London, United Kingdom) after consultation with the clinical team and multi-disciplinary team consensus. Patients aged ≥ 18 , were eligible for recruitment if they did not meet any of the listed major exclusion criteria listed in *appendix III*. Demographic details and relevant medical and drug history including the use of oral hypoglycaemic agents, insulin, length of T2D diagnosis and other relevant co-morbidities were documented and recorded prospectively. Changes to these were noted on subsequent follow up appointments. Surgical technique is detailed in *appendix IV* and post-operative dietary instructions are listed in *appendix V* and finally sample procurement and storage methods are listed in *appendix VI*.

Variations in age, sex, ethnicity are known investigative confounders, but the level of selectivity in recruitment in order to avoid demographic bias would not have been feasible in the initial proposed timeframe with the small team of investigators dedicated to this clinical research project. It was assumed that the longitudinal nature of the study and the widespread metabolic and physiological effect size of surgery (221) would outweigh these confounding variables. Participants were followed up in synchrony with their usual pre-operative and post-operative NHS appointments to improve study compliance. It was the

initial aim to collect serum, 24-hour urine and faecal samples at each of these timepoints, if possible.

Sample size was estimated at the outset of the study. However, due to the hypothesis generating nature of typical untargeted metabolomic studies generating more variables than samples, there is no gold standard technique for sample size calculations. The MetSizeR method was utilised by Mr Nicholas Penney (NP) to approximate sample size(222). For a study where there is an estimated 300 metabolites generated per analysis of which 20% differentiate between groups, and accounting for a $q=0.05$, it is estimated that 15 samples are needed for each group. With an estimated 30% rate (223) of patients not achieving surgical diabetes resolution and 20% drop out rate it was estimated that a minimum of 65 total patients were required to be recruited into the study which would include RYGB, VSG and non-diabetes control groups.

Initially, 210 patients were recruited into the study between July 2015 to March 2017 by NP. Eventually, 158 patients provided pre-operative clinical samples. I joined the study as a researcher in October 2017 and focused on data analysis, collecting post-operative data and post-operative biological samples. Although I have made modifications and amendments to the original study protocol and patient information booklet for ethics amendments, these were not originally authored by myself and thus have not been included in this thesis.

At the time of submission, all data presented within this thesis has not been used in any other thesis submissions or publications unless specified (see section: *Presentations and Publications*). In instances where data had not been acquired personally, the persons responsible have been appropriately credited. Otherwise, all data processing, statistical analysis, statistical modelling, and data visualisations are original work.

To be able to analyse metabolomic datasets, I learnt the coding language R (224) and respective packages to be able to pre-process data, perform univariate analysis and produce graphical visualisations of data. The computer software SIMCA (*Satorius Stedim Biotech, Sweden*) was used for purposes of multivariate analysis. I also ensured I learned how to run, condition, and troubleshoot LCMS experiments which is the primary metabolic profiling

technique employed to acquire data presented in this thesis with guidance from my post-doc mentor Dr Luke Whiley (LW) and Primary Co-Supervisor Professor Elaine Holmes.

3.1.2 *Thesis Overview*

I began this PhD journey with raw unprocessed and untargeted LCMS serum and urine datasets acquired in several different modes by NP which included 126 serum and 70 urine pre-operative samples; initial exploratory analysis of this dataset along with metabolite identification is presented in the latter part of this chapter. Metabolite identification proved to be extremely challenging for a researcher with minimal analytical chemistry background.

Multivariate exploratory modelling showed systematic differences in the serum and urinary metabolome pre-operatively between those with and without T2D, and showed that bariatric surgery induced a major effect on the metabolome at 3 months post operation.

The exploratory results from this untargeted data subsequently led to part II of this thesis where specific metabolite groups were investigated for their relationship with T2D using a targeted metabolomics approach. The effect of the two most common bariatric surgical procedures: RYGB and VSG on these metabolites were evaluated. Methodological development or comparisons, as well as biological interpretation components are incorporated into these chapters. The final part of the thesis aims to address the question of whether any of the findings presented have any clinical translational potential into clinical practice for prediction of surgical T2D resolution.

Metabolomic methods have previously been used to identify novel metabolites linked to mechanisms of Insulin Resistance (IR) and T2D(225, 226), these findings have also been used to predict incident T2D. By studying the systemic changes induced by surgical intervention and associated with T2D resolution, we may be able to identify some of the major mechanisms that contribute to the T2D phenotype that benefit from surgical intervention and its possible reversibility. This will enhance our understanding of the T2D phenotype and possibly identify novel targets of therapy for further investigation. From a translational perspective, I aim to investigate whether any of the findings may be used to help improve the prediction of surgical T2D resolution. This will assist in identifying patients who are most likely to benefit from surgical intervention, as well as guide clinicians in the clinical decision process. Finally, such predictors, if present, may also enhance the

pre-operative counselling and informed consent process for patients considering surgical intervention for obesity and T2D.

3.2 Metabolic Profiling Protocol summaries

Mass spectrometry data were acquired from several sources. As I only performed confirmatory MS and MS/MS experiments on urine samples, sample preparation methods are listed in *appendix VI*. The following methods are presented briefly for reference purposes. All urine and serum profiling and MS/MS data were acquired using a Waters Acquity Ultra Performance LC (UPLC) system (Waters, MA, USA) coupled with a Xevo G2 quadrupole- time of flight (Q-TOF) mass spectrometer (Waters, MA, USA) with an electrospray source using an established departmental protocol (207, 227). Nitrogen was used as desolvation gas and argon used as collision gas in all experiments.

3.2.1 *Untargeted LCMS*

For untargeted serum analysis, in brief, liquid chromatography was performed using a Waters ACQUITY C18 CSH column (1.7 μ m, 2.1mm x 100mm) at an operating temperature of 55°C. Mobile phases A & B were ACH/H₂O (60:40) mixed with 10mM ammonium formate and 0.1% formic acid; IPA/CAN (90:10) mixed with 10mM ammonium formate and 0.1% formic acid. The chromatographic gradients was as per departmental protocol (227). Mass spectrometry detection was performed with the following settings: Electron Spray Ionisation (ESI)+: Capillary voltage 2.5kV, cone voltage 30V, source temperature 120°C, desolvation temperature 400°C, desolvation gas flow 800L/h, cone gas flow 20L/h. ESI-: Capillary voltage 2.5kV, cone voltage 25V, source temperature 120°C, desolvation temperature 500°C, desolvation gas flow 800L/h, cone gas flow 25L/h. Leucine enkephalin solution [m/z 556.2771 in ESI+, m/z 554.2615 in ESI-] of 200 pg/ μ l concentration, was infused continuously at a flow rate of 30 μ l/min and used as lock mass correction.

For untargeted urine analysis, in brief, samples were analysed in random sequence with quality controls placed every ten samples. Urine was analysed with a reverse phase (RP) chromatographic methodology and both positive and negative MS ionisation detection. For liquid chromatography, a Waters ACQUITY UPLC HSS T3 column (1.8 μ m, 2.1 mm x 100mm) was used, with an operating temperature of 40°C. Mobile phases A & B were 0.1% formic acid in water and 0.1% formic acid in acetonitrile respectively. The chromatographic

gradients was as per established departmental protocol (207). Mass spectrometry detection was performed with the following settings: ESI+: Capillary voltage 1.5kV, cone voltage 20V, desolvation temperature 600°C, cone gas 150L/hr and desolvation gas 1000L/hr. ESI-: Capillary voltage 1kV, cone voltage 20V, source temperature 120°C, desolvation temperature 600°C, cone gas 150L/hr and desolvation gas 1000L/hr. Leucine enkephalin solution [m/z 556.2771 in ESI+, m/z 554.2615 in ESI-] of 200 pg/μl concentration, was infused continuously at a flow rate of 15 μl/min and used as lock mass correction.

3.2.2 Targeted LCMS

Profiling of urinary tryptophan metabolites was performed by LW adapting an established UHPLC-ESI-MS/MS protocol(228) designed for human serum and plasma samples as part of a yet to be published internal method development study. The subsequent raw UHPLC-ESI-MS/MS spectral data were processed by KTDY using the targetlynx application package found within masslynx (v4.1), Waters Corporation (MA, USA).

In brief, liquid chromatography was performed using a Waters ACQUITY UPLC HSS T₃ (1.8 μ m, 2.1mm x 150mm) column at an operating temperature of 45°C. Mobile phases A & B were 0.1% formic acid in water and 0.1% formic acid in acetonitrile respectively. The chromatographic gradients were as per established departmental protocol(228). Mass spectrometry detection was performed with the following settings: ESI+: Capillary voltage 2.5 kV, cone voltage 30V, desolvation temperature 600°C, source temperature 150°C, cone gas 150L/hr and desolvation gas flow 1000L/hr, collision gas 0.15ml/min. ESI -: capillary voltage changed to 0.25kV.

Detection was performed in ESI positive ion mode for all features except for tryptophan which was detected in ESI negative mode. Multiple reaction monitoring was used for quantification of each feature. The mass spectrometry conditions for each were determined via direct infusion of individual standard solutions.

Targeted serum amino acid quantification using the commercially available biocrates absoluteIDQ p190 kit was performed according to manufacturer guidelines by NP. In brief, liquid chromatography was performed using a Waters ACQUITY BEH C₁₈ (1.7 μ m, 2.1mm x 75mm) column at an operating temperature of 45°C. Mobile phases A & B were 0.1% formic acid in water and 0.1% formic acid in acetonitrile respectively. The chromatographic gradients were set per manufacturer protocol. Mass spectrometry detection was performed with the following settings: Capillary voltage 3.9 kV, cone voltage 20V, desolvation temperature 350°C, source temperature 150°C, cone gas 150L/hr and desolvation gas flow 650L/hr, collision gas 0.15ml/min. Raw spectral data were processed using the targetlynx

application package found within masslynx (v4.1), Waters Corporation (MA, USA) and METIDQ (Biocrates; version Carbon).

All other targeted serum amino acid and tryptophan metabolites profiling was otherwise performed with collaborators at the National Phenome Centre, Imperial College London using established protocols (228, 229). This was funded by an Imperial College Healthcare NHS Trust – Biomedical Research Centre Surgery & Surgical Technology Pilot Project 2019-2020 Grant of £10,000 awarded competitively to KTDY.

Targeted plasma lipid profiling was performed using a soon to be published UHPLC-MS/MS methodology by collaborators led by Prof Holmes from the Australian National Phenome Centre, Murdoch University, Perth, Australia. In brief, for extraction, plasma samples were placed in wells of a 96 well plate with 90 µl of propanol-2-ol extraction solvent which also contained stable isotope labelled internal standards (Lipidizer™ Internal Standards Kit from Sciex (MA, USA), SPLASH LipidoMIX™, Lyso PI 17:1, Lyso PG 17:1 and Lyso PS 17:1 (Avanti Polar Lipids)). Following on, the samples were shaken for 10 minutes and stored at -20 °C for 20 minutes prior to centrifugation at 3900 g for 15 minutes. The resulting supernatant was then transferred to a 96 well plate for analysis.

UHPLC-MS/MS was performed using an ExionLCTM coupled to a QTRAP 6500+ system (Sciex, MA, USA). Reversed-phase liquid chromatography was performed using a Waters ACQUITY BEH C18 (1.7 µm, 2.1 x 100 mm) column at an operating temperature of 60 °C. Mobile phase A was water/acetonitrile/propan-2-ol (50/30/20, v/v/v) containing 10 mM ammonium acetate and mobile phase B was propan-2-ol/acetonitrile/water (90/9/1, v/v/v) containing 10 mM ammonium acetate. The flow rate was 0.4 mL/min with gradient elution as follows: starting at 10 % B, increasing to 45 % B at 2.7 min, 53 % at 2.8 min, 60% B at 8.0 min, 80 % B at 8.1 min and holding at 80 % B until 11.5 min, 100 % B at 12.0 min for 1 min before returning to 10 % B for a 2 min re-equilibration for a total cycle time of 15 min. The injection volume was 5 µL.

The QTRAP 6500+ was operated with electrospray ionisation using polarity switching. Mass spectrometry detection was performed with the following settings: Capillary voltage 5.5 kV

(positive ion mode) and -4.5 kV (negative ion mode); temperature 300 °C; curtain gas, 20 psi; ion source gas 1, 40 psi; ion source gas 2, 60 psi. Time-scheduled multiple reaction monitoring (MRM) was used for data acquisition. Data were acquired using Analyst®1.7.1 and analysed using Sciex OS Analytics 1.7.0 software. Pre-processing and peak integration was performed using SkylineMS software(230)

A total of 835 named serum lipid species belonging to the following subclasses were quantified and passed pre-processing quality control using this method. Pre-processing quality control was performed in house at Murdoch university and consisted of 1) signal threshold filtering: removal of any peaks below a set threshold, to remove peaks that are actually noise. 2) Pooled QC filtering: features must return a %RSD of < 30% in replicate analysis of the pooled QC to be kept in the dataset. The pooled QC was analysed at frequent intervals throughout the data acquisition run.

3.2.3 NMR

¹H-NMR spectroscopy on urinary samples was performed by NP on a Bruker 600 MHz spectrometer (Bruker Biospin, Germany). Sample preparation and methodology was per departmental protocol and previously published (231).

3.2.4 Thesis statistics overview

For all statistical analysis presented within this thesis, following acquisition, any data points that were outside of 3 standard deviations of analytical subgroups were considered outliers and excluded. If any normalisation methods were used, exclusion was performed and the data re-normalised after exclusion. Any missing values due to their concentration being below the detection limit of targeted assays was imputed as half of the minimum value(232, 233).

Multivariate: LCMS datasets underwent multivariate modelling using SIMCA version 16.0 (*Satorius Stedim Biotech, Sweden*). Unsupervised PCA was used to assess metabolite profiles and screen for additional outliers. Supervised Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) analysis were used to explore variables contributing differentiation between labelled groups. The variable importance in projection (VIP) value indicated the importance of contribution towards the models. The goodness of fit R^2X and predictive ability Q^2Y values were used to judge the quality of models. All models displayed are cross validated with one aligned and one orthogonal (1 aligned + 1 orthogonal) component.

Univariate: The Shapiro Wilk Test was performed on the metabolites profiled and, as the majority were not normally distributed, for consistency throughout the thesis, differences between investigative groups were assessed for significant differences using Mann-Whitney U tests. Differences between longitudinally paired samples were assessed for significance using Wilcoxon rank test. In order to control the false discovery rate (FDR) in incidences of multiple testing, q values were generated using the method described by Benjamini and Hochberg(234). A q value threshold of ≤ 0.05 was considered to be significant and employed throughout the study where applicable.

Correlation Analysis: Correlations between variables was assessed using Spearman's Rank correlation unless specified otherwise. Ranked Cross-Corelations were evaluated using the R package: lares.

Random Forest: Random Forest modelling was performed using the following R packages: randomForest, caret, e1071, mlevel.

All other statistical analysis and graphical representations were run and produced with R (v 4.0.1)(224) run in R Studio (v1.3.959). R packages: MASS, dplyr, ggpur, tidyverse, corrplot, Hmisc, psych, GGally, corrr, reshape, ggplot2, gplots.

3.3 Exploratory Untargeted Analysis leading to Targeted Pathways

3.3.1 Preliminary Analysis of untargeted data – Urine

Pre-processing of raw untargeted urine LCMS data was performed using the XCMS (235) package on R. Filtration and peak identification using `xcmsSet` function and `centWave` method with the following settings: noise = 300, peakwidth 2-25, ppm=20, nslaves = 4, prefilter 8-3000, integrate = 2. Grouping was performed using the `group` function with the following settings: `mzwid` = 0.05, `bw`= 1, `minfrac` = 0.5, `minsamp` = 1. Retention time correction was performed using `retcor` function: `family` = s, `plottype` = m, `missing`= 0, `extra`=0. The data were then regrouped using the `group` function followed by filling of missing peak data using the `fillPeaks` function. Finally, median fold change normalisation(236) was applied to remove systematic variations due to various dilutions of urine. The pre-processed data were exported in csv form and imported into SIMCA for PCA and OPLS-DA exploratory multivariate analysis.

70 urinary samples pre-operative samples of patients with and without T2D were analysed comparing those with and without T2D. Untargeted data were acquired in Reverse Phase (RP) Positive and RP Negative modes. The respective PCA and cross validated OPLS-DA models are displayed below in *figure 3-1 & 3-2*.

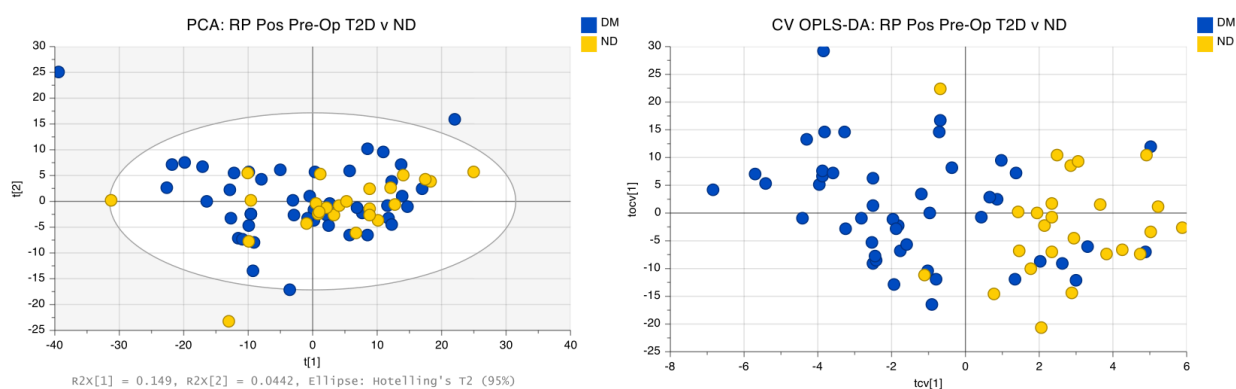


FIGURE 3-1 PCA(LEFT) AND CROSS VALIDATED OPLS-DA (RIGHT) PLOTS COMPARING THE PRE-OPERATIVE URINARY METABOLOME (RP POSITIVE MODE) OF PATIENTS WITH (BLUE) AND WITHOUT T2D (YELLOW)
CV OPLS-DA 1 ALIGNED + 1 ORTHOGONAL COMPONENTS $R^2Y = 0.805$ $Q^2Y = 0.389$

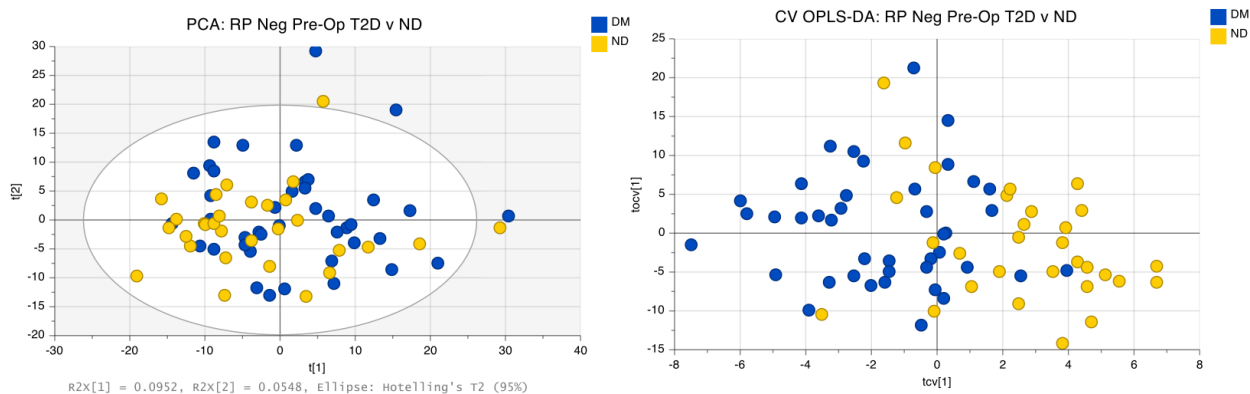


FIGURE 3-2 PCA(LEFT) AND CROSS VALIDATED OPLS-DA (RIGHT) PLOTS COMPARING THE PRE-OPERATIVE URINARY METABOLOME (RP NEGATIVE MODE) OF PATIENTS WITH (BLUE) AND WITHOUT T2D (YELLOW)
CV OPLS-DA 1 ALIGNED + 1 ORTHOGONAL COMPONENTS $R^2Y = 0.8899$ $Q^2Y = 0.406$

OPLS-DA modelling found Q^2Y predictive values of 0.389 and 0.406 respectively for RP positive and negative mode data. Both of which suggest the presence of biological differences between the baseline urinary metabolome between those with and without T2D.

3.3.2 Preliminary Analysis of untargeted data – Serum

Pre-processing of raw untargeted serum LCMS data was again performed using the XCMS (235) package in R with the same parameters listed above but without median fold normalisation. The pre-processed data were exported in csv form and imported into SIMCA for PCA and OPLS-DA exploratory multivariate analysis.

126 samples pre-operative serum samples were analysed comparing those with and without T2D. Untargeted data was acquired in Lipid Positive and Negative modes. The respective PCA and cross validated OPLS-DA models are displayed below in figure 3-3 & 3-4.

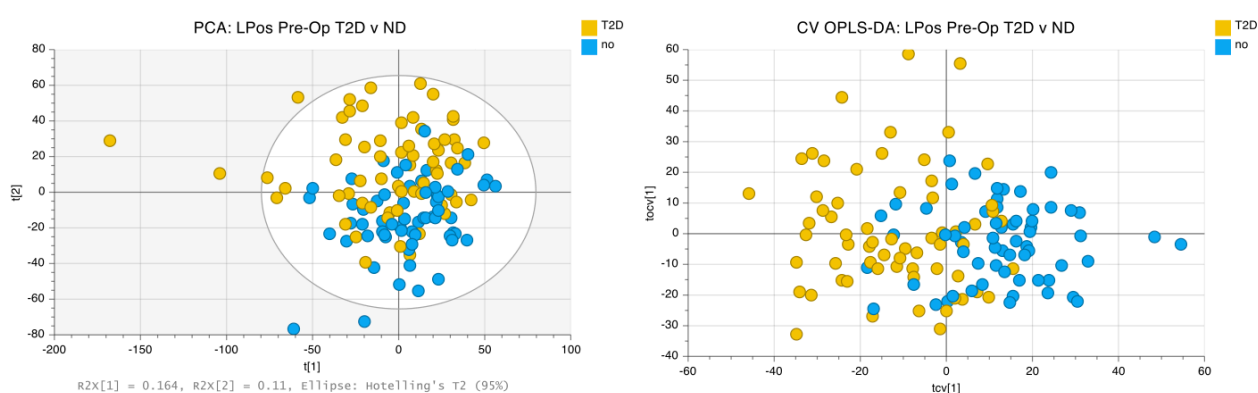


FIGURE 3-3 PCA(LEFT) AND CROSS VALIDATED OPLS-DA (RIGHT) PLOTS COMPARING THE PRE-OPERATIVE SERUM METABOLOME (LIPID POSITIVE MODE) OF PATIENTS WITH (YELLOW) AND WITHOUT T2D (BLUE)
CV OPLS-DA 1 ALIGNED + 1 ORTHOGONAL COMPONENTS $R^2Y = 0.688$ $Q^2Y = 0.498$

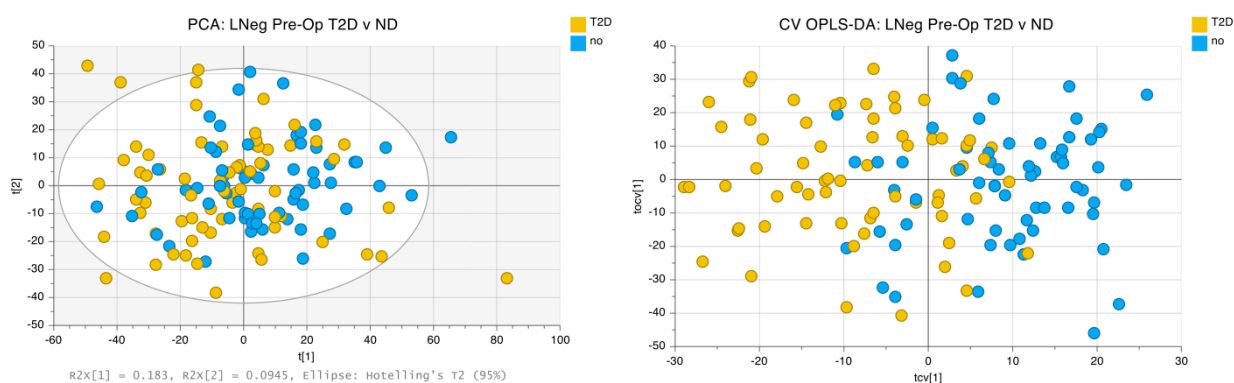


FIGURE 3-4 PCA(LEFT) AND CROSS VALIDATED OPLS-DA (RIGHT) PLOTS COMPARING THE PRE-OPERATIVE SERUM METABOLOME (LIPID NEGATIVE MODE) OF PATIENTS WITH (YELLOW) AND WITHOUT T2D (BLUE)
CV OPLS-DA 1 ALIGNED + 1 ORTHOGONAL COMPONENTS $R^2Y = 0.589$ $Q^2Y = 0.450$

Similarly, OPLS-DA modelling of the serum profiles found Q^2Y predictive values of 0.498 and 0.450 respectively for lipid positive and negative mode data. These are indicative of systematic biological differences between the baseline serum lipid metabolome between those with and without T2D.

Subsequently, 21 paired samples of pre-operative and 3-month post-operative from patients with T2D were analysed to assess the effect of surgery on the serum lipid metabolome. The respective PCA and cross validated OPLS-DA models are displayed below in *figure 3-5 & 3-6*.

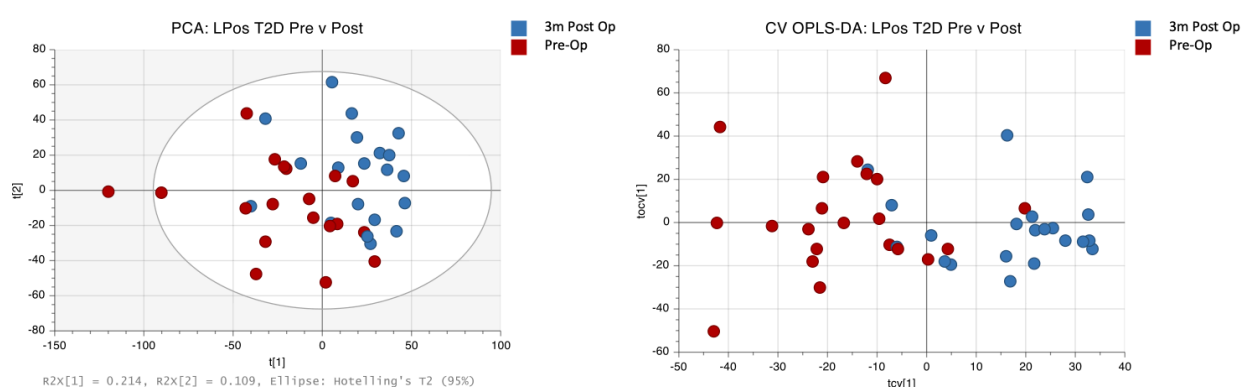


FIGURE 3-5 PCA(LEFT) AND CROSS VALIDATED OPLS-DA (RIGHT) PLOTS COMPARING THE PRE-OPERATIVE (RED) AND 3-MONTH POST-OPERATIVE (BLUE) SERUM METABOLOME (LIPID POSITIVE MODE) OF PATIENTS WITH T2D
CV OPLS-DA 1 ALIGNED + 1 ORTHOGONAL COMPONENTS $R^2Y = 0.795$ $Q^2Y = 0.605$

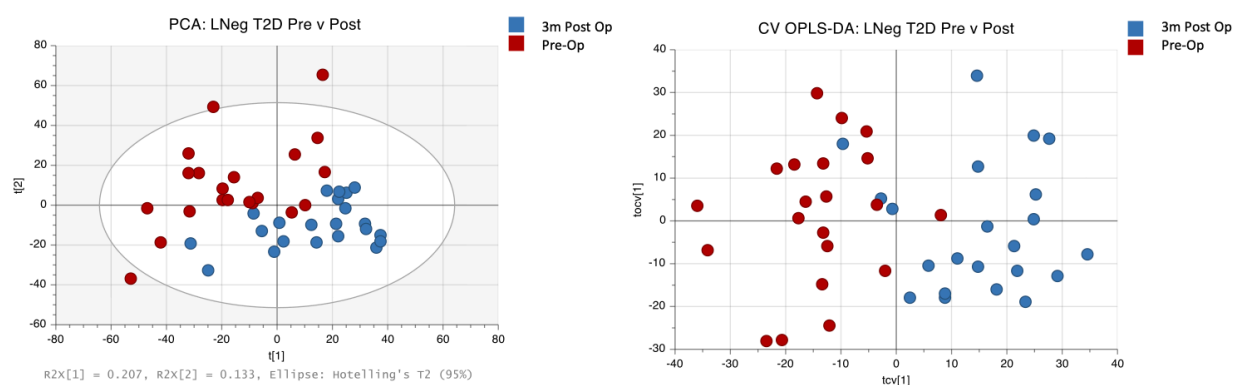


FIGURE 3-6 PCA(LEFT) AND CROSS VALIDATED OPLS-DA (RIGHT) PLOTS COMPARING THE PRE-OPERATIVE (RED) AND 3-MONTH POST-OPERATIVE (BLUE) SERUM METABOLOME (LIPID NEGATIVE MODE) OF PATIENTS WITH T2D
CV OPLS-D 1 ALIGNED + 1 ORTHOGONAL COMPONENTS $R^2Y = 0.797$ $Q^2Y = 0.657$

OPLS-DA modelling found Q²_Y predictive values of 0.605 and 0.657 respectively for lipid positive and negative mode data. These values indicate that the model differentiating patients lipidome pre and 3 months post-surgery is strong and reflects biological differences in those with obesity & T2D. These promising models led to the decision to perform full targeted lipid profiling which will be presented in detail in chapter six.

3.3.3 *Successful Metabolite Identification Workflow Example – Kynurenic Acid*

Work from this subchapter was presented at the 36th American Society of Metabolic and Bariatric Surgery (ASMBS) Obesity Week 2019 conference in Las Vegas, NV, USA.

An illustration of a successful MetID process using data obtained from this study is detailed below.

As an exploratory process, the 3-month post-operative urinary metabolome of 28 unpaired urine samples acquired in RP Positive mode was analysed comparing those who underwent RYGB and VSG. The data were first evaluated with PCA, followed by OPLS-DA see *figure 3-7* below. OPLS-DA modelling found a Q²_Y predictive value of 0.51 suggestive of relevant biological differences between these two groups.

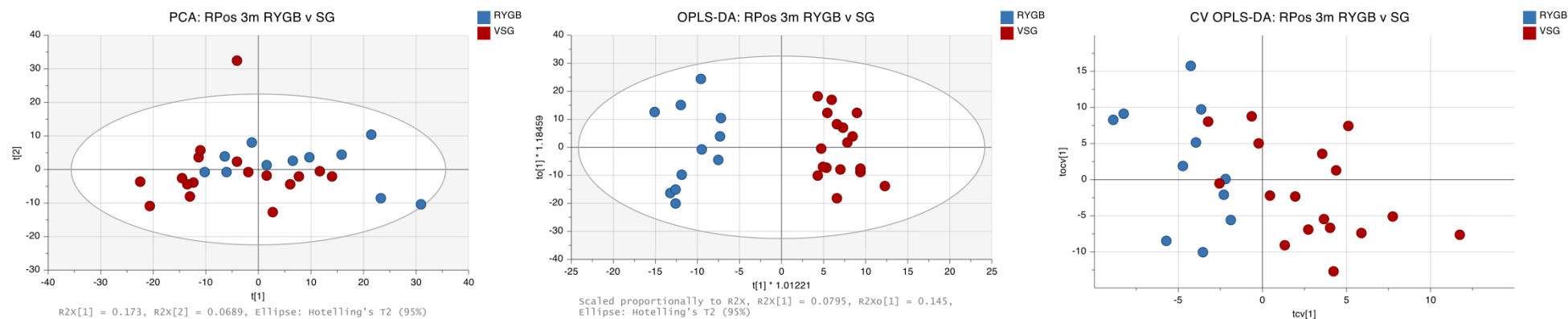


FIGURE 3-7 PCA (LEFT), OPLS-DA (CENTRE) AND CV OPLS-DA (RIGHT) PLOTS COMPARING THE 3-MONTH POST-OPERATIVE URINARY METABOLOME IN THOSE WHO UNDERWENT RYGB AND SG.

OPLS-DA 1 ALIGNED + 1 ORTHOGONAL COMPONENTS. $R^2X=0.934$ $Q^2X=0.51$

A list of differential metabolites between the two groups were identified from OPLS-DA modelling. To identify these features of interest, further MS/MS experimentation was conducted for over 50 statically relevant features. Following extensive manual searching, a match was found on the online HMDB library identifying the parent ion and fragmentation pattern of the feature with the mass 190m/z and RT of 205 seconds. This was identified as kynurenic acid (KYNA), a downstream metabolic product of the essential amino acid tryptophan.

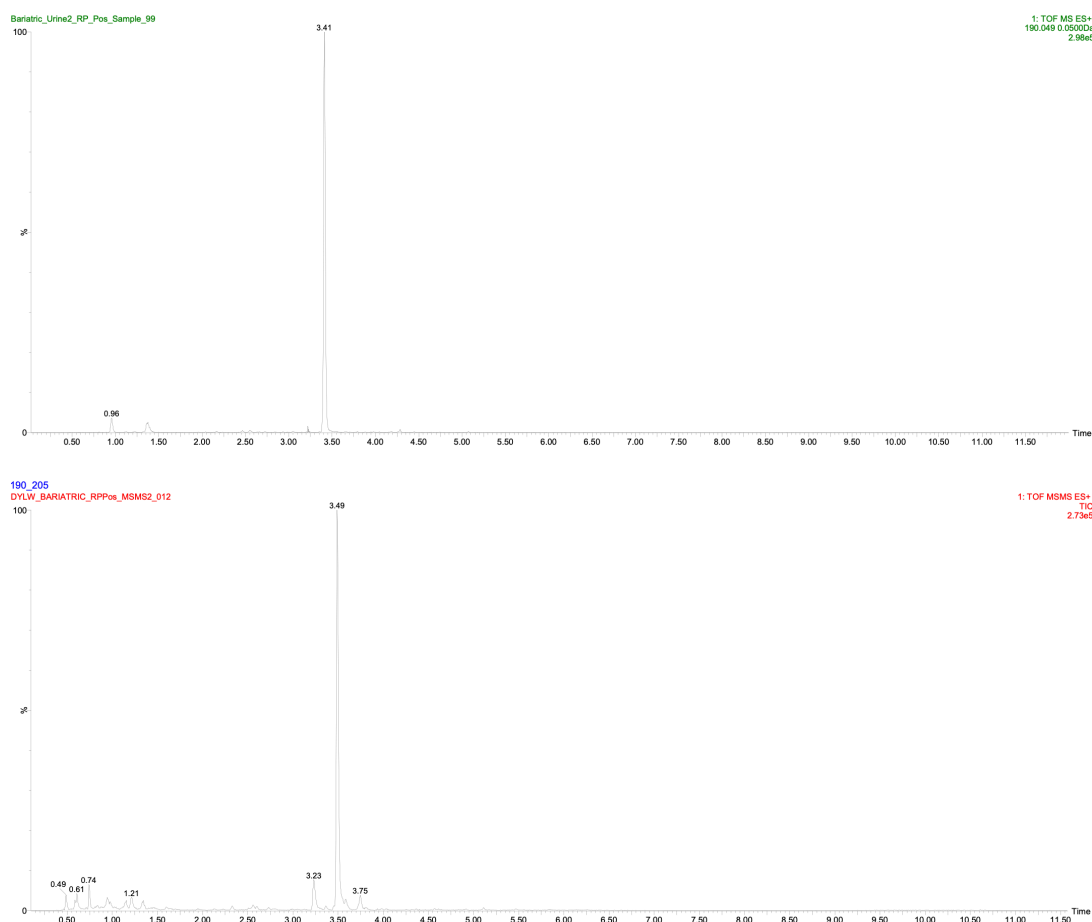


FIGURE 3-8 CHROMATOGRAM OF THE 190M/Z DIFFERENTIAL METABOLITE

ABOVE: CHROMATOGRAM FROM ORIGINAL MS DATA. BELOW: CHROMATOGRAM FROM MSMS EXPERIMENT. ILLUSTRATING INSTRUMENT DRIFT IN RT BETWEEN EXPERIMENTS.

Figure 3-8 illustrates the chromatogram of the feature with a mass of 190m/z. The chromatographic shape is almost identical but there is a drift in retention time between experiments. This is a common phenomenon seen in LC-MS experiments and it is necessary to establish whether the two peaks are from different molecules or are the same molecules but experiencing analytical drift. The MSMS fragmentation pattern with ramping 5-20 volts collision energy is presented below in *figure 3-9*.

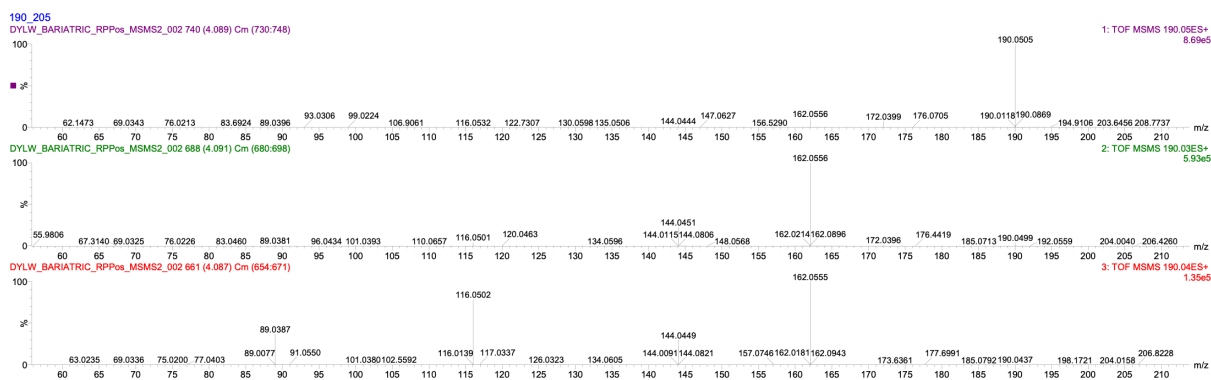


FIGURE 3-9 MS/MS RAMPING FRAGMENTATION SPECTRA OF FEATURE 190/205
RAMPING COLLISION ENERGY FROM TOP TO BOTTOM 10V,20V,30V.

To achieve a MSI level 1 identification, the chemical standard for KYNA was acquired. Using the same experimental conditions, the standard was ‘spiked’ into the same urine sample and dissolved into LCMS grade water in as a separate sample. Both samples underwent full MS profiling and MS/MS experiment and the chromatograms along with the original chromatogram are displayed below in *figure 3-10*.

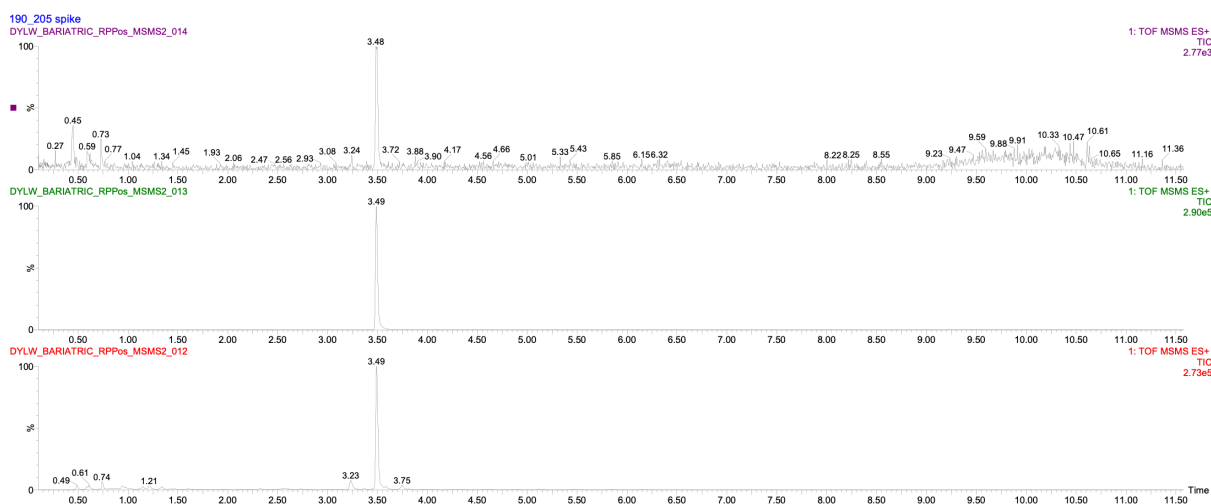


FIGURE 3-10 CHROMATOGRAMS OF 190M/Z
LCMS CHROMATOGRAMS- 190M/Z DIFFERENTIAL METABOLITE.
TOP TO BOTTOM: SPIKED SAMPLE, STANDARD DISSOLVED IN WATER, ORIGINAL EXPERIMENT CHROMATOGRAM.

The MS/MS chromatograms and fragments matched the original experimental data under identical experimental conditions, thus is classified as a level 1 annotation (refer to section 2.2.2). The relative intensities of KYNA within the comparative subgroups are shown in boxplots below *figure 3-11*.

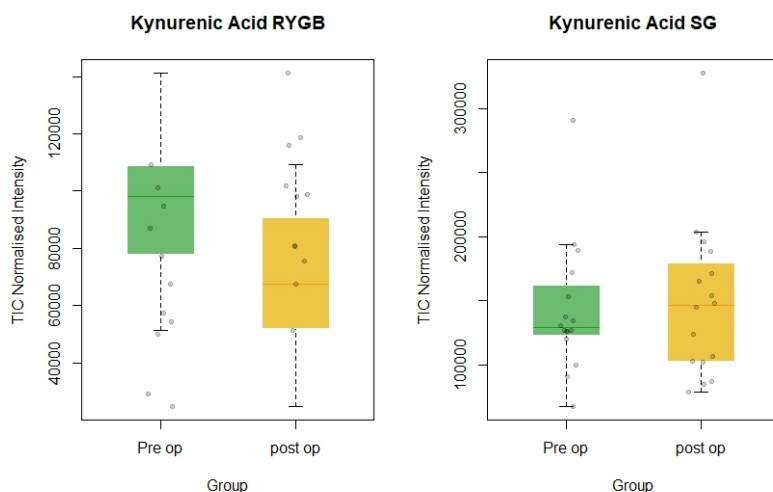


FIGURE 3-11 BOXPLOT DISPLAYING THE NORMALISED URINARY INTENSITY OF KYNURENIC ACID PRE (GREEN) AND 3 MONTHS POST-SURGERY (YELLOW) IN PATIENTS WHO UNDERWENT RYGB (LEFT) AND VSG (RIGHT)

Further univariate analysis indicated that those patients who underwent RYGB had a significant mean 1.37-fold decrease ($p=0.037$) in urinary KYNA levels at 3-months post-surgery, compared to those who underwent VSG, where a non-significant mean 1.04-fold decrease ($p=0.524$) was observed.

Amino acids have been associated with pre-diabetes states and have been found to be altered in those with established T2D(226). Kynurenic acid is a downstream metabolite of the essential amino acid tryptophan. Kynurenic acid along with other downstream tryptophan metabolites have been found to be elevated in the plasma of those with T2D and is hypothesised to be diabetogenic(237) with implications in systemic inflammation secondary to obesity. My initial analysis indicated that urinary levels of kynurenic acid were significantly reduced after RYGB but not VSG. This could insinuate different mechanisms of action on this pathway.

Considering the link between these metabolites and T2D. Amino acids and tryptophan pathway metabolites were selected for further targeted profiling analysis. The relationship of these metabolites with the T2D phenotype as well as the effect of bariatric surgery will be examined in detail in chapters 4 and 5 in an attempt to elucidate some of the mechanisms that may contribute to the resolution of T2D after surgery.

In summary, preliminary multivariate exploratory analysis of the untargeted urine and serum metabolomic datasets found promising models of differentiation between those with and without T2D. Bariatric surgery also seems to exert an effect on the urinary and serum metabolome, which would be worth further investigation. Using targeted mass spectrometry techniques, the following chapters examines the relationship of serum amino acids, serum & urinary tryptophan pathway metabolites, serum lipids and the T2D phenotype in details as well as exploring the effect of bariatric surgery on these metabolites.

Part II: Targeted Profiling of Metabolic Pathways

Chapter 4: Amino Acids

Some of the results presented in this chapter was supported by an Imperial College Healthcare NHS Trust – Biomedical Research Centre Surgery & Surgical Technology Pilot Project 2019-2020 Grant of £10,000 awarded to KTDY.

4.1 Background

4.1.1 Amino Acids and T2D

The association between amino acids (AA), insulin secretion and obesity was first reported in 1969(238). There are close links between glucose and amino acid metabolism, with the latter forming a large pool of metabolites that are both substrates and regulators of gluconeogenesis. AA modulate glucagon and insulin secretion through AA dependent mechanisms(239) and AA can interfere with insulin signals crucial for glucose uptake and regulation of gluconeogenesis(240). This has led to amino acids being one group of metabolites which have gained attention with their postulated role in T2D pathophysiology (241, 242).

Serum levels of AA are higher in those with obesity and worse metabolic health(243). Large scale and long-term studies have shown patterns of direct positive associations between branch chained (BCAA), aromatic amino acid (AAA) and other amino acid levels with levels of cardiometabolic risk factors (244), insulin secretion (245) and risk of incident T2D five to ten years later(246-251). These pre-diabetes signatures are present regardless of ethnicity(248, 252). Interestingly, some AA are also associated negatively with incident T2D, including serine, glycine, glutamine and asparagine(250, 253, 254). While elevated levels of other AA are observed in those with an established T2D(254-256) and AA levels track closely with the progression or resolution of insulin resistance and T2D (246, 257). Furthermore, under the metabolic syndrome spectrum, elevated BCAA levels were also positively correlated with severity of coronary artery disease(258, 259).

The 'chicken and egg' debate of whether amino acids are a causal agent in T2D pathophysiology or are they a marker of disease remains an open discussion. It is feasible that these complex relationships have simultaneous effects that lead to a vicious cycle. While the precise metabolic pathways leading to these metabolic signatures and association between the two remains at least partially unresolved, some theories along with existing evidence of their mechanistic contribution is explored below.

4.1.2 *Branch Chain and Aromatic Amino Acids*

BCAAs have attracted the most research attention due to a significant amount of published literature illustrating the predictive ability of serum BCAA and incident T2D suggesting possible causality, as well as its association with obesity and insulin resistance. Despite this attention, the molecular mechanisms leading to this association are not entirely understood.

BCAA are metabolised in a reversible process by the enzyme branch chain aminotransferase (BCAT) to form branched chain keto acids. Glutamate is metabolised to 2-ketoglutarate simultaneously in this reaction. In a non-reversible process catalysed by the enzyme branched-chain keto acid dehydrogenase (BCKDH), BCAAs are metabolised into acyl-coA intermediates which enter into the Krebs cycle(260).

BCAAs are also known to generate an insulin response. This is achieved by direct pancreatic beta cell stimulatory effects (261) or via incretins such as GLP-1 with orally rather than parentally administered BCAAs triggering an incretin response suggesting a gut sensing mechanism(262). Interestingly, the BCAAs leucine and isoleucine, along with alanine and arginine were found to be particularly important in stimulating pancreatic beta cell electrical activity which is essential for insulin secretion(263).

There are several postulated reasons as to why BCAAs are raised in obesity and T2D. Rapamycin molecular target (mTOR) can be activated by BCAA, insulin and glucose(264). mTOR is a regulator of cellular growth and metabolism, and it acts as an 'gatekeeper' of BCAA and insulin signalling pathways (265, 266). BCAA activation of mTOR upregulates BCAA catabolism, but chronic mTOR hyperactivation activation by BCAAs can also cause interference of the insulin signal by premature uncoupling(267), leading to insulin resistance. Increased BCAA also activate mTOR and S6 kinase leading to insulin resistance by phosphorylation and increased degradation of insulin receptor substrates (268). It is still not certain whether any of these mechanisms causes systemic changes in BCAA levels(242) but BCAA induced hyper-activation of mTOR have widespread effects on regulatory mechanisms of energy balance and metabolism in metabolically active tissues such as

adipose tissue, skeletal tissue, liver and pancreas, all of which contributes to the development of T2D(266)

Insulin is known to reduce BCAA turnover and increases AA uptake(242). Insulin has also been shown to reduce plasma BCAA levels by inducing hepatic expression of BCKDH. In obesity, liver branched chain keto acid dehydrogenase kinase is increased, which results in inhibition of BCKDH BCAA catabolic activity and increases plasma levels of BCAA(269). T2D is also associated with reduced expression of BCKDH. Rat models have demonstrated a direct hypothalamic insulin effect on this process. Overfeeding reduces the function of this axis, suggesting that hypothalamic insulin resistance may also partly be accountable for dysregulated BCAA metabolism in obesity and T2D(270).

BCAA utilisation may also be affected by several other factors. Key BCAA catabolic enzymes BCKDH and BCAT function may be impaired either by genetic predisposition(271) or as a result of raised systemic inflammatory cytokines, free fatty acids (FFA) and other toxic lipid metabolites such as ceramides and diacylglycerol(241). Mitochondrial BCKDH and BCAT are down regulated in visceral omental but not peripheral white adipose tissue in human tissues belonging to individuals with obesity and metabolic syndrome. Researchers hypothesise that is it the systemic disruption caused by metabolic syndrome such as systemic inflammation that disrupts BCAA enzymes in visceral tissue (272). Expression of skeletal muscle BCKDH and BCAT are also reduced in those with T2D(273). Others have also found that expression of BCAA catabolic enzymes and BCAA metabolism in obesity is downregulated in liver and adipose but not muscle tissue in obesity (269, 274). In obese mice, an increase in the insulin regulated glucose transporter type 4 (GLUT4) expression in adipose tissue is associated with a reduction in adipose tissue BCAA catabolic enzyme expression and oxidation capacity(274): this effect is not seen in muscles.

BCAA levels also increase in proportion to the severity of glucose intolerance(275). There are several possible explanations: increased circulatory concentration of BCAA in the circulation is secondary to protein catabolism as anti-proteolysis mechanisms are suppressed by insulin(267). Insulin resistance is also associated with impaired mitochondrial fuel

oxidation, leading to reduced amino acid clearance. There could also be a decrease in BCAA clearance through impaired catabolic pathways (267).

Individual BCAAs have also shown variations in the context of T2D. Impaired leucine catabolism appears to occur prior to isoleucine or valine impairment (276). There have also been specific BCAA led mechanisms that have been identified to contribute to hyperglycaemia. Isoleucine upregulates glucose transporters such as Sodium Glucose Transport Protein-1 (SGLT1) expression in the gut(277), promoting intestinal uptake of glucose. BCAA promotion of insulin secretion may also lead first to hyperinsulinemia but over time, hyperactivation and subsequently pancreatic beta-cell exhaustion(246).

In vivo studies found that increasing BCAA intake along with a high fat diet led to a linear increase in BCAA metabolites, obesity, and HOMA-IR(268). Reducing BCAA intake while maintaining an otherwise normal diet led to weight loss, reduction of adiposity and improved glucose control. This suggests that BCAAs have an influential role in the regulation of metabolic health and energy balance(278).

Using data from genome wide associate studies, genetic links to elevated IR were found to be associated with impaired BCAA metabolism (271) and a rise in BCAA and inflammatory markers, again, suggesting BCAA metabolism is causal to insulin resistance in T2D(279). Raised serum BCAA levels have also been linked to enhanced gut microbiota BCAA synthesis as well as a downregulated expression of bacterial BCAA transporters(280). The role of the gut microbiota in the regulation of BCAA/AAA is further emphasised by the observation that faecal transplant into germ free mice from a human donor with obesity led to an increase in serum AA and metabolites compared to the lean human twin donor, which did not induce any changes (281). All of the above most likely play an accumulative role leading to high serum levels of BCAA and subsequent physiological effects seen in obesity and T2D.

Interestingly, a clinical trial demonstrated increasing BCAA intake did not lead to increased BCAA catabolism or reduction in plasma BCAA concentrations (282). Some human studies

have reported a direct association with BCAA intake and incident T2D (283-285) but others have reported the opposite (286).

It has been suggested that metabolites of BCAA such as acylcarnitine and branch chain keto acids may be even stronger markers of insulin resistance and perhaps these products of BCAA catabolism may be in part associated with the T2D phenotype (287-289). Supporting this notion, in BCAT2 gene knockout mice which blocks an important first step in BCAA catabolism, plasma BCAA levels are higher than the corresponding wildtype. The study also found lower adiposity and body weight in such mice models despite an increased calorific oral intake. Improved glucose control and insulin tolerance was also observed(290). The leucine metabolite α -ketoisocaproate has also been shown to inhibit skeletal muscle insulin-stimulated glucose transport (291).

In summary, multiple mechanisms implicate BCAAs both as a cause and consequence of obesity and T2D. Elevated levels may be caused by decreased BCAA catabolism, increased protein catabolism and reduced insulin driven tissue uptake of BCAA. Genetic predisposition may also increase serum levels of BCAAs all of which subsequently lead to BCAA driven mechanisms of insulin resistance such as chronic activation of mTOR.

Aromatic amino acids (AAA) such as tryptophan, tyrosine and phenylalanine have also been associated with incident and established T2D(246, 251, 292). It is also uncertain why this occurs, one possible explanation is that liver dysfunction in those with metabolic syndrome reduces metabolism of AAA leading to increased serum levels(243). Increased serum BCAAs may also compete with AAA as both are transported into tissues through the large neutral amino acid transporter (LAT1)(268). However, a study investigating 80 children with obesity before and after lifestyle interventions found that tyrosine but not BCAA was associated with IR and thus concluded that tyrosine alterations occur prior to changes in BCAA metabolism in obesity (293). Another study found high tyrosine levels increased the odds of T2D particularly in the presence of low High Density Lipoprotein (HDL) (294). High tyrosine levels are also associated with increased risk of diabetes nephropathy(295). Interestingly, Interestingly, low levels of tyrosine have also been indicated as an

independent marker of microvascular risk in those with T2D (296). Since the precise reason for elevated levels of AAA is still incompletely understood, tryptophan, its downstream metabolites, and their relationship with obesity and T2D will be thoroughly explored in chapter five.

4.1.3 *Glycine and Serine*

Glycine is a non-essential amino acid and in humans it is mainly synthesised from serine in an inter-convertible process. It can also be synthesised from choline, sarcosine, glyoxylate and during L-carnitine synthesis by the liver and kidney but this process is often insufficient(297, 298). Glycine synthesis from serine is catalysed by serine hydroxymethyltransferase 1 (SHMT₁) and SHMT₂ found in the cytosol and mitochondria respectively (299) with the SHMT₂ being predominant and found in the liver. SHMT₁ and SHMT₂ gene expression has been found to be down regulated in non-alcoholic fatty liver disease (NAFLD) patients(300).

Lower levels of glycine are characteristic of individuals who are overweight and are associated with metabolic disorders including high insulin resistance (248, 256, 292, 301). Low glycine levels correlate with reduced insulin sensitivity(245) and are predictive of incident T2D(302). Glycine levels have also shown a strong positive correlation with glucose disposal rates, independent to BMI and gender. However this correlation was not found to be significant in T2D individuals (303). The underlying mechanistic role of these observations is again not entirely clear.

Phase I liver metabolism normally involves oxidation, reduction, or hydrolysis of compounds. Phase II metabolism involves chemical reactions that change the solubility of compounds to allow for urinary excretion. Glycine conjugation is an important factor in phase II metabolism(301, 304, 305). In the context of obesity, dysregulated amino acid and fatty acid metabolism leads to the production of acyl groups which are normally removed by glycine conjugation. Urinary acylglycines are increased and serum glycine levels are reduced in those with obesity (301, 306) indicating reduced bioavailability of glycine, perhaps due to increased utilisation of this detoxification process. This effect is reversed with exercise (306) or with a reduction in BCAA intake(307). Exercise increases circulating glycine and induces more efficient elimination of BCAA and AAA derived acyl groups by glycine conjugation in the liver rather than via BCAA oxidation (301), hence reducing the production of potentially toxic or diabetogenic metabolites.

Glycine is also closely associated with bile acids. In adults, BAs are primarily conjugated to glycine to form glycine-conjugated bile acids such as glycocholic acid(308). Glycocholic acid is found in bile in this conjugated form and secreted in response to food intake. In a simplistic model, in obesity, there is increased hepatic synthesis of BA. Insulin normally reduces circulating serum BAs, but this effect is found to be blunted in patients with obesity(309), which is partially contributing to reduction of serum glycine levels.

While the liver is the main site of glycine degradation, the gut also plays a role(297). Metagenomic studies of the faecal microbiota have shown that in those with T2D (not treated with metformin), there is an increased expression of genes involved in glycine degradation(310).

The lower levels of glycine characteristic of obesity and T2D probably result from a combination of factors which can be categorised into mechanism affecting gut absorption, decreased biosynthesis and increased catabolism and excretion (298). The reduced bioavailability of glycine may also contribute to beta cell damage. Glycine is converted to glutathione by the enzyme glutathione synthase (GS). In patients with T2D, reduced levels as well as reduced production rates of glutathione are observed (311). Glycine has been shown in vitro to protect pancreatic beta cells from oxidative damage by upregulation of GS and subsequent production of glutathione.

Emerging evidence also points to glycine having a possible neurotransmitter role by direct pancreatic signalling or via brain N-Methyl-D-aspartate (NMDA) receptors to influence insulin secretion(312). In summary, glycine is generally seen as a biomarker for metabolic health, but certain studies also point to potential mechanistic roles for glycine in the pathophysiology in obesity and T2D.

A recent study eloquently demonstrated a causal link between BCAAs, glycine and lipid oxidation. Zucker fatty rats were observed to have high circulating level of BCAA and lower levels of glycine when compared to lean controls. A reduction in liver BCKDH (BCAA catabolism) activity(313), compensated by increased skeletal muscle BCKDH activity was observed. This compensatory process in turn affects fatty acid oxidation within skeletal

muscle, leading to accumulation of fatty acyl CoAs within skeletal tissue. Fatty acyl CoA that accumulates contributes to skeletal muscle insulin resistance (306). Under normal circumstances, glycine conjugation allows for urinary excretion of these toxic products. The lower levels of glycine in obesity means this mechanism is exhausted and impaired, with the glycine pool depleted leading to further accumulation of insulin resistance inducing metabolites. Reducing BCAA intake led to normalisation of skeletal muscle glycine levels, improvement in insulin resistance and an increased excretion of urinary acetylglycine(307).

Serine is another non-essential amino acid. Serine can be absorbed from food sources or be derived from glycine, other proteins or phospholipids. It is less well studied but evidence of its potential pathological and therapeutic roles in T2D is beginning to emerge. Serine is reported by some to be reduced in obesity associated disorders(300, 314) and reduced levels are associated with reduced insulin secretion(245). However, the opposite has also been reported in other studies(315) with the underlying reason for the discrepancies unclear.

Phosphoserine aminotransferase (PSAT) is an enzyme that catalyses serine synthesis. Expression of hepatic PSAT and subsequent serine levels are reduced in diet induced obese mice. In vitro and in vivo experiments have shown PSAT overexpression using adenovirus expressing PSAT led to improved insulin signalling and insulin sensitivity suggesting a role for serine in insulin signalling(316).

Serine and palmitoyl-coA are the primary substrates in sphingolipid biosynthesis. 1-deoxysphingolipids are atypical sphingolipids which are synthesised in states of serine depletion and are implicated in T2D and diabetes neuropathy(317), this is explored in further detail in chapter six. Serine palmitoyltransferase [SPT] uses alanine or glycine instead of serine as an amino acid substrate. This dysregulated metabolic process is linked to the development of T2D with mechanisms linked to beta-cell signalling, cytokine secretion and apoptosis(318-320). Restoring serine levels seems reverse this process and increased serine directly inhibits the production of 1-deoxysphingolipids(321).

4.1.4 *Amino Acid Ratios*

Several ratios of amino acids have been described in the literature are used either as a direct marker of organ function or used to assess changes in metabolic pathways or function. Fischer's ratio is a molar ratio between BCAA and AAA and is a ratio associated with hepatic function primarily in cases of severe hepatic failure, linked to hepatic encephalopathy(322, 323). The BCAA to tyrosine ratio (BTR) has been shown to also correlate with biochemical indices of hepatic function(324) and considered as a marker of hepatic impairment in pathology affecting the liver(325). Similarly, low valine to phenylalanine ratio (VPR) has also been found to show strong correlation with mortality in end stage liver disease(326).

