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**THE USE OF METABOLOMICS TO INVESTIGATE BIOMARKER PROFILES AS  
POTENTIAL EARLY RISK FACTORS FOR DEVELOPMENT OF TYPE II DIABETES  
MELLITUS**

by

**JENNELLE ARNEW**

**THESIS**

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

**MASTER OF SCIENCE**

2012

MAJOR: NUTRITION AND FOOD SCIENCE

Approved by:

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Advisor

Date

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## **CHAPTER 1: INTRODUCTION**

A challenge facing many scientists today is the discovery of biomarkers for health benefits and disease diagnosis. Advances in this area have occurred from the realization that the focus should shift from the identification of single biomarkers to the identification of complex and dynamic biomarker patterns. Current research supports the capacity and need for molecular biomarkers that allow early detection of onset of disease or ideally, the pre-diseased state [1]. Identification of biomarker patterns, although complex to analyze, will be essential for answering questions related to the etiology and progression of disease states.

Technological advances in biological sciences over the past few years have created a new era of research including the emerging field of systems biology. Systems biology can be described as a “scientific field that endeavors to quantitatively characterize the genetic, transcription, protein, metabolic signaling and other information pathway responses to a clearly defined perturbation of a biological system and to assess their interactions and integrate that information into graphical network models that serve as predictive hypothesis to explain emergent behaviours” [2]. The comprehensive investigation of genomics, transcriptomics, proteomics, and metabolomics are important building blocks and will play a central role in studying organisms using a biological systems approach.

Metabolomics is an emerging field with tremendous potential for advancing our understanding of human health, disease progression and development of personalized medical interventions. Assessment of a biological system through metabolomic approaches provides the investigator with molecular information that is close to phenotypes in question because metabolites are a product of gene, mRNA and protein activity [3].



Metabolic disorders cause alteration in the metabolic regulatory system which are the cause for many human diseases such as obesity and type 2 diabetes mellitus. The pathological development involves altered expression of low molecular weight metabolites including lipids and amino acids, and carbohydrates such as glucose. Changes in the metabolic profile related to the progression of diabetes are complex, arising from the interaction between many genetic and environmental factors. An improved understanding of biological systems at the molecular level is anticipated to result from metabolomics.

## **METABOLOMICS**

Metabolomics is the rapidly evolving field of the comprehensive measurement of ideally all endogenous metabolites in a biological fluid [4][5][6][7][8][9]. One of the important main applications of metabolomics within the clinical and pharmacological disciplines lies in the discovery of biomarkers to diagnose health status, disease or outcome of pharmacological treatment [3]. The metabolome represents an immense number of components that belong to a wide variety of compound classes, such as amino acids, lipids, and organic acids. These compounds are diverse in their physical and chemical properties and are present in varying concentration range [10]. A major advantage in the application of metabolomics stems from an improved ability to detect up to many hundreds of metabolites in parallel, which provides an efficient method for monitoring altered biochemistry [11].

Changes in metabolic profiles are a potential source of such biomarkers [12][13][14][15]. The research shows that there is a general consensus that the metabolome describes the total sum of metabolites in a given biological sample. Due to the complex functions of a large number of different cellular components, a fundamental problem exists with the interpretation of the results from the analysis [16]. With emerging analytical techniques like nuclear magnetic resonance

(NMR) and mass spectroscopy (MS), data on metabolites, nutrients and other compounds in bio-fluids can be acquired with more accuracy and precision.

### *Metabolites*

Living cells have energy and building blocks required for growth and maintenance from the conversion of small chemical compounds to another set of chemical compounds with lower free energy content [17]. The metabolism involves the chemical reactions with many different chemical intermediates, and the chemicals involved in the metabolism are called metabolites. Despite the number of metabolites that participate in the metabolism, there is a high degree of organization of the different inter conversion processes.

Metabolites are intermediates for biochemical reactions and play an essential role in connecting many different pathways that operate within a living cell. They are the by-products of enzyme-catalyzed reactions that occur naturally within living cells [17]. As the intermediates of biochemical reactions, metabolites play an important role in connecting many different pathways that operate a living cell [17]. Thus, metabolites represent integrative information of the cellular function and it defines the phenotype of the cell or tissue in response to genetic or environmental changes [3].

The analysis of metabolites presents challenges because each metabolite is characterized by its individual structure. Metabolites are unique, their role is specific and metabolites from the same pathway can present different chemistry. Due to the diversity in physical and chemical properties currently an assortment of analytical techniques allowing the comprehensive analysis of metabolites is required.

Primary metabolites are found in all organisms and are an integrate part of essential life processes. These metabolites are involved in energy and production of core structures in the

cells for example, glycolysis, respiration or photosynthesis. These metabolites are building blocks and energy sources like proteins, nuclear acids or polysaccharides which belong to primary metabolism. These metabolites are influenced by the nutritional status of the organism and are similar across species. Secondary metabolites on the other hand, have only restricted distributions and often will have specific characteristics of individual organisms and species. Secondary metabolites do not participate in growth and development and therefore are not considered essential for life. These metabolites often have an unknown function and require genes to be produced [17].

It is thought that the human body contains approximately 3000-5000 detectable metabolites, a sizable fraction of which have been identified [18]. It is estimated the number of metabolites is lower than the number of all genes and protein in a cell. At this time, it is difficult to determine the number of metabolites and also other cell products such as transcripts and protons at a given time in a specific cell because the analytical techniques are not available [7].

The concentrations of metabolites are dependent on several factors including the environment, whether its an intermediate or end product, cell status, or turnover rate. Therefore, some metabolites will be highly abundant and others will be available in trace amounts which impacts the analytical technique used by the investigator. The dynamic range which exists in metabolites for any given biological system contributes to complexity of the analysis.

Metabolomic analysis includes the identification and quantification of all intracellular and extracellular molecules that have a mass lower than 1000 DA using different analytical techniques [16].

The metabolome consists of metabolites which vary widely in chemical structures and properties. The levels of each metabolite are dependent on the physiological, developmental and

pathological state of a cell, tissue or organism. Thus, the metabolic profile of biological specimens are affected by numerous factors such as age, diet, ethnicity, drugs, lifestyle or gut microfloral populations which contribute to the complexity to obtain information specific to a disease [19]. As, metabolites have a rapid turnover rate, sample preparation is a crucial step in the analysis due to high turnover rate.

Sample preparation is considered a limiting step in metabolomic analysis because the concentration of metabolites can change very rapidly. Because the metabolome represents a ‘snapshot’ of the *in vivo* metabolic state of a cell or organism in a specific developmental and environmental condition, rapid quenching is crucial to stop a reaction at a given time. This will minimize the formation or degradation of metabolites after sampling due to remaining enzymatic activity or oxidation. Quenching can usually be achieved through a change in pH and temperature [10]. One advantage of utilizing metabolomics in nutrition is that there are several accessible body fluids that contain possible biomarkers in the human body. Biofluids provide a window into the biochemical status of a living organism. Urine and blood (serum or plasma) are the most commonly used biofluids for metabolomics based studies because they contain detectable metabolites that can be obtained non or minimally invasively [10]. Compared to other biofluids, urine offers low concentration of protein and a high concentration of low molecular weight compounds which results in high quality measurements due to narrow line widths of the spectral peaks in the NMR spectra [11]. By offering this advantage, it improves the process of biomarkers identification by NMR for diagnostic and monitoring applications.

There are three approaches used in metabolomic research: target analysis, metabolic profiling and metabolic fingerprinting. Target analysis aims to quantitatively measure selected metabolites i.e., specific biomarkers. Metabolic profiling is a non-targeted strategy that focuses

on the study of a group of related metabolites or a specific metabolic pathway. Metabolite fingerprinting does not aim to identify all the metabolites, but rather to compare patterns of metabolites that change in response to the cellular pathway[10][16].

As a metabolite fingerprinting approach was employed for this project because more detailed description is provided on this analytical platform. The goal of this approach was not to identify each observed metabolite, rather to compare patterns or “fingerprints” of metabolites that change in response to disease or for the purpose of this study, different risk factors for diabetes. This technique can use a wide variety of biological matrices such as urine, plasma, serum, saliva tissue or cells. Metabolic fingerprinting is considered a true “omics” approach, because it can be applied to a wide range of metabolites [10]. The goal for many researchers is to use metabolic fingerprinting as a diagnostic tool for evaluating differences in the signature patterns between healthy and diseased subjects. This approach also allows for the evaluation of therapeutic interventions by observing if the metabolic phenotype shifts back to the healthy state after treatment. Although metabolic fingerprinting is an important platform to assess metabolic differences in sample groups - metabolomics should not be used exclusively for fingerprinting without identifying the metabolites that cause clustering of experimental groups. This will allow a greater understanding of underlying mechanisms of action. Thus, combining metabolite fingerprinting and profiling is an important approach so that the metabolic signature can be developed between different experimental groups and the metabolites causing the differences can be identified. In this study we will use a non targeted metabolomics approach to investigate the metabolite profiles of a heterogeneous non-diabetic human sample to determine which risk factors, if any, are correlated with clustering and/or separation of the data.

### **Nuclear Magnetic Resonance (NMR)**

NMR is a powerful analytical tool for the characterization of chemical structure and concentration based on nuclear properties of the atoms. NMR was first developed in 1946 by research groups at Stanford and M.I.T (USA) [20]. The data from the NMR analysis is expressed in a spectrum as a representation of the nuclei's chemical environment. When placed inside a magnetic field the nuclei aligns itself according to whether they oppose or are parallel to the applied magnetic field. The NMR spectrum shows at which shift the nuclei studied will absorb energy- the more shielded a nucleus is, the higher the chemical shift. An NMR spectrum is created by radiating the sample with a short pulse of high energy radio frequencies that excite all nuclei. Transition of atomic nuclei between the excited energy state and a lower energy is measured over a period of time as free induction decay (FID) signals. Since these FID signals are time domain signal files, it is very difficult to differentiate between spectra from different compounds so, the FID files are further transformed by Fourier transformation (Ft) (mathematical algorithm) to a frequency domain [18]. After Ft the acquired spectrum can be resolved into different peaks arising from different compounds or metabolites.

The NMR spectrum is plotted as a parts per million (ppm) value of the radiation frequency. The number of signals shows how many different kinds of protons are present and location of the signals shows how shielded or de-shielded the proton is. The intensity of the signal shows the number of protons of that type. The nuclei of most interest in biology are  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{31}\text{P}$ ,  $^{15}\text{N}$ ,  $^{19}\text{F}$  because they have an odd spin number. Transition between these 2 states can be brought about by absorption of energy which is provided by electro-magnetic radiation. When this magnetic field is proportional to the correct level then the nucleus can absorb the radio frequency. The amount of energy absorbed is proportional to the number of nuclei.

This analytical tool is a popular platform for metabolic profiling because the technique is non-destructive; requires little or no sample preparation; is rapid and provides highly reproducible results and the spectra contains a wealth of information [11]. This is in contrast to other techniques such as GC-MS, which requires pre-treatment of the specimen matrix, derivitization and then an on-column separation of the products before any molecular characterization can be done using the MS data [21]. In this study, we will use the  $^1\text{H}$  NMR to investigate the metabolic profiles for potential differences based on risk factors for diabetes.

### **Multivariate Data Analysis (MVDA)**

Data obtained from NMR is complex to analyze because it contains qualitative/quantitative information on several hundreds of metabolites. Multivariate data analysis (MVDA) is employed to obtain meaningful information on disease and healthy samples by differentiation of biofluid samples based on the difference in signals of multiple metabolites. Due to the huge data sets obtained in metabolomics analysis, it is necessary to transform that raw data into useful information. The most common data reduction technique is Principal Component Analysis (PCA). Data reduction using PCA is an example of an unsupervised method which determines correlation differences among data sets based on how the observations are different or similar to each other. This data reduction technique transforms the original data into a set of 'scores' for each sample, measured with respect to the principal component axes (loadings). The PC scores replace the original varieties and show maximum variability in the data set. These PC are compared in multidimensional space to corresponding score values [22].

The data is visualized as a PCA score plot where each score is one observation or in the case of NMR one score is one spectra [23]. The scores plots provide visualization and summarization of the data often revealing clustering of similar samples, separation of different

sample types, or the presence of outliers. The contribution of each individual variable to PC scores can be calculated as a loading plot. Loading plots represent the variables that are responsible for clustering or differentiating between the groups [24].

Partial Least Square (PLS) is a regression extension of PCA, which is used to connect two data matrices, X and Y, to each other by a linear multivariate model. This analysis is done to determine the patterns in the data sets based on specific knowledge about the data. In this supervised method one has to feed specific information about the data sets e.g. categorize the data sets into different groups. PLS has become a standard tool for modeling linear relations between multivariate measurements [22].

However, methods for data analysis and the sets of instruments used are diverse across laboratories that practice comprehensive metabolic profiling. This lack of standardization in the methodology increases the potential for reduced reproducibility of the findings. Often the metabolic platform is driven by factors such as cost, personal experience, and specific research goals [25]. Nevertheless, confidence in the obtained information is achieved by employing strict criteria for sample collection, methods and statistical algorithms.

### **Metabolite quantification (Chenomx)**

Chenomx is a metabolite database software that allows the identification and quantification of metabolites by comparing to the NMR spectra. Prior to the availability of the Chenomx software, once an unknown compound was determined, it was a challenging task to identify it and determine its biological significance of the metabolites. Chenomx provides a database consisting of 292 quantified urinary metabolites [14]. KEGG (*Kyoto Encyclopedia of Genes and Genomes*) is an online free website that explains all the known pathways that involve particular metabolites [26]. As well, the *Human Metabolome Database* [27] is an electronic



database containing information about the metabolites found in the human body. It is intended for metabolomics application in clinical chemistry, biomarker discovery and general education. This database contains over 7900 small molecules and provides information on metabolites from humans including both water-soluble and lipid soluble metabolites as well as metabolites that would be regarded as either abundant ( $> 1 \mu\text{M}$ ) or relatively rare ( $< 1 \text{nM}$ )[27].

To summarize, metabolomics is a quantitative and qualitative analytical technique that provides comprehensive information about metabolites. Samples for analysis can be obtained non invasively. Metabolomics can be used to assess early biomarkers and distinguish between healthy and diseased states. In the current study, we used metabolomics as a tool to evaluate the differences in the metabolic profiles between persons with risk factors for diabetes.

