Non-alcoholic fatty liver disease (NAFLD) is a term used to describe alcohol-like liver injury in the absence of alcohol abuse. It is being increasingly recognised worldwide as one of the commonest causes of chronic liver disease that may progress to end-stage liver disease.

The pathogenesis of NAFLD is poorly understood. A growing body of literature has documented its close relationship with the metabolic syndrome, which is characterised by abdominal obesity, insulin resistance with or without frank hyperglycaemia, dyslipidaemia, and hypertension.

The current pathogenetic hypothesis of NAFLD involves a ‘two-hit’ process in which an initial ‘first hit’ metabolic disturbance, namely insulin resistance, results in hepatic steatosis. This is followed by a ‘second hit’ involving increased mitochondrial β-oxidation of free fatty acids, resulting in increased free fatty acid availability and reactive oxygen species production, which promote inflammation and fibrosis.

**Aim.** Explore the possibility that increased gastrointestinal alcohol production may play a role in the pathogenesis of non-alcoholic fatty liver disease in patients with the metabolic syndrome.

**Methods.** Blood, urine and breath levels of alcohol measured in 20 patients with the metabolic syndrome were compared with those of 20 matched healthy controls.

**Results.** Eighty per cent of the patients had dyslipidaemia, 60% systemic hypertension and 70% type 2 diabetes mellitus. Seventy five per cent had ultrasonographic features of fatty liver disease, with mean serum aminotransferase activities being significantly higher in the patients than in the controls, alanine aminotransferase (ALT) 57.4±44.79 U/l versus 17.4±4.60 U/l (95% CI 18.02 - 61.42, p<0.001), and aspartate aminotransferase (AST) 52.5±36.21 U/l versus 23.4±4.86 U/l (95% CI 11.99 - 46.20, p<0.01). Adiponectin levels were lower (6 875 versus 15 475 ng/l; median value, p<0.01) in the patients with the metabolic syndrome and leptin levels significantly higher (13.56 versus 3.05 ng/l; median value, p<0.05). Alcohols were detected in body fluids of 60% of the patients, of which 35% tested positive for ethanol, 55% tested positive for methanol, and 30% tested positive for both. None of the controls tested positive for any alcohols.

**Conclusions.** Endogenous alcohol production may be involved in the pathogenesis of non-alcoholic fatty liver disease in patients with the metabolic syndrome.
in cellular oxidative stress due to the generation of reactive oxygen species. This in turn leads to lipid peroxidation which incites an inflammatory response and steatohepatitis.3

Abdominal obesity is associated with a decrease in gut motility, because of reduced sensitivity to neuropeptides such as cholecystokinin and bombesin.36 This may favour bacterial overgrowth and an increase in bacterial alcohol production. Therefore there is a possibility that obese individuals who do not consume alcohol are capable of endogenously producing and metabolising alcohol, and this may contribute to the pathogenesis of obesity-related fatty liver disease. It is even possible that small increases in gut-derived ethanol might chronically increase alcohol levels in the portal system to a sufficient extent to induce hepatic steatosis, given evidence that hepatic alcohol metabolism produces obligatory redox changes that promote the accumulation of triglycerides within hepatocytes.4

To the best of our knowledge, no studies in the English literature have been described regarding the role of increased gastrointestinal alcohol production in patients with the metabolic syndrome. The aim of this study was to examine patients with the clinical features of NAFLD for evidence of possible increased intestinal production of alcohol.

Patients, materials and methods

Patients

The study group consisted of 20 patients attending the endocrine and liver clinics at Johannesburg Hospital. Informed consent was obtained from each subject before enrollment into the study. Our study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in the approval obtained from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand.

Inclusion criteria were those of the National Cholesterol Education Panel: Adult Treatment Panel III (NCEP: ATP III) for the diagnosis of the metabolic syndrome. There had to be no history of alcohol use. The study group had to have deranged liver function tests and/or the presence of fatty liver disease on abdominal ultrasound. Twenty controls matched for age, gender and ethnic group were also studied. The inclusion criteria for the control group were absence of the metabolic syndrome as defined by the NCEP: ATP III criteria and no history of alcohol use. They had to have normal liver function tests (aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels <40 U/l) and no history of medical illness. Fasting blood, breath and urine samples were obtained from both the study group and the control group for measurements of the levels of alcohol in blood, breath and urine.