The glutamine to glutamate ratio (GGR) is a marker of energy metabolism. Lower levels of glutamine and higher levels of glutamate, leading to a lower GGR, has been associated with T2D risk(244, 327). An increased proline to citrulline ratio (ProCit) has been reported in patients with metabolic syndrome, it has been described as an indirect indicator of arginase/ nitric oxide synthase activity linked to endothelial dysfunction and a potential biomarker of metabolic syndrome risk(328). A high alanine to serine (AlaSer) ratio has been reported to be a marker of 1-deoxydihydroceramide formation. This is an atypical sphingolipid with diabetogenic effects and is potentially causal in diabetic neuropathy. AlaSer is also higher in those with T2D compared to lean controls(317).

Finally, the leucine and serine to threonine and tryptophan (LSTT) ratio has been associated with incident hypertension. The mechanisms leading to this association are not entirely clear but the reporting authors hypothesised an effect on neurotransmitter mediated blood pressure control(329). Most of these ratio markers apart from GGR have not been assessed in the context of obesity and T2D. Impaired hepatic function due to lipotoxicity occurs in obesity but it is often undiagnosed.

4.1.5 Urinary Metabolites of Microbiome and Endogenous Origin

Some metabolites produced by the gut microbiome can be detected in urine. Some of these metabolites also have links with obesity and T2D. In the context of this thesis, these metabolites may be useful in providing indirect information on the effect of bariatric surgery on metabolism and the metabolic function of the gut microbiome. Some of these metabolites, the metabolite of origin and its link to obesity and T2D are below in *table 4-1*.

Metabolite	Metabolite(s) of Origin	Obesity / T2D links
TMAO (microbial)	Choline, betaine, carnitine.	Increased in T2D and obesity. Positive correlation with endothelial dysfunction and inflammation (330, 331).
PAG (microbial)	Conjugate of phenylacetic acid which is derived from phenylalanine	Colonic gut metabolite of putrefaction of undigested proteins. Increased in T2D and obesity(332).
Indoxyl sulphate (microbial)	Tryptophan	Potentially toxic uremic solute. Linked to diabetes nephropathy, may induce arterial calcification(333, 334)
p-Cresol sulphate (microbial)	Tyrosine	Colonic gut metabolite of putrefaction of undigested proteins. Increased in T2D and obesity.(332)
Hippurate (microbial)	Glycine conjugate of Benzoic Acid	Potentially toxic uremic solute. Marker of gut microbiome diversity(335), reduced in obesity(336)
Acetate (microbial)	2 carbon short chain fatty acid from fermentation of indigestible fibre	A bacterial Fermentation end product that can promote metabolic syndrome via a microbiome-brain-pancreatic axis (337).
Formate (microbial)	One carbon metabolism intermediate metabolite and SCFA	Mediator of metabolic interactions. Host microbiome anaerobic fermentation end product. Reduced in obesity but its role is not fully defined(338) but hypothesised to be a metabolic signature of inflammation associated alteration of the gut microbiome (339).
Lactate	Product of anaerobic glycolysis	Produced by many microbes but more importantly, endogenous lactate originating from adipose tissue is linked to insulin resistance (340-342).
Carnitine	Mainly from the diet but can be synthesised endogenously using lysine and methionine	Essential role in energy metabolism, facilitates long chain fatty acid beta oxidation within mitochondria(343).

TABLE 4-1 URINARY METABOLITES OF MICROBIAL AND ENDOGENOUS ORIGIN

4.1.6 *Summary*

Serum amino acid metabolism is dysregulated in states of obesity and T2D. Human and animal studies have provided evidence that in most cases, this disruption can be both a cause and consequence of obesity and T2D. Studying the effect of bariatric surgery on these circulating metabolites and its association with surgical T2D resolution may allow us to further enhance our understanding of these underlying pathophysiological process.

4.2 Chapter Aims and Experimental Overview

This chapter first compares two metabolic profiling assays that can be used to quantify serum amino acids, then using data obtained by one of these assays, I examine the effect of bariatric surgery on circulating serum amino acids and amino acid ratios, its association with the T2D phenotype and surgical resolution of T2D. A small data subset of urinary metabolites of microbial origin related to amino acids obtained using ^1H -NMR spectroscopy is also presented and discussed.

4.3 Methods Comparison

4.3.1 *Commercial Metabolic Profiling Solutions*

There are now many commercial metabolic profiling solutions available to investigators. An example of this is a full metabolic profile available from companies such as Metabolon (Morrisville, NC, USA), which delivers quantitated metabolite values but retains the raw data. The MRC-NIHR National Phenome Centre (NPC) led by Imperial College and King's College London also offers metabolic profiling services to the wider UK research community on a collaborative or paid per service basis and uses published assays. As an intermediate solution, some companies such as Biocrates (Innsbruck, Austria) offer ready to use targeted mass spectrometry metabolomic kits such as the AbsoluteIDQ p180 kit that can be implemented on suitable LC-MS systems within the users own laboratory. The kit is ready to use out of the box and comes with sample preparation protocols along with instrument specific acquisition and quantification methods. Over 100 metabolites can be quantified including amino acids, acylcarnitine and lipid species.

The AbsoluteIDQ p180 kit was used within the research group for the wider study that included studies not presented within this thesis. While analysis of data acquired using the kit is not presented within this thesis, as a mini-methodological assessment, I compared the quantified amino acid values of equivalent samples obtained using the kit to values obtained through targeted UPLC-MS amino acid quantification performed with collaborators at the NPC (229) which uses a method validated against stable isotope labelled internal standards.

4.3.2 Comparison of Assays

A total of 107 matching samples were profiled using both assays and were available for direct comparison. For the precise methodology, please refer to *chapter 3.2*. A total of 19 quantified amino acids from the two respective assays were assessed for correlation.

Metabolite	R
Alanine	0.729
Arginine	0.477
Aspartic Acid	0.660
Asparagine	0.740
Glutamic Acid	0.703
Glutamine	0.562
Glycine	0.683
Histidine	0.620
Isoleucine	0.719
Leucine	0.722
Methionine	0.789
Phenylalanine	0.728
Proline	0.726
Serine	0.692
Threonine	0.803
Tryptophan	0.672
Tyrosine	0.725
Valine	0.742
Citrulline	0.693

TABLE 4-2 SPEARMAN CORRELATION COEFFICIENTS COMPARING QUANTIFIED VALUES OF BIOCRATES VS NPC AMINO ACID QUANTITATIVE METHOD

All quantified amino acids from the two assays were significantly ($p < 0.001$) correlated with its respective counterpart, the Spearman's correlation co-efficient are displayed in *table 4-2* and ranges from 0.477 to 0.803. Variations in the range of quantified amino acids from matching samples are illustrated as boxplots below in *figure 4-1*.

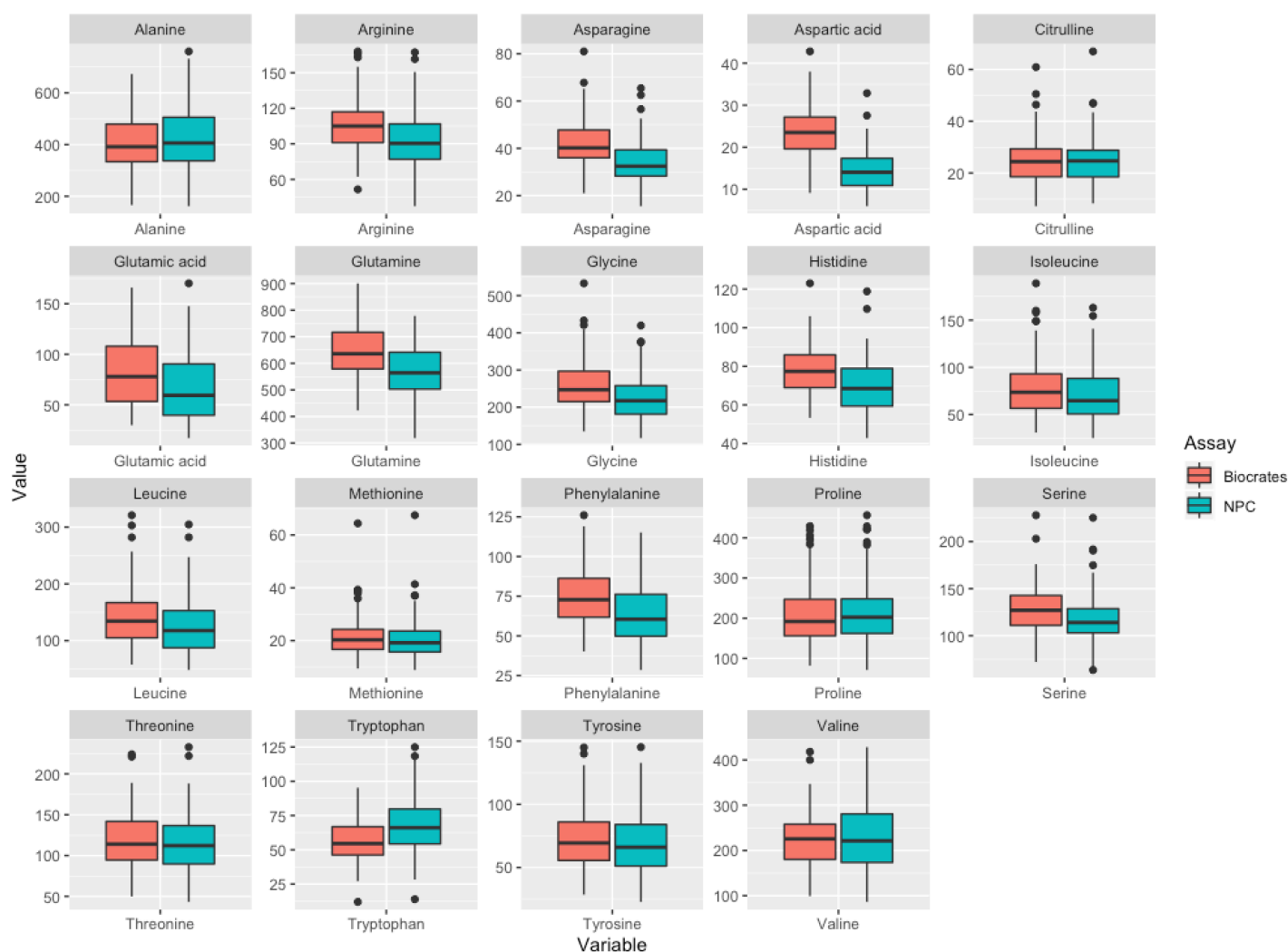


FIGURE 4-1 BOXPLOTS COMPARING BIOCRATES AND NPC AMINO ACID QUANTITATIVE METHODS

To evaluate if the variation in quantification may potentially impact biological interpretation, the quantified amino acid values of the respective assays were assessed for correlation with the clinical parameters BMI and HbA_{1c}, this is displayed below in *table 4-3*.

In incidences of significant correlation, most metabolites measured by the two assays showed agreeable results with the exception of proline, valine and citrulline when assessed for significant correlation with BMI. For correlations with HbA_{1c}, although there were slight differences in correlation co-efficient, the statistical significance of results did not differ.

Metabolite	BMI				HbA1c			
	Biocrates		NPC		Biocrates		NPC	
	R	q value	R	q value	R	q value	R	q value
Alanine	0.157	0.178	0.136	0.239	0.351	0.001	0.438	0.000
Arginine	-0.016	0.925	0.004	0.968	0.138	0.215	0.195	0.069
Aspartic Acid	0.242	0.040	0.226	0.047	-0.023	0.889	0.014	0.913
Asparagine	-0.092	0.424	-0.126	0.270	0.212	0.048	0.261	0.013
Glutamic Acid	0.280	0.020	0.363	0.002	0.375	0.000	0.462	0.000
Glutamine	-0.159	0.178	-0.106	0.377	0.026	0.883	0.016	0.913
Glycine	-0.376	0.002	-0.291	0.016	-0.190	0.075	-0.182	0.086
Histidine	0.015	0.925	0.067	0.570	0.093	0.450	0.081	0.518
Isoleucine	0.080	0.490	0.130	0.258	0.371	0.000	0.430	0.000
Leucine	0.097	0.411	0.152	0.182	0.349	0.001	0.431	0.000
Methionine	0.017	0.925	0.010	0.945	0.336	0.001	0.354	0.001
Phenylalanine	0.253	0.031	0.302	0.016	0.209	0.049	0.344	0.001
Proline	0.160	0.178	0.223	0.049	0.267	0.012	0.390	0.000
Serine	-0.237	0.042	-0.269	0.025	-0.029	0.883	-0.058	0.657
Threonine	0.291	0.016	0.262	0.028	0.247	0.019	0.263	0.013
Tryptophan	0.234	0.043	0.291	0.016	0.238	0.024	0.345	0.001
Tyrosine	0.152	0.182	0.205	0.070	0.312	0.003	0.379	0.000
Valine	0.201	0.073	0.227	0.047	0.417	0.000	0.491	0.000
Citrulline	0.253	0.031	0.210	0.069	-0.002	0.985	0.064	0.636

TABLE 4-3 TABLE COMPARING SPEARMAN'S CORRELATION COEFFICIENT AND BH CORRECTED Q VALUES OF BIOCRATES AND NPC AMINO ACID QUANTITATIVE METHOD AGAINST BMI AND HbA1c

4.3.3 Discussion

The choice of metabolic profiling methods chosen by any investigator may be influenced by many factors. This includes individual budget considerations, the scope of metabolites one may wish to investigate, the general availability of assays and access to laboratories which may be impacted by factors such as geographical location and academic collaborations.

It is not the aim of this mini evaluation to judge the accuracy of the two assays. To do so, both must be tested and compared by quantifying known standard reference materials obtained from sources such as the National Institute of Standards and Technology (NIST)(344).

The results above demonstrate that although there are some differences between the quantified values and ranges of amino acids measured by the two assays of the same samples. The values of each quantified amino acid from both assays do correlate to a significant degree. Generally agreeable correlations with clinical parameters such as BMI and HbA_{1c} are also demonstrated.

In conclusion, both assays would be appropriate for use in discovery or exploratory metabolomic studies and the choice of assay is often determined by many factors beyond scientific accuracy. The subsequent results presented below were obtained through targeted UPLC-MS amino acid quantification performed with collaborators at the NPC (229) unless otherwise specified.

4.4 Amino Acid Profiling Results

4.4.1 Baseline Comparison

Longitudinally paired pre-op and 3-month post-op serum samples (n=36) underwent targeted amino acid quantification and 21 amino acids were quantified using this methodology. A total of 17 patients were T2D before surgery, 18 patients underwent RYGB and VSG respectively. The baseline demographics divided into subgroups by T2D diagnosis are displayed in *table 4-4*. Those with T2D were older in age and had a higher recorded baseline HbA_{1c}, as expected.

Baseline Demographics	T2D	Non-Diabetes
n	17	19
Sex, n (M:F)	5;12	2;17
Age (Years)	49.65±9.27	41±8.77*
Weight (Kg)	125.69±26.41	132.11±19.93
BMI (kg/m²)	46.47±6.69	48.79±6.98
HbA_{1c} (mmols/mol)	60.76±16.79	36.47±3.53*
Metformin, n (%)	13 (76)	0 (0)
Insulin, n (%)	3 (18)	0 (0)

all listed data are means ± standard deviation unless stated otherwise

**p<0.05 subgroup comparison*

TABLE 4-4 BASELINE DEMOGRAPHICS OF AMINO ACID STUDY COHORT

All measured AA were elevated in those with T2D when compared to those without except for serine and citrulline. This difference was significant (q= BH adjusted p values) with BCAAs: leucine (q=0.005), isoleucine (q=0.004), valine (q=0.004) as well as alanine (q=0.005) and proline (q=0.02). These differences are visualised below in *figure4-2*.

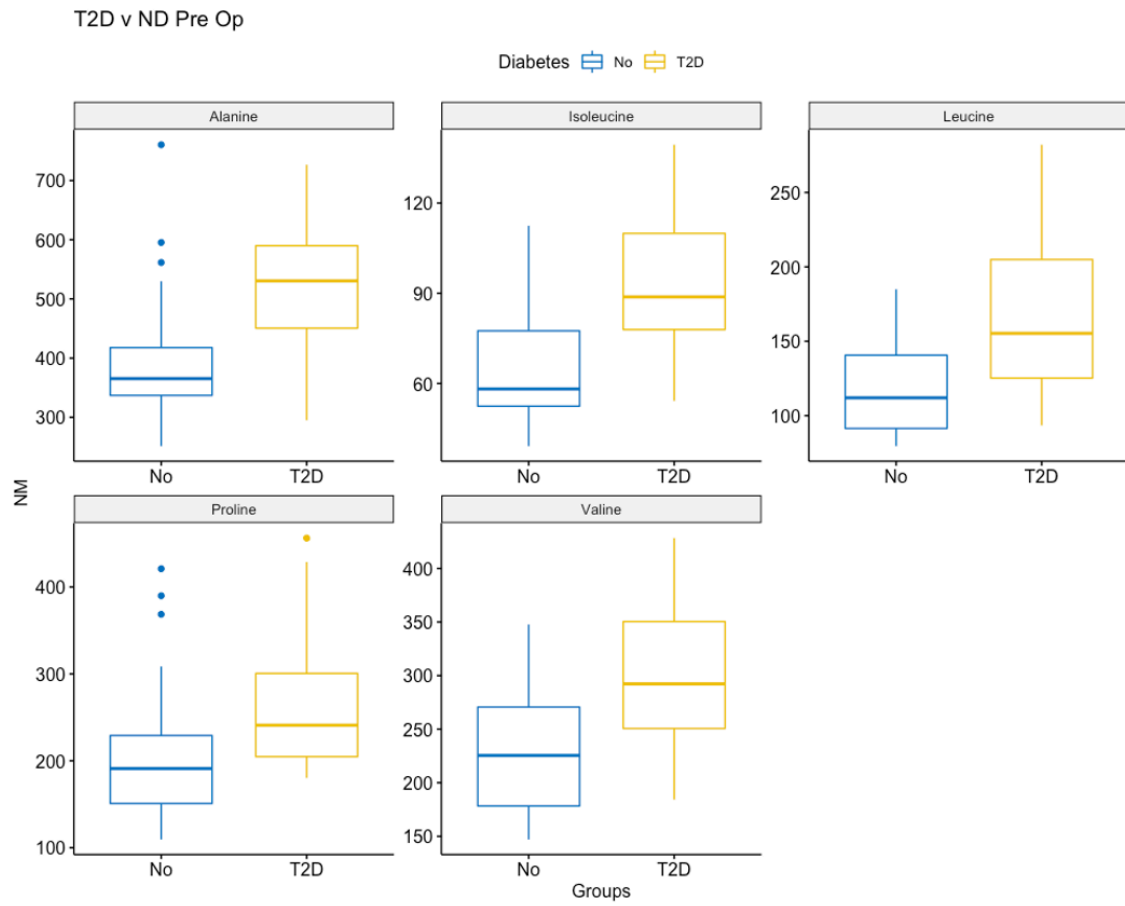


FIGURE 4-2 BOXPLOTS COMPARING SIGNIFICANTLY DIFFERENT T2D (YELLOW) AND NON-T2D (BLUE) PRE-OP VALUES OF MEASURED AMINO ACIDS

4.4.2 Pre- and Post-operative Comparison

Subgroups	RYGB		VSG		T2D		Non-Diabetes	
	Pre-Op	Post Op	Pre-Op	Post Op	Pre-Op	Post Op	Pre-Op	Post Op
n	18		18		17		19	
Sex, n (M:F)	1:17		5:13		5:12		2:17	
Age (Years)	45.83±10.97		44.33±8.96		49.65±9.27		41±8.77	
Weight (Kg)	126.66±17.13	100.87±12.82*	131.51±28.16	109.68±27*	125.69±26.41	100.72±21.95*	132.11±19.93	109.34±20.42*
BMI (kg/m²)	47.48±5.34	37.81±3.94*	47.91±8.24	39.89±8.12*	46.47±6.69	37.2±5.47*	48.79±6.98	40.33±6.9*
T2D, n (%)	10 (56)		7 (39)					
Metformin, n (%)	8 (44)	3 (16)	5 (28)	3 (17)	13 (76)	6 (35)	0 (0)	0 (0)
Insulin, n (%)	3 (17)	2 (11)	0 (0)	0 (0)	3 (18)	2 (12)	0 (0)	0 (0)
HbA1c (mmols/mol)	49.44±18.84	36.89±8.14*	46.44±15.17	38.22±5.64*	60.76±16.79	41.94±7.18*	36.47±3.53	33.63±3.64*

* $p < 0.05$ pre and post-op comparison

TABLE 4-5 PRE-OP V POST-OP DEMOGRAPHICS DISPLAYED BY OPERATIVE AND T2D SUBGROUPS

Following on, the effect of surgery at 3 months post-operative on subgroups divided by T2D diagnosis and surgical procedure are displayed above in *table 4-5*. Surgery led to significant reductions in weight, BMI and HbA1c across all subgroups at 3 months post-op when compared to baseline.

	RYGB				VSG			
	Pre-Op	Post-Op	p value	q value	Pre-Op	Post-Op	p value	q value
4-Hydroxyproline	13.49±8.92	13.09±5.36	0.420	0.467	13.43±6.58	11.74±2.51	0.628	0.628
Alanine	441.09±120.99	362.34±127.7	0.064	0.134	462.33±139.17	362.99±93.77	0.019	0.044
Arginine	83.76±20.95	78.6±17.78	0.728	0.728	104.22±27.47	89.1±22.68	0.085	0.137
Aspartic Acid	14.94±4.4	13.77±4.5	0.546	0.573	14.71±4.44	12.49±4.37	0.104	0.156
Asparagine	31.32±7.3	27.98±5.92	0.282	0.348	38.21±10.82	32.6±7.68	0.074	0.129
Glutamic Acid*	81.57±35.58	58.44±27.4	0.044	0.102	70.23±27.1	49.8±20.46	0.015	0.044
Glutamine	520.58±112.54	572.54±108.95	0.118	0.190	582.85±75.57	594.8±76.13	0.628	0.628
Glycine	194.59±35.62	257.73±61.98	0.001	0.005	208.54±38.27	253.58±78.27	0.192	0.252
Histidine	64.48±12.83	59.49±9.48	0.252	0.330	76.87±10.28	64.65±12.21	0.007	0.029
Isoleucine	71.3±27.19	51.68±17.64	0.011	0.032	87.28±30	61.07±19.15	0.008	0.029
Leucine	127.91±43.36	90.23±28.26	0.002	0.007	159.09±53.63	110.99±33.43	0.003	0.028
Methionine	19.26±6.41	16.1±3.97	0.214	0.300	25.76±8.32	19.39±4.38	0.022	0.047
Phenylalanine	65.55±12.8	47.39±10.74	0.000	0.001	75.64±15.37	53.5±10.88	0.000	0.002
Proline	227.91±79.38	187.48±74.47	0.111	0.190	222.34±75.04	176.72±44.76	0.055	0.105
Serine	97.07±19.44	123.84±21.77	0.000	0.002	111.16±20.93	118.74±22.06	0.214	0.265
Threonine	106.62±35	88.36±25.19	0.171	0.256	126.87±22.11	118.43±30.91	0.232	0.270
Tryptophan	66.23±15.47	47.81±16.58	0.001	0.005	78.16±18.27	62.13±17.44	0.019	0.044
Tyrosine	67.23±22.43	50.17±13.89	0.014	0.037	83.56±23.52	63.51±20.05	0.008	0.029
Valine	240.85±66.65	167.69±45.65	0.000	0.002	278.05±74.33	207.37±55.44	0.004	0.029
Citrulline	23.81±10.18	18.06±6.23	0.099	0.188	27.02±9.07	22.29±6.78	0.192	0.252
beta Alanine	5.33±2.64	4.37±1.38	0.423	0.467	6.12±3.33	4.87±1.57	0.428	0.473

UNITS LISTED μ M *GLUTAMIC ACID IONIC FORM IS KNOWN AS GLUTAMATE. Q VALUE = BH CORRECTED P VALUE

TABLE 4-6 PRE-OP V POST-OP VALUES OF MEASURED AMINO ACIDS DISPLAYED BY OPERATIVE SUBGROUP

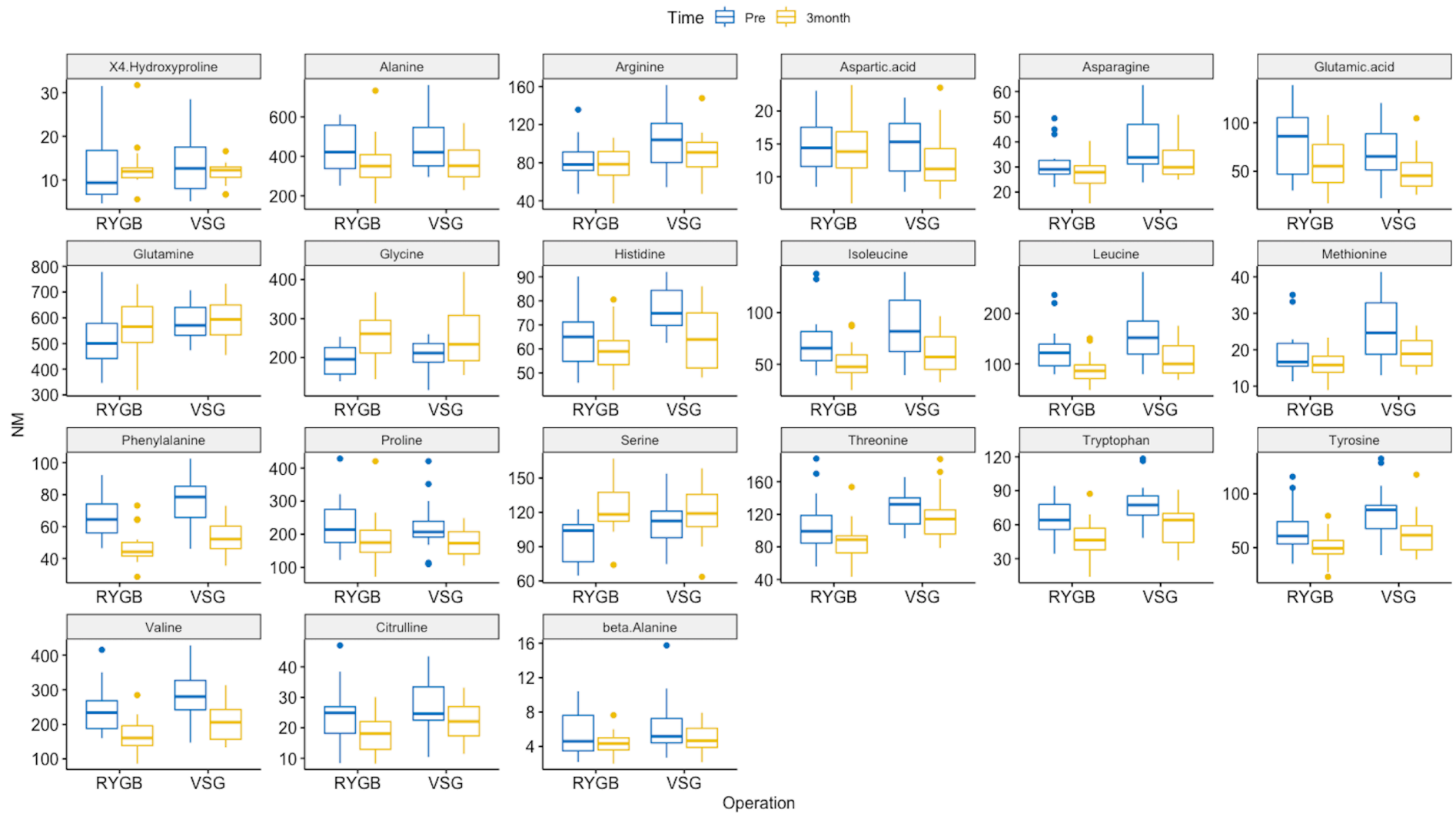


FIGURE 4-3 BOXPLOTS COMPARING PRE-OP V POST-OP VALUES OF MEASURED SERUM AMINO ACIDS DISPLAYED BY OPERATION SUBGROUP

	T2D				Non-Diabetes			
	Pre-Op	Post-Op	p value	q value	Pre-Op	Post-Op	p value	q value
4-Hydroxyproline	15.28±8.48	11.78±3.15	0.433	0.433	11.84±6.81	12.98±4.95	0.280	0.367
Alanine	505.56±114.33	393.23±120.59	0.003	0.009	403.53±124.64	335.31±95.43	0.116	0.202
Arginine	97.83±28.88	84.63±15.23	0.160	0.186	90.55±23.84	83.15±25.14	0.405	0.481
Aspartic Acid	14.66±3.99	12.54±4.1	0.160	0.186	14.97±4.79	13.66±4.74	0.425	0.481
Asparagine	36.11±9.53	31.64±7.55	0.153	0.186	33.55±10.04	29.08±6.75	0.130	0.210
Glutamic Acid	83.18±30.48	66.03±26.64	0.118	0.165	70.07±32.46	43.47±16.04	0.009	0.063
Glutamine	549.42±113.52	590.12±88.75	0.326	0.343	553.77±88.48	578.83±98.18	0.234	0.328
Glycine	197.21±40.69	240.69±73.21	0.114	0.165	205.46±34.25	269.05±65.24	0.001	0.012
Histidine	69.34±14.73	61.13±9.74	0.085	0.138	71.87±11.66	62.91±12.37	0.030	0.096
Isoleucine	92.87±31.51	57.35±17.27	0.001	0.002	67.43±21.97	55.51±20.43	0.091	0.200
Leucine	168.01±55.3	99.47±30.1	0.000	0.000	121.56±34.41	101.64±34.9	0.061	0.161
Methionine	25.26±7.31	18.36±3.65	0.005	0.012	20.05±8.01	17.19±5.09	0.435	0.481
Phenylalanine	73.83±15.45	51.2±11.42	0.000	0.000	67.7±14.07	49.76±11.07	0.000	0.007
Proline	255.9±67.12	202.46±72.25	0.016	0.034	197.58±74.73	163.88±42.5	0.184	0.276
Serine	106.05±22.64	124.7±16.31	0.022	0.041	102.39±20.19	118.23±25.75	0.032	0.096
Threonine	123.88±30.06	95.44±17.87	0.003	0.009	109.44±30.63	110.51±39.45	0.964	0.964
Tryptophan	79±17.8	56.55±17.7	0.003	0.009	66.1±15.74	53.07±19.13	0.029	0.096
Tyrosine	83.87±24.87	57.27±14.26	0.000	0.002	67.81±21.27	56.46±21.67	0.096	0.200
Valine	298.56±71.52	190.59±45.67	0.000	0.000	224.45±53.13	184.79±61.54	0.025	0.096
Citrulline	23.25±9.49	19.21±6.72	0.191	0.211	27.32±9.59	21.21±6.86	0.105	0.200
beta Alanine	5.57±2.16	4.34±1.35	0.086	0.138	5.86±3.62	4.87±1.58	0.779	0.818

UNITS LISTED μ M *GLUTAMIC ACID IONIC FORM IS KNOWN AS GLUTAMATE. Q VALUE = BH CORRECTED P VALUE
 TABLE 4-7 PRE-OP V POST-OP VALUES OF MEASURED AMINO ACIDS DISPLAYED BY T2D SUBGROUP

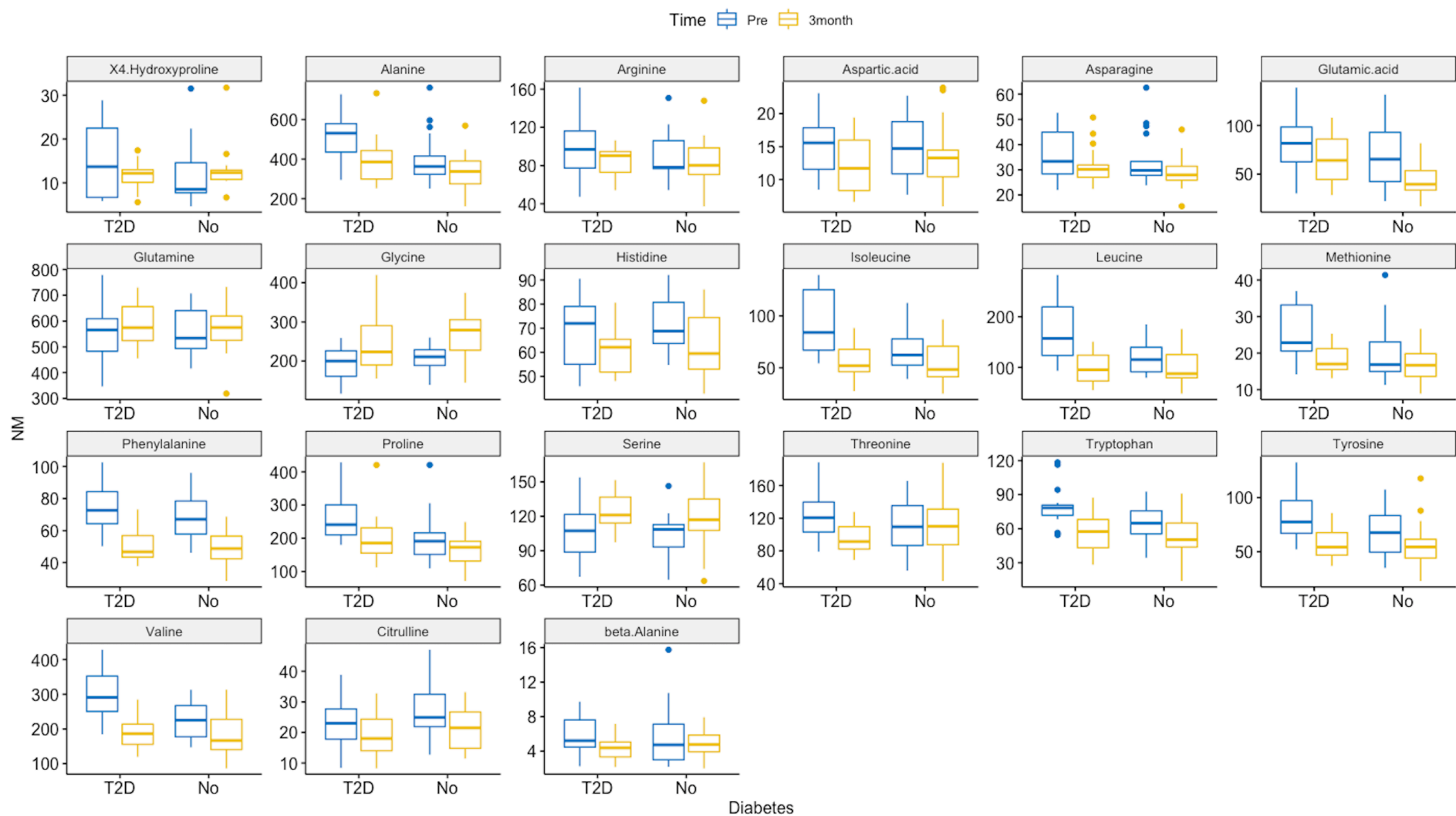


FIGURE 4-4 BOXPLOTS COMPARING PRE-OP V POST-OP VALUES OF MEASURED SERUM AMINO ACIDS DISPLAYED BY T2D SUBGROUP

When comparing the effect of RYGB and VSG on measured AA in this cohort at 3 months post-operative (*table 4-6 & figure 4-3*), surgery led significant reductions in BCAAs: isoleucine (q= RYGB 0.032 VSG 0.029), leucine (q= RYGB 0.007 VSG 0.028), valine (q= RYGB 0.002 VSG 0.029), AAA: phenylalanine (q= RYGB 0.001 VSG 0.002), tryptophan (q= RYGB 0.005 VSG 0.044) and tyrosine (q= RYGB 0.037 VSG 0.029). In addition, only RYGB led to a significant increase in glycine (q=0.005) and serine (q=0.002). Alanine (q=0.044), glutamic acid (q=0.044), histidine (q=0.029) and methionine (q=0.47) levels were reduced significantly after VSG only.

When comparing the effect of surgery on T2D and ND subgroups at 3 months post-operative (*table 4-7 and figure 4-4*) those with T2D had a significant reduction in BCAAs: isoleucine (q=0.002), leucine (q<0.001), valine (q<0.001), AAA: phenylalanine (q<0.001), tryptophan (q=0.009), tyrosine (q= 0.002) as well as alanine (q=0.009), methionine (q=0.012), threonine (q=0.009) and a significant increase in serine (q=0.041). Those without T2D had a significant reduction in phenylalanine (q=0.007) and significant increase in glycine (q=0.012) only.

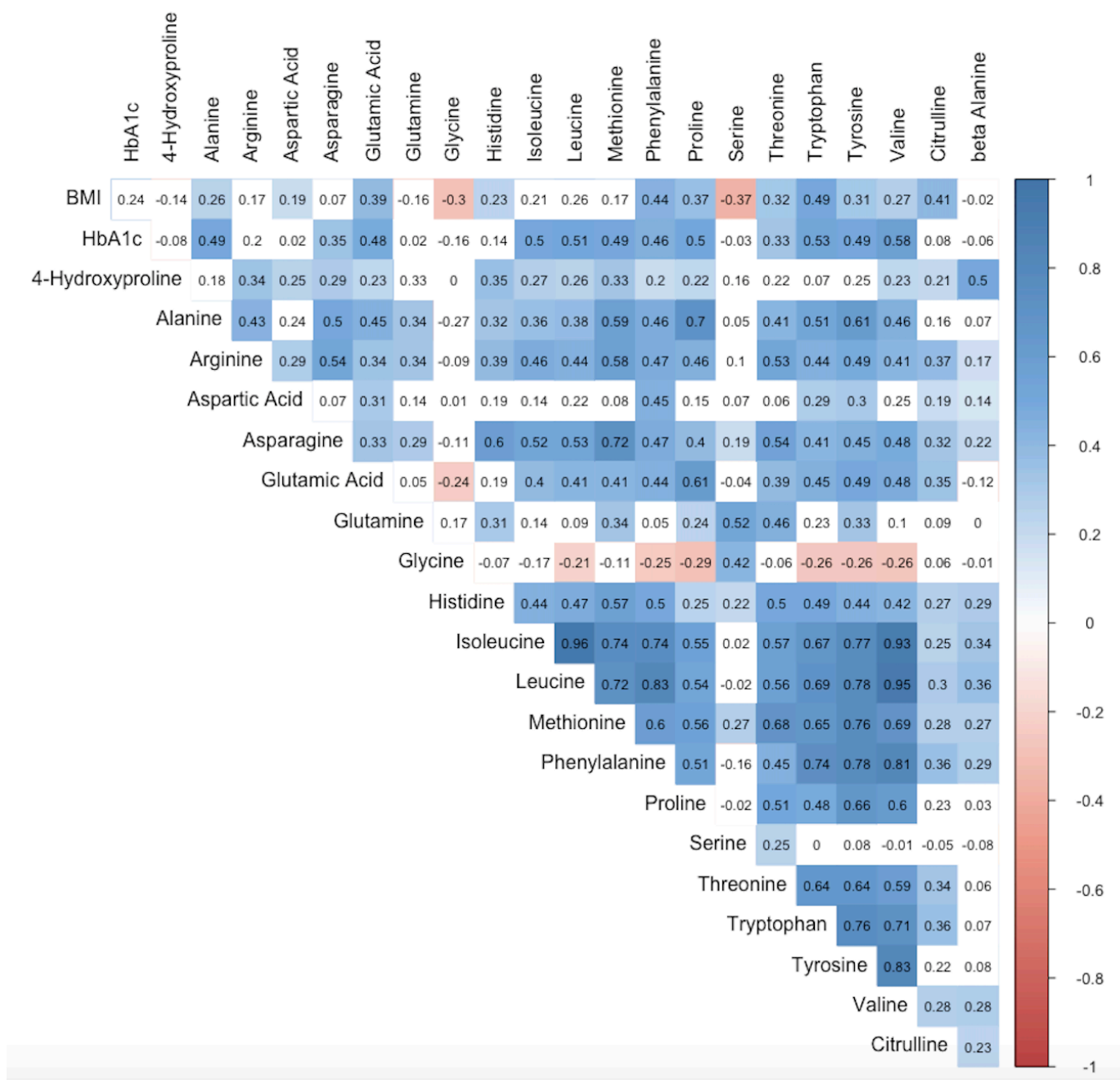


FIGURE 4-5 SPEARMAN'S CORRELATION CO-EFFICIENT HEATMAP BETWEEN MEASURED AMINO ACIDS, BMI & HbA1c. COLOURED SQUARES INDICATE SIGNIFICANT RESULTS WITH A BH CORRECTED Q VALUE OF <0.05

Figure 4-5 displays Spearman's Rank Correlation coefficients between measured AA, BMI and HbA1c throughout the dataset. BCAA: isoleucine ($r=0.50$), leucine ($r=0.51$), valine ($r=0.58$), AAA: phenylalanine ($r=0.44$), tryptophan ($r=0.49$), tyrosine ($r=0.31$) along with

alanine ($r=0.49$), asparagine ($r=0.35$), glutamic acid ($r=0.48$), methionine ($r=0.49$), proline ($r=0.5$), threonine ($r=0.33$) was found to be significant and positively correlated with corresponding serum HbA_{1c} at point of sampling.

AAA: phenylalanine ($r=0.44$), tryptophan ($r=0.49$), tyrosine ($r=0.31$), alanine ($r=0.26$), aspartic acid ($r=0.19$), glutamic acid ($r=0.39$), histidine ($r=0.23$), proline ($r=0.37$), threonine ($r=0.32$) and citrulline ($r=0.41$) showed a significant positive correlation relationship with BMI. Glycine ($r= -0.30$) and serine ($r= -0.37$) showed a significant negative correlation relationship with BMI. Only valine ($r=0.27$) from the BCAA subclass displayed any significant correlation with BMI. There were multiple inter-metabolite correlations. These correlation relationships are further visualised in a network plot shown in *figure 4-6*.

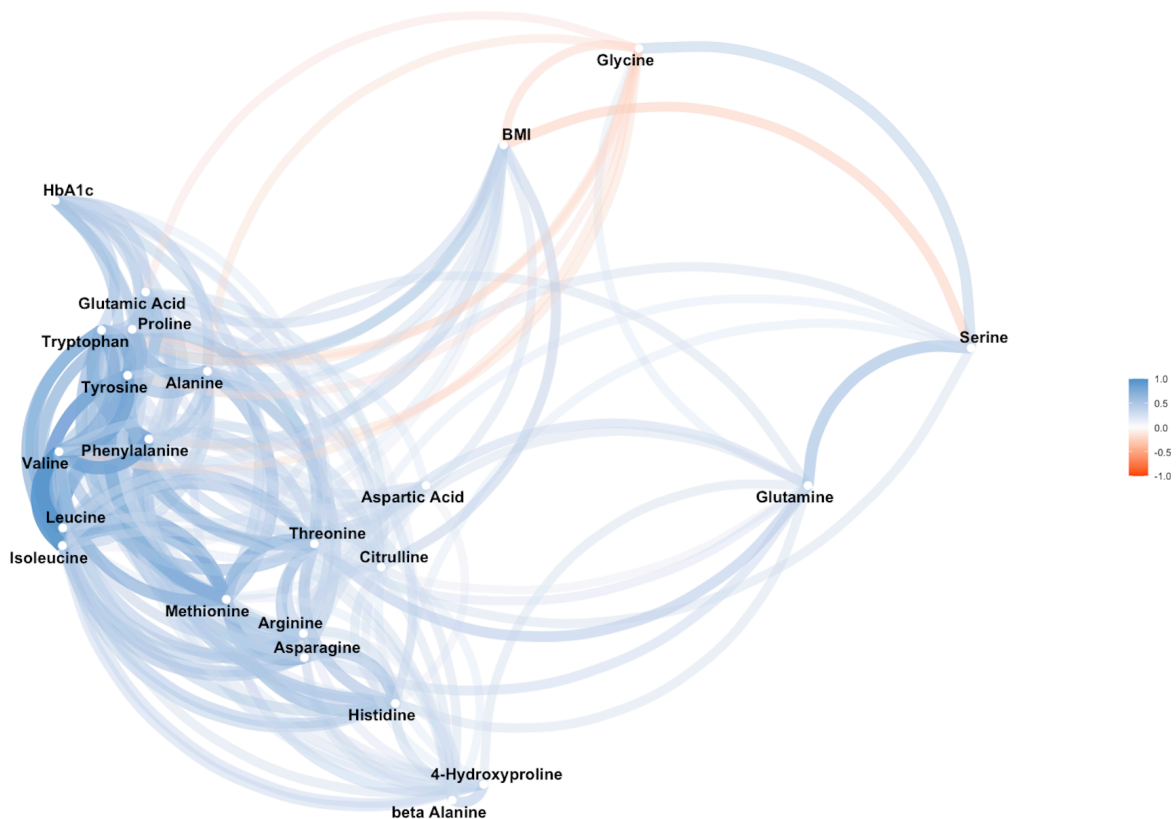


FIGURE 4-6 SPEARMAN'S CORRELATION NETWORK MAP BETWEEN MEASURED AMINO ACIDS, BMI & HbA_{1c}. CORRELATION STRENGTH AND DIRECTION INDICATED BY COLOUR AND PROXIMITIES OF CLUSTERS.

To further examine the effect of surgery, the pre-operative and 3-month post-operative Δ difference of measured metabolites were calculated and assessed for correlation with Δ BMI and Δ HbA_{1c}.

Δ HbA_{1c} was significantly correlated with Δ BCAA: Δ Isoleucine ($r=0.42$, $p=0.011$), Δ leucine ($r=0.46$, $p=0.005$) and Δ valine ($r=0.48$, $p=0.003$). Δ AAA: Δ tryptophan ($r=0.38$, $p=0.02$) and Δ tyrosine ($r=0.37$, $p=0.026$). As well as Δ asparagine ($r=0.33$, $p=0.049$), Δ methionine ($r=0.37$, $p=0.029$) and Δ threonine ($r=0.46$, $p=0.005$). Δ BMI showed significant positive correlation with Δ citrulline ($r=0.37$, $p=0.027$) and inverse relationships with Δ glutamine ($r= -0.38$, $p=0.023$) and Δ serine ($r= -0.52$, $p=0.001$). These relationships are further visualised in the heatmap below, *figure 4-7*.

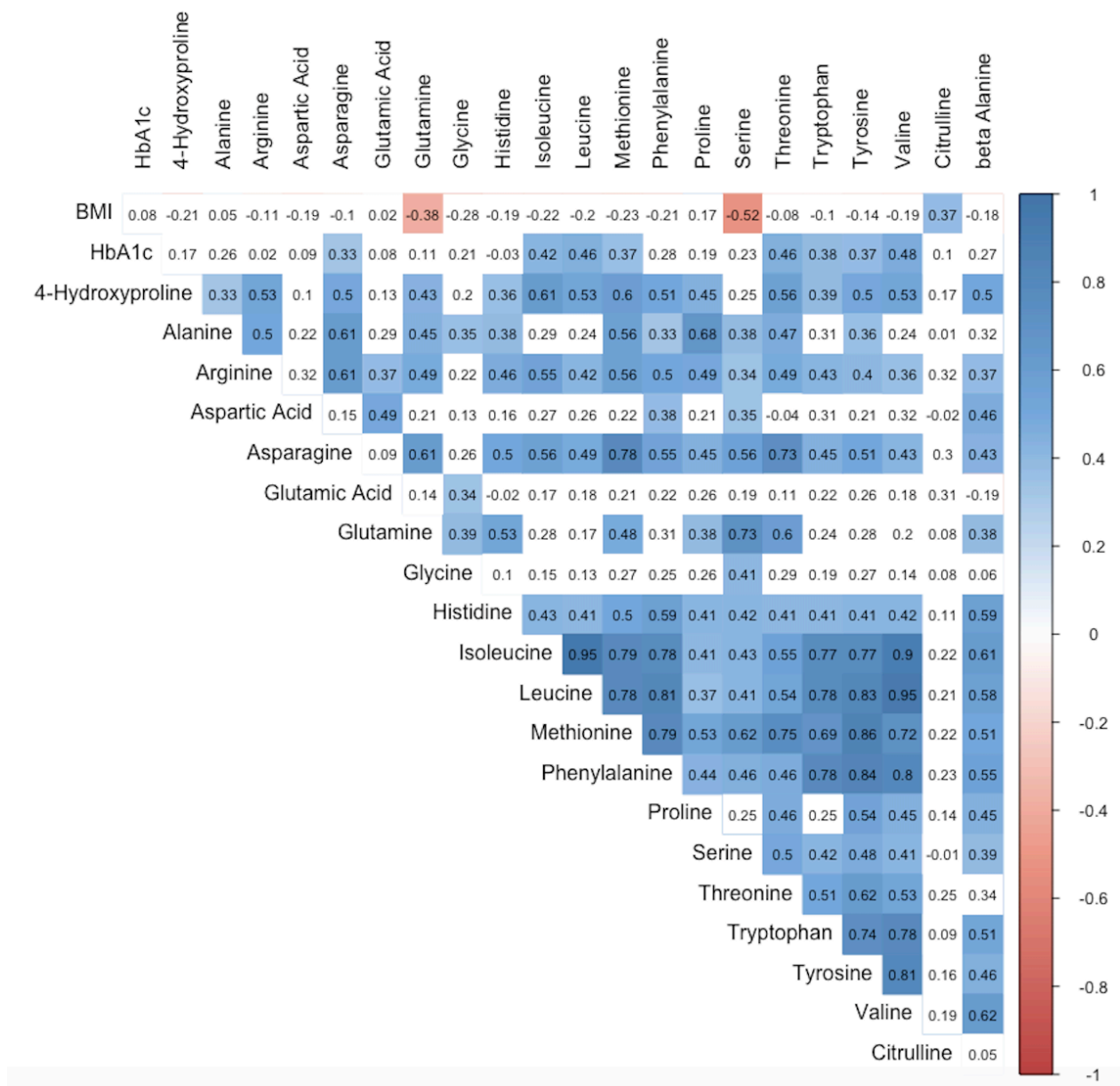


FIGURE 4-7 HEATMAP DISPLAYING SPEARMAN'S RANK CORRELATION COEFFICIENT OF Δ MEASURED AMINO ACIDS, Δ BMI AND Δ HbA1c. COLOURED SQUARES SIGNIFY A SIGNIFICANT CORRELATION, BLUE (POSITIVE) AND RED (NEGATIVE).

4.4.3 Amino acid Ratios

Fisher's ratio, BCAA to tyrosine ratio (BTR), valine to phenylalanine ratio (VPR), glutamine to glutamate ratio (GGR), proline to citrulline ratio (ProCit), alanine to serine (AlaSer) and leucine and serine to threonine and tryptophan ratio (LSTT) were first assessed in this cohort by comparing individuals with and without T2D at baseline (see boxplots *figure 4-8*). Fischer's ratio ($q=0.008$) along with VPR ($q<0.001$), ProCit ($q=0.017$) and AlaSer ($q=0.021$) were significantly increased in those with T2D.

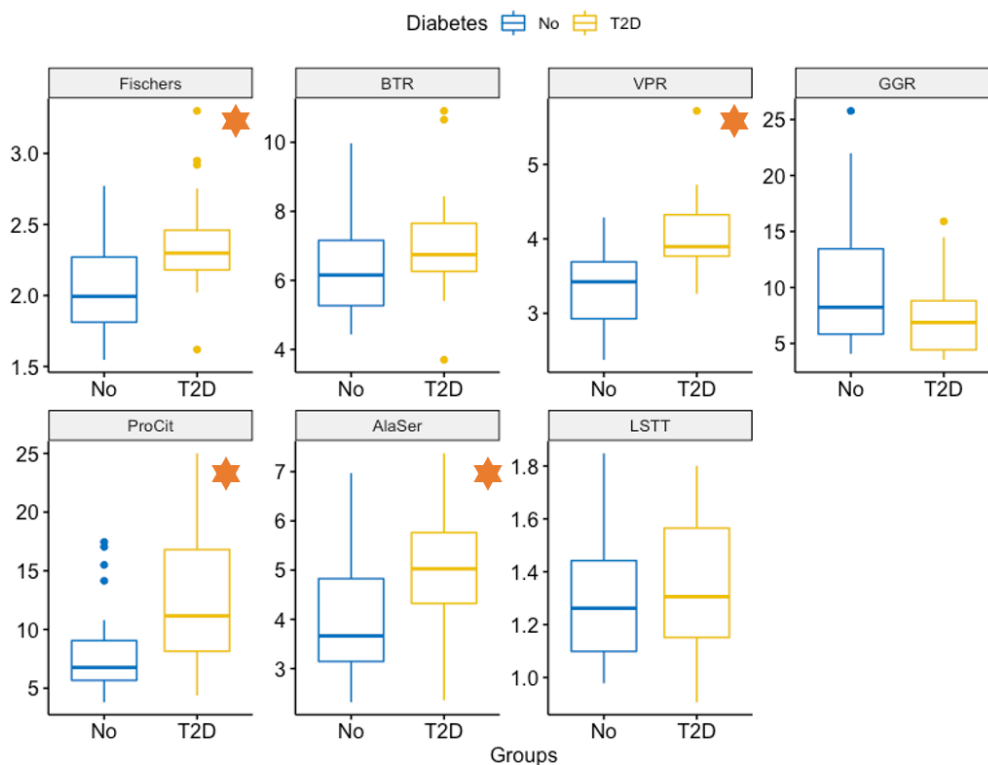


FIGURE 4-8 BOXPLOTS COMPARING PRE-OP VALUES OF AMINO ACID RATIOS

ORANGE STAR INDICATES SIGNIFICANT RESULTS WITH Q VALUE <0.05 , FISHER'S RATIO; BTR: BCAA TO TYROSINE RATIO; VPR: VALINE TO PHENYLALANINE RATIO; GGR: GLUTAMINE TO GLUTAMATE RATIO; PROCIT: PROLINE TO CITRULLINE RATIO; ALASER: ALANINE TO SERINE RATIO; LSTT: LEUCINE AND SERINE TO THREONINE AND TRYPTOPHAN RATIO

The effect of RYGB and VSG on amino acid ratios at 3 months post-op when compared to baseline were then assessed (see *figure 4-9*). VSG did not lead to any statistically significant changes to serum amino acid ratios. RYGB led to a significant decrease in alanine: serine

ratio ($q=0.003$) and significant increase in the glutamine: glutamate ratio ($q=0.032$) and leucine & serine to threonine & tryptophan ratio ($q=0.032$).

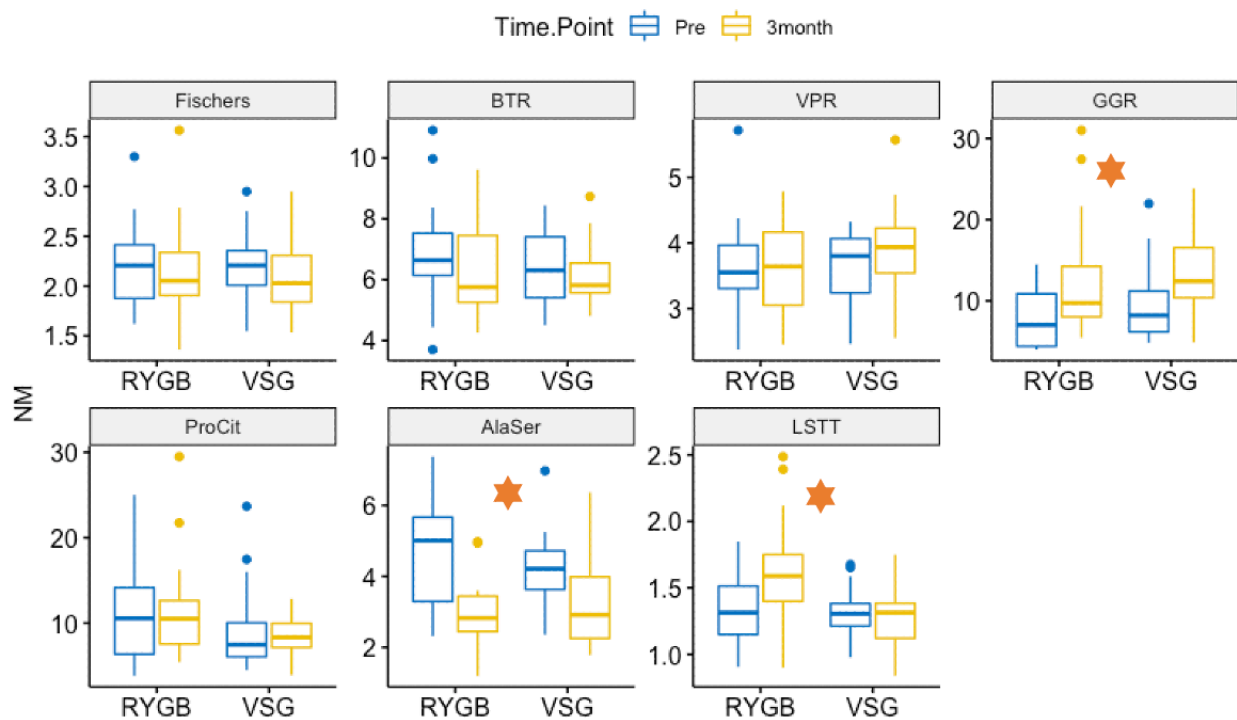


FIGURE 4-9 BOXPLOTS COMPARING PRE-OP V POST-OP VALUES OF AMINO ACIDS RATIOS DISPLAYED BY OPERATIVE SUBGROUP

ORANGE STAR INDICATES $q < 0.05$, BTR: BCAA TO TYROSINE RATIO; VPR: VALINE TO PHENYLALANINE RATIO; GGR: GLUTAMINE TO GLUTAMATE RATIO; PROCIT: PROLINE TO CITRULLINE RATIO; ALASER: ALANINE TO SERINE RATIO; LSTT: LEUCINE AND SERINE TO THREONINE AND TRYPTOPHAN RATIO

Amino acid ratio changes at 3 months post-op were also assessed in T2D and ND subgroups. Surgery led to a significant increase in glutamine: glutamate ratio (T2D $q=0.049$, ND $q=0.032$) and decrease in alanine: serine ratio (T2D $q < 0.001$, ND $q=0.034$) in both groups. In addition, Fischer's ratio was reduced significantly at 3 months post-operative in the T2D subgroup only ($q=0.049$), see *figure 4-10*.

