STUDY OF ISCHEMIA-REPERFUSION INDUCED METABOLITE CHANGES IN PORCINE KIDNEY MODEL AND THEIR POTENTIAL ROLE IN ACUTE KIDNEY INJURY

RAVINDRA VARMA POLISETTY

Thesis submitted to the University of Nottingham
for the degree of Master of Research
in
Advanced Genomic & Proteomic Sciences

September 2016

Abstract

Acute kidney injury (AKI) is a common and serious complication in patients undergoing surgery, chronic treatment or kidney transplantation. In AKI patients, the normal functioning of kidneys are suddenly compromised leading to morbidity and mortality. It is reported that annually more than 40,000 inpatient deaths and expenditure of one billion pounds by National Health Service (NHS) in England are associated with AKI. There is a critical need to understand the pathophysiology of ischemia-reperfusion induced acute kidney injury and develop biomarkers useful for clinical management of AKI.

In the present study, mass spectrometry based metabolomics approach was employed to investigate the metabolite changes in the kidney tissues of porcine AKI model. To ischemia or reperfusion induced porcine kidneys, two step solvent extraction followed by chromatography separation of metabolites was done prior to mass spectrometric analysis. The mass spectrometric data was generated and processed using IDEOM or Progenesis CoMet software algorithms. Further multivariate and univariate statistical analysis was done using SIMCA or Metaboanalyst software to identify key metabolites associated with Ischemia or reperfusion. Comparative analysis between control, ischemia and reperfusion kidneys revealed altered levels of several metabolites and majority of them belongs to amino acid, lipid and carbohydrate metabolisms. Degradation of several glucogenic and ketogenic amino acids was observed implicating their potential role in ischemia-reperfusion induced acute kidney injury. The combination of high throughput mass spectrometry, bioinformatics and statistical tools resulted in high confidence identification of metabolites and pathways associated with AKI and provide an opportunity for clinical intervention that may help prevent acute kidney injury.

Publications and Presentations

Participation in research conference:

- Attended "East Midlands Proteomics Workshop (EMPW) 2015" organised by University of Warwick on November 18, 2015.

Poster presentation:

- Polisetty, R.V., Gardner, D.S., (2016). A metabolomics approach to study the protective role of remote Ischaemic preconditioning in acute Kidney injury**.** Link 16 conference, University of Nottingham, UK, June 3, 2016.

Press release submission:

- A press release titled "Remote Ischemic preconditioning - Developing non-invasive, economical and effective 'tools' to fight Acute Kidney Injury" submitted to "Research Showcase-2016", University of Nottingham, UK, June 15, 2016.

Student essay prize competition:

- An essay titled "Hormone Replacement Therapy: In Pursuit Of Eternal Youth" was submitted to "Student essay prize competition-2016" conducted by Society for Endocrinology, UK and awarded with "Highly commended certificate" for the year 2016.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the test.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Signed ……..

Date……

Acknowledgements

As I start penning down my acknowledgements a cool breeze came through the window and reminded me of William Wordsworth poem “I wandered lonely as a cloud and came across hills, valleys and golden daffodils”.

In this one year of stay at University of Nottingham, I came across several golden people, who have given me knowledge, support, encouragement and happiness. As a mentor, Prof. David Gardner gave me a great opportunity to learn Metabolomics - a new cutting-edge technology in science. He encouraged me immensely to play with various data analysis softwares, supported me to collaborate with research team and enhanced my research skills. His cordial nature embraced me with motivation throughout the stay. Prof. Dave Barret at Pharmacy School gave me an opportunity to collaborate and learn Metabolomics. Working with his research team experts Prof. Hyun and Catherine enhanced my passion for science. Sergey and Mohammad are very jovial and nice friends and we had great time in exchanging new methods of data analysis. As success favors the prepared minds, I am confident that our research team continues and accomplish great things in future.

Albert Einstein quoted “It is the supreme art of the teacher to awaken joy in creative expression and knowledge.” At UoN, Prof. Chungui Lu, Charlie Hodgman, Ian Hardy, Dove Stekel and others influenced me with their enormous knowledge and creative teaching methods. They helped me in enhancing my creative and independent thinking. Life on campus is sparkling with friends and several enjoyable events. I am proud to have many friends and every moment with them is memorable. I had great time on campus with my friends Kamesh, Mukesh, Agasti, Umesh, Vandana, Priyanka, Kapila, Sneha, Vandana singh, Sanjeev, Ishan, Faraz, Yaseer, Azad, Gulio, Maria, Miguel and many others. I am lucky that Kamesh turned out to be my best friend and showed me new perspective in life. I realised the importance of extracurricular activities at every phase of life. We became members of Sutton Bonington (SB) cricket club and Tennis clubs and together played lots of Cricket, Badminton and Tennis adding many brighter moments. I got sweet big friends David, Di, John, Val and Ping from SB whom I believe as my well-wishers. I

sincerely acknowledge all these golden people from my heart for giving me more knowledge, happiness and making my stay wonderful.

This journey to explore new frontiers of science was supported by several golden people from my country. My Principal of Sri Venkateswara college, Dr.Hemalatha Reddy is a visionary person and encourages undergraduate research. Her support was enormous and encouraged me to pursue this “Faculty Training Program” organised by University of Delhi, India. My inspiring and passionate colleagues of Biochemistry Department, SVC, morally supported me throughout this journey. I sincerely acknowledge all these golden people who supported me for successful completion of this program.

I sincerely acknowledge University of Delhi, India for sponsoring this program. I am grateful to the Dean of International Relationships, DU for supporting my application to this program.

Every day I start acknowledging my family as they give me strength physically, morally and intellectually. My wife, Sravani supported and appreciated me in my achievements. My adorable son royal Reyansh keeps me ecstatic with his pretty acts. I love him with all my heart. My in-laws supported me immensely. I once again acknowledge these beautiful people.

As “all big things have small beginnings”, I hope that my short time association with UoN will progress into bigger collaboration in near future and help to accomplish great things in science.

Table of Contents

List of Figures

List of Tables

Abbreviations

A	
AKI	Acute kidney injury
ANOVA	Analysis of variance
ANP	Aqueous normal phase
APCI	Atmospheric pressure chemical ionisation
APCI-MS	Atmospheric pressure chemical ionisation- mass spectrometer
ATP	Adenosine triphosphate
C	
CKD	Chronic kidney disease
CoA	Coenzyme A
CRF	Chronic kidney failure
E, F	
EDTA	Ethylenediaminetetraacetic acid
EKHUFT	East Kent Hospitals University NHS foundation Trust
ESI	Electrospray ionisation
FDR	False discovery rate
G, H	
GC-MS	Gas chromatography-mass spectrometry
GFR	Glomerular filtration rate
HCA	Hierarchical clustering analysis
HES	Hospital Episode Statistics
HILIC	Hydrophilic interaction chromatography
HMDB	Human Metabolome Database
HPLC	High pressure liquid chromatography
HS-SPME	Headspace-solid phase micro-extraction

I, K

IR	Ischemia-Reperfusion
ICD	International Classification of Diseases
IL-6	Interleukin-6
IPA	Isopropyl alcohol
KEGG	Kyoto Encyclopedia of Genes and Genomes
KIM-1	Kidney injury molecule -1

L, M

LC-MS	Liquid chromatography-mass spectrometry
LLE	Liquid-liquid extraction
m/z	mass to charge
MALDI	Matrix assisted laser desorption ionisation
mM	milli molar
MS	Mass spectrometry
MS/MS	Tandem massspectrometry

N, O

NGAL	Neutrophil gelatinase associated lipocalin
NHS	National Health Service
NIH	National Institutes of Health
NO	Nitric oxide
OPLS-DA	Orthogonal partial least squares discriminant analysis

P, Q, R

PCA	Principle component analysis
QC	Quality control
RIFLE	Risk, Injury, Failure, Loss and End stage renal disease
RIPC	Remote ischemic preconditioning

ROS	Reactive oxygen species
RP	Reverse phase
S, T	
SIMCA	Soft independent method of class analogy
SPE	Solid phase extraction
TCA	Tricarboxylic acid cycle
TNF	Tumor necrosis factor
TOF	Time of Flight
U, V W	
UPLC	Ultra performance liquid chromatography
VBA	Visual Basic for Applications
VIP	Variable importance in projection
WHO	World Health Organization

1. Introduction

1.1 What is Acute Kidney Injury?

Acute kidney injury (AKI) is a common and serious clinical event that affects the normal functioning of kidneys in critically ill patients. Kidneys perform several physiological functions such as excretion of waste metabolic products, regulation of extracellular fluid volume, concentration of osmotically active substances, regulation of plasma pH, homeostasis of blood pressure and electrolytes (1). During AKI, a mild to complete loss of kidney function occurs due to kidney damage, although sometimes the damage is not clearly manifested at histopathological level (2). To define and classify AKI, RIFLE criteria (Risk, Injury, Failure, Loss and End stage renal disease) was developed which suggests that the increased serum creatinine levels from baseline and decreased urine output could be potential markers of failure of kidney glomerular function (3, 4). Serum creatinine levels are generally monitored to determine the stability of renal function (5). Serum creatinine is specific to renal function and it is easily measured to determine the changes in glomerular filtration rate (GFR). Changes in urine output are less specific to renal function however it is more sensitive and get detected even before the manifestation of biochemical changes relevant to AKI. Therefore the RIFLE system was based on these two markers for classification of AKI patients with adverse clinical outcomes. Further, Acute Kidney Injury Network group has modified the RIFLE criteria to include the patients with early or mild AKI and patients with chronic kidney disease (CKD) in this classification (6; **Figure 1)**. The current RIFLE classification system was validated in several multinational studies and at present used as a standard to classify AKI patients (7, 8, 9, 10). It is also used for predicting the duration of hospital stay, recovery of renal function, need of renal replacement therapy and hospital mortality.

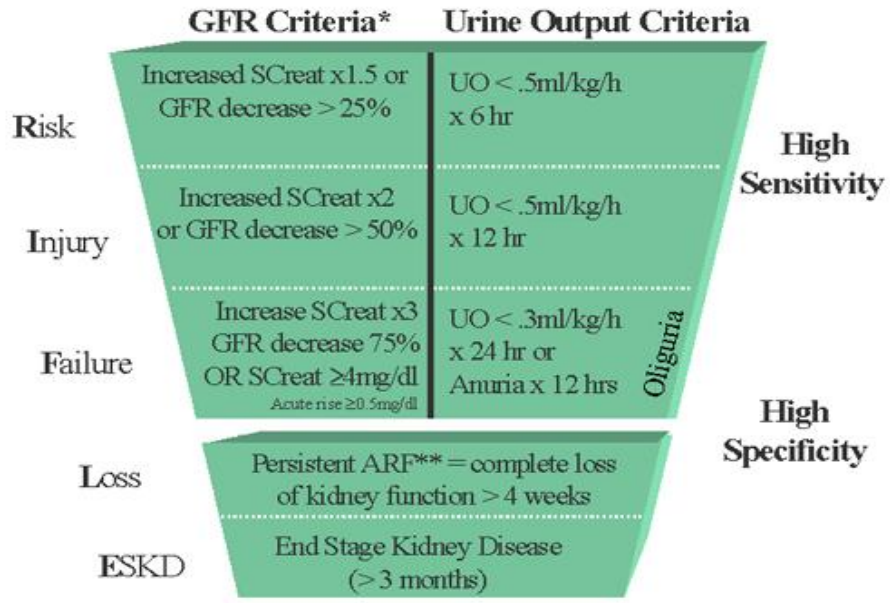

Figure 1. RIFLE criteria for classification of AKI cases. The RIFLE criteria include two clinical outcomes such as loss of kidney function and End stage kidney disease in addition to the three levels of kidney dysfunction based on serum creatinine levels and urine output in the AKI classification system. This classification detects patients with mildly affected kidneys as well as patients with severely affected kidneys.

1.2 Epidemiology of AKI and economic burden

The incidence of AKI is common in critically ill patients and is associated with morbidity and mortality (11, 12, 13). A population based study reported that the AKI incidence rate in hospitalised patients in china is between 3.2% and 20% and in intensive care units is reported to be between 22% and 67% (14, 15). The increase in serum creatinine levels correlated with increased length of stay in hospitals and significantly increased mortality. Using the International Classification of Diseases (ICD)-9 codes, the US centers for Disease Control and Prevention reported greater than 20-fold increase in AKI incidence based on analysis of hospital discharge sheets available from the year 1980 to 2005 (16). One of the major difficulties in assessing the AKI incidence is that it is not clear about the occurrence of AKI before or after hospitalisation. The US Renal Data system reported interesting findings on the AKI

incidence based on three different datasets available from 1995 to 2007 (12). The AKI incidence was observed to be higher in older individuals mainly at the age group of 85. Male sex, black race and patients with Chronic Kidney Disease (CKD) were identified as high risk groups of AKI. It is also observed that AKI accounts to nearly 23% of hospitalisations and twice the post-discharge mortality rate compared to those hospitalised without AKI. Over all, AKI is a serious disease and nearly 2 million people worldwide are expected to die every year.

In England, AKI prevalence in hospital inpatients is high and associated with high National Health Service (NHS) expenditure. A recent study analysed the Hospital Episode Statistics (HES) data obtained from patient records at each hospital and laboratory data from East Kent Hospitals University NHS foundation Trust (EKHUFT) to study the prevalence, mortality and economic burden of AKI for the year 2010-11 (17). HES data recorded 142705 (2.43%) patients with AKI out of 5881635 inpatient admissions. The occurrence of AKI is 0.32% in patients aged between 18-39 and 5.74% in patients aged 80 and above. Out of 142705 patients with AKI, 40109 (28.11%) patients died in the hospital and the mortality rates increased with age. EKHUFT data collected for a period of six months recorded 5521 (15.33%) patients with AKI out of 36015 admissions. The study reported that 38.10% of AKI patients had pre-existing CKD stage 3-5 and in 73.37% of AKI admissions, the patient already had AKI when admitted to hospital. Out of 5521 patients with AKI, 769 (13.93%) patients died in the hospital. Overall it is estimated that every year more than 40,000 inpatient deaths are associated with AKI in England and NHS expenditure is nearly one billion pounds on AKI inpatients which is a huge burden to economy.

1.3 Etiology of AKI

Several risk factors most often collectively or either independently contributes to occurrence of AKI. Acute kidney failures are generally categorised into pre renal, intrinsic and postrenal (18; **Figure 2**). Factors responsible for prerenal kidney failures are intrarenal vasoconstriction, a condition caused by drugs such as nonsteroidal anti-inflammatory drugs, angiotensin-converting enzyme inhibitors, and angiotensin receptor blockers. Syndromes such as abdominal compartment syndrome, hepatorenal syndrome, cardiorenal syndrome and hypercalcemia also results in vasoconstriction. Other factors include systemic vasodilation due to sepsis, volume depletion due to diuretic overuse and extrarenal losses from vomiting, diarrhoea, burns and blood loss. Prerenal failure can be reversed by treating the associated syndromes and diseases with appropriate therapies. In case of intrinsic renal failure, kidney gets injured at varying levels. Acute tubular necrosis is a common type of intrinsic acute kidney injury in hospital inpatients that occurs due to ischemic or nephrotoxic agents leading to tubular epithelial cell death. Acute inflammation of blood vessels and kidney glomeruli in case of glomerulonephritis also leads to intrinsic AKI. Acute interstitial nephritis leads to intrinsic renal failure and it occurs due to medications such as penicillin analogues, cephalosporins, and direct infection of renal parenchyma - due to viruses such as Epstein-Barr virus, cytomegalovirus, and human immunodeficiency virus; due to bacteria such as Streptococcus species, Legionella species; due to fungi such as candidiasis, histoplasmosis; and other systemic diseases such as sarcoidosis and systemic lupus erythematosus. Kidney vascular injury due to renal vein thrombosis, malignant hypertension, scleroderma renal crisis, renal atheroembolic disease and renal infarction leads to intrinsic AKI. Post renal failure occurs due to obstruction of the

urinary tract intrinsic by blood clots, stones or tumors and extrinsic by prostate hypertrophy, retroperitoneal fibrosis or by tumors in bladder, prostate, or cervix. All the above discussed factors generate ischemic condition directly or indirectly in the kidney leading to AKI. Oxidative stress or inflammation is also recently identified as additional factors that may lead to AKI (19).

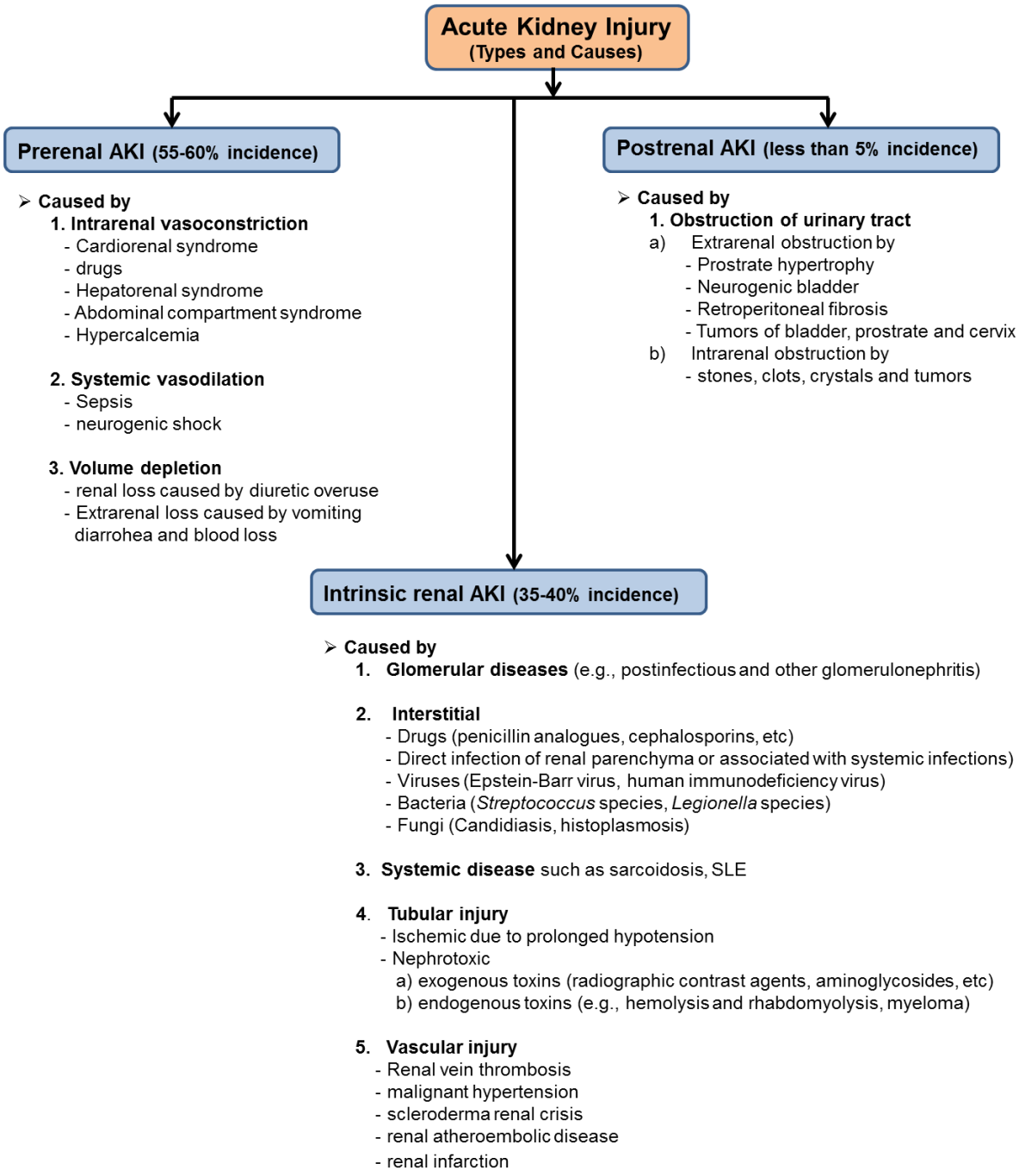

Figure 2. Types of AKI, incidence and causes. Adopted from Holley JL.Clinical approach to the diagnosis of acute renal failure. In: Greenberg A, Cheung AK, eds. Primer on Kidney Diseases. 5th ed. Philadelphia, Pa.: National Kidney Foundation; 2009:278. (Ref. 18)

1.4 Ischemic AKI

Ischemic AKI occurs due to several causes such as sepsis, major surgery, cardiogenic shock, vasoconstrictive drugs, hepatorenal syndrome and obstructive uropathy (20). Ischemia causes reduced delivery of oxygen and metabolic substrates to kidney cells leading to cellular injury. The pathophysiological changes associated with AKI include cellular, morphological and structural changes that have cumulative effect on kidney function. A brief description of the renal anatomy and physiology is done here to understand the pathophysiology of ischemic AKI.

1.4.1 Renal anatomy and Physiology

Kidneys are important organs of the body and play vital role in eliminating the waste products of metabolism and in maintaining body homeostasis. They filter the blood plasma and remove the metabolic waste products such as urea, creatinine, bilirubin etc. They also remove foreign chemicals and drugs by excreting them in the urine. Further, they regulate water and electrolyte balances for maintaining arterial pressure, acid-base balance etc. required by the body.