Diabetes mellitus was defined as a fasting blood glucose level of ≥7 mmol/l or if there was a previous diagnosis of diabetes. Hypertension was defined as an untreated blood pressure of ≥130/85 mmHg or if the patient was on antihypertensive therapy. Dyslipidaemia was defined as a fasting total cholesterol of >5 mmol/l, a triglyceride level of >1.7 mmol/l, and a high-density lipoprotein (HDL) cholesterol level of <1.2 mmol/l in women or <1.0 mmol/l in men and/or a low-density lipoprotein (LDL) cholesterol level of >3 mmol/l, or was assumed to be present if the patient was on lipid-modifying therapy.

Details of current and previous drugs the patients had taken to treat the above conditions, including antibiotics as well as supplemental medication, were noted. Patients were all physically examined, and anthropometric measurements which included weight, height, and waist circumference were recorded. The body mass index (BMI) was calculated using weight in kilograms divided by the square of the height in metres. All patients were examined for other target organ damage arising from their metabolic syndrome. Ultrasound scanning of the liver was performed to assess the presence and degree of steatosis. Serum leptin and adiponectin were included as surrogate markers for steatosis. Other causes of chronic liver disease were excluded.

Materials

Ethanol, methanol and n-propanol reagents of suprapure analytical grade were purchased from Merck Chemicals. Conical Eppendorff tubes (2 ml) for centrifuge work were also obtained from Merck Chemicals. Ten kD cut-off filters were purchased from Millipore. Enzyme-linked immunosorbent assay (ELISA) kits for adiponectin and human leptin were supplied by R&D systems. Roche Diagnostics supplied the colorimetric kit for measuring non-esterified free fatty acids (NEFA) and the automated latex kit for highly sensitive C-reactive protein (hsCRP). Two capillary gas chromatographic columns, 30 m x 0.32 mm internal diameter, one carbowax capillary column and one DB23 capillary column were purchased from Supelco and J & W respectively.

Sample collection

Following an overnight fast, each patient had two venous blood samples drawn: (i) 3 ml into a fluoride-oxalate tube for glucose analysis; and (ii) 10 ml into a sterile plain vacutainer. The latter sample was left to stand for approximately 1 hour to permit clot formation. Serum isolated from this sample was used for liver enzymes, hsCRP, a lipogram and serological tests for hepatitis B and C. Sera for analyses that...
were not carried out immediately were stored at -70°C.

Breath samples were collected by gently breathing through a straw for 60 seconds and bubbling the breath directly into 20 ml distilled water. Urine samples were collected at the same time that blood was drawn for glucose estimations. Both breath and urine samples were injected directly into the gas chromatograph without further treatment.

Gas chromatography
A Hewlett Packard 5890 gas chromatograph was fitted with either a carbowax or DB23 capillary column and used to determine the concentrations of the individual alcohols. The oven temperature was kept constant at 45°C. The temperatures of the injection port and flame ionisation detector were at 150°C and 250°C respectively. Pure nitrogen at a flow rate of 1 ml per minute was used as the carrier gas. Detection and area calculations of the signal tracings from the gas chromatograph were done on a Spectra Physics 4890 integrator.

Sample and standard preparation
Stock standards, 2% each of methanol, ethanol and n-propanol, were prepared in distilled water. A range of combined methanol and ethanol standards from 0.005% to 0.1% were prepared by diluting the stock solutions with distilled water to final volumes of 2 ml. An internal standard of 0.1% n-propanol was included in each standard.

Samples for alcohol estimations were prepared by pipetting 190 μl serum and 10 μl 2% n-propanol standard into a Millipore filter unit. This was then inserted into an Eppendorff tube and the unit centrifuged in a microfuge for 30 minutes at 12,000 rpm (~6,000 g). After centrifugation, the Millipore unit was removed from the tube and the collected filtrate injected directly into the gas chromatograph. Final results were corrected for the dilution of the sample using the factor of 200/190.

Standard curves
Half a microlitre of the combined aqueous alcohol standards were each injected directly into the gas chromatograph and the integrator activated at the same time. The ratio of the methanol area to propanol area as summed by the integrator was used to calculate a factor for each standard concentration. The inclusion of the ratios of the standards using the propanol as an internal standard negated any possible variation that may occur in the sample injection technique. Ratios for ethanol were calculated in a similar way. To verify the linearity of the technique, a graph was plotted of concentration (%) against the ratios as shown in Fig. 1 (a and b). The alcohol concentration in all the serum samples was calculated using this ratio technique.

