Obesity, adipocyte hypertrophy, fasting glucose, and resistin are potential contributors to nonalcoholic fatty liver disease in South Asian women

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Purpose: Nonalcoholic fatty liver disease (NAFLD) is often referred to as the hepatic manifestation of the metabolic syndrome. The relationship between body weight, NAFLD, and insulin resistance is not well characterized in humans. Additionally, it is unclear why South Asians develop these complications at lower levels of obesity compared to their Western counterparts.

Patients and methods: To address this question, we performed a cross-sectional study using a convenience sample of Sri Lankan adult females (n=34) and collected anthropometric data, adipose tissue specimens (for histology), and fasted serum samples (for metabolic and inflammatory markers). Hepatic steatosis was assessed by ultrasound scanning and used to classify participants as NAFL 0, NAFL 1, and NAFL 2.

Results: Waist circumference significantly increased with increasing NAFL grade. Participants with NAFL had significantly higher body mass index, hip circumference, and fasting plasma glucose, as well as a higher mean adipocyte area in both abdominal subcutaneous and visceral areas, indicating a higher degree of adipocyte hypertrophy associated with fatty liver. There were, however, no differences in measures of dyslipidemia. Of the multiple adipokines measured, resistin was the only proinflammatory adipokine significantly elevated in NAFL 2.

Conclusion: These findings indicate that measures of adiposity, fasting serum glucose, and resistin may be important indicators of NAFLD in South Asian women.

Keywords: adipokines, inflammation, metabolic syndrome

Introduction
Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease worldwide and is increasing in prevalence due to increasing obesity rates in both children and adults.¹⁻⁴ NAFLD represents a range of hepatic pathology in the absence of excessive alcohol consumption and/or other causes of chronic liver disease. Simple steatosis, or NAFL, characterized by the presence of >5% triglycerides (TGs) in hepatocytes progresses to nonalcoholic steatohepatitis (NASH) due to inflammation and collagen deposition,⁵ which is strongly linked to liver-related morbidity and mortality.⁶

NAFLD is often referred to as the hepatic manifestation of the metabolic syndrome, which is characterized by at least three of the following: hypertension,
hyperglycemia, abdominal obesity, elevated plasma TGs, and reduced plasma high-density lipoproteins (HDL). Although NAFLD pathogenesis is not well established, it is clear that obesity and insulin resistance are key contributors. Obesity increases the release of free fatty acids (FFAs) from adipose tissue into hepatocytes, contributing to more than two-thirds of FFAs in the liver, which aids in systemic insulin resistance. Furthermore, excessive adipose tissue increases proinflammatory adipokine secretion (eg, leptin, resistin, IL-6) that may 1) directly impact the liver and contribute to NAFLD pathogenesis and 2) cause systemic inflammation, which contributes to insulin resistance. Insulin resistance exacerbates metabolic abnormalities, including dyslipidemia and obesity, and thus induces NAFLD development.

Interestingly, not all individuals with metabolic syndrome will develop NAFLD and not all cases of fatty liver will progress to NASH or end-stage liver disease. Susceptibility to steatosis, and its associated metabolic consequences, as well as NAFLD progression have all been linked with ethnic and sex differences. Further, the association of NAFLD and obesity also varies by ethnicity, with Asian individuals at a higher risk of what is considered “nonobese NAFLD”, but that may be more strongly linked to central adiposity, genetic predisposition and diet.

Accordingly, 9.2% of Sri Lankan adults were obese, while 26.2% were considered centrally obese in 2010. A meta-analytic analyses of epidemiological data suggests NAFLD prevalence in Asia to be 27%. Compared to Caucasians, South Asians have a higher amount of total body fat, including subcutaneous adipose tissue and especially visceral adipose tissue for a given body mass index (BMI). At a comparatively low BMI, South Asians have an increased risk of hyperlipidemia, hypertension, glucose intolerance, and NAFLD, in part, due to excessive central adiposity but also due to genetic predisposition and excess energy consumption.