## **DIABETES**

Between 2010 and 2030, there will be an estimated 70% increase in the number of adults with diabetes in developing countries and 20% increase in developed countries around the world [28]. Diabetes is a complex disease [29], which is characterized by abnormal hepatic glucose output, insulin resistance and impaired insulin production [30][31]. A position statement published by *The American Diabetes Association (ADA)* (2011)[32] describes diabetes as a “chronic illness that requires continuing medical care and ongoing patient self management education and support to prevent acute complications and to reduce the risk of long term complications”. For many individuals who are unaware of their chronic state of high blood sugars, damage has started to occur before diagnosis. This chronic state of hyper-glycemia is associated with damage, dysfunction and failure of several organs including the kidneys, eyes, nerves and blood vessels [33].

Currently a small number of biomarkers are used for clinical assessment of diabetes and may be insufficient to reflect the global variation in the pathophysiology [34]. Changes in metabolic profiles which are a result of disease progression are a potential source of such biomarkers [14][15][35][36].

Diabetes is diagnosed by the fasting plasma glucose (FPG) or the 2-h 75-g oral tolerance test (OGTT) [37]. In 2010, ADA adopted the use of A1C test to diagnose diabetes of  $\geq 6.5\%$  [38]. In 1997 and 2003, the Expert Committee on Diagnosis and Classification of Diabetes Mellitus recognized an intermediate group of individuals whose glucose levels, did not meet the criteria for diabetes diagnosis but were too high to be considered normal [39][40]. Elevated blood glucose levels below the threshold for diabetes diagnosis also have clinical consequences. These persons are defined as “prediabetes” and have been tested with an impaired fasting glucose level of 100-125 mg/dl (5.6-6.9 mmol/l) or impaired glucose tolerance test 2 hours post glucose values of 140-199 mg/dl (7.8-11.0 mmol/l)[32]. Individuals with IFG and/or IGT do not have diabetes; however, they are at an increased risk for the future development of diabetes and CVD [41]. IFG and IGT are strongly associated with obesity, dyslipidemia with high triglycerides and/or low HDL cholesterol and hypertension [32].

In prospective studies, A1C has been used to predict the progression of diabetes. In a systematic review including 44, 203 participants from 16 cohort studies those with an A1C between 5.5 and 6.0% had a substantially increased risk of developing diabetes with a 5 year incidence ranging from 9-25%. This review found that an A1C range of 6.0-6.5% had a 5-year risk of developing diabetes 25-50% and relative risk 20 times higher compared with A1C of 5% [42]. ADA now recommends the A1C test as diagnostic tool for diabetes [32]. As with IFG and

IGT, elevated levels of A1C of 5.7-6.4% can be considered a predictor of increased risk for future diabetes as well as CVD [37].

The ADA recommends that Testing for Diabetes in asymptomatic persons include:

- Testing should be done at any age with a BMI  $\geq 25$  kg/m<sup>2</sup> and/or have one or more additional risk factors for diabetes which includes: physical inactivity, first-degree relative with diabetes, high risk ethnicity, women who delivered a baby weighing >9 lbs or were diagnosed with gestational diabetes, hypertension, HDL cholesterol level <35 mg/dl and/or a triglyceride level >250 mg/dl, women with polycystic ovarian syndrome, A1C $\geq$ 5.7%, IGT or IFG on previous testing, severe obesity, history of CVD.
- In those without the risk factors listed, testing should begin at age 45 years;
- To test for diabetes or assess risk for future diabetes, A1C, FPG, or 2-h 75 g OGTT is appropriate; and
- In those identified with increased risk for future diabetes, identify and if appropriate treat other CVD risk factors.

Fortunately, the same testing and clinical assessment can be done for screening, diagnosis and disease management. For many individuals, diagnosis of type 2 diabetes is not done until complications appear and according to the ADA [26], one-fourth of all people in the U.S. may be undiagnosed. Although the relatively low prevalence of diabetes in the general population makes it unlikely that mass screening will be cost effective, testing for people with risk factors for disease development is likely to result in more benefit than harm and will lead to overall cost savings [43][44]. Mathematical modeling studies suggest screening independent of risk factors beginning at age 30 or 45 years is a cost effective strategy [45]. Diabetes care accounts for about 2-7% of the total national health care budgets of western European countries [46].

There are intervention strategies for individuals who are at increased risk for developing diabetes that will significantly decrease the rate of onset for disease development. Risk factors for disease progression include physical inactivity, overweight, elevated lipid levels, unhealthy diet, ethnicity, family history of diabetes, smoking, ethnicity and family background. Many of these risks factors are modifiable which means that a person has the ability to delay the onset of disease progression. For many persons; however they do not display clinical symptoms of disease development until complications begin to occur.

Metabolomics has the potential in theory and based on observation from studies, to find early metabolic changes related to diabetes disease progression prior to many clinical symptoms. Although metabolic analysis is still considered to be in its infancy many believe that metabolomics strategies can have a pertinent impact on the discovery of clinical and pharmacological biomarkers. Given this emerging technology that's available, the increasing burden of the condition worldwide, earlier identification of 'at risk' individuals beyond current clinical markers is particularly important.

## **METABOLOMICS APPLIED TO DIABETES**

Individuals with diabetes have many metabolic pathways that are likely affected and presumably play a role in their overall metabolic dysfunction. Characterization of the pathophysiological alterations associated with the disease condition can be improved by identification of biomarkers and pathways related to the disease progression [47].

Assessment of a biological system by means of global and non-targeted metabolic profiling provides the investigator with molecular information that is close to phenotypes in question because metabolites are a product of gene, mRNA and protein activity.

According to Bain *et al.*[25], metabolomics application for diabetes provides advantages compared to other 'omics' technologies (genomics, transcriptomics, proteomics) because:

1. The Human Metabolome Database (HMDB)- Canada[27] currently lists approximately 6500 discrete small molecule metabolites which is a fraction of the estimated 25000 genes, 100 000 gene transcripts and 1 000 000 proteins;
2. Metabolite concentration alterations are the net result of genomic, transcriptomic and proteomic variability, therefore, providing the most integrated profile of biological status; and
3. Metabolomics is a precise tool for discerning mechanisms of action and possibly toxicological effects of drug therapies.

Thus, metabolomics can reveal crucial information that is closely related to the current disease state or therapeutic status.

Multivariate statistical analyses of the  $^1\text{H}$  NMR data from human, rat and mouse urinary profiles demonstrate metabolic similarities among the three species, including responses associated with disease progression of diabetes involving glucose metabolism, the tricarboxylic acid (TCA) cycle and the nucleotide and methylamine metabolisms [48]. In a study, using rats induced with diabetes NMR based metabolomics found differences in several metabolic pathways including glucose metabolism, TCA cycle, the alanine pathway, the Cori cycle, the acetate switch and choline metabolism [49]. Another study, with Zucker diabetic fatty rats, in the pre diabetic state showed that there were decreased levels of plasma concentrations for gluconeogenic amino acids, aspartate, serine, glutamine, glycine and histidine whereas, taurine, alpha-amino adipic acid, methionine, phenylalanine, tryptophans and branched chain amino acids were increased. At the diabetic stage, a larger number of gluconeogenic amino acids had

decreased plasma concentrations. The study concluded that there is evidence that insulin resistance alone is capable of bringing about many of the changes in amino acid metabolism observed in diabetes [50].

Cross-sectional studies have documented differences in blood metabolite profiles before and after glucose loading [25][27][51] and in obese versus lean human subjects [52]. These studies have noted differences in levels C3 and C5 acylcarnitines, glutamine/glutamate and additional amino acids and other small molecules. The observations from these studies demonstrated the possibilities that alteration in plasma metabolite levels are affected before the onset of diabetes and may be useful in the identification of 'at risk' individuals compared to clinical markers. For example, in a study conducted by, Doorn *et al.*, participants were given an oral glucose tolerance test, and endogenous metabolites were compared between participants with diagnosed diabetes compared to healthy individuals [53]. At baseline, there were disease-related metabolic differences between cases (diabetic subjects) and controls (non diabetic subjects). The cases underwent a 2 week 'wash-out' period prior to providing baseline urine and blood plasma samples. As a result, the differences in the metabolite concentrations were likely attributable to the disease related differences in the metabolic profile. In plasma, these differences were related to variation in lipid concentrations and lactate, and decreased levels of several amino acids. In urine, there were differences in the amino acid concentrations. Similarly, another study reported citrate and hippurate to be elevated in urine of diabetes subjects vs. healthy controls using NMR spectroscopy [54].

In a study conducted by Wang *et al.*, [55] metabolite profiles predicted the development of diabetes in a nested case control study. Among 2422 individuals who had normal glycemic levels, 201 developed diabetes over a 12 year period. Amino acids, amines and other polar

metabolites were provided in baseline specimens using liquid chromatography tandem mass spectroscopy. Cases and controls were matched for age, sex, body mass index and fasting blood glucose. In the paired analysis of the two groups, five metabolites had p-values of 0.0001 or smaller for the baseline differences. Of these metabolites the branched chain amino acids: leucine, isoleucine and valine were able to predict future risk of diabetes. Thus, the study observed that the fasting concentrations of amino acids were elevated up to 12 years prior to the onset of diabetes. One advantage of this study was that the 12 year observation period showed that the levels of amino acids were elevated prior to any alteration in insulin levels using standard biochemical measures. The major finding of this study was that despite normal fasting blood insulin levels amongst individuals, amino acid concentrations were predictive of diabetes development. The researchers noted that one drawback of this study was that the sample included individuals who were at “high” risk for diabetes disease progression because they displayed factors such as obesity and elevated fasting glucose. As a result, the findings may be a result of what would be expected in a high-risk cohort, not necessarily in a more heterogeneous sample. Hence, the study further investigated a random sample of 400 participants who did not have a diabetes or cardiovascular disease. This random cohort had a lower fasting glucose and BMI compared to the original case -control group. After repeating the amino acid analysis, the amino acid profile was still predictive of diabetes risk independent of standard diabetes risks; however the relative risk was not as significant suggesting that the amino acid profiling shows difference but may have greater value in ‘high’ risk individuals. The findings from this study do support the theory that hyper aminoacidemia could be an early manifestation of insulin resistance.

Studies of branched chain amino acid supplementation in both animals [55] and humans [56] observed that circulating amino acids may directly promote insulin resistance. The underlying cellular mechanisms may include activation of mTOR, JUN, IRS1 signaling pathways in skeletal muscle [52][57].

Insulin resistance and impaired insulin secretion play a critical role in the pathogenesis of type 2 diabetes and branch chain amino acids are modulators of insulin secretion [58][59][60]. Another mechanism that hyper aminoacidemia could promote diabetes is by hyperinsulinemia leading to pancreatic  $\alpha$  cell exhaustion.

A study published by *Lyssenko et al.*, [61] in the New England Journal of Medicine (2008), examined whether clinical or genetic factors or both could predict progression of diabetes. The study found that the inclusion of known polymorphisms of diabetes progression slightly improved the prediction of future disease development independent of clinical risk factors. A family history of diabetes, an increased BMI, current smoking status and elevated liver enzymes were independent predictors of future type 2 diabetes. Obesity was a strong risk factor for future diabetes, a risk that almost doubled in subjects with a family history.

A recent published study used a multiplatform approach of three different techniques covering NMR and tandem mass spectroscopy [62]. The researchers observed that this analytical approach detected perturbations of metabolic pathways linked to kidney dysfunction, lipid metabolism and interaction with the gut microflora. The differences in the biochemistry parameters between the diabetes and control group were modest; however with a limited sample size, the study did detect many differences in metabolic profiles.



A clinical challenge is to identify which individuals are 'at risk' and are most likely to develop diabetes. The goal of this project was to provide further insights on the risk factors that contribute to the differences in the metabolic profile of non-diagnosed adult subjects.

## **SUMMARY AND SPECIFIC AIMS**

To determine if metabolomics analytical techniques can differentiate between individuals with different risk levels for diabetes, a trial is needed to identify differences in the metabolic profiles of persons at risk for developing diabetes. The primary goal of this project is to conduct a pilot study using current metabolomics techniques to identify differences in the metabolites profiles of persons at risk for developing diabetes.

Before a definitive prospective trial is designed to answer these questions, we must first address important questions regarding feasibility, sample size, and preliminary evidence of differences in their metabolic profiles based on a person's clinical assessment for developing diabetes. Thus, we have conducted a pilot project to recruit subjects who pose a risk for developing diabetes based on clinical and lifestyle conditions including: BMI, physical inactivity, family history of diabetes, age, elevated blood pressure, elevated lipid profile, history of gestational diabetes in men and women older than 45 years of age.

The rationale for this proposed study is that metabolomics coupled with multivariate analysis will observe differences in the metabolic profiles of individuals with or without risk factors for development of type 2 diabetes.

In order to test the hypothesis, the following specific aims were established.

**Specific Aim 1:** To recruit and screen 50 persons with and without risk factors for development of type 2 diabetes to determine the correlation among these risk factors.

**Specific Aim 2:** To investigate metabolic profiles for potential differences based on risk factors for diabetes using metabolomics coupled with multivariate analysis.

This study will provide preliminary data to determine the differences, if any, in the metabolic profiles of individuals with risk factors for developing of diabetes using metabolomics analysis.

## **CHAPTER 2: MATERIALS AND METHODS**

The study was approved by the Humans Investigation Committee at Wayne State University and all participants were required to provide written consent (Appendix A). Confidentiality of response was assured by assigning code numbers to each questionnaire and biological samples, with the coding key only known to the project coordinator and principal investigator. All data was kept in a locked file and the master list of names and corresponding study numbers and consent forms will be shredded upon two years after completion of the study. Written data from which identifying information has been removed will be retained for potential future secondary analyses.

The first aim of the present study was to recruit and screen 50 persons with and without risk factors for development of type 2 diabetes to determine if there were differences in their metabolic profiles. A questionnaire was developed to collect information on risk factors for diabetes in asymptomatic adult individuals. The study collected data including: BMI; physical activity; diet, first-degree relative with diabetes; ethnicity; HDL lipid levels, fasting blood glucose, anthropometric measurements and medical history. ADA further recommends that in the absence of the above criteria, testing should begin at age 45 years. All participants did not have a clinical diagnosis of diabetes; however, all subjects were over the age of 45 years which is identified as a risk factor and many had other risk factors for diabetes.

### **Participant Recruitment**

Participants were recruited in the community of Chatham, ON by utilizing the services of local media and the Chatham-Kent Public Health Clinic Services to advertise the research project with flyers (Appendix B). The study recruited participants on a continuous in May, June and

July 2011. All assessments were conducted during 1- 60 minute visit. All assessments were conducted by the project coordinator.

