Adipocyte Fatty Acid Binding Protein Levels Relate to Inflammation and Fibrosis in Nonalcoholic Fatty Liver Disease

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Several circulating cytokines are increased with obesity and may combine with the influence of visceral fat to generate insulin resistance, inflammation, and fibrosis in nonalcoholic fatty liver disease (NAFLD). Little information exists in NAFLD about three recently recognized tissue-derived cytokines that are all lipid-binding and involved in inflammation, namely adipocyte fatty acid–binding protein (AFABP), lipocalin-2, and retinol-binding protein 4 (RBP4). We examined the association of these three peptides with hepatic steatosis, inflammation, and fibrosis plus indices of adiposity, insulin resistance, and dyslipidaemia in 100 subjects with NAFLD and 129 matched controls. Levels of AFABP and lipocalin-2, but not RBP4, were significantly elevated in NAFLD versus control (AFABP, 33.5 ± 14.4 versus 23.1 ± 12.1 ng/mL [$P < 0.001$]; lipocalin-2, 63.2 ± 26 versus 48.6 ± 20 ng/mL [$P < 0.001$]) and correlated with indices of adiposity. AFABP correlated with indices of subcutaneous rather than visceral fat. AFABP alone distinguished steatohepatitis from simple steatosis ($P = 0.02$). Elevated AFABP independently predicted increasing inflammation and fibrosis, even when insulin resistance and visceral fat were considered; this applied to lobular inflammation and ballooning (odds ratio 1.4, confidence interval 1.0-1.8) and fibrosis stage (odds ratio 1.3, confidence interval 1.0-1.7) ($P \leq 0.05$ for all). None of the cytokines correlated with steatosis grade. AFABP levels correlated with insulin resistance (homeostasis model assessment of insulin resistance) in controls and NAFLD, whereas lipocalin-2 and RBP4 only correlated positively with insulin resistance in controls. *Conclusion:* Circulating AFABP, produced by adipocytes and macrophages, and lipocalin-2, produced by multiple tissues, are elevated and may contribute to the metabolic syndrome in NAFLD. AFABP levels, which correlate with subcutaneous, but not visceral fat, independently predict inflammation and fibrosis in NAFLD and may have a direct pathogenic link to disease progression. (HEPATOLOGY 2009;49:1926-1934.)

Adiposity is clearly important to the pathogenesis of nonalcoholic fatty liver disease (NAFLD). However, while caloric excess, dietary composition and lack of physical activity are central to its development, intrahepatic triglyceride content correlates only moderately with body fat.[1,2] On the other hand, there is now considerable data to suggest that body fat topography, particularly central adiposity, is more closely linked with insulin resistance, hepatic steatosis, and other components of the metabolic syndrome, including atherosclerosis.[2,3] Consistent with this finding, surgical removal of visceral fat in rodents or humans improves insulin sensitivity.[4,5]

The factors that convert a proportion of fatty livers into ones with steatohepatitis are not completely understood.

Abbreviations: AFABP, adipocyte fatty acid–binding protein; BMI, body mass index; ELISA, enzyme-linked immunosorbent assay; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; RBP4, retinol binding protein 4; sTNFR2, soluble tumor necrosis factor receptor 2; TNF-α, tumor necrosis factor alpha; WHR, waist/hip ratio.

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We recently demonstrated that visceral obesity is a key determinant, with incremental increases in visceral fat substantially increasing the likelihood of hepatic inflammation and fibrosis.[1] From a mechanistic perspective, attention has focused on circulating adipokines and cytokines released by adipocytes, or macrophages infiltrating adipose tissues, as the drivers of local and systemic inflammation.[6] These studies are most convincing in rodent models of obesity, though their importance in mediating insulin resistance and systemic inflammation in humans is less clear.[7]

Several reports have examined the role of adipokines and cytokines in animal models and in correlative studies in humans with NAFLD. These findings indicate that leptin is antisteatotic, but proinflammatory and profibrogenic.[8-11] Conversely, adiponectin also enhances lipid oxidation yet has potent anti-inflammatory and antifibrotic effects.[12-14] We and others have demonstrated a correlation between low adiponectin levels and increasing hepatic steatosis and necroinflammation in NAFLD.[15,16] Likewise, tumor necrosis factor α (TNF-α), a major proinflammatory cytokine in the liver, and its p55 receptor are elevated in the liver of humans with nonalcoholic steatohepatitis (NASH). However, we have been unable to demonstrate an independent association between the expression of TNF-α or its soluble receptor 2 (sTNFR2) in serum, with the extent of hepatic inflammation in NAFLD.[15] These results are not altogether surprising, as TNF-α is considered to act principally in a paracrine and autocrine manner, with tissue levels and the TNF-α/adiponectin ratio being more important.[17]

