The metabolomics of acute alcohol abuse

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ABSTRACT

Alcohol is a substance used and abused by many individuals. The metabolic perturbations caused by excessive alcohol consumption are widespread throughout the human body. One of the primary consequences of alcohol abuse, particularly acute alcohol abuse, is very high levels of NADH formed from excessive ethanol oxidation. A high NADH:NAD+ ratio shifts the redox potential of the cells, shifting the normal physiological equilibrium, particularly within NAD-dependent dehydrogenase-catalyzed reactions. These particular reactions occur within various metabolic pathways, such as: citric acid cycle, glycolysis and branched-chain amino acid catabolism. As such, a disruptive effect within these metabolic pathways results in the slight accumulation of perturbation markers that can be associated with alcohol abuse. Isolation and identification of these widespread perturbation markers is difficult as they only occur in quantities only slightly higher than normal physiological values. Metabolomics makes for a very aptly used technique as it takes a holistic approach, taking into consideration the entire metabolic profile; and, with the aid of bioinformatics, is able to isolate and identify particular variables/metabolites of interest and accredit them as the variables responsible for the greatest variation between control and experimental groups. A novel approach used within this investigation effectively reduced the voluminous metabolomics data generated allowing for more efficient multivariate analysis. Application of three separate statistical models, namely: 1) Unfolding PCA, 2) Cross-sectional PCA, and 3) ANOVA-Simultaneous Component Analysis (ASCA), were used for analyzing the complex 3-dimensional data set created within this acute alcohol abuse investigation. Each model presented certain strengths and difficulties. Taking into consideration the results from all 3 models, the first phase of this investigation confidently illustrates the differentiation between control cases and individuals administered an acute alcohol dose and, subsequently allow for variables responsible for this separation to be: identified as variables of importance, selected and categorized into specific pathways and, finally, labeled as perturbation markers. Through experimental observation it was noted that a large number of perturbation markers associated with the branched-chain amino acid pathway were present within the experimental cases. A hypothesis was created from this observation, re-enforcing the principle that metabolomics is a hypothesis-generating system. The subsequent second phase of this investigation involves a targeted experimental protocol aimed at evaluating the proposed hypothesis, with a focus on three secondary metabolites of the isoleucine catabolism pathway (ethylhydracrylic acid, tiglylglycine and 2-methyl-3-hydroxybutyric acid). Results of this targeted approach show a definite perturbance, similar to a very minor inherited metabolic disorder, occurs within the isoleucine catabolism pathway in response to an acute alcohol dose. As to our knowledge, no information pertaining to the influences of acute alcohol abuse (or even chronic alcohol abuse) within the branched-chain amino acid pathway exists within the current literature, as of date. As such, the experimental observations presented and evaluated within this investigation provide a novel and more in-depth insight into the ethanol-induced perturbances within human metabolism.

CONTENTS

1. Introduction
2. Literature Overview of Ethanol Metabolism and Perturbances Induced by Acute Consumption
    - 2.1 Characteristics of Ethanol
    - 2.2 Primary Metabolism of Ethanol
    - 2.3 Ethanol-induced Toxicity
    - 2.4 Consequences of Increased NADH:NAD^+
        - 2.4.1 Depression of Glycolysis
        - 2.4.2 Depression of Citric Acid Cycle
        - 2.4.3 Hyperlacticacidemia
        - 2.4.4 Opposition toward Gluconeogenesis
        - 2.4.5 Ethanol-induced Ketoacidosis
        - 2.4.6 Altered Lipid Metabolism
        - 2.4.7 Hypoxia
        - 2.4.8 Hyperuricemia
    - 2.5 Additional Ethanol-induced Perturbances
    - 2.6 Metabolomics and Acute Alcohol Consumption
    - 2.7 Experimental Aim
3. Methodology of Metabolomics
    - 3.1 Experimental Design
    - 3.2 Organic Acid Analysis
    - 3.3 Orientation: Effects of Time and Repeatability on the Organic Acid Profile
    - 3.4 Amino Acid Analysis
    - 3.5 Acylcarnitine Analysis
    - 3.6 Software Analysis
    - 3.7 Biostatistics
    - 3.8 Determining Breath/Blood Ethanol Concentration
        - 3.8.1 Lion Alcometer® 500
        - 3.8.2 Quantichrom Ethanol Assay Kit (DIET-500)
4. Experimental Subjects
    - 4.1 Pilot Study (low alcohol dose)
    - 4.2 Acute Alcohol Dose & Controls
    - 4.3 Acute Alcohol Dose and/or Isoleucine Loading (large sample set)
    - 4.4 Acute Alcohol Dose and/or Valine/Leucine Loading
5. Time-dependent Analysis of Metabolic Perturbations Associated with Acute Alcohol Consumption
    - 5.1 Pilot Study (low alcohol dose)

5.1.1 Experimental Protocol
5.1.2 Results and Orientation
5.1.3 Determination of Breath Alcohol Concentration
5.2 Time-dependent Metabolomic Analysis of Acute Alcohol Consumption
5.2.1 Experimental Protocol
5.2.2 Data Reduction
5.2.2.1 Original Data
5.2.2.2 Statistical Reduction
5.2.2.3 Biological Filter
5.2.2.4 Final Consolidated Data Matrix
5.2.2.5 Evaluation
5.2.3 Application of Multivariate Models on Consolidated Reduced Data Matrix
5.2.3.1 Unfolding PCA
5.2.3.2 Cross-sectional PCA
5.2.3.3 ANOVA-Simultaneous Component Analysis (ASCA)
5.3 Hypothesis Formulation
6. Hypothesis Evaluation
6.1 Motivation for Hypothesis
6.2 Targeted Study into Ethanol-induced Perturbances within Isoleucine (Ile) Pathway
6.2.1 Experimental Protocol
6.2.2 Results
6.2.2.1 Determination of Blood Alcohol Concentration (via Ethanol Assay Kit)
6.2.2.2 Organic Acid Analysis
6.2.2.2.1 Postulated Results
6.2.2.2.2 Experimental Results
6.2.2.3 Amino Acid Analysis
6.2.2.4 Acylcarnitine Analysis
7. Discussion
8. Future Prospects
9. Acknowledgements
10. References
11. Appendix 1 – Additional Metabolite Information
12. Appendix 2 - Questionnaire
13. Appendix 3 – Informed Consent Form

1. INTRODUCTION

Alcohol is a widely available psychoactive drug that acts as a central nervous system (CNS) depressant and is used excessively by many individuals. According to the Diagnostic and Statistical Manual (revised third edition (DSM-III-R) and fourth edition (DSM-IV)) of the American Psychiatric Association (APA), two types of patterns have been assigned to excessive alcohol use: dependence and abuse. Dependence refers to the psychological and/or physiological factors associated with diminished volitional control over alcohol use, and abuse indicating consequences of alcohol use. More specifically, DSM-IV defines alcohol abuse as the: *"maladaptive pattern of alcohol use leading to clinically significant impairment or distress"* [1,2].

Alcohol abuse can be subdivided into two distinct drinking patterns: acute and chronic.

- **Acute** alcohol consumption, also termed as "binge drinking", is defined by the National Institute of Alcohol Abuse and Alcoholism (NIAAA) as the consumption of 5 or more drinks (males) or 4 or more drinks (females) within 2 hours, resulting in a blood alcohol concentration of approximately 0.08%. A standard 'drink' consisting of approximately 12.5g ethanol (e.g. 360ml beer, 150ml wine or 45ml 80-proof distilled spirits) [3].
- **Chronic** alcohol consumption is viewed as excessive alcohol consumption (more than 5 drinks (males) or 4 drinks (females) per day) over a long period of time [3].

The clinical consequences of acute alcohol abuse, and especially chronic alcohol abuse, has been addressed by numerous researchers over the years, resulting in multiple publications aimed at improving on the existing literature of ethanol-induced metabolic perturbations within the human body. Almost all of this research incorporates traditional methodology; however, due to numerous recent technological advances, a new scientific technique known as metabolomics has emerged, allowing more in depth analysis of metabolic perturbations.

The science of metabolomics is aimed at the simultaneous analysis of multiple metabolites, and per implication, their associated metabolic pathways, thereby capturing the status of the diverse biochemical pathways at a particular moment in time (i.e. a metabolic snapshot) defining all/any metabolic perturbations. Metabolomics thus incorporates a holistic approach for the identification and quantification of small metabolites within the metabolome. The metabolome being defined as the global collection of all low molecular weight molecules (metabolites) within a cell or organism [4,5,6,7,8,9].

Metabolomics encompasses various approaches and platforms that survey for global changes in numerous metabolic pathways. These approaches/platforms include: NMR-MS (nuclear magnetic resonance-mass spectroscopy), GC-MS (gas chromatography-mass spectroscopy), LC-MS (liquid chromatography-mass spectroscopy), CE-MS (capillary electrophoresis-mass spectroscopy) and LCECA

(liquid chromatography electrochemical array detection). Each of these hyphenated platforms involves a separation process (e.g. gas chromatography) prior to a detection process (e.g. mass spectroscopy). Each technique has its strengths and weaknesses. GC-MS, in particular, is a combined system where volatile and thermally stable compounds are first separated by gas chromatography and then eluting compounds are detected by electron impact mass spectroscopy. GC-MS allows rapid identification and quantification of volatile, non-polar metabolites, such as organic acids and metabolic derivatives of ethanol, with a high degree of sensitivity and chromatographic resolution [4,8,10].

Multivariate statistical analysis is used for the processing and interpretation of the large amounts of metabolic data generated by a metabolomics approach. Methods of analysis can be classified as either supervised or unsupervised pattern recognition methods. Supervised pattern recognition methods uses group membership information, as well as the metabolomic profile data, to build statistical models that attempt to explain the group separation. These models include, amongst others: partial least squares-discriminant analysis (PLS-DA) and ANOVA-simultaneous component analysis (ASCA). The objective of PLS-DA is to predict group membership (dependent variables) using the metabolite profile (independent variables), by reducing the dimension of the data matrix in such a way that the relationship with the dependent variable is retained [42]. When this model is used for prediction, a validation is required. The objective of the ASCA model is described in more detail later. In the case of unsupervised methods, which includes: principal component analysis (PCA) and hierarchical cluster analysis, no group membership is used in the statistical modeling of the metabolomic profile data; however, the group membership variable is used as a labeling variable to identify the natural grouping (perturbation) that exists in the data, and that are described and/or revealed by these statistical models. PCA is a powerful and important method of analysis and is best described by Coen *et al*: "*PCA groups data in an unbiased way and is an "unsupervised" approach; inherent clustering behavior of samples is ascertained with no prior knowledge of class membership. Of n principal components (PCs) identified by the analysis, the first (PC1) is a linear combination of the original input variables and describes the largest variation in the data set. The second (PC2) describes the next-largest variation. When 2 PCs have been defined, they constitute a plane. Projection of the observation vectors in the multidimensional space onto this plane enables the data to be visualized in a 2-dimensional map known as a "score-plot". This plot reveals inherent clustering of groups of data based on the closeness or similarity of their input coordinates*" [11].

Bioinformatics is a vitally important component for processing and interpreting the multitude of data generated by taking a metabolomics approach; however, the focus of this investigation is on the biochemical aspect of acute ethanol abuse. The unique characteristics of ethanol and the subsequent global perturbations within normal human metabolism, associated with excessive alcohol consumption, yields a perplexing problem as typically traditional methods would only be able to observe certain pathways and monitor major perturbations. The perturbations associated with ethanol abuse, particularly acute ethanol abuse, are often widespread across numerous, often minor, pathways (i.e. slightly outside normal physiological ranges). The scientific value of using a metabolomics approach in investigating the

perturbations associated with ethanol consumption was recently very clearly articulated by Harrigan *et al*: "*The most important value that metabolomics may add to alcohol-associated research is the increased number of individual metabolites within different metabolite classes that can be analyzed, thereby allowing researchers to gain a greater understanding of distinct biochemical processes associated with these metabolites*" [12].

Metabolomics has shown to be an aptly used technique for differentiating between alcoholics and abstainers (individuals who do not consume alcohol) in my BSc Honns study in 2008. That study also showed that a more structured, homogenous sampling protocol was needed in order to successfully identify metabolites of interest that could account for the differentiation. The experimental design of this current investigation is aimed at not only identifying particular metabolites associated with ethanol induced perturbations within the human body (i.e. perturbation markers), but also at differentiating between a physiological state void of alcohol vs. the perturbed physiological state induced by acute ethanol consumption within a defined, homogenous experimental group. An interesting area of focus will be the ethanol-induced metabolic perturbations as a consequence of increased NADH:NAD^+ ratio caused by ethanol abuse, as discussed in detail within the following investigation.

2. LITERATURE OVERVIEW OF ETHANOL METABOLISM AND PERTURBANCES INDUCED BY ACUTE CONSUMPTION

2.1 Characteristics of Ethanol

Ethanol (EtOH), also known as ethyl alcohol or simply alcohol, is colorless, aliphatic and weakly polar. As a chemical its characteristics include: -114.1 °C melting temperature, 78.5 °C boiling temperature, density of 0.789 g/mol at 20 °C and a molar mass of 46.068 g/mol [13]. Ethanol (CH_3CH_2OH) contains a hydrophobic hydrocarbon end and a hydrophilic hydroxyl end, making it miscible in both aqueous and organic solutions.

Ethanol provides a substantial source of metabolic energy, with 7.1 kcal (29.6 kJ) per gram, a value that exceeds the energy content of carbohydrates or proteins. In the case of heavy chronic ethanol consumers ethanol often constitutes approximately 50% of their total daily caloric intake. Ethanol, therefore, displaces important nutrients of a normal diet resulting in primary malnutrition (i.e. deficiencies of important vitamins (e.g. thiamine and folate)) and impairment of various normal physiological functions/pathways (e.g. perturbances within the gastrointestinal tract and liver), leading to malabsorption and ultimately secondary malnutrition [14,15,16].

The miscible nature of ethanol permits interactions with the phospholipid bilayer membrane of cells resulting in altered morphology, function and permeability. Ethanol is therefore able to diffuse easily across all cell membranes, the result being rapid absorption across the gastrointestinal tract, into the blood circulatory system and distribution throughout the body, exerting effects on most organ systems, even penetrating the blood brain barrier (BBB) and placenta.

2.2 Primary Metabolism of Ethanol

Only 2-10% of absorbed ethanol is eliminated through the kidneys and lungs [15], the rest (approximately 90%) is oxidized within the liver, making the liver the primary organ for the oxidative metabolism of ethanol and site for direct toxicity. Thus, it is not surprising that the abuse of ethanol and ethanol-associated disorders/diseases are traced back to perturbations within the liver morphology and physiology (i.e. alcohol-induced liver damage).

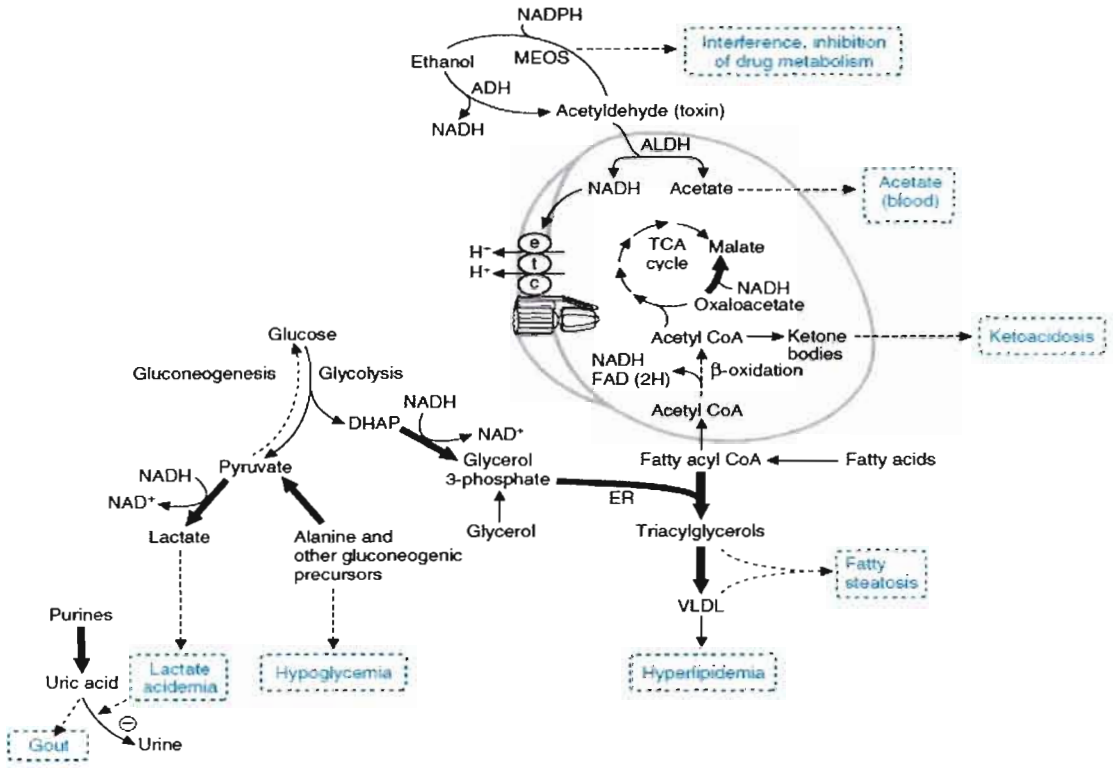
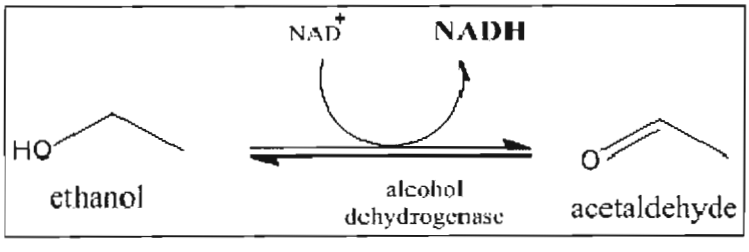


Figure 1: Perturbances and toxic effects linked to oxidation of ethanol within hepatocyte (Legend: dotted lines (- - -) indicates depression by ethanol, main arrows(➡) indicates stimulation, text in blue indicates disturbance induced by ethanol oxidation, MEOS = microsomal ethanol oxidation system, DHAP = dihydroxyacetone phosphate, VLDL = very low density lipoprotein) [17].

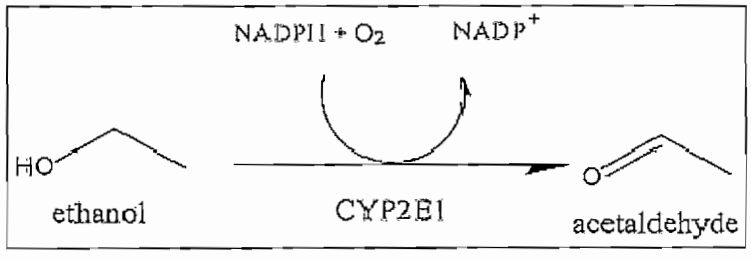
The model shown in Figure 1 is taken from the literature [Smith, C. Marks' Basic Medical Biochemistry, a Clinical Approach, 2nd Edition] and illustrates the primary hepatic pathways of ethanol metabolism as a consequence of acute alcohol consumption, including the consequential perturbations within other normal physiological pathways. Three main oxidative pathways of ethanol metabolism within the hepatocyte have been elucidated, as of date, each depending on different enzymes and cofactors, within different subcellular compartments. Each of these three pathways produces acetaldehyde, a reactive intermediate, which is further oxidized to produce the end product acetate.

The primary pathway of ethanol metabolism occurs within the cytosol and is catalyzed by alcohol dehydrogenase (ADH), a human dimeric metalloenzyme which occurs as various isoforms within the stomach and liver and with variable affinities toward ethanol:



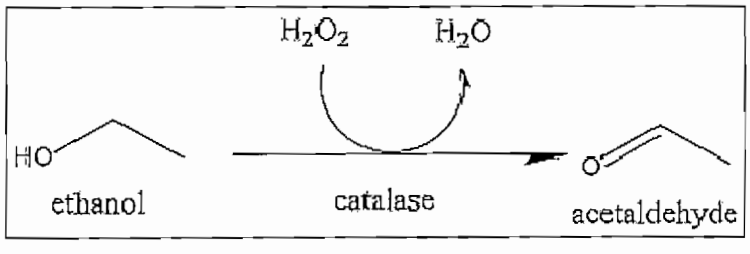
In this reaction ethanol loses a hydrogen ion (H^+) and causes the reduction of cofactor nicotinamide dinucleotide (NAD^+) to its reduced form NADH. Excess ethanol leads to the excess accumulation of reducing equivalents, a consequence of which is a noticeable shift in the reducing redox potential of the cytosol. This ADH-mediated pathway metabolizes the majority of ethanol when consumed in moderate doses.

At elevated concentrations of ethanol, particularly chronic ethanol consumption, the body cannot physiologically cope, the ADH-mediated pathway is overwhelmed and a second pathway becomes active. This second pathway is catalyzed by an ethanol-inducible isoform of cytochrome P450 known as cytochrome P450 2E1 (CYP2E1):



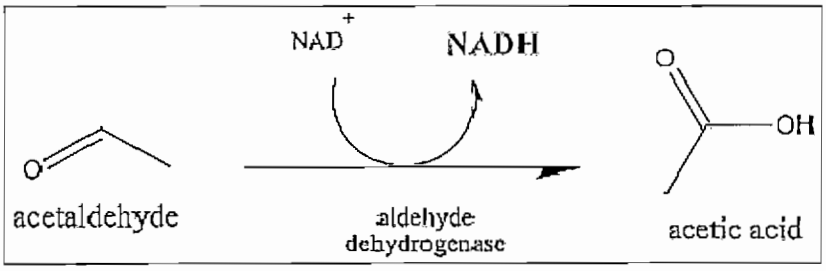
This NADPH-dependent pathway occurs within the microsome and is commonly referred to as the microsomal ethanol-oxidizing system (MEOS). CYP2E1 contributes to less than 10% of the overall hepatic oxidation of ethanol. The catalytic function of CYP2E1 depends on the transfer of electrons from NADPH to reduce the heme component of cytochrome P450 from its ferric state to its ferrous state. This is necessary to bind molecular oxygen to form an oxygenated CYP2E1 complex that catalyzes the above reaction. The oxygen activation of CYP2E1 results in the production of reactive oxygen species, which will be discussed more later.

In the case of heavy chronic ethanol consumers there is an accumulation of fatty acids in the liver, due to increased peroxisomal oxidation of fatty acids, which allows for a third prominent oxidative pathway of ethanol metabolism to be become active:



This catalase-catalyzed reaction requires a H_20_2-generating system. Kupffer cells, which are activated by endotoxins such as ethanol, activate this third ethanol metabolic pathway by production of mediators (e.g. prostaglandins) that inhibit lipoproteins lipase (i.e. inhibit fatty acid catabolism), resulting in an accumulation of fatty acids within the liver, necessary for the generation of H_2O_2 by means of peroxisomal β-oxidation. This catalase-dependent pathway can only occur within the peroxisome (i.e. in the absence of cytosolic ADH) as the NADH generated from cytosolic oxidation of ethanol by ADH inhibits β-oxidation of fatty acids.

Acetaldehyde, the toxic oxidative product of ethanol produced by all three of the above described pathways, is further oxidized in the mitochondria by aldehyde dehydrogenase (ALDH):



The oxidation of acetaldehyde involves the reduction of NAD^+, further increasing the NADH pool and contributing to the increased NADH:NAD^+ ratio.

Acetate, the end product of ethanol oxidation, needs to leave the liver and enter the blood circulation to be further metabolized to acetyl-CoA, as liver mitochondria lack the necessary enzyme (acetyl-coA synthase 2), which is abundant in other, extrahepatic (e.g. heart, muscle), tissue. Acetyl-CoA is consequently metabolized to CO_2 by way of the citric acid cycle [3,15-22].

2.3 Ethanol-Induced Toxicology

The metabolism of ethanol not only results in the oxidation of ethanol but also the consequential induction of several global cellular perturbations in response to the toxicological actions of the CYP2E1 pathway. Induction of CYP2E1 by ethanol in the microsomal ethanol-oxidizing system has little effect on the net clearance of ethanol; however, it contributes to forming a cellular environment favorable to oxidative stress.

The oxygenation of ethanol-inducible CYP2E1 into its catalytic active state results in the production of reactive oxygen species (ROS). ROS are small, high reactive, oxygen-containing molecules, such as: superoxide anion radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). These initial oxidants are converted to more powerful hydroxyl radicals and ferryl species in response to increased presence of transition metals (such as iron) due to ethanol consumption. High levels of ROS are toxic to cells because they react with cellular macromolecules, denature proteins (inactivate enzymes) and cause DNA damage (e.g. DNA strand breaks, base removal, base modifications) [18,21,23]. A major consequence of ROS is the formation of mutations and in particular the occurrence of lipid peroxidation. Ethanol-induced lipid peroxidation not only reduces the integrity of cellular membranes but it also results in the production of highly reactive products, such as malondialdehyde and 4-hydroxynonenal [23]. The function of P450 enzymes is to convert compounds into more polar forms that can be easily excreted directly or conjugated by phase II enzymes into more polar excretable metabolites. Ethanol-inducible CYP2E1 thus activates various other compounds into more polar toxic forms, in particular: analgesics (e.g. acetaminophens), anesthetics, hepatotoxins, solvents, carcinogens and various other exogenous drugs/chemicals, thereby increasing vulnerability to ubiquitous xenobiotics [16,21].

The increased occurrence of lipid peroxidation also results in a reduction in mitochondrial membrane potential and permeability. A consequence of which is not only increased ROS production but also: a) altered mitochondrial DNA (mDNA), b) diminished transport systems, c) altered mitochondrial morphology, d) enzyme inactivation (e.g. decreased cytochrome oxidase activity) and e) decreased ATP synthesis (uncoupling of oxidation with phosphorylation); all of which leads to an increased production of pro-apoptotic factors (e.g. caspase 3 activity) [18,23].

One of the diminished transport systems that occur within mitochondria as a result of ROS and lipid peroxidation is the mitochondrial glutathione transporter system. Reduced glutathione (GSH) is a powerful cellular antioxidant, especially against the toxic effects of ethanol. Thus, ethanol consumption not only reduces the levels of GSH by producing oxidants but also inhibits the glutathione transporter system, resulting in a condition that reduces the antioxidative capabilities of the cell [18,21,23]. Ethanol also directly, and indirectly, suppresses various other antioxidants (e.g. α-tocopherol) and antioxidative systems (e.g. superoxide dismutase activity). A ROS production rate that exceeds the rate at which ROS are removed ultimately leads to a state of oxidative stress.

This oxidative stress leads to characteristic, alcohol-associated, clinical injury to the liver. The presence of ROS and lipid peroxidation products acts as activators of hepatic stellate cells (HSC), leading to an increased production of extracellular matrix components (i.e. increased collagen synthesis), known as fibrogenesis, within the liver causing heptomegaly [23]. The induction of the ROS-producing CYP2E1 pathway is particularly damaging when combined with a high-fat diet or in the case of alcoholics, as both these cases will exhibit a fatty liver (steatosis) and the consequential lipid peroxidation will result in the early stages of liver damage. Acetaldehyde, a highly reactive toxic intermediate of ethanol oxidation, combined with a state of elevated lipid peroxidation and protein synthesis within the liver, results in the formation of highly unstable adducts (e.g. malondialdehyde-acetaldehyde protein adduct). These adducts stimulate immune responses by acting as neoantigens, interfere with normal physiological functions (inactivates enzymes, alters DNA repair mechanisms, impairs oxygen utilization) and further contributes to ethanol-induced fibrogenesis by inducing collagen accumulation [16,18]. All of these factors (increased ROS, lipid peroxidation, pro-apoptotic factors, fibrogenesis and acetaldehyde adducts) lead to a clinical state characteristic of ethanol abuse known as ethanol-induced liver damage.

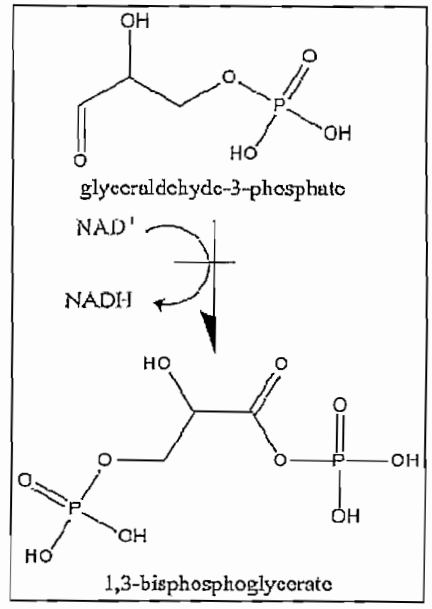
The hepatotoxic nature of the oxidative stress caused by ethanol consumption is typically prevalent in chronic ethanol abusers. This oxidative stress mediated toxicity contributes to one of the most significant global cellular perturbations caused by ethanol consumption (both chronic and acute); namely an increased ratio of NADH to NAD^+. The increased NADH found in alcohol abusers, particularly acute alcohol abuse, and the consequential effects will be the focus of this investigation.

2.4 Consequences of Increased NADH:NAD$^+$

The oxidation of ethanol to acetaldehyde and further oxidation, by ALDH, to acetate generates a large amount of NADH. This accumulation of reducing equivalent overwhelms the hepatocyte's ability to maintain redox homeostasis and a noticeable shift in the redox potential occurs within the cell [16]. A noticeable increase in NADH:NAD$^+$ occurs in both the cytosol and mitochondria. The excess NADH is reoxidized by the mitochondrial electron transport chain to a limited extent but the amount of excess NADH generated by ethanol oxidation overwhelms this system. The mitochondrial membranes are impermeable to NADH, therefore, cytosolic NADH is transported into the mitochondria via a malate-aspartate shuttle transport system [3,18]; however, mitochondrial, low Km ALDH generates the majority of the NADH within the mitochondria. The substantial increase in NADH:NAD$^+$ ratio results in widespread and noticeable global perturbations of numerous normal physiological pathways within the human body. Of notable interest are pathways catalyzed by oxidoreductase enzymes, particularly dehydrogenase catalyzed reactions; all of which are NAD-dependent and as such are directly influenced by alcohol abuse.

2.4.1 Depression of Glycolysis

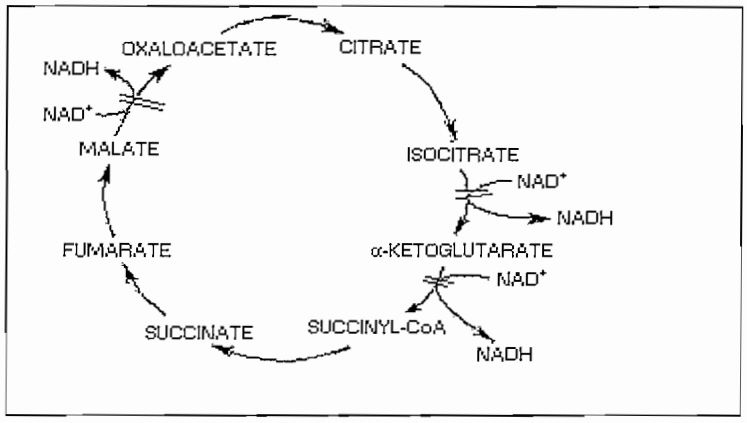
Excess levels of NADH suppress the sixth step of the glycolysis pathway (NAD-dependent dehydrogenation of glyceraldehyde-3-phosphate (G3P) to 1,3-bisphosphogylcerate (BPG)). The result of this inhibition being diminished ATP production within the glycolysis pathway as glucose is unable to be catabolised to appreciable values of cellular pyruvate concentrations. As such the second phase of glycolysis (the ATP-producing phase) becomes depressed and glucose no longer becomes a significant source for energy production. There is also an accumulation of certain glycolysis intermediates (e.g. G3P) and in some cases the occurrence of transient hyperglycemia when ethanol consumption occurs with a meal [17,22].



2.4.2 Depression of Citric Acid Cycle

Three NAD-dependent dehydrogenase-catalyzed reactions occur within the citric acid cycle/tricarboxylic acid (TCA) cycle, namely:

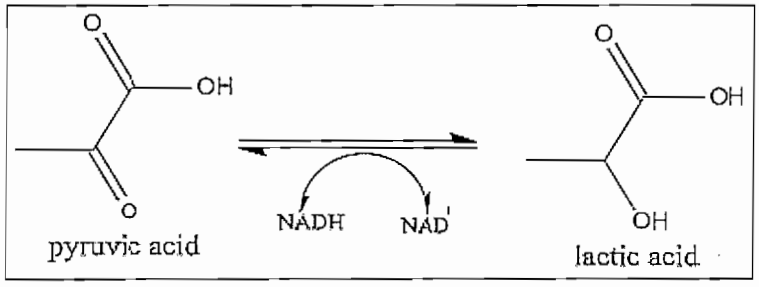
Isocitrate → α-ketoglutarate → succinyl-CoA and malate → oxaloacetate



The increase in mitochondrial NADH in the hepatocytes causes a feedback inhibition of these three dehydrogenase-catalyzed reactions, thereby slowing down the TCA cycle. A consequence of a depressed TCA cycle is not only diminished ATP production but also reduced capacity for the ability to oxidize excess acetyl-CoA created by ethanol oxidation into excretable CO_2, as well as an accumulation of certain TCA cycle intermediates [3,14,15]. In addition, very high NADH:NAD^+ ratios also shift all of the oxaloacetate in the TCA cycle to malate, leaving the oxaloacetate levels too low for citrate synthase to synthesize citrate [17].

