

# Lipocalin-2 Is an Inflammatory Marker Closely Associated with Obesity, Insulin Resistance, and Hyperglycemia in Humans

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**Background:** Lipocalin-2, a 25-kDa secreted glycoprotein, is a useful biomarker for early detection of various renal injuries. Because lipocalin-2 is abundantly expressed in adipose tissue and liver, we investigated its relevance to obesity-related pathologies.

**Methods:** We used real-time PCR and in-house immunoassays to quantify the mRNA and serum concentrations of lipocalin-2 in C57BL/KsJ *db/db* obese mice and their age- and sex-matched lean littermates. We analyzed the association between serum lipocalin-2 concentrations and various metabolic and inflammatory variables in 229 persons (121 men and 108 women) recruited from a previous cross-sectional study, and we evaluated the effect of the insulin-sensitizing drug rosiglitazone on serum lipocalin-2 concentrations in 32 diabetic patients (21 men and 11 women).

**Results:** Compared with the lean littermates, lipocalin-2 mRNA expression in adipose tissue and liver and its circulating concentrations were significantly increased in *db/db* diabetic/obese mice ( $P < 0.001$ ). These changes were normalized after rosiglitazone treatment. In humans, circulating lipocalin-2 concentrations were positively correlated ( $P < 0.005$ ) with adiposity, hypertriglyceridemia, hyperglycemia, and the insulin resistance index, but negatively correlated ( $P = 0.002$ ) with HDL cholesterol. There was also a strong positive association

between lipocalin-2 concentrations and high sensitivity C-reactive protein (hs-CRP), independent of age, sex, and adiposity ( $P = 0.007$ ). Furthermore, rosiglitazone-mediated decreases in lipocalin-2 concentrations correlated significantly with increases in insulin sensitivity ( $r = 0.527$ ;  $P = 0.002$ ) and decreases in hs-CRP concentrations ( $r = 0.509$ ;  $P = 0.003$ ).

**Conclusions:** Lipocalin-2 is an inflammatory marker closely related to obesity and its metabolic complications. Measurement of serum lipocalin-2 might be useful for evaluating the outcomes of various clinical interventions for obesity-related metabolic and cardiovascular diseases.

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Obesity is the most common risk factor for insulin resistance, type 2 diabetes mellitus (T2DM),<sup>6</sup> and cardiovascular disorders. Although the detailed molecular events that link obesity with its associated pathologies are not well understood, accumulating evidence suggests that systemic inflammation might be an important mediator (1, 2). Studies on both humans and animal models have demonstrated close associations between obesity and a state of low-grade, chronic inflammation characterized by macrophage infiltration in adipose tissue and increased circulating concentrations of proinflammatory molecules, including acute-phase proteins, cytokines, adipokines, and chemokines (3–6). In obese states, these proinflammatory factors are produced predominantly from enlarged adipocytes and activated macrophages in adipose

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<sup>6</sup> Nonstandard abbreviations: T2DM, type 2 diabetes mellitus; IL, interleukin; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; NGAL, neutrophil gelatinase-associated lipocalin; BMI, body mass index; PBS, phosphate-buffered saline; CRP, C-reactive protein; RBP4, retinol binding protein 4; HOMA-IR, homeostasis model assessment index for insulin resistance; hs-CRP, high-sensitivity C-reactive protein; A-FABP, adipocyte-fatty acid binding protein 4.

tissue and liver. Many of these inflammatory factors, such as interleukin (IL) 6, tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), and resistin, can directly induce glucose intolerance and insulin resistance by antagonizing insulin's metabolic actions at peripheral tissues, especially in liver and skeletal muscle (7, 8). On the other hand, several other adipokines produced from adipocytes, including adiponectin and visfatin, have demonstrated insulin-sensitizing activity and exertion of beneficial effects on glucose and lipid homeostasis (9–11).

Lipocalin-2, also known as 24p3 (12) and neutrophil gelatinase-associated lipocalin (NGAL) (13), is a 25-kDa secretory glycoprotein that was originally identified in mouse kidney cells and human neutrophil granules. This protein has been implicated in diversified functions such as apoptosis and innate immunity. In addition to neutrophils, lipocalin-2 is expressed in several other tissues, including liver, lung, kidney, adipocytes, and macrophages (14–16). Several inflammatory stimuli, such as lipopolysaccharides and IL 1 $\beta$ , can markedly induce lipocalin-2 expression and secretion in these cells (14, 17). Notably, the proinflammatory transcription factor NF- $\kappa$ B has been shown to transactivate lipocalin-2 expression through binding with a consensus motif in the promoter region of the lipocalin-2 gene (18), suggesting that this secretory protein might be involved in the inflammatory responses. Nevertheless, whether lipocalin-2 plays a role in the pathogenesis of obesity-related diseases has not been investigated to date.

Our laboratory has recently used a genome-wide interrogation strategy to identify differentially expressed genes during the development of obesity and its related pathologies in mice. As a result, we have found that the gene encoding lipocalin-2 was selectively induced in liver and adipose tissue of *db/db* obese/diabetic mice, and this increase was reversed by the insulin-sensitizing drug rosiglitazone. Accordingly, this study was designed to investigate the relationship between serum lipocalin-2 concentrations and obesity-related pathologies in mice and humans at baseline and after treatment with rosiglitazone (19).

