# Decreased Circulating Lactoferrin in Insulin Resistance and Altered Glucose Tolerance as a Possible Marker of Neutrophil Dysfunction in Type 2 Diabetes

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**Context:** Lactoferrin is an innate immune system protein with multiple beneficial health activities.

**Objective:** To gain insight in the interaction between innate immune system and metabolic disturbances (obesity and insulin resistance), we investigated the relationship between circulating lactoferrin and chronic inflammation-associated insulin resistance according glucose tolerance status in Caucasian population.

**Design, Setting, Participants, and Main Outcome Measures:** Circulating nonstressed lactoferrin (ELISA), metabolic variables, and inflammatory markers were measured in 229 men, 94 with normal (NGT) and 135 with altered glucose tolerance (AGT). Lactoferrin secretion by neutrophil was investigated in whole-blood culture (four young NGT subjects, four older NGT subjects, and four patients with type 2 diabetes) under microbial lipopolysaccharide (LPS) with IL-6 and rosiglitazone treatment. We also tested the lactoferrin action in THP-1 cells under LPS stimulus.

**Results:** Circulating lactoferrin was significantly decreased in patients with AGT (431.5 $\pm$ 187.5 *vs.* 493.5 $\pm$ 238.9 ng/ml, $P = 0.02$). In addition, circulating lactoferrin was negatively associated with hyperglycemia and obesity measures and positively with insulin sensitivity. Lactoferrin was negatively related to inflammatory markers, especially in AGT subjects. In *ex vivo* experiments, we found a significant decrease in LPS-induced lactoferrin release from neutrophils in subjects with type 2 diabetes. IL-6 coincubation decreased LPS-induced lactoferrin release in NGT subjects ($P < 0.001$). Finally, rosiglitazone treatment led to increased lactoferrin secretion (398 $\pm$ 193 *vs.* 280.1 $\pm$ 104.9 ng/ml, $P < 0.0001$). Lactoferrin decreased nuclear factor-$\kappa\beta$ activation and IL-6, IL-8, and macrophage chemoattractant protein-1 expression under LPS challenge.

**Conclusions:** Decreased circulating lactoferrin levels may play a role in chronic low level inflammation-associated insulin resistance. ***(J Clin Endocrinol Metab* 94: 4036–4044, 2009)**

Central to metabolic diseases is insulin resistance associated with a low-grade inflammatory status (1–3). The mechanisms through which proinflammatory cytokines, like TNF-$\alpha$, IL-6, and IL-1$\beta$ interact with cellular insulin signal transduction cascades have been described in the last years (4–7). *In vivo*, a direct correlation between increased circulating proinflammatory cytokines and insulin resistance has been well demonstrated (3). The origin of this increased inflammatory activity in obesity and type 2 diabetes is virtually unknown. Immune system ho-

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Abbreviations: AGT, Altered glucose tolerance; BMI, body mass index; BPI, bactericidal permeability increasing; HbA1c, glycosylated hemoglobin; LBP, LPS binding protein; LPS, lipopolysaccharide; MCP, macrophage chemoattractant protein; NF-$\kappa\beta$, nuclear factor-$\kappa\beta$; NGT, normal glucose tolerance; PB, peripheral blood; sCD14, soluble CD14.

meostasis is challenged by continuous external insults, like saturated fatty acid-rich diets (8), pathogen-associated molecular patterns like lipopolysaccharide (LPS) (9) and advanced glycation end products (10), burden of infection (11), and oxidative stress (12). These continuous insults could result in a chronic low-level inflammation that is associated with insulin resistance.

Lactoferrin is a pleiotropic glycoprotein of the innate immune system that is involved in LPS buffering. Lactoferrin is a monomeric, 80-kDa glycoprotein, with a single polypeptide chain of about 690 amino acid residues and two sialic acid molecules, which is produced by neutrophils and several epithelia types. Lactoferrin is folded into homologous N- and C-terminal lobes, each comprising two domains that enclose a conserved iron binding site. This protein is positively charged in N-terminal region (the first 60 amino acids) of N-lobe at physiological pH because it is rich in arginine (13).

In addition to LPS, lactoferrin is able to bind and buffer other pathogen-associated molecular patterns such as viral DNA and RNA, CpG sequences, and soluble components of the extracellular matrix (14). This ability is associated with the putative lactoferrin antiinflammatory activity, as demonstrated in several studies (15). Lactoferrin down-regulated proinflammatory cytokine production in cell lines acting via nuclear factor-$\kappa\beta$ (NF-$\kappa\beta$) (16), leading to decreased release of TNF-$\alpha$ and IL-6 in mice (17). In humans, lactoferrin gene polymorphisms and circulating lactoferrin were recently reported to be associated with dyslipidemia and vascular reactivity in subjects with altered glucose tolerance (18).

Despite this information, we are unaware of any study linking lactoferrin to insulin resistance-associated proinflammatory activity. With this background, we aimed to investigate: 1) the *in vivo* associations between circulating lactoferrin, insulin resistance, and markers of LPS action; 2) the effects of LPS, IL-6, and rosiglitazone on lactoferrin release *ex vivo* in normal glucose tolerance (NGT) and type 2 diabetes whole-blood culture; and 3) and test the antinflammatory activity of lactoferrin under LPS challenge in THP-1 cells.