Participants who responded to recruitment strategies were screened by telephone to determine eligibility. All participants were between the ages of 45 -65 years of age. The exclusion criteria included individuals who were insulin-dependent or taking medication required to control diabetes; had cancer requiring treatment in the past year; required use of lipid drugs; unwillingness to provide plasma and urine samples and/or unwillingness to provide informed consent.

### **Data Collection**

*Demographics and Medical Assessment-* A standardized questionnaire (Appendix C) was used to collect data on ethnicity, age, gender, medical history, family history of diabetes and medications at baseline. Participants were asked to bring in medications and supplements to the visit to verify medication and dosage.

*Dietary Assessment-* Participants completed a 24 hr diet recall with the project coordinator. From the information collected the project coordinator summarized the total number of servings consumed from each of the food groups as recommended in the *myplate* model produced by U.S Department of Agriculture [63].

*Anthropometry and Body Composition-* Height without shoes was measured using a wall-mounted stadiometer to the nearest 0.1 cm. Weight was measured to the nearest 0.1 kg using a calibrated and certified digital scale with the participant wearing light clothing without shoes. Height and weight was used to calculate BMI. Waist circumference was measured using a measuring tape placed around the subject at the level of the umbilicus. The subject was asked to take a normal breath and the waist circumference was recorded when the subject breathes out.

Blood Pressure was taken when the participant was relaxed and comfortably seated with the arm well supported at heart level.

*Blood and Urine Collection-* After an overnight food deprivation, blood was collected by a venous puncture and drawn by the project nurse into EDTA-containing tubes kept on ice. After each clinic, samples were labelled and brought on ice to the University of Windsor where the tubes were spun at 4500 rpm at 4°C for 20 minutes. Sodium azide (10%), gentamycin sulphate (0.01%) and EDTA (10%) were added to plasma samples, which were brought back to the Chatham-Kent Public Health Clinic Services and were stored at 4°C until study completion. Participants provided 10-20 mL of mid-stream urine and 10 mL of urine was transferred into a plastic tube where sodium azide (10%) was added to the sample. The urine samples were transferred into 3 – 2 mL tubes, labeled and stored at 4°C.

Once data collection was complete, blood and urine samples were shipped to Wayne State University on dry ice.

#### *Plasma Lipid Determination*

Plasma samples were assayed for Total Cholesterol, HDL-C and Triglycerides using enzymatic kits purchased from Pointe Scientific Inc. (Canton, MI). The procedure was followed as described in the enzymatic kits protocol.

A total of 50 persons were screened and recruited; however, some of the samples of plasma and/or urine were not used in the final analysis for the following reasons: the freezer was unthawed and 6 plasma and urine samples were unthawed for 2 days and discarded; 4 blood samples would not separate whole blood from plasma after 3 attempts; and 6 blood draws were unsuccessful after 2 attempts from the project nurse. A total of 34 subjects had all of the study parameters included in the analysis.

The second aim of the study was to investigate metabolic profile differences based on risk factors for diabetes using metabolomics coupled with multivariate analysis.

## **Statistical Analysis**

### **NMR Spectroscopy**

Urine samples were brought to room temperature and centrifuged at 10,000 rpm for 5 minutes. Samples were diluted with deuterium oxide (D<sub>2</sub>O) (Cambridge Isotope Inc., Andover, MA) in the ratio of 1:1. NMR solvent (in D<sub>2</sub>O) containing 0.5 mM 3-(trimethylsilyl) -1 propanesulfonic acid sodium salt (TMSPS) (Sigma Aldrich Inc. St. Louis, MO) and 1mM Imidazole (ACROS Organics, Thermo Fisher Scientific (NJ) were added to the diluted urine sample in 9:1 ratio. *Proton (1H) NMR Acquisition*- NMR spectra were acquired using a 500MHz Varian spectrophotometer. The free induction decay (FIDs) signals were acquired using a pulse sequence  $sw = 6009.6$ ,  $nt=64$ ,  $d1=10s$ ,  $satdly=990.0$  ms,  $pw =9.5$  us,  $mix-hst=100.0$  ms,  $pw=9.5$  us and 4.0 s acquisition.

*Spectral Processing*- FIDs were then processed using ACD/1D software (Advanced Chemistry Development, Inc., Toronto, ON, Canada). All the FIDs were stacked together as a group and then Fourier transformed to the spectra in the frequency domain. The whole spectra were edited, auto-phased, auto-base lined and binned. Intelligent binning was used to divide the edited spectra into 1000 bins. The spectra were digitized to a table of common integrals and exported as a non-negative value text file for *MultiVariate Analysis*- Multivariate data analysis (MVDA) (SIMCA P+ 12.0 Statistical software, Umetrics, Kinnelon, NJ).

Principle Component Analysis (PCA) and Partial Least Square (PLS) algorithms were used to evaluate the data for similarities and differences in metabolomic profiles between the groups.

## **CHAPTER THREE: RESULTS**

The first objective of the study was to recruit and screen a total of 50 persons with and without risk factors for development of type 2 diabetes to determine if there was a relationship between the risk factors from participants.

### **Participant Demographics**

A structured questionnaire was used to collect information on medical history and anthropometric measurements, which included height, weight, waist and hip circumferences. The total sample included 44 participants: 39 females and 5 male subjects. The age range was 45 to 64 years with an average age of 54 years. Of those participants, 98% were European Canadian and 2% were Native Canadian.

#### *Smoking Status*

The proportion of the participants who were current smokers was 11%. Of the non-smokers, 20% reported exposure to second hand in the home on a daily basis.

#### *Physical Activity*

On average, participants were physically activity for  $125 \pm 78$  minutes. This was assessed as a total for daily activity from vigorous, moderate or a combination of both. The percentage of participants meeting the daily physical activity guidelines of 150 minutes/wk of moderate activity, 75 minutes/wk of vigorous activity and/or combination of both activities was 71%.

#### *Fruit and Vegetable Intake*

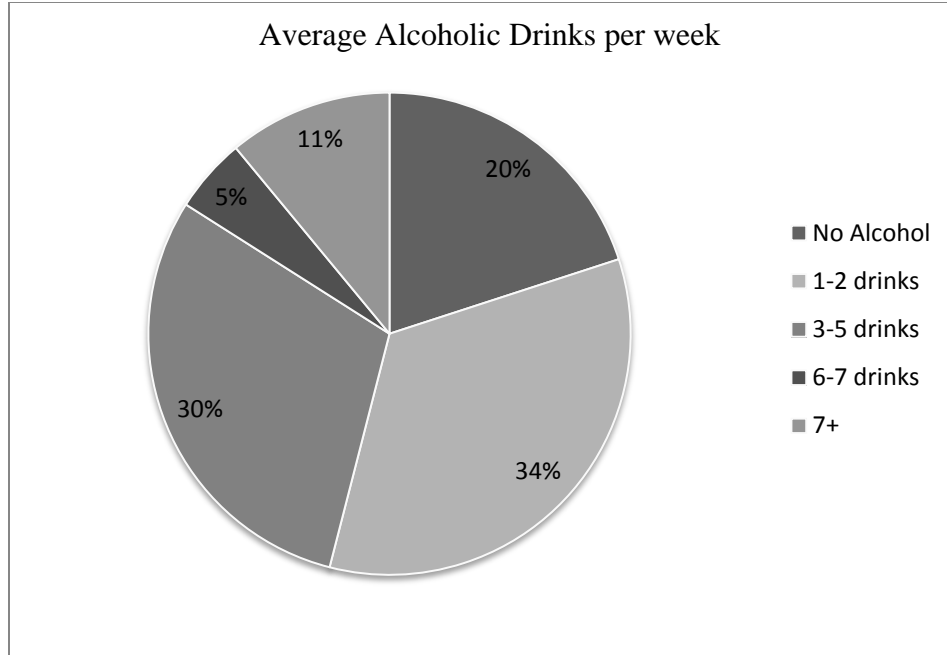
The findings from the study indicated that 48% of subjects consumed 5 servings or more of fruits and vegetables per day. The average intake was  $4.6 \pm 2.08$  servings per day.

#### *Alcohol Consumption*

According to the Low Risk Drinking Guidelines recommended by Health Canada, drinking in moderation is defined as having no more than 2 drinks per day for women and no more than 3 drinks per day for men. This definition is referring to the amount consumed on any single day and is not intended as an average over several days. The number of subjects meeting the low risk drinking guidelines was 89% (Figure 3:1). The mean weekly average for both males and females of the study was  $2.59 \pm 2.82$  standard drinks.

Physical activity, alcohol, smoking and fruit and vegetable consumption were analysed with bivariate correlations to determine if there was a relationship amongst these variables within the study group. There was a significant positive relationship ( $p < 0.05$ ) found between the average daily intake of fruit and vegetables and physical activity level ( $r = 0.33$ ). Physical activity was also inversely correlated to alcohol intake, therefore participants with higher average levels of physical activity were more likely to have lower alcohol consumption ( $r = -0.32$ ) and this correlation was significant ( $p < 0.05$ ).





**Figure 3:1 Average Alcoholic Drinks per week**

The proportion of subjects who reported consumption of alcoholic beverages in a typical week. From the information collected: 20% of participants reported consuming no alcohol on a weekly basis; 34% drank 1-2 standard drinks per week; 30% drank 3-5 drinks; 5% consumed 6-7 drinks per week; and 11% average consumption was seven or more drinks per week.

## **Anthropometric Measurement**

Body Mass Index (BMI) is a ratio of weight to height (kg/m<sup>2</sup>). Waist to Hip ratio estimates the distribution of subcutaneous and intra abdominal adipose and muscle tissue. Waist circumference provides an index of the absolute amount of abdominal fat and adds to the prediction of morbidity and mortality obtained from measuring BMI alone. BMI, waist to hip ratio and waist circumference (WC) are anthropometric screening tools that can be used together in order to identify individuals who are overweight or obese and are at an increased risk of diabetes, cardiovascular disease and other related conditions. These measures should be interpreted in conjunction with other measures of health risk as part of a complete individual assessment.

### *BMI*

The BMI provides ranges for a healthy weight (18.9-24.9kg/m<sup>2</sup>); overweight (25-29.9kg/m<sup>2</sup>) and obese (30kg/m<sup>2</sup> and above). BMI is a highly reliable and convenient measure used to screen for obesity, but it does have some limitations that restrict its usefulness in certain clinical settings. At the clinic visit participants' weight and height was collected and the BMI was calculated. The mean BMI was  $27.6 \pm 4.9$  (Figure 3:2).

The average BMI for subjects aged 45-49 yrs was 28 kg/m<sup>2</sup>; 26.5 kg/m<sup>2</sup> for those aged 50-59 and 30.3kg/m<sup>2</sup> for subjects over the age of 60 years (Figure 3:3). BMI and age were independent and the variables were unrelated to each other ( $r=0.20$ ) (Figure 3:4).

### *Waist Circumference*

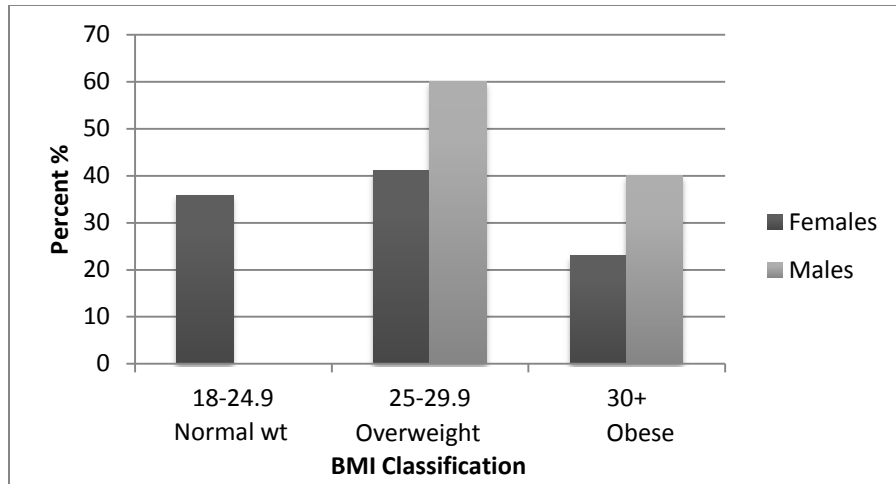
The waist circumference correlates with visceral fat stores. Values greater than 102 cm (40 inches) in men and 88 cm (35 inches) in women may indicate increased risk for

cardiovascular disease and diabetes [64]. The average waist circumference was 91 cm ( $\pm 14$ ) and 45% of subjects had a measurement higher than recommendations.

#### *Waist to Hip ratio*

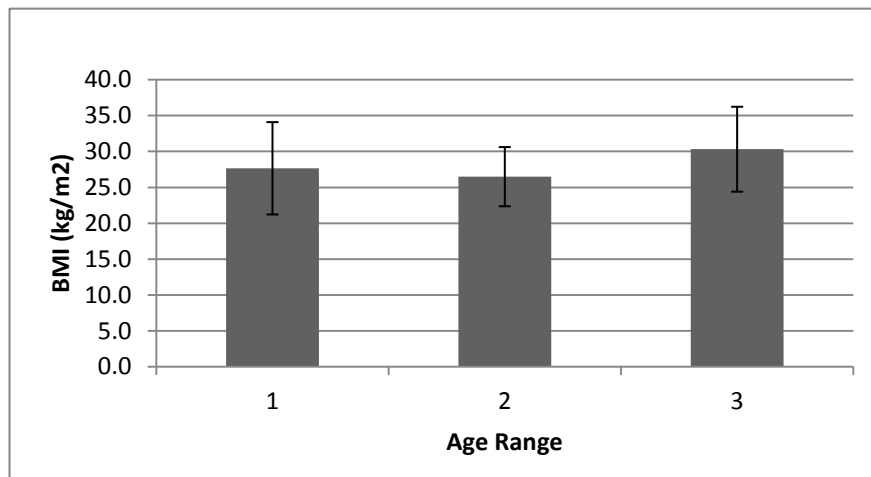
Waist to Hip ratio was calculated as an assessment of weight status for the subjects. Ratios  $>1.0$  in men and 0.8 in women may indicate increased risk for morbidity and mortality [65]. Sixty eight percent of the participants had a waist to hip ratio either  $>1.0$  in men or 0.8 in women. The mean waist to hip ratio was  $0.85 \pm 0.067$ .

As would be expected, anthropometric measurements of BMI, waist to hip ratio and waist circumference were strongly correlated with each other (Figure 3:5). This was more of an assessment to determine accuracy of the measurements.