## Materials and Methods

### ANIMAL STUDIES

The study included 15 C57BL/KsJ *db/db* diabetic mice and 15 lean littermates (The Jackson Laboratory; age 11–12 weeks). The metabolic characteristics of these mice are shown in Table 1. The mice were housed in a room at controlled temperature [mean (SD) 23 (1) °C], with free access to water and standard mouse chow. Rosiglitazone (a gift from GSK, Middlesex, UK) was administered to *db/db* mice by daily intragastric gavage (1 mg/kg body weight) for 2 weeks. All of the experiments were conducted in accordance with our institutional guidelines for the humane treatment of laboratory animals.

**Table 1. Metabolic parameters of *db/db* mice and lean mice used in this study.**

	Lean (n = 15)	<i>db/db</i> (n = 15)
Body weight, g	27.2 (1.3)	46.7 (2.9) <sup>a</sup>
Fasting glucose, mmol/L	7.3 (0.6)	23.6 (3.2) <sup>a</sup>
Fasting insulin, $\mu$ g/L	2.8 (1.5)	14.3 (6.7) <sup>a</sup>
Serum TG, mmol/L	0.93 (0.06)	2.34 (0.15) <sup>a</sup>

<sup>a</sup> P < 0.01 vs lean mice.

### HUMAN PARTICIPANTS

We measured lipocalin-2 concentrations in serum samples collected from a total of 229 adults (age range, 33–72 years) who were participants in our previous cross-sectional Hong Kong Cardiovascular Risk Factor Prevalence Study (20, 21). The clinical characteristics of these study participants, as described in our previous reports (20, 21), are shown in Table 2. Participants were selected from our database on the basis of their body mass index (BMI) and included 100 lean (BMI, <25 kg/m<sup>2</sup>), 80 overweight (BMI, 25–29.9 kg/m<sup>2</sup>), and 49 obese participants (BMI,  $\geq$ 30 kg/m<sup>2</sup>), so that a study sample covering a wide range of adiposity was available to evaluate the relationship between circulating adipokine concentrations and various cardio-metabolic parameters. In some analyses, a lower BMI (<23 kg/m<sup>2</sup>) was used as the cutoff for the lean group, according to guideline for Asian populations (22). Of 229 study participants, 69 had diabetes (fasting plasma glucose,  $\geq$ 7.0 mmol/L, or 2-h plasma glucose during a 75 g oral glucose tolerance test,  $\geq$ –11.1 mmol/L, as recommended by the WHO in 1998) and 76 had metabolic syndrome according to National Cholesterol Education Program criteria (23). Serum samples were stored at –70 °C until assayed.

For the rosiglitazone (Avandia) intervention study, 32 T2DM patients (21 men and 11 women; age range, 39–71 years) were treated with rosiglitazone (4 mg twice daily) for 8 weeks. The clinical characteristics of these individuals are listed in Table 2. The study protocol was approved by the Ethics Committee of the Medical Faculty, University of Hong Kong. All study participants provided written informed consent before participation.

### REAL TIME QUANTITATIVE PCR

We used the RNeasy reagent set (Qiagen) to isolate total RNA from mouse liver, epididymal adipose tissue, and soleus muscle. Subsequently, mRNA was reverse transcribed into cDNA with oligo(dT) primer (Roche). The relative gene abundance of lipocalin-2 was quantified by Taqman real-time PCR with predeveloped reagent sets (Applied BioSystems). The reactions were performed on the ABI 7000 sequence detection system.

### EXPRESSION AND PURIFICATION OF RECOMBINANT MURINE AND HUMAN LIPOCALIN-2

cDNA samples obtained from 3T3-L1 adipocytes were used as templates for amplification of the murine lipocalin-2.

**Table 2. Clinical characteristics of 229 subjects and correlation between serum lipocalin-2 concentrations and several study variables.**

Parameter	Value	r <sup>a</sup>	P <sup>a</sup>	r <sup>b</sup>	P <sup>b</sup>
BMI, kg/m <sup>2</sup>	27.1 (6.4)	0.394	<0.001		
WC, <sup>c</sup> cm	87.3 (15)	0.404	<0.001	0.111	0.085
Fat percentage, %	31.5 (10.0)	0.296	0.001	0.184	0.033
WHR	0.9 (0.1)	0.257	<0.001	0.052	0.423
Systolic blood pressure, mmHg	129.6 (21.2)	0.154	0.017	0.001	0.990
Diastolic blood pressure, mmHg	77.2 (11.0)	0.031	0.637	-0.088	0.173
Triglycerides, mmol/L	1.6 (1.1)	0.151	0.019	0.074	0.255
LDL cholesterol, mmol/L	3.3 (0.8)	-0.082	0.216	-0.075	0.259
HDL cholesterol, mmol/L	1.2 (0.3)	-0.200	0.002	-0.104	0.111
Fasting insulin, mIU/L	11.2 (7.3)	0.269	<0.001	0.069	0.286
Fasting glucose, mmol/L	6.3 (2.2)	0.148	0.021	0.142	0.027
2 h postprandial glucose, mmol/L	8.1 (3.9)	-0.063	0.451	-0.045	0.587
HOMA-IR	3.1 (2.3)	0.305	<0.001	0.145	0.025
hs-CRP, mg/L	2.2 (2.0)	0.236	0.002	0.202	0.007
Adiponectin, mg/L	6.4 (3.0)	0.05	0.507	0.115	0.098
A-FABP, ng/mL	24.3 (11.6)	0.245	<0.001	0.143	0.047

<sup>a</sup> Adjusted for sex and age.

<sup>b</sup> Adjusted for sex, age, and BMI. Note that the fat percentage was determined by bioelectric impedance analysis (Model TBF-410; Tanita).

