^{1}H NMR Spectroscopic Identification of Non-Invasive Biomarkers of Acute Rejection and Delayed Graft Function in Renal Transplantation

Paul Joseph Goldsmith

Submitted in accordance with the requirements for the degree of Doctor of Medicine

The University of Leeds

School of Medicine

December 2013

The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Published Work From Thesis

Chapter 1 of this thesis has been published in a peer-reviewed journal. The work is the individual effort of Paul Goldsmith who did the research and wrote the manuscript. All other authors commented on the manuscript prior to submission.

1. Metabonomics: A Useful Tool for the Future Surgeon. Goldsmith P, Fenton H, Morris-Stiff G, Ahmad N, Fisher J, Prasad KR. J Surg Res. 2010 May 1;160(1):122-32.

Acknowledgements

When I started this project the world of academic medicine was alien to me, but due to the hard work and endeavour of my three supervisors I managed to complete this project.

Mr K Rajendra Prasad, thank you for taking the chance and giving me the opportunity to work on the hepatobiliary and transplant unit and write this thesis. I will never forget the phone-call you made to me offering me the job. In the two and half years I worked for you I changed in many ways, I started the job unknowingly naïve, but over time I've learnt to appreciate the "bigger picture", listen to all the information available before making a decision and strive to achieve the goals I desire. The opportunity you afforded me will hold me in good stead for the future, something I'm eternally grateful for.

Mr Niaz Ahmad, your help has gone a long way into achieving this thesis. Understanding the process of renal transplantation, the issues medics have in the current era of transplantation and great moral support when not all things were going as planned.

Dr Julie Fisher, I'm not quite sure if I would ever have got here without your support. I didn't know much (perhaps anything) about metabonomics before I met you and your lab. From the moment I arrived everyone was friendly and incredibly helpful and I felt at ease straight away. You encouraged me patiently when experiments weren't going as well as hoped and a few years down the line we have managed to present and publish work and continue metabonomics based research in other fields with other MD/PhD students.

Abstract

Delayed graft function (DGF) and acute rejection (AR) are complications after renal transplantation. It is impossible to differentiate between these clinically. Renal biopsy is the gold standard for diagnosis but is invasive and associated with complications. We aimed to identify early biomarkers, of DGF and AR in renal transplantation, which could lead to a diagnostic test that has no morbidity or mortality associated with its use. In total 163 from twenty-four patients using blood samples over several different pre and post-operative time-points were analysed. Plasma was extracted and analysed by ^{1}H nuclear magnetic resonance spectroscopy (NMR). Spectra were interrogated using multivariate statistics, namely Principal component analysis (PCA) to reveal metabolites whose concentration varied as a function of kidney status. High performance liquid chromatography (HPLC) was used to validate and analyse molecules seen in the NMR studies. Until the third post-operative day, no differences were observed in the plasma metabolic profile of patients with DGF or AR in the NMR studies. From day four onwards molecules, trimethylamine-N-oxide and creatinine were found to vary in concentration across the patient groups in a way that correlated with the transplant outcome. In conclusion biomarkers exist in plasma which permit the discrimination between patients with DGF and AR, **from day four following renal transplantation**. These biomarkers are accessible, relatively non-invasive and without the morbidity associated with biopsy. A combination of these biomarkers presents the possibility of the development of a clinical diagnostic to improve clinical outcome.

Contents

References

Appendices

       Study recruitment

       Ethics

       Letter to patients

       Accepted abstracts

       Published paper

Figures

Figure 1.1 - The 'omics' family.

Figure 1.2 – Section of a plasma ^{1}H-NMR Spectrum.

Figure 1.3 Data Reduction (binning) of ^{1}H Spectra and the PCA Scores Plot from Such Data.

Figure 2.1 – Scores plot comparing all data for all time points and all patients.

Figure 2.2 – Scores plot comparing complete set of plasma for pre-operative, intra-operative and 2 hours post-operative samples.

Figure 2.3 – Section of a ^{1}H-NMR spectrum of intra-operative metabolic profile of a plasma sample.

Figure 2.4 - Scores Plot Comparing acute rejection, delayed graft function and primary function.

Figure 2.5 - ^{1}H-NMR spectrum of plasma from patient with acute rejection (AR) with expansion of area responsible for differentiation between rejected and non-rejected renal transplants

Figure 2.6 Scores Plot Comparing acute rejection versus primary function.

Figure 2.7 Scores Plot Comparing acute rejection versus delayed graft function.

Figure 2.8 – Loadings Plot. Values furthest from 0 are the greatest cause of discrimination within individual blood samples. These values correlate with the molecules creatinine, TMAO, and arginine.

Figure 2.9 – Plasma sample of LRD renal transplant (Patient 11).

Tables

Abbreviations

1D – one-dimensional

ala – alanine

Alem - Alemtuzumab

arg – arginine

AR – acute rejection

ATN - acute tubular necrosis

Bas - Basiliximab

°C – degrees Celsius

CIT - Cold ischaemia time

CNI – Calcineurin toxicity

CPMG – Carr-Purcell-Meiboom-Gill pulse sequence

Creat - Creatinine

δ – chemical shift (ppm)

Da – Daltons

DGF – delayed graft function

D_2O – Deuterium oxide

FID – free induction decay

HBD – Heart beating donor/deceased heart beating donors

HIF-1 - hypoxia-inducible factor-1

HOC – hyperosmolar citrate

HPLC – High Performance Liquid Chromatography

HR-MAS - high-resolution magic-angle spinning

ile – isoleucine

Immuno - Immunosuppression

lac-lactate

leu - leucine

lys - lysine

met – methionine

MMF - Mycophenolate mofetil

MP - Methylprednisolone

MR- magnetic resonance

MS – mass spectrometry

N/A - Not applicable

NHBD – non-heart beating donors/donation after cardiac death

NMR – nuclear magnetic resonance

OPA - σ-phthaldialdehyde

PC - principal components

PCA – principal components analysis

PF – primary function

Pred - prednisolone

PSA – prostate specific antigen

RAT - Renal artery thrombosis

RD – relaxation delay

RT – retention time

RVT - Renal vein thrombosis

SHY - Statistical heterospectroscopy

thr – threonine

Tac – Tacrolimus

TMA - Trimethylamine

TMAO – Trimethylamine-N-oxide

TSP – trimethylsilyl-propionic acid

tyr - tyrosine,

UW – University of Wisconsin solution

val – valine

WIT - Warm ischaemia time

Chapter 1

Introduction

1.1 Introduction

Over the past twenty years there has been a rapid expansion in the techniques available to analyse biological systems. Metabolomics is a relatively new term, having been coined in 1999 (Nicholson JK, 1999). It is defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiologic stimuli or genetic modification" and is concerned with the high throughput identification and quantification of the small molecule metabolites in the metabolome (Goodacre R *et al.*, 2004). The metabolome is the collection of the complete complement of all low molecular weight (<1500 Daltons [Da]) metabolites found in a system (cell, tissue, or organism) under a given set of conditions (Goodacre R, 2004; Wishart D, 2007). The metabolome constitutes the interaction between small molecules, such as metabolic substrates and products, lipids, small peptides, vitamins, and other protein co-factors (Ryals J, 2004; Beecher CWW, 2003).

A summary of metabolomic related definitions is provided in **Table 1.1**

 Table 1.1 Definitions related to metabolomics (Dettmer K *et al.*, 2007)

Metabolite	Small molecules that participate in general metabolic reactions and that are required for the maintenance, growth and normal function of a cell
Metabolome	The complete set of metabolites in an organism
Metabolomics	Identification and quantification of all metabolites in a biological system

Metabolomics is a rapidly expanding area of scientific research and is one of the new "omics" joining genomics, transcriptomics and proteomics, in an area of science trying to understand global systems biology (Schmidt C, 2004). This expansion has been reflected in the number of publications over the last 30-40 years with a steady increase since the year 2000 (Beecher CWW, 2003; Schmidt C, 2004). Further evidence of the rapid growth in the field is the recent launch of a dedicated journal *Metabolomics* (Springer, United Kingdom).

The profiling of metabolites was first reported in the literature in the 1950s but subsequent progress was slow, and only since the turn of the new millennium has metabolomics started to become a stand alone scientific entity.

The recent development of omic technologies and the analysis of the genome, transcriptome, proteome or metabolome, for understanding of complex systems and diseases at a global level, enable hypothesis generation and have occurred predominantly due to improvements in analytical techniques and data handling systems (Rochfort S, 2005). Whilst metabolomics is at the endpoint of the "omics cascade" and closest to the phenotype, which makes them a more appropriate target for phenotype- based research there is still no single-instrument platform that can currently analyse all metabolites (Dettmer K *et al.*, 2007), nor is there a standard complete metabolic database. When attaining a metabolic profile from a sample for analysis the process focuses on groups of metabolites. Often prior to the study the metabolites that may predict outcome are unknown and the method is akin to a "fishing expedition", as opposed to a "hypothesis driven" search which involves looking for trends that may foresee a result before it occurs. This can lead to problems; as with any high throughput

technology, metabolomics can be subject to high rates of false negative or false positive results.

The importance of metabolites in relation to the pathogenesis of disease is only just beginning to be fully understood, yet it is well appreciated that metabolites serve as markers for both the genome and proteome. Metabolites are not inert and are more than end products of metabolism. The study of single amino acid changes in a protein or a single base change in a gene can lead to as much as a 10,000 fold change in the concentration of certain metabolites (Bory C *et al.*, 1990). Under toxic stress, cells attempt to maintain homeostasis and metabolic control by varying the composition of the body fluids that either perfuse them or are secreted by them. This involves the simultaneous adjustment in the concentrations of hundreds or thousands of metabolites in order to maintain cell characteristics. Metabolites such as citrate, lactate, and glucose can reflect situations such as apoptotic alterations, hypoxia and oxidative stress. This metabolic adjustment is expressed as a metabolic fingerprint that can be sampled, analysed and compared (Lindon JC *et al 2004)*. Some authors (Katzmarzyk PT and Janssen L, 2004; Johnson JA, 2006) have also suggested that a large proportion of chronic disease, and therefore morbidity, arise from an adverse interaction between metabolite and the genome or proteome. Metabolites, such as those found in blood or urine or any biofluid contain in principle a comprehensive picture of the evolution of a patient's condition.

1.2 What Links the "Omics?"

Although metabolomics invariably deals with molecules less than 1500 Da in weight there is crossover in analysis and interaction between metabolomics and the better known proteomics and genomics **[Figure 1.1]**.
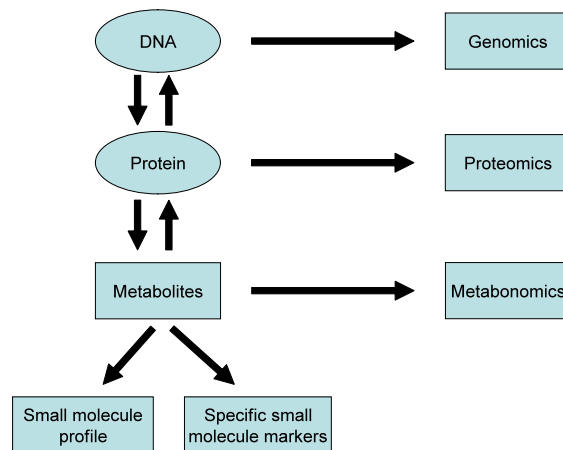
Figure 1.1 The 'omics' family

Genomics was derived from the term genome, which in turn was derived from a combination of the words; gene and chromosome to indicate the complete set of chromosomes, and the genes contained in them. The term "omics" has come to mean an approach capable of generating a comprehensive data set of whatever is being measured, be it transcripts (transcriptomics), proteins (proteomics), or metabolites (metabolomics) (Robertson DG, 2005). With these large comprehensive data sets correlations are sought with physiological and pathological conditions. It has been commented, that given the large amount of money spent on genomics and proteomics over the last decade there has been

surprisingly little impact on the numbers of new drugs making it into the clinical arena (Stumm G, 2002). A possible explanation for this observation is that genomics and proteomics do not provide evidence of endpoint markers for disease diagnosis, or evaluation of beneficial or adverse drug effects. With metabolomics, the ability to document the metabolites and therefore the phenotype, an absolute endpoint, may provide the ultimate diagnostic information (Lindon JC *et al*., 2004). It may well be easier to work from the phenotype through to the genotype than to try and speculate the significance of thousands of changes in transcript expression (Robertson DG, 2005).

The fields of proteomics and metabolomics use similar kinds of equipment for analysis, predominantly, mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy (Drysdale R and Bayraktaroglu L, 2005). In proteomics, problems arise from the sheer number of dynamically fluctuating proteins. In metabolomics, the general estimations of the size and number of species-specific metabolites are at a preliminary stage (Weckwerth W and Morgenthal K, 2005). The number of metabolites present in any given species is still unknown and estimates vary from 2000 in humans to up to 200 000 in plants (Dettmer K *et al*., 2007; Fiehn O., 2002).

All proteins are formed from amino acids that are themselves metabolites. Proteins use metabolites for their everyday function in the form of co-factors, signaling molecules or stabilizing agents, and when these proteins eventually decompose they return to a metabolite form. Proteins require metabolites and in return the metabolites require the proteins (Wishart D, 2007).

1.3 Analytical Techniques

In principle, the approach to metabolomics consists of two distinguishable parts: firstly, an experimental technique must be used to collect data from obtained samples. Secondly, a data processing technique must be applied to the dataset in order to sift out areas of interest from the millions of individual datapoints that are present. The end goal of the analytical technique being to accomplish a complete measurement of the metabolome and its responses to external stimuli whatever they may be.

1.4 Sample Collection

Metabolites are generally labile species, are chemically very diverse, and often present in a wide dynamic range. A major goal of all metabolite-profiling is to examine all metabolites within the biological system being sampled. Since body fluids such as plasma and urine contain huge numbers of small molecules this represents a considerable analytical challenge (Fiehn O, 2002). Indeed prior to any investigation careful consideration needs to be given to the type of sample, time taken to process, containers used in transit as the choice of vessel for sample collection is a particularly important requirement for successful metabolic profiling. Different tubes routinely used in the clinical setting contain different chemicals within them that can affect the resultant spectra and skew interpretation of results (Holland NT *et al.*, 2003).

At all times it is necessary to reduce as much biological variation that is not related to the hypothesis, this is because samples of biofluid can be very sensitive to diet, environmental factors, gender and geographical location.

Before any metabolites are measured it is important that any metabolic activity is stopped as quickly as possible to halt ongoing metabolic activity and reflect disease endpoint. With human tissue this can by done by snap freezing in liquid nitrogen (Hollywood K *et al*., 2006). At this point, samples are very stable as enzyme activity has stopped, and can be safely stored prior to analysis. Platelet or erythrocyte membrane samples may be analysed in other solvent media such as non-aqueous media enabling relatively non-water soluble lipid-like metabolites to be monitored.

1.5 Sample Analysis

There are two main technologies that may be used for analysis of the metabolome namely MS and NMR. NMR-based metabolomics has the advantage of being: non-destructive; non-selective (i.e. removes bias for a specific molecule); cost-effective; fast; and with minimal requirements for sample preparation. MS, however, is a much more sensitive technique and therefore the concentration detection limit is much lower than NMR (Goodacre R *et al*., 2004; Dettmer K *et al*., 2007; Dunn WB *et al*., 2005; Whitfield *et al*., 2004). MS requires a pre-separation technique of the metabolic components using either gas chromatography or liquid chromatography. The relative pros and cons of the two techniques are summarised in Table 1.2 (Robertson DG, 2005; Claudino WM *et al*., 2007). The reproducibility of both methods is not perfect and can result in variability in the data. MS based methods are more prone to this variability compared to NMR, mainly to the nature of the instrumentation and because the samples require more extensive pre-treatment and preparation.

Table 1.2 **The pros and cons of mass spectrometry (MS) and nuclear magnetic resonance (NMR) in relation to metabolomic studies** (Robertson DG, 2005; Claudino WM *et al.,* 2007)

Variable	NMR	MS
Equipment cost	High	High
Maintenance cost	High	High
Per sample cost	Low	High
Reproducibility (within or across labs)	High	Moderate
Identification of unknown metabolites	Time consuming	Can be Fast
Sensitivity	Lower than MS but approaches micromolar (10^{-3})	Higher than NMR
Quantitation	Routine	Difficult
Resolvable Metabolites	Possible	Possible
Potential for Sample Bias	No	Yes
Data Analysis Automation	Some	Yes

1.5.1 Nuclear Magnetic Resonance Spectroscopy

NMR is widely used in chemistry and was developed in the 1940s. It provides detailed information on the molecular structure of substrates. Its application to preclinical and medical imaging only really started in the 1980s as technology caught up.

It works on the basis that certain nuclei possess the property of spin. When placed in a magnetic field, these spins align themselves with respect to the magnetic field. Application of a radiofrequency pulse inverts the spins to a higher energy orientation and the magnetisation of the sample is deflected away from the direction of the magnetic field. When the radiofrequency (*rf*) is switched off, the spins undergo relaxation to their original lower energy orientations. During these processes a detectable NMR signal is produced that can be analysed with the aid of a computer in the form of readable spectra. Each molecule results in a set of lines in the spectrum, the positions of which (chemical shift) depends on chemical environment of the nuclei being observed [Figure 1.2] (Turner E *et al*., 2007). The area under the peak (any line for a nucleus from a particular molecule) is proportional to the concentration of that molecule present in the solution. Nuclei such as ^1H, ^{13}C, ^{15}N and ^{31}P may be observed but 1H is the most sensitive (non-radioactive) NMR nucleus and thus dominates NMR-based metabolomic studies.

The NMR spectrum of most metabolites has been analysed and hence it is possible to interpret new spectra on the basis of available data (Claudino WM *et al*., 2007; Griffin JL, 2004). If signals arise which have not previously been reported then other NMR experiments may be employed to unambiguously

assign these and thereby identify a new metabolite. Typically spectra of biofluids such as urine and plasma contain thousands of signals. With blood plasma and serum, protein and lipoproteins signals to the ^{1}H NMR spectrum and display broad lines, with sharp peaks from small molecules superimposed on them (Nicholson JK *et al*, 1995). Combinations of radiofrequency pulses maybe applied to remove these broad signals. Difficulties do occur not only in practicality but also interpretation of data. For example, the main component of any biofluid is water, which produces an NMR peak so huge that it can obscure other molecular information and cause dynamic range problems in the NMR detector (Lindon *et al*., 2004). Suppression techniques are possible to remove the water signal which does not interfere or destroy the sample. This is achieved by selective irradiation of the water signal with a weak *rf* field. In addition difficulty can also occur with visualization of low molecular weight metabolites in serum or plasma which can be obscured by the broad envelope of high molecular weight resonances of plasma proteins such as albumin (Lindon, *et al*., 2004).

Automatic sample preparation for NMR spectroscopy, is feasible. D_2O is added to act as a magnetic lock with a buffer solution to the biofluid and standard NMR spectra can take up to a few minutes to acquire. Using robotic flow injection methods, this process allows for larger and quicker throughput of samples. In this study a more conventional approach using 5mm diameter NMR capillary tubes loaded individually were used.
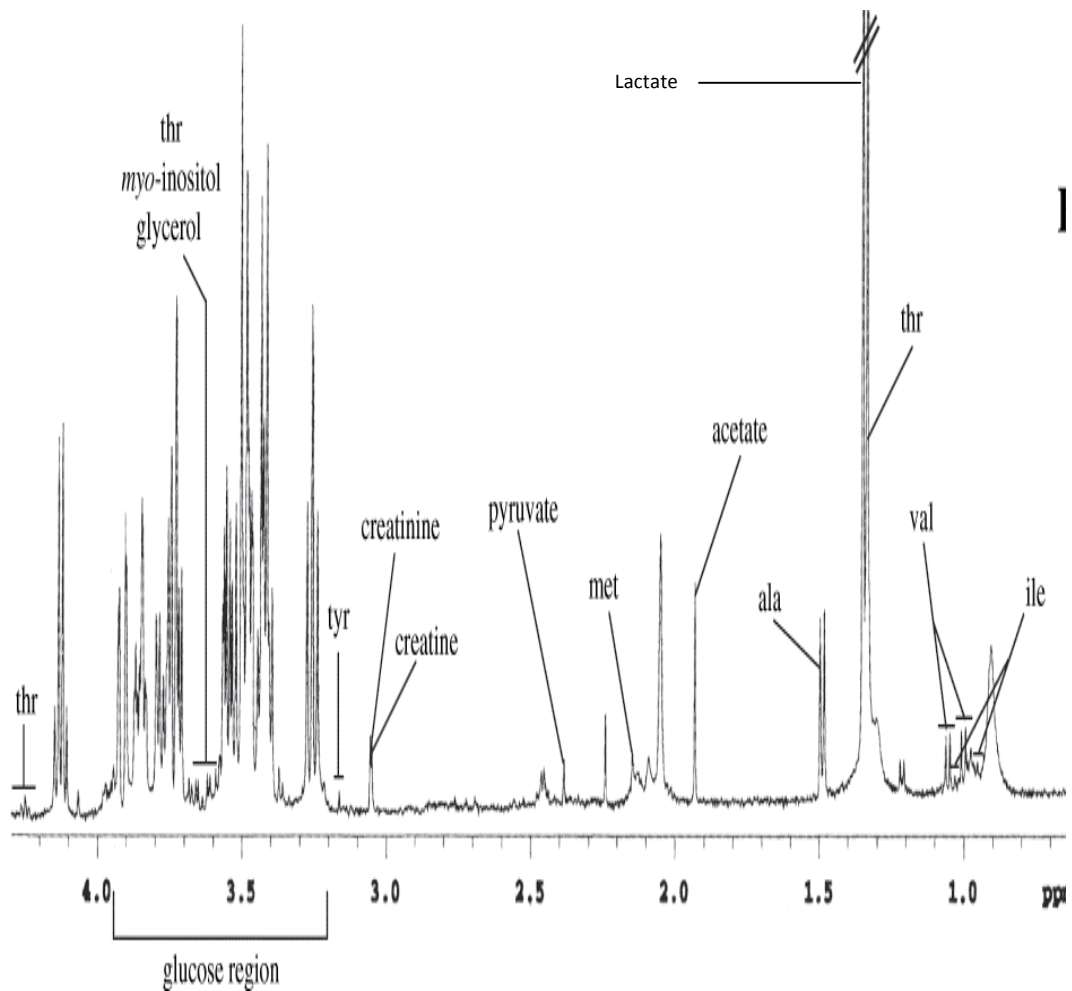
Figure 1.2 ^{1}H-**NMR Spectrum** Section of a plasma 1H-NMR spectrum ala =

alanine, ile = isoleucine, lys = lysine, met = methionine, thr = threonine, tyr =

tyrosine, val = valine (Turner E *et al.,* 2007)

1.5.2 Mass Spectrometry

MS is widely available and extensively used and is the standard technique in the pharmaceutical industry enabling identification and quantification of drug metabolites. MS offers high selectivity and sensitivity (Dettmer K *et al.,* 2007) and molecular formulae determination via accurate mass measurements (Dunn WB *et al.,* 2005). This makes it an incredibly powerful tool for analysis of large numbers of metabolites (Whitfield PD *et al*., 2004). MS based techniques are intrinsically more sensitive than NMR but usually require a sample preparation step, which unlike NMR can cause metabolite losses, and based on the sample introduction system and the ionization technique used, specific metabolite classes may be discriminated against (Dettmer K *et al*., 2007). Differing types of MS exist, with their varying advantages and disadvantages. Ion suppression and matrix effects can lead to difficulties in detecting certain compounds. These problems can be overcome by coupling a chromatography system to the mass spectrometer. The chromatographic step is able to reduce the number of competing analytes entering the mass spectrometer in addition to separating complex mixtures of metabolites. Derivatisation is required first in order to reduce the polarity of the analytes and enable chromatographic separation. (Griffin JL, 2004). As a result, liquid chromatography–MS is increasingly being used to profile low-molecular-weight metabolites (Pham-Tuan H *et al*., 2003; Plumb RS *et al*., 2002; Idborg-Bjorkman *et al*., 2003).Gas chromatography-MS is also growing in popularity for the profiling of volatile compounds (Dunn WB, 2008; Lenz ME & Wilson ID, 2007) This could be deemed a disadvantage, as most biofluids and tissue are involatile. Therefore treatment of samples needs to be undertaken to convert samples into volatile substances.

1.6 Statistical Analysis

Generally it is necessary to use data reduction and pattern recognition techniques in order to access the latent biochemical information present in the spectra and detect meaningful correlations once the data have been collected, as so much information is collated (Lindon *et al.,* 2004). Metabolomic studies result in complex multivariate data requiring a statistical approach to its analysis. Chemometrics is the term ascribed to data reduction and pattern recognition applied to chemical numerical data such as used in metabolomics. The aim being to assign and classify the spectral peaks, thus being able to identify data, which may contribute to a clinical condition. Chemometrics has been used to analyse "omic" data in other applications (Jellum E *et al*., 1981; Jiye A *et al*., 2005; Robertson DG *et al.,* 2000). It affords a well established, robust method of handling complicated data to give interpretable outputs. The most commonly used statistical approach at present is that of principal components analysis (PCA). In its simplest application to NMR data analysis, PCA requires the spectrum to be divided into many equally spaced regions (binning) and the area of the signal in each of these regions measured. Each region or 'bin' is between 0.01 to 0.04ppm. The bin is then interpreted for each clinical case. The regions with the biggest variation in area are likely to report of the status of the patient (healthy/unhealthy) and in this way provide a clinically useful biomarker of the condition (Dettmer K *et al*., 2007). PCA is an unsupervised statistical method which means that there is no knowledge of group/class of individual samples prior to analysis of the samples. The combination of NMR with statistical data analysis allows the measurement and interpretation of hundreds of metabolites,

quickly, easily and inexpensively (Dunn WB, 2005; Wishart D, 2006; Wishart D, 2005) **[Figure 1.3].**

Comparison of disease processes using chemometrics is to identify either a control group or compare different outcomes in patients with the disease. Hence in disease modeling a one-class or two-class system can be used.

One class: Only outcomes of disease define a group as patient group and control samples are very heterogeneous (gender, age, diet, lifestyle, disease process)

Two class: Control group and disease group define two separate classes

There is a limitation to the process in that it is rare for a peak not to overlap with another peak, thus it can be difficult to assign metabolites to overlapping peaks. Work is being done to address this problem in the form of library spectra (Wishart D, 2008), 2D NMR (Van QN *et al*, 2008) and statistical correlations (Sands CJ *et al*, 2009).

There are other methods also available for data reduction. Genetic algorithm aligns the peaks in automatically selected segments in each spectrum to the corresponding peaks in a preselected reference spectrum. A disadvantage to this process is that the reference spectrum cannot account for loss or appearance of new peaks (Forshed J *et al*., 2003). Statistical heterospectroscopy (SHY) allows data co-analysis and rapid structural information form metabolites by direct comparison between NMR and MS signals (Crockford DJ *et al*., 2006).
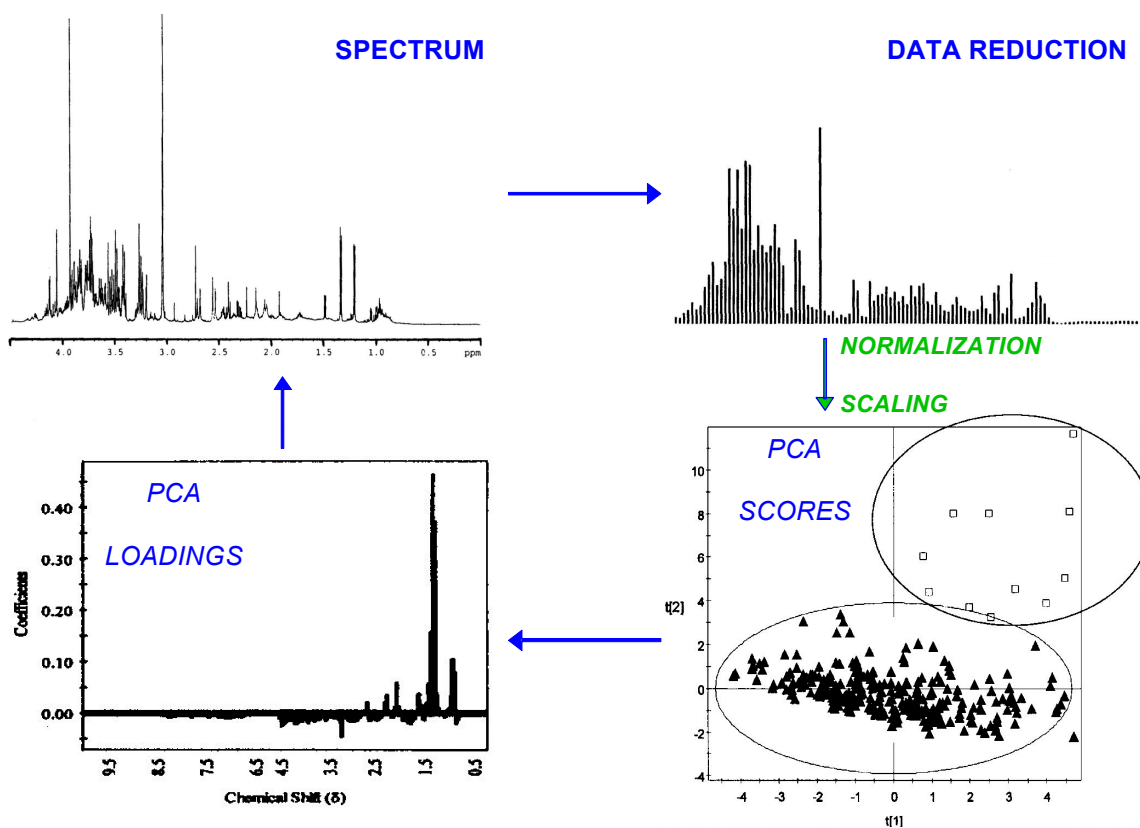
Figure 1.3 Data Reduction "binning" of ^{1}H **NMR Spectra and the PCA Scores Plot from such data**

1.7 Applications

Whilst still in its infancy, metabolomics is beginning to define its importance in the role of future medical advances. Omics technologies are currently used in two closely linked areas: biomarker detection and the elucidation of pathophysiologic processes to isolate novel therapeutic targets.

