IMMUNOMODULATION AND METABOLISM: POSSIBLE ROLE OF LACTOFERRIN

José María MORENO NAVARRETE

Dipòsit legal: GI-1082-2011
http://hdl.handle.net/10803/36737

Universitat de Girona

DOCTORAL THESIS

IMMUNOMODULATION AND METABOLISM:

POSSIBLE ROLE OF LACTOFERRIN

José María Moreno Navarrete

2011

DOCTORAL THESIS

IMMUNOMODULATION AND METABOLISM: POSSIBLE ROLE OF LACTOFERRIN

José María Moreno Navarrete

2011

PROGRAMA DE DOCTORAT: BIOTECNOLOGIA

Directed by : José Manuel Fernández-Real Lemos, Ph D MD

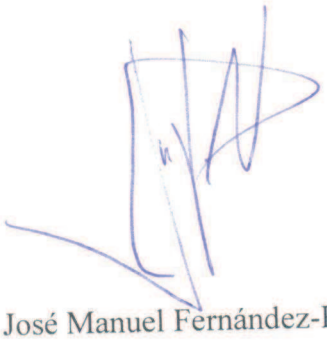
Memòria presentada per optar al títol de doctor per la Universitat de Girona

El Dr. José Manuel Fernández-Real Lemos, cap de secció del departament de endocrinologia i nutrició del Hospital Dr Josep Trueta,

CERTIFICO:

Que la tesi titulada "**Immunomodulation and metabolism: possible role of lactoferrin**", presentada per José María Moreno Navarrete per optar al grau de doctor per la Universitat de Girona, ha estat realitzat sota la meva direcció i, considerant aquesta acabada, autoritzo la seva presentació perquè sigui jutjada per la Comissió corresponent.

I per tal que així consti als efectes que correspongui, firmo la present a Girona el 14 de gener de 2011.



Dr José Manuel Fernández-Real Lemos

El doctorand

“If biologists have ignored self-organization, it is not because self-ordering is not pervasive and profound. It is because we biologists have yet to understand how to think about systems governed simultaneously by two sources of order. Yet who seeing the snowflake, who seeing simple lipid molecules cast adrift in water forming themselves into cell-like hollow lipid vesicles, who seeing the potential for the crystallization of life in swarms of reacting molecules, who seeing the stunning order for free in networks linking tens upon tens of thousands of variables, can fail to entertain a central thought: if ever we are to attain a final theory in biology, we will surely, surely have to understand the commingling of self-organization and selection. We will have to see that we are the natural expressions of a deeper order. Ultimately, we will discover in our creation myth that we are expected after all.”

Stuart Kauffman. *At Home in the Universe*

Agraïments

Per mi aquest apartat reflecteix la part del treball menys científica però no menys valuosa, ja que permet a l'autor expressar la seva gratitud a aquelles persones que l'han acompanyat durant tot el procés de planificació i construcció d'aquesta tesi, i que per tant han estat necessàries per la seva elaboració.

Inclouria en aquesta llista d'agraïments:

-Al Dr. José Manuel Fernández-Real per haver confiat en mi i donar-me la oportunitat de formar part d'aquest interessant projecte de recerca. També per haver-me guiat en el desenvolupament d'aquest treball.

-Al Dr. Wifredo Ricart pel seu recolzament i confiança en tot moment.

-A tots els companys del grup de recerca (UDEN) amb els que he anat coincidint durant tots aquests anys. En especial, la Mònica, la Judit, la Roser, la Carme, la Marta, la Mayca, la Berta, la Marcela, la Neus, en Paco, en Gerard, l'Òscar, l'Emili ...

- A tota la resta de companys del laboratori de recerca.

-A los compañeros del hospital Virgen de la Victoria de Málaga grupo liderado por el Dr. Francisco Tinahones.

-A tots els amics que m'han ajudat a desconnectar quan era necessari (Albert, Miquel, Pere, Paco, Ivan, ...).

-A tota la família (cosins, tiets, sogres, cunyats ...) i en especial al meu avi (Pedro José) que ens va deixar fa uns mesos i a la meva àvia (Beatriz).

Finalment, voldria destacar en aquests agraïments:

-Als meus pares (Leoncio i Beatriz) i a la meva germana (Bea) pels vostres savis consells (que tan m'han ajudat quan he hagut d'encarar situacions difícils) i per la vostra confiança i estimació.

-I sobretot, a la meva dona (Jèssica), per recórrer al meu costat tota aquesta odissea (sense la teva ajuda i comprensió no podria haver dedicat tantes hores a aquesta feina), per suportar i acceptar conviure amb els meus defectes, i per fer-me sentir cada dia l'home més feliç del món. Per això vull dedicar aquesta tesi a tu i a la filla (Celia) que esperem i que aviat formarà part de la nostra vida.

Bé acabats els agraïments, us desitjo una agradable, didàctica i profitosa lectura.

List of manuscripts

This thesis is presented as a compedium of five manuscripts:

Manuscript 1:
Moreno-Navarrete JM, Ortega FJ, Bassols J, Castro A, Ricart W, Fernandez-Real JM. Association of circulating lactoferrin concentration and 2 nonsynonymous LTF gene polymorphisms with dyslipidemia in men depends on glucose-tolerance status. *Clinical Chemistry* 54:2 301–309, 2008.
Clinical Chemistry has an impact factor of **6.263** and is the most cited journal in the category of medical laboratory technology (© 2009 Journal Citation Reports, published by Thomson Reuters).

Manuscript 2:
Moreno-Navarrete JM, Ortega FJ, Bassols J, Ricart W, Fernandez-Real JM. Decreased circulating lactoferrin in insulin resistance and altered glucose tolerance as a possible marker of neutrophil dysfunction in type 2 diabetes. *J Clin Endocrinol Metab* 94: 4036–4044, 2009.
Journal of Clinical Endocrinology and Metabolism has an impact factor of **6.202** and is ranked 10th (Q1) out of 105 journals in the category of Endocrinology and Metabolism (© 2009 Journal Citation Reports, published by Thomson Reuters).

Manuscript 3:
Fernández-Real JM, García-Fuentes E, Moreno-Navarrete JM, Murri-Pierri M, Garrido-Sánchez L, Ricart W, Tinahones FJ. Fat overload induces changes in circulating lactoferrin that are associated with postprandial lipemia and oxidative stress in severely obese subjects. *Obesity* 18:482-488, 2010.
Obesity has an impact factor of **3.366** and is ranked 13th (Q1) out of 66 journals in the category of Nutrition and Dietetics, and 39th (Q2) out of 105 journals in the category of Endocrinology and Metabolism (© 2009 Journal Citation Reports, published by Thomson Reuters).

Manuscript 4:
Moreno-Navarrete JM, Ortega FJ, Ricart W, Fernandez-Real JM. Lactoferrin increases ^{172Thr}AMPK phosphorylation and insulin-induced ^{p473Ser}AKT while impairing adipocyte differentiation. *Int J Obes (Lond)* 33:991-1000, 2009.
International Journal of Obesity has an impact factor of **4.343** and is ranked 4th (Q1) out of 66 journals in the category of Nutrition and Dietetics, and 23th (Q1) out of 105 journals in the category of Endocrinology and Metabolism (© 2009 Journal Citation Reports, published by Thomson Reuters).

Manuscript 5:
Moreno-Navarrete JM, Ortega FJ, Sabater M, Ricart W, Fernandez-Real JM. Pro-adipogenic effects of lactoferrin in human subcutaneous and visceral preadipocytes. *J Nutr Biochem*. Accepted (2010). In press.
Journal of Nutritional Biochemistry has an impact factor of **4.288** and is ranked 7th (Q1) out of 66 journals in the category of Nutrition and Dietetics (© 2009 Journal Citation Reports, published by Thomson Reuters).

CONTENTS

ABSTRACT

Insulin resistance and obesity-associated metabolic disturbances are associated with a low-level chronic inflammatory state. Lactoferrin is a pleiotropic glycoprotein with immunomodulatory activity. To gain insight in the relationship between innate immune system and metabolic disease, we aimed to investigate the effects of lactoferrin in insulin sensitivity and obesity-related metabolic disturbances on cross-sectional human studies and on *in vitro* experiments.

Circulating lactoferrin in plasma (by ELISA) and two non-synonymous miss-sense single nucleotide polymorphisms (SNPs) were studied in association with dyslipidemia, insulin resistance, obesity parameters and inflammatory markers according to glucose tolerance status. We tested the effects of lactoferrin on insulin action in HepG2 and 3T3-L1 cell lines during 24h treatment, and in adipogenesis on 3T3-L1 mouse cell line and human subcutaneous and visceral isolated preadipocytes. The effects of fat overload in circulating lactoferrin were also evaluated in obese morbid patients.

Circulating lactoferrin concentration was significantly decreased in subjects with altered glucose tolerance (AGT). Circulating lactoferrin associated negatively with fasting triglycerides, body mass index, and waist to hip ratio, fat mass, blood pressure, fasting glucose, glycated hemoglobin and inflammatory markers and positively with insulin sensitivity, HDL-cholesterol and Bactericidal Increasing Permeability (BPI). The SNPs-induced aminoacidic changes in lactoferrin N-terminus region (increasing its cationicity) were associated with a low atherogenic lipid profile. LPS-induced lactoferrin production in neutrophils decreased significatively in type 2 diabetic patients. IL-6 (5 ng/ml) co-incubation decreased significatively LPS-induced lactoferrin production in neutrophils in subjects with normal glucose tolerance (NGT).

Rosiglitazone (5 μM) treatment increased significatively baseline lactoferrin production in all subjects.

Lactoferrin (0.5 μM) buffered LPS (100 g/ml)-induced inflammatory effects in THP-1 cell line inhibiting LPS-induced NFκB activity. Lactoferrin increased insulin-induced ^{Ser473}AKT phosphorylation, even in inflammatory conditions and dexamethasone administration (two ways for increasing insulin resistance) in HepG2 and 3T3-L1 cell lines. Lactoferrin dose dependently blunted adipogenesis in 3T3-L1 and increased AMPK and Rb activity. Inversely, lactoferrin increased significantly adipogenesis and insulin-induced ^{Ser473}AKT phosphorylation in human isolated preadipocytes along the differentiation process. After acute fat overload, circulating lactoferrin concentration associated positively with the antioxidant proteins and negatively with lipid and inflammatory parameters.

In our knowledge, this is the first study where demonstrated that lactoferrin, a main component of innate immune system, may play a role in the metabolic control and insulin sensitivity.

RESUMEN

La resistencia a la insulina y los transtornos metabólicos asociados a obesidad están estrechamente asociados a un estado de inflamación subclínica. Lactoferrina es una glicoproteina pleiotrópica con actividad inmunomoduladora. Con el propósito de indagar en la interconexión entre el sistema inmune innato y los trastornos metabólicos, planteamos estudiar los efectos de la lactoferrina en la sensibilidad a la insulina y los transtornos metabólicos asociados a obesidad en estudios transversales en humanos y en experimentos *in vitro*.

Se ha estudiado la concentración circulante de lactoferrina (por ELISA) y dos polimorfismos, que producen un cambio aminoacídico en el fragmento funcional de lactoferrina, en asociación con dislipemia, resistencia a la insulina y parámetros relacionados con la obesidad y la inflamación de acuerdo con el grado de tolerancia a la glucosa. Se ha ensayado el efecto de la lactoferrina en la acción de la insulina en las líneas celulares HepG2 y 3T3-L1 durante 24 h, en condiciones normales y en condiciones que inducen resistencia a la insulina, y el efecto que tiene en la adipogénesis de 3T3-L1 mouse cell line y preadipocitos humanos de tejido adiposo visceral y subcutaneo. Finalmente, se ha investigado el efecto de una ingesta aguda de ácidos grasos en la concentración de lactoferrina circulante en pacientes obesos mórbidos.

Los niveles circulantes de lactoferrina se encontraban significativamente disminuidos en sujetos con la tolerancia a la glucosa alterada (AGT), y estos se correlacionaban negativamente con los triglicéridos basales, índice de masa corporal, índice cintura cadera, masa grasa corporal, tensión arterial, glucosa basal, hemoglobina glicosilada, y parámetros inflamatorios, y positivamente con la sensibilidad a la insulina, HDL

colesterol y la proteína antimicrobiana "Bactericidal Increasing Permeability (BPI)". Los cambios aminoacídicos en la región N-Terminal de la lactoferrina que incrementa su cationicidad, se asociaban a un perfil lipídico menos aterogénico.

La producción de lactoferrina en respuesta a endotoxina (LPS) disminuía significativamente en los neutrófilos de pacientes diabéticos tipo 2 y en los neutrófilos de sujetos con tolerancia normal a la glucosa (NGT) co-incubados con IL-6 (5 ng/ml). El tratamiento con rosiglitazona (5 μM) incrementaba significativamente la producción basal de lactoferrina en todos los sujetos.