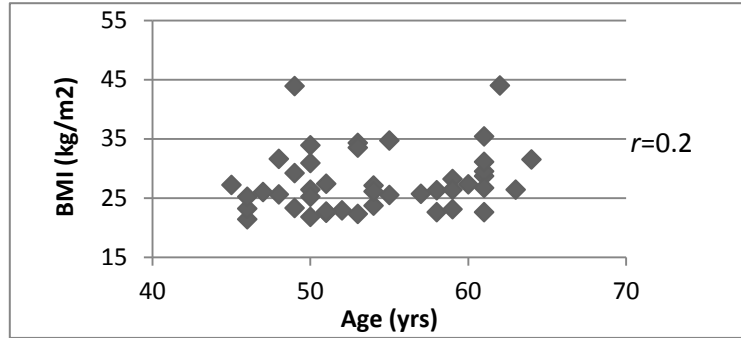
**Figure 3:2 Body Mass Index Classifications by Gender**

Figure illustrates the percentage of participants in each BMI classification by gender. The BMI distribution for females was 36% classified as a normal weight, 41% overweight and 23% classified as obese. The BMI distribution for males was that 60% of the males were in the overweight classification and 40% were classified as obese. Thus, 100% of the male sample was in the overweight or obese category



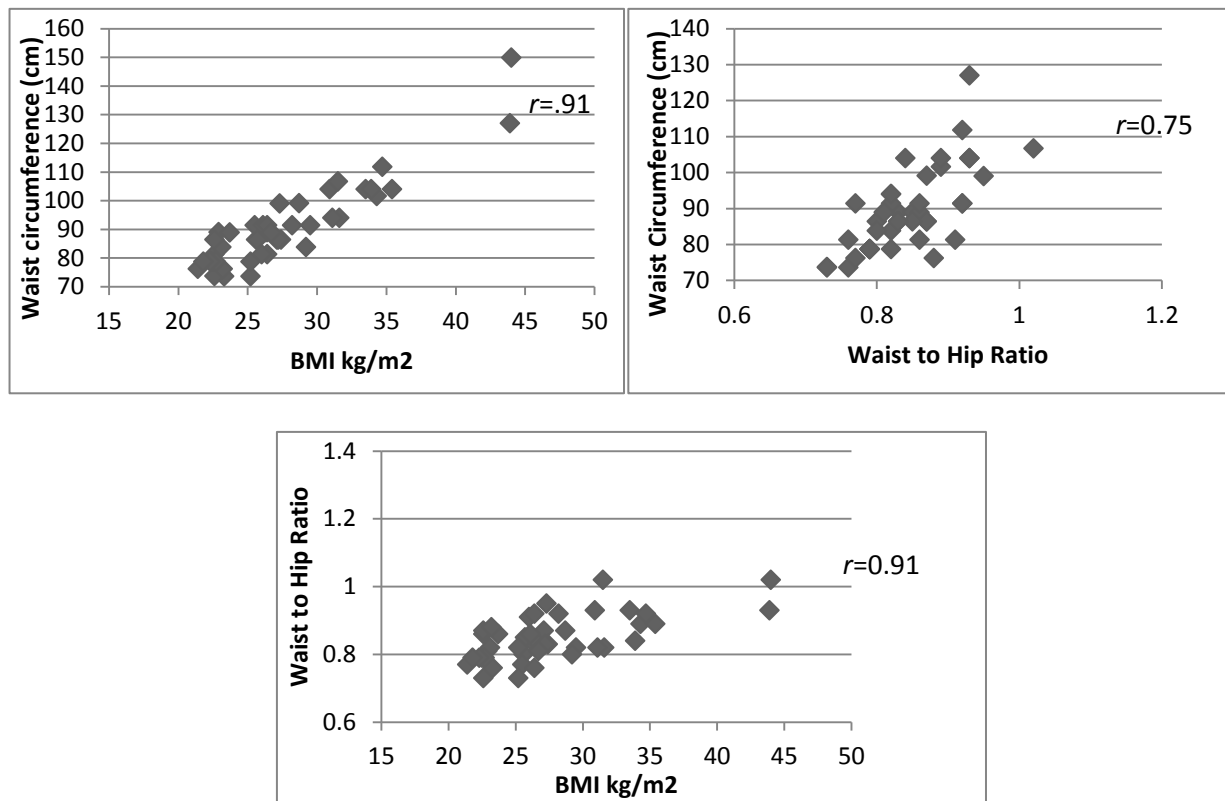
**Figure 3:3 Age and BMI**

This figure shows the BMI average for each age group. Participants were aged 45-49 in Group 1; group 2 50-59 and group 3 60-64 years.



**Figure 3:4 Correlation between Age and BMI**

The correlation between age and BMI for this sample was weak ( $r=0.2$ ). These variables were not related to each other.

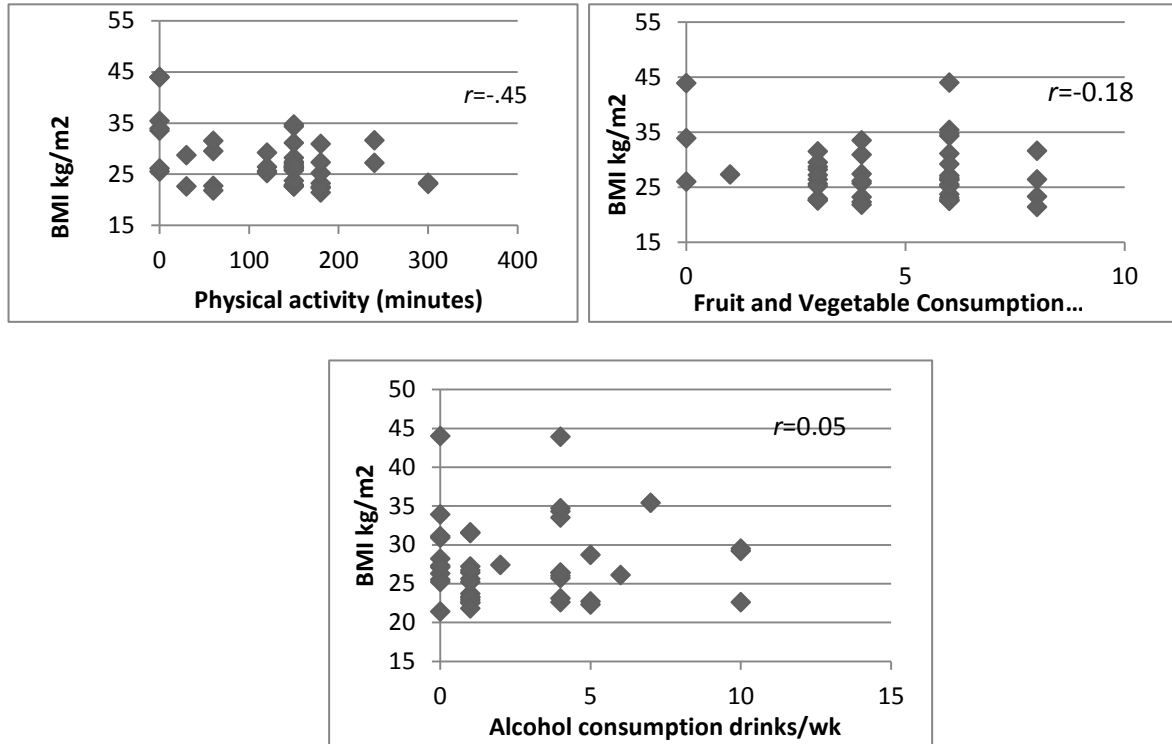


**Figure 3:5 Anthropometric measurements BMI, waist to hip and waist circumference**

Figure illustrates that the three measurements used to assess weight status were strongly correlated. Waist circumference and waist to hip ratio showed the strongest correlation with BMI. All of these correlations were statistically significant ( $p < 0.01$ ).

Factors such as physical activity and dietary intake contribute to a healthy weight. Correlational analysis was used to determine if these variables were related with weight measurements. As the weight measurements collected were strongly associated with each other, BMI was used for this analysis.

Physical activity showed a significant correlation with BMI ( $r=0.45$ )( $p<0.01$ ); however, fruit and vegetable and alcohol consumption were not associated with BMI (Figure 3:6).



**Figure 3:6 Lifestyle Behaviours and BMI**

Figure illustrates that BMI was negatively correlated with physical activity level which means that physical activity increased by the amount of minutes per week, BMI decreased. This correlation was significant ( $p < 0.01$ ). However, there was no correlation with fruit and vegetable intake and alcohol consumption.

## Lipids

Assessment of the lipid profile was included in the study because dyslipidemia is a risk factor for diabetes progression. When a person is diagnosed with diabetes, lipid management is important, as there is an increased prevalence of lipid abnormalities contributing to their risk of CVD [37]. Dyslipidemia refers to abnormal levels of lipids in the blood which includes high levels of total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), triglyceride (TG) and/or low levels of high density lipoprotein cholesterol (HDL-C). Dyslipidemia can also be described as hyperlipoproteinemia or hyperlipidemia (referring to abnormally high levels of TC, LDL-C or TG) or hypercholesterolemia (referring to elevated TC and/or LDL-C)[66].

During clinic visit, a fasting blood sample was collected to measure, total cholesterol, triglycerides and HDL cholesterol levels. Recommendations from the *American Heart Association* (AHA) were used as reference guidelines to assess normal and high values for lipid cut off points. The mean TC in the study was 237 mg/dl ( $\pm 55$ ) and the proportion of participants that were considered to have a high TC  $\geq 240$ mg/dl was approximately 50%. The average value for TG levels was 105 mg/dl ( $\pm 47$ ) and the proportion of persons with a high TG ( $\geq 150$  mg/dl) level was lower than those with high TC at 15%. The mean value for HDL-C was 65mg/dl ( $\pm 30$ ). The percentage of participants with a low HDL-C  $\leq 40$  mg/dl was 18%.

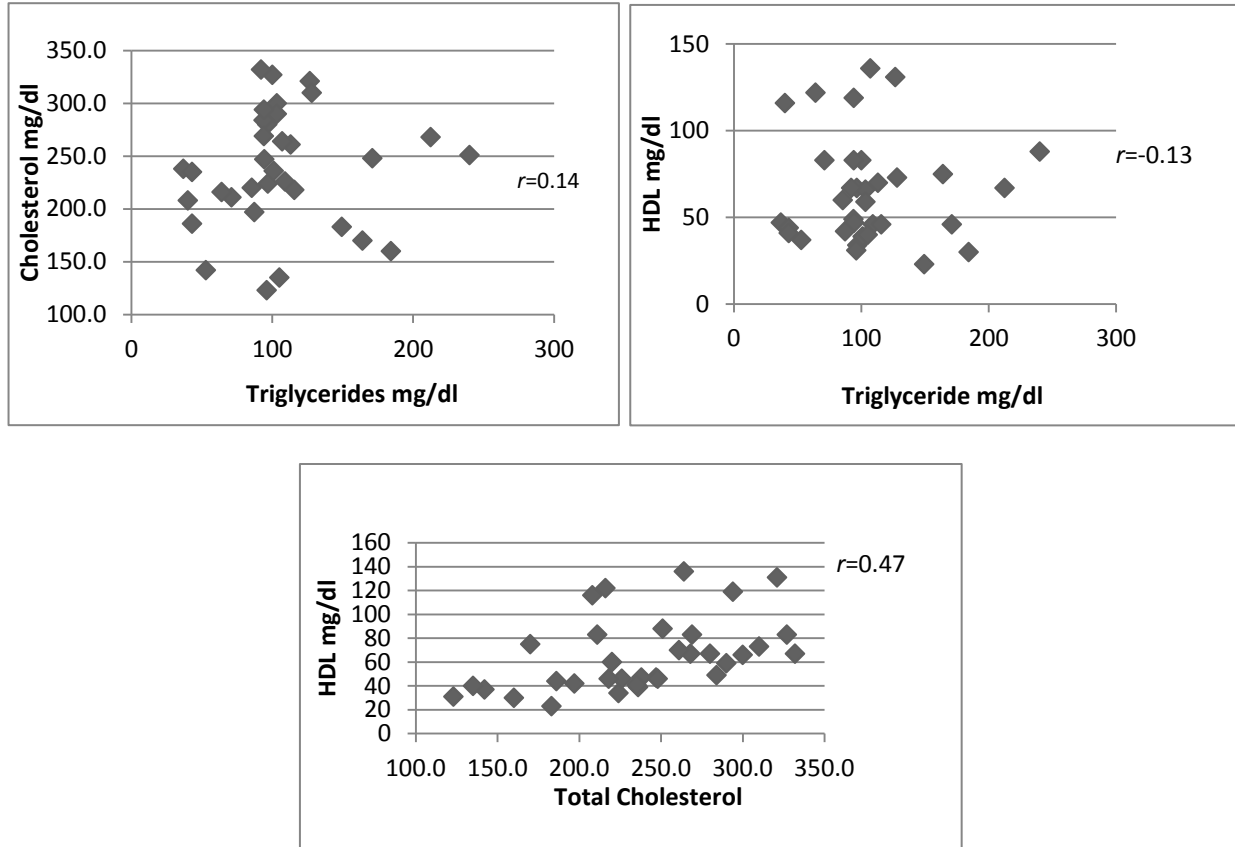
LDL-C levels (or “bad” cholesterol, in the bloodstream) were calculated indirectly using the Friedewald equation. This equation [ $LDL = \text{total cholesterol} - HDL - (\text{triglycerides}/5)$ ] calculates the concentration of LDL based upon the presence of total cholesterol, HDL and triglyceride levels. The mean concentration value for LDL-C was 151 mg/dl ( $\pm 49$ ). The concentration of TG did not correlate with HDL-C ( $r = -0.13$ ) or TC ( $r = 0.14$ ). However, HDL and TC showed a significant correlation ( $r = 0.47$ ) ( $p < 0.01$ ) (Figure 3:7). LDL was not included in



this regression analysis as the lipid value is derived from Cholesterol, HDL and triglyceride concentrations; and therefore not recommended for regression analysis.