Some important considerations include:

1. Over 95% of all diagnostic clinical assays look for small molecules (Tietz NW, 1995)

2. 89% of known drugs are small molecules (Drugbank)

3. 50% of all drugs are derived from pre-existing metabolites (Mahido *et al.,* 1998)

4. 30% of identified genetic disorders involve diseases of small molecule metabolism (Hamosh A *et al.,* 2002)

In the clinical setting, metabolomics can be used to test cohorts of patients with certain pathologies and compare their metabolic profile with a "normal" control cohort or to compare the metabolic profile within disease groups. This is known as metabolic fingerprinting. A number of complex diseases, such as atherosclerosis, type 2 diabetes mellitus and cancer (Wishart D, 2007), may result from a chronic imbalance of normal metabolism. Therefore, metabolomic based approaches may be of use in identifying surrogate biomarkers of pathological states (Whitfield PD *et al.,* 2004). Initially in this approach the intention is not to identify each observed metabolite, but to compare patterns or ''fingerprints'' of metabolites that change in response to disease, toxin exposure, environmental or genetic alterations (Dettmer K *et al.,* 2006). At present metabolic fingerprints have been reported for some disease states which include; pre-eclampsia (Claudino WM *et al.*, 2007) motor neurone disease (Rozen S *et al.,* 2005), Alzheimer's (Han XM, *et al*., 2002), coronary artery disease (Sabatine MS, *et al.,* 2005), breast cancer (Fan X *et al.,* 2005), ovarian cancer (Odunsi K *et al*., 2005) and hepatic cancer (Yang J, *et al.,* 2004).

1.7.1 Toxicology

Currently in the field of toxicology, NMR and MS have been used to assess the health risk of particular toxins/drugs and identifying metabolic differences in experimental animals and humans **[Table 1.3]** (Beckwith-Hall BM *et al*., 1998; Nicholson JK & Wilson ID, 1989; Robertson DG, *et al*., 2000; Lindon JC, *et al*., 2000; Bairaktari E, *et al*., 1998). The administration of a drug generally induces a series of metabolic events many in a time dependent manner, analysis of which can provide insight to toxicology/efficacy of the drug Nicholls AW, *et al*., 2001; Waters NJ *et al*., 2002; Lenz EM, *et al*., 2004; Lindon JC, 2004)

Table 1.3. Metabolic markers associated with toxins as determined by metabolomic analysis (Beckwith-Hall BM *et al.,* 1998; Nicholson JK & Wilson ID, 1989; Robertson DG, *et al.,* 2000; Lindon JC, *et al.*, 2000; Bairaktari E, *et al.*, 1998)

Toxin	Target organ/toxicity type	Associated biomarkers
Amiodarone	Phospholipidosis (lung)	↑Phenylacetylglycine, ↑DMG
a-Naphthylisothiocyanate (ANIT)	Liver (cholestasis)	↑acetate, ↑bile acids, ↓citrate, ↑glucose, ↓hippurate, ↓2-OG,↓succinate,
Chloroquine	Phospholipidosis & liver necrosis	↑Phenylacetylglycine, ↑DMG
Ethionine	Liver	↑glucose, ↓2-OG, ↑taurine
Galactosamine	Liver (hepatitis like lesion)	↑acetate, ↑betaine, ↑bile acids, ↑creatine, ↓hippurate, ↑organicacids, ↑taurine, ↑urocanic acid, ↓2-OG, ↓succinate

Lead acetate	Liver/lung/kidney	↑acetate, ↑creatine, ↓citrate, ↑glucose, ↓hippurate, ↑lactate, ↓2-OG, ↑N-acetyls, ↓succinate, ↑taurine
Paraquat	Kidney & lung	↑amino acids, ↓citrate ↓creatinine, ↑glucose, ↓hippurate, ↑lactate & organic acids, ↓valine
Puromycin aminonucleoside	Kidney (glomerulus & proximal tubular)	↑acetate, ↑alanine, ↓citrate, ↑creatine, ↑formate, ↑glucose,↑macromolecules (proteins and lipids), ↓2-OG ,↑taurine, ↑TMAO
Sodium chromate	Kidney (S1 proximal tubular)	↓citrate, ↑glucose, ↓hippurate, ↓2-OG
Sodium fluoride	Kidney (proximal tubular)	↑acetate, ↑amino acids, ↓citrate ↓creatinine, ↑glucose, ↓hippurate, ↑organic acids, ↓2-OG, ↓succinate, ↑threonine

1.7.2 Markers of Disease

Clinical medicine strives for non-invasive biomarkers that can diagnose clinically relevant processes. Biomarkers have a variety of functions, from screening to diagnostic to prognostic. As many diseases are indeed a result of metabolic disorders, it makes a great deal of sense to measure metabolites directly. The value of the glucose stick in diabetes or urine tests for the diagnosis of pregnancy show the worth of such simple bedside tests. Therefore a very active area of research within metabolomics is that of discovering which metabolites are indicative of disease (Hollywood K *et al*., 2006). The advantages of metabolomics in revealing a biomarker are shown in **[Table 1.4]** (Gwinner W, 2007).

Table 1.4 Advantages of metabolomics in identifying non-invasive biomarkers (Gwinner W, 2007).

Sampling
Easy access to samples – blood, urine, tissue biopsy
Repeatable/serial sampling possible
Marker stability– samples can be stored for long periods
Analysis
Reproducible process
Robustness against interfering substances in the sample – disease context specific
Explanatory power
Potential for good specificity and sensitivity – established in literature

Some biomarkers have already been identified. Sanins *et al*. identified taurine a metabolite specific to the liver as a marker of disease. They found that raised urinary levels of taurine were associated with necrosis and fatty liver. These results correlated with routine blood investigations (Sanins SM *et al.,* 1990). Liver dysfunction was also coincident with the appearance of certain types of bile acids present in both serum and urine. Beckwith-Hall *et al.* found that on ingestion of liver toxins, certain metabolites were present in high concentrations such as cholic, glycocholic, and taurocholic acids (Beckwith-Hall BM *et al.,* 1998).

Gartland *et al* also observed changes in taurine concentrations. They found that following exposure to hydrazine, taurine, citrate and lactate levels were affected Gartland KPR *et al.*, 1991). Nicholson *et al.* identified succinate, glycine, and dimethylamine in the blood that were found to be indicators of kidney damage (Nicholson JK, *et al.,* 2002). Measurement of urine metabolites has proven to be a good marker of kidney damage due to the nature of where urine is derived. Anthony *et al.* and Gartland *et al.* found metabolites such as glucose, lactate, alanine, lysine, glutamine, glutamate, and valine to be present in abundance when damage to the proximal tubule of the kidney has occurred. This is due to the inability of the damaged kidney to filter metabolites present in the blood (Anthony ML, *et al.,* 1994; Gartland KP, *et al*., 1989; Gartland KP, *et al.,* 1989).

The main concern in the area of biomarker identification from initial studies is that the same metabolites (2-oxoglutarate, acetate, citrate, creatine, creatinine, glucose, hippurate, lactate, succinate, taurine and trimethyl amine/trimethyl amine oxide (TMA/TMAO) (Robertson DG, 2005) are often present in response to drug administration, regardless of the nature of the drug, i.e. different drugs often result in similar metabolic profiles when analysing samples such as urine (Anthony ML *et al.,* 1994; Holmes E, *et al*., 1992; Nicholls AW, *et al.*, 2001; Robertson DG, *et al*., 2000). Importantly however, not all these molecules materialise in response to every toxicant nor do they appear in the same concentrations.

1.7.3 Transplantation

In the field of solid organ transplantation some of the early metabolomic studies have looked into primary non-function, delayed graft function and acute rejection. There is currently no single test to differentiate these pathologies. Some of the metabolite findings already observed in the field of transplant are summarised in **Table 1.5** (Wishart D, 2005).

In the field of hepatic transplantation, the widening of indications for liver transplant coupled with the shortage of donors, has lead to the increased utilisation of marginal grafts, which have higher rates of non-function and graft failure (Vilca Melendez H, *et al.,* 2000; Duarte IF, *et al*., 2005; Serkova NJ, *et al*., 2007a). The need for early diagnosis in these settings is becoming more apparent. The inability to diagnose primary graft non-function in a liver transplant patient at an earlier stage than is possible with currently available methods has far reaching implications. A delay in diagnosis can often mean that a patient is too ill for re-transplantation, whereas earlier detection within two to three hours of the implantation may result in better patient outcomes.

Serkova *et al.* reported on the use of metabolic profiling in a single patient who was re-transplanted successfully 8 days after the initial graft, which failed due to portal vein and hepatic artery thrombosis. They used NMR as a means of profiling the patient's blood: pre-operatively; 2 hours; 24 hours; and 48 hours post-operatively. Distinctly different profiles between the first unsuccessful and the second successful transplant were demonstrated. Variation was seen between the two operations in lactate, uric acid, glutamine, methionine and citrate, changes that were seen as early as two hours post operation. The patient's

metabolic profile following the successful transplant was found to be comparable with that of 5 healthy volunteer controls (Serkova NJ, *et al.*, 2007a).

Table 1.5. **Summary of metabolite measurement in organ transplantation or dysfunction** (Wishart D, 2005)

Organ	Condition	Metabolites Increased	Metabolites decreased
Kidney (Human)	Graft dysfunction	TMAO, dimethylamine, lactate, acetate, succinate, glycine, alanine	
Kidney (Rat)	Graft dysfunction or reperfusion injury	TMAO, dimethylamine, lactate, acetate, succinate, glycine, alanine	
Kidney (Rat)	Reperfusion injury (ischaemia)	TMAO, allantoin	
Kidney (Human)	Acute rejection		Nitrates, nitrites, nitric oxide metabolites (urine)
Liver (Human)	Reperfusion injury	Lactate, pyruvate, glycerol, alanine, glutamate, GABA,	Arginine <19hrs

		taurine, arginine >19hrs	
Liver (Rat)	Reperfusion injury	Citrate, succinate, ketone bodies (GOOD function)	Citrate, succinate, ketone bodies (POOR function)
Liver (Human)	Ischaemia	Methylarginine, dimethylarginine	
Liver (Human)	Graft dysfunction	Glutamine	Urea
Liver (Human)	Post-transplant	Phosphatidylcholine (bile)	

In renal transplantation, current monitoring of transplant recipients relies on technologies that have been in the clinical domain for many years, including measurement of serum creatinine levels, total urine output and blood pressure. Whilst identifying renal dysfunction, these tests do not provide sufficient specificity or sensitivity to determine the cause of impaired function and therefore may not allow appropriate and timely intervention. This is due to the presence of confounding factors such as hypertension, infection and immunosuppressive medications. In such cases, patients often require invasive biopsies and thus the definitive diagnosis of delayed graft function or acute rejection may be delayed (Wishart D, 2006; Gwinner W, 2007; Veronese FV, *et al*., 2005; Hauert, *et al*; 2000). Transplant biopsies are currently the only reliable

method of diagnosing the aetiology of transplant dysfunction, however they are not without complication and do present a risk both to patient and graft (Rush D, *et al*., 2000).

Rush *et al*. in 2000 examined the role of NMR in the identification of acute rejection following renal transplantation in patient urine samples (Rush D, *et al*., 2000). They reported 93% sensitivity and 96% specificity for the determination of acute rejection whilst the process was still at a subclinical level and therefore before the traditional methods of diagnosis. They predicted acute rejection three days earlier than with traditional methods of measuring serum creatinine allowing earlier treatment and improved long-term outcomes. The name of the metabolite identified was not revealed in the publication. The importance of early diagnosis of acute rejection at a preclinical level had previously been identified in a randomised study by the same group (Rush D, *et al*., 1998). They found early corticosteroid treatment of patients diagnosed with subclinical rejection based on invasive biopsy had better long-term outcomes. They showed a decrease in early and late clinical rejection, a lower serum creatinine at twenty-four months and improved long-term graft function in renal transplant patients subjected to protocol biopsies.

Foxall *et al* looked at patients with a diagnosis of either acute rejection or cyclosporin nephrotoxicity by analysing urine (Foxall PJ, *et al*., 1993). A significant clinical problem with transplant patients is differentiating between patients undergoing graft rejection and those suffering from cyclosporin toxicity. NMR was shown to clearly demonstrate and differentiate when a patient was undergoing graft rejection as apposed to cyclosporin nephrotoxicity (Foxall PJ, *et*

al., 1993). They noted a statistically significant increase in Trimethyl-N-oxide in those with graft dysfunction.

1.7.4 Oncology

In the vast area of oncology work is still surprisingly in its early stages. Yet cancer metabolism represents a field ideal to metabolic profiling because of the way its metabolism differs substantially from normal cells, especially that of the glucose and phospholipid metabolism (Serkova N, *et al*., 2007b).

Conventional treatment has a somewhat blunderbuss approach with treatment strategy based on tumour size and stage. A more individual approach targeting specific antigens and antibodies has led to the development of specific biomarkers to assess treatment progress. Linking metabolic profiles to cancer depends heavily on the analytical instruments available since in the aetiopathogenesis of cancers, abnormalities in gene expression, protein expression and biochemical pathways often coexist (Schmidt C, 2004). Traditional approaches using single molecule markers are often associated with a low predictive value (Foxall PJ, *et al.,* 1993). Current markers identified are shown in **Table 1.6**

Table 1.6 Oncological metabolite markers

(Marcotte EM, 2001; El-Sayed S, *et al.*, 2002; Moreno A, *et al*., 1998; Ben-
Yoseph O *et al.,* l1993; Beckonert O, *et al.,* 2003; Howells SL, *et al*., 1992;
Carmichael PL, *et al*., 1998; Chen JH, *et al.,* 2001; Millis K, *et al.,* 1999; Jordan
KW & Cheng LL, 2007)

Metabolite	Tumour Association
Taurine	Increased in squamous cell carcinoma, prostate cancer and liver metastases
Alanine	Increased in astrocytomas, gliomas, meningiomas and hepatomas
Myo-inositol	Increased in colonic and ovarian cancer. Decreased in breast cancer
Choline	Varying levels in hepatomas, prostate and brain tumours

NMR is able to assess and quantify all upregulated glycolytic metabolites. Genes
known to be associated with cancer p53, c-MYC and HIF-1 all cause an increase
in glucose metabolism. Metabolic profiling can assess this increase in glucose
uptake via the tumour cells (Matoba S, *et al*., 2006; Kim JW, *et al*., 2006; Shim
H, *et al.,* 1997)

Griffiths and Stubbs used NMR spectroscopy to measure metabolite changes in
mouse-derived tumour cells that were deficient in the transcription factor,

hypoxia-inducible factor-1 (HIF-1) (Griffiths JR, *et al.*, 2002). HIF-1 has been widely shown to play a direct role in accelerating tumour growth. They found that adenosine triphosphate production was impaired in HIF-1 deficient tumour cells. The spectra also revealed lower concentrations of glycine, betaine and some choline based metabolites. These metabolites are required for purine synthesis thus demonstrating possible avenues for future drug therapies in the bid to stop tumour growth (Griffiths JR, *et al.*, 2002; Griffiths JR, *et al.*, 2003).

Sitter *et al.* studied patients with breast cancer and looked at 88 tumour samples and 18 tissue samples from adjacent non-tumour tissue (Sitter B, *et al.*, 2006). With the use of high-resolution magic-angle spinning (HR-MAS) magnetic resonance spectroscopy they compared the fingerprints obtained from the two types of tissue. The concentrations of glycerophosphocholine, phosphocholine, and choline signals were recorded. They reported 82% sensitivity and 100% specificity. The study demonstrated that tumoral tissues when assessable by HR-MAS MR spectroscopy differed in their metabolic content from their benign counterparts (DeVita VT, *et al.*, 2005)

Odunsi *et al* compared 38 patients with epithelial ovarian cancer with 12 patients with benign ovarian cysts and 53 healthy women. Clear differentiation was observed between those with cancer and those without, although the nature of the metabolites giving rise to the discrimination has yet to be determined (Odunsi K, *et al.*, 2005).

Prostate cancer has a high incidence in men over 50 years old and current tests such as prostate specific antigen (PSA) and digital rectal examination (DRE) are not sufficiently specific. PSA although only seen in relation to prostatic

pathology, can be elevated in benign conditions such as benign prostatic hyperplasia and prostatitis. Abnormal PSA and DRE findings are only accurate for cancer in between 25-50% of cases (Jordan KW, *et al*., 2007). This can often lead onto prostate core biopsies, an invasive procedure not without its own morbidity, but nevertheless the gold standard in this field of cancer diagnosis. Sheidler *et al.* combined magnetic resonance spectroscopic imaging with traditional techniques of magnetic resonance imaging. This involved quantitative detection of choline, citrate and creatine via echo delay. They demonstrated an increase in sensitivity and specificity for the diagnosis of prostate cancer of 95% and 91% respectively, compared to established sensitivities and specificities of between 60-75% with current tests. The process noted that the three metabolites appeared in different ratios when compared between benign and malignant conditions (Sheidler J, *et al.,* 1999)

Excellent discrimination in lung cancer patients has been seen by Vautz *et al*. Analysis of 36 lung cancer patients versus 54 healthy controls using metabolic profiles of breath showed correct classification in 99% of the patients. The process involved using Ion mobility spectrometry (IMS) coupled to a multi-capillary column alongside an integrated medical software system for data processing (Vautz W & Baumbach JI, 2008).

1.7.5 Coronary Heart Disease

Brindle *et al.* used NMR analysis of serum to differentiate patients with triple vessel coronary heart disease from angiographically normal subjects (Brindle JT, *et al*., 2002). They were able to distinguish not only the presence of disease but also the severity of the coronary disease on the basis of NMR studies. Their study differentiated greater than 90% of patients with three vessel disease over patients with normal angiograms. From the study it was also possible to diagnose the severity of coronary stenosis. However the results of this work have been questioned (Kirschenlohr HL. *et al*., 2006). Acute events such as myocardial infarctions are by their nature unpredictable which precludes prior blood sampling for predictive purposes. The atherothrombotic plaques involved in coronary artery disease are varied in their proportions which may influence plasma biochemistry.

1.8 The Future

Metabolomics is a burgeoning science, usually combining high resolution NMR or MS with pattern recognition strategies to rapidly and accurately diagnose the metabolic status of the sample being studied. It has developed considerably in the last decade and is now recognized as an independent and widely used technique, as well as a proteomics-complementing technology (Weckwerth W, *et al*., 2003)

Metabolomics has the ability to evaluate onset, duration, severity and organ specificity of a disease or condition from easily available samples. As knowledge in this field improves, along with the evolution of the Human Metabolome Project (Wishart D, 2007) , the discovery of new metabolites and their interaction with the human genome and proteome will allow further advances in: drug

manufacture with enhanced drug targeting; development of biomarkers; and a better understanding of diseases processes. It has already been adopted by a number of pharmaceutical companies in their drug development initiatives (Lindon JC, *et al.*, 2004).

With regards to its clinical potential, metabolomics allows diagnosis of pathological conditions through identification of disease specific fingerprints, and changes in such profiles will assist in assessing disease progression and monitoring treatment (Turner E, *et al.,* 2007). The technology will also be able to predict drug toxicity and response following medical intervention such as administration of chemotherapy to oncology patients.

Metabolomics can provide new biomarkers, and with its ability to measure more than one metabolite at anyone time, this process will surely be more predictive than traditional single marker testing. Over time a powerful list of diagnostic markers will evolve, which can be measured using high-throughput assays enabling earlier diagnosis and quicker more patient specific treatment.

1.9 Objectives of This Project

The purpose of this study is to assess the metabolic activity of patients undergoing renal transplantation. Using NMR technology the study aims to analyse those patients who in the post-operative period develop acute rejection, delayed graft function or have no graft dysfunction and have primary function. The aim being to find a non-invasive biomarker that may predict graft outcome with the sensitivity and specificity of the current gold standard, a renal biopsy, but without the associated complications.

Chapter 2

^{1}H NMR Spectroscopy of Plasma of Renal Transplant Patients

2.1 Background

The clinical course immediately after renal transplantation is varied. Differential diagnosis of a graft dysfunction includes AR, acute tubular necrosis (ATN) manifesting as DGF, and calcineurin inhibitor (CNI) toxicity. All these can also co-exist and contribute to graft dysfunction. Clinical and laboratory findings can often be unhelpful and make differentiation between pathologies difficult. Reduced urine output, rising serum creatinine levels, hypertension, graft tenderness and peripheral oedema (Wishart, 2006) are non-specific diagnostic markers. The gold standard is a histological diagnosis obtained from renal biopsy. Invasive biopsies are not without their risks. Complications include haematuria (35%), haematoma (65%), arteriovenous malformation (AVM), and injury to bowel. Whilst most biopsies are asymptomatic, transfusion is required in less than 1%, some of these requiring re-exploration (0.3%) and renal loss occurs in less than 0.1% of cases. Loss of life is reported but is extremely rare (Wishart D, 2006; Hergesell O, *et al*., 1998; Parrish AE, 1992; Whittier WL & Korbet SM, 2004; Lin WC, *et al*., 2006; Al Rasheed SA, *et al*., 1990). AR is a key factor in long-term graft function and survival in renal transplant patients. Timely detection and treatment of rejection is therefore, an important goal in post-transplant surveillance (Hergesell O, *et al*., 1998). Extensive effort has been directed towards developing a non-invasive test, which remains as specific and sensitive as a biopsy but can be used in the immediate postoperative period without risk of injury to patient or transplant, for monitoring of AR (Wishart D, 2006; Gwinner W, 2007; O'Riordan E, *et al*., 2004; Clarke W, *et al.,* 2003; Wittke S, *et al*., 2005).

Work in this field has concentrated on blood, urine and individual immune and injury markers therein. Potential markers identified have included neopterin (Carlson IH, 1992), sCD30 (Pelzl S, *et al*., 2003), monokines induced by IFN-γ(MIG) (Hauser IA, *et al*., 2005) and mRNA for perforin granzyme B (Vasconcellos LM, *et al.*, 1998) but none of these are in current clinical use.

We describe in this chapter a NMR spectroscopy-based metabolomics approach to the analysis of plasma which has permitted the identification of small molecule (metabolite) biomarkers which could act as diagnostics of DGF and AR. NMR allows for minimal sample preparation, non-destructive analysis and can identify molecules at millimolar concentration. Such hypothesis generating metabolomic studies have been performed in the clinical setting previously; in transplantation (Serkova NJ, *et al.,* 2006), pre-eclampsia (Turner E, *et al,* 2007), motor neurone disease (Rozen S, *et al*., 2005), Alzheimer's (Han XM, *et al*., 2002), coronary artery disease (Sabatine MS, *et al*., 2005), breast cancer (Fan X, *et al*., 2005), ovarian cancer (Odunsi K, *et al*., 2005), hepatic cancer (Yang J, *et al*., 2004), nephrotoxicity in animal models (Boudonck KJ, *et al*., 2009), and indeed in renal transplantation (Wishart DS, 2005; Hauet T, *et al*., 2000). In this study analysis of 24 patients' plasma over several post-operative time points by proton (^{1}H) NMR spectroscopy has revealed biomarkers present in all classes of graft outcome, but their concentrations vary in a graft-status-dependant manner such that it is possible to differentiate between DGF, AR and PF from day four post-operation. These biomarkers may provide a diagnostic that could aid post-operative management of renal transplant patients.

The following chapter focuses on the analysis of plasma of 24 consecutive patients, who underwent renal transplantation, using ^{1}H NMR spectroscopy.

Samples were taken at nine separate time points where possible. Pre-operatively, one to ten minutes post reperfusion, two hours post operation and post-operative days, one, two, three, four, five and seven. In some cases nine blood tests were not possible per patient if haemolysis of the sample or a delay of greater than one hour from patient's blood test to freezer occurred.

2.2 Patient Selection

Twenty-four consecutive patients undergoing renal transplantation who were admitted to St James's University Hospital a part of The Leeds Teaching Hospitals Trust were recruited into the study. **Table 2.1** presents the donor and recipient demographics.

For the purposes of this study patients were divided into three groups according to clinical outcome.

1. Primary function (PF)
2. Delayed graft function (DGF)
3. Acute rejection (AR)

If a patient had both DGF and AR, or PF and then AR they were assigned to the group with AR. DGF was defined as the need for dialysis in the first week post-renal transplantation. Diagnosis of DGF or AR was based on histology from a biopsy. All biopsies were performed between days six and nine. No patient in this study had biopsy proven CNI toxicity. Demographics are shown in **Table 2.1**.

Table 2.1 – Recipient and Donor Demographics

Patient	Recipient Age (yrs)	Recipient Gender	Aetiology of renal failure	Donor Age (yrs)	Type of donor	Cause of donor death	HLA	Induction Agent	Immuno	CIT (mins)	WIT (mins)	Biopsy Proven DGF	Biopsy Proven AR
1	76	M	Unknown	78	Deceased donor	Skull fracture	1:1:1	Bas + MP	Tac + MMF	1060	35	N	N
2	55	M	Autosomal dominant interstitial nephritis	44	Deceased donor	Intracranial haemorrhage	1:1:1	Bas + MP	Tac + MMF	957	30	N	N
3	52	M	Fanconi's	59	Deceased donor	Cerebral infarct	1:1:1	Bas + MP	Tac + MMF	750	45	Y	N
4	25	F	Ureteric reflux	28	Living Donation	N/A	2:1:2	Bas + MP	Tac + MMF	258	18	N	N
5	78	M	Unknown	55	Donation after cardiac death	Intracranial haemorrhage	1:1:1	Bas + MP	Tac + MMF	895	30	N	N

6	52	F	Unknown	55	Donation after cardiac death	Intracranial haemorrhage	2:1:1	Bas + MP	Tac + MMF	659	30	Y	N
7	65	M	Adult polycystic kidney disease	37	Donation after cardiac death	Hypoxic brain damage	1:1:1	Bas + MP	Tac + MMF	965	32	Y	Y
8	52	M	Adult polycystic kidney disease	37	Donation after cardiac death	Hypoxic brain damage	1:1:0	Bas + MP	Tac + MMF	675	33	Y	N
9	24	M	Malignant hypertension	42	Living related	N/A	1:1:1	Bas + MP	Tac + MMF	160	18	N	N
10	53	M	Unknown	35	Deceased donor	Intracranial haemorrhage	1:1:0	Bas + MP	Tac + MMF	1680	20	N	N
11	42	F	Unknown	46	Living Related	N/A	0:2:1	Bas + MP	Tac + MMF	212	41	N	Y
12	68	M	Hypertension	69	Deceased donor	Road traffic accident	1:1:0	Bas + MP	Tac + MMF	802	25	N	N
13	64	M	Hypertension	53	Deceased donor	Intracranial haemorrhage	2:1:1	Bas + MP	Tac + MMF	1080	35	Y	Y

14	53	M	Hypertension	47	Living related	N/A	1:2:1	Alem + MP	Tac + MMF	180	21	N	N
15	51	M	Adult polycystic kidney disease	63	Living related	N/A	1:2:2	Bas + MP	Tac + MMF	184	36	Y	Y
16	53	M	Adult polycystic kidney disease	21	Donation after cardiac death	Subarachnoid haemorrhage	2:1:0	Bas + MP	Tac + MMF	870	42	Y	N
17	23	F	Renal Tuberculosis	17	Donation after cardiac death	Hypoxic brain damage	1:1:2	Bas + MP	Tac + MMF	683	35	Y	N
18	26	M	Dysplastic Kidneys	17	Donation after cardiac death	Hypoxic brain damage	1:1:1	Alem + MP	Tac + MMF	952	36	Y	N
19	61	M	Adult polycystic kidney disease	48	Deceased donor	Hypoxic brain damage secondary to pulmonary embolus	0:0:0	Bas + MP	Tac + MMF	1275	45	Y	N
20	36	M	Hypertension	48	Deceased donor	Hypoxic brain damage	0:0:0	Bas + MP	Tac + MMF	1090	55	Y	N

						secondary to pulmonary embolus							
21	63	M	Membranous nephropathy	49	Deceased donor	Intracranial bleed	0:0:0	Bas + MP	Tac + MMF	816	55	N	N
22	45	M	Unknown	43	Living related	N/A		Bas + MP	Tac , MMF + pred	42	45	N	N
23	63	M	Unknown	45	Living related	N/A		Bas + MP	Tac + MMF	154	40	N	N
24	29	F	Pyelonephritis	38	Deceased donor	Glioma	0:1:0	Bas + MP	Tac + MMF	768	25	N	N

Table 2.2 – Statistical Significance of Recipient and Donor Characteristics

	AR	DGF	PF	Statistical Significance
Recipient Age (years)	55.5 ± 11	43 ± 14.3	54 ± 20.7	NS
Sex M/F	3/1	6/2	10/2	NS
Donor Age (years)	49.8 ± 11	34.3 ± 19.1	49.8 ± 16.6	NS
Total HLA Mismatches	3.7 ± 1	3.2 ± 0.7	3.1 ± 1	NS

Values = mean ± standard deviation

NS = Not significant

2.3 Blood sample collection

Venous blood was collected in lithium heparinised anticoagulant tubes. The blood samples were taken at the same time as routine clinical blood tests. The blood was centrifuged at 2000 rpm for ten minutes at 4°C all within one hour of sample collection from the patient. The plasma was removed from the sample, and stored in Eppendorf tubes at −80°C until NMR measurements were performed. In this study plasma was used as the risk of uncontrolled and incomplete clotting is very low, allowing excellent reproducibility amongst samples.