La lactoferrina atenuaba la respuesta inflamatoria a LPS (100 ng/ml) en la línea celular monocitaria, THP-1, inhibiendo la activación de NFκB en respuesta a LPS. La lactoferrina incrementaba la fosforilación de ^{Ser473}AKT en respuesta a insulina, incluso en condiciones inflamatorias y con administración de dexametasona (dos vías de incremento de resistencia a la insulina) en HepG2 y 3T3-L1. La lactoferrina inhibía la adipogénesis en 3T3-L1 disminuyendo de forma dosis-dependiente las proteínas adipogénicas (FASN, ACC, PPARγ y la acumulación de vacuolas lipídicas) y aumentando la activación de AMPK y de Rb. Contrariamente, la lactoferrina aumentaba significantemente la adipogénesis y la fosforilación de ^{Ser473}AKT en respuesta a insulina en preadipocitos humanos durante el proceso de diferenciación. El aumento de lactoferrina circulante después de la ingesta aguda de ácidos grasos se asociaba positivamente con el incremento de proteínas antioxidantes y negativamente con parámetros inflamatorios y lípidicos.

En base a nuestro conocimiento, es la primera vez que se describe que la lactoferrina, un importante componente del sistema inmune innato, podría participar positivamente en el control metabólico y en sensibilidad a la insulina.

RESUM

La resistència a la insulina sistémica i els desordres metabòlics associats a l'obesitat estan associats a un estat d'inflammació subclínica. La lactoferrina és una glicoproteïna pleiotròpica amb activitat immunomoduladora. Amb l'objectiu d'investigar la interconnexió entre el sistema immunològic innat i els trastorns metabólics, ens plantegem estudiar l'efecte de la lactoferrina en la sensibilitat a la insulina i en els desordres metabòlics associats a l'obesitat en estudis transversals en humans i en experiments *in vitro*.

S'ha estudiat la concentració circulant de lactoferrina (per ELISA) i dos polimorfismes, que produeixen un canvi aminoacídic en el fragment funcional de lactoferrina, en associació amb dislipèmia, resistència a la insulina i paràmetres relacionats amb l'obesitat i la inflamació d'acord amb el grau de tolerancia a la glucosa. S'ha assajat l'efecte de la lactoferrina en l'acció de la insulina en les línies cel·lulars HepG2 i 3T3-L1 durant 24 h, en condicions normals i en condicions que indueixen resistència a la insulina, i l'efecte que té en l'adipogènesis en 3T3-L1 i en preadipocits humans de teixit adipós subcutani i visceral. Finalment, s'ha investigat l'efecte d'una ingesta aguda d'àcids grassos en la concentració de lactoferrina circulant en pacients obesos mòrbids.

Els nivells circulants de lactoferrina es trobaven significativament disminuits en subjetes amb la tolerància a la glucosa alterada (AGT), i aquests es correlacionaven negativament amb els triglicèrids basals, índex de massa corporal, índex cintura cadera, massa de greix corporal, tensió arterial, glucosa basal, hemoglobina glicosilada, i paràmetres inflamatoris, i positivament amb la sensibilitat a la insulina, HDL colesterol i la proteína antimicrobiana " Bactericidal Increasing Permeability (BPI)". Els canvis aminoacídics en la regió N-Terminal de la lactoferrina que incrementa la seva cationicitat s'associaven a un perfil lipídic menys aterogènic.

La producció de lactoferrina en resposta a endotoxina (LPS) reduïa significativament en els neutròfils de pacients diabètics tipus 2 i en subjectes amb la tolerància normal a la glucosa (NGT) aquesta es veia afectada negativament per una co-incubació de IL-6 (5 ng/ml). El tractament amb rosiglitazona (5 μM) incrementava significativament la producció basal de lactoferrina en els neutròfils de tots els subjectes estudiats.

La lactoferrina atenuava la resposta inflamatòria a LPS (100 ng/ml) en la línia cel·lular monocitària, THP-1, inhibint l'activació de NFκB en resposta a LPS. La lactoferrina incrementava la fosforil·lació de ^{Ser473}AKT en resposta a insulina, inclús en condicions inflamatòries i amb l'administració de dexametasona (dos vies d'increment de resistència a la insulina) en HepG2 i 3T3-L1. La lactoferrina inhibia l'adipogènesi en 3T3-L1 reduint de forma dosi-dependent les proteïnas adipogèniques (FASN, ACC, PPARγ y la acumulació de vacuoles lipídiques) y incrementant la activació de AMPK y de Rb. Contrariament, la lactoferrina incrementava la adipogénesis i la fosforil·lació de ^{Ser473}AKT en resposta a insulina en preadipòcits humans durant el procés de diferenciació. L'increment de lactoferrina circulant després de la ingesta aguda d'àcids grassos s'associava positivament amb l'increment de proteïnes antioxidants y negativament amb paràmetres inflamatoris i lipídics.

En base al nostre coneixement, és la primera vegada que es descriu que la lactoferrina, un important component del sistema immunològic innat, podria participar positivament en el control metabòlic i en la sensibilitat a la insulina.

INTRODUCTION

Metabolic disease is an important health problem. Obesity is the underlying common factor to many metabolic disorders. Obesity is a condition that is characterized by excessive accumulation and storage of fat in the body and that in an adult Caucasian is diagnosed by a body mass index of 30 (kg/m^2) or greater.

The incidence of obesity worldwide has increased drastically during recent decades. Consequently, obesity and associated disorders now constitute a serious threat to the current and future health of all populations on Earth. The World Health Organization estimates that more than 1 billion adults worldwide are overweight, 300 million of who are clinically obese. Obesity is closely associated with the development of chronic diseases, including increased risk of insulin resistance, atherosclerosis, endothelial dysfunction, nonalcoholic fatty liver disease, dyslipidemia, hypertension, type 2 diabetes, degenerative disorders including dementia, airway disease and some cancers (1).

Fat distribution in adipose tissue is an important factor in the development of obesity-associated metabolic disturbances. The adipose tissue comprises one of the largest organs in the body. Even lean adult men and women have at least 3.0-4.5 kg of adipose tissue, and in individuals with severe obesity, adipose tissue can constitute 45 kg or more of body weight. The adipose organ is complex, with multiple depots of white adipose tissue involved in energy storage, hormone (adipokine) production and local tissue architecture, as well as small depots of brown adipose tissue, required for energy expenditure to create heat. Excessive accumulation of white adipose tissue in individuals with obesity causes insulin resistance and increases the risk of many metabolic disorders. White adipose tissue is distributed throughout the body in the form

of two major divisions or types: subcutaneous adipose tissue and the intra-abdominal visceral adipose tissue. These two major adipose tissue depots have differential metabolic effects. Epidemiological studies have found that increased visceral adipose tissue, that is, central obesity (determined by large waist circumference or high waist-hip ratio), is associated with adverse health risks, such as insulin resistance, type 2 diabetes mellitus, dyslipidemia (elevated plasma triglycerides (TG) and low levels of high-density lipoprotein (HDL) cholesterol), hypertension, atherosclerosis, hepatic steatosis, cholesterol gallstones and overall mortality (2-4). In this sense, the waist-to-hip ratio has been shown to be a stronger predictor of cardiovascular events than body mass index (5,6). The protective effects of subcutaneous adipose tissue, that is, peripheral obesity (determined by low waist-hip ratio) are done by the higher lipogenic and anti-lipolitic and lipogenic activity in response to insulin (adipogenic capacity), the higher expansion (proliferation and differentiation) capacity, the lower responsiveness to the lipolitic action of catecholamines, the lower levels of macrophages, T cells, and natural killer cells and the decreased release of inflammatory cytokines than visceral adipose tissue (7,8).

Insulin resistance is a central feature of the pathophysiology of most obesity-related disorders including type 2 diabetes, and is defined as a subnormal response of tissues to the actions of insulin (9). Several possible mediators of insulin resistance are associated with obesity, including alteration of lipid metabolism (10) and low-grade inflammation (11). Insulin resistance in skeletal muscle manifests primarily as a reduction in insulin-stimulated glycogen synthesis, which is in turn a consequence of reduced glucose transport. In the liver, insulin resistance seems to be somewhat paradoxically associated with a reduced ability of insulin signaling to inhibit glucose production, whereas insulin-stimulated lipogenesis is enhanced. When the β-cell is healthy, the adaptive

response to insulin resistance involves changes in both function and mass, and is so efficient that normal glucose tolerance is maintained. But when β-cell dysfunction is present, impaired glucose tolerance, impaired fasting glucose and, at the extreme, type 2 diabetes results (12).

The magnitude of the reduction in β-cell function in type 2 diabetes is compatible with a failure of the cell to respond adequately to secretagogue stimulation, an important contributor to reduced insulin release. Type 2 diabetes is progressive, and one of the main factors responsible for this is a continued decline in β-cell function. As a result of β-cell dysfunction and inadequate insulin secretion, postprandial and subsequently fasting glucose levels increase owing to incomplete suppression of hepatic glucose production and decreased efficiency of liver and muscle glucose uptake. The extremely elevated blood glucose levels frequently observed in diabetes might contribute to further disease progression through glucotoxic effects on the β-cell and harmful effects on insulin sensitivity, both of which can be ameliorated by therapeutically lowering the glucose level (12).

Lipid accumulation within skeletal muscle is associated with serine phosphorylation on critical sites on IRS-1 and reduced tyrosine phosphorylation of IRS-1. This in turn inhibits binding and activation of PI 3-kinase. A number of different serine kinases could be responsible for serine phosphorylation of IRS-1. Candidates include members of the nPKC family, which may be activated by accumulation of lipid intermediates (particularly DAGs), as well as inflammatory intermediates such as IKKβ, JNK1, and TNF-α. The latter may be activated within adipose tissue in obese states. Lipid accumulation in skeletal muscle and liver may be a result of increased delivery/synthesis of fatty acids to/in these tissues in states in which energy intake exceeds adipose tissue storage capacity (as seen in obesity and lipodystrophy), or a consequence of either

acquired or inherited mitochondrial dysfunction (10). Insulin resistance in adipose tissue decreases its capacity to storage triglycerides inhibiting the adipogenesis process and increasing the expression of proinflammatory cytokines (11,12).

Endothelial dysfunction is a systemic pathological state of the endothelium (the inner lining of our blood vessels) and can be broadly defined as an imbalance between vasodilating and vasoconstricting substances produced by (or acting on) the endothelium. Endothelial dysfunction is associated with reduced anticoagulant properties as well as increased adhesion molecule expression, chemokine and other cytokine release, and reactive oxygen species production from the endothelium, all of which play important roles in the development of atherosclerosis. In fact, endothelial dysfunction has been shown to be of prognostic significance in predicting vascular events including stroke and heart attacks. A key and quantifiable feature of endothelial dysfunction is the inability of arteries to dilate fully in response to an appropriate stimulus that stimulates release of vasodilators from the endothelium like nitric oxide (NO). Endothelial dysfunction is commonly associated with decreased NO bioavailability, which is due to impaired NO production by the endothelium and/or increased inactivation of NO by reactive oxygen species. Endothelial dysfunction might also increase the risk for insulin resistance and T2DM (14,15). The relationship among endothelial dysfunction, insulin resistance and type 2 diabetes (16,17), could underlie the association between cardiovascular disease and inflammation. Recently, circulating IL-6 has been reported to be linked to endothelial dysfunction independently of insulin sensitivity in apparently healthy men (18).

This study has been focused in the relationship between innate immune system and these obesity-related metabolic disturbances.

1- The relationship between innate immune system and metabolic processes

The high frequency of metabolic disorders and the strong relationship between they and inflammatory processes may reside in the ability to withstand starvation and the capacity to mount an effective immune response to pathogens. Formerly, the lifestyle selected genotypes for energy efficiency and favours the storage of excess calories when access to food was intermittent. However, in the presence of a continuous nutritional addition, this once advantageous metabolic state could set the stage for excess adiposity and its associated problems. The ability to fight off an infection has also led to selection of strong immune responses, particularly after massive population declines during periods of infectious disease epidemics and pandemics. The combination of these attributes is likely to have given rise to a biological organization that is highly capable of processing and storing energy and is also equipped with a powerful, and perhaps at times overly sensitive, immune response (1).

There is also a strong relationship between the immune and metabolic response systems that has many evolutionary underpinnings. For example, the Drosophila fat body, which incorporates the mammalian homologues of the liver and the hematopoietic and immune systems and carries out a crucial function in sensing energy and nutrient availability, and coordinates the appropriate metabolic and survival responses (19). In higher organisms, the adipose tissue, liver and hematopoietic system have specialized into distinct functional organs. Thus, it is possible to imagine metabolic and immune functions regulated by common or overlapping through common key regulatory molecules and signaling systems. It has been reported that nutrients can induce inflammatory responses through pathogen-sensing systems such as Toll-like receptors (TLRs) (20-22).