## Subjects and Methods

### Participant recruitment

Two hundred twenty-nine Caucasian men were recruited and studied. One hundred forty-nine of these were recruited in an ongoing study dealing with nonclassical cardiovascular risk factors in northern Spain. Subjects were randomly localized from a census and they were invited to participate. The participation rate was 71%. A 75-g oral glucose tolerance test according to the American Diabetes Association Criteria was performed in all subjects. All subjects with NGT (n = 94) had fasting plasma glucose less than 7.0 mM and 2-h postload plasma glucose less than 7.8 mM after a 75-g oral glucose tolerance test. Glucose intolerance was diagnosed in 43 subjects according to the American Diabetes Association Criteria (postload glucose between 7.8 and 11.1 mmol/liter). Previously unknown type 2 diabetes was diagnosed in 12 of these 92 subjects (postload glucose higher than 11.1 mmol/liter). Inclusion criteria were detailed in supplemental data, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.

To increase the statistical power of the group of patients with type 2 diabetes, 80 patients were prospectively recruited from diabetes outpatient clinics on the basis of a stable metabolic control in the previous 6 months, as defined by stable glycosylated hemoglobin (HbA1c) values. Data from these patients were merged with those from the recently diagnosed type 2 diabetic patients. Exclusion criteria and pharmacological treatment for patients with type 2 diabetes were detailed in supplemental data (2). The institutional review board of the institution approved the protocol.

### Measurements

Subjects were studied in the postabsorptive state. Anthropometric measurements were detailed in supplemental data (3).

### Study of insulin sensitivity

Insulin sensitivity was measured using the frequently sampled iv glucose tolerance test on a different day in those subjects who agreed (n = 114). In brief, basal blood samples were drawn at −15 and −5 min, after which glucose (300 mg/kg body weight) was injected over 1 min starting at time 0. At 20 min, regular insulin (Actrapid; Novo Nordisk A/S, Bagsværd, Denmark; 0.03 U/kg) was injected as a bolus. Additional samples were obtained from a contralateral antecubital vein at times 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 20, 22, 23, 24, 25, 27, 30, 40, 50, 60,70, 80, 90, 100, 120, 140, 160, and 180 min. Samples were rapidly collected via a three-way stopcock connected to the butterfly needle. Data from the frequently sampled iv glucose tolerance test were submitted to computer programs that calculate the characteristic metabolic parameters by fitting glucose and insulin to the minimal model that describes the times course of glucose and insulin concentrations. The glucose disappearance model, by accounting for the effect of insulin and glucose on glucose disappearance, provides the parameters insulin sensitivity ($10^{-4}$) per minute per microunit per milliliter) or the insulin sensitivity index, a measure of the effect of insulin concentrations above the basal level to enhance glucose disappearance. The estimation of model parameters was performed according to the MINMOD computer program (19).

### Analytical methods

Serum glucose concentrations were measured in duplicate by the glucose oxidase method using a Beckman glucose analyzer II (Beckman Instruments, Brea, CA). HbA1c was measured by the HPLC method (Bio-Rad, Muenchen, Germany, and autoanalyzer Jokoh HS-10, respectively). Intraassay and interassay coefficients of variation were less than 4% for all these tests.

Serum insulin was measured in duplicate by monoclonal immunoradiometric assay (Medgenix Diagnostics, Fleunes, Belgium). The intraassay coefficient of variation was 5.2% at a concentration of 10 mU/liter and 3.4% at 130 mU/liter. The interassay coefficients of variation were 6.9 and 4.5% at 14 and 89 mU/liter, respectively.

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Total serum triglycerides were measured through the reaction of glycerol-phosphate-oxidase and peroxidase on a Hitachi 917 instrument (Roche, Mannheim, Germany).

Plasma lactoferrin levels were measured by Bioxytech Lactof enzyme immunoassay (OxisResearch, Beverly Hills, CA). Soluble TNF receptor 2 (sTNFR2) concentration was measured by sTNF-RII EASIATM ELISA kit (Biosource Europe S.A., Nivelles, Belgium); plasma bactericidal permeability increasing (BPI) protein levels were measured by human BPI ELISA kit (HyCult Biotechnology b.v., PB Uden, The Netherlands); serum soluble CD14 (sCD14) levels were measured by human sCD14 ELISA kit (HyCult Biotechnology); serum LPS binding protein (LBP) levels were measured by human LBP ELISA kit (HyCult Biotechnology). Plasma and serum samples were diluted and assayed according to the manufacturer's instructions. Intra- and interassay coefficients of variation for all these determinations were between 5 and 10%. To prevent the activation of neutrophils, blood was drawn in tubes containing EDTA as anticoagulant and kept at 4 C. Whole blood was centrifuged within 3 h at 3000 × *g* for 10 min at 4 C, and finally the plasma supernatant was removed and stored at −80 C.

Endotoxin determination was detailed in supplemental data (4).

### Whole-blood culture

Citrate anticoagulated peripheral blood (PB) samples from a total of 12 adult volunteers [four young NGT subjects 31 ± 4.6 yr old with body mass index (BMI) of 24.3 ± 3.4 kg/m$^2$, four older NGT subjects 56 ± 1 yr old with BMI of 26 ± 2.6 kg/m$^2$, and four patients with type 2 diabetes 60.5 ± 9.6 yr old with BMI of 28.3 ± 1.96 kg/m$^2$] were obtained after informed consent according to the guidelines of the Ethics Committee of the University Hospital Dr. Josep Trueta of Girona (Girona, Spain). In all experiments, PB samples were prepared and cultured *in vitro* within a maximum period of 1 h after they were collected (20, 21). Leukocyte, monocyte, and neutrophil counts (EDTA sample; Coulter Electronics, Hialeah, FL) were determined by routine laboratory tests.