Samples were discarded if contaminated in the blood letting process or were delayed by greater than one hour before being frozen. Ethical consideration in this study did not allow for a patient to have another blood sample taken solely for the study as this would amount to additional intervention in the normal clinical care of the patient.

2.4 NMR sample preparation

Samples were prepared with a 0.17 % w/v solution of the sodium salt of 3-(trimethylsilyl)propionic- 2,2,3,3-d_4 acid (TSP) (Sigma-Aldrich, UK) in deuterium oxide (D_2O) (Fluorochem, UK). TSP was used as a chemical shift reference (0 ppm). 350 μL of this was added to 300 μL of the plasma in an Eppendorf tube. The mixture was vortexed to ensure adequate mixing before transferring all 650 μL to a 5 mm NMR tube (528PP-WILMAD, Sigma-Aldrich, UK).

2.5 Preparation of samples for metabolomic analysis of plasma

Prior to NMR data collection, plasma samples were thawed to room temperature and then centrifuged at 4000 rpm for 10 minutes.

2.6 NMR experiments

^{1}H-NMR spectra were acquired at 499.97 MHz on a Varian Unity Inova 500 spectrometer, at 20°C

2.6.1 CPMG.

The Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence [RD – 90° – (τ – 180° – τ)n – acq] was used for data collection . This technique removes signals from high molecular weight molecules (such as proteins) enabling those for small molecules to be resolved and quantified. A relaxation delay (RD) of 2 seconds was used, during which the water resonance was selectively irradiated to suppress it. The spin-spin relaxation delay, 2 nτ, of 450 ms was used for all samples; τ of 1.5 ms and n of 150. 256 transients were collected into 32,768 data points for each spectrum with a spectral width of 6499.84 Hz.

An exponential line broadening of 1 Hz was applied to each free induction decay (FID), prior to zero filling to 65,536 points, followed by Fourier transformation. Resultant spectra were phased and baseline corrected using VnmrJ 1.1D (Varian Inc., Palo Alto, California, USA).

2.7 Results

Spectra were "binned" into 225 segments, each with a width of 0.04 ppm, over the range of −1 to 8 ppm. The integrals in each binned segment were measured (Turner E, *et al.,* 2007; Turner E, *et al*., 2008). These relate directly to the concentration of the molecules giving rise to the signal. These integrals were normalised to unit total sum of the spectral integral to ensure that the spectra were directly comparable, and used for multivariate analysis. The chemical shift reference molecule, TSP, may also be used as a concentration reference and thus for normalisation. However, this charged molecule is known to interact with albumin in the plasma, affecting its NMR response. For this reason the

alternative approach of normalising to total spectral integral was utilised (Lindon JC, *et al.,* 2000; Kriat M, *et al.,*1992).

PCA was performed on mean-centred Pareto-scaled data using SIMCA-P + 11 (Umetrics, Umeå, Sweden). PCA is an unsupervised statistical method able to simplify, distinguish and compare between groups of subjects based solely on the characteristics of their NMR spectrum, with no knowledge of the 'preformed' groups (Lindon JC, *et al*., 2003) and present the data in two or three dimensions.

Chemical shift ranges δ 4.44 to 5.92 (water) and δ 3.44 to 3.96 (glucose), were removed from the analysis of plasma. Lactate found at δ 1.32 to 1.36 (lactate methyl (CH_3) group) and δ 4.08 to 4.12 (lactate CH_2 group) was removed in all analyses except as described in section 2.7.4 where lactate was included for discrimination purposes. Removal of water was to eliminate the effects of varying efficiency in water suppression during NMR data acquisition. It is common to remove signals from glucose as these can vary in response to differences in the food intake of the individuals' involved (Holmes E, *et al.,* 1994). Lactate signals were large and preliminary statistical analyses were dominated by these. Whilst Pareto scaling is used to accommodate such wide dynamic range within sample metabolite concentrations, it can be beneficial to tighten the range (by removing the larger signals) to investigate lower concentration components (Turner E, *et al*., 2007; Tiziani S, *et al*., 2008). **Table 2.3** shows the common metabolites found in a typical sample of plasma and the chemical shift at which it occurs (Nicholson JK *et al*., 1995).

Table 2.3 - Resonance assignments with chemical shifts in spectra of normal human blood plasma.

1H **Shift (δ)**	**Multiplicity**	**Molecule**
1.33, 4.11	D(1.33), Q(4.11)	Lactate
1.46	D	Alanine
1.68, 1.91, 3.24	M(1.68, 1.91) T (3.24)	Arginine
1.69	M	Lysine
1.99, 2.05, 2.36, 3.34, 3.45	M	Proline
2.52, 2.69	D	Citrate
2.84, 2.94	DD	Asparagine
3.05, 4.05	S	Creatinine
3.06, 3.16	DD	Tyrosine
3.26	S	TMAO

Key – S =singlet peak, D = doublet, T = triplet, Q= quartet M = complex multiplet, DD= doublet of doublets

The statistical significance of demographic data was analysed using SPSS v13 for Windows (SPSS Inc., Chicago, IL), using Kruskall Wallis and Mann-

Whitney tests. Data was tabulated using Excel (Microsoft ® Excel 2004 for Mac 2004 .Version 11.0). p values <0.05 were defined as statistically significant.

Analysis was performed on;

1. All data for all time points and all patients

2. With removal of pre-operative, post perfusion and two hour post operation samples

3. Individual patient sets

4. Spectra with lactate included

5. Donor type categories

2.7.1 Analysis of all data for all time points and all patients

In total, analysis was made of 163 samples, taken pre-operatively, intra-operatively, two hours post-operatively and on days, 1, 2, 3, 4, 5 and 7.

Pre-operative samples were taken on admission of the patient for renal transplant along with routine pre-operative bloods. Intra-operative blood samples were taken one to ten minutes post perfusion of the transplant kidney. The variation in time was due to safety and stability of the patient being paramount once reperfusion had taken place, so the blood sample was only taken once the patient had recovered from the initial insult of reperfusion. Post-operative samples were taken two hours after the anaesthesia had been withdrawn. Day one to seven bloods were taken early morning with routine samples for clinical practice.

Figure 2.1 is a typical output from the PCA of the NMR data. Each point on the figure represents an individual time point for an individual patient. The first and second principal components PC1, PC2 are depicted; these relate to the two most variable bins. Clustering of a majority of samples as shown in **figure 2.1** is seen. There is no discrimination in terms of metabolite profile between the separate clinical groups Metabolic composition analysed by 1H NMR spectroscopy can be influenced by lifestyle factors including smoking (Vulimiri SV *et al.*, 2009), diet (Holmes E *et al.,* 2008) and medications (Sands CJ, *et al.,* 2009). With this in mind, the samples included in the study were reviewed. The pre, intra and 2 hour post-operative period samples were considered separately (**Figure 2.2**). Within this sample set again no differentiation was seen between those pre, intra and two hour postoperative samples. However this series illustrates the natural differences between individuals from the outset.

Pre-operative samples were taken when the patient arrives on the ward for their transplant. The very nature of renal transplantation means that patients can turn up at anytime of day having eaten, just taken their medications or had a cigarette or have been exposed to cigarette smoke. These factors would influence the metabolic profiles of the patients rendering spectra difficult to interpret.

Anesthesia seems to have contributed to the metabolic spectra of blood samples taken at the intra-operative and post-operative time points (**Figure 2.3**). No obvious discrimination was seen between the different clinical outcomes at these particular time points.

As it appeared that the pre-operative, intra-operative and 2 hour blood test were being effected by pre-operative fasting/non-fasting state, anaesthesia and drugs

used at induction, there-by effecting analysis, it was decided to procede to further analysis without these three timepoints included in the statistical analaysis.

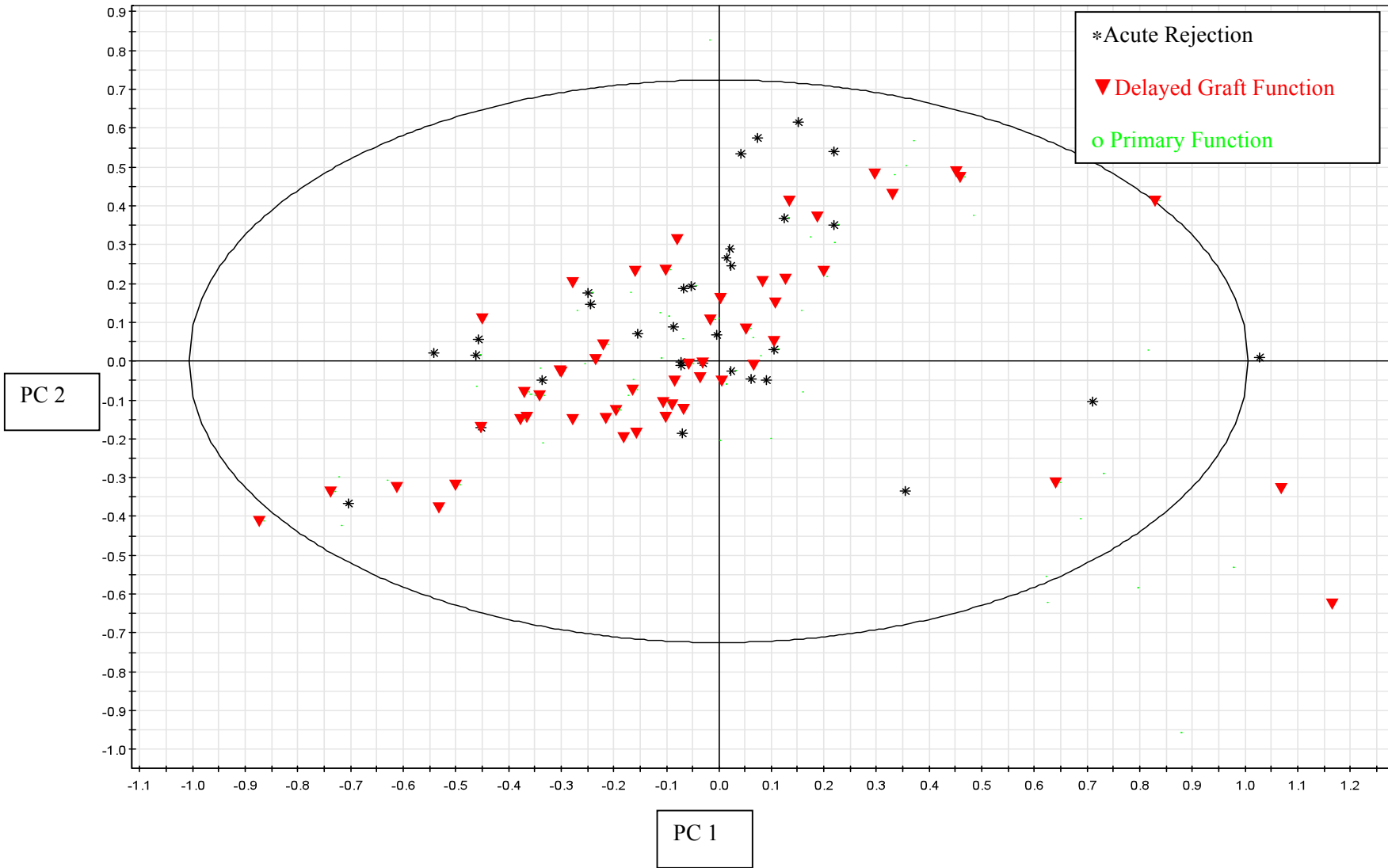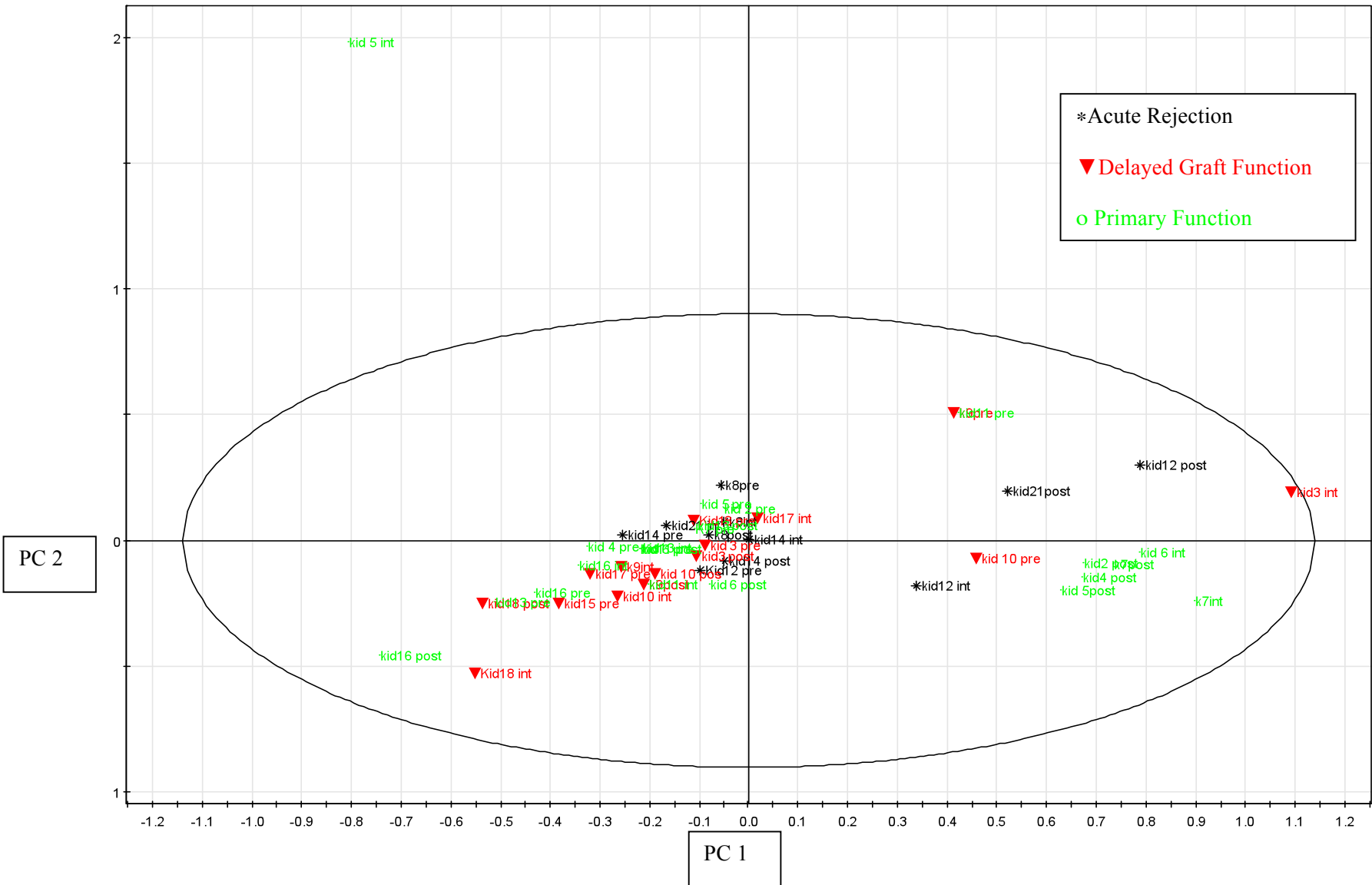**Figure 2.1** – Scores plot comparing all data for all time points and all patients

Legend:
* Acute Rejection
▼ Delayed Graft Function
o Primary Function

PC 2

PC 1

Figure 2.2 – Scores plot comparing complete set of plasma for pre-operative, intra-operative and 2 hours post-operative samples
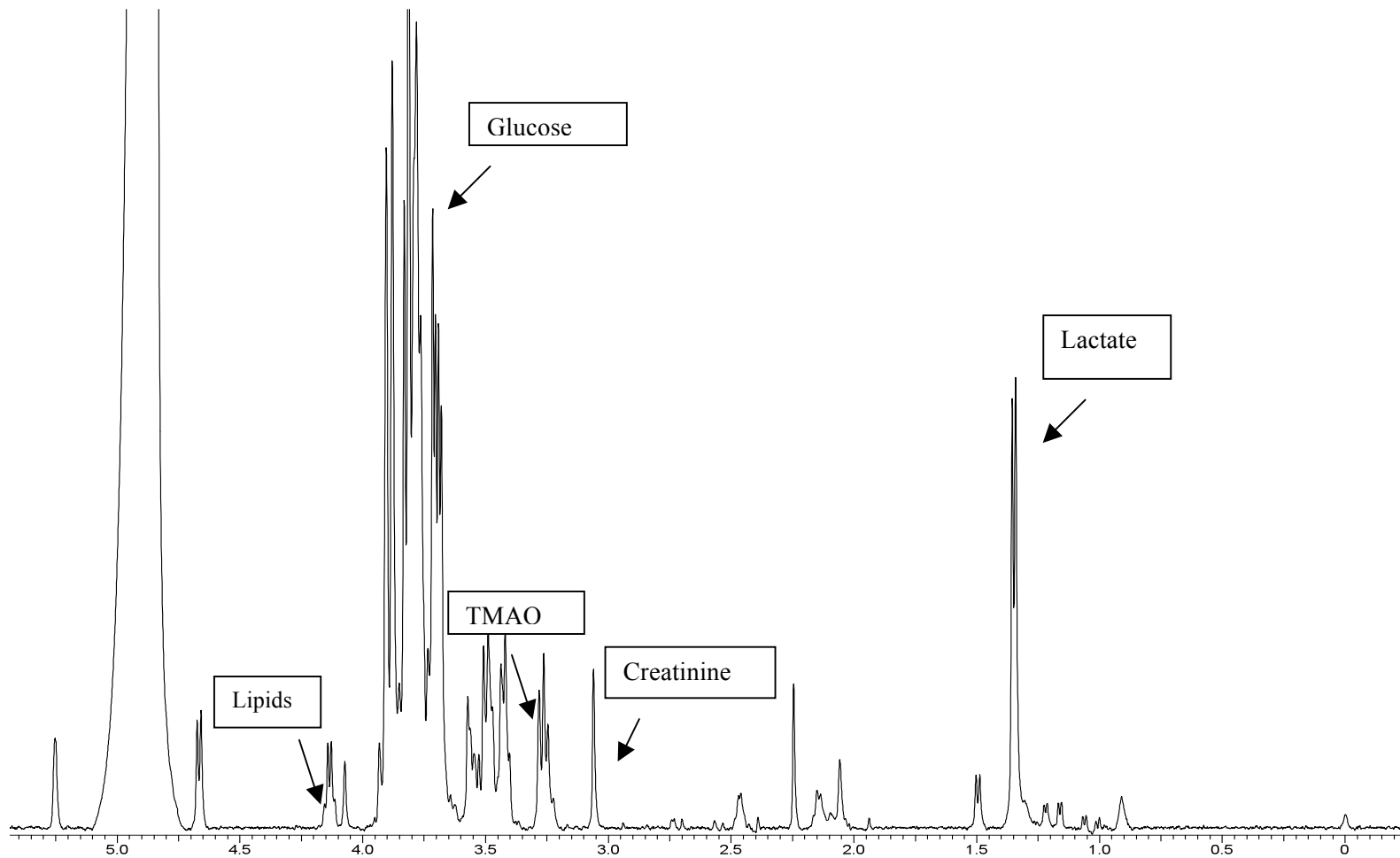
51

Figure 2.3 – Section of a 1H-NMR spectrum of intra-operative metabolic profile of a plasma sample

2.7.2 Analysis of samples from days one to seven

In total 117 samples taken on days one, two, three, four, five and seven were available for full analysis and comparison.

The PCA plot shown in **figure 2.4** includes all patient plasma samples and shows clustering of those patients with acute rejection in the left lower of the scores plot (ellipse 1). Also within this area are patients with DGF and a scattering of patient with PF. Importantly it is patient samples from days one, two and three in the DGF patients found in **ellipse 1** but from day four onwards these move trajectory to **ellipse 2** where a majority of patients with primary function can be found and only two plasma samples of a patient with acute rejection; whom incidentally was found to have another ongoing pathology related to the kidney (renal vein thrombosis) which may account for the differences seen in the metabolic profile of this patient. From **figure 2.4** no differences in plasma profile can be seen between patients with AR or DGF from days one to three, differentiation becomes apparent from day four onwards. **Figure 2.5** shows on the 1H NMR spectrum of plasma of a patient with acute rejection with the magnification of the area responsible for differentiation between AR, DGF and PF. Metabolites were identified with the aid of reference to a chemical shift database (Nicholson JK *et al* 1995, www.hmdb.ca).

Figures 2.6 and **2.7** use the same data as in **figure 2.4** but concentrate on direct comparison of two clinical outcomes not all three. **Figure 2.6** compares AR and primary function. Whilst there is a small amount of overlap of data, those with AR appear in a line, with those with PF moving away from these data points.

Figure 2.7 compares AR with DGF. Those with AR cluster in the left lower

quadrant of the scores plot, with some overlapping of those patients with DGF. The plasma samples of those patients with DGF that overlap with those patients with AR again echo the finding of **figure 2.5** and are the samples taken on days one, two and three post-operatively. From day four onwards the metabolic profiles of those patients with DGF move away from those samples with AR as indicated in the plot.

The loadings plot (**Figure 2.8)** identifies regions of the spectrum (ppm/bins) giving rise to the discrimination; 3.00 ppm to 3.40 ppm resulting from the presence of arginine, trimethylamine-N-oxide (TMAO) and creatinine and 4.00 to 4.04 ppm (**Figure 2.3**) also due to creatinine (an additional creatinine signal).

In days five and seven the metabolic profile of those patients with only DGF and no rejection continue to move into the PF group.

From day one prior to any clinical signs of acute rejection, patients with DGF and AR had less intense peaks for arginine and more intense peaks for TMAO than those patients with PF. By day 4 arginine peak intensities increase in those with only DGF, but remain low in the AR group. DGF patients see a decrease in signal intensity for (and thus concentration of) TMAO from day four whilst those with AR show a slight increase.

The statistical significance of the metabolite concentration variations was tested using Kruskal–Wallis. Creatinine (3.04 and 4 - 4.04 ppm) was found at a higher concentration in the PF compared to either the AR or DGF group (PF versus AR $p<0.001$ and PF versus delayed graft function $p=0.079$). In the clinical setting one would expect the creatinine level to be lower in patients with a primary functioning graft. This apparent anomaly may be the result of some DGF and AR

patients having undergone dialysis during the study period. The creatinine levels would be lowered by this. Trimethylamine-N-oxide (TMAO at 3.27 ppm) was responsible for some of the differentiation seen between AR, DGF and PF. Statistical significance was almost reached when comparing AR to PF (p=0.068). The concentration variation of arginine did not achieve statistical significance.

Concentration from day three to four when differentiation is seen between AR and DGF a 1.53 fold change decrease in the concentration of TMAO in the DGF patients versus an increase of 1.26 in the AR group is revealed. A summary of fold change of three metabolites contributing to change are shown in **table 2.**