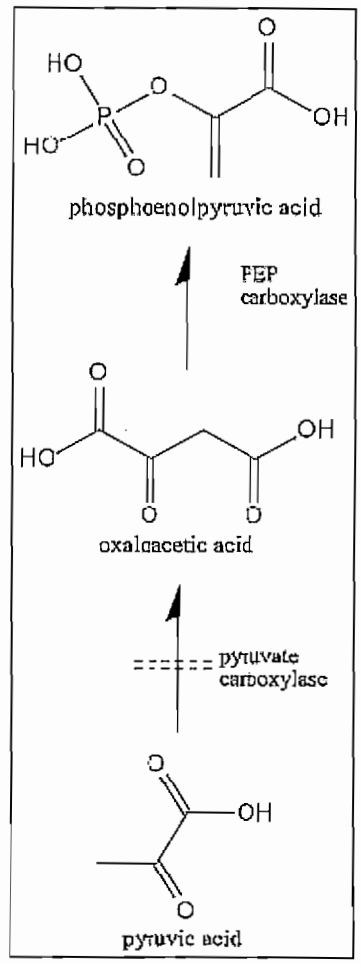
2.4.3 Hyperlacticacidemia

Excess NADH stimulates the conversion of low cellular concentrations of existing pyruvate (depressed glycolysis results in reduced levels of produced pyruvate) into lactate, by means of a NAD-dependent lactate dehydrogenase-catalyzed reaction, by shifting the equilibrium from pyruvate to lactate.



The consequence of which is even further reduced levels of pyruvate and increased levels of lactate (i.e. increased lactate:pyruvate ratio), contributing to a state of lactic acidosis. The reverse reaction of lactate → pyruvate is inhibited by the presence of excess NADH [3,14,15,18,19].

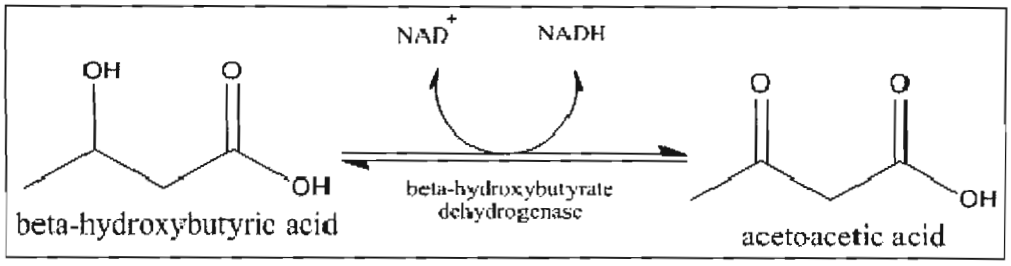
2.4.4 Opposition toward Gluconeogenesis



Excess NADH depresses the glycolysis pathway, as noted previously, and promotes the conversion of existing pyruvate into lactate. These two perturbances, caused by increased NADH:NAD^+, result in low cellular concentrations of pyruvate. The initial step in gluconeogenesis is the carboxylation of pyruvate to oxaloacetate by pyruvate carboxylase. The low concentrations of pyruvate substrate, as well as the low concentrations of oxaloacetate substrate, opposes gluconeogenesis. The shift in equilibrium of lactate dehydrogenase toward lactate consequently blocks all major gluconeogenesis precursors from entering the gluconeogenesis pathway as the pyruvate formed from these precursors is quickly converted into lactate [3,14]. In addition, pyruvate carboxylase, a biotin-dependent enzyme, is allosterically activated by acetyl-CoA. Low concentrations of substrate pyruvate and excessive levels of NADH inhibits the NAD-dependent pyruvate dehydrogenase complex catalyzed reaction of pyruvate → acetyl-CoA within the hepatic mitochondria. Also, as mentioned previously, hepatic mitochondria lack the enzyme acetyl-CoA synthase 2 necessary for synthesis of acetyl-CoA from the excessive amounts of acetate produced by the oxidation of acetaldehyde by ALDH within the mitochondria [3,14,15]. Thus, insufficient activator (acetyl-CoA), reduced bioavailability of dietary biotin due to ethanol-induced malnutrition/malabsorption (prevalent in chronic alcohol abuse cases), a shift in equilibrium of lactate dehydrogenase toward lactate and relatively low concentrations of substrate pyruvate within hepatic mitochondria ensures the suppression of the initial steps of gluconeogenesis. One of the major consequences of reduced gluconeogenesis within individuals in a fasted state is a state of hypoglycemia. This ethanol-induced hypoglycemia consequently leads to a depletion of glycogen reserves, resulting in ketoacidosis, taking into account that the hyperglycemia/hypoglycemia profile is dependent upon the dietary state of the individual [17].

2.4.5 Ethanol-induced Ketoacidosis

The hypoglycemic state induced by reduced gluconeogenesis stimulates an adrenergic response (similar to the effects caused by epinephrine). This hormonal response results in: a subsequent, rapid depletion of glycogen reserves, an increase in fatty acid levels and a massive, but temporary, increase in blood glucose levels. Once the glycogen stores and blood glucose levels are depleted the body turns to an alternative source of energy, namely fatty acids and ketones, and ketoacidosis occurs. An increased ratio of beta-hydroxybutyrate:acetoacetate, a characteristic seen in chronic alcoholics, is indicative of ethanol-induced ketoacidosis.

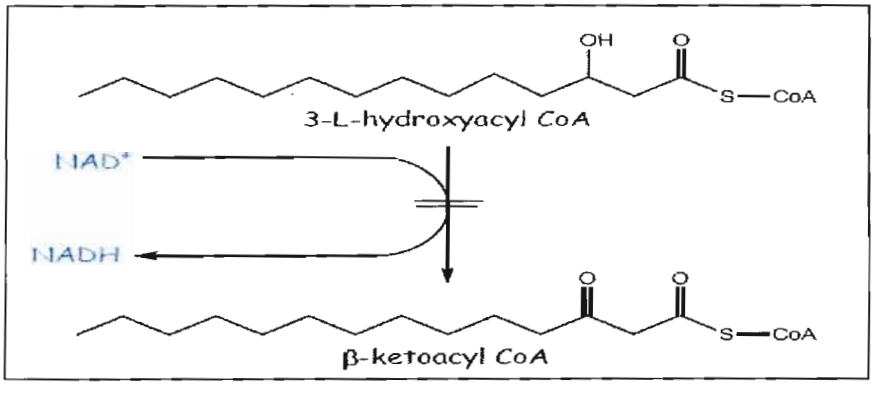


The elevated levels of beta-hydroxybutyrate is a consequence of excess NADH shifting the equilibrium of beta-hydroxybutyrate dehydrogenase from acetoacetate toward beta-hydroxybutyrate. Hypoglycemic stimulated ketoacidosis also ensures a supply of acetoacetate is available. High concentrations of circulating acetyl-CoA, an end product of ethanol metabolism, also contributes to ethanol-induced ketoacidosis by increased synthesis of ketone bodies.

An additional factor contributing to a state of ketoacidosis within ethanol abusers, particularly chronic ethanol users, is the fasted state simulated by malnutrition and malabsorption caused by ethanol abuse. The occurrence of ketoacidosis, combined with hyperlacticacidemia, leads to a state of massive metabolic acidosis [3,18,19, 22,24,25].

2.4.6 Altered Lipid Metabolism

A major perturbance that occurs within individuals that consume excessive amounts of ethanol is an altered lipid metabolism, primarily as a result of high NADH:NAD^+ levels but also due to other ethanol-associated reasons. As noted previously, the presence of ethanol not only causes an accumulation of fatty acid due to the adrenergic response but also activates the Kupffer cells within the liver, which releases mediators (e.g. prostaglandins) that inhibit the actions of lipoprotein lipase (i.e. reduces the degradation of fatty acids). Altered mitochondrial morphology and function, caused by ROS-mediated oxidative stress in response to ethanol abuse, induces altered lipid metabolism. Ethanol-induced hyperlipidemia also occurs as a result of increased levels of α-glycerophosphate, which favors the accumulation of hepatic triglycerides by trapping fatty acids. Increased NADH:NAD^+ ratio, however, is the most prominent cause of altered lipid metabolism as a result of ethanol abuse. The massive shift in the redox state caused by excess NADH not only promotes the synthesis of fatty acids but also inhibits the mitochondrial β-oxidation of existing fatty acids [3,14,15,16,18].



Thus, activated Kupffer cells, dysfunctional mitochondrial physiology, increased levels of α-glycerophosphate, increased NADH:NAD^+ ratio and the fact that ethanol displaces dietary fat as an

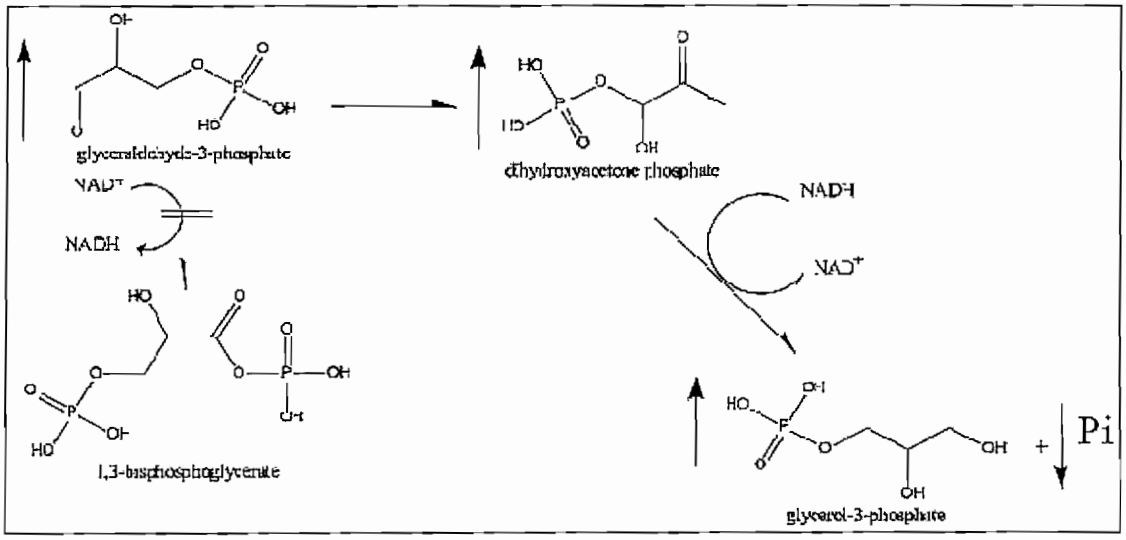
energy source, favors hepatic lipogenesis and causes a state of ethanol-induced hyperlipidemia. The consequences of altered lipid metabolism includes: 1) dysfunctional acylation of membrane phospholipids, 2) hypertriglyceridemia (i.e. increased levels of circulating triglycerides), 3) high HDL (high density lipoprotein) cholesterol in the blood, 4) increased microsomal induction, 5) defective lipoprotein metabolism (increased levels of certain lipoproteins (e.g. chylomicrons, LDL (low density lipoproteins) and VLDL (very low density lipoproteins)) and conformational changes of apolipoproteins from alpha to beta state) and 6) ethanol induced steatosis (fatty liver), hepatitis (necrosis and inflammation of liver) and cirrhosis (fibrosis and distorted hepatic morphology). Ethanol-induced liver damage is a consequence of enhanced lipogenesis and increased ROS production and lipid peroxidation [22,26,27].

2.4.7 Hypoxia

Ethanol directly increases the uptake of molecular oxygen by inducing the CYP2E1 pathway of ethanol oxidation, which requires molecular oxygen for the catalytic activation of CYP2E1, and also by inducing mitochondrial electron transport chain activity (i.e. massive burst of respiration) caused by increased levels of NADH. This increased uptake of molecular oxygen leads to a downstream state of insufficient oxygen known as hypoxia. Ethanol also indirectly increases oxygen utilization by hepatocytes by increasing hepatic metabolic activity through activation of Kupffer cells, further contributing to a state of hypoxia within the hepatocyte. Severe, prolonged hypoxia, a characteristic feature in acute and chronic ethanol abusers, initiates oxidative stress which contributes to ethanol-induced liver damage [3,22].

2.4.8 Hyperuricemia

Decreased glycolysis, due to an increased ratio of NADH:NAD^+, results in an increase in certain glycolysis intermediates, especially glyceraldehyde-3-phosphate (G3P):



An accumulation of G3P leads to an accumulation of dihydroxyacetone phosphate (DHAP), which is metabolized by a NADH-mediated reaction to produce increased levels of glycerol-3-phosphate and decreased levels of inorganic phosphate (Pi). Inorganic phosphate acts as an inhibitor of AMP

deaminase, a rate-limiting enzyme in the adenine nucleotide catabolism pathway. Decreased levels of inorganic phosphate thus removes the inhibition of AMP deaminase and results in increased adenine nucleotide catabolism. Increased levels of NADH also inhibits xanthine dehydrogenase activity resulting in increased levels of xanthine and hypoxanthine, intermediates within the adenine nucleotide catabolism pathway. The end product of adenine nucleotide catabolism is uric acid. The removal of inhibition of rate-limiting AMP deaminase and increased levels of xanthine and hypoxanthine, both of which are a result of ethanol-induced increased NADH:NAD^+ ratio, leads to increased uric acid production and subsequently primary hyperuricemia. Overproduction of acetyl-CoA, as a result of excessive ethanol oxidation, also contributes to enhanced adenine nucleotide catabolism by the subsequent degradation of acetyl-CoA to AMP, which is the initial substrate for the adenine nucleotide catabolism pathway [3,14,15].

In addition to increased uric acid production, elevated levels of NADH also causes decreased excretion and increased reabsorption of uric acid within the kidneys. The shift in the redox potential created by increased NADH:NAD^+ ratio results in hyperlacticacidemia, as noted previously. Both uric acid and lactic acid are transported in co-ordination with each other by means of urate transporter 1 (URAT1) within the kidneys. Excessive levels of lactic acid, as a result of increased NADH levels, results in increased excretion of lactic acid. Transport of lactic acid by URAT1 from the proximal tubular cells to the proximal tubular lumen operates in co-ordination with transport of uric acid from proximal tubular lumen to proximal tubular cells [19,28]. The end results being increased reabsorption of uric acid and competitive inhibition of uric acid excretion, both of which leads to secondary hyperuricemia. Ketoacids are also transported in co-ordination with uric acid. Thus excessive ketoacids formed from ethanol-induced ketoacidosis also contributes to secondary hyperuricemia. Hyperuricemia is not only a consequence but also a response of ethanol abuse. Uric acid scavenges free radicals by chelating transition metal ions and prevents the degradation of extracellular superoxide dismutase, a physiologically important enzyme that functions to maintain levels of oxidants. Thus uric acid can also be viewed as an antioxidant counteracting the oxidative stress produced by ethanol abuse [29]. NOTE: hydrogen peroxide is also formed in the xanthine oxidase reactions used to synthesize uric acid and should also be considered when discussing the oxidative consequences of ethanol abuse.

Figure 2 depicts a model of a summary of the perturbances caused by a high NADH pool as a result of oxidation of ethanol to acetaldehyde by ADH and further oxidation to acetate by ALDH. Excessive ethanol oxidation increases the NADH:NAD^+ ratio, causing a massive accumulation of reducing equivalent NADH, which affects numerous other normal physiological pathways (i.e. global perturbances), in particular NAD-dependent dehydrogenase-catalyzed reactions. Although a large number of ethanol-induced perturbances can be attributed to increased NADH:NAD^+ ratio, several other pathological consequences of alcohol abuse have varying sources of origin.

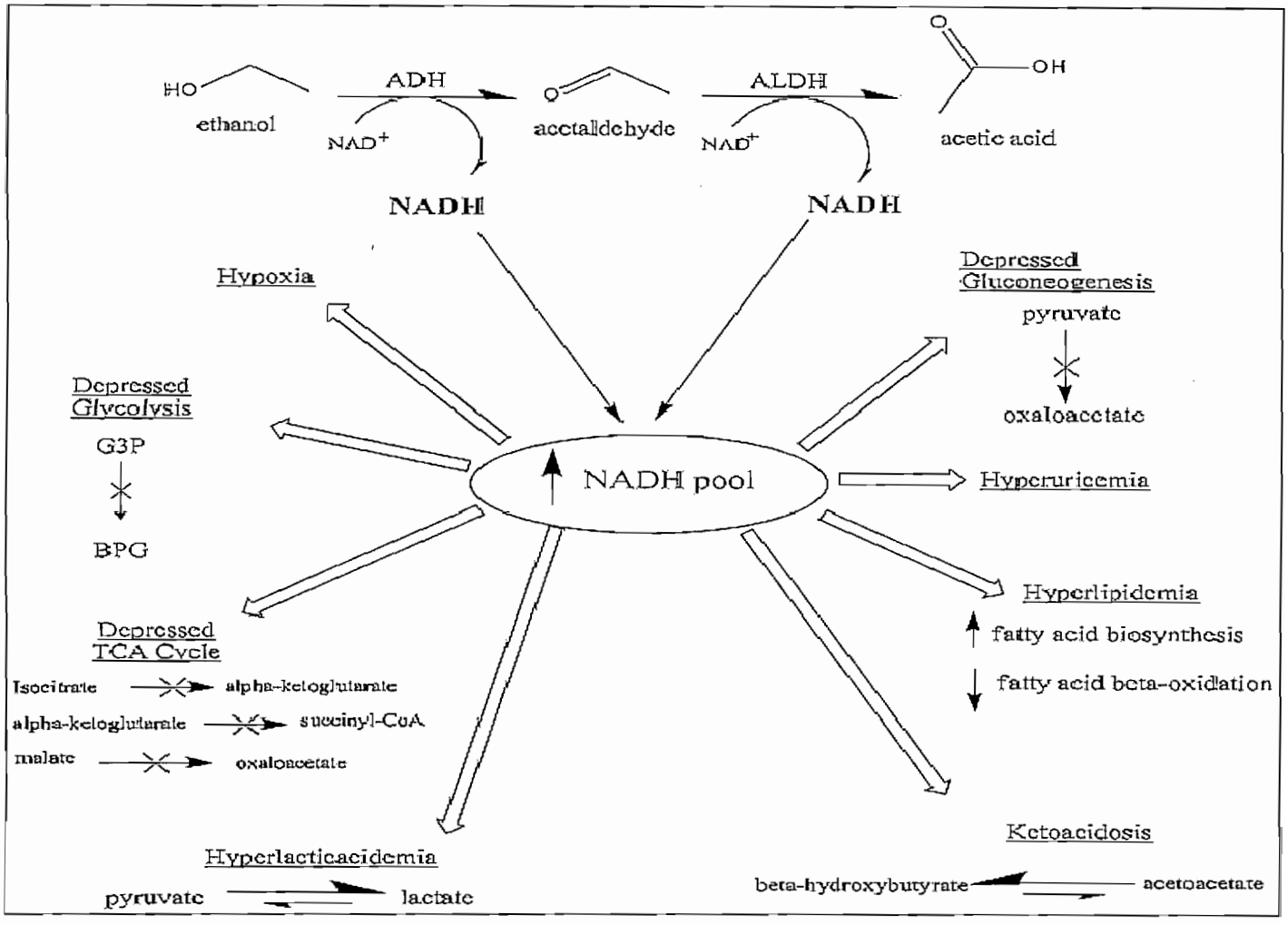


Figure 2: Summarized model of global perturbances induced by excessive NADH as a result of ethanol oxidation (Legend: ⇌ indicates perturbed reaction equilibrium, ⤫ indicates depressed reaction, G3P = glyceraldehydes-3-phosphate, BPG = 1,3-bisphosphoglycerate)

2.5 Additional Ethanol-induced Perturbances

Various other global perturbations occur within the human body in response to ethanol abuse, such as: altered metabolism of vitamins [16], altered hormone and steroid metabolism [19], inflammatory cytokines and immunological derangements, carcinogenesis [30], contributing hepatotoxic elements (e.g. cigarette smoking, pharmaceutical drugs), comorbid conditions (e.g. hepatitis, AIDS, diabetes) [3], fetal alcohol syndrome, alterations in various transport systems etc. These factors, albeit important consequences of ethanol abuse, fall outside the scope of this investigation and consequently are not addressed within this literature overview. In addition, several biomarkers (e.g. CDT, GGT etc.) currently exist to assess alcohol intake [52], these biomarkers, however, while noted, will not be discussed in this investigation.

2.6 Metabolomics and Acute Alcohol Consumption

As of date, all of the information regarding ethanol metabolism and the perturbances induced by acute consumption, as described within the literature overview, has been founded upon research from

numerous sources; however, all of this research involved a traditional (conventional) mindset where focus was placed upon one particular perturbation/pathway and analyzed accordingly. Harrigan *et al.* very clearly highlighted the possible value of metabolomics in alcohol research, as quoted previously, by suggesting that metabolomics could potentially increase our knowledge on metabolic perturbations associated with alcohol abuse by increasing the number and type of metabolites measured within a biological sample. Metabolomics has many definitions.

- Nicholson (2006) [43] defines metabolomics as the quantitative analysis of all the metabolites, commonly referred to as the metabolome. The term metabolome was first used by Olivier *et al.* (1998) [44] to describe the set of metabolites synthesized by an organism, in a fashion analogous to that of the genome and proteome. This definition has been limited to: "*the quantitative complement of all of the low molecular weight molecules present in cells in a particular physiological or developmental state*".
- The term metabolomics was first used by Fiehn [45] and defined as: "*a comprehensive analysis in which all metabolites of a biological system were identified and quantified*".

From these two definitions it is clear that metabolomics is a broad, non-targeted approach toward analyzing the metabolome. Metabolites, however, are linked as the biological consequences of the transcription of genes. Thus, metabolomics can also be defined as the characterization of metabolic phenotypes and the linking of these phenotypes to their correspondent genotypes [46]. Metabolomics is thus only one of the 'omics' (others include: genomics, transcriptomics, proteomics [47]) when considering the complete physiological profile of an individual. As such, based upon the understanding of metabolomics, a holistic approach toward understanding the metabolic perturbations associated with acute alcohol consumption should yield a large range of metabolites from various different metabolic pathways and, through the application of bioinformatics, allow for a more in depth and novel understanding of acute alcohol abuse.

2.7 Experimental Aim

Based on this overview, the focus of the investigation will be on: the metabolic consequences of an acute dose of alcohol, the isolation of particular variables/metabolites of interest by statistical and biochemical means and linking/associating these metabolites of interest to their respective pathways. The aim of this investigation can hence be addressed by proposing four basic questions:

1) Can a metabolomics approach be successful in differentiating between a non-intoxicated physiological state and an alcohol-intoxicated state? (i.e. can metabolomics be used to identify individuals that have recently consumed an acute dose of alcohol?)
2) If so, can the variables responsible for the differentiation be statistically isolated, identified and given biochemical significance?
3) Can these variables of importance (VIPs) be biochemically assigned as ethanol-induced perturbation markers? (i.e. can these metabolites be validated as a consequence of an acute alcohol dose?)

NOTE: the term VIP is more precisely defined as variables important in projection (VIP) used for various statistical models; however, it is loosely referred to as variables of importance (VIPs) within this investigation.

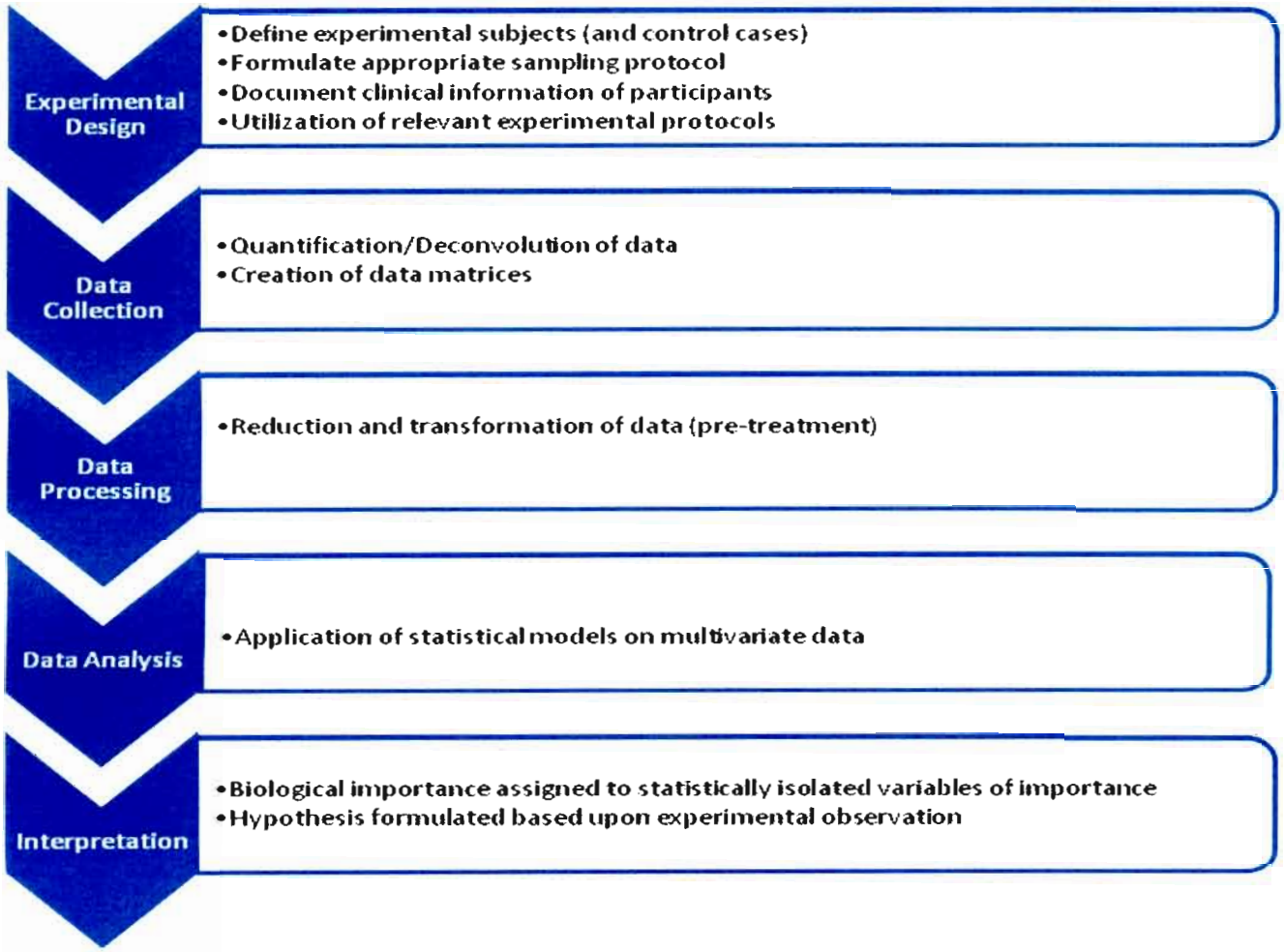
4) Can these results be used to generate a relevant hypothesis that can be subsequently tested?

   NOTE: it is generally accepted that metabolomics is a hypothesis-generating scientific method, rather than a hypothesis verification method.

The defined and methodical metabolomic approach taken within this investigation, aimed at addressing these four proposed questions, is described in detail in the following section.

3. METHODOLOGY OF METABOLOMICS

A very important aspect when approaching a metabolomics study is the formulation of a well-structured experimental design, the subsequent chronological collection, processing, analysis and interpretation of metabolomic data and finally formulation and testing of relevant hypotheses.



This linear flow of information within a metabolomics study was very clearly described by Goodacre *et al.* (2007) [48]. The purpose of documenting and reporting all the steps within a metabolomic study being to account accurately for any subsequent conclusions. As such, the experimental design and all subsequent experimental protocols used within this investigation are described in detail.

3.1 Experimental Design

In an attempt to answer the four proposed questions stated within the experimental aim, an ethically approved (all experiments done involving human experimental subjects involves ethical risks, as such, ethical approval was obtained through standard procedures from University Ethics Committee) experimental design was formulated. This experimental design was developed to consist of two phases. The first phase being an open-minded approach by analyzing the organic profiles of urine samples obtained from individuals administered a defined alcohol dose. An initial pilot experiment within the first

phase allows for orientation, formulation of a more structured consequent experiment and the subsequent articulation of a provisional hypothesis. The second phase can be considered a more targeted approach with a focused look at the branched-chain amino acids (specifically isoleucine), aimed at evaluating our generated hypothesis.

The initial experiment or pilot study within the first phase was structured as a broad and non-targeted approach, aimed at determining an ideal, medically safe "acute" alcohol dose, an optimal sampling timeframe and what, if any, metabolic perturbations could be identified. The small, defined, homogenous experimental group chosen for this investigation consisted of young males loosely classified as "social drinkers" (i.e. they consume on average 1-3 drinks per week) in their early 20's (ranging from approximately 20 to 24 years old), with no medical problems or under no chronic medication, who have abstained from any consumption of alcohol at least 48-hours prior to the initiation of the experiment and whom are in an overnight fasted state. A relatively low alcohol dose (0.5g EtOH per kg body weight) was administered under medically supervised conditions, urine samples collected periodically over a 24-hour period and an open-minded analysis of the organic acid profiles conducted. The intent of the pilot study being to determine:

- if any metabolic perturbances occur and if any VIPs of biochemical significance can be identified.
- if the low alcohol dose caused any medical complications and ascertain if it would be safe to proceed with an additional high alcohol dose experiment.
- the timeframe/duration required for an individual to return to a "normal" physiological state, as well as identify the time range of most interest.

The results of the pilot study allowed for a more defined and structured protocol to be formulated for an experiment involving a relatively high alcohol dose (1.5g/kg body weight) that had been medically ascertained as safe. The acute alcohol dose was administered to a similar experimental group and urine samples were collected over a shorter timeframe (hourly samples over first 5 hours). Similarly, analysis of the organic acid profiles was conducted.

The results of the acute alcohol dose experiment allowed for the formulation of a hypothesis and subsequent evaluation of this hypothesis by means of a more targeted, larger scale protocol into the isoleucine degradation pathway, involving a similar homogenous experimental group administered a 1.5g/kg alcohol dose and/or 100mg isoleucine load per kg of body weight. Both urine and blood samples were collected and analysis of amino acid and acylcarnitine profiles included, as well as organic acid profiles. A brief, small sample set, additional study was also made to determine what, if any, ethanol-induced perturbances occur within the other two branched-chain amino acids (valine and leucine). Thus, initially, a very broad, non-targeted approach was taken for determining the metabolic perturbances associated with acute alcohol abuse and the experimental protocols made more targeted and defined for each subsequent experiment.

The open-minded mentality by which metabolomics is approached incorporates the utilization of numerous and various experimental protocols, as well as various types of biological samples. Each protocol has its advantages and limitations (e.g. an organic acid protocol is specific toward organic acids only and is not suitable for analysis of amino acids). By utilizing and amalgamating several protocols, multiple aspects of the metabolic profile can be considered, providing a broad and concise image of the metabolic state of an individual at a particular point in time (i.e. their metabolic flux).

As noted in the literature, a major consequence of ethanol abuse is the altered production of organic acids, such as: lactic acidosis, depressed citric acid cycle, ketoacidosis, increased fatty acid synthesis, hyperuricemia and various other perturbed metabolic pathways. Thus, a logical and valid initial approach toward the investigation of the metabolic effects of acute alcohol abuse would be the analysis of the organic acid profile.

3.2 Organic Acid Analysis

The protocol employed within this investigation for the analysis of organic acids is composed of three principle steps, namely:

1) Isolation of organic acids from physiological sample by means of a liquid-liquid extraction technique (ethylacetate and diethyl ether extraction)
2) Decreasing the polarity of the organic acids by formation of thermally stable, volatile derivatives (i.e. silylation with trimethylsilyl (TMS) and N,O-bis-(trimethylsilyl) trifluoraceteamine (BSTFA))
3) Ionization, separation and detection within the GC-MS.

GC-MS is thus able to separate the highly volatile organic acids through gas chromatography, followed by detection by means of mass spectroscopy, allowing rapid identification and quantification of organic acids with a high degree of sensitivity and chromatographic resolution. A standard operation procedure (SOP) for analysis of organic acids, shown below, has been compiled and authorized by the Metabolic Screening Unit of the Biochemistry Department within the North-West University, Potchefstroom Campus and used within this investigation.

A: URINE

Storage: short term (5°C), long term (-20°C)
Volume urine used according to creatinine (creat) values;

Creatinine	*<*	*100*	*mg%*	*use 1 ml urine*
Creatinine	*>*	*100*	*mg%*	*use 0.5 ml urine*
Creatinine	*<*	*5*	*mg%*	*use 2 ml urine*
Creatinine	*<*	*2*	*mg%*	*use 3 ml urine*

Add 6 drops 5M HCl to adjust pH 1.
Add internal standard (IS): 5X creatinine mg% = volume in µl
(IS = 3-Phenylbutyric acid)

B: SERUM

Whole blood centrifuged at 40,000 rpm for 20 mins. Supernatant (serum) collected.
Serum storage: short term (5°C), long term (-20°C)
1 ml Serum
Add 100 µl internal standard

Add 6 drops 5M HCl to adjust pH1

Procedure continues for urine and serum

1. *Add 6 ml Ethyl acetate (first extraction step)*
2. *Shake 30 min (Roto-torque)*
3. *Centrifuge ± 3 min at 40,000 rpm (separates organic phase from aqueous phase)*
4. *Aspirate the organic phase into a clean tube*
5. *Add 3 ml Diethyl ether to the aqueous (lower) phase (second extraction step)*
6. *Shake 10 min*
7. *Centrifuge ± 3 min at 40,000 rpm*
8. *Aspirate the organic phase & add to the ethyl acetate phase*
9. *Add two spatula (pasteur pipette) desiccating agent Na_2SO_4 (removes any remaining water)*
10. *Vortex*
11. *Note: The Na_sSO_4 must now be powder & not flakes. Can add more.*
12. *Centrifuge ± 1 min at 40,000 rpm*
13. *Pour the organic phase into a clean smaller kimax test tube*
14. *Evaporate to dryness under Nitrogen at 40°C ± 1 hour*
15. *Add BSTFA A: (2X creat mg% = volume in µl) for urine*

    B: 40 µl for serum

    Add TMCS A: (0.4X creat mg% = volume in µl) for urine

    B: 8 µl for serum
16. *Incubate at 60°C for 1 hour (45 min - 70°C)*
17. *Inject sample into GC-MS*

Deconvolution of subsequent GC-MS profile and identification of organic acids done using a software program called AMDIS (Automated Mass Spectral Deconvolution and Identification System), which will be discussed in more detail in section 3.6.