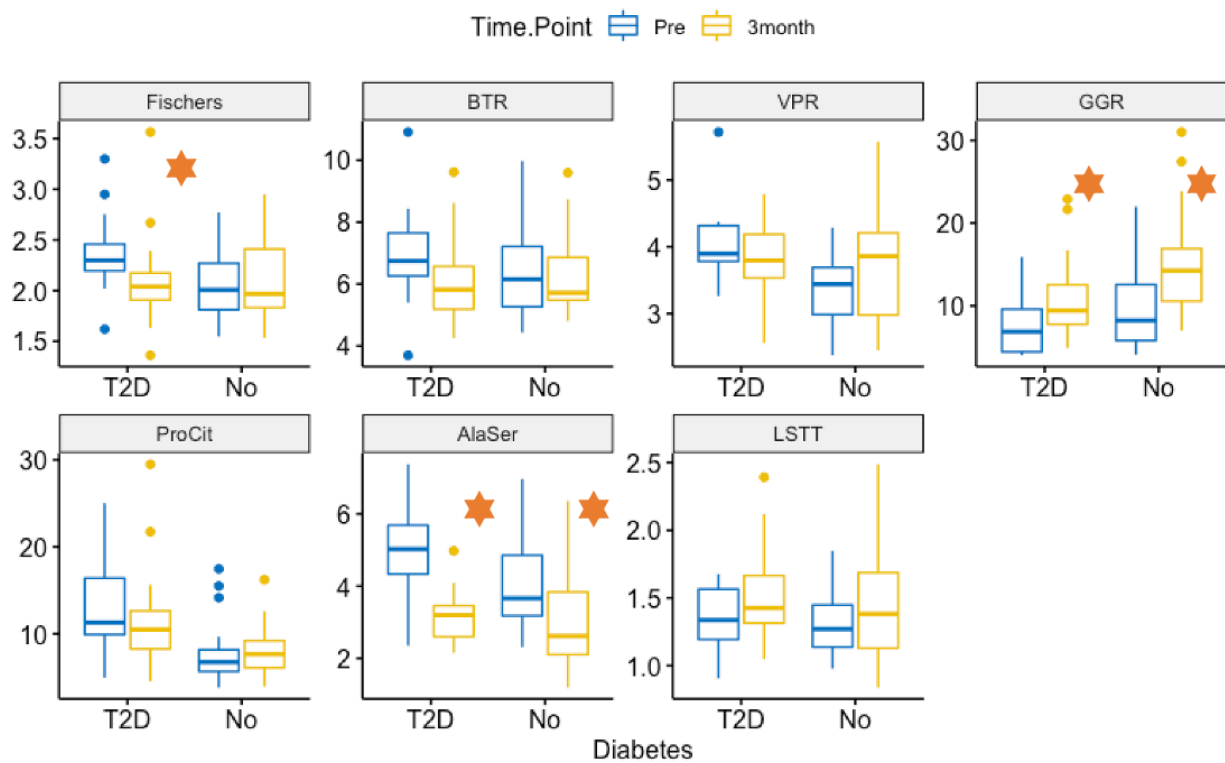


FIGURE 4-10 BOXPLOTS COMPARING PRE-OP V POST-OP VALUES OF AMINO ACIDS RATIOS DISPLAYED BY T2D SUBGROUPS

ORANGE STAR INDICATES $Q < 0.05$, BTR: BCAA TO TYROSINE RATIO; VPR: VALINE TO PHENYLALANINE RATIO; GGR: GLUTAMINE TO GLUTAMATE RATIO; ProCit: PROLINE TO CITRULLINE RATIO; AlaSer: ALANINE TO SERINE RATIO; LSTT: LEUCINE AND SERINE TO THREONINE AND TRYPTOPHAN RATIO

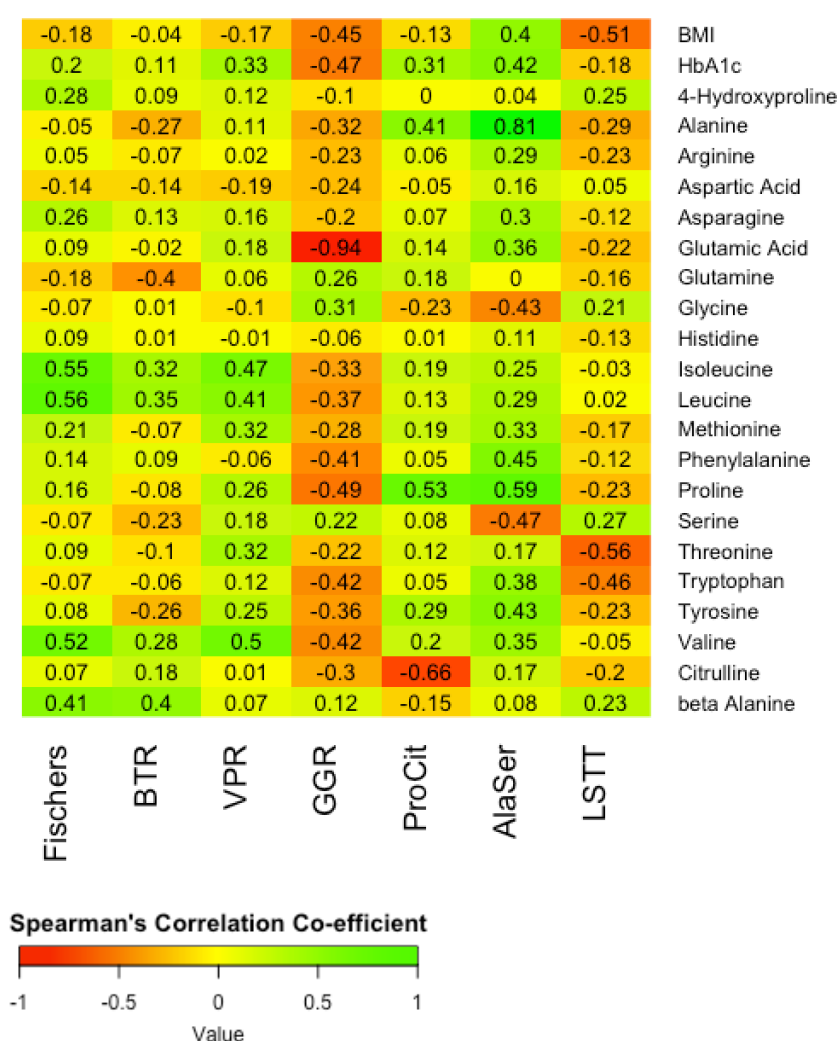


FIGURE 4-11 AMINO ACID TO AMINO ACID RATIO SPEARMAN RANK CORRELATION HEATMAP

Amino acid ratios were also assessed against BMI, HbA1c and measured amino acids using spearman's rank correlation (*figure 4-11*). HbA1c was significantly positively correlated with VPR ($r=0.33$, $p=0.004$), ProCit ($r=0.31$, $p=0.01$), AlaSer ($r=0.42$, $p<0.001$) and significantly negatively correlated with GGR ($r= -0.47$, $p<0.001$). BMI was significantly and positively correlated with AlaSer ($r=0.40$, $p<0.001$) and significantly and negatively correlated with GGR ($r= -0.45$, $p<0.001$) and LSTT ($r= -0.51$, $p<0.001$).

Alanine to Serine and Glutamine to Glutamate Ratio both have close associations with T2D and obesity which will be discussed later, and their correlation relationship with BMI and HbA1c are further visualised in scatter plots in *figure 4-12*.

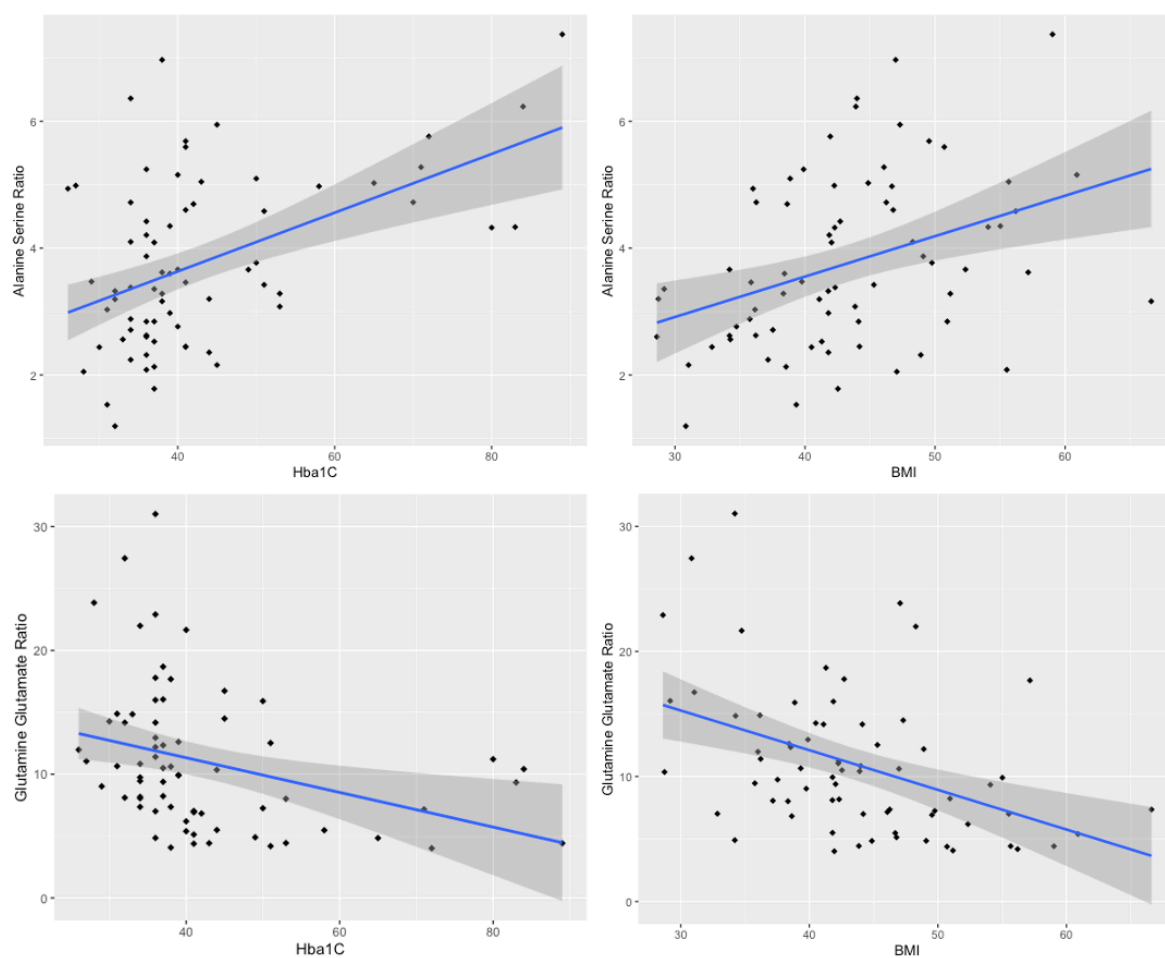


FIGURE 4-12 SCATTER PLOTS OF SELECTED AMINO ACID RATIOS, HbA1c AND BMI

TOP: SCATTER PLOTS OF ALANINE: SERINE RATIO AGAINST HbA1c ($r=0.41$, $p<0.001$) AND BMI ($r=0.40$, $p<0.001$)

BOTTOM: SCATTER PLOTS OF GLUTAMINE: GLUTAMATE RATIO AGAINST HbA1c ($r=-0.46$, $p<0.001$) & BMI ($r=-0.45$, $p<0.001$)

4.4.4 Urinary Metabolites of Microbiome and Endogenous Origin

Raw & unpublished 24-hour urinary metabolic data obtained using ¹H-NMR spectroscopy (by Nicholas Penney) from a subset of 21 pairs of patients within this cohort was also available for analysis. These are briefly presented below to aid discussion. The results were not quantified against pre-prepared chemical standard calibration ranges, but analysis of NMR intensities can still provide useful and relevant 'fold change' metabolic information as it is an inherently quantitative technique.

Features identified and available for analysis include the amino acids alanine, leucine, valine and glycine. Known urinary metabolites of gut microbiome origin including p-Cresol sulphate, trimethylamine N-oxide (TMAO), phenylacetate glutamine (PAG), Indoxyl sulphate (IS), acetate, Hippurate and formate. Markers of metabolism including lactate and carnitine were also included. Urinary NMR intensities were normalised using the respective 24-hour urine creatinine values.

T2D (n=10) and ND (n=11) subgroups were compared. BMI and HbA_{1c} are displayed for comparison purposes. At baseline, there were no significant statistical differences between the measured urinary metabolites between the two groups except for HbA_{1c} (see *figure 4-13*) Comparison of the effect of surgery at 3-months by T2D and surgical procedure subgroups on these urinary metabolites are displayed in *figure 4-14 and 4-15*.

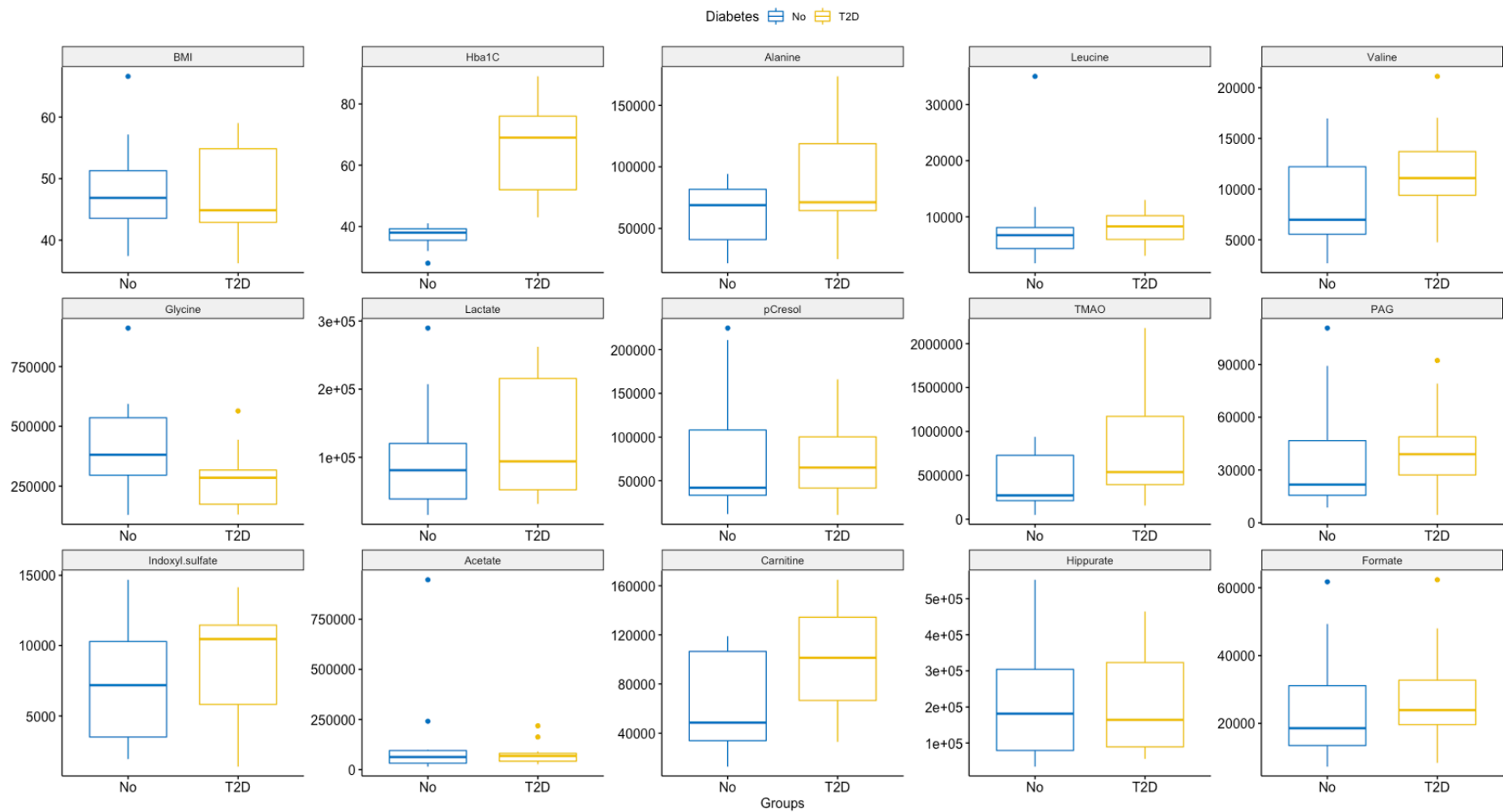


FIGURE 4-13 BOXPLOTS COMPARING PRE-OP VALUES OF MEASURED URINARY METABOLITES

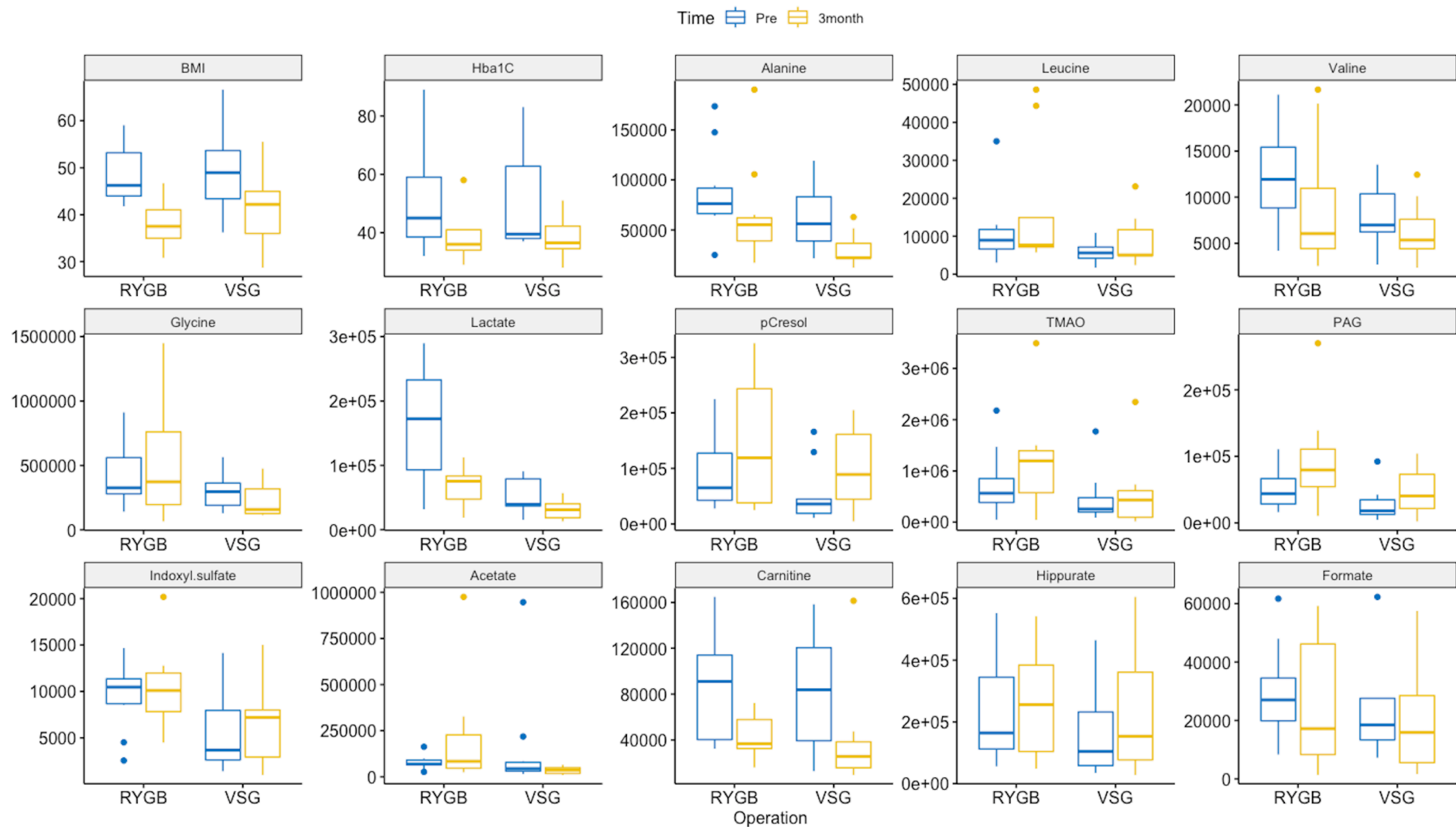


FIGURE 4-14 BOXPLOTS COMPARING PRE-OP V POST-OP VALUES OF MEASURED URINARY METABOLITES DISPLAYED BY OPERATIVE SUBGROUP

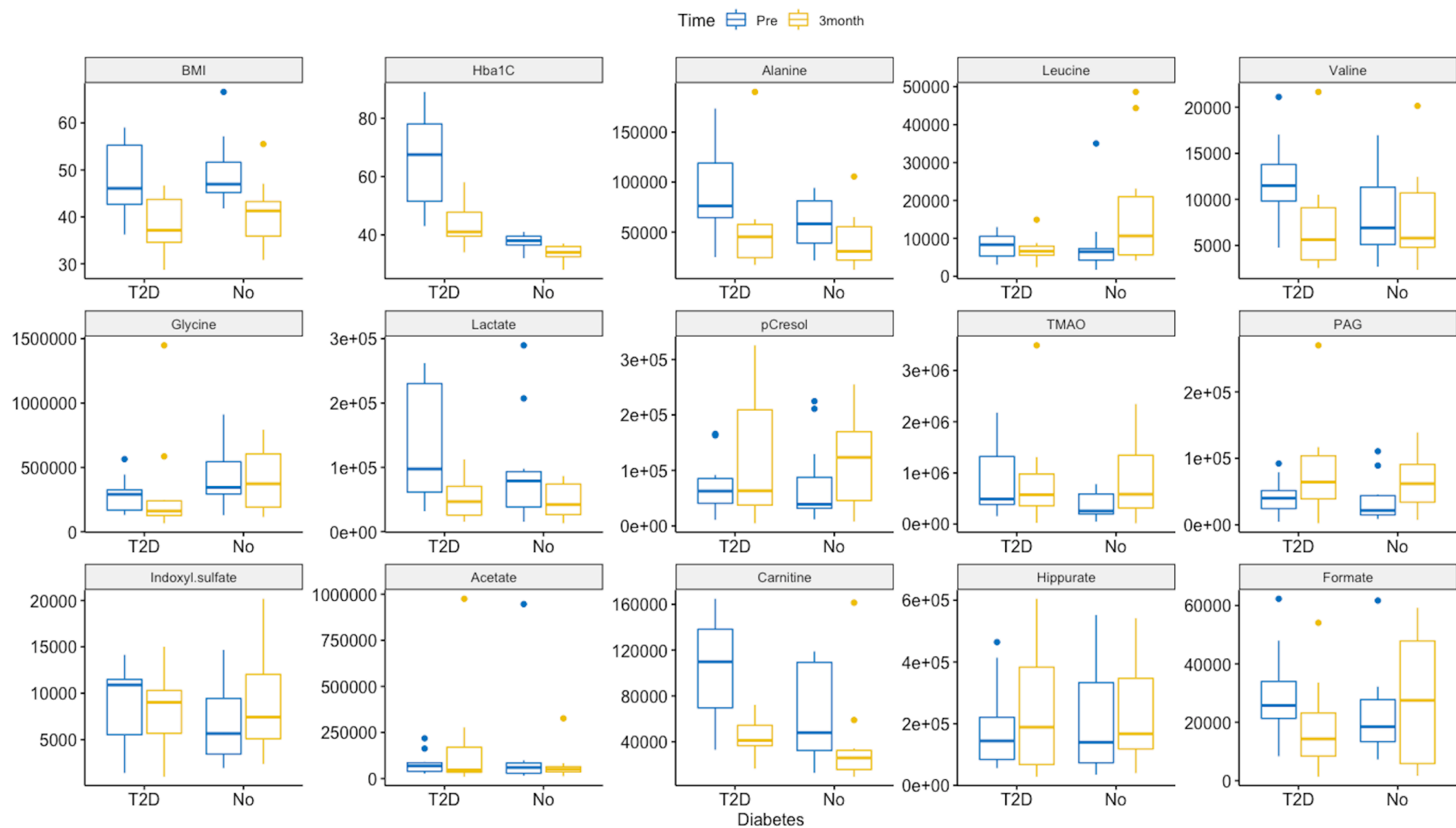


FIGURE 4-15 BOXPLOTS COMPARING PRE-OP V POST-OP VALUES OF MEASURED URINARY METABOLITES DISPLAYED BY OPERATIVE SUBGROUP

After controlling for FDR using the Benjamini-Hochberg method, no significant changes were observed when comparing the pre and 3-month post-operative intensities of the measured urinary metabolites.

For discussion purposes, the following metabolites were significant prior to controlling for FDR. RYGB: lactate ($p=0.010$, $q=0.135$), PAG ($p=0.039$, $q=0.166$) & carnitine ($p=0.028$, $q=0.166$). VSG: alanine ($p=0.023$, $q=0.301$). T2D: alanine ($p=0.009$, $q=0.058$), valine ($p=0.019$, $q=0.080$), lactate ($p=0.035$, $q=0.113$) & carnitine ($p=0.007$, $q=0.058$). There were no notable differences observed within the ND subgroup.

The measured urinary metabolites were also assessed for correlation against HbA_{1c} and BMI. Significant correlations were observed for HbA_{1c} with glycine ($r=-0.36$, $p=0.024$) and carnitine ($r=0.42$, $p=0.006$) only. These correlations are visualised as scatter plots below in figure 4-16. Significant correlations were observed for BMI with lactate ($r=0.32$, $p=0.041$), PAG ($r=-0.32$, $p=0.037$) and carnitine ($r=0.35$, $p=0.025$). These correlations are visualised as scatter plots below in figure 4-17.

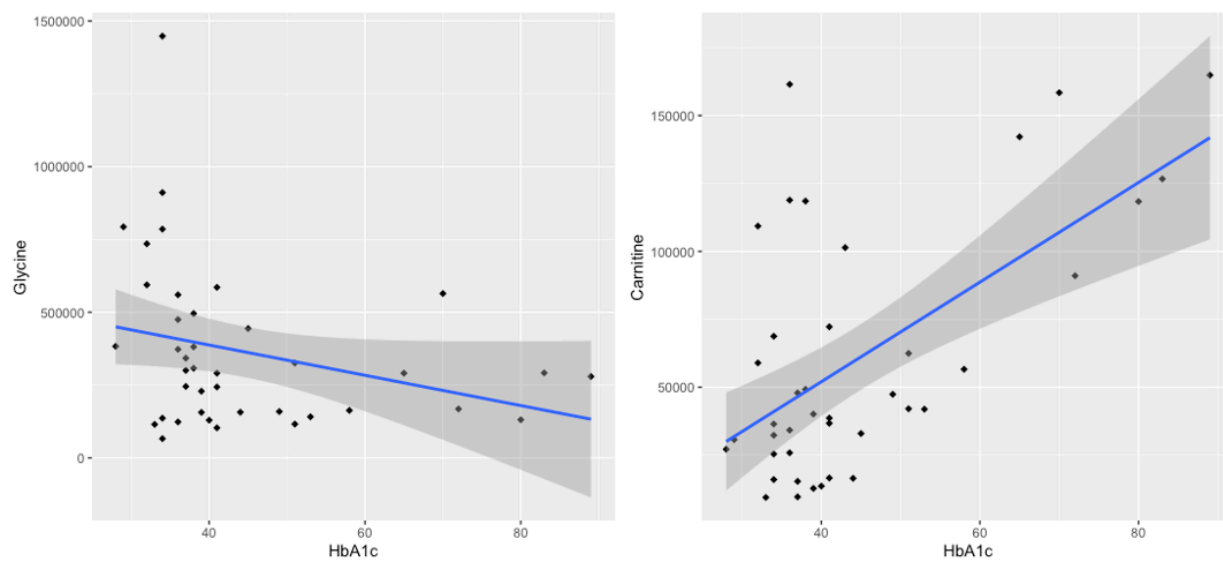


FIGURE 4-16 SCATTER PLOTS OF URINARY GLYCINE ($r=-0.36$, $p=0.024$) AND CARNITINE ($r=0.42$, $p=0.006$) AGAINST HbA1c

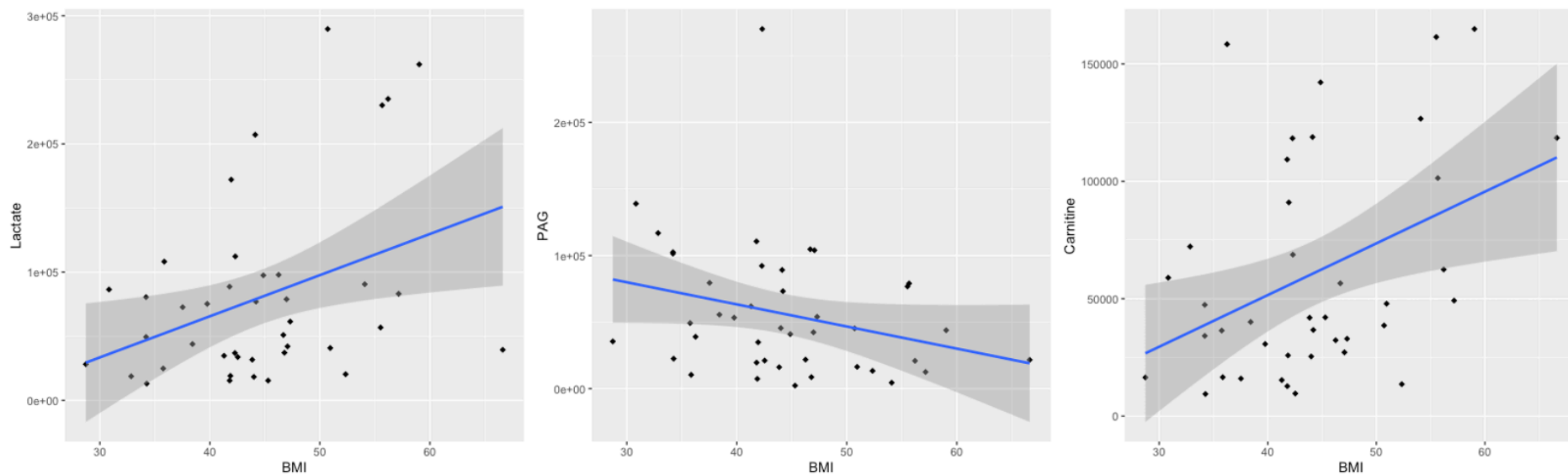


FIGURE 4-17 SCATTER PLOTS OF URINARY LACTATE ($r=0.32$, $p=0.041$), PAG ($r=-0.32$, $p=0.037$) AND CARNITINE ($r=0.35$, $p=0.025$) AGAINST BMI

Next, urinary metabolites of gut microbiome origin were compared to serum levels of their amino acid precursor measured at the time of sampling. Urinary PAG ($r = -0.47$, $p = 0.002$) demonstrated significant negative correlations with serum levels of its precursor phenylalanine. Changes in urinary pCresol sulphate ($r = -0.28$, $p = 0.076$), indoxyl sulphate ($r = -0.22$, $p = 0.17$) and Hippurate ($r = 0.17$, $p = 0.29$) were not significantly correlated with their serum precursor, see *figure 4-18*.

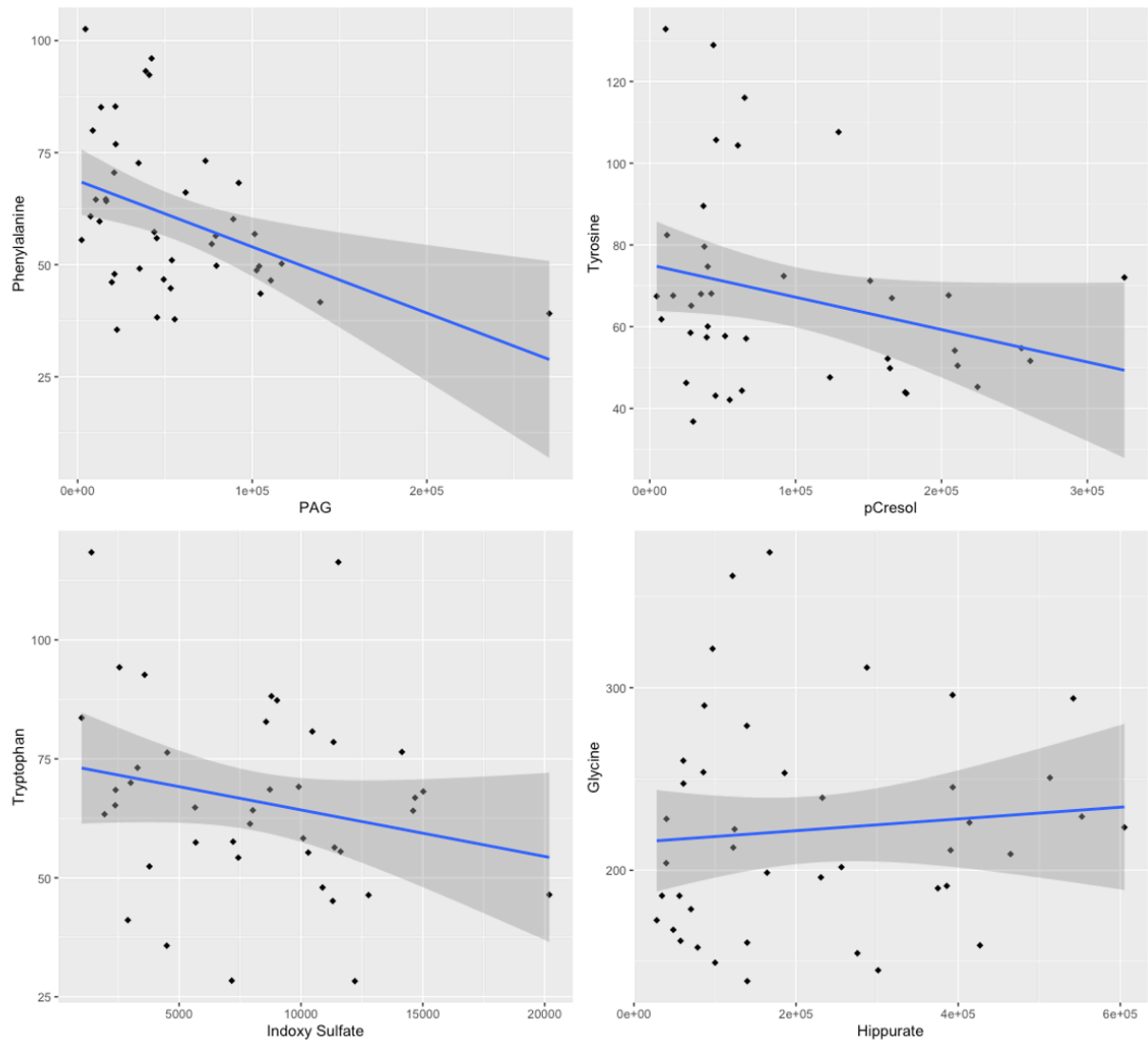


FIGURE 4-18 SCATTER PLOTS ASSESSING CORRELATION OF URINARY METABOLITES WITH ITS CORRESPONDING SERUM PRECURSOR.

TOP: URINARY PAG: SERUM PHENYLALANINE ($r = -0.47$, $p = 0.002$); URINARY P-CRESOL SULPHATE: SERUM TYROSINE ($r = -0.28$, $p = 0.076$)

BOTTOM: URINARY INDOXYL SULPHATE: SERUM TRYPTOPHAN ($r = -0.22$, $p = 0.17$); URINARY HIPPURATE: SERUM GLYCINE ($r = 0.17$, $p = 0.29$)

4.4.5 Type 2 Diabetes Resolution Analysis

Of the 17 patients with T2D, 11 (65%) achieved a status of resolution defined as HbA_{1c} < 42 mmol/mol and cessation of all T2D medications in accordance with American Diabetes Association (ADA) guidelines(345) at 3 months post-op. There were no significant differences observed when comparing the pre-operative values of BMI, HbA_{1c}, amino acids, amino acid ratios or measured urinary metabolites between those with and without T2D resolution.

When divided into T2D resolution and non-resolution subgroups, BMI and HbA_{1c} were reduced at 3 months post-op in both groups when compared to baseline. This was only significant in all groups except for BMI in those without T2D resolution, (Resolution: BMI $p=0.0006$, HbA_{1c} $p=0.0001$) vs (Non-Resolution: BMI $p=0.132$, HbA_{1c} $p=0.015$).

When comparing the pre-op and 3-month post-operative serum amino acid profiles of those with and without T2D resolution, significant reductions were observed in both groups for leucine ($q=0.028$, $q=0.023$), phenylalanine ($q=0.027$, $q=0.023$) and valine ($q=0.028$, $q=0.023$). In addition, significant reductions were observed with the T2D resolution group for threonine ($q=0.027$) and tyrosine ($q=0.027$) only. In the non-resolution group, significant reductions were observed for isoleucine ($q=0.023$) and beta alanine ($q=0.033$).

Post-operative changes in serum amino acids for the two groups followed the same direction trend except for glutamic acid (glutamate) where those without T2D resolution($q=0.904$) had increased levels post-operatively compared to a decreased trend in those with T2D resolution ($q=0.065$), see *figure 4- 19*.

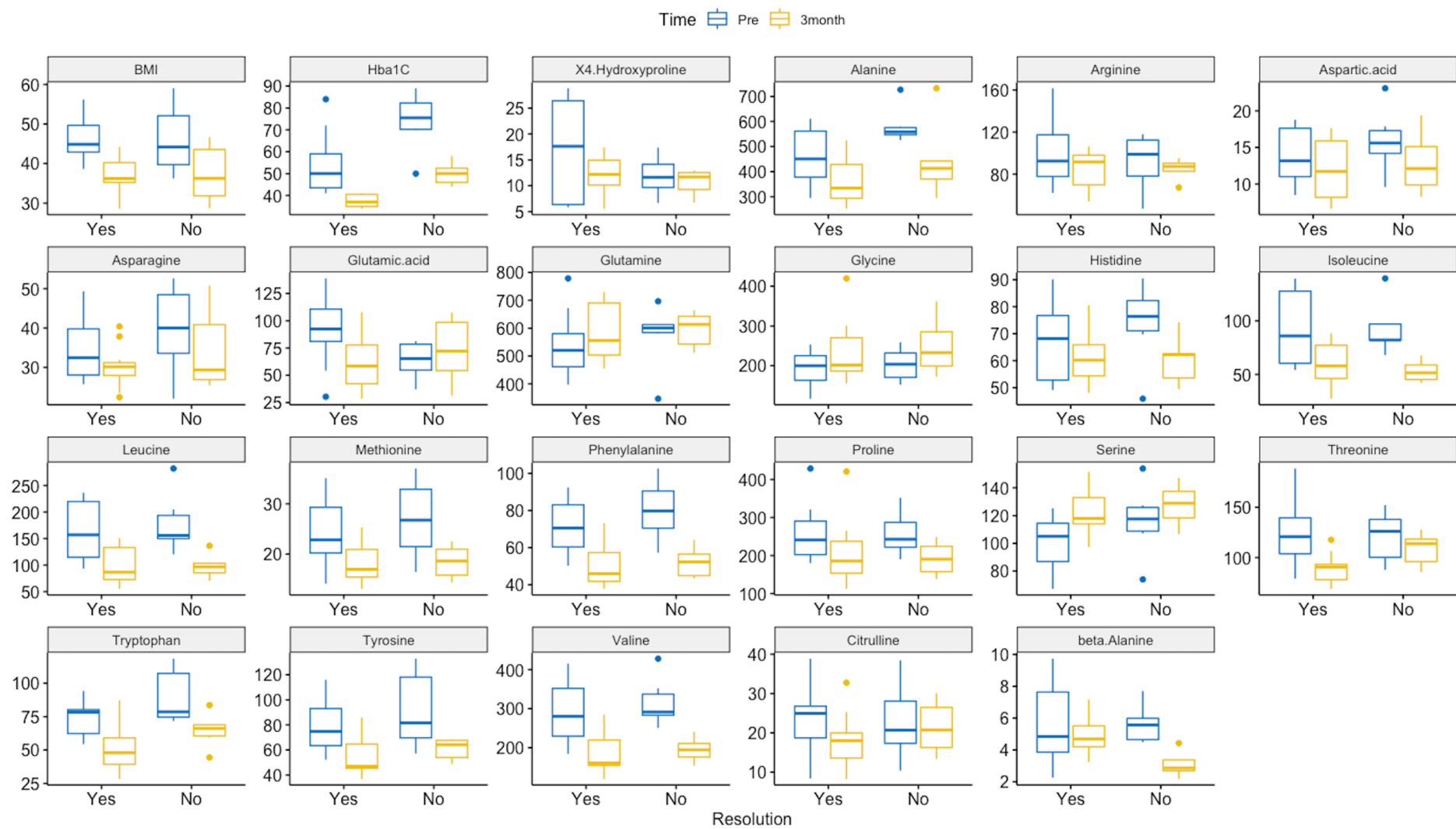


FIGURE 4-19 PRE-OP V POST-OP VALUES OF MEASURED AMINO ACIDS DISPLAYED BY T2D RESOLUTION SUBGROUP

Similarly, the same T2D resolution subgroup comparisons were performed for measured amino acid ratios. Again, no statistically significant differences were observed when comparing pre-operative values of AA ratio between the two groups. When comparing pre-op and 3-month post-operative changes, the non-resolution group did not have any significant changes. However, in those with T2D resolution, a significant increase in GGR ($q=0.017$) and a significant decrease in AlaSer ($q=0.013$) was observed, see *figure 4-20*. Both ratios demonstrated significant and strong correlation with HbA_{1c} in earlier results.

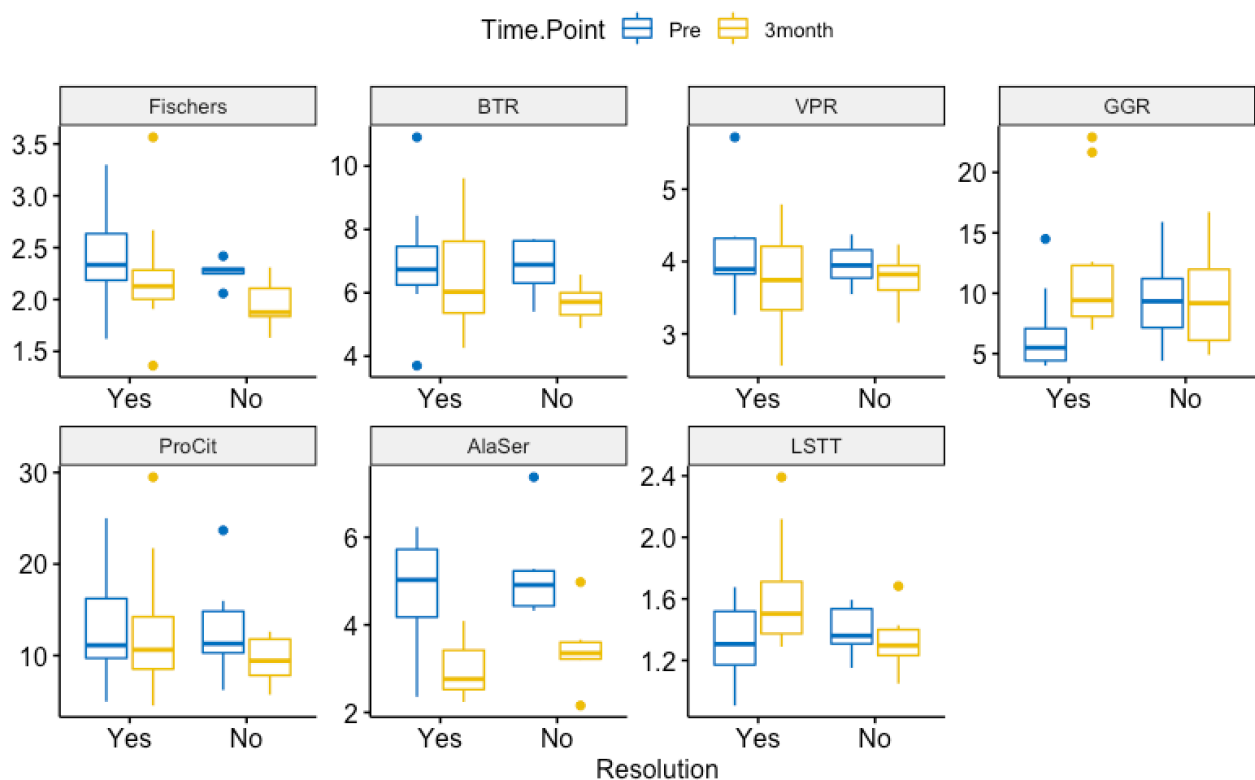


FIGURE 4-20 PRE-OP V POST-OP VALUES OF MEASURED AMINO ACID RATIOS DISPLAYED BY T2D RESOLUTION SUBGROUP

Of the ten T2D patients with urinary NMR data presented above, six had T2D resolution and four did not. There were no significant differences when comparing the pre-operative values of measured urinary metabolites between the two groups. No significant changes were observed when comparing pre- and post-operative values of

urinary metabolites in each subgroup either. *Figure 4-21* displays the trend changes for discussion purposes.

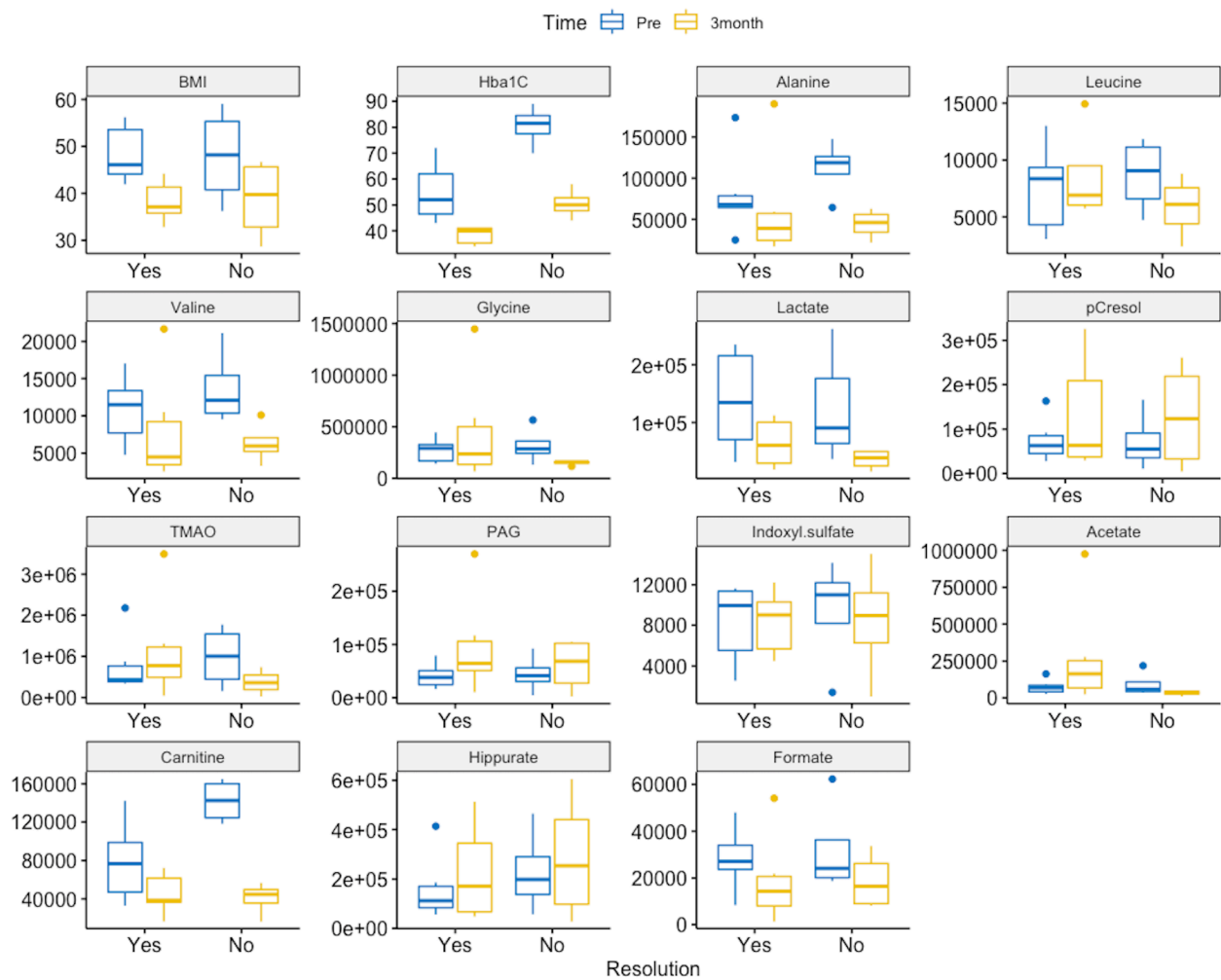


FIGURE 4-21 PRE-OP V POST-OP NMR INTENSITIES OF MEASURED URINARY METABOLITES DISPLAYED BY T2D RESOLUTION SUBGROUP

4.4.6 *Summary of Results*

In serum, all measured AA were raised in those with T2D except for serine and citrulline. These differences were significant for BCAA, alanine and proline.

Surgery led to reduction of serum levels of all BCAA and AAA. RYGB led to significant increases in glycine & serine, both markers of improved metabolic health. VSG led to significant reduction of alanine, glutamic acid, histidine and methionine. In the T2D cohort, significant reductions of BCAA, AAA, alanine, methionine, threonine were observed at 3-months post-operative irrespective of surgical procedure. As was a significant increase in serine. In the ND group, there was a significant reduction of phenylalanine and increase in glycine levels only. ΔHbA_{1c} correlated significantly with several amino acids including ΔBCAAs and some ΔAAA . ΔBMI negatively correlated with $\Delta\text{glutamine}$ and Δserine .

The serum glutamine: glutamate ratio showed significant negative correlation with BCAA levels. The serum glutamine: glutamate ratio (negative) and alanine: serine ratio (positive) showed significant correlation with both HbA_{1c} and BMI. Both of which implicate states of disturbed metabolism linked to T2D. RYGB induced significant changes to both of these ratios in keeping with an improvement in glycaemic control at the 3 months post-operative time point.

When evaluating urinary metabolites of microbial origin, no significant changes were observed between measured urinary metabolites when comparing T2D and ND groups at baseline. Similarly, no significant changes were observed when comparing the pre and 3-month post-operative levels of measured urinary metabolites in T2D, ND, RYGB and VSG subgroups.

Significant correlations were identified between BMI to urinary lactate, PAG & carnitine and HbA_{1c} to glycine and carnitine. Only PAG demonstrated a significant negative correlation with its serum precursor phenylalanine.

In cases of T2D resolution at 3 months, no significant differences in baseline serum amino acids, amino acid ratios or measured urinary metabolites were observed between those with

and without T2D resolution. Surgery, leading to reductions of serum amino acids, correlated with HbA1c in both groups. In addition, significant reductions were observed in threonine and tyrosine in the T2D resolution group only. Those with T2D resolution had a significant increase in the glutamine: glutamate ratio and significant decrease in the alanine: serine ratio. Both of these ratios demonstrate strong and significant correlation with BMI and HbA1c. No significant differences were observed for measured urinary metabolites when comparing those with and without T2D resolution at 3 months post-operative.

4.5 Discussion

4.5.1 *Serum Amino Acids*

The study results demonstrated that bariatric surgery led to widespread reductions of serum BCAA, these changes a 3-months post-op were particularly significant in those with T2D and were associated with an improvement in glycaemic control. Reduction of serum BCAA after bariatric surgery is a commonly reported finding (346). In a separate study, reductions in serum AA and in particular BCAA after RYGB has been shown to occur irrespective of weight loss or the presence of T2D (347). In those with T2D, improved glycaemic control is associated with a reduction in BCAAs but this also occurs in the absence of weight loss. Similarly, BCAA reductions after weight loss intervention can also predict reduction in HOMA-IR irrespective of weight loss(348). This implies mechanisms other than weight loss contribute to the glucose control effects of RYGB (275, 347). Similar weight independent effects on circulating BCAA and glucose tolerance have been demonstrated in mouse models after VSG (349). However, the impact of surgery on BCAA is only one of the many overarching beneficial effects of surgery resulting in improved glycaemic control.

A recent study in animal models was conducted where authors blocked the surgical reduction of BCAA levels with diet or genetic manipulation. Improvement of glycaemic control and weight loss still occurred after VSG further acknowledging that the reduction of BCAA is not necessary for metabolic benefits and that other mechanisms induced by surgery also contribute to these beneficial effects(349).

To further corroborate this idea, researchers comparing weight loss interventions found RYGB and not gastric band or very low-calorie diets (VLCD) reduced serum BCAA. Weight loss occurred in all three interventions, indicating effects beyond just weight loss, or diet restriction leading to reduced serum BCAA(275). Others have found both RYGB and gastric band induced weight loss was associated with reduced serum BCAA(350). A similar procedure, one anastomosis or mini gastric bypass also led to decreased serum BCAA(257, 351). Perhaps some degree of this effect may be due to the post-surgical influence on the gut

microbiome, which was particularly apparent after RYGB (352). The gut microbiome metabolite PAG demonstrated significant negative correlation with its serum precursor phenylalanine, indicating gut microbial metabolism may partly account for the reduction in serum levels. Similar but weaker and non-significant negative correlations were also demonstrated for p-Cresol sulphate & tyrosine and indoxyl sulphate & tryptophan.

Another possible mechanism is linked to the downregulation of adipose tissue BCAA catabolic enzymes in obesity. This effect is reversed by treatment such as bariatric surgery(269), which also coincides with improved glucose control. A reduction in dietary intake in the weeks and months following surgery is also likely to be contributory to these beneficial effects (*appendix V and VII*). Although in states of starvation, serum BCAA levels are found to increase (353), so thus this is further indication that bariatric surgery induces these changes via other powerful metabolic effects. A dedicated study controlling and studying the effects of diet on serum amino acid and its metabolites would be of interest.

Surgery-induced change is one of the ways in which serum BCAAs are reduced. This can also happen via other interventions. Restoration of metabolic health has been demonstrated by decreasing consumption of branched-chain amino acids(278). In a randomised control trial comparing short term (four week) BCAA reduction to placebo in T2D patients, reducing BCAA consumption reduced post-meal insulin secretion and improved white adipose tissue metabolism; measured by the reduction of MTOR expression. This effect has been demonstrated previously in mouse models(354). Interestingly, the reduction of insulin secretion did not result in an increase in blood glucose suggesting either an improvement in insulin sensitivity or reduction in mechanisms of BCAA mediated hyperglycaemia. The faecal microbiome was also modified by enrichment of Bacteroidetes and reduction of firmicutes, reversing obesity and T2D associated changes(355)

BCAA concentrations are also reduced after mono insulin sensitizer therapy(356, 357). Similar effects were observed with dual oral insulin sensitiser therapy using a combination of metformin & pioglitazone(358) or metformin & glipizide(359). In the former study, the

same effect on BCAA was seen with insulin infusion leading to a hypothesis that insulin sensitivity and action, rather than direct pharmacotherapy, affects circulating serum AA levels. In the latter study, the extent of BCAA change was linked to insulin resistance status, which leads to the possibility of using serum BCAA or AA levels as a biomarker to monitor and quantify the progress of intervention. Like the multiple causes of increased BCAA in obesity and T2D, surgery and other interventions reverse this process by acting on several fronts. These changes ultimately lead to reduction of BCAA levels and its associated diabetogenic effects.

Alanine is a non-essential amino acid that can be synthesised from pyruvate and BCAAs. Within human blood, alanine is one of the most abundant amino acids. Serum alanine levels were observed to be significantly higher in those with T2D, and positively correlated with HbA_{1c} levels. Those with T2D were observed to have a significant reduction in serum alanine levels at 3 months post-op.

Alanine levels have been shown to have a strong association with HOMA-IR and incident T2D(360). Circulating alanine has been proposed as an indicator of pyruvate production(361). Alanine is converted to pyruvate through a series of reactions catalysed by the enzyme alanine aminotransferase (ALT). Alterations in the alanine cycle is linked to T2D and leads to an increase in ALT. ALT can also be a marker of fat accumulation within the liver in cases of non-alcoholic steatohepatitis, a condition commonly seen with metabolic syndrome.

Pyruvate is the precursor of lactate in the lactate dehydrogenase reaction. Glucose loads have been shown to increase circulating lactate, pyruvate and alanine in a response that is dependent on insulin sensitivity(362). Thus, hyperglycaemia in T2D accounts for elevated levels of alanine and associated metabolites. I observed a reduction in alanine after surgery as well as a reduction of urinary lactate, both of which is in keeping with improved glycaemic control. No data on pyruvate levels were available to further corroborate this hypothesis.

In vitro experiments have shown that in appropriate conditions, alanine promotes insulin secretion (363) and is protective to pancreatic beta cells(364). The protective effect is conferred by enhancement of genes involved in processes such as cellular signalling, apoptosis and cellular stress responses(364). Alanine is also metabolised by oxidation in beta cells producing glutamate and aspartate. Alanine enhances glucose metabolism (oxidative and non-oxidative) with increased production of lactate, in the presence of glucose(365). Increasing oral intake of alanine has been shown to increase cellular glucose uptake leading to metabolic improvements of reduced fat storage and amelioration of glucose tolerance. It has been suggested that alanine also acts to suppress lipogenesis (366).

Up to 40% of non-glycogen derived hepatic glucose production originates via the glucose-alanine cycle (367). Glutamine is metabolised into glutamate and alanine, both of which are gluconeogenic(368). Glutamine loading has been shown to be associated with increased production of alanine in T2D rat models(369). Another experiment found that alanine supplementation of high fat diet mice led to suppression of weight gain, lower expression of liver fatty acid synthase, and higher adipose expression of triglyceride lipase(370), suggesting a potential regulatory role for alanine in lipid metabolism.

I observed that surgery led to increased serum glutamine and decreased serum alanine concentrations across all subgroups. Glutamate was reduced when compared to baseline in those with T2D resolution at 3-month post-operative however, the opposite was observed in those with non-resolution. This suggests a difference in the underlying metabolic response to surgery between these two groups of patients, which is worth further investigation as a differentiator or predictor of response. The mechanism leading to this effect is not immediately clear.

I reported that RYGB led to a significant increase in serum glycine and serine. As discussed in the introduction, the chronically low levels of serum glycine in obesity and T2D is probably partially as a result of its enhanced utilisation to buffer excess acyl metabolites formed in the peripheral metabolism of BCAA/AAA and lipids. Surgery leads to a reduction or reversal of this process resulting in a restoration or increase in the glycine pool.

Interestingly, regardless of intervention type (dieting, exercise, pharmacological or surgical) in patients with obesity or T2D, after intervention, increased plasma glycine was associated with improvement in insulin sensitivity(301, 350, 358, 371, 372) suggesting that this is an indicator of improved metabolic health rather than a surgically induced beneficial effect.

Glycine is also an essential feature of collagen. Collagen which lacks glycine in arterial walls are predisposed to degradation, which subsequently promotes calcification (373). With lower concentrations of glycine reported in those with obesity and T2D, this could be one of the mechanisms which contribute to increased cardiovascular atherosclerotic disease risk in T2D. Serine and glycine metabolism are also implicated in chronic kidney disease, so this mechanism may also be causal in arterial calcifications and increased cardiovascular mortality commonly seen with cohort of patients with obesity(373). The significant increase in serum glycine and serine after RYGB may be one mechanism which contributes to improved cardiovascular and renal outcomes after bariatric surgery. I observed that serum serine levels were significantly and negatively correlated with BMI in this cohort. This would suggest that metabolic changes due to surgery that impact on serine levels are weight dependant.

Furthermore, oral glycine supplementation has been shown to improve insulin response (374) and glucose tolerance (375) and may even protect from oxidative stress in the context of systemic inflammation in metabolic syndrome(376). From the discussion above, non-surgical interventions aimed at increasing serum glycine and serine concentrations would be worth further investigating to whether a causal effect could be established.

4.5.2 *Amino Acid Ratios*

Glutamine is the most abundant non-essential amino acid within human plasma, amongst many roles, it regulates pancreatic beta cell and insulin secretion (327). Glutamate (glutamic acid in non-ionised form) is found in many proteins rich foods and also gives a pleasurable taste sensation when consumed. BCAAs are essential as a nitrogen provider to maintain glutamine, glutamate and alanine stores within skeletal muscle(377). The conversion of glutamine to glutamate is important for many cellular metabolic functions(377), and dysregulation between the two may contribute to the development of T2D. Increased glutamate levels are associated with chronic inflammation and oxidative stress, which is common in individuals with obesity. Glutamate enables an increase of transamination of pyruvate to alanine, which then promotes gluconeogenesis(268, 378). It is hypothesised that increased glutamate levels act accumulatively with factors such as hyperglycaemia and increased FFAs contributing to pancreatic beta cell dysfunction and apoptosis(379). Under normal circumstances, intracellular glutamate is a neurotransmitter which promotes glucose stimulated insulin secretion(380) by amplification of the calcium messenger signal for insulin release in pancreatic beta cells(381). Extracellular glutamate on the other hand, reduces insulin secretion. Excess activation of pancreatic islet glutamate receptors such as NMDA receptors in beta cells(382) can also cause dysregulation and increase glutamate induces susceptibility of oxidative beta cell injury leading also to effects on cell mortality.

Serum glutamine and glutamate both provide carbon for gluconeogenesis in the kidney and liver. The levels are respectively negatively and positively associated with T2D(254), a higher glutamine to glutamate ratio has also been reported to be associated with a lower risk of incident T2D(244, 327) illustrating the important of metabolic balance between the two. Glutamate has also been proposed as a marker of T2D and sensitive marker of glycaemic control(383). Our results demonstrate significant positive associations between glutamate levels, BMI and HbA_{1c}, which would be in keeping with this suggestion. As previously discussed, those with T2D resolution were observed to have a reduction in glutamate levels

at 3-month post-operative when compared to baseline. Those with non-resolution were observed to have an increased level when compared to baseline.

The glutamine: glutamate ratio is reported to reflect the state of energy metabolism and balance of these two metabolites within an individual(327). In this study cohort, while not significant after controlling for FDR ($q=0.06$) those with T2D had a lower GGR compared to those without which is also in keeping with current literature. RYGB led to a significant increase in GGR. GGR was also negatively correlated with HbA_{1c} and BMI ($r= -0.4$). GGR was also negatively correlated with all BCAA levels, which the metabolism of both is intricately linked. BCAA catabolism by BCAT utilises glutamine as a nitrogen receiver. Leucine also activates the enzyme responsible for glutamate oxidative deamination (327). This study also reported a significant increase in the GGR for those who had T2D resolution at 3 months, where those who did not have T2D resolution had similar levels of GGR pre- and post-op. This may point to underlying mechanisms contributing to energy metabolism differences in those with and without T2D resolution and perhaps GGR may be used as a measure of response to surgery.

In an RCT, 30g/day of oral glutamine supplementation in patients with T2D was associated with an improvement in cardiovascular risk factors such as reduction of fasting glucose, mean HbA_{1c} as well as increase of fat free mass(384). In another RCT glutamine was shown to regulate GLP-1 and subsequent insulin secretion, leading to reduced blood glucose(385). However, a recent systematic review concluded that while glutamine supplementation seems to be associated with increased insulin production, increased serum GLP-1 levels and reduced blood sugar, its effect on HbA_{1c} are inconclusive and further dose dependant clinical trials are required to obtain T2D specific outcomes(386).

β -cell regeneration or neogenesis from α -cells has also been suggested as a potential therapeutic avenue in instances of T2D β -cell depletion(387). α -Cell proliferation in animal models required mTOR activation by AA, in a process which requires glutamine(388). It is hypothesised that metabolic imbalance inferred by the glutamine to glutamate ratio affects this process(327).

The effect of surgery on glutamine, glutamate, GGR and their associations with improved glycaemic control and T2D resolution make investigating oral glutamine supplementation and other methods of influencing this metabolic axis a potentially valuable pursuit and is an example of how studying mechanisms of surgical T2D resolution may pave the way for other non-surgical treatments for T2D.