Figure 2.4 - Scores Plot Comparing Acute Rejection, delayed graft function and Primary Function. The direction of the arrow is indicative of change in patient metabolic profiles as day's progress from surgery
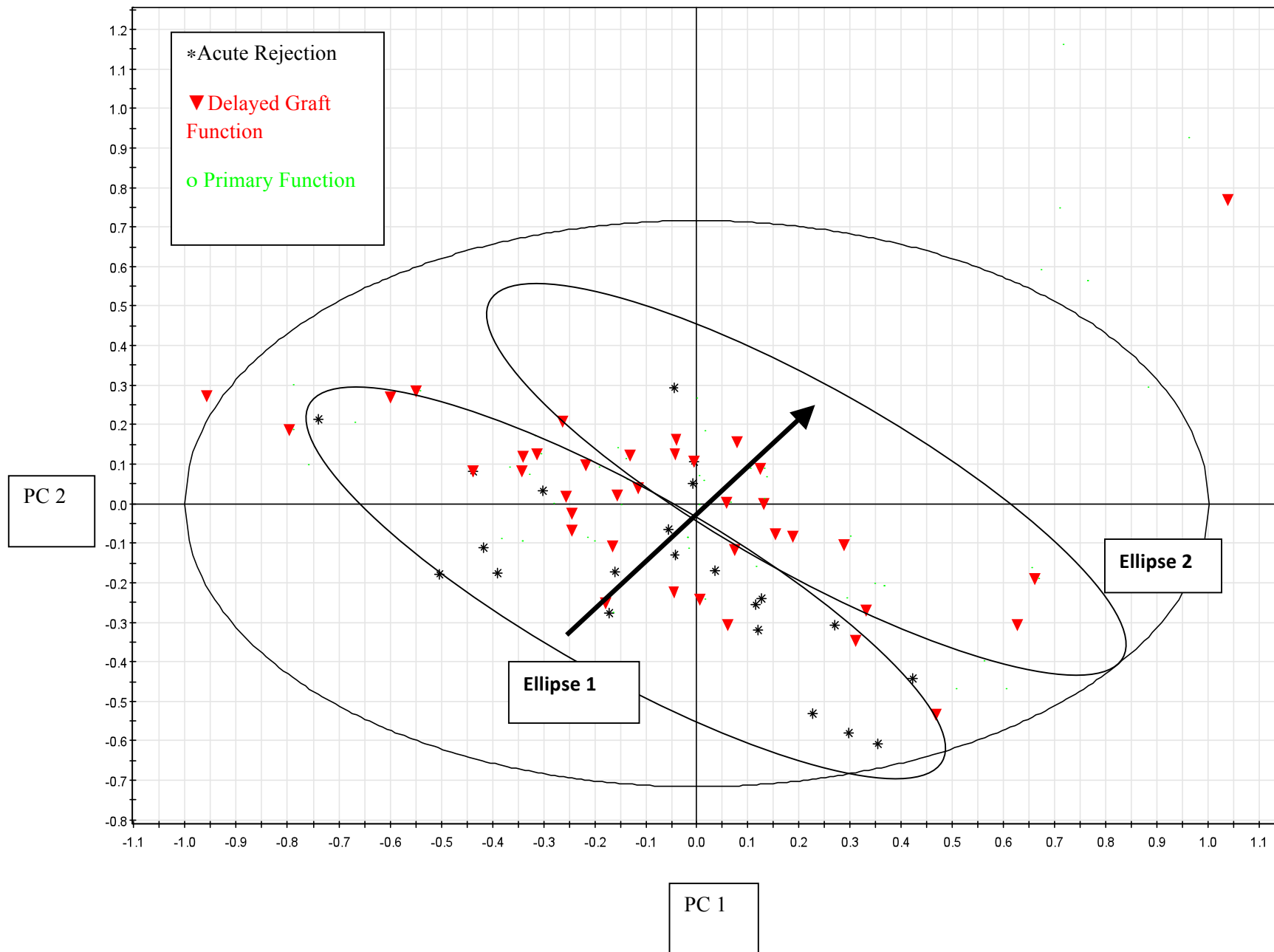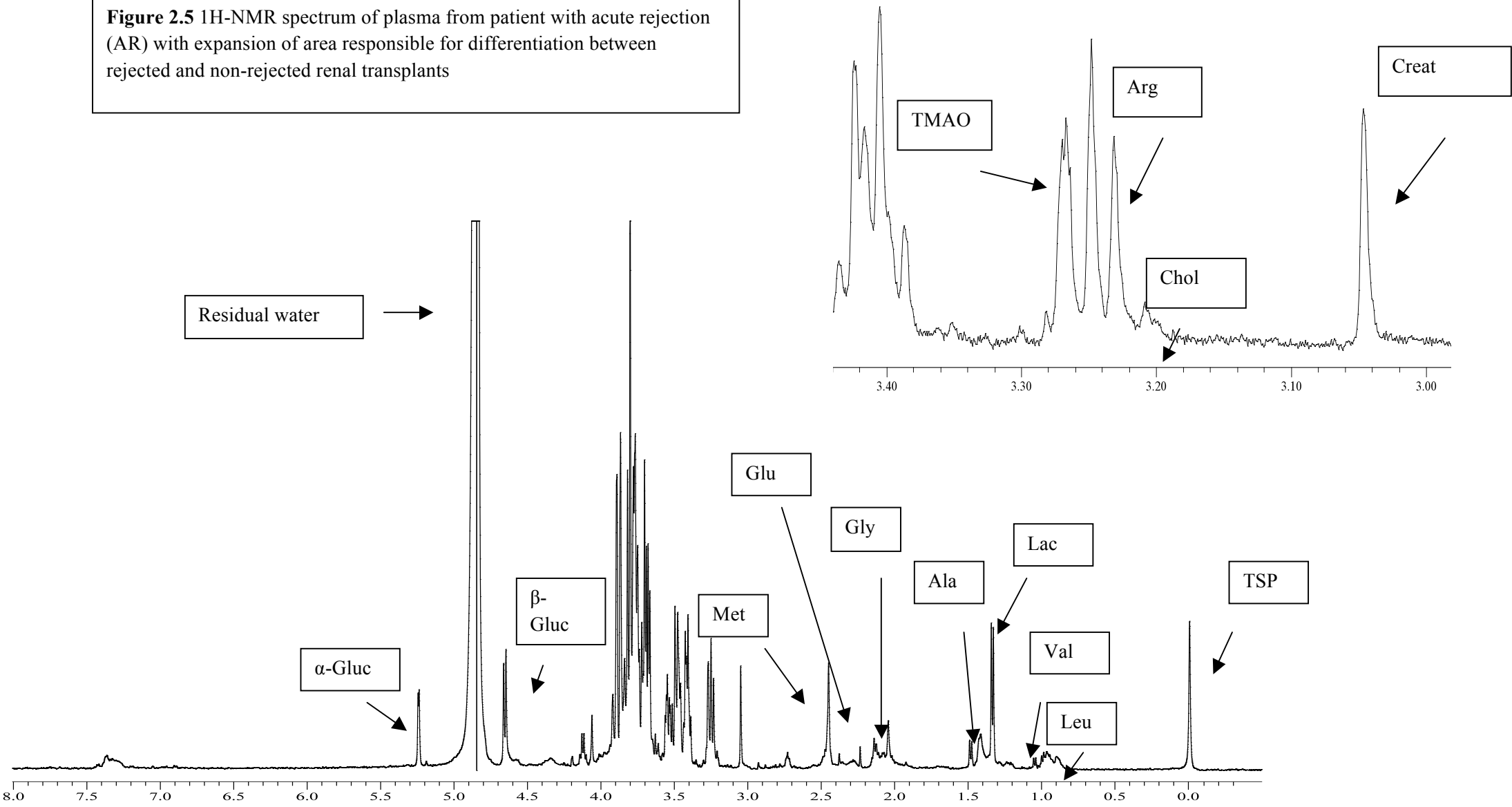
Figure 2.5 1H-NMR spectrum of plasma from patient with acute rejection (AR) with expansion of area responsible for differentiation between rejected and non-rejected renal transplants
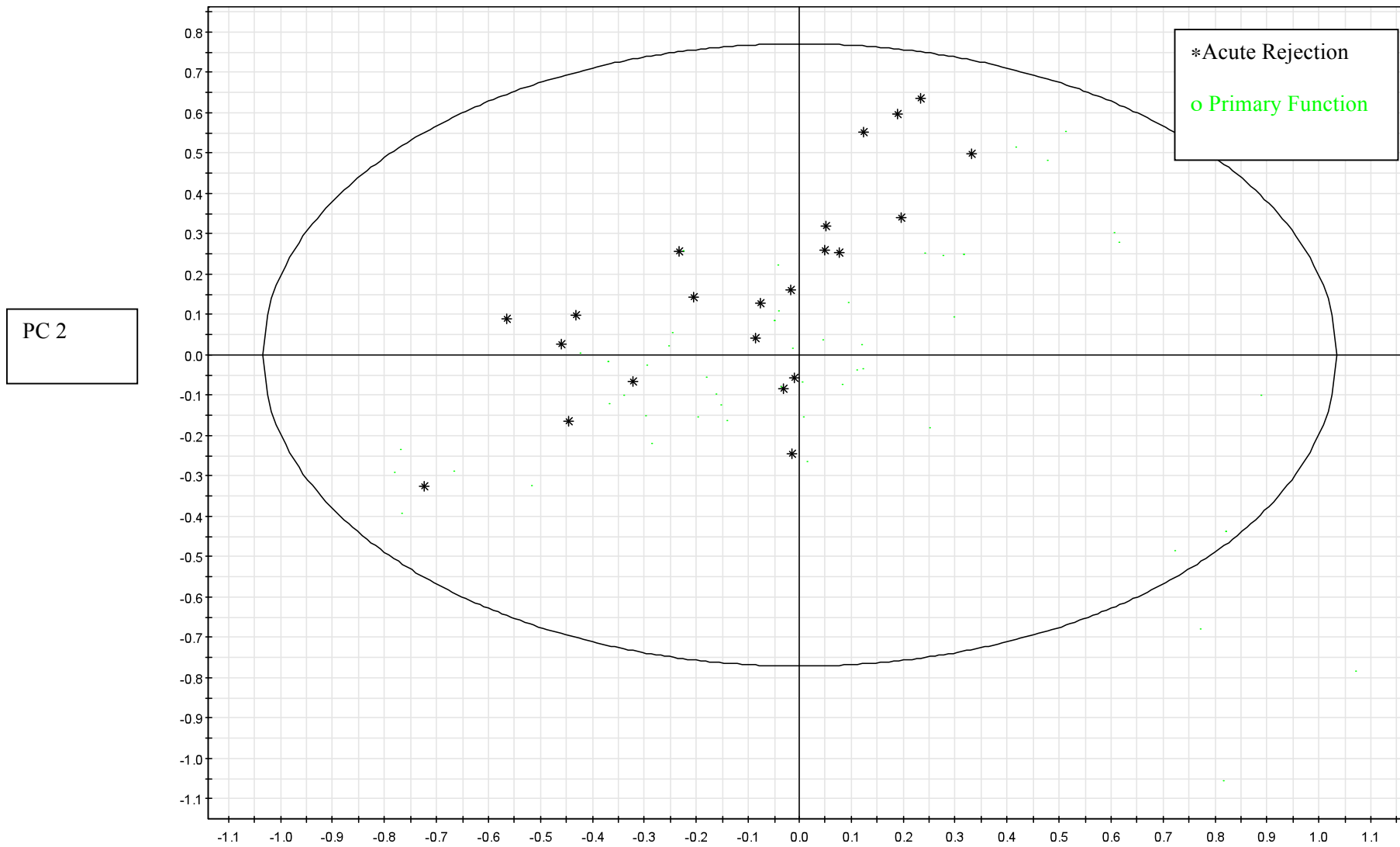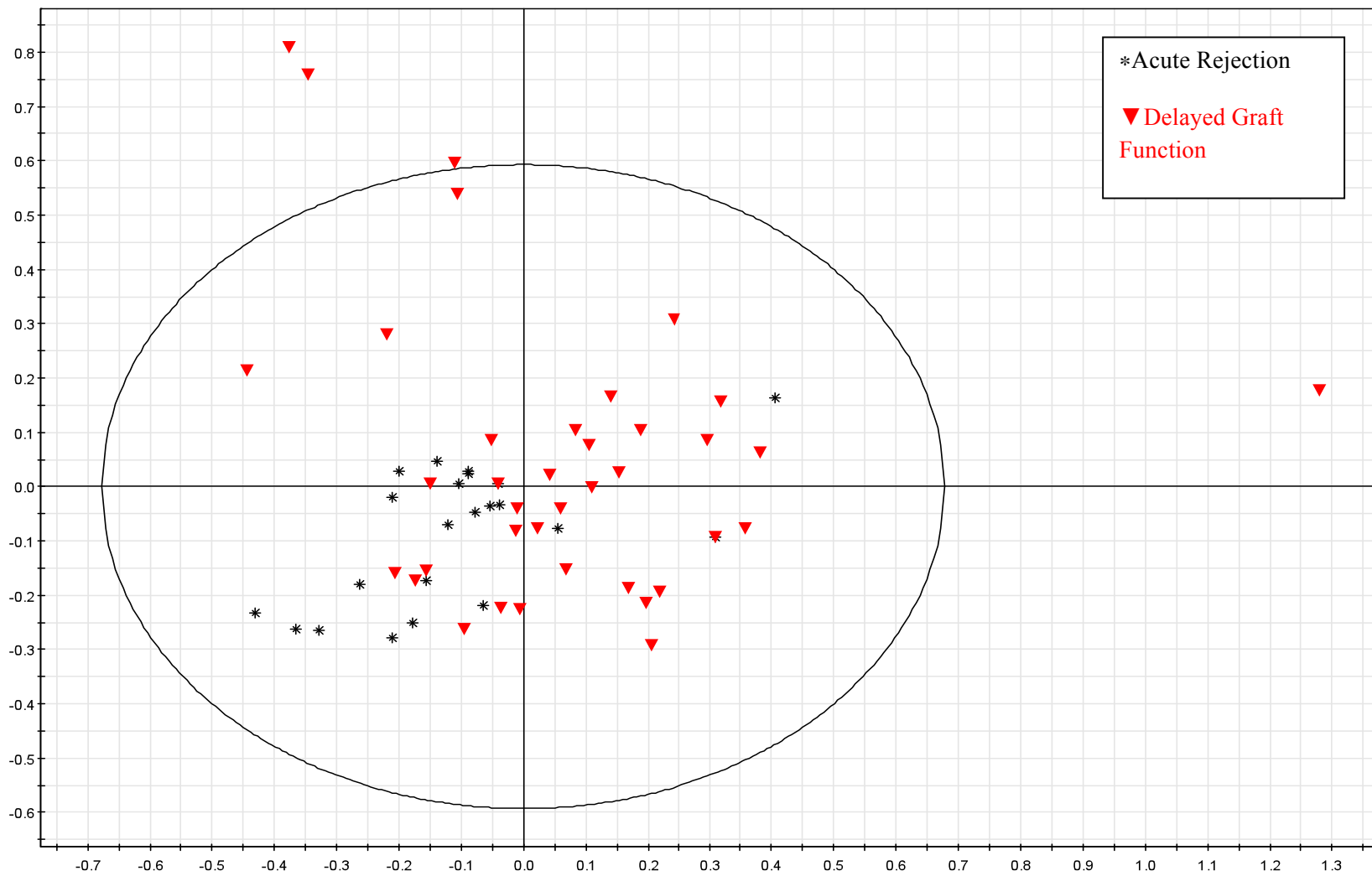
Figure 2.6 Scores Plot Comparing Acute Rejection (AR) versus Primary Function (PF)

58

Figure 2.7 - Scores Plot Comparing Acute Rejection (AR) versus Delayed Graft Function (DGF)

PC 3

PC 2

59

Figure 2.8 - Loadings Plot. Values furthest from 0 are the greatest cause of discrimination within individual blood samples These values correlate with the molecules creatinine, TMAO, and arginine.
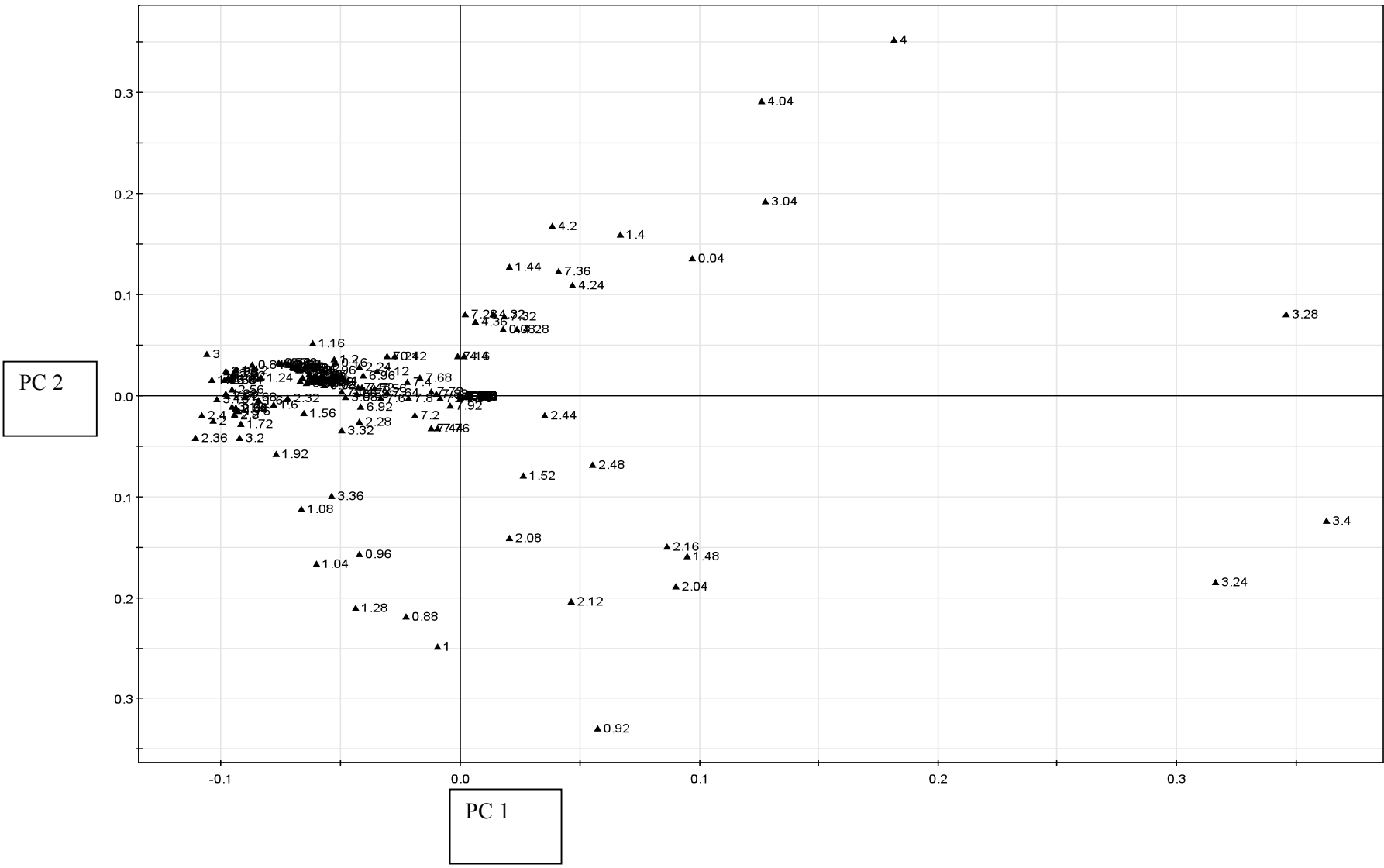
Table 2.4 - Comparison of concentration fold change from day 3 to day 4 in metabolites responsible for discrimination between transplant outcomes

	Primary Function	Delayed Graft Function	Acute Rejection
Trimethylamine-N-Oxide	↓ (0.80)	↓ (1.53)	↑ (1.26)
Arginine	↓ (0.90)	↑ (1.23)	↑ (1.17)
Creatinine	↓ (0.95)	↓ (1.94)	↑ (1.20)

2.7.3 Individual patient sets

The aim of this analysis was to compare the metabolic profile of individual patient sets undergoing renal transplant. All plasma samples taken of patients were directly compared to each other to see if there was a trend where-by day seven the patient's metabolic profile returned to their pre-operative sample in those patients with PF when compared to those patients who had post-operative DGF or AR.

Figure 2.9 is the scores plot of a patient receiving a live-related renal transplant. This patient (patient 11) developed AR as diagnosed on biopsy which was successfully treated with methylprednisolone. Analysing the scores plot

unsurprisingly as shown in section 2.7.1 the intra and post-operative samples are far removed from the samples taken from day one onwards. Referring to the loadings plot (**figure 2.10**) for differentiation between the samples creatinine and β-glucose are contributing to the differences seen between pre-operative, intra-operative and post-operative samples. Whereas TMAO is found to be contributing to the differences seen with samples taken on day three, four and five, this is not replicated in the metabolic profile of day seven when AR had been proven on biopsy. If this individual plasma set is then compared to another patient who underwent a live related renal (LRD) transplant which had primary function (patient 9) no obvious trend is apparent, with overlap of samples from intra-operative and post-operative to days one through to five. These results are in keeping with previous work in this study where those patients with primary function have very little difference seen in their metabolic profile from day one onwards.
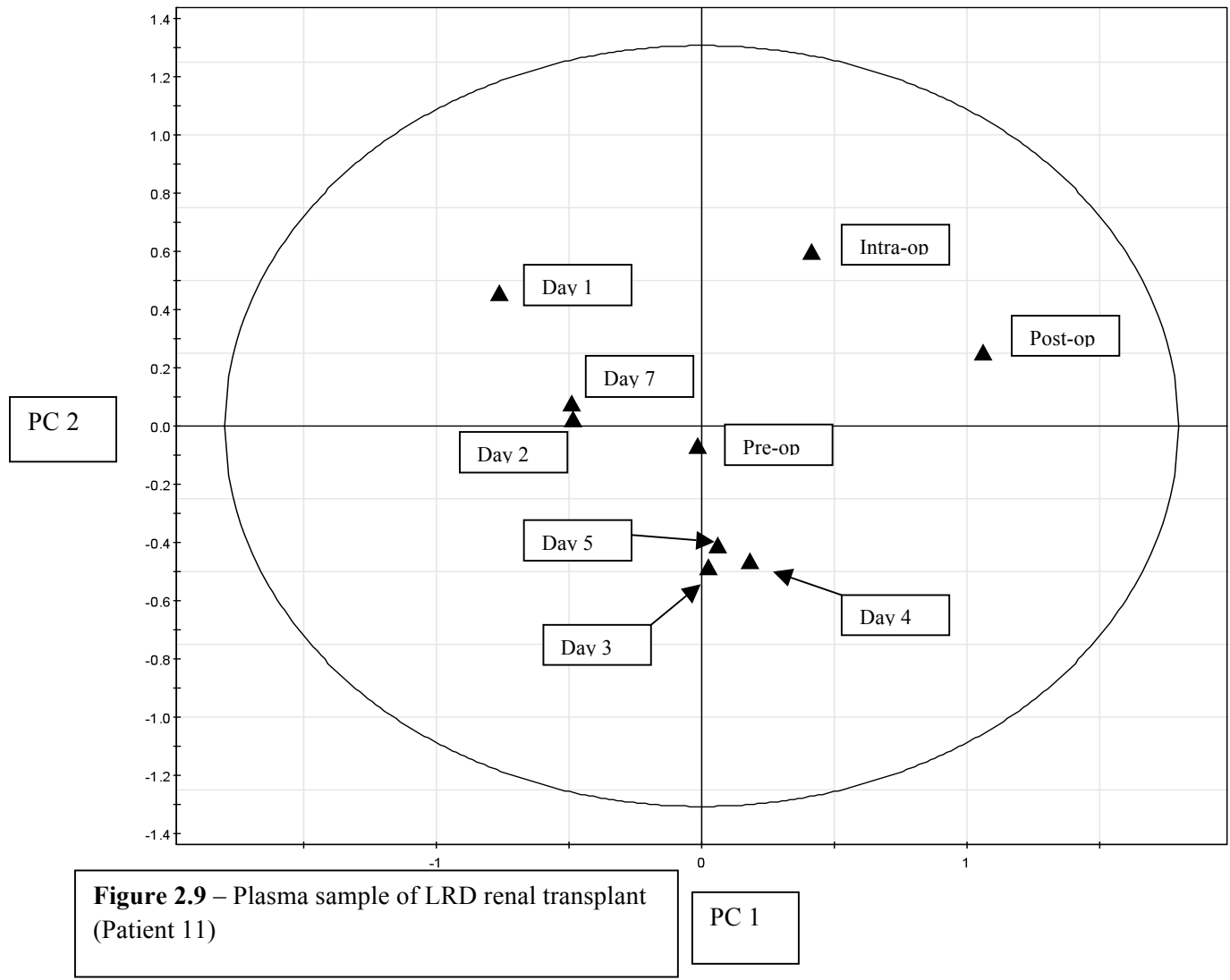
Figure 2.9 – Plasma sample of LRD renal transplant (Patient 11)

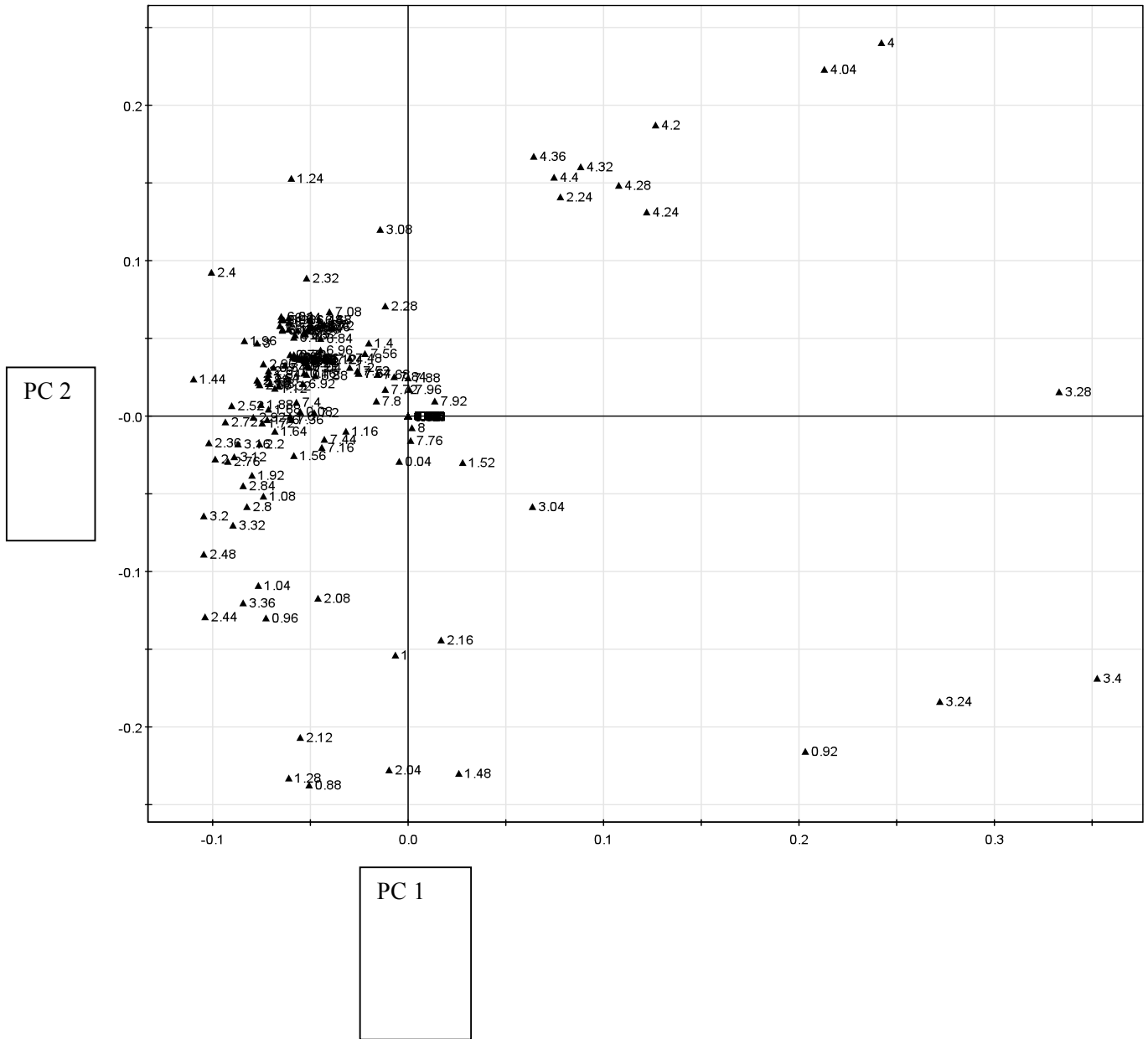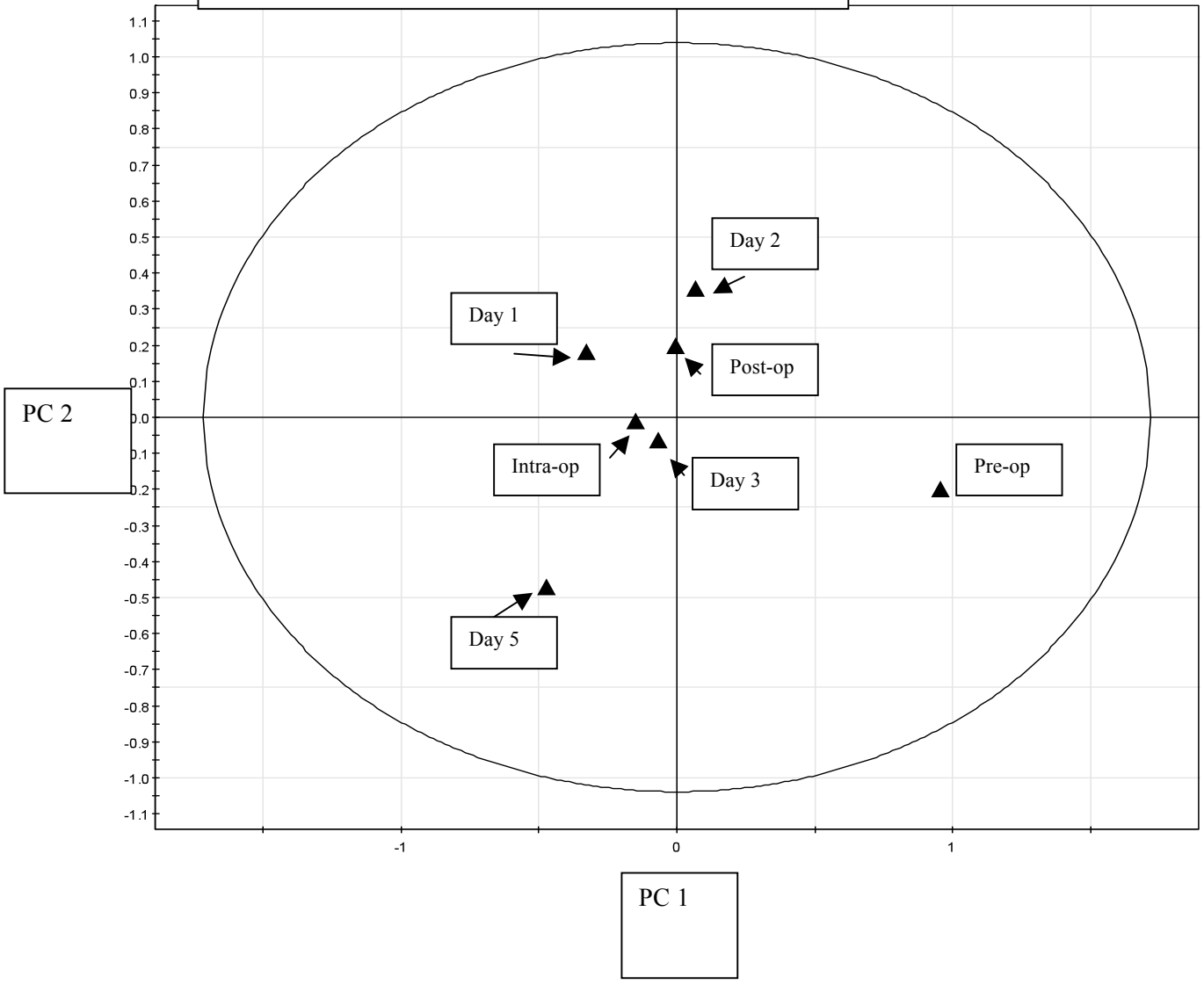**Figure 2.10** – Loadings plot of plasma samples of patient 11

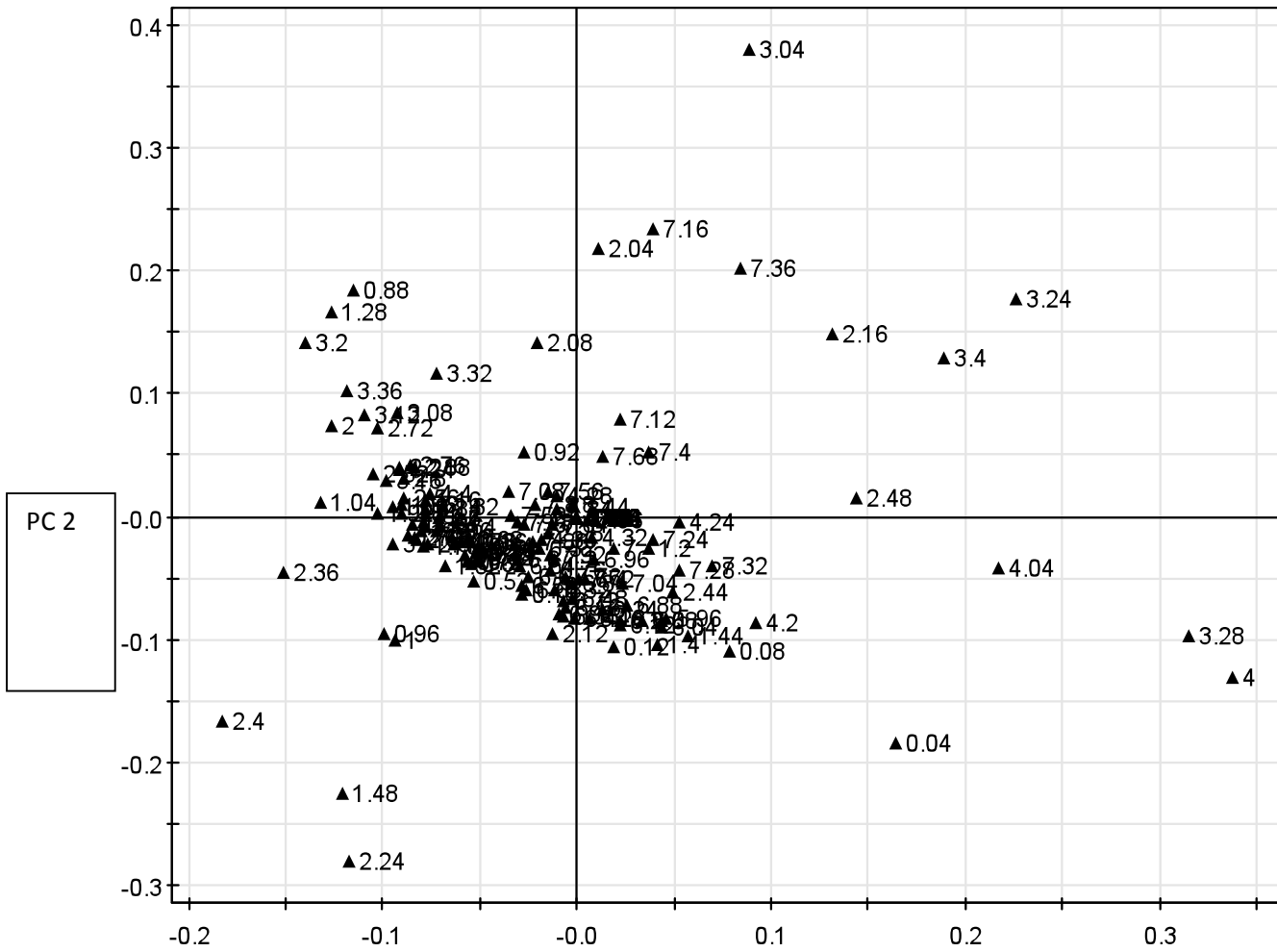**Figure 2.11** – Plasma sample of LRD patient 9

Figure 2.12 - Loadings plot of plasma samples of patient 9

2.7.4 With inclusion of lactate in the spectra

The aim of this analysis was to observe the role of lactate across the time period of blood samples taken in the pre and post-operative period of the renal transplant.