Anatomically, two kidneys are present at the posterior side of the abdomen (**Figure 3**). The kidney is surrounded by a tough, fibrous capsule. The outer region of the kidney is called cortex and inner region is called medulla. The medulla is divided into several cone shaped structures called renal pyramids. Each pyramid starts at the border of cortex and medulla and terminates in the papilla. Papilla enters the funnel shaped continuation of the ureter called renal pelvis. The outer border of the renal pelvis is divided into major calyces and further divided into minor calyces. Calyces are open ended pouch like structures that collect urine from tubules of each papilla.

The concave side of the kidney is called hilum and renal artery, renal vein, lymphatics, nerve supply and ureter passes through it. The renal artery enters the kidney and branches to form the interlobar arteries, arcuate arteries, interlobular arteries and afferent arterioles and then lead to the glomerular capillaries. In glomerular capillaries urine is formed by glomerular filtration. The ureter carries the urine from the kidney to the urinary bladder. The distal ends of the capillaries of each glomerulus merge to form the efferent arteriole, which leads to the peritubular capillaries, a second capillary network. Both the glomerular and peritubular capillaries are separated by the efferent arterioles, which help to regulate the hydrostatic pressure in both sets of capillaries. High hydrostatic pressure in the glomerular capillaries causes rapid fluid filtration, whereas a lower hydrostatic pressure in the peritubular capillaries causes rapid fluid reabsorption. The peritubular capillaries empty into the vessels of the venous system, that run parallel to the arteriolar vessels and form the interlobular vein, arcuate vein, interlobar vein, and renal vein that carries the blood away from the kidney.

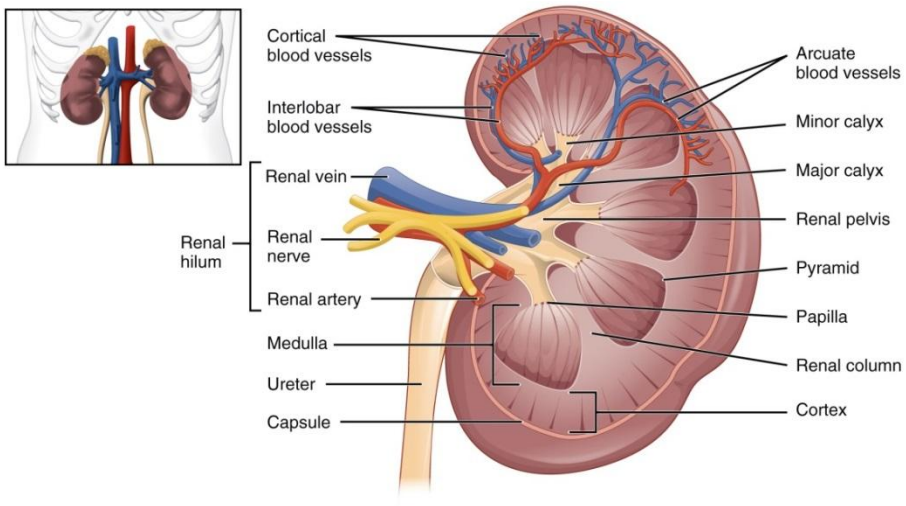

Figure 3. The anatomical structure of the human kidney. Adopted from "https://openstax.org/details/anatomy-and-physiology".

The functional unit of the kidney is called nephron. Each human kidney contains nearly one million nephrons. Each nephron contains a bunch of glomerular capillaries called the glomerulus. The glomerular capillaries are covered by epithelial cells, and the glomerulus is encased in Bowman's capsule. Fluid filtered from the blood in glomerular capillaries flows into Bowman's capsule and then into the proximal tubule, which lies in the cortex of the kidney (**Figure 4**). From the proximal tubule, fluid flows into the loop of Henle, which enters into the renal medulla. Each loop consists of a descending and an ascending limb. The walls of the descending limb and the lower end of the ascending limb are very thin and therefore are called the thin segment of the loop of Henle. The wall of ascending limb of the loop that enters the cortex is much thicker, and it is referred to as the thick segment of the ascending limb. At the end of the thick ascending limb is a short segment, is known as the macula densa which plays an important role in controlling nephron function. Beyond the macula densa, fluid enters the distal tubule that lies in the renal cortex. This is followed by the connecting tubule and the cortical collecting tubule, which lead to the cortical collecting duct. The cortical collecting ducts join to form medullary collecting duct. The collecting ducts merge to form progressively larger ducts and empty into the renal pelvis through the tips of the renal papillae.

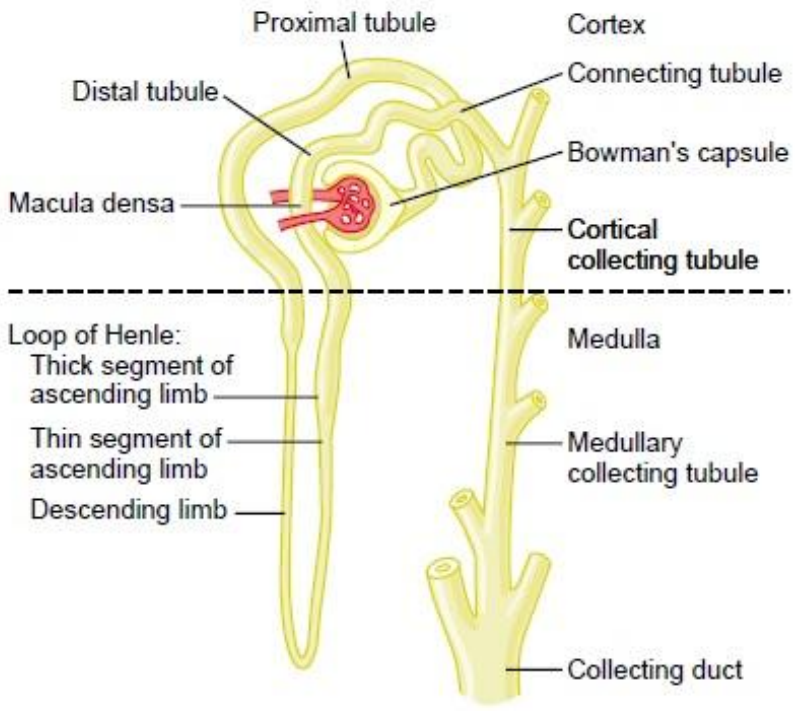

Figure 4. Basic tubular segments of the nephron. Adopted from Textbook of medical physiology, Guyton AC, Hall JE, 11th edition, Elsevier Saunders, Philadelphia, 2006.

1.4.2 Pathophysiology of ischemic AKI

Various types of renal cells undergo injury or cell death under ischemic conditions. Among epithelial cells, the proximal tubular cells are highly susceptible to ischemic injury as they need more oxygen to exhibit high metabolic rate required for ion transport. Reduced oxygen and metabolite substrates cause inadequate intracellular ATP levels leading to cell injury. Injury to proximal tubular epithelial cells causes afferent arteriolar vasoconstriction which occurs due to tubuloglomerular feedback, luminal obstruction, back leak of filtrate across injured proximal tubular cells leading to defective glomerular filtration and reduced glomerular filtration rate (GFR) (21, 22). The endothelial cells located in the S3 segment of the nephron also undergo injury due to microvascular hypoperfusion which persists even after recovery of

cortical blood flow in this region (23). The epithelial cells present in the medullary thick ascending limb of nephron also undergo injury during ischemic AKI.

Morphologically, injured glomerular epithelial cells disrupt microvilli that get detached from the apical cell surface. Apoptosis is commonly observed in both proximal and distal tubular cells (24). It leads to formation of membrane bound blebs that are released into the tubular lumen. Loss of tubular cells results in formation of proximal tubular dilation and distal tubular casts (25). Loss of apical brush border of proximal tubular cells is considered to be the hallmark of ischemic AKI. These detached tubular cells, brush border vesicle remnants and cellular debris form granular casts with other compounds and block the tubular lumen leading to ineffective GFR in these regions. Histological analysis revealed the loss of glomerular epithelial cells after ischemic injury.

Several cytoskeletal and structural changes have been observed in proximal tubular cells that affect the glomerular filtration rate. Inadequate cellular ATP levels lead to depolymerisation of F-actin resulting in rapid disruption and redistribution of cytoskeletal F-actin core. Proteins such as cofilin, tropomyosin and ezrin mediate depolymerisation of F-actin core. Further, depolymerisation of F-actin causes structural instability of cell membranes and formation of membrane blebs that get detached and released into tubular lumen (26, 27). Changes in actin cytoskeleton also results in changes in epithelial cell polarity and function. Cytoskeletal changes causes redistribution of Na^+/K^+-ATPase pumps and affects the bidirectional transport of sodium and water across the apical, basolateral and epithelial cell membrane. High fractional excretion of sodium in AKI patients is due to abnormal localisation of

Na^+/K^+-ATPase pumps caused by ischemic condition (28). Ischemia also causes loss of tight junctions and adherens junctions leading to back leak of glomerular filtrate into the interstitium (29). Ischemia in epithelial cells also causes relocalisation of β-integrins from the basal membrane to apical membrane resulting in detachment of epithelial cells from basement membrane and release into tubular lumen (30). Overall the less injured epithelial cells tend to recover if alleviated from acute ischemic condition while the highly injured cells undergo cell death by apoptosis or necrosis. In AKI, both the intrinsic and extrinsic apoptotic pathways are known to be activated and inhibition of caspase activity, *in vivo*, showed protection against injury in AKI (31, 32, 33). Ischemic injury also causes increased production of reactive oxygen species in epithelial cells leading to cell death by necrosis. These reactive oxygen species have been reported to have vasoconstrictive effects by scavenging nitric oxide (34).

Ischemia also alters various endothelial cell processes in kidney such as vascular tone, permeability and modulation of coagulation and inflammation. Histopathological studies revealed vascular congestion, edema, decreased microvascular blood flow to local tissue beds and adhesion of inflammatory cells to the endothelium upon ischemia. It leads to extended ischemic injury in kidneys due to decreased perfusion. It was observed that the post ischemic kidneys in rats could not autoregulate blood flow immediately and requires longer time for recovery (35). Microvascular permeability was also observed to be increased in AKI due to breakdown of the barrier function of renal endothelium by various factors such as disruption of actin cytoskeleton, alterations in contacts between endothelial cells, loss of integrity of tight junctions and binding of leukocytes to endothelial cells.

Damaged endothelial cells undergo apoptosis and stimulate inflammatory response which induces microvascular coagulation (36). Inflammation and activation of leukocytes have been identified as major mediators of endothelial and tubular cell injury. During early inflammation, leukocytes bind to vascular endothelium and migrate into the interstitium. Several leukocytes get activated and accumulate at the site of ischemic injury. They include neutrophils, macrophages, B-cells and T-cells and mediate injury and inflammation. These cells act synergistically and mediate tubular injury at various phases. Several cytokines secreted by injured endothelial or epithelial cells enhance the inflammatory response during ischemia (37). Macrophages and dendritic cells also secrete various proinflammatory cytokines such as TNF, IL-6 that activate other leukocytes and promote ischemic injury (38). Postischemic kidneys have increased number of infiltrating regulatory T-cells and their depletion increased tubular damage suggesting their protective role against Ischemic injury by modulating the proinflammatory cytokine production of other T-cells. Overall, the interactions between endothelial and leukocytes due to inflammatory response extends hypoxia and further enhances the injury and dysfunction of epithelial and endothelial cells.

1.5 Biomarker of AKI

Biomarkers provide the disease status enabling intervention with the appropriate therapeutic agent and early decisions for disease treatment. Thus, use of biomarkers to study human diseases has gained significance. Specifically, molecular biomarkers have been found useful in early detection and in the prediction of treatment efficacy and clinical outcomes. Till date, biomarkers have been defined in several ways. According to the US National Institutes of Health's (NIH) Working Group and the

Biomarkers Consortium (http://www.biomarkersconsortium.org), a biomarker is defined as "a characteristic that is objectively measured as an indicator of normal biological processes, pathogenic processes, or a pharmacological response to a therapeutic intervention". The United Nations' World Health Organization (WHO) defines biomarker as "any substance, structure or process that can be measured in the body or its products and influences or predicts the incidence of outcome or disease" (Biomarkers in Risk Assessment: Validity and Validation, Environmental Health Criteria Series, No222, WHO).

Biomarkers are mainly classified into four major categories.

- **Diagnostic** markers assess the presence or absence of disease.
- **Prognostic** biomarkers allow the natural course of a disease to be predicted, distinguishing 'good outcome' from 'poor outcome', and they guide in disease treatment.
- **Predictive/response** biomarkers are used to assess the probability that a patient will benefit from a particular treatment.
- **Pharmacodynamic** biomarkers measure the near-term treatment effects of a drug on the host and can be used to guide dose selection in the early stages of clinical development of a new drug.

AKI occurs in a variety of clinical settings and is mostly asymptomatic. The inhospital deaths associated with AKI is increasing every year inspite of significant advances in clinical medicine. The poor outcome of patients with AKI is mainly due to delay in the diagnosis of AKI that subsequently affect the AKI disease management. The commonly used diagnostic markers include measuring the rise in serum creatinine and reduced urine output as indicators of decreased of kidney function. However, the use of serum creatinine and urine output as AKI diagnostic

marker has known limitations. Serum creatinine is a suboptimal AKI marker as serum creatinine levels are influenced by several systemic factors and do not clearly represent accurate glomerular filtration rate. The increased rate of serum creatinine during AKI is influenced by factors such as rate of creatinine generation, rate of tubular secretion, and volume of distribution (39, 40). The detectable increase in serum creatinine levels occurs after several hours and when 50% of the kidney is injured due to ischemia. Therefore it fails to estimate the timing of injury and degree of kidney dysfunction during AKI. The sensitivity of serum creatinine levels is low in some patients with liver disease or older patients with low muscle mass. Also, the increased serum creatinine levels fail to differentiate between prerenal, intrinsic and post-renal AKI. Moreover, the serum creatinine is a marker of steady state kidney function while AKI is an unstable pathological condition. Therefore, rise in serum creatinine levels is currently understood as retrospective, insensitive and inaccurate diagnostic marker of AKI (41).

The need for effective AKI disease management has lead to discovery of new biomolecules with biomarker properties. These include neutrophil gelatinase associated lipocalin (NGAL), kidney injury molecule -1 (KIM-1), cystatin C and several other molecules (42). Discussion on some of these promising biomarkers of AKI is merited here.

1.5.1 Neutrophil Gelatinase Associated lipocalin:

Neutrophil gelatinase-associated lipocalin (NGAL) is a 25-kDa protein, synthesized during granulocyte maturation in the bone marrow (43). It is also expressed in epithelial cells during inflammation (44). In AKI mouse model study, NGAL levels

were found to be highly increased within few hours of renal injury (45). Further, several experimental and clinical studies in AKI revealed the increased levels of NGAL in urine and suggested to be potential early diagnostic marker of AKI (46, 47, 48). Experimental studies in mice also revealed the protective effect of NGAL against AKI (49). Together, urinary NGAL is currently identified as a novel early diagnostic marker for AKI. Large multicenter studies in validating the clinical applications of urinary NGAL is in progress.

1.5.2 Kidney injury molecule-1

KIM-1 is a membrane glycoprotein with receptor activity. It is found to be expressed on renal epithelial cells in postischemic kidney and play important role in phagocytosis of apoptotic cells (50). A genomic study revealed the significant upregulation of KIM-1 gene in post ischemic kidney injury in rat treated with cisplatin (51). Increased expression of KIM-1 protein is specifically observed on apical membrane of proximal tubules which are highly susceptible to ischemic injury. Further, the KIM-1 protein is released into urine and suggested to be potential diagnostic marker for AKI (52). Several clinical studies showed that the urinary KIM-1 to be potential early diagnostic marker of kidney injury compared with other conventional markers (52, 53). In several AKI studies, significant upregulation of KIM-1 in kidney biopsy specimens, especially in S3 segment of proximal tubule, and elevated urinary levels of KIM-1 was observed in early periods of kidney injury (52, 54). Together, the expression of KIM-1 at early stages of kidney injury due to ischaemia and its detectability in urine makes it a potential diagnostic marker for AKI. Further, a direct correlation of urinary KIM-1 concentration with severity of

kidney injury was observed suggesting its biomarker potential for use in disease diagnosis and treatment monitoring (55).

1.5.3 Cystatin C

It is a 13-kDa protein with protease inhibitor activity. Experimental and clinical studies suggest that the serum levels of cystatin C increase with renal injury and could be potential marker of AKI (56). Thus cystatin C is a potential specific marker of AKI than serum creatinine. Further, the elevated levels of cystatin C in urine were also observed in patients with tubular dysfunction indicating its potential as specific marker renal injury (57, 58). Higher urinary cystatin C levels also reported to predict the poor outcome in patients with nonoliguric AKI (59).

Further characterisation of these molecules as diagnostic, prognostic and treatment monitoring markers of AKI is in progress. Urinary biomarkers of AKI have certain limitations. The urinary biomarker levels in AKI are influenced by hydration status, diuretic therapy or primary polyuric AKI. Till date, there is no accurate method for estimating the urinary markers of AKI. Further, the markers like NGAL is not yet ideal for using as diagnostic marker for sepsis patients without AKI. Therefore further large scale studies need to be designed to validate the potential use of these serum or urinary markers for differential diagnosis of AKI. As single biomarker may fail to diagnose various types of AKI, the discovery of multiple or panel of markers is recommended.

1.6 Metabolomics

Metabolomics is one of the high throughput 'omics' approaches to study the metabolites in a given biological system. The metabolome includes an array of metabolites such as aminoacids, nucloetides, lipids, organic acids, etc. that play diverse biological roles in the organism. Two approaches that are widely used in metabolomics analysis are metabolic fingerprinting and metabolic profiling (60). Metabolic fingerprinting is used to identify all the metabolites in the given biological sample. Further, the metabolite patterns or fingerprints are identified that is specific to the group or condition. Metabolic fingerprinting is used to identify panel of markers useful for classification of different groups, disease diagnosis or treatment monitoring or in determining efficacy of treatment. Metabolic profiling is an alternative method in which a group or class of metabolites are identified and quantitated based on comparative analysis between groups. It is a hypothesis driven approach and could be used for discovery of novel disease biomarkers.

Several analytical platforms have been developed for metabolomics studies. Of them, liquid chromatography-mass spectrometry (LC-MS) is generally employed for metabolic profiling or metabolic fingerprinting. It is highly sensitive technique and has the potential to identify metabolites. A typical mass spectrometry based metabolomics study includes sampling, sample preparation, sample separation and detection, data extraction and data analysis.

1.6.1 Sampling and sample preparation methods

Sampling is an important step in which sample number and method of sample collection is chosen to meet the requirements of the experimental design. Sufficient

number of samples is required to overcome biological variability, a common issue observed in metabolic fingerprinting analysis. Large number of samples helps to provide statistically significant data useful for classification of groups in metabolic fingerprinting studies.

Sample preparation methods involves extraction of various metabolites from diverse samples such as serum, plasma, tissue or cell lysates, saliva and urine using various chemical methods. For qualitative and quantitative analysis, the metabolites/compounds will be isolated from samples, fractionated or directly subjected to mass spectrometer. Depending on nature of metabolites and study objectives, various sampling methods were developed. Head space sampling was used for isolation of highly volatile metabolites from urine samples (61). In this method, sampling is done above the samples in the headspace compartment. The volatile compounds partitioned between sample and headspace are removed from the headspace by passing inert gases or captured using various techniques such as solvent traps or adsorbent matrix and then analysed directly using mass spectrometry. Another method used for extraction of volatile and semi-volatile compounds is headspace-solid phase micro-extraction (HS-SPME). In HS-SPME method, a fiber coated with extracting phase (liquid or solid) extracts different kinds of analytes (volatile or semi-volatile) from various samples (liquid or gas media) (62). The extraction is based on partition coefficient of analytes from samples between extracting phase and headspace and then subjected to gas chromatography. Solid phase extraction (SPE) method was widely used for extraction of metabolites from biological fluids based on their distribution between liquid sample and solid sorbent (63). It is commonly used for metabolic target or profiling studies. For

metabolite extraction from tissue samples, liquid-liquid extraction (LLE) method is preferred in which homogenised tissues are vortexed with mixture of organic solvents to separate metabolites based on their degree of polarity (64). After extraction, metabolites are either injected directly or prefractionated and then detected by mass spectrometer.

1.6.2 Sample preparation and mass spectrometric methods

Direct injection is a quick method as all the compounds (mixture) get ionised and enters the mass spectrometer together. However, it is less sensitive and identifies only abundant species in case of complex mixtures in a given time period. Therefore for complex mixtures, pre-fractionation is preferred for better qualitative and quantitative analysis of compounds.

For metabolite separation by prefractionation, chromatography technique is commonly employed in metabolomics. Some of the commonly used chromatography methods in metabolomics include reverse phase chromatography, hydrophilic interaction chromatography, and gas chromatography. Reverse phase liquid chromatography is used for separation of non-polar and medium polar compounds. In this technique, C-18 micro columns are generally used for metabolomic analysis. Hydrophilic interaction chromatography (HILIC) is used for separation of highly polar compounds. In this technique, columns are made of silica or derivatized silica columns. Both the reverse phase and hydrophilic interaction chromatography techniques coupled with electrospray ionisation (ESI) and different mass analysers such as ion trap, triple quadrupole or quadrupole time of flight is used routinely in various metabolomics studies. Gas chromatography is used for separation of

compounds based on their specific boiling points (65). In this technique, samples are derivatized to increase the volatility and thermal stability. The active hydrogens in –COOH, -OH, -NH and –SH functional groups are alkylated, acylated or silylated with various derivatization agents. Gas chromatography coupled with electron impact ionisation and Time of Flight (TOF) mass analysers is commonly used for metabolomics investigations. Capillary electrophoresis, although less reported so far, is another method used for separation of polar and charged metabolites (66).