RPMI 1640-diluted PB samples were supplemented with 1% glutamine, 1% sodium pyruvate, and 80 μg/ml gentamicin, with a dilution of 1:1. Four experimental conditions were used: 1) a control treatment with RPMI 1640; an LPS from *Escherichia coli* O$^2$6:B6 (LPS) treatment with 10 ng/ml LPS (Sigma-Aldrich, St. Louis, MO); a LPS-IL-6 [cotreatment with 10 ng/ml LPS and 5 ng/ml IL-6 (MBL International Corporation, Woburn, MA)]; and finally a rosiglitazone treatment (5 μM), it was kindly provided by Margarita Lorenzo (Department of Biochemistry, Faculty of Pharmacy, Universidad Complutense, Madrid, Spain). All treatments were made in duplicate for 12 h. Cells were maintained at 37 C in 5% $CO_2$ humidified atmosphere.

To test the functional activity of cells in this whole-blood culture, we evaluated a profile of 42 cytokines by means of arrays in a control experiment (in triplicate, RayBio cytokine antibody array; RayBiotech, Norcross, GA) that was carried out following the manufacturer's instructions. The supernatants were collected, centrifuged at 900 × *g*, aliquoted, and stored at −80 C until testing. Lactoferrin supernatants levels were measured by Bioxytech Lactof enzyme immunoassay (OxisResearch).

### Lactoferrin effect in LPS-stimulated THP-1

Human monocytic THP-1 cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 containing 10% fetal calf serum and differentiated 48 h with 100 nM phorbol-12-myristate-13-acetate. After differentiation, we performed several treatments for 24 h: control (RPMI 1640 deprived), LPS (100 ng/ml), and LPS (100 ng/ml) + lactoferrin (0.5 μM). The physiological concentration of lactoferrin is 300–2,000 ng/ml under basal conditions, and it may increase 20-fold under acute response (6,000–40,000 ng/ml). We tested the concentration of 40,000 ng/ml (0.5 μM) because we aimed to test the lactoferrin effects in a scenario compatible with physiological acute response.

Treatment response of THP-1 was evaluated analyzing the IL-6, IL-8, and macrophage chemoattractant protein (MCP)-1 expression, which was detailed in supplemental data (5). Quantitative real-time TaqMan PCR was performed with a commercial inventoried primers and FAM dye-labeled TaqMan minor groove binder probes (Applied Biosystems, Foster City, CA) for IL-6, MCP-1, IL-8, and cyclophilin A, which is used as endogenous control, and 2× universal PCR master mix (Applied Biosystems). In parallel, we made the same treatments for 10 min to analyze the effects of these in NF-κβ(p65) activation, with NF-κβ(p65), and $_{p536}$NF-κβ(p65) antibodies (Cell Signaling Technology, Inc., Beverly, MA) by Western blot. This experiment was repeated three different times. NF-κB activation was assayed measuring p536Ser-NF-κβ (p65) by Western blot analysis. LPS-induced pathway leads to phosphorylation of the p65 subunit of NFκβ on serine 536 after NF-κβ translocation to the nucleus. Doyle *et al.* reported that in response to LPS stimulation, Bruton's tyrosine kinase functions on the pathway leading to enhanced transactivation by p65 via phosphorylation of serine 536 but not inhibitory-κBα phosphorylation and degradation (22).

### Statistical methods

Statistical analyses were performed using SPSS 12.0 software (SPSS Inc., Chicago, IL). Unless otherwise stated, descriptive results of continuous variables are expressed as mean and SD for Gaussian variables. Parameters that did not fulfill normal distribution were mathematically transformed to improve symmetry for subsequent analyses. The relation between variables was analyzed by simple correlation (Pearson's test) and multiple regression analyses. Unpaired *t* tests were used to compare subjects with NGT and altered glucose tolerance (AGT) subjects. Paired *t* tests were used to compare the effects of treatment on lactoferrin release. Levels of statistical significance were set at $P < 0.05$. For a given value of $P = 0.05$, the study had an 98% power to detect significant correlations between plasma lactoferrin and metabolic parameters (Pearson's coefficient of at least 0.30) in bilateral tests in all subjects studied and a 91% power in AGT subjects.

## Results

### Cross-sectional study of circulating lactoferrin with metabolic parameters

We evaluated circulating lactoferrin in subjects with varying degrees of insulin action (NGT and AGT). Neutrophils are the only leukocytes that contribute to significant amounts of circulating lactoferrin in blood stream (13). The neutrophil count was significantly higher in AGT men as a group

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**TABLE 1.** Anthropometrical and biochemical variables of study subjects (cross-sectional study)

| | Normal glucose tolerance | Altered glucose tolerance | ANOVA *P* |
|---|---|---|---|
| n | 94 | 135 | |
| Age (yr) | 49.80 ± 11.3 | 58.18 ± 10.7 | <0.001 |
| BMI (kg/m$^2$) | 27.02 ± 3.6 | 28.9 ± 4.06 | <0.001 |
| Waist to hip ratio | 0.92 ± 0.06 | 0.97 ± 0.07 | <0.001 |
| Fat-free mass (kg) | 73.03 ± 9.9 | 70.9 ± 8.7 | 0.17 |
| Fat mass (kg) | 8.2 ± 14.4 | 12.3 ± 11.1 | 0.05 |
| Fasting glucose (mg/dl) | 92.8 ± 7.6 | 144.4 ± 68.3 | <0.001 |
| HbA1C (%) | 4.8 ± 0.32 | 6.3 ± 1.8 | <0.001 |
| Insulin sensitivity ($10^{-4}$/min$^{-1}$ · mU per liter)[a] | 0.57 ± 0.22 | 0.34 ± 0.19 | <0.001 |
| Neutrophil count | 3645.2 ± 1470.8 | 4647.7 ± 1718.4 | <0.001 |
| sTNFR2 (ng/ml) | 6.8 ± 3.76 | 7.7 ± 5.04 | 0.17 |
| LBP (μg/ml) | 19.75 ± 16.9 | 43.1 ± 27.9 | <0.001 |
| sCD14 (μg/ml) | 4.26 ± 1.13 | 5.2 ± 2.6 | 0.005 |
| BPI (ng/ml) | 26.6 ± 22.6 | 19.7 ± 19.8 | 0.02 |
| Lactoferrin (ng/ml) | 493.5 ± 238.9 | 431.5 ± 187.5 | 0.02 |
| Lactoferrin/neutrophil count | 0.12 (0.08–0.2) | 0.08 (0.06–0.13) | <0.001 |