Figure 2.13 shows no statistical differentiation between samples from the three different clinical outcomes. When compared on the loadings plot, lactate was responsible for the differentiation at ppm 1.32 and 1.36 and also at 4.12 and 4.16, but this was regardless of type of donor or clinical outcome. This was felt to be due to the large quantity of lactate seen compared to other metabolites, thereby dampening down the significance of any of the other metabolites.

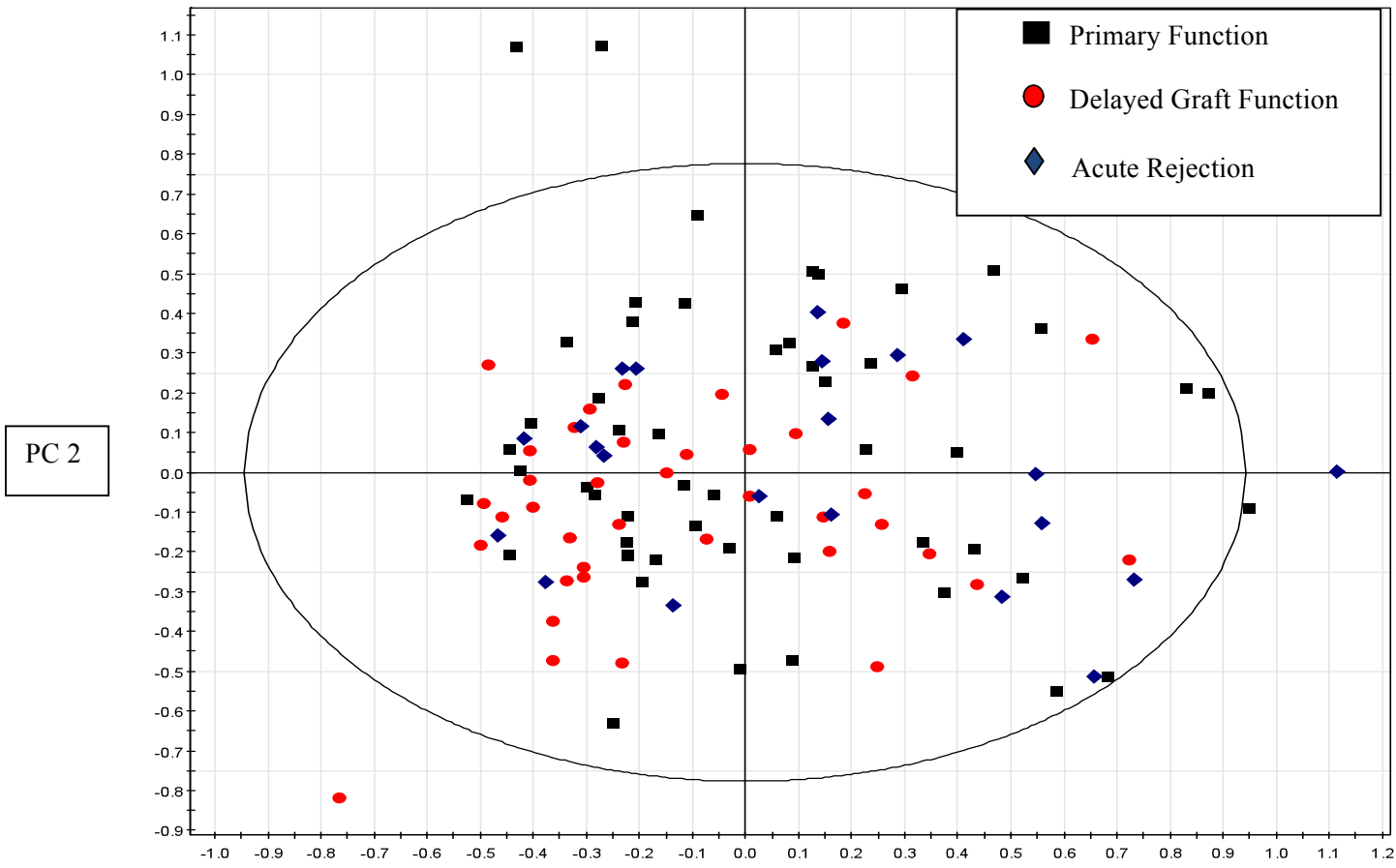**Figure 2.13** - Comparison of different clinical outcomes with inclusion of lactate in metabolic profiles

Figure 2.14 – Loadings plot of figure 2.13

69

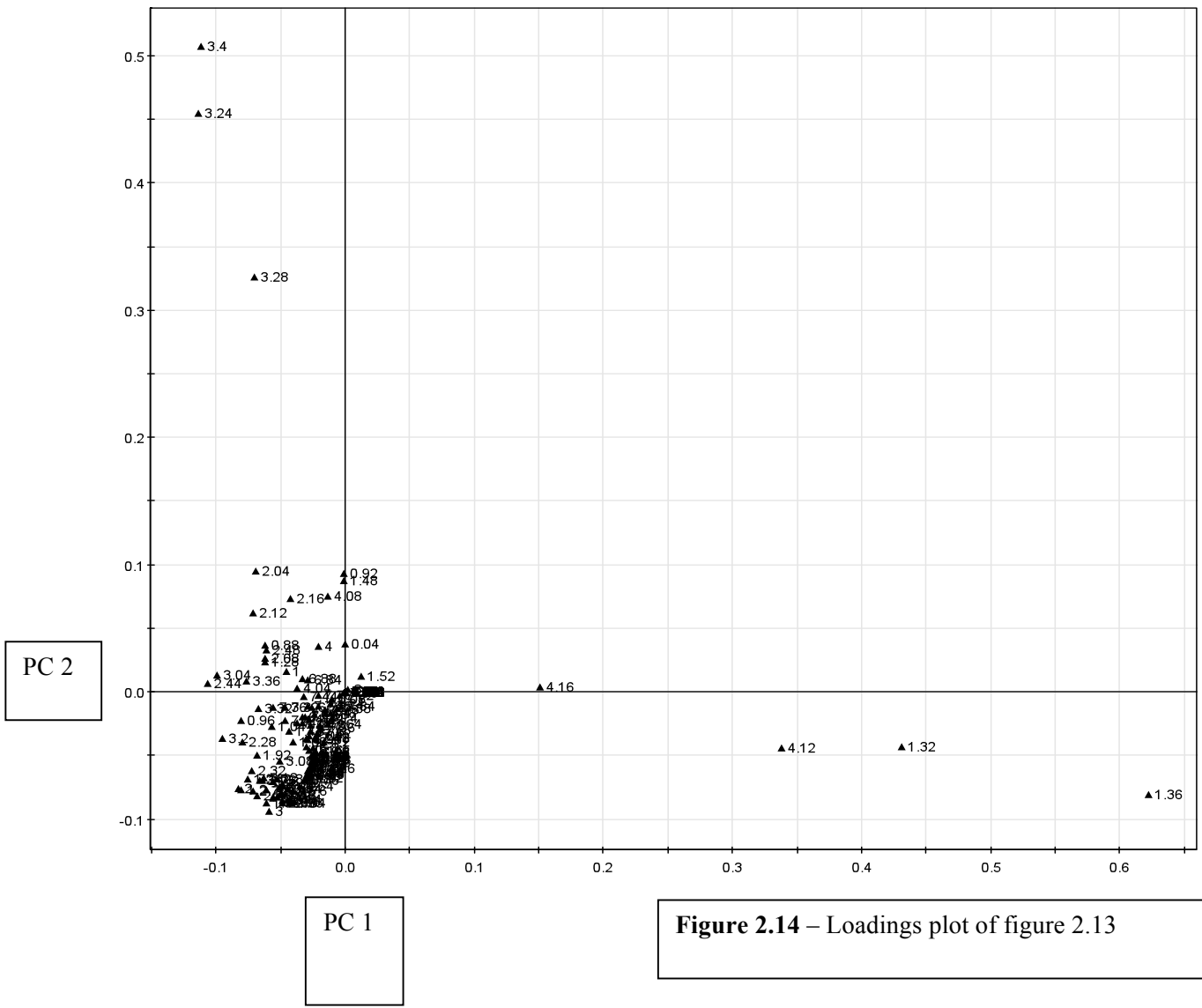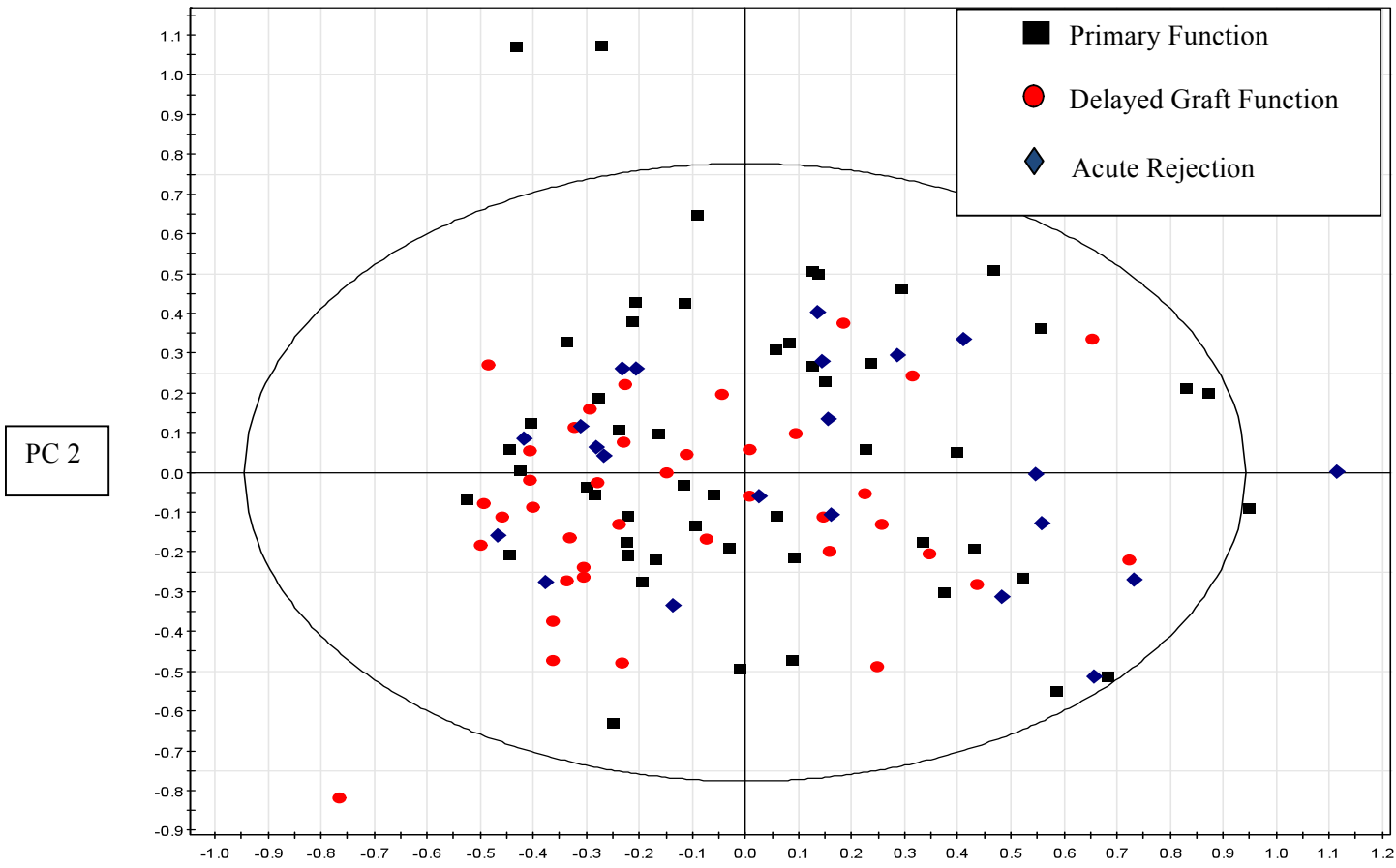**Figure 2.13** - Comparison of different clinical outcomes with inclusion of lactate in metabolic profiles
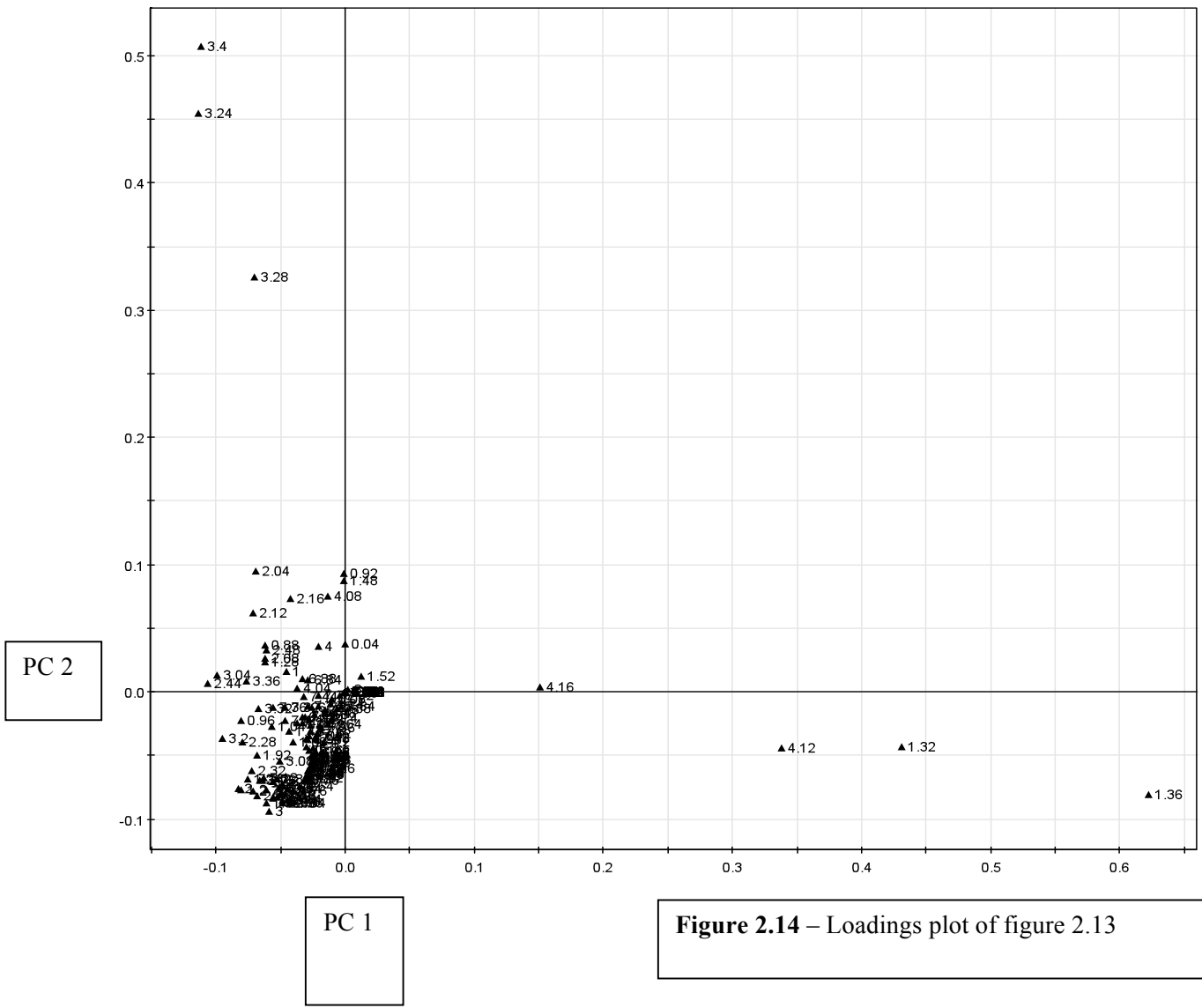
68

Figure 2.14 – Loadings plot of figure 2.13

2.7.5 Comparison of Different Donor Types

To assess the reliability of ^{1}H NMR across all modes of transplantation (LRD, HB, Non-HB) a direct comparison was made between these different groups.

Figure 2.13 shows the scores plot in principal components 1 and 2 accounting for 82% (R2X 0.826) of the variability in the study population of a direct comparison of these transplant types. As can been seen in this figure there is no distinguishable difference between the three different group types. This suggests that no matter what type of transplantation takes place the metabolic profiles of patients in the post-operative period varies depending on transplant outcome not on donor type.

Figure 2.15 – Direct comparison of LRD, HB and non-HB plasma samples

PC 2

PC 1

Live Related

Non-heart beating

Heart beating
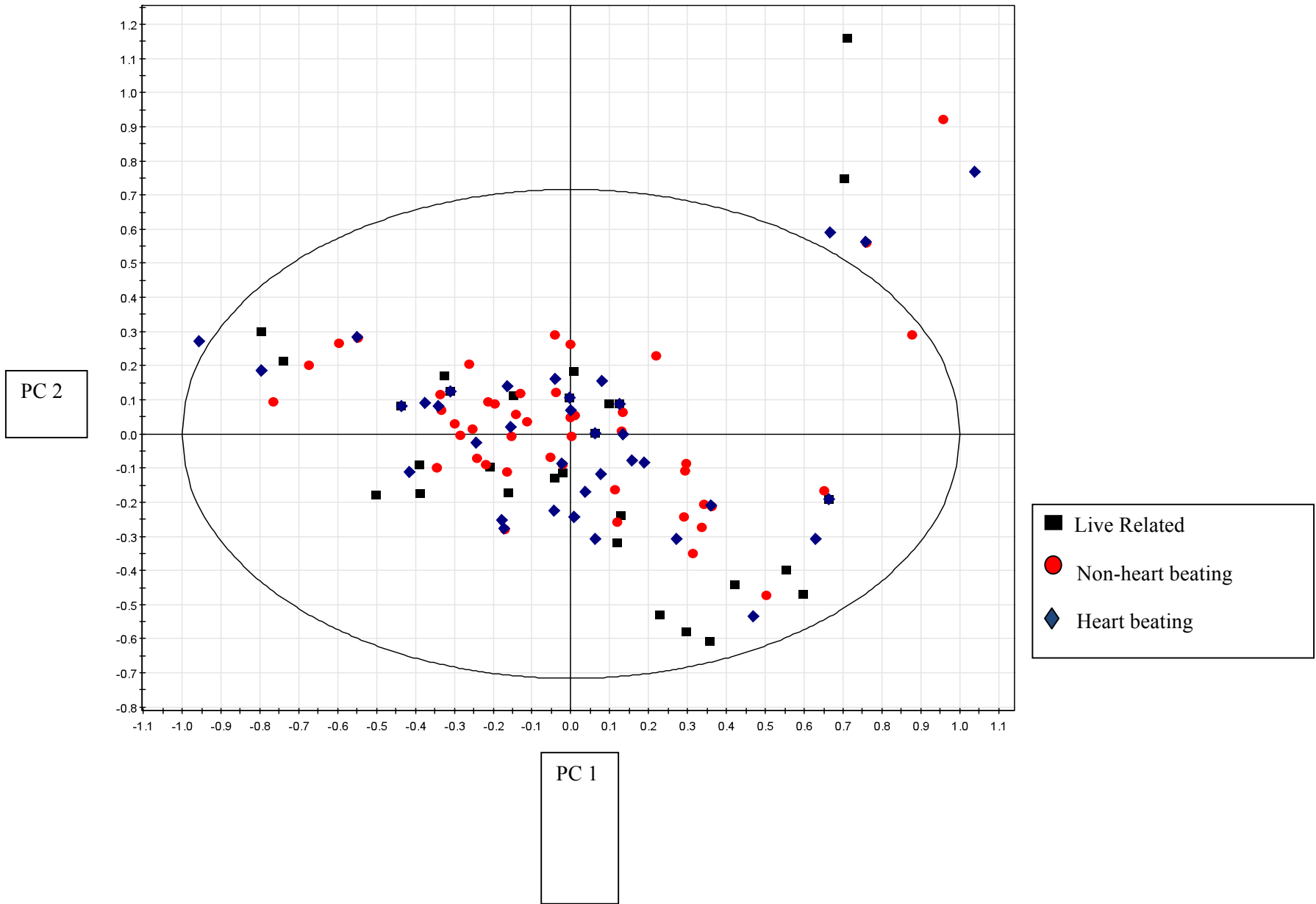
Chapter 3

Quantification of Concentration of Plasma

Metabolites using NMR

3.1 Introduction

To work out the concentration of TMAO present in the plasma samples calibration curves using ^{1}H NMR were acquired. In this part of the study 1D NMR was used for quantification of metabolite concentration. Whilst the benefits of 2D NMR to quantify metabolites have been put forward, it is not without its problems (Gronwald W *et al.,* 2008). 1D uses 64k data points but 2D only 1k points and therefore has much lower digital resolution meaning the signals are not well resolved making interpretation of intensity and baseline noise more difficult to resolve. This could prove a problem when interpreting plasma samples as the signals for molecules overlap.

The same high resolution afforded in 1D would be needed with 2D experiments but this is not possible in a reasonable time frame, especially with multiple samples being analysed. Gronwald *et al* mentioned the overlap in 2D spectra so not all signals could be used for quantification and multidimensional peak intensities are influenced by many factors, listed in the paper, meaning calibration curves result with very different slopes depending on the signal used, hence each signal needs to calibrated individually before achieving an average calibration for the metabolite. The lowest concentration of any possible molecule to identify will vary depending on the signal to noise ratio. In their study the most dilute concentration of 19.5 µM was detected for some metabolites including TMAO but the limit of detection for many metabolites was higher. The lower limit of quantification as defined by less than 20% relative standard deviation from six triplicates was often much higher.

For quantification of the TMAO concentration in this study 1D NMR was used due to the problems associated with 2D NMR highlighted in the previous paragraph.

3.2 Method

As TMAO was identified as a potential biomarker of renal transplant AR in NMR studies (chapter 2), the same experimental parameters were used in sample preparation and pulse sequences to allow for direct relationship with these previous samples (sections 2.6 and 2.6.1).

An aliquot of blood was taken from a control to refine the technique and work out the concentrations of TMAO and centrifuged at 4000 rpm for 10 minutes before the plasma was aliquotted into 300 μl amounts. The aliquots were stored at $-80^{o}C$ for 8 days until the start of the experiment. The maximum time stored at $4^{o}C$ between sample make-up and acquisition was 1 hour. Defrosted samples were centrifuged at 4000 rpm for 10 minutes and the whole 300 μl added to 350 μl D_2O containing TMAO, then vortexed for approximately 5 seconds and 650 μl transferred to NMR tubes. All tubes used were of the same specification from the same manufacturer.

Samples were prepared with a 0.17 % w/v solution of the sodium salt of 3-(trimethylsilyl)propionic- 2,2,3,3-d_4 acid (TSP) (Sigma-Aldrich, UK) in deuterium oxide (D_2O) (Fluorochem, UK). TSP was used as a chemical shift reference (0 ppm).

Data for all 10 samples containing TMAO were acquired on the same day. Two control samples were analysed the following morning using the same preparation method.

To work out the concentration and volumes of TMAO required the following methodology was used.

TMAO Concentration:

75.11g in 1litre	= 1M

0.7511g in 1litre   = 10mM

0.7511g in 100ml   = 100mM

300µl plasma + 350µl D_2O (containing TMAO) = 650µl total volume

New TMAO concentration = 350 / 650 = 0.538 of original

Amount of TMAO required relative to original to give same concentration = 1 / 0.538 = 1.857

TMAO required = 1.857 x 0.7511g = 1.3949g

Only 1g TMAO available so 0.6975g in 50ml = 100mM = stock solution 1

Stock 1 (µl)	D$_2$O (µl)	Concentration (µM)
35.00 +	315.00 =	10000
17.50 +	332.50 =	5000
8.75 +	341.25 =	2500
4.38 +	345.62 =	1250

and

10.00 +   990.00 =   1000   = stock solution 2

Need 625μM so 0.625 x 350 = 218.75 stock solution 2

Stock 2 (μl)   D$_2$O (μl)   Concentration (μM)

218.75 +   131.25 =   625

109.38 +   240.62 =   312.5

54.69 +   295.31 =   156.3

27.34 +   322.66 =   78.1

13.67 +   336.33 =   39.1

6.84 +   343.16 =   19.5

0.00 +   350.00 =   0.0 = control

3.3 Results

From early investigation it was clear that the shimming was not as good for the very high concentrations, e.g. 10000, 5000 and 2500 μM, but this is likely to be due to the sample constitution being changed enormously as these concentrations provided gigantic peaks and were far larger than the clinical sample run, typical TMAO peaks in the plasma samples of those patients with DGF and AR were less than 20 μl.

This raises the question of sensitivity and accuracy of quantification as these are minute changes. The main problem identified when analysing the TMAO peaks

which had been spiked with varying concentration of TMAO was the overlapping signals found under the TMAO peak.

With the higher concentration of TMAO in the plasma samples it was hoped that there would be a distinct signal from which analysis could take place, however the triplet peak of arginine overlapped with the TMAO peak, and deconvolution was not possible. Deconvolution is subtraction of an underlying peak from the one in question leaving an area that only represents the metabolite of interest. This effectively rules out quantification of metabolites using conventional techniques as most have some degree of overlap.