An increased alanine: serine ratio is associated with T2D(314), this ratio has also recently been demonstrated to be associated with T2D severity (317). Increased alanine: serine ratio contributes to the formation of 1-deoxysphingolipid(1-deoxySL)(389). 1-deoxySL are atypical lipids formed during sphingolipid formation. These are a class of phospholipid and are discussed in further detail in chapter six. Serine deficiency is observed in those with T2D, the key enzyme in the sphingolipid formation process: serine palmitoyltransferase (SPT). In conditions of serine deficiency, this enzyme uses alanine or glycine instead of serine as amino acid substrate, leading to the formation of 1-dexoySL. Serum 1-deoxySL levels are higher in those with T2D but not T1D when compared to controls(390). This suggests that 1-deoxySL is an independent causative agent in T2D or is a result of metabolic changes associated with T2D only. In this study cohort, an increased alanine: serine ratio was associated with T2D, and surgery led to a reduction of this ratio. The alanine serine ratio was also significantly negatively correlated with HbA1c ($r=-0.415$), and BMI ($r=-0.411$). Furthermore, those with T2D resolution were found to have a significant decrease in alanine: serine ratio compared to those without T2D resolution, suggesting that this pathway may have a significant role in the T2D phenotype. Both AA ratios discussed above can be used as novel surrogate markers of mechanisms related to T2D resolution.

4.5.3 *Urinary Metabolites*

Carnitines play an essential role in energy metabolism. It is essential for the transfer of long chain fatty acids (FA) across inner mitochondria membrane for beta oxidation. FA and CoA esters cannot cross the mitochondrial membrane without the formation of carnitine esters(343).

$\frac{3}{4}$ of daily carnitine requirements come from the diet and another $\frac{1}{4}$ is synthesised from lysine and methionine. There is also some degree of renal reabsorption, if required to maintain homeostasis (391). Plasma levels of carnitine are dependent on physiological requirements and the rate of renal excretion and reabsorption changes according to plasma concentrations of carnitine(392).

Carnitine has anti-inflammatory and antioxidant properties(393, 394). It also has a role as a scavenger of acyl residues derived from glucose, amino acid and lipid metabolism(395). This process of conjugation producing acylcarnitine, reduces the number of acyl residues attached to CoA, which is important as mitochondria fatty acid oxidation leads to accumulation of fatty acyl-CoA. A component of the signature of obesity and IR is the accumulation of acylcarnitines in peripheral tissues(306, 396). In certain organs such as the heart, accumulation of such toxic metabolites induces apoptosis and inflammation(397). Elevated levels of serum carnitine may be a response to metabolic changes associated with obesity and T2D.

Microbial metabolism of carnitine is also known to have physiological effects and is known to generate TMAO in a process stimulated by low fibre intake(398). Production of TMAO contributes to atherosclerotic and cardiovascular risk(399). To investigate this, further correlation analysis was performed, and I found no significant correlation between urinary carnitine and TMAO ($r=0.11$, $p=0.52$) in this cohort.

While in humans, carnitine is essential for FA oxidation, this does not apply to microbes, where carnitine is both a nutrient source and protects against environmental stress(400). A recent study demonstrated two novel carnitine structure analogues of gut microbial origin

that antagonises carnitine function. This inhibited brain cell mitochondrial function, demonstrating direct microbial influence on brain cells(401). Further work is required to investigate the role of gut microbiome metabolites and the effect of mitochondria function on other tissue types.

Carnitines do not have a known physiological role within the plasma and to an extent, urine levels maybe be an indirect measure for metabolic activity(343). In the case of obesity and T2D, data from this cohort demonstrated significant positive correlation between BMI, HbA1c and urinary carnitines levels. Surgery also led to a reducing trend of urinary carnitines levels. As serum carnitine levels are dependent on its metabolic requirement, and urinary levels being a surrogate of serum levels, these findings would certainly support the notion that increased peripheral AA and FFA metabolic activity is associated with obesity and T2D. Surgery leading to reduction in BMI and HbA1c correlated with a reduction in both urinary and serum carnitine levels, possibly indicating a reduction of the number of acyl residues produced in peripheral tissues, thus one of the beneficial metabolic changes conferred by surgical intervention.

Urinary levels of lactate were found to be significantly positively correlated with BMI in this study. Lactate is a well-known product of anaerobic glycolysis, and a marker of oxidative capacity and serum levels rises in instances of ischaemia or exercise. It is less well-known that during a rested and fasted state, raised lactate represent the balance between gluconeogenesis and extra hepatic glycolysis and glucose disposal(402). While skeletal muscle accounts of most plasma lactate, in those with obesity, increased lactate concentrations originate from adipose tissue(403), in keeping with states of oxidative stress and underlying inflammation. Factors such as increased adipocyte size, increased intracellular hypoxia and inadequate vascularisation(404) all add to this oxidative stress. Acute elevations in glucose, insulin levels or anaerobic glycolysis can also contribute to elevated plasma lactate levels (403).

Circulating lactate has been identified as a predictor for incident T2D (405). Elevated serum lactate has been observed when there is impairment of mitochondrial oxidative and

phosphorylation activity. These changes are associated to insulin resistance (340-342) and may be a marker of progression of severity of T2D(406). It has been shown that normal changes in circulating serum lactate levels during oral glucose tolerance testing is blunted in the presence of insulin resistance(407). Lactate is also a precursor in hepatic gluconeogenesis and the major carbon substrate for mitochondria TCA in most tissues(408). GLUT 4, is an insulin dependent glucose transporter which moves glucose into muscle and fat cells for glycolysis in response to insulin secretion(409). One mechanism in which lactate can induce insulin resistance is by suppression of GLUT4 expression in skeletal muscle(410). It may also inhibit lipolysis by activation of G-protein receptor 81 in adipocytes via an autocrine like loop(411).

Increased insulin sensitivity after weight loss and an associated reduction in plasma lactate has previously been reported(412). BMI was significantly positively correlated with urinary lactate in this cohort. Although not statistically significant, I found higher levels of urinary lactate in those with T2D compared to those without. Surgery also led to a trend of reduction in urinary lactate which correlated with a reduction in BMI but not HbA1c in this cohort. This would be in keeping with weight loss leading to a reduction in peripheral oxidative stress.

4.5.4 Urinary Metabolites of Microbiome Origin

When considering the urinary metabolome, the metabolites reported in the study were not exhaustive and were reported for the purposes of a wider discussion. The derangement of such metabolites has previously been linked to disturbed metabolism in obesity or T2D. In particular, it has been demonstrated that gut microbial metabolism of tryptophan, phenylalanine and tyrosine metabolism is dysregulated in instances of obesity(413). While not statistically significant, I demonstrated relatively increased urinary levels of indoxyl sulphate, PAG, p-Cresol sulphate and decreased Hippurate in those with T2D when compared to those without. This is in keeping with current literature(413).

Both p-Cresol sulphate and indoxyl sulphate are pro-inflammatory and pro-oxidative metabolites produced by the gut microbiome which contribute to chronic kidney

disease(414) and arterial wall stiffness(415). Increasing levels of both are associated with worsening cardiovascular disease and mortality in Chronic Kidney Disease (CKD) patients (416-418). It is thought that the pathological mechanism involves generation of reactive oxygen species in endothelial cells leading to endothelial dysfunction(419). It also activates the NF-kB pathway which causes oxidative stress and the release of systemic inflammatory cytokines(420, 421).

Bariatric surgery, particularly malabsorptive procedures such as RYGB, has also been demonstrated to cause changes in metabolic pathways affecting BCAA and AAA metabolism and subsequent serum levels. These changes include a decrease of excreted urinary BCAA and increased urinary excretion of microbial metabolites of protein putrefaction – PAG, p-cresol sulphate and indoxyl sulphate(221). Elevated levels of urinary PAG are associated with reduced insulin resistance (422). The trends reported in this study cohort would agree with these findings, but the relatively low sample numbers and lack of statistical significance means no significant conclusions could be drawn.

It is worth nothing raised serum PAG is also associated with overall mortality in cardiovascular disease risk in patients with CKD (423), perhaps via similar mechanisms seen in cases of elevated serum PAG in those with obesity and T2D. PAG is dependent on renal tubular excretion and 24-hour urine PAG levels can be used as a surrogate for PAG intestinal uptake and renal clearance (417). In patients with CKD, serum PAG is elevated but this deference is not accounted for simply with eGFR deficiency. Unfortunately, I did not have serum PAG levels to corroborate this, nor was I able to comment on the effect of bariatric surgery on serum PAG levels. The results presented also suggest that at 3 months after bariatric surgery, there are elevated trends of increased urinary p-cresol sulphate, indoxyl sulphate and PAG. Again, I did not have the data to compare whether increased urinary excretion corresponded to changes in serum levels. If it does, then perhaps not all metabolic changes brought about by bariatric surgery has beneficial metabolic effects.

However, in patients with CKD is it not clear whether elevated levels of p-cresol sulphate or indoxyl sulphate and PAG are purely because of dysregulated microbiome function or

compounded by renal dysfunction. All three metabolites require active tubular secretion rather than glomerular filtration for renal clearance. So thus, toxicity may be due to renal impairment of excretion. Renal replacement therapy cannot replace tubular function and p-Cresol sulphate, indoxyl sulphate and PAG levels have been found to be over one hundred times above normal in predialysis patients when compared to normal controls(424). There are potentially clinical implications here that require further investigation. If surgery does increase these potentially harmful metabolites, then perhaps those with CKD should not undergo procedures that result in elevated serum levels of PAG, p-Cresol sulphate or indoxyl sulphate.

Serum levels of phenylalanine and tyrosine are known to decrease after bariatric surgery, however a recently comprehensive systematic review found no direct evidence that these changes are a result of the changing gut microbiome(425). A significant negative correlation was observed between phenylalanine and its relative downstream microbial metabolite PAG in this study cohort, although causality cannot be determined by correlation, it would not be unreasonable to hypothesise that increased microbial metabolism induced by changes to the microbiome by surgery may partially contribute to this effect.

A cross sectional study comparing high and low compliance to Mediterranean diet found urinary PAG and p-Cresol sulphate levels to be highly differentiative of the two groups. Both have links to glucose metabolism, highlighting the fact that dietary interventions can also have metabolic modulatory effects on the gut microbiome(426).

Overall, although it is not the primary focus of this study, the gut microbiota is still seen to have an influence on systemic insulin resistance. Bariatric surgery also alleviates insulin resistance partly through mechanisms conferred via the gut microbiome(425). Another important point is that changes in the gut microbiota may also play a direct role in the reduction of adiposity after bariatric surgery(68) or *vice versa*, and with or without influence on glucose homeostasis. As in the case of this cohort, downstream urinary metabolites of microbiome origin only correlated with BMI and not HbA_{1c}. Ultimately, the precise underlying mechanisms contributing to these clinical benefits are yet to be fully identified.

Understanding these mechanistic pathways will be important for allow for a more personalised surgical approach or the development of gut microbial or dietary interventions.

4.6 Conclusion

In conclusion, this chapter has identified established serum amino acid markers related to T2D, investigated the immediate effects of bariatric surgery on these metabolites and identified amino acid and novel amino acid ratio signatures of T2D resolution.

The glutamine to glutamate and alanine to serine ratios both highlight and represent pathophysiological pathways which lead to T2D. Improvement in glycaemic control after surgery corresponds strongly to a change with these markers and raises the question of whether other non-surgical interventions targeting these pathways may have possible metabolic benefits. The association of these amino acid ratios to surgical T2D resolution are previously unreported.

Chapter 5: The Tryptophan Pathway

Sections of this chapter are adapted from “The impact of bariatric surgery on serum tryptophan – kynurenine pathway metabolites: KTD Yeung, N Penney, L Whiley, H Ashrafian, M Lewis, S Purkayastha, A Darzi & E Holmes”, published in Scientific Reports 12, 294, 2022 (427).

5.1 The Tryptophan Pathway, Inflammation and Type 2 Diabetes

5.1.1 Introduction

Pro-inflammatory factors are secreted by immune cells that accumulate within excess visceral adipose tissue(6). This contributes to a state of chronic low-grade systemic inflammation in patients with obesity. Chronic systemic inflammation is reflected in an individual's metabolic phenotype (188, 428) and metabolites from the tryptophan-kynurenine pathway are thought to be potentially mechanistic in systemic pathways that contribute to clinical insulin resistance(428).

Tryptophan (TRP) is an essential amino acid obtained from the diet and is found in common protein food sources including milk, meat, fish and eggs. Tryptophan is an important substrate for endogenous protein synthesis (429) but its catabolic products which include kynurenines, serotonin and indoles are bioactive and exert effects systemically on the gut, brain(430-432), central nervous system, gut microbiome(433) and has regulatory roles in systemic inflammation (428, 434). Tryptophan and its downstream products of metabolism are generally excreted in urine(435).

The precise pathway dynamics remain elusive but dysregulation of this pathway has been implicated in a wide range of conditions including, obesity, metabolic syndrome (428, 436), type 2 diabetes(437, 438), irritable bowel syndrome(439), inflammatory bowel disease(440) and neurological conditions such Alzheimer's disease (441, 442), schizophrenia(443), depression(433, 444) and multiple sclerosis(445).

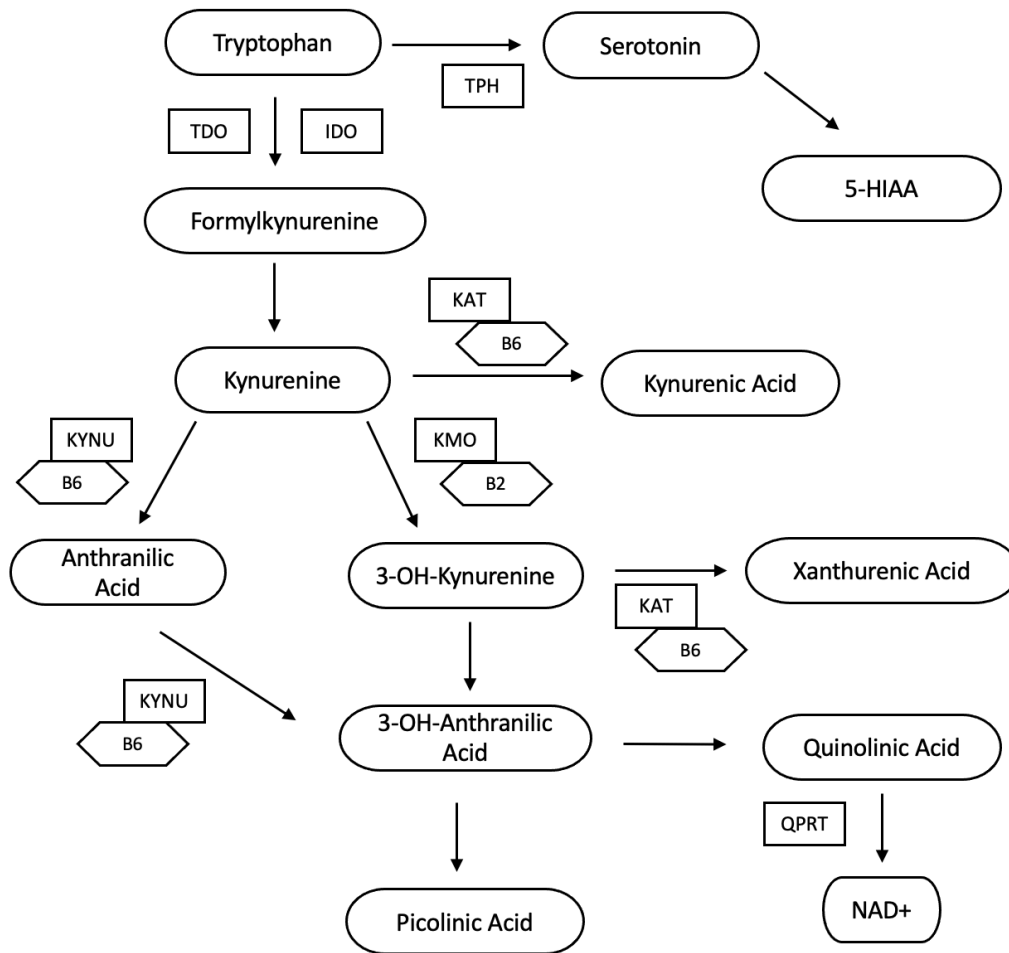


FIGURE 5-1 TRYPTOPHAN METABOLISM VIA KYNURENINE AND SEROTONIN METABOLIC PATHWAYS

KEY: TPH – TRYPTOPHAN HYDROXYLASE; TDO – TRYPTOPHAN 2,3-DIOXYGENASE; IDO- INDOLEAMINE 2,3-DIOXYGENASE; KAT- KYNURENINE AMINOTRANSAMINASE; KMO – KYNURENINE 3 MONOOXYGENASE; KYNU – KYNURENINASE, PLP (VIT B6 – PYRIDOXAL 5—PHOSPHATE; FAD – B2 – FLAVIN ADENINE DINUCLEOTIDE; 5-HIAA – 5-HYDROXYINDOLEACETIC ACID; NAD – NICOTINAMIDE ADENINE DINUCLEOTIDE; QPRT- QUINOLINATE PHOSPHORIBOSYL TRANSFERASE. ADAPTED WITH PERMISSION UNDER OPEN ACCESS CREATIVE COMMONS LICENSE(427)

95% of TRP absorbed is metabolised to kynurenine (KYN) by oxidation in the first step down the kynurenine pathway (see *figure 5-1*) within the liver by the enzyme tryptophan 2,3-dioxygenase (TDO) or in extrahepatic tissues by the enzyme indoleamine 2,3-dioxygenase (IDO) (446). Other minor routes of TRP catabolism by hydroxylation produces serotonin, decarboxylation produces tryptamine and transamination produces indolepyruvic acid (446).

Under normal physiological conditions within the kynurenine pathway, IDO activity is minimal. In those with chronic obesity, pro-inflammatory macrophages found within adipose tissue(6) secrete inflammatory cytokines such as IFN- γ (447) that preferentially induces and significantly enhances IDO action(448), which in turn, alters tryptophan availability. This association drives the interest between obesity, systemic inflammation and the TRP – KYN pathway.

The kynurenine tryptophan ratio (KTR) is used by some as a measure of IDO activation and a marker of cellular immune activation (449) but other factors such as substrate availability and cytokine interactions can also affect this metric (450). Higher levels of cellular immune activation have been associated with systemic insulin resistance (451).

KYN is then metabolised via hydroxylation to 3-hydroxy-kynurenine (3OH-KYN) within adipose tissue by the enzyme kynurenine 3-monooxygenase (KMO)(452). KMO is not present in primary adipocytes. Following on, 3OH-KYN is metabolised by different divisions of this pathway leading to production of kynurenic acid (KYNA), xanthurenic acid (XA), picolinic acid (PA) or quinolinic acid (QA) & nicotinamide adenine dinucleotide (NAD⁺). Vitamin B2 and B6 have important cofactor roles and a pathway shift towards over-production of XA has been linked to the reduced production, release and systemic effects of insulin (451).

5.2 Chapter Aims and Experimental Overview

This chapter first describes the analytical adoption of a targeted LC-MS method for use in urine to measure metabolites of the TRP-KYN pathway. I then evaluated whether the use of urine could be an appropriate biofluid surrogate in place of blood serum or plasma for use in studying the tryptophan pathway.

As bariatric surgery leads to rapid and sustained resolution of obesity and related co-morbidities, I subsequently used univariate statistics and correlation analysis to explore the powerful metabolic effects of bariatric surgery on serum and urine tryptophan-kynurenine metabolites with the aim of providing insights on the link between the pathway, T2D and chronic obesity induced inflammation.

5.3 Methods

5.3.1 Serum

The research group previously published a targeted ultrahigh-performance liquid chromatography tandem mass spectrometry with electrospray ionization (UHPLC-ESI-MS/MS) method to quantify metabolites linked to tryptophan metabolism and gut health(228). This had been extensively validated for use in blood serum and plasma and was used to generate the data presented below in collaboration with the National Phenome Centre, funded by an Imperial College Healthcare NHS Trust – Biomedical Research Centre Surgery & Surgical Technology Pilot Project 2019-2020 Grant of £10,000 awarded to KTDY.

TRP, KYN, 3OH-KYN, PA, QA, KYNA and XA were measured and quantified. Serotonin was measured but not quantified, however its downstream metabolite 5-hydroxyindole acetic acid (5-HIAA) was quantified. Neopterin is produced by IFN- γ stimulated macrophages and was measured and quantified as a marker of cellular inflammation. Citrulline was measured and quantified as a marker of gut health related to enterocyte mass reduction(453). Indole-3-acetic acid (I3AA) is a gut microbiota-derived metabolite from dietary TRP(454) and was measured and quantified as an arbitrary marker of microbiome influence on the indole pathway.

5.3.2 *Urine*

Profiling of urinary tryptophan metabolites was performed by Dr Luke Whiley adapting an established UHPLC-ESI-MS/MS protocol(228) designed for human serum and plasma samples as part of a yet to be published internal method development study. The subsequent raw UHPLC-ESI-MS/MS spectral data were processed by KTDY using the targetlynx application package found within masslynx (v4.1), Waters Corporation (MA, USA). The adaption and measurement of urinary TRP-KYN metabolites are described below. TRP, KYN, 3OH-KYN, PA, QA, KYNA, XA, I3AA, 5-HIAA, 3HAA, Citrulline (CIT), Neopterin, Dopamine, Serotonin, Nicotinic Riboside (NR) and Beta-nicotinamide mononucleotide (NMN) were measured and quantified in urine. 24-hour urine volumes were recorded on the day of collection. Osmolality was measured using freezing point depression osmometer-OsmoTECH Single-Sample Micro-Osmometer, Advanced Instruments (MA, USA). Creatinine was measured using an Alinity c Creatinine Reagent Kit, Abbott Diagnostics (IL, USA). Both osmolality and creatinine values were measured at Imperial College Healthcare NHS clinical biochemistry laboratories.

5.4 Development of Urine Profiling Method

5.4.1 Background

The collection of blood or serum samples is not always easy or practical. A trained phlebotomist is required to collect the sample in a designated clinical space and the availability of a clinical laboratory is then required to process the sample. Patients may find blood tests invasive or inconvenient if not timed with their clinical visits and concerns such as needle phobia may further lessen the desirability of blood draws. Studies operating to limited time and budget may find a significant reduction of samples collected, leading to a reduction in statistical power.

When compared to blood derived samples, collection of urine is straight forward, non-invasive and can be performed acceptably in a home environment. Subsequent steps of storage and preparation are relatively simple and thus urine is a common biofluid used in metabolic profiling and biomarker discovery studies. The drawback with urine is that there is significant natural variability in metabolite concentrations depending on the hydration status and renal function of the participant. This has implications for the analysis and biological interpretation of data generated from urine profiling.

Many have reported various methods of normalisation each with their own drawbacks and there is yet to be an agreed 'gold standard' (455-458). Normalisation methods chosen may also depend on the expertise or measured parameters available to investigators. Normalisation may occur prior or after acquisition of data using measured parameters including osmolality, creatinine, total urine volume, total signal intensity or dry mass. Advanced mathematical computational methods such as creatine ratio, quantile normalisation, probabilistic quotient, median fold change or weight scatter plot smoothing have also been proposed (236, 459).

With the points above in mind, I evaluated whether urine collected over 24 hours could be adopted as an appropriate biofluid surrogate in place of blood serum or plasma for use in the study of tryptophan pathways metabolites. I also evaluated the effect of using 24-hour urine volumes, creatinine and osmolality as a normalisation factor of results.

5.4.2 Comparison of Normalisation Methods

Ten 24-hour pre- and 3-month post-operative paired samples were obtained from participants who did not have T2D or chronic kidney disease (one male, nine females, age 27-56, mean = 42.7). Bariatric surgery led to a significant reduction in BMI (kg/m^2) at 3 months post-operative (47.85 ± 9.05 , 39.09 ± 8.14 , $p=0.035$). Osmolality and creatinine values showed strong positive correlation ($r=0.941$, $p<0.001$) and these values showed a negative correlation with total urine volume ($r=-0.671$, $p<0.01$; $r=-0.822$, $p<0.001$ respectively). There were no statistical differences between the pre- and post-operative groups for all parameters, see *figure 5-2*.

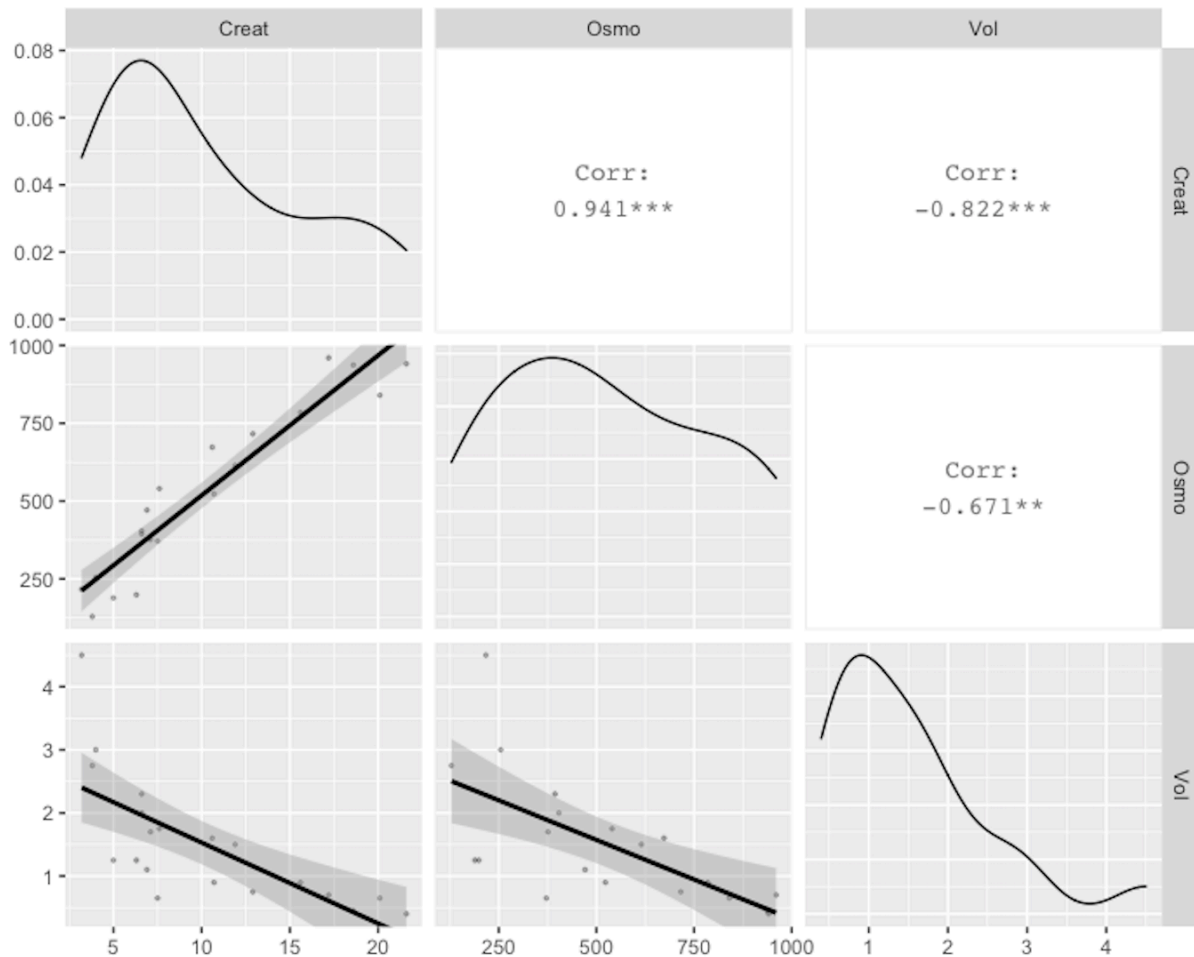


FIGURE 5-2 URINE CORRELATION RELATIONSHIP

CORRELATION RELATIONSHIP AND SPEARMAN'S CORRELATION COEFFICIENTS BETWEEN VALUES OBTAINED FOR OSMOLALITY (OSMO/KG), CREATININE (MMOL/L), AND TOTAL URINE VOLUME (L).

The concentration values of measured metabolites were normalised by dividing metabolite (ng/ml) by creatinine (mmol/L) or osmolality (osmo/kg) or volume (L). OPLS-DA using 1 aligned + 1 orthogonal components were performed and compared to raw and normalised data, the respective cross validated models, coefficient of variation plots along with R^2X and Q^2Y values are displayed in *figures 5-3 & 5-4*.

In summary, the models showed good separation between the pre-operative and post-operative groups, demonstrating the effect of surgery on these metabolites. Normalisation using osmolality ($R^2X = 0.856$, $Q^2Y = 0.583$) or creatinine ($R^2X = 0.872$, $Q^2Y = 0.667$) improved the models of separation when compared to raw data ($R^2X = 0.756$, $Q^2Y = 0.533$). Normalisation using total urine volume produced a non-predictive model ($R^2X = 0.0354$, $Q^2Y = 0.099$).

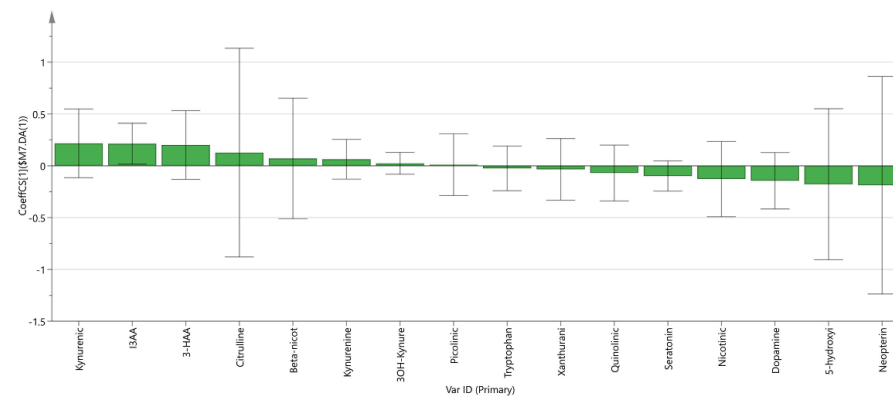
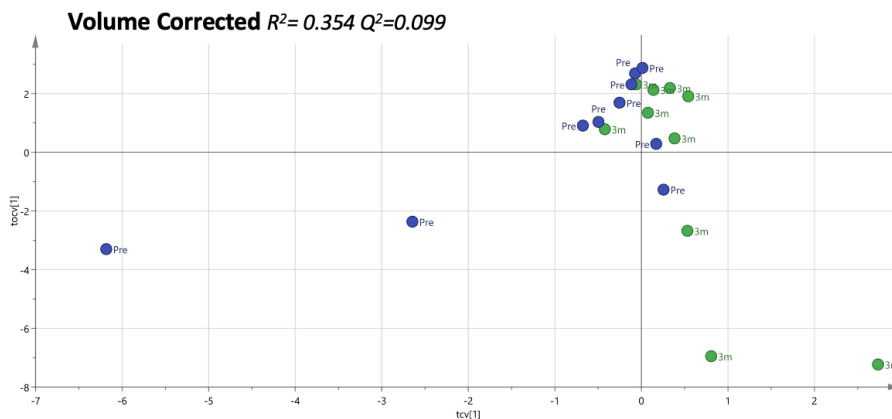
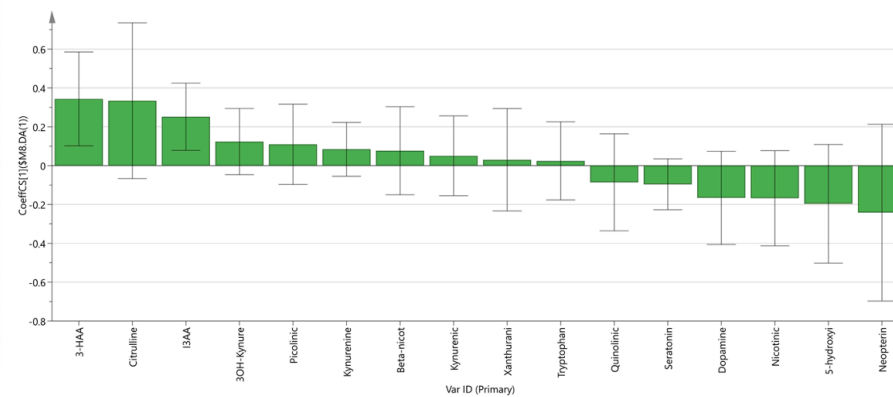
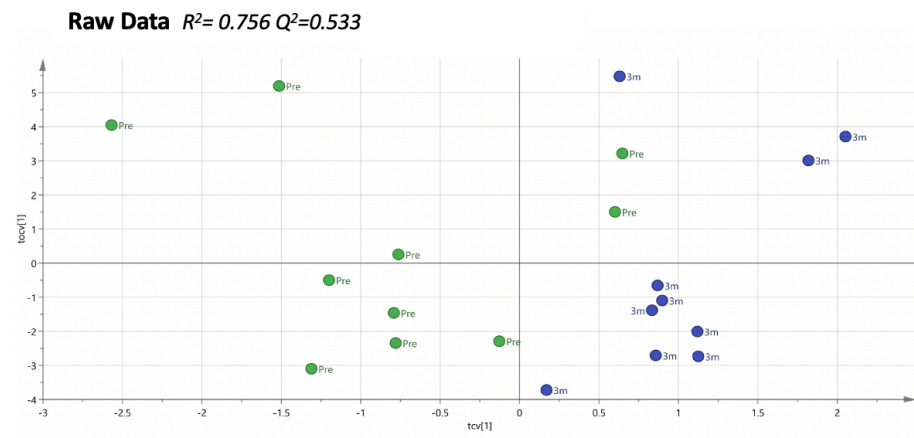


FIGURE 5-3 CROSS VALIDATED OPLS-DA MODELS

R^2 X AND Q^2 Y VALUES DISPLAYED WITH RESPECTIVE NORMALISED DATASET CROSS VALIDATED OPLS-DA MODELS WITH 1 ALIGNED +1 ORTHOGONAL COMPONENTS AND COEFFICIENT OF VARIATION PLOT.

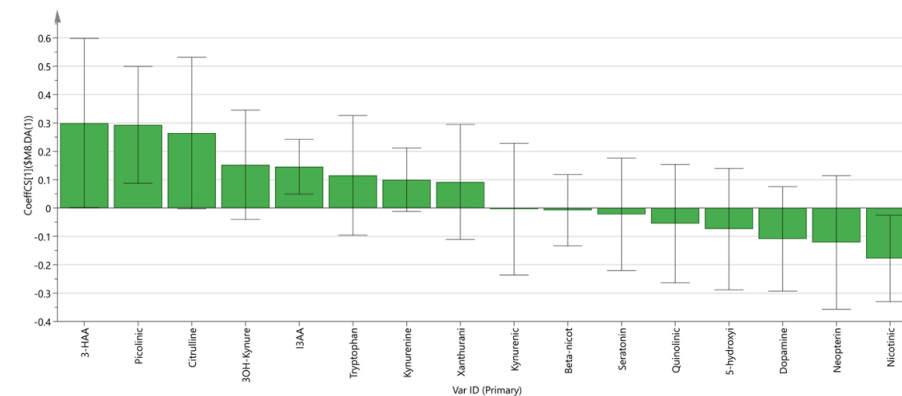
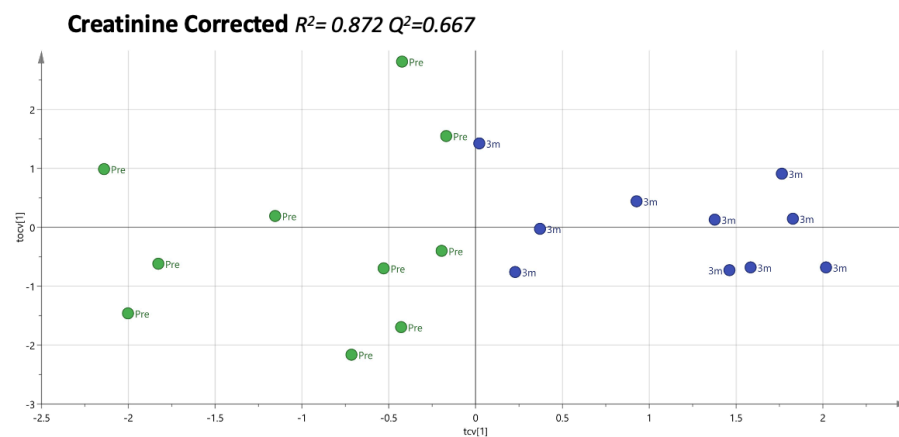
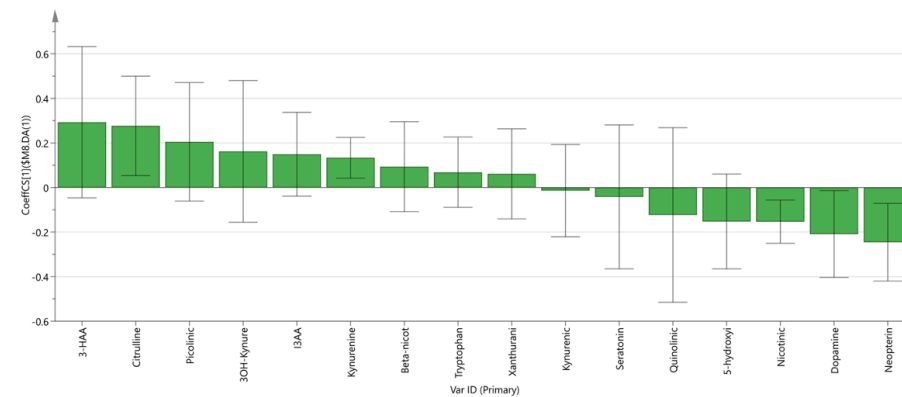
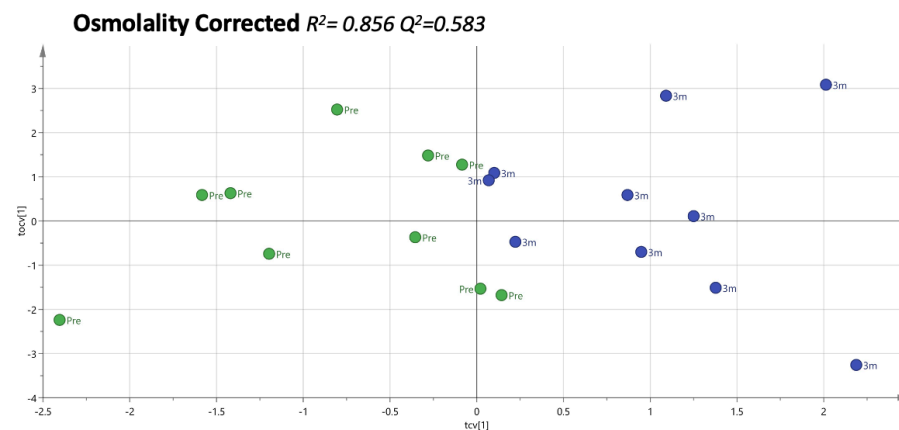


FIGURE 5-4 CROSS VALIDATED OPLS-DA MODELS

R^2X AND Q^2Y VALUES DISPLAYED WITH RESPECTIVE NORMALISED DATASET CROSS VALIDATED OPLS-DA MODELS WITH 1 ALIGNED +1 ORTHOGONAL COMPONENTS AND COEFFICIENT OF VARIATION PLOT.

5.4.3 Serum versus Urine Correlation Analysis

This analysis included 46 matched urine and serum samples collected from 20 participants. (20 pre-operative, 20 three-month post-operative and six one-year post-operative): ten were T2D, none had chronic kidney disease (five male, ten females, age 28-66, mean = 47.05). All available quantified metabolites were used to assess for correlation between serum and urine metabolite concentrations.

KYN (R=0.52-0.61, q=0.0184), 3OH-KYN (R= 0.49-0.55, q=0.0184) and KYNA (R= 0.43-0.7, q=0.0017) were found to display strong and significant correlation between serum and 24-hour urine levels utilising osmolality as a normalisation factor. In addition, of these metabolites, TRP (R=0.52, q=0.0053) and citrulline (R= 0.47, q=0.0135) displayed significant positive correlation when values were adjusted using creatinine (*table 1-2*). Normalisation using total urine volume led to no significant correlation relationships. The respective scatter plots are displayed in *figure 5-5*.

Urine Normalisation	Raw Data		Osmolality		Creatinine		Total Volume	
Metabolite	R	q value	R	q value	R	q value	R	q value
Picolinic Acid	0.09	0.9620	0.02	0.9620	0.08	0.8663	0.07	0.9226
Serotonin	-0.23	0.9620	-0.04	0.9620	-0.02	0.9698	-0.43	0.4368
Quinolinic Acid	0.34	0.8272	0.1	0.8272	0.34	0.1604	0.14	0.7778
Citrulline	0.41	0.0666	0.42	0.0666	0.47	0.0135	0.3	0.4368
Indole-3-acetic-acid	0.24	0.7363	0.15	0.7363	0.09	0.7981	0.31	0.4368
Kynurenic Acid	0.43	0.0017	0.61	0.0017	0.7	1.3E-05	0.15	0.7778
5-hydroxyindole-acetic-acid	0.06	0.8109	0.11	0.8109	0.28	0.2838	-0.12	0.7891
Xanthurenic Acid	-0.01	0.7911	0.17	0.7911	0.16	0.7869	-0.17	0.7778
Kynurenine	0.53	0.0184	0.52	0.0184	0.61	0.0016	0.22	0.7778
3OH-Kynurenine	0.55	0.0184	0.49	0.0184	0.58	0.0016	0.14	0.7778
Neopterin	0.46	0.3530	0.26	0.3530	0.29	0.2672	0.39	0.4368
Tryptophan	0.41	0.0972	0.26	0.0972	0.52	0.0053	0.34	0.4368

TABLE 5-1 URINE V SERUM TRYPTOPHAN CORRELATION

SPEARMAN'S' CORRELATION CO-EFFICIENT AND BH ADJUSTED Q VALUES COMPARING MEASURED 24-HOUR URINE AND SERUM LEVELS OF MEASURED METABOLITES WITH DISPLAYED NORMALISATION METHOD. RED VALUES INDICATE A SIGNIFICANT CORRELATION

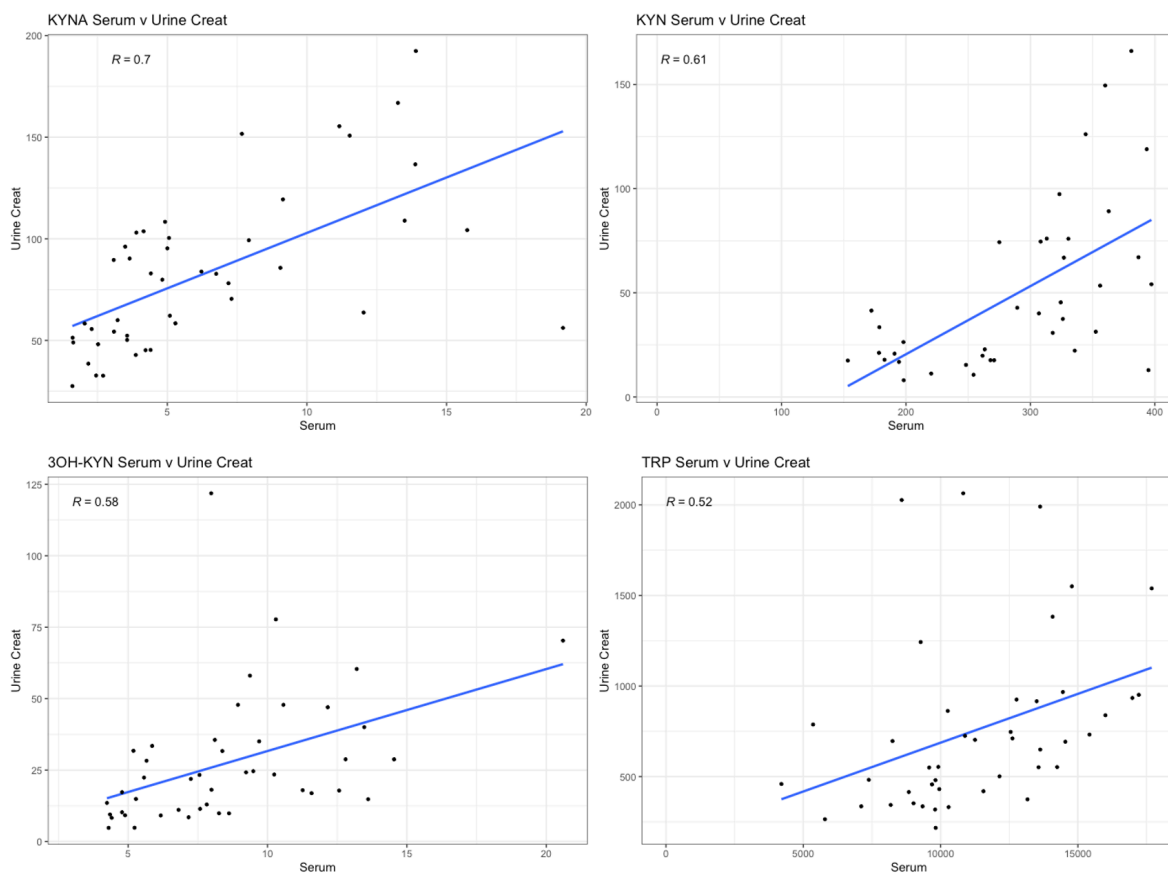


FIGURE 5-5 SCATTER PLOTS DEMONSTRATING SPEARMAN'S CORRELATION RELATIONSHIP BETWEEN SERUM AND 24-HOUR URINE CONCENTRATIONS NORMALISED USING CREATINE OF KYNURENIC ACID (TOP LEFT), KYNURENINE (TOP RIGHT), 3OH-KYNURENINE (BOTTOM LEFT) AND TRYPTOPHAN (BOTTOM RIGHT)

5.3.4 *Urine Method Discussion*

To assess the application of a urine based UHPLC-ESI-MS/MS tryptophan pathway assay, I applied the assay to samples collected from patients with obesity before and 3-months after bariatric surgery. This cohort was seen as an ideal exemplar application as in those with obesity, pro-inflammatory cytokines secreted by immune cells found in excess visceral adipose tissue leads to activation of the key TRP-KYN pathway enzyme IDO. This is postulated to be one of the mechanisms leading to dysregulation of the pathway. It is also hypothesised that one of the mechanisms by which bariatric surgery confers its benefits, is modulation of this pathway.

When compared to raw data, normalisation with creatinine or osmolality improved the predictive models in this example. However, a significant number of factors can affect the findings of urinary metabolomic studies (460, 461). Some have reported normalisation with creatinine to be not reliable (461), while others found it to improve models even when applied to patients with acute kidney injury (462). Creatinine values can also vary depending on the participant's gender, race, age, diet and muscle mass.

Normalisation with osmolality has also been reported to lead to improved statistical separation (463). Others have demonstrated that there are no significant differences when normalising samples to either creatinine or osmolality on overall diagnostic accuracy or biomarker identification (457, 464). A degree of this, may be explained by the effect size of the disease process on metabolites investigated. Nevertheless, care must be taken when designing urinary metabolomic studies. Adoption of any method needs to take into account the characteristics of study participants and the metabolic pathways linked to the pathological process being investigated.

The results demonstrate that using measurements of both creatinine and osmolality but not total urine volume as a post-acquisition normalisation factor improved the resultant statical OPLS-DA models of separation when compared to raw data. When comparing both models, normalisation led to minor changes to coefficient of variation values but the differentiator metabolites (3HAA, PA, Citrulline & 3OH-KYN) that contributed most

significantly to model separation did not change. This signifies that the use of osmolality and creatinine for normalisation can be straightforward. While it must be acknowledged that this may not be the most precise method of normalisation, it is practical and adequate for any first line screening or preliminary discovery studies for investigators wishing to investigate tryptophan metabolites in clinical studies.

The feasibility of using TRP/ KYN pathway metabolites measured in 24-hour urine as surrogates to its corresponding serum metabolites was also examined. Most metabolomic study designs aspire to produce longitudinal multi-biofluid profiles. However, investigators are often limited by budget, time, and physical barriers to collecting samples in order to perform full scale studies. Collection of urine is easy and non-invasive which improves the likelihood of collecting a higher number of samples within a short space of time.

5.3.5 *Conclusion*

TRP and its downstream metabolites of KYN, 3OH-KYN and KYNA measured in 24-hour urine and subsequently normalised using creatinine measurements displays strong positive correlation with their respective serum values indicating urine's suitability when used as a surrogate biofluid. This would be an appropriate consideration when designing screening or preliminary discovery metabolomic studies of the pathway. To my knowledge, this is the first demonstration of this relationship.

5.5 Results

5.5.1 Serum Tryptophan Kynurenine Metabolites

Baseline Characteristics

Twenty paired samples were included in this analysis. Half the patients were T2D, eleven and nine patients underwent RYGB and VSG respectively. The baseline demographics divided into subgroups by T2D diagnosis are displayed in *table 5-2*.

Baseline Demographics	T2D	Non-Diabetes
n	10	10
Sex, n (M:F)	4:6	1:9
Age (Years)	51.7±6.29	42.20±9.59
Weight (Kg)	130.40±22.00	131.60±21.83
BMI (kg/m²)	48.14±7.60	49.85±7.66
HbA_{1c} (mmols/mol)	65.10±16.41	37.60±2.95
Metformin, n (%)	9 (90)	0 (0)
Insulin, n (%)	2 (20)	0 (0)

all listed data are means ± standard deviation unless stated otherwise

TABLE 5-2 BASELINE DEMOGRAPHICS

Except for serum tryptophan ($p=0.005$) and xanthurenic levels ($p=0.015$) levels, there were no differences in the metabolites measured in those with and without T2D at baseline. However, these were not significant after adjusting for multiple testing ($q=0.10$, $q=0.14$ respectively). Boxplots comparing these metabolites in those with and without T2D are displayed below in *figure 5-6*.

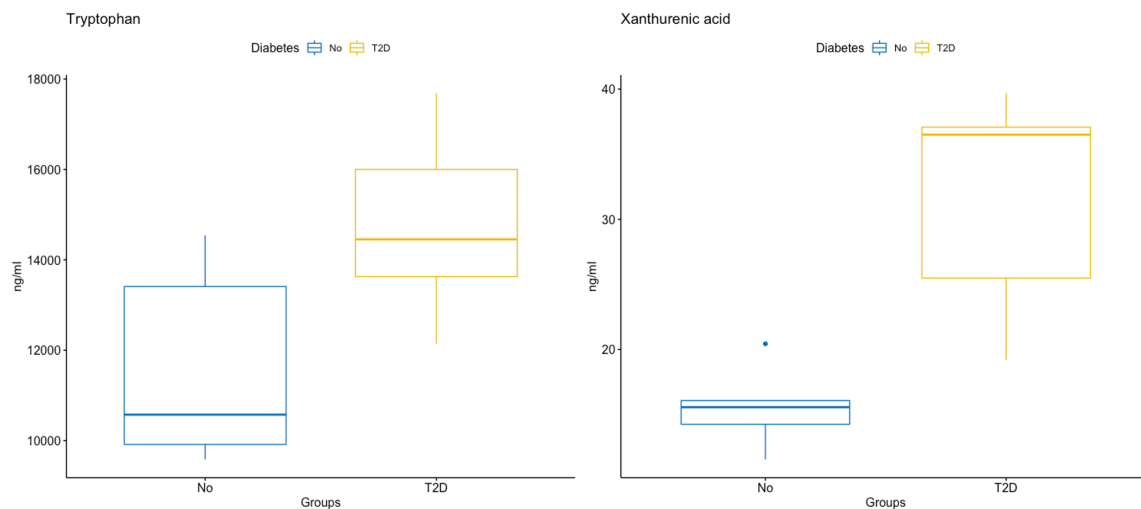


FIGURE 5-6 BOXPLOTS DISPLAYING BASELINE TRYPTOPHAN AND XANTHURENIC ACID LEVELS IN THOSE WITH (YELLOW) AND WITHOUT T2D (BLUE)

Pre-Operative and 3-month Post-Operative Demographic Comparisons

The study cohort was then separated into four subgroups for analysis: those with and without T2D at baseline, and those stratified by subsequent surgical procedure (RYGB or VSG). There were no significant statistical differences in baseline demographics between the subgroups except for an expected higher HbA1c in those with T2D. The effect of surgery on each subgroup is displayed below in *table 5-3*.

Subgroups	RYGB		VSG		T2D		Non-Diabetes	
	Pre-Op	Post-Op	Pre-Op	Post-Op	Pre-Op	Post-Op	Pre-Op	Post-Op
n	11		9		10		10	
Sex, n (M:F)	1:10		4:5		4:6		1:9	
Age (Years)	48.27±9.65		45.56±9.00		51.7±6.29		42.20±9.59	
Weight (Kg)	124.73±18.80	98.03±14.04*	138.67±22.78	114.80±20.63*	130.40±22.00	104.06±17.45*	131.60±21.83	107.06±21.02*
BMI (kg/m²)	48.32±6.12	38.03±4.88*	49.81±9.20	41.19±7.99	48.14±7.60	38.43±5.97*	49.85±7.66	40.48±7.12*
T2D, n (%)	7 (64)		3 (33)					
Metformin, n (%)	6 (55)	4 (36)	3 (33)	3 (33)	9 (90)	7 (70)	0 (0)	0 (0)
Insulin, n (%)	2 (18)	1 (9)	0 (0)	0 (0)	2 (20)	1 (10)	0 (0)	0 (0)
Hba1c (mmols/mol)	51±17.78	38.09±7.73*	51.78±19.75	39.00±7.52	65.10±16.41†	43.20±7.57*	37.60±2.95	33.80±3.26*

†/significant difference between baseline demographics

* $p < 0.05$ pre- and post-op comparison

TABLE 5-3 PRE-OP V POST-OP DEMOGRAPHICS DISPLAYED BY OPERATIVE AND T2D SUBGROUPS

Significant weight and BMI reductions were observed across subgroups at 3 months. HbA1c reduction was also significant in RYGB, T2D and ND but not VSG subgroups.

Pre-Operative and 3-month Post-Operative Metabolite Comparisons – Surgical and T2D Subgroups

	RYGB				VSG			
	Pre-Op	Post-Op	p value	q value	Pre-Op	Post Op	p value	q value
Picolinic Acid	7.39±3.93	5.98±1.51	0.933	0.933	5.32±1.28	6.58±1.73	0.181	0.699
3 Hydroxyanthranilic Acid	15.67±8.77	14.79±0.59	0.533	0.747	15.31±3.1	13.32±NA	0.857	0.923
Quinolinic Acid	67.49±15.35	53.95±24.84	0.043	0.121	68.44±20.14	64.55±17.87	0.605	0.804
Citrulline	3548.68±1608.63	2790.11±840.02	0.217	0.434	4264.1±1337.04	3296.14±880.96	0.113	0.699
Indole 3 Acetic Acid	319.93±269.57	425.13±350.29	0.401	0.624	264.97±154.65	361.19±268.67	0.666	0.804
Kynurenic Acid	7.06±4.75	3.34±1.87	0.010	0.048	8.89±4.48	7.5±4.83	0.596	0.804
5-hydroxyindole acetic acid	9.53±5.06	8.33±2.49	0.847	0.912	8.09±3.82	6.36±1.49	0.340	0.794
Xanthurenic Acid	24.06±10.53	7.33±4.78	0.001	0.017	22.47±12.68	16.19±8.23	0.267	0.747
Kynurenine	317.55±69.72	248.94±77.3	0.065	0.152	317.64±73.11	297.82±65.44	0.606	0.804
3OH Kynurenine	10.56±4.35	6.81±2.65	0.023	0.082	10.13±3.13	7.35±2.03	0.063	0.699
Neopterin	2.67±1.59	2.91±1.93	0.844	0.912	2.35±0.94	2.44±0.65	0.489	0.804
Tryptophan	13242.5±2443.76	8711.05±3482.81	0.002	0.017	12892.19±2741.4	11291.87±2413.88	0.200	0.699
Kynurenine/Tryptophan Ratio	0.025±0.006	0.026±0.007	0.842	0.912	0.024±0.004	0.026±0.004	0.689	0.804

Units of all of metabolites displayed - ng/ml

KTR expressed as a ratio

Red indicates statistical significance after controlling for FDR

TABLE 5-4 PRE-OP V POST-OP VALUES OF MEASURED TRYPTOPHAN METABOLITES DISPLAYED BY OPERATIVE SUBGROUP

	T2D				ND			
	Pre-Op	Post Op	p value	q value	Pre-Op	Post Op	p value	q value
Picolinic Acid	7.42±3.82	5.83±1.08	0.683	0.796	5.29±1.56	6.68±1.86	0.100	0.325
3 Hydroxyanthranilic Acid	16.19±7.56	14.3±0.94	0.937	0.937	13.85±4.39	NA±NA	1.000	1.000
Quinolinic Acid	66.57±11.77	58.16±25.84	0.113	0.318	69.18±21.68	59.7±19.11	0.353	0.675
Citrulline	3479.83±1516.31	2950.59±918.29	0.353	0.634	4261.41±1451.63	3085.06±872.6	0.063	0.301
Indole 3 Acetic Acid	318.45±276.86	350.77±238.45	0.579	0.737	271.94±160.66	441.95±375.87	0.529	0.820
Kynurenic Acid	8.22±4.91	4.38±3.79	0.011	0.054	7.54±4.51	6.04±4.27	0.579	0.820
5-hydroxyindole acetic acid	9.98±4.92	8.28±2.32	0.571	0.737	7.78±3.95	6.62±2.02	0.631	0.820
Xanthurenic Acid	31.59±8.81	8.19±5.33	0.004	0.030	15.58±3.23	13.65±8.73	0.364	0.675
Kynurenine	330.23±49.48	282.51±79.95	0.370	0.634	304.95±85.57	262.71±71.78	0.274	0.675
3OH Kynurenine	11±3.98	7.33±2.85	0.029	0.101	9.74±3.63	6.78±1.83	0.070	0.301
Neopterin	2.61±1.72	3.03±2.01	0.739	0.796	2.45±0.84	2.37±0.57	0.796	0.862
Tryptophan	14794.55±1848.93	9922.06±3971.6	0.003	0.030	11565.41±2004.27	9822.78±2557.61	0.052	0.301
Kynurenine/Tryptophan Ratio	0.023±0.004	0.026±0.006	0.408	0.634	0.027±0.006	0.027±0.006	0.762	0.862

Units of all of metabolites displayed - ng/ml

KTR expressed as a ratio

Red indicates statistical significance after controlling for FDR

TABLE 5-5 PRE-OP V POST-OP VALUES OF MEASURED TRYPTOPHAN METABOLITES DISPLAYED BY T2D SUBGROUP

Reductions of TRP-KYN pathway metabolites were observed at 3 months post-operative. There was a significant reduction in serum levels of TRP (p=0.002, q=0.017), kynurenic acid (KYNA) (p=0.010, q=0.048) and XA (p=0.001, q=0.017) in those who underwent RYGB, see *table 5-4 and figure 5-7*. Significant reduction in serum levels of TRP (p=0.003, q=0.03) and XA (p=0.004, q=0.03) were also observed in those

with T2D pre-operatively see *table 5-5 and figure 5-8*. Total TRP and KYN levels decreased at 3 months post-op regardless of subgroup, but no significant change was observed in KTR after surgery.