[a] Performed in 64 NGT and 50 AGT subjects.

and was inversely associated with insulin sensitivity ($r = -0.27$, $P = 0.005$) (Table 1 and Fig. 1).

In all subjects, circulating lactoferrin was significantly and positively associated with neutrophil count, in both NGT ($r = 0.27$, $P = 0.01$) and AGT ($r = 0.21$, $P = 0.015$). Interestingly, the differences in lactoferrin concentration between NGT and AGT subjects were increased after normalizing by neutrophil count [0.12 (0.08–0.2) *vs.* 0.08 (0.06–0.13) ng/ml per cell count, $P < 0.001$].

For that reason, we analyzed the results with normalized lactoferrin (relative to neutrophil count) to adjust for this increase and with lactoferrin only. Mean circulating lactoferrin and normalized lactoferrin was significantly higher in NGT than AGT men (Table 1 and Fig. 1). Plasma normalized lactoferrin concentration correlated positively with insulin sensitivity ($r = 0.33$, $P < 0.001$, Fig. 1) and negatively with age, BMI, waist to hip ratio, fasting glucose, and glycated hemoglobin (Table 2). We then ex-

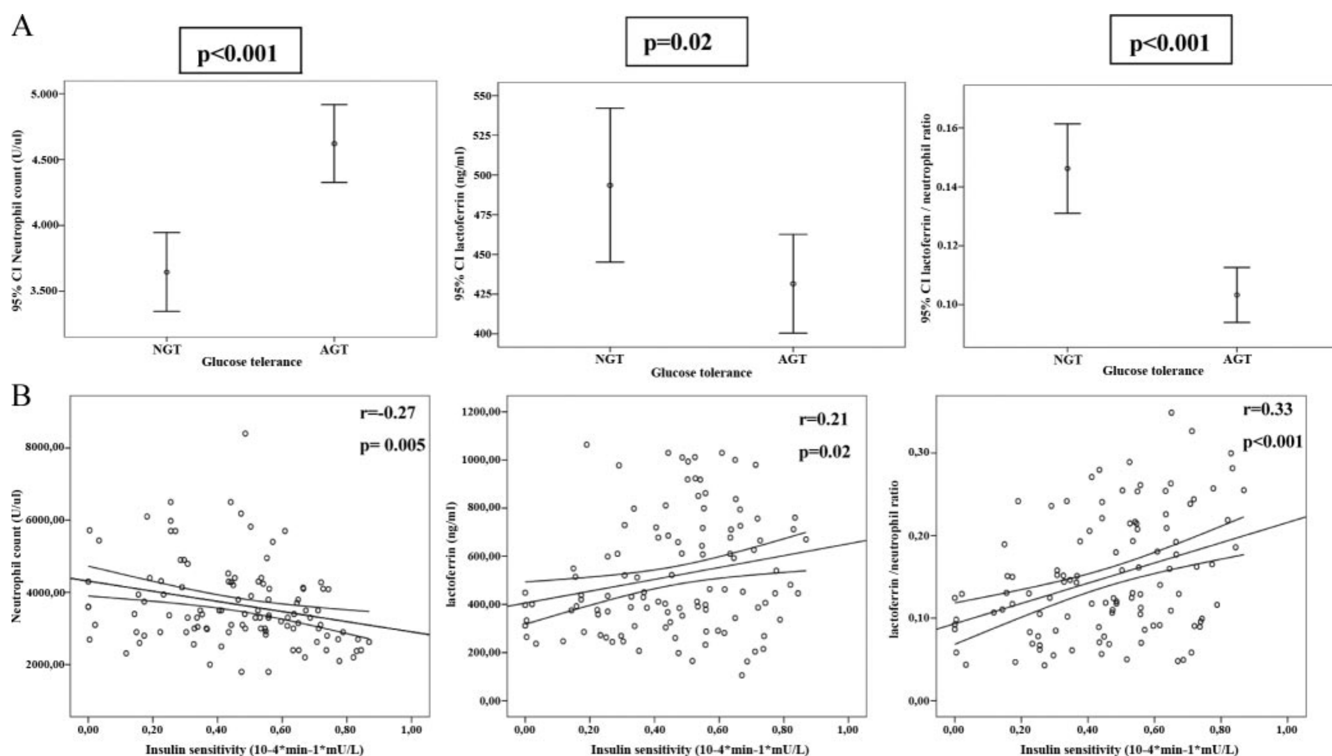


**FIG. 1.** A, 95% confidence interval for the mean of neutrophil, circulating lactoferrin and normalized lactoferrin according to glucose tolerance status. B, Linear relationship between lactoferrin, neutrophil count, and normalized lactoferrin, respectively, and insulin sensitivity.