For very high concentrations approximations can be made provided underlying peaks are small but this is not scientifically sound as the underlying peak area and concentration cannot be accurately determined.
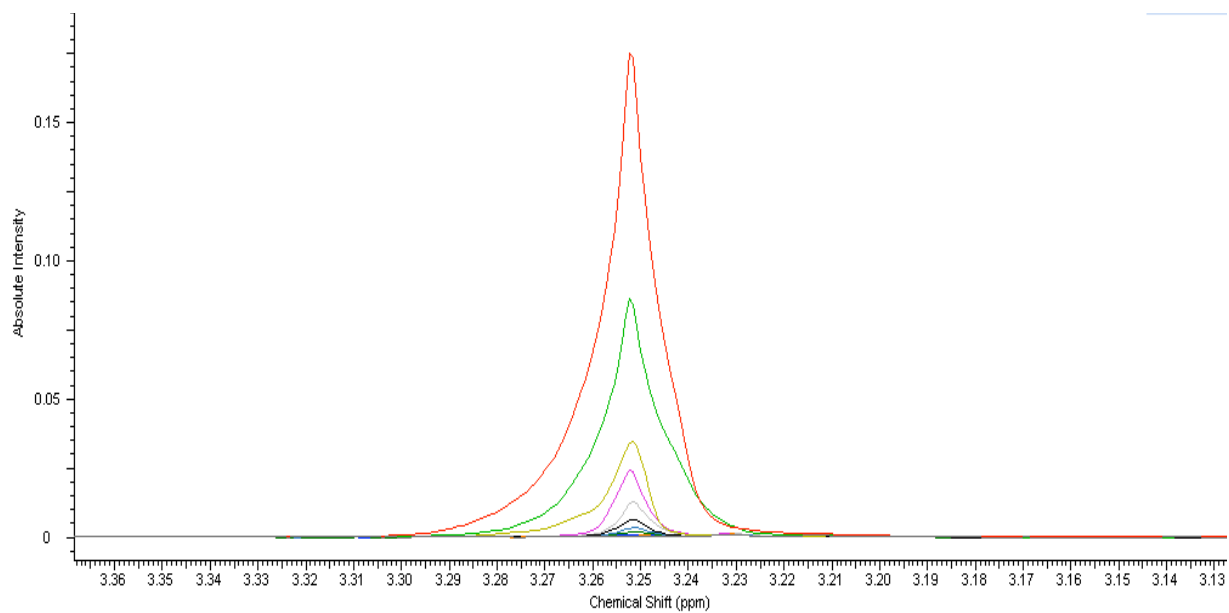
Figure 3.1a - Spectra showing TMAO at 3.25ppm with a concentration range from 10000 µM to 19.5 µM referenced to lactate at 1.317 ppm. Underlying peaks become clear at lower concentrations
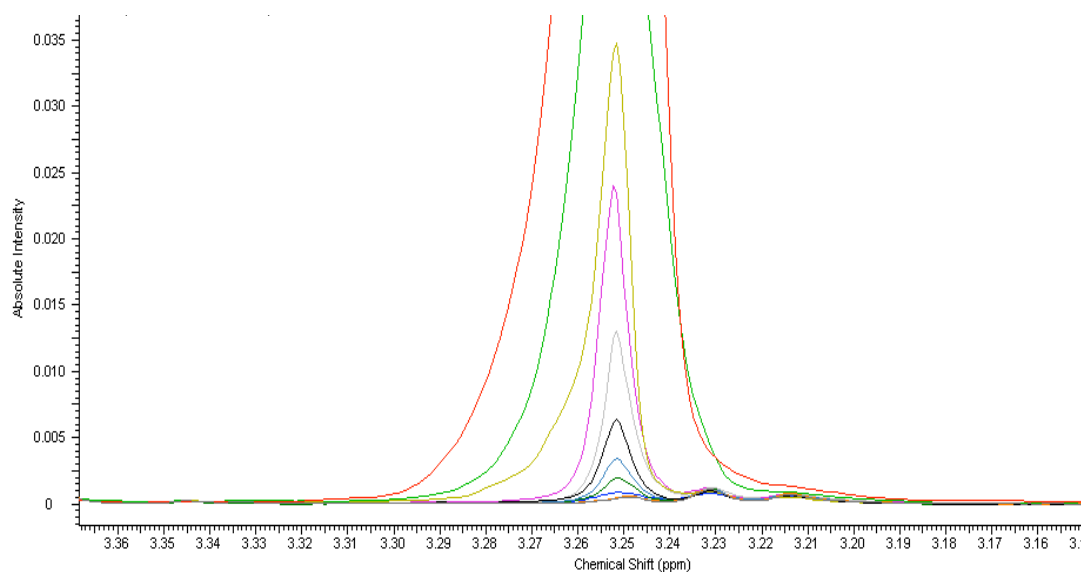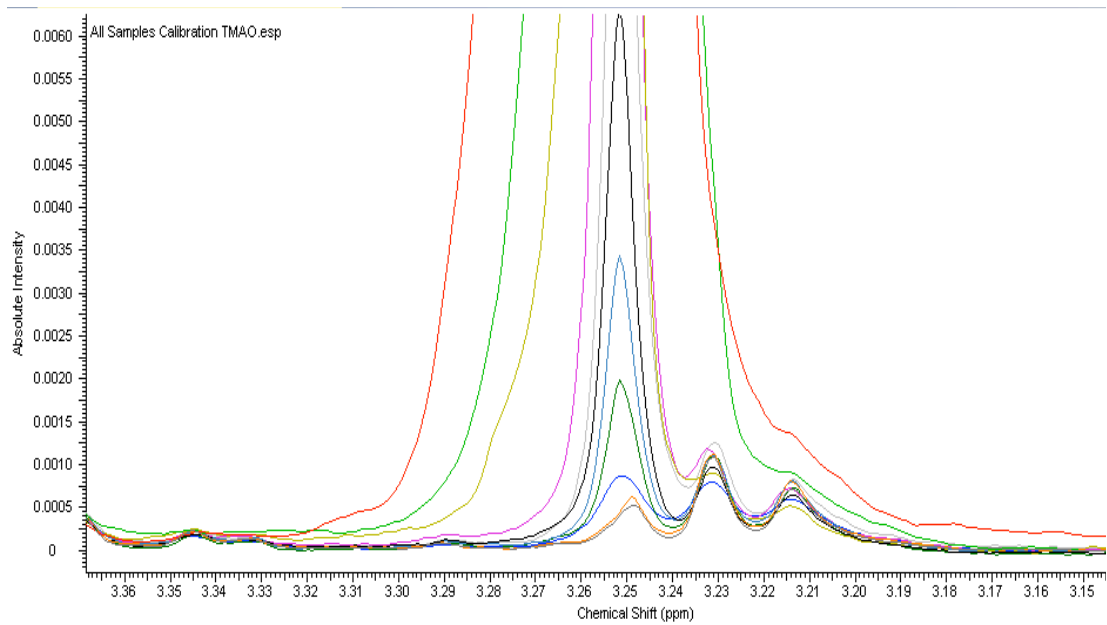
Figure 3.1b & c – Further TMAO peaks. Underlying peaks becoming more visible as concentration of TMAO is reduced

Arginine peak overlapping TMAO peak, rendering concentration analysis of TMAO not feasible
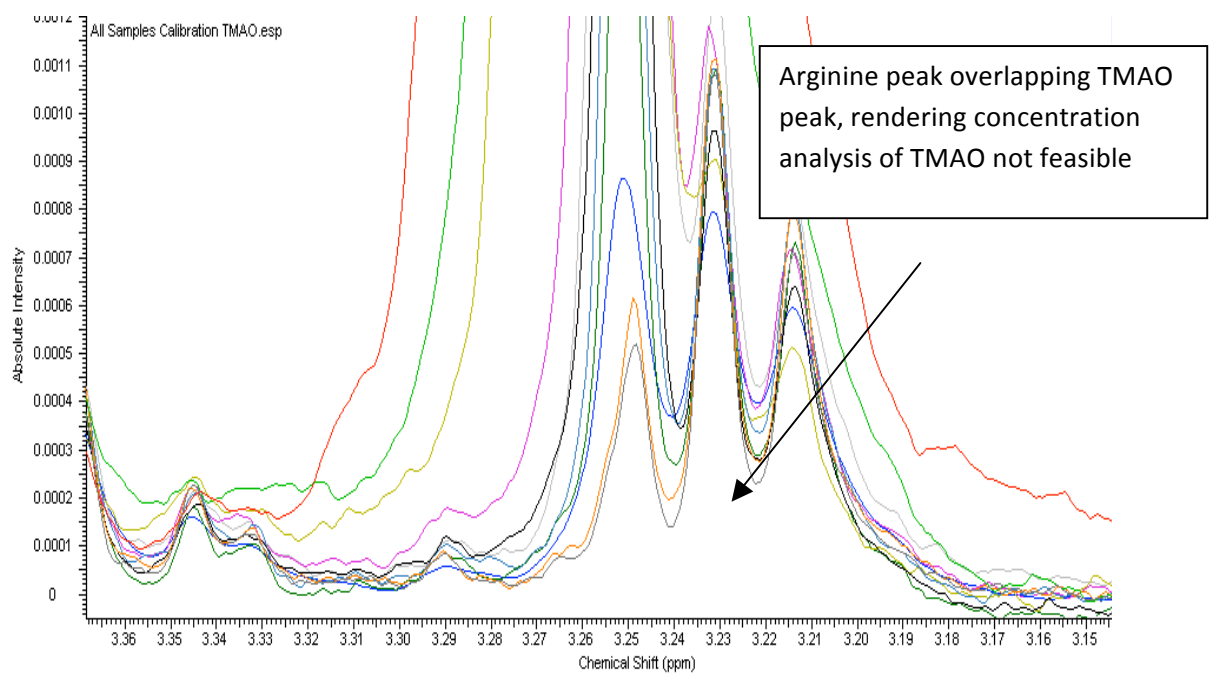
Figure 3.1d – Spectra with 19.5 µM of TMAO added. Increasing clarity of underlying peaks offset by overlapping from Arginine

Figure 3.1 a-d shows that as the as the intensity of the spectra increases the overlapping nature of the arginine triplicate and TMAO peaks become more apparent.

Based on the above experiment quantification of TMAO was not possible.

Chapter 4

Validation

4.1 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a chromatographic technique that can separate a mixture of compounds according to their affinity for the chromatographic medium to identify, quantify and purify the individual components of the mixture (Lough WJ, & Wainer LW, 1997).

HPLC operates under high pressure conditions and has two main phases. The mobile phase (eluent) is a liquid (a single solvent or mixture of solvents) which is moving through the chromatographic column and carrying analytes. The stationary phase (adsorbent) is solid porous media which consists of the rigid porous particles, usually silica based, with specific surface properties. Differing affinities of the mixtures components for the stationary and mobile phases leads to their separation, since certain components will be more attracted to the mobile phase and will elute quickly whilst others will be retained by the stationary phase for longer and therefore will elute more slowly, i.e. have a longer retention time (RT) (Allwood JW and Goodacre R., 2010).

Solvent reservoirs are used, one for each of the mobile phase eluents (solvent A and B), as well as further reservoirs for autosampler syringe and line washes. A high-pressure pump forms the solvent delivery system, which generates and metres a specified flow rate (typically millimetres per min). A solvent partitioning valve allows the mixing of solvents A and B at specified ratios and time gaps, thus permitting the operator to programme the HPLC to perform gradient elution steps (Meyer, 2004).

4.2 Method

In this study the concentration of the amino acids in the plasma was determined with reverse phase HPLC in an attempt to identify compounds that overlapped in the NMR studies, especially in the region of 3.20 to 3.30 ppm where the molecule TMAO is located as well as arginine and choline. σ-phthaldialdehyde (OPA) was used as a pre-column derivatising agent, in the presence of a strong reducing agent, such as β-mercaptoethanol, reacts rapidly with primary amines to form highly fluorescent thio-substituted isoindoles.
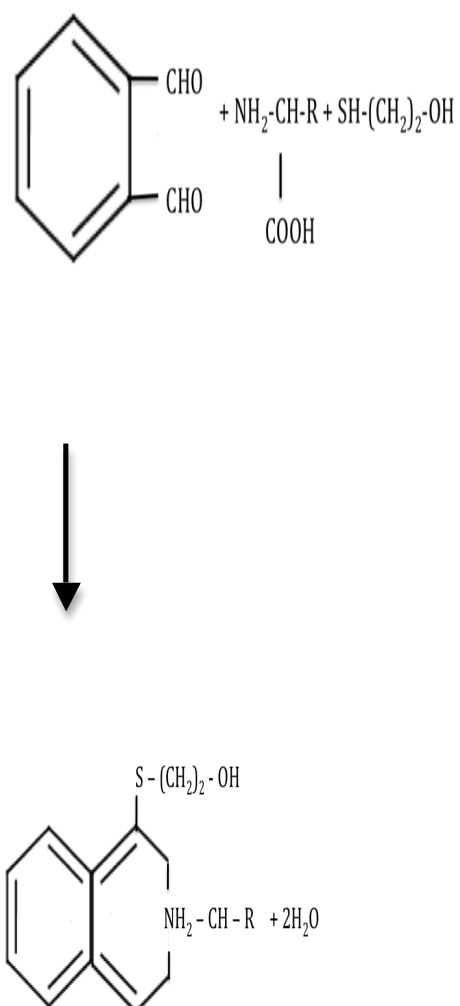
Figure 4.1 – Pre-column derivitisation with OPA

These can then be separated and analysed by HPLC.

A Kontron 500 series automated HPLC system fitted with a Jasco fluorescence detector, and a 4.5mm Hypersil ODS-16 was used. The system used an automated injector, with gradient control, degasser, sample cooler and integrator.

4.2.1 Solvents A and B

A solvent system gradient elution programme was run with 18 ml tetrahydrofuran, 200 ml methanol and 800 ml sodium acetate as solvent A and 800 ml methanol and 200 ml sodium acetate as solvent B. 6 M acetic acid was used to adjust pH to 5.9. The solution was stored at 4 ^{o}C until required. 2 μl β-mercaptoethanol was added for every 1ml of OPA. The resultant mixture was stored in quantities of 1.5 ml at -20 ^{o}C until required.

Table 4.1 – Composition of solvent systems A and B

Component	Solvent A (ml)	Solvent B (ml)
83mM Sodium acetate	800	200
HPLC – grade methanol	200	800
Tetrahydofuran	10-18[*]	-

[*]Tetrahydrofuran was added to buffer A according to chromatogram drift and peak separation

4.2.2 Reference amino acid composition

Sigma AA-S-18 amino acid standard solution (alanine, arginine, aspartate, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine at 2.5 mM and cysteine at 1.25 mM) was diluted in a ratio of 1:100 with sterile water to give a final concentration of 25 μM for all amino acids, except cysteine which had a final concentration of 12.5 μM. The mix (AA-18) was stored at -20 ^{o}C until required.

A second standard mix was made of 4 amino acids (AA-4), 33.02 mg asparagine, 35.53 mg glutamine, 12.75 mg tryptophan and 15.15 mg cysteine in 100 ml of sterile water. This was diluted to give final concentration of 25 μM for asparagine, glutamine and tryptophan and 12.5 μM of cysteine. The solution (AA-4) was stored at -20 ^{o}C until required.

Prior to use, a standard amino acid solution was made up containing 200 μL of AA-18 and 200 μL of AA-4. 25 μL of this standard was aliquoted into 250 μL polypropylene HPLC inserts and placed in the loading tray.

At the start of every new HPLC analysis a standard was run to ensure adequate peak separation. A new standard was run every 3 to 7 experimental plasma samples. This was performed to ensure quality control and any changes in the fluorescence detection of any amino acids over the period of the assays. The control samples of amino acids during the analysis allowed for direct comparison with plasma samples as analysis took place and account for column changes during a certain time period.

An analytical run was performed for the designated samples to identify and quantify the amino acids within the plasma using 25 µl with an equal volume of reagent. Flow rate was maintained at 1.5 ml/min throughout.

Separation took 34 minutes. Chromatogram peaks were integrated automatically by Kontron 2000 software.

Each individual plasma sample taken from day 1 to 7 was analysed. At this point only 89 samples were available for HPLC analysis as all other samples had been utilised with the NMR studies.

4.3 Results

In total eight amino acids were isolated from a 34 minute HPLC run. The amino acids are listed below with associated retention time.

Aspartate – 5 minutes

Glutamate – 7 minutes 10 seconds

Asparagine – 11 minutes

Histadine – 15 minutes

Sereine – 19 minutes

Glutamine – 25 minutes

Threonine – 26 minutes

Arginine – 32minutes

The concentration for each amino acid present was calculated using the following formula.

From the standard solution we know that the concentration of an amino acid like arginine from the area under the curve is equivalent to 10μmmol, the concentration of each amino acid from 1μL of plasma in 24μL of water can be calculated:

If the area under the curve is 205.21, this equals 10μmol of arginine. If in the plasma sample the area under the curve is 3.22, the concentration of arginine is 205.21/3.22 =63.72. 10/63.72 =0.156 μmmol of arginine.

To work out concentration in 25μL:

0.156 x 25 = 3.925μmol of arginine in the plasma.

4.3.1 Univariate Statistics

Test of normality revealed that data was not normal so ANOVA was not applicable. Therefore Kruskal-Wallis test was used for non-parametric data.

Table 4.2 - p values for multiple comparisons of amino acids

Amino acid	Bonferroni corrected p-value
Aspartate	0.168
Glutamate	1.000
Asparagine	1.000
Histidine	0.752
Serine	0.224
Glutamine	0.224
Threonine	0.072
Arginine	0.372
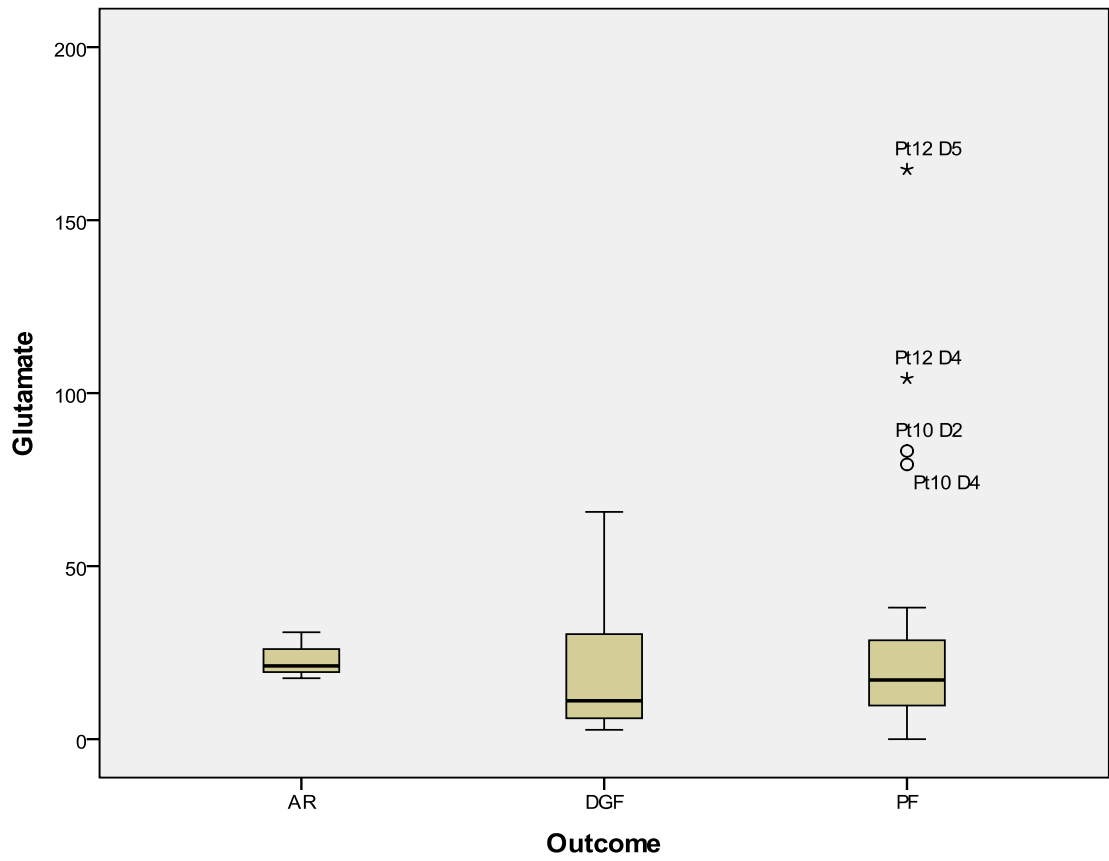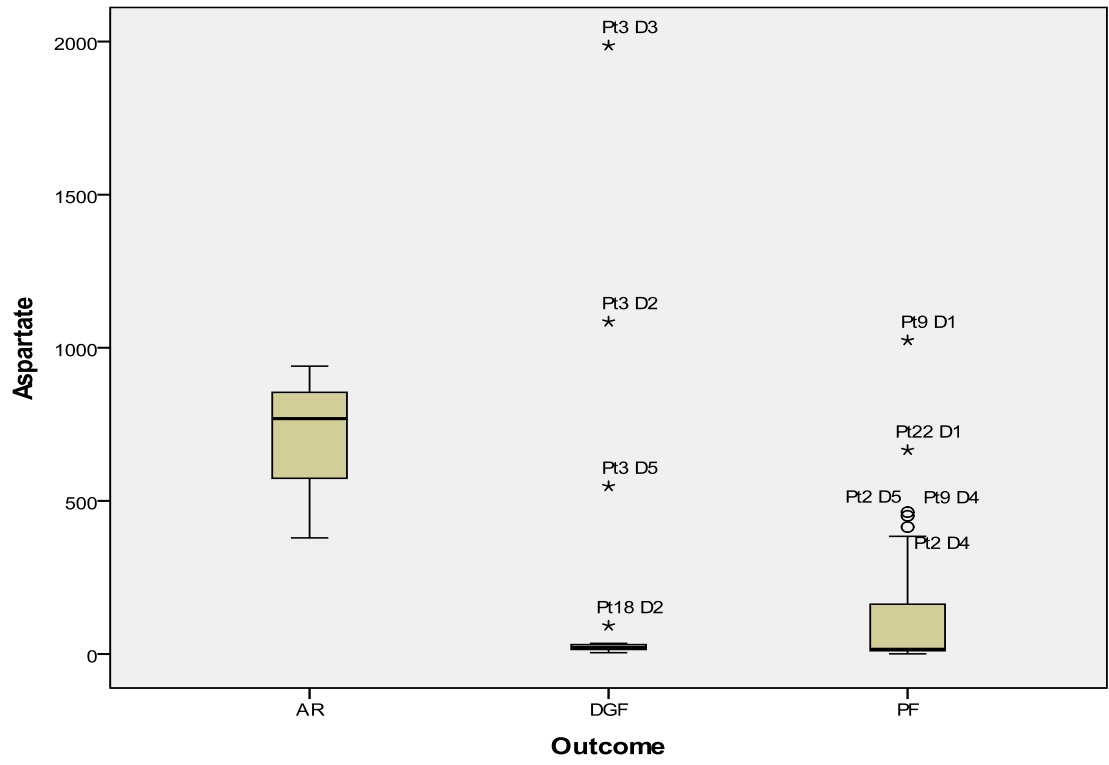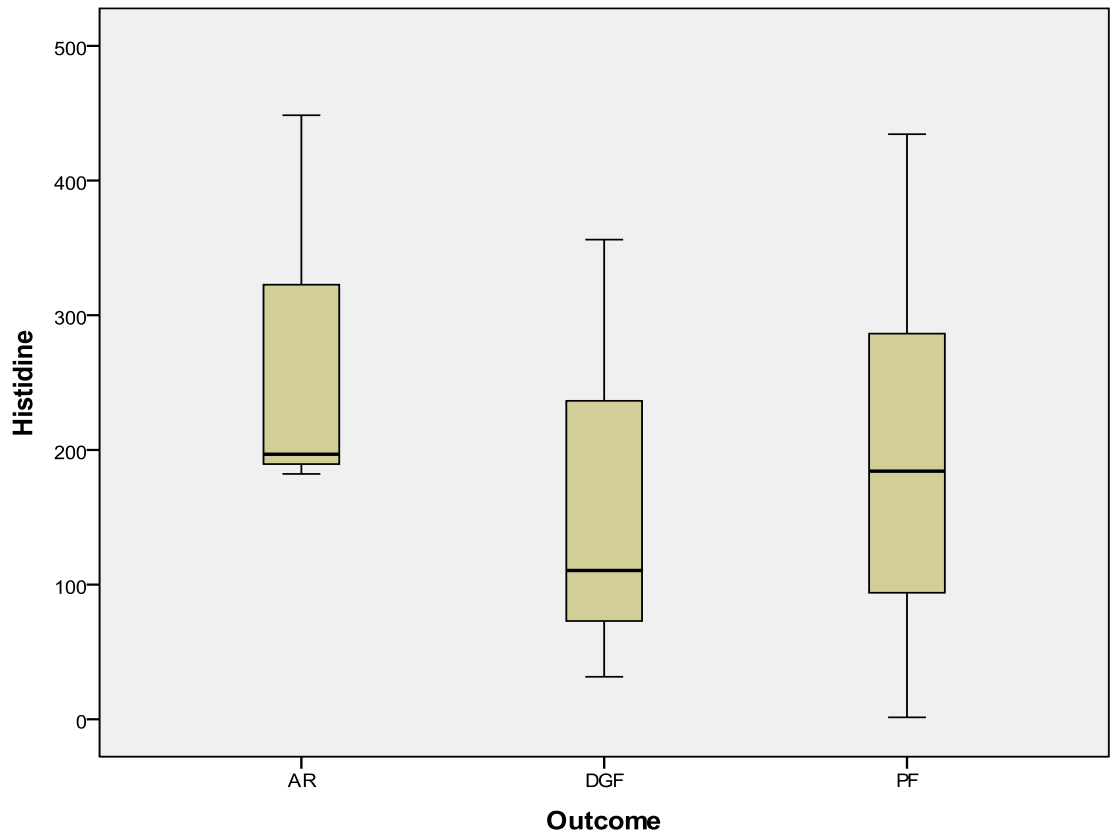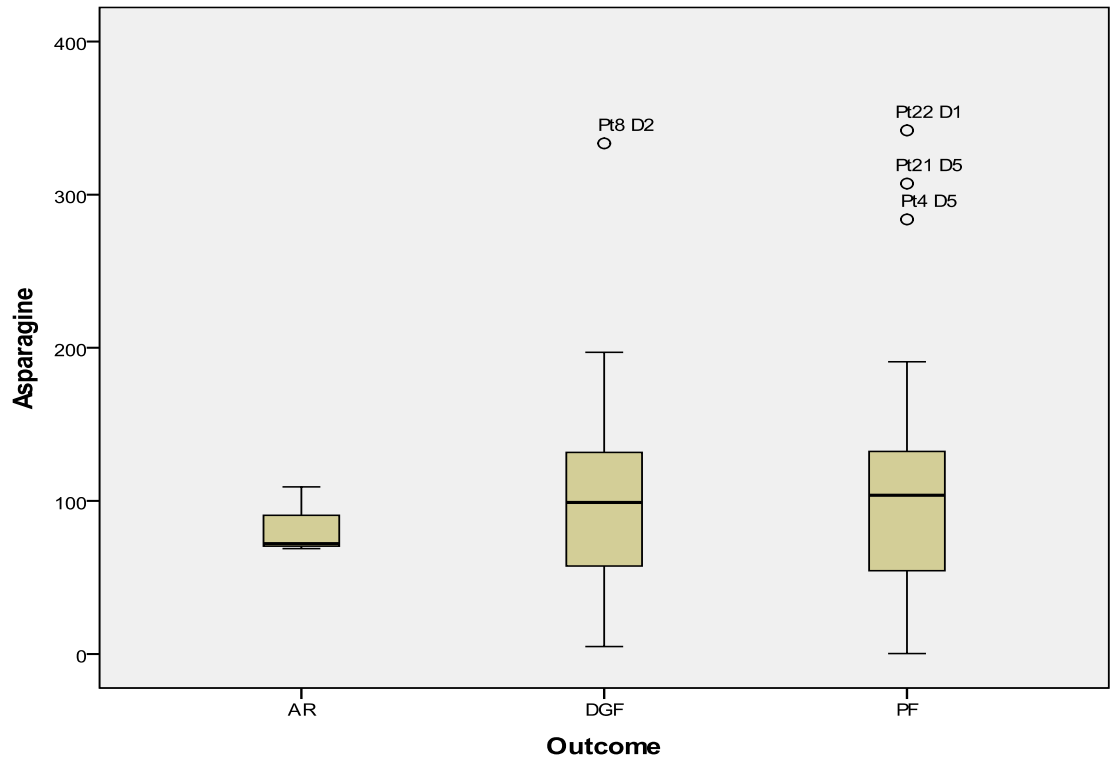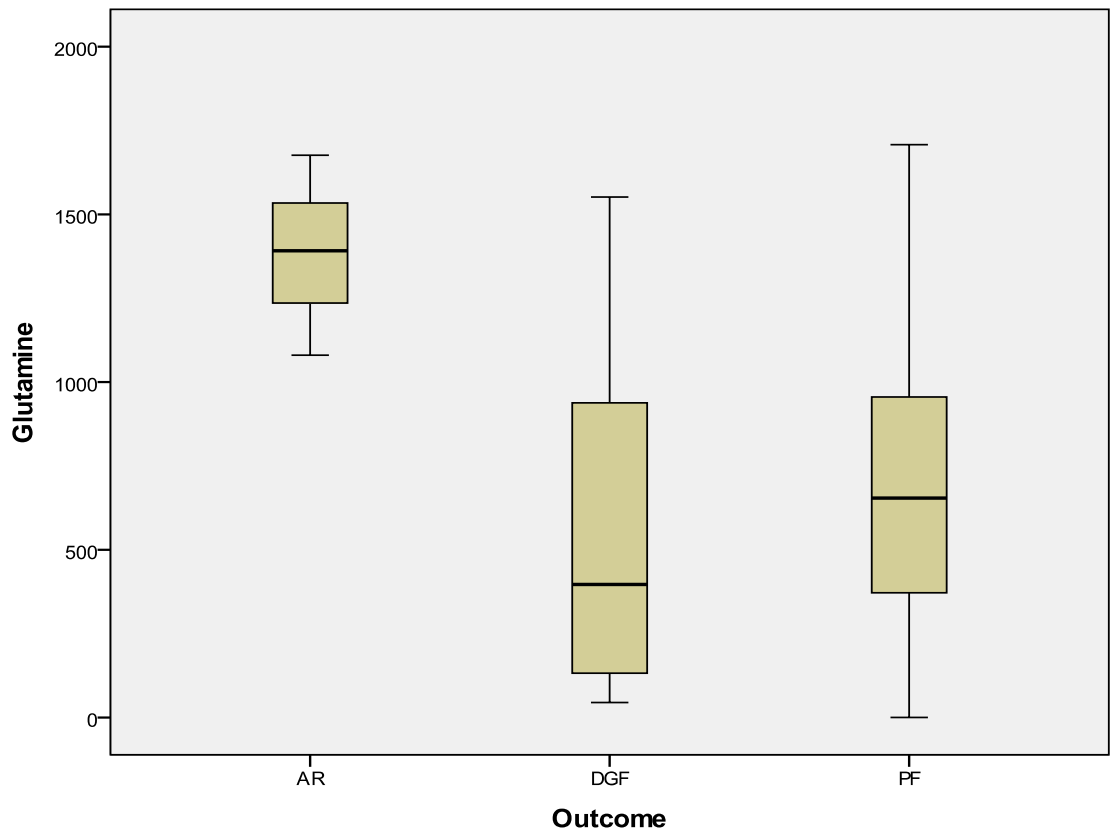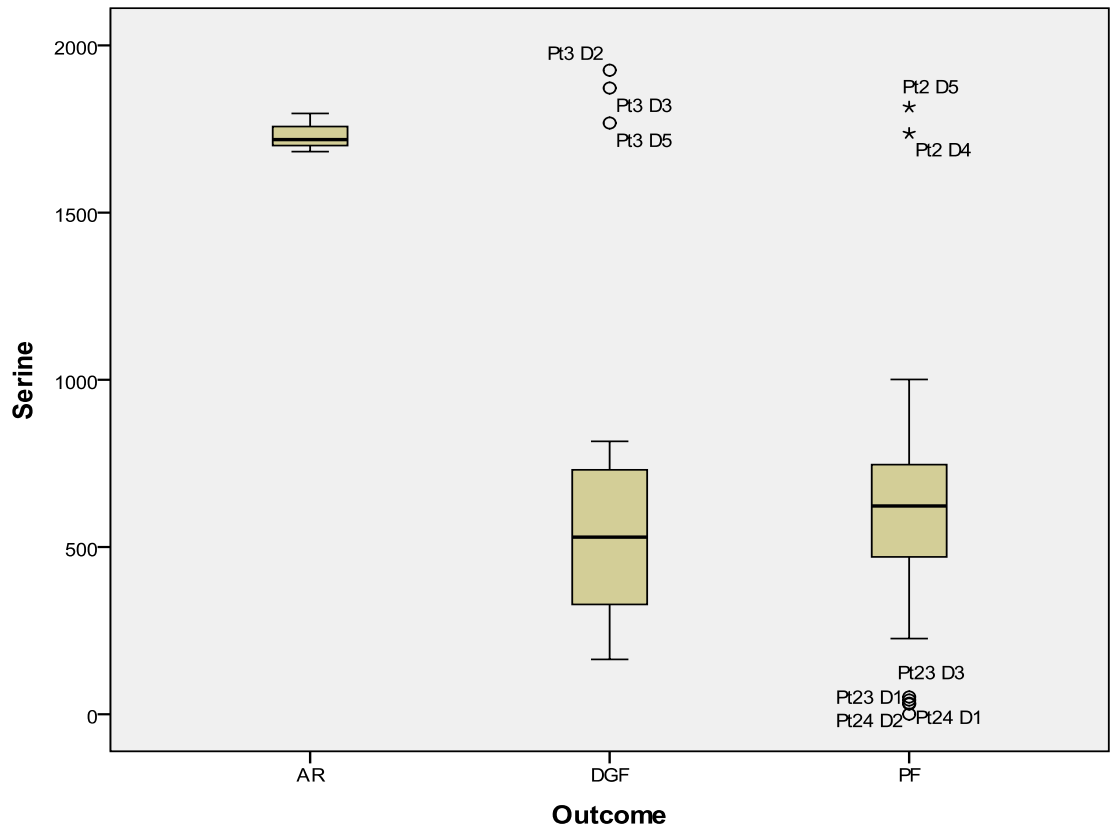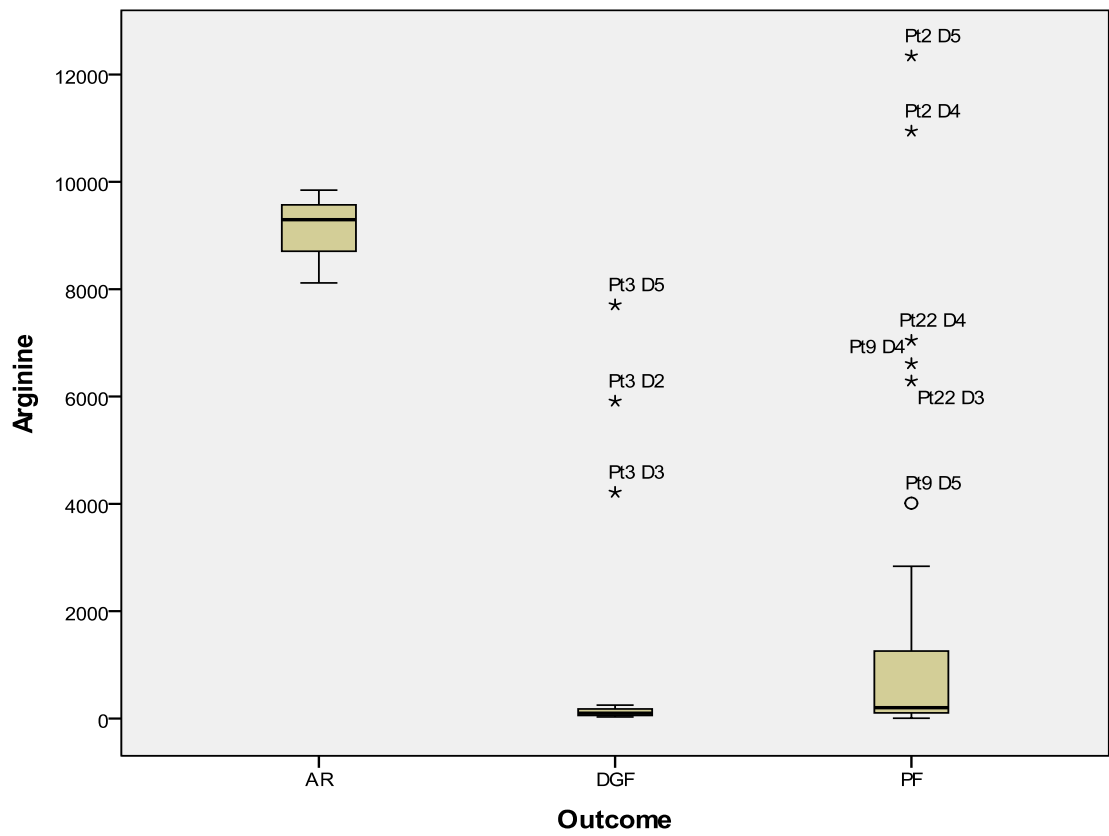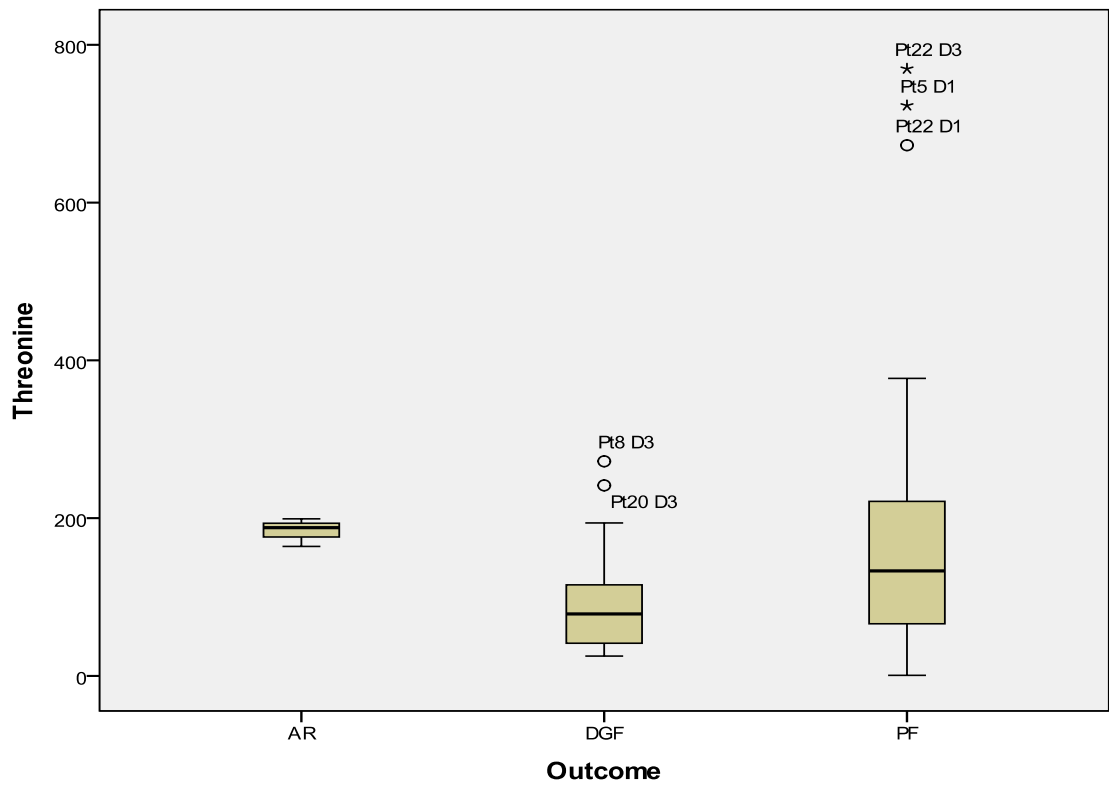
Figure 4.2 a-h – Box plot analysis of amino acids dependent on clinical outcome