Retention times of a solute are uniquely affected by the stationary phase employed in a chromatographic column. Since methanol is not commonly found in serum samples, verification of its presence had to be confirmed. To verify that the alcohols present in the sera were indeed methanol and ethanol, two gas chromatograph capillary columns, each with a stationary phase of different polarity, was employed. A standard mixture of methanol, ethanol and propanol displayed consistent retention times for each alcohol when employing the Carbowax column, but the retention times for each differed from the Carbowax column when using the DB23 column. The comparative difference of the retention times in the two columns of each alcohol in spiked serum samples confirmed the presence of both methanol and ethanol.

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

The study group and the control group were comparable in terms of age, gender and ethnicity (Table I). All the study patients were already on treatment for their medical conditions. Although 45% of the control group were found to have dyslipidaemia, this was mild in all cases (total cholesterol <7.5 mmol/l) and none was on lipid modifying treatment. There was a significant difference between the two groups with regard to waist circumference and body mass index (p<0.0001).

HDL cholesterol levels were significantly lower and NEFA concentrations significantly higher in the study group than in the control group (Table II). Total cholesterol and triglyceride levels were also higher in the study group, but this was not statistically significant. The differences between the patient and control groups’ serum aminotransferase activities were significant, with the study group’s ALT levels being 57.4±44.79 as opposed to 17.4±6.6 U/l in the control group (95% CI 18.02 - 61.42, p<0.001) and the study group’s AST levels being 52.5±36.21 versus 23.4±8.6 U/l (95% CI 11.99 - 46.20, p<0.01). There was a significant difference between the two groups with regard to adiponectin and leptin levels, the adiponectin levels being significantly lower and the leptin levels significantly higher in the study group than in the controls. The hsCRP levels were significantly higher in the patients than in the controls (Table II). Seventy-five per cent of the patients with the metabolic syndrome had increased hepatic echogenicity on ultrasonographic examination, in keeping with fatty liver disease. Five of them had had liver biopsies confirming the diagnosis of NAFLD. These were done before their inclusion into the study, as liver biopsy was not part of the study protocol.

Of the 20 study patients with the metabolic syndrome screened for alcohol in body fluids, 60% tested positive. Fifty-five per cent tested positive for methanol and 35% positive for ethanol (Table III). Of note, no alcohol was detected in any of the control subjects. The methanol concentration was highest in the blood and significantly higher when compared with that in the urine and breath (Table IV).

Discussion

NAFLD is considered to represent the hepatic expression of the metabolic syndrome. The study examined the possibility that as part of the ‘second-hit’ theory, endogenous production of alcohol may contribute to the pathogenesis of NAFLD in patients with the metabolic syndrome. The fact that alcohol was detected in blood, urine and breath is important evidence of its role in the development of NAFLD. This was in keeping with previous studies on obese animals and humans.6,9

A significantly higher percentage of patients had detectable levels of methanol than ethanol, 55% compared with 35%. Alcohol was not detected in the controls, which was in keeping with their reported non-consumption of alcohol. Of note, no ethanol was detected in the breath of the study subjects, which

Table I. Characteristics of the study population

| Characteristics          | Study group (N=20) | Control group (N=20) | p-value |
|--------------------------|--------------------|----------------------|---------|
| Gender (M/F)             | 8/12               | 8/12                 | ND      |
| Age (yrs) (mean ± SD)    | 48±15.3            | 48±16.2              | 0.968   |
| Waist circumference (cm) | 111±14.9           | 82±8.8               | 0.0001  |
| Body mass index (kg/m²)  | 35.1±8.2           | 23.3±2.1             | <0.0001 |
| Hypertension             | 12 (60%)           | 0(0%)                | <0.01   |
| Dyslipidaemia            | 16 (80%)           | 9 (45%)              | <0.01   |
| Diabetes/impaired fasting glucose (IFG) | 14 (70%) | 0(0%) | <0.01 |

ND = not done.