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**TABLE 2.** Correlations between circulating lactoferrin (corrected by neutrophil count) and clinical and biochemical variables

| | All subjects | P | NGT | P | AGT | P |
|---|---|---|---|---|---|---|
| n | 229 | | 94 | | 135 | |
| Age (yr) | −0.15 | 0.02 | −0.08 | 0.4 | −0.11 | 0.19 |
| BMI (kg/m$^2$) | −0.15 | 0.02 | 0.02 | 0.8 | −0.21 | 0.01 |
| Waist to hip ratio | −0.22 | 0.002 | −0.1 | 0.3 | −0.37 | 0.001 |
| Fat-free mass (kg) | 0.08 | 0.3 | −0.035 | 0.7 | 0.25 | 0.005 |
| Fat mass (kg) | −0.12 | 0.1 | 0.05 | 0.6 | −0.4 | <0.001 |
| HbA1c (%) | −0.26 | <0.001 | −0.21 | 0.03 | −0.26 | 0.003 |
| Fasting glucose (mg/dl) | −0.19 | 0.005 | −0.05 | 0.62 | −0.17 | 0.04 |
| Insulin sensitivity[a] | 0.33 | <0.001 | 0.34 | 0.005 | 0.28 | 0.05 |
| sTNFR2 (ng/ml) | −0.03 | 0.6 | 0.1 | 0.3 | −0.16 | 0.05 |
| LBP (ng/ml) | −0.43 | <0.001 | −0.4 | <0.001 | −0.38 | <0.001 |
| sCD14 ($\mu$g/ml) | 0.03 | 0.7 | 0.17 | 0.1 | −0.01 | 0.9 |
| BPI (ng/ml) | 0.53 | <0.001 | 0.5 | <0.001 | 0.5 | <0.001 |

[a] Performed in 64 NGT and 50 AGT subjects.

plored whether these associations could be mediated by interaction with metabolic endotoxemia. As surrogates of this, we used circulating levels of LBP and BPI, known to directly interact with LPS. We observed an association with BPI (r = 0.53, $P < 0.001$) and LBP (r = −0.43, $P < 0.001$) in all subjects as a whole and separately in NGT and AGT subjects (Table 2). We did not observe any association between circulating normalized lactoferrin and sCD14.

In AGT subjects, circulating normalized lactoferrin was also associated a marker of inflammatory activity such as sTNFR2 (r = −0.16, $P = 0.05$).

When circulating lactoferrin was not normalized by neutrophil the main associations were maintained (Table 3 and Fig. 1).

As shown in Table 4, LBP ($P = 0.02$) and insulin sensitivity ($P = 0.01$) contributed independently to circulating lactoferrin variance, after controlling for the effects of age, waist to hip ratio, and HbA1c in NGT subjects. LBP ($P = 0.03$) and waist to hip ratio ($P = 0.03$) contributed independently to circulating lactoferrin variance, after controlling for the effects of age, HbA1c, and insulin sensitivity in AGT subjects.

In other multivariant linear regression model, glucose tolerance status ($P = 0.04$) and fasting triglycerides ($P = 0.01$) contributed independently to circulating lactoferrin variance, after controlling for the effects of age, BMI, and systolic blood pressure.

Interestingly, serum endotoxin concentration was significantly associated with circulating lactoferrin (r = −0.37, $P = 0.03$, n = 33) and LBP (r = 0.6, $P = 0.001$, n = 33) in all subjects. Serum endotoxin concentration tended to be increased in AGT (n = 15) *vs.* NGT (n = 18) subjects (0.21 ± 0.09 *vs.* 0.18 ± 0.03 EU/ml, respectively, $P = 0.1$).

Circulating lactoferrin was significantly decreased only in treated diabetic patients (424.7 ± 210 *vs.* 493.5 ± 238.9 ng/ml, $P = 0.01$), in whom HbA1c was significantly increased (7.3 ± 1.7 *vs.* 4.8 ± 0.32%, $P < 0.001$). Circulating lactoferrin concentration (483 ± 230.5 *vs.* 493.5 ± 238.9 ng/ml, $P = 0.4$) and HbA1c (5.7 ± 1.4 *vs.*

**TABLE 3.** Correlations between circulating lactoferrin (absolute concentration) and clinical and biochemical variables

| | All subjects | P | NGT | P | AGT | P |
|---|---|---|---|---|---|---|
| n | 229 | | 94 | | 135 | |
| Age (yr) | −0.18 | 0.007 | −0.1 | 0.3 | −0.15 | 0.06 |
| BMI (kg/m$^2$) | −0.13 | 0.04 | 0.03 | 0.7 | −0.18 | 0.03 |
| Waist to hip ratio | −0.2 | 0.003 | −0.1 | 0.3 | −0.3 | 0.005 |
| Fat-free mass (kg) | 0.07 | 0.3 | 0.001 | 0.9 | 0.21 | 0.02 |
| Fat mass (kg) | −0.07 | 0.4 | 0.05 | 0.6 | −0.29 | 0.007 |
| HbA1c (%) | −0.15 | 0.02 | −0.17 | 0.1 | −0.11 | 0.2 |
| Fasting glucose (mg/dl) | −0.09 | 0.1 | −0.08 | 0.4 | −0.03 | 0.6 |
| Insulin sensitivity[a] | 0.21 | 0.02 | 0.12 | 0.3 | 0.26 | 0.07 |
| Neutrophil count | 0.18 | 0.007 | 0.27 | 0.01 | 0.21 | 0.015 |
| sTNFR2 (ng/ml) | −0.01 | 0.8 | 0.09 | 0.4 | −0.06 | 0.4 |
| LBP (ng/ml) | −0.33 | <0.001 | −0.38 | <0.001 | −0.23 | 0.01 |
| sCD14 ($\mu$g/ml) | 0.15 | 0.05 | 0.21 | 0.04 | 0.13 | 0.28 |
| BPI (ng/ml) | 0.52 | <0.001 | 0.53 | <0.001 | 0.45 | <0.001 |

[a] performed in 64 NGT and 50 AGT subjects.