<sup>c</sup> WC, waist circumference; WHR, waist-hip ratio.

lin-2 gene with 5'-AGT AGG ATC CCA GGA CTC AAC TCA GAA CTT G-3' and 5'-AGT ACT CGA GTC AGT TGT CAA TGC ATT GGT C-3' as upstream and downstream primers, respectively. The amplified PCR products were double-digested by the restriction enzymes BamHI and XhoI and subsequently subcloned into the pPROEX-HTb vector (Invitrogen), leading to an expression vector pPRO-His-mLCN2, which encodes NH<sub>2</sub>-terminal His<sub>6</sub>-tagged murine lipocalin-2 with 181 amino acid residues starting immediately after secretory signal peptide. The gene encoding human lipocalin-2 was amplified from cDNA derived from human HepG2 cells, with 5'-TGC AGG ATC CCA GGA CTC CAC CTC AGA CC-3' and 5'-TAG CCT CGA GTC AGC CGT CGA TAC ACT GGT CG-3' as sense and antisense primers, respectively. After digestion with BamHI and XhoI, the PCR fragments were cloned into a pPROEX-HTb vector to generate the construct pPRO-His-hLCN2 for prokaryotic expression of NH<sub>2</sub> terminal His<sub>6</sub>-tagged human lipocalin-2 with secretory signal peptide removed. The inserted human or murine lipocalin-2 cDNA was verified by DNA sequencing.

The expression of His-tagged murine or human lipocalin-2 in BL21 cells was induced by the addition of 0.5 mmol/L of isopropyl-β-D-thiogalactopyranoside to the growth medium. The recombinant proteins were purified from the bacterial lysates using the Ni<sup>2+</sup>-nitrilotriacetic acid-agarose column as we described previously (24). After purification, the NH<sub>2</sub>-terminal His<sub>6</sub> tag was removed by cleavage with recombinant TEV protease, and the endotoxin was removed by use of the Detoxi-gel Endotoxin-Removal Kit (Pierce). The purity of the protein was confirmed by sodium dodecyl sulfate-polyacrylam-

ide gel electrophoresis and fast protein liquid chromatography analysis.

#### ANTIBODY PRODUCTION AND DEVELOPMENT OF SANDWICH ELISA FOR QUANTIFICATION OF MURINE AND HUMAN LIPOCALIN-2

The polyclonal antibodies against human or murine recombinant lipocalin-2 were generated in New Zealand female rabbits as described previously (25). Antihuman or antimurine lipocalin-2 IgG was purified from the immunized rabbit serum with protein A/G beads, followed by affinity chromatography with their respective antigens as the ligands.

The affinity-purified antihuman or antimurine lipocalin-2 IgG was biotinylated with a reagent set from Pierce and used as the detection antibody. Unlabeled antihuman or antimurine lipocalin-2 IgG was used for coating a 96-well microtiter plate overnight at 4 °C. Human or mouse serum was diluted (1:50) into phosphate-buffered saline (PBS) (10 mmol/L sodium phosphate, 137 mmol/L NaCl, 2.7 mmol/L KCl, pH 7.4), and 100 μL of the diluted samples or recombinant standards were applied to each well and incubated at 37 °C for 1 h. The plates were washed 3 times and then incubated with 100 μL of the detection antibody for 2 h. After being washed 3 more times with PBS, the wells were incubated with streptavidin-conjugated horseradish peroxidase for 1 h and subsequently reacted with tetramethyl-benzidine reagent for 15 min. A total of 100 μL of 2 mol/L H<sub>2</sub>SO<sub>4</sub> was added to each well to stop the reaction, and the absorbance at 450 nm was measured. Five human serum samples with lipocalin-2 concentration ranges above (150–180 μg/L), within (60–80 μg/L), and below the reference interval

(20–40  $\mu\text{g/L}$ ) were used to determine the intra- and interassay CVs. These samples were tested 12 times on 1 plate and in 12 separate assays to assess intraassay and interassay variations, respectively.

#### STATISTICAL ANALYSIS

All the statistical calculations were performed with the SPSS 11.5 statistical software package (SPSS Inc). We used the Kolmogorov–Smirnov test to test variables for normality. The Student unpaired *t*-test was used for comparison between 2 groups, and a paired *t*-test for intragroup comparison before and after rosiglitazone treatment. Partial Pearson correlation coefficients were used to establish the relationship between lipocalin-2 concentrations and various clinical characteristics, with adjustment for sex, age, and/or BMI. We also stratified human participants into 5 groups according to number of components of the metabolic syndrome (0, 1, 2, 3,  $\geq 4$ ) and used 1-way ANOVA with Scheffé-type multiple comparison test to compare serum lipocalin-2 concentrations among these groups. In all statistical comparisons, a *P* value  $<0.05$  was used to indicate a significant difference.

### Results

#### LIPOCALIN-2 EXPRESSION IS INCREASED IN DB/DB DIABETIC/OBESE MICE AND IS SUPPRESSED AFTER TREATMENT WITH THE PPAR $\gamma$ AGONIST ROSIGLITAZONE

We used microarray analysis for systematic identification of genes differentially expressed in *db/db* obese/diabetic mice and their lean littermates. Our results showed that the expression of a gene encoding lipocalin-2, a secretory glycoprotein, was markedly induced in the liver tissue of *db/db* obese/diabetic mice. To explore the relevance of lipocalin-2 with obesity, we used real-time quantitative PCR analysis to further compare the expression profiles of lipocalin-2 in male *db/db* mice and their age- and sex-matched lean littermates in various tissues. In both liver and adipose tissue, the steady-state mRNA concentrations of the lipocalin-2 gene in obese/diabetic *db/db* mice were substantially higher than those in their age- and sex-matched lean littermates (Fig. 1), and these changes were reversed after 2 weeks of treatment with rosiglitazone. On the other hand, the concentrations of lipocalin-2 mRNA in several other tissues, including lung, kidney, and spleen were comparable between the obese and lean mice.