Even though there is now good evidence for the role of adiponectin and visceral fat as predictors of liver inflammation in NAFLD, only a proportion of the observed variance can be accounted for by these factors. Adipocyte fatty acid–binding protein (AFABP) (also known as aP2, FABP4, and adipocyte lipid-binding protein), a member of the lipid chaperone fatty acid–binding protein family, is produced in adipocytes and macrophages, with its gene expression being several orders of magnitude higher in adipocytes.[18,19] Disruption or pharmacological blockade of AFABP protects mice from dyslipidaemia, atherosclerosis, insulin resistance, and fatty liver in the context of either a high-fat diet or genetically induced obesity.[20,21] Though once thought to be a purely intracellular protein, recent studies have shown AFABP to be abundantly present in human serum.[18,19,22] Lipocalin-2 is produced in many tissues and is involved in impairment of insulin action, is induced by inflammatory stimuli, and has been implicated in the generation of atherosclerosis.[23] Its serum levels correlate with obesity, dyslipidaemia, and metabolic disturbance. Finally, retinol-binding protein 4 (RBP4), produced from both adipocytes and hepatocytes, has been implicated in the generation of insulin resistance in animals and in humans.[24]

Although these newer lipid binding peptides are all involved in inflammation in the metabolic syndrome, and one recent report has shown elevated AFABP levels to be associated with sonographically determined hepatic steatosis in type 2 diabetes,[25] the relationship of AFABP and lipocalin-2 to hepatic histopathology has not been characterized. In attempting to further dissect these relationships, we assessed AFABP, lipocalin-2, and RBP4, in addition to measures of leptin, adiponection, TNF-α and sTNFR2, hepatic fat, inflammation and fibrosis, as well as insulin resistance and lipids, in a cohort with NAFLD, in comparison to a control cohort. Our results indicate that both AFABP and lipocalin-2 are elevated and may contribute to the metabolic syndrome in NAFLD, whereas AFABP levels may have a direct pathogenic link to disease progression.

Patients and Methods

Patients and Data Collection. We studied 100 patients (60 men and 40 women) with biopsy-proven NAFLD recruited from a tertiary liver clinic, 31 with steatosis alone and 69 with steatohepatitis. Some of the patients were the subject of previous reports.[1] Patients were referred for the assessment of abnormal liver function tests or hepatic steatosis detected by ultrasonography. In all patients, current and past daily alcohol intake was less than 40 g/week, confirmed by at least two physicians and close family members. All had a normal serum albumin level, prothrombin time, and renal function. The diagnosis of type 2 diabetes mellitus was based on World

Received October 20, 2008; accepted February 1, 2009.

Supported in part by Hong Kong Research Council CRF Grant HKU 2/07C (to A. X.), National Health and Medical Research Council of Australia Grant 358398, a GESA postgraduate medical research scholarship, a Robert W. Storr Bequest to the University of Sydney, and a University of Sydney Grant.

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.22896

Potential conflict of interest: Nothing to report.

Health Organization criteria.[26] Subjects with type 2 diabetes were taking metformin (n = 14), sulfonylureas (n = 7), and insulin (n = 7); however, patients using thiazolidinediones were excluded. Secondary causes of steatohepatitis and other causes of liver disease were excluded by appropriate serological and biochemical tests. One hundred twenty-nine controls (71 men and 58 women) were recruited through advertisements in local newspapers and at the hospital. All had normal physical examinations and liver function tests, negative serology for viral hepatitis, and no history of liver disease. The study protocol was approved by the Human Ethics Committee of the Western Sydney Area Health Service, and written informed consent was obtained.

Pathology. Liver tissues were stained with hematoxylin-eosin, reticulin, and Gomori trichrome stains and scored by an experienced hepatopathologist (J. G. K). All cases showed macrovesicular steatosis affecting at least 5% of hepatocytes and were classified as either steatosis or steatohepatitis (NASH). In addition to steatosis, the minimum criteria for the diagnosis of NASH included the presence of lobular inflammation and either ballooning cells or perisinusoidal/pericellular fibrosis in zone 3 of the hepatic acinus.[27,28] Subjects with cirrhosis were either "definite" or "probable" cases of NASH-associated cirrhosis according to a recently proposed clinico-pathological classification.[29] All cases were scored using the methods proposed by Brunt et al.[30] and Kleiner et al.[31] Steatosis was graded from 1 to 3 (1 = 5%-33%; 2 = 34%-66%; 3 = 67%-100%), lobular inflammation from 0 to 3, and fibrosis stage from 0 to 4. Patients with both advanced lobular inflammation (grades 2-3) and advanced fibrosis (stages 3-4) were grouped together for statistical analysis.