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**TABLE 4.** Multiple linear regression analysis with circulating lactoferrin normalized as dependent variable

| | All subjects (n = 114) | | NGT (n = 64) | | AGT (n = 50) | |
|---|---|---|---|---|---|---|
| | t | P | t | P | t | P |
| Age (yr) | −0.05 | 0.9 | 1.15 | 0.25 | −1.23 | 0.2 |
| Waist to hip ratio | −2.1 | 0.04 | −1.2 | 0.2 | −2.2 | 0.03 |
| HbA1c (%) | −0.7 | 0.5 | −1.54 | 0.1 | −0.03 | 0.9 |
| LBP (ng/ml) | −3.01 | 0.003 | −2.4 | 0.02 | −2.2 | 0.03 |
| Insulin sensitivity | 2.8 | 0.005 | 2.6 | 0.01 | 0.7 | 0.4 |
| Adjusted $R^2 \pm$ SE | 0.24 ± 0.06 | | 0.22 ± 0.07 | | 0.27 ± 0.05 | |

4.8 ± 0.32%, $P = 0.1$) did not significantly differ between untreated diabetic patients *vs.* NGT subjects.

### Lactoferrin secretion in whole-blood culture

To corroborate the cross-sectional findings in humans, we aimed to evaluate the production of lactoferrin after LPS stimuli. As expected, cytokine antibody arrays showed a response of the whole-blood culture to LPS, with an increase of IL-6, IL-1$\beta$, TNF-$\alpha$, IL-8, MCP-1, and MCP-2 in response to LPS (supplemental Fig. S1). Again, AGT status and age significantly influenced lactoferrin secretion in this model. Significantly increased lactoferrin secretion was observed in NGT compared with type 2 diabetes subjects. Aged NGT men showed significantly increased lactoferrin secretion compared with aged type 2 diabetics [5.2 ± 0.87- (from 296.7 ± 152.9 to 1542.6 ± 572.7 ng/ml) *vs.* 3.24 ± 0.27-fold increase (from 316.9 ± 73.3 to 1026.5 ± 231 ng/ml), $P = 0.008$]. The neutrophil lactoferrin response was even higher among young NGT *vs.* aged type 2 diabetic subjects [7.24 ± 1.59- (from 226.5 ± 88.6 to 1639.4 ± 735.3 ng/ml) *vs.* 3.24 ± 0.27-fold increase, $P = 0.003$, Fig. 2-A].

LPS-induced lactoferrin release was decreased by 38% in the presence of interleukin-6 but this decrease was observed only in NGT subjects (1453 ± 572 *vs.* 882 ± 336 ng/ml, $P = 0.03$, Fig. 2B).

Finally, we also tested whether an insulin sensitizer could modify basal lactoferrin secretion in this model. Rosiglitazone treatment led to increased basal lactoferrin secretion (398 ± 193 *vs.* 280.1 ± 104.9 ng/ml, $P < 0.0001$).

### Human lactoferrin effects on LPS-stimulated THP-1 cells

Coincubation with human lactoferrin (0.5 $\mu$M) led to decreased IL-6, IL-8, and MCP-1 relative expression (66.6, 47, and 50%, respectively) in LPS-stimulated THP-1 cells compared with LPS-stimulated cells (Fig. 3A). Similarly, p536Ser-NF-$\kappa\beta$(p65) decreased by 45% when cotreatment of human lactoferrin (0.5 $\mu$M) and LPS (100 ng/ml) was compared with LPS treatment alone ($P < 0.01$) (Fig. 3B).