None of these systems have evolved and adapted to be beneficial in the presence of continuous nutrient abundance such as we are now experiencing. These historically advantageous traits and the juxtaposition of nutrient and pathogen responses have established the groundwork for chronic metabolic diseases. Obesity, insulin resistance and type 2 diabetes are closely associated with chronic 'inflammation' characterized by abnormal cytokine production, increased acute-phase reactants and other mediators, and activation of a network of inflammatory signaling pathways (23).

1.1- Insulin resistance and chronic low level inflammation

Obesity-associated metabolic alterations are increasingly envisioned as a chronic inflammatory disease in which macrophage infiltration into the adipose tissue may play a role in insulin resistance-associated inflammatory activity. In this sense, macrophage-secreted factors block adipogenesis and insulin action in adipocytes and hepatocytes, via down regulation of insulin receptor sustrate-1 (IRS-1), leading to a decrease in Akt phosphorylation (24-27). Central to metabolic diseases is insulin resistance associated with a low-grade inflammatory status (28-30). The mechanisms through which proinflammatory cytokines, like tumor necrosis factor α (TNF-α), interleukin-6 (IL-6) and interleukin 1-β (IL-1β) interact with cellular insulin signal transduction cascades have been described in the last years (31-34). *In vivo*, a direct correlation between increased circulating proinflammatory cytokines and insulin resistance has been well-demonstrated (30). Insulin stimulates tyrosine phosphorylation of IRS proteins, which is a crucial event in mediating insulin action. This step in insulin-receptor signaling is defective in most cases of systemic insulin resistance, both in experimental models and

in humans. TNF-α-induced insulin resistance is produced through inhibitory serine phosphorylation of IRS-1 (35).

It has now been established that IRS-1 is phosphorylated at serine residues by various kinases that interfere with the ability of this protein to engage in insulin-receptor signaling and result in alterations in insulin action (36-38). Among these IRS-modifying enzymes, mounting evidence indicates that activation of JNK, IKK and conventional protein kinase C (PKC) is central to mediating insulin resistance in response to various stresses that occur in obesity and other conditions of insulin resistance.

We here summarize the role of these cytokines:

TNF-α

The finding a little over a fifteen years ago that tumour necrosis factor-α (TNF-α) is over-expressed in the adipose tissue of obese mice provided the first clear link between obesity, diabetes and chronic inflammation (39). TNF-α is a proinflammatory cytokine that activates various signal transduction cascades, including critical inhibitors of insulin action. In obese mouse models a lack of TNF-α function results in improved insulin sensitivity and glucose homeostasis, confirming that this inflammatory response has a critical role in the regulation of insulin action in obesity (40,41). TNF-α is also over-expressed in the adipose and muscle tissues of obese humans, and when administered exogenously leads to insulin resistance (42).

Interestingly, the widespread use of anti-TNF-α treatments in inflammatory diseases such as rheumatoid arthritis have produced clear secondary results supporting a role for TNF-α in systemic insulin sensitivity in humans (43).

Various other inflammatory mediators and cytokines, such as IL-6, IL-1B, IL-8, NGAL and MCP-1 are also over-expressed in adipose and other tissues contributing to insulin resistance and metabolic disturbances.

IL-6

IL-6 has been described as a proinflammatory cytokine that can contribute to insulin resistance in peripheral tissues when overproduced by adipose tissue. The adipose tissue plays a key role in IL-6 production and is a major target of the cytokine. Adipose cells responded to IL-6 and TNF-α by increasing the production and secretion of IL-6. IL-6 altered insulin signaling after a chronic exposure (8 days) to differentiating (3T3-F442A) or fully differentiated (3T3-L1) adipocytes. In fact IL-6 had dual effects on insulin action. It acutely (30–180 min) mimicked insulin action on ERK 1/2 and Akt activation and chronically induced insulin resistance. Indeed, the inhibition of ERK1/2 and Akt/PKB phosphorylation appeared after a 24-h exposure and aggravated after 4 days of exposure. This was confirmed by the adverse effects of IL-6 on the markers of the mature adipocyte involved in glucose and lipid metabolism (FASN, GAPDH, GLUT4, and aP2) and insulin sensitivity (IRS-1 and IR-β). Rosiglitazone prevented all the adverse effects of IL-6 on both differentiating (3T3-F442A) and fully differentiated (3T3-L1) adipocytes. IL-6 had adverse effects at both the receptor (IR-β and IRS-1 tyrosine phosphorylation) and post-receptor levels (ERK 1/2, Akt/PKB, glucose transport, and lipogenesis). SOCS-3 can bind to phosphorylated tyrosine of IR-β and inhibit insulin signaling. The negative effect of IL-6 on insulin signaling is linked to the up-regulation of SOCS-3 mRNA expression. Rosiglitazone-treated cells had a decreased SOCS-3 and IL-6 mRNA expression that could result from the decreased IL-6 secretion or from a direct effect of rosiglitazone on IL-6 signaling (44, 45).

IL-6 is also expressed by skeletal muscle during exercise, with positive metabolic effects that can modulate insulin action. IL-6 per se activated glucose uptake, a dual effect on insulin action was observed: short-term IL-6 treatment was additive to insulin on activating glucose uptake and AS160 phosphorylation, which resulted in an

improvement in glucose tolerance and insulin sensitivity in mice, whereas chronic exposure produced insulin resistance both *in vitro* and *in vivo*. IL-6 induces the sequential phosphorylation of LKB1, AMPK, and AS160. LKB1 was phosphorylated by IL-6 at Ser431, although the state of phosphorylation of this kinase did not significantly affect LKB1 catalytic activity or its cellular location, as described previously. Short-term (3 h) pretreatment with IL-6 followed by acute insulin stimulation produced an additive increase in glucose uptake in C2C12 myotubes. This increase is a consequence of the activation of AMPK and Akt by IL-6 and insulin, respectively, and is additive to AS160 phosphorylation, as observed in C2C12 cells and in skeletal muscle, in agreement with other reports (46). Chronic exposure (24 h) to IL-6 impaired insulin-stimulated glucose uptake and GLUT4 translocation in both C2C12 and neonatal myotubes. Accordingly, insulin-stimulated IRS-1 and Akt phosphorylation was inhibited by IL-6. Moreover, no phosphorylation of AMPK or AS160 was detectable, a fact that indicates a reciprocal negative cross talk in the signaling pathways elicited by insulin and IL-6 under chronic treatment with the cytokine. The molecular mechanism underlying IL-6–mediated insulin resistance could involve activation of JNK1/2, accumulation of *socs3* mRNA, and increases in *ptp1b* mRNA and activity in murine myotubes. IL-6 impairs insulin signaling at the level of IRS-1 by three mechanisms that involve *1*) serine phosphorylation by JNK, *2*) impairment of tyrosine phosphorylation by SOCS3, and *3*) tyrosine dephosphorylation by PTP1B (47).

Recently, it has been proposed that mTOR may play a key role in IL-6-induced hepatic insulin resistance by regulating STAT3 activation and subsequent SOCS3 expression (48).

IL-1β

IL-1β exerts its biological function by binding to IL-1 type I receptor and activates the IKK/NF-κB pathway and the three types of mitogen-activated protein (MAP) kinases ERK, JNK and p38MAPK. Recent studies suggest that IL-1β could also belong to the network of cytokines involved in insulin resistance (49). Indeed, in a case/control study, individuals with detectable circulating levels of IL-1β and elevated levels of IL-6 have an increased risk to develop type 2 diabetes compared with individuals with increased concentrations of IL-6 but undetectable levels of IL-1β. Further, IL-1β concentration are elevated in non-diabetic offspring of diabetic individuals and are correlated with the metabolic syndrome. Expression of both IL-1β and its receptor is increased in visceral adipose tissue of obese subjects (49).

Prolonged IL-1β treatment induces an inhibition of insulin effect on glucose uptake in murine and human adipocytes. A sustained increase in the expression of IL-1β in adipose tissue during obesity could thus participate in the development of the insulin resistance. This inhibitory effect was mainly due to the down-regulation of the expression of IRS-1 and to a lesser degree of Glut 4. IL-1β for 6 days did not modify their differentiation state, whereas addition of the cytokine during the differentiation process markedly altered the adipocyte phenotype (50).

IL-8

IL-8 is produced and released from human adipose tissue and from isolated adipocytes *in vitro*, mainly under proinflammatory conditions (IL-1β and TNF-α treatment) (51). The role of IL-8 in insulin resistance has been poorly investigated. Recently, it has been reported that the IL-8 administration in acute and chronic exposure inhibited insulin-

induced Akt phosphorylation in human adipocytes via the MEK and p38 MAPK pathway (52).

NGAL

A recently characterized factor produced by the adipose tissue is lipocalin 2 (also known as 24p3, and neutrophil gelatinase-associated lipocalin (NGAL), siderocalin). NGAL is a 25-kDa secretory glycoprotein that belongs to the lipocalin family. The members of the lipocalin family contain a common tertiary structure with an eight-stranded B-barrel surrounding a cup-shaped ligand binding interior, covered with hydrophobic amino acid residues. This structure confers lipocalins the ability to bind and transport a wide variety of small lipophilic molecules (53). Known ligands for lipocalins include retinol, steroids, odorants, pheromones, and, in the case of NGAL, siderophores (54). NGAL is expressed in many tissues and cells in addition to adipose tissue, including kidney, liver, lung, thymus, small intestine, mammary tissue and leucocytes (macrophages and neutrophils). Expression of NGAL in liver, macrophages and adipocytes is markedly induced by a variety of pro-inflammatory stimuli through activation of NF-κB (55). NGAL was elevated in multiple murine models of obesity and reduction of NGAL in cultured adipocytes improved insulin sensitivity. Data from *db/db* mice (56-57) indicated an elevated NGAL expression in the liver, while in high-fat-fed mice liver NGAL expression tended to be lower. The authors concluded that the contribution of extra-adipose sources of NGAL to serum was unclear and may differ between obesity models. Studies in humans showed a positive relationship between circulating NGAL concentrations and fasting insulin and HOMA values. However, the origin of increased circulating NGAL in humans is poorly known. As NGAL concentrations were positively correlated with several adiposity variables, including

BMI, waist circumference, and fat percentage, some authors suggested that the increased fat mass might also account for the increased circulating concentrations of this protein in obese humans (58). Recently, it has been reported that metabolic endotoxemia (metabolic LPS concentration, which was not enough to produce acute endotoxemia) and saturated fat intake might contribute to circulating NGAL concentrations in patients with insulin resistance (59). Law *et al.* reported that NGAL increases insulin resistance stimulating the expression and activity of 12-lipoxygenase (increasing the amounts of arachidonic acid) and TNF-α production in fat tissues (60).

MCP-1

Monocyte chemoattractant protein 1 expression decreased during adipocyte differentiation, but increased significantly under proinflammatory stimuli (TNF-α and IL-6) and other factors (GH and insulin) that induce insulin resistance at least in part mediated by Janus kinase 2 and p44/42 mitogen-activated protein kinase (61). The MCP-1 gene expression in adipose tissue was increased both in genetically obese diabetic (*db/db*) mice and in WT mice with obesity induced by a high-fat diet. The increase of MCP-1 gene expression in adipose tissue contributes to the macrophage infiltration into this tissue, insulin resistance, and hepatic steatosis associated with obesity in mice (62).

MCP-1 alone impaired insulin signaling in skeletal muscle cells at doses similar to its physiological plasma concentrations (200 pg/ml). MCP-1 significantly reduced insulin-stimulated glucose uptake in the myocytes. Expression analysis of chemokine receptors in skeletal muscle cells revealed the presence of chemokine CXC motif receptor 1/2 and chemokine CC motif receptor 1/2/4/5/10. The action of MCP-1 on insulin signaling in

skeletal muscle cells occurs via ERK1/2 activation but does not involve activation of the NF-κB pathway (63).

1.2- Neutrophil dysfunction in metabolic disease

Given that 60–70% of blood leukocytes are granulocytes and over 90% of granulocytes are neutrophils, polymorphonuclear cells (PMNs) are the largest fraction of white blood cells. PMNs possess a variety of functions including chemotaxis, adhesion to the endothelium and foreign agents, phagocytosis, and microbicidal activity. PMNs are able to penetrate and migrate into infected tissues and destroy invading microorganisms after internalization by producing multiple toxic agents such as reactive oxygen species (ROS), proteases (elastase), and proteins interfering with bacterial development.

Chronic disease (such as type 2 diabetes), age-associated insulin resistance, nutrition, and lifestyle have a significant effect on PMN function. Of note, the risk of infectious diseases is 2-fold to 4-fold higher in patients with diabetes, or even impaired glucose tolerance without hyperglycemia, than in healthy subjects (64). The neutrophils of diabetic patients show enhanced production of reactive oxygen species, increased apoptosis, and significantly lower neutrophil chemotactic responses. It is notable that the circulating levels of proinflammatory cytokines are elevated in diabetic patients, and it has been suggested that the impaired functions of neutrophils contribute to the increased susceptibility to infections observed in these patients. Hyperglycemia, or the presence of AGEs, leads to persistent activation of neutrophils, as evidenced by the increased activity of neutrophil alkaline phosphatase (65), Furthermore, both an increased basal release of TNF-α, IL-8, and IL-6 (65,66) and a low secretion of some granular proteins by neutrophils from patients with type 2 diabetes (T2DM) (67)

(T2DM) has been reported. In addition, the impaired actin polymerisation in neutrophils from T2DM patients was a main factor in the inability of neutrophils to down-regulate integrin CD11b/CD18 and to exocytose primary granules (CD69), altering neutrophil exocitosis (67).