Quantification:

Urine:
*Organic acid (mg/g creatinine) = Area organic acid / Area IS*262.5*
*Organic acid (mmol/mol creatinine) = Area organic acid / Area IS*180*
Serum:
*Organic acid (mg/L) = Area organic acid / Area IS*56.5*

Urine and serum samples each provide a characteristic organic acid profile; however, urine yields a higher collection of organic acids, when compared to serum, and is often the preferred biological sample used within organic acid analysis.

3.3 *Orientation*: Effects of Time and Repeatability on the Organic Acid Profile

An investigation performed as an orientation study within my BSc Honn in 2008 involved the early morning (fasted state), mid-stream diurnal urine analysis of two independent abstainers (non-alcohol drinkers), one male and one female. Collection of urine samples occurred over a similar time period (3-4 week period) and each set of urine samples analyzed independently of each other by two separate, independent analysts. Each diurnal urine sample was analyzed in triplicate (sample # 1, 2 & 3) by means of the above described organic acid SOP. The intended aim of this orientation study was to determine if there existed any effects of time on the organic acid profile, as well as to determine the repeatability of

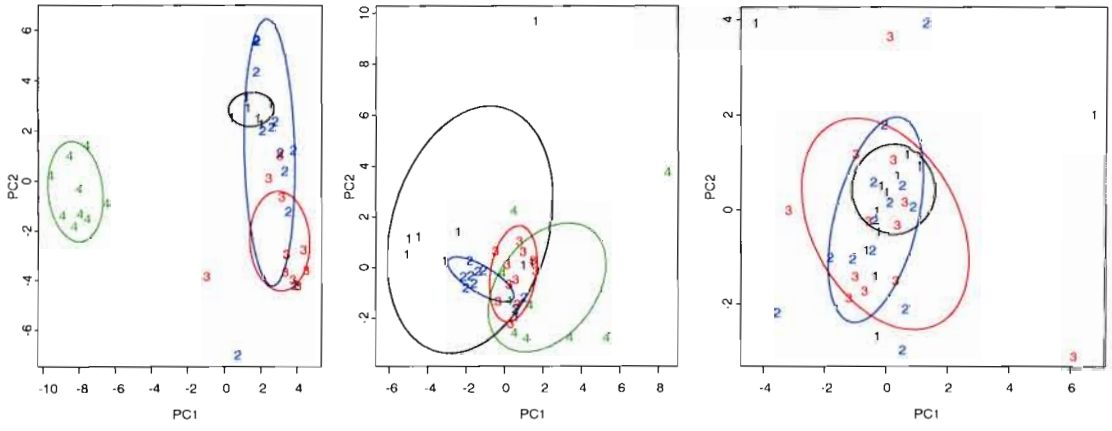
the organic acid protocol and ascertain if the protocol was standardized. Although this was part of my BSc Honn study it is reported again here due to its importance for the present investigation.

Prior to the multivariate analysis the data was suitably scaled and centered, as discussed later. The various variation components that were investigated were isolated, after which PCA was performed. The resulting PCA score plots, as illustrated, of the organic profiles of both the male and female abstainer produced very similar results.

Male Abstainer:

PCA score plots from transformed data:

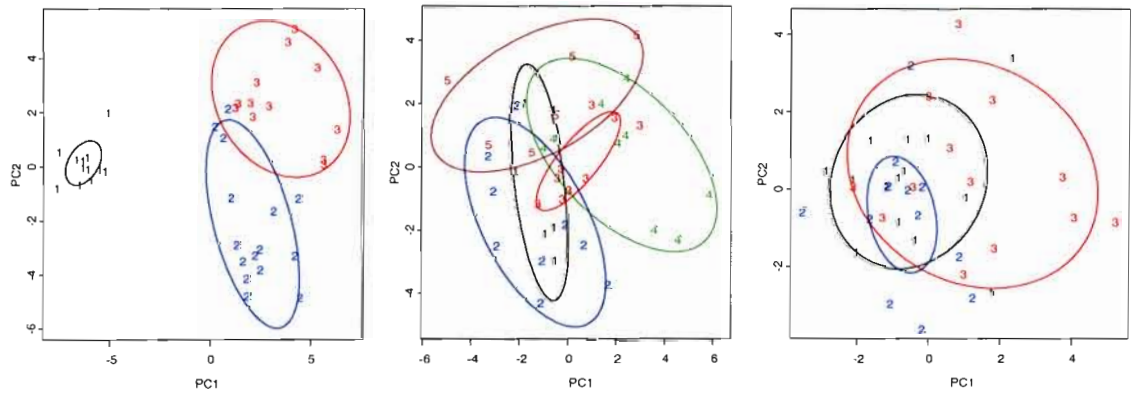
A-Week Effect **B– Day Effect** **C– Sample Number Effect**



Female Abstainer:

PCA score plots from transformed data:

A-Week effect **B – Day effect** **C – Sample number effect**



In both situations there exists very little variation between days within a week (i.e. there exists no daily effect on the sampling); however, in both individuals one week stood out as different from the other weeks suggesting that there is a week effect. There exists little/no separation between sample numbers within each triplicate set, as shown in the PCA score plots. This confirms that the results obtained are reproducible and that the organic acid protocol used can be considered as standardized. Another conclusion of importance for the present investigation is that time is an integral component in the sampling process. The effects of time within the sampling procedure was thus taken into account during the formulation of the experimental protocols and collection of samples, as discussed later.

The metabolic consequences of alcohol abuse extends beyond organic acid profiles. Perturbations also occur within amino acid and detoxification pathways. These perturbations cannot be detected by organic acid analysis and, as such, require their own independent protocols.

3.4 Amino Acid Analysis

Analysis of the amino acid profile allows for an additional viewpoint into understanding the perturbances associated with acute alcohol abuse; in particular, the use of an amino acid specific protocol is especially important in the latter part of this investigation as a more targeted approach is taken by focusing on the branched-chain amino acid pathways. The analysis of amino acids within this investigation is done by means of the *EZ:faast Amino Acid Analysis* kit. The protocol of this analysis kit consists of a solid phase extraction, followed by a derivatization and a liquid-liquid extraction and the derivatized samples are analyzed by GC-MS. The solid phase extraction is performed via a sorbent packed tip that binds amino acids while allowing interfering compounds to flow through. Amino acids on the sorbent tip are extruded into a sample vial and derivatized, which concomitantly migrate to the organic layer for additional separation from interfering compounds. The following amino acid SOP, used for the determination of the amino acid concentrations in serum and urine samples within this investigation, was taken from the EZ:faast user manual:

Sample Preparation:

1. *For each sample, line up one glass sample preparation vial in the vial rack.*
2. *Pipette sample (100µl urine or 50µl plasma/serum) and 100µl Reagent 1 (internal standard solution (IS=Norvaline 0.2mM)) into each sample preparation vial.*
3. *Attach a sorbent tip to a 1.5ml syringe and loosen the syringe piston; immerse the tip and let the solution in the sample preparation vial pass through the sorbent tip by slowly pulling back the syringe piston, in small steps.*
4. *Pipette 200µl Reagent 2 (washing solution) into the same sample preparation vial. Pass the solution slowly through the sorbent tip and into the syringe barrel. Drain the liquid from the sorbent bed by pulling air through the sorbent tip. Detach the sorbent tip, and leave it in the sample preparation vial, then discard the liquid accumulated in the syringe.*
5. *Prepare the eluting medium as follows:*

Number of Samples	Reagent 3A Eluting Medium Component I	Reagent 3B Eluting Medium Component II
2	300µl	200µl
4	600µl	400µl
7	900µl	600µl
12	1.5ml	1.0ml
14	1.8ml	1.2ml
19	2.4ml	1.6ml
24	3.0ml	2.0ml
29	3.6ml	2.4ml
34	4.2ml	2.8ml

6. *Pipette 200µl freshly prepared eluting medium into same sample preparation vial.*
7. *Pull back the piston of a 0.6ml syringe halfway up the barrel and attach the sorbent tip used in steps 3-6.*
8. *Wet the sorbent tip with the eluting medium; watch as the liquid rises through the sorbent particles and stop when the liquid reaches the filter plug in the sorbent tip.*
9. *Eject the liquid and sorbent particles out of the tip and into the sample preparation vial. Repeat step 7 and 8 until the sorbent particles in the tip are expelled into the sample preparation vial.*
10. *Pipette 50µl Reagent 4 (organic solution I) into the sample preparation vial.*
11. *Emulsify the liquid in the vial by repeatedly vortexing for 5-8 seconds, allow to stand for 1 min and re-emulsify by vortexing for another 5 seconds.*
12. *Pipette 100µl Reagent 5 (organic solution II) into the sample preparation vial and repeat vortexing procedure in step 11.*
13. *After allowing the reaction to proceed for 1 min; transfer part of the (upper) organic layer (about 50-100µl) using a Pasteur pipette into an autosampler vial for GC-MS analysis.*

The EZ:faast Amino Acid Analysis kit allows for the analysis of approximately 60 aliphatic and aromatic amino acids, including primary and secondary amines.

3.5 Acylcarnitine Analysis

Another aspect to consider, besides the metabolic consequences, with acute alcohol abuse is the detoxification state within an individual. Determination of the detoxification state is done by analyzing the acylcarnitine profile. Acylcarnitine analysis thus serves as a third protocol used within this investigation, in addition to the organic acid analysis protocol and amino acid analysis protocol. The protocol used within this investigation involves the use of isotope-diluted samples that have been chemically derivatized (butylated) and subjected to analysis by electrospray tandem MS. The acylcarnitine analysis SOP used was compiled and authorized by the Metabolic Screening Unit of the Biochemistry Department within the North-West University, Potchefstroom Campus and used within this investigation.

Carnitine isotopes (stock solutions):

A. Acetylcarnitine: 5mg/100ml (20.83 µmol)

B. Propionylcarnitine, isovalerylcarnitine and octanoylcarnitine:

Propionylcarnitine 5mg/100ml is 19.69 µmol

Isovalerylcarnitine 5mg/100ml is 17.73 µmol

Octanoylcarnitine 5mg/100ml is 15.43 µmol

C. Palmitoylcarnitine: 5mg/100ml (11.47 µmol)

D. Free carnitine: 5mg/100ml (30.446 µmol)

Dilutions of the different carnitine isotopes:

Take 1 ml of A stocksolution

Take 2 ml of D stocksolution

Take 125 µl of B stocksolution for serum, 250 µl of B for urine

Take 1 ml of C stocksolution

Sample preparation:

In the case of a urine sample -

Take 100µl of urine and centrifuge (30 minutes) to get rid of crystals in the urine
Take 10µl of urine sample
Add 400µl of carnitine isotopes mixture
Centrifuge sample for 20 minutes
Transfer supernatant to new tube

In the case of a serum sample -

Take 10 µl of serum sample
Add 400µl of carnitine isotope mixture
Centrifuge sample for 30 minutes
Transfer supernatant to new tube
Centrifuge sample again for 20 minutes
Transfer sample to new tube

Continue with the following steps:

1 *Evaporate to dryness under nitrogen at 55 C for 30 minutes.*
2 *Add 200 µl 3N butanolic HCl and incubate at 55 C for 20 minutes (closed caps).*
3 *The solvent is then again evaporated under nitrogen (35-40 minutes).*
4 *Reconstitute the residue in acetonitril:water and 1% formic acid (80:20)*
5 *Inject the samples into the VG Quatro tandem mass-spectrometer*

3.6 Software Analysis

The *resulting* data taken from all three independent protocols was analyzed by AMDIS (Automated Mass Spectral Deconvolution and Identification System); a software program for spectral extraction and compound identification of GC-MS data and acylcarnitine data from electrospray tandem MS. Deconvolution thus allows overlapping signals to be separated into individual chemical peaks. Subsequent chromatographic peaks are identified according to relevant recorded spectral libraries, based upon similarities of spectra. The data matrices compiled from this software (done on Microsoft Excel) were subjected to various types of biostatistical analysis. All metabolites occurring in multiple quantities within each sample were considered as stereo-isomers of the same metabolite and combined into one value to ensure no duplicate variables existed within the data matrix.

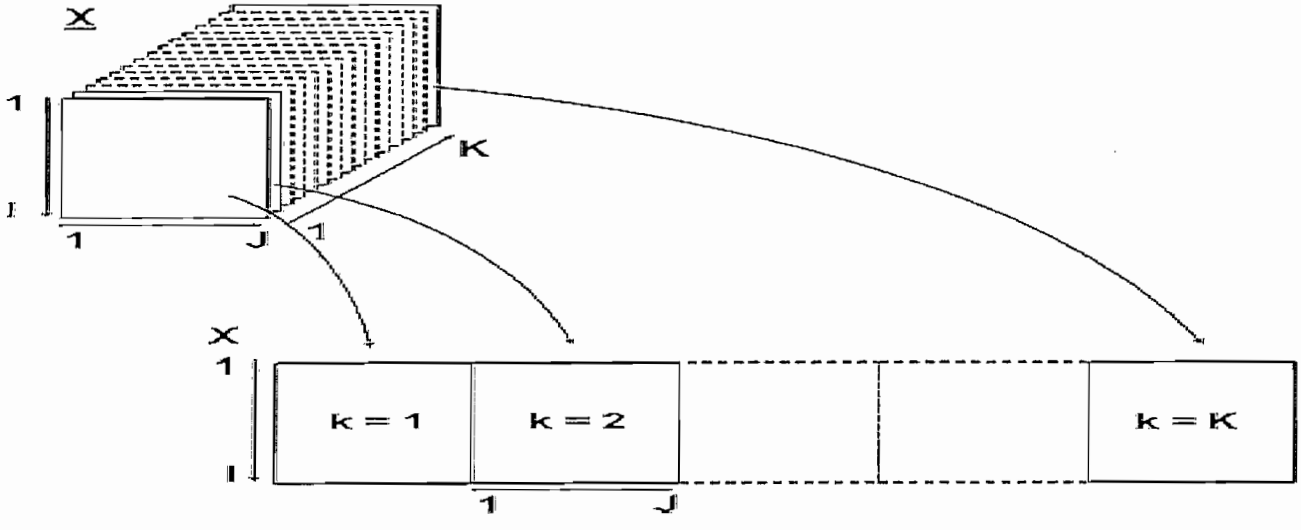
3.7 Biostatistics

The data generated in metabolomics investigations typically consists of a matrix of experimental subjects and variables in the following configuration:

$$\underset{(n \times p)}{X} = \begin{bmatrix} X_{11} & X_{12} & \dots & X_{1p} \\ X_{21} & X_{22} & \dots & X_{2p} \\ \vdots & \vdots & \vdots & \vdots \\ X_{n1} & X_{n2} & \dots & X_{np} \end{bmatrix}$$

where 1,..., *n* = cases (e.g. controls and experimental subjects who consumed alcohol) and 1,...,*p* = variables (e.g. concentration of metabolites, e.g. mg (or mmol) hippuric acid per gm (or mol) creatinine). A third dimension (time) also needs to be considered within the time-dependent data collected within this investigation. The following statistical tools were thus applied to accommodate analyses of data in this format:

- Median discrepancy measure – variable filter reduction method that compares the median values of metabolites at specific time points to time 1. All variables that show, on expectation, some movement over time were considered for further analysis; all other variables were disregarded.
- Logarithmic (log) and nonparametric transformation (Koekemoer and Swanepoel [40]) – data pre-treatment methods that pulls smaller data values apart and bring the larger data values together and closer to the smaller data values (shrinkage effect). The end result being more comparable variables. Data pre-treatment is typically used prior to multivariate pattern recognition with variable scales that vary quite significantly.
- Unfolding PCA – statistical tool that transforms data that is in a 3-dimensional format (X) into a 2-dimensional matrix (X), with dimensions I (cases), J (variables/metabolites) & K (time), as illustrated below:



Unfolding allows PCA, which is only applicable to 2-dimensional data, to be applied to a 3-dimensional data array. The resulting trajectory scores plots characterizes the movement over time. (NOTE: refer to Villez *et al.* (2009) [41] for a more thorough discussion on this method)

- Cross-sectional PCA – modeling of time-dependent data at different cross-sectional time points (snapshots). Biochemical interpretation is then used to determine the effect of time.
- ANOVA-Simultaneous Component Analysis (ASCA) – published model (Smilde *et al.* (2005) [36]) that directly compares groups together by separating the variation into independent, orthogonal parts (dimension reduction) and assigning it to factors (e.g. time, treatment). Interpretation of the resulting components subsequently operates in the same manner as an ordinary PCA.

3.8 Determining Breath/Blood Ethanol Concentration

Determination of the ethanol concentration within breath and/or blood of the experimental individuals allows for an estimation of the degree of alcohol intoxication. The blood alcohol content of an individual gives an idea of the degree of expected associated metabolic perturbances (the higher the blood alcohol concentration, the more severe the expected metabolic perturbances). Two separate methods were employed within this investigation.

3.8.1 Lion Alcometer® 500 (Lion Laboratories Ltd., Barry, UK)

This breath alcohol analytical instrument was utilized in the first phase of this investigation as it is a rapid, non-invasive method for determining the breath alcohol concentration of individuals and, if necessary, subsequently estimates their blood alcohol concentration by means of a conversion table.

3.8.2 Quantichrom Ethanol Assay Kit (DIET-500)

This ethanol assay kit allows for the colorimetric determination of ethanol at a wavelength of 580nm by incorporating the traditional dichromate method in which dichromate is reduced by ethanol to a bluish chromic (Cr^{3+}) product. The intensity of the color is a direct measure of the alcohol concentration of the sample. This method is more invasive than the Lion Alcometer® 500 as blood samples are required. The protocol, as provided within the kit, was adapted for use within this investigation and is as follows:

1. *Deproteination of serum sample by addition of 1 volume of sample to 2 volumes of 10% trichloroacetic acid (TCA), followed by centrifugation at 14000 rpm for 5 min and transfer supernatant for assay.*
2. *Prepare 600 µL 2% Premix by mixing 120 µL 10% Standard and 480 µL distilled water. Prepare dilution series as follows:*

No	Premix + H2O (µl)	Vol (µl)	Ethanol (%)
1	35 + 115	150	0.469
2	30 + 120	150	0.400
3	25 + 125	150	0.334
4	20 + 130	150	0.240
5	15 + 135	150	0.200
6	10 + 140	150	0.133
7	5 + 145	150	0.067
8	0 + 150	150	0

3. *Transfer 100 µL dilutions and samples into wells of a clear bottom 96-well plate. Add 100 µL Reagent A quickly using a multi-channel pipettor. Tap plate lightly to mix.*

4. *Incubate 30 min at room temperature. The reagent color changes from yellow to visibly bluish in wells 1-4. Add 100 µL Stop Reagent B quickly using a multi-channel pipettor. Tap plate to mix.*
5. *Read optical density (OD) at 580nm.*

The detection range of the ethanol assay kit is 0.04% to 4% alcohol.

4. EXPERIMENTAL SUBJECTS

A vital component for a successful metabolomics study is the selection of appropriate experimental subjects and obtaining a concise summary of all/any relevant clinical information. This factor was a clear conclusion as stated within my 2008 Honn study: "*due to the heterogeneity of the experimental groups used, the lack of sample structure and patient clinical information and insufficient quantity of samples, this investigation was unable to clearly isolate any particular metabolites of interest, with a significant degree of confidence*". This conclusion was based upon the fact that there existed too many qualitative differences between the control and experimental group, as well as within the control group; differences not related to the effects of alcohol (e.g. age, gender, medical state, medications etc.). The samples obtained from the control and experimental groups also differed quantitatively. Thus, the degree of heterogeneity within and between the experimental and control group was very large. In addition, a lack of clinical information on the individuals within the experimental group meant it was impossible to determine: at what time the urine sample was collected, the metabolic state of the individual at the time of sampling (i.e. directly after meal or fasted state), what, if any, types of medication used by the individual, when they had last consumed any alcohol and numerous other unanswered questions which provided insurmountable complications. A clear conclusion was thus the importance of the quantitative (weight, alcohol dose etc.) and qualitative (health, age, gender etc.) requirements in the selection of experimental subjects and stressed the need of homogeneity for the selection of experimental subjects.

Thus, the experimental subjects selected for this investigation were chosen to be part of a controlled, defined group with a reasonable degree of homogeneity. This group consisted of young males in their early 20's, who generally classified themselves as "social drinkers" (i.e. they consumed moderate amounts of alcohol on a regular basis). All relevant clinical information was collected from each participant in the form of a questionnaire prior to initiation of the experiment (copy of questionnaire presented within Appendix 2). A physician, who was present for each experimental sampling event, reviewed all questionnaires and excluded any individuals under age, under chronic medication that may react adversely with alcohol and any individuals deemed medically unfit for participation (e.g. asthma and diabetes was included as an exclusion criteria). The physician was present during the first 2 hours of each experiment, which was conducted within a university health clinic, to ensure that if any possible medical complications arose that it would be dealt with expediently. After the first 2 hours the physician remained on-call for the remainder of the experiment and the participants were allowed to leave the clinic provided they abstained from alcohol and provided all necessary subsequent urine samples. Thus, the first 2 hours of each experiment was conducted in a highly controlled manner under the supervision of medically trained personnel.

Each participant was also required to read and sign an informed consent form, prior to participation, which stated that: participation was completely voluntary, that the participant needed to be in an over-night fasted state (i.e. experiment conducted first thing in morning and without breakfast being consumed), no alcohol should have been consumed for at least 48 hours prior to initiation of the experiment and no additional alcohol may be consumed for the duration of the experiment (example of informed consent form, including all amended forms for each experimental sampling event, presented in Appendix 3). Also, participants were told that they may only consume food after their third urine sample. Each participant needed to provide a "0" hour urine/blood sample immediately before the dose/load was consumed. The alcohol used was triple distilled Smirnoff Vodka (approximately 43% v/v alcohol) which was mixed with lemon flavored carbonated water. The sampling timeframe and dose/load used within each experiment differs per experiment; these details are given below, as well as the clinical information obtained from the participants from each experiment:

4.1 Pilot Study (low alcohol dose)

Participants: 10

Alcohol dose: 0.5g per kg body weight

Samples collected: urine and breath

Urine sample required every hour up until 4 hours, after which additional urine samples collected at 6hrs, 8hrs, 12hrs and a final urine sample at 24hrs after alcohol consumption (total of 9 urine samples). Breath samples collected approximately every 5min for the first hour after consumption of alcohol for 3 of the participants only.

Table 1: Clinical information of participants in pilot (low alcohol dose) experiment

Pilot (Low Alcohol Dose)								
Participant	Age	Weight (kg)	Alcohol dose (g)	Medical issues	Medication	# drinks (per week)	Preferred Drink	Smoker
			[dose: 0.5g/kg]					
1	20	69	34.5	none	none	1 to 3	brandy	no
2	23	73.5	36.75	none	none	1	beer	no
3	22	70.5	35.25	none	none	1 to 5	wine	no
4	23	83	41.5	Hayfever, Gilbert Syndrome	none	1 to 10	beer/spirits/wine	no
5	22	72	36	none	none	1 to 3	beer/brandy	no
6	24	117	58.5	Epilepsy	Trileptil	6	beer/southern comfort	no
7	24	68	34	none	none	6 to 12	beer/spirits	yes
8	20	86	43	none	none	5 to 10	brandy	yes
9	20	65	32.5	none	none	6 to 12	beer/brandy	no
10	19	83	41.5	none	none	6 to 20	beer/rum	yes
Range	19 - 24	65 - 117	32.5 - 58.5			1 to 20		
Avg	21.7	78.7	39.35			5.6		

Participant 4 declared a previous medical diagnosis of Gilbert's Syndrome. Gilbert's Syndrome is a mild, hereditary disorder found within 5% of the population, where the main symptom is increased unconjugated bilirubin in the blood (hyperbilirubinemia) as a result of reduced activity of glucoronyltransferase. Elevated levels of bilirubin may cause some level of insignificant jaundice but otherwise it is considered a harmless condition (absence of liver disease and overt hemolysis) and typically does not require treatment [31].

Participant 6 declared a medical history of mild epilepsy and takes Trileptil as medication. Epilepsy is a common chronic neurological disorder characterized by recurrent unprovoked seizures. These seizures are transient signs and/or symptoms of abnormal, excessive or synchronous neuronal activity in the brain. Epilepsy is usually controlled, but not cured, with medication. Trileptal® (oxcarbazepine) is an antiepileptic drug which is cleared from the body mostly in the form of metabolites which are predominantly excreted by the kidneys. More than 95% of the dose appears in the urine, with less than 1% as unchanged oxcarbazepine. Oxcarbazepine, which is activated to eslicarbazepine in the liver, has had no reported adverse reactions with alcohol [32].

Upon review the physician determined that all participants were medically stable for participation and that no adverse reaction with the alcohol dose was expected.

NOTE: exclusion criteria included for acute alcohol dose experiment but not for pilot study.

4.2 Acute Alcohol Dose & Controls

Participants: 8

Alcohol dose: 1.5g per kg body weight (intervention by administration of a single alcohol dose)

Samples collected: urine and breath

Acute alcohol dose: Urine samples collected approximately every hour up until 5 hrs after initial alcohol dose (total of 6 urine samples). Three breath samples collected periodically from each participant during the first hour after alcohol dose consumed

Control: Urine samples collected approximately every hour up until 5 hrs following consumption of lemon flavored carbonated water (total of 6 urine samples).

A control experiment was done, aimed at testing the vehicle (flavored water) and fasted state of participants in comparison to acute alcohol dose.

NOTE: vehicle control experiment conducted with 7 of the 8 participants (excluding participant 3, who was unable to provide control samples). Also, removal of participant 3 further improves the homogeneity of the experimental subjects for this study, with respect to weight.

Table 2: Clinical information of participants in acute alcohol dose experiment, including control experiment

Acute Alcohol Dose & Controls (excluding participant 3)								
Participant	Age	Weight (kg)	Alcohol dose (g)	Medical issues	Medication	# drinks (per week)	Preferred Drink	Smoker
			[dose: 1.5g/kg]					
1	23	82	123	Hayfever, Gilbert Syndrome	none	1 to 10	beer/spirits/wine	no
2	22	74	111	none	none	1 to 3	beer/brandy	no
3	24	114	171	Epilepsy	Trileptil	6	beer/southern comfort	no
4	22	73	109.5	none	none	2 to 6	wine	no
5	21	82	123	none	none	1	beer	no
6	21	72	108	none	none	1 to 2	brandy	no
7	22	72	108	none	none	1 to 5	wine	no
8	24	70	105	none	none	6 to 12	beer/spirits	yes
Range	21 - 24	70 - 114	105 - 171			1 to 12		
Avg	22.375	79.875	119.8125			3.8125		

4.3 Acute Alcohol Dose and/or Isoleucine Loading (large sample set)

Participants: 27

NOTE: 4 individuals excluded due to medically severe asthma, 1 individual excluded due to being diabetic and potentially adverse affects of diabetic medication (glucophage) mixed with alcohol and 2 individuals chose not to take part due to time constraints.

Alcohol dose: 1.5g per kg body weight and/or Isoleucine load: 100mg per kg body weight [34].

Samples collected: urine and EDTA-treated blood

NOTE: limited time-dependent analysis of organic acids in blood was done.

First blood and urine samples collected immediately before alcohol dose and/or isoleucine load; second samples collected approximately 1 hour after consumption of alcohol and/or isoleucine load (total of 2 blood samples). Additional urine samples collected at 2hrs, 4hrs, 6hrs and 8hrs after initial dose/load (total of 6 urine samples).

Table 3: Clinical information of participants in isoleucine targeted experiment

Group 1 - Alcohol + Isoleucine Load									
Participant	Age	Weight (kg)	Alcohol dose (g) [dose: 1.5g/kg]	Isoleucine load (g) [dose: 100mg/kg]	Medical issues	Medication	Regular Social drinker	# drinks (per week)	Participant Comments
1	21	85	127.5	8.5	none	none	yes	2	Dizzy after initial consumption
2	25	73	109.5	7.3	none	none	no	1	No problems
3	21	85	127.5	8.5	none	none	yes	20	Partially disorientated for 1 hour
4	20	75	112.5	7.5	none	none	yes	20	Slightly light-headed but no problems
5	20	105	157.5	10.5	none	none	yes	20	Light-headed during first hour
6	21	95	142.5	9.5	none	none	yes	20	Slightly drunk but no problems
7	19	87	130.5	8.7	none	none	yes	6	Light-headed for 10 min and hungry 30 mins later
8	19	84	126	8.4	none	none	yes	8	No effects
9	20	105	157.5	10.5	none	none	yes	20	No major effects
10	19	80	120	8	none	none	yes	1	No problems
11	21	95	142.5	9.5	none	none	no	1	Drunk with headache
Range	19 - 25	73 - 105	109.5 - 157.5	7.3 - 10.5				1 to 20	
Avg	20.55	88.09	132.14	8.81				10.82	

Group 2 - Isoleucine Load								
Participant	Age	Weight (kg)	Isoleucine load (g) [dose: 100mg/kg]	Medical issues	Medication	Regular Social drinker	# drinks (per week)	Participant Comments
1	21	86	8.6	none	none	yes	20	Felt hungry with bitter taste in mouth
2	22	84	8.4	Hayfever, Gilbert Syndrome	none	yes	5	Hunger pains but no problems
3	24	68	6.8	none	none	yes	24	Upset stomach
4	22	73	7.3	none	none	yes	2	No problems
5	21	67	6.7	none	none	yes	10	No negative effects
6	20	86	8.6	none	none	no	1	No effects
7	19	90	9	none	none	yes	2	Dizzy and upset stomach
8	21	88	8.8	none	none	yes	20	No effects
Range	19 - 24	67 -90	6.7 - 9				1 to 24	
Avg	21.25	80.25	8.025				10.5	

Group 3 - Alcohol load								
Participant	Age	Weight (kg)	Alcohol dose (g) [dose: 1.5g/kg]	Medical issues	Medication	Regular Social drinker	# drinks (per week)	Participant Comments
1	22	78	117	none	none	yes	5	Slightly drunk at first but fine after a while
2	20	66	99	none	none	yes	1	No effects
3	22	60	90	none	none	no	1	Slightly drunk for about an hour
4	25	117	175.5	Epilepsy	Trileptil	yes	6	Felt normal
5	22	80	120	none	none	yes	10	Little drunk with slight headache later
6	20	101	151.5	none	none	yes	13	Dizzy after 30min and stomach pains from no breakfast
7	24	110	165	none	none	yes	20	Felt drunk for first hour, felt better after 2 hours
8	20	80	120	none	none	yes	7	Slightly drunk
Range	20 - 25	60 - 117	90 - 175.5				1 to 20	
Avg	21.875	86.5	129.75				7.875	

4.4 Acute Alcohol Dose and/or Valine/Leucine Loading

Participants: 4

Alcohol dose: 1.5g per kg body weight and/or Leucine/Valine load: 100mg per kg body weight.

Samples collected: urine and EDTA-treated blood

First blood and urine samples collected immediately before alcohol dose and/or leucine/valine load; second samples collected approximately 1 hour after consumption of alcohol and/or leucine/valine load (total of 2 blood samples). Additional urine samples collected at 2hrs and 5hrs after initial dose/load (total of 4 urine samples).

Table 4: Clinical information of participants in brief study into valine and leucine pathways

Leucine and Valine load Trial										
Participant	Age	Weight (kg)	Alcohol dose (g) [dose: 1.5g/kg]	Leucine Load (g) [dose: 100mg/kg]	Valine Load (g) [dose: 100mg/kg]	Medical issues	Medication	# drinks (per week)	Preferred Drink	Smoker
1	22	72	108		7.2	none	none	1 to 5	wine	no
2	22	74	111	7.4		none	none	1 to 3	beer/brandy	no
3	27	70		7		none	none	0		yes
4	67	65			6.5	Hypertension	Antihypertension & Anticholesterol	2	wine	no

Thus, a defined, homogenous group of young males in their early 20's were given a defined dose/load, periodic samples taken under relatively controlled conditions, relevant analytical protocols applied to allow for a biochemical view of the metabolic fingerprint of each individual at a specific time and selected biostatistical tools and models applied to identify important variables based upon the most significant variances. The recorded results, shown in the following section, depict how aptly metabolomics is used as a hypothesis-generating system, as opposed to the traditional hypothesis-testing systems [49,50].

5. TIME-DEPENDENT ANALYSIS OF METABOLIC PERTURBATIONS ASSOCIATED WITH ACUTE ALCOHOL CONSUMPTION

The results presented within this section depict an unsupervised, unbiased and methodical approach toward the generation of metabolomic data regarding the metabolic implications of acute alcohol consumption. The metabolic perturbations caused by a single alcohol dose within this section are studied by analyzing the excreted urinary metabolites, more specifically the organic acid profiles of collected urine samples, over a defined period of time. The chronological progression toward understanding these metabolic implications followed the following format:

1) Pilot study aimed at determining an appropriate alcohol dose that can be considered acute enough to cause sufficient metabolic perturbation and isolating the sampling timeframe best suited for this investigation.
2) Formulation of a more structured experimental protocol, based upon the results of pilot study, allowing for a more precise open examination of the metabolic perturbations of an acute alcohol dose and the development of time-dependent statistical models for the analysis of generated metabolomics data.
3) Generation of a provisional hypothesis based upon the experimental observations of the effects of an acute alcohol dose, and the subsequent evaluation of this proposed hypothesis through additional experimental design.