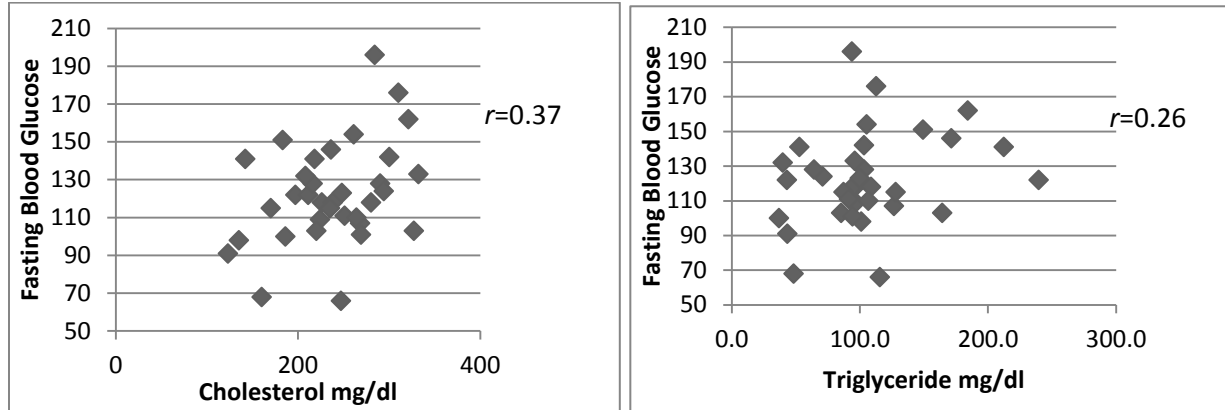
The changes in the lipid profiles are often accompanied by hypertension, obesity and insulin resistance. The data collected in the study showed that there was a significant correlation ( $p < 0.05$ ) between a fasting blood glucose and LDL-C ( $r = 0.34$ ), TC ( $r = 0.37$ ). The fasting blood glucose was positively correlated TG ( $r = 0.26$ ); however this relationship was not significant (Figure 3:8).

A systolic blood pressure reading showed a positive correlation with TG; however this relationship was unrelated (Figure 3:9).



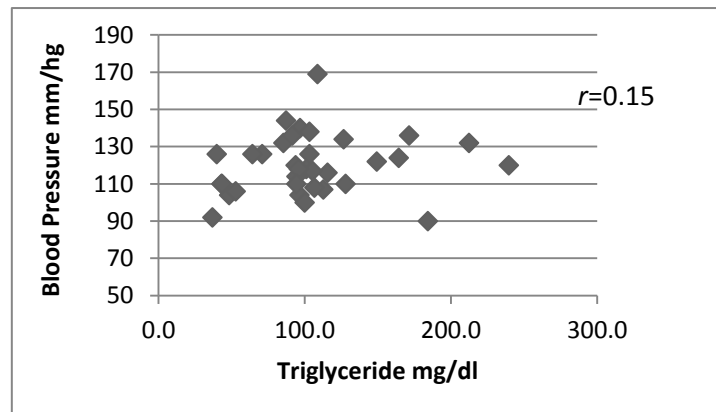
**Figure 3:7 The correlation between lipid values: TG, TC and HDL-C**

As shown in this figure there was a very weak relationship between TG and HDL-C and TC concentrations. HDL-C and TC values were significantly correlated ( $p<0.01$ ).



**Figure 3:8 The Correlation between Fasting blood glucose and LDL-C, TC and TG concentrations**

LDL and cholesterol were found to be significantly correlated ( $p < 0.05$ ) with fasting blood glucose. TG and fasting blood glucose concentrations were correlated; however this relationship was not significant.

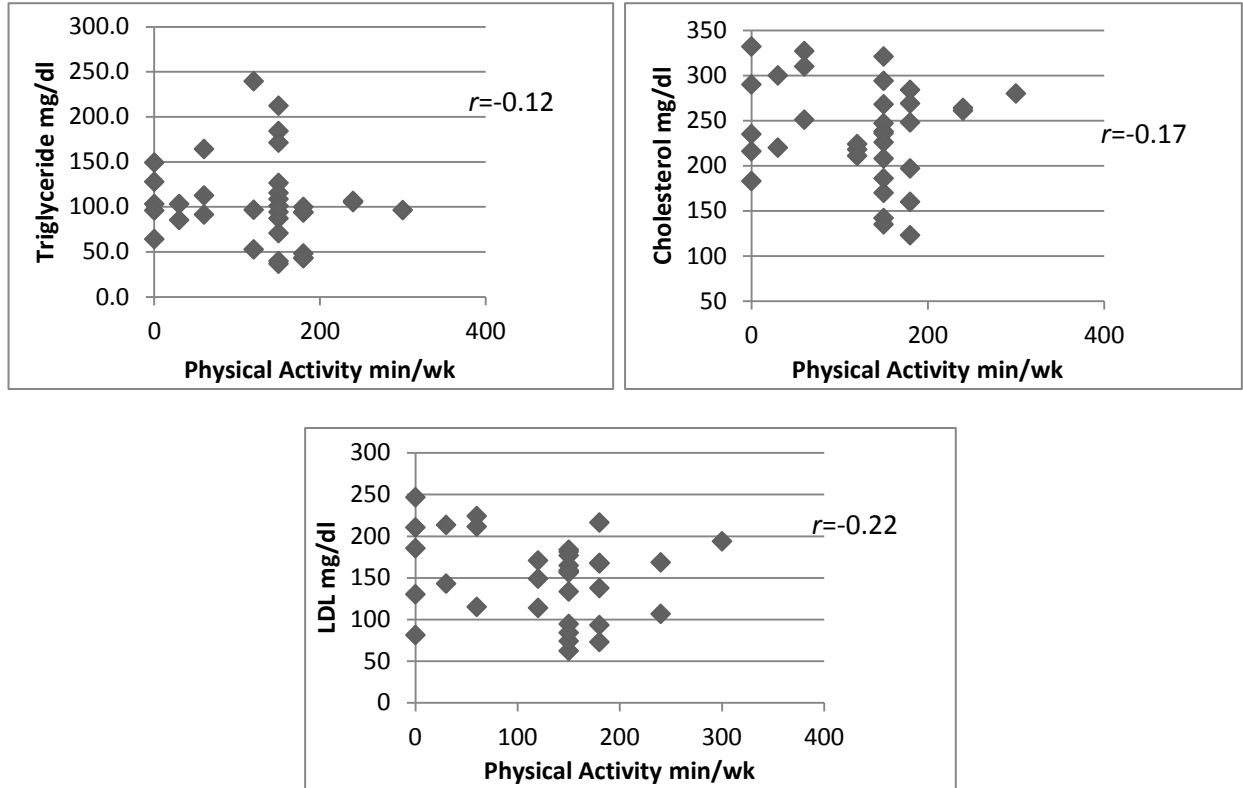


**Figure 3:9 The Correlation between Systolic blood pressure reading and TG concentrations**

Blood pressure and TG levels are unrelated ( $r=0.25$ ,  $p > 0.05$ )

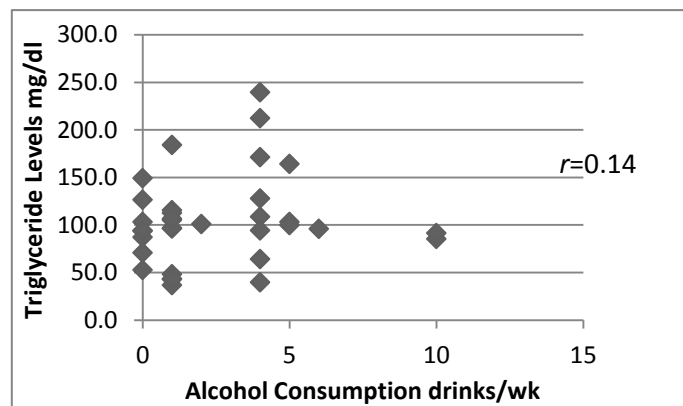
Higher levels of physical activity have been related to improved lipid profile and are recommended to prevent the onset of diabetes and heart disease. From the analysis, there was a slight; however, a negligible correlation between the level of physical activity and lipid values of TG ( $r=-0.12$ ), TC ( $r=-0.17$ ) and LDL-C ( $r=-0.22$ ) (Figure 3:10).

Increased blood pressure combined with hyperlipidemia contributes to an increased risk of heart disease mortality compared to hyperlipidemia alone. There was an unrelated correlation ( $r=0.14$ ) found between triglyceride level and alcohol consumption for this population (Figure 3:11).



**Figure 3:10 The Correlation between physical activity and concentration of LDL-C, TC and TG concentrations**

The relationship between physical activity measured in minutes per week showed slight, negligible correlations to the concentration level for lipids. This analysis did show an inverse relationship showing that as the number of minutes increased being physical active, the concentration levels for LDL, cholesterol and triglycerides decreased. The strongest correlation was noted to be LDL with an  $r = -0.22$ ; however this correlation was not statistically significant.



**Figure 3:11 The correlation between average weekly alcohol consumption and TG levels**  
This relationship between triglycerides and alcohol consumption was unrelated  $r = 0.14$ .

The 2006 AHA diet and lifestyle recommendations to improve lipid levels, advise that individuals consume a diet rich in vegetables and fruits as these foods are rich in nutrients, low in calories and high in fibre, therefore contributing to nutrient requirements without adding substantial calories [67]. It is unknown whether CVD risk reduction is due to vegetables and fruits themselves or the absence of other foods displaced from the diet. Diets rich in vegetables and fruits have been demonstrated to lower blood pressure and improve other CVD risk factors in short-term randomized trials. Furthermore, in longitudinal observational studies, regular consumption of diets high in vegetables and fruits lower risk of developing CVD. The correlation between fruit and vegetable intake and lipid values was conflicting in this study. Anticipated results were that as fruit and vegetable consumption increased, HDL would be higher; however, this was not the relationship found from the results. This correlation value; indicated that the relationship between the variables were unrelated (Figure 3:12).



## **Blood Pressure**

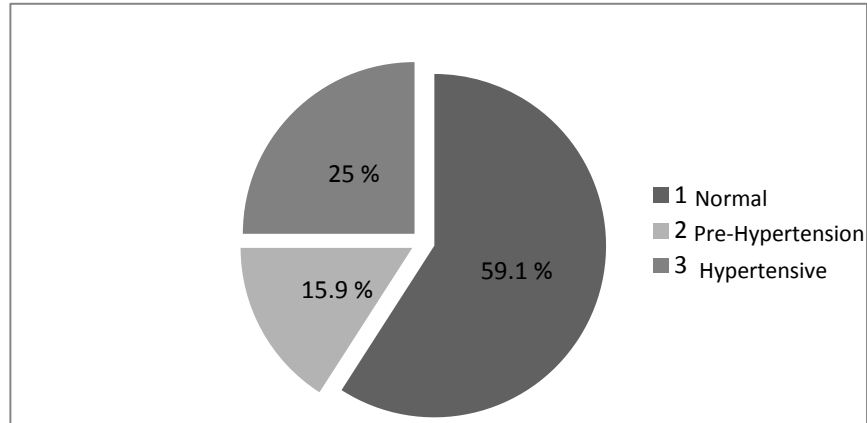
The ADA identifies hypertension as a risk factor for developing diabetes. According to the *AHA* there are several factors that can increase a person's risk of developing high blood pressure: family history, age, gender, physical inactivity, diet, overweight and obesity and alcohol consumption. Other factors such as smoking, stress and sleep apnea may also be associated with increased blood pressure [68]. Normal blood pressure was defined as a systolic pressure below 120 mm HG and a diastolic pressure below 80 mm HG. Pre-hypertension was defined as a systolic pressure between 120-139 mm HG and a diastolic between 80-89 mm HG. High blood pressure was defined as systolic pressure of 140 mm HG or more, a diastolic pressure 90 mm HG or more, or both. Hypertension diagnosis requires an average of several readings; however for the purpose of this study, participants were categorized into Normal, Pre-Hypertensive and Hypertensive based on the reading at the clinic visit or if they were taking blood pressure lowering medication (Figure 3:13).

### *Blood Pressure and Age*

Blood vessels lose flexibility with age which can contribute to increasing pressure throughout the system, thus high blood pressure is typically associated with advancing age. For subjects aged 45-49 years, 82% had a normal reading and 9% had a read which was considered pre-hypertensive and 9% hypertensive. For participants aged 50-55 years, the proportion of persons with a normal blood pressure reading was 67%, and 27% had a read which would classify as hypertension. For those aged 55-59 years, 43% had a normal blood pressure reading, 43% pre-hypertensive and 14% hypertension. For individuals aged 60-64 years, 40% had a normal BP read, 20% pre-hypertension and 40% hypertensive. Correlational analysis

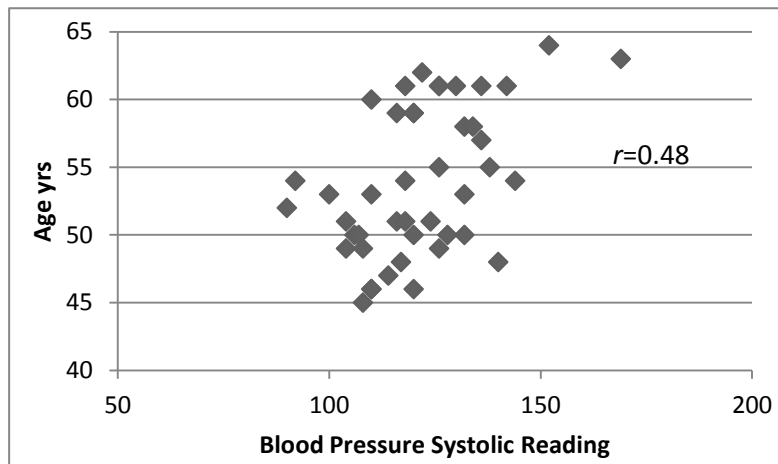


demonstrated that there was a moderate correlation between blood pressure reading using the systolic measurement and age ( $r=0.48$ ) which was significant ( $p<0.01$ ) (Figure 3:14).



**Figure 3:13 Proportion of participants classified as Normal, Pre-Hypertension and Hypertensive**

As illustrated in the graph, over half 59% of the participants had a normal blood pressure, 16% were considered pre-hypertensive and 25% had a high blood pressure.



**Figure 3:14: Correlation between age and blood pressure**

As age increased amongst the participants in the study, blood pressure readings for the systolic measurement were higher. This correlation was statistically significant ( $p<0.01$ ).

### *Blood Pressure and Weight*

Maintenance of a healthy BMI (18.5-24.9 kg/m<sup>2</sup>) and waist circumference (WC) (<88 cm Female; <102 cm Male) is recommended to reduce blood pressure in hypertensive individuals. Overweight, hypertensive individuals are advised to lose weight to decrease blood pressure. The results from this study showed a relationship between BMI and blood pressure (Figure 3:15). The correlation between BMI and blood pressure ( $r=0.30$ ) was weakly positive and statistically significant. (Figure 3:16).