To investigate whether altered mRNA expression in adipose tissue and liver leads to changes in circulating concentrations of lipocalin-2, we developed a sandwich ELISA method for measuring this protein in serum samples. To this end, we generated a polyclonal antibody against mouse lipocalin-2, using the recombinant protein as the antigen (Fig. 2). The specificity of this antibody was validated by its selective immunoprecipitation of recombinant lipocalin-2 from bacterial lysate and serum samples (data not shown). The sandwich ELISA standard curve generated with recombinant murine lipocalin-2

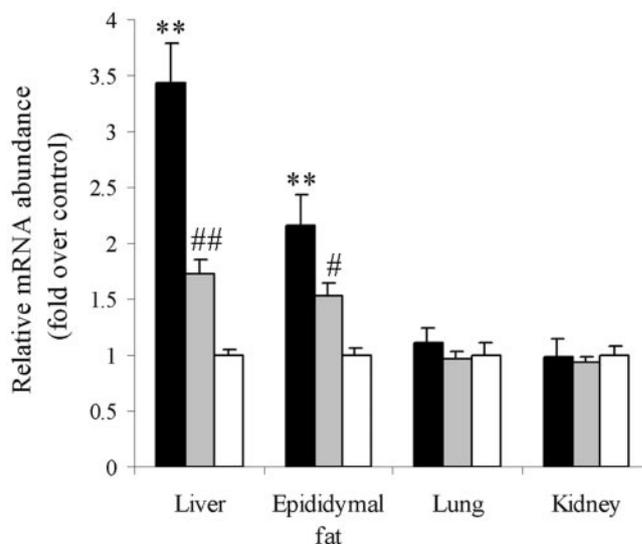


Fig. 1. The relative mRNA abundance of the lipocalin-2 gene in lean mice and *db/db* obese mice treated without or with rosiglitazone (Rosi).

Total RNA was extracted from male lean mice or age- and sex-matched *db/db* mice treated without or with rosiglitazone for 2 weeks. The steady-state mRNA concentrations of lipocalin-2 in liver, epididymal fat pads, lung, and kidney were quantified with real-time PCR and normalized against 18S RNA. \*\*, *P*  $<0.01$  vs lean control; #, *P*  $<0.05$  vs *db/db* mice without treatment; ##, *P*  $<0.01$  vs *db/db* mice without treatment. ■, *db/db* (*n* = 15); ▒, *db/db* + Rosi (*n* = 13); □, lean (*n* = 15).

yielded a consistent *r* value  $>0.985$ . This analysis revealed that circulating concentrations of lipocalin-2 in *db/db* mice were significantly higher than those in their lean littermates. On the other hand, rosiglitazone treatment decreased circulating lipocalin-2 concentrations in *db/db* mice.

#### SERUM CONCENTRATIONS OF LIPOCALIN-2 ARE INCREASED IN OBESE HUMANS AND ARE CLOSELY ASSOCIATED WITH SEVERAL ANTHROPOMETRIC, BIOCHEMICAL, AND INFLAMMATORY VARIABLES

To investigate the relationship between lipocalin-2 and obesity in humans, we also developed an in-house immunoassay for measurement of human lipocalin-2. Western blot analysis and immunoprecipitation revealed that the affinity-purified antihuman lipocalin-2 antibody selectively recognized an  $\sim 25$ -kDa protein in human serum, which was subsequently confirmed by tandem mass spectrometry analysis to be human lipocalin-2 (data not shown). The immunoassay based on this antibody was highly specific to human lipocalin-2, with no detectable cross-reactivity to several other human adipokines and cytokines, including adiponectin, leptin, resistin, TNF $\alpha$ , C-reactive protein (CRP), IL-6, and other lipocalin family members, including retinol-binding protein 4 (RBP4) and adipocyte fatty acid binding protein (data not shown). The intra- and interassay CVs were 3.8%–6.0% and 3.1%–5.2%, respectively. This analysis showed that the circulating concentrations of lipocalin-2 were 20.9–182.5  $\mu\text{g/L}$  in the 229 study participants and exhibited gaussian distri-