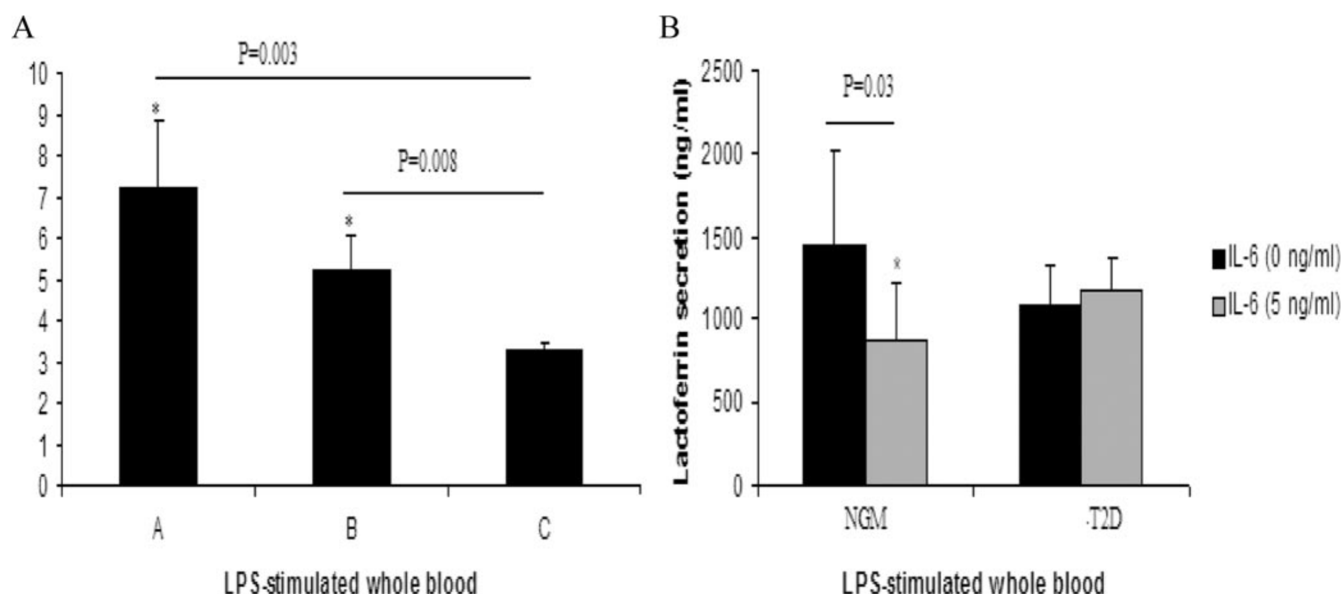


**FIG. 2.** A, LPS-induced lactoferrin secretion in different groups according to glucose metabolism status and age (A, NGT subjects aged 32 ± 3 yr; B, NGT subjects aged 55 ± 2 yr; C, type 2 diabetes subjects aged 57 ± 3 yr). B, LPS-induced lactoferrin secretion in the presence of IL-6 in NGT and type 2 diabetes subjects.

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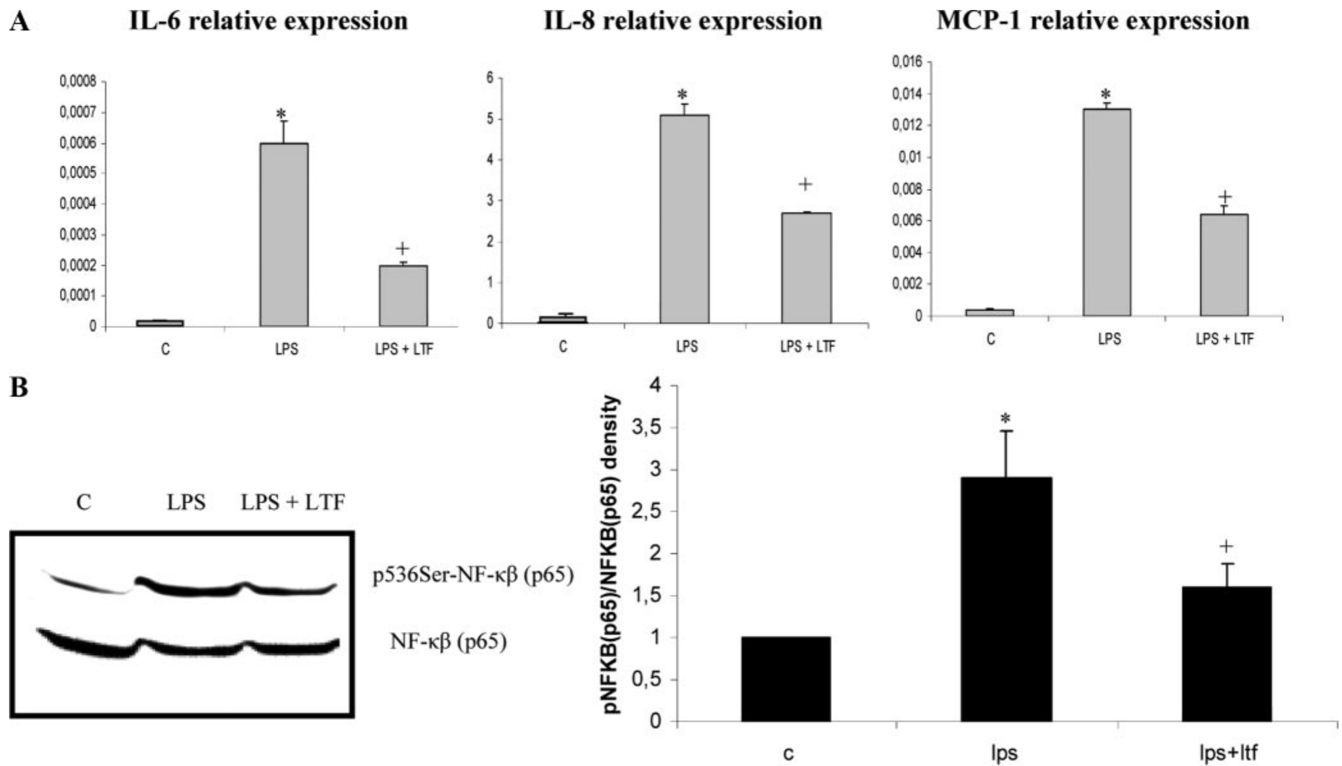


**FIG. 3.** A, IL-6, IL-8, and MCP-1 relative expression in LPS-stimulated THP-1 with and without lactoferrin (0.5 $\mu$M). *, $P < 0.01$ *vs.* control (C); +, $P < 0.01$ *vs.* LPS stimuli. B, Buffering effects of lactoferrin in $_{pSer536}$NF-$\kappa\beta$(p65) by LPS stimulation. *, $P < 0.01$ *vs.* control; +, $P < 0.01$ *vs.* LPS stimuli.

## Discussion

High-fat diet increases the circulating concentration of plasma LPS at a concentration sufficient to increase body weight, fasted glycemia, and inflammation. This increase in plasma LPS concentration induced by high-fat feeding has been recently defined as metabolic endotoxemia (23). LPS infusion in normal diet-fed mice causes a metabolic response similar, to some extent, to high-fat feeding. In these mice, the body and adipose depot weights and fasted glycemia were increased to the same extent as during high-fat feeding. Furthermore, the chronic LPS infusion induced liver insulin resistance and was associated with fasted hyperinsulinemia. LPS-treated mice developed inflammation and increased expression of genes coding for cytokines, IL-6, TNF-$\alpha$, IL-1, and plasminogen activator inhibitor-1 in adipose depots, liver, and muscle.