Clinical and Laboratory Evaluation. A complete physical examination was performed on each patient. Anthropometric evaluation included measures of body mass index (BMI) and central obesity (waist and hip circumferences and waist/hip ratio [WHR]). On the morning of liver biopsy, venous blood was drawn after an overnight 12-hour fast to determine the levels of serum alanine aminotransferase, bilirubin, albumin, total cholesterol, triglycerides, glucose, insulin, adiponectin, leptin, TNF-α, and sTNFR2. Serum insulin was determined via radio-immunoassay (Phadeseph Insulin RIA; Pharmacia and Upjohn Diagnostics AB, Uppsala, Sweden). Leptin, TNF-α, and sTNFR2 were measured in duplicate using enzyme-linked immunosorbent assays (ELISAs) (Diagnostic Systems Laboratories, Webster, TX; R&D Systems, Minneapolis, MN). Serum AFABP and lipocalin-2 were measured with immunoassays as we described previously.[18,23] The intra-assay and interassay coefficients of variation for A-FABP were 3.7%-6.4% and 2.6%-5.3%, respectively. The intra-assay and interassay coefficients of variation for lipocalin-2 were 3.8%-6.0% and 3.1%-5.2%, respectively. Serum RBP4 was measured using an in-house monoclonal antibody-based ELISA. The capture antibody specific to RBP4 was diluted to a concentration of 2 μg/mL, added to each well of a microtiter plate, and incubated overnight at 4°C. The coated plate was washed three times with phosphate-buffered saline containing 1% bovine serum albumin and blocked with 100 μL of phosphate-buffered saline containing 1% bovine serum albumin and 0.05% Tween for 2 hours. Human serum was diluted at 1:1,000, and 100 μL of the diluted samples were applied to each well along with the standard, incubated at 37°C for 1 hour, washed three times with phosphate-buffered saline–Tween, and then incubated with 100 μL of the horseradish peroxidase–conjugated antibody (2 μg/mL) for another 1 hour. After washing three times, the wells were incubated with tetramethylbenzidine reagent for 15 minutes. One hundred microliters of 2 M H_2SO_4 was added to each well to stop the reaction, and the absorbance at 450 nm was measured. The assay is highly specific to human RBP4 and does not cross-react with AFABP, lipocalin-2, or a panel of other adipokines tested. The intra-assay and interassay coefficients of variation for this assay were 4.1%-6.8% and 3.8%-7.2%, respectively. Different RBP4 assays have reported different absolute circulating levels of this adipokine. In the present study, our method was compared with the Millipore (Billerica) RBP4 ELISA assay, which yields higher RBP4 levels, but there is excellent correlation between the two assays (r^2 = 0.93, n = 86). Serum total adiponectin was quantified by a monoclonal antibody-based sandwich ELISA as we previously described.[32] The intra-assay and interassay coefficients of variation for this assay were 4.4%-6.9% and 3.9%-7.1%, respectively. All other biochemical tests were performed using a conventional automated analyzer within the Department of Clinical Chemistry at Westmead Hospital. Insulin resistance was calculated by the homeostasis model assessment of insulin resistance (HOMA-IR) using the following formula: HOMA-IR = fasting plasma insulin (mU/L) × fasting plasma glucose (mmol/L)/22.5.

Quantitation of Fat Mass and Abdominal Fat. Total fat mass and lean mass was determined in 69 NAFLD subjects via dual X ray absorptiometry (Norland XR-36, Fort Atkinson, WI). Magnetic resonance examinations were performed using a Siemens Magnetom Vision 1.5 T system (Siemens, Erlangen, Germany) on 36 NAFLD patients within 2 weeks of liver biopsy. Nineteen transverse T1-weighted and T2-weighted images were acquired from L5/S1 upward with a slice thickness of 10 mm and an interslice spacing of 2.5 mm. Visceral and subcutaneous abdominal fat volumes were quantified using a validated automated fitting routine (Hippo Fat, Pisa, Italy).[33]

Statistical Analysis. Statistical analysis was per-

Table 1. Baseline Characteristics of Study Cohort

	Control (n = 129)	*P* Value*	Steatosis (n = 31)	*P* Value†	NASH (n = 69)
BMI (kg/m^2)	26.9 (4.8)	<0.001	29.6 (4.9)	NS	31 (5.0)
Age (years)	47.4 (10.7)	NS	44.5 (12.6)	0.04	49.8 (11.5)
Males/Females (n, %)	71/58 (55/45)	NS	22/9 (71/29)	NS	38/31 (55/45)
Waist (cm)	88.0 (11.7)	<0.001	100.4 (12.9)	NS	103.8 (11.6)
WHR	0.9 (0.1)	<0.001	1.0 (0.08)	0.04	1.0 (0.07)
Diabetics (n, %)	0	<0.001	6 (19.4)	NS	22 (31.9)
Fasting glucose (mmol/L)	5.0 (0.4)	<0.001	5.8 (1.4)	0.02	6.8 (2.8)
Fasting Insulin (mU/L)	8.6 (4.8)	<0.001	13.9 (5.6)	0.003	20.6 (11.8)
HOMA-IR	1.9 (1.1)	<0.001	3.6 (1.8)	0.02	6.3 (6.5)
C-peptide (mmol/L)	0.6 (0.2)	<0.001	0.8 (0.3)	0.01	1.1 (0.4)
Cholesterol (mmol/L)	5.2 (1.0)	NS	4.9 (0.8)	0.01	5.4 (1.2)
LDL (mmol/L)	3.0 (0.9)	NS	2.7 (0.7)	0.04	3.1 (1.0)
Triglycerides (mmol/L)	1.4 (0.9)	<0.001	1.8 (1)	NS	2.3 (1.5)
HDL (mmol/L)	1.6 (0.4)	<0.001	1.3 (0.4)	NS	1.3 (0.4)
ALT (U/L)	24.6 (9.9)	<0.001	66.6 (34)	NS	82.1 (53.8)
Adiponectin (μg/mL)	11.9 (8.6)	NS	12.1 (7.8)	NS	10.1 (8.5)
Leptin (ng/mL)	–		20.4 (16.8)	NS	27.4 (20.6)
sTNFR2 (ng/mL)	–		2.4 (0.7)	NS	2.6 (0.8)
TNF-α (pg/mL)	–		2.5 (0.8)	NS	3.0 (1.7)