Table II. Biochemical results

| Biochemistry          | Study group (N=20) | Control group (N=20) | p-value |
|-----------------------|--------------------|----------------------|---------|
| Total cholesterol (mmol/l) (mean ± SD) | 5.71±3.25 | 5.27±1.10 | 0.587   |
| Triglycerides (mmol/l) (mean ± SD)       | 7.16±18.50      | 1.11±0.58 | 0.16    |
| HDL (mmol/l) (mean ± SD) Fairly Low Cholesterol Levels | 1.23±0.36 | 1.58±0.42 | <0.01   |
| NEFA (mmol/l) (mean ± SD)                | 86±5±6.46       | 432±9±16 | <0.0001 |
| ALT (U/l) (mean ± SD)                    | 57.4±44.79      | 17.7±4±60 | <0.001  |
| AST (U/l) (mean ± SD)                    | 52.5±36.21      | 23.4±4±86 | <0.01   |
| hsCRP (mg/l) (median, range)             | 6.46 (0.36 - 141.8) | 0.86 (0.17 - 16) | <0.01   |
| Adiponectin (ng/l) (median, range)       | 6 875 (1 090 - 29 600) | 15 475 (4 750 - 95 200) | <0.01   |
| Leptin (ng/l)                           | 13.56 (0 - 78.1) | 3.05 (0 - 35.3) | <0.006  |
is not consistent with previous studies; this may be explained by the fact that the intestinally derived ethanol indeed underwent hepatic metabolism, and could not reach the lungs. Methanol was detected in all body fluids measured. The methanol concentration was highest in blood and significantly higher when compared with that in urine and breath. Methanol is oxidised in the human liver by alcohol dehydrogenase to formaldehyde and formic acid. The rate of metabolism of methanol is much slower than that of ethanol and is independent of the concentrations in blood. The slow degradation of methanol in the blood could possibly account for its presence in blood, urine and breath. Formic acid is responsible for the toxic effects of methanol.

Although methanol is not normally found in serum, it is derived from the diet, sourced principally from vegetables, fresh fruits and fruit juices that contain pectin. After consuming fruit, the concentration of methanol in the human body increases because of the degradation of natural pectin, which is esterified to methyl alcohol in the human gut. Other sources include diet drinks, in which the artificial sweetener aspartame is widely used; when hydrolysed this is partly converted to methanol. The patient volunteers had the metabolic syndrome and consumed diet drinks and fruits for the purpose of weight control.

Intestinal bacterial overgrowth has been implicated in increased production of endogenous alcohol. Alterations in gut motility associated with obesity, and complications associated with diabetes such as autonomic neuropathy, can be responsible for an increase in the intestinal bacterial overgrowth. In our study, alcohol concentrations were no higher in the diabetics than in the non-diabetics with NAFLD. This may be because of the small sample size.

The level of hepatic exposure to intestinally derived endogenous alcohols is best determined by portal venous sampling to avoid the influence of the first-pass effect, but this would be invasive and impractical. The metabolism of alcohols is likely to increase hepatocyte oxidant stress and could conceivable be an important risk factor in determining the eventual development of non-alcoholic steatohepatitis. Whether this risk is greater for ethanol, methanol or the combination is currently not known.

NAFLD is usually suspected in persons with asymptomatic elevation of aminotransferase levels, radiological findings of fatty liver, or unexplained persistent hepatomegaly. Even though only 75% of our patients had ultrasonographic features of NAFLD, and only 5% of them had biopsy-proven diagnoses, they all had elevated levels of transaminases and adipokines in keeping with the diagnoses.

| Table III. | Alcohol measurements |
|-----------|----------------------|
| Alcohol present | Study group (N=20) | Control group (N=20) | p-value* |
| Ethanol  | 7 (35%) | 0 (0%) | 0.0084 |
| Methanol | 11 (55%) | 0 (0%) | 0.0000 |
| Ethanol and methanol | 6 (30%) | 0 (0%) | 0.0022 |
| Any alcohol present | 12 (60%) | 0 (0%) | 0.0000 |
| *2-tailed Fisher’s exact test. |

| Table IV. | Study subjects with detectable alcohol levels |
|-----------|-----------------------------------------------|
| Patient No. | Ethanol (mg/dl) | Methanol (mg/dl) |
|           | Blood | Urine | Breath | Blood | Urine | Breath |
| 3        | 9     | ND    | ND     | 2     | 1.6   | 2.4    |
| 4        | 2     | ND    | ND     | 1.8   | ND    | ND     |
| 6        | ND    | ND    | ND     | 50    | ND    | ND     |
| 7        | 8     | 44.6  | ND     | 29    | 32.3  | ND     |
| 8        | 8     | ND    | ND     | 2     | ND    | 8      |
| 9        | 10    | ND    | ND     | ND    | ND    | ND     |
| 10       | 10    | ND    | ND     | 21    | ND    | 8      |
| 11       | ND    | ND    | ND     | 7.1   | ND    | ND     |
| 12       | ND    | ND    | ND     | 3     | ND    | ND     |
| 13       | 3     | ND    | ND     | 9     | 3.8   | 1      |
| 14       | ND    | ND    | ND     | 7     | ND    | ND     |
| 15       | ND    | ND    | ND     | 46    | 9.7   | 5.3    |
| Mean ± SD | 7.1± 3.71± 0 | 16.17± 6.8± 2.05±  |
|           | 3.28  | 12.87 | 17.95  | 13.58 | 3.19  |