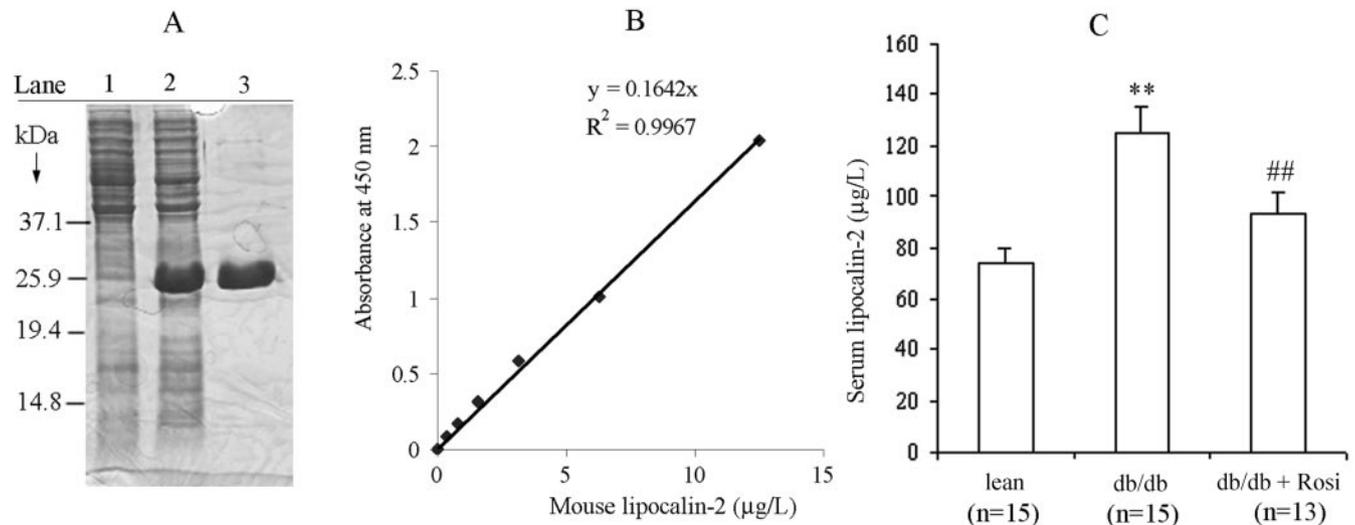


Fig. 2. Effect of rosiglitazone on increased serum concentrations of lipocalin-2 in *db/db* mice.

(A), purification of recombinant lipocalin-2 from *Escherichia coli* as an antigen for antibody production. Thirty  $\mu\text{g}$  of proteins from bacterial lysate before (lane 1) or after induction with isopropylthio- $\beta$ -D-galactoside (lane 2) or with 5  $\mu\text{g}$  of the purified protein (lane 3) was separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue R250. (B), the ELISA standard curve was generated with different concentrations of the recombinant protein. (C), serum concentrations of lipocalin-2 in heterozygous lean mice and *db/db* mice treated without or with rosiglitazone, as described in *Materials and Methods*. \*\*,  $P < 0.01$  vs lean control; ##,  $P < 0.01$  vs *db/db* mice without treatment.

bution by Kolmogorov–Smirnov test. There was a trend toward a positive association between serum concentrations of lipocalin-2 and age ( $P = 0.055$  and  $0.068$  before and after adjustment for sex, respectively).

Serum lipocalin-2 concentrations in males were significantly higher than those in females in both obese and lean groups (Table 3). Notably, the circulating concentrations of lipocalin-2 in obese persons were significantly higher than those in lean persons, and this difference was significant for both sexes. A strong positive correlation was observed between serum lipocalin-2 concentrations and BMI (Table 2). After adjustment for sex and age, serum concentrations of lipocalin-2 were positively correlated with waist-to-hip ratio; waist circumference; fat percentage; systolic blood pressure; fasting serum concentrations of insulin, triglycerides, and glucose; and the insulin resistance index by homeostasis model assessment (HOMA-IR) (26). On the other hand, lipocalin-2 concentrations were negatively correlated with fasting serum concentrations of HDL cholesterol. Notably, the positive correlations of serum lipocalin-2 with fasting glucose and HOMA-IR were still significant even after adjustment for

BMI, suggesting that lipocalin-2 might be an independent risk factor for insulin resistance and hyperglycemia in humans. Notably, circulating lipocalin-2 concentrations in current smokers were much higher than those in non-smokers [mean (SD)  $93.5$  ( $45.0$ ) vs  $66.3$  ( $36.0$ )  $\mu\text{g/L}$ ;  $P = 0.001$ ], and this significance remained even after adjustment for age, sex, and BMI ( $P = 0.031$ ).

Several previous *in vitro* studies have suggested lipocalin-2 to be an acute-phase protein that can be induced by a variety of inflammatory stimuli (27, 28). Consistent with these experimental data, we observed a strong positive correlation between circulating lipocalin-2 and high-sensitivity CRP (hs-CRP), an established serum marker for chronic inflammation. This correlation remained significant even after adjusting for age, sex, and adiposity, suggesting that lipocalin-2 is independently associated with inflammation. In addition, serum lipocalin-2 concentrations were positively associated with adipocyte-fatty acid binding protein (A-FABP), a novel serum marker for adiposity and metabolic syndrome (20). On the other hand, there was no significant correlation between serum concentrations of lipocalin-2 and adiponectin (Table 2).

Patients with diabetes had higher mean (SD) serum concentrations of lipocalin-2 than those without this disease [ $82.4$  ( $43.6$ ) vs  $69.6$  ( $32.9$ )  $\mu\text{g/L}$ ;  $P = 0.039$  after adjustment for age and sex], but this significance disappeared after adjustment for BMI ( $P = 0.122$ ). Mean (SD) serum lipocalin-2 concentrations in patients with metabolic syndrome were significantly higher than those without metabolic syndrome [ $83.2$  ( $39.6$ ) vs  $67.5$  ( $32.7$ )  $\mu\text{g/L}$ ;  $P = 0.024$  after adjustment for age and sex]. When study patients were further stratified according to the number of

**Table 3. Comparison of serum lipocalin-2 concentrations ( $\mu\text{g/L}$ ) in lean (BMI,  $<23$   $\text{kg/m}^2$ ) and obese (BMI,  $>30$   $\text{kg/m}^2$ ) humans.**

	Obese	Lean
Female	92.9 (46.6) ( $n = 25$ )	57.6 (29.3) <sup>a</sup> ( $n = 31$ )
Male	117.7 (48.7) <sup>b</sup> ( $n = 24$ )	72.1 (36.8) <sup>b, c</sup> ( $n = 32$ )
Total	105.0 (48.8) ( $n = 49$ )	65.6 (33.7) <sup>c</sup> ( $n = 63$ )

<sup>a</sup>  $P < 0.05$  vs obese group.