In mass spectrometry based metabolomics, mass spectrometer plays vital role for identification of metabolites based on their mass to charge ratio. The basic components of mass spectrometer are sample source, ionisation source, mass analyser and detector as shown in **Figure 5**. The sample source inject sample to mass spectrometer. The commonly used sample sources include MALDI-plate, direct injection by syringe or chromatography. From the sample source, the samples enter ionisation source at which sample is ionised into gaseous ions. After ionisation, the gaseous ions enter the mass analyser at which they are separated according to their mass-to-charge ratio. The resolved ions then get detected and recorded in the detector system.

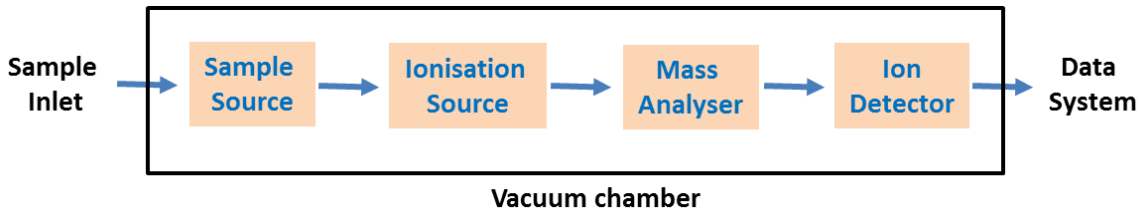

Figure 5. Basic components of Mass spectrometer.

Different ionisation methods and mass analysers are used depending on their sensitivity and accuracy for detecting metabolites. Atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) are the most commonly used ionisation methods. In APCI ionisation method solvent and analyte molecules get vaporized when passed through corona discharge electrode (67; **Figure 6**). Solvent ions then react with analyte molecules by transferring a proton or removing proton from it. It is very efficient method for ionisation at atmospheric pressure as it reduces the thermal decomposition of compound resulting in soft ionisation. These ions directly enter the mass spectrometer and detected based on their mass to charge ratio. This ionisation method provides mass spectra without fragmentation patterns and reveals molecular weight information of the parent ions.

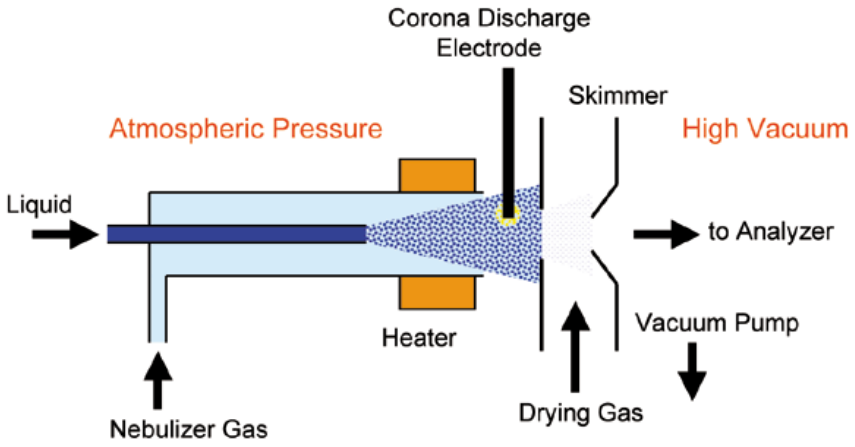

Figure 6. Atmospheric pressure chemical ionisation method.

For volatile compounds extracted by headspace sampling method, atmospheric pressure chemical ionisation- mass spectrometer (APCI-MS) is commonly used for identification of metabolites. Electron impact ionisation is another method for ionisation of volatile compounds. After pre-fractionation with gas chromatography,

the sample is bombarded with high energetic electrons resulting in ionisation and fragmentation of molecules (68; **Figure 7**).

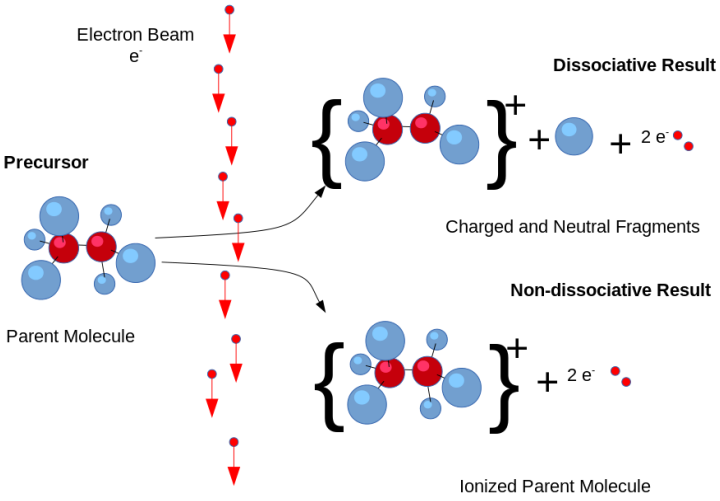

Figure 7. Electron impact ionisation method.

After ionisation, the ions are guided to the quadrupole mass analyser and get separated in the electric field on the basis of their masses. The mass spectra generated were then searched against the spectral libraries for accurate identification of compounds. This method is applicable to all volatile compounds, provides reproducible mass spectra, and fragmented ions provides structural information which is more reliable than chemical ionisation method. It is used in identification of metabolites by GC-MS. These are some of the commonly used methods for ionisation of volatile compounds.

Electrospray ionisation (ESI) is another method widely used for ionisation of non-volatile compounds (69, **Figure 8**). In this method, ionic species in solution is transferred to gaseous phase using high electric energy. The sample is sprayed

through the capillary tube (electron spray tip) which is maintained at a high voltage generating highly charged droplets. These charged droplets get evaporated at high temperatures and release ions into gaseous phase and move towards the mass analyser with the help of nebulising gas. ESI is commonly used in LC-MS based metabolomics investigations.

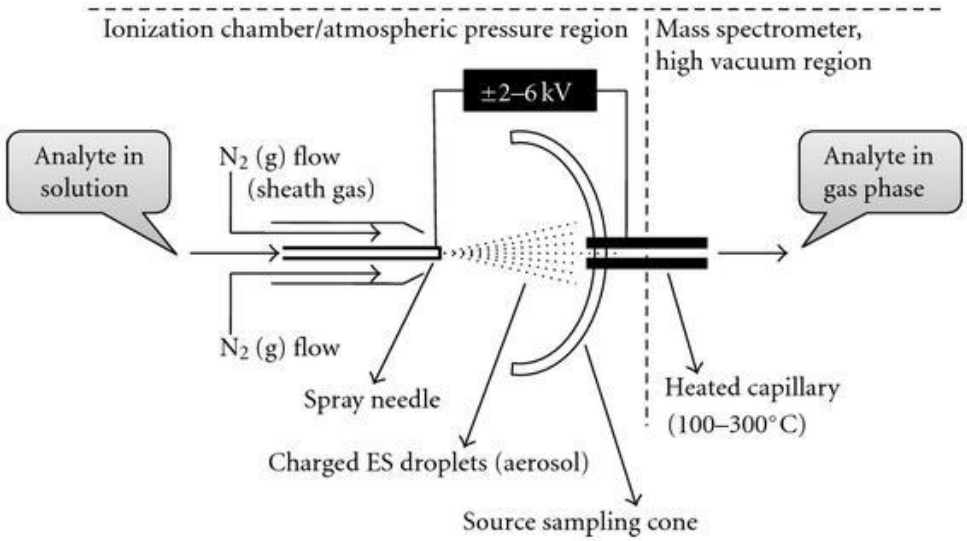

Figure 8. Electrospray ionisation method

Various mass analysers are used in metabolomics investigations. In mass analysers, the ions with different mass-to-charge ratio move differently and get resolved in the given electric field. Triple quadrupole or Time of Flight mass analysers are highly accurate and commonly used for structural analysis of biomolecules at the level of precursor ion and product ions (70). Ion trap mass analysers are highly sensitive and commonly used for tandem mass analysis of metabolites.

1.6.3 Data Processing and data analysis methods

In metabolomics, the mass spectrometric data is processed and then analysed in numerous ways to extract the biological information with high confidence. The molecular information of the metabolites is available in file formats. There are different raw data file formats available that are specific to each analytical platform. Of them, mzXML and netCDF are widely used file formats (71). The basic steps involved in data processing are filtering, feature detection, alignment and normalisation. For efficient processing, centroiding and binning of data is done as it allows better mass assignment and improves data quality. Mass spectrometric data contains either chemical noise or baseline drift in the data and random analytical noise. Data filtering reduces the random analytical noise by employing signal processing techniques such as moving-window filtering or wavelet transforms. Baseline drift is corrected by subtracting the shape of baseline from the data. Peak or feature detection from false signals or noise is done several ways. Signal with intensity above a defined noise level is considered as true ion peak. The peak shape or intensity is then associated with the corresponding mass-to-charge and retention time data, independently (72, 73). Alternatively, the single ion chromatograms can be processed independently either with Gaussian-filter to detect the apex and inflection points for integration or searching for the areas in chromatogram above the defined threshold levels (74, 75). Another approach is application of model fitting against raw signal which includes isotope detection (76, 77). In mass spec data, deconvolution algorithms are employed to group fragment ions from same compound. Alignment is done at mass-to-charge and retention time levels to overcome the variation that occurs during sample runs. The alignment algorithm depends on reference chromatogram to correct the retention time differences caused

by factors such as changes or fluctuations in temperature, pH, pump pressure etc. Alignment helps to combine the results from multiple sample runs. Alignment is done either by binning data along the chromatographic dimension or by compressing or stretching the retention time axis of samples to a common reference (78, 79). Mapping of total ion chromatogram and warping are commonly used alignment methods in metabolomics. Normalisation is also done to remove the systematic bias in peak intensities between samples without affecting the actual biological variation. Normalisation is done using statistical models or by inclusion of internal standards that cover the retention time range in the experiment (72, 80). For high throughput analysis, metabolomics softwares are used for data processing/extraction. These softwares perform noise filtering, peak detection, alignment, normalisation and visualisation. Some of the softwares used for processing of mass spectrometric metabolomics data are MetAlign, XCMS, and MZmine which are freely available (81, 82, 83). Metabolomics data analysis involves the use of statistics to extract relevant biological information from large datasets. Several statistical tools are available for metabolomics data analysis. The selection of these tools depends on the goal of the study. Supervised and unsupervised methods are employed for classification of samples. For samples with unknown identity, unsupervised methods such as principle component analysis (PCA) and hierarchical clustering analysis (HCA) are used for classification of samples. For samples with known identity, supervised methods such as principal least square (PLS) or soft independent method of class analogy (SIMCA) is used to identify biomarkers. Univariate significance tests such as Student's t-test, ANOVA are used to identify variables with significant p-value which distinguishes different known groups.

For identification and information about the metabolites such as structure, function, biochemical pathways and spectral information, various databases are available. Some of the widely used databases (http://metabolomicssociety.org) are KEGG and BioCyc for metabolites and their biochemical pathways; PubChem and CAS for information about physiochemical properties of metabolites; METLIN, HMDB and Golm for metabolite mass spectral database. The metabolomics mass spectral data at MS level is generally searched against these databases and known metabolites are identified from samples based on their mass-to-charge ratio and retention time. However, in some cases, two or more metabolites get detected with same mass-to-charge ratio. To overcome this MS/MS is performed and database search is done to identify the actual metabolite based on its structural information. The combinatorial use of high throughput mass spectrometric and bioinformatics tools in metabolomics may help to elucidate altered metabolic pathways associated with acute kidney injury and for development of biomarkers with various clinical applications.

The metabolomics study includes experimental design, data collection, data storage, data pre-processing, data analysis and interpretation. There are several sampling and sample preparation methods, chromatography and mass spectrometric tools, data processing algorithms, data analysis softwares and databases used by the scientific community at different levels of pipeline in a metabolomics experiment. However, the information about their usage is not properly reported or reported in different ways causing concern regarding the reliability of the experimental data. To overcome such situation, the information about the biological samples and data analysis performed in metabolomics experiments should be clearly explained or reported in the publications. Recently, metabolomics Society - metabolomics standards initiative

proposed a standard reporting requirements in metabolomics experiments (84). For pre-clinical studies which include animal experiments, the following requirements need to be reported for biological samples. For the experimental subjects the information such as species or strain name, age range and weight range; housing information such as group/individual, cage type, cleaning frequency, environmental conditions, light cycle; water and feed information such as feed type, supplements, suppliers name, and water source; veterinary treatment information such as use of anaesthesia type and dosage, acclimation duration. Information about experimental design such as group size, gender, physical examination details, treatment information such as chemicals, dose, route of administration, fasting conditions, euthanasia method, tissue collection list, tissue processing method, clinical observations, blood and urine analysis details such as volume, location, frequency and time of blood collection, tissue amount, processing methods, storage conditions and other assays used for the study should be reported. Similarly, standard reporting requirements have been proposed for data analysis methods in metabolomics investigations (85). These include information about number of replicates (experimental or technical), protocols for loading samples into instruments, instrument parameters, duration of experiment, data collection methods and formats, deconvolution methods, pre-processing workflows, softwares or algorithms used for preprocessing, pre-treatment, software version number, details about datasets used for training, validating and testing softwares, statistical models, threshold values or prediction scores, outliers, details about data analysis criteria, interpretation and visualisation. Detailed information about various software tools, algorithms and parameters applied for data analysis is recommended to report in research

publications for the benefit of scientific community. It helps to reproduce the results convincingly and enhance the confidence of the metabolomics data.

1.7 Animal models of ischemic AKI

Experimental models have been known to be essential for understanding the pathophysiology of several complex diseases, including AKI. In vitro models such as renal tubular cell cultures, isolated renal tubules, and isolated perfused kidneys have been used in research for studying the disease mechanisms of ischemic AKI. Renal tubular cell cultures have been employed to study the cellular mechanisms associated with acute tubular necrosis (86). However, it is known that cultured cells undergo phenotypic changes such as resistance to hypoxic injury to adapt to in vitro culture conditions, which is problematic to study ischemic AKI. To overcome this, freshly isolated proximal tubules have been widely used for studying tubular cell injury (87, 88). Several studies employed proximal tubules for the metabolic and structural investigations as they maintain the biochemical properties, structural integrity and fully differentiated state resembling in vivo conditions. However, in vitro models could not mimic the complexity of human body and therefore limited their use to attain cellular and mechanistic insights of ischemic AKI. To reveal the complex interactions of hemodynamic and immunological factors involved in AKI pathogenesis, various whole animal models have been developed. Two commonly used AKI models are bilateral renal ischemic reperfusion and unilateral renal ischemic reperfusion (89). In bilateral ischemic AKI model, the blood supply is blocked to both the kidneys and is considered more relevant model representing human pathological condition. Both the small and large animal ischemic AKI models have been developed to study kidney pathophysiology. Ischemic AKI was induced in

dogs by clamping the left renal artery for one hour (90). In rabbits, Ischemic AKI was induced by occlusion of the bilateral renal arteries for one hour (91). Ischemic AKI was induced in pigs using standard angled arterial cross-clamp for 150 minutes (92). For small animal models, rats and mice were used for studying ischemic AKI. Ischemic AKI rat models have been extensively used since 1960 and several hundreds of research articles have been published. Ischemic AKI was induced in rats by clamping both the kidneys for 60 min (93). In another study, ischemic AKI was induced in rat by clamping unilateral left renal artery for 40 minutes (94). Ischemic AKI was also induced in rats by clamping both the right renal artery and vein for 60 minutes (95). With the advent of transgenic mice, mice models have been widely used to study ischemic AKI. Several recent studies employed ischemic AKI mice models and research articles have been published reporting novel molecular insights of AKI. Ischemic Aki was induced in mice models by blocking left renal artery by using an atraumatic microvascular clamp for 37 minutes (96). In general ischemia was induced in mice models by clamping the kidneys for 40 minutes. Mice models have been accepted in biomedical research for studying several complex diseases including AKI due to their small size, fast reproductive cycles, short life span, easy to breed in lab and availability of transgenic mice. However, it is known mice models have bigger variations and they do not represent features of human disorders in several cases (97). For studying AKI, porcine models are effective as they share many similarities with humans such as size, physiology, anatomy, metabolic profile and longer life span (98). Especially, the renal physiology and anatomy of pig is highly similar to humans and therefore valuable models for pharmacological studies (99). Further, the progress in assisted reproduction, somatic cell cloning, stem cell culture and transgenesis has resulted in development of new porcine models useful

for biomedical research. Recently, new ischemic AKI porcine models have been developed to understand the biochemical mechanisms of kidney pathophysiology and several studies are in progress.

1.8 Aims and Hypotheses

1.8.1 Aim of the study

To identify the early metabolite changes associated with ischemia and reperfusion induced AKI using an untargeted metabolomics approach.

1.8.2 Objectives of the study

1. To identify significantly altered metabolites and metabolite pathways in ischemic and reperfused porcine kidneys using mass spectrometry.
2. To use a bioinformatics approach to reveal key metabolic pathways associated with acute kidney injury.

1.8.3 Hypotheses of the study

Ischemia or reperfusion induced AKI is characterised by several biochemical and structural changes in proximal tubular epithelial cells leading to loss of kidney function. It is also reported that AKI induces a wide range of metabolic changes in the kidney tissues implicating their association with AKI. Identifying these AKI induced early metabolic changes in kidney tissues may help to better understand their possible role in kidney pathophysiology. Further, these early altered metabolites could be investigated as potential biomarkers for various clinical applications.

A metabolomics approach is ideal for studying the metabolite changes associated with AKI. In various kidney diseases, metabolomics approaches have been used already to study drug induced nephrotoxicity, renal cell carcinoma and diabetes mellitus nephropathy. Few metabolomics studies have been reported in ischemic AKI. For example, Liu et al. reported metabolomic changes in a rat AKI model by

employing an LC-MS approach (100). They reported that changes in lipid metabolism leading to oxidative stress could be the possible cause of ischemia or reperfusion induced AKI. Wei et al. reported global metabolic changes in kidney tissues and plasma of a murine AKI model. They suggested that altered metabolites such as citrulline, tryptophan and pathways such as TCA cycle, lipid metabolism are associated with early injury, inflammation and late phase kidney recovery (101). In another metabolomics study on mice AKI model by Chouchani et al. reported that accumulation of succinate in ischemic kidney could be the major contributor of reperfusion injury through mitochondrial ROS production (102). Interestingly, these metabolomics studies were carried out on rat or mice AKI models whose basal metabolic rate is reported to be several times higher than humans. As an alternative to the mouse model, pig is believed to be a better model whose kidneys are histologically and functionally close to human kidneys. Few studies already reported the use of porcine kidney models in understanding sepsis and catheter induced AKI. Recently, Gardner et al developed porcine model of ischemic AKI and investigated the renoprotective mechanisms induced by remote ischemic preconditioning (RIPC) to reduce AKI during surgery (103). Till date, metabolomics studies on ischemia/reperfusion induced AKI were not reported in porcine models. Therefore, in the present study, a mass spectrometry based metabolomics approach will be employed to study ischemia /reperfusion induced early metabolite changes in porcine AKI model. This study would provide a resource of altered metabolites associated with early stages of AKI pathogenesis and subsequently reveal the early molecular events of AKI.

2. Methods and Materials

2.1 Study design

The workflow of the study is shown below in **Figure 9**.