ND = not detected.
Generally, histological examination of a liver biopsy specimen is the gold standard. While the typical features on liver biopsy are well described, given the anticipated high numbers of patients with NAFLD in the context of a high prevalence of both obesity and diabetes, it could be argued that an enormous number of patients would be likely to warrant a biopsy. However, a liver biopsy may be considered when other hepatic abnormalities need to be excluded, and it may also be helpful in ascertaining the extent of inflammation so as to distinguish simple steatosis from non-alcoholic steatohepatitis (NASH), which may have important prognostic implications. A biopsy is also expensive, invasive and associated with the risk of complications. It has therefore been argued that although there are potential new treatments, the only effective therapy at present for the obese patient is weight loss, and it would be needless to subject them to a liver biopsy. There is also the opportunity for sampling errors. Histological lesions of NASH are unevenly distributed throughout the liver parenchyma, so a sampling error when performing a liver biopsy can result in substantial misdiagnosis and staging inaccuracies. Each case therefore has to be assessed individually. Measurements of the serum concentration of adipocytokines have recently been used to distinguish bland steatosis from NASH, suggesting that liver biopsies may become unnecessary in the majority of patients with suspected NAFLD. Studies have found that serum levels of leptin and resistin were significantly higher in patients with NASH than in those with simple steatosis, whereas serum levels of adiponectin were significantly lower, suggesting that these adipocytokines may play a role in the pathogenesis of NAFLD. Leptin is a mediator of hepatic inflammation and fibrosis in NAFLD, and resistin and adiponectin are important modulators of insulin resistance, a central factor in the pathogenesis of NASH. In this study, serum adipocytokines were used as surrogate markers in addition to other markers.

Our patients also had significantly higher levels of hs-CRP than the control group, in keeping with recent studies confirming that the metabolic syndrome is an inflammatory condition. Therefore, it is of paramount importance to also control an individual's obesity as well as the accompanying diabetes, dyslipidaemia or hypertension. Multiple interventions have been advocated with variable success results. Strategies advocated include lifestyle interventions such as diet and exercise, the use of dyslipidaemia therapy and insulin-sensitising drugs. Other interventions include the use of cytoprotective agents and antioxidants. These include ursodeoxycholic acid, vitamin E, lecgin, B carotene, selenium, S-adenosyl-methionine, metadoxine, silimarín and lipid-lowering agents. Recent trials of glucose-lowering agents such as metformin and thiazolidinediones have shown promising results leading to metabolic and histological...
improvement in subjects with non-alcoholic steatohepatitis.\textsuperscript{24}

Because bacterial overgrowth has been implicated in NAFLD, oral antibiotics such as metronidazole may be efficacious in reverting steatosis, and in some cases inflammation and fibrosis.\textsuperscript{25,26} Doses may have to be reduced in severe liver disease. However, side-effects such as gastrointestinal intolerance and an unpleasant taste may affect long-term compliance. Prolonged use of metronidazole may also cause a reversible peripheral neuropathy, and it is contraindicated in the first trimester of pregnancy. Probiotics may be a better alternative.\textsuperscript{27,28} They may interfere with the development of NAFLD by decreasing proinflammatory cytokines, altering the inflammatory effects of pathogenic strains of intestinal bacteria, through changes in cytokine signalling, and by improving epithelial barrier function, thereby avoiding excessive exposure of the liver to cytokines and bacterial alcohol. They have shown promising results, and have also been shown to improve liver enzymes in recent studies.\textsuperscript{27,28}

Our study patient sample size was small, and further studies with larger patient numbers are required to confirm these results. The fact that S controls had high cholesterol levels may also have affected our statistical outcomes.

In conclusion, this study therefore suggests that endogenous alcohol production may indeed be responsible for the ‘second hit’ and may well be involved in the pathogenesis of NAFLD.

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