It has previously been shown that insulin has a strong regulating effect on the functional activities of immune cells (68,69). Generally speaking, the priming action of insulin on PMN activity may be seen as the body providing a global defense to support primary immune response against exposure to antigens, which is enhanced by food intake (70). Walrand *et al* showed that aging-induced reduction in insulin sensitivity plays a role in the age-related weakening of the immune system (71), particularly after food intake (70). Therefore, alterations in immune cell function may partly explain the higher prevalence of infective episodes in the type 2 diabetes and older population. Previous studies have shown that the clearly altered PMN functions of diabetic subjects could be restored by controlling hyperglycemia with insulin. Interestingly, although PMNs do not require insulin to uptake glucose, glucose use and glycogen metabolism inside PMNs are both insulin dependent. In addition, insulin receptor expression was correlated with PMN chemotaxis in both young and elderly subjects after the insulin treatment (71). Antimicrobial protein production in PMNs is also altered in association with insulin resistance and in the elderly (71) (as reviewed below), and decreased under hyperglycemia conditions in humans after intravenous endotoxin administration (72).

1.3- Immunomodulation as a therapeutic target in inflammatory-induced insulin resistance

Recently, anti-inflammatory treatment has been postulated to be a therapeutic tool treating inflammation-induced insulin resistance (73). The most important anti-

inflammatory molecules studied *in vivo* and *in vitro* experiments were adiponectin, salicylates and IL-10.

Adiponectin

Adiponectin (ADIPOQ) is almost exclusively produced by adipocytes and abundantly present in serum, where it circulates in two higher-order forms: a low-molecular weight dimers or trimers and a larger high-molecular weight complex of 12-18 subunits (74). ADIPOQ is known to affect LPS-mediated inflammatory events. It inhibits LPS-induced NF-κB activation and IL-6 production, and increases PPAR-γ expression in adipocytes, while in macrophages it suppresses both LPS-induced TNF-α and IL-6 production. Peake *et al.* suggested that adiponectin may have anti-inflammatory potential by directly binding to LPS (75). It is well known that adiponectin expression is reduced in obesity and insulin resistance states. Plasma levels of adiponectin have also been reported to be significantly reduced in obese/diabetic mice and humans, and in patients with cardiovascular diseases, hypertension or metabolic syndrome (76). A direct insulin-sensitizing effect of adiponectin *in vivo* has been extensively reported. The main mechanism of action of adiponectin in insulin-sensitizing actions are mediated through a reduction of tissue triglyceride content and activation of PPAR-α (77) and AMP kinase (78,79) leading to the up-regulation of insulin signaling. However, the anti-inflammatory effects of adiponectin and LPS neutralizing action cannot be forgotten, as two indirect ways to improve insulin sensitivity.

IL-10

Co-treatment of the anti-inflammatory cytokine IL-10 completely prevented IL-6–induced defects in both hepatic and skeletal muscle insulin action. The protective effects of IL-10 on IL-6–induced insulin resistance involved normalization of insulin signaling and reduction in intramuscular fatty acyl-CoA levels compared with the IL-6–treated mice (80). Recently, Hong et al have shown that IL-10 increases insulin sensitivity and protects skeletal muscle from obesity-associated macrophage infiltration (81).

Salicylates

Several studies showed that salicylates improved the glucose levels in subjects with type 2 diabetes or in glucose intolerant subjects through the combined effects of increased peripheral insulin sensitivity (inhibiting IKKβ activity) (82-84) and higher plasma insulin concentrations (increasing insulin secretion) (85).

Two other molecules, insulin (86) and rosiglitazone (87,88) used in the insulin resistance – associated metabolic disturbances therapy also have a significant anti-inflammatory activity.

2- Lactoferrin

2.1- Biochemical characteristics and lactoferrin gene

The ‘‘moonlighting’’ proteins are those proteins that can have different job descriptions in different environments. In this context, lactoferrin may be identified as a classic multifunctional moonlighting protein. Lactoferrin was first discovered more than 60 years ago as a “red protein” from milk, and has been the subject of intensive structural and functional studies since it was first purified from human and bovine milks, in 1960. The characteristic intense red color is caused by the iron binding property. Intriguingly,

Lf was found not only in milk (1g/l) and in colostrums (7g/l), but also in other external secretions with lower concentration, such as saliva, tears, semen, and mucosal secretions, and as an important constituent of the neutrophilic granules of leucocytes. Lactoferrin concentration in plasma is 0.4 – 1 mg/l in normal condition and in sepsis state can increase until 200 mg/l (89). In several cancers, lactoferrin expression was decreased or blunted by epigenetic processes (90).

Lactoferrin consists of a single polypeptide chain of 692 amino acids (80 kDa) and is a member of the transferrin family. Lf differs from transferrin by its higher affinity for iron (Ka of about 10^{-24}M) and its ability to retain iron at a pH lower than 4 (89).

Two notable features of the Lf sequence are:

-Its two fold internal sequence repeat, indicative of an evolutionary gene duplication event.

-Its highly basic character. Lf haves an isoelectric point of 9 in physiological conditions. This feature is extremely important to many of the biological activities of Lf. For example, a region rich in basic amino acid residues near the N-terminus of lactoferrin was responsible for the interaction with ac- or ox-LDL. This cationic fragment of lactoferrin was strongly bound on modified LDLs by electrostatic interaction (91). Arginine residues provided this positive charge because they have an isoelectric point of 9, which is higher than the physiologic pH (89). Two non-synonymous single nucleotide polymorphisms (SNPs) have been described to be associated with amino acid changes in the N-terminus region of lactoferrin. These SNPs lead to Lys to Arg change in the 47 aminoacidic position (rs1126478), and to Ala change by Tre in the 29 aminoacidic position (rs1126477).

Structural studies on the Lfs from other species and on Tf have shown that the same basic structure is shared across the whole Tf family. The single polypeptide chain is

folded into two globular lobes (Figure 1), representing its N- and C-terminal halves (residues 1-333 and 345-691 in human Lf). The two lobes are connected by a peptide of 10-15 residues (residues 334-344 in human Lf), which in Lfs forms a 3-turn a-helix, but in Tfs is irregular and flexible, and are stabilized in their association by a ‘‘cushion’’ of non-covalent interactions, mostly hydrophobic, between them. Both lobes have the same fold, consistent with their sequence homology. In each lobe, two a/b domains, referred to as N1 and N2, or C1 and C2, enclose a deep cleft within which is the iron binding site (89).

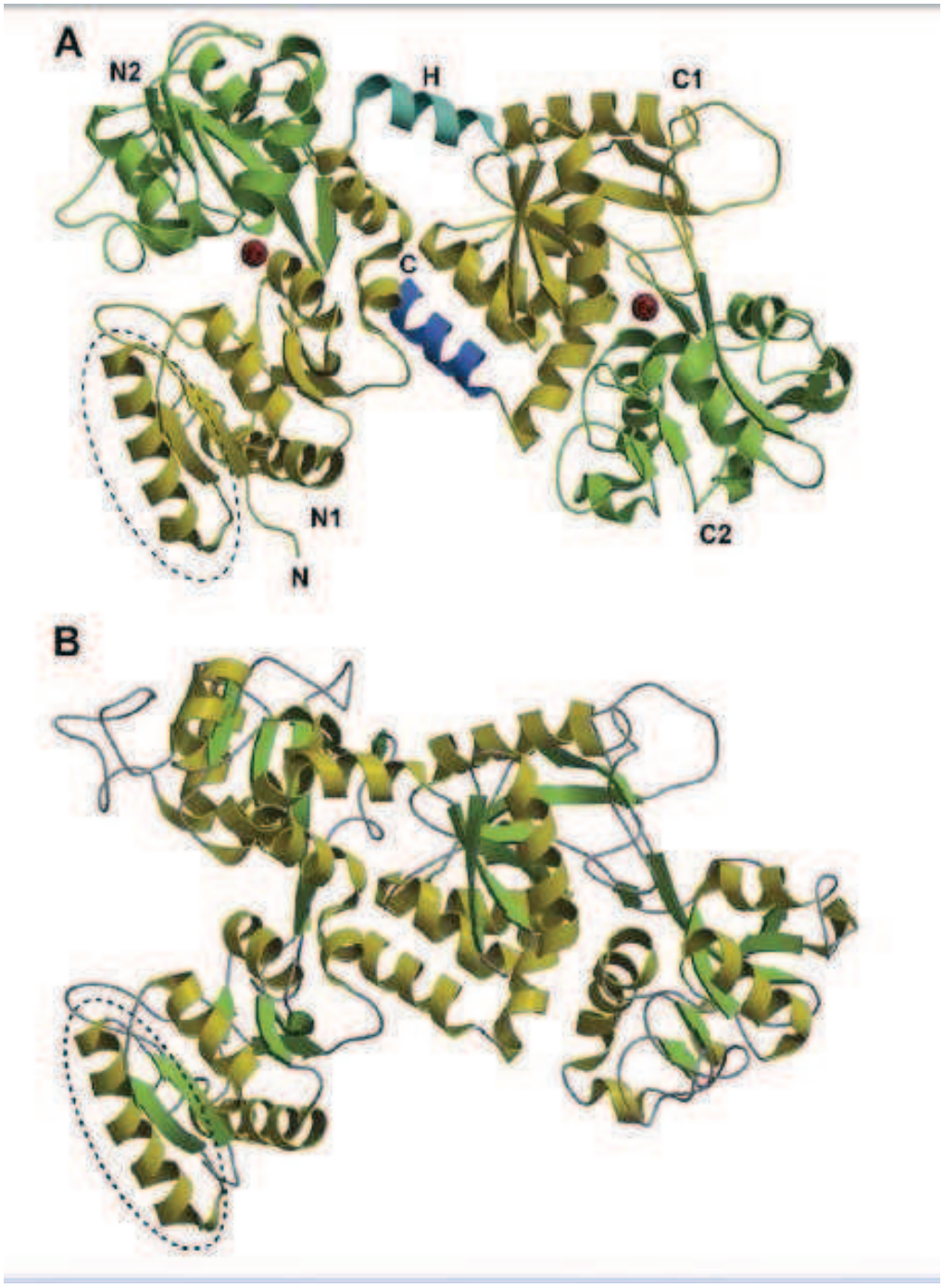


Figure 1. Polypeptide folding of lactoferrin. (A) Structure of the iron-bound (holo) form of human Lf. The N-lobe is on the left and the C-lobe is on the right, with the N- and C-termini of the polypeptide chain labeled N and C. The four domains are labeled N1, N2, C1, C2, with the N1 and C1 domains shown in gold and the N2 and C2 domains shown in green. The helix which joins the two lobes (H) and the C-terminal helix are shown in blue. The two iron binding sites are shown with red spheres.
(B) Structure of the iron-free (apo) form of Lf, shown for one of the two crystal structures of apo-Lf. In this structure, the N-lobe (left) is wide open following a 54º rotation about a hinge behind the iron site, whereas the C-lobe is closed, albeit with no metal bound. Other apo-Lf crystal structures have been solved with both lobes open or both lobes closed. In both diagrams, the location of the antibacterial lactoferricin domain is outlined by a broken line.

E.N. Baker, H.M. Baker / Biochimie 91 (2009) 3-10

The first helix (residues 12-31 in human Lf) forms the major part of the bactericidal domain identified by Bellamy *et al.* (92), described as the lactoferricin (Lfcn) domain. The Lfcn peptide, when released by proteolysis of the intact protein, is a potent bactericidal agent, probably because it is able to form amphipathic structures that disrupt cell membranes. This region is also a major factor in the antibacterial activity of intact Lf, probably in a similar manner through disruption of cell membranes by the basic residues (Lys and Arg) arrayed along the outside of this helix, which is highly solvent-exposed on the surface of the N-lobe (Fig. 1) (89,93).

Lactoferrin is expressed in three distinct patterns during embryogenesis. First, lactoferrin is expressed at the 2-cell stage in the preimplantation embryo where it continues to be expressed until the blastocyst stage when expression ceases. The second phase of lactoferrin expression is not detected until the latter half of gestation when the protein is detected in the myeloid cells, beginning in the fetal liver at embryonic day 11 and later in the spleen and bone marrow coinciding with the onset and diversification of myelopoiesis in these organs during embryogenesis. Finally, lactoferrin is detected in a variety of glandular epithelial cells and/or their secretions, including respiratory and oral epithelia which is consistent with the expression pattern observed for this protein in the adult where it plays an important role in host defense at the mucosal surface (94).