Since the presence of NALFD is linked to abdominal obesity and South Asians are at an increased risk of developing obesity-related metabolic abnormalities, it is of interest to dissect alterations in the metabolic profile that exist with varying degrees of NAFLD in this population. Furthermore, no single biomarker or group of metabolites has been related to the progression of NAFLD in human serum, and thus proper medical care for NAFLD is limited.

Therefore, the purpose of the current study was to determine the association between degree of adiposity, plasma adipokine levels, and markers of inflammation and insulin resistance with the presence of fatty liver in a South Asian population. We hypothesized that an increase in insulin resistance, markers of inflammation, and serum adipokines would exist proportional to the degree of fatty liver.

Material and methods

This was a cross-sectional study to determine the association between anthropometric measures, measures of adiposity, indices of systemic insulin resistance, and plasma adipokine levels with degree of hepatic steatosis in Sri Lankan adults. Ethical clearance for this study was granted by the Faculty of Medicine, University of Peradeniya and informed written consent was obtained from all participants. De-identified serum samples were processed at Texas Tech University under an exempt Institutional Review Board (IRB) Approval (IRB #503875).

Participants

A convenience sample of adult women (age 18 to 65 years) undergoing routine abdominal surgery (hernia repair n=4; hysterectomy n=10; laparoscopy n=6; laparotomy n=3; other n=11) at the Teaching Hospital, Peradeniya, Sri Lanka, were recruited (n=34). Individuals with inflammatory conditions, cancer, or previous abdominal surgery were excluded from the study. Demographic data, diet, and physical activity history were recorded using an interviewer-administered questionnaire.

On the day of the surgery, a fasting blood sample was collected, and a radiologist performed an ultrasound scan of the abdomen. Blood was collected into microcentrifuge tubes and allowed to clot on ice. Serum was separated via centrifugation and supernatant was stored in −80°C for subsequent analyses.

Anthropometric measurements

Height was measured to the nearest millimeter using a stadiometer. Weight was measured to the nearest 100 g with a digital scale. Waist circumference (WC) was measured midway between the lowest rib and the superior border of the iliac crest in the mid-axillary line, with an inelastic measuring tape at the end of normal expiration to the nearest millimeter. Hip circumference (HC) was measured around the widest portion of the buttocks. BMI was calculated with the weight (wt) and height (ht) data [BMI = wt (kg)/ht² (m²)].

Liver ultrasonography

A radiologist performed an ultrasound scan of the liver. A 3.5-MHz transducer was used to obtain the following
images: sagittal view of the right lobe of the liver and right kidney, transverse view of the left lateral segment of the liver and spleen, transverse view of the liver and pancreas, and any focal areas of altered echotexture (Philips EnVisor C HD M2540A-USA-Diagnostic Ultrasound Imaging System, Andover, MA, USA). The severity of echogenicity was graded as follows: fatty liver grade 0 (NAFL 0, normal echogenicity); fatty liver grade 1 (NAFL 1, slight, diffuse increase in fine echoes in liver parenchyma with normal visualization of diaphragm and intrahepatic vessel borders); fatty liver grade 2 (NAFL 2, moderate, diffuse increase in fine echoes with slightly impaired visualization of intrahepatic vessels and diaphragm); and fatty liver grade 3 (NAFL 3, marked increase in fine echoes with poor or nonvisualization of the intrahepatic vessel borders, diaphragm, and posterior right lobe of the liver).26

Adipose tissue histology

Adipose tissue samples were obtained from the anterior abdominal wall close to the umbilicus (subcutaneous adipose tissue) and from the greater omentum (visceral adipose tissue). Samples were formalin fixed, and a routine paraffin embedded tissue section was prepared. Adipocyte size was measured using the ImageJ (NIH, http://imagej.nih.gov/ij/) software (mean surface area of 100 adipocytes in each biopsy).

Serum biomarkers and adipokines

Enzymes

Enzymes indicative of liver damage, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes, were assayed using IFCC method in an automated biochemical analyzer (Thermo Scientific Konelab Analyzer Holliston, MA, USA).

Measures of insulin resistance

Blood glucose was measured by the glucose oxidase method. HOMA index was calculated as fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5.