Serum endotoxin levels were significantly associated with circulating LBP concentration (r = 0.6, $P = 0.001$). Circulating LBP concentration could be a specific marker of metabolic endotoxemia. Endotoxin levels also were inversely associated with circulating lactoferrin association. Lactoferrin has been demonstrated to interact with LPS (24), avoiding the LPS-LBP complex formation (25). Lactoferrin also interacts with CD14, preventing the LPS-LBP complex interactions with toll-like receptor, which amplify the inflammatory signal (26). Thus, high lactoferrin concentration lead to decreased free LPS, which down-regulates LBP expression (25). We tested the buffering effect of lactoferrin in the LPS-stimulated THP-1 cell line, and we corroborated the antiinflammatory effect of lactoferrin by decreasing NF-$\kappa\beta$ activation and IL-6, IL-8, and MCP-1 expression under LPS challenge.

Even though neutrophil count was significantly higher in subjects with AGT and was negatively associated with insulin sensitivity (27–29), circulating lactoferrin in plasma (secreted by neutrophils to blood stream) was significantly decreased in patients with AGT and positively associated with insulin sensitivity. In addition, circulating lactoferrin was negatively associated with obesity measures (BMI, waist to hip ratio), hyperglycemia, and insulin resistance. The fact that insulin sensitivity did not enter into the multiple linear regression model (Table 3) was probably due to the relatively low range of insulin sensitivity in AGT subjects. The mechanisms for these associations were studied cross-sectionally by evaluating the associations of lactoferrin with markers of LPS action. The strong relationships with LBP and BPI suggest that LPS could underlie the low lactoferrin-insulin resistance relationship. Recently Stegenga *et al.* (30) reported that hyperglycemia impaired neutrophil degranulation in humans with systemic inflammation induced by iv LPS administration. For this reason, we cannot exclude the hyperglycemia effects on circulating lactoferrin concentrations.

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Lactoferrin was positively and strongly correlated with BPI in all subjects, in both NGT and AGT subjects. BPI is released by the primary granules of neutrophils, by monocytes and several epithelia (31). As lactoferrin, BPI has a high cationic fragment and has been reported to behave as an antiinflammatory molecule. The associations of BPI with several metabolic variables, including insulin sensitivity, were similar to those found with lactoferrin (31).

Circulating sTNFR2 was negatively associated with lactoferrin in AGT subjects. TNF-$\alpha$ has been demonstrated to stimulate lactoferrin secretion from neutrophils (32), but high TNF-$\alpha$ levels led to decreased lactoferrin (33, 34). Thus, the inverse association between lactoferrin and sTNFR2 (plasma concentrations of TNFR2 are thought to reflect the degree of activation of the TNF system) found in AGT subjects could reflect the alteration in immune response associated to chronic inflammation (1, 35).

In different animal models of inflammation, like gut inflammation (17) and skin inflammation (25), lactoferrin showed important antiinflammatory and immunoregulatory functions, restoring immune defense system homeostasis (36).

The relationship between lactoferrin and metabolic inflammation was also explored mechanistically. A recent study showed that insulin was a strong modulator of some neutrophil function in nondiabetic healthy subjects (37). Neutrophil activity could be restored by controlling hyperglycemia with insulin. Moreover, although neutrophils do not require insulin to uptake glucose, glucose use and glycogen metabolism inside these cells are both insulin dependent (37).

Our results in whole-blood experiment support these hypotheses because we found a significant decrease in lactoferrin release from neutrophils (whole blood) in type 2 diabetic subjects. IL-6 is a proinflammatory cytokine associated with insulin resistance *in vivo* and *in vitro* (5, 6). IL-6 coincubation possibly affected neutrophil function in NGT subjects (acting through impaired cytoskeleton action?), leading to decreased lactoferrin secretion in response to LPS. In subjects with type 2 diabetes and already compromised neutrophil function, IL-6 had no additive effects.

Although the preliminary data showed that rosiglitazone could increase lactoferrin production and improve the neutrophil function, further research will be necessary. In fact, a stimulatory effect of insulin on the absolute number of neutrophils expressing lactoferrin has been recently demonstrated (37). Insulin under strict euglycemia is able to prime neutrophil function in adult healthy humans and modulates neutrophil activity not only by gaining a better metabolic control but also through a direct effect of the hormone (38). Thus, improved insulin action [by the administration of insulin (37) or an insulin-sensitizer as rosiglitazone] may act as an immunoregulatory agent to turn immune cells to a primed state, which prepares the cell for a greater immune response. It has also recently reported that high protein kinase B (a major downstream phosphatidylinositol 3-kinase effector) activity was found to promote neutrophil and monocyte development (39). The antiinflammatory activities of insulin and rosiglitazone have been extensively reported (40–42). Increased insulin action or sensitivity would allow a more balanced neutrophil response, increasing the efficiency of the immune system. Increased insulin action would also allow repairing the day-to-day continuous insults preventing from an enhanced immune response and increased cytokine secretion.

Furthermore, our results were in concordance with several studies, which suggested that an alteration in neutrophil functionality (decreasing bactericidal capacity and increasing neutrophil count) were associated with type 2 diabetes (27–29).

In summary, this is the first report showing decreased circulating lactoferrin in association with insulin resistance and type 2 diabetes. This primary or secondary abnormality could amplify the chronic inflammatory response caused by external insults worsen insulin resistance. Furthermore, these findings support the loss of neutrophil efficiency associated with type 2 diabetes. Further studies on animal models are necessary to confirm a protective role of lactoferrin in front of metabolic disturbances (through its buffering activity).

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