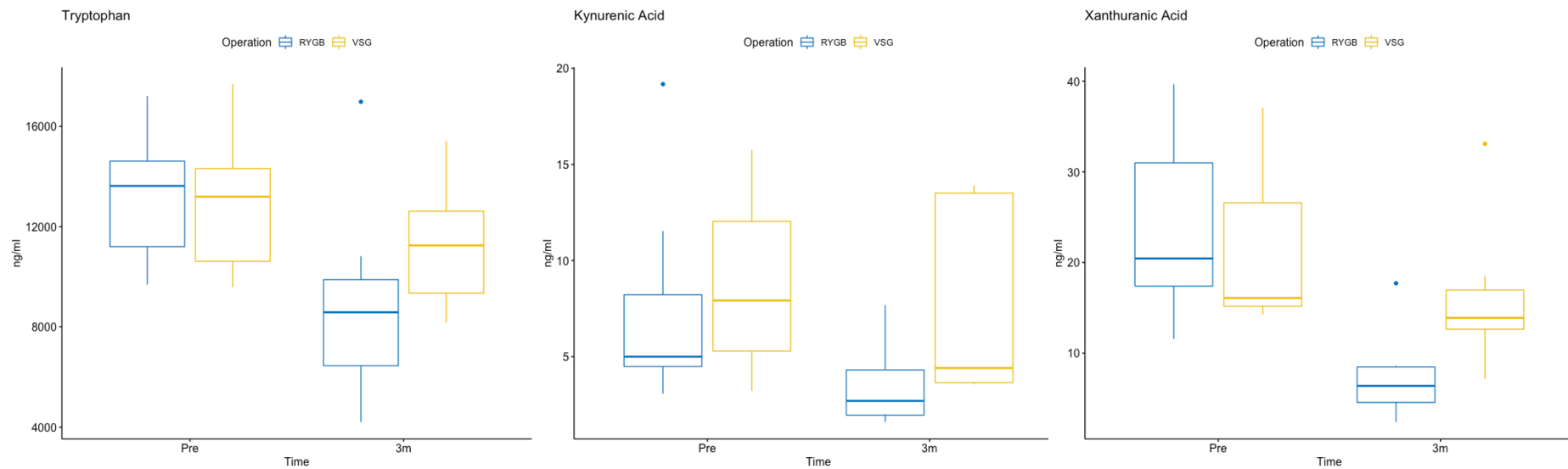


FIGURE 5-7 BOXPLOTS DISPLAYING PRE AND 3-MONTH POST-OPERATIVE LEVELS OF TRYPTOPHAN (LEFT), KYNURENIC ACID (CENTRE) AND XANTHURENIC ACID(RIGHT) COMPARING SURGICAL INTERVENTION SUBGROUPS

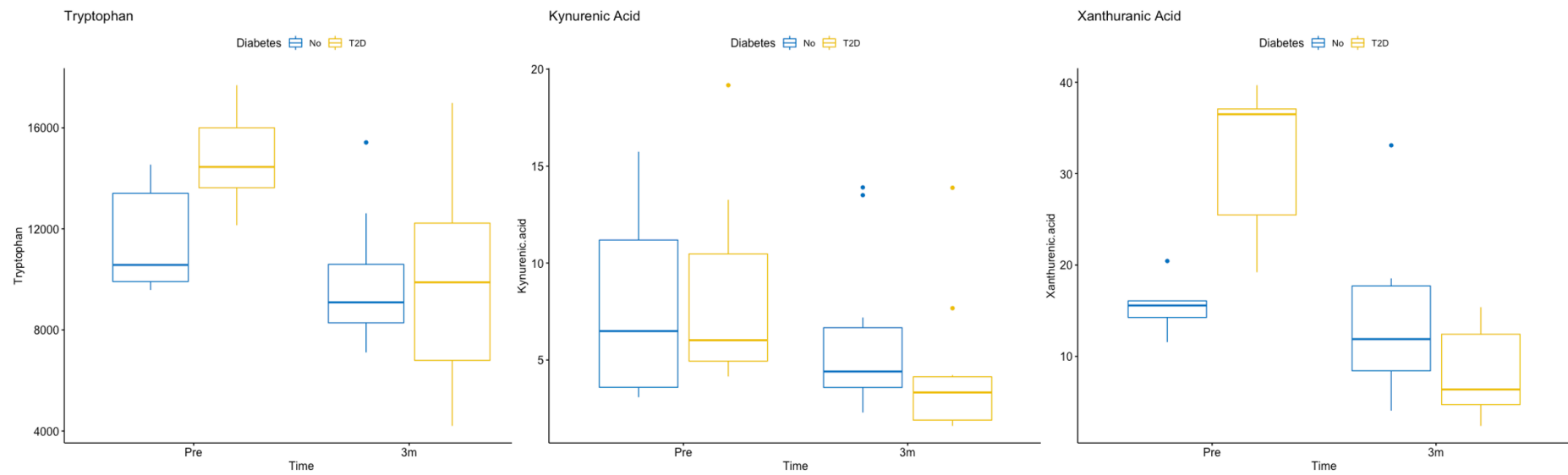


FIGURE 5-8 BOXPLOTS DISPLAYING PRE AND 3-MONTH POST-OPERATIVE LEVELS OF TRYPTOPHAN (LEFT), KYNURENIC ACID (CENTRE) AND XANTHURENIC ACID(RIGHT) COMPARING T2D SUBGROUPS

TRP ($R=0.57$, $p<0.001$), KYNA ($R=0.33$, $p=0.024$) and XA ($R=0.57$, $p=0.001$) were also found to be positively correlated with serum Hba1c levels at respective sample collection timepoints, see *figure 5-9*.

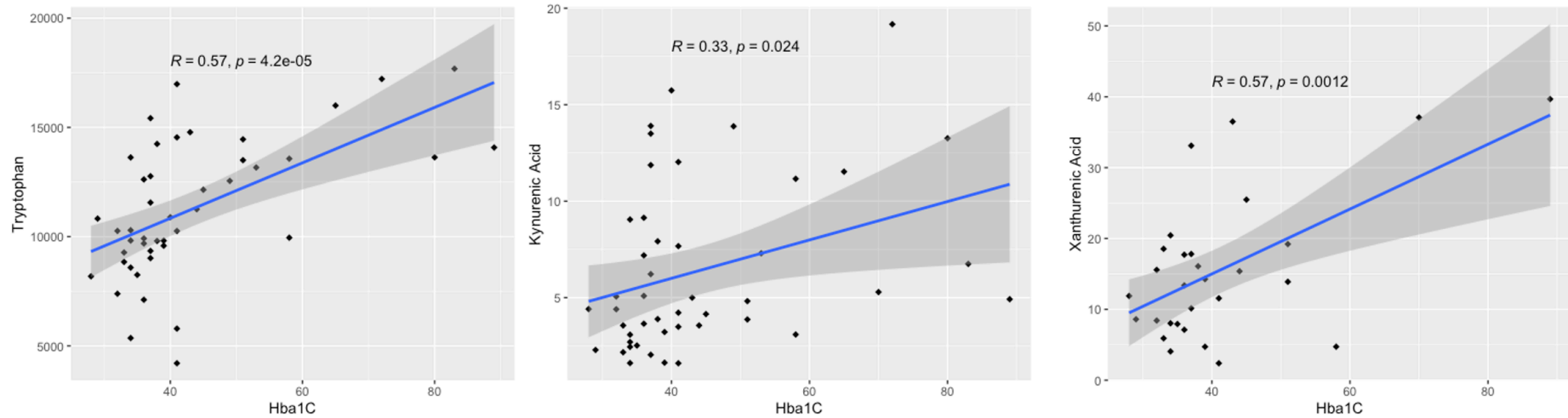


FIGURE 5-9 SCATTER CHARTS DISPLAYING CORRELATION RELATIONSHIP BETWEEN HBA1C AND TRYPTOPHAN (LEFT), KYNURENIC ACID (CENTRE) AND XANTHURENIC ACID (RIGHT)

In order to further examine the effect on surgery on this metabolic pathway, the pre- and post-operative Δ change in concentration of measured metabolites was calculated. The correlation coefficient for each metabolite within the pathway was calculated against the Δ BMI and Δ Hba1c. A correlation heatmap displaying these relationships are displayed below in *figure 5-10*.

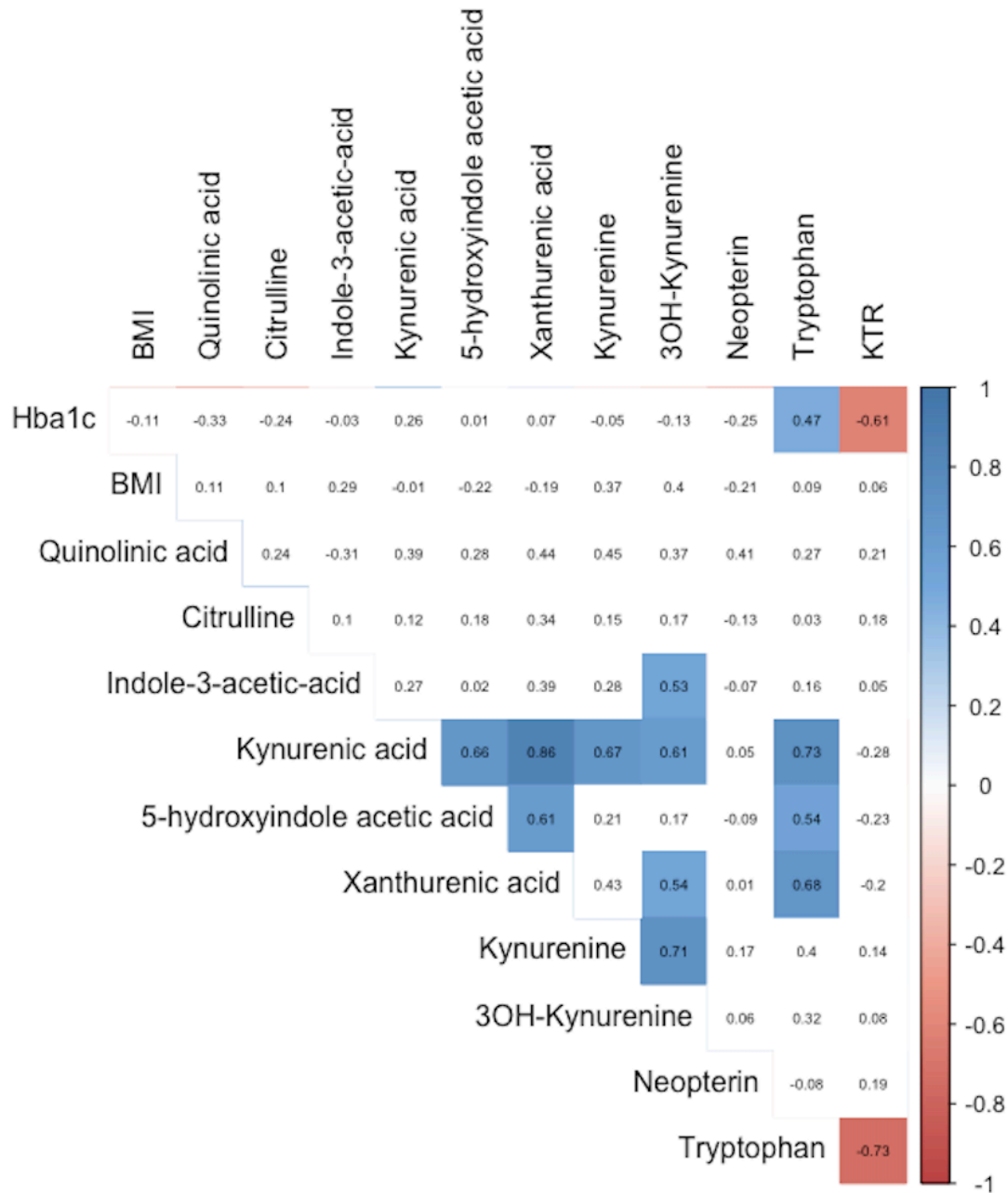


FIGURE 5-10 HEATMAP DISPLAYING SPEARMAN'S RANK CORRELATION (NUMBERS) CO-EFFICIENT BETWEEN Δ OF MEASURED PARAMETERS AND PATHWAY METABOLITES. COLOURED SQUARES INDICATE SIGNIFICANT CORRELATION RELATIONSHIP WITH P VALUE ≤ 0.05

Δ Hba1c was observed to be significantly and positively correlated to Δ Tryptophan ($r=0.57$, $p= 0.003$) and significant negatively correlated with Δ KTR ($r=-0.61$, $p= 0.004$). Changes in

BMI were not found to be significantly correlated with any of the Δ of measured metabolites.

5.5.2 Urine Tryptophan Kynurenine Metabolites

Baseline Characteristics

Based on the earlier methodological analysis (chapter 5.4), 24-hour urinary creatinine levels were used as a normalisation factor for the following analysis. 24 paired samples were available for analysis. Eleven patients were T2D, 11 and 13 patients underwent RYGB and VSG respectively. The baseline demographics divided into subgroups by T2D diagnosis are displayed in *table 5-6*.

Baseline Demographics	T2D	Non-Diabetes
n	11	13
Sex, n (M:F)	5:6	1:12
Age (Years)	50.27±7.61	41.77±9.73*
Weight (Kg)	132.2±21.68	128.69±20.41
BMI (kg/m²)	48.32±7.23	48.22±7.50
HbA_{1c} (mmols/mol)	64.18±15.86	37.38±2.63*
Metformin, n (%)	10	0
Insulin, n (%)	3	0

TABLE 5-6 BASELINE DEMOGRAPHICS

all listed data are means ± standard deviation unless stated otherwise

** p<0.05 subgroup comparison*

Those who were T2D were older in age and as expected had a significantly higher HbA_{1c}. Except for urinary QA (p=0.014) and 5HIAA (p=0.03) levels, there were no significant differences observed in urinary metabolites measured in individuals with and without T2D at baseline. However, these differences presented BH values of over 0.05 when controlling for FDR (q=0.264, q=0.271 respectively), see *figure 5-11*.

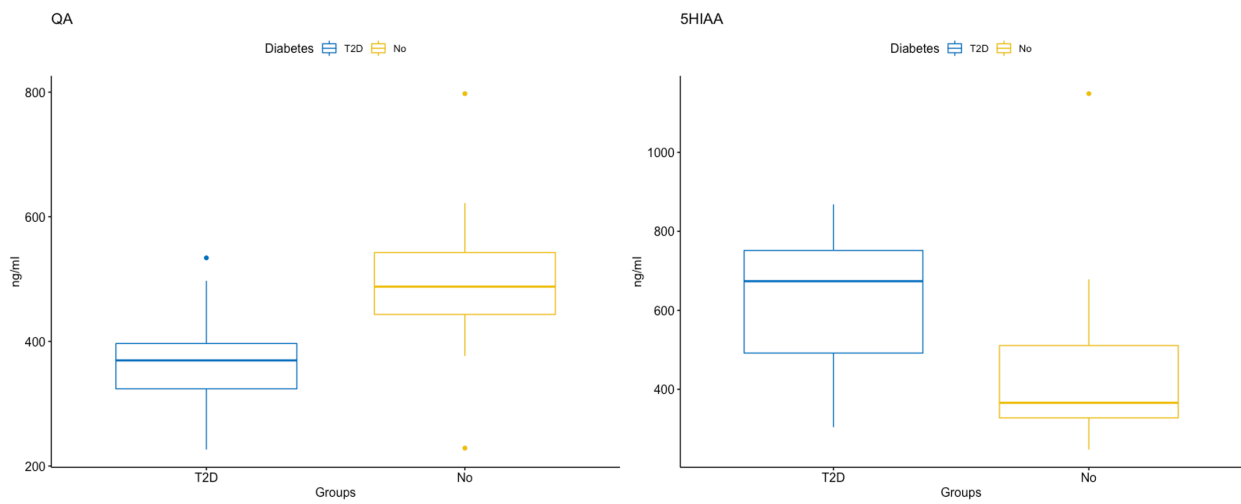


FIGURE 5-11BOXPLOTS DISPLAYING BASELINE QUINOLINIC ACID AND 5-HIAA LEVELS IN THOSE WITH (YELLOW) AND WITHOUT (BLUE) T2D

Pre-Operative and 3-month Post-Operative Comparisons – Demographics

The effect of surgery on the baseline demographics on the T2D and surgical procedure subgroups are displayed in *table 5-7*. Significant weight, BMI and HbA1c reductions were observed across all subgroups at 3 months post-operative.

Subgroups	RYGB		VSG		T2D		Non-Diabetes	
	Pre-Op	Post-Op	Pre-Op	Post-Op	Pre-Op	Post-Op	Pre-Op	Post Op
n	11		13		11		13	
Sex, n (M:F)	1:10		5:8		5:6		1:12	
Age (Years)	48.27±9.65		43.46±9.47		50.27±7.61		41.77±9.73	
Weight (Kg)	124.73±18.8	98.03±14.04*	135±21.62	112.48±19.21*	132.18±21.69	105.41±17.15*	128.69±20.41	106.24±19.79*
BMI (kg/m²)	48.34±6.12	38.03±4.88*	48.21±8.28	40.16±7.21*	48.32±7.23	38.54±5.67*	48.22±7.5	39.73±6.83*
T2D, n (%)	7 (64)		4					
Metformin, n (%)	6 (55)	4 (36)	4 (31)	4 (31)	10 (91)	8 (73)	0 (0)	0 (0)
Insulin, n (%)	2 (18)	2 (18)	1 (8)	0 (0)	3 (27)	2 (18)	0 (0)	0 (0)
Hba1c (mmols/mol)	51±17.78	38.09±7.73*	48.54±17.51	37.15±6.85*	64.18±15.86	42.36±7.7*	37.38±2.63	33.54±3.02*

* $p < 0.05$ pre- and post-op comparison

TABLE 5-7 EFFECT OF SURGERY ON T2D AND SURGICAL SUBGROUPS

Pre-Operative and 3-month Post-Operative Metabolite Comparisons – Surgical and T2D Subgroups

	RYGB				VSG			
	Pre-Op	Post-Op	p value	q value	Pre-Op	Post-Op	p value	q value
Picolinic Acid	16.29±3.98	10.33±3.64	0.003	0.029	14.08±4.43	10.3±4.07	0.014	0.098
Nicotinic Acid	11.88±7.96	8.65±6.95	0.476	0.576	5.16±2.63	4.83±2.69	0.99	0.99
3 Hydroxy Anthranilic Acid	151.81±104.18	83.28±66.9	0.113	0.406	96.15±47.66	44.7±26.48	0.005	0.091
Dopamine	28.04±12.33	30.79±12.67	0.426	0.576	21.76±7.01	19.84±6.8	0.545	0.7
Serotonin	95.42±74.38	74.77±49.97	0.756	0.796	97.04±62.63	96.95±55.31	0.894	0.946
Quinolinic Acid	461.37±150.19	510.69±299.41	0.512	0.576	422.29±121.25	378.6±130.43	0.223	0.445
Citrulline	52.63±34.81	33.45±16.95	0.201	0.518	52.06±34.14	27.91±16.23	0.059	0.192
Indole 3 Acetic Acid	267.7±246.75	212.98±234.86	0.436	0.576	140.39±89.87	107.43±79.7	0.169	0.38
Kynurenic Acid	97.29±25.1	55.19±33.74	0.001	0.014	98.24±41.32	85.78±47.05	0.376	0.677
5-hydroxyindole acetic acid	613.7±251	566.11±198.52	0.512	0.576	461.54±213.43	424.17±124.38	0.801	0.901
Xanthurenic Acid	95.68±61.65	74.65±69.8	0.349	0.576	73.92±32.86	53.48±24.84	0.169	0.38
Kynurenine	80.54±56.79	55.73±38.56	0.247	0.557	58.76±31.03	33.79±15.01	0.016	0.098
3OH Kynurenine	47.12±33.9	24.17±15.68	0.095	0.406	24.93±13.51	14.77±6.03	0.064	0.192
Neopterin	46.23±27.78	60.2±25.2	0.193	0.518	28.68±9.23	32.29±11.44	0.479	0.7
Nicotinic Riboside	6.27±2.51	8.19±4.7	0.426	0.576	6.02±3.62	5.99±1.55	0.545	0.7
Beta Nicotinamide Mononucleotide	39.92±65.28	40.75±55.32	0.468	0.576	22.68±27.59	12.03±8.84	0.611	0.734
Tryptophan	987.59±517.75	487.71±234.52	0.013	0.08	844.51±348.21	570.77±179.53	0.057	0.192
Kynurenine/Tryptophan Ratio	0.08±0.04	0.1±0.08	0.796	0.796	0.07±0.03	0.06±0.02	0.418	0.685

Units of all of metabolites displayed - ng/ml/creatinine

KTR expressed as a ratio

Red indicates statistical significance after controlling for FDR

TABLE 5-8 EFFECT OF SURGERY BY SURGICAL PROCEDURE SUBGROUP ON MEASURED URINARY METABOLITES

Urinary PA was reduced at 3-months post-operative amongst all groups, but this was only significant in those who underwent RYGB ($p=0.003$, $q=0.029$) and in those who didn't not have T2D ($p=0.009$, $q=0.009$). Urinary KYNA levels were reduced at 3-months post-operative amongst all groups but was only significant within the RYGB subgroup ($p=0.001$, $q=0.014$), see *table 5-8*. 3HAA levels were also significant reduced in the non-diabetes subgroup only ($p=0.005$, $q=0.049$) see *table 5-9*. There were no significant changes to any of the measured urinary tryptophan metabolites within VSG and T2D subgroups.

	T2D				ND			
	Pre-Op	Post-Op	p value	q value	Pre-Op	Post-Op	p value	q value
Picolinic Acid	15.94±5.36	11.02±4.99	0.047	0.231	14.37±3.17	9.71±2.44	0.009	0.009
Nicotinic Acid	11.01±10.3	7.96±7.24	0.857	0.941	6.33±3.51	6.32±4.93	0.99	0.99
3 Hydroxy Anthranilic Acid	119.76±92.68	67.18±53	0.165	0.372	120.81±73.69	54.9±48.64	0.005	0.049
Dopamine	23.63±10.22	24.89±12.87	0.863	0.941	25.15±10.12	24.83±10.08	0.99	0.99
Serotonin	93.51±84.19	88.76±47.6	0.654	0.905	98.63±51.35	85.12±59.08	0.437	0.656
Quinolinic Acid	367.89±98.23	447.36±287.24	0.918	0.941	494.2±132.61	432.19±177.51	0.064	0.192
Citrulline	54.31±41.49	31.48±19.16	0.23	0.46	50.8±28.1	29.88±14.12	0.032	0.143
Indole 3 Acetic Acid	152.86±106.13	138.91±134.56	0.28	0.491	228.73±224.09	164.41±197.69	0.223	0.401
Kynurenic Acid	93.43±39.08	60.45±42.69	0.019	0.231	101.78±29.25	81.33±43.49	0.06	0.192
5-hydroxyindole acetic acid	623.16±195.9	543.66±202.44	0.3	0.491	453.54±251.56	432.92±125.77	0.65	0.775
Xanthurenic Acid	86.12±46.79	54.2±29.68	0.114	0.342	81.28±50	70.78±63.47	0.153	0.307
Kynurenine	83.11±50.84	50.77±25.64	0.132	0.342	56.79±36.6	36.51±31.72	0.123	0.307
3OH Kynurenine	45.44±34.8	18.81±9.53	0.051	0.231	26.35±13.95	18.45±13.67	0.152	0.307
Neopterin	36.1±25.44	47.6±25.27	0.133	0.342	37.24±18.48	42.95±22.46	0.614	0.775
Nicotinic Riboside	6.21±2.62	7.73±4.49	0.401	0.601	6.06±3.56	6.23±1.92	0.437	0.656
Beta Nicotinamide Mononucleotide	47.96±66.65	42.14±59.13	0.941	0.941	15.88±15.71	13.27±6.48	0.614	0.775
Tryptophan	953.14±441.71	578.7±250.22	0.043	0.231	871.02±427.13	494.89±140.96	0.011	0.064
Kynurenine/Tryptophan Ratio	0.08±0.03	0.1±0.07	0.918	0.941	0.07±0.04	0.06±0.02	0.689	0.775

Units of all of metabolites displayed - ng/ml/creatinine

KTR expressed as a ratio

Red indicates statistical significance after controlling for FDR

TABLE 5-9 EFFECT OF SURGERY BY T2D SUBGROUP ON MEASURED URINARY METABOLITES

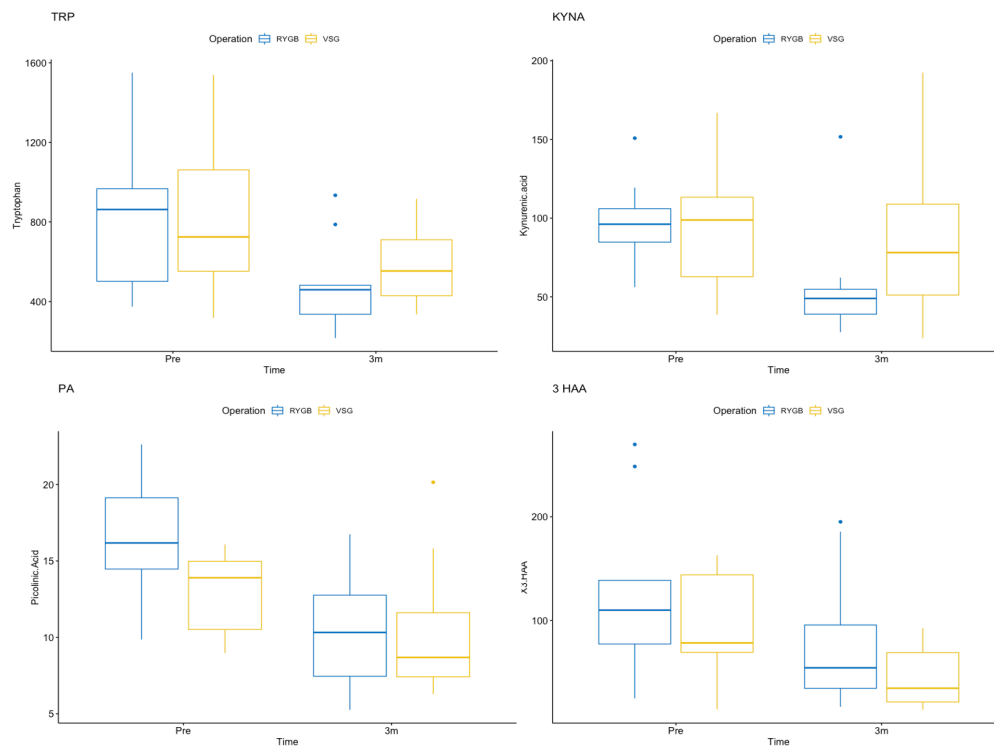


FIGURE 5-12 BOXPLOTS DISPLAYING PRE AND 3-MONTH POST-OPERATIVE URINARY LEVELS OF TRYPTOPHAN (TOP LEFT), KYNURENIC ACID (TOP RIGHT), PICOLINIC ACID (BOTTOM LEFT) AND 3-HAA (BOTTOM RIGHT) COMPARING SURGICAL INTERVENTION SUBGROUPS

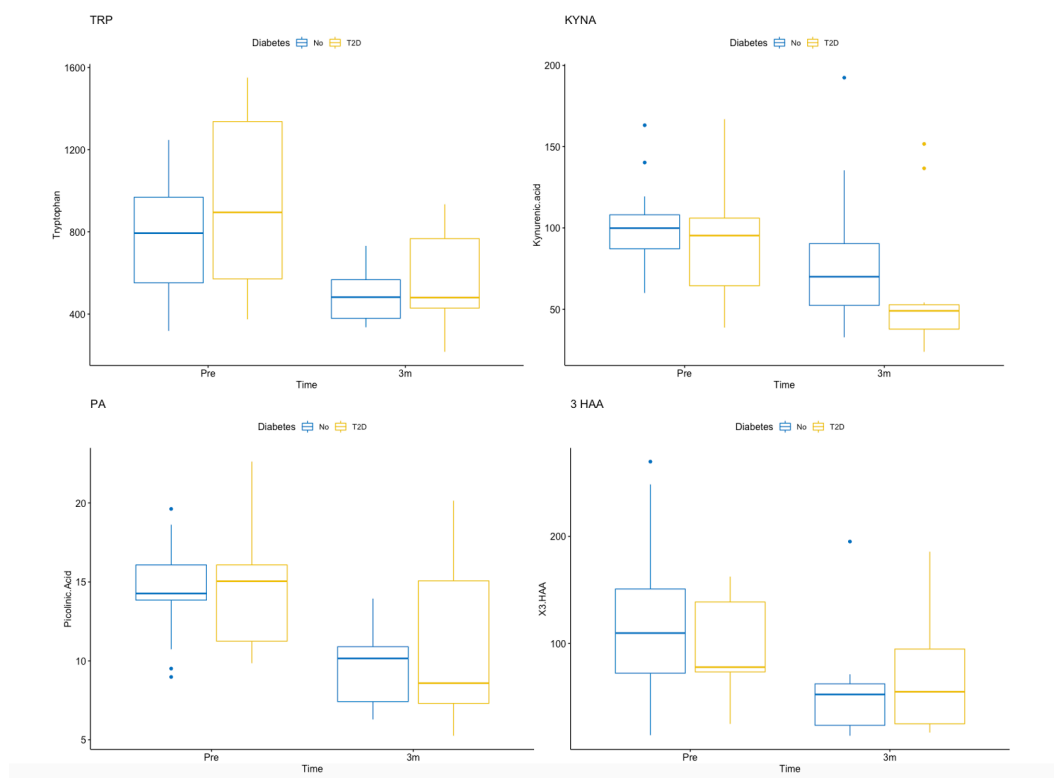


FIGURE 5-13 BOXPLOTS DISPLAYING PRE AND 3-MONTH POST-OPERATIVE URINARY LEVELS OF TRYPTOPHAN (TOP LEFT), KYNURENIC ACID (TOP RIGHT), PICOLINIC ACID (BOTTOM LEFT) AND 3-HAA (BOTTOM RIGHT) COMPARING T2D SUBGROUPS

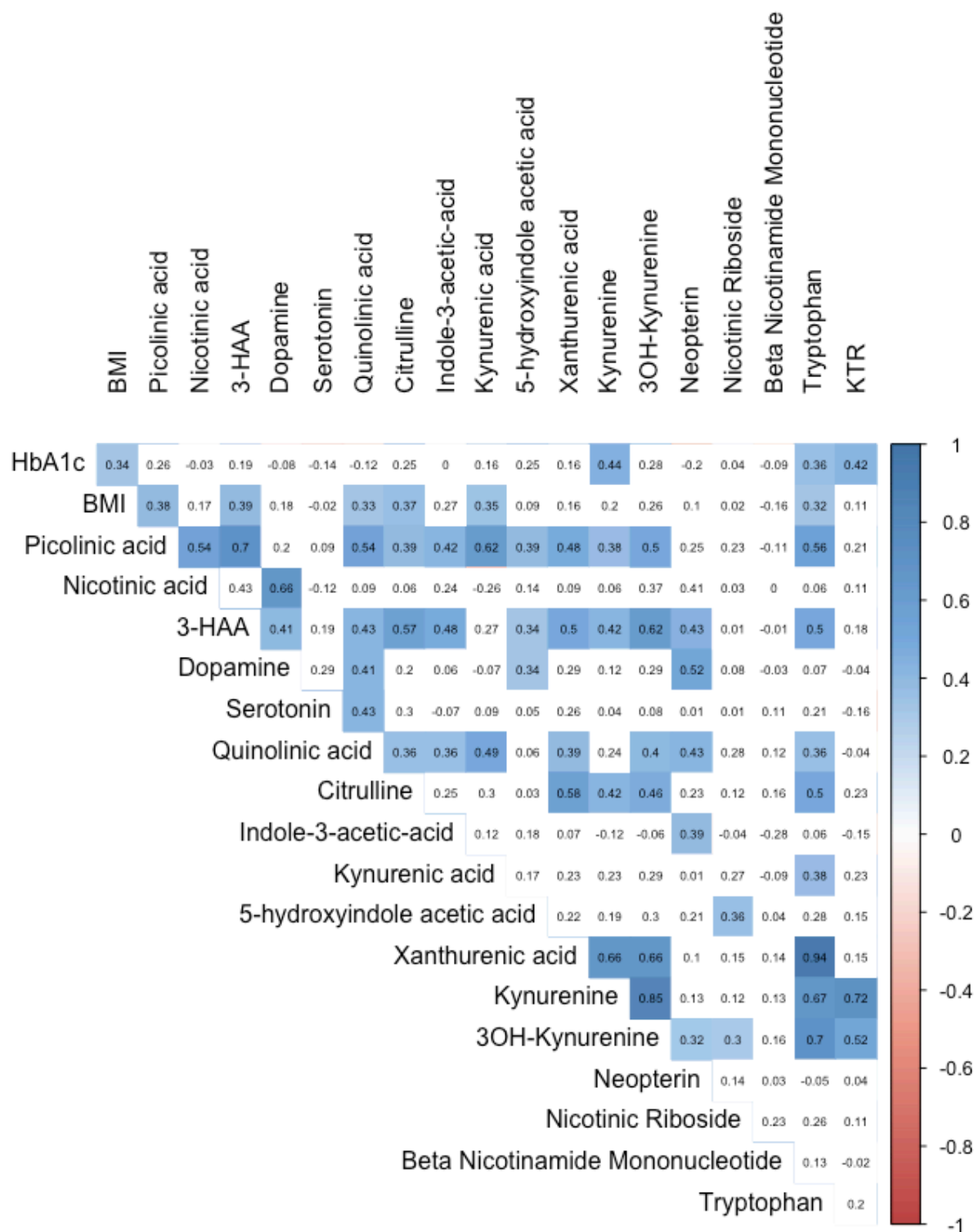


FIGURE 5-14 HEATMAP DISPLAYING SPEARMAN'S RANK CORRELATION (NUMBERS) CO-EFFICIENT BETWEEN HbA1c, BMI AND MEASURED URINARY TRP-KYN PATHWAY METABOLITES. COLOURED SQUARES INDICATE SIGNIFICANT CORRELATION RELATIONSHIP WITH P VALUE OF ≤ 0.05

Correlation analysis found that HbA1C was significant correlated with urinary TRP ($r=0.36$, $p=0.015$), KYN ($r=0.44$, $p=0.002$), and KTR ($r=0.35$, $p=0.004$), at the respective collection timepoints. BMI was correlated with PA ($r=0.39$, $p=0.008$), 3-HAA ($r=0.39$, $p=0.008$), QA ($r=0.33$, $p=0.024$), Citrulline ($r=0.37$, $p=0.0150$), KYNA ($r=0.35$, $p=0.017$) and TRP ($r=0.32$, $p=0.033$), see *figure 5-14*.

TRP values correlated strongly with metabolites found the TRP-KYN pathway, KYN ($r=0.67$, $p<0.0001$), 3OH KYN ($r=0.70$, $p<0.0001$), PA, ($r=0.56$, $p=0.0001$), XA ($r=0.94$, $p<0.0001$), 3HAA ($r=0.5$, $p=0.0008$), QA ($r=0.36$, $p=0.018$) and KYNA ($r=0.38$, $p=0.013$). These relationships are further visualised in correlation network map *figure 5-15*. Examination of the pre and 3-month post operative Δ of measured urinary metabolites did not yield any further significant insights.

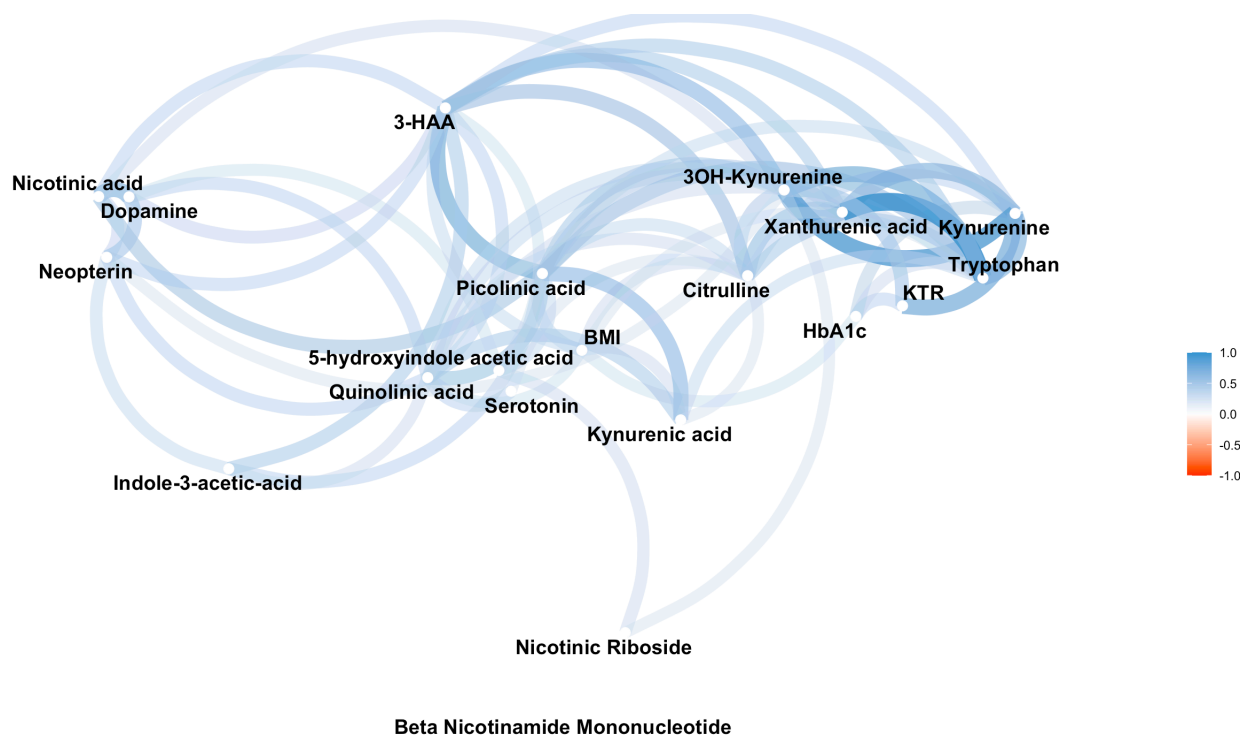


FIGURE 5-15 NETWORK MAP OF SPEARMAN CORRELATION BETWEEN TRP-KYN METABOLITES
CORRELATION STRENGTH AND DIRECTION INDICATED BY COLOUR AND PROXIMITIES OF CLUSTERS.

5.5.3 *T2D Resolution*

Out of the ten patients with T2D within the serum study cohort, only 3 achieved a status of resolution defined as HbA_{1c} < 42 mmol/mol and cessation of all T2D medications at 3 months post-op. This was similar in the urine study cohort where only 3 out of 11 T2D patients achieved T2D resolution.

There were no significant differences observed in both serum or urine TRP-KYN pathway metabolites when comparing 1) pre-operative profiles of those with and T2D resolution at 3 months and 2) pre-operative vs post-operative profiles of subgroups divided by those with and without T2D resolution.

5.6 Discussion

5.6.1 Serum Tryptophan Kynurenine Metabolites

Bariatric surgery leads to weight loss and improves glycaemic control. One of the mechanisms which confer these beneficial effects is thought to be via the modulation of the TRP-KYN pathway. It is reported in literature that elevated serum levels of TRP and its downstream metabolites is associated with T2D (237, 246, 437, 438, 465). This analysis was consistent with these findings, observing increased serum levels of TRP and XA when comparing those with and without T2D.

Chronic inflammation and immunomodulation (466) in those living with chronic obesity is believed to be one of the factors connecting T2D and the TRP-KYN pathway (434). Obesity is linked to increased gene expression of all key enzymes found within this pathway: IDO, KMO, KYNU and KAT found within adipose tissue and proinflammatory macrophages (436). Increased KMO expression and activation is reported to be positively correlated with HbA_{1c} levels (436). Activation of KMO leads to pathway diversion towards increased production of XA and decreased production of NAD which is associated with the T2D phenotype. Our data support this finding with higher concentrations of XA reported in the pre-operative groups compared to the 3 month post bariatric surgery samples.

XA is described as diabetogenic and has been reported to be elevated in the urine(467, 468) and serum(237) of patients with T2D. Reduction of XA after bariatric surgery has also been associated with improved glycaemic control a year post-operative (469). This was in keeping with our reported findings. Those in the T2D group were observed to have higher baseline serum XA concentrations when compared to those without T2D. Bariatric surgery led to significant reductions of XA concentration at 3-month post-surgery in the T2D but not the ND group. Of note, only surgical intervention with RYGB and not VSG led to a significant reduction of XA at 3-months,

XA also significantly correlated with HbA_{1c} (*figure 5-9*), again highlighting the association between XA and T2D. While this observation does not demonstrate causality, the

significant relationship supports the postulated contributory role of TRP-KYN pathway dysregulation in the pathogenesis of T2D.

Several possible mechanisms of action have been proposed, this includes zinc chelation of XA leading to molecular complexes that present similarly to insulin antigens(470-472). XA may also be directly toxic to pancreatic islet cells (472, 473) or have a directly inhibitive action on insulin secretion from islet cells(474). The role of XA in T2D resolution warrants further investigation, with modulation of this pathway a possible target for future drug targets or personalised surgical intervention.

The enzymes KYNU and KAT also plays a role in the link between the TRP-KYN pathway, obesity and T2D. The enzymes KYNU and KAT both depend on B6 as an important co-factor (see *figure 5-1*). While not assessed in this study, vitamin B6 deficiency is also associated with obesity and chronic inflammation (475, 476). KYNU is more sensitive to vitamin B6 deficiency when compared to KAT and B6 deficiency first causes downregulation of KYNU activity. This in turn produces a shift within the pathway leading to increased production of XA & KYNA both of which are reported to be diabetogenic(451). Higher concentrations of XA and KYNA were both observed in the pre-operative groups in this study. Post-operatively, reduction in BMI and systemic inflammation could potentially account for some of the changes observed in XA and KYNA levels.

Modulation of the TRP-KYN pathway metabolites by bariatric surgery may also have benefits and unintended effects beyond glycaemic control. Bariatric surgery has been shown to reduce cardiovascular risk in those with obesity(477, 478) but elevated levels of KYNA have been associated with an increased risk of acute coronary events in individuals with chronic heart disease (479). In this study, surgery led to a reduction of KYNA levels, which could be one of the mechanisms modulating this risk. TRP metabolites may also have direct nephrotoxic effects on the kidney. These metabolites have been shown to display a positive correlation relationship with severity of chronic kidney disease (480), a relationship which could be explained by worsening renal function and protein clearance. However, as

renal function improves after bariatric surgery(481), modulation TRP-KYN pathway may in part be responsible for these beneficial metabolic effects.

The TRP-KYN pathway also has links to the central nervous system. Cytokine mediated inflammation present in those with obesity is associated with dysregulation of the hypothalamus-pituitary-adrenal axis and has been implicated in depression (444). KYNA and QA are both neuroactive metabolites modulated by bariatric surgery and are of interest in neuro-gastroenterology. KYNA, a neuroprotective metabolite, is a NMDA antagonist and QA, a neurotoxic metabolite(482), is a NMDA agonist (433). Both can influence central and peripheral neuronal activity. The link between TRP metabolism and the central nervous system may be due modulation of the gut microbiome (483). While an evolving concept, it is hypothesised that the gut microbiota influence brain function, mood and cognitive function(484). A recent study reported kynurenic acid and serotonin levels measured within small bowel mucosa to be directly correlated with anxiety and depression scores in patients with irritable bowel syndrome(485). The authors hypothesise that changes in the GI tract may affect TRP-KYN pathway metabolism and subsequently influence psychological states (485). A chronically low tryptophan state also seems to accelerate the metabolism of serotonin to 5-HIAA (465). As bariatric surgery leads to a reduction in TRP-KYN metabolites and other micronutrients, it remains to be seen whether this may be contributory to the increased incidence of depression after bariatric surgery (486) and certainly warrants further investigation.

Gut microbes metabolise tryptophan to indole species, which in turn play a mechanistic role in microbiota to host signalling (433, 454). Indoles, including IAA is produced by the microbial metabolism of TRP by the microbial enzyme tryptophan monooxygenase and indole-3-acetamide hydrolase. IAA is an aryl hydrocarbon receptor (AHR) ligand which has recognised roles in human host immunity(487), gut permeability (488) and interkingdom crosstalk (454, 489). Tryptophan derived indoles have been reported to increase after gastric bypass (488). IAA is also reported to modulate AHR mediated responses in the human colonic carcinoma Caco2 cell line(490) and AHR activation has also been linked with increased risk of cardiovascular disease(491). No significant change in IAA

concentrations before or 3-months after bariatric surgery were observed in our study cohort but bariatric surgery has been associated with an increased risk of developing colonic cancer (492-494). It has been reported that bariatric surgery increases putrefaction within the colon; a process considered to have a negative effect on health(495). Researchers have also reported increased abundances of anaerobic gram negative enterobacteria after RYGB (221, 422), these organisms are found in low abundance within the normal gut and increased abundances are associated with inflammatory bowel disease (496)and colon cancer(497). While beyond the scope of this study, the effect of surgery on such microbial metabolites and their potential unintended actions certainly warrants further investigation.

Within this cohort, a larger reduction in TRP-KYN metabolites was observed in those who underwent RYGB, which raises the question as to whether this may be one of the factors the favours RYGB over VSG in T2D resolution.

This study also reported no significant correlation relationships between Δ BMI and Δ TRP-KYN pathway metabolites but an association between reduction in BMI and kynurenine has been observed in patients who have undergone bariatric surgery or low-calorie dieting (436, 498, 499). TRP-KYN pathway metabolites' association with glycaemic control has also been shown to be evident after adjusting for weight loss(469) which adds to the growing body of evidence that the anti-diabetes effect of surgery is not limited to just weight loss.

Finally, the reduction of nutritional intake after surgery may also be a factor contributing to these results. Eleven patients provided dietary information before and after surgery using an online recall questionnaire (see appendix VII). There was a significant reduction of protein intake from 80.6g/day to 49.7g/day ($p=0.0014$) which is consistent with similar studies investigating nutrient intake after bariatric surgery (500). However, if the findings were solely due to dietary limitations, it would in turn be expected that all metabolites within the TRP-KYN pathway to be uniformly reduced after surgery which was not the case. Thus, it would be reasonable to assume that the changes observed in this study were associated with specific enzymatic steps in the pathway.

5.6.2 *Urine Tryptophan Kynurenine Metabolites*

In summary, there were no significant differences in the baseline urinary TRP-KYN metabolites when comparing those with and without T2D in this study cohort. When analysed as an entire cohort, surgery led to significant reductions in PA, 3-HAA, KYNA and TRP.

However, with further subgroup analysis, urinary PA levels were reduced significantly in those who underwent RYGB and in those who did not have T2D only. Only RYGB led to a significant reduction in urinary KYNA. Urinary 3-HAA reduced significantly in those without T2D only. Surgery did not cause any significant changes to the measured urinary metabolites within T2D and VSG sub-groups. PA, KYNA and 3HAA levels did not correlate with BMI or HbA_{1c}. Trend changes were observed across all metabolites but did not reach significance after adjusting for FDR (q values of >0.05). The relatively small sample may limit the generalisability of the result and incurs the risk of a type II error which decrease the power of the study.

PA has not been extensively studied but it has links to pathogenesis of inflammatory disorders. In vitro studies have demonstrated PA can act independently (501) or synergistically with IFN gamma as an activator of macrophage inflammatory functions (502, 503). Interestingly, PA's most reported link to T2D seems to be associated with its role as an efficient metal chelator. The oral supplement chromium appears to regulate glucose and lipid metabolism and in those with T2D, this has been reported to reduce hypercholesterolemia and enhances insulin function & sensitivity (504-506). In vivo studies suggest chromium picolinate could even ameliorate diabetes nephropathy via antioxidative stress mechanisms (507) but this effect has not been replicated in human studies.

PA's role as a neuroactive agent has been explored more thoroughly (508), it is known to be neuroprotective and has immune and antiproliferative effects (509). PA also has antagonistic properties against the neurotoxic QA (508) described earlier, but the precise mechanisms by which this effect is achieved, are unknown.

The relationship between serum TRP-KYN metabolites and T2D has been thoroughly discussed in the previous section. However, the relationship between T2D and urinary TRP-KYN metabolites is not as clear nor established (510, 511). Elevated concentration of urinary KYN, KYNA, XA, QA and TRP indole metabolites as well as a high KTR have been reported in patients with metabolic syndrome (468, 512) and T2D rodent models(468). Urine KTR is reported to be a strong predictor for incident T2D in a cohort of patients with coronary artery disease(513), but this study did not observe any difference in baseline urinary KTR between those with and without T2D with was in keeping with our findings.

Surgery did not seem to lead to a significant effect on urinary TRP-KYN metabolites at 3 months post-surgery. However, TRP, KYN and KTR values demonstrated a significant positive correlation relationship with HbA1c at their respective collection timepoints. TRP, KYNA, Citrulline, QA, 3-HAA and PA demonstrated a significant positive correlation relationship with BMI at their respective collection timepoints

Beta Nicotinamide Mononucleotide (NMN) is a nucleotide and a source of cellular energy in human cells (514). It is also an intermediate of nicotinamide adenine dinucleotide (NAD⁺) synthesis. NAD⁺ can only be synthesised de novo from TRP through QA (514). Although not significant, this study found a trend reduction in urinary QA in those with T2D compared to those without. NAD⁺ is an important coenzyme with roles in metabolism, immunity, aging, neurodegeneration and DNA repair(514). Down regulation and depletion of NAD⁺ has been found in instances of inflammation, insulin resistance and impaired mitochondrial function(515). These features are linked to cancer, neurodegeneration, and metabolic disease such as T2D and obesity(516, 517). Oxidative stress, altered systemic inflammation and impaired lipid metabolism leading to insulin resistance in T2D, can be improved by NAD⁺ supplementation(518). However, this study did not find any significant alteration to urinary concentration of these metabolites suggesting that these associations are due to other metabolic pathways.

Gestational diabetes (GDM) develops during pregnancy and generally resolves soon after delivery. It is traditionally thought to be caused by placental hormones such as oestrogen

progesterone, cortisol and prolactin(519). It is hypothesised that pancreatic beta cells are unable to match the increased insulin demands of pregnancy(519). GDM is considered as a different type of diabetes but urinary metabolomic studies investigating this disease have generated metabolic insights and revealed close links to pathophysiological dysregulation of the TRP-KYN pathway seen in T2D.

A longitudinal study exploring the urinary metabolome through the course of pregnancy identified TRP-KYN pathway activation early in the first trimester, before the placenta or fetoplacental unit could have produced any hormonal physiological effects(520). This observation challenges the idea that GDM is caused by hormones related to pregnancy. Alternatively, this could suggest that some individuals have underlying metabolic disturbance related to the TRP-KYN pathway which may predispose them to the development of GDM. Another study identified fourteen metabolites upregulated in the urinary metabolome of GDM patients including TRP pathway metabolites (521). It is believed that this upregulation of TRP pathway metabolites in GDM is due to systemic oxidative stress and inflammation and could lead to higher systemic levels of insulin resistance (521).

Low grade inflammation (522) and increased levels of pro-inflammatory TNF alpha is found in women with GDM in similar fashion to those with T2D. TNF- α levels in women with GDM also correlated with HbA_{1c} levels (523). IDO can be activated by TNF- α and may lead to up regulation of TRP-KYN pathway contributing to a 'diabetes effect' similar to T2D previously described. Our study demonstrated up regulation of serum but not urinary TRP-KYN pathway metabolites in those with T2D and obesity but lacked comparison to a healthy control cohort.

Serotonin has an autocrine role in increasing β -cell mass in anticipation of increased insulin demand during pregnancy(524) and has inhibitory effects on alpha cell glucagon secretion (525). Serotonin levels are also found to be higher in women with GDM(526) suggesting that serotonin has a compensatory role in hyperglycaemia, which may be overcome by other factors in GDM. Women with GDM are seven times more likely to develop T2D within ten

years of pregnancy and have a higher risk of developing associated end organ disease(527). A meta-analysis of GDM metabolomic studies found several linking key metabolites involved in both GDM and T2D (528). These insights illustrate the ability of metabolomic studies to enhance our understand of current disease. While not yet fully elucidated, there are possibly several similar underlying metabolic disturbances between T2D and GDM, further investigation may yield markers found in urinary test which could be useful for identifying those at risk and could benefit from primary preventative interventions.

5.7 Conclusion

Bariatric surgery, in particular RYGB led to significant reduction of serum tryptophan and downstream kynurenine metabolites including kynurenic acid and xanthurenic acid at three months post-surgery. These metabolites are implicated in T2D, and its reduction correlates with a reduction in HbA_{1c} levels and BMI. However, whether these findings are a cause or consequence remains to be established. The modulation of serum TRP-KYN pathway metabolites may also have a wide range of beneficial and unintended effects beyond weight loss and glycaemic control.

On the other hand, surgery did not cause significant changes in urinary TRP and downstream KYN metabolites at 3 months post-surgery except for a significant reduction in urinary KYNA for those who underwent RYGB. These metabolites however, correlated with measured HbA_{1c} levels at respective timepoints. While surgery was not demonstrated to significantly influence these urinary metabolites, a correlation with glycaemic control was observed.

Chapter 6: Serum Lipids

6.1 Background

6.1.1 *The Lipidome & Lipidomics*

Lipids have three crucial roles in human biology, they are the main structural components of cellular membranes, they regulate energy metabolism as storage molecules and also play important roles in cellular signalling(529). The lipidome is the complete lipid profile found within cells, tissues, or an organism. There are currently hundreds of known molecular lipid species that make up the human plasma lipidome(530). Lipidomics is the study of lipidomes found within a biological system (531) and is important for understanding molecular mechanisms that contribute to metabolic states of disease. Lipidomics commonly utilises mass spectrometry based analytical techniques and recent advances in mass spectrometry instrument sensitivity (532) has allowed the detection and identification of closely related lipid species. Annotation and classification of lipids is complex and is constantly evolving as lipids can have relatively long carbon chains with double bonds in multiple places and therefore the exact structural configuration and identification of isomers can be challenging (533).

Lipid metabolism and the lipidome is complex and not fully understood but disruptions to the lipidome can have significant physiological effects such as the promotion of insulin resistance (534). Which and how lipid species contribute to states of healthy metabolic homeostasis, and which lead to states of metabolic disorders are yet to be fully uncovered(529). For example, cellular membranes are formed from a diverse range of lipids and the functions of these lipids remain poorly understood. However, even minor lipid structural changes has been shown to be associated with significant effects on important biological signalling and function in conditions such as type 2 diabetes and Alzheimer's disease (535).

6.1.2 *Lipidomic Profiles, Obesity & Type 2 Diabetes*

The human lipidome, like any biological system is an equilibrium of lipid intake, biosynthesis, and degradation. Metabolic influx due to hyper calorific diets leads to excess fat, and obesity. Excess lipids are stored as triglycerides (TAG) within adipocytes but when storage capacity is exceeded, an imbalance of lipid metabolites is observed within the serum and peripheral tissues. Some of these lipid metabolites such as diacylglycerols (DAGS) and ceramides (CER) are reported to be bioactive and may be toxic (536). These metabolic products contribute to systemic inflammation, altering insulin signals leading to the development of insulin resistance (536) and expression of the T2D phenotype(537). Interestingly, there is no established association between total fat intake and incidence of T2D(538, 539) suggestive of a more complex relationship between diet and T2D. However, the specific type of fatty acid ingested (i.e. saturated vs mono or poly-unsaturated) may have differentiating mechanistic diabetogenic roles(540).

Plasma lipidomic changes are found to be present long before clinical T2D diagnosis (541) and lipids profiles can be used to predict incident T2D (541). This raises the potential for early primary preventative interventions before T2D pathophysiology occurs(541). Assessing the lipidomic associations with T2D and changes before and after surgery may help further elucidate the biological mechanisms underlying obesity and T2D. By understanding these processes more clearly, perhaps novel findings can also assist in the prediction of future resolution, or risk stratification, or response of T2D and other obesity associated co-morbidities to surgery.

Current routine clinical practice only measures total triacylglycerols (TGs), total cholesterol, high density lipoprotein (HDL) and low density lipoprotein LDL. The imbalance of such lipids and lipid containing molecules is otherwise known as dyslipidaemia, which is often associated with T2D as part of the metabolic syndrome leading to increased risk of atherosclerotic cardiovascular disease(542). However, these markers are insufficient in predicting disease risk. For example, within the triglycerides' family, there are a variety of molecular species with links to the T2D phenotype, but the precise mechanisms by which these molecules contribute to diabetogenesis are not

clear⁽⁵⁴³⁾. Globally elevated TG is linked to elevated cardiometabolic risk, but it has been suggested that precise subcellular level localisation and compositions of lipid species is a superior predictor of T2D rather than sum total measurements⁽⁵⁴⁴⁾ illustrating an area of research interest.

Longitudinal observational studies investigating the link between lipidomic markers to T2D⁽⁵⁴⁵⁾ and risk of T2D incidence have begun to emerge ^(541, 543, 546-551). These association studies have utilised long term cohorts (four years PREDIMED, 5 years Finnish, 12 years Framingham and 20 years Mamo cohorts) and have provided useful insights. Collectively these studies have reported that long term-lipid signatures characteristic of T2D precede diagnosis and that lipid profiles may be used to predict incident T2D. However, although all these studies demonstrate significant associative links, causality is yet to be proven. Nevertheless, these markers have the potential to be used as a predictive biomarker for incident T2D and perhaps response to surgery. Lipidomic signatures including several Lyso-phosphatidylcholines (LPC), PC and TAG species have been reported to identify groups where there is a high risk of progression to T2D when standard risk factors such as age, obesity, gender, blood pressure and HbA1c were not helpful at distinguishing progression of disease⁽⁵⁴⁷⁾. Lipid signatures may perhaps outperform traditional measures used in clinical practice such as BMI as predictors of future T2D⁽⁵⁴¹⁾ but these findings require further validation.

The lipids family is diverse and different lipid species have been associated with T2D. For example, a large association study found that higher levels of phosphatidylcholines (PC), sphingomyelin (SM) and cholesterol esters (CE) were associated with lower T2D risk. Triacylglycerols (TAGS), diacylglycerols (DAGS) and phosphatidylethanolamine (PE) on the other hand are associated with higher T2D Risk ^(276, 543, 547, 548, 551). These lipid species will be discussed in detail below along with their association and postulated mechanism contributing to the T2D phenotype.

6.1.3 *Phospholipids*

Phospholipids have a hydrophilic phosphate head group and two fatty acid hydrophobic tails. They are key cell membrane structural components with the phospholipid bilayer as the dominant structure of most cell membranes(552). Lysophospholipid is where lyso- indicates species where one of the fatty acid chains are removed by hydrolysis. Lysophospholipids are lipid signalling molecules that signal via G protein coupled receptor and are involved in a wide range of biological process(553). The current understanding of lysophospholipids is not extensive but these molecules are known to have roles in cellular stress signalling and systemic inflammation(553).

Phospholipid signatures have been identified to be associated with obesity and T2D(202), PC (40 to 50%) and phosphatidylethanolamine (PE) (up to 40%) are the two most abundant lipid species found in biological phospholipid membranes(554, 555). Disrupted PC: PE ratios are linked to multiple metabolic disease states(555) such as non-alcoholic fatty liver disease (NAFLD) (556) and T2D(545). Decreased levels of Lyso-phosphatidylcholines (LPC) have been reported to be lower in those with obesity(557), and this was found to not increase back to normal even after weight loss(557). LPC levels also negatively correlate with CRP and BMI leading to a postulated anti-inflammatory role (557, 558) which is reduced in obesity associated chronic systemic inflammation.

LPC levels are also negatively correlated with insulin sensitivity(559) and are reported to lower serum glucose concentrations by inducing insulin release(559). Plasma LPC and Lyso-phosphatidylethanolamine (LPE) are reported to be lower in those with T2D (276, 547, 560, 561) and low concentrations have been identified as a predictor of incident Impaired Glucose Tolerance (IGT) and T2D(250, 302). LPC levels can also discriminate lean individuals without diabetes from other cohorts (562). Lower levels of LPC were observed in a stepwise fashion in those with obesity but without T2D, followed by lean T2D and lowest in those with obesity and T2D(562). This progressive signature led to the authors postulating a role of causality. When comparing cohorts diagnosed with T2D, lower levels of LPC and LPE are also associated with a higher risk of subsequent cardiovascular disease(541, 547, 563). The specific mechanisms leading to these effects are unknown. An

anti-oxidant effect where LPCs act as sacrificial antioxidant, sparing oxidation of other lipids and scavenger of reactive oxygen species(564) is one possible explanation of this effect.

Plasma levels of PE are reported to be highest in individuals with T2D and obesity (545, 562) and can discriminate this cohort from those who have the lean T2D and obese non-diabetes phenotypes. Lower levels of PC and SM also discriminates lean T2D from other cohorts, but the obese T2D lipid signature is not homogenous. Signatures of lower concentrations of 32-44 carbon chain lengthed PC species differentiated individuals who were living with obesity and T2D from those who were lean with T2D (562). SM, CER and Hexosylceramide (HCER) species were lower in lean T2D individuals when compared to lean non-diabetes individuals (562) suggestive of a lipid signature linked to T2D.

6.1.4 *Neutral Glycerol Lipids*

TAG and DAG and other glycolipids are found in human plasma within chylomicrons and very low-density lipoprotein. The sum concentration of this lipid group is directly related to dietary intake with secondary factors such as genetic predisposition, T2D and obesity also contributory to plasma concentrations (552). These neutral lipids have an important role in mammalian evolutionary history as energy storage molecules where historically, nutrients were not regularly available (565).

Higher levels of circulating DAG, short and odd chains TAGs are associated with T2D (541, 543, 545, 548). Current evidence suggests that DAGs have a causal role in lipid induced insulin resistance. This effect on insulin resistance appears to be mediated by activation of Protein Kinase C (PKC) by affecting key steps in the insulin signalling pathway (537) in different tissue types, however, the full extent to which this axis influences insulin resistance is not fully elucidated yet. Hepatic DAG accumulation due to high circulating levels of DAG activates PKC- ϵ , which impairs insulin receptor activation and glycogen synthesis (566). In contrast to TAG, although elevated plasma levels are associated with insulin resistance, there are currently no mechanistic models to suggest TAG contributes directly to the pathogenesis of T2D directly or if it only represents an obesity related metabolic state(255).

One possible unifying mechanism is linked to the metabolite glycerol-3-phosphate (G₃P), which is a precursor for TAG, DAG and LPCs. G₃P is produced during glycolysis when six-carbon glucose is converted into two three-carbon G₃P and serum levels of G₃P increase when there are increased plasma concentrations of glucose (567). In such cases, it is possible that upregulation of TAG synthesis (lipogenesis) causes downregulation of LPC synthesis due to substrate competition. Altogether, increased TAGs, decreased phospholipid and sphingolipids could be a metabolic signature of upregulated lipogenesis seen in T2D(567).