<sup>b</sup>  $P < 0.05$  vs female group.

<sup>c</sup>  $P < 0.001$  vs obese group.

the components of metabolic syndrome, mean (SD) serum concentrations of lipocalin-2 for those with 0, 1, 2, 3 and  $\geq 4$  components of metabolic syndrome were 63.7 (29.4)  $\mu\text{g/L}$  ( $n = 43$ ), 68.9 (34.6)  $\mu\text{g/L}$  ( $n = 61$ ), 69.1 (37.7)  $\mu\text{g/L}$  ( $n = 49$ ), 79.3 (36.9)  $\mu\text{g/L}$  ( $n = 40$ ), and 87.7 (43.5)  $\mu\text{g/L}$  ( $n = 36$ ), respectively. However, the correlation between serum lipocalin concentrations and the number of the metabolic syndrome criteria was not significant ( $P = 0.097$ ).

#### CHANGES IN SERUM LIPOCALIN-2 CONCENTRATIONS ARE CORRELATED WITH THE OUTCOMES OF CLINICAL INTERVENTIONS IN T2DM PATIENTS

We next investigated whether serum lipocalin-2 is a useful surrogate marker for monitoring the therapeutic responses to the PPAR $\gamma$  agonist rosiglitazone, a drug with insulin-sensitizing and antiinflammatory activities. To this end, we analyzed serum samples from 32 T2DM patients treated with rosiglitazone (4 mg twice daily) for 8 weeks. The clinical features of these patients are listed in Table 2. During the 8-week intervention, there was no significant change in BMI, waist circumference, or serum triglycerides, whereas fasting glucose, fasting insulin, and HOMA-IR were all significantly decreased (Table 4). Rosiglitazone treatment was also associated with a marked decrease in circulating concentrations of lipocalin-2 and hs-CRP. Furthermore, rosiglitazone-mediated relative changes in serum lipocalin-2 concentrations correlated well with relative changes in insulin sensitivity ( $r = 0.527$ ;  $P = 0.002$ ) and CRP ( $r = 0.509$ ;  $P = 0.003$ ). Serum concentrations of adiponectin were increased after rosiglitazone treatment (Table 4), but there was no significant correlation between changes in serum adiponectin and lipocalin-2 concentrations.

#### Discussion

Although lipocalin-2 was identified more than a decade ago, the physiologic functions of this protein remain

poorly understood. Previous studies have focused on the role of this protein in the innate immune response to bacterial infection (29) and in apoptosis (30). Several recent reports suggest that lipocalin-2 might be a sensitive biomarker for early renal injury (31, 32). In cardiopulmonary bypass-induced acute renal injury and cisplatin-induced nephrotoxic injury, increased de novo synthesis of lipocalin-2 in proximal tubule cells leads to sharply increased concentrations of this protein in both urine and serum.

Our study provides both clinical and experimental evidence demonstrating that circulating lipocalin-2 is a marker for obesity and its associated pathologies. Although many tissues express lipocalin-2, our results suggest that adipose tissue and liver are probably the 2 principal sources that contribute to the increased circulating concentrations of this protein in obesity states. In *db/db* obese mice, increased serum concentrations of lipocalin-2 are associated with the selective elevation of its mRNA expression in adipose tissue and liver (Fig. 1). In humans, lipocalin-2 concentrations are positively correlated with several adiposity variables, including BMI, waist circumference, and fat percentage (Table 2), suggesting that the increased fat mass might also account for the increased circulating concentrations of this protein in obese humans.

In line with our results, several previous *in vitro* studies have demonstrated the abundant expression of lipocalin-2 in adipocytes (15) and macrophages (16), the 2 major cell populations in obese adipose tissue. Furthermore, lipocalin-2 expression is sharply increased after conversion of preadipocytes to mature adipocytes or inflammatory stimulation (33). Expression of lipocalin-2 in both adipose tissue and liver can be induced by lipopolysaccharides, suggesting lipocalin-2 to be an acute-phase protein (27). Consistent with our data from *db/db* diabetic/obese mice, an earlier microarray-based study also found a marked increase in lipocalin-2 expression in white adipose tissue of *ob/ob* obese mice (34). In contrast, another study of *ob/ob* mice demonstrated decreased lipocalin-2 expression in adipose tissue but increased expression in liver (27). Although we cannot explain the observed differences between these studies, these data collectively support the role of lipocalin-2 as an adipokine linked to the development of obesity-related metabolic disorders in animal models.