Branched-chain amino acids

Colorimetric Assay Kit (BioVision Incorporated, Milpitas, CA, USA) was used to measure branched-chain amino acids (BCAAs), leucine, isoleucine, and valine (Cat. #K564–100; range =0–10 nmol).27

Lipids

TGs, HDL, very-low-density lipoprotein (VLDL), and low-density lipoprotein (LDL) were assessed by CovenantHealth (Lubbock, TX, USA).

Hormones and adipokines

Luminex XMAP technology Magpix™, Human Adipokine Magnetic Bead Panel I (Millipore, Billerica, MA, USA; Cat. # HADK1MAG-61K) was used to measure adiponectin (range =26–400,000 ng/mL) and resistin (range =6.4–100,000 pg/mL).28 Milliplex® Human Adipokine Magnetic Bead Panel II (EMD Millipore, Billerica, MA, USA; Cat. # HADK2MAG-61K) was used to measure IL-6 (range =0.96–15,000 pg/mL), leptin (range =38–600,000 pg/mL), and monocyte chemoattractant protein-1 (MCP-1; range =1.3–20,000 pg/mL).29 BioTek Cytation imaging reader technology, ELISA (EMD Millipore) was used to measure high-molecular-weight (HMW) adiponectin (Cat. #SPREZHMWADPN65K; range =1.56–200.88 ng/mL) and insulin (Cat. #EZHI-14K; range =2–200 μU/mL).30 Angiotensinogen (AGT) (Cat. #ELH-AGT; range =1.229–300 ng/mL) was measured using a kit from RayBiotech (Norcross, GA, USA). High-sensitivity C-reactive protein (hsCRP) was measured using a kit from MP Biomedicals (Solon, OH, USA; Cat. # 07BC-1119; range =0–0.1 mg/L). When appropriate, serum dilutions were performed.

Statistical analysis

Data were entered using Microsoft Excel (2010) and an ANOVA was performed using R data analysis software, version 20 (Statistical and Products Service Solutions, Chicago, IL, USA). Bonferroni–Holm corrections were performed. When necessary, post-hoc comparisons were made. Differences were considered significant at \( p<0.05 \) after the aforementioned correction.

Results

Participants

Of the 34 female participants, 11 were categorized as fatty liver grade 0 (NAFL 0), 13 were categorized as fatty liver grade 1 (NAFL 1), 10 were categorized as fatty liver grade 2 (NAFL 2), and 0 were categorized as fatty liver grade 3 (NAFL 3). Participant characteristics are presented in Table 1. Age of the participants was not significantly different based on the degree of steatosis. The number of participants with hypertension did not vary among fatty liver groups.

NAFL and measures of adiposity

After Bonferroni–Holm corrections, WC \( (p=0.0027) \) was the only significant variable increasing across NAFL grade:
post-hoc analyses indicate an increase between NAFL 0 and 1 ($p=0.006$), NAFL 1 and NAFL 2 ($p=0.027$), and also between NAFL 0 and 2 ($p=0.001$) (Figure 1A). BMI ($p=0.0043$) and HC ($p=0.013$) were nominally significant based on the degree of NAFL. Post-hoc analyses for BMI indicate a significant increase between NAFL 0 and NAFL 1 ($p=0.0014$) and NAFL 0 and NAFL 2 ($p=0.011$), but not between NAFL 1 and NAFL 2 ($p=0.13$) (Figure 1B). Similarly, post-hoc analyses for HC indicate a significant increase between NAFL 0 and NAFL 1 ($p=0.002$) and NAFL 0 and NAFL 2 ($p=0.022$), but not between NAFL 1 and NAFL 2 ($p=0.27$) (Figure 1C). There was not a significant difference in waist-to-hip ratio among the groups ($p=0.17$) (Figure 1D). These findings indicate that increasing adiposity, especially in the visceral compartment, is associated with NAFLD.