6.1.5 *Free Fatty Acids*

Short and medium chains Free Fatty Acids (FFA) are ingested from food and are directly absorbed by gut enterocytes. Longer chain FFAs (>c12) are transported within lipoprotein (HDL, LDL, VLDL, chylomicrons) particles(568). Transmembrane proteins mediate uptake of these particles across all cell types and, in adipocytes, FFAs are stored by conversion into TAG droplets(569). Under normal conditions, this is an efficient method of energy storage. When there is an excess of FFA and the storage reservoir as TAGs is saturated, excess FFAs from lipolysis spills into the blood stream (570).

FFAs are also synthesised *de novo* from carbohydrates, in a process otherwise known as lipogenesis(569). This process is upregulated by insulin predominantly in the liver, adipose tissue, and mammary glands. The opposite process: lipolysis occurs when there is an increase in adrenaline, catecholamines and reduction of insulin illustrating a tightly regulated homeostatic cycle(571) and in T2D, the pathological inability to suppress lipolysis is a significant contributor to the development of hyperglycaemia (572). As an energy source, FFAs undergo Beta-oxidation within the mitochondria (572).

Within this subclass, various FFAs have important biological roles in the regulation of lipid homeostasis, inflammation(573), endocrine signals(574) and are involved in various functions of cell membranes(575). Metabolic dysregulation and an influx of lipids in the context of obesity leads to increased serum FFA and contributing to the dysregulation of serum FFA concentrations. Increased serum FFAs causes obesity-associated T2D and cardiovascular disease(576). Insulin resistance and T2D then causes further dysregulation of FFA levels (577).

When there are excess FFAs and cells are not able to Beta-oxidate or convert to TAGs, toxic lipids such as DAGs and ceramides (CER) are generated (575). These toxic metabolites cause endoplasmic stress and mitochondrial dysfunction, leading to the generation of reactive oxygen species (ROS) (575). This then leads to a cascade of systemic inflammation, insulin resistance in adipocytes and skeletal muscles, and apoptosis of pancreatic beta-cells(578-580). Excess fat storage with adipocytes also leads to hypoxia and triggers macrophage

infiltration of adipose tissue. Both factors lead to increased secretion of inflammatory cytokines, contributing to systemic inflammation(575). Excess FFAs entering systemic circulation then accumulate in organs such as the pancreas and liver leading to local lipotoxic effects (575).

Long chain FFAs regulate GLP-1 and subsequent beta-cell insulin secretion through G-protein-coupled receptor 120 (GPR120) found in the gut(581). GPR 120 activation in adipocytes and macrophages downregulates inflammation(582). FFAs also bind to GPR 40 found in pancreatic cells, increasing insulin secretion(583). Gut sensing of FFAs also influences the hypothalamic appetite centre illustrating a gut-brain axis relationship (584). Peroxisome proliferator activated receptors (PPARs) are nuclear receptors that sense FFA signals. Binding of PPARs are thought to be a significant link between FFAs and signalling leading to the regulation of inflammation(585).

Saturated FFAs also induces inflammation via the activation of toll-like-receptor 4 (TLR-4). TLR-4 activation leads to the production of pro-inflammatory cytokines such as IL-6 and TNF- α (586, 587). This affects insulin signalling, reducing glucose tolerance and insulin sensitivity(588). FFAs also activate an inflammasome multi-protein complex known as NLRP3 which mediates IL-6. In the context of T2D, NLRP3 activation in pancreatic beta-cells has been shown to lead to impaired insulin secretion and cellular apoptosis (589). In animal models, β -cell apoptosis was also found to be induced by increased FFA via ceramide and nitrous oxide production pathways (590). Finally, ROS generated from abnormal Beta-oxidation also contributes to mitochondrial dysfunction, ER stress and is thought to be contributory to end stage T2D pancreatic beta-cell death(575, 591).

The literature regarding the role of dietary fat intake in relation to FFAs and the incidence of T2D is still inconsistent(592) as plasma levels of FFAs are also influenced by de novo synthesis and TAG lipolysis. While higher levels plasma FFAs are generally associated with lower insulin secretion and T2D, the evidence regarding individual FFA species is heterogeneous and inconclusive(575). For example, high plasma levels of total FFA or saturated (i.e., palmitic or stearic acid) FFAs were also associated with a higher future risk

of T2D(593, 594). FFAs have also been reported to correlate directly with HbA_{1c}(595). Plasma linoleic acid however, was found have an inverse relationship with risk of T2D(594, 596) and has been proposed as a potential candidate biomarker for T2D risk and glycaemic control(597). Untargeted metabolomic analyses has identified increased longer 20-22 carbon chain length fatty acids to be elevated in individuals with impaired glucose tolerance (289) when compared to healthy controls, and depletion of shorter chained fatty acids in individuals with T2D when compared to healthy controls (289). It has also been reported that higher serum levels of TG containing longer chain length fatty acids and double bonds were associated with decreased T2D risk with the opposite of TG containing shorter chain length FA and less double bonds to be associated with an increased risk of T2D(548).

Excess FFAs also impact hypothalamic feeding centres and increase appetite through a series of inflammation and hormone induced mechanisms(598, 599). This effect was seen to be reversed in mouse models when plasma unsaturated FA levels were increased. In the GI tract, FA metabolism influences the gut microbiome composition, and a change in composition influences the metabolic products produced by the microbiome(600). Reduced short chain fatty acids (SCFAs) have roles in insulin sensitivity and reduced production is associated with T2D(601). Increased dietary saturated FFAs leads to increased production of bacterial endotoxins, which may contribute to systemic inflammation(602). This effect has been shown to be reversible(603).

In summary, elevated plasma FFAs result from dysregulation of the lipid pool. Excess FFAs results in widespread metabolic changes both directly and indirectly influencing systemic inflammation and insulin sensitivity. Importantly, some of these effects have been shown to be reversible.

6.1.6 *Sphingolipids*

Sphingolipids (SLs) are a diverse and biologically active class of lipids that are differentiated from other lipids due a backbone formed of sphingoid bases(604) including sphingosine; an amino alcohol with 18 carbon chain length. The SL class includes ceramides (CER), sphingomyelins (SMs) and glycosphingolipids (HCER/LCER). Currently, there over 600 confirmed species (605) and these lipids forms a significant portion of the known human serum lipidome(606), SMs contribute to almost 87% of plasma sphingolipids. SLs are known as bioactive lipids and have roles in cell cycle processes such as cell death, cellular apoptosis cell differentiation, autophagy and well as systemic effects in the regulation of inflammation and insulin resistance(604).

The relationship between sphingolipids and T2D has not been entirely established yet but sphingolipids are thought to be the molecular signalling link between inflammation and insulin resistance(607-609). Inhibiting glycosphingolipid synthesis in T2D mice models have also shown to significantly improve insulin sensitivity by increased insulin signalling in muscle cells (610). However, the association of sphingolipids and glucose regulation is non-linear, suggesting a complicated association with many factors involved(611).

Previous understanding of sphingolipids has been limited due to instrument sensitivity of mass spectrometers and other analytical platforms for characterising lipid composition but advances in mass spectrometry have enabled accurate discrimination of multiple species. Recent evidence has shown that sphingolipids can contribute to lipotoxicity associated with diseases such as NAFLD(612), and to mechanisms leading to insulin resistance and other metabolic disorders such as obesity, T2D and cardiovascular disease (613-615). However, to date, very few human studies have investigated the effect of select sphingolipids on insulin resistance and beta cell dysfunction and it has been suggested that sphingolipids have a stronger mediation effect on beta cells rather than directly towards insulin resistance (616, 617).

Altered sphingolipid metabolism is also reported to contribute to the proinflammatory state seen in those with obesity and the pathogenesis of associated cardiovascular and metabolic diseases(618).

Studies reporting on associations between pre-diabetes(619), T2D and selected sphingolipids have reached different and sometimes contrasting conclusions, albeit measured in different geographic and ethnically composed cohorts (250, 543, 617, 620-622), illustrating the difficulty in generalisability and reproducibility of reported findings when applied globally.

Ceramides (CER) are important structural membrane lipids in lipid bilayer cellular membranes(623); this subclass is pathologically relevant to T2D and has so far gained the most research attention. They are composed of sphingosine and a fatty acid. Ceramides are bioactive lipid intermediates similar to diacylglycerols (DAG) and have roles in regulation of pathways affecting cell signalling, oxidative stress and apoptosis(624, 625). Ceramides are bioactive lipid intermediates similar to diacylglycerols (DAG). Ceramides primarily originate from the liver and are transported within LDL & VLDL (606). In healthy individuals approximately 60% of CER is found within LDL(626). Biologically, newly synthesised ceramides from adipocytes are nutrient signals that regulate body-wide inflammation and energy expenditure (627). Related to pathology, ceramides can promote inflammation, impair beta-cell function and induce insulin resistance(628). Ceramides are also linked to endothelial dysfunction(629), chronic renal disease (630),cardiovascular diseases(628) and have reported roles in regulation of cell growth and proliferation in the context of cancer(631).

Ceramide levels have been reported in some studies to be elevated in individuals with T2D(545, 632) but the inverse has been observed in other studies (543). This inconsistency could be explained by different CER species exerting biological effects or a different T2D phenotype. Ceramides are also associated and influence the severity of insulin resistance. This is postulated to be caused by modification of insulin signalling, mitochondrial function and by activation of inflammation associated mediators such as TNF- α (632, 633). LDL

containing ceramide also induces a pro-inflammatory response in macrophages and activates Nuclear Factor Kappa B (NF- κ B) increasing cytokine production and leading to systemic inflammation(634). Interestingly, ceramide levels are independent of dietary intake. Suggesting that de novo production and its changes are influenced by enzymatic activity (604, 635).

In addition to CER being transported in LDL particles, the proportion of CER is elevated in LDL particles in those with T2D(636). In one study where LDL containing CER was infused into lean mice, a reduction in insulin stimulated uptake of glucose in the skeletal muscle was observed as well as an associated reduction in skeletal Akt phosphorylation and glucose transported type 4 (GLUT4) translocation (636), this impairment of insulin action was specific to skeletal muscle. Skeletal CER also impairs insulin signalling by activating protein phosphatase enzyme (PP2A), altering an insulin signalling intermediate responsible for skeletal muscle glucose uptake(637).

Ectopic fat deposits, a common occurrence in those with obesity are also associated with tissue production of ceramide. C16:0 ceramide which is produced by the enzyme ceramide synthase 6 (CerS6) has been linked to insulin resistance. The blocking of CerS6 in animal models resulted in lower levels of C16:0 ceramide and improved insulin sensitivity(638).

In vivo models of decreased serum ceramide, created by either genetically ablating enzymes critical to de novo CER synthesis or by pharmacological blockade(639) led to improve glucose homeostasis (612, 640). Improvements were seen with insulin signalling, mitochondria function along with decreased lipogenesis and lipid uptake. To illustrate the opposite, finding, another in vivo experiment that increased de novo CER synthesis observed a reduction in beta cell function.

The degradation of sphingomyelin (SM) to ceramide increases the proportion of ceramide contained within lipid rafts. In the pancreas, this leads to a signal promoting beta cell apoptosis and well as a reduction in expression of the insulin gene (641). Increased CER also enhances ROS leading to mitochondria dysfunction and beta-cell apoptosis(319, 642).

As ceramides are synthesised by adipocytes, it is no surprise that ceramide levels correlate positively with BMI but, hexosylceramide (HCER), a glycosphingolipid, correlates negatively with both BMI and HOMA-IR. This demonstrates an intricate role between ceramide species and expressed phenotypes. It is thought that sphingolipid metabolism imbalance rather than solely CER accumulation is associated with obesity(620).

In another large-scale study spanning nine years, dihydroceramides (DCERs) which are precursors of CER were observed to be another early predictor of T2D (619). Inhibition of DCER to CER in obese mice models led to reduced CER levels and subsequent T2D. DCER has also been suggested as a strong predictor of metabolic dysfunction(643) and it is thought that DCER is a marker of FFA biosynthesis activation(612).

The consensus on the relationship between CER and SM-induced insulin resistance is still being explored. The influence of ceramide on hepatic mitochondrial function and/or lipid oxidation may be tissue specific and requires further investigation(644). Pharmacological inhibition of ceramide synthase is potentially an attractive drug target in the treatment of metabolic disease(645). Further work is required to identify the mechanisms of action which influences these metabolites influence insulin resistance.

1-deoxysphingolipid(1-dexySLs) are atypical sphingolipids formed during de-novo sphingolipid synthesis when alanine or glycine is used as an amino acid substrate instead of serine(321). States of serine depletion are seen in those with obesity (317). This group of metabolites has also been observed to be biomarkers for prediction of incident T2D in individuals with and without obesity(646, 647), 1-deoxySLs are also directly cytotoxic to insulin producing cells (320).

The toxic effects of 1-dexySL may also be contributory to T2D related complications but has been found to be reversible. L-serine has a direct suppressive influence on the production of 1-deoxySL and subsequent plasma levels (648). Demonstrated in a rat model, oral administration of L-serine led to reduced plasma levels of 1-dexySL and was found to improve diabetic neuropathy.

To illustrate the biological significance of 1-deoxySL, patients with primary mitochondrial disorders also exhibit similar profiles of increased alanine and reduced serine (649). This leads also to increase in 1-deoxySL levels, which in turn causes peripheral neuropathy. Treatment by serine supplementation in this scenario was also found to reduce deoxysphingolipid levels(649). In T2D the same amino acid derangements are present so the same mechanism may potentially be attributed to the development of diabetic neuropathy(321).

Cytotoxic 1-deoxyceramide accumulate in visceral adipose tissue and serum in T2D. This occurs in both lean and obese T2D. In vitro experiments have revealed that these higher concentrations of the toxic metabolites are due to synthesis by adipocytes, rather than these tissues acting as a sink reservoir (562). However, the precise signalling mechanisms leading to toxicity are currently unknown (650).

Sphingomyelins (SMs) are another abundant subclass of lipids and structurally consist of a CER with a phosphocholine or phosphoethanolamine head group. Some studies have reported an inverse relationship between serum SM levels and T2D (250, 543, 651). The enzyme SM synthase (SMS) converts ceramide to sphingomyelin. Mice models found knockout of SMS led to mitochondrial dysfunction, and severe impairment of the ability to release insulin in response to glucose (642). Some of this effect was subsequently successfully treated with the antioxidant N-Acetyl-Cysteine. This therapeutic effect perhaps suggests that pancreatic beta-cell dysfunction in SMS knockout mice may in part be due to ROS overproduction, and that the enzyme is required for healthy mitochondrial function and pancreatic beta-cell insulin secretion(642). SMS also generates DAG during sphingomyelin synthesis and DAG has been shown to inhibit insulin signalling through c-Jun N-terminal-kinase (JNK) activation(652).

In a separate experiment, SMS-2 deficient mice were observed to have reduced plasma SM levels and modified NF- κ B activation. This in turn led to improved insulin sensitivity and glucose tolerance(614) suggesting links between SMs and inflammatory pathways.

The precise signalling mechanism associating SM, insulin resistance and T2D are again yet to be fully elucidated. One hypothesis is that T2D arises due to an imbalance in phospholipid membrane composition of SM (653), with reduced plasma membrane SM observed to lead to increases in insulin sensitivity (654). Similar to CER, the altered composition of lipid rafts is reported to prevent insulin receptor dimerization, contributing to the development of insulin resistance (654). The fact that we currently do not know whether serum SM concentrations directly reflect alterations in cell membranes or whether this effect is different throughout various tissue types also limits our current understanding of the role of SMs.

In summary, serum lipids and their association with obesity and T2D is an expanding area of research. Serum lipid profiles have already been used to predict incident T2D but more importantly, research has begun to associate lipid subclasses such as phospholipids, FFA, and SL directly with systemic inflammation, insulin resistance and well as direct effects on pancreatic beta cell insulin secretion and apoptosis. While the mechanisms contributing to these effects are not well understood yet, this presents an exciting area of research and by exploring to effects of bariatric surgery on these metabolites and the T2D phenotype there is the potential to highlight areas of further interest.

6.2 Chapter Aims and Experimental Overview

This chapter utilises multivariate modelling, univariate statistics, and correlation analysis as a method of exploratory analysis to:

- 1) establish if lipidomic differences exist between individuals living with obesity and T2D
- 2) determine the effect of bariatric surgery on the serum lipidome and its association with the T2D phenotype and surgical resolution of T2D
- 3) Compare the quantitative lipids is compared with the amino acid data from chapter 4 to evaluate their associations with the T2D phenotype

6.3 Methods

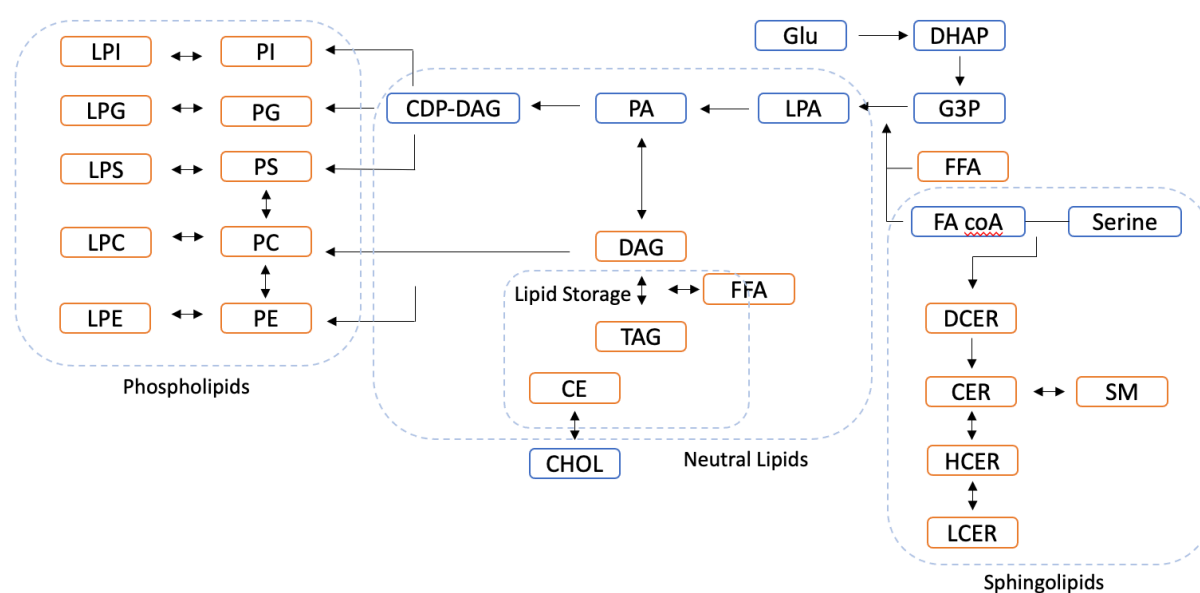
Data for the results from this chapter were acquired a part of an academic collaboration with the Centre for Computational & Systems Medicine, Health Futures Institute, Murdoch University, Perth, Australia, where Professor Holmes is centre director. Upon ethical approval (AMo2) and completion of contracts. Coded non identifiable aliquoted samples were shipped on dry ice to Murdoch university for lipidomic analysis.

Targeted serum lipid profiling was performed using a soon to be published UHPLC-MS/MS methodology by collaborators led by Dr Luke Whiley. In brief, for extraction, plasma samples were placed in wells of a 96 well plate with 90µl of propanol-2-ol extraction solvent which also contained stable isotope labelled internal standards (Lipidizer™ Internal Standards Kit from Sciex (MA, USA), SPLASH LipidoMIX™, Lyso PI 17:1, Lyso PG 17:1 and Lyso PS 17:1 (Avanti Polar Lipids)). Following on, the samples were shaken for 10 minutes and stored at -20°C for 20 minutes prior to centrifugation at 3900 g for 15 minutes. The resulting supernatant was then transferred to a 96 well plate for analysis.

UHPLC-MS/MS was performed using an ExionLCTM coupled to a QTRAP 6500+ system (Sciex, MA, USA). Reversed-phase liquid chromatography was performed using a Waters ACQUITY BEH BEH C18 (1.7 mm, 2.1 x 100 mm) column (Waters, MA, USA), at an operating temperature of 60 °C. Mobile phase A was water/acetonitrile/propan-2-ol (50/30/20, v/v/v) containing 10 mM ammonium acetate and mobile phase B was propan-2-ol/acetonitrile/water (90/9/1, v/v/v) containing 10 mM ammonium acetate. The flow rate was 0.4 mL/min with gradient elution as follows: starting at 10 % B, increasing to 45 % B at 2.7 min, 53 % at 2.8 min, 60% B at 8.0 min, 80 % B at 8.1 min and holding at 80 % B until 11.5 min, 100 % B at 12.0 min for 1 min before returning to 10 % B for a 2 min re-equilibration for a total cycle time of 15 min. The injection volume was 5 µL.

The QTRAP 6500+ was operated with electrospray ionisation using polarity switching. Mass spectrometry detection was performed with the following settings: Capillary voltage 5.5 kV (positive ion mode) and -4.5 kV (negative ion mode); temperature 300 °C; curtain gas, 20 psi; ion source gas 1, 40 psi; ion source gas 2, 60 psi. Time-scheduled multiple reaction

monitoring (MRM) for each lipid target was used for data acquisition. Data were acquired using Analyst®1.7.1 and analysed using Sciex OS Analytics 1.7.0 software. Pre-processing and peak integration was performed using SkylineMS software(230)



6.4 Results

6.4.1 Multivariate Exploratory Analysis

Study participants and samples utilised in this analysis are identical to those reported in chapter 4. For demographics and the effect of surgery on these parameters, please refer to chapter 4.4, *table 4-5*. In brief, 36 paired pre-operative and 3-month post-operative samples were included in this analysis. Within this cohort, 17 patients were T2D, and 18 patients underwent RYGB and VSG respectively.

Multivariate analysis was deployed for preliminary data exploration purposes. PCA and OPLS-DA models with 1 aligned + 1 orthogonal components were generated. Groups were analysed in a similar fashion to the results presented in this thesis. 1) Pre-op comparison of T2D v ND groups. 2) Pre-op v 3-month post-operative comparisons of T2D, ND, RYGB and VSG subgroups.

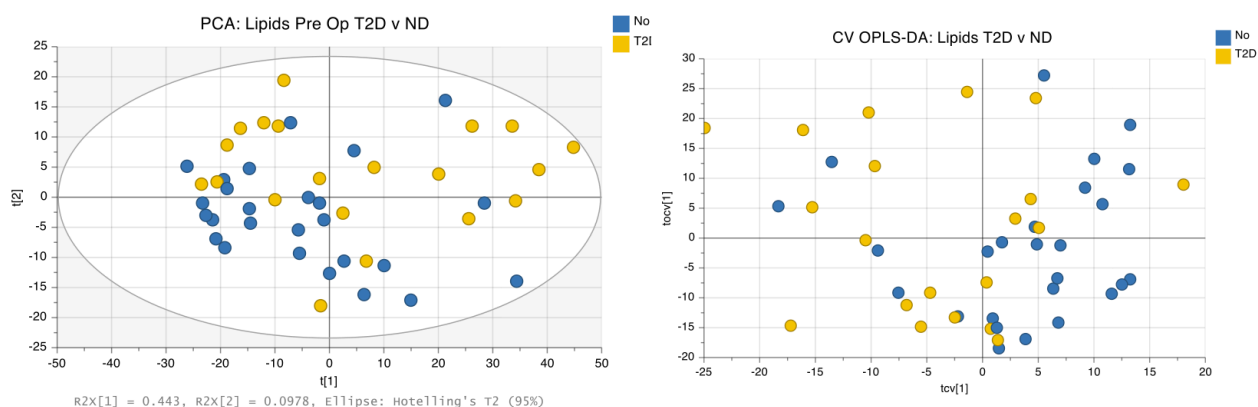


FIGURE 6-2 PCA & OPLS-DA PRE-OP T2D v ND

OPLS-DA MODEL WITH 1 ALIGNED + 1 ORTHOGONAL COMPONENTS COMPARING LIPID PROFILES OF THOSE WITH (YELLOW) AND WITHOUT (BLUE) T2D. $R^2X=0.627$ $Q^2Y=0.060$

PCA and OPLS-DA ($Q^2Y=0.060$) modelling did not uncover any substantial separation between the lipid profiles of those with and without T2D at the pre-operative baseline timepoint (*Figure 6-2*).

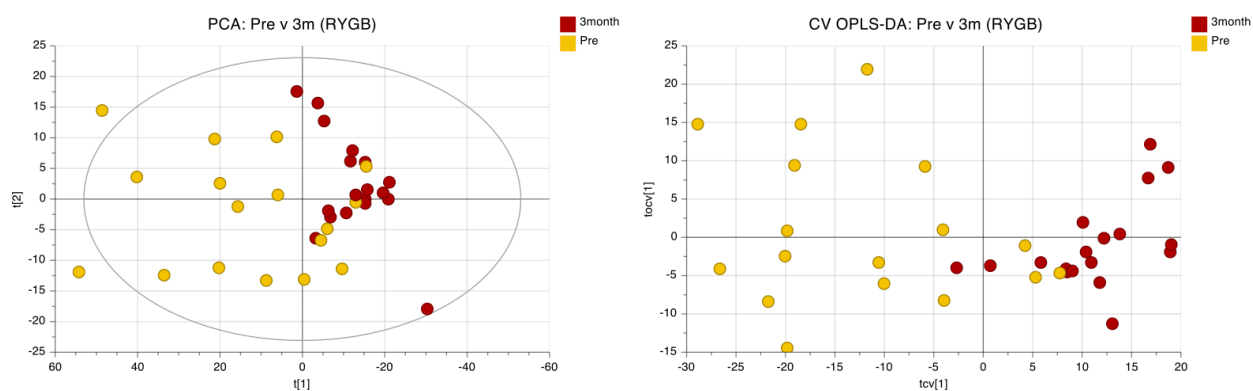


FIGURE 6-3 PCA & OPLS-DA RYGB PRE v POST
OPLS-DA MODEL WITH 1 ALIGNED + 1 ORTHOGONAL COMPONENTS COMPARING LIPID PROFILES OF PRE-OP (YELLOW)
AND 3 MONTH POST-RYGB (RED), $R^2X = 0.561$ $Q^2Y = 0.640$

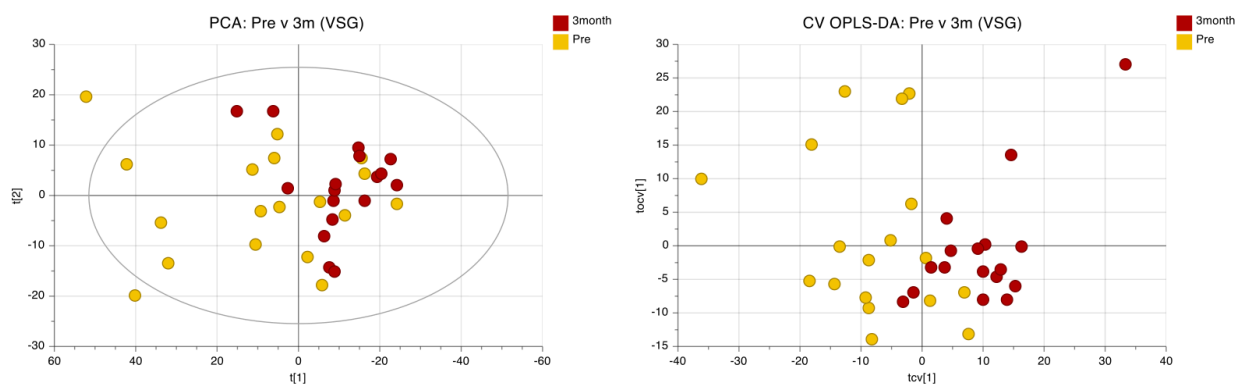


FIGURE 6-4 PCA & OPLS-DA VSG PRE v POST
OPLS-DA MODEL WITH 1 ALIGNED + 1 ORTHOGONAL COMPONENTS COMPARING LIPID PROFILES OF PRE-OP (YELLOW)
AND 3 MONTH POST-VSG (RED), $R^2X = 0.545$ $Q^2Y = 0.447$

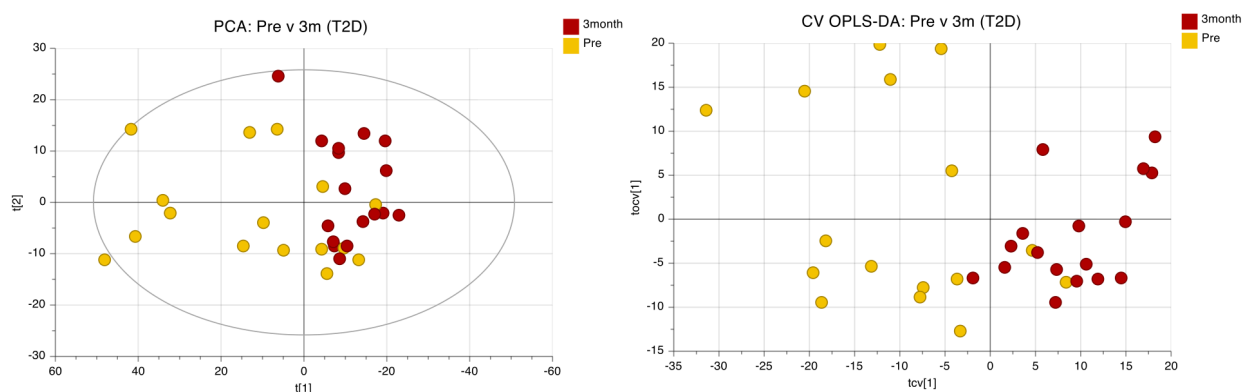


FIGURE 6-5 PCA & OPLS-DA T2D PRE v POST

OPLS-DA MODEL WITH 1 ALIGNED + 1 ORTHOGONAL COMPONENTS COMPARING LIPID PROFILES OF THOSE WITH T2D PRE-OP (YELLOW) AND 3 MONTH POST-OP (RED), $R^2X=0.530$ $Q^2Y=0.593$

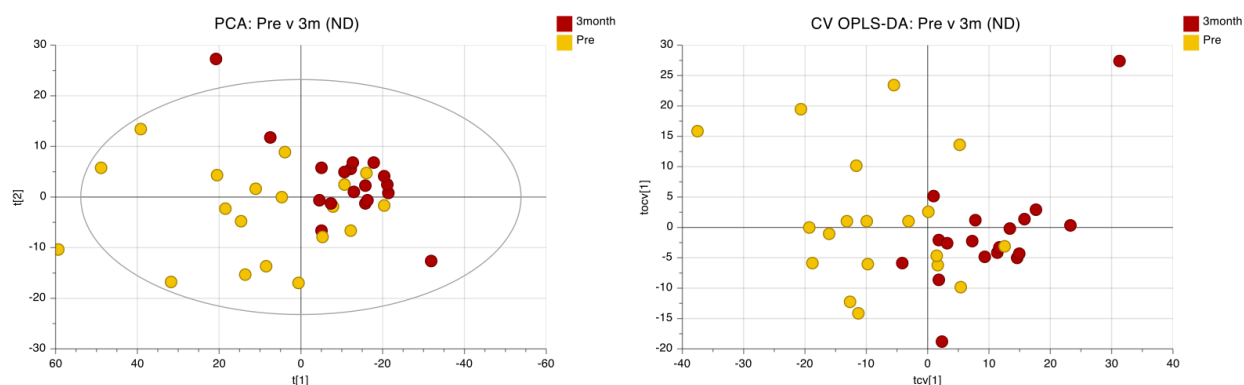


FIGURE 6-6 PCA & OPLS-DA Non T2D PRE v POST

OPLS-DA MODEL WITH 1 ALIGNED + 1 ORTHOGONAL COMPONENTS COMPARING LIPID PROFILES OF THOSE WITHOUT T2D PRE-OP (YELLOW) AND 3 MONTH POST-OP (RED), $R^2X=0.583$ $Q^2Y=0.426$

However, when comparing the pre-operative and 3-month post-operative lipid profiles of participants who underwent RYGB ($Q^2Y=0.640$, Figure 6-3) or VSG ($Q^2Y=0.447$, Figure 6-4) and participants with T2D ($Q^2Y=0.593$, Figure 6-5) and without T2D ($Q^2Y=0.426$, Figure 6-6), both PCA and OPLS-DA models demonstrates a large effect caused by surgery on serum lipid profiles.

6.4.2 Univariate Analysis at Lipid Class Level

Typically, lipids are highly correlated within their own species subclasses. Therefore, for a more interpretable visualisation and for discovery analysis, the subsequent sub-chapters present analysis performed using the sum-total of each lipid subclass rather than each individually quantified lipid.

Baseline comparison of lipids between those with and without T2D

When comparing baseline sum-total values of lipid subclasses using univariate analysis, those with T2D had significantly lower levels summed of hexosylceramide (HCER) ($p=0.005$, $q=0.028$), summed lactocylceramide (LCER) ($p<0.001$, $q<0.001$) and summed sphingomyelin (SM) ($p=0.004$, $q=0.028$) when compared to those without T2D. The relative boxplot comparisons are displayed in *figure 6-7*.

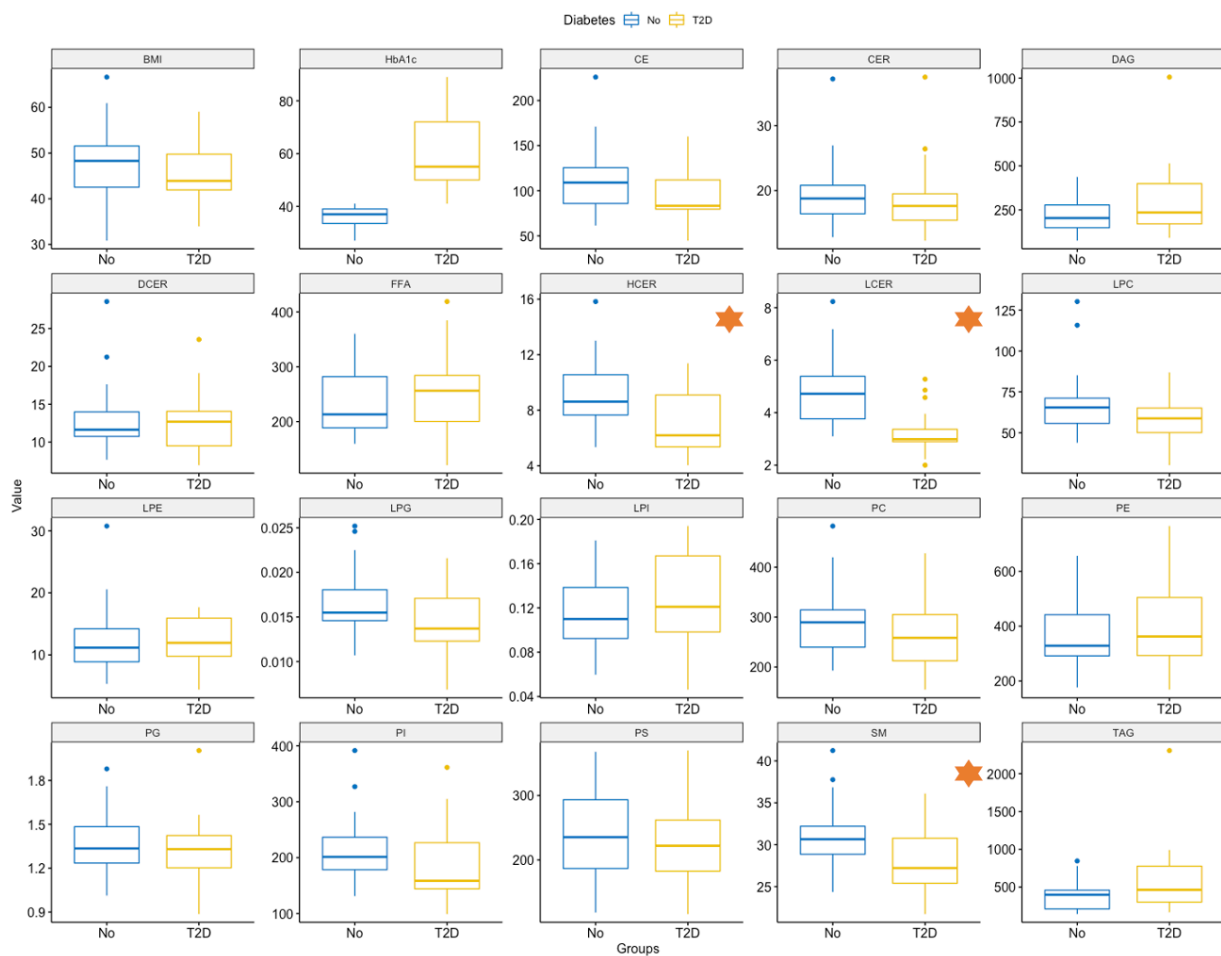


FIGURE 6-7 BOXPLOTS COMPARING PRE-OP V POST-OP VALUES OF MEASURED SERUM LIPIDS DISPLAYED BY T2D SUBGROUP, NON-T2D (BLUE) AND T2D (YELLOW). ORANGE STAR INDICATES SIGNIFICANT RESULTS WITH Q VALUE <0.05

6.4.3 Pre-operative and 3-month Post-operative analysis

RYGB and VSG

When comparing the effect of RYGB or VSG on measured lipid subclasses in this cohort at 3 months post-operative compared to baseline (*table 6-1 & Figure 6-8*), VSG did not cause any significant changes. RYGB led to reductions in summed CE ($p=0.0007$, $q=0.002$), summed CER ($p=0.0009$, $q=0.002$), summed DAG ($p=0.03$, $q=0.049$), summed DCER ($p<0.0001$, $q<0.001$), summed LPC ($p=0.002$, $q=0.005$), summed LPE ($p=0.003$, $q=0.006$), summed LPI ($p=0.0003$, $q=0.001$), summed PC ($p<0.0001$, $q<0.001$), summed PE ($p=0.0002$, $q=0.001$), summed PI ($p=0.008$, $q=0.013$), summed PS ($p=0.0001$, $q=0.001$), summed TAG ($p=0.0004$, $q=0.001$) and an increase in summed FFA ($p=0.003$, $q=0.005$) at 3-months post-operation.

T2D and Non-T2D

When comparing the effect of surgery on T2D and ND subgroups at 3 months post-operative (*table 6-2 & Figure 6-9*), significant reductions were observed in both groups for summed DCER ($p=0.002$, 0.025 ; $q=0.012$, 0.042), summed LPE ($p=0.009$, 0.004 ; $q=0.018$, 0.013), summed LPI ($p=0.009$, 0.027 ; $q=0.018$, 0.042), summed PC ($p=0.002$, 0.0001 ; $q=0.010$, 0.004), summed PE ($p=0.04$, 0.003 ; $q=0.012$, 0.013), summed PS ($p=0.004$, 0.008 ; $q=0.012$, 0.021) and summed TAG ($p=0.007$, 0.003 ; $q=0.017$, 0.013). Summed FFA ($p=0.016$, $q=0.029$) and summed LCER ($p=0.004$, $q=0.012$) were significantly increased post-operatively in those with T2D only. Summed CE ($p=0.015$, $q=0.033$), summed CER ($p=0.0198$, $q=0.036$) and summed LPC ($p=0.008$, $q=0.021$) were significantly reduced at 3-months post-operative in those without T2D only.

	RYGB				VSG			
	Pre-Op	Post Op	p value	q value	Pre-Op	Post Op	p value	q value
CE	108.22±27.79	75.97±18.81	0.001	0.002	109.45±41.27	100.4±31.65	0.569	0.599
CER	19.65±5.34	15.15±4.1	0.001	0.002	19.56±5.68	18.92±5.63	0.462	0.513
DAG	294.71±207.47	183.68±58.56	0.037	0.049	249.42±112.18	203.13±88.48	0.161	0.238
DCER	12.64±4.09	7.72±2.05	0.000	0.000	13.47±4.94	11.46±3.59	0.226	0.281
FFA	258.36±60.39	333.53±74.93	0.003	0.005	239.21±86.07	309.69±88.24	0.013	0.057
HCER	7.96±2.42	8.13±2.86	0.815	0.815	8.38±2.67	9.99±3.79	0.152	0.238
LCER	3.93±1.17	4.71±1.43	0.064	0.080	4.44±1.53	5.89±2.14	0.019	0.06
LPC	61.51±19.43	45.37±10.55	0.002	0.005	66.25±21.01	58.22±15.32	0.171	0.238
LPE	11.6±4.57	7.44±2.36	0.003	0.006	13.48±5.55	9.11±3.58	0.007	0.057
LPG	0.02±0	0.01±0	0.088	0.103	0.02±0	0.02±0	0.716	0.716
LPI	0.13±0.04	0.08±0.03	0.000	0.001	0.12±0.05	0.1±0.03	0.179	0.238
PC	279.94±64.56	184.4±38.52	0.000	0.000	285.76±70.39	235.58±52.05	0.014	0.057
PE	367.89±152.73	207.52±72.77	0.000	0.001	404.27±130.97	304.11±73.09	0.011	0.057
PG	1.31±0.24	1.37±0.28	0.239	0.265	1.38±0.24	1.55±0.24	0.064	0.134
PI	198.87±62.94	138.97±54.66	0.009	0.013	218.44±72.87	189.49±63.82	0.239	0.281
PS	251.33±73.61	160.13±52.41	0.000	0.001	225.32±58.55	188.51±66.02	0.068	0.134
SM	28.93±3.7	28.79±4.28	0.584	0.615	30.15±4.61	32.85±5.68	0.074	0.134
TAG	560.78±479.65	257.9±84.81	0.000	0.001	473.12±231.87	308.88±127.41	0.027	0.066

TABLE 6-1 PRE-OP V POST-OP VALUES OF MEASURED LIPIDS DISPLAYED BY OPERATIVE SUBGROUP

RED INDICATES STATISTICALLY SIGNIFICANT RESULT

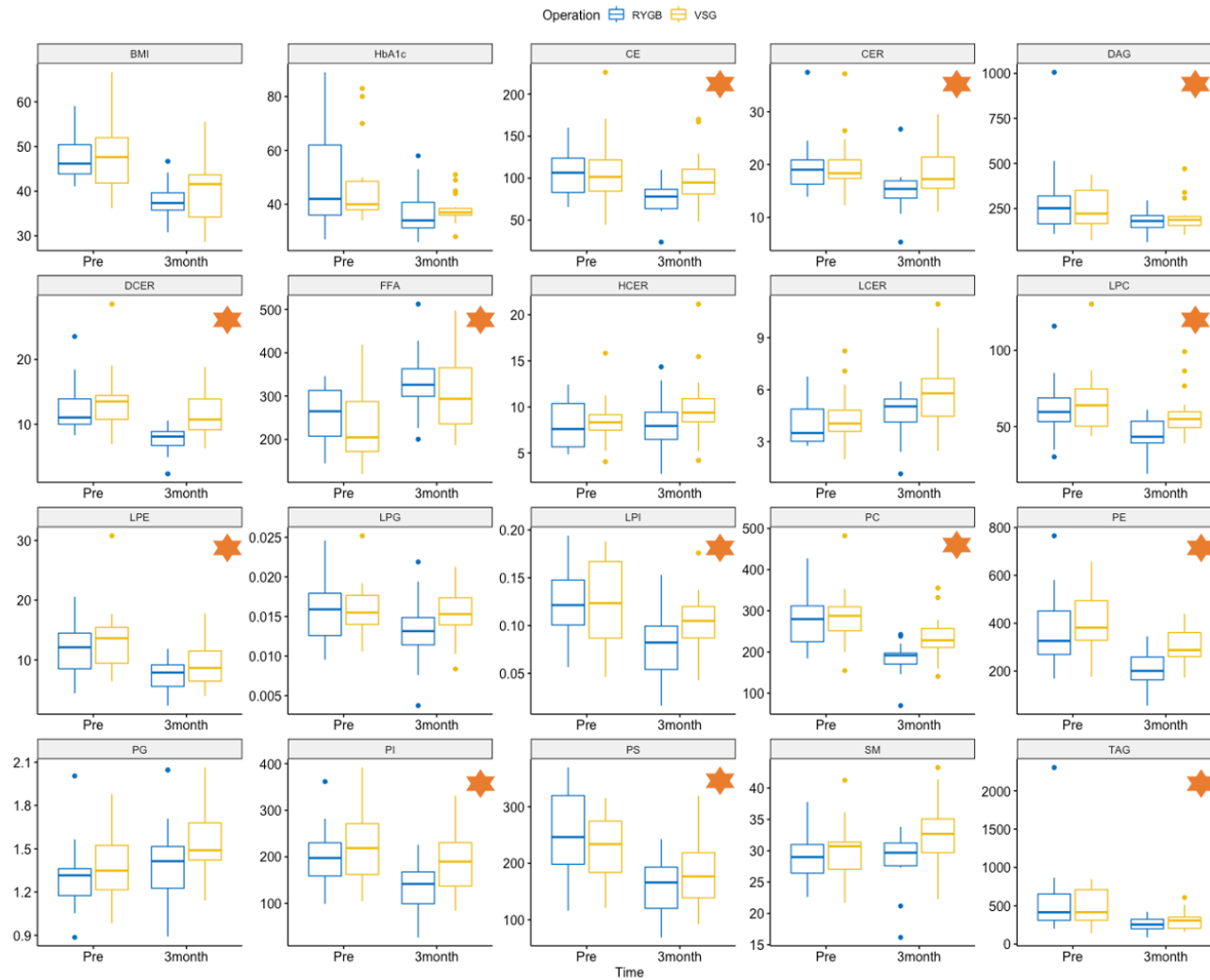


FIGURE 6-8 BOXPLOTS COMPARING PRE-OP V POST-OP VALUES OF MEASURED SERUM LIPIDS DISPLAYED BY OPERATION SUBGROUP, RYGB (BLUE) AND VSG (YELLOW). ORANGE STAR INDICATES SIGNIFICANT RESULT WITH Q VALUE <0.05 FOR RYGB SUBGROUP

	T2D				Non-Diabetes			
	Pre-Op	Post Op	p value	q value	Pre-Op	Post Op	p value	q value
CE	99.85±32.19	84.73±29.78	0.099	0.132	116.88±35.68	91.28±27.73	0.015	0.033
CER	19.15±5.82	17.28±5.43	0.122	0.153	20.01±5.2	16.82±5.16	0.020	0.036
DAG	313.17±212.42	202.08±55.67	0.062	0.096	235.29±102.13	185.65±89.07	0.091	0.107
DCER	13.08±4.51	9.04±3.05	0.003	0.012	13.03±4.6	10.08±3.79	0.025	0.042
FFA	263.05±84.31	333.6±81.72	0.016	0.029	236.02±62.77	310.87±82.14	0.004	0.013
HCER	7.4±2.38	8.7±4.15	0.474	0.474	8.86±2.5	9.38±2.73	0.354	0.354
LCER	3.39±0.84	4.92±1.82	0.004	0.012	4.9±1.36	5.64±1.94	0.065	0.082
LPC	57.64±14.5	50.79±12.52	0.085	0.122	69.46±22.98	52.69±16.37	0.008	0.021
LPE	12.26±4.28	8.57±2.4	0.009	0.018	12.79±5.84	8.01±3.68	0.004	0.013
LPG	0.02±0	0.01±0	0.153	0.180	0.02±0	0.02±0	0.314	0.348
LPI	0.13±0.05	0.09±0.03	0.009	0.018	0.12±0.03	0.09±0.03	0.027	0.042
PC	271.98±67.17	204.79±49.53	0.002	0.010	292.57±66.41	214.64±55.14	0.000	0.004
PE	393.71±165.93	241.07±76.12	0.004	0.012	379.26±119.65	269.01±95.77	0.003	0.013
PG	1.32±0.25	1.45±0.34	0.193	0.203	1.36±0.23	1.48±0.2	0.065	0.082
PI	198.09±76.21	148.03±57.43	0.038	0.063	218.12±59.88	178.72±67.4	0.046	0.066
PS	235.12±72.25	170.95±51.28	0.004	0.012	241.19±63.53	177.33±68.96	0.008	0.021
SM	27.69±3.74	29.9±5.59	0.193	0.203	31.2±3.9	31.65±5.18	0.339	0.354
TAG	608.47±489.5	313.04±86	0.007	0.017	435.06±209.58	256.86±123.6	0.003	0.013

TABLE 6-2 PRE-OP V POST-OP VALUES OF MEASURED LIPIDS DISPLAYED BY T2D SUBGROUP
RED INDICATES STATISTICALLY SIGNIFICANT RESULT



FIGURE 6-9 BOXPLOTS COMPARING PRE-OP V POST-OP VALUES OF MEASURED SERUM LIPIDS DISPLAYED BY T2D SUBGROUP, NON-T2D (BLUE) AND T2D (YELLOW). STARS INDICATE STATISTICALLY SIGNIFICANT RESULT OF $Q < 0.05$ FOR T2D (ORANGE) AND NON-T2D (GREEN) SUBGROUPS

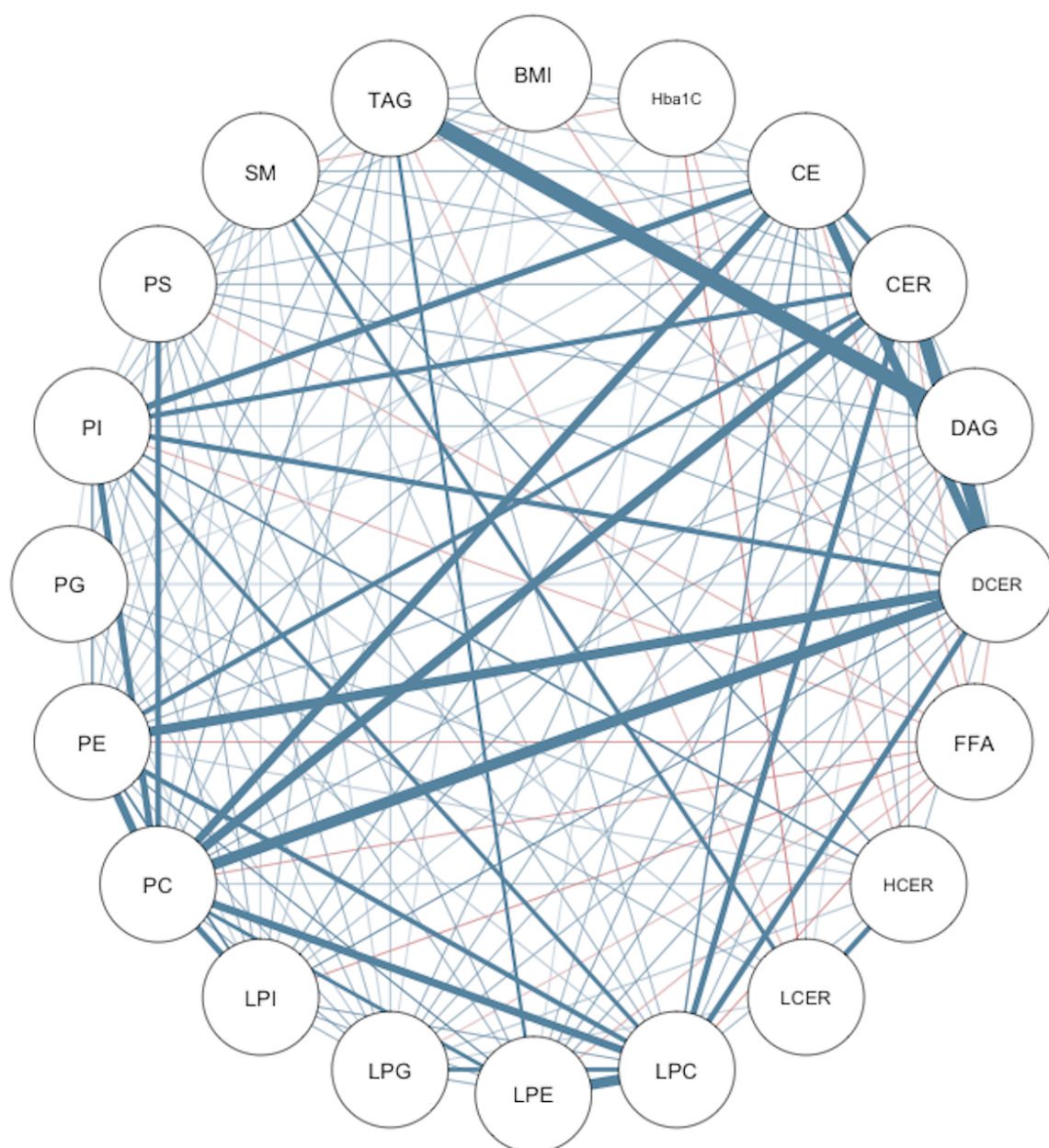


FIGURE 6-10 INTRA LIPID SUBGROUP CORRELATION NETWORK

CORRELATION NETWORK DEMONSTRATING THE CORRELATION RELATIONSHIP BETWEEN EACH LIPID AT CLASS LEVEL, BMI AND HBA1C ACROSS THE DATASET. ONLY SIGNIFICANT (BH $Q < 0.05$) SPEARMAN'S CORRELATIONS BETWEEN SUMMED LIPID SUBGROUPS ARE DISPLAYED. THICKER LINES DENOTES A HIGHER DEGREE OF CORRELATION. BLUE AND RED INDICATED POSITIVE AND NEGATIVE CORRELATION RESPECTIVELY.

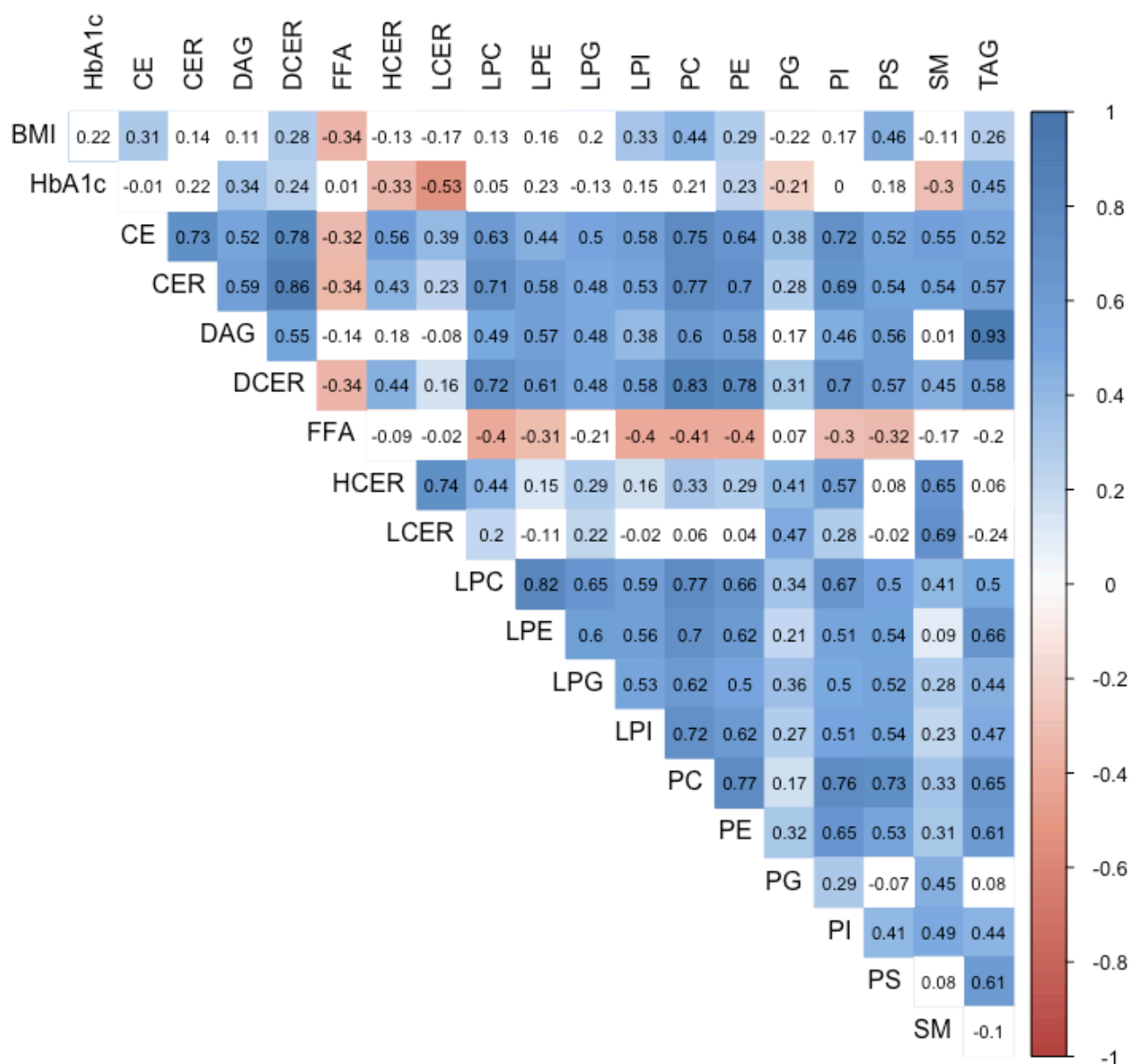


FIGURE 6-11 LIPID SUBGROUP SPEARMAN RANK CORRELATION HEAT MAP

CORRELATION HEATMAP DEMONSTRATING THE CORRELATION RELATIONSHIP BETWEEN EACH LIPID AT CLASS, BMI AND HbA1c ACROSS THE DATASET. SIGNIFICANT (BH $Q < 0.05$) SPEARMAN'S CORRELATIONS BETWEEN SUMMED LIPID SUBGROUPS ARE COLOURED. CORRELATION COEFFICIENTS ARE INDICATED. BLUE AND RED INDICATES POSITIVE AND NEGATIVE CORRELATION RESPECTIVELY.

The measured serum lipids at class level were assessed for correlation with BMI, HbA_{1c} within the dataset. These relationships along with interclass associations are visualised as a correlation network (*figure 6-10*) and correlation heatmap (*figure 6-11*). In summary, BMI demonstrated significant positive correlation with CE ($r=0.31$, $p=0.005$), DCER ($r=0.28$, $p=0.010$), LPI ($r=0.33$, $p=0.003$), PC ($r=0.44$, $p<0.0001$), PE ($r=0.29$, $p=0.001$), PS ($r=0.46$, $p<0.0001$) & TAG ($r=0.26$, $p=0.018$) and significant negative correlation with FFA ($r=-0.34$, $p=0.0019$). HbA_{1c} demonstrated significant positive correlation with DAG ($r=0.34$, $p=0.002$), DCER ($r=0.24$, $p=0.030$), PE ($r=0.23$, $p=0.039$) & TAG ($r=0.45$, $p<0.0001$) and significant inverse correlation with HCER ($r=-0.33$, $p=0.003$), LCER ($r=-0.53$, $p<0.0001$), PG ($r=-0.21$, $p=0.05$) & SM ($r=-0.30$, $p=0.007$). Several strong inter-class correlations are also demonstrated reaffirming that many of these lipids are closely associated.

To further examine the effect of surgery, the Δ difference of measured lipids before and 3 months after surgery were calculated and assessed for correlation with Δ BMI and Δ HbA_{1c}. There were no significant correlations between Δ BMI and the Δ of measured lipids. Δ HbA_{1c} was significantly inversely correlated with Δ LCER ($r=-0.23$, $p=0.042$), Δ LPC ($r=-0.38$, $p=0.028$) and Δ SM ($r=-0.39$, $p=0.045$) only, these relationships are demonstrated as scatter plots in *figure 6-12*. Other lipid subclasses which were significantly correlated with HbA_{1c} but were not significant in Δ analysis included: Δ HCER ($r=-0.3$, $p=0.057$), Δ DAG ($r=0.05$, $p=0.245$), Δ TAG ($r=0.08$, $p=0.216$), Δ DCER ($r=-0.05$, $p=0.555$), Δ PE ($r=0.02$, $p=0.302$), Δ PG ($r=-0.36$, $p=0.147$).