2.2-Lactoferrin activities

Lactoferrin and iron status

Lactoferrin (Lf) and transferrin (Tf) ensure that the proper 10^{-18} M free iron concentration in human fluids is maintained, thus avoiding iron precipitation, ROS induction and microbial colonization (95, 96).

Oral administration of 100 mg of Lf (about 30% iron-saturated) twice a day before the meal, increased the total serum iron and hemoglobin concentrations at a greater extent than that observed after oral administration of ferrous sulphate. In contrast to the administration of ferrous sulphate, Lf oral administration did not result in any side effect (97). Oral administration of Lf also increases the number of red blood cells and serum ferritin concentrations at a higher extent with respect to orally administered ferrous sulphate. Lactoferrin administration also decreased IL-6 concentration, avoiding the increase of IL-6 produced by ferrous sulphate (97,98).

Antimicrobial activity

The widespread distribution of the molecule in all the body suggests that Lf is in the front line of host defense. The type of Lf secretion is correlated with its function: direct anti-microbial activities in secretions and at the surface of epithelia or regulation of the inflammatory response.

The antibacterial functions of lactoferrin have been substantiated by both *in vitro* and *in vivo* evidence. It appears that two different mechanisms involving two separate domains of the protein contribute to the antimicrobial functions of lactoferrin. The first mechanism is a bacteriostatic effect related to the high iron binding affinity of the protein that deprives iron-requiring bacteria of this essential growth nutrient. The

second antibacterial property of lactoferrin is due to a direct bactericidal function within the protein. A cationic domain at the N-terminus of lactoferrin is responsible for its bactericidal properties, damaging the outer membrane of gram negative bacteria (99,100).

Anti-inflammatory activity

Its expression is up-regulated in response to inflammatory stimuli (101). Lactoferrin is able to bind and buffer several pathogens associated molecular patterns such as lipopolysaccharide (LPS), viral components and soluble components of the extracellular matrix (102). This ability is associated with the putative lactoferrin anti-inflammatory activity, as demonstrated in several studies (103). Lactoferrin administration led to decreased release of TNF-α and IL-6 in mice (104,105), to down-regulated pro-inflammatory cytokine production in different cell lines acting via NF-κβ (106), and to decreased LPS-induced binding of NF-κB to the TNF-alpha promoter (106).

Antioxidant activity

The high affinity and stability of iron binding by Lf make the protein not only a powerful bacteriostatic agent but also an antioxidant protective molecule. Experimental studies in cells demonstrated that lactoferrin significantly inhibited/decreased intracellular reactive oxygen species levels in a dose-dependent manner and protected from oxidative stress (107, 108).

Lactoferrin and cell proliferation and differentiation

In primary osteoblasts, lactoferrin stimulated proliferation and differentiation and acted as a survival factor, inhibiting apoptosis induced by serum withdrawal, and inhibiting

osteoclastogenesis in a murine bone marrow culture (109). LF was shown to enhance osteoblasts differentiation and inhibit osteoclastogenesis. The anabolic effects on bone growth were substantiated by *in vivo* studies where subcutaneous administration of LF (4 mg daily for 5 days) to mice resulted in a four-fold increase in bone mass (110). In a follow-up study, it was shown that the mitogenic response of LF in osteoblasts is mediated in part by binding and signaling through the low-density lipoprotein receptor-related protein-1 (LRP-1) (111). LF has also been demonstrated to have mitogenic effects on other cell types, including rat and human enterocytes (112-114) B and T lymphocytes (115) and macrophages (116).

Anti-atherosclerotic activity

Bovine lactoferrin has been described to display beneficial effects on plasma lipid concentrations. Its administration led to increased plasma HDL-cholesterol concentration, decreased plasma triacylglycerol and NEFA accompanied by decreased hepatic cholesterol and triacylglycerol contents in rodents (117-122).

Anticancer activity

A growing number of rodent studies have demonstrated a protective effect of LF against chemically induced carcinogenesis, tumor growth and/or metastasis in several organs, including the esophagus, tongue, lung, liver, colon and bladder (123-127). Moreover, it appears that like many of the biological functions of LF, the anti-cancer role of this protein may be multifaceted. A direct effect on tumor cell growth was first suggested by the observation that LF and a splice variant thereof are down-regulated or absent in many cancer cell lines and in experimental tumors (128-130). The negative effect on cellular proliferation appears to be due to LF-induced alterations in the expression

and/or activity of critical cell cycle regulatory proteins including the Cdk inhibitors p21 and p27, which may be mediated in part by modulation of the Akt and MAPK pathways (131). LF stimulates the production and/or activation of several immune cells, including lymphocytes and NK cells (132,133), in addition to increasing the target cell sensitivity to NK lysis (134). In this regard, up-regulation and/or enhanced activation of NK cells, CD4+ T lymphocytes and CD8+ T-lymphocytes were observed upon LF administration. Lactoferrin has also been implicated in the modulation of retinoblastoma (Rb, a suppressor tumor gene) activity and expression (135).

HYPOTHESIS

Lactoferrin may modulate proinflammatory–induced insulin resistance and obesity-related metabolic disturbances.

OBJECTIVES

1- **To evaluate the relationship among lactoferrin gene polymorphisms, circulating lactoferrin concentrations, plasma lipid concentrations, vascular reactivity (an endothelial dysfunction measure) and obesity according to glucose tolerance status.**

2- **To evaluate the relationship between circulating lactoferrin and insulin sensitivity.**

3- **To evaluate the effects of acute fat intake in circulating lactoferrin levels in association with baseline and postprandial plasma lipid concentrations, parameters of oxidative stress and inflammation in subjects with morbid obesity.**

4- **To test the effects of lactoferrin on 3T3-L1 adipocyte differentiation and on insulin-induced ^{473Ser}AKT phosphorylation in the 3T3-L1 fibroblast mouse cell line.**

5- **To test the effects of lactoferrin on subcutaneous and visceral human pre-adipocyte differentiation.**

MANUSCRIPTS

Manuscript 1

Moreno-Navarrete JM, Ortega FJ, Bassols J, Castro A, Ricart W, Fernandez-Real JM. Association of circulating lactoferrin concentration and 2 nonsynonymous LTF gene polymorphisms with dyslipidemia in men depends on glucose-tolerance status. *Clinical Chemistry* 54:2 301–309, 2008.

José Maria Moreno-Navarrete, Francisco José Ortega, Judit Bassols, Antoni Castro, Wifredo Ricart, and José Manuel Fernández-Real. "Association of circulating lactoferrin concentration and 2 nonsynonymous *LTF* gene polymorphisms with dyslipidemia in men depends on glucose-tolerance status". *Clinical Chemistry*. Vol. 54, issue 2 (February 2008) : p. 301-309

http://dx.doi.org/10.1373/clinchem.2007.095943

http://www.clinchem.org/cgi/content/abstract/54/2/301

Background: Lactoferrin, an innate immune protein with antiinflammatory properties, shows considerable antiatherosclerosis activity in animal studies. We investigated the relationship between circulating lactoferrin, lactoferrin gene (*LTF*, lactotransferrin) polymorphisms, dyslipidemia, and vascular reactivity in the context of glucose-tolerance status in men.

Methods: We evaluated 2 nonsynonymous *LTF* polymorphisms (rs1126477 and rs1126478) and measured circulating lactoferrin concentrations by ELISA under nonstressed conditions in healthy Caucasian men (n = 188) and male patients with an altered glucose tolerance (n = 202). We also studied the association of lactoferrin concentration with vascular reactivity via high-resolution ultrasound analysis of the brachial artery in a subsample of study participants.

Results: Circulating lactoferrin concentration was inversely associated with fasting triglyceride concentration ($r = –0.24$; $P = 0.001$), body mass index (BMI) ($r = –0.20$; $P = 0.007$), waist-to-hip ratio ($r = –0.35$; $P < 0.001$), and fasting glucose concentration ($r = –0.18$; $P = 0.01$), and directly correlated with HDL cholesterol concentration ($r = 0.21$; $P = 0.004$). Control AG heterozygotes for rs1126477 had significantly decreased fasting triglyceride concentrations ($P = 0.001$). Similarly, control individuals who were G carriers for rs1126478 had significantly lower fasting triglyceride concentrations ($P = 0.044$) and significantly higher HDL cholesterol concentrations ($P = 0.028$) than AA homozygotes. These associations remained significant after controlling for age, BMI, waist-to-hip ratio, fasting glucose concentration, smoking status, and alcohol intake. Circulating lactoferrin concentration was not significantly associated with endothelium-dependent vasodilatation (EDVD) in the individuals studied (n = 95); however, lactoferrin was positively associated with EDVD in obese participants with an altered glucose tolerance ($r = 0.54$; $P = 0.04$).

Conclusions: We have identified associations among *LTF* polymorphisms, circulating lactoferrin concentration, fasting triglyceride concentration, and vascular reactivity in humans.

Manuscript 2

Moreno-Navarrete JM, Ortega FJ, Bassols J, Ricart W, Fernandez-Real JM. Decreased circulating lactoferrin in insulin resistance and altered glucose tolerance as a possible marker of neutrophil dysfunction in type 2 diabetes. *J Clin Endocrinol Metab* 94: 4036–4044, 2009.

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

J. M. Moreno-Navarrete, F. J. Ortega, J. Bassols, W. Ricart, and J. M. Fernández-Real

Section of Diabetes, Endocrinology, and Nutrition, Institut d'Investigació Biomédica de Girona, 17007 Girona, Spain; and CIBER Fisiopatologia Obesidad y Nutricion (CB06/03/010), Instituto de Salud Carlos III, 28029 Madrid, Spain

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-α, IL-6, and IL-1β 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-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2009-0215 Received January 29, 2009. Accepted June 29, 2009.
First Published Online July 7, 2009

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-α 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.

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

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-

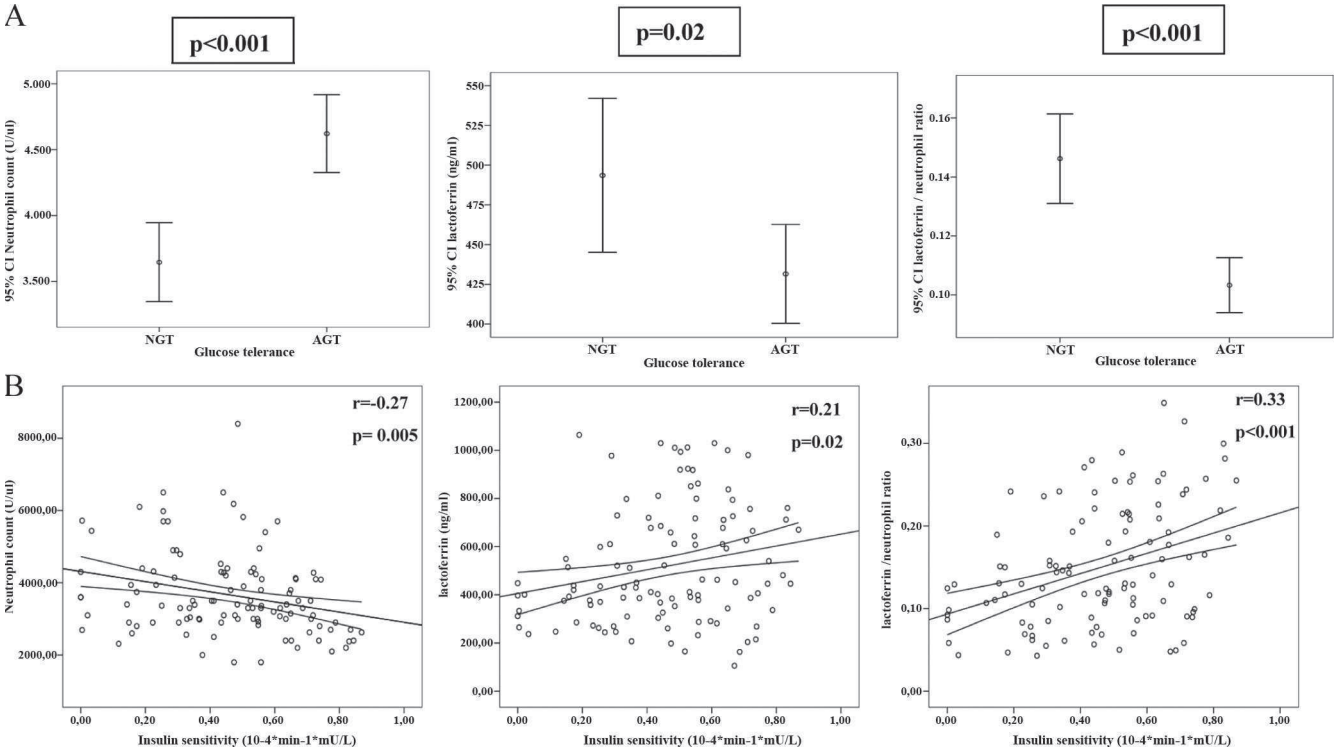


FIG. 1. A, 95% confidence interval for the mean of neutrophil, circulating lactoferrin and normalized lactoferrin according to glucose tolerance status. B, Linear relationship between lactoferrin, neutrophil count, and normalized lactoferrin, respectively, and insulin sensitivity.