### *Waist to Hip Ratio*

Individuals who met the criteria for the recommended waist to hip ratio were more likely to have a normal blood pressure reading. Eighty six percent of subjects who met the recommendations for the waist to hip ratio had a normal blood pressure; whereas 48% of participants who were above the recommendations were classified as pre-hypertensive or having hypertension (Figure 3:17). This correlation was not statistically significant.

### *Alcohol Consumption*

Individuals with hypertension are at increased risk of developing diabetes. Similarly, good systolic blood pressure control in individuals with diabetes reduces vascular complications. Following the low risk drinking guidelines is recommended to reduce blood pressure for individuals with hypertension. The results from the study showed that there was an unrelated correlation between alcohol consumption and blood pressure measurements (Figure 3:18).

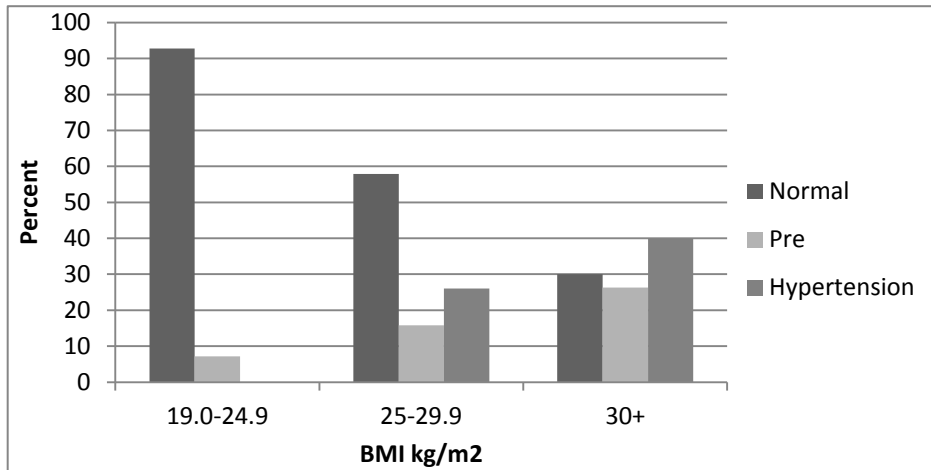
### *Physical Activity*

Accumulation of 30-60 minutes of dynamic exercise (such as walking, jogging, cycling or swimming), at least four times per week in addition to routine activities of daily living is associated with a reduction in blood pressure and may reduce the possibility of becoming

hypertensive. The number of minutes was indicated as a weekly average and a positive relationship was determined with blood pressure ( $r=0.32$ ) which was statistically significant ( $p<0.05$ ).

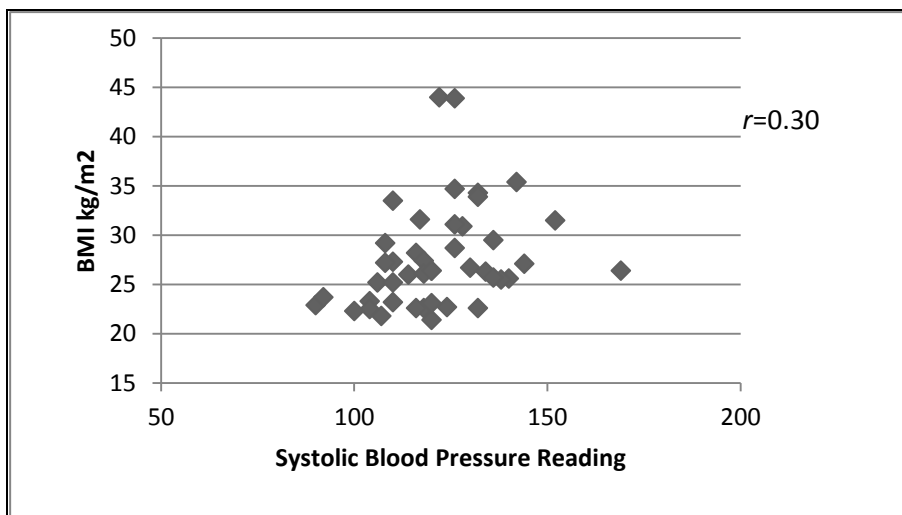
#### *Fruit and Vegetable Consumption*

A recently published study found that higher intake of fruits and vegetables, as part of a healthy dietary pattern, may only contribute a modest beneficial effect to hypertension prevention, possibly through improvement in body weight regulation [69]. The low sodium DASH diet (i.e. high in fruit, vegetables and low fat dairy products; an emphasis on whole grains; a low intake of cholesterol, saturated fat, red meats, and refined sugar; and the inclusion of nuts, seeds and legumes several times a week) lowers blood pressure in normotensive individuals [70]. The 24 hour dietary recall collected from the participants was not compared to the recommendations of the DASH diet. However, information on daily fruit and vegetable intake was obtained in the standard questionnaire. From the results, there was no correlation between fruit and vegetable intake and blood pressure ( $r=0.03$ ).



**Figure 3:15: The proportion of persons with normal, pre-hypertension and hypertension classified by BMI**

For subjects with a normal weight BMI classification, 93% had a recommended blood pressure read and 7% were considered pre hypertensive. Participants who had a BMI in the overweight classification were more likely to have a pre hypertensive or hypertensive reading; with 16% pre hypertensive and 26% hypertensive. The proportion of subjects classified as obese an increased prevalence of pre-hypertension 28% and hypertension 40%.



**Figure 3:16 Correlation between blood pressure and BMI**

As illustrated in this figure there was a correlation between BMI and blood pressure reading. Correlation was significant ( $p < 0.05$ ).





### *Fasting blood glucose*

The mean blood glucose level was  $123 \pm 27$  mg/dl. The two variables that were statically correlated with the fasting blood glucose were LDL and cholesterol. Other variables such as physical activity, BMI, triglycerides and blood pressure showed weak correlations.

### **Summary of Aim 1**

Studies have highlighted the association between insulin resistance (IR) and several cardiovascular (CV) risk factors, including hypertension, obesity, dyslipidemia (i.e. high triglyceride and low HDL-cholesterol) and glucose intolerance, in a cluster known as the metabolic syndrome (MS). Many variables such as weight, family history, smoking status, diet patterns, physical activity, sodium intake and alcohol consumption contribute to this cluster known as metabolic syndrome increasing the complexity of the analysis and determination of which factors are most connected. As shown in this study, it is difficult to identify which individuals are most 'at risk' for developing diabetes as the risk factors can be independent to one another but are often connected and overlapped.

The purpose of this project was to provide further insight on the risk factors that contribute to the differences in the metabolic profile for non diabetic subjects. As a first step, bivariate correlations were determined to investigate the linear relationship between the risk factors and to determine how strong the relationship was between the variables.

### **Specific Aim 2**

The objective of this aim was to study changes in urinary metabolism profiles based on the varying risk factors for diabetes using 1D proton NMR. Urine was collected at base line and stored at 4°C until further use. The samples were prepared and NMR FID files were acquired



using 500 MHz Magnet. The files were processed in ADC 1D NMR software and MVDA was conducted using SIMCA P+ software.

### **Multivariate Data Analysis**

The data files acquired from NMR were ready for MVDA analysis after processing. PCA and PLS showed significant differences between the groups.

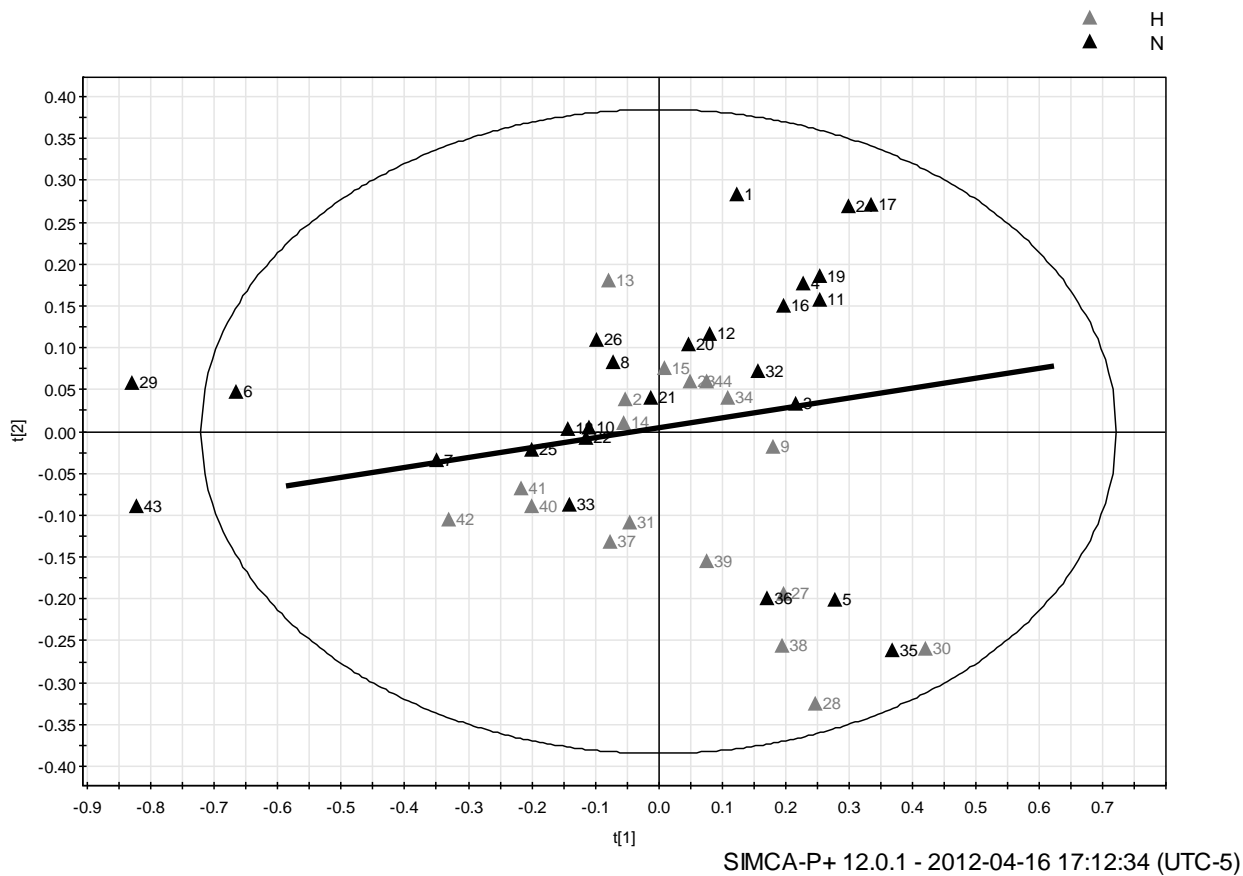
#### **PCA**

Unsupervised PCA was completed as a first step to determine if there were correlated factors without the removal of outliers. This analysis is exploratory in nature to determine which factors caused the greatest separation between the variables. There was a clustering of subjects observed based on the dominating variable BMI. The score plot shows a separation when subjects were divided into 2 groups: a BMI <27 kg/m<sup>2</sup> and BMI ≥ 27 kg/m<sup>2</sup> (Figure 3-20).

#### **Partial Least Squares Regression Discriminate analysis (PLS-DA)**

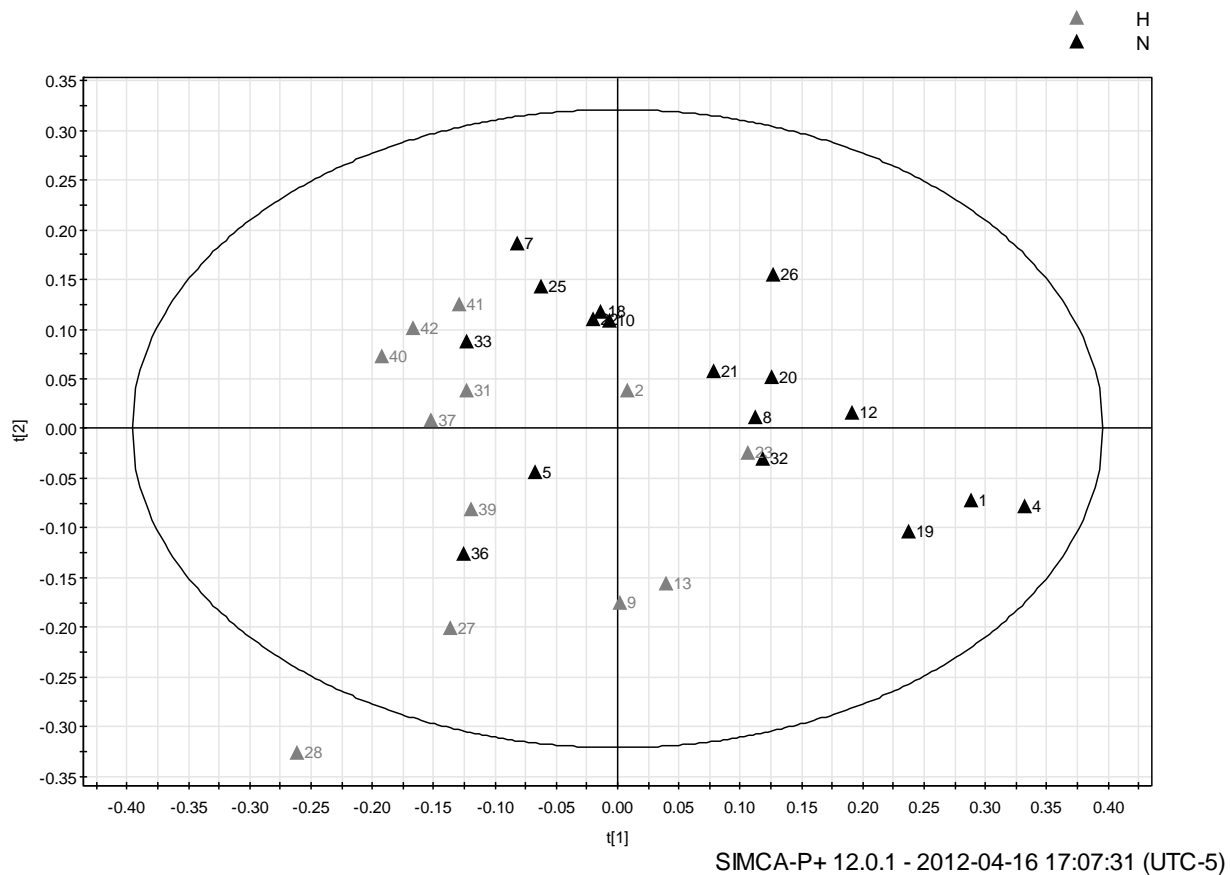
As the unsupervised analysis showed correlation with BMI, we further analyzed this with PLS-DA, a supervised multivariate analysis algorithm. As shown in Figure 3-21, the somewhat loose BMI separated clusters in PCA, became clearly distinct. Results indicate that in the inter-individual urinary profiles are dominated by differences in their BMI. Thus, there are some metabolites in the urine samples that are correlating with obesity/BMI.