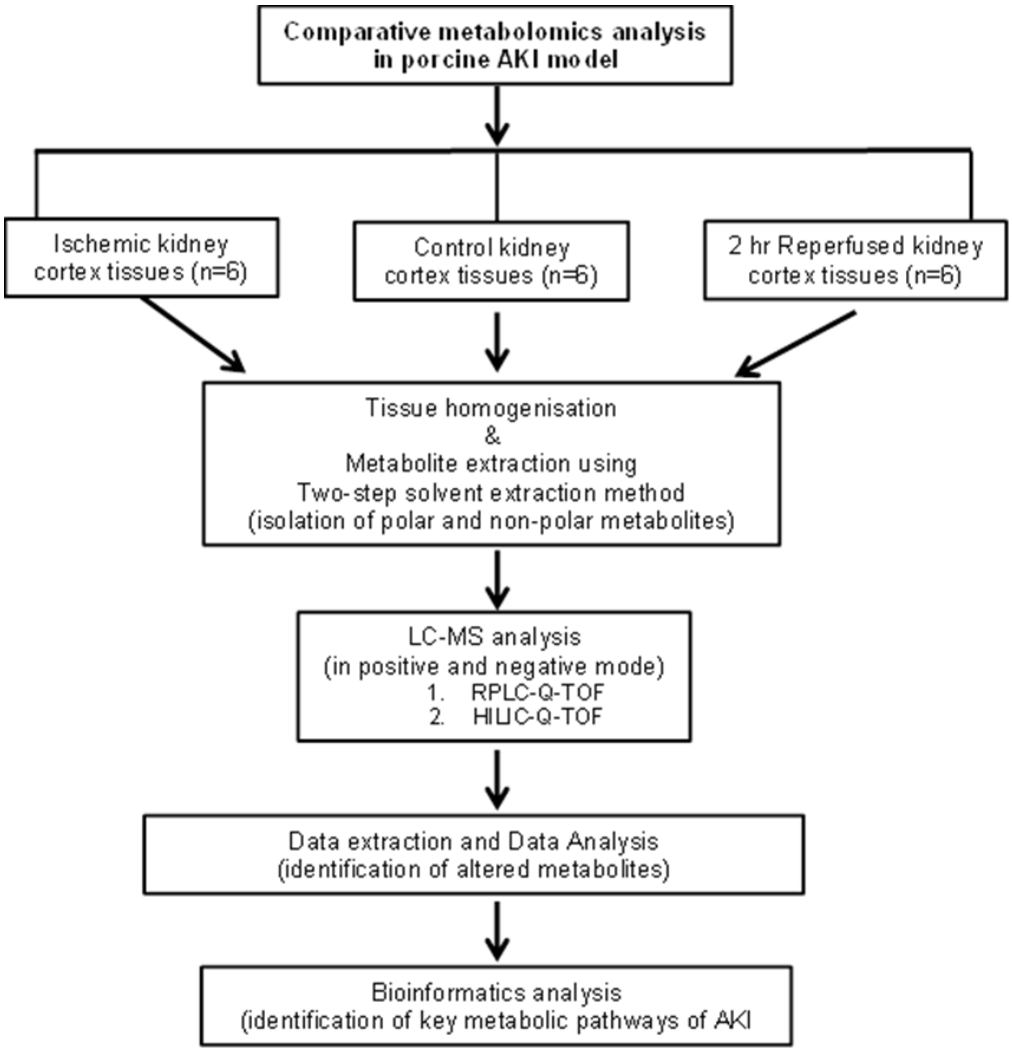

Figure 9. The workflow of the metabolomics study

2.2 Development of porcine AKI model

Female Canberra-12 pigs (supplied by Manor Farm feeds, Melton Mowbray) of 10-12 weeks age range and weighing approximately 55-60 kgs were selected for the study. Pigs were randomly grouped into sham control (n=6) or ischemia/reperfusion group (n=6). They are housed individually in floor pen type cages ($3m^2$) and fed with standard pig diet and tap water. The light cycle was 12 hours light and 12 hours dark. They are acclimatised for 7 days and found to be in good health. Animals are kept for 18-24 hours fasting before surgery. Pigs were anaesthetised for general surgery by giving buprenorphine (0.05 mg/kg), ketamine (5mg/kg), and detomidine (0.1 mg/kg)] with alfaxalone (0.7–2.4 mg/kg), dosed to effect for intubation. Isoflurane (1–2% in O_2) was also administered through ventilator using a tidal volume of 10–12ml/kg. A venous catheter was inserted for supportive fluid delivery and vascular access throughout the surgery. A bladder catheter (12 Fr) was inserted for urine collection. The experimental animal details such as strain type, housing, diet, treatment and experimental design was provided **in Table 1**. All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act, (1986). The experiments were approved by the local ethical review committee of the University of Nottingham, UK.

Table 1. Details of experimental animal such as strain type, housing, diet, treatment and experimental design employed in the present study**.**

Animal information		
Species or strain name	:	Canberra 12 (pig)
Age range	:	10-12 weeks
Weight range	:	55-60 Kgs
Housing informationsuch as		
Group/individual	:	Individual
Cage type	:	Home Office approved floor pen ($3m^2$)
Cleaning frequency	:	Daily
Environmental conditions		
Light cycle	:	12h 6am to 6pm light: dark
Water and feed information such as		
Feed type	:	Standard pig diet
Supplements	:	None
Suppliers name	:	Manor Farm feeds, Melton Mowbray
Water source	:	Tap
Acclimation duration	:	7 days
Veterinary treatment information such as		
Use of anaesthesia type and dosage:		buprenorphine (0.05 mg/kg), ketamine (5mg/kg), and detomidine (0.1 mg/kg)] with alfaxalone (0.7–2.4 mg/kg), dosed to effect for intubation. Isoflurane (1–2% in O_2) was also administered through ventilator using a tidal volume of 10–12ml/kg.
Information about experimental design such as		
Group size	:	12
Gender	:	Female
Physical examination details	:	all in good health
Treatment information such as		
Chemicals/dose/ administration route :		N/A
Fasting conditions	:	18-24h prior to surgery
Euthanasia method	:	overdose of euthatal (200mg/kg)
Clinical observations	:	N/A
Blood and urine analysis details such as		
Blood volume	:	10 ml
Blood collection	:	Venous
Frequency/time of blood collection:		0 hr, 0.5 hr, 1.5 hr, 2.5 hr
Tissue amount	:	Cortex region of kidney, 200mg
Processing methods	:	Solvent extraction methods suitable for metabolomics study
Storage conditions	:	-80^0C storage - tissue, blood and urine
Other assays used for the study	:	Serum creatinine assay

2.3 Experimental design

Porcine AKI model was developed in David Gardner's lab (103). Briefly, for ischemia/reperfusion group, a midline laparotomy was performed and renal artery cross-clamping was done for both the right and left kidneys. Blood samples were collected before and after ischemia, and after reperfusion. Blood sample of 10ml volume was collected in EDTA coated tubes at 0 hr, 0.5 hr, 1.5 hr and 2.5 hr time points. Plasma was isolated by centrifuging at 1500g for 10 minutes, aliquoted in 1ml cryovials, snap frozen in liquid nitrogen and then stored at -80^{0}C refrigerator, until use. After cross clamping of renal artery for 40 minutes, the left kidney was immediately removed surgically and considered as ischemic kidney. For the right kidney, the cross clamping of renal artery was removed after 40 minutes and allowed to reperfuse for 2 hours. After 2 hours of reperfusion, the right kidney was removed surgically and considered as reperfused kidney. For sham control group, both the left and right kidneys were removed without cross clamping of renal arteries. Kidney cortex tissues were collected from control and treated kidneys, immediately snap frozen in liquid nitrogen and stored at -80^{0}C refrigerator, for metabolomics studies. All the pigs were humanely euthanized by administering lethal dose of euthatal (200mg/Kg).

2.4 Sample preparation

Sample preparation was done appropriate for metabolomics study. For metabolomics studies, kidney cortex tissues stored at -80^{0}C freezer were taken out and thawed on ice. Equal amounts (200mg) of wet weight kidney cortex tissues have been taken and homogenised using Pistil and mortar. Two-step solvent extraction method published by Want el al. in Nature Protocols, had been adapted and modified for optimal

isolation of both polar and non-polar compounds (104, **Figure 10**). Briefly, the first step involves extraction of aqueous or polar metabolites by homogenisation of tissues in 1.5 ml methanol/water (1:1) solvent. The tissue homogenates were centrifuged at 16,000g for 10 minutes at 4^0C, and collected the supernatants. The second subsequent step involves extraction of non-polar metabolites from the pellet (collected during the first step), by manually resuspending in 1.5ml of chloroform/methanol (2:1) solvent. The supernatant was collected by centrifugation at 16,000g for 10 minutes at 4^0C, vacuum dried, resuspended in 120µl of isopropyl alcohol (IPA) to create the lipid fraction for analysis. Both the polar and non-polar fractions were centrifuged to remove any particulates and then subjected to LC-MS analysis. This two-step solvent extraction method allows prefractionation of sample based on polarity, thus reducing the sample complexity.

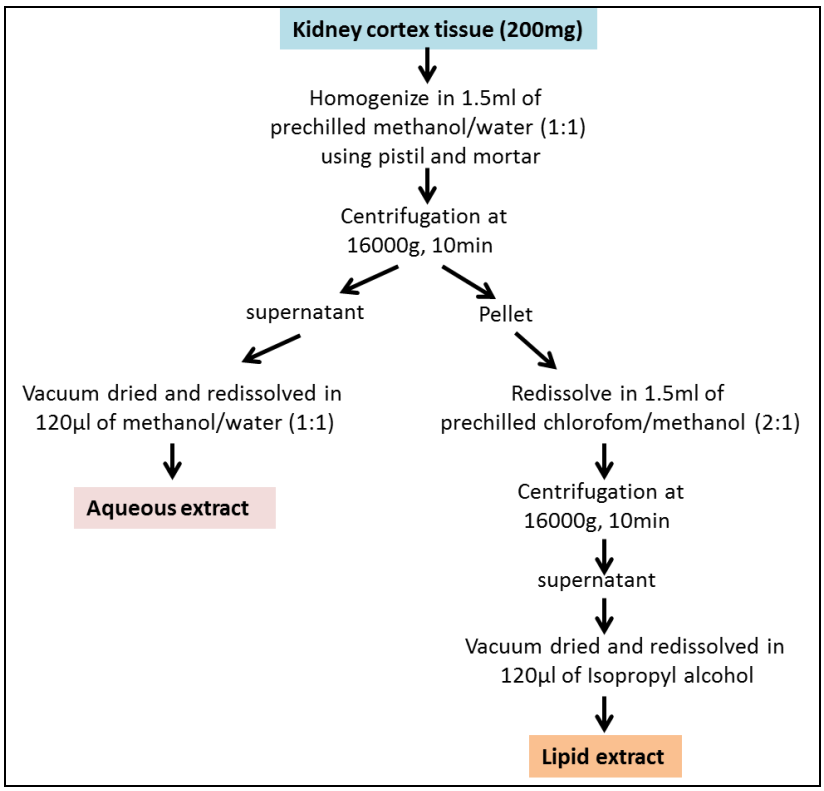

Figure 10. Two-step solvent extraction method. This method was employed in the present metabolomics study to isolate aqueous and lipid fractions and then subjected to LC-MS analysis. Aqueous and lipid fraction was isolated from porcine kidney cortex tissues.

2.5 Quality control

Two series of quality control (QC) types were created. Pre-extraction pooling of the crude suspensions was done for serving the purpose of extraction QC. Similarly, post extraction pooling of the samples was done for checking of the instrument performance respectively. The pooled QCs were injected as a series of six at the beginning and end of the sample sets and between every 6 sample injections. Further, both the samples extraction batches and analysis sequence were randomised to overcome experimental bias.

2.6 Liquid chromatography coupled mass spectrometry analysis (LC-MS)

Metabolomics profiling was performed for both the lipid and aqueous fractions independently using aqueous normal phase (ANP) and reverse phase (RP) chromatographic separations methods followed by Exactive series high resolution accurate mass spectrometer from ThermoScientific, Hemel Hempstead, UK. A modular Accela HPLC containing cooled autosampler, column oven, vacuum degasser and quaternary pump was used for the study. The lipid samples were analysed using an Ace Excel 2µm Super C18 HPLC column (50mm x 2.1mm) held at 45^{0}C. The mobile phase 'A' composition was 60% water and 40% acetonitrile and mobile phase 'B' composition was 10% water, 10% acetonitrile and 80% isopropanol. In each case the modifier was 0.1% ammonium acetate. The gradient flow profile was shown in **Table 2.**

Table 2. HPLC gradient method used for lipid fraction separation

Time (min)	B%	Flow µl/min
0	30	300
2	35	300
8	100	300
13	100	300
17	100	500
18	20	300
20	20	300

The LC flow was coupled to mass spectrometer directly. The mass spectrometer (MS) parameters was optimised for intact lipids of the mass range 600-1200 daltons by infusing a mix of the QC pooled sample and mobile phase while running an automated macro for total ion count. The instrument was then calibrated and sample analysis was done for a 150 to 1500 daltons m/z range in both positive and negative modes at a resolution setting of 25,000. The desolvation temperature was set at 300 ^{0}C. Supplementary lipid analysis was performed on a newly installed Q-Exactive using identical LC and matching MS source parameters.

For aqueous samples, Waters Acquity UPLC BEH HILIC 1.7 µm column (100mm x 2.1mm) held at 40^{0}C was used for the analysis. The mobile phase 'A' composition was 90% acetonitrile and 10% water while mobile phase 'B' composition was 100% water. Both mobile phases used 10mM ammonium acetate as a modifier. The gradient flow profile was shown in **Table 3**.

Table 3. HPLC gradient method used for aqueous fraction separation

Time (min)	B%	Flow µl/min
0	0	250
1.3	0	250
5	5	250
10	15	250
12.5	30	250
16	80	500
17	80	500
17.5	0	500
20	0	500

The LC flow was coupled to mass spectrometer directly. The mass spectrometer was calibrated and sample analysis was done for a 60 to 1000 m/z range in both positive and negative modes at a resolution setting of 25,000. The desolvation temperature was set at 150^0C.

2.7 Data processing

Mass spectrometric raw data was generated and stored for each sample in .raw file format. Data processing was performed using a combination of IDEOM, Progenesis CoMet (NonLinear Dynamics) and LipidSearch softwares. Mass spectrometric raw data generated from analysis of aqueous fraction was processed using IDEOM software. IDEOM is a Microsoft Excel template having VBA macros enabled for automatic processing of LC-MS data (105). It allows removal of noise from mass spectrometric data, metabolite identification and data visualisation. IDEOM software is associated with data processing methods such as mzMatch (106) which is used for conversion of raw LC-MS data and XCMS (107) for filtering the data and then annotated into list of putative metabolites having information about their confidence levels and relative concentrations in samples. The raw data processing using IDEOM

is briefly explained here. The six biological replicate files (raw LC-MS data files) from each group - control, ischemia and reperfusion were pooled into their respective folders and then imported into the online available IDEOM software (version 19). Using msconvert, a proteowizard tool, all the '.raw' files were converted into an open-source file format '.mzXML'. The data was then centroided and split into positive and negative polarities. Chromatographic peak detection was done using the centWave algorithm of XCMS and saved in the peakML format. Noise was removed using the default parameters. Using XCMS, peak picking was done by applying the following parameters: 3 ppm mass deviation, peak width range of 10-100S, signal to noise ratio of 3, and minimum 3 scans (prefilter) with peak intensity threshold of 1000. Peak matching and annotation of related peaks was done using mzMatch.R based on retention time, peak shape and peak intensities across sample groups. Default parameters include 5ppm mass deviation and 30 seconds retention time (RT) window for grouping peaks from replicate samples, relative standard deviation (RSD) of 0.5, noise filter of 0.8 based on peak shape, peak intensity filter of 5000 was applied. In IDEOM, metabolite identification was based on matching retention times and exact masses of detected peaks with the authentic standards (MSI confidence level 1). Other metabolites used predicted retention times and masses based on KEGG, metaCyc and LIPIDMAPS database (MSI confidence level 2). For reliable annotation of metabolites, the databases were updated with retention times of known standards generated by running a mixture of 148 authentic standards in the LC-MS system and externally using ToxID software. Since the data was generated on Q-Exactive mass spectrometer in both positive and negative ion mode, the pre-processed positive and negative datasets were combined and comparison list was generated for all the groups. The confidence of metabolite identification was verified

by looking into confidence score and presence of associated metabolites (KEGG pathways). Lack of such information for a hit will be considered as putatively-annotated hit and MS/MS fragmentation was performed for metabolite identification. Multivariate analysis OPLS-DA was done for all the three groups using SIMCA 14.1 (Umetrics, Umea, Sweden) to identify the biological variations between the groups. Further, univariate statistical analysis ANOVA was done on the putatively identified metabolites data using Metaboanalyst software (108). In addition, group wise comparison analysis was also done by applying T-test (0.05) to identify metabolites that distinguish between groups. Volcano plot analysis was done to identify significantly altered metabolites. Pathway analysis was done using Pathos software (109) by exporting metabolites identified from IDEOM.

Mass spectrometric raw data generated from analysis of lipid fraction was processed using Progenesis CoMet (NonLinear Dynamics) by applying default parameters. Progenesis CoMet is a new generation of bioinformatics tool used for metabolomics investigations (110). It allows processing of different types of file formats of MS data with highly visualized and guided workflows. It is used for both identification and quantitation of potential biomarkers. Lipid profile differences between experimental classes were highlighted using Simca 14.1. Multivariate analysis OPLS-DA was done for all the three groups using SIMCA 14.1 to identify the biological variations between the groups.

3. Results

3.1 Induction of ischemia-reperfusion causes AKI in porcine model

Each pig was subjected to renal ischemia by cross clamping the renal artery of both the kidneys, for 40 minutes. At this time, the left kidney was immediately excised surgically, with the right kidney reperfused, after removal of the clamp, for a further 2h. At this time, the right kidney was also excised surgically and the animal euthanized under anaesthesia. Cross clamping of kidneys for 40 minutes resulted in mild to moderate renal ischemia. Both the kidneys showed change of colour from red to dark purple, indicating effective renal ischemia (**Figure 11A**). The right kidney after reperfusion for 2 hours changed back to normal red colour (**Figure 11B**). All six animals showed similar renal phenotypic change.

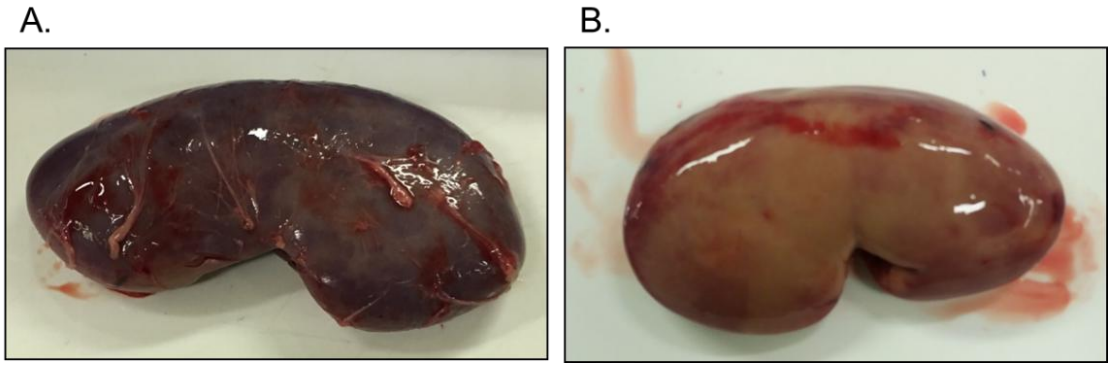

Figure 11. Ischemia or reperfused porcine kidneys. A) The left kidney showing dark purple colour after 40 minutes of cross clamping of renal artery. **B)** The right kidney showing normal red colour after reperfusion for 2 hours.

3.2 Metabolic profiles for aqueous fraction

To study the global metabolic changes as a result of ischemia and subsequent reperfusion (IR), kidneys from animals not subject to IR (control) and to IR (left kidney) with reperfusion (right kidney) were processed accordingly for analysis by LC-MS. Polar fractions were isolated as described in methods and untargeted mass spectrometric analysis was performed, independently.

3.2.1 Multivariate analysis

LC-MS data generated from aqueous fractions was preprocessed using IDEOM software as described in methods. The pMZmatch and nMZmatch ions were combined and OPLS-DA was done using SIMCA software version 14.1 to determine the biological variations between control, ischemia and reperfused groups.

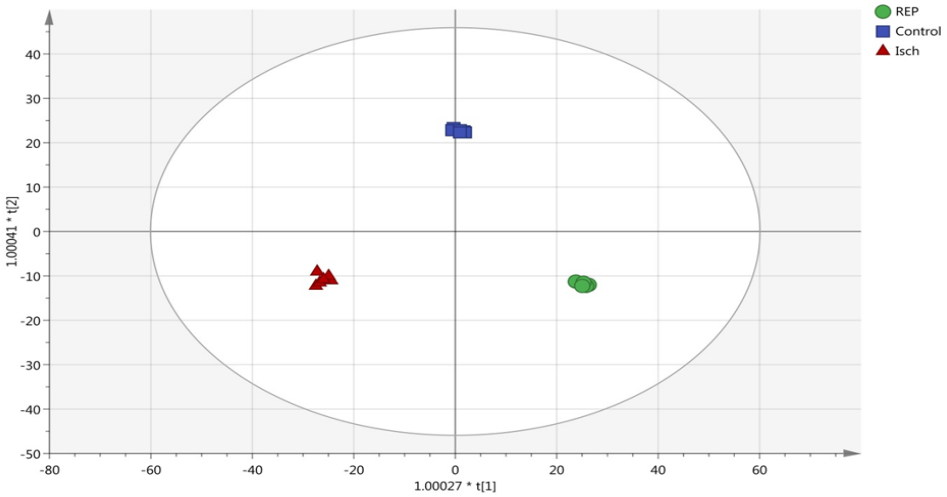

Figure 12. OPLS-DA score scatter plot for control, ischemic and reperfused sample groups. Control group (n=6) is shown in blue square boxes; ischemia group (n=6) is shown in red triangles; and reperfusion group (n=6) is shown in green circles.

The score plot showed clustering of biological replicates for each sample group (n=6 per group) indicating good separation between groups (**Figure 12**). The R2 and Q2 values of each sample group were 0.998 and 0.566 for control, 0.998 and 0.547 for Ischemia, and 0.997 and 0.527 for reperfusion, respectively. These values are indicative of OPLS-DA as an acceptable model. The clear separation of these sample groups suggest that biological variation between them is clearly evident and this metabolomics data could be further investigated for identification of key metabolites and pathways associated with AKI.

3.2.2 IDEOM analysis

For metabolite identification, the processed peak list from IDEOM was searched against authentic standards as well as against KEGG, metaCyc and LIPIDMAPS databases. A total of 374 ions were matched to databases and resulted in identification of 374 significantly altered metabolites with high confidence (score 5 and above). Sixteen metabolites matched to known standards suggesting the confidence of metabolomics data. They include biologically important metabolites such as Xanthosine, Oxalate, Hippurate, Adenine and Mannitol. Metabolite classification shows that 82 metabolites belong to pathways associated with amino acid metabolism, 65 belong to various lipid pathways, 26 belong to pathways of carbohydrate metabolism, 15 belong to pathways of nucleotide metabolism and 25 belong to metabolism of oligopeptides. 130 metabolites were unclassified (**Figure 13**).