NAFL and adipose tissue dysfunction

Adipose tissue dysfunction, indicated by dysregulated adipokine secretion and chronic low-grade inflammation, is mechanistically linked to the pathogenesis of obesity-associated metabolic disorders. Dysfunctional adipose tissue is characterized by large, insulin-resistant adipocytes. Thus, next, we measured adipocyte size in visceral and subcutaneous compartments to identify a link with NAFL. Abdominal subcutaneous adipocyte area was nominally significant among different NAFL groups ($p=0.0034$), with post-hoc analyses indicating a significant increase between NAFL 0 and NAFL 2 ($p=0.005$) and trending increases between NAFL 0 and NAFL 1 ($p=0.052$) and NAFL 1 and NAFL 2 ($p=0.054$) (Figure 2A). Representative images of subcutaneous abdominal adipocyte area are shown in Figure 2B. Visceral (omenta...
adipocyte area was also nominally significant among different NAFL groups \((p=0.0042)\), with post-hoc analyses indicating a significant increase between NAFL 0 and NAFL 1 \((p=0.022)\) and NAFL 0 and NAFL 2 \((p=0.006)\), but not between NAFL 1 and NAFL 2 \((p=0.22)\) (Figure 2C).

**Serum biomarkers and adipokines**

**NAFL and liver enzymes**

NAFL can lead to elevated liver enzymes.\(^{31,32}\) ALT was not statistically different among participants with varying degrees of NAFLD. There was a trend toward nominal significance for AST \((p=0.066)\) among NAFL groups, with post-hoc analyses indicating a significant increase in AST between NAFL 0 and NAFL 2 \((p=0.035)\), but not between NAFL 0 and NAFL 1 \((p=0.46)\) or NAFL 1 and NAFL 2 \((p=0.10)\) (Table 1).

**NAFL and insulin resistance**

NAFLD is associated with insulin resistance. However, it is not clear whether the latter is a cause or effect of NAFLD.\(^{14}\) We found that fasting blood glucose (FBG) \((p=0.0052)\) nominally increased among NAFL groups (Figure 3). Post-hoc analyses for FBG indicated a significant increase between NAFL 0 and NAFL 1 \((p=0.011)\) and NAFL 0 and NAFL 2 \((p=0.003)\), but not between NAFL 1 and NAFL 2 \((p=0.26)\) (Figure 3). Accordingly, participants in the NAFL 1 and NAFL 2 groups met criteria for prediabetes (5.6 to 6.9 mmol/L).\(^{33}\) However, neither serum insulin nor HOMA-IR were significantly different among participants with varying degrees of NAFL (Table 1). Since BCAAs are altered with insulin resistance,\(^{34}\) they were also assessed in our study but were not significantly different based on the degree of NAFLD (Table 1). Taken together, these findings suggest that insulin resistance is unlikely to be the major mechanism responsible for NAFL in this population.

**NAFL and lipids**

All lipid panel measures, including VLDL, LDL, HDL, and serum TGs, were not significantly different based on the degree of NAFLD (Table 1).
NAFL, hormones, and adipokines

Obesity is characterized by a chronic low-grade inflammation. However, all markers of inflammation evaluated in our study, including hsCRP, MCP-1, and IL-6, were not significantly different based on the degree of NAFLD (Table 1), indicating that systemic inflammation is unlikely to be associated with NAFLD in this population.

Furthermore, adipokines, including adiponectin, HMW adiponectin, and leptin, were not significantly different based on NAFL degree (Table 1). There was a trend toward nominal significance for resistin ($p=0.063$) among NAFL groups, with post-hoc analyses indicating a significant increase in serum resistin between NAFL 0 and NAFL 2 ($p=0.025$) and a trend toward significance between NAFL 1 and NAFL 2 ($p=0.052$), but not between NAFL 0 and NAFL 1 ($p=0.81$) (Figure 4).

Activation of the renin–angiotensin system favors NAFLD development and progression. However, AGT was not significantly different based on the degree of NAFL in our sample population (Table 1).