Results are expressed as the mean (standard deviation) or frequency (percentage).

Abbreviations: NASH, nonalcoholic steatohepatitis; BMI, body mass index; WHR, waist/hip ratio; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; HDL, high-density lipoprotein; ALT, alanine aminotransferase; sTNFR2, soluble tumor necrosis factor receptor 2; TNF-α, tumor necrosis factor; NS, not significant.

*Comparison between NASH patients and controls.

†Comparison between NASH patients and steatosis.

formed using SPSS version 15.0 (SPSS, Chicago, IL). Results are reported as the mean ± standard deviation. Student *t* tests were used to compare means of continuous variables. The strength of association between continuous variables was reported using Spearman rank correlation. Univariate analysis of variance was used to examine the association between adipocytokines and increasing histology grades/stages among all subjects with NAFLD (simple steatosis and NASH). Multiple ordinal regression analysis with backward stepwise variable selection was performed to identify independent predictors for lobular inflammation, ballooning, and fibrosis stage.

Results

Patient Characteristics. The baseline patient characteristics are given in Table 1 and are separated into control subjects (without NAFLD), subjects with steatosis alone, and subjects with steatosis plus inflammation and/or fibrosis (NASH). The three groups were well matched for age, but as would be expected, indices of adiposity (BMI, waist, and WHR) and insulin resistance were significantly greater in the NAFLD groups. There were no patients with type 2 diabetes in the control group, but six patients in the steatosis group and 22 patients in the NASH group had type 2 diabetes.

Adipocyte Fatty Acid Binding Protein. AFABP levels were elevated in NAFLD compared with controls (33.5 versus 23.1 ng/mL, $P < 0.001$) and were significantly higher in NASH than in those with steatosis alone (35.8 versus 28.5 ng/mL, $P = 0.02$) (Fig. 1A and Table 2). AFABP levels were higher in females compared with males (33.7 versus 23.2 ng/mL, $P < 0.001$). AFABP levels correlated strongly within the whole cohort and in the NAFLD cohort with measures of adiposity and insulin resistance (Table 3). AFABP levels also correlated with triglyceride levels but not with cholesterol, low-density lipoprotein (LDL) or high-density lipoprotein (HDL) levels. Figure 2A shows the highly significant relationship between AFABP and HOMA-IR by quartiles ($P < 0.001$). Within the NAFLD cohort, AFABP levels were associated with increasing lobular inflammation ($P = 0.02$), ballooning ($P = 0.01$), and fibrosis stage ($P = 0.01$), but not with steatosis grade (Table 2). To determine if AFABP levels were independently associated with advanced inflammation, ballooning, and fibrosis in NASH, or simply markers of overall adiposity and insulin resistance, we created multivariate models for each of these histological endpoints. Factors significant on univariate analysis were entered into multiple ordinal regression models with backward stepwise elimination of variables to determine independent risk factors for each. For increasing lobular inflammatory grade, the univariate predictors were AFABP, WHR, HOMA-IR, cholesterol, and female sex. Multivariate analysis revealed that only