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 (μg/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.* $4.8 \pm 0.32\%$, $P < 0.001$). 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 (μg/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.

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-1β, TNF-α, 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 μM) 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 μM) and LPS (100 ng/ml) was compared with LPS treatment alone ($P < 0.01$) (Fig. 3B).

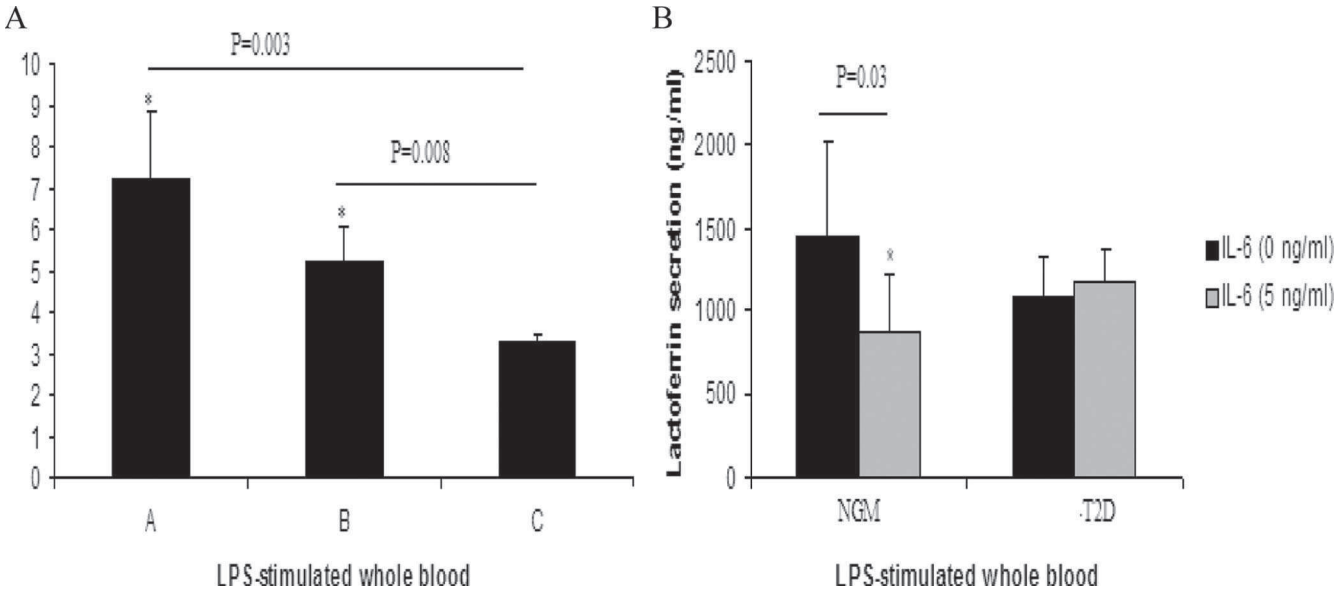


FIG. 2. A, LPS-induced lactoferrin secretion in different groups according to glucose metabolism status and age (A, NGT subjects aged 32 ± 3 yr; B, NGT subjects aged 55 ± 2 yr; C, type 2 diabetes subjects aged 57 ± 3 yr). B, LPS-induced lactoferrin secretion in the presence of IL-6 in NGT and type 2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

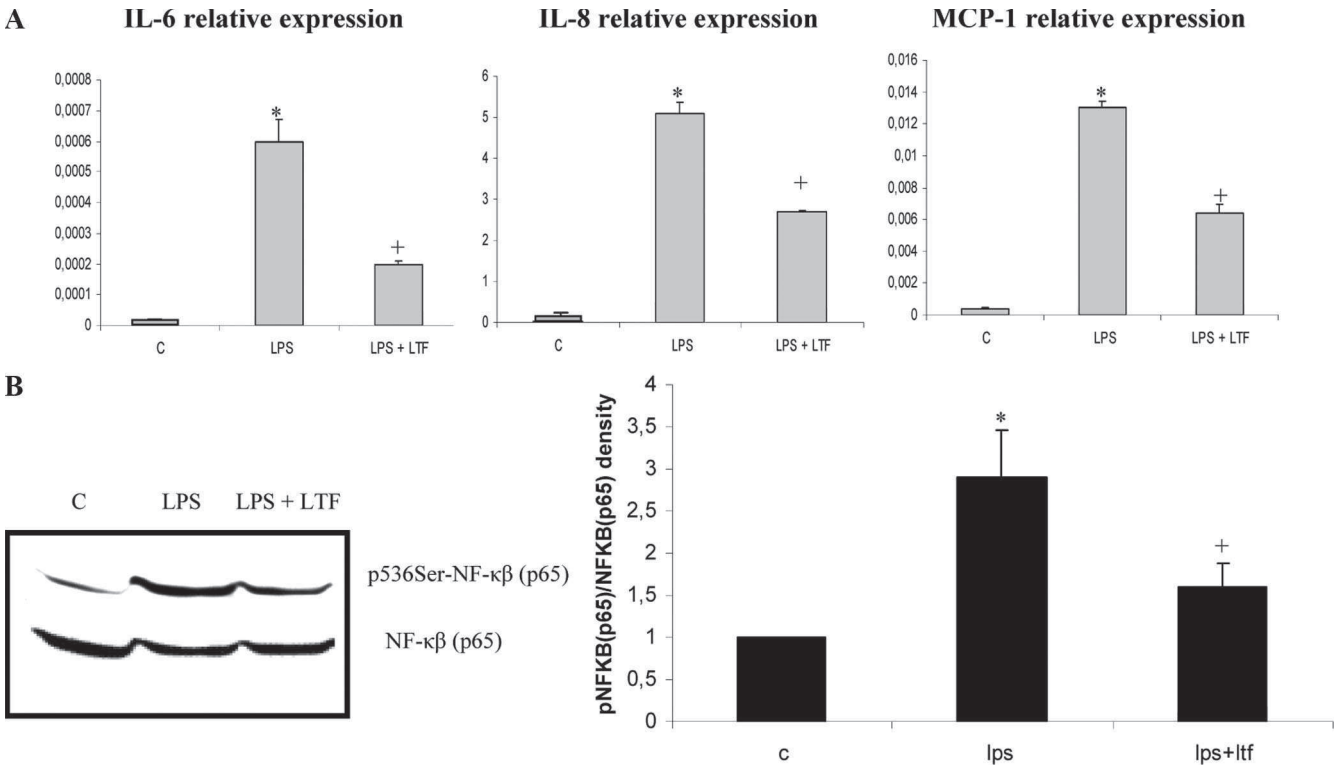


FIG. 3. A, IL-6, IL-8, and MCP-1 relative expression in LPS-stimulated THP-1 with and without lactoferrin (0.5 μM). *, $P < 0.01$ *vs.* control (C); +, $P < 0.01$ *vs.* LPS stimuli. B, Buffering effects of lactoferrin in $_{pSer536}$-NF-$\kappa\beta$(p65) by LPS stimulation. *, $P < 0.01$ *vs.* control;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

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-α has been demonstrated to stimulate lactoferrin secretion from neutrophils (32), but high TNF-α 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).

Acknowledgments

Address all correspondence and requests for reprints to: J. M. Fernández-Real, M.D., Ph.D., Unit of Diabetes, Endocrinology, and Nutrition, Hospital de Girona "Dr. Josep Trueta," Ctra. França s/n, 17007 Girona, Spain. E-mail: jmmorsim@hotmail.com.

This work was partially supported by research grants from the Ministerio de Educación y Ciencia (SAF2008-0273).

Disclosure Summary: J.M.M.-N., F.J.O., J.B., W.R., J.M.F.-R. have nothing to declare.

References

1. **Fernandez-Real JM, Ricart W** 2003 Insulin resistance and chronic cardiovascular inflammatory syndrome. Endocr Rev 24:278–301
2. **Hotamisligil GS** 2003 Inflammatory pathways and insulin action. Int J Obes Relat Metab Disord 27(Suppl 3):S53–S55
3. **Dandona P, Aljada A, Bandyopadhyay A** 2004 Inflammation: the link between insulin resistance, obesity and diabetes. Trends Immunol 25:4–7
4. **He J, Usui I, Ishizuka K, Kanatani Y, Hiratani K, Iwata M, Bukhari A, Haruta T, Sasaoka T, Kobayashi M** 2006 Interleukin-1α inhibits insulin signaling with phosphorylating insulin receptor substrate-1 on serine residues in 3T3-L1 adipocytes. Mol Endocrinol 20:114–124
5. **Rotter V, Nagaev I, Smith U** 2003 Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor

necrosis factor-α, overexpressed in human fat cells from insulin-resistant subjects. J Biol Chem 278:45777–45784
6. **Senn JJ, Klover PJ, Nowak IA, Mooney RA** 2002 Interleukin-6 induces cellular insulin resistance in hepatocytes. Diabetes 51:3391–3399
7. **Stephens JM, Lee J, Pilch PF** 1997 Tumor necrosis factor-α-induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. J Biol Chem 272: 971–976
8. **Lee JY, Hwang DH** 2006 The modulation of inflammatory gene expression by lipids: mediation through Toll-like receptors. Mol Cells 21:174–185
9. **Song MJ, Kim KH, Yoon JM, Kim JB** 2006 Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes. Biochem Biophys Res Commun 346:739–745
10. **Tan KC, Chow WS, Tam S, Bucala R, Betteridge J** 2004 Association between acute-phase reactants and advanced glycation end products in type 2 diabetes. Diabetes Care 27:223–228
11. **Fernandez-Real JM, Lopez-Bermejo A, Vendrell J, Ferri MJ, Recasens M, Ricart W** 2006 Burden of infection and insulin resistance in healthy middle-aged men. Diabetes Care 29:1058–1064
12. **Ceriello A, Motz E** 2004 Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. Arterioscler Thromb Vasc Biol 24:816–823
13. **Baker EN, Baker HM** 2005 Molecular structure, binding properties and dynamics of lactoferrin. Cell Mol Life Sci 62:2531–2539
14. **Otsuki K, Yakuwa K, Sawada M, Hasegawa A, Sasaki Y, Mitsukawa K, Chiba H, Nagatsuka M, Saito H, Okai T** 2005 Recombinant human lactoferrin has preventive effects on lipopolysaccharide-induced preterm delivery and production of inflammatory cytokines in mice. J Perinat Med 33:320–323
15. **Conneely OM** 2001 Antiinflammatory activities of lactoferrin. J Am Coll Nutr 20:389S–395S; discussion 396S–397S
16. **Haversen L, Ohlsson BG, Hahn-Zoric M, Hanson LA, Mattsby-Baltzer I** 2002 Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-κB. Cell Immunol 220:83–95
17. **Kruzel ML, Harari Y, Chen CY, Castro GA** 1998 The gut. A key metabolic organ protected by lactoferrin during experimental systemic inflammation in mice. Adv Exp Med Biol 443:167–173
18. **Moreno-Navarrete JM, Ortega FJ, Bassols J, Castro A, Ricart W, Fernandez-Real JM** 2008 Association of circulating lactoferrin concentration and 2 nonsynonymous LTF gene polymorphisms with dyslipidemia in men depends on glucose-tolerance status. Clin Chem 54:301–309
19. **Bergman RN, Prager R, Volund A, Olefsky JM** 1987 Equivalence of the insulin sensitivity index in man derived by the minimal model method and euglycaemic glucose clamp. J Clin Invest 79:790–800
20. **Beckmann I, Efraim SB, Vervoort M, Visser W, Wallenburg HC** 2004 Tumor necrosis factor-α in whole blood cultures of preeclamptic patients and healthy pregnant and nonpregnant women. Hypertens Pregnancy 23:319–329
21. **Langezaal I, Coecke S, Hartung T** 2001 Whole blood cytokine response as a measure of immunotoxicity. Toxicol In Vitro 15:313–318
22. **Doyle SL, Jefferies CA, O'Neill LA** 2005 Bruton's tyrosine kinase is involved in p65-mediated transactivation and phosphorylation of p65 on serine 536 during NFκB activation by lipopolysaccharide. J Biol Chem 280:23496–23501
23. **Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmée E, Cousin B, Sulpice T, Chamontin B, Ferrières J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, Burcelin R** 2007 Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 56: 1761–1772
24. **Japelj B, Pristovsek P, Majerle A, Jerala R** 2005 Structural origin of endotoxin neutralization and antimicrobial activity of a lactoferrin-based peptide. J Biol Chem 280:16955–16961
25. **Ward PP, Uribe-Luna S, Conneely OM** 2002 Lactoferrin and host defense. Biochem Cell Biol 80:95–102
26. **Baveye S, Elass E, Fernig DG, Blanquart C, Mazurier J, Legrand D** 2000 Human lactoferrin interacts with soluble CD14 and inhibits expression of endothelial adhesion molecules, E-selectin and ICAM-1, induced by the CD14-lipopolysaccharide complex. Infect Immun 68:6519–6525
27. **Shurtz-Swirski R, Sela S, Herskovits AT, Shasha SM, Shapiro G, Nasser L, Kristal B** 2001 Involvement of peripheral polymorphonuclear leukocytes in oxidative stress and inflammation in type 2 diabetic patients. Diabetes Care 24:104–110
28. **Advani A, Marshall SM, Thomas TH** 2002 Impaired neutrophil actin assembly causes persistent CD11b expression and reduced primary granule exocytosis in type II diabetes. Diabetologia 45:719–727
29. **Shim WS, Kim HJ, Kang ES, Ahn CW, Lim SK, Lee HC, Cha BS** 2006 The association of total and differential white blood cell count with metabolic syndrome in type 2 diabetic patients. Diabetes Res Clin Pract 73:284–291
30. **Stegenga ME, van der Crabben SN, Blümer RM, Levi M, Meijers JC, Serlie MJ, Tanck MW, Sauerwein HP, van der Poll T** 2008 Hyperglycemia enhances coagulation and reduces neutrophil degranulation, whereas hyperinsulinemia inhibits fibrinolysis during human endotoxemia. Blood 112:82–89
31. **Gubern C, Lopez-Bermejo A, Biarnes J, Vendrell J, Ricart W, Fernandez-Real JM** 2006 Natural antibiotics and insulin sensitivity: the role of bactericidal/permeability-increasing protein. Diabetes 55:216–224
32. **Koivuranta-Vaara P, Banda D, Goldstein IM** 1987 Bacterial-lipopolysaccharide-induced release of lactoferrin from human polymorphonuclear leukocytes: role of monocyte-derived tumor necrosis factor α. Infect Immun 55:2956–2961
33. **Srivastava CH, Rado TA, Bauerle D, Broxmeyer HE** 1991 Regulation of human bone marrow lactoferrin and myeloperoxidase gene expression by tumor necrosis factor-α. J Immunol 146:1014–1019
34. **Zimecki M, Chodaczek G, Kocieba M, Kruzel ML** 2004 Lethality in LPS-induced endotoxemia in C3H/HeCr mice is associated with prevalence of proinflammatory cytokines: lack of protective action of lactoferrin. FEMS Immunol Med Microbiol 42:167–172
35. **Fernández-Real JM, Broch M, Ricart W, Casamitjana R, Gutierrez C, Vendrell J, Richart C** 1998 Plasma levels of the soluble fraction of tumor necrosis factor receptor 2 and insulin resistance. Diabetes 47:1757–1762
36. **Legrand D, Elass E, Carpentier M, Mazurier J** 2005 Lactoferrin: a modulator of immune and inflammatory responses. Cell Mol Life Sci 62:2549–2559
37. **Walrand S, Guillet C, Boirie Y, Vasson MP** 2006 Insulin differentially regulates monocyte and polymorphonuclear neutrophil functions in healthy young and elderly humans. J Clin Endocrinol Metab 91:2738–2748
38. **Walrand S, Guillet C, Boirie Y, Vasson MP** 2004 *In vivo* evidences that insulin regulates human polymorphonuclear neutrophil functions. J Leukoc Biol 76:1104–1110
39. **Buitenhuis M, Verhagen LP, van Deutekom HW, Castor A, Verploegen S, Koenderman L, Jacobsen SE, Coffer PJ** 2008 Protein kinase B (c-akt) regulates hematopoietic lineage choice decisions during myelopoiesis. Blood 111:112–121
40. **Mohanty P, Aljada A, Ghanim H, Hofmeyer D, Tripathy D, Syed T, Al-Haddad W, Dhindsa S, Dandona P** 2004 Evidence for a potent antiinflammatory effect of rosiglitazone. J Clin Endocrinol Metab 89:2728–2735
41. **Dandona P, Aljada A, Mohanty P, Ghanim H, Hamouda W, Assian E, Ahmad S** 2001 Insulin inhibits intranuclear nuclear factor κB and stimulates IκB in mononuclear cells in obese subjects: evidence for an anti-inflammatory effect? J Clin Endocrinol Metab 86:3257–3265
42. **Jeschke MG, Klein D, Thasler WE, Bolder U, Schlitt HJ, Jauch KW, Weiss TS** 2008 Insulin decreases inflammatory signal transcription factor expression in primary human liver cells after LPS challenge. Mol Med 14:11–19

Supplemental data

1- Inclusion criteria for subjects recruited in ongoing study dealing on non-classical cardiovascular risk factors in Northern Spain:

1) BMI < 40 kg/m^2, 2) absence of systemic disease, and 3) absence of infection within the previous month. None of the control subjects were under medication or had evidence of metabolic disease other than obesity. Alcohol and caffeine were withheld within 12 h of performing the insulin sensitivity test. Liver disease and thyroid dysfunction were specifically excluded by biochemical work-up.

2- Exclusion criteria for patients with type 2 diabetes:

Exclusion criteria for patients with type 2 diabetes included the following: 1) clinically significant hepatic, neurological, endocrinologic, or other major systemic disease, including malignancy; 2) history or current clinical evidence of hemochromatosis; 3) history of drug or alcohol abuse, defined as >80 g/day in men and >40 g/day in women, or serum transaminase activity more than twice the upper limit of normal; 4) an elevated serum creatinine concentration; 5) acute major cardiovascular event in the previous 6 months; 6) acute illnesses and current evidence of acute or chronic inflammatory or infective diseases; and 7) mental illness rendering the subjects unable to understand the nature, scope, and possible consequences of the study.

Pharmacological treatment for these patients was: Insulin: 29 patients; metformin: 35 patients; sulfonylureas, 43 patients; statins: 28 patients; fibrates: 9 patients; blood pressure lowering agents: 32 patients; aspirin: 16 patients; and allopurinol: 3 patients. All subjects gave written informed consent after the purpose of the study was explained to them.

3- Anthropometric measurements:

BMI was calculated as weight (in kilograms) divided by height (in meters) squared. Subjects' waists were measured with a soft tape midway between the lowest rib and the iliac crest; hip circumference was measured at the widest part of the gluteal region; and waist-to-hip ratio (WHR) was accordingly calculated. Fat mass, percent fat mass and fat-free mass were calculated using bioelectric impedance (Holtain BC Analyzer, Cambridge, UK). Blood pressure was measured in the supine position on the right arm after a 10-min rest; a standard sphygmomanometer of appropriate cuff size was used and the first and fifth phases were recorded. Values used in the analysis are the average of three readings taken at 5-min intervals.

4- Endotoxin determination:

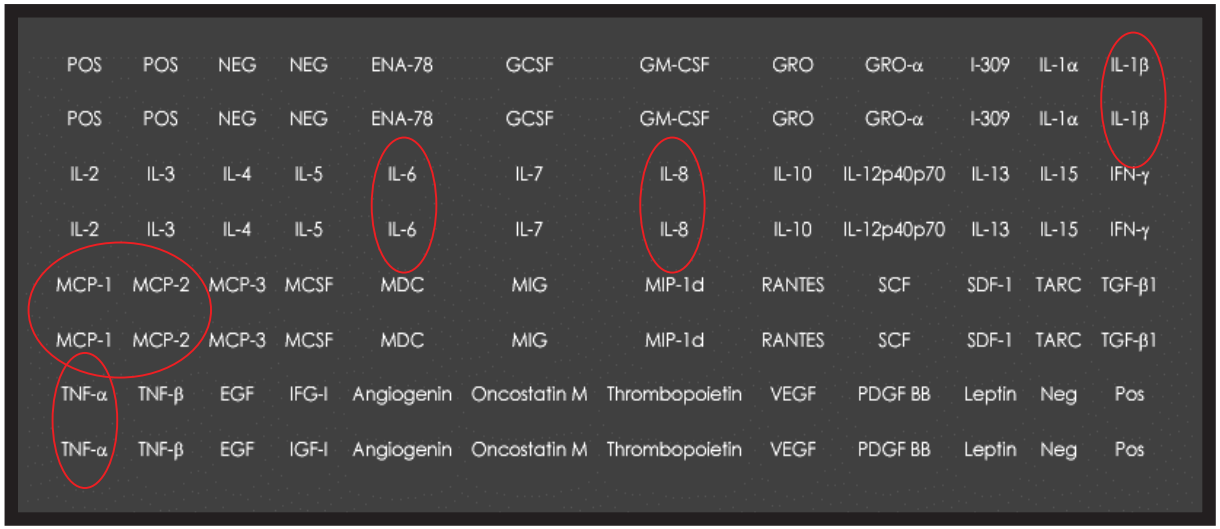
Serum endotoxin was measured in 33 consecutive participants of the cross-sectional study, 18 NGT and 15 AGT subjects using a limulus amebocyte lysate test (Pyrochrome; Cape Cod, Falmouth, MA). Plasma was collected in nonpyrogenic EDTA tubes and frozen at -20°C until assay. Plasma was diluted 1:20 or 1:40 and heat inactivated at 75°C for 10 min. The reaction was read using kinetics, i.e., measuring the time to reach a given absorbance at 405 nm. Recovery of spiked lipopolysaccharide was between 50 and 200%. Sensitivity of the assay was 0.005 Ehrlich units/ml (0.5pg/ml).

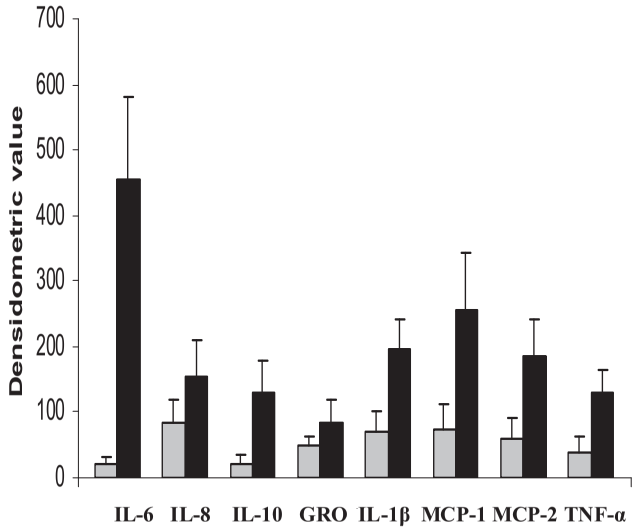
5- IL-6, IL-8 and MCP-1 expression analysis:

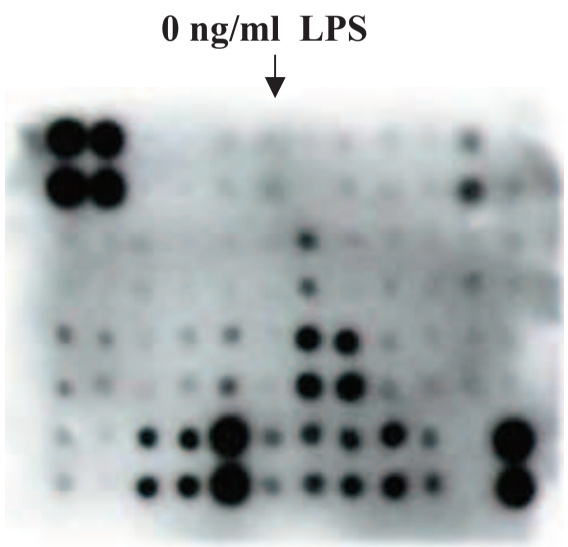
Total RNA from THP-1 was isolated using (Nucleospin RNA II, McNacherey-Nagel, Maryland), according to the manufacturer's protocol. Quantity and integrity of RNA was measured with 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany), the integrity was above 8.5 in all samples. First strand cDNA was synthesized from 1 μg total RNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Quantitative real-time TaqMan PCR was performed with a commercial inventoried primers and FAM dye-labeled TaqMan minor groove binder

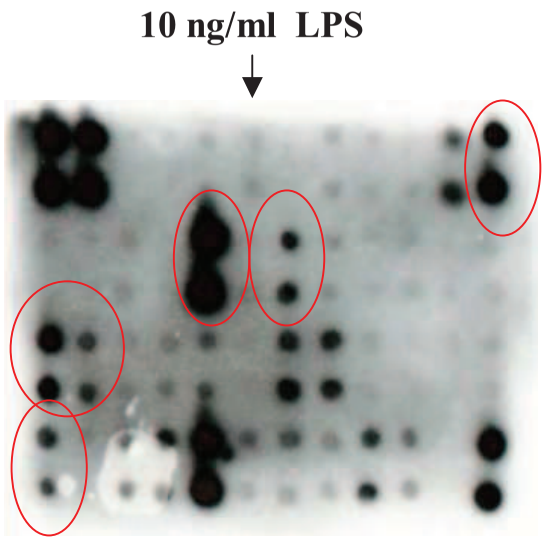
(MGB)-probes (Applied Biosystems, Foster City, CA) for IL-6, MCP-1, IL-8 and Cyclophilin A, which is used as endogenous control, and 2x Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Each TaqMan reaction contained 4 µl of cDNA, corresponding to 100 ng of total RNA, in a total reaction volume of 25 µl. The relative expression was determined by the comparative threshold method as described in the ABI Prism 7700 User Bulletin (P/N 4303859) from Applied Biosystems, Foster City, CA.