In agreement with the animal data, our clinical study results showed a marked increase of circulating lipocalin-2 in obese humans (Table 3), findings similar to those occurring with many insulin resistance-inducing and inflammatory adipokines and cytokines, such as TNF $\alpha$ , IL6, and resistin (1, 2). Furthermore, we observed a significant positive correlation between lipocalin-2 concentrations and several variables associated with obesity-related metabolic disorders, including adverse lipid profiles (increased serum triglyceride and decreased HDL cholesterol), hyperinsulinemia, fasting glucose concentrations,

**Table 4. Effects of rosiglitazone treatment on serum concentrations of lipocalin-2 and several other metabolic and inflammatory parameters in 32 T2DM patients (21 male and 11 female).**

	Baseline	8 weeks
Age, years	55.4 (9.8)	
BMI, kg/m <sup>2</sup>	25.4 (3.6)	25.5 (3.6)
Waist circumference, cm	86.8 (10.1)	86.9 (10.3)
Triglycerides, mmol/L	1.7 (1.0)	1.8 (1.1)
Fasting glucose, mmol/L	8.7 (1.5)	6.8 (1.2) <sup>a</sup>
Fasting insulin, mIU/L	10.2 (5.0)	7.9 (3.5) <sup>b</sup>
HOMA-IR	4.0 (2.2)	2.4 (1.2) <sup>a</sup>
CRP, mg/L	2.0 (1.9)	1.2 (1.3) <sup>a</sup>
Serum adiponectin, mg/L	7.7 (5.5)	17.1 (13.0) <sup>a</sup>
Lipocalin 2, $\mu\text{g/L}$	84.8 (25.2)	57.1 (22.0) <sup>a</sup>

<sup>a</sup>  $P < 0.01$  vs baseline.

<sup>b</sup>  $P < 0.05$ .

and the insulin resistance index (HOMA-IR). The association of lipocalin-2 with fasting glucose and HOMA-IR remained significant even after adjustment for age, sex, and adiposity, suggesting that this protein might be an independent risk factor for hyperglycemia and insulin resistance in humans.

Chronic low-grade inflammation is now recognized to be a key mediator in the development of obesity-related metabolic and cardiovascular diseases. Numerous epidemiologic studies have demonstrated that circulating hs-CRP, an established marker of chronic inflammation, is an independent risk factor for T2DM as well as atherosclerotic heart diseases (35, 36). In this study, we have demonstrated a close association between lipocalin-2 and hs-CRP, independent of age, sex, and adiposity. Both lipocalin-2 and hs-CRP are increased in obese individuals. Furthermore, the relative changes in serum lipocalin-2 concentrations correlate well with the relative changes in CRP concentrations after treatment with rosiglitazone, a drug with antiinflammatory activities (19). These results suggest that lipocalin-2 can be considered a marker for monitoring obesity-related low-grade inflammation in humans.

The physiologic relevance of lipocalin-2 with respect to obesity and its related pathologies remains to be determined. Notably, 2 other members of the lipocalin family, RBP4 and A-FABP, which are also produced from adipocytes, have recently been reported to play important roles in regulating systemic energy homeostasis, insulin sensitivity, and inflammation in animal models. Transgenic overexpression of RBP4 or injection of recombinant RBP4 in normal mice causes insulin resistance (37). Conversely, genetic deletion of RBP4 enhances insulin sensitivity. Several independent studies have shown that targeted disruption of the A-FABP gene provides significant protection from both dietary and genetic obesity-associated insulin resistance, hyperglycemia, and fatty liver diseases and also leads to marked alleviation of inflammation and atherosclerosis associated with ApoE-deficient mice (38–40). Our preliminary results showed that administration of a neutralizing antibody that blocks the actions of lipocalin-2 can alleviate insulin resistance, hyperglycemia, and inflammation in *db/db* mice (unpublished observations), suggesting that increased lipocalin-2 might play a etiologic role in the development of obesity-related pathologies, a possibility that is currently under active investigation in our laboratory.

In summary, our study provides the first clinical evidence demonstrating that serum concentrations of lipocalin-2 are closely associated with obesity and its related chronic inflammation and metabolic complications. Our findings that rosiglitazone-mediated decreases in serum lipocalin-2 concentrations correlate well with decreases in inflammation and insulin sensitivity suggest that serum lipocalin-2 can be a useful marker for evaluating the therapeutic outcomes of clinical interventions for obesity-

related metabolic and cardiovascular diseases. The major limitation of this study, however, is the lack of data providing insight into the mechanisms that underlie the increase of serum lipocalin-2 concentrations in obesity and its related pathologies. In addition, we cannot address the question of whether increased lipocalin-2 is a causative factor or simply an irrelevant bystander in the pathogenesis of obesity-associated metabolic abnormalities. Further longitudinal studies should allow us to determine the direction of the observed associations, the regulatory factors that modulate serum lipocalin-2, and the predictive value of this biomarker for obesity-associated metabolic and cardiovascular diseases.

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## References

1. Berg AH, Scherer PE. Adipose tissue, inflammation, and cardiovascular disease. *Circ Res* 2005;96:939–49.
2. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. *J Clin Invest* 2005;115:1111–9.
3. Tataranni PA, Ortega E. A burning question: does an adipokine-induced activation of the immune system mediate the effect of overnutrition on type 2 diabetes? *Diabetes* 2005;54:917–27.
4. Canello R, Henegar C, Viguier N, Taleb S, Poitou C, Rouault C, et al. Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes* 2005; 54:2277–86.
5. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 2003;112:1821–30.
6. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003;112:1796–808.
7. Trayhurn P, Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. *Br J Nutr* 2004;92:347–55.
8. Fantuzzi G. Adipose tissue, adipokines, and inflammation. *J Allergy Clin Immunol* 2005;115:911–9.
9. Kobayashi K. Adipokines: therapeutic targets for metabolic syndrome. *Curr Drug Targets* 2005;6:525–9.
10. Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. *Endocr Rev* 2005;26:439–51.
11. Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, et al. Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science* 2005;307:426–30.
12. Hrabá-Renevey S, Turler H, Kress M, Salomon C, Weil R. SV40-induced expression of mouse gene 24p3 involves a post-transcriptional mechanism. *Oncogene* 1989;4:601–8.
13. Kjeldsen L, Johnsen AH, Sengelov H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J Biol Chem* 1993;268:10425–32.
14. Liu Q, Nilsen-Hamilton M. Identification of a new acute phase protein. *J Biol Chem* 1995;270:22565–70.
15. Kratchmarova I, Kalume DE, Blagoev B, Scherer PE, Podtelejnikov AV, Molina H, et al. A proteomic approach for identification of

- secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. *Mol Cell Proteomics* 2002;1:213–22.
16. Meheus LA, Fransen LM, Raymackers JG, Blockx HA, Van Beeumen JJ, Van Bun SM, et al. Identification by microsequencing of lipopolysaccharide-induced proteins secreted by mouse macrophages. *J Immunol* 1993;151:1535–47.
  17. Jayaraman A, Roberts KA, Yoon J, Yarmush DM, Duan X, Lee K, et al. Identification of neutrophil gelatinase-associated lipocalin (NGAL) as a discriminatory marker of the hepatocyte-secreted protein response to IL-1 $\beta$ : a proteomic analysis. *Biotechnol Bioeng* 2005;91:502–15.
  18. Fujino RS, Tanaka K, Morimatsu M, Tamura K, Kogo H, Hara T. Spermatogonial Cell-mediated Activation of An IkappaBzeta-independent NF-kappaB Pathway in Sertoli Cells Induces Transcription of the Lipocalin-2 Gene. *Mol Endocrinol* 2006;4:904–15.
  19. Staels B, Fruchart JC. Therapeutic roles of peroxisome proliferator-activated receptor agonists. *Diabetes* 2005;54:2460–70.
  20. Xu A, Wang Y, Xu JY, Stejskal D, Tam S, Zhang J, et al. Adipocyte fatty acid binding protein is a plasma biomarker closely associated with obesity and metabolic syndrome. *Clin Chem* 2006;52:405–13.
  21. Tan KC, Wat NM, Tam SC, Janus ED, Lam TH, Lam KS. C-reactive protein predicts the deterioration of glycemia in Chinese subjects with impaired glucose tolerance. *Diabetes Care* 2003;26:2323–8.
  22. Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. *Lancet* 2004;363:157–63.
  23. Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA* 2001;285:2486–97.
  24. Wang Y, Xu A, Knight C, Xu LY, Cooper GJ. Hydroxylation and glycosylation of the four conserved lysine residues in the collagenous domain of adiponectin. Potential role in the modulation of its insulin-sensitizing activity. *J Biol Chem* 2002;277:19521–9.
  25. Xu A, Lam MC, Chan KW, Wang Y, Zhang J, Hoo RL, et al. Angiotensin-like protein 4 decreases blood glucose and improves glucose tolerance but induces hyperlipidemia and hepatic steatosis in mice. *Proc Natl Acad Sci U S A* 2005;102:6086–91.
  26. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and  $\beta$ -cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985;28:412–9.
  27. Lin Y, Rajala MW, Berger JP, Moller DE, Barzilai N, Scherer PE. Hyperglycemia-induced production of acute phase reactants in adipose tissue. *J Biol Chem* 2001;276:42077–83.
  28. Cowland JB, Muta T, Borregaard N. IL-1 $\beta$ -specific upregulation of neutrophil gelatinase-associated lipocalin is controlled by IkappaB-zeta. *J Immunol* 2006;176:5559–66.
  29. Flo TH, Smith KD, Sato S, Rodriguez DJ, Holmes MA, Strong RK, et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestering iron. *Nature* 2004;432:917–21.
  30. Devireddy LR, Teodoro JG, Richard FA, Green MR. Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation. *Science* 2001;293:829–34.
  31. Mishra J, Mori K, Ma Q, Kelly C, Barasch J, Devarajan P. Neutrophil gelatinase-associated lipocalin: a novel early urinary biomarker for cisplatin nephrotoxicity. *Am J Nephrol* 2004;24:307–15.
  32. Mishra J, Dent C, Tarabishi R, Mitsnefes MM, Ma Q, Kelly C, et al. Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. *Lancet* 2005;365:1231–8.
  33. Baudry A, Yang ZZ, Hemmings BA. PKB $\alpha$  is required for adipose differentiation of mouse embryonic fibroblasts. *J Cell Sci* 2006;119:889–97.
  34. Soukas A, Cohen P, Socci ND, Friedman JM. Leptin-specific patterns of gene expression in white adipose tissue. *Genes Dev* 2000;14:963–80.
  35. Haffner SM. The metabolic syndrome: inflammation, diabetes mellitus, and cardiovascular disease. *Am J Cardiol* 2006;97:3A–11A.
  36. Tsimikas S, Willerson JT, Ridker PM. C-reactive protein and other emerging blood biomarkers to optimize risk stratification of vulnerable patients. *J Am Coll Cardiol* 2006;47:C19–31.
  37. Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, et al. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* 2005;436:356–62.
  38. Maeda K, Cao H, Kono K, Gorgun CZ, Furuhashi M, Uysal KT, et al. Adipocyte/macrophage fatty acid binding proteins control integrated metabolic responses in obesity and diabetes. *Cell Metab* 2005;1:107–19.
  39. Hotamisligil GS, Johnson RS, Distel RJ, Ellis R, Papaioannou VE, Spiegelman BM. Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. *Science* 1996;274:1377–9.
  40. Boord JB, Maeda K, Makowski L, Babaev VR, Fazio S, Linton MF, et al. Adipocyte fatty acid-binding protein, aP2, alters late atherosclerotic lesion formation in severe hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 2002;22:1686–91.