Discussion

In this study, we found that in female Sri Lankan participants, fatty liver grade increases with obesity, as indicated by anthropometric measures and adipocyte size. Compared to Caucasians, South Asians have a higher amount of total body

Figure 2 Mean adipocyte size in participants with varying degree of nonalcoholic fatty liver (NAFL). (A) Participants with NAFL 2 had a significantly higher abdominal adipocyte area as shown by post-hoc analyses, which also indicate increases in abdominal adipocyte area between NAFL 0 and NAFL 1 and NAFL 1 and NAFL 2 that are trending significance. (B) Representative H&E-stained sections of abdominal adipose tissue (subcutaneous) are shown for each group. (C) Participants with fatty liver had a significantly higher omental (visceral) adipocyte area as shown by post-hoc analyses, which indicate a significant increase in adipocyte area between NAFL 0 and NAFL 1, but not between NAFL 1 and NAFL 2. Hepatic steatosis was assessed using ultrasound. Adipocyte area was measured using the ImageJ software. $n=10–13$ per group. ANOVA with Bonferroni–Holm corrections analyses was performed.

Figure 3 Elevated fasting blood glucose with degree of nonalcoholic fatty liver (NAFL). Participants with fatty liver had significantly higher fasting blood glucose (FBG) as shown by post-hoc analyses, which indicate a significant increase in FBG between NAFL 0 and NAFL 1, but not between NAFL 1 and NAFL 2. Hepatic steatosis was assessed using ultrasound. $n=10–13$ per group. ANOVA with Bonferroni–Holm corrections analyses was performed.
fat for a given BMI, and thus lower values were established for BMI classifications (overweight, BMI ≥23 kg/m², obese BMI ≥27.49 kg/m²) in this population. Furthermore, at a given total body fat, South Asians have higher total adipose tissue, including subcutaneous adipose tissue and visceral adipose tissue, compared to Caucasians.

There is strong evidence that obesity-associated metabolic abnormalities occur at lower absolute amounts of total body fat in this population. It has been suggested that South Asians develop abdominal obesity earlier than Caucasians due to inefficient TG storage in the superficial subcutaneous adipose tissue compartment, which is the primary storage compartment. Increases in other compartments—the deep subcutaneous and visceral adipose tissue—as seen in South Asians, increases fatty acid flux and thus, the amount of fatty acids delivered to the liver. Indeed, liver steatosis is linked with increased adipocyte size and overall increased visceral adiposity.

Earlier utilization of the secondary adipose tissue compartments explains greater values for anthropometric measures and justifies an increase in BMI, WC, HC, and both abdominal subcutaneous and visceral (omental) adipocyte size in our participants with NAFLD. The significant increase in subcutaneous abdominal adipocyte size with NAFLD is novel, especially the trending increase between NAFL 1 and NAFL 2 (p=0.054) seen in our participants.

Interestingly, waist-to-hip ratio did not increase with increasing fatty liver. Invariably, previous studies have shown a weaker correlation with waist-to-hip ratio and metabolic abnormalities in this population.

Surprisingly, lipoprotein profiles of our participants did not increase with the degree of fatty liver. This was unexpected since an atherogenic lipoprotein profile is often seen in South Asians with increased abdominal adiposity and has been linked with fatty liver in previous studies. Lipid values of our participants do compare to those reported in other studies, showing lower LDL in South Asians compared to Caucasians. Furthermore, low HDL and high TGs are common to dyslipidemia in South Asians. Consistent with previous studies, HDL averages for our participants were low (<40 mg/dL); however, interestingly, TG levels were not elevated (normal ≤150 mg/dL) in our participants. Perhaps, the most interesting, when considering lipid panel results, is the lower TG levels and lack of correlation with a fatty liver degree in our participants. However, this has been reported in other studies and could be due to overall decreased VLDL hepatic output and increased hepatic TG storage.

With excessive nutrient intake, impaired adipogenesis leads to adipose tissue metabolic and immunologic dysfunction. Despite increasing adipocyte size with increasing NAFL grade, we did not see significant increases in the proinflammatory markers (IL-6, MCP-1, and hsCRP) evaluated in our study. CRP, which is strongly associated with central obesity in South Asians, was identified as a weak predictor of NAFLD. Accordingly, others have also reported no difference in hsCRP with NAFLD. Taking into account the insignificant findings regarding inflammation and AGT as well as enzymes for liver health, including ALT and AST, we suggest that individuals in our study must have stages of NAFLD that are not considered severe. This is supported in our findings for AST, which showed the largest increase in the NAFL 2 group. Furthermore, it may be that the threshold at which enlarged adipocytes contribute to systemic inflammation has not been reached.