5.1 Pilot Study ("Low Alcohol Dose")

5.1.1 Experimental Protocol

The pilot study, also referred to as the "low alcohol dose" experiment, embraces the true nature of metabolomics by allowing for a broad, open-minded examination of the organic acid profile of the experimental subjects based upon a low dose of alcohol.

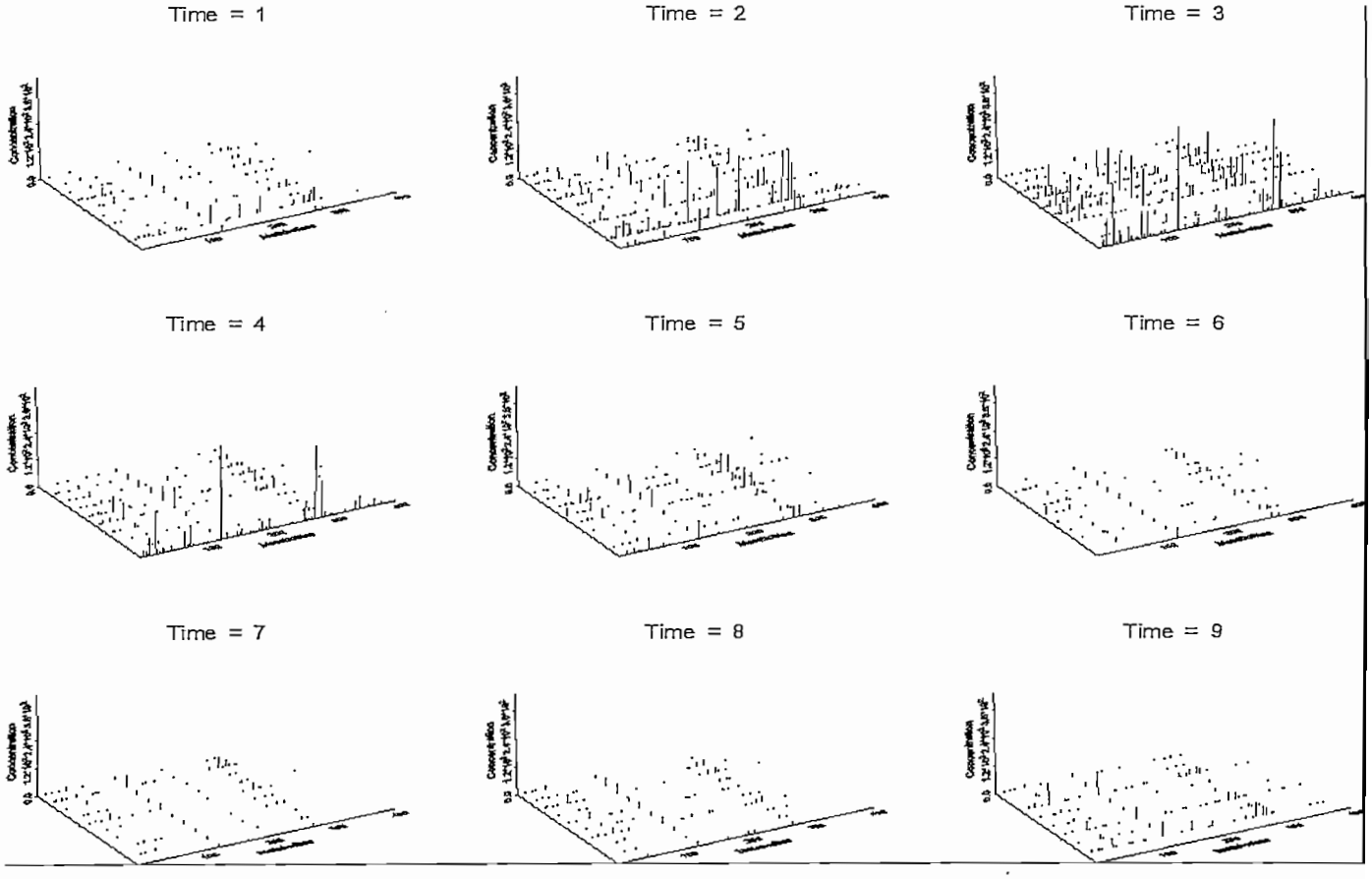
- The alcohol dose used (0.5g alcohol per kg of body weight) was based upon published values deemed as a "low" dose according to Høiseth *et al.* (2007) [33].
- The experimental protocol used was an adapted form of the protocol used within Høiseth *et al.* A total of 10 individuals volunteered to participate within this pilot study.
- Each individual was asked to provide mid-stream urine samples at timed intervals (0 hrs, 1 hrs, 2 hrs, 3 hrs, 4 hrs, 6 hrs, 8 hrs, 12 hrs and 24 hrs), with a 0 hour sample, classified as the baseline sample (i.e. sample taken at "normal" physiological state), being taken immediately before the alcohol dose was administered.
- Initiation of the experiment began at approximately 7am and each participant was required to be in an overnight fasted state (i.e. none of the participants were allowed to consume anything immediately prior to the experiment and during the first 2 hours of the experiment). Therefore, the controlled state, or point of departure, within this pilot study was the organic acid profile obtained from the experimental subjects following a phase of over-night fasting (i.e. 0 hr sample).

Thus, a total of 9 urine samples were obtained from 10 participants over a 24 hour period, resulting in a total of 90 urine samples. The organic acid analysis protocol, as described in section 3.2, was used to produce a time-dependent data matrix of the organic acid profiles of each participant and multivariate analysis was applied to the data to determine if a low alcohol dose can be considered sufficient to investigate the effect of alcohol on human metabolism over time (i.e. ethanol-induced metabolic perturbation).

5.1.2 Results and Orientation

Figure 4 provides a graphical overview of the concentrations (z-axis) of the various metabolites/variables (x-axis) of each participant (y-axis) at each time point. This overview is of all the raw data.

Figure 4: A 3D-scatterplot with drop line of the raw data, depicting concentrations of various metabolites/variables of each participant at each time point (z-axis = metabolite concentration, x-axis = metabolite, y-axis = participant)



A mild, but noticeable, increase in metabolite concentrations can be seen over the first 3 times (0 hrs, 1 hrs and 2 hrs samples), with a noticeable variance being between time 1 (0 hrs) and time 3 (2 hrs). At time 4 (3 hrs) metabolite concentrations are reduced and by time 6 (6 hrs) have almost completely returned to "normal" status (i.e. back to the profile shown in time 1). A notable observation was that one participant showed particularly high peaks at time 3 and time 4, the cause of which is unknown. The time dependency of each participant was ignored in order to ascertain if the low alcohol dose used was

sufficient to produce a metabolic perturbation. Data was scaled by nonparametric data pre-treatment (Figure 5) and presented in a PCA score plot (Figure 6).

Figure 5: Nonparametric transformation of data

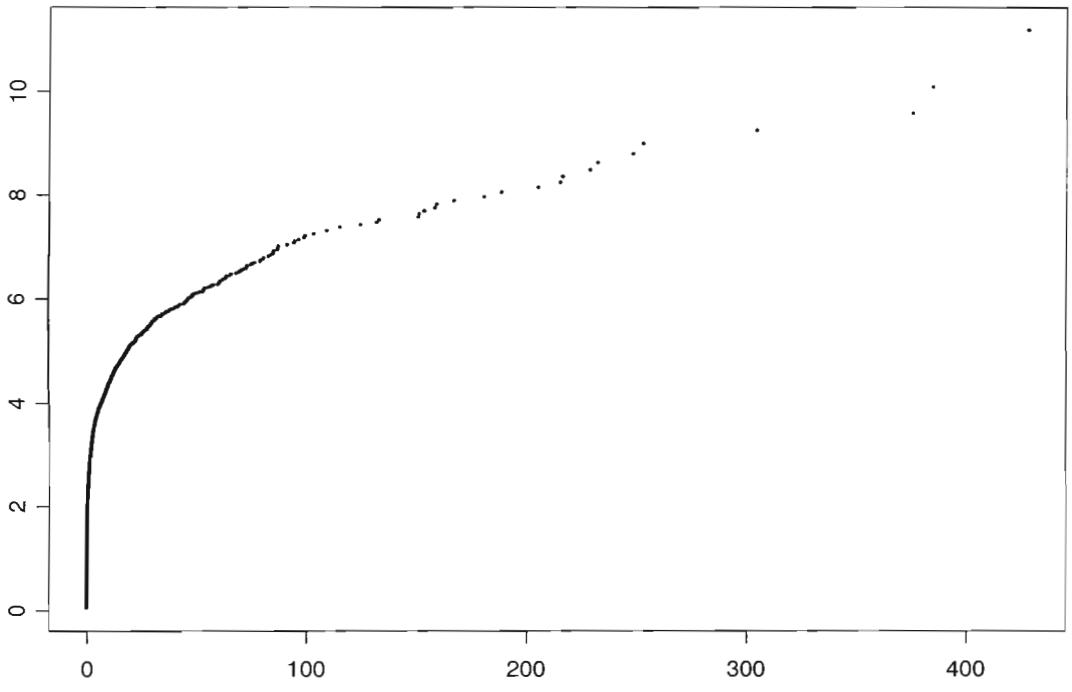
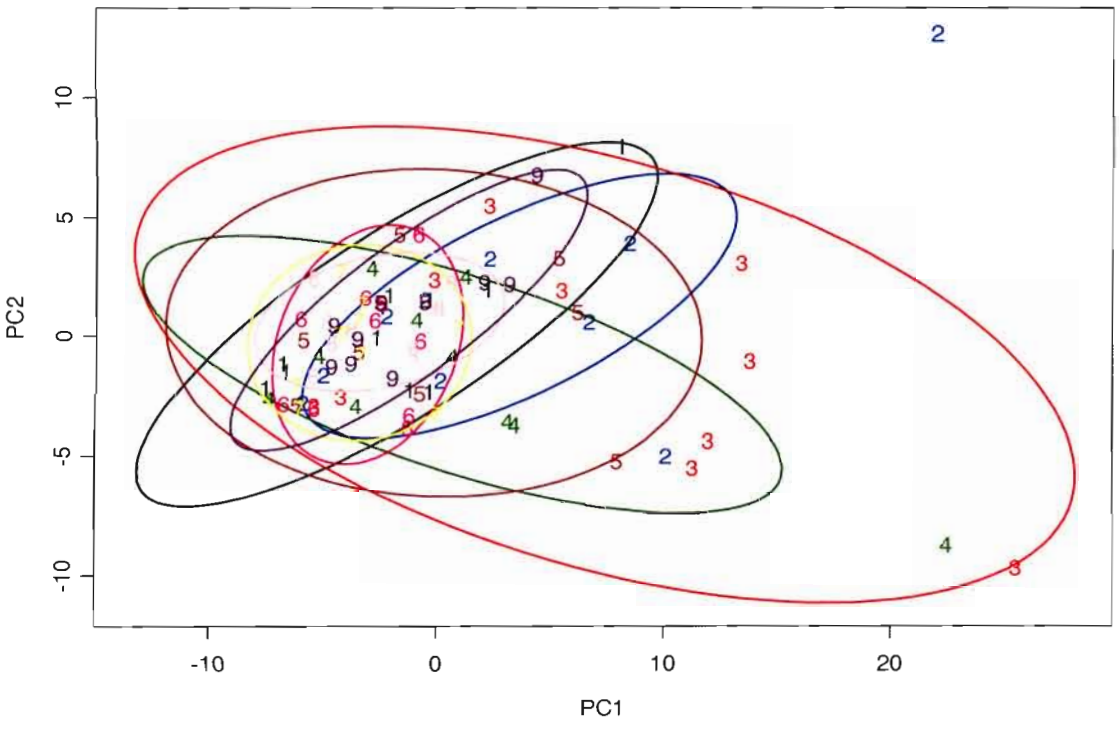


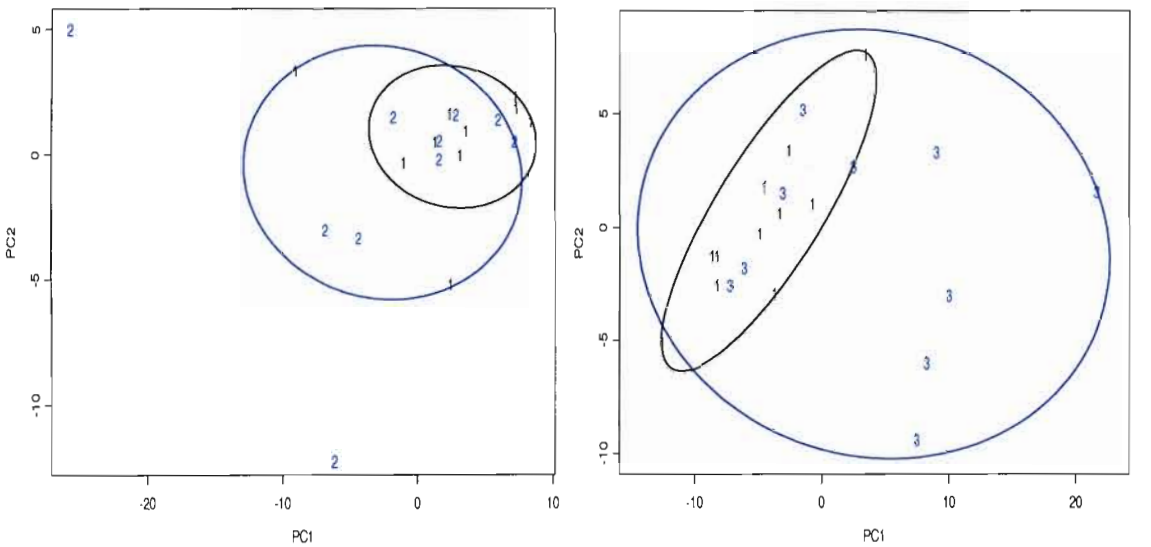
Figure 6: Score plot of all 9 times



As can be seen, PCA of all 9 times of each participant in one comprehensive score plot (Figure 6) does not provide sufficient separation to identify perturbances, if any, associated with alcohol consumption. Since the graphical overview (Figure 4) of all the metabolite concentrations over time illustrates that a mild perturbation does occur over the first 3 times, a more specific PCA was done by comparing time 1 with time 2 and time 1 with time 3.

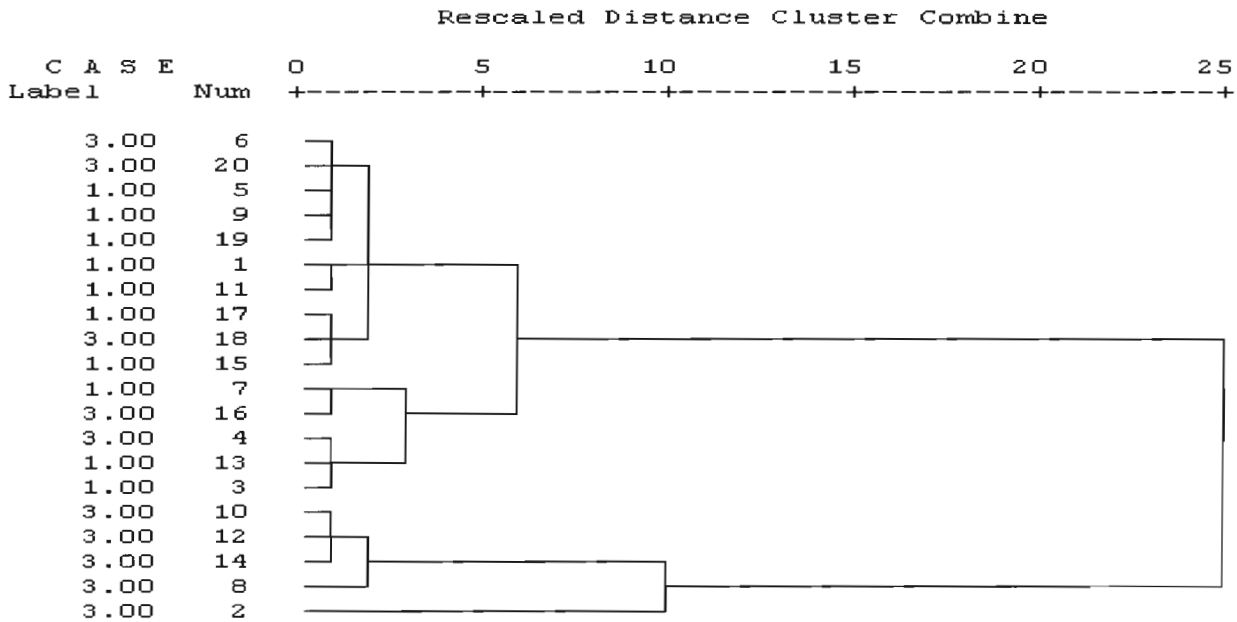
Figure 7: PCA score plots comparing time 1 to time 2 and time 3

Time 1 vs. Time 2 **Time 1 vs. Time 3**



Some separation is seen in both score plots, however, there is also overlapping values where no separation is seen. This suggests that some individuals experienced deviation from their normal metabolic state as a result of the alcohol dose, while others showed little deviation. In addition, hierarchical cluster analysis using Ward's method with a squared Euclidian distance measure was used to further investigate the separation between time 1 and 3. The resulting dendogram of time 1 vs. time 3 (Figure 8) confirms that there is indeed a small grouping in time 3, suggesting that at least 5 individuals are in a different physiological state compared to the other participants; however, as with the PCA, no complete separation can be made between time 1 and time 3.

Figure 8: Dendogram using Ward's method of time 1 vs. time 3



It can be concluded from the above multivariate analysis that there is some mild perturbation as a result of alcohol consumption; however, this perturbation only occurred in some participants and isn't significant enough to produce a list of variables of importance (VIPs) with biochemical significance.

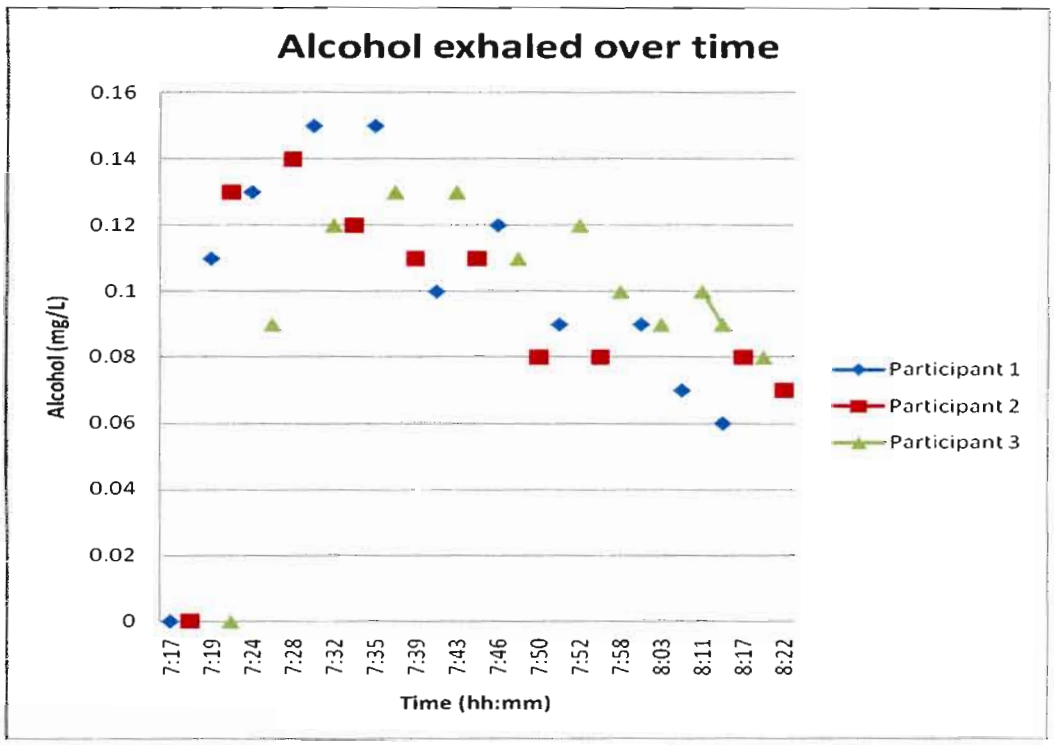
5.1.3 Determination of Breath Alcohol Concentration

Of the 10 participants, 3 were also asked to provide periodic breath samples with the Lion Alcometer® 500(Lion Laboratories Ltd., Barry, UK), as described in section 3.8.1, for a time period of approximately 1 hour after consumption of alcohol dose. The following results were recorded:

Table 5: Periodic breath samples (mg/L) taken from 3 participants over approximately 1 hour

	Alcohol (mg/L)		
Time	Participant 1	Participant 2	Participant 3
7:17	0		
7:18		0	
7:19	0.11		
7:20		0.13	0
7:24	0.13		
7:26			0.09
7:28		0.14	
7:30	0.15		
7:32			0.12
7:34		0.12	
7:35	0.15		
7:38			0.13
7:39		0.11	
7:41	0.1		
7:43			0.13
7:45		0.11	
7:46	0.12		
7:47			0.11
7:50		0.08	
7:51	0.09		
7:52			0.12
7:55		0.08	
7:58			0.1
8:01	0.09		
8:03			0.09
8:09	0.07		
8:11			0.1
8:13	0.06		0.09
8:17		0.08	
8:20			0.08
8:22		0.07	

Figure 9: Plotted graph of alcohol exhaled over time of 3 participants



All 3 participants had a breath alcohol concentration level (BrAC) of 0 at the start of the experiment. The analysis of the amount of alcohol exhaled over time allows for an estimation of the degree of intoxication and to illustrate that there is indeed an increase in alcohol content within each individual. A trend can be seen where the breath alcohol concentration peaks very quickly and gradually declines with time. According to the Lion Alcometer® 500 handbook: "*social drinkers with a breath alcohol concentration below 0.15mg/L will show no obvious effects and can be considered in a stage of sobriety*". Although the analysis of the breath alcohol concentration only provides an estimate of the degree of intoxication within the individual, it can be safely concluded from the above results that the alcohol dose used was indeed very low.

The results of the pilot study confirms that an alcohol dose of 0.5g per kg body weight, as used in Høiseth *et al*, is indeed low. Analysis of the breath alcohol concentration showed no significantly high values and graphical analysis of the raw data showed variances over the first 6 hours; however, insufficient perturbance and inconsistent occurrence of perturbances (i.e. only some participants depicted deviation from a normal physiological state) was noted and the additional component of time made the use of 2-dimensional-specific PCA difficult to apply on a 3-dimensional data matrix. Also, the vast amount of variables presented complications.

From the outcomes of this pilot study it was predicted that:

- ✓ 1) a shorter sampling timeframe (the first 6 hours after alcohol consumption) would be sufficient for analyzing the metabolic perturbances associated with alcohol consumption,
- ✓ 2) a moderate increase in amount of alcohol administered would not only be medically safe, as no noticeably significant clinical effects of alcohol was seen within the pilot study, but could also potentially cause more defined and identifiable variances over time, and
- ✓ 3) a unique statistical approach would have to be developed to effectively reduce the number of variables within the data set and apply a method that would allow for time-dependent multivariate analysis of this 3-dimensional reduced data set.

This pilot study can thus be considered as an orientation, with the results allowing for the formulation of a more structured experimental protocol involving a higher alcohol dose that can be considered acute and the application of alternative statistical tools and models.

5.2 Time-dependent Metabolomic Analysis of Acute Alcohol Consumption

5.2.1 Experimental Protocol

The outcomes of the pilot study provided sufficient supporting information to allow for the formulation of a structured experimental protocol that would potentially provide sufficient and adequate data that could be used in the development of time-dependent models for acute alcohol consumption. In accordance to a protocol similar to that used within the pilot study, a defined, homogenous experimental group of 8 young males, in an overnight fasted-state, was used. The alcohol dose administered was increased to an acute dose of 1.5g alcohol per kg of body weight. The alcohol used was Smirnoff Vodka (43% v/v alcohol) mixed with lemon flavored carbonated water. Urine samples were collected at: 0 hrs, 1 hrs, 2 hrs, 3 hrs, 4 hrs and 5 hrs. Analysis of periodic breath samples during the first hour confirmed that all 8 participants were in an intoxicated state.

An additional follow-up control experiment was also conducted using 7 of the 8 participants used within the acute alcohol dose experiment (patient 3 was unable to take part in the vehicle control experiment). The exact same protocol was followed except that the participants were asked to consume an amount of lemon flavored carbonated water only, without any alcohol, in an overnight fasted state. Thus, this vehicle control experiment was aimed at determining what, if any, effects the fasted state and lemon flavored carbonated water would have on the metabolic state of each individual and validate any significant metabolic perturbances as being a consequence of acute alcohol consumption. The organic acid analysis protocol, as described in section 3.2, was used to produce a time-dependent data matrix of the organic acid profiles of each participant. Since participant 3 was unable to provide control samples, he was removed from the data matrix in order to maintain homogeneity between the experimental and control groups. Thus, the final data matrix consisted of experimental (acute alcohol dose) and control variables of 7 male participants over all 6 times (0-5 hrs). This original data set consisted of 347 variables/metabolites.

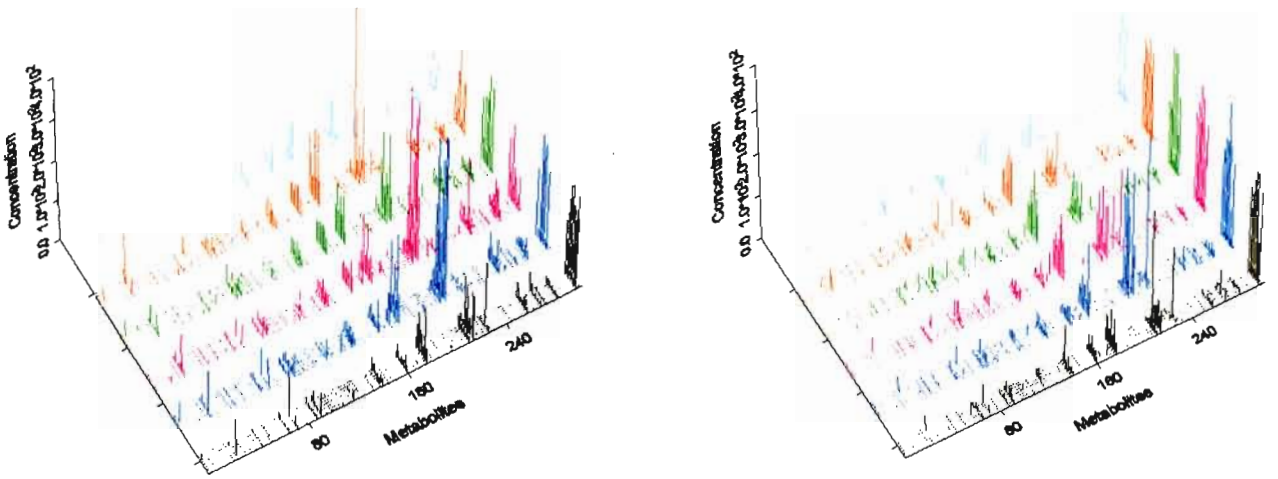
5.2.2 Data Reduction

5.2.2.1 Original Data

To facilitate analysis, variables unidentified by AMDIS were removed from the data matrix, as well as the internal standard (3-phenylbutyric acid), which is a constant used as a reference value for the quantification of the organic acids. The resulting data matrix consisted of 302 variables/metabolites which were used for statistical analysis. An overview plot of the raw data (shown in Fig 10), for both experimental and control cases, provides a graphical overview of variances for each variable over all 6 times within each participant.

Figure 10: Comprehensive time-dependent plot of raw data (302 variables) for experimental (A) and control (B) cases. (black=0hr, blue=1hr, magenta=2hr, green=3hr, orange=4hr, light blue=5hr)

A **B**

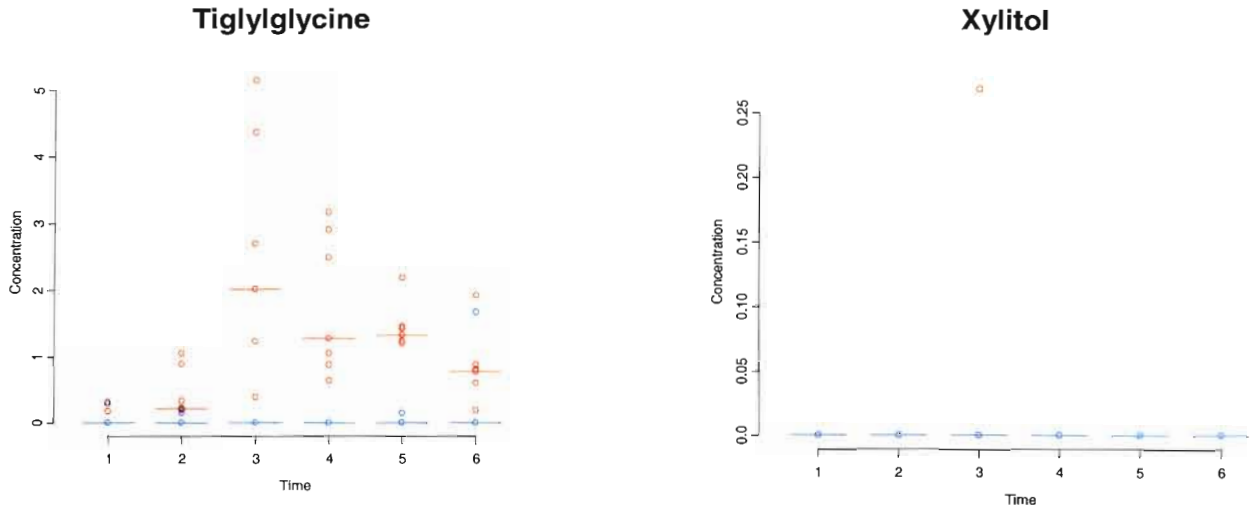


The large number of variables, however, makes graphical comparative analysis very difficult. This difficulty extends into the statistical analysis of this data as well, where the vast amount of variables in relation to the limited number of experimental subjects makes application of multivariate analysis, aimed at determining variances, less effective. To remedy this, a median discrepancy measure was developed that assesses the variation of each variable over time.

5.2.2.2 Statistical Reduction

The principle behind this discrepancy measure was to remove all variables that did not show any variation/movement over time, both in the experimental and control cases. Variables with no movement over time were removed from the data set and will be considered background metabolites (i.e. trace compounds, contaminants and/or metabolites not associated with alcohol metabolism). To illustrate the difference in movement over time, two variables taken from the control and experimental data sets are shown as an example below in Fig 11:

Figure 11: Comparison of movement over time of 2 selected variables: Tiglylglycine and Xylitol (red = experimental, blue=control)



Within this example, tiglylglycine shows definite movement over time and was included into a reduced data set, while xylitol showed no movement over time and was excluded from further analysis. Movement over time in these cases is assessed according to time 1 (0 hr), which is used as the baseline (the point from which other points are calculated from). Within each graph, the value of the variable (concentration) of each individual is plotted at each time and the median metabolite concentration is determined accordingly. This median value at each time point is then compared to the median value of time 1. The formula used is shown below:

$$d_j = \sum_{i=2}^{6} \left| \tilde{X}_{i,j} - \tilde{X}_{1,j} \right|$$

j = variable 1, 2, ... 302

where $\tilde{X}$ is the median concentration value of variable j and time point i.

This discrepancy value (d_j) is calculated for both the experimental and control groups independently. In the experimental group, 124 variables had a d_j value greater than zero (as illustrated with tiglylglycine), indicative of movement over time. For the control group, there were 113 variables for which the d_j values were greater than zero. These 124 and 113 variables (i.e. the variables that showed some movement over time) were thus selected based upon a statistical measure (NOTE: these 2 independently generated lists contained numerous common variables).

5.2.2.3 Biological Filter

In order to further reduce this data set, these variables were inspected and a biological filter applied to remove variables/metabolites of little/no significant biological importance for this investigation (e.g. metabolites from exogenous sources such as medication/diet and/or microbes/contamination). Urine is a biological fluid that is used by the body for the excretion of numerous waste products from various processes. As such, its contents will contain compounds of processes (both human and microbial) with

insufficient association with ethanol metabolism and deemed of no biological value for subsequent evaluation within this study (i.e. eliminated from the data matrix). Thus, the biological filter is a selective method, whereby human biological understanding of urinary metabolites is used in order to assess which variables can be removed from the data matrix. The final result was a consolidated data matrix of a total of 78 variables.

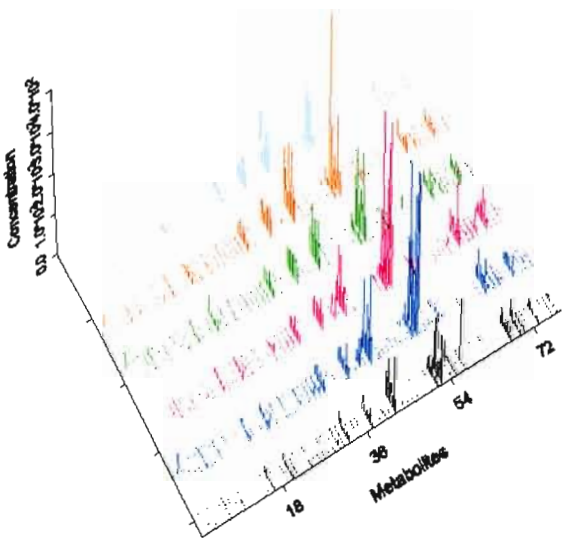
5.2.2.4 Final Consolidated Data Matrix

Thus, a large complicated data matrix of 302 variables was reduced using a statistical discrepancy measure to give 2 data sets consisting of 124 variables and 113 variables for the experimental and control cases respectively. This measure, using the stated formula, selected variables that showed variation/movement over time. These 2 data sets were then consolidated into 1 final reduced data matrix using biological interpretation as a consolidator.

Figures 12 and 13 shows the data of the 78 variables selected (12A and 13A), as well as the remaining variables not selected (12B and 13B), for the final reduced data matrix of the experimental and control cases respectively.