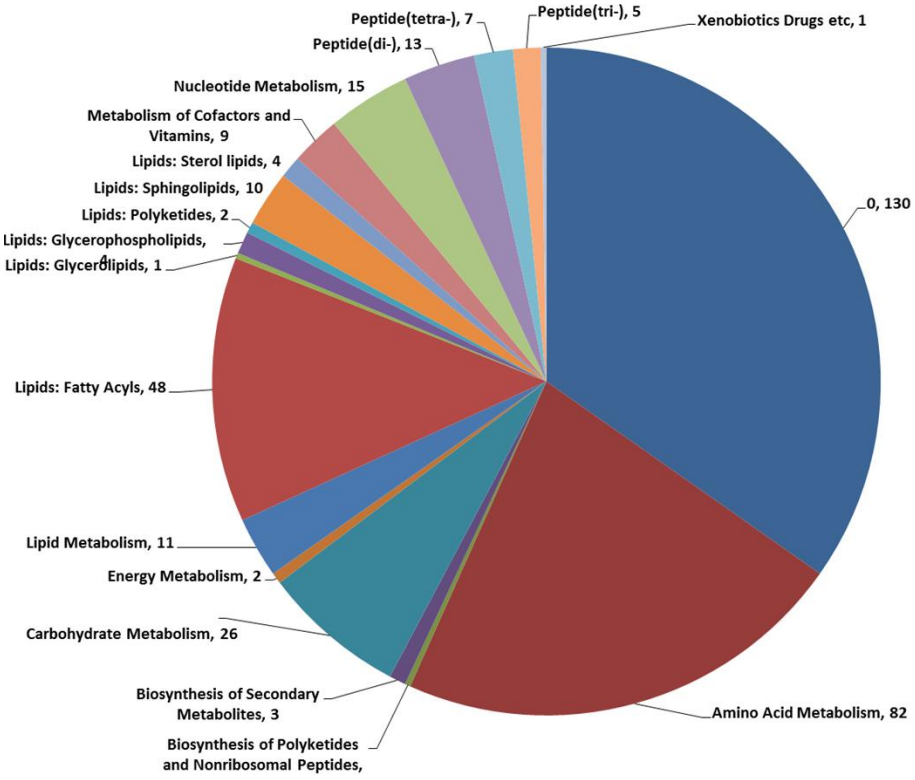

Figure 13. Functional classification of metabolites identified from aqueous fraction using IDEOM software. Metabolites belonging to various pathways were identified in aqueous fraction.

Further, to identify the key altered metabolomic pathways, the peaklist from IDEOM was exported to Pathos software for mapping the pathways. A total of 30 pathways were found to be identified and each pathway was mapped with a minimum of three metabolites. Some of the important pathways include pathways related to amino acid metabolism such as tryptophan metabolism, arginine and proline metabolism, glycine serine and threonine metabolism, purine and pyrimidine metabolism (**Table 4**).

Table 4. List of top metabolic pathways altered in ischemic and reperfused kidneys compared to control kidneys. Number of metabolites identified in the present study out of total metabolites reported in the literature.

Metabolic Pathways	**Number of metabolites mapped to corresponding pathways**
Tryptophan metabolism	5 metabolites out of 80
Pantothenate and CoA biosynthesis	3 metabolites out of 25
Beta-Alanine metabolism	5 metabolites out of 32
Arginine and proline metabolism	14 metabolites out of 67
Lysine degradation	11 metabolites out of 42
Biosynthesis of unsaturated fatty acids	10 metabolites out of 49
Pyrimidine metabolism	8 metabolites out of 55
Glycine, serine and threonine metabolism	10 metabolites out of 45

3.2.3 Univariate Student's t-test analysis

To identify the mass ions that majorly contributed to distinguish between control, ischemia and reperfusion, group wise comparisons were performed between control and ischemia, control and reperfusion, as well as between ischemia and reperfusion within the OPLS-DA models (**Figure 14**). Separation was clearly observed between the sample groups suggesting possible biological variation.

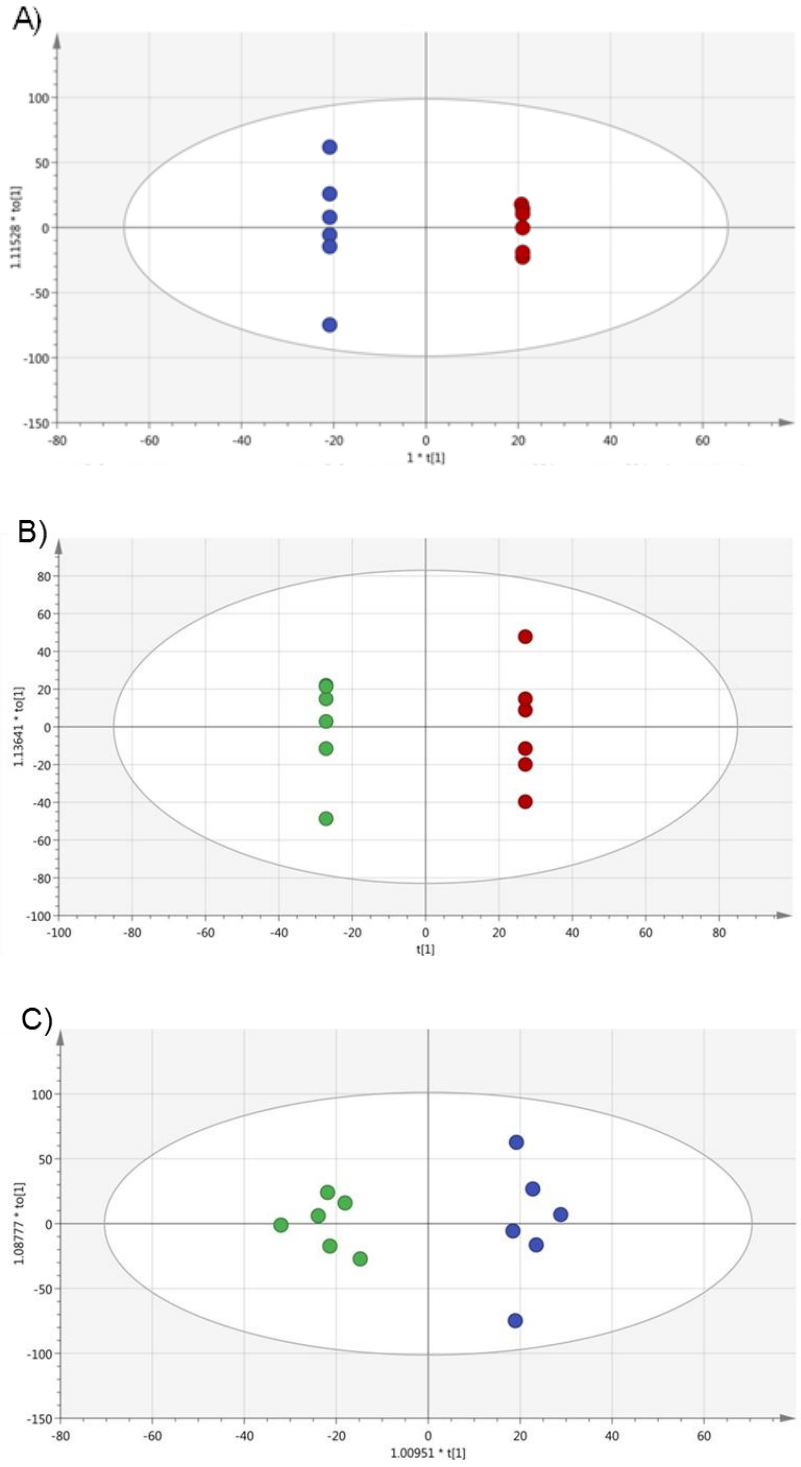

Figure 14. OPLS-DA score scatter plots for groupwise comparision. A) control and ischemia sample groups, B) ischemia and reperfusion sample groups, and C) control and reperfusion sample groups. Control group (n=6) is shown in blue circles; ischemia group (n=6) is shown in red circles; and reperfusion group (n=6) is shown in green circles.

To identify the key metabolites that distinguish between groups, the Students T-test was performed using Metaboanalyst software. Only mass ions with a VIP score 1.0 and above were considered as potential mass ions (determined using SIMCA). T-test analysis for control and ischemia sample groups resulted in identification of 49 mass ions but with more than 5% FDR. Volcano plot analysis revealed that 37 of them were altered with P-value less than 0.05. T-test analysis was also performed between control and reperfusion sample groups and resulted in identification of 394 mass ions. Many of these mass ions (n=57) were identified with less than 5% FDR. Volcano plot analysis revealed that 336 out of 394 metabolites were altered with P-value less than 0.05.Similarly, T-test analysis for ischemia and reperfused sample groups resulted in identification of 825 mass ions. Many of these mass ions (n=265) were identified with less than 5% FDR. Volcano plot analysis revealed that 260 out of 825 metabolites were significantly altered with P-value less than 0.05.

3.3 Altered amino acid metabolisms

3.3.1 Altered Tryptophan metabolism

Amino acid metabolism was observed to be majorly affected between ischemia and reperfusion samples. It includes amino acids such as Tryptophan, Arginine, Proline, Lysine, Glycine, Serine, Threonine and Tyrosine. In Tryptophan metabolism, several metabolites were identified such as L-Formylkynurenine, 2-aminomuconate, 5-Hydroxyindoleacetylglycine, 2, 3-Dihydroxyindole and Indole (**Table 6**). L-Tryptophan levels were also found to decrease in reperfusion samples by more than 3-fold change compared to ischemia samples (**Figure 15**).

Table 5. List of metabolites associated with tryptophan metabolism. Exact mass, name of the putative metabolite and their fold changes were shown. 1.5 fold change and above were highlighted in bold. Control–con, ischemia-isch, and reperfusion-rep.

Tryptophan metabolism				
Exact mass	**putative metabolite**	**con/isch**	**isch/rep**	**rep/con**
204.0900	L-Tryptophan	0.7	**3.6**	**0.4**
236.0791	L-Formylkynurenine	**0.3**	**0.4**	**8.8**
157.0370	2-Aminomuconate	0.7	1.1	1.2
248.0797	5-Hydroxyindoleacetylglycine	**0.5**	**0.4**	**6.0**
149.0477	2,3-Dihydroxyindole	**0.5**	**2.3**	0.8
117.0579	Indole	1.0	**0.3**	**3.0**

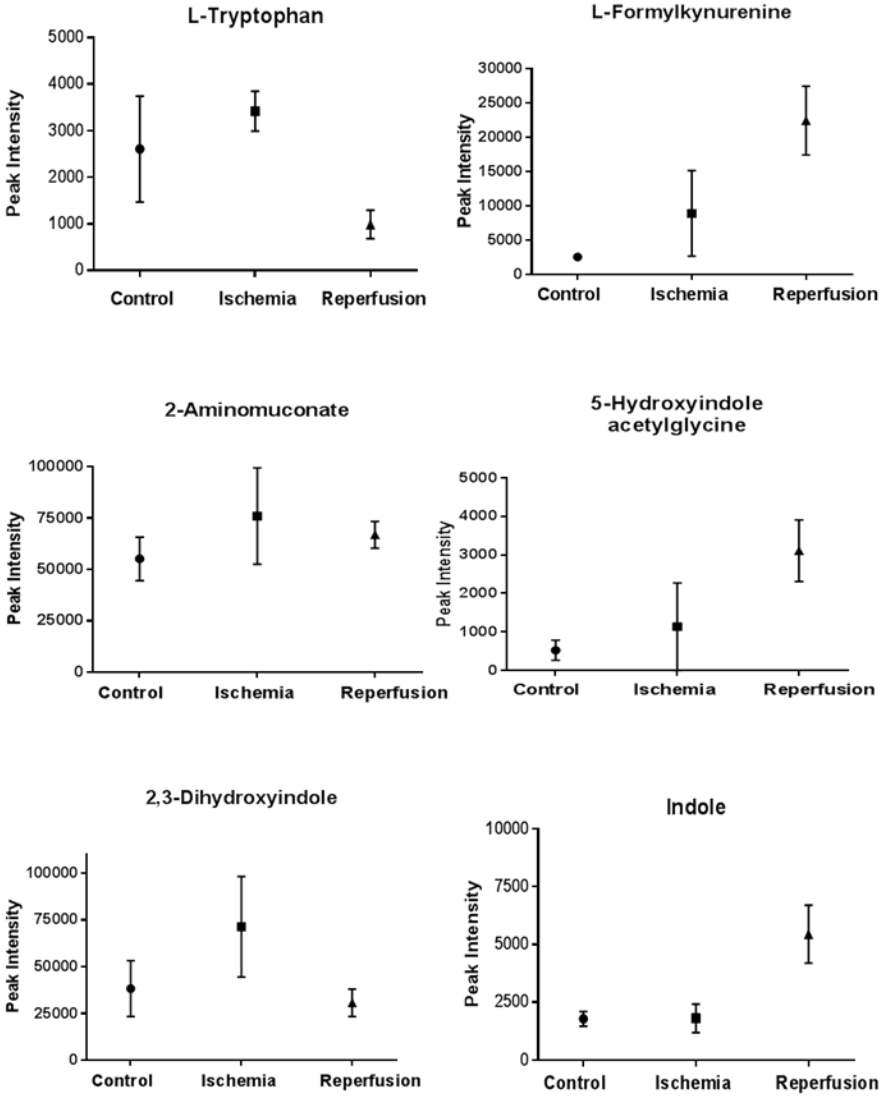

Figure 15. Altered Tryptophan metabolism in AKI. Altered levels of L-Tryptophan and other Tryptophan metabolites in control, ischemia and reperfusion samples. Standard error mean bars were shown.

3.3.2 Altered Arginine and Proline metabolism

In Arginine and Proline metabolism, several metabolite have been identified such as L-proline, Pyrroline-5-carboxylate, 5-Amino pentothenate, L-4-Hydroxyglutamate semialdehyde, L-Glutamate 5-semialdehyde and N-Carbamoylsarcosine (**Table 7**). In the present study decreased levels of L-Proline was observed significantly in reperfusion samples compared to ischemia or control samples (**Figure 16 and 17**).

Table 6. List of metabolites associated with Arginine and Proline metabolism. Exact mass, name of the putative metabolite and their fold changes were shown. 1.5 fold change and above were highlighted in bold. Control–con, ischemia-isch, and reperfusion-rep.

Arginine and proline metabolism				
Exact mass	**putative metabolite**	**con/isch**	**isch/rep**	**rep/con**
115.0633	L-Proline	0.8	**1.9**	**0.6**
113.0473	(S)-1-Pyrroline-5-carboxylate	1.1	0.8	1.2
117.0790	5-Aminopentanoate	1.3	0.9	0.9
147.0531	L-4-Hydroxyglutamate semialdehyde	1.1	0.8	1.2
131.0583	L-Glutamate 5-semialdehyde	**0.6**	**2.3**	0.7
132.0534	N-Carbamoylsarcosine	0.7	**2.2**	0.7
130.0263	2,5-Dioxopentanoate	1.1	**1.8**	**0.5**
131.0695	Creatine	1.1	1.2	0.8
117.0538	Guanidinoacetate	0.9	1.3	0.8
259.1167	Linatine	0.7	0.8	1.8
231.0738	N-Succinyl-L-glutamate 5-semialdehyde	0.7	1.0	1.4
246.1327	N2-(D-1-Carboxyethyl)-L-arginine	**0.6**	1.8	0.9

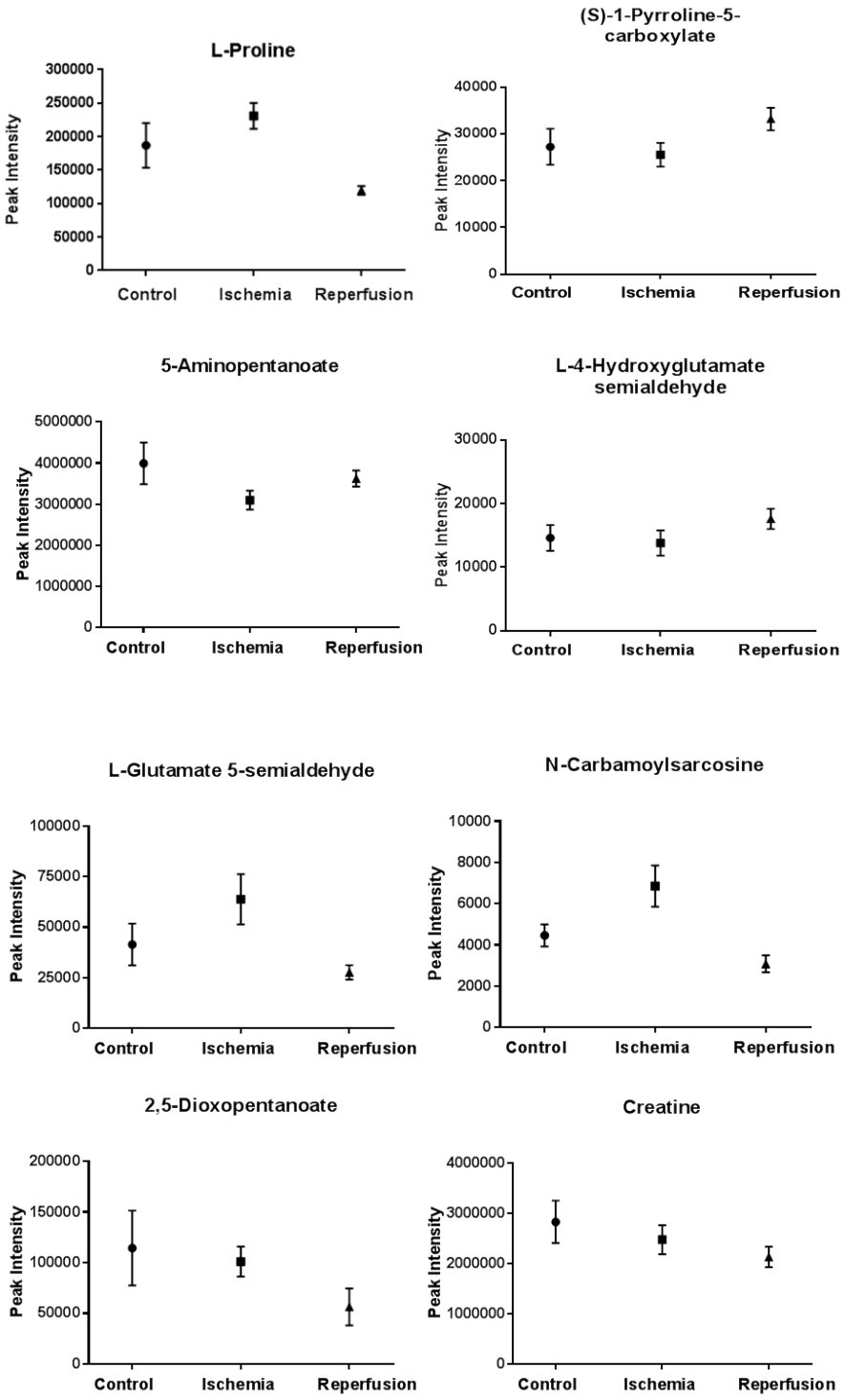

Figure 16. Altered Arginine and Proline metabolism in AKI. Altered levels of proline and other metabolites associated with Arginine and Proline metabolism were observed in control, ischemia and reperfusion sample groups. Standard error mean bars were shown.

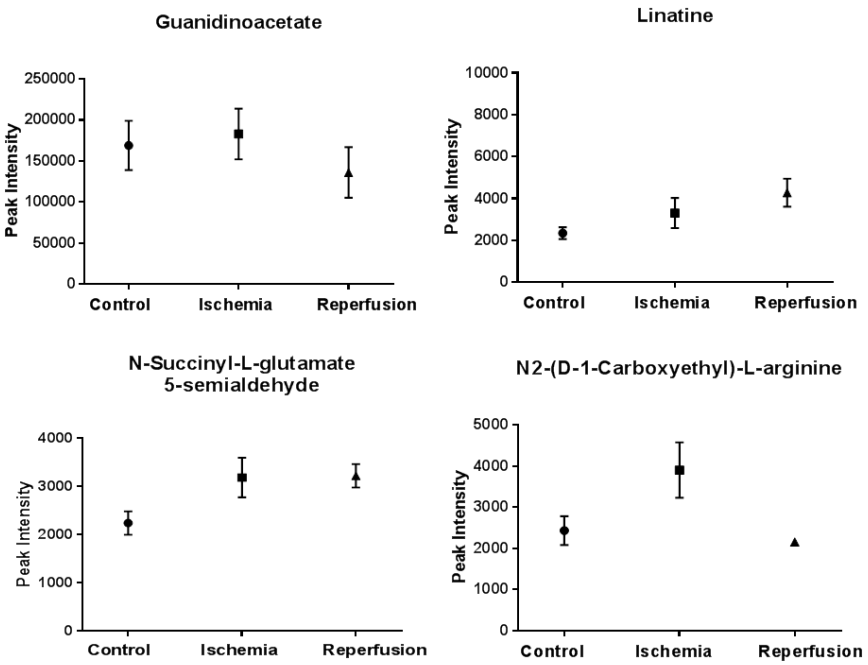

Figure 17. Altered Arginine and Proline metabolism in AKI. Altered levels of various metabolites associated with Arginine and Proline metabolism were observed in control, ischemia and reperfusion sample groups. Standard error mean bars were shown.