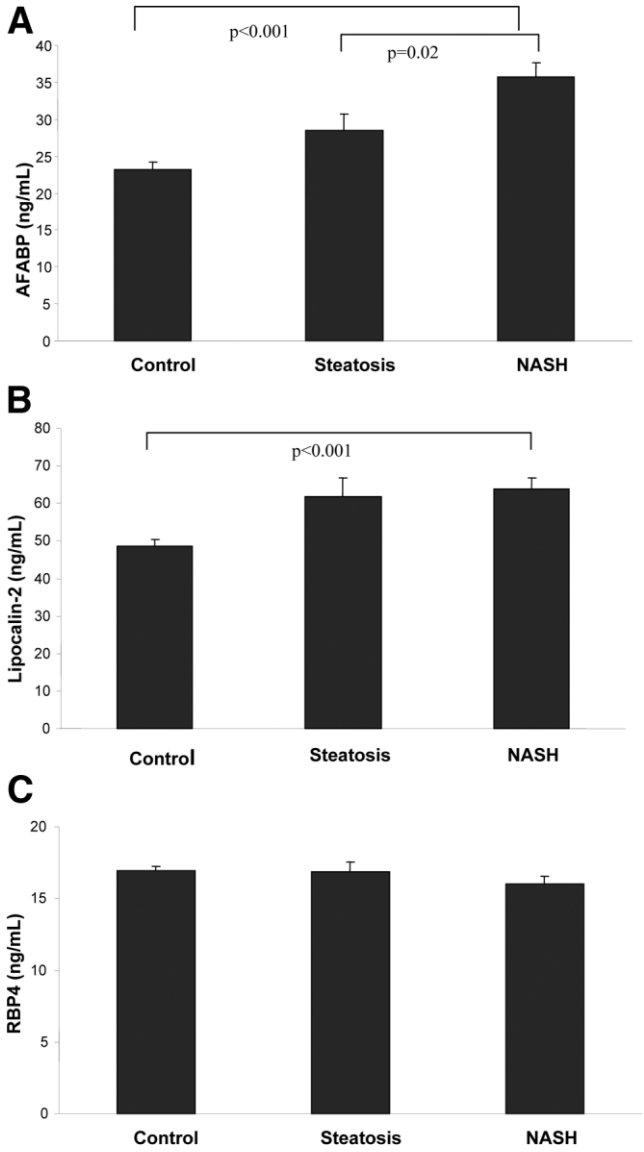

Fig. 1. Adipocytokine levels in control, steatosis, and nonalcoholic steatohepatitis (NASH) subjects. (A) AFABP serum levels increased significantly between control and NASH subjects ($P < 0.001$) and steatosis and NASH subjects ($P = 0.02$). (B) Lipocalin-2 levels increased significantly between control and NASH subjects ($P < 0.001$). (C) No difference in RBP4 levels between control and NASH subjects and steatosis and NASH subjects.

AFABP was independently associated with increasing inflammation, with an odds ratio of 1.4 (confidence interval 1.0-1.8, $P = 0.03$) for each 10-ng/mL increase (Table 4). For increasing ballooning grade, the univariate predictors were AFABP, HOMA-IR, adiponectin, female sex, and increasing age. After multivariate analysis, AFABP remained an independent predictor of ballooning (odds ratio 1.4, confidence interval 1.0-1.8, $P = 0.05$), as did increasing age and decreasing adiponectin levels (Table 4). Finally, for increasing fibrosis stage, the univariate predictors were AFABP, WHR, decreasing HDL, and increasing age. Multivariate analysis revealed that both AFABP (odds ratio 1.3, confidence interval 1.0-1.7, $P = 0.03$) and WHR independently predicted increasing fibrosis (Table 4).

Lipocalin-2. Lipocalin-2 levels were significantly higher in NAFLD compared with controls (63.2 versus 48.6 ng/mL, $P < 0.001$), but there was no difference between those with NASH versus those with steatosis alone (63.9 versus 61.8 ng/mL, $P = 0.7$) (Fig. 1B and Table 2). Levels were similar in females and males (57.2 versus 53.3 ng/mL, $P = 0.4$). Lipocalin-2 levels correlated significantly in both the whole cohort and the NAFLD cohort with AFABP (all $r = 0.342$, $P < 0.001$, NAFLD $r = 0.416$, $P < 0.001$), waist measurement, and triglycerides, but the associations with BMI, HOMA-IR, and HDL were significant only when the whole cohort was considered (Table 3). Figure 2B shows the significant relationship between lipocalin-2 and HOMA-IR by quartiles ($P = 0.001$). Within the NAFLD group, lipocalin-2 was not associated with increasing steatosis, lobular inflammation, ballooning, or fibrosis (Table 2).

Retinol Binding Protein 4. RBP4 levels were similar in all the groups studied with no difference between control and NAFLD (16.9 versus 16.3 ng/mL, $P = 0.24$) or steatosis and NASH (16.9 versus 16.0 ng/mL, $P = 0.36$) (Fig. 1C and Table 2). RBP4 levels were lower in females compared with males (15.8 versus 17.3 ng/mL, $P = 0.003$) as previously described.[34] RBP4 levels did not correlate with lipocalin-2, leptin levels, or with the degree of liver steatosis, inflammation, or fibrosis ($P > 0.15$ for each) (Table 2) and in the whole cohort only correlated marginally with AFABP ($r = 0.131$, $P = 0.05$), BMI, and waist circumference (Table 3). RBP4 correlated positively with HOMA-IR only in the control group ($r = 0.239$, $P = 0.007$) and had a weak negative correlation with HOMA-IR in the NAFLD group ($r = -0.2$, $P = 0.046$). There was a significant correlation with triglycerides and HDL cholesterol (only in the whole cohort) and none with cholesterol and LDL levels.

