CHAPTER 2

THE ROLE OF INCREASED GASTROINTESTINAL ALCOHOL PRODUCTION IN PATIENTS WITH THE METABOLIC SYNDROME

2.1 AIMS OF THE STUDY

It has been shown that small increases in gut derived alcohol might increase portal blood alcohol levels which are enough to induce hepatic steatosis. Animal studies have demonstrated the presence of increased gut alcohol production by intestinal bacteria which has probably lead to the pathogenesis of NAFLD.

The main aim of this study was to evaluate patients with the metabolic syndrome who had clinical features of non-alcoholic fatty liver disease (NAFLD) for the presence of increased intestinal production of alcohol. The study was done in an attempt to increase our level of understanding of the aetiology and pathogenesis of NALFD, and thereby possibly identify new approaches to the management of the disease.

2.2 SUBJECTS UNDER STUDY

The study group included 20 patients attending both state and private

Endocrine and Liver clinics at the Johannesburg Hospital. The control group included 20 healthy hospital staff members or their relatives. Informed consent was obtained from every subject before enrollment into the study. Ethics Committee clearance was obtained from the University of the Witwatersrand (M03-09-25, see Appendices).

Inclusion criteria included:

1. **NCEP:ATP III criteria for the diagnosis of the metabolic syndrome.**
2. No history of alcohol use.
3. Deranged liver function tests.
4. The presence of fatty liver disease on abdominal ultrasound.

The inclusion criteria for the control group were:

1. **Absence of the metabolic syndrome as defined by the NCEP: ATP III criteria.**
2. No history of alcohol use/ abuse.
3. Normal liver function tests.
4. No history of medical illness.

Fasting blood, breath, and urine samples were obtained from the study group and the control group to assess the levels of alcohol in blood, breath and urine.

2.3 DATA COLLECTION

2.3.1 Data

Data were collected from patients' records. This included details of risk factors for NAFLD such as type 2 diabetes, hypertension and dyslipidaemia. Diabetes mellitus was defined as a fasting blood glucose >/= to 5.6 mmol/L or a previous diagnosis of diabetes. Hypertension was defined as a blood pressure of >/= to 130/85 or on antihypertensive therapy. Dyslipidaemia was defined as a fasting total-cholesterol of > 5 mmol/l, a triglyceride of > 1.5 mmol/l, a HDL-cholesterol of < 1.2 mmol/l and/or a LDL-cholesterol of > 3 mmol/l, or was assumed to be present if the subject was on lipid modifying therapy (Appendix 9).

Details of current and previous drug as well as supplement medication was also taken.

They were all physically examined, and anthropometric measurements

included weight, height and waist circumference. BMI was calculated using weight in kilograms divided by height in metres squared. All subjects were also examined for complications of their medical problems. Blood tests included the total cholesterol, HDL-C, TG, calculated LDL-C, HbA1C levels, fasting glucose levels, AST as well as ALT levels.

Ultrasound scanning of the liver was also performed to assess the degree of steatosis. Healthy control subjects were age, sex and race matched. Blood samples were obtained for liver enzymes, a fasting lipogram and a fasting glucose. Both the study and control subjects were screened to exclude other causes of chronic liver disease. This included screening for Hepatitis B and C infection.

2.4 SPECIMEN ANALYSIS

2.4.1 Alcohol Analysis

Blood, urine and breath samples were collected and analysed for alcohol levels. This was performed using Gas Chromatography (GC), which has been accepted as a worldwide standard for forensically estimating blood alcohol concentrations.

The columns used for alcohol GC analyses were

mainly supplied by Sigma-Aldrich being one of them. Their recommendations (Sigma-Aldrich catalogue, 2004/2005, page 318) were adapted to suit the experimental procedures and sensitivity required. Sources of error were greatly reduced by limiting the number of steps in the protocol for the sample procedure. For this reason the direct injection method was used.[62]

Most samples were analysed on the same day. If not possible, the samples were stored at –20ºC and thawed slowly at ambient room temperature and mixed well before analysis. In order to verify the stability of these preserved samples, the samples that were analysed on the day of collection were aliquoted and frozen. After 30 days, these samples were thawed to room temperature and the alcohol concentration estimated by GC. This was a standard practice in the estimation of volatile substances such as alcohol.