3.3.3 Altered Lysine metabolism

In Lysine metabolism, several metabolites have been identified that were known to be degradation products of Lysine (**Table 8**). L-Pipicolate was significantly reduced in reperfusion samples and known to be major degradation product of Lysine metabolism (**Figure 18 and 19**) (114).

Table 7. List of metabolites associated with Lysine metabolism. Exact mass, name of the putative metabolite and their fold changes were shown. 1.5 fold change and above were highlighted in bold. Control–con, ischemia-isch, and reperfusion-rep.

Lysine degradation metabolism				
Exact mass	**putative metabolite**	**con/isch**	**isch/rep**	**rep/con**
145.0738	(S)-5-Amino-3-oxohexanoic acid	0.8	**2.2**	**0.6**
159.0891	5-Acetamidopentanoate	1.4	0.9	0.8
117.0790	5-Aminopentanoate	1.3	0.9	0.9
242.0666	5-Phosphonooxy-L-lysine	0.9	1.2	0.9
129.0790	L-Pipecolate	1.0	**1.6**	**0.6**
218.1267	N2-(D-1-Carboxyethyl)-L-lysine	0.9	**2.2**	**0.5**
276.1322	N6-(L-1,3-Dicarboxypropyl)-L-lysine	1.0	1.0	1.0
188.1161	N6-Acetyl-L-lysine	1.2	1.0	0.8
204.1111	N6-Acetyl-N6-hydroxy-L-lysine	0.7	**1.7**	0.8
162.1004	N6-Hydroxy-L-lysine	0.9	**1.6**	0.7
83.0735	Piperideine	**1.5**	0.8	0.9

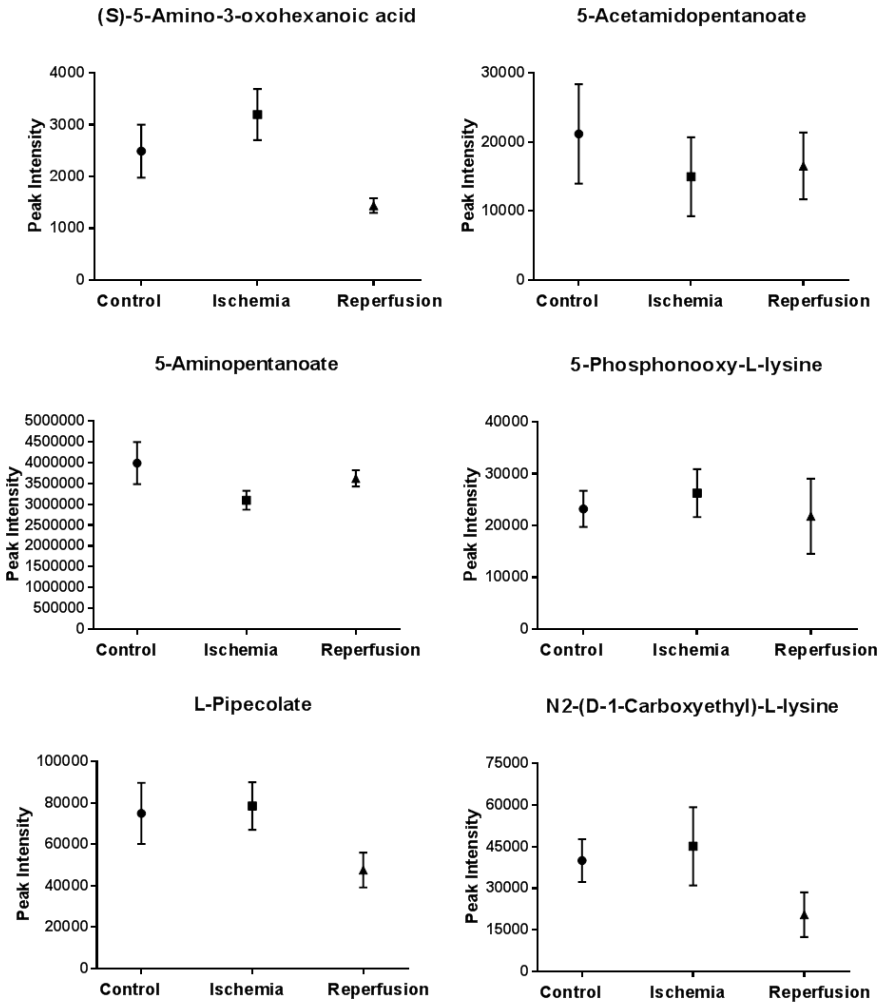

Figure 18. Altered Lysine metabolism in AKI. Altered levels of various metabolites of Lysine metabolism were observed in control, ischemia and reperfusion sample groups. Standard error mean bars were shown.

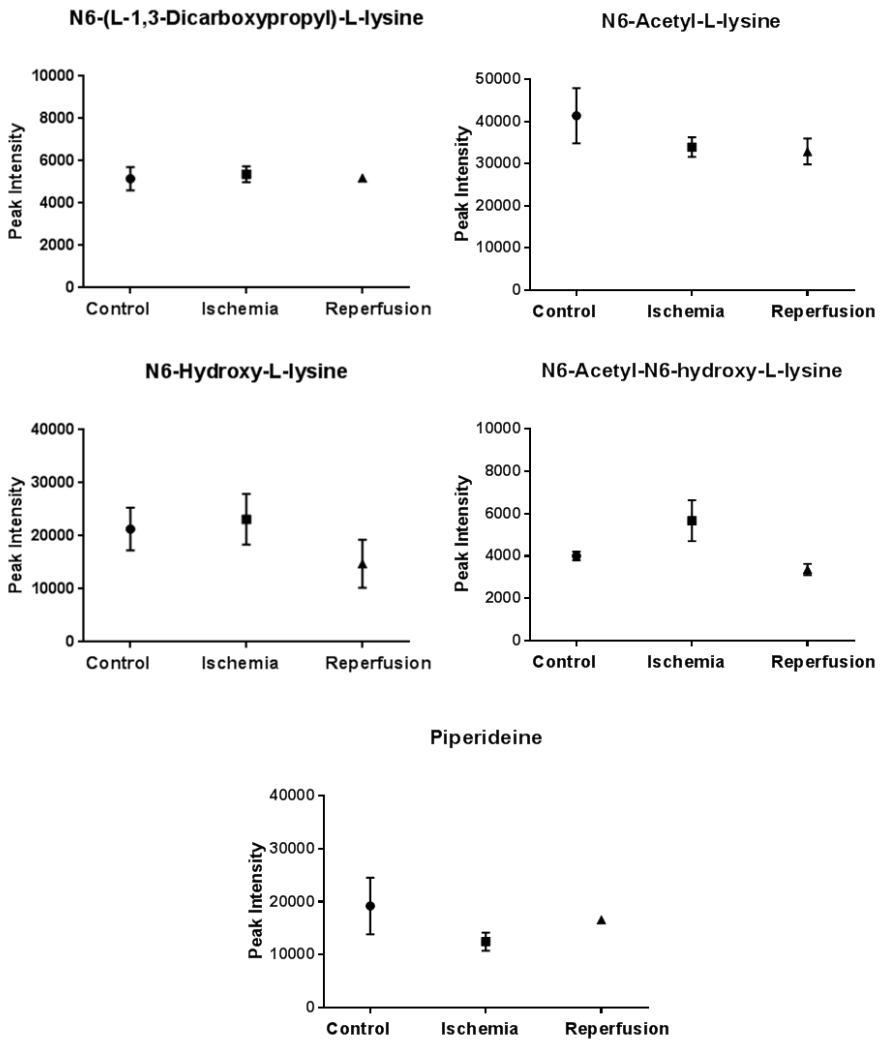

Figure 19. Altered Lysine metabolism in AKI. Altered levels of various metabolites of Lysine metabolism were observed in control, ischemia and reperfusion sample groups. Standard error mean bars were shown.

3.3.4 Altered Glycine, Serine and Threonine metabolism

Glycine, Serine and Threonine metabolism was found to be significantly altered in ischemia and reperfusion samples. Several degradation products of this metabolism were identified (**Table 9**). L-Serine was significantly low in reperfusion samples compared to ischemia (**Figure 20 and 21**). Aminoacetone and L-Allothreonine was significantly low in reperfusion samples compared to ischemia or control samples.

Table 8. List of metabolites associated with Glycine, Serine and Threonine metabolism. Exact mass, name of the putative metabolite and their fold changes were shown. 1.5 fold change and above were highlighted in bold. Control–con, ischemia-isch, and reperfusion-rep.

Glycine serine and Threonine metabolism				
Exact mass	**putative metabolite**	**con/isch**	**isch/rep**	**rep/con**
105.0426	L-Serine	0.9	**1.7**	0.7
119.0583	L-Allothreonine	0.9	**2.2**	**0.5**
75.0684	(R)-1-Aminopropan-2-ol	1.1	0.8	1.1
73.0528	Aminoacetone	0.8	**2.1**	**0.6**
117.0790	Betaine	1.1	1.0	1.0
131.0695	Creatine	1.1	1.2	0.8
142.0742	Ectoine	0.9	1.3	0.9
117.0538	Guanidinoacetate	0.9	1.3	0.8
117.0425	L-2-Amino-3-oxobutanoic acid	0.7	**1.5**	1.0
103.0633	N,N-Dimethylglycine	1.2	1.0	0.9

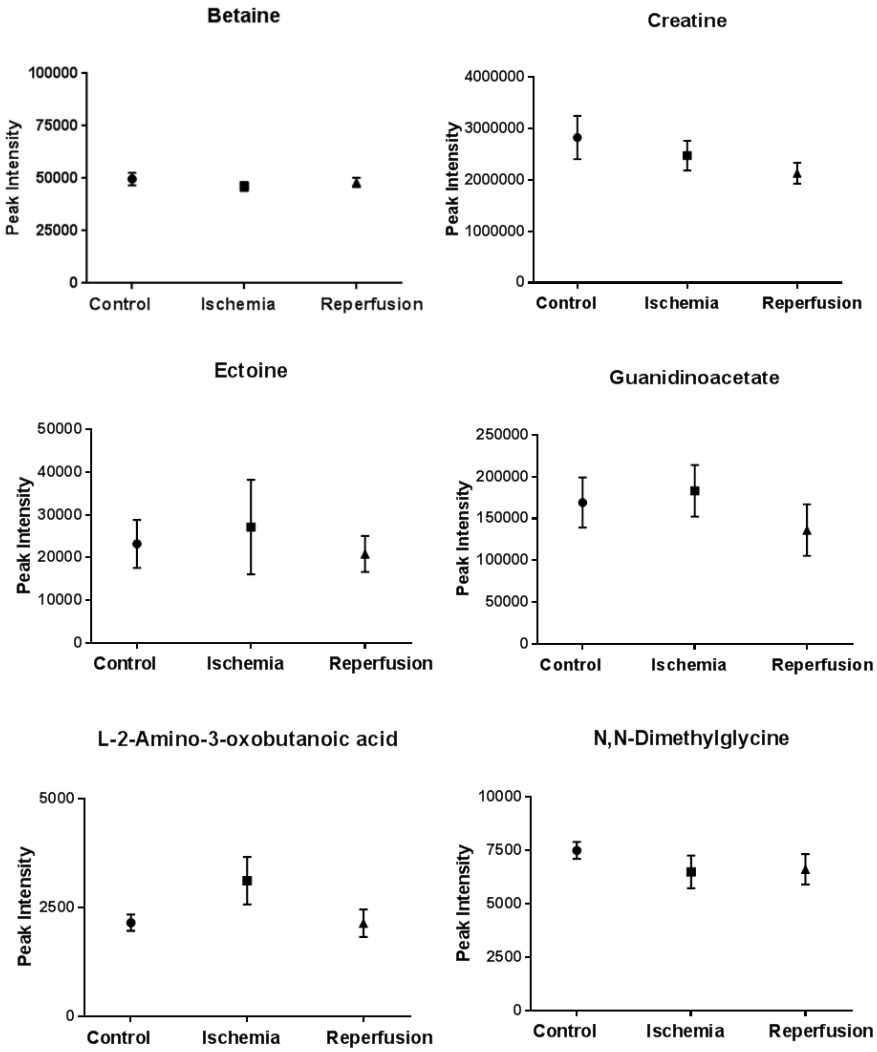

Figure 20. Altered Glycine, serine and Threonine metabolism in AKI. Altered levels of various metabolites of Glycine, serine and Threonine metabolism was observed in control, ischemia and reperfusion sample groups. Standard error mean bars were shown.

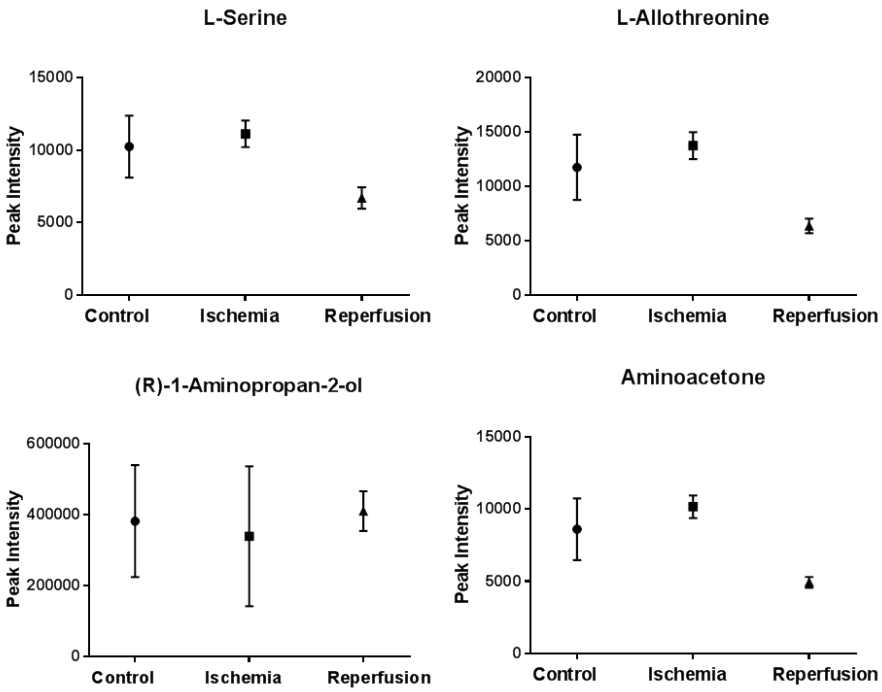

Figure 21. Altered Glycine, serine and Threonine metabolism in AKI. Altered levels of Serine and other metabolites of Glycine, serine and Threonine metabolism was observed in control, ischemia and reperfusion sample groups. Standard error mean bars were shown.

3.3.5 Altered phenylalanine and Tyrosine metabolism

Phenylalanine and Tyrosine metabolism was observed to be significantly altered between ischemia and reperfusion as well as between control and reperfusion sample groups. Phenylalanine and Tyrosine were found in low levels at 2 hour reperfusion compared to ischemia or control samples (**Table 10**). In addition, the major degradation product Hippuric acid was found to be increased in reperfusion samples suggesting degradation of Phenylalanine or Tyrosine (**Figure 22**).

Table 9. List of metabolites associated with Phenylalanine and Tyrosine metabolism. Exact mass, name of the putative metabolite and their fold changes were shown. 1.5 fold change and above were highlighted in bold. Control–con, ischemia-isch, and reperfusion-rep.

Phenylalanine and Tyrosine metabolism				
Exact mass	**putative metabolite**	**con/isch**	**isch/rep**	**rep/con**
165.0790	D-Phenylalanine	0.8	**2.1**	**0.6**
181.0740	D-Tyrosine	0.8	**2.2**	**0.6**
114.0314	2-Hydroxy-2,4-pentadienoate	1.1	**1.6**	**0.5**
179.0578	Hippurate	1.1	**0.3**	**3.0**
226.0590	Nitrotyrosine	**0.6**	**1.6**	1.1

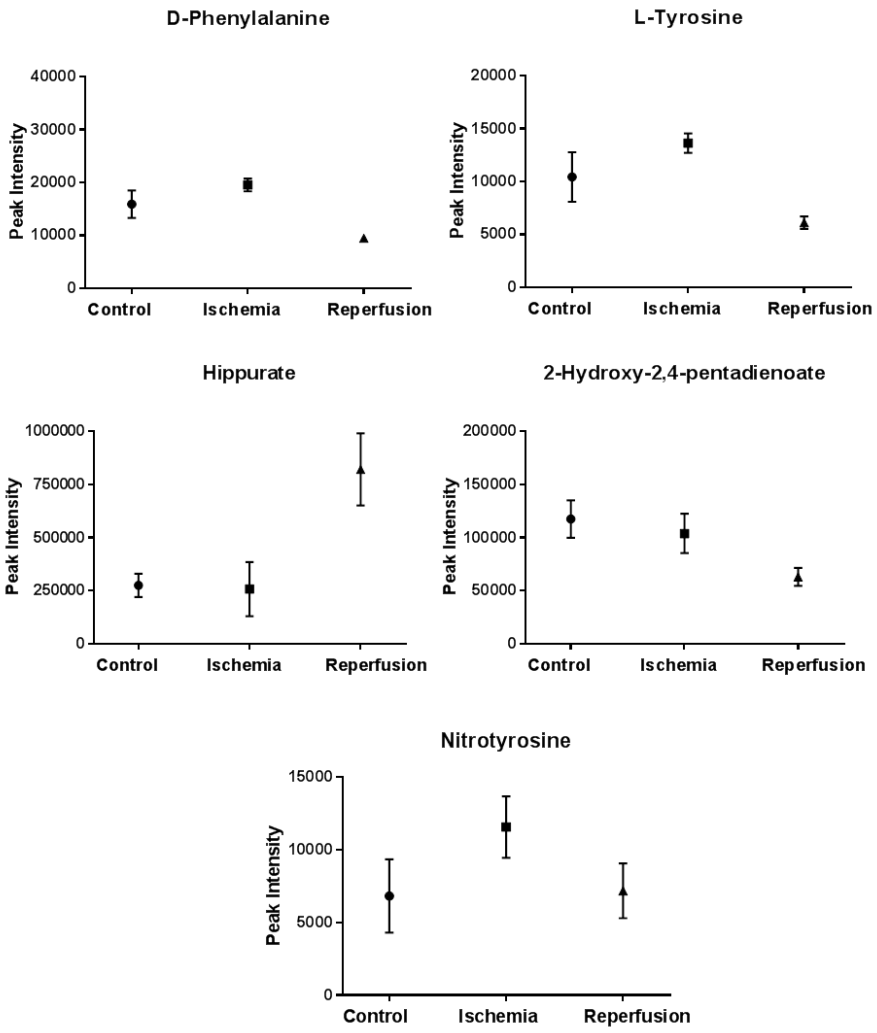

Figure 22. Altered Phenylalanine and Tyrosine metabolism in AKI. Altered levels of Phenylalanine, Tyrosine and metabolites in control, ischemia and reperfusion sample groups. Standard error mean bars were shown.

3.4 Metabolic profiles for lipid fraction

To study the lipid profile changes as a result of ischemia-reperfusion, lipid fraction was isolated as described in methods and untargeted mass spectrometric analysis was performed.

3.4.1 Multivariate analysis

LC-MS data generated from lipid fractions was preprocessed using Progenesis software as described in methods section. The processed lipid data was analysed by applying OPLS-DA on SIMCA 14.1 to determine the biological variations between control, ischemia and reperfused groups.

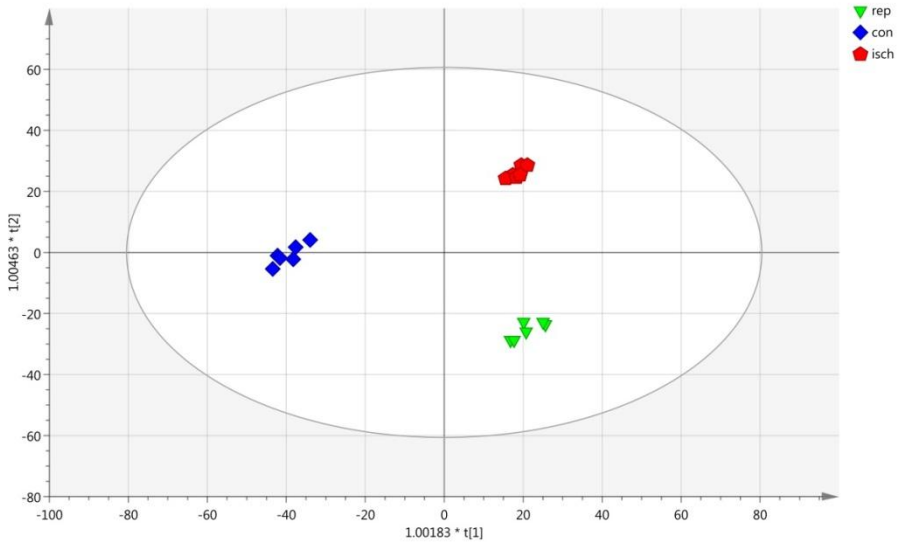

Figure 23. OPLS-DA score plot of control, ischemic and reperfused sample groups. Control group (n=6) is shown in blue square boxes; ischemia group (n=6) is shown in red pentagons; and reperfusion group (n=6) is shown in green triangles.