Relationship of AFABP, Lipocalin-2, and RBP4 with Other Adipocytokines in the NAFLD Group. Leptin correlated strongly with AFABP ($r = 0.59$, $P < 0.001$) and lipocalin-2 ($r = 0.49$, $P < 0.001$). There was no correlation between adiponectin and these three adipokines. sTNFR2 correlated strongly with lipocalin-2 ($r = 0.38$, $P < 0.001$), but more modestly with AFABP ($r = 0.29$, $P = 0.01$). TNF-α levels did not correlate with any of the measured adipokines and as described[15] did not correlate with the degree of inflammation or fibrosis.

Correlations with Body Fat. Sixty-nine NAFLD patients had a dual X ray absorptiometry measurement of total fat mass and a body fat percentage that showed a strong correlation with AFABP and lipocalin-2 but no

Table 2. Comparison of Adipocytokines Levels with Severity of Liver Histology in NAFLD

	Adipocyte Fatty Acid Binding Protein (ng/mL)	Lipocalin-2 (ng/mL)	Retinol Binding Protein-4 (ng/mL)
Diagnosis			
Steatosis (n = 31)	28.5 (11.8)	61.8 (27.9)	16.9 (3.6)
NASH (n = 69)	35.8 (15.0)	63.9 (25.2)	16.0 (4.6)
P value*	0.02	NS	NS
Steatosis grade			
1 (n = 47)	32.2 (14.3)	65.6 (28.5)	17.0 (3.8)
2 (n = 37)	36.0 (15.3)	59.3 (24.7)	15.7 (4.8)
3 (n = 14)	31.6 (13.6)	67.1 (21.8)	15.9 (4.9)
P value†	NS	NS	NS
Lobular inflammatory grade			
0 (n = 33)	28.1 (11.6)	58.0 (24.4)	17.0 (4.4)
1 (n = 56)	35.7 (15.5)	63.6 (25.1)	15.9 (4.1)
2-3 (n = 11)	38.9 (12.0)	77.4 (31.8)	16.5 (5.4)
P value†	0.02	NS	NS
Ballooning grade			
0 (n = 45)	27.8 (8.4)	60.2 (26.5)	17.0 (3.6)
1 (n = 42)	37.7 (14.8)	65.8 (26.9)	15.7 (4.7)
2 (n = 13)	39.6 (16.1)	65.6 (21.9)	15.6 (5.4)
P value†	0.01	NS	NS
Fibrosis stage			
0 (n = 30)	27.1 (10.5)	61.8 (29.6)	16.5 (3.7)
1 (n = 35)	35.5 (15.3)	65.0 (22.1)	16.7 (4.7)
2 (n = 17)	35.9 (14.7)	65.7 (30.2)	15.4 (3.8)
3-4 (n = 18)	38.1 (15.2)	59.9 (24.0)	16.0 (5.1)
P value†	0.01	NS	NS

All values expressed as the mean (standard deviation).
*P values for independent variable t tests.
†P values for analysis of variance.

relationship to RBP4 levels (AFABP: total fat $r = 0.578$, $P < 0.001$; percent body fat $r = 0.654$, $P < 0.001$; lipocalin-2: total fat $r = 0.413$, $P < 0.001$, percent body fat $r = 0.339$, $P = 0.004$). In the 36 NAFLD patients who had a magnetic resonance imaging assessment of abdominal fat, there was a strong correlation of AFABP and lipocalin-2 levels with total abdominal ($r = 0.510$, $P < 0.001$ and $r = 0.435$, $P < 0.008$, respectively) and abdominal subcutaneous fat ($r = 0.577$, $P < 0.001$, and $r = 0.419$, $P = 0.01$, respectively), but there was no correlation with visceral fat. RBP4 levels did not correlate with any measurements of body fat in this cohort.

Exclusion of Subjects with Type 2 Diabetes. When subjects with type 2 diabetes were excluded, AFABP and lipocalin-2 levels remained significantly higher in NAFLD subjects compared with controls (31.6 versus 23.1 ng/mL, $P < 0.001$, and 62.3 versus 48.6 ng/mL, $P < 0.001$, respectively). RBP-4 levels were similar between NAFLD patients and controls (16.3 versus 16.9 ng/mL, $P = 0.2$).

Discussion

This report focuses on the three relatively recently described adipocytokines—AFABP, lipocalin-2, and

Table 3. Correlations between AFABP, Lipocalin-2, RBP4, and Adiposity, Insulin Resistance, and Lipids

	Adipocyte Fatty Acid Binding Protein (ng/mL)		Lipocalin-2 (ng/mL)		Retinol Binding Protein-4 (ng/mL)	
	NAFLD (n = 100)	Whole Cohort (n = 229)	NAFLD (n = 100)	Whole Cohort (n = 229)	NAFLD (n = 100)	Whole Cohort (n = 229)
BMI (kg/m^2)	0.42*	0.57*	0.09	0.27*	0.14	0.16†
Waist (cm)	0.37*	0.53*	0.23†	0.33*	0.14	0.15†
HOMA-IR	0.26†	0.42*	0.15	0.34*	−0.20†	−0.03
Triglycerides (mmol/L)	0.23†	0.27*	0.20†	0.29*	0.23†	0.29*
HDL (mmol/L)	0.08	−0.12	0.03	−0.24*	−0.07	−0.18*

All values are expressed as an r correlation coefficient (P value).
*$P < 0.01$.
†$P < 0.05$.