(Any outliers are marked with a circle and extreme cases with an asterisk.)

4.3.2 Multivariate Analysis

Data is scaled to unit variance in order to ensure that if one amino acid is particularly high in concentration compared to others, it does not dominate.

Figure 4.3 – Scores plot comparing primary function, delayed function and acute rejection when comparing Amino acids using HPLC
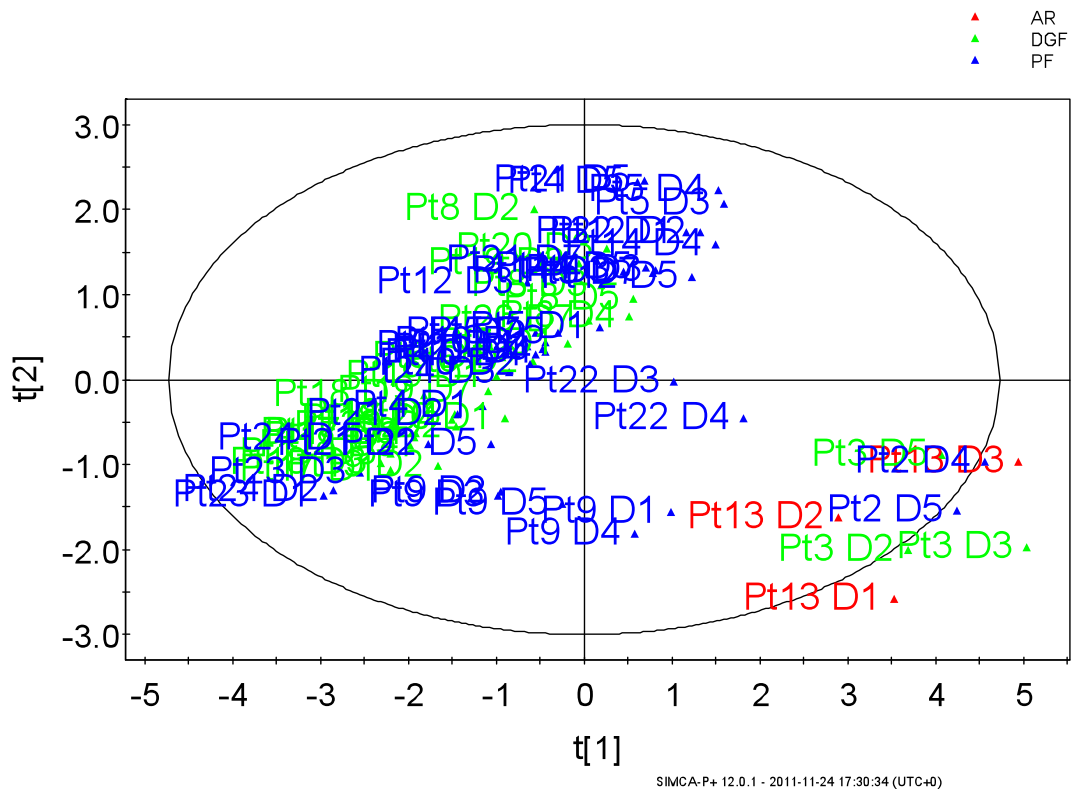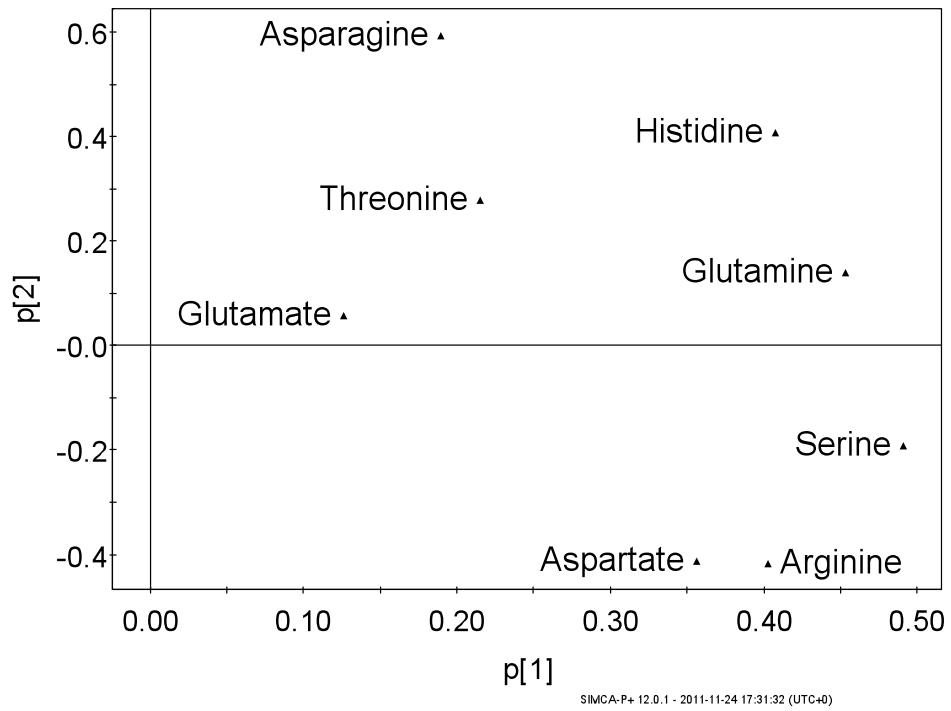
Figure 4.4 – Loadings plot Amino acids responsible for differences seen in **Figure 4.3**

Chapter 5

Discussion

Currently only few tests are capable of rapidly assessing the function of a donor graft in the immediate post-operative period. The clinical course following renal transplantation is varied and early allograft dysfunction can often cause a diagnostic dilemma. Definitive diagnosis of AR over other pathology especially DGF or CNI toxicity relies on invasive biopsy, which can be complicated by haematuria, haematoma, AVM and other injuries often requiring transfusion, re-exploration or, rarely, graft loss. The NMR method used in this study uses readily available blood samples and requires little or no pre-treatment of the sample.

The importance of early diagnosis of AR at a preclinical level has been shown in studies using protocol biopsies (Rush D, *et al.,* 1998; Wilkinson A, *et al*., 2006). Protocol biopsy screening is used to detect subclinical pathologic events, where earlier treatment may improve long-term outcome. The idea being that regular biopsy in a clinically normal transplant would reveal the pathologic process early in its course, allowing time for effective treatment of AR prior to detection with traditional methods. The finding of subclinical rejection has been associated with decreased graft survival at 10 years, and there is evidence that treatment of subclinical rejection improves long-term results. Rush *et al.* used early corticosteroid treatment of patients diagnosed with subclinical rejection based on invasive biopsy, even when in the presence of normal serum creatinine. They found a decrease in early and late clinical rejection, a lower serum creatinine level at twenty-four months and improved long-term graft function (Rush D, *et al*., 1998).

There is clearly a need for an early non-invasive biomarker of graft function and NMR-based metabolomics has the potential to provide this.

A similar approach has recently been utilised in liver transplantation although in this instance extracts of serum were investigated. A single patient was monitored pre-operatively and post-operatively at 3 time points. Different metabolite profiles were observed for the first unsuccessful and the second successful liver transplants. Lactate, uric acid, glutamine, methionine and citrate were identified at different concentrations as early as two hours post operation. The patient's metabolic profile following the successful transplant was found to be comparable with that of 5 healthy volunteers two hours post-operation (Serkova NJ, *et al.,* 2007).

The targeted profiling of metabolites using ^{1}H-NMR spectroscopy and multivariate analysis allowed identification and evaluation of a wide range of metabolites in plasma. In section 2.7.2 the results of a metabolome investigation involving plasma from 24 patients and samples taken at different time points were described. Several molecules were highlighted whose concentration seems to reflect the status of the renal graft. One of these, creatinine, is the breakdown product of creatine phosphate. Creatinine levels would usually be expected to increase in a non-functioning kidney as in DGF or AR but because of the use of dialysis in some of these patients creatinine levels were found to be artificially lower. It seems from this current study that whilst creatinine was affected by the dialysis, the spectra were not altered in terms of metabolic profile or concentration of other metabolites.

TMAO is an oxidation product of trimethylamine and is found in animals and humans (Figure 5.1). TMAO decomposes to TMA, which is the main odorant that is characteristic of degrading seafood (Human Metabolome Database). TMAO is an osmolyte and acts as a homeostatic agent that the body utilises in

Figure 5.1 Chemical structure of TMAO
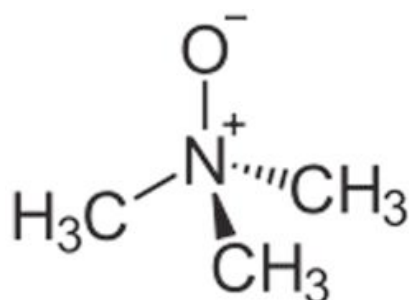
kidney failure to offset and stabilise the effects of increased concentrations of urea, that arise during renal failure or renal stress or in this study with the effects of a failing transplant. TMAO which is secreted from the renal medulla and has been correlated with renal injury in the transplant and chronic renal failure setting previously when in the presence of a raised urea (Hauet T, *et al*., 2000; Richer J, *et al*., 2000). Foxall *et al.* analysed urine samples with NMR to differentiate nephrotoxicity secondary to cyclosporin to those who developed AR. For each patient in this study NMR data was compared with clinical outcomes, graft biopsy and standard renal blood tests. TMAO was found to be significantly increased in urine in patients with AR (Foxall PJ, *et al.,* 1993). The study concluded that NMR spectroscopy was useful in this setting and that urinary metabolite profiles analysed with NMR can follow the course of renal transplant function. Whilst a statistical significance was seen with an increase in urinary TMAO in this study in patients with graft dysfunction, it could not differentiate entirely those patients with graft dysfunction, normal controls or patients with good graft function. We found TMAO to be elevated in plasma, where differentiation in patient groups was seen from day 4 onwards using

TMAO as a marker. Le Moyec et al. have also seen these findings on analysis of urine and plasma in 39 renal transplant patients (Le Moyec L, *et al*., 1993). TMAO should not usually appear in the plasma of a healthy individual as it is effectively filtered by the healthy kidney (Bell JD, *et al*., 1991). Bell *et al*. in their study of 16 plasma and urine samples in 16 chronic renal failure patients showed that TMAO was closely correlated with rising urea and creatinine, a similar process in those patients with non-functioning renal transplant. Results from this study show to almost statistical significance that changes in TMAO concentration are associated with AR and DGF as diagnosed on biopsy with no evidence of CNI toxicity. The studies performed so far that have observed a rise in TMAO (urinary) in the dysfunctioning kidney, whether as a renal transplant or in the chronic failure group, however they have concluded that the sensitivity and specificity are not good enough for clinical diagnosis.

Arginine arises in the urea cycle and its pathway is altered in chronic renal failure where patients with little normal functional renal mass see a reduction in arginine (Tizianello A, *et al.,* 1980). In transplantation changes in arginine production may be due to the ischaemia/reperfusion injury associated with transplant (Becker T, *et al.,* 2009). Arginine deficiency could develop because of decreased arginine output from the proximal tubules of the kidney; these provide the major endogenous supply throughout the body (Wu G & Morris SM, 1998) and may be affected by AR or DGF. When interpreting the NMR results initial arginine concentration in the PF group is high and concentration levels change little over the separate time points of this study. This differs from the AR group where levels start low and remain low for the duration of the study. Change is seen in the DGF group where initial low levels of arginine start to increase the

further out from the operation the patient is, resembling the PF group. However in the NMR spectrum TMAO and arginine appear at similar positions and further tests were required to differentiate between the two metabolites. Validation was performed utilizing HPLC (Chapter 4) and has shown that changes in arginine is not significant across differing renal transplant outcomes (p=0.372). No other metabolite using the HPLC method in this study revealed any metabolite contributing to discrimination between the differing types of graft outcome.

The **key to our findings** is the stage at which it is possible to discriminate amongst the groups and the implications this could have for clinical practice. Based on our results treatment could have been initiated for the AR group a minimum of 48 hours earlier than is the norm with currently used tests. Earlier treatment of AR would allow for better long-term outcomes, additionally it could be argued that in those patients with only DGF who underwent biopsy, this was an unnecessary invasive and potentially transplant threatening procedure. A non-invasive plasma biomarker that suggests DGF in a non-functioning graft may instill confidence in the clinician to "sit tight" for a further 24 to 48 hours and wait to see if the renal transplant starts clearing on its own. The biomarkers identified could have established those patients with DGF who will not go onto develop AR and those with DGF who will go onto develop AR, from day 4 and thus avoid unnecessary biopsies.

In summary we have demonstrated, using high resolution 1H-NMR spectroscopy of plasma, coupled with multivariate statistical analysis, early discrimination in metabolic profiles of patients with DGF, AR and PF in the renal transplant setting.

Our data shows:

- Metabolic profiles from day 4 can differentiate between AR and DGF

- Metabolic profiles of those with PF differ from day 1 compared to those with AR or DGF

- Patients with PF who then develop AR have profiles different to those with PF alone from day 1

- Those with DGF who then develop AR have metabolic profiles the same as those who just have AR

Whilst we may be some time from these tests being performed in the transplant outpatient setting, our data from 24 patients with 6 separate post-operative time points are encouraging. NMR spectroscopy could become a technique for discrimination of graft outcome in the renal transplant setting without any disadvantage to the patient. From a practical stand point this method allows analysis of plasma by a non-destructive process enabling identification of TMAO, amino acids and other small molecules all at the same time facilitating identification of novel biomarkers for graft outcome.

Future work in this field would be necessary before any clinical application could be utilized from the findings here. There would need to be an increase in number of patients analysed and the methods performed in other laboratories.

References

- Al Rasheed SA, Al Mugeiren MM, Abdurrahman MB, Elidrissy ATH. The outcome of percutaneous renal biopsy in children: an analysis of 120 consecutive cases. *Pediatr Nephrol*. 1990;4:600–603.

- Allwood JW, Goodacre R. An introduction to liquid chromatography-mass spectrometry instrumentation applied in plant metabolomic analyses. *Phytochem Anal*. 2010 Jan;21(1):33-47.

- Anthony ML, Gartland KP, Beddell CR, Lindon JC, Nicholson JK. Studies of the biochemical toxicology of uranyl nitrate in the rat. *Arch. Toxicol*. 1994; **68**: 43–53.

- Bairaktari E, Katopodis K, Siamopoulos KC, Tsolas O. Paraquat-induced renal injury studied by ^{1}H NMR spectroscopy of urine. *Clinical Chemistry*. 1998; **44**(6): 1256-1261.

- Becker T, Mevius I, de Vries DK, Schaapherder AF, zu Vilsendorf AM, Klempnauer J, Frölich JC, Tsikas D.Nitric Oxide. The L-arginine/NO pathway in end-stage liver disease and during orthotopic liver and kidney transplantation: biological and analytical ramifications. *Nitric Oxide*.2009Feb;**20**(1):61-7.

- Beckonert O, Monnerjahn J, Bonk U, Leibfritz D. Visualizing metabolic changes in breast-cancer tissue using ^{1}H-NMR spectroscopy and self-organizing maps. *NMR Biomed* 2003; **16**: 1–11.

- Beckwith-Hall BM, Nicholson JK, Nicholls A, Foxall PJD, Lindon JC, Connor SC, Abdi M, Connelly J, Holmes E. Nuclear magnetic resonance spectroscopic and principal components analysis investigations into

biochemical effects of three model hepatotoxins. *Chem. Res. Toxicol.* 1998; **11**: 260-272.

- Beecher CWW: The human metabolome, in Harrigan GG, Goodacre R (eds): Metabolic Profiling: Its Role in Biomarker Discovery and Gene Function Analysis. Norwell, MA, Kluwer Academic Publishers. 2003; **17**: 1-8.

- Bell JD, Lee JA, Lee HA, Sadler PJ, Wilkie DR, Woodham RH. Nuclear magnetic resonance studies of blood plasma and urine from subjects with chronic renal failure: identification of trimethylamine-N-oxide. *Biochim. Biophys. Acta.* 1991 Feb **22**;1096(2):101-7.

- Ben-Yoseph O, Badar-Goffer RS, Morris PG, Bachelard HS. Glycerol 3-phosphate and lactate as indicators of the cerebral cytoplasmic redox state in severe and mild hypoxia respectively: a 13C- and 31P-n. m. r. study. *Biochem. J.* 1993; **291**: 915–919.

- Bory C, Boulieu R, Chantin C, Mathieu M. Diagnosis of alcaptonuria: rapid analysis of homogentisic acid by HPLC. *Clin. Chim. Acta.*1990 Jul; **189**(1):7-11.

- Boudonck KJ, Mitchell MW, Német L *et al.* Discovery of metabolomics biomarkers for early detection of nephrotoxicity. *Toxicol. Pathol.* 2009;**37**(3):280-92

- Brindle JT, Antti H, Holmes E, Tranter G, Nicholson JK, Bethell HWL, Clarke S, Schofield PM, McKilligan, E, Mosedale DE, Grainger DJ. Rapid and non-invasive diagnosis of the presence and severity of coronary heart disease using 1H NMR-based metabonomics. *Nat. Med.* 2002; **8:** 1439 – 1445.

- Carlson IH. New markers in serum for lymphocyte activation for predicting allograft rejection. Neopterin and soluble interleukin-2 receptor. *Clin. Lab. Med.* 1992;12:99–111 32

- Carmichael PL. Mechanisms of action of antiestrogens: relevance to clinical benefits and risks. *Cancer. Invest.* 1998; **16**: 604–611.

- Chen JH, Enloe BM, Fletcher CD, Cory DG, Singer S. Biochemical analysis using high-resolution magic angle spinning NMR spectroscopy distinguishes lipoma-like well-differentiated liposarcoma from normal fat. *J. Am. Chem.* Soc. 2001; **123**: 9200–9201.

- Clarke W, Silverman BC, Zhang Z, Chan DW, Klein AS, Molmenti EP. Characterization of renal allograft rejection by urinary proteomic analysis. *Ann. Surg.* 2003; **237**:660–664 (discussion 664–665)

- Claudino WM, Quattrone A, Biganzoli L, Pestrin M, Bertini I, Angelo DL. Metabolomics: Available Results, Current Research Projects in Breast Cancer, and Future Applications. *J. of Clin. Onc*. 2007; **25**(19): 2840-2846.

- Crockford DJ, Holmes E, Lindon JC, Plumb RS, Zirah S, Bruce SJ, Rainville P, Stumpf CL, Nicholson JK. Statistical Heterospectroscopy, an Approach to the Integrated Analysis of NMR and UPLC-MS Data Sets: Application in Metabonomic Toxicology Studies. *Anal. Chem.* 2006; **78**: 363-371

- Dettmer K, Aronov AP, Hammock BD. Mass Spectometry-Based Metabolomics. *Mass Spectrometry Reviews*. 2007; **26**:51-78.

- DeVita VT, Hellman S, Rosenberg SA. Cancer Principles and Practice of Oncology. 2005. Lippincott. Williams & Wilkins PA USA.

- Drugbank. www.drugbank.ca

- Drysdale R, Bayraktaroglu L. Current awareness. *Yeast* 2000; **17**: 159-166.

- Duarte IF, Stanley, EG, Holmes E, Lindon JC, Gil AM, Tang H, Ferdinand R, McKee, CG, Nicholson JK, Vilca-Melendez H, Heaton N, Murphy GM. Metabolic assessment of Human Liver Transplants from Biopsy samples at the Donor and Recipient Stages Using High-Resolution Magic Angle Spinning 1H-NMR Spectroscopy. *Anal. Chem.* 2005; **77**:5570-5578.

- Dunn WB, Bailey NJ, Johnson HE. Measuring the Metabolome: Current Analytical Technologies. *Analyst* 2005; **130**:606-625.

- Dunn WB. Current trends and future requirements for the mass spectrometric investigation of microbial mammalian and plant metabolomes. Phys. Biol.,2008, **5**(1),1-24

- El-Sayed S, Bezabeh T, Odlum O, Patel R, Ahing S, MacDonald K, Somorjai RL, Smith IC. An ex vivo study exploring the diagnostic potential of 1H magnetic resonance spectroscopy in squamous cell carcinoma of the head and neck region. *Head Neck* 2002; **24**:766–772.

- Fan X, Bai J, Shen P. Diagnosis of breast cancer using HPLC metabonomics fingerprints coupled with computational methods. *Conf Proc IEEE Eng Med Biol Soc* 2005; **6**:6081–84

- Fiehn O. Metabolomics – the link between genotypes and phenotypes. *Plant Mol. Biol.* 2002; **48**: 155–171.