To see the effect of BMI on various risk factors, we used PLS to correlate the urinary metabolomic profiles with the risk factors collected. In the individual analysis, under Aim 1 BMI showed a strong correlation with the lipid profile ( $r^2=0.52$ )(Figure 3-22). The urinary NMR profile of the subjects showed good correlation between triglyceride concentration levels and the lipid profile which included cholesterol, HDL, LDL and triglycerides.



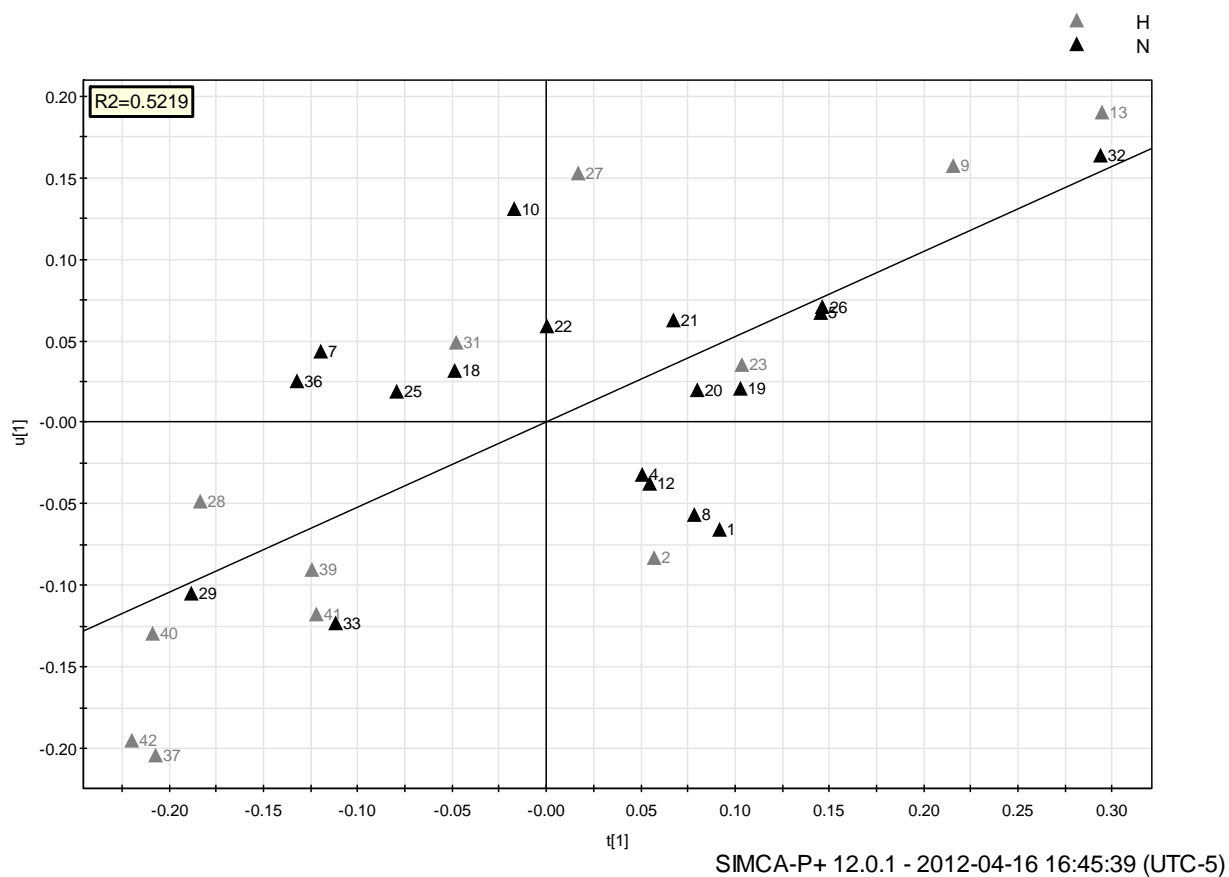
**Figure 3:20 The PCA score plot BMI**

The PCA score plot is showing some separation between the urinary metabolomics profiles of individuals with normal ( $<27 \text{ kg/m}^2$ ) or high BMI ( $>27 \text{ kg/m}^2$ ). Scores within the eclipse are significant to the model. Two outliers (29, 43) are seen in this plot.



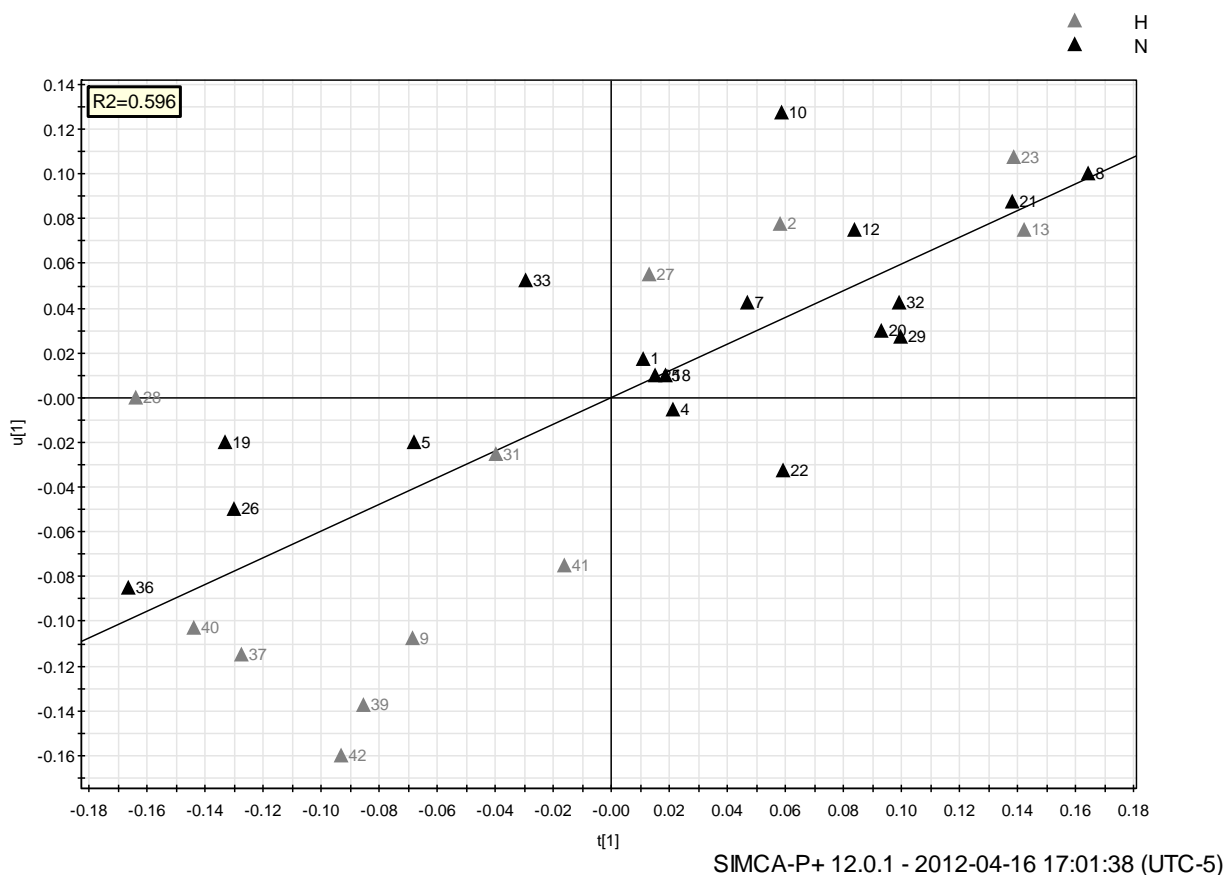
**Figure 3-21 PLS-DA Score Plot for BMI**

PLS-DA score plot showing a clear separation between the urinary metabolomics profiles of subjects with normal and high BMI.



**Figure 3:22 PLS Correlation Plot of 1 NMR Urinary metabolomics Profiles with BMI and Lipids**

The figure shows a good correlation between the urinary metabolomics profile (x-axis) and Lipid concentration (y-axis). Since the metabolomic profiles were separated into two clusters based on BMI, this further indicated that metabolite in the urinary profile related to BMI or obesity correlates with plasma lipid concentrations.



**Figure 3:23 PLS Correlation Plot of 1 HNMR Urinary Metabolomic Profiles with BMI and Blood Glucose**

The figure shows correlation of urinary profiles between BMI and blood sugar in a non-diabetic population. The figure depicts a good correlation of the metabolites in the urinary profile between BMI and blood glucose levels. This shows that some metabolites in urine based on BMI could predict or correlate with fasting blood glucose, the current biomarker for prediction of diabetes.

**Summary of Specific Aim 2**

Utilizing NMR technique and use of SIMCA P+ (PCA score plot) differences were observed between the urinary profiles based on BMI. Therefore, variability in the urinary profiles for this study sample was evident for persons with a BMI  $>27$  kg/m<sup>2</sup>.

## CHAPTER FOUR: DISCUSSION

The prevalence of diabetes is increasing epidemic proportions; with an estimated 17.5 million type 2 diabetic individuals in the United States. This costs the USA \$174 billion [71] and is considered to be one of the most pressing public health issues. To date, the exact cause of type 2 diabetes remains unclear, however, it is considered to be an interplay between environmental and genetic factors [72]. Emerging technologies for metabolomics analysis increases the capacity to detect the onset of disease or ideally, the pre-diseased state.

The pathophysiology of diabetes is complex, and multifactorial. In this relatively, heterogeneous sample group of participants, we set out to investigate the impact of identified risk factors related to the pathophysiology of diabetes in the metabolic profiles. Risk factors such as age, obesity, physical inactivity, first degree relative with diabetes, high risk ethnicity, hypertension, dyslipidemia, or impaired fasting glucose are factors related to the onset of diabetes. The ADA recommends that individuals over the age of 45 years have testing completed to assess for diabetes. For this study, a standard questionnaire was developed to collect information on these risk factors. The sample group did not have a diagnosis of diabetes, but in addition to age, many of the participants had at least one risk factor increasing the risk for future onset. Individuals may have risk factors such as obesity, dyslipidemia, elevated blood sugars; however, often there is long pre-symptomatic phase prior to diagnosis. Early diagnosis is imperative to improve a person's outcome. In addition to current clinical markers, metabolomics provides an opportunity to expand our knowledge of the causes and progression of this disease and enhance treatment options for individuals. This is possible, by simultaneously investigating a large and diverse set of metabolites. This integrated approach allows the analysis and

classification of metabolic states, reveals new pathways, and potentially improves the sensitivity for detection of the altered state.

A first step in the analysis was to determine correlations among risk factors. Key findings from this analysis were that there was a significant relationship between average daily intake of fruit and vegetables and physical activity levels. Physical activity was also significantly correlated with decreased alcohol consumption. This was an expected finding, as individuals who are physically active often display other healthy behaviours such increased fruit and vegetable intake and moderate alcohol consumption. Physical activity was also strongly associated with BMI which was an expected association, as individuals who report being active, are more likely to have a lower body weight.

The characteristic features of dyslipidemia in humans with diabetes are high TG and LDL-C concentrations and low HDL-C concentrations, whereas TC levels are not typically increased in patients with diabetes. We conducted lipid analysis to determine the correlation to the fasting blood sugar. The fasting blood sugar was significantly correlated with LDL-C and TC concentrations.

Hypertension affects 60% of diabetic persons. High blood pressure often increases the severity of diabetic complications such as diabetic retinopathy and kidney disease. Having diabetes increases your risk of developing high blood pressure and other cardiovascular problems, because diabetes adversely affects the arteries, predisposing individuals to atherosclerosis, it can lead to high blood pressure, which if not treated, can lead to blood vessel damage, stroke, heart failure, heart attack, or kidney failure. In this study sample, 41% of participants were considered to be pre-hypertensive or have hypertension. Blood pressure was significantly correlated to other measurements such as age, BMI and average weekly physical



activity. However, in this study sample, blood pressure and fasting blood sugar were not found to be strongly correlated.

The second part of the study was to conduct a non-targeted metabolomic analysis to determine if there were differences in the metabolic profile and if so, what risk factors were having the greatest impact on the metabolic signatures. The results showed that although this group did not have a clinical diagnosis of diabetes, MVDA of  $^1\text{H}$  NMR data showed that the greatest degree of differentiation was based on a BMI  $>27$  kg/m $^2$ . The PCA scores plot showed significant separation between the groups based on BMI. Each score or observation in the score plot represents each individual urine  $^1\text{H}$  NMR data signifying that these groups were different (PCA score plot) because of differences in the metabolite concentrations. The PLS technique was used to assess correlation between the observed  $^1\text{H}$  NMR data and other risk factors such as age. Our findings indicated that in the non-diabetic population being studied, differences in the inter-individual urinary profiles were dominated by differences in the BMI. The PLS correlation plot shows a good correlation of the urinary profiles between BMI and blood sugar in a non-diabetic population. Based on current clinical practices, this may be a relevant model for prediction of risk for diabetes because BMI is a reliable and convenient measure and is currently recommended as part of assessing diabetes risk. The correlation plots also demonstrated a good correlation between the urinary metabolic profiles related to obesity also with triglycerides and the lipid profile which included all of the lipid measurements.

$^1\text{H}$  NMR proved to differentiate between the urinary metabolic profiles of individuals who are overweight with a BMI  $> 27$  kg/m $^2$ . To our knowledge this is the first example of  $^1\text{H}$  NMR showing a strong separation between metabolic profiles based on a BMI of 27 kg/m $^2$ .

Obesity showed the greatest degree of discrimination between the urinary profiles compared to other risk factors.

It is understood, that the metabolite profile differs in lean individuals compared to obese individuals; however, our study showed that metabolite profiles show differentiation when individuals are in overweight classification (BMI 25-29.9 kg/m<sup>2</sup>) with a BMI >27 kg/m<sup>2</sup>.