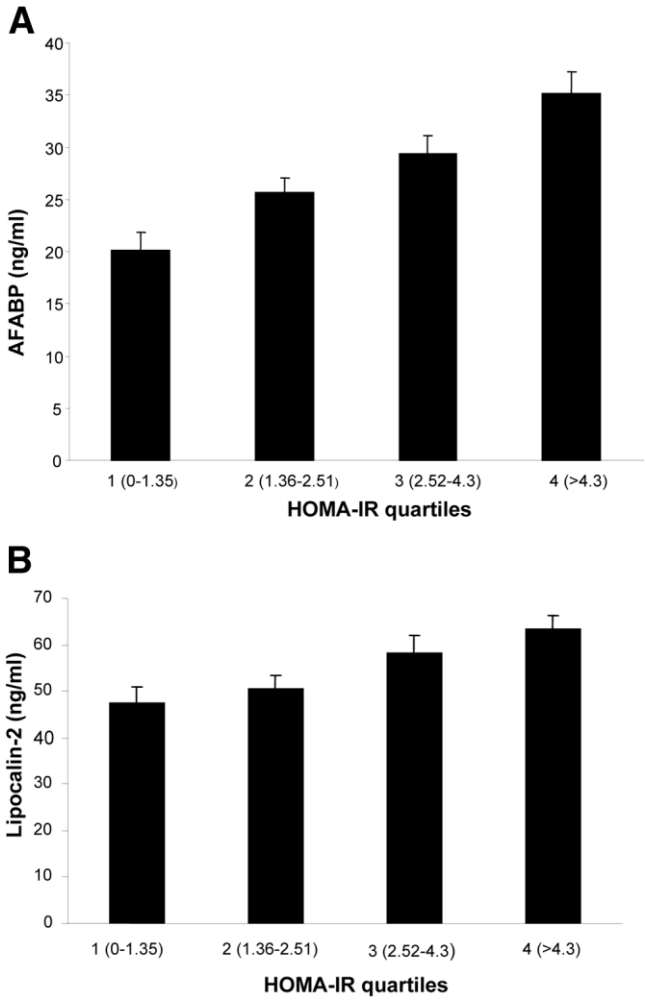

Fig. 2. Relationship of HOMA-IR by quartiles to (A) AFABP ($P < 0.001$) and (B) lipocalin-2 ($P = 0.001$).

RBP4—in patients with NAFLD. Serum levels of each of these peptides have been reported to be elevated in obesity and have been implicated in the inflammatory pathogenesis of the metabolic syndrome. In our cohort of control and NAFLD patients, we found a strong correlation between AFABP levels and all indices of adiposity (BMI, waist, and leptin levels), whereas lipocalin-2 and RBP4 correlated with BMI and waist circumference. We were unable to discern any association between serum AFABP, lipocalin-2, or RBP4 and the degree of steatosis in the NAFLD cohort ($P > 0.35$ in each case). The finding of elevated levels of AFABP in NAFLD is consistent with a recent report by Koh et al.[25] in a cohort with type 2 diabetes and liver steatosis on ultrasound. However, this study did not assess the relationship of AFABP to the severity of steatosis or to indices of inflammation/fibrosis, because biopsies were not part of the study protocol. In our study, AFABP (but not lipocalin-2 or RBP4) predicted fibrosis, even when measurements of insulin resistance, visceral fat, and other adipokines were considered. Our finding that RBP4 did not correlate with steatosis or inflammation/fibrosis in NAFLD agrees with a recent report by Petta et al.[35] in a small cohort with NAFLD, which did, however, suggest a relationship of this protein with steatosis in patients with genotype 1 chronic hepatitis C infection.

The AFABP relationship has substantial potential pathogenic significance, as in vitro and animal in vivo experiments have shown that FABPs are involved in a powerful interaction between adipocytes and macrophages, generating inflammation and insulin resistance.[19,36] Combined deficiency of AFABP and epidermal FABP (another minor form of FABP expressed in adipocytes and macrophages) in rodents, completely protects against fatty liver disease.[21,37] Moreover, absence or blockade of AFABP prevents bacterial lipopolysaccharide activation of nuclear factor kappa B and Jun N-terminal kinase 1 in macrophages.[38,39] Whereas the expression levels of AFABP in adipocytes are at least 100-fold higher than those in monocytes/macrophages, inflammatory stimuli, such as the Toll-like receptor 2 and Toll-like receptor 4 (lipopolysaccharide) ligands, cause over 1,000-fold increases in AFABP expression in macrophages.[40] Therefore, the precise source of elevated circulating serum AFABP in NAFLD patients remains unclear and requires further study. Although our cross-sectional study design prevents firm conclusions in humans about a cause-and-effect relationship, increased AFABP production could contribute to macrophage activity in the liver, possibly by enhancing fatty acid availability to hepatocytes and/or macrophages, with activation of inflammatory pathways, including nuclear factor kappa B,[17,19,36] resulting in inflammation and fibrosis. Whether this is mediated via circulating AFABP or via local production within the liver is at present unclear. The former is possible but not yet supported by published data.[19] An alternative hypothesis more in line with published evidence is that the elevated circulating AFABP reflects increased cellular production