Figure 12: Plots of raw data showing the 78 selected variables exhibiting movement/variance over time (A), as well as the 178 variables not selected (B), within experimental cases

A



B

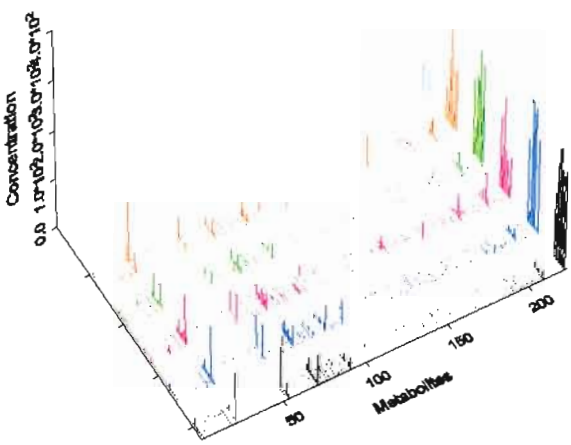
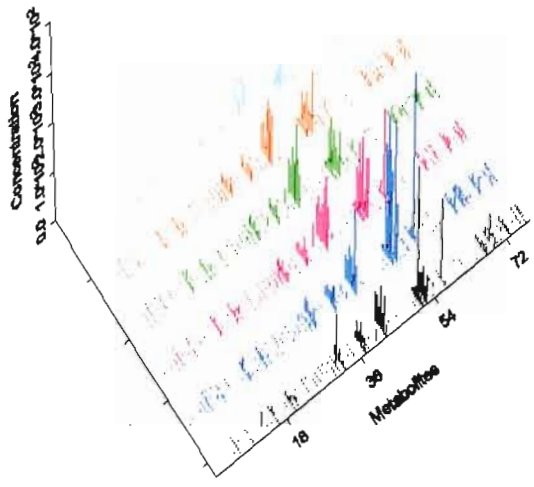
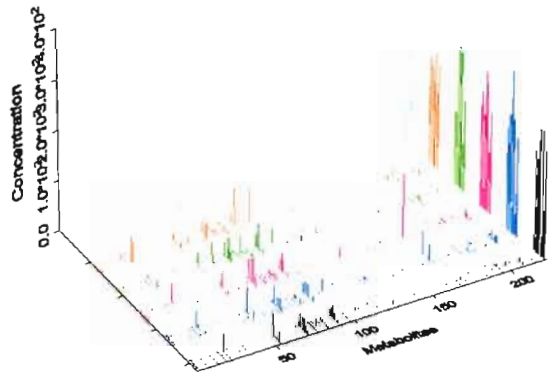


Figure 13: Plots of raw data showing the 78 selected variables exhibiting movement/variance over time (A), as well as the 189 variables not selected (B), within control cases

A



B

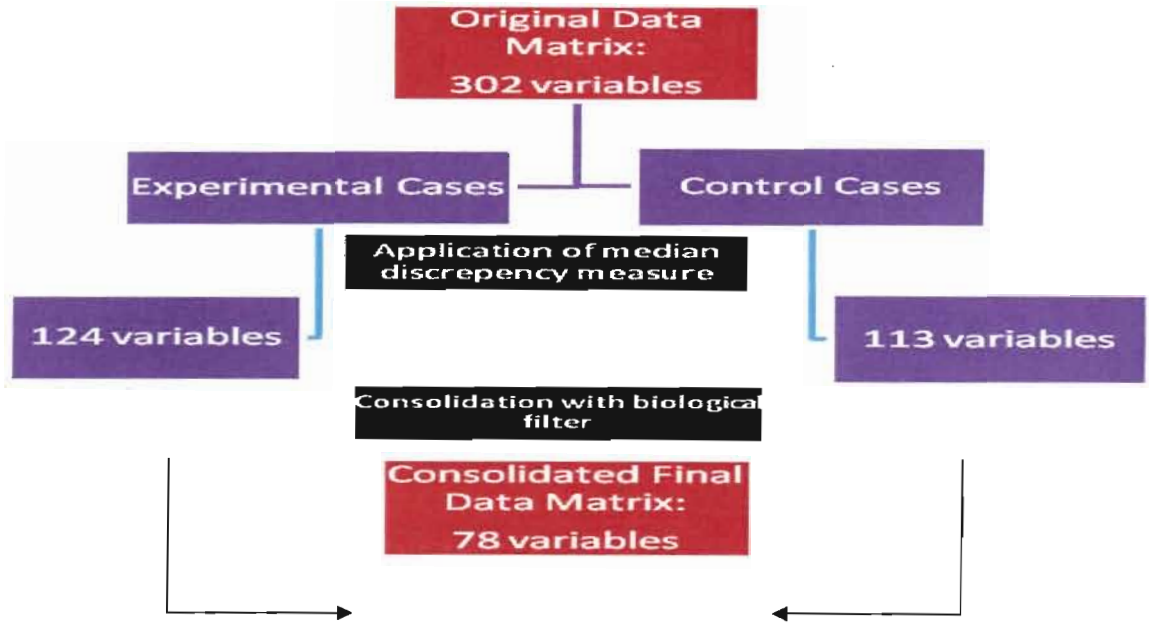


This illustrates the principle of movement/variance over time where an observable variance between each time point for each variable can be noted within the selected, reduced data set; whereas little/no variance can be seen within the nonselected variables. Also, the reduction of data allows for more effective assessment of variation over time. An important observation within Figures 12B and 13B is that one particular metabolite occurs in high concentration (approximately the last metabolite present in both 12B and 13B), with detectable variation, for both the experimental and control cases; however was not selected to be part of the reduced data set. This particular metabolite is urea and provides a very good example of the implementation and value of the biological filter applied during data reduction. Urea, an excretory product of nitrogen metabolism, is a common urinary metabolite and typically occurs in high concentrations within the urine. The urea concentrations present in both the experimental and control cases were neither pathologically high nor low and no significantly major variation was observed. Urea was thus considered as a normal urinary metabolite of normal concentration and deemed of no biological importance for this investigation and subsequently removed from the data set.

5.2.2.5 Evaluation

The method employed in the selection of variables based upon the median discrepancy measure and application of a biological filter is summarized in a schematic illustration within figure 15.

Figure 15: Schematic overview of reduction of data



This method aimed toward reducing the voluminous metabolomics data generated by this investigation is a novel approach, uniquely designed for this investigation. Graphical comparison of Figure 12 and 13 with Figure 10 clearly illustrates how reducing the number of variables within the data matrix allows for variations to be more clearly discernable. The reduced consolidated data matrix, created by this novel approach, thus allows for the effective use of multivariate analysis to isolate and identify variables of statistical and biological importance.

5.2.3 Application of Multivariate Models on Consolidated Reduced Data Matrix

Data pre-treatment was performed prior to multivariate analysis of the consolidated data matrix. Both the log and nonparametric transformation functions were applied, which made the variable scales more comparable. The statistical output resulted in similar graphical interpretation for both transformation methods; however, the lists of important variables produced by the log transformation yielded more meaningful biological interpretation. As such, only the results of the log transformed data was reported.

Three different multivariate modeling approaches were utilized in an attempt to explain the differences between the experimental and control groups, whilst also modeling time. These 3 models are:

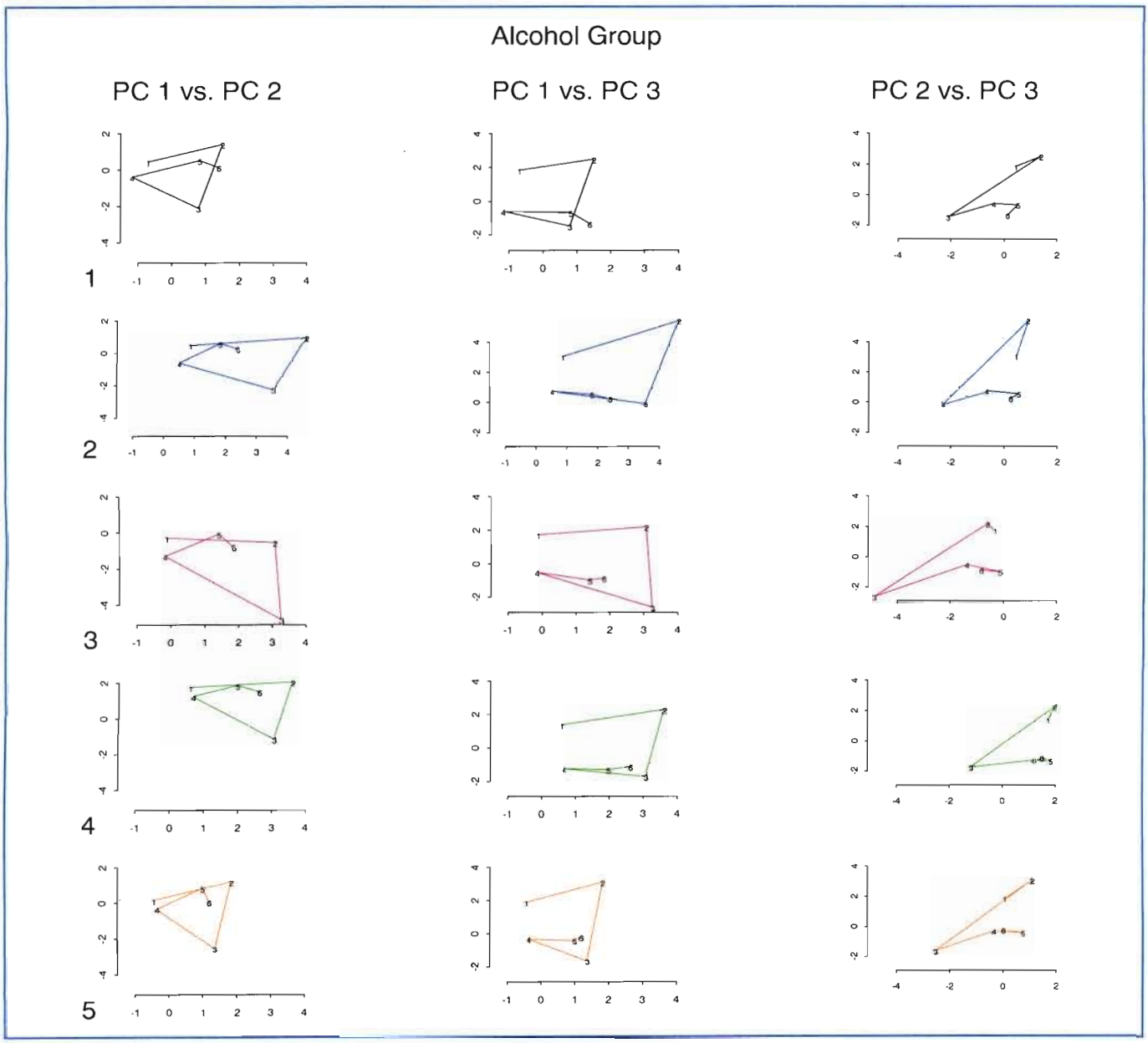
- Unfolding PCA
- Cross-sectional PCA
- ASCA

Each of these models presents a different interpretation of the time-dependent data by taking a different modeling viewpoint.

5.2.3.1 Unfolding PCA

Unfolding of the data was done to determine if any distinguishing characteristics/patterns, within and between experimental and control cases, could be elucidated. Unfolding allows application of 2-dimensional analysis (PCA) on 3-dimensional (time-dependent) data, and is explained in greater detail in section 3.7. The trajectory plots (shown in both figure 16 and 17) plot the respective principal components at a specific time point for each individual. In addition, the mean trajectory plots, as well as a 3-dimensional representation of the mean trajectories. The first 3 PCs explained 69% and 66% of the total variation for the control and experimental groups respectively.

Figure 16: Trajectory plots between selected principal components for each of the 7 participants within the experimental cases



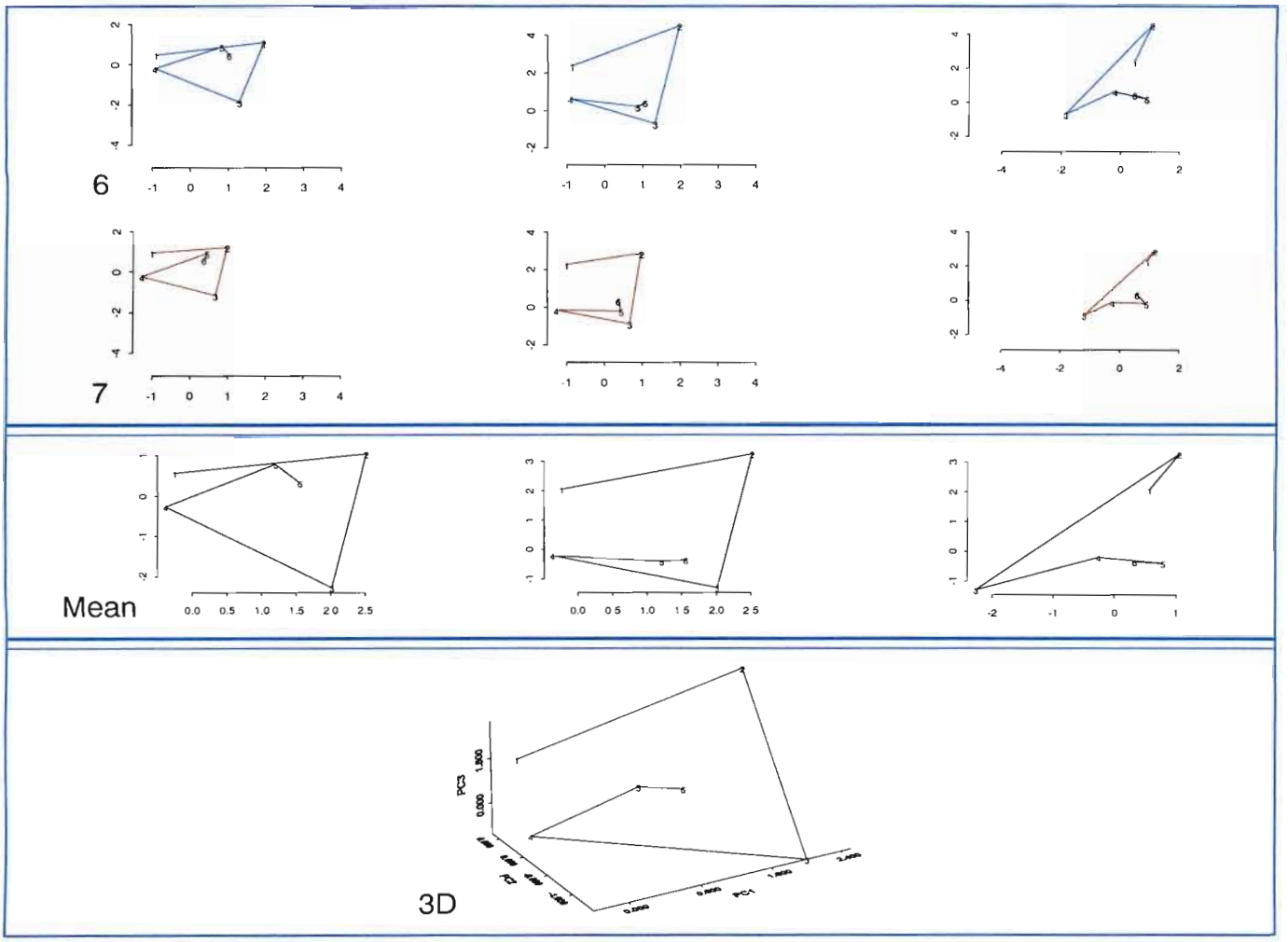
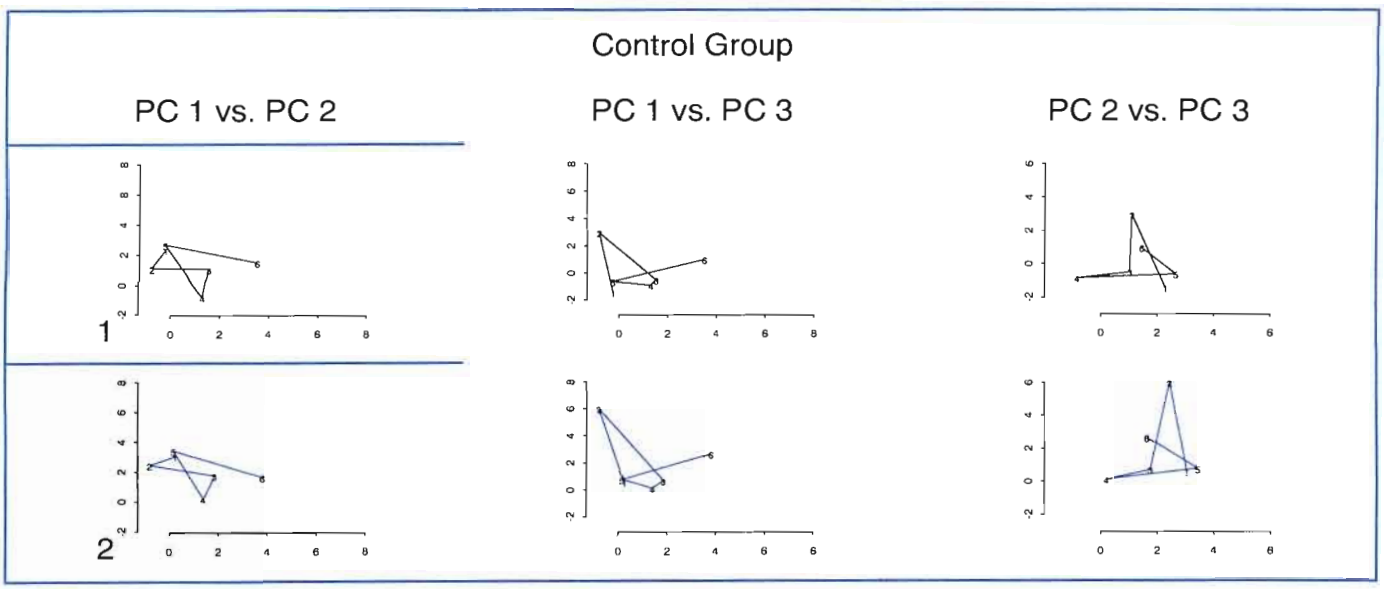
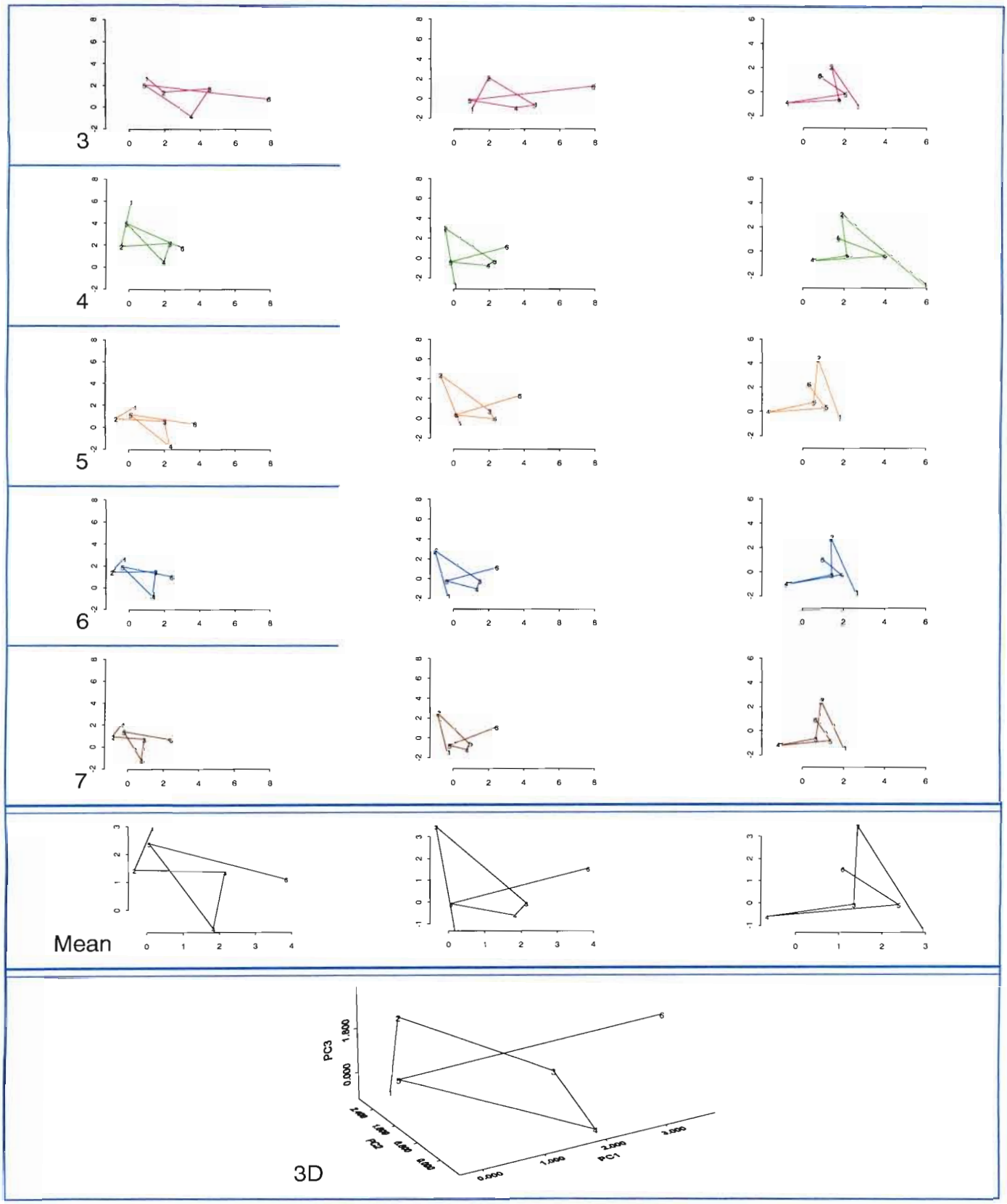


Figure 17: Trajectory plots between selected principal components for each of the 7 participants within the control cases





As can be seen, the trajectory plots within the experimental cases follow the same pattern for each individual when comparing PC1 vs. PC2, same for PC1 vs. PC3 and PC2 vs. PC3. Also, a similar pattern within the control cases is seen; however, the trajectory plots differ when comparing the experimental cases with the control cases, sufficiently enough to suggest that the pattern of variance seen within the

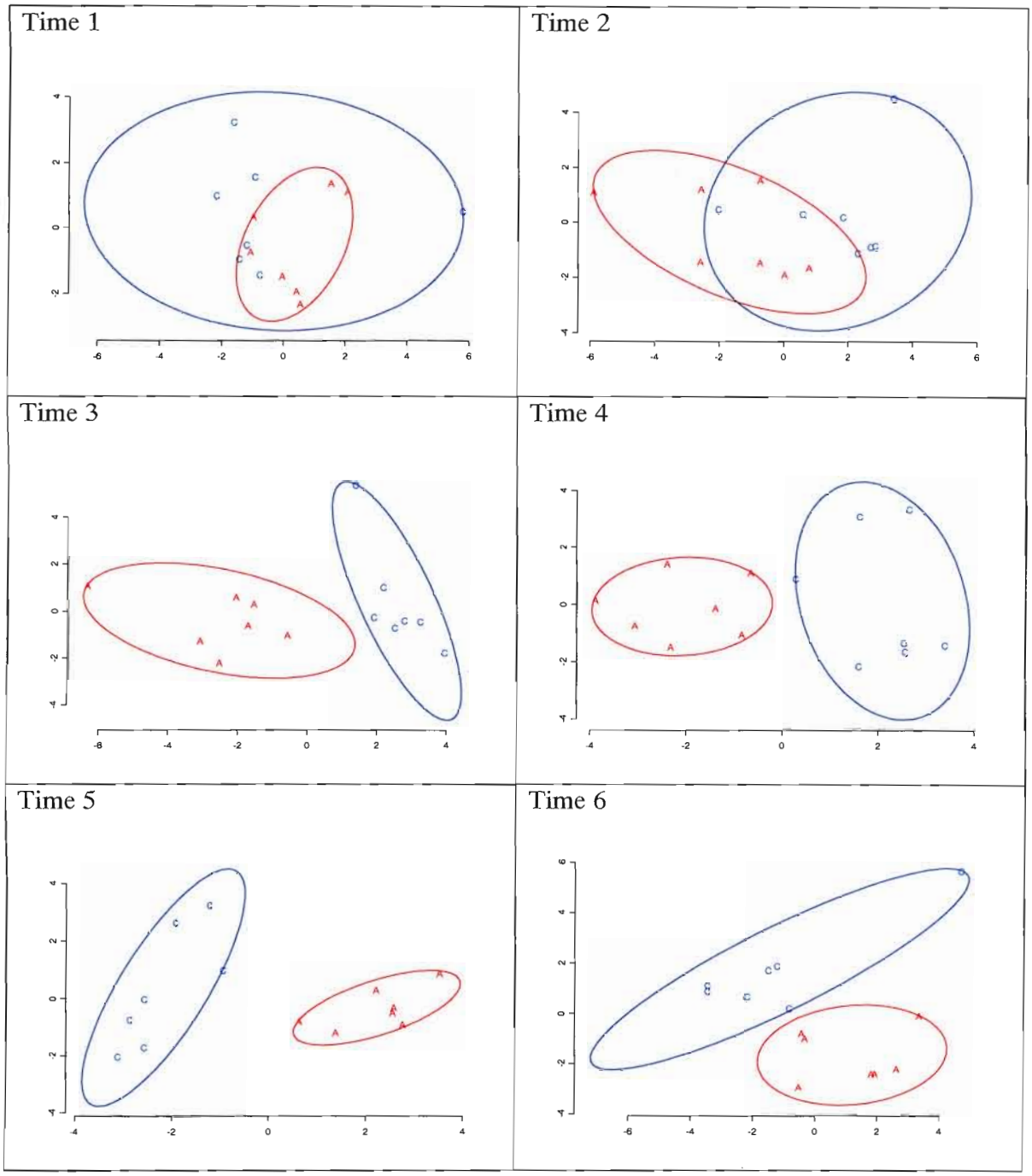
experimental cases is different to the variance seen within the control cases. The patterns seen within each group indicates that the movement/variation over time is not random and that a similar variation is seen within each individual. Thus, each of the control cases exhibited the same chronological variation in their metabolic profiles, while the experimental cases exhibited an altered metabolic profile compared to the control cases. Since each experimental case exhibited the same altered metabolic profile, the cause of the variation can be accredited to a common anomaly, namely the acute alcohol dose.

To provide a better graphical understanding of the movement seen over time the mean PC plots were placed into a 3-dimensional plot shown in both figure 16 and 17. Comparing the respective mean trajectory plots, as well as the 3-dimensional trajectory plots, between the two groups further confirms the observation that the patterns of deviation seen within the experimental and control cases differ from each other. Thus, the trajectory plots provide a clear pattern of deviation, however, successful isolation and identification of variables responsible for the variation over time proved difficult. The major weakness of the time-dependent unfolding model was that by looking at the data matrix of 78 variables of all 6 times at the same time actually meant that the model was considering 468 (78 x 6) variables and this vast number of variables resulted in complications when attempting to extract identities of variables of interest.

5.2.3.2 Cross-sectional PCA

In order to isolate and identify the variables causing the greatest variation over time a more conventional PCA approach was taken, where each time point was treated independently as a snapshot of the metabolic profile (see section 3.7). Cross-sectional PCA thus separates the data according to specific times and allows PC score plots to be created, comparing the experimental and control groups in order to determine at which time the separation between the two groups is the greatest. From the scree plots (not shown), 4 components were extracted for calculation of the VIPS. The variance explained for each of the time points (time 1, 2, 3, 4, 5 and 6) are 62%, 68%, 74%, 65%, 66% and 68% respectively; however, only the scores of the first 2 components are displayed graphically below in Figure 18.

Figure 18: PC score plots comparing experimental (red A's) and control (blue C's) groups for each time point independently (x-axis = PC1, y-axis = PC2)



As Figure 18 illustrates, no initial separation is seen at time 1 (0 hr), which is expected as this is the baseline time point. A gradual separation begins during time 2, with a definite separation occurring within time 3. That separation persists for the remaining time points.

Using the PC loadings as input a list of variables of importance (VIPs) can be created, listing the variables responsible for the greatest variation between the experimental and control group at each specific time point (shown in table 6).

Table 6: VIPs of PC score plots comparing experimental and control groups for each time point (VIP > 0.40)

Time 1	
Metabolite	VIP
LACTIC.ACID.t1	0.65
X4.HYDROXYMANDELIC.ACID.t1	0.58
X2.3.BUTANEDIOL.t1	0.57
PHOSPHORIC.ACID.t1	0.56
X3.HYDROXYPROPIONIC.ACID.t1	0.55
X2.3.4.TRIHYDROXYBUTYRIC.ACID.t1	0.54
ADIPIC.ACID.t1	0.53
HIPPURIC.ACID.t1	0.52
FUMARIC.ACID.t1	0.51
SUBERIC.ACID.t1	0.51
BENZOIC.ACID.t1	0.50
HYDANTOINPROPIONIC.ACID.t1	0.49
ISOVANILGLYCOLIC.ACID.t1	0.48
X2.METHYL.3.HYDROXYBUTYRIC.ACID.t1	0.47
ETHYLHYDRACRYLIC.ACID.t1	0.46
PYROGLUTAMIC.ACID.t1	0.45
X4.HYDROXYHIPPURIC.ACID.t1	0.44
XANTHURENIC.ACID.t1	0.44
X3.HYDROXYISOBUTYRIC.ACID.t1	0.43
X3.HYDROXYPHENYLACETIC.ACID.t1	0.43

Time 2	
Metabolite	VIP
BENZOIC.ACID.t2	0.79
X4.KETOVALERIC.ACID.t2	0.76
GLYCERIC.ACID.t2	0.75
X3.4.DIHYDROXYBUTANOIC.ACID.t2	0.74
HIPPURIC.ACID.t2	0.70
X2.HYDROXYBUTYRIC.ACID.t2	0.67
METHYLSUCCINIC.ACID.t2	0.62
SUCCINIC.ACID.t2	0.62
PYROGLUTAMIC.ACID.t2	0.61
OXALIC.ACID.t2	0.61
LACTIC.ACID.t2	0.60
NONANOIC.ACID.t2	0.60
PHENYLACETYLGLUTAMINE.t2	0.59
X3.HYDROXYPROPIONIC.ACID.t2	0.58
X2.3.4.TRIHYDROXYBUTYRIC.ACID.t2	0.58
FUMARIC.ACID.t2	0.57
ISOVANILGLYCOLIC.ACID.t2	0.56
X2.KETOGLUTARIC.ACID.t2	0.53
ACONITIC.ACID.t2	0.53
MALIC.ACID.t2	0.52
CITRIC.ACID.t2	0.50
GLYCEROL.t2	0.50
ETHYLHYDRACRYLIC.ACID.t2	0.49
CITRAMALIC.ACID.t2	0.48
GLYCOLIC.ACID.t2	0.46
X1.2.DIHYDROXYBUTANE.t2	0.46
STEARIC.ACID.t2	0.45
X1.2.DIHYDROXYETHANE.t2	0.45
URACIL.t2	0.44
GLUTARIC.ACID.t2	0.43
ISOVANILYLGLYCINE.t2	0.42
X2.HYDROXYGLUTARIC.ACID.t2	0.42
TIGLYLGLYCINE.t2	0.41
GLUCURONIC.ACID.t2	0.41

(Legend: green=hippuric/benzoic acid (selected for quantitative analysis), red=citric acid cycle intermediates, blue=catecholamine metabolites, purple=metabolites of branched-chain amino acid pathway)

NOTE: the 'x' in the front of the names of some of the variables presented in table 6 is a consequence of the statistical package (S-PLUS) used and is not of any biochemical significance. Also, the greater the VIP value, the greater the importance of that particular metabolite.