The score plot showed clustering of biological replicates for each sample group and also indicated significant separation between these groups (**Figure 23**). The clear separation suggests the possible existence of biological variation within these sample groups.

4. Discussion

The application of high throughput mass spectrometry in combination with various bioinformatics and statistical tools resulted in identification of significantly altered metabolites with high confidence. Pathway mapping revealed several biologically important metabolites associated with AKI that have potential to investigate further in a targeted way for developing various therapeutic applications.

In the present study, metabolomics analysis between control, ischemia and reperfusion samples was done to identify metabolite changes associated with AKI. Majority of metabolites altered in this study belongs to amino acid metabolism, lipid metabolism and carbohydrate metabolism (**Figure 13**). Significant changes in metabolites/metabolic pathways have been observed mainly between ischemia and reperfusion samples. Several important metabolites have been identified with high confidence (**Table 5)**.

Comprehensive analysis of amino acid metabolism showed that Tryptophan, Arginine, Proline, Lysine, Glycine, Serine, Threonine and Tyrosine were found to undergo degradation significantly in reperfusion samples compared to ischemia or control samples. In Tryptophan metabolism, L-Tryptophan levels were found to decrease in reperfusion samples (**Figure 15**). Further, its degradation product L-Formylkynurenine showed increased levels in reperfusion sample groups compared to ischemia or control groups confirming the Tryptophan degradation in porcine AKI model. Tryptophan degradation in AKI was reported earlier in mice models (101). Increased levels of Kynurenine, a degradation product of Tryptophan metabolism

Table 10. Important list of metabolites identified with high confidence and significantly altered in ischemia or reperfusion samples with less than 5% FDR. Exact mass, retention time (RT), name of the putative metabolite, IDEOM score, pathway name and their fold changes shown. ischemia-isch, and reperfusion-rep.

Exact mass	RT (min)	Putative metabolite	ID score	Metabolic Pathway	isch/rep
182.079	3.36	Mannitol	10	Fructose and mannose metabolism	2.4
284.075	4.11	Xanthosine	10	Purine metabolism	2.6
136.064	12.58	1-Methylnicotinamide	8	Nicotinate and nicotinamide metabolism	0.4
115.063	12.33	L-Proline	8	Arginine and proline metabolism	1.9
204.09	10.16	L-Tryptophan	8	Tryptophan metabolism	3.4
145.074	13.30	[FA oxo,amino(6:0)] 3-oxo-5S-amino-hexanoate	8	Lysine degradation	2.2
172.013	14.76	sn-Glycerol 3-phosphate	8	Glycerophospholipid metabolism	1.9
104.047	5.02	4-Hydroxybutanoate	8	Butanoate metabolism	1.8
244.069	14.26	Pseudouridine	8	Pyrimidine metabolism	2.8
299.076	14.90	D-4'-Phosphopantothenate	8	Pantothenate and CoA biosynthesis	2.3
215.055	11.09	sn-glycero-3-Phosphoethanolamine	8	Glycerophospholipid metabolism	1.9
85.0891	14.68	Piperidine	7	Piperine biosynthesis	1.8
248.054	10.46	Pentane-1,3,4,5-tetracarboxylate	7	Methanofuran biosynthesis	1.9
138.105	1.49	4E,8-Nonadienal	7	Fatty aldehydes	0.5
97.9769	14.77	Orthophosphate	6	Oxidative phosphorylation	2.2
165.079	11.42	L-Phenylalanine	6	Phenylalanine metabolism	2.1
181.074	11.52	L-Tyrosine	6	Tyrosine metabolism	2.2
119.058	13.66	L-Allothreonine	6	Glycine, serine and threonine metabolism	2.2
73.0528	13.53	Aminoacetone	6	Glycine, serine and threonine metabolism	2.1
105.043	13.90	L-Serine	6	Glycine, serine and threonine metabolism	1.7
169.05	16.03	Phosphodimethylethanolamine	6	Glycerophospholipid metabolism	1.7
132.042	12.99	2-(Hydroxymethyl)-4-oxobutanoate	5	Vitamin B6 metabolism	4.9
722.509	1.60	PG(32:0)	5	Glycerophosphoglycerols	4.2
228.136	4.78	[FA dioxo(12:0)] 9,12-dioxo-dodecanoic acid	5	Fatty Acids and Conjugates	0.3

was observed at 2 hr reperfusion in mice and further increased significantly at 1 week reperfusion. Biologically, Tryptophan degradation was known to be associated with inflammation in kidney following ischemia and reperfusion. Tryptophan degradation was also reported to be associated with chronic kidney disease (111). Tryptophan degradation observed in the reperfusion samples suggests induction of inflammation in kidneys during 2 hr reperfusion condition.

In Arginine and Proline metabolism, decreased levels of L-Proline or increased levels of its degradation product, (S)-1-Pyrroline-5-carboxylate, was observed in reperfusion samples compared to ischemia or control samples confirming Arginine or Proline degradation (**Figure 16 and 17**). Earlier studies reported accumulation of Citrulline, a degradation product of Arginine, in cortex tissues of AKI mice models at 2 hr reperfusion (101). They also reported decreased Proline levels in plasma of mice at 2 hr reperfusion. Arginine is known to play role in inflammation by synthesising nitric oxide (112). It is also predicted that L-arginine promotes renal injury in some disease models of glomerulonephritis. Together, the degradation of Arginine or Proline observed in the study further confirms reperfusion induced inflammation in kidneys. In contrast, experimental studies also showed that administration of Arginine reduces renal injury in ischemic acute renal failure and other animal models of preeclampsia (113). The definite physiological role of Arginine in AKI pathogenesis needs to be further investigated.

Lysine degradation products were observed in ischemia or reperfusion sample groups (**Figure 18 and 19**). L-Pipicolate was known to be major degradation product of Lysine metabolism (114). Pipecolate, 5-Aminopentanoate and N6-Acetyl-L-lysine

formed from lysine degradation were known to be further metabolised to glutarate and glutaryl Co-A. Lysine degradation leads to formation of succinate and its role in AKI through reactive oxygen species (ROS) biosynthesis was recently reported (115). Investigation of Lysine degradation pathway may provide new insights in ischemia or reperfusion induced AKI. Glycine, Serine and Threonine degradation was significantly observed in reperfusion samples. Modification in this metabolism was reported in a recent metabolomics study in chronic kidney disease implicating their potential role in kidney injury (116).

Phenylalanine and Tyrosine levels were found to be significantly decreased in reperfusion samples compared to ischemia or control samples. Further, increased levels of degradation product hippuric acid in reperfusion samples confirm degradation of Tyrosine. It was reported that conversion of Phenylalanine to Tyrosine was affected in chronic kidney failure (CRF) leading to reduced Tyrosine levels in plasma and tissues of these patients (117). Increased formation of nitrotyrosine was reported in CRF, diabetic nephropathy or allograft nephropathy patients. Nitrotyrosine is formed by nitration of tyrosine and it occurs in presence of active metabolite nitric oxide (NO) leading to oxidative stress. In the present study, nitrotyrosine levels were elevated to some extent in ischemia samples compared to control samples and then decreased at 2 hr reperfusion samples. It is possible that nitric oxide synthesised during Arginine degradation might contributed in nitrotyrosine formation.

Together, amino acid degradation was identified as one of the early metabolic changes in IR induced AKI. During ischemia, minute increase or no significant

changes in amino acid levels were observed in this study. Whereas after 2 hr reperfusion, there was significant decrease in amino acid levels compared to ischemia or control sample groups. It clearly suggests that these amino acids were utilised in unknown renal cellular processes associated with AKI. The association of amino acid degradation with inflammation was already known and suggest tissue inflammation could be one of the early responses in IR induced AKI. Also, the amino acid degradation could be associated with ROS production through TCA cycle. Succinate accumulation and ROS production in IR tissues was recently reported in mice models (115). Amino acid degradation could also be the cause of increased blood urea nitrogen (BUN) formation which is commonly observed during renal failure.

Along with amino acid metabolism, Lipid, carbohydrate, purine and pyrimidine metabolisms were also found to be altered in AKI. Decreased levels of sn-Glycerol 3-phosphate, sn-glycero-3-Phosphoethanolamine, Phosphodimethylethanolamine, and PG(32:0) were identified in reperfusion samples compared to ischemia. Increased levels of 1-Methylnicotinamide, a metabolite of Nicotinate and nicotinamide metabolism, were identified in reperfusion samples compared to ischemia or control samples. 1-Methylnicotinamide is known to be an anti-apoptotic and anti-necrotic factor and play important role in lipotoxicity mediated renal injury (118). Decreased levels of Xanthosine and Pseudouridine, metabolites involved in purine and pyrimidine metabolism, were identified in reperfusion samples compared to ischemia. These metabolisms need to be further analysed in the context of AKI.

5. Conclusion and Future work

The present study revealed increased amino acid degradation of tryptophan and arginine as an early event marking 2hr reperfusion after AKI in a porcine model. Lack of these amino acids may suggest a mechanism for the cell injury and inflammation that can occur during AKI. Future studies will be focussed on absolute quantitative analysis of these nine amino acid pathways to identify the key metabolites and their potential role associated with inflammation in IR induced AKI. Subsequently, amino acid analysis in urine and plasma of AKI patients may provide potential biomarkers useful for clinical diagnosis. Further, lipidomics data generated in the present study need to be analysed to discover novel mechanisms associated with AKI pathogenesis. To my knowledge, using large animal model, this is the first high throughput mass spectrometry based metabolomics study to report ischemia or reperfusion associated metabolomics changes in porcine AKI model.

6. Limitations of the study

Here, we applied untargeted mass spectrometry based metabolomics approach to study early metabolite changes in a porcine AKI model. This high throughput metabolomics approach is highly sensitive and resulted in identification of metabolites that were altered in AKI kidney tissues. Further bioinformatics data analysis resulted in identification of key metabolic pathways and helps to interpret these metabolic changes in the context of AKI. Together, the goal of this study was achieved and the early molecular events of AKI pathogenesis were revealed in a porcine model. However, there are a few limitations of this untargeted metabolomics approach that needs to be discussed here:

First, for large scale identification of metabolites, a sample prefractionation step has to be included before subjecting the sample to LC-MS. Also, many unknown metabolites have been detected that could not be matched to the existing metabolome databases. These metabolites could be important in the context of the current study and is a general concern for the developing field of metabolomics. Furthermore, the present study provides only semi-quantitative information about metabolite abundance as the metabolite samples extracted from control, ischemia and reperfused kidneys were individually analysed using LC-MS and quantified relative to the reference pool and to the other groups. To overcome this limitation, a more advanced, but ultimately very time-consuming method for quantitative metabolite profiling could be through post extraction derivatization with differentially labeled derivatizing reagents. This method provides isotopic internal standards for all the metabolites in the given sample and facilitates the quantification efficacy. In the present metabolomics study, altered levels of several amino acids and their

intermediates have been identified implicating their association with AKI. However, to confirm their altered levels, absolute quantification of these specific metabolites needs to be done in a targeted way. Effective generation of both qualitative and quantitative information of these metabolites helps to better understand the molecular insights of AKI pathophysiology which is essential for clinical management of AKI.

7. Bibliography

1. Natochin, Y.V., 1996. Evolutionary aspects of renal function. Kidney Int., 49 (6), 1539-42.
2. Dear, J.W., Kobayashi, H., Jo, S.K., Holly, M.K., Hu, X., Yuen, P.S., Brechbiel, M.W., Star, R.A., 2005. Dendrimer-enhanced MRI as a diagnostic and prognostic biomarker of sepsis-induced acute renal failure in aged mice. Kidney Int, 67 (6), 2159-67.
3. Kellum, J.A., Levin, N., Bouman, C., Lameire, N., 2002. Developing a consensus classification system for acute renal failure. Curr Opin Crit Care, 8 (6), 509-14.
4. Bellomo, R., Ronco, C., Kellum, J.A., Mehta, R.L., Palevsky, P., Acute Dialysis Quality Initiative workgroup. 2004. Acute renal failure - definition, outcome measures, animal models, fluid therapy and information technology needs: the Second International Consensus Conference of the Acute Dialysis Quality Initiative (ADQI) Group. Crit Care, 8 (4), R204-12.
5. Kim, K.E., Onesti, G., Ramirez, O., 1969, Creatinine clearance in renal disease. A reappraisal. BMJ, 14, 11-19.
6. Mehta, R.L., Kellum, J.A., Shah, S.V., Molitoris, B.A., Ronco, C., Warnock, D.G., Levin, A., Acute Kidney Injury Network, 2007. Acute Kidney Injury Network: report of an initiative to improve outcomes in acute kidney injury. Crit Care, 11 (2), R31.
7. Uchino, S., Bellomo, R., Goldsmith, D., Bates, S., Ronco, C., 2006. An assessment of the RIFLE criteria for acute renal failure in hospitalized patients. Crit Care Med, 34, 1913–1917.
8. Ostermann, M., Chang, R.W., 2007. Acute kidney injury in the intensive care unit according to RIFLE. Crit Care Med, 35, 1837–1843.
9. Bagshaw, S.M., George, C., Dinu, I., Bellomo, R., 2008. A multi-centre evaluation of the RIFLE criteria for early acute kidney injury in critically ill patients. Nephrol Dial Transplant, 23, 1203–1210.
10. Murugan, R., Karajala-Subramanyam, V., Lee, M., Yende, S., Kong, L., Carter, M., Angus, D.C., Kellum, J.A., Genetic and Inflammatory Markers of Sepsis (GenIMS) Investigators, 2010. Acute kidney injury in non-severe

pneumonia is associated with an increased immune response and lower survival. Kidney Int, 77, 527-35.

11. Ali, T., Khan, I., Simpson, W., Prescott, G., Townend, J., Smith, W., Macleod, A., 2007. Incidence and outcomes in acute kidney injury: a comprehensive population-based study. J Am Soc Nephrol, 18 (4), 1292-8.
12. Acute Kidney Injury—United States Renal Data System 2009 Annual Data Report. United States Renal Data System. 2009. [online], http://www.usrds.org/2009/pdf/V1_08_09.PDF
13. Waikar, S.S., Wald, R., Chertow, G.M., Curhan, G.C., Winkelmayer, W.C., Liangos, O., Sosa, M.A., Jaber, B.L., 2006. Validity of International Classification of Diseases, Ninth Revision, Clinical Modification Codes for Acute Renal Failure. J Am Soc Nephrol, 17 (6), 1688-94.
14. Fang, Y., Ding, X., Zhong, Y., Zou, J., Teng, J., Tang, Y., Lin, J., Lin, P., 2010. Acute kidney injury in a Chinese hospitalized population. Blood Purif, 30 (2), 120-6.
15. Thakar, C.V., Christianson, A., Freyberg, R., Almenoff, P., Render, M.L., 2009. Incidence and outcomes of acute kidney injury in intensive care units: a Veterans Administration study. Crit Care Med, 37, 2552–2558.
16. Centers for Disease Control and Prevention (CDC), 2005. Hospitalization discharge diagnoses for kidney disease–United States, 1980–2005. MMWR Morb Mortal Wkly, 57, 309–312.
17. Kerr, M., Bedford, M., Matthews, B., O'Donoghue, D., 2014. The economic impact of acute kidney injury in England. Nephrol Dial Transplant, 29 (7), 1362-8.
18. Holley, J.L., 2009. Clinical approach to the diagnosis of acute renal failure. In: Greenberg A, Cheung AK, eds. Primer on Kidney Diseases. 5th ed. Philadelphia, Pa.: National Kidney Foundation.
19. Himmelfarb, J., 2009. Acute kidney injury in the elderly: problems and prospects. Semin Nephrol, 29 (6), 658-64.
20. Uchino, S., Kellum, J.A., Bellomo, R., Doig, G.S., Morimatsu, H., Morgera, S., Schetz, M., Tan, I., Bouman, C., Macedo, E., Gibney, N., Tolwani, A., Ronco, C., Beginning and Ending Supportive Therapy for the Kidney (BEST Kidney) Investigators., 2005. Acute renal failure in critically ill patients: a multinational, multicentre study. JAMA, 294 (7), 813-8.

21. Alejandro, V., Scandling, J.D. Jr., Sibley, R.K., Dafoe, D., Alfrey, E., Deen, W., Myers, B.D., 1995. Mechanisms of filtration failure during postischemic injury of the human kidney. A study of the reperfused renal allograft. J Clin Invest, 95 (2), 820-31.
22. Ramaswamy, D., Corrigan, G., Polhemus, C., Boothroyd, D., Scandling, J., Sommer, F.G., Alfrey, E., Higgins, J., Deen, W.M., Olshen, R., Myers, B.D., 2002. Maintenance and recovery stages of postischemic acute renal failure in humans. Am J Physiol Renal Physiol, 282 (2), F271-80.
23. Molitoris, B.A., Sutton, T.A., 2004. Endothelial injury and dysfunction: role in the extension phase of acute renal failure. Kidney Int, 66 (2), 496-9.
24. Saikumar, P., Venkatachalam, M.A., 2003. Role of apoptosis in hypoxic/ischemic damage in the kidney. Semin Nephrol, 23 (6), 511-21.
25. Solez, K., Morel-Maroger, L., Sraer, J. D., 1979. The morphology of "acute tubular necrosis" in man: analysis of 57 renal biopsies and a comparison with the glycerol model. Medicine (Baltimore) 58, 362–376.
26. Molitoris, B.A., 2004. Actin cytoskeleton in ischemic acute renal failure. Kidney Int, 66 (2), 871-83.
27. Atkinson, S.J., Hosford, M.A., Molitoris, B.A., 2004. Mechanism of actin polymerization in cellular ATP depletion. J Biol Chem, 279 (7), 5194-9.
28. Molitoris, B. A., 1993. Na+-K+-ATPase that redistributes to apical membrane during ATP depletion remains functional. Am. J. Physiol, 265, F693–F697.
29. Molitoris, B.A., Marrs, J., 1999. The role of cell adhesion molecules in ischemic acute renal failure. Am J Med, 106 (5), 583-92.
30. Zuk, A., Bonventre, J. V., Brown, D., atlin, K. S., 1998. Polarity, integrin, and extracellular matrix dynamics in the postischemic rat kidney. Am. J. Physiol, 275, C711–C731.
31. Bonegio, R., Lieberthal, W., 2002. Role of apoptosis in the pathogenesis of acute renal failure. Curr Opin Nephrol Hypertens, 11 (3), 301-8.
32. Guo, R., Wang, Y., Minto, A.W., Quigg, R.J., Cunningham, P.N., 2004. Acute renal failure in endotoxemia is dependent on caspase activation. J Am Soc Nephrol, 15 (12), 3093-102.
33. Nicholson, D.W., 2000. From bench to clinic with apoptosis-based therapeutic agents. Nature, 407 (6805), 810-6.

34. Galli, F., Piroddi, M., Annetti, C., Aisa, C., Floridi, E., Floridi, A., 2005. Oxidative stress and reactive oxygen species. Contrib Nephrol, 149, 240-60.
35. Conger, J.D., Robinette, J.B., Hammond, W.S, 1991. Differences in vascular reactivity in models of ischemic acute renal failure. Kidney Int, 39 (6), 1087-97.
36. Mizutani, A., Okajima, K., Uchiba, M., Noguchi, T., 2000. Activated protein C reduces ischemia/reperfusion-induced renal injury in rats by inhibiting leukocyte activation. Blood, 95, 3781–3787.
37. Kinsey, G. R., Li, L., Okusa, M. D., 2008. Inflammation in acute kidney injury. Nephron Exp. Nephrol, 109, e102–e107.
38. Day, Y. J., Huang, L., Ye, H., Linden, J., Okusa, M. D., 2005. Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: role of macrophages. Am. J. Physiol. Renal Physiol, 288, F722–F731.
39. Moran, S.M., Myers, B.D., 1985. Course of acute renal failure studied by a model of creatinine kinetics. Kidney Int, 27 (6), 928-37.
40. Star, R.A., 1998. Treatment of acute renal failure. Kidney Int, 54 (6), 1817-31.
41. Doi, K., Yuen, P.S., Eisner, C., Hu, X., Leelahavanichkul, A., Schnermann, J., Star, R.A., 2009. Reduced production of creatinine limits its use as marker of kidney injury in sepsis. J Am Soc Nephrol, 20 (6), 1217-21.
42. Vaidya, V.S., Ferguson, M.A., Bonventre, J.V., 2008. Biomarkers of acute kidney injury. Annu Rev Pharmacol Toxicol, 48, 463-93.
43. Borregaard, N., Sehested, M., Nielsen, B.S., Sengeløv, H., Kjeldsen, L., 1995. Biosynthesis of granule proteins in normal human bone marrow cells. Gelatinase is a marker of terminal neutrophil differentiation. Blood, 85 (3), 812-7.
44. Nielsen, B.S., Borregaard, N., Bundgaard, J.R., Timshel, S., Sehested, M., Kjeldsen, L., 1996. Induction of NGAL synthesis in epithelial cells of human colorectal neoplasia and inflammatory bowel diseases. Gut, 38 (3), 414-20.
45. Mishra, J., Ma, Q., Prada, A., Mitsnefes, M., Zahedi, K., Yang, J., Barasch, J., Devarajan, P., 2003. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. J Am Soc Nephrol, 14 (10), 2534-43.