Table 4. Multiple Ordinal Regression Analysis for Factors Associated with Lobular Inflammatory Grade, Ballooning, and Fibrosis Stage in NAFLD

Factor	Odds Ratio	95% Confidence Interval	*P* Value
Lobular inflammatory grade			
AFABP (per 10-ng/mL increase)	1.4	1.0–1.8	0.03
HOMA-IR (per unit increase)	1.1	1.0–1.2	0.06
Ballooning grade			
AFABP (per 10 ng/mL increase)	1.4	1.0–1.8	0.05
Age (per 5-year increase)	1.3	1.0–1.5	0.02
Adiponectin (per 5-μg/mL decrease)	1.4	1.0–1.9	0.05
Fibrosis stage			
AFABP (per 10-ng/mL increase)	1.3	1.0–1.7	0.03
WHR (per 1% increase)	1.1	1.0–1.2	0.03

in both adipocytes and macrophages in response to increased lipid availability, with increased Kupffer cell production of AFABP mediating an increased inflammatory response in the liver.

We recently reported that visceral fat is a strong predictor of liver inflammation and fibrosis in NAFLD[1] and have previously provided data suggesting that low adiponectin levels may be an important contributor.[13,15] The lack of correlation of AFABP with visceral fat in the subgroup (n = 36) of NAFLD subjects with magnetic resonance imaging measurement of subcutaneous and visceral abdominal fat is consistent with evidence that AFABP production is substantially greater in subcutaneous compared with visceral fat and clearly indicates that AFABP is not the mediator of the visceral fat effect.[41] Rather, AFABP and lipocalin-2 are closely related to overall and subcutaneous adiposity. Furthermore, the relationship of AFABP and visceral fat[1] with liver inflammation was independent of adiponectin levels. This raises important conceptual issues regarding the pathogenesis of inflammation in NASH. Thus, while recent evidence suggests that visceral fat and factors released from visceral adipose tissue are critical to the transition from steatosis to steatohepatitits, the present data regarding AFABP indicates that peripheral fat stores may also contribute to the development of steatohepatitis. Subcutaneous fat has been previously shown to contain increased macrophages and ceramides in those with increased liver fat[42,43] and to be a major contributor to systemic free fatty acid delivery,[44] suggesting its role in the pathogenesis of inflammation in the liver.

Insulin resistance is known to be an intrinsic defect in NAFLD[45] closely associated with steatosis, inflammation, and disease progression in NASH. Several clinical studies have supported this association by demonstrating a strong independent relationship between insulin resistance and inflammation and fibrosis severity in well-characterized NASH cohorts.[15,46,47] Moreover, insulin resistance and hyperinsulinemia can stimulate fibrogenesis via up-regulation of connective tissue growth factor and direct interactions with hepatic stellate cells, effects that are particularly marked in the presence of hyperglycemia.[48,49] Within this NAFLD cohort, AFABP correlated strongly with insulin resistance. Although the AFABP association with inflammation and fibrosis was independent of insulin resistance, it is likely that insulin resistance created the milieu in which specific AFABP (macrophage and adipocyte) interactions would promote disease progression.

All the measured novel adipocytokines in this study correlated in the whole population with indices of dyslipidemia. Lipocalin-2 and RBP4 both predicted increased triglycerides and reduced HDL, whereas AFABP predicted only elevated triglycerides. This is the typical dyslipidemia associated with the metabolic syndrome. Likewise, in all subjects AFABP and lipocalin-2 (but not RBP4) correlated strongly with the HOMA-IR measurement of insulin resistance. The fact that RBP4 only correlated positively with insulin resistance in controls is surprising, but adds to the growing uncertainty about the importance of this relationship.[50-52]

In conclusion, examination of AFABP, lipocalin-2 and RBP4 levels in control and NAFLD subjects has shown that AFABP and lipocalin-2 levels are elevated within both cohorts in relation to adiposity and AFABP is strongly related to insulin resistance. In the NAFLD cohort, none of these adipocytokines correlated with hepatic steatosis; however, AFABP correlated strongly with hepatic inflammation and fibrosis, a relationship that may indicate a causative effect. From this and other recent animal and human studies on adipose tissue contributions to the pathogenesis of hepatic steatosis and steatohepatitits, it is suggested that elevated leptin levels (or more likely leptin resistance), and reduced adiponectin could play a role in relation to steatosis, but that visceral fat volume, elevated AFABP levels, and decreased adiponectin levels may each contribute to the inflammation and fibrosis.

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