Time 3	
Metabolite	VIP
ETHYLHYDRACRYLIC.ACID.t3	0.86
STEARIC.ACID.t3	0.79
BENZOIC.ACID.t3	0.77
X3.4.DIHYDROXYBUTANOIC.ACID.t3	0.74
OCTADECENOIC.ACID.t3	0.74
MYRISTIC.ACID.t3	0.72
GLYCERIC.ACID.t3	0.70
GLYCOLIC.ACID.t3	0.69
METHYLSUCCINIC.ACID.t3	0.68
MALIC.ACID.t3	0.68
X1.2.DIHYDROXYETHANE.t3	0.66
OXALIC.ACID.t3	0.65
LACTIC.ACID.t3	0.64
VANILLYLMANDELIC.ACID.t3	0.62
X3.METHYLGLUTACONIC.ACID.t3	0.61
FUMARIC.ACID.t3	0.61
CITRAMALIC.ACID.t3	0.61
X2.HYDROXYBUTYRIC.ACID.t3	0.60
NONANOIC.ACID.t3	0.56
ACONITIC.ACID.t3	0.55
GLUCURONIC.ACID.t3	0.55
X5.HYDROXYINDOLACETIC.ACID.t3	0.53
X2.HYDROXYGLUTARIC.ACID.t3	0.51
URACIL.t3	0.50
SUCCINIC.ACID.t3	0.50
INDOL.3.ACETIC.ACID.t3	0.49
GLYCEROL.t3	0.49
GLUTARIC.ACID.t3	0.48
HIPPURIC.ACID.t3	0.48
X3.HYDROXYPHENYLACETIC.ACID.t3	0.48
URIC.ACID.t3	0.47
X4.HYDROXYPHENYLLACTIC.ACID.t3	0.43
METHYLMALONIC.ACID.t3	0.42
AZELAIC.ACID.t3	0.42
OROTIC.ACID.t3	0.42

Time 4	
Metabolite	VIP
X3.HYDROXYISOBUTYRIC.ACID.t4	0.74
X2.HYDROXYISOBUTYRIC.ACID.t4	0.71
VANILLYLMANDELIC.ACID.t4	0.71
ETHYLHYDRACRYLIC.ACID.t4	0.69
OXALIC.ACID.t4	0.66
INDOL.3.ACETIC.ACID.t4	0.65
FUMARIC.ACID.t4	0.63
URACIL.t4	0.61
GLUCURONIC.ACID.t4	0.59
PHOSPHORIC.ACID.t4	0.57
X5.HYDROXYINDOLACETIC.ACID.t4	0.56
X3.4.DIHYDROXYPHENYLACETIC.ACID.t4	0.56
ACONITIC.ACID.t4	0.55
SUCCINIC.ACID.t4	0.54
BENZOIC.ACID.t4	0.53
X2.HYDROXYGLUTARIC.ACID.t4	0.52
X3.4.DIHYDROXYBUTANOIC.ACID.t4	0.51
X2.3.DIHYDROXYBUTANOIC.ACID.t4	0.50
X4.HYDROXYHIPPURIC.ACID.t4	0.49
LACTIC.ACID.t4	0.47
MALIC.ACID.t4	0.47
X2.HYDROXYBUTYRIC.ACID.t4	0.47
GLYCERIC.ACID.t4	0.47
GLUTARIC.ACID.t4	0.46
X3.HYDROXYVALERIC.ACID.t4	0.46
X3.HYDROXY.3.METHYLGLUTARIC.ACID.t4	0.45
ISOVANILYLGLYCINE.t4	0.45
GLYCOLIC.ACID.t4	0.45
OROTIC.ACID.t4	0.44
TIGLYLGLYCINE.t4	0.43
X4.HYDROXYPHENYLLACTIC.ACID.t4	0.42
METHYLMALONIC.ACID.t4	0.42
X3.HYDROXYPROPIONIC.ACID.t4	0.42
METHYLCITRIC.ACID.t4	0.41
URIC.ACID.t4	0.41

Time 5	
Metabolite	VIP
VANILLYLMANDELIC.ACID.t5	0.72
OXALIC.ACID.t5	0.70
X3.4.DIHYDROXYBUTANOIC.ACID.t5	0.67
X3.HYDROXYISOBUTYRIC.ACID.t5	0.66
X3.4.DIHYDROXYPHENYLACETIC.ACID.t5	0.65
ISOVANILGLYCOLIC.ACID.t5	0.62
PHOSPHORIC.ACID.t5	0.60
X5.HYDROXYINDOLACETIC.ACID.t5	0.59
FUMARIC.ACID.t5	0.59
GLYCEROL.t5	0.59
ACONITIC.ACID.t5	0.57
X1.2.DIHYDROXYBUTANE.t5	0.55
GLYCERIC.ACID.t5	0.55
SUCCINIC.ACID.t5	0.54
URIC.ACID.t5	0.54
X2.HYDROXYBUTYRIC.ACID.t5	0.54
MALIC.ACID.t5	0.53
GLYCOLIC.ACID.t5	0.51
NONANOIC.ACID.t5	0.49
URACIL.t5	0.49
X3.METHYLADIPIC.ACID.t5	0.48
X2.HYDROXYGLUTARIC.ACID.t5	0.46
MYRISTIC.ACID.t5	0.46
X2.KETOGLUTARIC.ACID.t5	0.45
TIGLYLGLYCINE.t5	0.44
STEARIC.ACID.t5	0.43
BENZOIC.ACID.t5	0.41
X2.3.BUTANEDIOL.t5	0.41
GLUCURONIC.ACID.t5	0.41
X2.3.DIHYDROXYBUTANOIC.ACID.t5	0.40

Time 6	
Metabolite	VIP
X2.HYDROXYBUTYRIC.ACID.t6	0.78
X3.HYDROXYISOBUTYRIC.ACID.t6	0.73
ETHYLHYDRACRYLIC.ACID.t6	0.72
BENZOIC.ACID.t6	0.68
GLUTARIC.ACID.t6	0.67
MALIC.ACID.t6	0.64
VANILLYLMANDELIC.ACID.t6	0.59
METHYLMALONIC.ACID.t6	0.55
X3.HYDROXYPROPIONIC.ACID.t6	0.54
X4.HYDROXYPHENYLACETIC.ACID.t6	0.54
FUMARIC.ACID.t6	0.54
X4.HYDROXYMANDELIC.ACID.t6	0.53
X4.HYDROXYPHENYLLACTIC.ACID.t6	0.51
SUCCINIC.ACID.t6	0.51
X2.3.DIHYDROXY.2.METHYLBUTYRIC.ACID.t6	0.51
LACTIC.ACID.t6	0.51
X4.HYDROXYBENZOIC.ACID.t6	0.51
X3.HYDROXYPHENYLACETIC.ACID.t6	0.50
X2.HYDROXYGLUTARIC.ACID.t6	0.49
GLYCOLIC.ACID.t6	0.49
GLUCURONIC.ACID.t6	0.47
HYDANTOINPROPIONIC.ACID.t6	0.47
X2.METHYL.2.3.DIHYDROXYBUTYRIC.ACID.t6	0.45
X3.METHYLADIPIC.ACID.t6	0.45
ACONITIC.ACID.t6	0.45
X3.4.DIHYDROXYPHENYLACETIC.ACID.t6	0.44
X2.HYDROXYPHENYLACETIC.ACID.t6	0.43
X3.HYDROXY.3.METHYLGLUTARIC.ACID.t6	0.43
X5.HYDROXYINDOLACETIC.ACID.t6	0.42
METHYLSUCCINIC.ACID.t6	0.42
XANTHURENIC.ACID.t6	0.42
INDOL.3.ACETIC.ACID.t6	0.42
X2.HYDROXYISOBUTYRIC.ACID.t6	0.41
HIPPURIC.ACID.t6	0.41
X2.METHYL.3.HYDROXYBUTYRIC.ACID.t6	0.40

Certain metabolites have been color coded within table 6 and categorized into specific perturbed pathways, shown below (NOTE: additional metabolite characteristics described in Appendix 1):

Citric acid cycle: aconitic acid, citric acid, citramalic acid, fumaric acid, malic acid, succinic acid

Neurological/catecholamine: isovanilglycolic acid, isovanilylglycine, 5-hydroxyindoleacetic acid, indol-3-acetic acid, vanillylmandelic acid

Branched-chain amino acid: 2-hydroxybutyric acid, 2-hydroxyisobutyric acid, 3-hydroxyisobutyric acid, ethylhydracrylic acid, tiglylglycine, 2-methyl-3-hydroxybutyric acid, methylmalonic acid, 3-hydroxyvaleric acid

The selected color coded metabolites can be associated with three primary pathways and can be loosely classified as perturbation markers associated with acute alcohol consumption. There exists several other unselected metabolites within the VIP lists, some of which can be associated with acute alcohol consumption (e.g. fatty acids, such as stearic acid, and their derivatives (glycerol), indicating abnormal lipid metabolism); however, these metabolites fall outside the focus of this investigation and as such only 3 predominantly observed perturbed pathways are noted. Two additional metabolites within the VIP lists have also been selected (in green), namely: benzoic acid and hippuric acid. These two particular metabolites were highlighted as an example to allow for additional quantitative analysis of the metabolomics data.

		Control		Experimental	
		Benzoic acid	Hippuric acid	Benzoic acid	Hippuric acid
Mean concentration (mg/g creat)	0 hr	4.120	74.425	1.734	81.427
	1 hr	3.810	182.053	25.481	309.074
	2 hr	7.971	101.956	17.851	268.511
	3 hr	5.982	65.050	1.016	123.496
	4 hr	2.151	61.314	2.651	143.225
	5 hr	6.260	75.143	1.705	122.790

Hippuric acid is the detoxification product of benzoic acid. Benzoic acid is a dietary compound that occurs as a preservative, in the form of sodium benzoate, within the lemon flavored carbonated water consumed within both the experimental and control cases, accounting for its occurrence within this study. Quantitative analysis of these 2 metabolites shows that a greater mean concentration of both benzoic acid and hippuric acid occurs within the experimental cases when compared to the control cases. According to Siquiera *et al.* (2002) [51], the normal urinary reference value of hippuric acid within individuals aged between 18 and 35 years old is 160 +/- 9 milligram per gram creatinine. The concentrations of hippuric acid within the experimental cases noticeably exceed this published normal reference value. The implications and cause of this observation exceeds the scope of this investigation, however, these 2 metabolites were selected to illustrate the quantitative differences existing between the experimental and control cases as the VIP lists only provide qualitative information. NOTE: univariate analysis was not deemed necessary for these quantitative results given the deterministic nature of the values shown.

Another significant observation of the VIP lists is that a significant degree of disruption is occurring within the branched-chain amino acid pathway as a vast number of metabolites (both intermediate and secondary) are present from this pathway and are also ranked highly on the VIP lists, particularly ethylhydracrylic acid. The presence of metabolites associated with perturbed citric acid cycle and catecholamine metabolism is consistent with the published literature.

Thus, unfolding PCA reveals a unique pattern of deviation within each independent group, with clear separation being made between the experimental and control group; however, since this model considers all the times at once it is difficult to isolate and identify the variables responsible for the different trajectories of each group. Cross-sectional PCA supports the observations seen within unfolding PCA by illustrating definite separation between the 2 independent groups, seen within the PC score plots (Fig 18). Cross-sectional PCA also overcomes the difficulties presented within unfolding PCA by successfully generating lists of variables of importance for each time point.
Both of these models however treat the experimental and control groups independently. A third and final model (ASCA model) was employed within this investigation to directly compare these two groups and allow a comparison with cross-sectional PCA to determine if the noted VIPs are common between both models.

5.2.3.3 ANOVA-Simultaneous Component Analysis (ASCA)

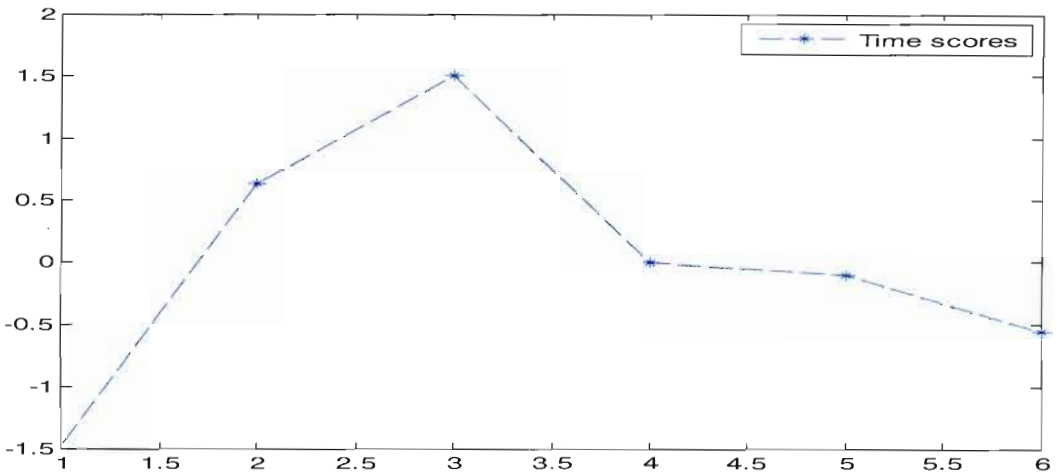
Analysis of variance (or also known as ANOVA) is a well-used technique within the world of statistics as it is a global test that examines variances within sample sets and between different sample groups. ANOVA on its own, however, is best suited for univariate data. ASCA (ANOVA-Simultaneous Component Analysis), was recently described by Smilde et al. [36], where the objective is to apply an ANOVA-like method to time-dependent data where the effect of a treatment factor on multiple dependent variables is investigated. The ASCA model allows us to directly compare both groups (experimental and control) together by separating the variation into independent, orthogonal parts (dimension reduction) and assigning it to factors (e.g. time, treatment). Interpretation of the resulting components subsequently operates in the same manner as an ordinary PCA.
The model consists of the following components:

- An overall mean,
- A simultaneous component analysis (SCA) model describing the overall (common) effect of the factor time
- A SCA model describing the important interaction between time and treatment and lastly,
- A SCA model describing the 3-way interaction between the time, treatment and participant.

Thus, the ASCA model ultimately deconstructs a complicated multivariate data set into separate components. To apply the ASCA model, one simultaneous component (SC) was extracted for the factor time, whilst 2 SCs were extracted for the interaction of the factor time and treatment and time, treatment and participant. These components explained 9%, 21% and 70% of the isolated variation of time, time

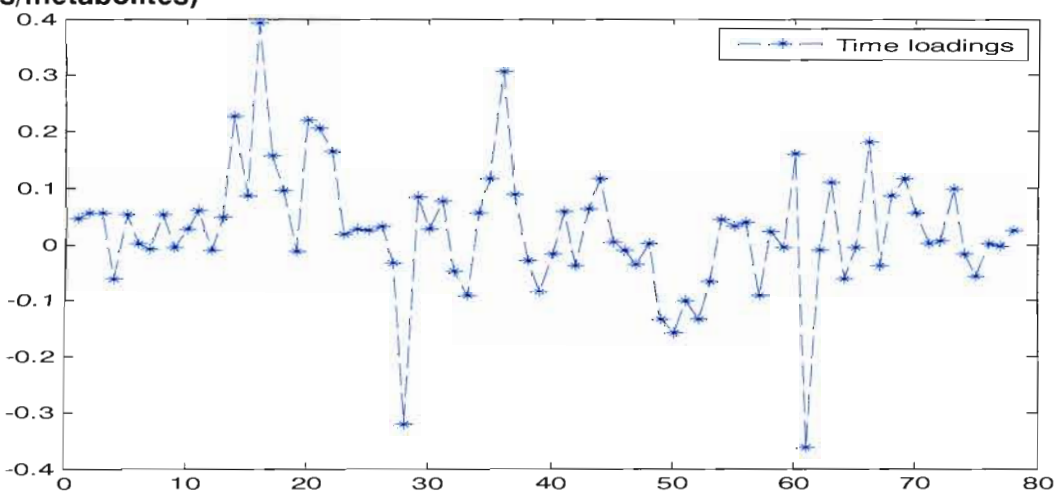
and treatment interaction and time, treatment and participant interaction respectively. The common contribution of the factor time is displayed below in terms of the ASCA scores produced for this factor.

Figure 19: Time factor scores on the first component (y-axis = SC1, x-axis = time)



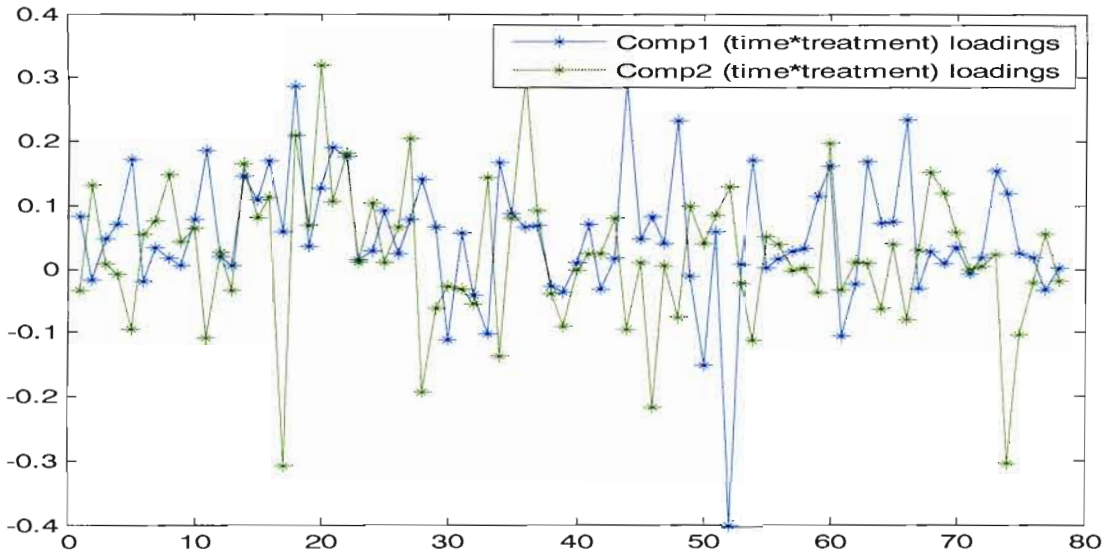
The scores in figure 19 thus indicate an initially increasing and then decreasing and leveling of the time profile. This indicates that a common variation is indeed present over time within the data, with a large variance within the first 4 time points and a leveling within the last 2 time points. The ASCA loadings of the factor time, belonging to the first component (shown in Fig 20), illustrates the contribution of each variable to the model. The variables with high absolute loading values can be considered as more important when describing the common time factor. These variables were identified but not reported since the loadings of time and treatment interaction seems to be more informative.

Figure 20: Time factor loadings on the first component (y-axis = SC1, x-axis = variables/metabolites)



The SC loadings of the 2 SCs extracted for the analysis of the second contribution factor in the ASCA model, namely the interaction between the factors of time and treatment (acute alcohol dose), are presented in Fig 21 below:

Figure 21: PC loadings of PC1 and PC2 for the factor of interaction between time and treatment

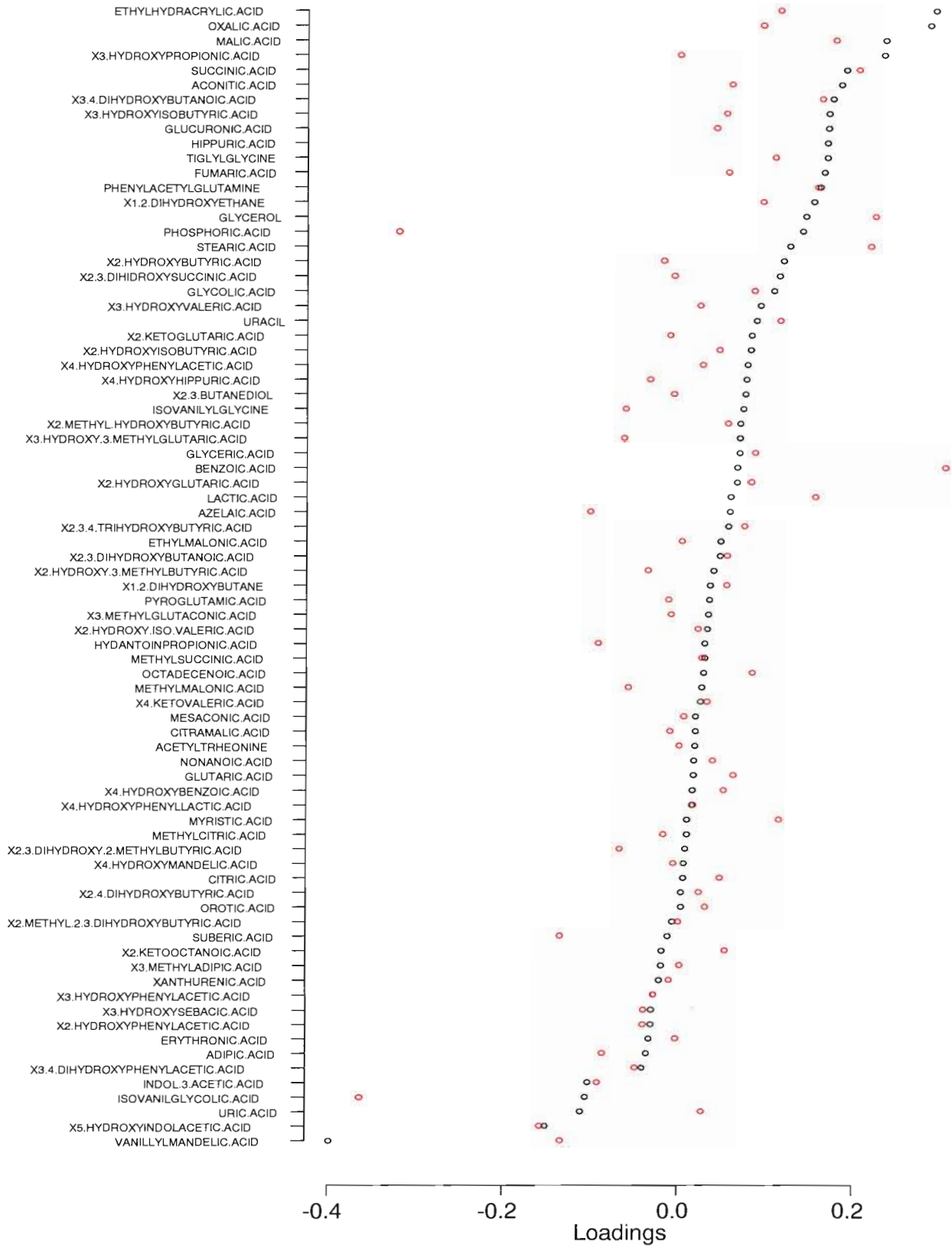


From this plot we can conclude that those variables with higher absolute loadings are of greater importance when describing the treatment and time interaction.

The SC loadings, shown in Fig 21, were then transposed into an S-form graph (Fig 22) and variable names included to allow for easier identification of variables; and rearranged in ascending order from variables with the greatest negative SC1 ranking (vanillylmandelic acid) to variables with the greatest positive SC1 ranking (ethylhydracrylic acid).

Variables around the axis where SC1=0 are variables with a small contribution to the time*treatment interaction. Thus, from this graph variables with the greatest variance over time (identified from both positive and negative SC rankings), as a consequence of an acute alcohol dose, can be clearly identified and labeled as perturbance markers of acute alcohol abuse. (NOTE: the details of selected variable/metabolite characteristics and origins are given in Appendix 1)

Figure 22: Loadings of interaction between time and treatment on the first (shown in black) and second (shown in red) component



Variables of greatest positive ranking:

- Ethylhydracrylic acid
- Oxalic acid
- Malic acid
- 3-Hydroxypropionic acid
- Succinic acid
- Aconitic acid
- 3,4-Dihydroxybutanoic acid
- 3-Hydroxyisobutyric acid
- Glucuronic acid
- Hippuric acid
- Tiglylglycine
- Fumaric acid
- Phenylacetylglutamine
- 1,2-Dihydroxyethane
- Glycerol
- Phosphoric acid
- Stearic acid

Variables of greatest negative ranking:

- Vanillylmandelic acid
- 5-Hydroxyindoleacetic acid
- Uric acid
- Isovanilglycolic acid
- Indole-3-acetic acid

These variables can be categorized (according to their metabolic information given in Appendix 1) into specific primary perturbed pathways:

- Citric Acid Cycle: malic acid, succinic acid, fumaric acid, aconitic acid
- Neurological/Catecholamines - isovanilglycolic acid, vanillylmandelic acid, 5-hydroxyindoleacetic acid, indole-3-acetic acid (also possibly microbe product)
- Branched-chain amino acid: ethylhydracrylic acid, 3-hydroxyisobutyric acid, 3-hydroxypropionic acid, tiglylglycine
- Lipid metabolism: glycerol, stearic acid
- Other: hippuric acid, phosphoric acid, 3,4-dihydroxybutanoic acid, oxalic acid, 1,2-dihydroxyethane, uric acid, phenylacetylglutamine, glucuronic acid

These selected variables show the greatest variance over time and can be confidently accredited as perturbance markers associated with acute alcohol consumption. The variables categorized as "other"

includes metabolites formed by perturbances within numerous other biological systems as a consequence of excessive ethanol oxidation (e.g. phenylacetylglutamine is a detoxification product and an indicator of liver damage) and their presence is supported by the literature, as noted within Appendix 1. Oxalic acid, however, is a dietary component (exogenous source) and the reason for its variance is uncertain.

Experimental observation of the loadings plot within Figure 22 and the subsequent categorization of predominant perturbance markers provide clear qualitative information. For a better quantitative understanding of the data, 4 of the highest ranking (both positive and negative) metabolites were selected and compared to reference values:

Table 7: Quantitative analysis of 4 greatest positive and negative ranking metabolites and comparison with reference values (n.d = not determined, reference values obtained from Blau *et al.* [39])

			Ethylhydracrylic acid	Malic acid	3-Hydroxy propionic acid	Succinic acid
Mean concentration (mg/g creat)	Control	0 hr	1.519	0.000	0.629	9.321
		1 hr	0.970	0.047	0.259	12.471
		2 hr	3.076	0.488	0.454	13.456
		3 hr	0.509	0.000	0.561	14.926
		4 hr	1.217	0.000	0.329	11.383
		5 hr	0.960	0.000	0.544	14.051
	Experimental	0 hr	2.362	0.000	1.264	14.014
		1 hr	5.248	2.250	2.845	26.742
		2 hr	5.876	3.428	2.680	42.901
		3 hr	5.701	1.896	3.459	23.868
		4 hr	5.144	2.349	3.211	22.107
		5 hr	5.935	2.441	3.744	20.085
Reference (mmol/mol creat)			<5	n.d	<5	n.d
Reference (mg/g creat)			<7.3	n.d	<7.3	n.d

			Vanillylmandelic acid	5-Hydroxy indoleacetic acid	Uric acid	Isovanilglycolic acid
Mean concentration (mg/g creat)	Control	0 hr	6.862	1.979	3.798	4.243
		1 hr	5.538	0.847	5.243	4.500
		2 hr	7.017	1.930	7.353	2.014
		3 hr	5.445	3.650	5.940	2.085
		4 hr	6.633	1.501	6.087	3.893
		5 hr	7.802	2.816	5.259	4.133
	Experimental	0 hr	5.663	1.366	3.337	7.849
		1 hr	2.742	0.543	3.186	2.623
		2 hr	0.566	0.026	3.787	0.000
		3 hr	0.000	0.232	3.336	0.662
		4 hr	0.000	0.260	3.775	0.612
		5 hr	1.106	0.613	2.968	2.429
Reference (mmol/mol creat)			0.8-2.2	0.3-5.1	n.d	n.d
Reference (mg/g creat)			1.17-3.21	0.44-7.44	n.d	n.d

As can be seen within table 7, the four selected variables with the greatest positive ranking exhibit higher mean concentrations within the experimental cases when compared to the control cases; and, vice versa, the four selected variables with the greatest negative ranking exhibit lower mean concentrations within the experimental cases when compared to the control cases. Comparison of mean concentrations to published reference values in a quantitative manner thus illustrates how, although these variables offer the most significant qualitative information for this study, the variables still remain, on the most part, within the normal physiological range. The cause of the slightly increased levels of vanillylmandelic acid (VMA) within the control cases is uncertain and beyond the scope of this investigation.

An observation of notable interest, as was seen within the cross-sectional PCA model, is the occurrence of numerous perturbance markers from the branched-chain amino acids pathway. As of our knowledge, there currently exists no information within the literature regarding the branched-chain amino acids pathway with respect to perturbances associated with acute (or chronic) alcohol abuse and warrants additional investigation. This observed experimental anomaly thus sustains the development of the notion that metabolomics is a hypothesis-generating system, not a hypothesis-testing system, which is demonstrated within the second phase of this investigation. (NOTE: the loadings of the time, treatment and participant interaction are not reported since these are of less interest to the current investigation.)

5.3 Hypothesis Formulation

A hypothesis is a provisional but possible explanation of a distinct feature that is observed through experimentation and must be phrased in such a manner that allows for experimental verification through the development and execution of a practical and relevant experimental protocol. Through the application of numerous statistical models in the open-minded assessment of the metabolic perturbations associated with acute alcohol consumption, an evident perturbation was observed within the branched-chain amino acid catabolism pathway, particularly isoleucine catabolism. This led to the articulation of the following hypothesis:

> **The presence of perturbance markers of isoleucine catabolism indicates that acute alcohol consumption stimulates secondary metabolic pathways resembling, on a minor scale, inherited metabolic disorders of branched-chain amino acids (specifically isoleucine).**

The experimental protocol designed for the second phase of this investigation is thus aimed at evaluating the validity of this proposed hypothesis.

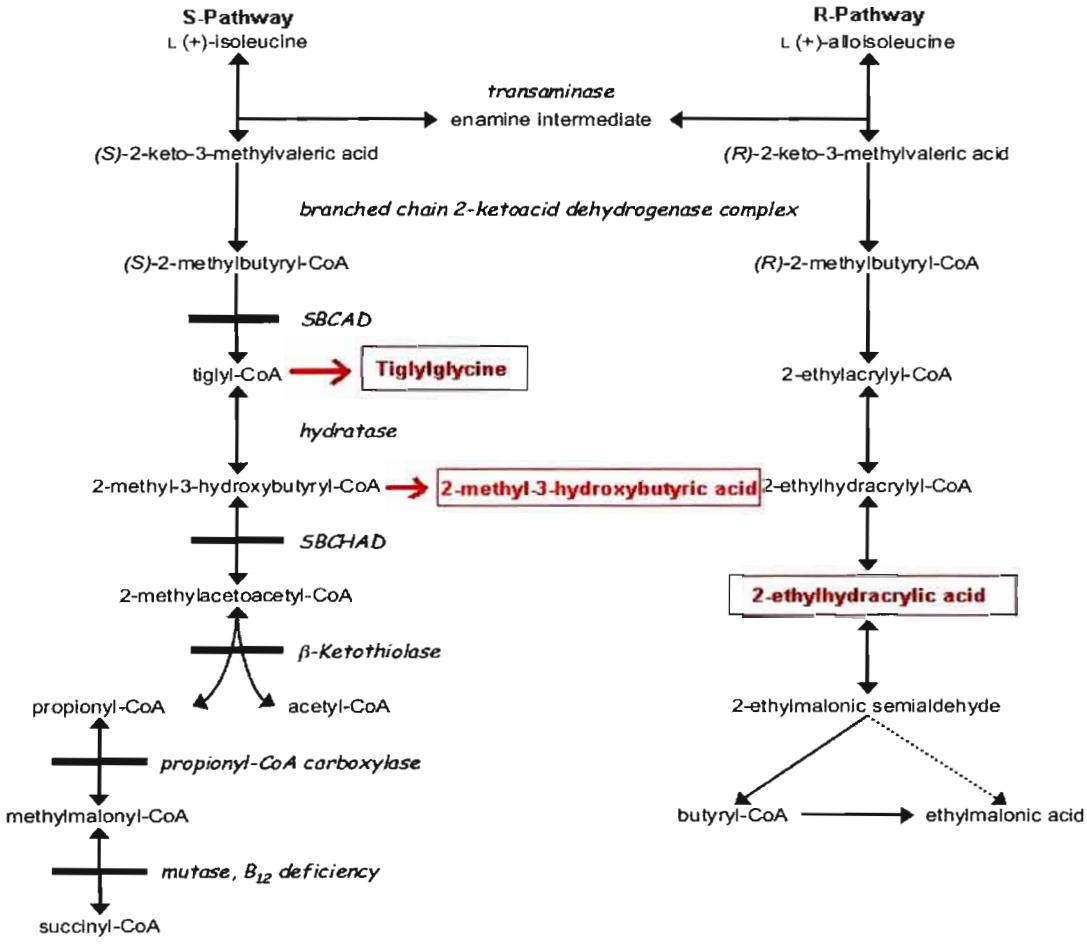
6. HYPOTHESIS EVALUATION

The application of three different statistical models in the multivariate analysis of time-dependent organic profiles of individuals administered an acute alcohol dose, as described in section 5, culminated in a list of variables with the most significant variance over time and of relevant biological value to be accredited to alcohol-associated metabolic perturbances. Categorization of these variables of importance (primary and secondary metabolites) resulted in identifying three primary perturbed pathways of interest, namely: the citric acid cycle, metabolism of catecholamines and branched-chain amino acids pathways. The literature, as of date, recognizes the presence of metabolites due to alcohol-induced perturbances within the citric acid cycle and catecholamines pathway; however, there exists no information relating alcohol metabolism and branched-chain amino acid catabolism, specifically for isoleucine degradation. The hypothesis, which was formulated from the untargeted metabolomics data, thus relates to the latter metabolic pathway.

6.1 Motivation for Hypothesis

Under normal physiological conditions, catabolism of the branched-chain amino acid isoleucine favors the S-pathway. When under distress (e.g. enzyme inactivity in the case of an inherited metabolic disorder, such as propionyl-CoA decarboxylase deficiency) there is increased flux through the minor R-pathway (Figure 23). The stimulation of this R-pathway and the presence of specific secondary metabolites (such as tiglylglycine) serve as perturbance markers indicating abnormal physiological activity within the isoleucine catabolic pathway. Within the previously described experiment on acute alcohol consumption and the associated metabolic perturbances, three metabolites of interest (ethylhydracrylic acid, tiglylglycine and, on a lesser scale, 2-methyl-3-hydroxybutyric acid) were noted and can be attributed to abnormal isoleucine catabolism.

Figure 23: The S- & R-Pathways of Isoleucine Catabolism; including: 1) enzyme names (shown in italics), 2) locations of possible inherited metabolic disorders (indicated by —) and 3) observed perturbance markers of acute alcohol consumption (shown in red) (Legend: SBCAD=short branched-chain acyl-CoA dehydrogenase, SBCHAD=short branched-chain hydroxyl acyl-CoA dehydrogenase)



2-Ethylhydracrylic acid is an intermediate metabolite within the R-pathway of isoleucine catabolism. Elevated levels of this metabolite are typically found within individuals suffering from SBCADD (Short Branched-Chain Acyl-Coa Dehydrogenase Deficiency), an inherited metabolic disorder [38]. Tiglylglycine and 2-methyl-3-hydroxybutyric acid are overflow/secondary metabolites, not present (or only present in trace amounts) under normal physiological conditions, that typically only occur in elevated amounts in the urine of individuals with defects in SBCHAD (Short Branched-Chain Hydroxy Acyl-CoA Dehydrogenase), β-ketothiolase and/or propionyl-CoA carboxylase (propionic aciduria) [39]. SBCAD and SBCHAD are both NAD-dependent. Following the trend set within the literature, it is probable that the NAD-dependent dehydrogenase-catalyzed reactions will be the primary sites of perturbance as an increased NADH:NAD^+ ratio, caused by excessive ethanol oxidation from acute alcohol consumption, will disrupt normal physiological equilibrium.