46. Mishra, J., Mori, K., Ma, Q., Kelly, C., Barasch, J., Devarajan, P., 2004. Neutrophil gelatinase-associated lipocalin: a novel early urinary biomarker for cisplatin nephrotoxicity. Am. J. Nephrol, 24, 307–15.
47. Wagener, G., Jan, M., Kim, M., Mori, K., Barasch, J.M., Sladen, R.N., Lee, H.T., 2006. Association between increases in urinary neutrophil gelatinase-associated lipocalin and acute renal dysfunction after adult cardiac surgery. Anesthesiology, 105 (3), 485-91.
48. Trachtman, H., Christen, E., Cnaan, A., Patrick, J., Mai, V., Mishra, J., Jain, A., Bullington, N., Devarajan, P., 2006. Urinary neutrophil gelatinase-associated lipocalcin in D+HUS: a novel marker of renal injury. Pediatr Nephrol, 21 (7), 989-94.
49. Mori, K., Lee, H.T., Rapoport, D., Drexler, I.R., Foster, K., Yang, J., Schmidt-Ott, K.M., Chen, X., Li, J.Y., Weiss, S., Mishra, J., Cheema, F.H., Markowitz, G., Suganami, T., Sawai, K., Mukoyama, M., Kunis, C., D'Agati, V., Devarajan, P., Barasch, J., 2005. Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. J Clin Invest, 115 (3), 610-21.
50. Bailly, V., Zhang, Z., Meier, W., Cate, R., Sanicola, M., Bonventre, J.V., 2002. Shedding of kidney injury molecule-1, a putative adhesion protein involved in renal regeneration. J Biol Chem, 277 (42), 39739-48.
51. Amin, R.P., Vickers, A.E., Sistare, F., Thompson, K.L., Roman, R.J., Lawton, M., Kramer, J., Hamadeh, H.K., Collins, J., Grissom, S., Bennett, L., Tucker, C.J., Wild, S., Kind, C., Oreffo, V., Davis, J.W., Curtiss, S., Naciff, J.M., Cunningham, M., Tennant, R., Stevens, J., Car, B., Bertram, T.A., Afshari, C.A., 2004. Identification of putative gene based markers of renal toxicity. Environ Health Perspect, 112 (4), 465-79.
52. Han, W.K., Bailly, V., Abichandani, R., Thadhani, R., Bonventre, J.V., 2002. Kidney Injury Molecule-1 (KIM-1): a novel biomarker for human renal proximal tubule injury. Kidney Int, 62 (1), 237-44.
53. Vaidya, V.S., Ramirez, V., Ichimura, T., Bobadilla, N.A., Bonventre, J.V., 2006. Urinary kidney injury molecule-1: a sensitive quantitative biomarker for early detection of kidney tubular injury. Am J Physiol Renal Physiol, 290 (2), F517-29.

54. Liangos, O., Perianayagam, M.C., Vaidya, V.S., Han, W.K., Wald, R., Tighiouart, H., MacKinnon, R.W., Li, L., Balakrishnan, V.S., Pereira, B.J., Bonventre, J.V., Jaber, B.L., 2007. Urinary N-acetyl-beta-(D)-glucosaminidase activity and kidney injury molecule-1 level are associated with adverse outcomes in acute renal failure. J Am Soc Nephrol, 18 (3), 904-12.
55. Huo, W., Zhang, K., Nie, Z., Li, Q., Jin, F., 2010. Kidney injury molecule-1 (KIM-1): a novel kidney-specific injury molecule playing potential double-edged functions in kidney injury. Transplant Rev (Orlando), 24 (3), 143-6.
56. Bökenkamp, A., Ciarimboli, G., Dieterich, C., 2001. Cystatin C in a rat model of end-stage renal failure. Ren Fail, 23 (3-4), 431-8.
57. Uchida, K., Gotoh, A., 2002. Measurement of cystatin-C and creatinine in urine. Clin Chim Acta, 323 (1-2), 121-8.
58. Conti, M., Moutereau, S., Zater, M., Lallali, K., Durrbach, A., Manivet, P., Eschwège, P., Loric, S., 2006. Urinary cystatin C as a specific marker of tubular dysfunction. Clin Chem Lab Med, 44 (3), 288-91.
59. Herget-Rosenthal, S., Pietruck, F., Volbracht, L., Philipp, T., Kribben, A., 2005. Serum cystatin C--a superior marker of rapidly reduced glomerular filtration after uninephrectomy in kidney donors compared to creatinine. Clin Nephrol, 64 (1), 41-6.
60. Dettmer, K., Hammock, B.D., 2004. Metabolomics--a new exciting field within the "omics" sciences. Environ Health Perspect, 112 (7), A396-7.
61. Wahl, H.G., Hoffmann, A., Luft, D., Liebich, H.M., 1999. Analysis of volatile organic compounds in human urine by headspace gas chromatography-mass spectrometry with a multipurpose sampler. J Chromatogr A, 847 (1-2), pp 117 - 25.
62. Agata, S., Michał, P., Adam, K., Jacek, N., 2010. "Current trends in solid-phase microextraction (SPME) fibre coatings". Chemical Society Reviews, 39, 4524.
63. Idborg, H., Edlund, P.O., Jacobsson, S.P., 2004. Multivariate approaches for efficient detection of potential metabolites from liquid chromatography/mass spectrometry data. Rapid Commun Mass Spectrom, 18 (9), pp 944 - 54.
64. Gullberg, J., Jonsson, P., Nordström, A., Sjöström, M., Moritz, T., 2004. Design of experiments: an efficient strategy to identify factors influencing

extraction and derivatization of Arabidopsis thaliana samples in metabolomics studies with gas chromatography/mass spectrometry. Anal Biochem, 331 (2), pp 283 - 95.

65. Patti, G.J., 2011. Separation strategies for untargeted metabolomics. J Sep Sci, 34 (24), pp 3460 - 9.
66. Ramautar, R., Somsen, G.W., de Jong, G.J., 2009. CE-MS in metabolomics. Electrophoresis. 30 (1), 276-91.
67. Chapman, J. R., 1995. Practical Organic Mass Spectrometry: A Guide for Chemical and Biochemical Analysis. John Wiley & Sons. ISBN 978-0-471-95831-4.
68. Robinson, J.W., Skelly Frame, E.M., Frame II, G.M., 2005. Undergraduate Instrumental Analysis, 6th ed. Marcel Drekker, New York.
69. Ho, C.S., Lam, C.W., Chan, M.H., Cheung, R.C., Law, L.K., Lit, L.C., Ng, K.F., Suen, M.W., Tai, H.L., 2003. Electrospray ionisation mass spectrometry: principles and clinical applications. Clin Biochem Rev, 24 (1), 3-12.
70. Lu, W., Bennett, B.D., Rabinowitz, J.D., 2008. Analytical strategies for LC-MS-based targeted metabolomics. J Chromatogr B Analyt Technol Biomed Life Sci, 871 (2), pp 236 – 42.
71. Pedrioli, P.G., Eng, J.K., Hubley, R., Vogelzang, M., Deutsch, E.W., Raught, B., Pratt, B., Nilsson, E., Angeletti, R.H., Apweiler, R., Cheung, K., Costello, C.E., Hermjakob, H., Huang, S., Julian, R.K., Kapp, E., McComb, M.E., Oliver, S.G., Omenn, G., Paton, N.W., Simpson, R.,Smith, R., Taylor, C.F., Zhu, W., Aebersold, R., 2004. A common open representation of mass spectrometry data and its application to proteomics research. Nat Biotechnol, 22 (11), 1459-66.
72. Wang, W., Zhou, H., Lin, H., Roy, S., Shaler, T.A., Hill, L.R., Norton, S., Kumar, P., Anderle, M., Becker, C.H., 2003. Quantification of proteins and metabolites by mass spectrometry without isotopic labeling or spiked standards. Anal Chem, 75 (18), 4818-26.
73. Hastings, C.A., Norton, S.M., Roy, S., 2002. New algorithms for processing and peak detection in liquid chromatography/mass spectrometry data. Rapid Commun Mass Spectrom. 16 (5), 462-7.

74. Smith, C.A., Want, E.J., O'Maille, G., Abagyan, R., Siuzdak, G., 2006. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Anal Chem, 78 (3), 779-87.
75. Eanes, R. C., Marcus, R. K., 2000. Peakfitter - an integrated Excel-based Visual Basic program for processing multiple skewed and shifting Gaussian-like spectral peaks simultaneously: Application to radio frequency glow discharge ion trap mass spectrometry. Spectrochim. Acta B. At. Spectrosc. 55B, 403–428.
76. Hermansson, M., Uphoff, A., Käkelä, R., Somerharju, P., 2005. Automated quantitative analysis of complex lipidomes by liquid chromatography/mass spectrometry. Anal Chem, 77 (7), 2166-75.
77. Leptos, K.C., Sarracino, D.A., Jaffe, J.D., Krastins, B., Church, G.M., 2006. MapQuant: open-source software for large-scale protein quantification. Proteomics, 6 (6), 1770-82.
78. Nordström, A., O'Maille, G., Qin, C., Siuzdak, G., Nonlinear data alignment for UPLC-MS and HPLC-MS based metabolomics: quantitative analysis of endogenous and exogenous metabolites in human serum. Anal Chem, 78 (10), 3289-95.
79. Johnson, K.J., Wright, B.W., Jarman, K.H., Synovec, R.E., 2003. High-speed peak matching algorithm for retention time alignment of gas chromatographic data for chemometric analysis. J Chromatogr A, 996 (1-2), 141-55.
80. Scholz, M., Gatzek, S., Sterling, A., Fiehn, O., Selbig, J., 2004. Metabolite fingerprinting: detecting biological features by independent component analysis. Bioinformatics, 20 (15), 2447-54.
81. Katajamaa, M., Miettinen, J., Oresic, M., 2006. MZmine: toolbox for processing and visualization of mass spectrometry based molecular profile data. Bioinformatics, 22 (5), pp 634 - 6.
82. Smith, C.A., Want, E.J., O'Maille, G., Abagyan, R., Siuzdak, G., 2006. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Anal Chem, 78 (3), pp 779 - 87.

83. Lommen, A., 2009. MetAlign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. Anal Chem, 81 (8), 3079-86.
84. Julian, L.G., Andrew, W. N., Clare, A. D., Sarah, H. Hector, C. K., Ina, S.K., John, R. G., Leo, L. C., Philippe, R.S., Denis, V. R., Donald, R., 2007. Standard reporting requirements for biological samples in metabolomics experiments: mammalian/in vivo experiments.Metabolomics, 3 (3), pp 179-188.
85. Royston, G., David, B., Age, K. S., Bruce, S. K., Baker, J. D.,Richard, B.,Conrad, B.,Susan, C.,Giorgio, C.,Andrew, C., Tim, E.,Douglas, B.,Cesare, M.,Jack, N.,Giovanni, P.,Ray, S.,Michael, S.,Joh an, T.,Florian, W., 2007. Proposed minimum reporting standards for data analysis in metabolomics. Metabolomics, 3 (3), pp 231–241.
86. Canfield, P.E., Geerdes, A.M., Molitoris, B.A., 1991. Effect of reversible ATP depletion on tight-junction integrity in LLC-PK1 cells. Am J Physiol, 261 (6 Pt 2), F1038-45.
87. Weinberg, J.M., Davis, J.A., Venkatachalam, M.A., 1997. Cytosolic-free calcium increases to greater than 100 micromolar in ATP-depleted proximal tubules. J Clin Invest, 100 (3), 713-22.
88. Weinberg, J.M., 1985. Oxygen deprivation-induced injury to isolated rabbit kidney tubules. J. Clin Invest, 76 (3), 1193–1208.
89. Wei, Q., Dong, Z., 2012. Mouse model of ischemic acute kidney injury: technical notes and tricks. Am J Physiol Renal Physiol, 303 (11), F1487-94.
90. Yatsu, T., Arai, Y., Takizawa, K., Kasai-Nakagawa, C., Takanashi, M., Uchida, W., Inagaki, O., Tanaka, A., Takenaka, T., 1998. Effect of YM435, a dopamine DA1 receptor agonist, in a canine model of ischemic acute renal failure. Gen Pharmacol, 31 (5), 803-7.
91. Kim, S.J., Lim, Y.T., Kim, B.S., Cho, S.I., Woo, J.S., Jung, J.S., Kim, Y.K., 2000. Mechanism of reduced GFR in rabbits with ischemic acute renal failure. Ren Fail, 22 (2), 129-41.
92. Baker, R.C., Armstrong, M.A., Young, I.S., Mc-Clean, E., O'Rourke, D., Campbell, F.C., D'Sa, A.A., McBride, W.T., 2006. Methyl prednisolone increases urinary nitrate concentrations and reduces subclinical renal injury during infrarenal aortic ischemia reperfusion. Ann Surg, 244 (5), 821–826

93. Bhalodia, Y., Kanzariya, N., Patel, R., Patel, N., Vaghasiya, J., Jivani, N., Raval, H., 2009. Renoprotective activity of benincasa cerifera fruit extract on ischemia/reperfusion-induced renal damage in rat. Iran J Kidney Dis, 3 (2), 80-5.
94. Damianovich, M., Ziv, I., Heyman, S.N., Rosen, S., Shina, A., Kidron, D., Aloya, T., Grimberg, H., Levin, G., Reshef, A., Bentolila, A., Cohen, A., Shirvan, A., 2006. ApoSense: a novel technology for functional molecular imaging of cell death in models of acute renal tubular necrosis. Eur J Nucl Med Mol Imaging, 33 (3), 281-91.
95. Foglieni, C., Fulgenzi, A., Ticozzi, P., Pellegatta, F., Sciorati, C., Belloni, D., Ferrero, E., Ferrero, M.E., 2006. Protective effect of EDTA preadministration on renal ischemia. BMC Nephrol, 15, 7, 5.
96. Susa, D., Mitchell, J.R., Verweij, M., van de Ven, M., Roest, H., van den Engel, S., Bajema, I., Mangundap, K., Ijzermans, J.N., Hoeijmakers, J.H., de Bruin, R.W., 2009. Congenital DNA repair deficiency results in protection against renal ischemia reperfusion injury in mice. Aging Cell, 8 (2), 192-200.
97. Seok, J., Warren, H.S., Cuenca, A.G., Mindrinos, M.N., Baker, H.V., Xu, W., Richards, D.R., McDonald-Smith, G.P., Gao, H., Hennessy, L., Finnerty, C.C., López, C.M., Honari, S., Moore, E.E., Minei, J.P., Cuschieri, J., Bankey, P.E., Johnson, J.L., Sperry, J., Nathens, A.B., Billiar, T.R., West, M.A., Jeschke, M.G., Klein, M.B., Gamelli, R.L., Gibran, N.S., Brownstein, B.H., Miller-Graziano, C., Calvano, S.E., Mason, P.H., Cobb, J.P., Rahme, L.G., Lowry, S.F., Maier, R.V., Moldawer, L.L., Herndon, D.N., Davis, R.W., Xiao, W., Tompkins, R.G., 2013. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A., 110 (9), 3507-12.
98. Gutierrez, K., Dicks, N., Glanzner, W.G., Agellon, L.B., Bordignon, V., 2015. Efficacy of the porcine species in biomedical research. Front Genet, 6, 293.
99. Huppertz, N.D., Tolba, R.H., Grosse, J.O., Micturition in Göttingen minipigs: first reference in vivo data for urological research and review of literature. Lab Anim, 49 (4), 336-44.

100. Liu, Y., Yan, S., Ji, C., Dai, W., Hu, W., Zhang, W., Mei, C., 2012. Metabolomic changes and protective effect of (L)-carnitine in rat kidney ischemia/reperfusion injury. Kidney Blood Press Res, 35 (5), pp 373 – 81.
101. Wei, Q., Xiao, X., Fogle, P., Dong, Z., 2014. Changes in metabolic profiles during acute kidney injury and recovery following ischemia/reperfusion. PLoS One, 9 (9), e106647.
102. Chouchani, E.T., Pell, V.R., Gaude, E., Aksentijević, D., Sundier, S.Y., Robb, E.L., Logan, A., Nadtochiy, S.M., Ord, E.N., Smith, A.C., Eyassu, F., Shirley, R., Hu, C.H., Dare, A.J., James, A.M., Rogatti, S., Hartley, R.C., Eaton, S., Costa, A.S., Brookes, P.S., Davidson, S.M., Duchen, M.R., Saeb-Parsy, K., Shattock, M.J., Robinson, A.J., Work, L.M., Frezza, C., Krieg, T., Murphy, M.P., 2014. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature, 515 (7527), pp 431 – 5.
103. Gardner, D.S., Welham, S.J., Dunford, L.J., McCulloch, T.A., Hodi, Z., Sleeman, P., O'Sullivan, S., Devonald, M.A., 2014. Remote conditioning or erythropoietin before surgery primes kidneys to clear ischemia-reperfusion-damaged cells: a renoprotective mechanism?. Am J Physiol Renal Physiol, 306 (8), F 873 - 84.
104. Want, E.J., Masson, P., Michopoulos, F., Wilson, I.D., Theodoridis, G., Plumb, R.S., Shockcor, J., Loftus, N., Holmes, E., Nicholson, J.K., 2013. Global metabolic profiling of animal and human tissues via UPLC-MS. Nat Protoc, 8 (1), pp17-32.
105. Creek, D.J., Jankevics, A., Burgess, K.E., Breitling, R., Barrett, M.P., 2012. IDEOM: an Excel interface for analysis of LC-MS-based metabolomics data. Bioinformatics, 28 (7), 1048-9.
106. Scheltema, R.A., Jankevics, A., Jansen, R.C., Swertz, M.A., Breitling, R., 2011. PeakML/mzMatch: a file format, Java library, R library, and tool-chain for mass spectrometry data analysis. Anal Chem, 83 (7), 2786-93.
107. Smith, C.A., Want, E.J., O'Maille, G., Abagyan, R., Siuzdak, G., 2006. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Anal Chem, 78 (3), 779-87.

108. Xia, J., Psychogios, N., Young, N., Wishart, D.S., 2009. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Res, 37(Web Server issue), W652-60.
109. Leader, D.P., Burgess, K., Creek, D., Barrett, M.P., 2011. Pathos: a web facility that uses metabolic maps to display experimental changes in metabolites identified by mass spectrometry. Rapid Commun Mass Spectrom, 25 (22), 3422-6.
110. Burch, T.C., Isaac, G., Booher, C.L., Rhim, J.S., Rainville, P., Langridge, J., Baker, A., Nyalwidhe, J.O., 2015. Comparative Metabolomic and Lipidomic Analysis of Phenotype Stratified Prostate Cells. PLoS One, 10 (8), e0134206.
111. Schulman, G., 2012. A nexus of progression of chronic kidney disease: tryptophan, profibrotic cytokines, and charcoal. J Ren Nutr, 22 (1), 107-13.
112. Lau, T., Owen, W., Yu, Y.M., Noviski, N., Lyons, J., Zurakowski, D., Tsay, R., Ajami, A., Young, V.R., Castillo, L., 2000. Arginine, citrulline, and nitric oxide metabolism in end-stage renal disease patients. J Clin Invest, 105 (9), 1217-25.
113. Cherla, G., Jaimes, E.A., 2004. Role of L-arginine in the pathogenesis and treatment of renal disease. J Nutr, 134 (10 Suppl), 2801S-2806S, discussion 2818S-2819S.
114. Posset, R., Opp, S., Struys, E.A., Völkl, A., Mohr, H., Hoffmann, G.F., Kölker, S., Sauer, S.W., Okun, J.G., 2014. Understanding cerebral L-lysine metabolism: the role of L-pipecolate metabolism in Gcdh-deficient mice as a model for glutaric aciduria type I. J Inherit Metab Dis, 38 (2), 265-72.
115. Chouchani, E.T., Pell, V.R., Gaude, E., Aksentijević, D., Sundier, S.Y., Robb, E.L., Logan, A., Nadtochiy, S.M., Ord, E.N., Smith, A.C., Eyassu, F., Shirley, R., Hu, C.H., Dare, A.J., James, A.M., Rogatti, S., Hartley, R.C., Eaton, S., Costa, A.S., Brookes, P.S., Davidson, S.M., Duchen, M.R., Saeb-Parsy, K., Shattock, M.J., Robinson, A.J., Work, L.M., Frezza, C., Krieg, T., Murphy, M.P., 2014. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature. 515 (7527), 431-5.
116. Zhang, Z.H., Wei, F., Vaziri, N.D., Cheng, X.L., Bai, X., Lin, R.C., Zhao, Y.Y., 2015. Metabolomics insights into chronic kidney disease and

modulatory effect of rhubarb against tubulointerstitial fibrosis. Sci Rep, 28, 5, 14472.

117. Kopple, J.D., 2007. Phenylalanine and tyrosine metabolism in chronic kidney failure. J Nutr, 137 (6 Suppl 1), 1586S-1590S; discussion 1597S-1598S.
118. Zacharias H.U., Schley, G., Hochrein, J., Klein, M.S., Köberle, C., Eckardt, K., Willam, C., Oefner, P.J., Gronwald. W., 2013. Analysis of human urine reveals metabolic changes related to the development of acute kidney injury following cardiac surgery. Metabolomics, 9, 697.
119. Tanaka, Y., Kume, S., Araki, H., Nakazawa, J., Chin-Kanasaki, M., Araki, S., Nakagawa, F., Koya, D., Haneda, M., Maegawa, H., Uzu, T., 2015. 1-Methylnicotinamide ameliorates lipotoxicity-induced oxidative stress and cell death in kidney proximal tubular cells. Free Radic Biol Med, 89, 831-41.