BMI is a highly reliable and convenient measure used to screen for obesity. The effect of obesity on the metabolic state; however, is still considered in its infancy. Obese individuals typically have a greater degree of adipose tissue. This tissue releases bioactive molecules that influence body weight, insulin sensitivity, blood pressure, circulating lipids and inflammation which leads to metabolic diseases [73].

Diabetes can lead to pathological concentrations of several metabolites that can be detected in urine. Analysis of <sup>1</sup>H NMR in urine has identified a number of significantly changing metabolites such as high lactate citrate, glycine, alanine, hippurate, trimethylamine-N-oxide, dimethylaminecreatinine, acetate, betaine and ketone bodies in diabetes [74]. Comparison between human and animal models (rat and mice) have shown similarities in the levels of glucose, TCA cycle intermediate, polyols, amines and amino acids [48]. Studies have shown a separation between T2DM patients compared with that of healthy subjects and identified the metabolites contributing to the separation. For example, a study analyzed the differences in the metabolite profile between healthy and non-medicated diabetic individuals who maintained good daily blood glucose control by following ADA dietary guidelines. Metabolites that caused significant separation between the groups included amino acids; methylamines; fatty acids and nucleotides. This study highlighted the metabolic pathways that were affected which included:

methylamine metabolism; TCA cycle; fatty acid metabolism; amino acid metabolism; and nucleotide metabolism [74].

As shown in our study, a BMI  $>27$  kg/m<sup>2</sup> was the risk factor that caused the greatest separation. There are several recent studies that have analyzed how obesity is affecting the metabolite profile of humans. For example, one study analyzed differences between obese insulin resistance and sensitive participants [75], another study compared the metabolome between lean and diabetic individuals [62] and recently a comparison was conducted between healthy lean and healthy obese individuals [55]. The benefit of the latter study, comparing healthy lean versus healthy obese was that the risks factors that co-exist with obesity were controlled so that this was a direct comparison between obesity biomarkers. The study revealed novel and confirmed previously discovered metabolites that are markers for body fat mass changes.

There are some challenges when utilizing metabolomics to study human population. Firstly, unlike animal models which are in controlled laboratory conditions, human samples display greater heterogeneity in their metabolic make-up. Factors such as genetics, environment and lifestyle factors such as diet, physical activity levels, smoking status and intake of vitamins and medications contribute to the heterogeneous nature when investigating human metabolite profiles. Other limitations to this study are that a small sample limits generalizability of the findings and the inability to conclude causality between the variables due to the use of a correlational design. Despite these challenges and in a limited sample, metabolomics analysis was able to differentiate between the metabolic profiles of these subjects. This project supports the growing body of evidence that metabolomics using NMR spectroscopy is a sensitive measurement to identify altered metabolite chemistry based on a person's BMI. NMR analysis

has its advantages in studying the metabolite profile as this tool offers a high through put method at relatively low costs for high concentration biomarkers using non-invasive biofluids such as urine. In a review of  $^1\text{H}$  NMR based metabolomics for investigating diabetes it concluded that: “NMR spectroscopy is a reproducible and reliable means of measuring and understanding the metabolic status of pathologies such as diabetes.” In future, this may be tool used to identify and monitor people at risk for diabetes.

The ultimate goal of metabolomics is to define the metabolic signature of disease, versus normality and use those signatures to gain insight into how the disease came to be and how to treat. Further insight is needed to identify risk factors which have the greatest impact on the metabolic signature and how these risk factors affect the biomarkers.

### **Future Directions**

The next step is to identify and quantify the metabolites in chenomx. Further application would be required to determine how obesity affects the chemistry of these metabolites. Validation and estimation of these metabolites in the urinary profile would provide important information about the progression of diabetes. The primary goal of this project was to obtain preliminary findings to examine differences in the metabolomics profile of individuals with varying level of risk for development of the diabetes. This study was considered a pilot for a future project and was not designed to provide a high degree precision. However, this project does provide a greater insight that metabolomics is a sensitive tool that is able to differentiate between the metabolic profiles based on BMI, thus making it important to use the project characteristics for a larger trial.

## APPENDIX A

### Research Informed Consent

Title of Study: Use of Metabolomics to Investigate biomarker profiles as early risk factors for development of T2DM/metabolic syndrome

Principal Investigator (PI): Dr. Smiti Gupta, PhD  
Department of Nutrition and Food Science  
3009 Science Hall, Wayne State University  
Detroit, MI 48202  
(313) 577 2344

When we say “you” in this consent form, we mean you; “we” means the doctors and other staff.

#### Purpose

You are being asked to be in a research study that measures patterns in the markers for diseases such as type II diabetes mellitus. You are eligible to take part in this study, based on your age, health conditions and other health factors that are needed from participants. This study is being conducted at Wayne State University in collaboration with the Chatham-Kent Public Health Unit. The estimated number of study participants to be enrolled is about 50 people throughout the Chatham-Kent area.

**Please read this form and ask any questions you may have before agreeing to be in the study.**

In this research study, the goal is to identify metabolites, which are in the body as a product of metabolism. Metabolites can be used to predict the progression of diseases such as type II diabetes mellitus. From the blood and urine samples, a technique will be used to analyze the metabolites to see if there are differences in these biomarkers for diseases. Using the analytical technique, Nuclear Magnetic Resonance, we hope to discover similarities between participants based on their risk factors for developing type II diabetes.

#### Study Procedures

If you agree to take part in this research study, you will be asked to visit the Chatham-Kent Clinic Services for one 60-minute clinic visit. You will be asked to complete a questionnaire with the research assistant and provide a blood and urine sample. The blood will be drawn and blood pressure taken by a qualified Registered Nurse at the Chatham-Kent Public Health Clinic Services. The study will collect 10 ml urine and 8 ml of blood. Other physical measures that will be collected are: height, weight and hip circumference. The questionnaire will also require that you provide information on medical history, dietary intake.

The blood and urine samples will be analyzed at Wayne State University Chemistry Department using the technique, Nuclear Magnetic Resonance spectroscopy.

**Benefits**

As a participant in this research study, there may/will be no direct benefit for you; however, information from this study may benefit other people with similar health issues now or in the future.

**Risks**

Blood samples will be obtained from your veins. Possible side effects of obtaining blood samples are pain, bruising, bleeding, or infection at the blood draw site. Occasionally nausea, lightheadedness or fainting may occur.

There may also be risks involved from taking part in this study that are not known to researchers at this time.

**Study Costs**

Participation in this study will be of no cost to you.

**Compensation**

For taking part in this research study, you will be paid for your time and inconvenience by receiving a \$20 Gift Certificate to the local grocery store.

**Research Related Injuries**

In the event that this research related activity results in an injury, treatment will be made available including first aid, emergency treatment, and follow-up care as needed. Cost for such care will be billed in the ordinary manner to you or your insurance company. Wayne State University or Chatham-Kent Public Health Clinic Services offers no reimbursement, compensation, or free medical care. If you think that you have suffered a research related injury, contact the PI right away at (313) 577 2344.

**Confidentiality**

All information collected about you during the course of this study will be kept confidential to the extent permitted by law. You will be identified in the research records by a code name or number. Information that identifies you personally will not be released without your written permission. However, the study sponsor, the Human Investigation Committee (HIC) at Wayne State University, or federal agencies with appropriate regulatory oversight [e.g., Food and Drug Administration (FDA), Office for Human Research Protections (OHRP), Office of Civil Rights (OCR), etc.] may review your records.

When the results of this research are published or discussed in conferences, no information will be included that would reveal your identity.

### **Voluntary Participation/Withdrawal**

Taking part in this study is voluntary. You have the right to choose not to take part in this study.

### **Questions**

If you have any questions about this study now or in the future, you may contact Dr. Smiti Gupta or one of her research team members at the following phone number (313) 577 2344. If you have questions or concerns about your rights as a research participant, the Chair of the Human Investigation Committee can be contacted at (313) 577-1628. If you are unable to contact the research staff, or if you want to talk to someone other than the research staff, you may also call (313) 577-1628 to ask questions or voice concerns or complaints.

### **Consent to Participate in a Research Study**

To voluntarily agree to take part in this study, you must sign on the line below. If you choose to take part in this study you may withdraw at any time. You are not giving up any of your legal rights by signing this form. Your signature below indicates that you have read, or had read to you, this entire consent form, including the risks and benefits, and have had all of your questions answered. You will be given a copy of this consent form.

\_\_\_\_\_  
Signature of participant

\_\_\_\_\_  
Date

\_\_\_\_\_  
Printed name of participant

\_\_\_\_\_  
Time

\_\_\_\_\_  
Signature of person obtaining consent

\_\_\_\_\_  
Date

\_\_\_\_\_  
Printed name of person obtaining consent

\_\_\_\_\_  
Time

Research Informed Consent





## APPENDIX C

Study #: \_\_\_\_\_

Gender: M / F

Date of Birth: \_\_\_\_\_  
M/D/Y

Ethnicity: \_\_\_\_\_

## HEALTH RISK FACTORS

Do you smoke? Y  N  Have you smoked in the past 12 months? Y  N 

If yes, how many cigarettes per day? \_\_\_\_\_

Please indicate if you are exposed to second hand smoke ? Y  N How often do you participate in vigorous physical activity for 30-60 minutes?  
(Vigorous activities include aerobics, fast cycling, walking, running, fast swimming or moving heavy objects)5-6 times/week  3-4 times/week  1-2 times/week  0-3 times/month How often are you physically active for more than 30-60 minutes of moderate activity?  
(Moderate activities include brisk walking, cycling, swimming, golfing and heavy gardening)5-6 times/week  3-4 times/week  1-2 times/week  0-3 times/month 

Is there anything that prevents you from being active? \_\_\_\_\_

On average, how many servings of fruits and vegetables do you typical eat everyday?  
(1/2 cup of fresh or frozen, 1 cup leafy vegetables)0-1 servings  2-4 servings  5-7 servings  8 or more servings How many alcoholic drinks do you consume in a typical week?  
(The serving size for 1 drink is as follows: a regular 12 oz beer; 1.5 oz of hard liquor; a 5 oz glass of wine; a regular 12 oz cooler)None  1-2 drinks  3-6 drinks  7-10 drinks  10 + drinks 

## MEDICAL HISTORY

Heart disease  Cancer  Diabetes  Stroke  Lung Disease   
(family history)

Other: \_\_\_\_\_

Current medications? If yes, please list:

Allergies? If yes, please list:

**ANTHROPOMETRIC DATA**

Weight \_\_\_\_\_ Height \_\_\_\_\_ BMI \_\_\_\_\_

Waist Circumference \_\_\_\_\_ Hip Circumference \_\_\_\_\_ Waist/Hip Ratio \_\_\_\_\_

Systolic blood pressure (mmHg) \_\_\_\_\_

**BIOCHEMICAL DATA**

Fasting Blood Glucose \_\_\_\_\_ HDL \_\_\_\_\_ LDL \_\_\_\_\_ Total Cholesterol \_\_\_\_\_

Triglycerides \_\_\_\_\_

<b>Food Group</b>	<b>Breakfast</b>	<b>AM</b>	<b>Lunch</b>	<b>PM</b>	<b>Dinner</b>	<b>HS</b>	<b>TOTAL</b>
<b>Fruit and Vegetables</b>							
<b>Grains Refined/Whole</b>							
<b>Dairy and Alternatives</b>							
<b>Meat and Alternatives</b>							
<b>Other</b>							

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**ABSTRACT****THE USE OF METABOLOMICS TO INVESTIGATE BIOMARKERS PROFILES AS POTENTIAL EARLY RISK FACTORS FOR DEVELOPMENT OF TYPE II DIABETES**

by

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Type 2 Diabetes affects an estimated 17.5 million individuals in the United States and is considered to be one of the most pressing public health issues of our time. To date, the exact cause of type 2 diabetes remains unclear, however, it is considered to be an interplay between environmental and genetic factors. Emerging technologies for metabolomics analysis increases the capacity to detect the onset of disease or ideally, the pre-diseased state. Metabolomics has the potential to find early metabolic changes related to diabetes progression prior to many clinical symptoms. Although this technology is considered to be in its infancy many believe that metabolomics strategies can have an impact on the discovery of pathological biomarkers for diabetes progression. Given this emerging technology that is available, the increasing burden of diabetes, earlier identification of ‘at risk’ individuals is particularly important.

The objective of this study was to determine if metabolomics analytical techniques could identify differences in the metabolic profiles of persons at risk for developing diabetes. A heterogeneous non-diabetic sample of persons with and without risk factors for development of diabetes were recruited for this pilot study. A standard questionnaire was conducted to assess

risk factors for diabetes. Fasting blood and urine samples were collected and frozen at  $-80^{\circ}\text{C}$ . Bivariate correlations were determined to investigate the linear relationship between the risk factors. Urinary metabolite profiles were analyzed by proton nuclear magnetic resonance ( $^1\text{H}$  NMR) spectra. The processed, digitized NMR spectral data was analyzed using multivariate data analysis software, SIMCA P+. Partial Least Squares Discriminant Analysis (PLS-DA) Score Plot showed a clear separation between the urinary metabolomic profiles of subject based on a BMI  $\geq 27\text{ kg/m}^2$ . PLS correlation plot showed a significant correlation between the urinary profiles between BMI and fasting blood sugar in this non-diabetic population. The data suggests and in agreement with the hypothesis, that  $^1\text{H}$  NMR was able to detect changes in the urinary profiles of a non-heterogeneous non-diabetic population with the greatest degree of discrimination based on BMI of  $27\text{ kg/m}^2$ . Based on current clinical practices, the identification of the metabolites causing discriminating in the urinary profiles based on obesity may be a relevant focus for predicting risk in a non-diabetic population.

This study was considered a pilot for a future project and was not designed to provide a high degree of precision. However, this project does provide greater insight that metabolomics is a sensitive tool that is able to differentiate between the metabolic profiles of human urinary profiles based on BMI, thus making it important to use the project characteristics for a larger trial.

**AUTOBIOGRAPHICAL STATEMENT**

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The author received a Bachelors in Science- Honors undergraduate degree in Food and Nutrition at the University of Western Ontario. In 2006, she completed a Dietetic Internship at the London Health Sciences Centre. Currently she is working as a Registered Dietitian at the Chatham-Kent Public Health Unit. Jennelle Arnew is continuing her academic studies in nutrition towards accomplishment of a Masters degree in Nutrition and Food Science at Wayne State University.