Table 8 below compares the mean concentration values of our 3 targeted metabolites in individuals administered an acute alcohol dose with control values and reference values. The control and experimental mean concentrations presented within table 8 were taken from the results of the untargeted acute alcohol investigation at time 2 (the time point at which urinary metabolites are expected to be at their peak).

Table 8: Mean concentrations of 3 targeted metabolites (at time point 2) for experimental and control cases in comparison to reference values (reference values obtained from Blau et al. [39])

		Ethylhydracrylic acid	Tiglylglycine	2-Methyl-3-hydroxybutyric acid
Mean Concentration (mg/g creat)	Controls	3.076	0.000	0.734
	Experimental	5.876	2.264	1.404
	Reference value (s)	<7.3	<2.9	0 - 16

The elevated presence of these 3 metabolites (ethylhydracrylic acid, tiglylglycine and 2-methyl-3-hydroxybutyric acid), albeit in only marginally elevated levels, within the urine of individuals whom have consumed an acute dose of alcohol resemble certain inherited metabolic disorders of the isoleucine catabolism pathway, but only in a very minor scale in comparison with the inherited enzyme disorders. These 3 perturbance markers and the isoleucine degradation pathway (both R- & S-pathways) will be the focus within this second phase of this investigation. In order to evaluate the proposed hypothesis, an extended and more targeted experimental protocol was designed, aimed at validating the presence of these 3 perturbance markers by means of an experiment involving an alcohol dose and/or isoleucine loading.

NOTE: the effect of the vehicle (carbonated, flavored water) was extensively investigated, as described in the previous chapter as part of acute alcohol dose experiment (particularly within the trajectory plots in Fig 17). From these results, as well as from the ASCA-model, it is clear that no appreciable values of tiglylglycine, ethylhydracrylic acid nor 2-methyl-3-hydroxybutyric acid were detected under these conditions. The importance of the control samples was thus deemed sufficiently investigated based upon the results observed and thus a control experiment was not considered necessary for inclusion for the loading tests performed within this targeted study.

6.2 Targeted Study into Ethanol-induced Perturbances within Isoleucine (Ile) Pathway

6.2.1 Experimental Protocol

To evaluate the validity of the proposed hypothesis, 3 defined experimental groups were used and given the following:

Group 1: Isoleucine load + acute alcohol dose

Group 2: Isoleucine load only

Group 3: Alcohol dose only

Participants were selected according to the same inclusion and exclusion criteria used in the previous experiments (i.e. similar homogenous group of young males). Eleven participants were assigned to

group 1, while eight participants were assigned to group 2 and to group 3. As described previously, the alcohol dose used was 1.5g per kg body weight (43% v/v Smirnoff triple-distilled vodka) and the isoleucine load used was 100mg per kg body weight [34]. All protocol procedures used within the previous acute alcohol consumption protocol were retained within this experiment, including the requirement of all participants to be in an overnight fasted state. A "0 hour" blood and urine sample was collected immediately before administration of alcohol and/or isoleucine. A second blood and urine sample was collected approximately one hour later. Additional urine samples were required at 2 hrs, 4 hrs, 6 hrs and 8 hrs after initial consumption of alcohol dose and/or isoleucine load. Thus, each participant was required to supply a total of 2 blood samples and 6 urine samples.

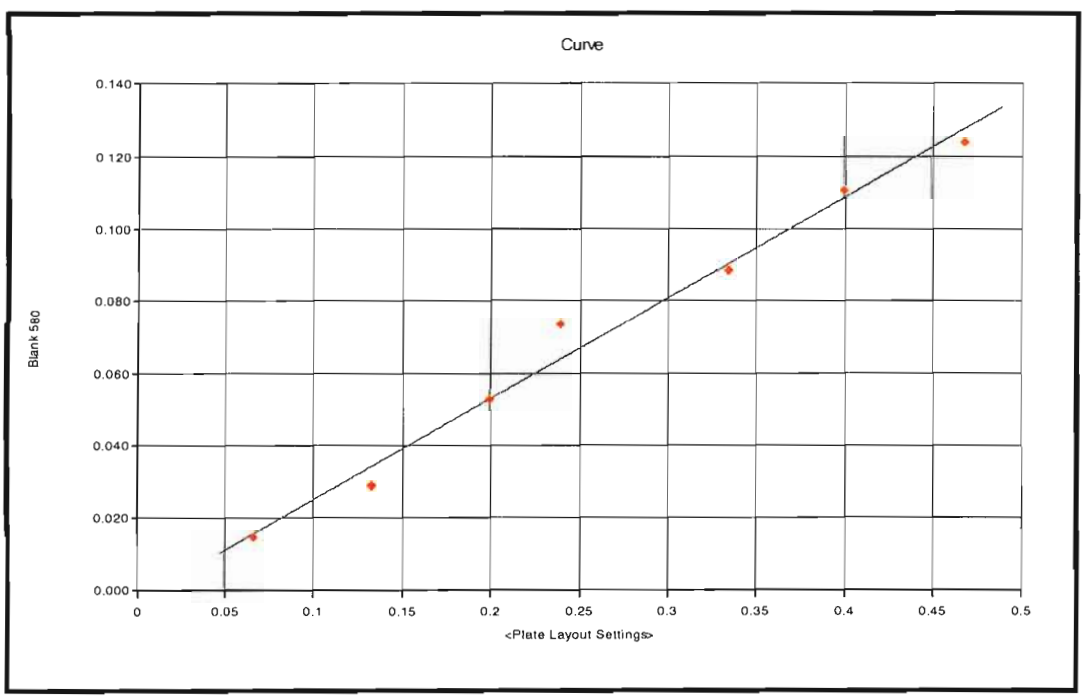
The analytical protocols applied to these collected biological samples were extended from only organic acid analysis and determination of ethanol concentration in breath/blood, to include amino acid and acylcarnitine analysis.

6.2.2 Results

6.2.2.1Determination of Blood Alcohol Concentration (via Ethanol Assay Kit)

The expected peak of blood alcohol concentration occurs at approximately 1 hour after consumption of alcohol, according to Høiseth *et al.* [33]. As such, a 1 hour blood sample was deemed sufficient for determining peak in blood alcohol concentration for our targeted study. Protocol specific to ethanol assay kit (described in section 3.8.2) was applied to the blood samples and read at 580nm. An ethanol standard, supplied with the ethanol assay kit, was used to create an ethanol dilution series. From this dilution series a standard curve was drawn:

Figure 24: Standard curve from ethanol standard dilution series (y-axis represents OD (580nm minus blank), x-axis represents concentration (EtOH %)



All unknown concentration values were thus calculated from this standard curve according to their recorded OD. Conversion of ethanol concentration values from EtOH % into mg/L blood was done by use of the following conversion equation: 1% EtOH = 7.85g/L. Each blood sample was done in duplicate and the average of the two results was used to determine an approximate blood alcohol concentration. A mean blood alcohol concentration value was determined for each group (table 9).

Table 9: Mean blood alcohol concentration for each group at 1 hour, calculated as EtOH % (determined as average from duplicate results) and as gram per L blood (calculated from conversion ratio: 1% EtOH = 7.85 g/L)

	EtOH %	[EtOH] (g/L)
Group 1	0.0968	0.760
Group 2	0.0432	0.339
Group 3	0.0863	0.678

Group 1 (EtOH + Ile) had individuals ranging in blood alcohol concentrations from approximately 0.6g/L to 1.2g/L. Group 2 (Ile) weren't administered any alcohol however low concentrations of alcohol in the blood were still detectable. Group 3 (EtOH) had blood alcohol concentration values ranging from approximately 0.6g/L to 1.0g/L, similar to group 1. These results confirm the presence of increased alcohol in the blood of the individuals administered an alcohol dose, as expected. These values are also consistent with published acute alcohol dose cases [35]. However, the detection of alcohol within the individuals not administered any alcohol suggests that the protocol of the ethanol assay kit used is not fully suitable for this investigation. Our area of focus for blood alcohol concentration within this investigation is approximately 0.06 – 0.08%. The range of the ethanol assay kit is 0.04% - 4%. As such, an assay kit with a lower minimum range would be more suitable. The results from the determination of the blood alcohol concentrations, however, were sufficient enough to indicate that group 1 and group 3 had indeed been given an acute dose of alcohol.

6.2.2.2 Organic Acids Analysis

Organic acid analysis on the urine of all 3 experimental groups yielded a large data matrix of metabolites. The first attempt to validate the proposed hypothesis was a targeted analysis of minor metabolites formed due to the alcohol perturbation. The metabolites related to the pathway of isoleucine degradation were targeted and examined. They are:

1. Ethylhydracrylic acid
2. Tiglylglycine – detoxification product of tiglyl-CoA
3. 2-Methyl-3-hydroxybutyric acid

These metabolites were targeted based upon their association, as perturbation markers, with inherited metabolic disorders within the isoleucine degradation pathway (as stated earlier). Based upon the understanding of the literature on ethanol-induced metabolic perturbations, an acute dose of alcohol should increase the NADH:NAD^+ ratio and hence depresses NAD-dependent dehydrogenase-catalyzed reactions. According to Figure 23, several dehydrogenase-catalyzed reactions exist within the isoleucine

degradation pathway, specifically: SBCAD and SBCHAD. As such, disruption at these two sites should cause an accumulation of metabolites upstream of these sites, as well as increasing the flux through the R-pathway.

6.2.2.2.1 Postulated Results

A defect at SBCHAD, as per an inherited metabolic disorder of this enzyme, is expected to cause an accumulation of tiglylglycine and 2-methyl-3-hydroxybutyric acid, secondary metabolites of tiglyl-CoA and 2-methyl-3-hydroxybutyryl-CoA respectively. A defect at SBCAD causes an accumulation of R-pathway specific metabolites of the isoleucine catabolism pathway. One such expected accumulated abnormal metabolite, amongst others, would be ethylhydracrylic acid. In the case where a defect exists at both SBCAD and SBCHAD it is expected that a higher accumulation of R-pathway metabolites (i.e. ethylhydracrylic acid) would be present as there would be decreased precursors available for the formation of the secondary metabolites associated with defective SBCHAD (i.e. lower levels of tiglylglycine and 2-methyl-3-hydroxybutyric acid). As such, Table 10 depicts the expected results of this targeted study into the ethanol-induced perturbances of isoleucine catabolism:

Table 10: Expected results of targeted study into ethanol-induced perturbances of isoleucine catabolism (legend: ++++=very high,+++=high,++=moderate and +=low increase)

Targeted Metabolite	Group 1 (ETOH+Isoleucine)	Group 2 (Isoleucine)	Group 3 (ETOH)
Ethylhydracrylic acid	++++	+++	++
Tiglylglycine	++	+	+
2-Methyl-3-hydroxybutyric acid	++	+	

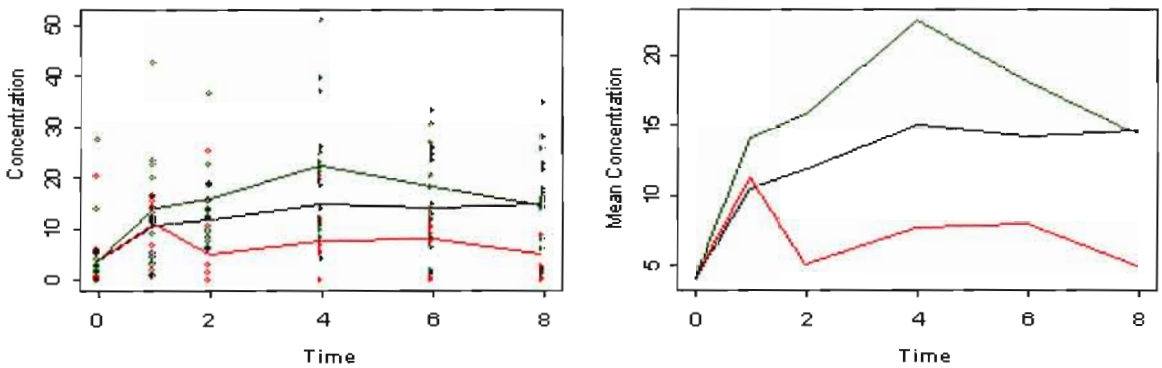
It is expected that an isoleucine load will increase flux into the R-pathway of isoleucine catabolism, as well as cause the presence of secondary metabolites of the S-pathway. Thus an increase of our targeted metabolites is expected in group 2, with a higher expected value of ethylhydracrylic acid. As shown within the results of the previous acute alcohol dose experiment, the slightly elevated presence of ethylhydracrylic acid and tiglylglycine (perturbation markers of isoleucine degradation) is expected in group 3. 2-Methyl-3-hydroxybutyric acid did not rank as a significant perturbation marker within the untargeted study and accordingly is not expected to be present in any appreciably elevated amount in group 3 within this targeted study. A combination of an acute alcohol dose and an isoleucine load, as administered within group 1, is expected to produce levels of our targeted metabolites higher than group 2.

6.2.2.2.2 Experimental Results

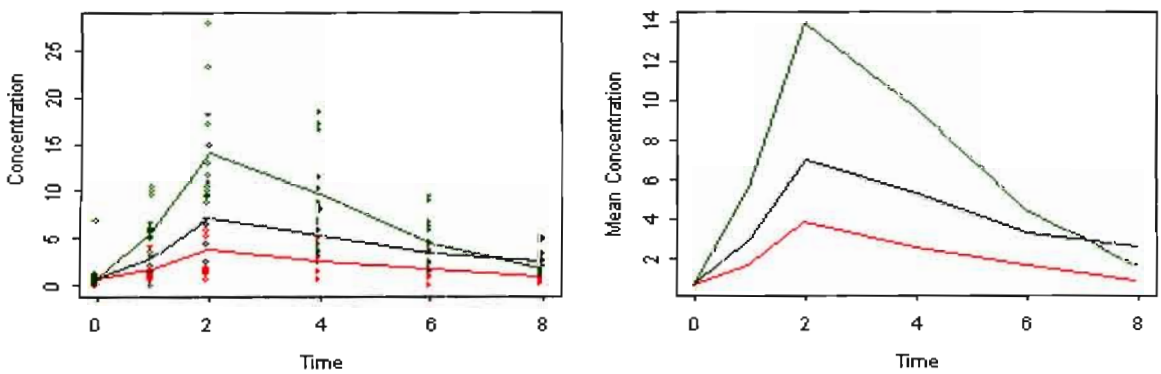
The results of this targeted time-dependent analysis are given in Figure 25. The figure includes the individual values observed for each experimental subject over the 8 hour experimental period with the mean concentration curve for each group (left panel), as well as the mean concentration curves of all three groups over the 8 hour period only, on a smaller, comparable scale (right panel).

Figure 25: Time-dependent mean concentrations (mg per g creatinine) of selected organic acids within urine: a) ethylhydracrylic acid, b) tiglylglycine, and c) 2-methyl-3-hydroxybutyric acid (green = group 1(EtOH + Ile), black = group 2 (Ile), red = group 3 (EtOH))

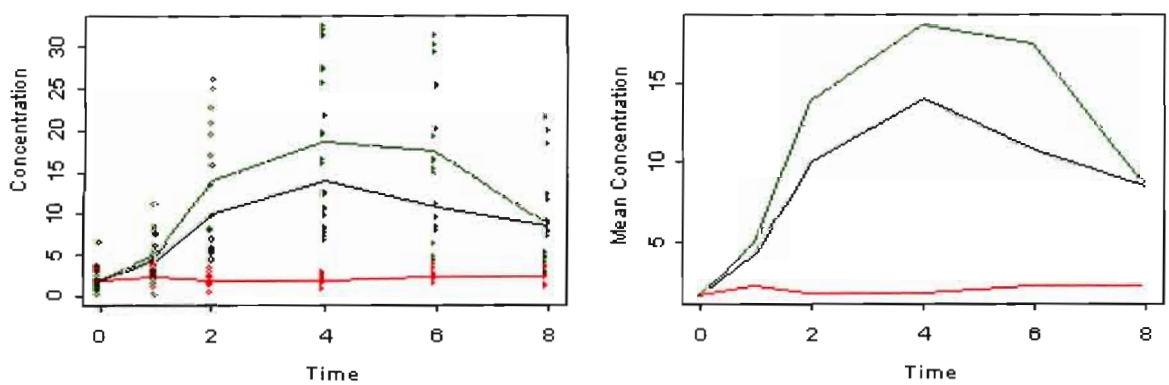
a) Ethylhydracrylic acid



b) Tiglylglycine



c) 2-Methyl-3-hydroxybutyric acid



In accordance with our reasoning above, an overload within the isoleucine catabolism pathway resulted in an increased flux into the R-pathway (an overflow pathway), resulting in subsequent elevated ethylhydracrylic acid production, seen in group 2. Further elevated concentrations of ethylhydracrylic

acid are noted within individuals given an isoleucine load and an acute alcohol dose in group 1. Only a minor, brief increase in ethylhydracrylic acid is noted within group 3.

Tiglylglycine, the secondary metabolite of tiglyl-CoA (an intermediate metabolite in isoleucine degradation), showed slightly increased concentrations within group 2 and 3; however, occurred in much higher concentrations within group 1.

2-Methyl-3-hydroxybutyric acid, the secondary metabolite of 2-methyl-3-hydroxybutyryl-CoA (another intermediate metabolite in isoleucine degradation), occurred in increased concentrations in group 2 and even higher concentrations in group 1; however, no occurrence in group 3.

Only a minor change within these 3 targeted metabolites was detectable within the blood over the first hour. The results (mean concentrations) for the targeted metabolites are presented in table 11 below:

Table 11: Mean concentrations (mg per g creatinine) of ethylhydracrylic acid, tiglylglycine and 2-methyl-3-hydroxybutyric acid recorded in blood for all 3 experimental groups at 0 hour and 1 hour

	Ethylhydracrylic acid		Tiglylglycine		2-Methyl-3-hydroxybutyric acid	
	0hr	1hr	0hr	1hr	0hr	1hr
group 1	0.711	1.461	0.000	0.000	0.018	0.306
group 2	0.476	1.817	0.000	0.000	0.011	0.238
group 3	0.636	0.465	0.000	0.000	0.011	0.037

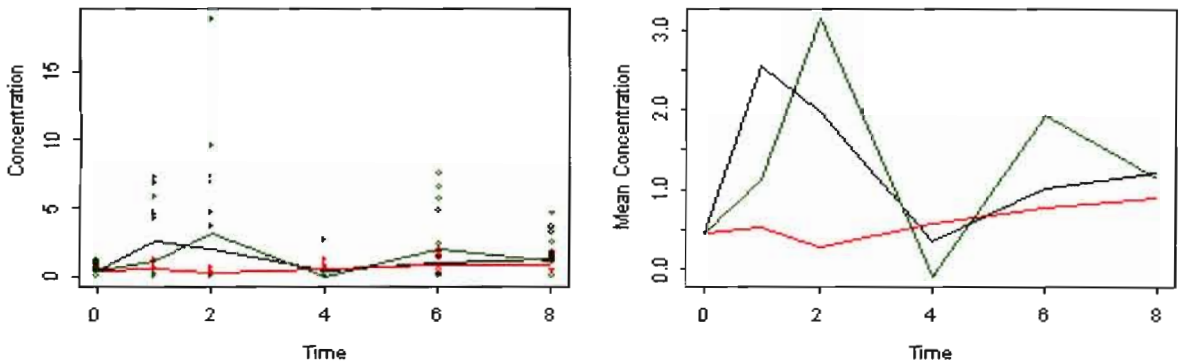
As can be seen within table 11, very low concentrations of ethylhydracrylic acid and 2-methyl-3-hydroxybutyric acid are present within the blood at 0hr, with a minor increase occurring in group 1 and group 2. Group 3 showed little/no deviation of blood organic acid profile of these 2 specific metabolites in response to an acute alcohol dose. Tiglylglycine is a detoxification product that is excreted through the urine only thus it is not detectable in the blood. The variances seen within the blood are minor (not detectable for tiglylglycine and no variances seen for group 3), and supports the experimental observations seen within the urinary organic acid profiles.

6.2.2.3 Amino Acid Analysis

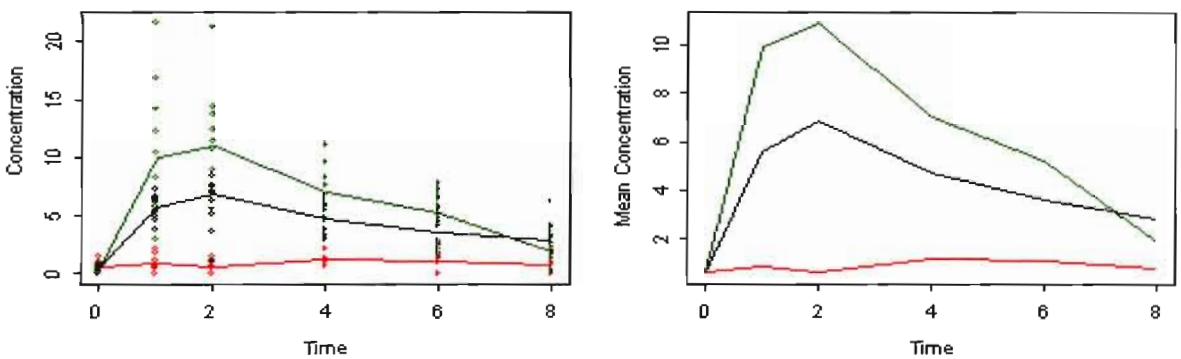
Amino acid analysis of urine of all 3 groups yielded an expansive profile of all amino acids, of which only isoleucine and it's R-form, allo-isoleucine, were targeted for analysis. Analysis of these 2 targeted amino acids (Figure 26) was done to determine if a discernable increase in isoleucine and allo-isoleucine could be detected, and to assess the relative ratio between the S-form isoleucine and its R-form allo-isoleucine in an attempt to determine which pathway has the greater flux.

Figure 26: Time-dependent mean concentrations (mg per g creatinine) of selected amino acids within urine: a) Isoleucine and b) Allo-isoleucine (green = group 1(EtOH + Ile), black = group 2 (Ile), red = group 3 (EtOH))

a) Isoleucine



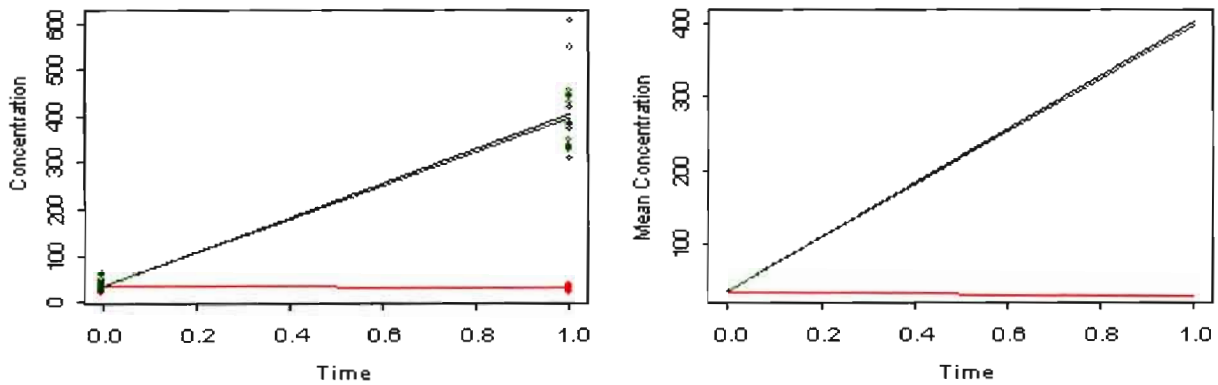
b) Allo-isoleucine



As expected, there was only change over time within groups 1 and 2 for isoleucine and allo-isoleucine as individuals within group 3 were not given any isoleucine loading. For both cases, isoleucine and allo-isoleucine occur in higher elevated amounts in group 1. Also, a higher concentration of allo-isoleucine is present compared to isoleucine (i.e. increased flux into R-pathway).

This high elevation of allo-isoleucine is especially noticeable within the blood, as a massive increase in the concentration of allo-isoleucine is detected in groups 1 and 2, shown in Figure 27, with no detectable values of allo-isoleucine present in group 3.

Figure 27: Mean concentration (mg per L) of allo-isoleucine in blood of all 3 groups (green = group 1(EtOH + Ile), black = group 2 (Ile), red = group 3 (EtOH))



The results of the organic acid and amino acid analysis of all 3 groups can be thus summarized in the following table:

Table 12: Overview of perturbance markers within isoleucine degradation pathway

	Organic Acids					
	Ethylhydracrylic acid		Tiglylglycine		2-Methyl-3-hydroxybutyric acid	
	Urine	Blood	Urine	Blood	Urine	Blood
Group 1	+++	+	+++	-	+++	+
Group 2	++	+	++	-	++	+
Group 3	+	-	+	-	-	-

	Amino Acids			
	Isoleucine		Allo-isoleucine	
	Urine	Blood	Urine	Blood
Group 1	+	-	++	+++
Group 2	+	-	++	+++
Group 3	-	-	-	-

These results confidently confirm that the presence of alcohol further depresses the S-pathway of isoleucine catabolism (most likely at the NAD-dependent dehydrogenase-catalyzed reactions (e.g. SBCAD and/or SBCHAD)) resulting in increased flux into R-pathway (evident by increased ethylhydracrylic acid), as well as increasing the production of secondary metabolites, particularly tiglylglycine and 2-methyl-3-hydroxybuyric acid. These experimental results are also consistent with our expected results.

6.2.2.4 Acylcarnitine Analysis

In addition to the organic acid and amino acid analysis of the experimental data collected within this targeted study, acylcarnitine analysis was also conducted. The purpose of this particular analysis was aimed at assessing what, if any, perturbances could be detected within the detoxification pathway of the participants. According to the reported literature, excessive consumption of alcohol leads to the formation of ROS and other oxidative compounds. Physiological responses to increased toxic oxidative species typically involves initiation of detoxification pathways, the end result being conjugated, less toxic compounds (e.g. tiglylglycine) that are excreted from the body. As such, it is expected that a noticeably increased level of detoxification products found within either urine or blood samples (detectable by acylcarnitine analysis) is indicative of a perturbed detoxification pathway. The results of the acylcarnitine profiles of both blood and urine samples of all 3 experimental groups, however, showed no discernibly signific :nt characteristics or deviation over time. It can be speculated that the acute alcohol dose used within this investigation was not sufficient enough to induce a significantly perturbed detoxification profile and that perhaps a larger alcohol dose, administered at regular intervals over a defined period of time (as would be evident within chronic alcohol users), would produce sufficient toxic oxidative products to induce an altered and detectable detoxification profile.

7. DISCUSSION

Through the results of the metabolomics approach used within this investigation we were able to make a number of statements substansiating the aims of this investigation:

- ✓ Metabolomics supports successful differentiation between a physiological state void of alcohol from an alcohol-intoxicated state within an individual.

This differentiation was clearly illustrated through the application of statistical models. Unfolding PCA yielded trajectory plots displaying unique patterns of deviation that differed between the experimental and control cases (Figure 16 and Figure 17). Cross-sectional PCA supported this observed deviation by clearly illustrating separation between the experimental cases and control cases between time 3 and time 6 (Figure 18). From the VIP lists created from cross-sectional PCA (table 6) we are able to state that:

- ✓ Multivariate analysis of metabolomics data allows for successful isolation and identification of variables responsible for this differentiation.

Application of an additional independent statistical model (ASCA model) to the generated metabolomics data supported the results obtained from cross-sectional PCA by isolating and identifying similar variables of importance (table 7). Thus, we were successfully able to identify the variables responsible for differentiating between a physiological state void of alcohol from an alcohol-intoxicated state. From these two independently generated lists of variables of importance it was noted that there were numerous common perturbation markers present from several different metabolic pathways. Of these perturbation markers it was evident, through selection and assigning biochemical significance, that a large majority of these ethanol-induced perturbation markers could be categorized into three primary perturbed pathways, namely: the citric acid cycle, metabolism of catecholamines and branched-chain amino acid pathway. Thus, by assigning biochemical importance to selected variables of importance we were able to:

- ✓ Biologically verify certain metabolites as perturbation markers of acute alcohol consumption.

This investigation also adds new insights on the use of multivariate analysis in metabolomics investigations:

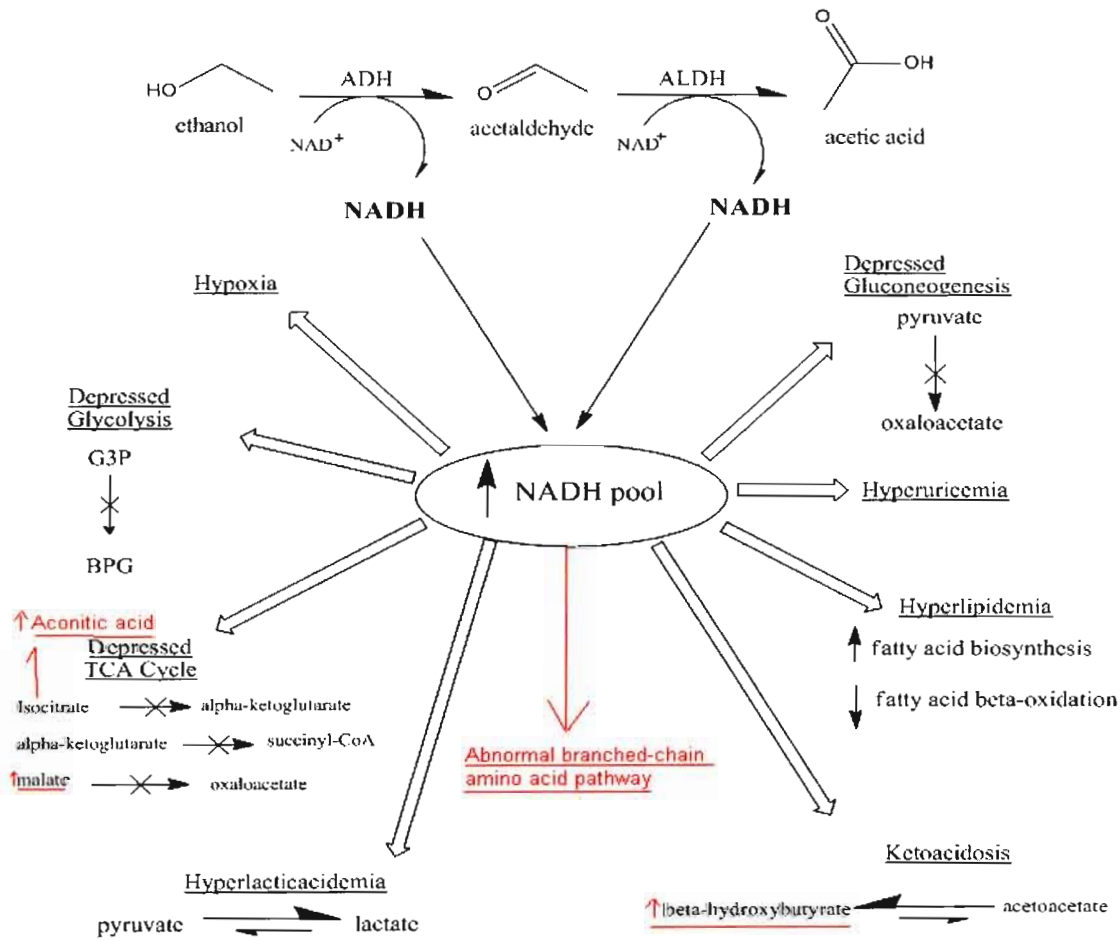
- ✓ The method of data reduction method used for the pre-treatment of our generated metabolomics data is a novel approach, unique to data of the kind generated within this investigation.

This method successfully allowed for the statistical selection of variables of most importance for our investigation (based upon deviation of variables from time 1 (baseline value)), disregarding all other less significant variables, by means of a median discrepancy measure. Application of a supervised biological filter, which selected variables of biological importance for our investigation and removed variables deemed as no biological importance (e.g. microbial products and normal physiological levels of urea), further reduced our data. The final result was reduction an original data matrix consisting of 302 variables to a consolidated reduced data matrix consisting of 78 variables used for further analysis. This

method of data reduction thus allowed for the effective use of multivariate analysis to successfully produce lists of VIPs for analysis.

Comparison of the VIP lists generated by the ASCA model and cross-sectional PCA show a definite similarity, thus supporting the labeling of certain metabolites as perturbation markers. The categorization of perturbation markers into specific pathways also allows a comparison of our metabolomics generated data with the summarized model (Figure 2) created within the overview of the current literature of ethanol-induced metabolic perturbances. The experimental data obtained within this investigation thus supported much of the model shown in Figure 28.

Figure 28: Expanded model of the summary of ethanol-induced metabolic perturbances



According to the results obtained from our investigation, the following correlates with the model:

- Elevated urinary concentrations of citric acid cycle intermediates
    - Malic acid – accumulation due to decreased activity of NAD-dependent malate dehydrogenase and the shifting of normal physiological equilibrium from oxaloacetate to malic acid due to high levels of NADH.

- Succinic acid – accumulation due to decreased activity of FAD- dependent succinate dehydrogenase. Could also be linked to mitochondrial dysfunction associated with excessive ethanol oxidation.
- Citric acid and aconitic acid – accumulation due to decreased activity of NAD-dependent isocitrate dehydrogenase. Isocitrate is the isomerization product of citric acid, during the formation of isocitrate an intermediate is formed (aconitate) by means of a dehydration step of citric acid, followed by rehydration to produce isocitrate. Thus, an accumulation of isocitrate results in accumulation of these two upstream metabolites.

The presence of elevated citric acid cycle intermediates thus correlates with published literature regarding depressed citric acid cycle activity within individuals that consume acute doses of alcohol.