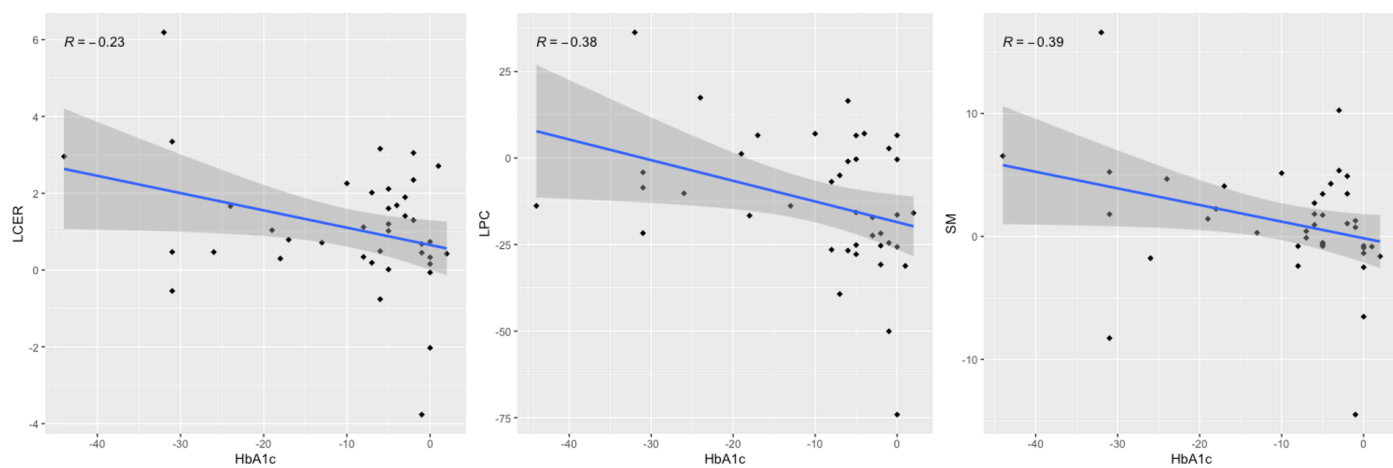


FIGURE 6-12 SCATTER CHARTS OF SIGNIFICANT ΔLIPID AGAINST ΔHbA1c : LCER, LPC AND SM

6.4.4 T2D Resolution

As per chapter 4, of the subset of 17 patients with T2D, 11 (65%) achieved a status of resolution defined as HbA_{1c} < 42 mmol/mol and cessation of all T2D medications at 3 months post-op. There were no significant differences observed when comparing the pre-operative values of BMI, HbA_{1c} and lipid subclass sum totals of those with and without T2D resolution at 3-months post-operative.

When divided into T2D resolution and non-resolution subgroups, BMI and HbA_{1c} were reduced at 3 months post-op in both groups when compared to baseline. This was only significant in all groups except for BMI in those without T2D resolution, (Resolution: BMI $p=0.0006$, HbA_{1c} $p=0.0001$) vs (Non-Resolution: BMI $p=0.132$, HbA_{1c} $p=0.015$).

When comparing the pre-op and 3-month post-operative serum lipid profiles of those with and without T2D resolution (*Figure 6-13*), no significant changes were observed in the non-resolution subgroup. Significant reductions in DCER ($p=0.008$, $q=0.049$), PC ($p=0.003$, $q=0.029$) and significant increase in LCER ($p=0.0007$, $q=0.014$) were observed in the group with T2D resolution at 3-months post-operative.

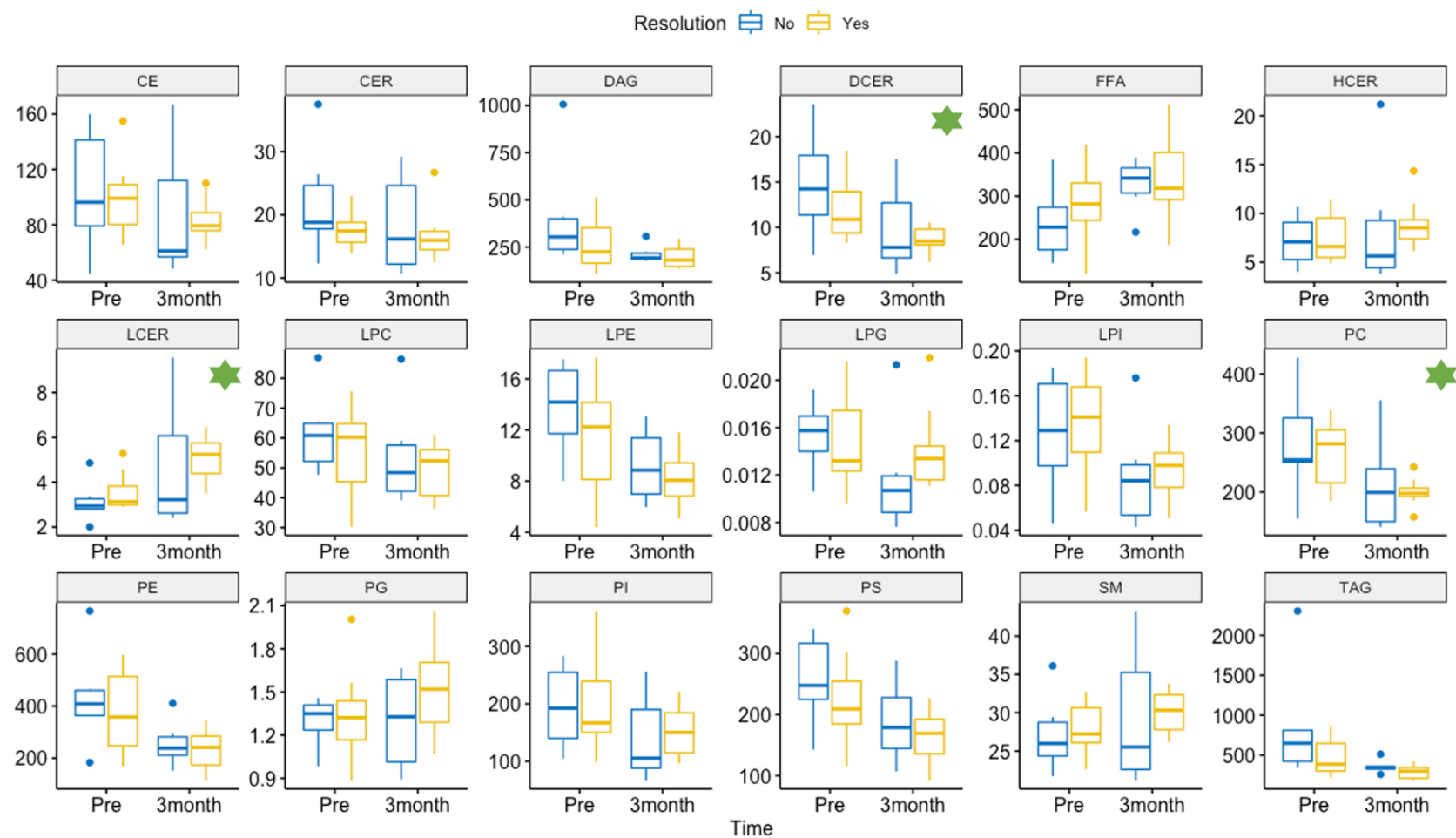


FIGURE 6-13 PRE-OP V POST-OP VALUES OF MEASURED LIPIDS BY T2D RESOLUTION SUBGROUP, NON RESOLUTION (BLUE) AND T2D RESOLUTION (YELLOW). GREEN STAR INDICATES SIGNIFICANT RESULT WITH Q VALUE <0.05 FOR T2D RESOLUTION SUBGROUP

6.4.5 Amino Acid and Lipid Correlations

Earlier sections of this thesis explored the association between T2D, serum amino acids and serum lipids respectively. However, while there are some linking hypothesis, direct links between these two major metabolite groups in this context are not obvious although there have been some suggestions. For example, in the context of T2D, some have hypothesised that one of the mechanisms leading to altered systemic metabolism is the increased accumulation of BCAA catabolic products within skeletal muscle, which subsequently alters lipid metabolism (655).

Correlation Analysis

As a further exploratory investigation, correlations between matching serum lipid and amino acid concentrations for the 36 patients investigated in chapter 4 and 6 are analysed and visualised in a Euclidean clustered heatmap below (*figure 6-14*). Euclidean hierarchical clustering is utilised to identify closely correlated clusters within a large dataset. BMI and HbA_{1c} are included in this visualisation.

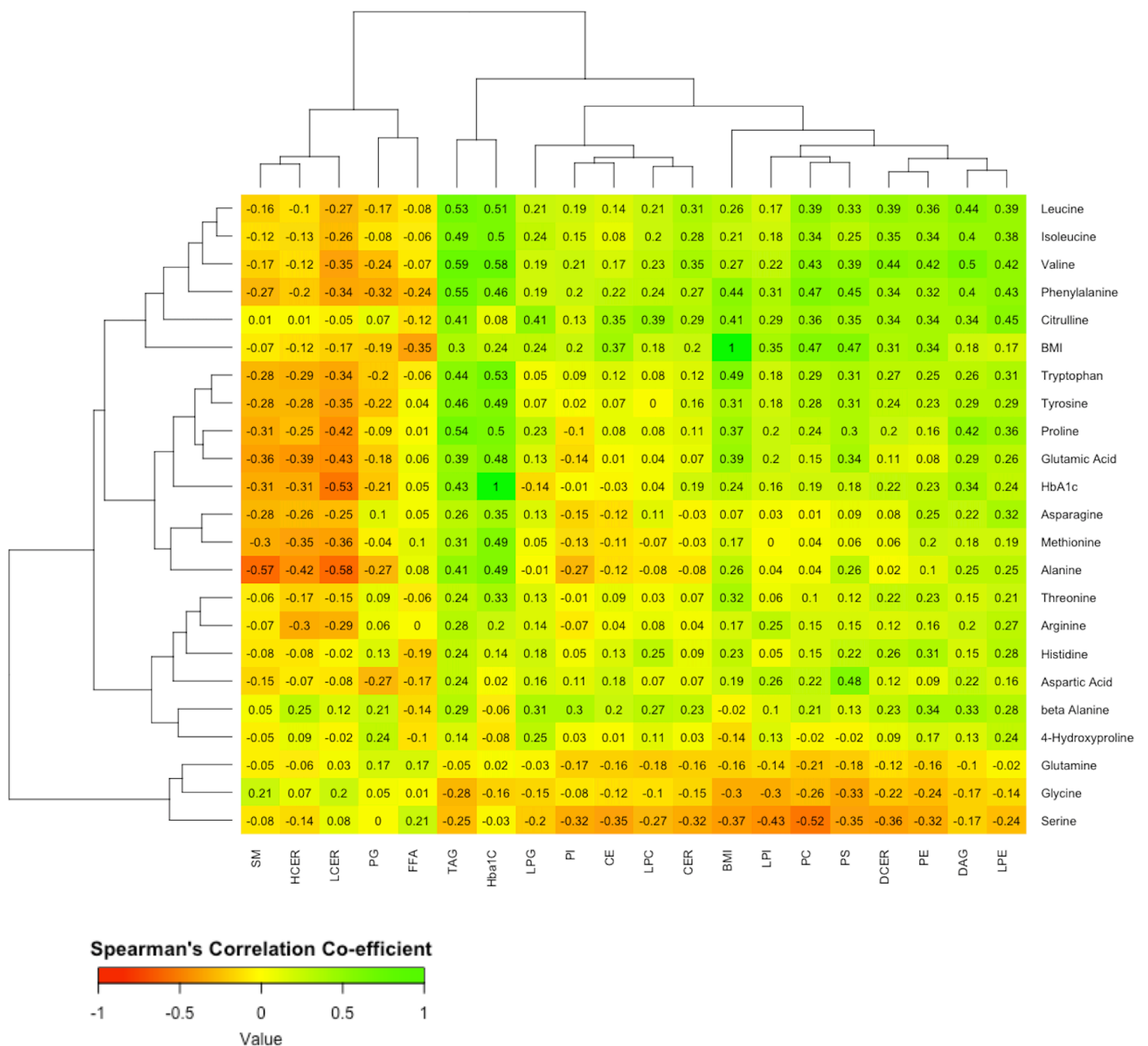


FIGURE 6-14 AMINO ACID AND LIPID SPEARMAN RANK CORRELATION HEATMAP WITH EUCLIDEAN CLUSTERING

Ranked Cross Correlation Analysis

With a large dataset containing both serum AA and lipid variables, ranked cross correlation analysis was employed to identify variables with the highest correlation associations and are displayed in *figure 6-15*. This method selects variables with the highest correlation

associations as well as calculates the relative strength of association between each pair. The data are displayed in an easy-to-read ranked graphical format.

Interestingly, out of all the strongest ranked inter-metabolite correlations, none were correlations between AA and lipids, suggesting no strong direct association between individual AA and lipids within this dataset.

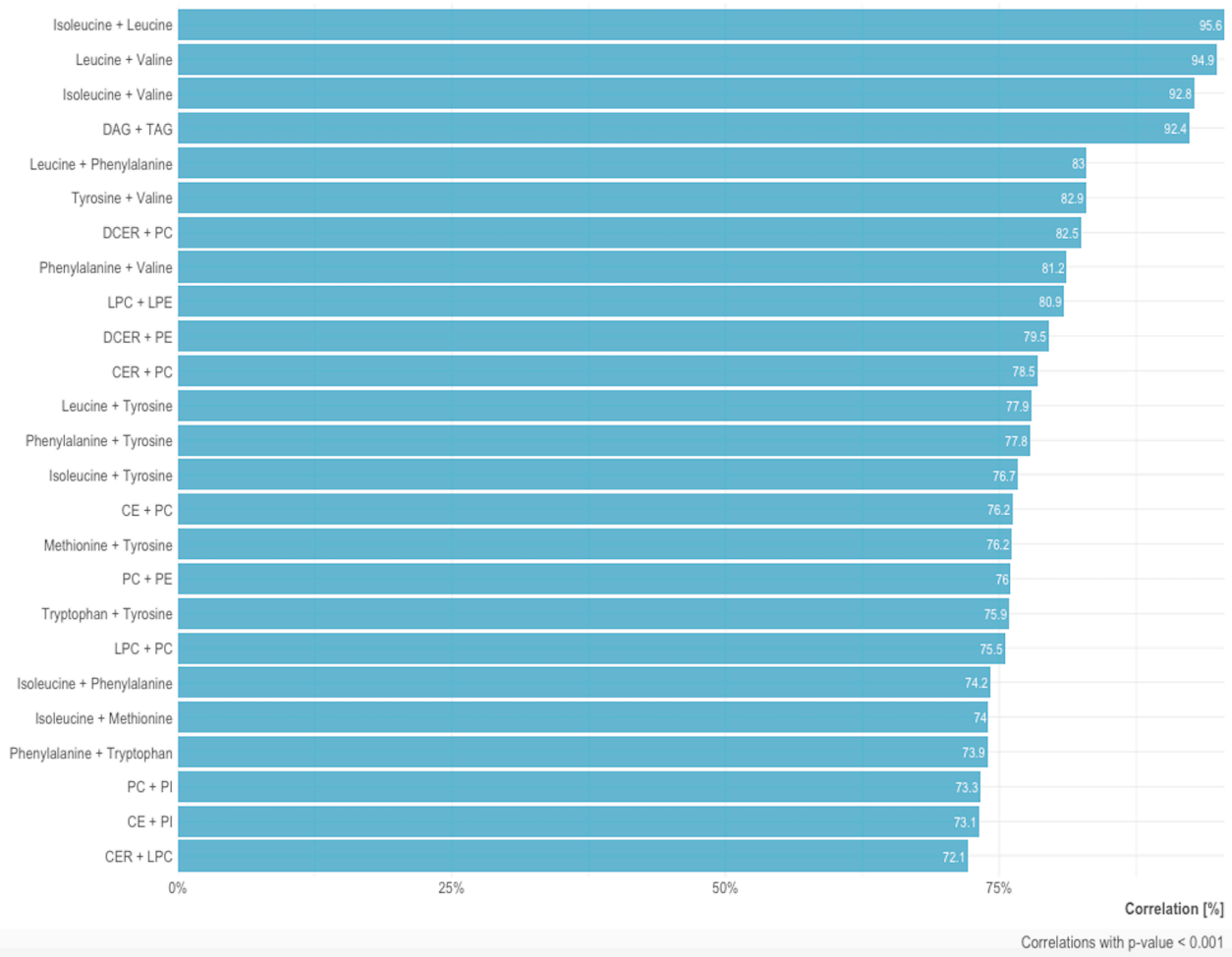


FIGURE 6-15 RANKED CROSS CORRELATIONS ANALYSIS OF MEASURED SERUM AMINO ACID AND LIPIDS, DISPLAYING TOP TWENTY-FIVE PAIRS OF METABOLITES WITH THE STRONGEST CORRELATION RELATIONSHIPS

To compare the relative strength of correlations between AA and lipids with BMI and HbA1c within this cohort, the respective ranked cross correlations are displayed below in *figure 6-16 & 6-17*. Four out of the top ten top ranked correlated features with BMI were lipid species

(PC, PS, CE and LPI). Only one out of the top ten ranked features with HbA1c were lipid species (LCER).

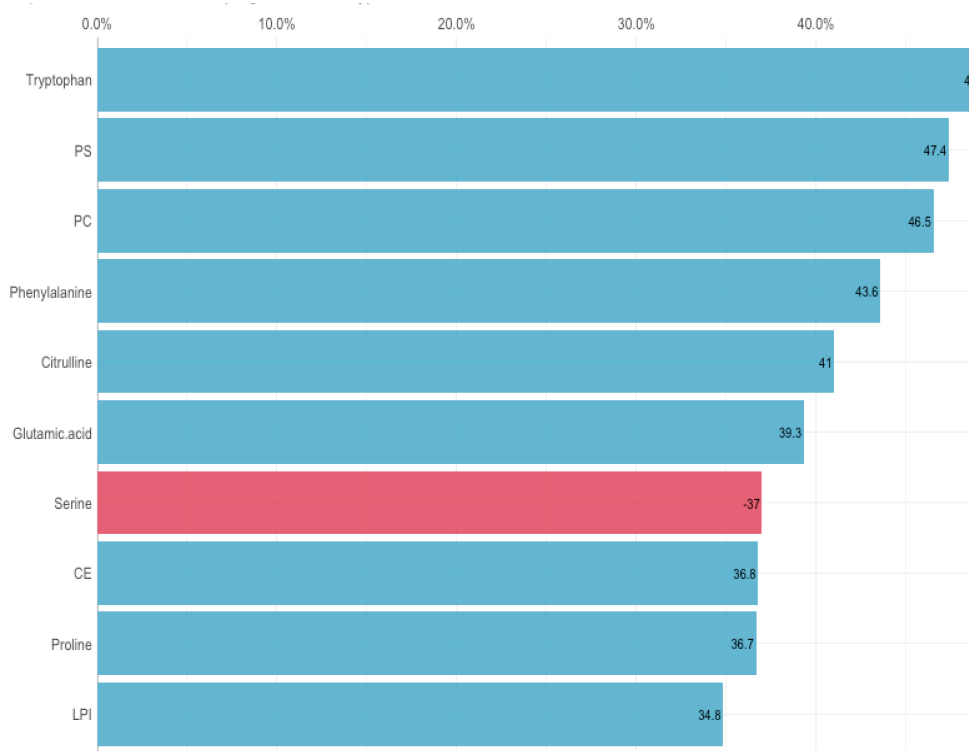


FIGURE 6-16 RANKED CROSS CORRELATIONS ANALYSIS OF MEASURED METABOLITES AND BMI, DISPLAYING TOP TEN METABOLITES WITH THE STRONGEST CORRELATION RELATIONSHIP WITH BMI

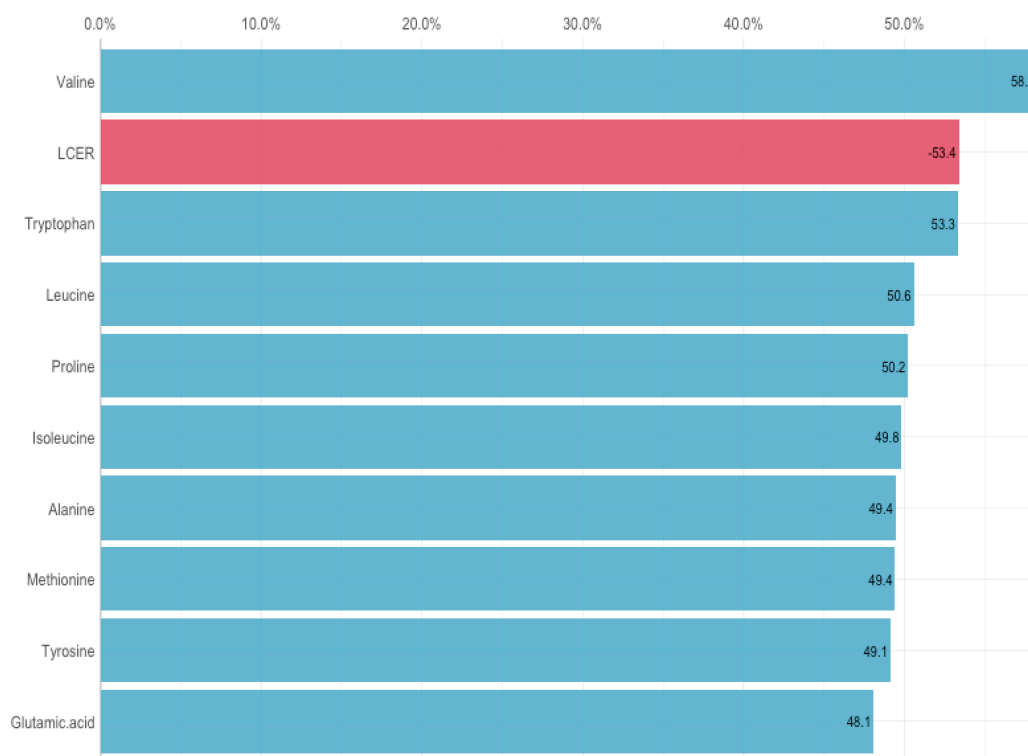


FIGURE 6-17 RANKED CROSS CORRELATIONS ANALYSIS OF MEASURED METABOLITES AND HbA1c, DISPLAYING TOP TEN METABOLITES WITH THE STRONGEST CORRELATION RELATIONSHIP WITH HbA1c

6.4.6 Summary of Results

A summary chart of the results is presented below (*figure 6-18*). In summary, those with T2D had significantly lower serum levels of summed HCER, LCER and SM when compared to those without.

RYGB induced widespread significant reductions in summed serum levels of CE, CER, DCER, DAG, TAG, multiple lysophosphatidyl (LPC, LPE) and phosphatidyl (PC, PE, PS) subspecies while increasing serum FFA and PI levels. VSG did not cause any significant changes in circulating lipids. When comparing T2D and ND subgroups, significant reduction in summed DCER, TAG, LPE, LPI, PC and increased in FFA were observed for both groups. Significant increase in summed LCER and decrease in PS and PE were observed in those with T2D only. CE, CER, and LPC were significantly reduced in those without T2D.

Many of the lipid subclasses showed significant correlation with BMI and HbA_{1c}. With BMI, the strongest significant correlation relationships were with PC ($r=0.44$), PS ($r=0.46$), CE ($r=0.31$), LPI ($r=0.33$), FFA ($r=-0.34$) PE ($r=0.29$) and TAG ($r=0.26$). For HbA_{1c}, the strongest significant correlation relationships were with DAG ($r=0.34$), TAG ($r=0.45$), HCER ($r=-0.33$), LCER ($r=-0.53$) and SM ($r=-0.30$). Thus, the correlation patterns between BMI and lipids were different from those patterns observed for HbA_{1c} and lipids.

Δ BMI after surgery did not significantly correlate with the Δ of measured lipids subclasses. Δ HbA_{1c} was significantly negatively correlated with Δ LCER ($r=-0.23$), Δ LPC ($r=-0.38$) and Δ SM ($r=-0.39$) only. T2D resolution was associated with significant reductions in serum DCER, PC and an increase in LCER levels.

When comparing the strength of relative lipid and AA correlations to BMI and HbA_{1c}, LCER was the only lipid within the top ten ranked correlations with HbA_{1c}. For BMI, four lipids (PS, PC, CE, LPI) were in the top ten.

	Pre-Op		3-months Post-Op			Corr Coeff	
	T2D	RYGB	VSG	DM	ND	HbA1c	BMI
BMI		↓	↓	↓	↓		
HbA1c	↑	↓	↓	↓	↓		0.24
CE	↓	↓	↓	↓	↓	-0.01	0.31
CER	↓	↓	↓	↓	↓	0.22	0.14
DAG	↑	↓	↓	↓	↓	0.34	0.11
DCER	↑	↓	↓	↓	↓	0.24	0.28
FFA	↑	↑	↑	↑	↑	0.01	-0.34
HCER	↓	↑	↑	↑	↑	-0.33	-0.13
LCER	↓	↑	↑	↑	↑	-0.53	-0.17
LPC	↓	↓	↓	↓	↓	0.05	0.13
LPE	↑	↓	↓	↓	↓	0.23	0.16
LPG	↓	↓	↓	↓	↓	-0.13	0.20
LPI	↑	↓	↓	↓	↓	0.15	0.33
PC	↓	↓	↓	↓	↓	0.21	0.44
PE	↑	↓	↓	↓	↓	0.23	0.29
PG	↔	↑	↑	↑	↑	-0.21	-0.22
PI	↓	↑	↑	↓	↓	0.00	0.17
PS	↓	↓	↓	↓	↓	0.18	0.46
SM	↓	↑	↑	↑	↑	-0.30	-0.11
TAG	↑	↓	↓	↓	↓	0.45	0.26

FIGURE 6-18 LIPIDS ANALYSIS RESULTS SUMMARY CHART. ARROWS INDICATE IN THE PRE-OPERATIVE SETTING THE TRENDS SEEN IN THOSE WITH T2D AND SUBSEQUENTLY DIRECTION OF METABOLITE LEVEL CHANGE 3 MONTH AFTER SURGERY IN RESPECTIVE RYGB, VSG, T2D AND ND SUBGROUPS. COLOURED SQUARES INDICATES SIGNIFICANCE OF $Q < 0.05$. SPEARMAN'S CORRELATION COEFFICIENT FOR METABOLITE GROUP WITH HbA1c AND BMI ALSO DISPLAYED.

6.5 Discussion

6.5.1 *Pre-Operative Lipid Signatures of T2D*

When comparing study participants with and without T2D prior to surgery, global multivariate modelling did not demonstrate obvious differentiation between the two groups. However, when each lipid subgroup was analysed using univariate analysis, significantly lower levels of summed serum sphingolipids: HCER, LCER and SM in those participants with T2D were observed. All three lipid classes were also demonstrated to be significantly and inversely correlated with HbA_{1c}. HCERs and LCERs are glycosphingolipids and are products of ceramide glycosylation. SMs are generated by CER and phosphatidylcholine.

I did not find statistically significant differences in other known lipid markers that have been discussed within the introduction and reported in literature to differentiate between those with and without T2D including DAG, TAG, CER, PC, LPC, and LPE, although the trend differences were all in keeping with known data.

Serum ceramides are diabetogenic and known to be elevated in those with obesity and T2D(545, 616, 632), with direct effects leading to pancreatic beta cell dysfunction and apoptosis(625). However, large population-based studies have demonstrated that sphingolipid correlations with BMI and markers of glycaemic control are not uniform. SMs which are downstream products of the sphingolipid pathway have been found to be inversely associated with T2D(250, 543, 656), in keeping with our findings. The dynamic changes within the lipidome of those with obesity are not universally upregulated and is not explained simply by an increase in lipid storage. Complex metabolic lipid dysregulation that is yet to be fully elucidated contributes to obesity, T2D and subsequent metabolic complications (657).

Sphingolipids can be synthesised de novo from FFAs. Higher saturated fatty acid intake was reported to be associated with higher plasma levels of CER, HCER and SM(658), although this was not the case with polyunsaturated fatty acids(659). I reported lower trend levels of

summed CER, SM and significantly lower levels of summed HCER in those with T2D when compared to those without T2D.

Data on the absorption of complex sphingolipids are lacking, but a significant effect solely due to diet is thought to be unlikely as SM and CER must first be degraded into smaller molecules before intestinal absorption(660). So therefore, any systemic changes to these lipids are likely to stem from processes of metabolic dysregulation.

Our findings of lower summed glycosphingolipids are consistent with several large-scale studies. HCER has been reported to be negatively correlated to BMI, biochemical features of obesity and T2D and incident T2D (616, 657, 661). On the other hand, lower serum levels of LCER and its association with T2D has only been otherwise reported once recently in 2021 (317). However, higher serum levels of HCER have also been associated with cardiovascular disease (CVD) risk(662) demonstrating a complex dynamic relationship that is yet to be understood.

Inhibition of HCER synthesis in mice and rat models was found to improve glycaemic control and insulin sensitivity(610, 663), which is in contrast to findings discussed above. However, in vitro studies have also demonstrated that the effects on insulin signalling by CER and HCER differ according to tissue types. While CER antagonises insulin signalling in both muscle cells and adipose tissue, HCER enhances insulin signalling in muscles cells but suppresses insulin signalling in adipocytes (664). Furthermore, in rat T2D models, muscle concentrations of CER, HCER, LCER were found to be significantly higher in T2D when compared to controls(665). In the same study, muscle LCERs were found to be decreased in T1D models suggesting a difference in underlying pathophysiologic pathways (i.e., T2D inflammation association). Another study comparing calorie restricted vs regular diet rats found that after 6 months of caloric restriction, the total skeletal muscle levels of ceramide increased but the products of ceramide metabolism: HCER and LCER decreased significantly (666). There are currently no published studies that have reported changes in human serum levels of HCER or LCER in the context of T2D.

LCER is synthesised by LCER synthase, this enzyme is activated by many agonists relevant to metabolic disease including platelet derived growth factor (PDGF), TNF- α , vascular endothelial growth factor (VEGF) and oxidised-LDL(667). LCER can directly activate cytosolic phospholipase A_{2a} (CPLA_{2a}), which is part of the TNF alpha inflammatory pathway with activation leading to a cascade of inflammatory molecules (668). LCER also activates NADPH oxidase and leads to a state of oxidative stress. This in turn is linked to a variety of disease phenotypes including T2D(667). Elevated levels of mitochondrial LCER have also been associated with mitochondrial metabolic dysfunction in T2D(667). Skeletal muscle LCER expression has been demonstrated to play a role in inflammation and development of IR (665). Both HCER and LCER have also been shown to reduce skeletal insulin signals by dephosphorylation of the Akt protein(663, 669). However, whether reduction in serum levels of LCER represents increased utilisation of LCER or expression of LCER within other tissue types or how this occurs, is again an area that requires further investigation.

Patients with CKD have also been reported to have higher levels of pro-inflammatory factors which are hypothesised to activate sphingomyelinases leading to the production of ceramides(630). The unifying theme of systemic inflammation in obesity and CKD hints at some similarities of underlying lipid metabolism disturbance which may be mechanistic

In the case of LCER, Fridman et al elegantly demonstrated that plasma levels were found to decrease in stepwise fashion when comparing lean healthy, overweight but healthy, T2D with obesity and T2D individuals with obesity and diabetic neuropathy(317). The authors speculate two opposing mechanisms may cause this effect 1) LCER is required for the formation of ganglioside, which is essential for axon-myelin interactions. Thus, a reduction in LCER leads to nerve injury. 2) increased ganglioside formation in individuals with T2D and obesity depletes the LCER pool.

I reported lower summed levels of SM in those with T2D however the association may not be as simple. Various SMs of specific chain length have been reported to be positively and inversely associated with T2D risk (657) suggesting that it is the balance of sphingolipid

metabolism rather than just a simple model of pure metabolite accumulation or consumption being responsible for pathological effects in obesity and T2D.

This study demonstrated, low summed serum levels of LCER, HCER and SM are associated with obesity T2D. At the same time, high skeletal muscle levels of LCER and HCER have also been associated with the same pathology. What is uncertain is the dynamic relationship between serum, muscle, and other organic levels of such metabolites. There is currently no published data on serum and multiple tissue types measured in synchrony, which would be important next step of investigation.

Finally, from OPLS-DA modelling comparing T2D v ND (see chapter 6.4.1, *figure 6-2*), while individual results were not presented, of the top 20 differentiating metabolites generated by the models, only three were not LCER/HCER/SM, namely LPC (20:0), LPC (20:1) and LPC (20:2). Although not significantly different when assessed as a group using univariate analysis, decreased circulating sum LPC concentrations have been reported within the literature to be associated with obesity, T2D and higher levels of inflammation(560). LPC binds to G protein coupled receptor (GPR) and may have a modulatory role in inflammatory pathways associated with immune cells such as neutrophils and macrophages(670). However, the precise link between LPC, inflammation and obesity are still currently not well understood. LPC concentrations also do not appear to normalise after weight loss interventions(557, 671), in keeping with our findings that surgery, in particular RYGB led to significant reductions of serum sum LPC levels.

6.5.2 *Surgery induced serum lipid changes*

This study demonstrated that bariatric surgery, in particularly RYGB leads to widespread and significant changes to serum lipids at 3 months post-op. Some, although not all, of these changes are due to weight loss and some of these changes also reverse trends reported in obesity and T2D (see chapter 6.4.6, summary *figure 6-8*). These changes cannot be explained solely by changes in post-surgery dietary intake alone (see *appendix V and VII*).

Lipids with BMI only correlation

Circulating concentrations of DAG, TAG, DCER and PE (541, 543, 545, 548) are known to be elevated in those with T2D. Although the difference was not significant when comparing the study participants with T2D and without T2D pre-operatively, all four of these lipid groups were significantly positively correlated with HbA_{1c} and BMI. RYGB led to significant reduction in the summed serum levels for all four lipid classes and in those participants with T2D, the reduction was significant for DCER, PE and TAG. The correlations with BMI suggest changes to DAG, TAG, DCER and PE due to surgery are associated with changes in BMI.

CE, LPI, PC, and PS were all positively and significantly correlated with BMI but not HbA_{1c} suggesting that these lipids are not associated with glycaemic control. In particular, CE, PC and PS were significantly reduced following RYGB.

Changes to pre-operative lipid markers of T2D

On the other hand, surgery led to increases in the circulating concentrations of sphingolipids HCER, LCER and SM at 3 months post-op, although this was only significant for LCER in the T2D subgroup. All three metabolites were previously highlighted as significant components of the T2D plasma metabolome. As all three groups were negatively correlated with HbA_{1c} but not BMI, is it possible that changes to these lipids due to surgery are induced by mechanisms beyond weight loss or that they confer an effect on glycaemic control, independent of weight.

Furthermore, SM degradation occurs at the proximal jejunum, relying on bile as digestion is via alkaline enzymatic pathways(672, 673). In theory, as RYGB bypasses the proximal gut, this procedure may exert an effect on SM metabolism, but the precise implications of this need to be further investigated. I did not find any significant changes in serum SM levels following either RYGB or VSG in the current study although both showed an increasing trend at 3 months post-operative, reversing the pre-operative finding of lower summed SM in those with T2D.

Free Fatty Acids

Excess plasma FFAs are observed in those with T2D. This is probably a result of increased fat intake but also a result of impaired insulin driven suppression of lipolysis which contributes to hyperglycaemia(575). Excess plasma FFAs also contributes to negative effects on insulin signalling (576) and leads to the formation of toxic metabolites such as DAG and CER, which also exert local lipotoxic effects when deposited in organs such as the liver and pancreas. Interestingly, I observed that RYGB led to a significant increase in serum FFAs at 3 months post-operative in this study and this effect occurred in all subgroups, although it was not significant in those who underwent VSG. There was no correlation between FFA and HbA_{1c} in the current study but significant negative correlation with BMI ($r=-0.35$). The changes in FFAs could be as a result of weight loss and the degradation of excess TAGs. This effect on FFAs in the early months after surgery has been reported in other cohort studies (674-676), although I lack longer term data, these studies have reported normalisation of FFA concentrations six months after surgery. These results insinuate that changes in FFAs may not directly mechanistically interfere with glycaemic control in an accumulative manner and that elevated serum FFAs in T2D may be a sign of systemic lipidomic dysregulation. This corroborates the hypothesis that is it lipid metabolism dysregulation rather than accumulation of metabolites that directly contribute to the pathophysiology of obesity and T2D.

RYGB vs VSG

It is very striking that only RYGB led to significant changes in almost all measured lipids and not VSG despite very similar patient subgroup size. Patients in both groups had significant reductions in BMI and HbA_{1c} at 3 months post-op and follow the same post-operative diet plan (see *appendix V*). This perhaps insinuates that RYGB induces a stronger effect and more significant changes to the serum lipidome and lipid metabolism, but whether all or which of these changes lead to an improvement in glycaemic control, or other metabolic benefits remains yet to be fully established.

Lipidomic changes after RYGB have also been examined in other studies. RYGB has been reported to lead to reduction of routinely measured serum cholesterol, LDL, and TG(677), our results also agree with reported generalised reductions of TAG, CE, CER, LPC, PC, PE (678, 679) after RYGB. However, various studies report different specific individual carbon chain length and lipid types to be positively or inversely correlated with HbA_{1c}, with no real consensus between studies (676, 678, 680). Analysis of specific carbon length lipids is beyond the scope of this thesis.

Serum changes in PC, SM and CER, have been found to occur independent of weight loss after RYGB(680), another studying comparing RYGB to gastric band found that RYGB led to early and persistent change in serum concentrations of PC, SM and CER, again independent of weight loss. These changes were not observed in those with gastric band implying mechanism beyond weight loss, dietary restriction and linked to RYGB. The authors also concluded that RYGB leads to alterations of sphingolipid metabolism (680). Although lower levels of CER were found in participants with T₂D in this study, this is not in keeping with published literature. however, significant reductions of CER were noted after surgery, which would be more in keeping with reported mechanisms.

While the precise molecular mechanisms are yet to be fully elucidated, evidence is now also emerging from other studies linking these lipids to systemic inflammation. The reduction in ceramide levels after RYGB have been reported to be inversely correlated with insulin sensitivity and TNF- α levels (677). As discussed in the introduction, CERs have been

hypothesised to be mediators of proinflammatory state, associated with activation of inflammatory mediators such as TNF- α (632, 633) and modification of serum levels may be in part responsible for improved insulin sensitivity after gastric bypass surgery (677).

Reported changes in the literature are not always in agreement, Carlsson et al followed up 220 patients 2 years after RYGB, in keeping with our results, they reported lower pre-operative levels of SMs in individuals with T2D compared to those without. At 2 years post-op, those without T2D had a significant reduction in SM levels but in those with T2D, SM levels did not change significantly despite HbA_{1c} normalisation. All groups had an increase in serum SMs when adjusted for weight suggesting the effect of RYGB on SM metabolism occurs irrespective of T2D status (656). Both our studies agreed that no direct correlation between SM and BMI was found.

Although RYGB seems to induce widespread changes to the lipidome, the beneficial reduction of serum SMs (681, 682) and increase in HCERs (557) have both been demonstrated after non-surgical weight loss too. This may indicate signalling mechanisms linked to these changes are responsible for the health benefits conferred by weight loss. Lipidomic changes induced by RYGB, in particular, alterations to sphingolipid metabolism seem to be associated with improved glycaemic control. The most pressing question to arise is what are the molecular mechanisms through which RYGB induces these lipidomic changes? And how or which of these changes specifically confer glycaemic and metabolic benefits?

6.5.3 Δ Delta Analysis

To further differentiate the effect of surgery and the potential influence on BMI and HbA_{1c}, the Δ difference of measured lipids were calculated and assessed for correlation with Δ BMI and Δ HbA_{1c}. I found no statistically significant correlations between Δ BMI and the Δ of measured lipids. However, Δ LCER ($r=-0.23$), Δ LPC ($r=-0.38$) and Δ SM ($r=-0.39$) were found to be inversely and significantly correlated to Δ HbA_{1c}. Although, causality cannot be demonstrated by this study alone, these findings add to the collection of evidence that points towards an important association of sphingolipid and LPC metabolism in the pathophysiology of obesity and T2D.

All three lipid groups have been previously discussed. Reduced serum levels of LCERs and SMs were associated with T2D (632) and bariatric surgery led to an increase in serum levels along with an associated decrease in HbA_{1c}. The association reported with LCER makes investigating interventions targeting LCER synthesis even more interesting, whether pharmacological or via gene manipulation, the LCER pathway potentially has a significant role in T2D and metabolic disease.

On the other hand, LPC levels were found to be lower in those with T2D, although this difference was not significant in our analysis. Surgery led to a reduction of LPC however, while Δ LPC inversely correlated with Δ HbA_{1c}, overall LPC levels did not correlate with HbA_{1c} or BMI. Reduced levels of serum LPC has been reported as a marker of obesity (560, 626) with specific chain lengthened LPCs inversely associated with dysglycaemia (683). Low serum LPC concentrations have also been associated with CVD (684).

LPC metabolism involves a complex enzymatic cascade in which LPC is derived from hydrolysis of PC by the enzyme phospholipase A₂ (PLA₂) or conversion from PC by the enzyme lecithin-cholesterol acyltransferase (LCAT). LPCs are transported back to the liver via albumin and alpha 1 acid glycoprotein. In the liver, LPC is converted back to PC by lysophosphatidylcholine acyltransferase (LPCAT) in the presence of Acyl-CoA. Over expression of PLA₂ increases LPC content in LDL which induces harmful inflammatory signals via several receptors such as G protein-coupled receptor (685). An excess LPCs also

causes atherosclerotic plaques and can lead to endothelial dysfunction. No enzyme that can degrade LPC into harmless metabolites has been identified in human circulation(685, 686). It would be reasonable to hypothesize that the association observed between Δ LPC and Δ HbA_{1c} is again via mediation of systemic inflammation due to the effects of bariatric surgery. Interestingly, metformin treatment also leads to reduced levels of circulating LPCs(356).

In vivo experiments have found LPC to be elevated in liver and skeletal muscles of mice with obesity and T2D (687). Researchers found that blocking Palmitic acid's (PA) conversion to LPC using calcium dependent phospholipase inhibitors prevented insulin resistance induced by PA. The effect also insinuates that LPCs are an important lipid intermediate linking saturated fatty acids to IR(687).

6.5.4 *T2D Resolution*

When comparing those with and without T2D resolution at 3 months, I found no significant differences in the respective pre-operative lipidomes. Significant reductions were observed in the T2D resolution group for the lipids DCER, LCER and PC at 3 months. The importance of ceramides and their likely role in obesity, inflammation and T2D has already been extensively discussed.

A similar study which followed 17 patients with T2D who underwent RYGB found contrasting pre- and post-operative lipid profiles between those with and without T2D remission. Higher pre-operative levels of long chain fatty acid triglycerides and long chain phospholipids (PC/PE) were associated with T2D remission at 2 years post-op(676). The authors also report that patients T2D remission had higher levels of metabolites associated with the TCA cycle suggesting a higher level of baseline metabolic activity. These results are in contrast to ours, but direct comparison is difficult due to the shorter follow up window in our study.

DCER the precursor to CER is also a known predictor of T2D(619) but the mechanisms are poorly understood. A hint towards the link between DCER and T2D prediction may be found by considering NAFLD. NAFLD is often associated with the umbrella of disease encompassing metabolic syndrome including T2D. There is increased secretion of VLDL enriched with DCER by the NAFLD liver and VLDL levels are also associated with insulin resistance(688). Plasma DCER levels positively correlated with severity of NAFLD (689), and liver biopsies taken from patients with NAFLD found that the increased expression of mRNA enzymes associated with sphingolipid metabolism to also be positively correlated with the severity of hepatic steatosis and inflammation (689). So, it is hypothesised that the increase in plasma DCER found in both NAFLD and T2D dyslipidaemia is due to altered sphingolipid metabolism (689). Within our cohort, RYGB led to significant reduction in DCER levels, and it would be reasonable to hypothesise that the modulation of DCER levels by bariatric surgery may be in part responsible for the improvement in obesity associated co-morbidities such as NAFLD.

A significant reduction in PCs was observed across T2D, ND and RYGB groups but not in those who underwent VSG at 3 months post-operative. Serum levels of this lipid group was strongly correlated to BMI ($r=0.47$) but not HbA_{1c} or Δ HbA_{1c}. Although BMI was reduced in both T2D resolution and non-resolution subgroups, this was only significant in those with T2D resolution. The close association between PC and BMI would explain our findings, and it does not seem reductions in PC are directly associated with glycaemic control. A study comparing a small group ($n=10$) of matched lean and individuals living with obesity also reported similar associations between some PC species and body weight(690). The significant reduction in BMI for those with T2D resolution would also suggest that there are other weight dependent factors which are important for surgical T2D resolution.

6.5.5 *Beyond Type 2 Diabetes*

The overwhelming lipid changes secondary to bariatric surgery may also explain the benefits beyond the improvement of T2D and glycaemic control. NAFLD in obesity is reversed by bariatric surgery including the reduction of hepatic volume and fat content (691). The reduction of serum TGs/PC may be responsible for this in both DM and ND patients (691). Originally, it was believed that fatty acids originating from adipose tissue were responsible for liver TG deposits. However, it has now been revealed that PCs which form key parts of structural membranes and plasma lipoproteins are significant precursors to hepatic TGs (692). Surgery may induce this pathology by reduction of serum PC concentrations, this association may be weight dependent, suggested by the significant correlation relationship between PC levels and BMI.

Some serum SM species have also been identified as markers of progression from NAFLD (fatty liver) to non-alcoholic steatohepatitis (NASH). Following VSG, those with resolution of NASH resolution display a SM profile similar to those with NAFLD suggestive of a degree of reversibility. There is potential for these SM signatures to be used as serum markers to monitor NASH progression (693). Also, if mechanistic drivers of this process are identified and found to contribute to the progression of disease, then there is potential for the development of other targeted treatments such as drugs aimed at enzymatic blockade.

I previously discussed possible overlapping mechanisms that underly gestational diabetes (GDM) and T2D. Over 1/3 of those with GDM develop T2D within ten years, and estimated lifetime risk of over 50%(694). A large prospective lipidomic study of over 1000 women reported an upregulation in TAG/DAG biosynthesis prior to onset of T2D. Downregulation of sphingolipid metabolism leading to a reduction in SM, HCER and LCER were also observed, which is a T2D signature consistent with our findings. The authors conclude underlying lipid dysregulation is present in GDM women who subsequently progress to T2D even in the early post-partum period and that impaired lipid metabolism is contributory to incident T2D(567). Identifying these signatures may be useful to stratify those at risk of progressing to T2D where preventative interventions may be utilised.

Ceramides have also been implicated in diabetes kidney disease (DKD). Rodent models have found renal ceramides to be local inducers of DKD. Genetic and pharmacological techniques were employed to lower renal ceramide led an improvement of renal function and the reversal of histological changes found in DKD. In humans, studies have shown that sphingolipid levels can be used to differentiate between those with T2D and those with T2D renal nephropathy (695). The significant influence of bariatric surgery on these metabolites and their effect on DKD may be beneficial or used to identify further targets of non-surgical intervention.

Finally, microbial carbohydrate metabolism also influences lipid metabolism within the gut by promoting expression of genes necessary for lipid oxidation(696). Microbial diversity has been demonstrated to affect BMI, serum TG and HDL irrespective of age and gender(697). The microbiome can also have a direct influential role in host lipid metabolism. Sphingolipids, sterols, fatty acyls and glycerolipids have all been shown to have species of microbial origin(698), which can affect host lipid metabolism(699). Although beyond the scope of this thesis, evaluation of the faecal metabolome/lipidome before and after surgery would provide an indication of the functional influence of the gut microbiome(698, 700). This topic has been extensively reviewed recently by Lamichhane et al (698) but in summary, the human gut microbiome gives rise to bioactive lipid metabolites that very likely plays a role in host lipid homeostasis and by extension, influences states of disease. Surgery leads to increased gut microbiota richness which in turn leads to control of adiposity by regulation of lipid metabolism(701). However, methodological challenges currently inhibit full and informative sampling, analysis & interpretation of faecal metabolomic and metagenomic data.

6.5.6 *Lipids and Amino Acids*

So far, the effect of bariatric surgery on AA and lipids metabolites and the associated changes related to T2D has been demonstrated separately throughout this thesis. Human metabolism is a complex and dynamic network, so while investigating the metabolite groups separately allows for manageable experiments and can generate good hypotheses, it was of research interest to evaluate the relative associations and changes between these two major metabolite groups.

Metabolomic studies reported throughout the literature and including this study have found that AA, in particularly BCAA & AAA and metabolites may have a larger effect on glycaemic control and insulin resistance than lipids. However, the metabolism of the two are still closely linked. In animal studies, BCAA supplementation promoted insulin resistance only when associated with a high fat diet(702). Within this study cohort, I demonstrated strong correlations between TAG/DAG and BCAA and AAA (Chapter 6.4.5, *Figure 6-14*) but interestingly not with FFA.

The cause and effect of increased BCAA levels in T2D have been thoroughly discussed in chapter 4, and BCAA levels are also elevated in states of starvation (703). Intriguingly, although these are contrasting metabolic states, the underlying mechanisms are not dissimilar and illustrate the association between AAs and lipids. Both are a result of decreased glycolysis (reduced insulin secretion in starvation and insulin resistance in obesity) and increased muscular fatty acid oxidation (alternate energy source in starvation and excess in obesity (703)).

When assessed with Euclidean hierarchal clustering, (see *Figure 6-14*) BMI was found to be most closely correlated in clusters with BCAA (leucine, isoleucine, Valine) as well as AAA-phenylalanine. BMI was found to be closed correlated with the lipids LPI, PC, PS, DCER, PE DAG and LPE. HbA_{1c} is most closely correlated with AAA (tryptophan, tyrosine) as well as proline and glutamic acid (glutamate). HbA_{1c} was most closely correlated with TAGs.

When assessing lipid and AA metabolites for combined ranked cross correlations, valine, LCER and tryptophan were identified as the top ranked metabolites for correlation with HbA_{1c}. Implying significant association for these metabolites and their associated pathways with glycaemic control compared to all the metabolites reports within the data chapters. Interestingly, LCER was the only lipid within the top ten ranked correlations with HbA_{1c} signifying that the link between glycaemic control and amino acids were greater than lipids within this cohort.

Tryptophan, PS and PC were top ranked for correlations with BMI suggesting a close association between weight and these metabolites. In total, only four out of the ten top ranked compounds were lipids suggesting the strongest weight related correlation relationships are with serum amino acids.

Information obtained for one metabolite group may also yield information regarding another. The associations described between alanine to serine ratio to 1-deoxysphingolipids formation is a good example of this. I have previously discussed that 1-deoxysphingolipid(1-deoxySLs) are atypical sphingolipids formed when alanine or glycine is used as an amino acid substrate when there is depletion of the serine pool(321). Although quantification of 1-deoxySLs was beyond the scope of this study, I found the alanine: serine ratio to strongly correlate with HbA_{1c} ($r=0.48$). Its reduction after surgery was significantly associated with T2D resolution.

The use of amino acid ratios as proxies to mechanisms leading expression of the T2D phenotype may perhaps be useful in exploratory studies. The precise mechanisms leading to these relationships still need to be investigated and prospectively evaluated but it also has the potential to be used in clinical practice either to identify individuals at risk of T2D, predict response or monitor progression of treatment.

6.6 Conclusion

In conclusion, within this study cohort, significantly lower serum levels of the sphingolipids HCER, LCER and SM differentiated between those with and without T2D at baseline. These sphingolipid metabolites in particular ceramide, have been implicated in T2D with direct effects on pancreatic beta cell function and cellular apoptosis. Ceramide is also implicated in the activation of the systemic inflammatory cascade contributing to systemic inflammation.

Bariatric surgery, in particular RYGB led to widespread changes to serum lipids at 3 months post-operative. Some of these changes were associated with weight and not glycaemic control, whilst the opposite scenario was observed for other metabolites. It appears that sphingolipids, in particular LCER and HCER are significantly associated with mechanisms that are either directly or indirectly related to obesity, systemic inflammation and glycaemic control. Importantly, changes in these lipids are also observed in individuals who undergo non-surgical weight loss interventions further suggesting that modification of these metabolites by surgery led to the observed metabolic benefits. Changes in serum sphingolipid metabolism due to bariatric surgery also reverse directional trends in serum lipids dysregulation observed in obesity associated diseases such as NAFLD, diabetic kidney disease and diabetes neuropathy.

The modulation of serum lipids by bariatric surgery and its associated improvement in glycaemic control and obesity related co-morbidities suggest modulation of lipids metabolism is likely to be an important mechanism of action of the health benefits conferred by bariatric surgery.

Part III: Clinical Applicability of Findings

Chapter 7: Predictors of Surgical Diabetes Resolution & Random Forest Modelling

7.1 Current Predictors of Surgical Diabetes Resolution

7.1.1 *Prediction Models*

Clinical prediction models or scores are a useful tool for guiding clinicians in selecting appropriate treatment options and can also be used to counsel and involve patients in a shared decision-making process. In the context of this thesis, metabolic profiles that include serum amino acid and lipid information have previously been shown to successfully predict pre-diabetes and progression to T2D (704, 705). An example is the amino acid index risk score, which was generated using correlation-based network analysis and can be used to predict risk of T2D and T2D complications(706). This score is now used in Japan to provide early risk assessment within populations and to select individuals for early intervention(707). However, while there are various models proposed within the current literature, there is still no effective or reliable method of predicting the probability of surgical resolution of T2D and the potential of subsequent relapse. Current models include clinical predictors such as duration of T2D, severity of T2D and patient characteristics such as age, sex, waist circumference, weight and BMI (97).

7.1.2 Current Risk Prediction Models of T2D Remission after Surgery

Scoring models derived from a variety of cohorts using various predictor variables that predict the probability of T2D remission after bariatric surgery have been reported in literature and are summarised below in *table 7-1*. The follow-up time frame, operations that participants underwent and the T2D remission criteria used in conceiving the models are also listed.

Name of Model	Predictor Variables	Follow up Prediction Timeframe	Operations	Remission Criteria
ABCD Score(708)	• Age • BMI • T2D Duration • C Peptide Level	At least 3 Years	RYGB/MGB	American Diabetes Association (ADA)(345)
Diabetes Remission Score (DRS) (709)	• Age • BMI • T2D Duration • Microvascular Complications • Macrovascular Complications • Pre-Op Insulin use • Stimulated C Peptide	At least 1 Year	IISG / IIDSG	ADA
DiaRem Score(710)	• Age • HbA1c • T2D Medications • Insulin Use	Within 5 Years	RYGB	ADA
DiaRem2 (711)	• Age • T2D Duration • HbA1c • Insulin Use	At least 1 Year	RYGB	ADA
Ad-DiaRem(712)	• Age • T2D Duration • Hba1c • Insulin Use • T2D Medication	1 Year	RYGB	ADA

	• Number of T2D Medications			
5y-Ad-DiaRem(713)	• Pre-Op ○ T2D Duration ○ T2D Medication ○ HbA1C • 1 Year Post op ○ Medications ○ Fasting Glucose ○ % Weight Loss ○ T2D Remission Status	5 Years	RYGB	ADA
DiaBetter(714)	• Duration of T2D • HbA1C • T2D Medication and Insulin	At least 2 Years	RYGB/VSG	DSS-II Consensus group Criteria
Individualized Metabolic Surgery Score (IMS)(715)	• Duration of T2D • HbA1C • T2D Medications • Insulin Use	At least 5 Years	RYGB/VSG	ADA
Metabolic Surgery Diabetes Remission (MDR) Score(716)	• Age • T2D Duration • Hba1C • HOMA2-B ○ (Fasting Glucose & C Peptide)	At least 1 Year	RYGB/VSG	ADA

TABLE 7-1 T2D REMISSION PREDICTION MODELS

IISG -ILEAL INTERPOSITION WITH SLEEVE GASTRECTOMY. IISDG- ILEAL INTERPOSITION WITH DIVERTED SLEEVE GASTRECTOMY. HOMA-2 HOMEOSTASIS MODEL ASSESSMENT INDEX – ESTIMATED USING FASTING GLUCOSE AND C PEPTIDE

All scoring models are built retrospectively and there is great heterogeneity within the selected patient groups, with significant differences in baseline participant characteristics including weight, BMI and type of operation. Most scoring systems described in the table

are aimed at the prediction of T2D remission one year after surgery and rarely longer term (>5 years) except for IMS, which was developed as a tool to help inform the choice of procedure depending on the severity of disease(715).

A recent systematic review and meta-analysis performed in 2021 of models predicting T2D remission after surgery found the ABCD and DiaRem scores to have been widely validated in different populations (717). While some of the newer prediction models such as DiaRem2, 5yr Diarem and MDR showed better discrimination, these models lack external validation studies. A lack of high quality models for long term T2D remission were also noted (717).

Plaek et al also recently comprehensively reviewed eleven predictive scoring and eight regression models including those listed above by applying the scoring models to a cohort of 250 western patients to explore the predictive value of each for T2D remission 1-year after surgery. This study reported the IMS (Area Under the Receiver Operating Characteristic curve: AUROC = 0.912), DiaBetter (AUROC = 0.907) and Ad-DiaRem (AUROC = 0.903) scores all predicted 1-year T2D remission reliably (718). In general, most scores predicted short term T2D remission well with all the highly performing models including at least three predictor variables that are correlated and are indicative of the severity of T2D.

This is no surprise when considering it is now well reported that those who are younger in age, with a shorter duration of T2D, less severe T2D (indicated by lower HbA_{1c}, lower number of medications and the absence of insulin use along with higher C peptide levels) and more weight loss have a higher probability of achieving T2D remission and lower probability of future relapse (719-722). Some report that baseline BMI does not seem to be strongly associated with T2D remission(97) but others suggest weight loss is strongly correlated with T2D remission(718). The positive prognostic factors for T2D resolution listed above are associated with the degree of irreversible beta-cell death(716), which to our current understanding is the most significant underlying mechanism influencing post-operative T2D remission and relapse despite the powerful anti-diabetes effects of surgery(123, 130, 723).

Existing prediction model and scores have also been evaluated in cohorts of East Asian, Chinese, Hispanic and Latino populations with contrasting results(724-729) signalling a lack of consistency and incomplete understanding of the influence related to population characteristics on T2D and these models. When tested in an ethnically diverse cohort, authors found models predicted those likely to have T2D remission well but was poor in the prediction of those that did not(730). Models also need to be validated against individual procedures, Ad-DiaRem was found to perform better in predicting 5 Year T2D remission after RYGB compared to DiaRem but not after VSG(731). Authors have also applied prediction models to patients who underwent BPD/DS and found T2D remission to be reliably predicted (732). The 5-year Diarem score incorporates post-operative markers, which are obviously not available at the time of consultation for bariatric surgical interventions, so while it shows promise in predicting longer term T2D resolution, its true clinical applicability is limited.

None of the existing scoring methods include data obtained by metabolomic analysis. Highly performing models can only be developed if large and high-quality datasets are available, and models developed require both extensive internal and external validation. Parameters currently utilised in existing prediction models are widely available and thus is widely applicable in the primary care or bedside clinical setting. This is both an advantage and a limitation. The inclusion of metabolomic data linked to underlying T2D pathophysiology may yield stronger prediction models if applied appropriately.

Some have attempted to incorporate other biomarkers into current prediction models and found variables such as serum amino acid profiles(733, 734) or post-op GLP-1 levels (735) have an enhancing effect on existing prediction models of weight loss or T2D resolution but these models have yet to be validated amongst larger and more diverse populations. Ultimately, no single model is perfect, and caution must be exercised when applying models to populations different to those used to derive the scores.

When considering long (>5 years) and longer term (>10 years) prediction, current literature is less robust. There is not a high amount of longer-term data available yet, therefore it is

not possible to build high quality models. Current models tested on longer term data have shown their predictive power to decrease significantly with time(736).

8546 patients from the Swedish Obese Subjects (SOS) cohort were evaluated retrospectively and complete T2D remission at 5 years was again found to be associated with shorter duration of T2D, insulin treatment, age and baseline HbA_{1c}(737). However, when extrapolated to longer term, 222 individuals were assessed for T2D outcomes at 10 and 15 years after surgery. The authors found that DiaRem, Ad-DiaRem and DiaBetter performed poorly when used in attempts to predict longer term T2D remission. Within this cohort, it was reported that 2-year weight change alone was a superior metric in predicting T2D long term remission than any score(738), highlighting the potentially significant role of weight loss in achieving long term glycaemic control or the fact that research has yet to identify key differentiating markers in those with long term T2D resolution. A similar study found that using T2D duration alone as a predictor variable performed to the same predicative ability compared to risk scores when used to prediction of 10-year T2D remission(736).

All current models have their limitations but in simplified terms most only measure T2D severity and do not incorporate or explain any other factors. In time with advancing understanding of the underlying pathological mechanisms and risk factors along with the availability of long-term and larger clinical trial datasets, prediction models will improve. This is also where multi-‘omics’ precision-based medicine incorporating genetic, metabolic, functional, biochemical and clinical information has the ability to further enhance current practice. As we stand, the key clinical point is early intervention in those with obesity and T2D improves chances of long term T2D resolution(721). Consideration for surgery should not be delayed until T2D is poorly controlled(22) in order to reduce to risk to long-term diabetes complications.

Several amino acids and lipidomic signatures associated with T2D and surgical T2D resolution have been identified and presented in earlier chapters of this thesis. The following chapter is a proof-of-concept study and first tests the applicability of a machine learning algorithm to differentiate between those with and without T2D. Subsequently, this

method is applied to the dataset generated from the studies within this thesis with the aim of evaluating whether any of the metabolites profiled pre-operatively may have a role in prediction of surgical T2D resolution.

7.2 Random Forest

7.2.1 *Introduction to Random Forest Modelling*

Random Forest (RF) (739) is a powerful and popular non-parametric ensemble based machine learning algorithm which can perform classification or regression analysis. RF constructs a forest of decision trees to define the output; it is a mean of all outputs in regression or majority in classification trees. RF selects the most relevant features out of all possible features in a large dataset and ranks features based on predictive ability for classification or regression (740).