Additionally, insulin resistance is a common metabolic dysfunction associated with low-grade inflammation and impaired adipogenesis. However, serum insulin and HOMA calculations were not significantly increased with increasing NAFLD grade in our study. Hyperglycemia, indicated by increased FBG, was seen in our participants with NAFLD. This is in contradiction to others who have reported no difference in fasting glucose but increased

Figure 4 Elevated resistin associated with nonalcoholic fatty liver (NAFL) grade 2. Participants with fatty liver had serum resistin values that were trending significance. Participants with NAFL 2 had a significantly higher serum resistin as shown by post-hoc analyses, which also indicate increases in serum resistin between NAFL 1 and NAFL 2. No difference was found between NAFL 0 and NAFL 1. Hepatic steatosis was assessed using ultrasound. n=10–13 per group. ANOVA with Bonferroni–Holm corrections analyses was performed.
insulin and overall insulin resistance susceptibility in individuals with NAFLD. Others have shown BCAA alterations, decreased adiponectin, and increased leptin to be associated with insulin resistance in South Asians; thus, it is not surprising that we did not see differences in our study.

Resistin is a hormone produced primarily by macrophages in humans and is positively correlated with obesity and insulin resistance due to its proinflammatory properties. The role of resistin in the pathogenesis of hepatic and systemic insulin resistance is well established in animals but is controversial in humans. Others have reported higher serum resistin levels in individuals with fatty liver and levels that parallel increasing fatty liver grade. Thus, resistin may participate in the pathways underlying liver damage and the progression of simple steatosis to steatohepatitis. Interestingly, in our study, resistin was trending significance, with the biggest increase in the NAFL 2 group. Since resistin stimulates hepatic glucose output, it may contribute to the elevated FBG seen in our study.

There are a few noteworthy limitations of the current study. The first limitation in our study is the small size of the study population. Despite this, several findings reached statistical significance when adjusting for the multiple comparisons performed. Sample size was calculated a priori, and we estimated 40 participants in each NAFL group would be needed in order to reach significance in those variables found nominally significant. Further, our convenience sample only included female participants, thus limiting the translation of our findings to males within this population. Third, liver biopsy is considered the gold standard for NAFLD diagnosis. Liver ultrasonography is a limitation in this study because it cannot differentiate between fibrosis and fatty changes. However, TG deposit is the most common pathological feature of NAFLD and most likely the dividing factor among our grades of NAFL. Interestingly, results from our study are similar if statistical analyses are conducted based on grouping (ie, the control [NAFL 0], normal liver echogenicity vs NAFL [both NAFL 1 and NAFL 2 group combined]) or alterations in liver echogenicity. Fourth, body composition analysis would have strengthened our discussion on adipocyte size and its relation to increasing NAFL grade. Additionally, utilizing the hyperinsulinemic–euglycemic clamp method or performing a glucose tolerance test on participants in our study would have aided in our understanding of dysglycemia in relation to increasing NAFL grade. Last, while dietary intake was collected, our interpretation and application to NAFL groups were limited. Diet could have significant implications in this population, given the shift to higher fat and fructose consumption in this population, as well as the known benefits of a Mediterranean diet and antioxidant consumption in NAFLD.

**Conclusion**

It is well established that the associations of adiposity and health outcomes may differ between Asian and European populations. In the current study, we have shown that increases in adiposity are associated with NAFLD in South Asian women. Specifically, WC may be the best indicator of NAFLD progression in this population. Furthermore, increased subcutaneous as well as visceral fat deposition, elevated fasting glucose, and serum resistin could be important in the pathogenesis of NAFLD in South Asian women.

**Abbreviation list**

AGT, angiotensinogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBG, fasting blood glucose; FFA, free fatty acids; HMW, high molecular weight; hsCRP, high-sensitivity C-reactive protein; HC, hip circumference; MCP-1, monocyte chemoattractant protein 1; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; TG, triglyceride; WC, waist circumference.

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