Samples were measured using a Hewlett Packard 5890A GC, Spectra Physics 4820 Integrator, Supelco Carbowax Capillary column (30 metre x 0.32 mm ID), Hamilton 5 µL syringe, Amican Ultra- MC centrifugal filter.

Laboratory pure ethanol, methanol and n-Propanol standards were used for calibration.The carbowax column was inserted into the GC as per the

manufacturer’s instructions.

Nitrogen was used as the carrier gas and set to a flow rate of approximately 1.0mL/min. The oven was controlled at 45ºC. Stock standard solutions of methanol, ethanol and n-propanol were made to 2% in distilled water.

A 190µL aliquot was placed in a Millipore filter with a cut-off of 10kD and 10µL of the internal standard of 2% n-propanol was added. This was then centrifuged in a microfuge at 12rpm (xG) for 30 minutes.

Four microlitres of the filtrate was then injected into the GC and the integrator activated at the same time. Both methanol and ethanol were quantified by preparing a range of concentrations between 0.01%-0. 1% and including n-propanol at a fixed concentration of 0.1% for each standard.

The calculation of the ratios of the integrated area of each alcohol to the area of the 0.1% n-propanol gave a figure that could be plotted on a graph, the vertical y-axis being the ratio and the horizontal x-axis, the alcohol concentration. The graph thus depicted yielded a straight line indicating that the ratio of alcohol to n-propanol was linear, confirming a directly propotional relationship. Incorporation of the 0.1% n-propanol in

each unknown sample permitted the direct calculation of the alcohol being sought.

The carbowax column separated the alcohols presented according to their chain length, it therefore followed that methanol was the first to be eluted, this was followed by ethanol and then the n-propanol standard. Once the ratio to n-propanol to either the methanol or the ethanol standards was established, one was able to directly calculate the concentration of the alcohol eluted. Since methanol had a molecular mass of only 32, it became fairly difficult to establish its presence using mass spectrometry. However, by fitting a less polar capillary column (DB24) in the GC, the elution profile of the different alcohols did vary with time. This was definitive in identifying the alcohols eluted when compared with the known alcohol standards. Analytes that appeared to be methanol were identified and confirmed using this technique.

Of note, urine and breath samples were injected directly onto the GC column without preparation, however serum samples have a more complex matrix and the proteins present could form deposits on the capillary column in the GC thus affecting the efficiency of the column. It was therefore deemed necessary to remove most of the protein prior to

analysis by employing Millipore cut-off filters that excluded any molecules greater than 10kD. Alcohols have a molecular masses much lower than the cut-off and would therefore pass freely through the filter. This was verified by incorporating the propanol as the internal standard into the serum samples to be tested. The propanol from these serum samples gave results comparable to the aqueous propanol standard.

2.4.2 Analysis of Blood

Blood samples were collected from the study and control subjects for liver enzymes, a fasting lipogram and a fasting glucose. Hepatitis B and C serologies were also done.

Blood was assessed for adiponectin, leptin, NEFA, and hsCRP levels. Blood specimens were centrifuged and their supernatants (serum) were dispensed into 5ml labelled plastic tubes and stored at -70°C for batch analysis at a later stage.

Serum NEFA analysis was performed manually using the Half-micro test (Roche), an enzymatic colorimetric assay. Leptin analysis was performed using the Quantikine® Human Leptin Immmunoassay (R&D systems) while

Adiponectin analysis was done using the Quantikine® Adiponectin Immunoassay (R&D systems). These assays employed the quantitative sandwich enzyme immunoassay technique.

Immunoturbidimetric analysis for the in vitro quantitative determination of hs-CRP in serum was carried out using the Tina-quant (Roche) C-reactive (latex) high sensitive assay.

All tests were performed following the manufacturer's instructions and the concentrations were calculated as per instructions. To avoid inter-assay variability, all assays were performed as single batch assays. All assays had an inter-assay variability of less than 5%.

2.5 STATISTICAL ANALYSIS

Data was analysed using the GB- Stat v4 program (Dynamic Microsystems, Inc. Silver Springs, MD 20904). Quantitative or continuous data were either expressed as the mean ± SD, with data that was normally distributed, or as median with a range for data that was non-parametric. Categorical variables which were expressed as percentages were evaluated using the Fisher's 2-tailed exact test. Continuous variables were analysed with the unpaired Student's t test. All reported P values were considered as significant at a level of $P< 0.05$.