- Presence of ketones (elevated urinary 2-hydroxybutyric acid), indicating ketoacidosis, can also be supported and correlated with published literature. The formation of increased 2-hydroxybutyric acid can be associated with a shift in equilibrium within the 2-hydroxybutyrate dehydrogenase catalyzed reaction from acetoacetate to 2-hydroxybutyric acid.

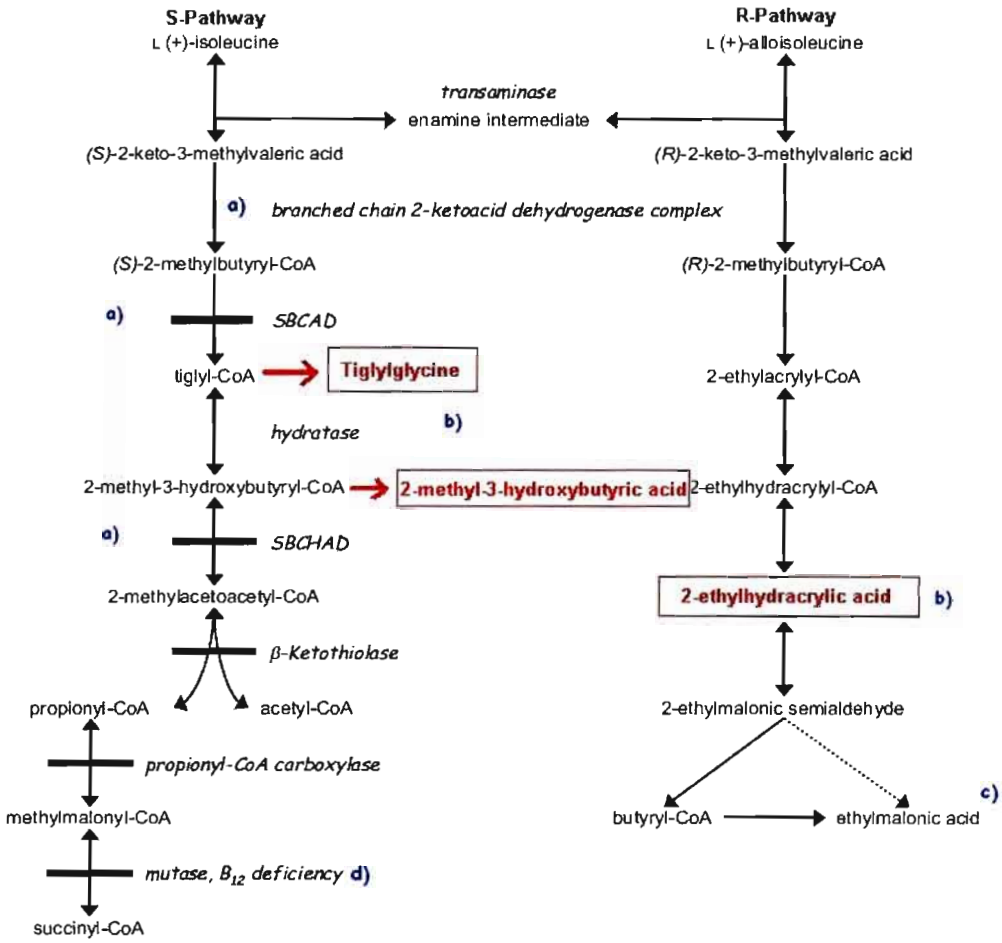
Other aspects of the model, however, were difficult to relate to the experimental data, for example, depressed glycolysis and gluconeogenesis is difficult to assess based upon urinary metabolites alone and requires additional testing of enzyme activity. No protocols specific toward lipids were used so we were unable to ascertain if there was increased fatty acid biosynthesis/decreased fatty oxidation beta-oxidation, however, the presence of abnormal glycerol and stearic acid within experimental cases suggests abnormal lipid metabolism. Hyperlacticacidemia and hyperuricemia would be more prevalent within chronic cases and detection within blood would require a more extensive blood sample collection protocol than was used within this investigation. The data obtained from this study thus supports, and can be correlated with, much of the current literature information. In addition, from the information obtained within this investigation we were able to expand on the current model of the summary of the ethanol-induced perturbances of acute alcohol consumption to include abnormal branched-chain amino acid pathway (Figure 28).

The detection of perturbation markers from the isoleucine degradation pathway was a significant result within this investigation. From this experimental observation we were able to formulate a final important statement:

- ✓ A hypothesis, derived from the results of our acute alcohol dose metabolomics study, could be generated and subsequently successfully verified (through a targeted experimental approach), namely: that acute alcohol consumption stimulates secondary metabolic pathways resembling, on a minor scale, inherited metabolic disorders of isoleucine catabolism.

As to our knowledge, this is a novel observation not previously reported in the current literature. Based upon the model of inherited metabolic disorders within the isoleucine pathway (shown in Figure 23 and presented again below in Figure 29), certain comments and speculations can be made.

Figure 29: Points of interest (shown in blue) regarding perturbation markers observed within this investigation in relation to known inherited metabolic disorders of isoleucine degradation pathway



a) In accordance to the literature, an acute alcohol dose causes an increased level of NADH, reducing the activity of the NAD-dependent dehydrogenase-catalyzed enzyme reactions within the isoleucine catabolism pathway. Decreased activity of these specific enzymes resembles, on a minor scale, inherited metabolic disorders of these enzymes. Comparison of published normal urinary reference values with published inherited metabolic disorder values and experimental values obtained from this investigation are illustrated in table 13 below.

Table 13: Published reference values (normal/abnormal) for urinary excretion of ethylhydracrylic acid, tiglylglycine and 2-methyl-3-hydroxybutyric acid compared to experimental values induced by acute alcohol dose and/or isoleucine load. (reference values obtained from Korman et al. [38] and Blau et al. [39])

			ethylhydracrylic acid	tiglylglycine	2-methyl-3-hydroxybutyric acid
Normal (mmol/mol creat)			< 5	< 2	0 - 11
Inherited Metabolic Disorders			methylmalonate semialdehyde dehydrogenase deficiency: 19 - 85 mmol/mol creat	propionic aciduria: 100 - 1000 X normal 3-ketothiolase deficiency: 5 - 100 X normal	3-ketothiolase deficiency: 10 - 450 X normal 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency: 10 - 30 X normal
Acute Alcohol Dose (mean concentration, mmol/mol creat)	Group 1	0 hr	3.38	0.82	1.27
		1 hr	10.43	4.22	3.53
		2 hr	11.53	9.93	9.66
		4 hr	16.14	6.99	12.90
		6 hr	13.17	3.43	12.14
		8 hr	10.51	1.47	6.05
	Group 2	0 hr	1.93	0.31	0.87
		1 hr	6.45	1.90	2.69
		2 hr	7.37	4.66	6.65
		4 hr	9.50	3.45	9.35
		6 hr	8.95	2.10	7.10
		8 hr	9.26	1.60	5.58
	Group 3	0 hr	2.67	0.23	1.27
		1 hr	7.67	0.94	1.68
		2 hr	3.48	2.40	1.34
		4 hr	5.22	1.51	1.30
		6 hr	5.47	0.92	1.65
		8 hr	3.35	0.38	1.69

As can be seen in table 13, concentrations of tiglylglycine and ethylhydracrylic acid, in response to an acute alcohol dose, exceed the normal reference values but still remain less than those recorded within inherited metabolic disorders. Interestingly, ethylhydracrylic acid concentrations found within group 1 almost approach the minimum reference values of those associated with the respective inherited metabolic disorders published within the literature. Thus, an acute alcohol dose does indeed induce the formation of secondary metabolites of the isoleucine degradation pathway, consistent with a mild form of an inherited metabolic disorder of the isoleucine catabolism pathway.

b) Ethylhydracrylic acid, tiglylglycine and 2-methyl-3-hydroxybutyric acid are highly toxic, secondary metabolites. Persistent and elevated levels of these secondary metabolites have been linked to neurodegeneration within cases of inherited metabolic disorders (such as 2-methyl-3-hydroxybutyric aciduria) [37]. An acute alcohol dose, however, only causes slightly, and transitory, elevated levels of these secondary metabolites, as shown in table 13. As such, little/no neurodegenration can be

expected within individuals consuming occasional acute doses of alcohol; however, if highly acute doses of alcohol are consumed within short intervals of each dose over a prolonged period (such as for severe chronic alcoholics) it can be speculated that the constant, elevated presence of these toxic secondary metabolites would most probably cause some severe, or even irreversible, neurological consequences, not generally unknown to occur in alcoholics.

c) While not present within our experimental findings, elevated levels of ethylmalonic acid would be expected due to increased levels of its upstream precursor, ethylhydracrylic acid. Ethylmalonic acid is another toxic metabolite, linked to neurodegenration, if present in elevated amounts. It can be speculated that sufficiently high doses of alcohol for prolonged periods of time could produce appreciable amounts of ethylmalonic acid and, as such, cause symptoms similar to those of inherited metabolic disorders associated with increased ethylmalonic acid (such as short-chain acyl-CoA dehydrogenase deficiency (SCADD)).
d) It can also be speculated that depression of the isoleucine degradation pathway, as well as depression of numerous other pathways (such as citric acid cycle), results in reduced end products of these respective pathways, many of which act as precursors/intermediates for other pathways. Thus the implications of these perturbed pathways extends further into other pathways, further exasperating the metabolic complications associated with acute alcohol consumption.

In conclusion, the value of the holistic metabolomic approach taken within this investigation is very aptly described by quoting Aristotle: "*the whole is more than the sum of its parts*". Thus, from the experimental observations of an open-minded nontargeted study into the metabolic effects of an acute alcohol dose and the subsequent formulation of additional experimental protocols, we were able to present and verify novel information, improving on the existing knowledge of ethanol-induced metabolic perturbations associated with acute alcohol consumption. In addition, a novel data pre-treatment method (data reduction) was utilized and, based upon our experimental findings, proved to be a very successful and aptly used method

8. FUTURE PROSPECTS

Following the experimental observations presented and validated within this investigation, a very brief additional pilot study was performed to ascertain if the metabolic perturbances induced by acute alcohol consumption, seen within the isoleucine pathway, were present within the other two branched-chain amino acids (valine and leucine). An experimental protocol similar to our targeted isoleucine experiment was followed involving 4 participants. The doses/loadings administered to each participant are shown in table 4, in section 4.4. Targeted analysis of the urinary organic acid profiles yielded two metabolites of interest, namely: 3-hydroxyisobutyric acid (also present as a variable of interest within our untargeted study in section 5) and isovalerylglycine. Isovalerylglycine is a secondary metabolite (detoxification product) of isovaleryl-CoA found within the leucine degradation pathway. 3-Hydroxyisobutyric acid occurs as an intermediate within the valine degradation pathway and accumulation of 3-hydroxyisobutyric acid acts as a diagnostic marker of a known inherited metabolic disorder (3-hydroxisobutyric aciduria), which is caused by defective 3-hydroxyisobutyric acid dehydrogenase. The presence of both of these metabolites indicates that abnormal activity occurred, within both the leucine and valine degradation pathways, in response to an acute alcohol dose. This observation suggests that the influences of acute alcohol consumption affects all of the branched-chain amino acids and is not only localized to the isoleucine degradation pathway; however, additional investigation (more structured protocol and larger number of participants) is needed to verify these observations. As such, this brief additional pilot study was only meant to serve as a foundation for future investigation into acute alcohol consumption.

Other interpretations as the one describe above may also be quite plausible. One such interpretation which was brought to our attention, for example, focuses upon the inhibition of SBCAD and SBCHAD by alcohol, namely:

"An inhibition of both these enzymes, [*shown in Fig 29*], would not result in the metabolites seen. Inhibition of SBCHAD alone would, however, do so. If SBCAD is inhibited, no accumulation of tiglylglycine or 2-methyl-3-hydroxybutyric acid could occur. One would, however, expect flow into the R-pathway. [*The interpretation discussed within this investigation does not take into consideration*] that some of the enzymes in the R-pathway are NADH dehydrogenases, and, hence, also inhibited. In fact, R-methylbutyryl-CoA is converted to 2-ethylacryl-CoA using the same SBCAD at the S-pathway, and hence, if this enzyme is inhibited as [*assumed by the interpretation discussed within this investigation*], no 2-ethylhydracrylic acid will be formed either. [... *The mechanism*] by which this abnormal profile can exist is through inhibition of only SBCHAD, and this may occur through a metabolite being formed which is structurally similar to that of 2-methyl-3-hydroxybutyryl-CoA, and hence inhibits the enzyme. This unknown metabolite was not detected. However, the nature of the study design being more targeted, only identifying compounds known to occur in existing libraries, does not allow for novel compound detection, hence, this would not be detected."

This proposed interpretation is quite valid as an alternative to the interpretation presented and discussed within this investigation, and might be considered as an example to direct future studies. It should be noted, however, that the discussion described in section 7 is based on the results of this investigation as interpreted from the paradigm that the NADH/NAD ratio affects individual enzymes (e.g. as theoretically reflected by Michaelis-Menten parameters, for example Km-values for substrates (organic acids) and cofactors (NADH or NAD)). Although this paradigm was not explicitly investigated, it may form an important concept in future evaluations of our hypothesis.

In addition, according to the literature a major cause of metabolic perturbances in acute alcohol users is an increased NADH:NAD^+ ratio caused by excessive alcohol oxidation. The isoleucine pathway contains several NAD-dependent dehydrogenase-catalyzed reactions and it can be speculated that the observed ethanol-induced perturbances seen within the isoleucine pathway are most likely at these enzyme reaction sites (high NADH levels shifts the equilibrium of these normal physiological reactions); however, in order to confirm this speculation additional studies need to be conducted, aimed at measuring relative concentrations of NAD and NADH within biological samples, particularly blood samples. Thus, an ideal follow-up study for this investigation would involve including both valine and leucine into the investigation and the utilization of a protocol aimed at testing the relative concentrations of NAD and NADH over time, following an acute alcohol dose and/or isoleucine/leucine/valine load, in order to associate any possible metabolic perturbances with a shift in the normal concentrations of these cofactors of interest.

9. ACKNOWLEDGEMENTS

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11. APPENDIX 1 – Additional Metabolite Information

NOTE: the following information was mostly compiled from the Human Metabolome Database, *http://hmdb.ca*

Aconitic acid - an intermediate in the citric acid cycle produced by the dehydration of citric acid.

Citramalic acid – compound structurally similar to malic acid, an intermediate in the citric acid cycle.

Ethylhydracrylic acid – formed as a secondary metabolite of accumulated R-form isoleucine. Urinary excretion of ethylhydracrylic acid is variably increased in defects of isoleucine oxidation at distal steps in the catabolic pathway.

Fumaric acid - a precursor to L-malate in the citric acid cycle. It is formed by the oxidation of succinate by succinate dehydrogenase. Fumarate is converted by fumarase to malate. Also formed from tyrosine, arginine and proline catabolism.

Glucuronic acid – glucuronic acid is a carboxylic acid that has the structure of a glucose molecule that has had its sixth carbon atom (of six total) oxidized. It is often linked to poisonous substances to allow for subsequent elimination, and to hormones to allow for easier transport. Activated form acts as detoxification agent that removes toxic substances (e.g. acetaldehyde and it derivatives, ROS and ethanol) by conjugation and subsequent elimination.

Glycerol - Glycerol is an important component of triglycerides (i.e. fats and oils) and of phospholipids. Glycerol is a three-carbon substance that forms the backbone of fatty acids in fats. When the body uses stored fat as a source of energy, glycerol and fatty acids are released into the bloodstream. The glycerol component can be converted to glucose by the liver and provides energy for cellular metabolism.

Hippuric acid - Hippuric acid is an acyl glycine formed by the conjugation of benzoic acid with glycine. Presence of elevated benzoic acid and subsequently increased hippuric acid is a dietary consequence of consuming flavored carbonated water used within experimental protocol (contains sodium benzoate preservative). (Dietary source as well as detoxification product)

Indole-3-acetic acid - Indoleacetic acid (IAA) is a breakdown product of tryptophan metabolism and is often produced by the action of bacteria in the mammalian gut. It may be produced by the decarboxylation of tryptamine or the oxidative deamination of tryptophan. (Consequence of perturbed catecholamine pathway or a product of normal gut microbes)

Isovanilglycolic acid – Also known as isovanilhydroxyacetic acid. Derivative of homovanillic acid (vanilacetic acid). A metabolite of dopamine catabolism.

Isovanilylglycine – detoxification product formed by conjugation of isovanilglycolic acid with glycine.

Malic acid - In its ionized form it is malate, an intermediate of the TCA cycle along with fumarate. It can also be formed from pyruvate as one of the anaplerotic reactions.

Methylmalonic acid - A malonic acid derivative which is a vital intermediate in the metabolism of fat and protein. Abnormalities in methylmalonic acid metabolism lead to methylmalonic aciduria. This metabolic disease is attributed to a block in the enzymatic conversion of methylmalonyl CoA to succinyl CoA within the propanoate pathway. (Propanoate pathway constitutes later part of isoleucine degradation pathway)

Oxalic acid - A strong dicarboxylic acid occurring in many plants and vegetables. It is produced in the body by metabolism of exogenous glyoxylic acid or ascorbic acid.. (Dietary source)

Phenylacetylglutamine - product formed by the conjugation of phenylacetate and glutamine. Part of phase II detoxification. Phenylacetic acid is formed as a decarboxylation product of phenylpyruvic acid, which is the transamination product of excess phenylalanine/tyrosine. High urinary concentrations associated with liver dysfunction.

Phosphoric acid – a component of several important cellular transportation systems (e.g. ABC transporters, oxidative phosphorylation). Excessive urinary levels suggest possible transmembrane transportation and mitochondrial systems dysfunctions.

Stearic acid - Stearic acid, also called octadecanoic acid, is a saturated fatty acid. Reason for increased urinary stearic acid in acute alcohol cases is uncertain; however, several hypotheses can be assigned to increased stearic acid: alternative energy source (hypoglycemia), increased blood clotting (reduce thrombogenic state), participation in signaling pathways (e.g. apoptosis, proliferation) and neuroprotection against oxidative stress (*Ze-Jian Wang, Guang-Mei Li, Bao-Ming Nie, Yang Lu, Ming Yin. Neuroprotective effect of the stearic acid against oxidative stress via phosphatidylinositol 3-kinase pathway (Chemico-Biological Interactions 160 (2006) 80–87*).

Succinic acid - Succinic acid is a dicarboxylic acid. The anion, succinate, is a component of the citric acid cycle capable of donating electrons to the electron transfer chain. Succinate dehydrogenase (SDH) plays an important role in the mitochondria, being both part of the respiratory chain and the citric acid cycle. Also occurs as intermediate of several other pathways.

Tiglylglycine - Tiglylglycine is an intermediate product of the catabolism of isoleucine. An elevated level of tiglylglycine is identified in urine of patients with beta-ketothiolase deficiency or with disorders of propionate metabolism. Secondary metabolite present due to perturbances within isoleucine catabolic pathway.

Uric acid – Uric acid is a heterocyclic purine derivative that is the final oxidation product of purine metabolism. Produced in small quantities with excess accumulation leading to a type of arthritis known as gout. (As stated within section 2.4.8, both uric acid and lactic acid are transported in co-ordination with each other by means of urate transporter 1 (URAT1) within the kidneys. The shift in the redox potential created by increased NADH:NAD^+ ratio results in excessive levels of lactic acid and subsequently less excretion and more reabsorption of uric acid)

Vanillylmandelic acid - The major urinary catecholamine catabolic metabolites of L(2)-norepinephrine (NE) and L(2)-epinephrine (E) in humans are vanillylmandelic acid (VMA) and 3-methoxy-4-hydroxyphenylethylene glycol (MHPG). *(Tarana, F., Bernard, H., Valleix, A., Cre´minon, C., Grassi, J., Olichon, D. , Deverre, J.R., Pradelles, P. Competitive enzyme immunoassay for urinary vanillylmandelic acid (Clinica Chimica Acta 264 (1997) 177–192)).* High levels suggests increased: level of stress/oxidation, level of epinephrine and/or catabolism of epinephrine.

3,4-Dihydroxybutanoic acid – present within individuals with succinate-semialdehyde dehydrogenase deficiency, associated with a disorder of GABA (gamma-aminobutyric acid) metabolism *(Maneesh Gupta, Erwin E. W. Jansen, Henry Senephansiri, Cornelis Jakobs, O. Carter Snead,*

Markus Grompe and K. Michael Gibson. Liver-Directed Adenoviral Gene Transfer in Murine Succinate Semialdehyde Dehydrogenase Deficiency (Molecular Therapy (2004) 9, 527–539; doi: 10.1016/j.ymthe.2004.01.013)). High levels of NADH shifts the equilibrium from succinate toward succinate semialdehyde; and together with high levels of GABA, result in formation of secondary metabolite 4-hydroxybutyrate and its derivative 3,4-dihydroxybutyrate.

3-Hydroxyvaleric acid - product of the condensation of propionyl-CoA with acetyl-CoA catalyzed by 3-oxoacyl-CoA thiolases. An increase amount of 3-hydroxyvaleric acid can be found in methylmalonic acidemia and propionic acidemia.

2-Hydroxybutyric acid - 2-Hydroxybutyric acid is an organic acid that is involved in propanoate metabolism. It is produced in mammalian tissues (principally hepatic) that catabolize L-threonine or synthesize glutathione. Oxidative stress or detoxification demands can dramatically increase the rate of hepatic glutathione synthesis. 2-Hydroxybutyrate is released as a by-product when cystathionine is cleaved to cysteine that is incorporated into glutathione. 2-Hydroxybutyric acid is often found in the urine of patients suffering from lactic acidosis and ketoacidosis. 2-Hydroxybutyric acid generally appears at high concentrations in situations related to deficient energy metabolism. Elevated NADH levels considered the most important factor for the increased production of 2-hydroxybutyric. Secondary metabolite that can be associated with numerous pathways placed under oxidative stress.

2-Hydroxyisobutyric acid – metabolite present in elevated amounts within individuals suffering from 3-hydroxyisobutyric aciduria or methylmalonate semialdehyde dehydrogenase deficiency.

3-Hydroxyisobutyric acid - 3-Hydroxyisobutyric (3-HIBA) acid is an intermediate in the metabolic pathways of L-valine and L-thymine amino acids. Increased levels caused by disorder of NAD-dependent 3-hydroxyisobutyrate dehydrogenase. Neurodegenerative symptoms associated with tissue accumulation of 3-HIBA *(Loupatty, FJ., van der Steen, A., IJlst, L., Ruiter, JPN., Ofman, R., Baumgartner, MR., Ballhausen, D., Yamaguchi, S., Duran, M., Wanders, RJA. Clinical, biochemical, and molecular Findings in three patients with 3-hydroxyisobutyric aciduria (Molecular Genetics and Metabolism 87 (2006) 243–248)).* Also linked to altered energy metabolism (mitochondrial dysfunction) *(Carolina Maso Viegas, Gustavo da Costa Ferreira, Patrícia Fernanda Schuck, Anelise Miotti Tonin, Ângela Zanatta, Angela Terezinha de Souza Wyse, Carlos Severo Dutra-Filho, Clóvis Milton Duval Wannmacher and Moacir Wajner Evidence that 3-hydroxyisobutyric acid inhibits key enzymes of energy metabolism in cerebral cortex of young rats (International Journal of Developmental Neuroscience Volume 26, Issues 3-4,, May-June 2008, Pages 293-299).*

3-Hydroxypropionic acid – chemical structure similar to L-serine and lactic acid. Produced by certain microbes; as well as a secondary metabolite of propanoate/alanine/valine pathway. Also known as hydracrylic acid.

1,2-Dihydroxyethane – direct conjugate derivative of ethanol

5-Hydroxyindoleacetic acid - 5-hydroxyindoleacetic acid (5HIAA) is a breakdown product of serotonin (5-hydroxytryptamine) that is excreted in the urine. Serotonin is a hormone/catecholamine that acts as a vasoactive substance. 5-HT and 5HIAA are used as indicators of various pathological conditions ranging

from appendicitis to carcinoid tumors. Abnormal values attributed to perturbances within catecholamine metabolism.

2-Methyl-3-hydroxybutyric acid – metabolite involved in isoleucine degradation pathway, as well as being associated with beta-oxidation of fatty acids and ketogenesis. Elevated urinary concentrations present in individuals with beta-ketothiolase deficiency. Secondary metabolite associated with abnormal isoleucine catabolism.

12. APPENDIX 2 - Questionnaire

Questionnaire of Clinical Information of Participant in Pilot Experiment to Determine Possible Perturbations within Metabolism of Young Males Caused by Alcohol Consumption Over a Defined Time Period

1. Full Name: _____
2. Age: _____
3. Gender: _____
4. Ethnicity: _____
5. Weight: _____
6. Height: _____
7. Medical History (any diagnosed medical problems, e.g.: diabetes, allergies, heart disease):
   _____
   _____
   _____
   _____
8. History of Alcohol Use:
   a. Have you consumed alcohol before? _____
      (NOTE: if answered "NO" then 6b-6e can be ignored)
   b. How often do you consume alcohol? _____
   c. How long have you been drinking alcohol? _____
   d. What is your preferred alcoholic beverage? (can include multiple choices)
      _____
      _____
      _____
      _____
   e. How much alcohol do you typically consume? (per week)
      i. At home: _____
      ii. In a relaxed social situation: _____
      iii. At a bar/club/celebration: _____
9. Are you a Smoker? _____
   a. If so, how much do you smoke? _____

10. Do you currently take any chronic medication? _____
    a. If so please name:

_____

_____

_____

11. Would you classify your normal diet as:
    a. Balanced? _____
    b. High carbohydrate (breads, pastas, rice etc)? _____
    c. High fat (take-away foods)? _____

12. Do you suffer from any deficiencies?

_____

_____

_____ _____

SIGNED DATE

13. APPENDIX 3 – Informed Consent Form

INFORMED CONSENT TO PARTICIPATE IN (PILOT) EXPERIMENT WITH ETHICAL APPROVAL FROM THE NORTH-WEST UNIVERSITY

TITLE: The metabolomics of acute alcohol abuse

AIM OF PILOT EXPERIMENT: The determination of possible metabolic perturbations in young males over a defined time period, caused by the consumption of a fixed dose of alcohol in a fasted state, as investigated by a metabolomics methodology.

INVESTIGATOR'S NAME: Shayne Mason

SUPERVISOR: Prof. C.J. Reinecke, (Head (Acting): Centre for Human Metabonomics)

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INTRODUCTION

The North-West University recently established a Centre for Human Metabolomics aimed at investigating perturbations associated with human metabolism by means of a metabolomics approach. ***Metabolomics*** is a biochemical technique, involving a comprehensive study of low molecular weight bio-molecules, commonly known as metabolites. Biochemical analysis of biological samples (such as urine or blood) provides a large comprehensive matrix of metabolites. This matrix of data is analysed by means of bioinformatics, a field of science incorporating statistical multivariate analysis, providing information used to determine/distinguish any potential irregularities within the metabolic profile. The focus being the identification of very specific metabolites that can statistically discriminate between normal and abnormal metabolic situations. These metabolites are known as ***biomarkers***.

PURPOSE OF THE EXPERIMENT

This (pilot) experiment constitutes part of the investigator's M.Sc. thesis involving the study into the metabolic perturbations associated with alcohol consumption. This (pilot) experiment is aimed at determining if there are any possible perturbations that occur within the metabolic profile of a homogenous, defined experimental group, namely young males between the ages of 20 and 30 years, caused by the consumption of a fixed, (moderate/acute) alcohol dose in an overnight fasted state. The biological specimens used in this experiment will be urine samples taken at defined intervals of time, followed by a metabolomics analysis.

PROTOCOL OF EXPERIMENT

For Pilot Study:

- Participants are required to abstain from alcohol consumption at least 48 hours preceding this experiment; as well as abstaining from consumption of food prior to initiation of the experiment (i.e. overnight fasted state)
- A questionnaire involving the basic clinical profile, including history of alcohol consumption, of the participant needs to be completed.
- An initial urine sample will be taken and labelled "o hour" before alcohol dose consumed.
- The participant will consume an amount of alcohol (Smirnoff vodka 43%, v/v, mixed with flavored water) over a 15-min period at a fixed dose of 0.5 g/kg body weight.
- Urine samples will be needed approximately every hour up until 4 hours, after which urine samples will be needed at 6 hours, 8 hours, 12 hours and a final urine sample at 24 hours after alcohol consumption (consumption of water 15 minutes before a voiding is needed will facilitate obtaining urine samples).
- The experiment will be done in an environment compatible with clinical and ethical requirements
- A physician will attend the early phases of the experiment (first 2 hours) and will be on call for the

remaining part of the experiment.

NOTE: Urine samples must be labelled clearly and precisely and stored in a refrigerated environment and no additional alcohol should be consumed during the experimental time frame.

For Acute Alcohol Dose Study:

- Participants are required to abstain from alcohol consumption at least 48 hours preceding this experiment; as well as abstaining from consumption of food prior to initiation of the experiment (i.e. overnight fasted state)
- A questionnaire involving the basic clinical profile, including history of alcohol consumption, of the participant needs to be completed.
- An initial urine sample will be taken and labelled "o hour" before alcohol dose consumed.
- The participant will consume an amount of alcohol (Smirnoff vodka 43%, v/v, mixed with lemon flavored water) over a 15-min period at a fixed dose of 1.5 g/kg body weight.
- Urine samples will be needed approximately every hour up until 5 hours after alcohol dose first consumed (consumption of water 15 minutes before a voiding is needed to facilitate obtaining urine samples).
- The experiment will be done in an environment compatible with clinical and ethical requirements
- A physician will attend the early phases of the experiment (first 2 hours) and will be on call for the remaining part of the experiment.

NOTE: Urine samples must be labelled clearly and precisely and stored in a refrigerated environment and no additional alcohol should be consumed during the experimental time frame.

For Targeted Study into Ethanol-induced Perturbances within Isoleucine Pathway:

Group1:

- Participants are required to abstain from alcohol consumption at least 24 hours preceding this experiment; as well as abstaining from consumption of food prior to initiation of the experiment (i.e. overnight fasted state)
- An initial blood and urine sample will be taken and labelled "0 hour" before alcohol dose or isoleucine load is consumed.
- The participant will consume: (1) an amount of alcohol (Smirnoff vodka 43%, v/v) at a fixed dose of 1.5 g/kg body weight, and (2) 100 mg isoleucine load per kg of body weight. Both amounts may be consumed with water.
- A second blood and urine sample will be taken approximately 1 hour after consumption of alcohol and isoleucine load.
- Additional urine samples will be taken at 2, 4, 6 and 8 hours after '0 hour'.

The experiment will be done in an environment compatible with clinical and ethical requirements, supervised by medical staff.

Group2:

- Participants are required to abstain from alcohol consumption at least 24 hours preceding this experiment; as well as abstaining from consumption of food prior to initiation of the experiment (i.e. overnight fasted state)
- An initial blood and urine sample will be taken and labelled "0 hour" before isoleucine dose is consumed.
- The participant will consume: 100 mg isoleucine load per kg of body weight, mixed with water.
- A second blood and urine sample will be taken approximately 1 hour after consumption of isoleucine load.
- Additional urine samples will be taken at 2, 4, 6 and 8 hours after '0 hour'.
- The experiment will be done in an environment compatible with clinical and ethical requirements, supervised by medical staff.

Group3:

- Participants are required to abstain from alcohol consumption at least 24 hours preceding this experiment; as well as abstaining from consumption of food prior to initiation of the experiment (i.e. overnight fasted state)
- An initial blood and urine sample will be taken and labelled "0 hour" before alcohol dose is consumed.
- The participant will consume an amount of alcohol (Smirnoff vodka 43%, v/v) at a fixed dose of 1.5 g/kg body weight. Dose may be consumed with water.
- A second blood and urine sample will be taken approximately 1 hour after consumption of alcohol.
- Additional urine samples will be taken at 2, 4, 6 and 8 hours after '0 hour'.
- The experiment will be done in an environment compatible with clinical and ethical requirements, supervised by medical staff.

INFORMED CONSENT PROCEDURES

Participation in the project is fully voluntary. You are free to enquire on the experiment through the investigator and/or supervisor and if agreed to participate, the participant will be asked to sign this informed consent form. Should any participant request feedback on the outcomes of this (pilot) study, such information can be made available to them.

It is required from all participants in this (pilot) study to complete a questionnaire which provides information which is essential for the project. The participating physician will evaluate the information of all participants, and will approve their participation based on the information given in the questionnaire.

BENEFITS ASSOCIATED WITH THE STUDY

The Masters study, to which this experiment will contribute toward, is a research project aimed at better understanding the biological relationship between chronic/acute alcohol consumption and metabolic perturbations within humans. As in all cases, improved knowledge of the normal physiology will eventually yield to a better understanding and treatment of any deviation from the normal physiology. The outcome of this research will be used by the researcher for a M.Sc.-thesis and no reference will be included in the thesis regarding any individual who participated in the study.

PAYMENT OR REIMBURSEMENT

Participants will not be paid for their participation and don't contribute to the costs of the study.

CONFIDENTIALITY

All research records are confidential unless law requires disclosure. No name or other personal identifying information of the participants will be used in any reports or publications resulting from this study. Data from this study will be used in an anonymous statistical analysis and reported as such by the NWU. No patient identification detail will be reported or made known to other parties.

VOLUNTARY PARTICIPATION AND CONDITIONS OF WITHDRAWAL

Your participation in this study is completely voluntary. You may choose not to participate in this study to which you are otherwise entitled.

CONSENT

I, _____, have read and understood the preceding information describing this research study and my questions have been answered to my satisfaction. I voluntary consent to participate in this research study. I do not waive my legal rights by signing this consent form. I will receive a signed and dated copy of this consent form.

PARTICIPANT:

_____	_____	_____
Printed name	***Signature***	***Date***

INVESTIGATOR:

_____	_____	_____
Printed name	***Signature***	***Date***

PHYSICIAN:

_____	_____	_____
Printed name	***Signature***	***Date***