- Forshed J, Schuppe-Koistinen I, Jacobsson SP. Peak alignment of NMR signals by means of a genetic algorithm. Anal. Chim. Act. 2003; **487**(2):189–199.

- Foxall PJ, Mellotte G J, Bending MR, Lindon JC, Nicholson JK. NMR spectroscopy as a novel approach to the monitoring of renal transplant function. *Kidney Int*. 1993; **43**: 234–245.

- Gartland KP, Bonner FW, Nicholson JK. Investigations into the biochemical effects of region-specific nephrotoxins. *Mol. Pharmacol* 1989; **35**(2): 242–250.

- Gartland KP, Bonner FW, Timbrell JA Nicholson JK. Biochemical characterisation of para-aminophenol-induced nephrotoxic lesions in the F344 rat. *Arch. Toxicol.* 1989; **63**(2): 97– 106.

- Gartland KPR, Beddell CR, Lindon JC, Nicholson JK. Application of pattern recognition methods to the analysis and classification of toxicological data derived from proton nuclear magnetic resonance spectroscopy of urine. *Mol. Pharmacol.* 1991; **39**: 629–642.

- Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG and Kell DB. Metabolomics by numbers: acquiring and understanding global metabolite data. *Trends Biotechnol.* 2004; **22**: 245-252.

- Griffin JL. Metabolic profiles to define the genome: Can we hear the phenotypes? *Philos. TransR. Soc. Lond. B. Biol. Sci*. 2004; **359**:857-871.

- Griffiths JR, Stubbs M. Opportunities for studying cancer by metabolomics: preliminary observations on tumors deficient in hypoxia-inducible factor 1. *Adv. Enzyme Regul*. 2003; **43**: 67–76.

- Griffiths JR, McSheehy PM, Robinson SP, Troy H, Chung YL, Leek RD, Williams KJ, Stratford IJ, Harris AL, Stubbs M. Metabolic changes detected by in vivo magnetic resonance studies of HEPA-1 wild-type tumors and

tumors deficient in hypoxia-inducible factor-1b (HIF-1b): evidence of an anabolic role for the HIF-1 pathway. *Cancer Res*. 2002; **62**: 688–695.

- Gronwald W, Klein MS, Kaspar H, Fagerer SR, Nürnberger N, Dettmer K, Bertsch T, Oefner PJ. Urinary metabolite quantification employing 2D NMR spectroscopy. *Anal. Chem.* 2008 Dec 1;**80**(23):9288-97.

- Gwinner W. Renal Transplant Rejection Markers. *World. J. Urol.* 2007; **25**: 445-455.

- Hamosh A, Scott AF, Amberger J Bocchini C, Valle D, McKusick VA. Online Mendelian Inheritance in Man (OMIM) a knowledge base of human genes and genetic disorders. *Nucleic Acid Res.* 2002; **30**: 52-55.

- Han XM, Holtzman D, McKeel DW, Kelley J, Morris JC. Substantial sulfatide deficiency and ceramide elevation in very early Alzheimer's disease: potential role in disease pathogenesis. J. *Neurochem.* 2002; **82**:809–18

- Hauet T, Gibelin H, Richer JP, *et al*: Influence of retrieval conditions on renal medulla injury: Evaluation by proton NMR spectroscopy in an isolated perfused pig kidney model. *J. Surg. Res.* 2000; **93**:1–8.

- Hauert T, Baumert H, Gibelin H, Hameury F, Goujon JM, Carretier M, Eugene M. Noninvasive Monitoring of Citrate, Acetate, Lactate and Renal Medullary Osmolyte Excretion in Urine as Biomarkers of Exposure to Ischaemic Reperfusion injury. *Cryobiology* 2000; **41**:280-291.

- Hauser IA, Spiegler S, Kiss E, *et al*. Prediction of acute renal allograft rejection by urinary monokine induced by IFN-gamma (MIG). *J. Am. Soc. Nephrol.* 2005;**16**:1849–1858

- Hergesell O, Felten H, Andrassy K, Kuhn K, Ritz E. Safety of ultrasound guided percutaneous renal biopsy—retrospective analysis of 1090 consecutive cases. *Nephrol. Dial. Transplant*. 1998;**13**:975–977.

- Holland NT, Smith MT, Eskenazi B, Bastaki M. Biological sample collection and processing for molecular epidemiological studies. *Mutat. Res*. 2003 Jun;**543**(3):217-34.

- Hollywood K, Brison DR, Goodacre R. Metabolomics: Current technologies and future trends. *Proteomics* 2006; **6**, 4716–4723.

- Holmes E, Bonner FW, Sweatman BC, Lindon JC, Beddell CR, Rahr E, Nicholson JK. Nuclear magnetic resonance spectroscopy and pattern recognition analysis of the biochemical processes associated with the progression of and recovery from nephrotoxic lesions in the rat induced by mercury(II) chloride and 2-bromoethanamine. *Mol. Pharmacol.* 1992; **42**: 922–930.

- Holmes E, Foxall PJD, Nicholson JK, Neild GH, Brown SM, Beddell CR, Sweatman BC, Rahr E, Lindon JC, *et al*. Automatic data reduction and pattern recognition methods for analysis of 1H nuclear magnetic resonance spectra of human urine from normal and pathological states. *Anal Biochem* 1994; **220**(2):284–296.

- Holmes E, Loo RL, Stamler J, Bictash M, Yap IK, Chan Q, Ebbels T, De Iorio M, Brown IJ, Veselkov KA, Daviglus ML, Kesteloot H, Ueshima H, Zhao L, Nicholson JK, Elliott P. Human metabolic phenotype diversity and its association with diet and blood pressure. *Nature*. 2008 May 15;**453**(7193):396-400.

- Howells SL, Maxwell RJ, Peet AC, Griffiths JR. An investigation of tumor 1H nuclear magnetic resonance spectra by the application of chemometric techniques. *Magn. Reson. Med.* 1992; **28**: 214–236.

- Jellum E, Bjørnson I, Nesbakken R, Johansson E, Wold S. Classification of human cancer cells by means of capillary gas chromatography and pattern recognition analysis. J. Chromatogr. 1981 Nov 6;**217**:231-7.

- Jiye A, Trygg J, Gullberg J, Johansson AI, Jonsson P, Antti H, Marklund SL, Moritz T. Extraction and GC/MS analysis of the human blood plasma metabolome. *Anal. Chem.* 2005 Dec 15;**77**(24):8086-94.

- Johnson JA, Pohar, Sl, Majumdar SR. Health Care Use and Costs in the Decade after the Identification of Type 1 and Type 2 Diabetes. A Population Based Study. *Diabetes Care* 2006; **29**: 2043-2408.

- Jordan KW, Cheng LL. NMR Based Metabolomics Approach to Target Biomarkers for Human Prostate Cancer. *Expert Rev. in Proteomics*. 2007; **4**(3): 389-400.

- Lough WJ, Wainer LW. Introduction in High Performance Liquid Chromatography. Fundamental Principles and Practice. 1997. Chapman and Hall

- Idborg-Bjorkman H, Edlund PO, Kvalheim OM, Schuppe-Koistinen I & Jacobsson SP. Screening of biomarkers in rat urine using LC/electrospray ionisation-MS and two-way data analysis. *Anal. Chem*. 2003; **75**: 4784–4792.

- Katzmarzyk PT, Janssen L. The Economic Costs Associated with Physical Inactivity and Obesity. *Can J Appl Physiol*. 2004; **29**: 90-115.

- Kim JW, Tchernyshyov I, Semenza GL, Dang CV: HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. *Cell. Metab.* 2006; **3**(3):177-185.

- Kirschenlohr HL, Griffen JL, Clarke SC Rhydwen R, Grace AA, Schofield PM, Brindle KM, Metcalfe JC. Proton NMR analysis of plasma is a weak predictor of coronary artery disease. *Nat. Med.* 2006; **12**: 705-710.

- Kriat M, Confort-Gouny S, Vion-Dury J, Sciaky M, Viout P, Cozzone PJ. Quantitation of metabolites in human blood serum by proton magnetic resonance spectroscopy. A comparative study of the use of formate and TSP as concentration standards. *NMR in Biomed.* 1992; **5**:179-184.

- Le Moyec L, Pruna A, Eugène M, Bedrossian J, Idatte JM, Huneau JF, Tomé D.Proton nuclear magnetic resonance spectroscopy of urine and plasma in renal transplantation follow-up. *Nephron*. 1993;**65**(3):433-9.

- Lenz EM, Bright J, Knight R, Wilson ID, Major H. Cyclosporin A-induced changes in endogenous metabolites in rat urine: a metabonomic investigation using high field 1HNMR spectroscopy, HPLC-TOF/MS and chemometrics. *J. Pharm. Biomed. Anal*. 2004; **35**: 599–608.

- Lenz ME, Wilson ID. Analytical strategies in metabonomics. *Journal of proteome research,*. 2007; **6**(2): 443-446

- Lin WC, Yang Y, Wen YK, Chang CC. Outpatient versus inpatient renal biopsy: a retrospective study. *Clin. Nephrol*. 2006;**66**:17–24.

- Lindon JC, Nicholson JK, Holmes E, Everett JR. Metabonomics: Metabolic processes studied by NMR spectroscopy of biofluids. *Concepts in Magnetic Resonance*. 2000; **12**(5): 289-320.

- Lindon JC, Nicholson JK, Holmes E. So what's the deal with metabolomics? *Anal. Chem.* 2003; **75**(17):384A–391A.

- Lindon JC, Holmes E, Bollard ME, Stanley EG, Nicholson JK. Metabonomics technologies and their applications in physiological monitoring, drug safety assessment and disease diagnosis. *Biomarkers* 2004; **9** (1): 1-31.

- Lindon JC. Metabonomics – Techniques and Applications. Business Briefing: Future Drug Discovery 2004.

- Mahido C, Ruchiwarat S, Prawat H, Pisutjaroenpong S, Engprasert S, Chumsri P. Biodiversity and Natural Product Drug Discovery. *Pure. Appl. Chem.* 1998; **70**: 2065-2072

- Marcotte EM. The path not taken. *Nat. Biotechnol*. 2001; **19**:626-627.

- Matoba S, Kang JG, Patino WD, Wragg A, Boehm M, Gavrilova O, Hurley PJ, Bunz F, Hwang PM: p53 regulates mitochondrial respiration. *Science* 2006; **312**(5780):1650-1653.

- Meyer V. 2004. Practical High-performance Liquid Chromatography, 2nd edn. Wiley: New York.

- Millis K. Weybright P, Campbell N, Fletcher JA, Fletcher CD, Cory DG, Singer S. Classification of human liposarcoma and lipoma using ex vivoproton NMR spectroscopy. *Magn. Reson Med* 1999; **41**: 257–267

- Moreno A, Lopez LA, Fabra A, Arus C. 1H MRS markers of tumour growth in intrasplenic tumours and liver metastasis induced by injection of HT-29 cells in nude mice spleen. *NMR Biomed.* 1998; **11**: 93–106.

- Nicholls AW, Holmes E, Lindon JC, Shockcor JP, Farrant RD,Haselden JN, Damment SJ, Waterfield CJ, Nicholson JK. Metabonomic investigations into hydrazine toxicity in the rat. *Chem. Res. Toxicol*. 2001; **14**: 975–987.

- Nicholson JK, Wilson ID. High resolution proton NMR spectroscopy of biological fluids. *Prog. NMR Spectrosc*. 1989; **21**: 444-501.

- Nicholson JK, Foxall PLD, Spraul M, Farrant RD, Lindon JC. 750 MHz 1H and 1H-13C NMR Spectroscopy of Human Blood Plasma. *Anal. Chem*. 1995;**67**(5): 793-811

- Nicholson JK; Lindon JC; Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica*, 1999;**29**(11): 1181-1189

- Nicholson JK, Connelly J, Lindon JC, Holmes E Metabonomics: a platform for studying drug toxicity and gene function. *Nat. Rev. Drug Discovery* 2002; **1**: 153– 161.

- Odunsi K, Wollman RM, Ambrosone CB, Hutson A, McCann SE, Tammela J, Geisler JP, Miller G, Sellers T, Cliby W, Qian F, Keitz B, Intengan M, Lele S, Alderfer JL. Detection of epithelial ovarian cancer using 1H-NMR-based metabonomics. *Int. J. Cancer* 2005; **113**:782–88

- O'Riordan E, Orlova TN, Mei JJ, *et al*. Bioinformatic analysis of the urine proteome of acute allograft rejection. *J. Am. Soc. Nephrol.* 2004;15:3240–3248

- Parrish AE. Complications of percutaneous renal biopsy: a review of 37 years experience. *Clin. Nephrol*. 1992;**38**:135–141.

- Pelzl S, Opelz G, Daniel V, *et al* Evaluation of post-transplantation soluble CD30 for diagnosis of acute renal allograft rejection. *Transplantation* 2003;**75**:421–423

- Pham-Tuan H, Kashavelis L, Daykin CA, Janssen HG. Method development in high-performance liquid chromatography for high throughput profiling and metabonomic studies of biofluid samples. *J. Chromatogr.* 2003; **789B**: 283–301.

- Plumb RS, Stumpf CL, Gorenstein MV, Castro-Perez JM, Dear GJ, Anthony M, Sweatman BC, Connor SC, Haselden JN. Metabonomics: the use of electrospray mass spectrometry coupled to reversed-phase liquid chromatography shows potential for the screening of rat urine in drug development. *Rapid Commun Mass Spectrom.* 2002; **16**: 1991–1996.

- Richer J, Baumer TH, Gibelin H, *et al*: Evaluation of renal medulla injury after cold preservation and transplantation: noninvasive determination of medullar damage by proton nuclear magnetic resonance spectroscopy of urine and plasma. *Transplant. Proc.* 2000; **32**:47–482

- Robertson DG. Metabonomics in Toxicology: *A Review Toxicological Sciences*. 2005; **85**: 809–822.

- Robertson DG, Reily MD, Sigler RE, Wells DF, Paterson DA, Braden TK. Metabonomics: Evaluation of nuclear magnetic resonance (NMR) and pattern recognition technology for rapid in vivo screening of liver and kidney toxicants. *Toxicological. Sciences*. 2000; **57**(2): 326-337.

- Rochfort S. Metabolomics Reviewed: A New "Omics" Platform Technology for Systems Biology and Implications for Natural Products *Research. J. Nat. Prod* 2005; **68**: 1813-1820.

- Rozen S, Cudkowicz ME, Bogdanov M, Matson WR, Kristal BS, Beecher C, Harrison S, Vouros P, Flarakos J, Vigneau-Callahan K, Matson TD, Newhall KM, Beal MF, Brown RH, Kaddurah-Daouk R. Metabolomic analysis and signatures in motor neuron disease. *Metabolomics* 2005; **1**:101–8

- Rush D, Nickerson P, Gough J, Mckenna R, Grimm P, Cheang M, Trpkov K, Solez K, Jeffery J. Beneficial Effects of Treatment of Early Subclinical Rejection: A Randomised Study. *J. Am. Soc. Nephrology.* 1998; **9**:2129-2134.

- Rush D, Somorjai RL, Deslauriers R, Shaw A, Jeffery J, Nickerson P. Subclinical rejection—a potential surrogate marker for chronic rejection - may be diagnosed by protocol biopsy or urine spectroscopy. *Ann. Transplant.* 2000; **5**: 44 – 49.

- Ryals J. Metabolomics: An important emerging science. *Business Briefing Pharmatech* 2004: 51-54, http://www.touchbriefings.com.

- Sabatine MS, Liu E, Morrow DA, Heller E, McCarroll R, Wiegand R, Berriz GF, Roth FP, Gerszten RE. Metabolomic identification of novel biomarkers of myocardial ischemia. *Circulation* 2005; **112**(25):3868–75

- Sands CJ, Coen M, Maher AD, Ebbels TM, Holmes E, Lindon JC, Nicholson JK. Statistical Total Correlation Spectroscopy Editing of (^{1})H NMR Spectra of Biofluids: Application to Drug Metabolite Profile Identification and Enhanced Information Recovery. *Anal Chem*. 2009 Aug 1;**81**(15):6458-66.

- Sanins SM, Nicholson JK, Elcombe C, Timbrell JA. Hepatotoxin-induced hypertaurinuria: a proton NMR study. *Arch. Toxicol.* 1990; **64**(5): 407 – 411.

- Schmidt C. Metabolomics Takes Its Place as Latest Up-and-Coming "Omic" Science. *Journal of the National Cancer Institute*. 2004; **96** (10) May 19:732-734.

- Serkova NJ, Zhang Y, Coatney, JL, Hunter L, Wachs ME, Niemann CU, Mandell S. Early Detection of Graft Failure Using the Blood Metabolic Profile of a Liver Recipient. *Transplantation* 2007a; **83**(4): 517-521

- Serkova N, Spratlin J, Eckhardt S. NMR-based metabolomics: Translational application and treatment of cancer. *Current Opinion in Molecular Therapeutics* 2007b; **9**(6):572-585

- Sheidler J, Hricak H, Vigneron DB Yu KK, Sokolov DL, Huang RL, Zaloudek CJ, Nelson SJ, Carroll PR, Kurhanewicz J. Prostate Cancer: Localisation with three-dimensional proton MR spectroscopic imaging – clinicopathologic study. *Radiology*. 1999; **213:**473-480.

- Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, Dalla-Favera R, Dang CV: c-Myc transactivation of LDH-A: Implications for tumor metabolism and growth. *Proc. Natl. Acad. Sci. USA* 1997; **94**(13):6658-6663.

- Sitter B, Lundgren S, Bathen TF, Halgunset J, Fjosne HE, Gribbestad IS. Comparison of HR MAS MR spectroscopic profiles of breast cancer tissue with clinical parameters. *NMR Biomed*. 2006; **19**:30-40.

- Stumm G, Russ A, Nehls M. Deductive genomics - a functional approach to identify innovative drug targets in the post-genomic era. *American Journal of Pharmacogenomics* 2002; **2**: 263-271.

- Tietz NW. Clinical Guide to Laboratory Tests. 3rd Edition WB Saunders Press, Pa, USA, 1995.

- Tizianello A, De Ferrari G, Garibotto G, Gurreri G, Robaudo C. Renal metabolism of amino acids, and ammonia in subjects with normal renal function and in patients with chronic renal insufficiency. *J. Clin. Invest.* 1980; **65**: 1162–1173.

- Tiziani S, Emwas AH, Lodi A, Ludwig C, Bunce CM, Viant MR, Günther UL. Optimized metabolite extraction from blood serum for 1H nuclear magnetic resonance spectroscopy. *Anal. Biochem*. 2008 Jun 1;**377**(1):16-23.

- Turner E, Brewster JA, Simpson NAB, *et al*. Plasma From Women with Preeclampsia Has a Low Lipid and Ketone Body Content—A Nuclear Magnetic Resonance Study. *Hypertension in Pregnancy*. 2007; **26**: 329-342.

- Turner E, Brewster JA, Simpson NAB *et al*. Aromatic Amino Acid Biomarkers of Preeclampsia—A Nuclear Magnetic Resonance Investigation. *Hypertension in Pregnancy* 2008;**27**:3,225-235.

- Van, Q. N., Issaq, H. J., Jiang, Q., Li, Q., Muschik, G. M., Waybright, T. J., Lou, H., Dean, M., Uitto, J., and Veenstra, T. D. Comparison of 1D and 2D NMR spectroscopy for metabolic profiling. *J. Proteome Res*. 2008:**7**; 630–639.

- Vasconcellos LM, Schachter AD, Zheng XX *et al*. Cytotoxic lymphocyte gene expression in peripheral blood leukocytes correlates with rejecting renal allografts. *Transplantation.* 1998; **66**:562–566

- Vautz W, Baumbach JI. Exemplar application of multi-capillary column ion mobility spectrometry for biological and medical purpose. *International Journal of Ion Mobility Spectrometry* 2008. Online publication date: 6-Jul-2008.

- Veronese FV, Manfro RC, Roman FR, Edelweiss MI, Rush DN, Dancea S, Goldberg J and Gonçalves LF. Reproducibility of the Banff Classification in Subclinical Kidney transplant Rejection. *Clin. Transplant* 2005; **19**:518-521.

- Vilca Melendez H, Rela M, Murphy G, Heaton N. Assessment of Graft Function Before Liver Transplantation: Quest for the Lost Ark? *Transplantation* 2000; **70**:560-565.

- Vulimiri SV, Misra M, Hamm JT, Mitchell M, Berger A.Effects of Mainstream Cigarette Smoke on the Global Metabolome of Human Lung Epithelial Cells. *Chem Res Toxicol.* 2009 Mar 16;**22**(3):492-503.

- Waters NJ, Holmes E, Waterfield CJ, Farrant RD, Nicholson JK. NMR and pattern recognition studies on liver extracts and in livers from rats treated with alpha-naphthylisothiocyanate. *Biochem. Pharmacol.* 2002; **64**: 67–77.

- Weckwerth W. Metabolomics in systems biology. *Annu. Rev. Plant Biol.* 2003; **54**: 669–689.

- Weckwerth W, Morgenthal K. Metabolomics: from pattern recognition to biological interpretation. *DDT* 2005; **22**(10): 1551-1558.

- Whitfield PD, German AJ, Noble PJM. Metabolomics: an emerging post-genomic tool for nutrition. *British Journal of Nutrition* 2004; **92**: 549–555.

- Whittier WL, Korbet SM. Timing of complications in percutaneous renal biopsy. *J Am Soc Nephrol.* 2004;**15**:142–147.

- Wilkinson A. Protocol transplant biopsies: are they really needed? *Clin. J. Am. Soc. Nephrol.* 2006;**1**:130–137.

- Wishart D. Metabonomics: The Principles and Potential Applications to Transplantation. *American Journal of Transplantation* 2005; **5**: 2814-820.

- Wishart D. Metabonomics in Monitoring Kidney Transplants. *Curr. Op. in. Nephrology & Hypertension*. Nov 2006; **15**(6): 637-642.

- Wishart D. Proteomics and The Human Metabolome Project. *Expert. Rev. Proteomics* 2007; **4**(3):333-335.

- Wishart D. Metabolomics in Monitoring Kidney Transplants. *Curr. Op. in Nephrology & Hypertension*. Nov 2006;**15**(6):637-642.

- Wishart, D. S. Quantitative metabolomics using NMR. *Trends Analyt. Chem.* 2008; **27**:228–237.

- Wittke S, Haubitz M, Walden M, Rohde F, Schwarz A, Mengel M, Mischak H, Haller H, Gwinner W. Detection of acute tubulointerstitial rejection by proteomic analysis of urinary samples in renal transplant recipients. *Am. J. Transplant.* 2005; 5:2479–2488

- Wu G, Morris SM. Arginine metabolism: beyond nitric oxide. *Biochem* J.1998;**33**:1–17.

- Yang J, Xu G, Zheng Y, Kong H, Pang T, Lv S, Yang Q. Diagnosis of liver cancer using HPLC-based metabonomics avoiding false-positive result from hepatitis and hepatocirrhosis diseases. *J. Chromatogr. B. Analy. Technol Biomed Life Sci* 2004; **813**:59–65

Appendices

Study recruitment

Ethics

Letter to patients

Accepted abstracts

Published paper

Patient information leaflet, consent form and letter to General Practionner

A Study to Identify Early Markers for Delayed Graft

Function and Transplant Rejection

You have been invited today to participate in a study looking into ways of detecting why some transplants take longer to start working and ways of detecting transplant rejection earlier than the current methods. The hope being that earlier detection may mean quicker treatment of the problem and therefore improve the outcome for the patient and their transplant.

Thank you for considering participation in this study. You are under absolutely NO obligation to take part and if you chose not to take part your medical management will in no way be effected.

Please read this leaflet carefully and at your own leisure, feel free to discuss it with friends and family, your GP or consultant.

What is the Study About?

You may be aware that by having a liver or kidney transplant one of the risks associated with this is rejection or delayed working. This is where your body fights against the transplant that they have been given. Rejection and delayed function can be difficult to diagnose and other conditions (e.g. infection) can often present like rejection. There is no one test that can diagnose rejection. Our aim is to try and find a substance in blood that might predict transplant rejection.

Why Me?

As you are going ahead to receive a liver or kidney transplant you are an ideal person for this study.

Who is Organising the Study?

The study will be taking place at St James's University Hospital with the help of The University of Leeds under the guidance of the Liver and Kidney Unit. Mr R Prasad and Mr N Ahmad are the two lead consultants with an interest in this topic. The research fellow is Mr Paul Goldsmith who will be spending 2 years on this topic with the aim of achieving a research qualification.

How will it Affect Me?

If you agree to take part you will be required to sign a consent from to say that you are happy to go ahead. The study will NOT require you to visit hospital more regularly and your hospital stay will hardly be any different than if you didn't take part. On the day you arrive to hospital for your transplant a single sample of 4mls of blood will be taken along with your other routine blood tests so you won't need an extra blood test. During your operation again another sample of blood will be taken whilst you are asleep. After the operation a sample of blood will need to be taken at 2, 24 and 48 hours and then on days 3, 4, 5 and 7. All these blood tests will be taken along with other routine blood tests. In some instances the post operative blood sample may require an extra blood test.

What Will Happen to the Blood Samples?

With the blood samples tests will be carried out in the laboratory looking for any signs that might predict transplant rejection.

At the end of the study that will take a total of 2 years all samples will be destroyed.

Are There Any Risks?

There are no risks in taking part. You should not notice anything different in your management as blood tests are often a routine part of treatment, but by being in this study some of you blood once taken will be analysed for any signs that may predict rejection or delayed functioning of your transplant.

Are There any Benefits?

You will not benefit from the study but it is hoped that a few years down the line patients in a similar position to you will.

Is The Study Confidential?

At no time will your name appear on blood samples, just your date of birth and unit number. A letter of confirmation would be sent to your GP and consultant so that they are aware that you wish to participate in the study.

Final Outcome

If it is ok with you, once the study is finished a letter will be sent to your home address telling you of how the study went and if any break through in has been made.

You are under absolutely no obligation to take part in this study and if you have any questions please feel free to email me at goldsmithpaul@hotmail.com or phone in office hours to 07787 113 150 which are the details of the doctor running the study.

Patient Consent Form

A Study to Identify Early Markers for Delayed Graft

Function and Transplant Rejection

Name

Date of Birth

Unit Number

Study Number

Consultant

Please initial the boxes once you are satisfied to take part in the study

□

- I have read the patient information leaflet

□

- I have had the opportunity to discuss the study

  with family, friends, GP or consultant

□

- I understand the purpose of the study and how

I am involved

☐

- I understand by taking part in this study I

  will not gain any direct personal gain

- I understand that all information collected will be

  confidential

☐

- I confirm that I am happy to take part in the is study

  of my own free will and can withdraw at any time I wish.

  It will not have any effect on my medical management

☐

I agree to take part in the study:

Signed…………………………………….   **Date………..**

Print…………………………………..

Person Taking Consent………………………   **Date………**

Dear Dr GP

Your patient is about to undergo a liver/kidney transplant and has kindly agreed to participate in a trial looking at biomarkers for delayed graft function and acute transplant rejection. As you may be aware the diagnosis of delayed graft function and acute rejection is often difficult to make and no-one test can make a definite diagnosis.

The trial involves taking a sample of blood preoperatively, perioperatively and samples at 2, 24 and 48 hours, day 3,4,5, and 7 postoperatively. The samples will be then centrifuged and stored at -80^0C until analysis at The Leeds University Chemistry Laboratories. At this point the plasma will be placed in a spectrometer where an electromagnetic pulse will be fired at the samples. The end product is a spectrum identifying all metabolites within the sample.

These spectra will be analysed and the metabolites compared. We will then look at the progress the patients make on the ward and in the outpatient setting during routine follow up. It is hoped that the spectra may reveal differing metabolic intensities in those patients who go onto graft dysfunction or acute rejection.

The study will not alter patient management.

If you have any questions please don't hesitate to contact me on 07787113150 or email at goldsmithpaul@hotmail.com and I will help as best I can with any further enquiries.

Kind Regards

Paul Goldsmith

Research Fellow

St James's University Hospital

Abstracts accepted to meetings

1. Non-Invasive Biomarkers For Acute Rejection In Renal Transplantation Using 1H-NMR Spectrometry. Goldsmith PJ, Fenton H, Turner E, Fisher J, Ahmad N, Prasad KR Transplant Int. 2009; 22:245

2. 1H-NMR Spectrometric Identification of Acute Rejection in Renal Transplantation. Goldsmith PJ, Fenton H, Turner E, Fisher J, Ahmad N, Prasad KR. Br J Surg. 2009 (96) suppl 4:83

3. Society of Academic & Research Surgery Annual Meeting (SARS). Proton Nuclear Magnetic Resonance Spectroscopy of Plasma in Renal Transplantation. Identification of Biomarkers for Delayed Graft Function and Acute Rejection. Goldsmith PJ

4. The Leeds Regional Surgical Club. 1H NMR Spectroscopy of Plasma in Renal Transplantation. Identification of Biomarkers for Delayed Graft Function and Acute Rejection. Goldsmith PJ

5. XXIII International Congress of the Transplant Society. Metabolic profiling of plasma in the search of a biomarker for graft function in renal transplantation. P.J. Goldsmith, H. Fenton, R.P. Hopton, N. Ahmad, J. Fisher, K.R. Prasad

6. The British Transplant Society. 1H-NMR Spectrometric Identification of Acute Rejection in Renal Transplantation. Goldsmith PJ, Fenton H, Turner E, Fisher J, Ahmad N, Prasad KR