In brief, a collection or 'forest' of individual decision trees are constructed from a dataset. A training set is created randomly from a subset of samples and variables. Around $2/3$ of the data is included and is termed the training or 'bootstrap' set. The remaining $1/3$ are termed the 'out-of-bag' (OOB) samples. Decision trees are grown using the training data by recurrent partitioning. In each tree 'node', variables are selected randomly from the dataset and assessed for their ability to classify the data. At each split it asks what features will split the observations in a way that will lead to two groups that are as different from each other as possible. With the resulting members of the subgroups being similar to each other.

This splitting process continues repeatedly until terminal or pure nodes (also termed leaf) contain samples belonging to the same class only. The modelling or training process is complete when the tree is fully grown, and the model can be used to predict the class of new samples. This collective method makes RF models a strong classifier with the fundamental ensemble concept of 'wisdom of crowds' as a collection of uncorrelated decision trees operating as a unit will outperform any single individual models. Over training is reduced as the random splitting of variables leads to low correlation between trees.

The RF output is a list of selected features with an estimated variable importance measure to explain the contributory importance of the feature in the model (740) and is useful in selecting which features are useful for classification. RF has been used in the prediction of factors associated with clinical outcomes successfully in cases of T2D, stroke and

cardiovascular disease (741-744). It is a popular method due to its accuracy and the ability to handle large datasets where there are often more potential predictor variables than samples, such as the case with many metabolomic datasets. RF also handles non-linear data, and the resulting models tend to not overfit (745, 746). RF have also been shown to outperform other ML methods for imputing missing LCMS data(747), here the *RFimpute* function from randomForest R package was used to impute missing values. For the reasons listed above, random forest-based modelling was selected as an appropriate method in this exploratory study.

A further advantage is that cross validation is built into RF as each tree has its own bootstrap (training) and OOB (test) data. The out-of-bag (OOB) error is calculated to help avoid the need for a further independent validation step(740). The Receiver Operating Characteristic (ROC) curve and the area under curve (AUC) statistic is commonly calculated and used to evaluate and compare different machine learning classification models. This statistic describes the sensitivity of the model; in other words the ability of the model to differentiate between individuals with and without the predicted outcome(748). The ROC curve is a probability curve with the true positive rate and false positive rate on the respective x and y axis. The closer the AUC to 1, the better the model is at classify those with and without disease, AUC of 0.5 means the model is unable to differentiate between groups.

7.2.2 *Random Forest Applied to T2D and Surgical T2D Remission*

Most current predictor models utilise parameters that are easily available within routine clinical practice. Recent trends and efforts have driven the idea of more personalised care and precision-based medicine. Although metabolomic markers are currently not commonly measured in a clinical environment, if identified, they have the potential to be powerful in contributing to the predictive power of models. Exploratory work has already demonstrated the addition of metabolic data such as amino acid profiles may enhance the predictive ability of existing prediction models of T2D remission after bariatric surgery (733), although some have also shown otherwise(225). Within a similar theme, metabolic signatures linked to AA dysmetabolism have been identified and used to predict progression in patients with gestational diabetes to T2D and the model was demonstrated to be superior to current models using only clinical parameters(567). Lipid profiles have also been utilised in prediction of future cardiovascular events in T2D cohorts(749).

T2D pathogenesis involves complex dysregulation of lipid and amino acid metabolic pathways and earlier chapters have demonstrated bariatric surgery leads to modulation of these changes. As a final exploratory study, the following chapter first tests the applicability of this machine learning algorithm when applied to the data derived from this study cohort. Then using this method, I evaluated whether any of the metabolites profiled might have a role in prediction of surgical T2D resolution.

7.3 Results

7.3.1 Type 2 Diabetes v Non-Diabetes

To demonstrate the applicability of random forest in this cohort, I first applied classification modelling to compare those with and without T2D pre-operatively. RF are traditionally reported with two measures, mean decrease in accuracy and mean decrease in gini importance. The gini importance is calculated to represent the purity of nodes, meaning the probability that the variable has been wrongly classified if it is chosen randomly(750).

The *varImp* function within the *caret* R package takes both into account and estimates the variable importance contributing to the model, plotting a rank of features by importance scaled to 100, with 100 being the most important. This improves interpretability of models and is used to present the results below. Resampling with three repeats of ten-fold cross validation using the *train* function in *caret* R package was used to evaluate the effect of model tuning parameters and to choose the optimal parameters for best model performance. The resulting models were evaluated using the *evalm* function from *MLeval* R Package: computing sensitivity, specificity and AUC-ROC statistic.

RF models were used to identify variables of importance when differentiating between those with and without T2D using groups of serum metabolites previous presented in this thesis. This was performed to first test the applicability of RF in this study cohort and then to explore whether this method of data exploration would yield further differentiator insights compared to univariate analysis previous presented. Urine metabolomic data was not used due to a low number of observations. Each metabolite group was first assessed independently and then as a combined dataset. *Table 7-2* summarises the contributors to each model, the RF parameters and performance statistics. *Figure 7-1* presents AUC-ROC for the models.

Model	Metabolites	n	n variables	ntree	mtry	Sensitivity	Specificity	AUC-ROC
1	AA	48	21	1000	11	0.762	0.741	0.77
2	AA Ratios	48	7	1000	7	0.762	0.852	0.85
3	Lipid	48	18	1000	18	0.762	0.889	0.85
4	All (1-3)	48	46	1000	46	0.762	0.852	0.83
5	Tryptophan	20	15	1000	2	0.700	0.600	0.62
6	All (1-3) & BMI	48	47	1000	46	0.762	0.889	0.84
7	All (1-3 & 5)	20	61	1000	30	0.800	0.800	0.91

TABLE 7-2 RANDOM FOREST MODELS TRAINED ON VARIOUS DATASET IN CLASSIFICATION OF T2D V NON-DIABETES
 NTREE = NUMBER OF TREES; MTRY = NUMBER OF VARIABLES RANDOMLY SAMPLED AT EACH SPLIT

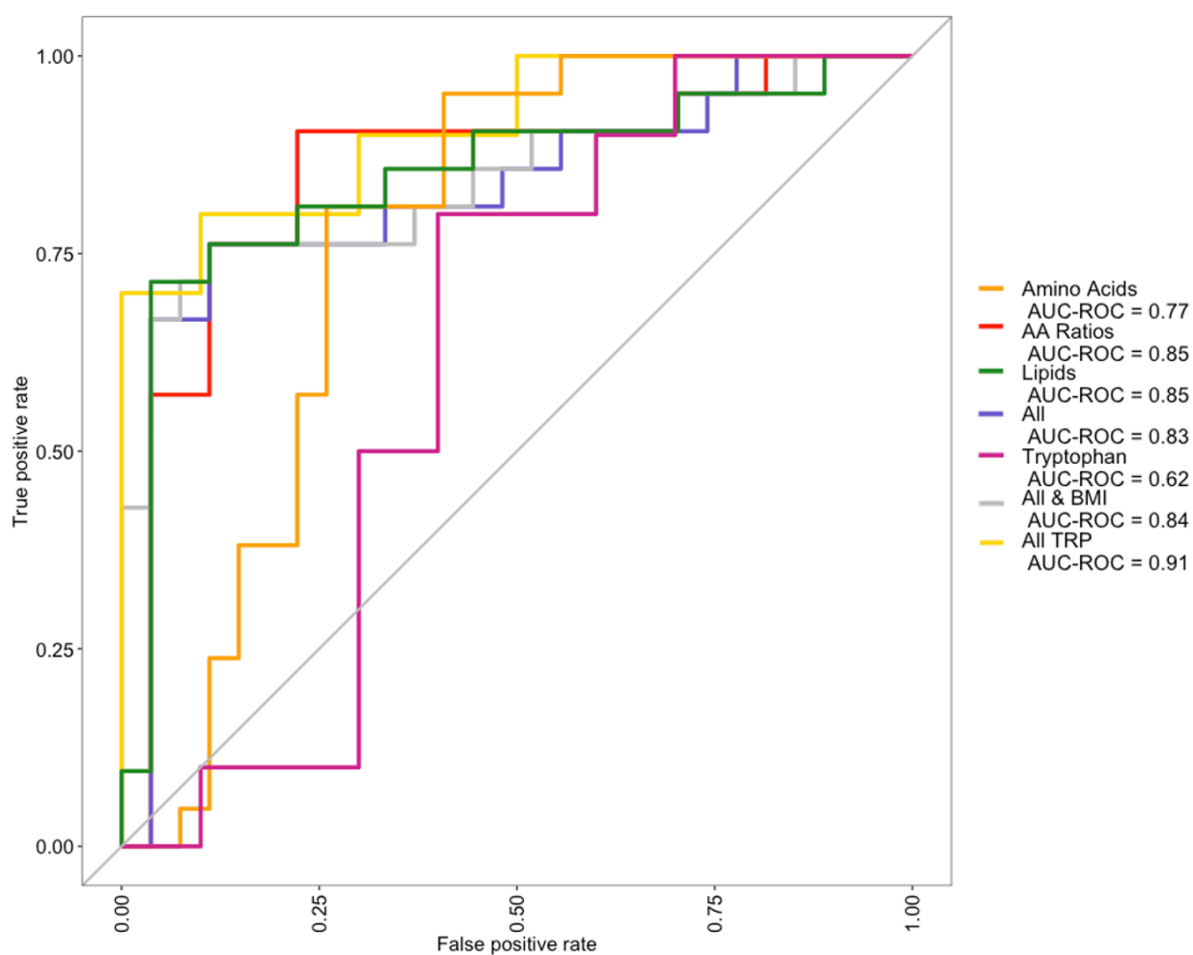


FIGURE 7-1 RANDOM FOREST AUC-ROC PLOTS OF MODELS PRESENTED IN TABLE 7-2

Modelling using individual metabolite groups was first performed. AA only data produced a model with AUC-ROC 0.77 (Sensitivity 0.762, Specificity 0.741). Models using AA ratio only data or lipid only data produced a higher performing model with AUC-ROC 0.85 (Sensitivity 0.762, Specificity 0.852; and Sensitivity 0.762, Specificity 0.889 respectively). Tryptophan only data produced the weakest model AUC-ROC 0.62 (Sensitivity 0.700, Specificity 0.600). The variable of importance plots for each model are displayed below, see *figure 7-2 & 7-3*.

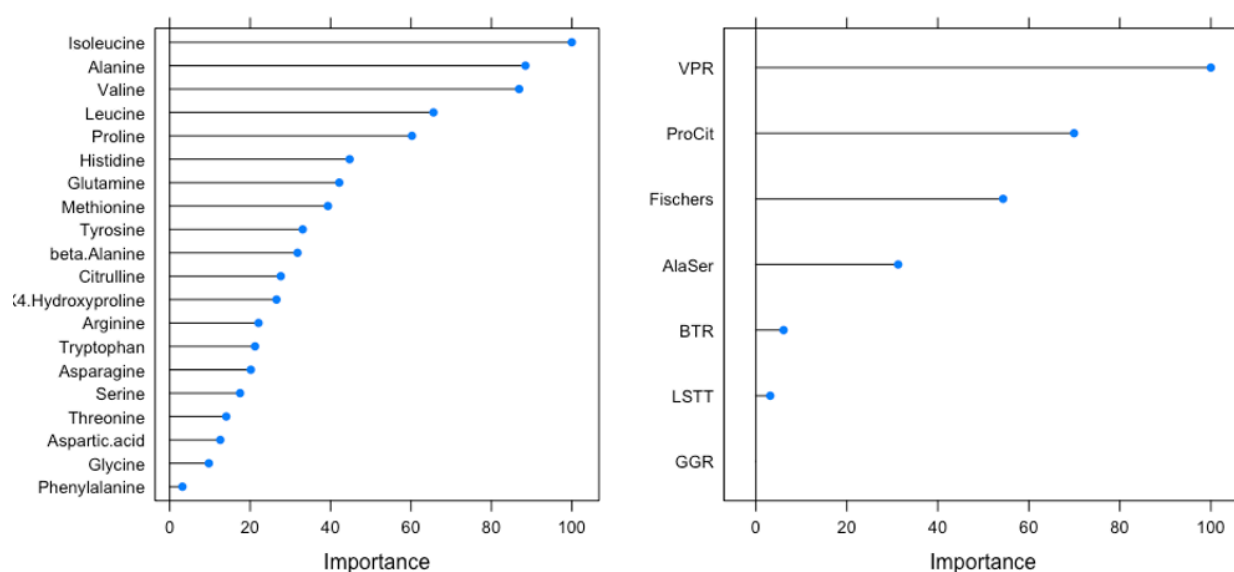


FIGURE 7-2 VARIABLE OF IMPORTANCE PLOT FOR RF MODELS NUMBER 1 (LEFT) & 2 (RIGHT)

KEY: (RIGHT) VPR, VALINE: PROLINE RATIO; PROCIT, PROLINE: CITRULLINE RATIO; ALASER, ALANINE: SERINE RATIO; BTR, BRANCHED CHAIN AMINO ACID TO TRYPTOPHAN RATIO; LSTT, LEUCINE AND SERINE TO THREONINE AND TRYPTOPHAN; GGR, GLUTAMINE TO GLUTAMATE RATIO

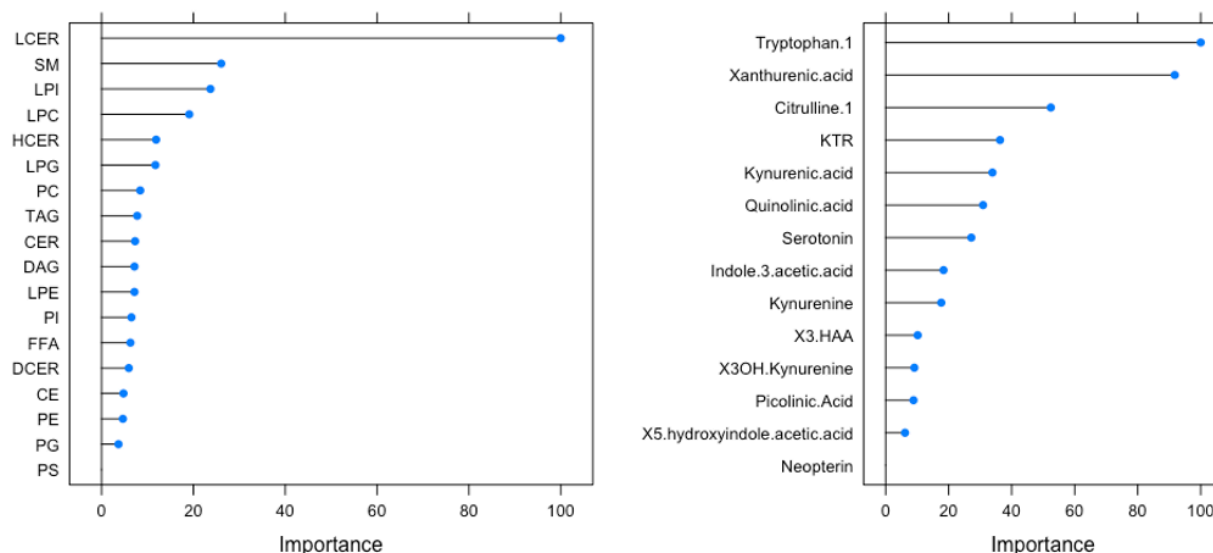


FIGURE 7-3 VARIABLE OF IMPORTANCE PLOT FOR RF MODELS NUMBER 3 (LEFT) & 5 (RIGHT)

KEY: (LEFT) LCER, LACTOSYLCERAMIDE; SM, SPHINGOMYELIN; LPI ,LYSO-PHOSPHATIDYLINOSITOL; LPC , LYSO-PHOSPHATIDYLCHOLINE; HCER, HEXOSYLCERAMIDE; LPG , LYSO-PHOSPHATIDYLGLYCEROL; PC, PHOSPHATIDYLCHOLINE; TAG, TRIACYLGLYCERIDE; CER, CERAMIDE; DAG, DIACYLGLYCEROL; LPE, LYSO-PHOSPHATIDYLETHANOLAMINE; PI, PHOSPHATIDYLINOSITOL; FFA, FREE FATTY ACID; DCER, DIHYDROCERAMIDE; CE, CHOLESTEROL ESTERS; PE, PHOSPHATIDYLETHANOLAMINE; PG, PHOSPHATIDYLGLYCEROL; PS, PHOSPHATIDYLSERINE (RIGHT) KTR, KYNURENINE: TRYPTOPHAN RATIO; 3-HAA, 3-HYDROXYANTHRANILIC ACID

All four models generally reported features of importance that differentiated between those with and without T2D in agreement with earlier results generated using univariate statistics. However, some variations do exist and will be discussed later. Three further models were trained using combined datasets of: 1) AA, AA ratio and lipid data; 2) AA, AA ratio, lipid data and BMI; 3) AA, AA ratio, lipid and tryptophan data. The final model only had 20 observations due to data availability. Interestingly, the model that performed best with AUC-ROC 0.91 included profiling data from all metabolite groups albeit a lower number of observations. The importance plots for each model number 4 & 7 are displayed below (*figure 7-4*). Plot for model 6 not displayed as BMI was not reported to a top 20 variable of importance.

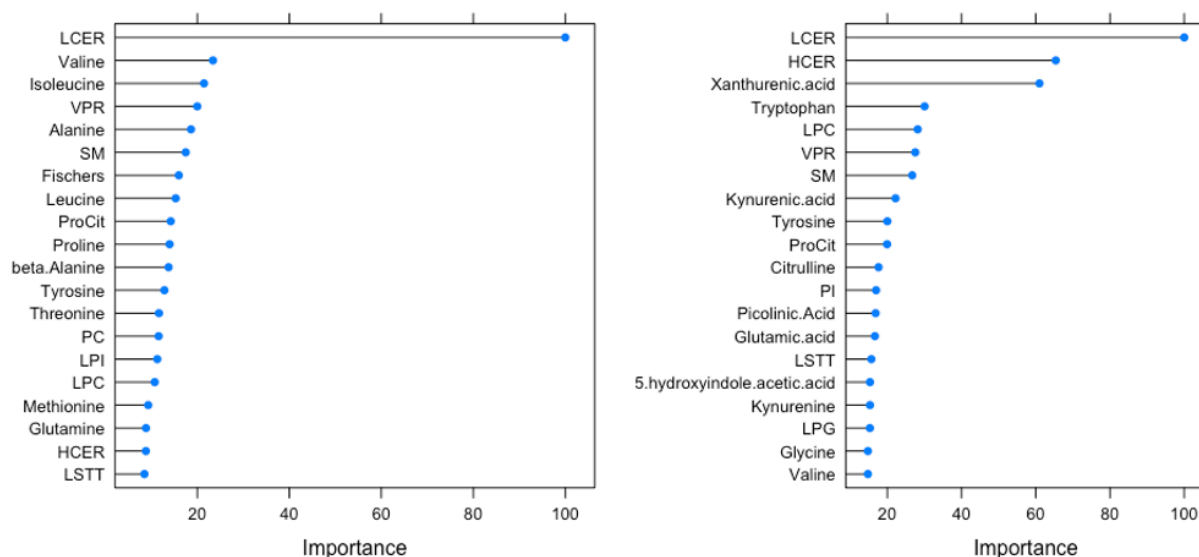


FIGURE 7-4 VARIABLE OF IMPORTANCE PLOT FOR RF MODELS NUMBER 4 (LEFT) &7 (RIGHT)

KEY: (LEFT) LCER, LACTOSYLCERAMIDE; VPR, VALINE: PROLINE RATIO; SM, SPHINGOMYELIN; PROCIT, PROLINE: CITRULLINE RATIO; PC, PHOSPHATIDYLCHOLINE; LPI, LYSO-PHOSPHATIDYLINOSITOL; LPC, LYSO- PHOSPHATIDYLCHOLINE; HCER, HEXOSYLCERAMIDE; LSTT, LEUCINE AND SERINE TO THREONINE AND TRYPTOPHAN (RIGHT) LCER, LACTOSYLCERAMIDE; HCER, HEXOSYLCERAMIDE; LPC, LYSO- PHOSPHATIDYLCHOLINE; VPR, VALINE: PROLINE RATIO; SM, SPHINGOMYELIN; PROCIT, PROLINE: CITRULLINE RATIO; PI, PHOSPHATIDYLINOSITOL; LPG, LYSO-PHOSPHATIDYLGLYCEROL

The top differentiators in these models have been identified throughout this thesis. Interestingly, the additional of tryptophan and metabolites data led to a reduction in the relative importance of serum amino acids as T2D differentiators.

7.3.2 T2D Resolution v Non-Resolution

Next, RF modelling was used to investigate whether any pre-operative serum metabolic markers differentiated between those with and without T2D resolution at 3-month post-op with the aim of seeking out potential markers that could be used to contribute to predictor models of surgical T2D resolution, see *table 7-3* and *figure 7-5*.

Model	Metabolites	n	n variables	ntree	mtry	Sensitivity	Specificity	AUC-ROC
8	All (1-3)	17	46	1000	2	0.846	0	0.32
9	All & Clinical Metrics	17	52	1000	51	0.846	0	0.38

TABLE 7-3 RANDOM FOREST MODELS TRAINED IN CLASSIFICATION OF T2D RESOLUTION AT 3 MONTHS POST OP
CLINICAL METRICS INCLUDED BMI, HbA1c, T2D DURATION (YEARS), DIAREM SCORE, DIAREM2 SCOREHbA1C
REGRESSION. NTREE = NUMBER OF TREES; MTRY = NUMBER OF VARIABLES RANDOMLY SAMPLED AT EACH SPLIT

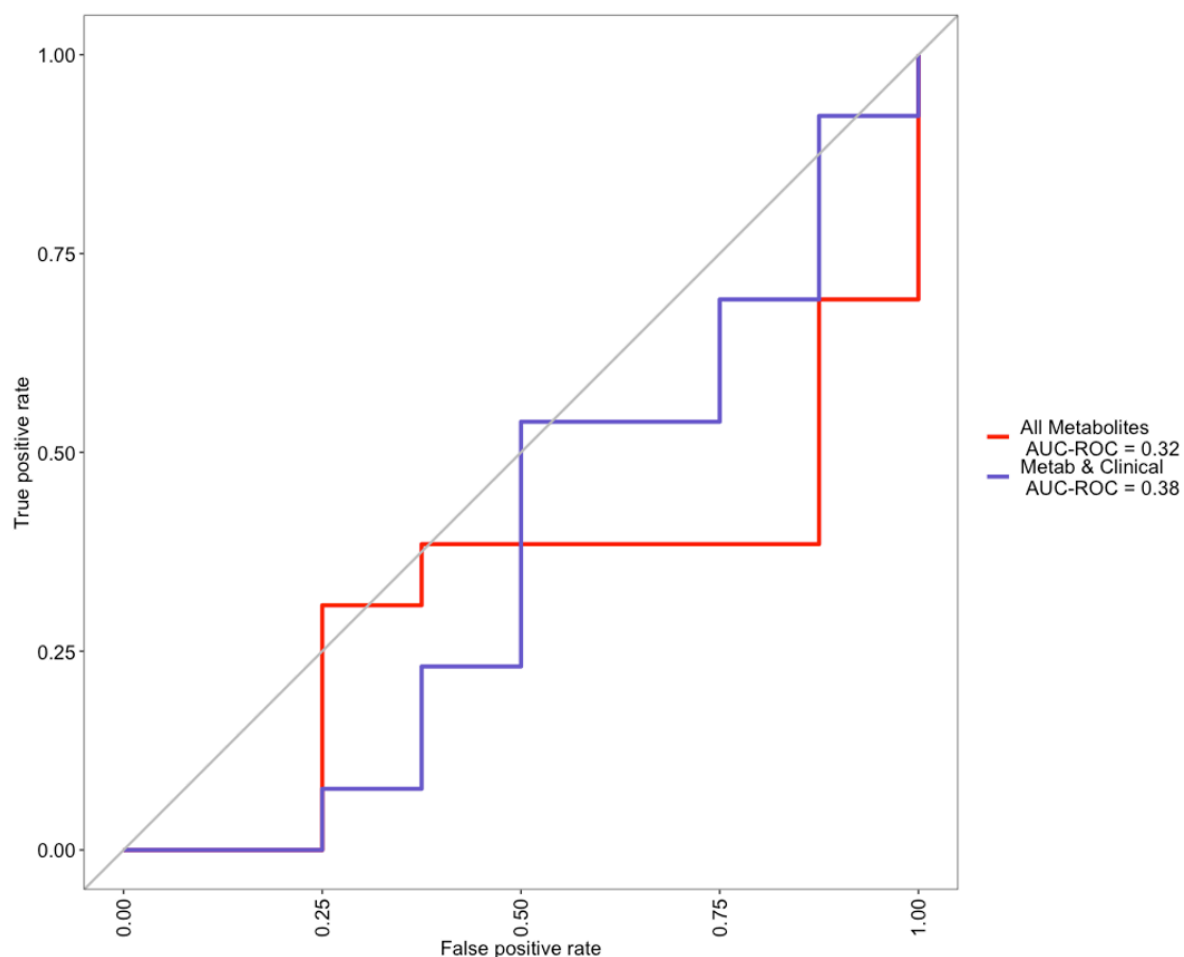


FIGURE 7-5 RANDOM FOREST AUC-ROC PLOTS OF MODELS PRESENTED IN TABLE 7-3

The RF model trained using only metabolic data produced a non-predictive model with AUC-ROC 0.32 (Sensitivity 0.846, Specificity 0.0). The addition of clinical metrics including pre-operative BMI, HbA1c, T2D duration in years as well as Diarem and Diarem 2 scores did not improve the model significantly with AUC-ROC 0.38 (Sensitivity 0.846, Specificity 0.0). Due to the poor nature of the models, the variable importance plots are not displayed. Tryptophan and metabolites data were not included in this analysis due to the low number of observations collected.

7.3.3 HbA1c Random Forest Regression

Finally, random forests can also be used to perform regression analysis. Two models were trained, first a regression model of HbA1c against a collective dataset of BMI, AA, amino acid ratio and lipid data. The second, aimed at evaluating the effect of surgery was a regression model of Δ HbA1c against Δ of measured metabolites. The prediction accuracy of regression models was determined using coefficient of determination R^2 which is computed on out of the bag data.

Model	n	n variables	ntree	mtry	R ² (OOB)
HbA1c regression	74	47	500	24	0.54
HbA1c Δ regression	37	47	500	2	-0.01

TABLE 7-4 RANDOM FOREST REGRESSION MODELS

NTREE = NUMBER OF TREES; MTRY = NUMBER OF VARIABLES RANDOMLY SAMPLED AT EACH SPLIT

RF regression of HbA1c against measured metabolites did not generate a strong predictive model ($R^2 = 0.54$), the variable importance plot is displayed below (*figure 7-6*). RF regression of Δ HbA1c against Δ of measured metabolites produced a non-predictive model ($R^2 = -0.01$).

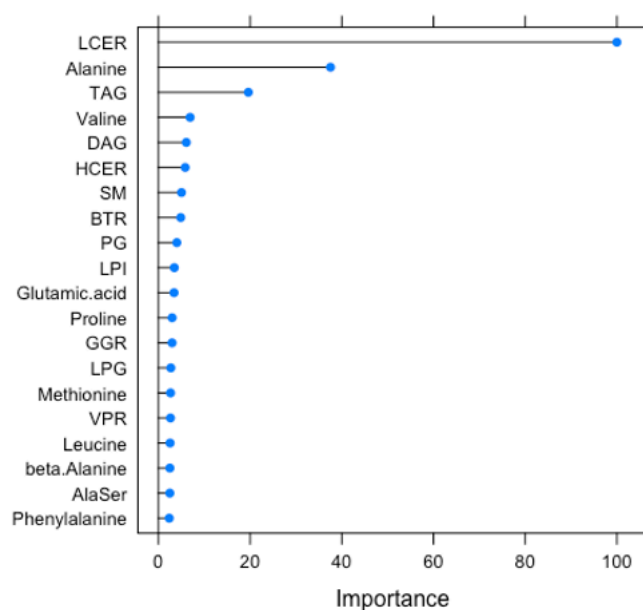


FIGURE 7-6 VARIABLE OF IMPORTANCE PLOT RF MODEL OF HbA1c REGRESSION

KEY: LCER, LACTOSYLCERAMIDE; TAG, TRIACYLGLYCERIDE; DAG, DIACYLGLYCEROL; HCER, HEXOSYLCERAMIDE; SM, SPHINGOMYELIN; BTR, BRANCHED CHAIN AMINO ACID TO TRYPTOPHAN RATIO; PG, PHOSPHATIDYLGLYCEROL; LPI, LYSO-PHOSPHATIDYLINOSITOL; GGR, GLUTAMINE TO GLUTAMATE RATIO; LPG, LYSO-PHOSPHATIDYLGLYCEROL; VPR, VALINE: PROLINE RATIO; ALASER, ALANINE: SERINE RATIO

7.4 Discussion

7.4.1 Type 2 Diabetes v Non-Diabetes

Earlier univariate analysis identified five serum amino acids that significantly differed when comparing those with and without T2D: leucine, isoleucine, valine, alanine and proline. This matched the top five variables of importance identified by the RF model, confirming the applicability of the method when applied to the study cohort. An additional benefit of RF when compared to univariate analysis is that the variables identified are also ranked by importance. Interestingly OPLS-DA analysis (results not shown) of the same data identified similar but not identical features: leucine, isoleucine, valine, tyrosine and methionine to be the top differentiators between the two groups. Univariate and RF analyses of amino acid ratios also yielded the same variables of importance.

For serum lipids, SM, HCER and LCER were identified by univariate analysis to be significantly different between those with and without T2D. Despite low values reported in OPLS-DA modelling reported in chapter six ($Q_2=0.115$ see *chapter 6.4.1, figure 6-2*) several LPC species were found to differentiate between T2D and ND. Interestingly, RF trained on the same lipid dataset to differentiate between those with and without T2D performed well (AUC-ROC=0.85, Sensitivity 0.762, Specificity 0.889) and found LCER to be the most important differentiator followed by SM, LPI, LPC and HCER. This adds to the results which have been shown previously: Low serum LCER levels are found in those with T2D. LCER strongly inversely correlates with HbA_{1c} ($r=-0.53$) and its increase after surgery differentiated between those with and without surgical T2D resolution.

RF trained on tryptophan pathway metabolites only data did not perform well as a predictive model (AUC-ROC=0.62, Sensitivity 0.700, Specificity 0.600). Combining amino acid, amino acid ratio and lipid data produced a reasonable model with (AUC-ROC=0.83, Sensitivity 0.762, Specificity 0.852). The addition of BMI to this combined dataset weakened the model but considering all study participants had high BMI, it was not expected that BMI would differentiate between the two groups. However, the addition of tryptophan and

downstream metabolites data to the combined dataset, led to an improved model (AUC-ROC = 0.91, Sensitivity 0.800, Specificity 0.800). There was, however, a reduction in the number of observations (from n=48 to 20) due to sparsity in the tryptophan dataset so direct comparison needs to be taken with caution. Interestingly, xanthurenic acid and tryptophan were found to be more important than BCAAs in differentiating between those with and without T2D. Much focus has been centred on the role and association of BCAA/ AAA in T2D research but in combination with the previously reported cross ranked correlations with BMI and HbA1c in chapter six (figure 72&73), our results suggest that in this cohort, metabolic pathways involving the sphingolipid species LCER, HCER along with tryptophan and its downstream pathway metabolite xanthurenic acid may have a higher influence on the underlying pathology of obesity and T2D. It would be important to explore this further in larger cohorts of patients. If these associations are further validated, then the research focus should shift to understanding the mechanisms underlying these associations. Ultimately, this may also yield further therapeutic targets.

7.4.2 *T2D Resolution*

Disappointingly, RF trained using pre-operative serum markers profiled within this thesis did not produce a predictive model for surgical T2D resolution at 3 months post-op (AUC-ROC = 0.32). The addition of clinical metrics only improved the model slightly (AUC-ROC = 0.38).

There could be several explanations for this. Although I identified AA and lipid signatures that were significantly associated with T2D resolution, univariate analysis did not identify any of the metabolites reported throughout this thesis to be significantly different at baseline between those with and without T2D resolution. Currently, as previously discussed, it is established that those with a higher probability of T2D remission have more significant weight loss, a shorter duration of T2D and less severe T2D. In addition, higher C peptide levels – a marker of residual beta cell function has also been reported to be associated with a lower rate of subsequent T2D relapse, so although surgery does induce some powerful anti-diabetes effects, sustained remission is closely linked to the degree of irreversible beta cell death. The metabolites investigated throughout this thesis do not directly correspond to any of these factors.

It is also important to acknowledge that our understanding of the heterogeneous T2D classification is still inadequate (751). The addition of clinical metrics did not significantly improve the prediction model further highlighting the fact that currently, strong markers that predict T2D resolution have not yet been identified. Nevertheless, it was still important to investigate and report the predictive potential of serum amino acid and lipid metabolites. There are also a significant number of other pathways and metabolites with reported links to obesity, T2D and surgical T2D resolution that have not been included in this analysis and should form a part of further work which will be discussed later.

7.4.3 Regression

The final RF models were trained to perform regression of HbA_{1c} against measured metabolites at the time of sampling. This model did not achieve high predictive value ($R^2 = 0.54$) but identified LCER, alanine, TAG, valine and DAG to be the topmost variables of importance. These results are comparable to the ranked correlations reported in chapter 6.4.5 (*figure 6-17*) which found LCER, valine, tryptophan, leucine and proline to be the top five ranked variables with the strongest correlation to HbA_{1c}. RF regression model trained using Δ HbA_{1c} against Δ of measured metabolites produced a non-predictive model suggesting that single changes to these measured metabolites do not directly explain the associated change in HbA_{1c}.

7.5 Conclusion

RF models trained using data presented throughout this thesis supported and strengthened previously identified metabolic signatures of T2D in individuals with obesity. In particular, the relative importance of serum sphingolipids and tryptophan pathway metabolites in glycaemic control was further highlighted. It was disappointing that RF models trained using the pre-operative serum markers did not produce models of substantial predictive value for T2D resolution and thus did not identify any significant pre-operative features with predictive potential but nevertheless these results will help inform the ongoing search for a reliable predictor model of surgical T2D resolution.

Chapter 8: Conclusion, Limitations and Further Work

8.1 Summary of findings

8.1.1 *Summary of Clinical Findings*

Throughout this thesis, predominantly LC-MS based metabolic profiling was utilised to explore the short-term post-surgical effects of bariatric surgery on serum amino acids, serum & urine tryptophan metabolites and serum lipids profiles in a cohort of participants with and without obesity and type 2 diabetes. Differentiating metabolic signatures were identified when comparing those with and without T2D prior to surgery and in those with and without surgical resolution of T2D at the 3 months post-operative time point. Finally, using random forest modelling, I explored whether any pre-operative serum amino acid or lipid metabolic signatures could be used as markers to predict surgical T2D resolution at 3 months post-surgery.

Pre-operatively, significant elevations in serum BCAA (Leucine, Isoleucine & Valine), alanine and proline differentiated those with and without T2D. Surgery reduced serum levels of all BCAA and AAA. RYGB led to significant increases in glycine & serine, both of which are markers of improved metabolic health. In particular, ΔHbA_{1c} significantly and positively correlated with Δ of all three BCAA. ΔBMI was associated with significant and inverse correlation with $\Delta\text{glutamine}$ and Δserine .

Serum glutamine: glutamate (negative) and alanine: serine ratios (positive) were significant and strong correlated with HbA_{1c} and BMI, both of which implicate states of disturbed metabolism linked to T2D. RYGB induced significant changes to both markers, in keeping with an improvement in glycaemic control at 3 months post-surgery. When comparing those with and without surgical T2D resolution, I observed the novel finding of a significant increase in glutamine: glutamate ratio and a significant decrease in alanine: serine ratio in those with T2D resolution only.

The amino acid associated urinary metabolites profiled in this study did not significantly differentiate between those with and without T2D, and surgery did not lead to significant

changes to these metabolites. However, BMI did demonstrate correlation with urinary lactate, PAG and carnitine, possibly linked to reduced systemic inflammation secondary to weight loss. HbA_{1c} demonstrated significant correlations to urinary glycine and carnitine.

Bariatric surgery led to a reduction of serum tryptophan and downstream kynurenine metabolites associated with T₂D at 3 months post-operative. In particular, significant reductions of serum tryptophan, kynurenic acid and xanthurenic acid was observed for those who underwent RYGB. Significant reductions in serum tryptophan and xanthurenic acid levels at 3 months post-surgery were also observed in those with T₂D. Serum tryptophan, kynurenic acid and xanthurenic acid levels are significantly and positively correlated with HbA_{1c} and the pre- and post-operative Δ HbA_{1c} was significantly and positively correlated with Δ tryptophan.

On the other hand, the influence of surgery on urinary tryptophan and downstream kynurenine pathway metabolites were less pronounced. Only RYGB led to significant reductions in urinary kynurenic acid levels. However, urinary tryptophan, kynurenine and KTR all demonstrated significant positive correlation relationships with serum HbA_{1c}.

Significantly lower serum levels of sphingolipids (HCER, LCER and SM) differentiated between those with and without T₂D prior to surgery. RYGB and not VSG induced widespread and significant changes to serum levels of measured lipids. Some of these were related to weight loss and some were not. In particular, Δ HbA_{1c} was significantly negatively correlated with Δ LCER, Δ LPC and Δ SM only. T₂D resolution was associated with significant reductions in serum DCER, PC and an increase in LCER. DCER and PC were observed to be significantly and positively correlated with both HbA_{1c} and BMI while LCER inversely and strongly correlated with HbA_{1c} which may signify mechanisms of action independent of weight loss.

When assessing lipid and AA metabolites for combined ranked cross correlations, valine, LCER and tryptophan were identified as the top ranked metabolites for correlation with HbA_{1c}, inferring significant roles for these metabolites and their associated pathways with

glycaemic control within this study cohort. Tryptophan, PS and PC were top ranked for correlations with BMI.

Random forest modelling further corroborated the relative importance of sphingolipids: LCER & HCER and tryptophan and its downstream metabolites to the pathophysiology underlying obesity and T2D but disappointingly models trained using pre-operative serum markers profiled in the studies above did not produce models of significant predictive ability for surgical resolution of T2D at 3 months post-operative.

8.1.2 *Summary of Methodological Findings*

Two targeted LC-MS methods of serum amino acid quantification were compared. The first was a commercially available targeted kit (AbsoluteIDQ p180) and second an established UPLC-MS quantification method validated against stable isotope labelled internal standards. The accuracy of these assays was not assessed but both produced comparable results that did not alter results of significance when assessed for correlation against clinical measurements applicable to this cohort.

Simple post-acquisition normalisation methods of raw data acquired using a targeted profiling assay for tryptophan and kynurenine metabolites in urine was also assessed. Normalisation using creatinine or osmolality but not urine volume as a post-acquisition normalisation factor improved the resulting OPLS-DA models of separation when compared to raw data. While this may not be the most precise method of normalisation, it is practical and adequate for any first line screening or preliminary discovery studies for investigators wishing to investigate tryptophan metabolites in clinical studies.

Finally, the 24-hour urine values of TRP, KYN, 3OH-KYNA and KA with creatinine used as a post-acquisition normalisation factor displayed strong positive correlation with their respective serum values as reported, indicating urine's suitability to be used as a surrogate biofluid in screening or preliminary discovery metabolomics studies covering these metabolites.

8.2 Study Limitations

The studies reported within this thesis generated a plethora of data. The data were paired and longitudinal, albeit short term (3 months post-operative), in nature. The studies also lacked a cohort of normal controls for further comparison and validation of the findings. The small sample size was a result of budget, personnel, and physical constraints, which limits the generalisability and the statistical power of some of the reported findings. However, significant physiological and metabolic effects were still observed, illustrating the powerful nature of bariatric surgery. The strong effect of bariatric surgery reduced the impact of inter-individual variability and the effects of a smaller sample size.

BMI and HbA_{1c} were the main clinical metrics utilised in the studies. Other markers and clinical tests associated with glycaemic control such as serum insulin levels, c-peptide levels, fasting glucose, oral glucose tolerance test, HOMA-IR measures were not utilised. The 3:1 female to male ratio of study participants also requires recognition but this ratio is in keeping with the distribution of patients undergoing primary bariatric surgery in the United Kingdom(58).

The complete metabolic pathways for the molecules profiled were not always analysed. For example, I investigated serum amino acids, but the study did not extend into the intermediate metabolites associated with these pathways. Similarly, I investigated some downstream amino acid metabolites of gut microbial origin within urine samples but did not have the corresponding data for the same metabolites in serum samples. I was also unable to include downstream lipid metabolites of microbial origin in urine, faeces and serum into this work. Some investigations that may have extended our understanding of the metabolic perturbations, such as metagenomic profiling to characterise the microbiome, were also beyond the scope of this thesis. Other metabolites such as short chain fatty acids, bile acids, acylcarnitine and further subspecies of lipids have reported links to T2D and also warrant further investigation.

Regarding the tryptophan kynurenine pathway, other factors such as vitamin B levels as well as other derivatives of tryptophan, beyond the metabolites measured using the methodology utilised in this study, merits further investigation.

Analysis of serum lipid profiles at class level respective to the aims of this thesis led to some interesting and novel findings. However, the dynamic nature of the human lipidome is not well understood yet. Serum levels of each lipid species may not necessarily correspond directly with concentrations found in metabolically active tissues such as the liver and muscle, which could account for some of the discrepancies in the results reported within this thesis and between published studies. The precise mechanisms by which these metabolites influence health and disease is also not well understood yet. Further advanced statistical models would permit simpler analysis and interpretation of individual chain length lipid species to be assessed in relation to the study objectives. It is possible that while some metabolites at class level displayed constant levels, specific chain lengthed species of lipids within the class group may have increased or decreased but still led to an overall neutral effect.

As previously discussed, the reduction in overall dietary intake after surgery may also be a driver for many of the observations reported. However, if diet was the only factor influencing the results, one would assume all observations to be reduced at the post-operative time points. This was not the case and thus indicates that the changes observed in the studies were associated with specific enzymatic processes and changes to metabolic pathways.

Analytical methods employed throughout this thesis were mainly correlation based. While this lends itself to hypothesis generation, it has notable limitations for the mechanistic implication of the results. The effect of confounding factors such as gender, ethnicity and age on these results also needs to be explored and evaluated with larger datasets.

This study targeted several metabolic pathways of interest, but other metabolic pathways will also need to be investigated for their postulated roles in those with T2D to provide a comprehensive evaluation. Nevertheless, the exploratory and discovery nature of this thesis

has confirmed existing and identified several novel amino acid and lipid signatures linked to metabolic dysfunction in T2D and its association with T2D surgical resolution. Some of the findings also explain a subset of the beneficial effects of surgery for other obesity associated conditions. The results from these studies give clues to mechanistic drivers and can form the basis of future studies to establish causality, and discovery of potential targets of therapeutic intervention.

8.3 Future Work

Following on from the discovery nature of the work presented within this thesis, the observations from the studies contained within this thesis require further investigation and validation. The potential for further work is vast but in the ideal world without time or budget constraints, the following areas of future work would be important to consolidate and develop the findings.

8.3.1 Study Design

Longer term (one to 5 years) clinical and metabolic data would be prudent to establish the sustained nature of the findings. Increasing the sample size would significantly increase the power and generalisability of the study. It would also be of interest to include comparative controls including healthy lean individuals, lean individuals with T2D and T2D individuals that have not undergone surgery to provide more robust comparative groups. This would help ascertain pathological signatures that are similar but also differentiating of disease and T2D resolution that could be useful in future risk stratification and identification of disease. Extending the criteria to those with Type 1 Diabetes may also find some overlapping metabolic disturbances, which may help identify those Type 1 Diabetes patients where metabolic surgery would be of therapeutic benefit.

A separate dedicated diet-controlled study aimed at investigating the effects of dietary change and surgically induced dietary change on the reported metabolites and its effects on T2D would be of importance and tremendous interest and could potentially be used to tailor targeted metabolic intervention in those individuals who are unsuitable for surgery.

The widespread metabolic effects of newer surgical therapies such as one anastomosis or mini gastric bypass, other newer endobariatric therapies (752) including endoscopic sleeve gastropasty(753) and newer successful pharmacological interventions such as Tirzepatide (754): a dual glucose-dependent insulinotropic polypeptide GLP-1 receptor agonist or Semaglutide (755): a selective GLP-1 receptor agonist, should also be assessed. Similarities

in metabolic changes induced by treatment would corroborate key mechanistic metabolic pathways of T2D and T2D resolution and promote understanding of the variation in action of each treatment to allow for a more personalised precision and personalised based therapeutic approach in the future, particularly for those who don't wish to undergo surgery or are unsuitable for surgical intervention.

8.3.2 *Metabolites and Metabolomics*

To further the findings previously reported, the catabolic products of amino acids should be investigated due to their potential effect on systemic glycaemic control. Urinary metabolites of amino acids should also be investigated as urinary tests are much easier to collect in the clinical environment. With regards to the tryptophan – kynurenine pathway, other tryptophan derivatives such as serum and urinary indoles would be of interest due to the bioactive nature of such compounds as well the fact that many of these compounds are produced and influenced by changes in the gut microbiome.

Currently, lipid metabolism and the human lipidome is complex and not yet well understood. Observations presented in this thesis only featured analysis at lipid class level so further in-depth individual lipid species analysis is warranted once improved analytical methods are available. Future lipidomic analysis would ideally require simultaneous sampling of biofluids and tissues to further establish the dynamic nature and effect of changing lipid metabolism in response to therapeutic interventions.

The implications of changing serum lipid levels on body-wide insulin action and glycaemic control needs to be further evaluated and understood before molecular mechanisms can be elucidated. The reported changes in serum lipids may be due to an alteration in metabolism in response to disease or surgical intervention with subsequent implications to the complex lipid signalling network. Lipidomic molecular mechanisms and signalling pathways may influence metabolically active tissues such as the liver, adipocytes, and skeletal muscle in different ways.

8.3.3 *Statistical modelling and Data Integration*

One major drawback of metabolomic studies particularly untargeted profiling, is this method generates significantly large datasets which are not easy nor straightforward to interpret. This is an evolving field and the ability to utilise and analyse these large metabolomic datasets requires further advancement in statistical and computational methods.

The development and incorporation of deep learning, machine learning and even artificial intelligence technologies (756) in the future will no doubt be able to aid both scientific and clinical research. Areas which urgently need progression and are of importance include the current bottlenecks in metabolomic research such as metabolite identification and data analysis. Until the integration and analysis of genomic, proteomic and other multi-“omic” dynamic datasets leading to clinically relevant outputs is possible, the true potential utilisation of metabolomic data is yet to be fulfilled (757) . With regard to research relevant to topics discussed in this thesis, the identification of key metabolic pathways contributing to the T2D phenotype and subsequent surgical T2D resolution will allow for better patient selection but also offer potential targets for drug discovery.

The quality of random forest models utilised in chapter seven and any machine learning method is reliant on the quality of datasets which are used to train the models. With larger datasets containing a wider spectrum of metabolites and study participants these methods will be able to produce more useful prediction models, which would then need to be validated against a wider population.

8.3.4 *Future Clinical Applications*

Metabolomics and multi-omics studies provide a new perspective of research with a systems wide biological approach. The historical singular cause and effect model on pathophysiological processes and ‘one size fits all’ treatment mentality is now beginning to be superseded by advancements in this field. The overarching trend of metabolomic research is also now shifting from widespread profiling and descriptive studies to more detailed mechanistic investigation of pathways implicated in states of disease.

Ultimately, the primary objective is to be able to translate and apply findings into day-to-day clinical practice but currently in depth metabolomic profiling is still limited to research-based studies. In the future, I envision the development of custom built and miniaturised clinical platforms that could be utilised in advancing the understanding of disease, disease screening, prevention, diagnosis, biomarker discovery, drug discovery and other therapeutic developments

Particularly with metabolomics research in T2D, there are several areas in which this type of research would be particularly beneficial.

The first is the stratification of future disease risks based on metabolic profiles. Metabolic disturbances are present before clinical manifestation of the disease. Once key mechanistic pathways contributing to T2D can be identified and key metabolites measured, a personalised risk score based on these metabolic findings may be produced. Those identified as at risk of disease in screening programs can be offered preventative interventions at a much earlier stage than currently available. Biofluids such as urine which are easily obtainable without invasive venepuncture would be an attractive option in screening studies if urinary metabolites can be identified, correlated with corresponding serum metabolites, and associated with mechanisms which contribute or result in disease.

In those with established metabolic disease such as T2D, a personalised precision-based method targeted at specific metabolic disturbances may be applied when offering both pharmacological and surgical treatment options. As an extension, using metabolic or

microbiome profiles, in the future it may be possible to predict the likelihood of an individual's potential response to treatment such as medication or surgery.

Examples of such applications have already begun to appear, amino acid (246-251), and lipidomic (546) profiles have been investigated and used to predict risk of future T2D (546). Other specific markers such as serum ceramide levels have also been used to risk stratify patient's cardiometabolic risk (758). The goal would be to combine these metabolic markers into a wider prediction model which could be used universally in disease prevention.

Targeted therapies aimed specifically at modifying key pathological metabolites or metabolic pathways can be derived from metabolomic research. Drug therapies may be able to target enzymatic steps linked to metabolite synthesis or other salvage pathways. Pharmacological blockade of receptors which act as signalling channels or control influx of cellular substrates could all influence build up to toxic metabolites and would be of tremendous research interest. An example of this is the development of drug therapies targeting the reduction of ceramide synthesis or reduction of hepatic oxidative stress in T2D and NAFLD (613).

Currently, there are still limitations with widespread metabolic profiling. Cost of metabolic profiling remains high; methodologies still require extensive manpower, computational processing, and time. Technological advances have already allowed high throughput methods to be developed but further improvements may be required so that tests can be run in routine medical laboratories or even as clinical "point of care" bedside instruments.

Nevertheless, technology and software will continue to improve. As will our scientific understanding of the pathologies described and once these barriers can be overcome then the full potential of metabolomic research will be increasingly applicable in clinical medical practice. Ultimately, contributing to better health screening, a more personalised therapeutic approach and eventually, better health outcomes.

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Appendices

Appendix I: Ethics

Ethical Approval: This study was approved by East of Scotland Research Ethics Committee (15/ES/0026).

Project Title: “Longitudinal Phenotyping of Bariatric Surgery Patients”

Joint Research Compliance Reference Number: 15SM2479

IRAS project ID: 169767

The original ethical approval had been obtained by supervisor NP in 2015, with subsequent amendments beyond Oct 2017 written, submitted and obtained by KTDY (AMo2 Feb 2019, NSA1 Nov 2019, NSA 2 Feb 2021).

Ethical application for the collaboration with Murdoch University providing data for chapter 6 was co-written with post-doctoral mentor LW and granted in August 2020. Murdoch University Human Research Ethics Committee Project No 2020/042.

Project Title: “Longitudinal Phenotyping of Bariatric Surgery Patients.”

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Appendix II: GDPR Transparency Wording Document

Study title: Longitudinal Phenotyping of Bariatric Surgery Patients

IRAS number: 169767

Imperial College London is the sponsor for this study based in the United Kingdom. We will be using information from you and your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. Imperial College London will keep identifiable information about you for a minimum of 10 years after the study has finished.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally identifiable information possible.

You can find out more about how we use your information:

Principle investigator: Professor Ara Darzi

Co-Investigators: Mr Nicholas Penney & Mr Kai Tai Derek Yeung

Contact Email: k.yeung@imperial.ac.uk

Imperial College London will collect information from you and your medical records for this research study in accordance with our instructions.

Imperial College London will use your name, hospital number, date of birth and contact details to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Individuals from Imperial College London and regulatory organisations may look at your medical and research records to check the accuracy of the research study. Imperial College NHS Trust will pass these details to Imperial College London along with the information collected from you and your medical records. The only people in Imperial College London who will have access to information that identifies you will be people who need to contact you to regarding follow up and sample collection or audit the data collection process. The people who analyse the information will not be able to identify you and will not be able to find out your name, hospital number, date of birth and contact details

Imperial College London will keep identifiable information about you from this study for a minimum of 10 years after the study has finished.

When you agree to take part in a research study, the information about your health and care may be provided to researchers running other research studies in this organisation and in other organisations. These organisations may be universities, NHS organisations or companies involved in health and care research in this country or abroad. Your information

will only be used by organisations and researchers to conduct research in accordance with the UK Policy Framework for Health and Social Care Research.

Your information could be used for research in any aspect of health or care and could be combined with information about you from other sources held by researchers, the NHS or government.

Where this information could identify you, the information will be held securely with strict arrangements about who can access the information. The information will only be used for the purpose of health and care research, or to contact you about future opportunities to participate in research. It will not be used to make decisions about future services available to you, such as insurance.

Where there is a risk that you can be identified your data will only be used in research that has been independently reviewed by an ethics committee.

Appendix III: Inclusion and Exclusion Criteria

Inclusion Criteria:

Referred to the Imperial Weight Centre (NHS) Tier 4 service team for consideration bariatric surgery (RYGB / VSG / mRYGB / LAGB), BMI >30kg/m², Age ≥18, failure of efforts at lifestyle modification and dieting, fitness for anaesthesia and procedure, willingness to comply with the trial protocol.

Exclusion Criteria:

Previous bariatric surgery, previous major abdominal surgery*, pregnancy or intention to become pregnant during trial period, lack of capacity to consent.

* = Small or large bowel resection, liver, pancreatic or splenic surgery, as these will influence the bacteria in the gut and / or the patient's metabolic state, which we are studying. It will not include patients that have previously had an appendicectomy, cholecystectomy, hernia repairs or surgery on other organs not listed above.

Appendix IV: Surgical Technique

Operations were performed in a standardised fashion by five consultant surgeons and the key steps are summarised below. Surgical antibiotic prophylaxis of choice is Co-Amoxiclav unless penicillin allergic, then Clindamycin & Metronidazole is administered.

Roux-En-Y Gastric Bypass:

A 15 to 30 cc gastric pouch is first formed using laparoscopic staplers sized using a 34F Ewald tube. A sided-to-side stapled gastrojejunostomy is formed using a 30mm linear stapler with the resulting enterotomy closed using a continuous 3-0 vicryl suture followed by a second layer of interrupted 3-0 vicryl sutures. An underwater leak test is used to check the anastomosis. The Jejunal- jejunal anastomosis is formed in a side-to-side fashion using a linear 45mm stapler with the resulting enterotomy closed using a continuous 3-0 vicryl suture. The biliary-pancreatic limb is 75cm and roux limb 100cm.

Vertical Sleeve Gastrectomy:

A gastric sleeve is formed over 34F Ewald orogastric tube. Stapling commences 3cm proximal from the pylorus. A total of 4 to 5 firings of 45/60mm staplers with staple line reinforcement is used. Underwater leak test is again used to check the staple line.

Appendix V: Dietary Instructions

Pre-Operative

All patients underwent an intensive “liver shrinkage” diet for two weeks prior to surgical intervention. Patients were limited to 800-1000 kcal intake per day using a meal plan that is low in carbohydrates (100g), low in fat and moderate in protein.

Post-Operative

Patients are prescribed multivitamin ForcevalTM, once daily. Oral intake is progressed in a stepwise fashion: liquid diet for the first ten days, followed by a puree diet for three to four weeks and finally a soft diet for six weeks. Consumption of normal food is advised twelve weeks post-operatively.

Patients are given extensive dietary advice and asked to aim for a minimum intake of 60-70g protein and 800-1200 kcal per day.

Appendix VI: Sample Collection, Storage and Preparation for metabolomic analysis

Sample Procurement and Storage

Blood samples were obtained as part of the routine pre and 3-month post-operative outpatient clinical review appointments using additive free red top BD Vacutainer® (New Jersey, USA) tubes. After 45 minutes at room temperature, the tubes were centrifuged at 12,000 x g for fifteen minutes. The resultant serum supernatant was aliquoted.

Urine Samples were collected for 24 hours prior to the participants' outpatient appointment. The samples were aliquoted immediately without additives.

All samples were stored at -80°C until analysis.

Sample Preparation

Urine LCMS and MS/MS: Follows established departmental protocol (207). In brief summary, following thawing, urine samples were vortexed and centrifuged for 10 minutes at 1600 x g for removal of precipitated proteins and particulates. 200 µl of the resulting supernatant was diluted in equal portions with HPLC grade water, vortexed and centrifuged for 20 minutes at 2700 x g then aliquoted for analysis.

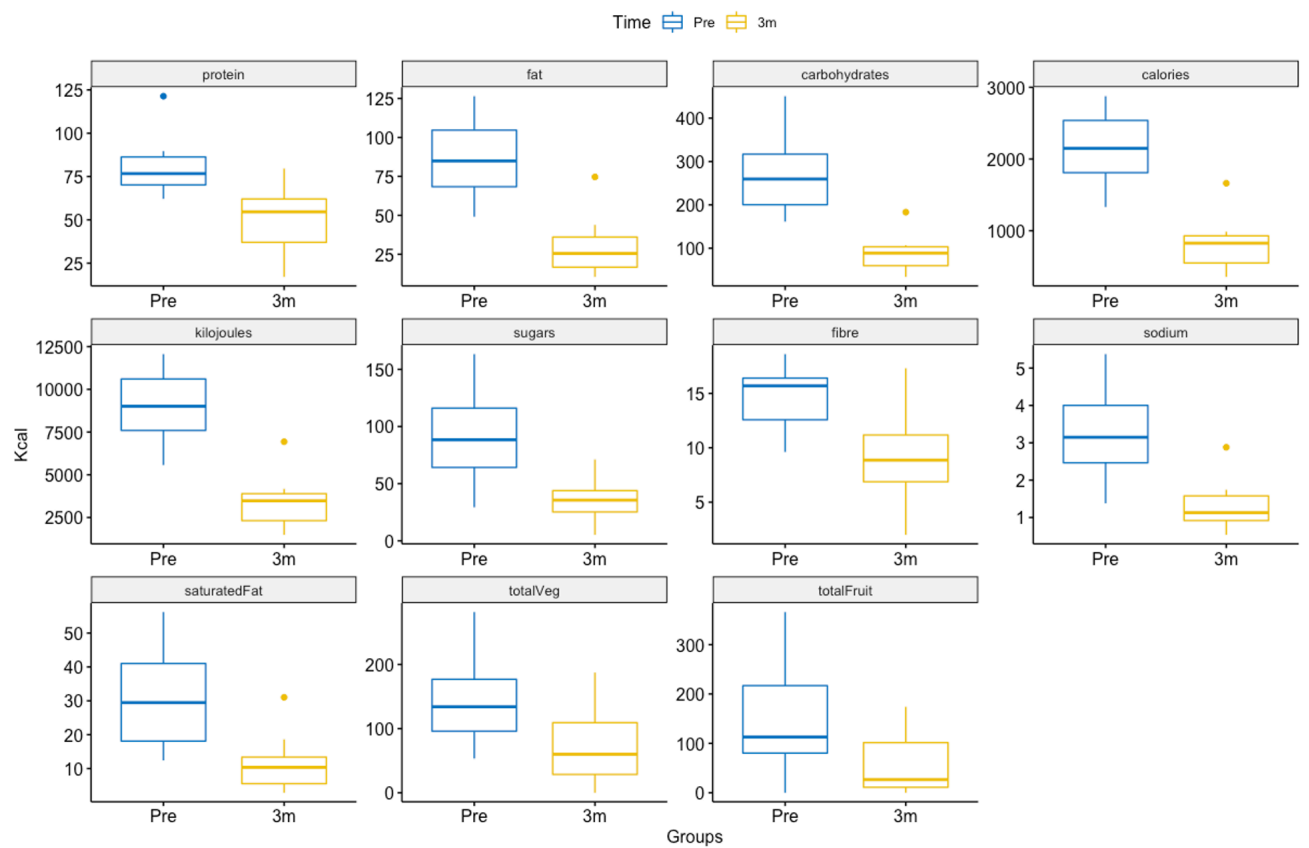
Appendix VII: Dietary Questionnaire

A subset of 11 patients provided dietary information. Information was collated using a self-reporting online recall questionnaire (www.myfood24.org) on three separate occasions pre-operatively and post-operatively for dietary changes after surgery to be explored. The raw data from this questionnaire was collected by NP and subsequently statistical analysis performed by KTDY.

Macronutrient	Pre-Op	Post-Op	p value
Protein	80.64±16.37	49.67±18.2	0.014
Fat	85.77±27.15	29.25±18.37	0.003
Carbohydrates	270.06±89.63	86.42±41.69	0.002
Calories (kcal)	2151.34±537.85	806.59±361.42	0.002
Kilojoules (kj)	9009.07±2236.89	3383.23±1507.35	0.002
Sugars	92.96±42.38	35.13±19.71	0.002
Fibre	14.74±2.78	9.1±4.53	0.007
Sodium	3.22±1.21	1.3±0.66	0.001
Saturated Fat	30.96±15.45	11.31±8.16	0.014
Total Veg	141.27±69.29	75.45±62.45	0.175
Total Fruit	144.13±110.1	60.15±65.91	0.103

all listed data are means ± standard deviation (g/day) unless stated otherwise

TABLE COMPARING PRE AND 3 MONTH POST OPERATIVE DIETARY INTAKE BASED ON AN ONLINE SELF-REPORTING RECALL QUESTIONNAIRE



BOXPLOT COMPARING PRE AND 3 MONTH POST OPERATIVE DIETARY INTAKE BASED ON AN ONLINE SELF-REPORTING RECALL QUESTIONNAIRE