Supplemental Figure.









Manuscript 3

Fernández-Real JM, García-Fuentes E, Moreno-Navarrete JM, Murri-Pierri M, Garrido-Sánchez L, Ricart W, Tinahones FJ. Fat overload induces changes in circulating lactoferrin that are associated with postprandial lipemia and oxidative stress in severely obese subjects. *Obesity* 18:482-488, 2010.

José M. Fernández-Real, Eduardo García-Fuentes, José M. Moreno-Navarrete, Mora Murri-Pierri, Lourdes Garrido-Sánchez, Wifredo Ricart and Francisco Tinahones. "Fat overload induces changes in circulating lactoferrin that are associated with postprandial lipemia and oxidative stress in severely obese subjects". *Obesity*. Vol. 18, issue 3 (March 2010) : p. 482-488

http://dx.doi.org/10.1038/oby.2009.266

http://www.nature.com/oby/journal/v18/n3/abs/oby2009266a.html

Received 4 December 2008; Accepted 2 July 2009; Published online 20 August 2009.

Abstract: Lactoferrin is an innate immune system protein with anti-inflammatory and antioxidant activities. We aimed to evaluate circulating lactoferrin levels in association with lipid concentrations, and parameters of oxidative stress and inflammation in subjects with morbid obesity after an acute fat intake. The effects of a 60 g fat overload on circulating lactoferrin and antioxidant activities were evaluated in 45 severely obese patients (15 men and 30 women, BMI 53.4 ± 7.2 kg/m^2). The change in circulating lactoferrin after fat overload was significantly and inversely associated with the free fatty acid (FFA) change. In those subjects with the highest increase in lactoferrin (in the highest quartile), high-density lipoprotein (HDL)-cholesterol decreased after fat overload to a lesser extent (P = 0.03). In parallel to lipid changes, circulating lactoferrin concentrations were inversely linked to the variations in catalase (CAT) and glutathione reductase (GSH-Rd). Baseline circulating lactoferrin concentration was also inversely associated with the absolute change in antioxidant activity after fat overload, and with the change in C-reactive protein (CRP). Furthermore, those subjects with higher than the median value of homeostasis model assessment of insulin secretion ($HOMA_{IS}$) had significantly increased lactoferrin concentration after fat load (885 ± 262 vs. 700 ± 286 ng/ml, P = 0.03). Finally, we further explored the action of lactoferrin *in vitro*. Lactoferrin (10 µmol/l) led to significantly lower triglyceride (TG) concentrations and lactate dehydrogenase activity (as expression of cell viability) in the media from adipose explants obtained from severely obese subjects. In conclusion, circulating lactoferrin concentrations, both at baseline and fat-stimulated, were inversely associated with postprandial lipemia, and parameters of oxidative stress and fat-induced inflammation in severely obese subjects.

Manuscript 4

Moreno-Navarrete JM, Ortega FJ, Ricart W, Fernandez-Real JM. Lactoferrin increases ^{172Thr}AMPK phosphorylation and insulin-induced ^{p473Ser}AKT while impairing adipocyte differentiation. *Int J Obes (Lond)* 33:991-1000, 2009.

Moreno-Navarrete, J M; Ortega, F J; Ricart, W; Fernandez-Real, J M. “Lactoferrin increases 172ThrAMPK phosphorylation and insulin-induced p473SerAKT while impairing adipocyte differentiation”. *International Journal of Obesity*. Vol. 33 (September 2009) : p. 301-309

http://search.proquest.com/docview/219177495/131C32874BE22E58D6E/6?accountid=15295

http://dx.doi.org/10.1038/ijo.2009.143

OBJECTIVE: Lactoferrin is a pleiotropic glycoprotein of the innate immune system with known effects on immunomodulation and cell differentiation. To gain an insight into the interaction among obesity, inflammation and insulin action, we aimed to examine the effects of lactoferrin on adipogenesis and the response to insulin in human hepatocarcinoma (HepG2) and 3T3-L1 cell lines. DESIGN: The cells were cultured with increasing lactoferrin concentration under non-inflammatory, inflammatory and standard conditions. The response to insulin was evaluated through (473Ser)AKT phosphorylation. The effects of lactoferrin on adipogenesis were studied through the expression of different lipogenic markers, AMP-activated protein kinase (AMPK) activation, retinoblastoma (Rb) activity and Oil Red O staining in 3T3-L1 cells. RESULTS: Lactoferrin increased dose-dependent insulin-induced (473Ser)AKT phosphorylation in both cell lines. Inflammation-induced decreased (473Ser)AKT phosphorylation was also rescued by lactoferrin. In addition, lactoferrin led to increased (p172Thr)AMPK during 3T3-L1 differentiation and to decreased adipogenesis (as shown by decreased expression of fatty acid synthase, acetyl-coenzyme A carboxylase-alpha and peroxisome proliferator-activated receptor-gamma in parallel with decreased formation of lipid droplets). Lactoferrin also increased dose-dependent Rb activity (expression and hypophosphorylation) during 3T3-L1 differentiation. CONCLUSION: Lactoferrin administration increased insulin-induced (473Ser)AKT phosphorylation, even in those conditions wherein the response to insulin was downregulated, and led to blunted adipogenesis in the context of increased (p172Thr)AMPK and Rb activity.

Manuscript 5

Moreno-Navarrete JM, Ortega FJ, Sabater M, Ricart W, Fernandez-Real JM. Pro-adipogenic effects of lactoferrin in human subcutaneous and visceral preadipocytes. *The Journal of Nutritional Biochemistry*. Accepted (2010).

ARTICLE IN PRESS

Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry xx (2011) xxx–xxx

Journal of Nutritional Biochemistry

Proadipogenic effects of lactoferrin in human subcutaneous and visceral preadipocytes☆

José María Moreno-Navarrete, Francisco Ortega, Mònica Sabater, Wifredo Ricart, José Manuel Fernández-Real, MD, PhD*

Department of Diabetes, Endocrinology and Nutrition, CIBEROBN Fisiopatología de la Obesidad y Nutrición CB06/03/010 and Girona Biomedical Research Institute [IdIBGi], Girona, Spain

Received 6 June 2010; received in revised form 20 September 2010; accepted 23 September 2010

Abstract

Lactoferrin has been associated with insulin sensitivity in vivo and in vitro studies. We aimed to test the effects of lactoferrin on human subcutaneous and visceral preadipocytes. Human subcutaneous and visceral preadipocytes were cultured with increasing lactoferrin (hLf, 0.1, 1, 10 μM) under differentiation conditions. The effects of lactoferrin on adipogenesis were studied through the expression of different adipogenic and inflammatory markers, AMPK activation and Retinoblastoma 1 (RB1) activity. The response to insulin was evaluated through ^{Ser473}AKT phosphorylation. In both subcutaneous and visceral preadipocytes, lactoferrin (1 and 10 μM) increased adipogenic gene expressions and protein levels (fatty acid synthase, PPARγ, FABP4, ADIPOQ, ACC and STAMP2) and decreased inflammatory markers (IL8, IL6 and MCP1) dose-dependently in parallel to increased insulin-induced ^{Ser473}AKT phosphorylation. In addition to these adipogenic effects, lactoferrin decreased significantly AMPK activity (reducing ^{pThr172}AMPK and ^{pSer79}ACC) and RB1 activity (increasing the $^{pser807/811}$RB1/RB1 ratio). In conclusion, these results suggest that lactoferrin promotes adipogenesis in human adipocytes by enhancing insulin signaling and inhibiting RB1 and AMPK activities.

Keywords: Lactoferrin; Adipogenesis and insulin action

1. Introduction

Adipose tissue is recognized as an endocrine organ that plays an important role in human diseases such as type 2 diabetes and cancer. Adipogenesis can be divided in two stages: adipocyte commitment of multipotent stem cells and terminal differentiation to mature adipocytes. Adipocyte commitment under tissue culture conditions is achieved by cell confluency. Cells under high confluency can be further induced to become mature adipocytes by various adipogenic inducers. Transcriptional pathways important for the later stages of adipogenesis have been extensively studied. In particular, several key transcription factors such as PPARγ and members of the C/EBP protein family play pivotal roles in dictating cascades of transcriptional activation required for establishing the terminally differentiated state [1,2]. The negative effects of inflammatory mediators on adipocyte differentiation has been extensively reported [3,4], leading to concomitant blunting of insulin action.

Lactoferrin is a pleiotropic glycoprotein (80 kDa) and a prominent component of the first line of mammalian host defense, acting on specific lactoferrin receptors that exist in a variety of cells, like monocytes, lymphocytes and adipocytes [5]. Its expression is up-regulated in response to inflammatory stimuli [5]. Lactoferrin is able to bind and buffer several pathogen-associated molecular patterns such as lipopolysaccharide, viral components and soluble components of the extracellular matrix [6]. This ability is associated with the putative lactoferrin anti-inflammatory activity, as demonstrated in several studies [7-10]. Lactoferrin also participates in the regulation of cellular growth and differentiation [11,12]. In primary osteoblasts, lactoferrin stimulated proliferation and differentiation and acted as a survival factor, activating PI3 kinase-dependent AKT signaling, inhibiting apoptosis induced by serum withdrawal and inhibiting osteoclastogenesis in a murine bone marrow culture [13]. Recently, we have reported that lactoferrin is associated with insulin sensitivity in vivo in humans and in vitro (increasing ^{473Ser}AKT phosphorylation) in HepG2 and 3T3-L1 cell lines. We also showed that lactoferrin inhibited adipogenesis in 3T3-L1 cell line through the increase of AMPK and Retinoblastoma 1 (RB1) activity [14,15].

Lactoferrin is found in considerable concentrations in breast milk (1 mg/ml) and in colostrum (7 mg/ml). LF is a natural component of breast milk which is ingested by infants. Lactoferrin administration in

☆ This work was partially supported by research grants from the Ministerio de Educación y Ciencia (SAF2008-0273). The authors declared no conflict of interest.

* Corresponding author. Unit of Diabetes, Endocrinology and Nutrition. Hospital de Girona "Dr Josep Trueta" Ctra. França s/n, 17007 Girona, Spain. Tel.: +34 972 94 02 00; fax: +34 972 94 02 70.

E-mail address: jmfernandezreal.girona.ics@gencat.cat (J.M. Fernández-Real).

0955-2863/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2010.09.015

GENERAL DISCUSSION

This study showed new findings in the relationship among innate immune system, insulin resistance and obesity-associated metabolic disturbances. The main findings were as follows:

- Circulating lactoferrin was negatively associated with obesity, dyslipemia and vascular reactivity.
- Circulating lactoferrin was negative associated with insulin resistance, and its production was reduced in neutrophils from type 2 diabetic subjects.
- After fat over-load, the increase of circulating lactoferrin was favorably associated with lipid levels and parameters of oxidative stress and inflammation.
- hLf improved insulin action on mouse 3T3-L1 cell line.
- hLf inhibited mouse 3T3-L1 cell line differentiation increasing AMPK and Rb activity.
- hLf led to enhanced human subcutaneous and visceral preadipocyte differentiation increasing insulin action and decreasing AMPK and Rb activity.

Even though neutrophil count was significantly higher in subjects with AGT and was negatively associated with insulin sensitivity (67,136,137), 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, fasting triglycerides and insulin resistance. 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.* reported that hyperglycemia impaired neutrophil degranulation in humans with systemic inflammation induced by intravenous LPS administration (72). For this reason, we can not exclude the hyperglycemia effects on circulating lactoferrin concentrations. Lactoferrin was positively and strongly correlated with BPI in all subjects, both in NGT and AGT subjects (138). BPI is released by the primary granules of neutrophils, by monocytes and several epithelia. As lactoferrin, BPI has a high cationic fragment and has been reported to behave as an anti-inflammatory molecule. The associations of BPI with several metabolic variables, including insulin sensitivity, were similar to those found with lactoferrin (138). Endotoxin levels also were inversely associated with circulating lactoferrin association. Several studies confirmed that 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 (139). Circulating sTNFR2 was negatively associated with lactoferrin in AGT subjects. TNF-α has been demonstrated to stimulate lactoferrin secretion from neutrophils (140), but high TNF-α levels led to decreased lactoferrin (141,142). 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 (26,143).

In different animal models of inflammation, like gut inflammation (102) and skin inflammation (144), lactoferrin showed important anti-inflammatory and immunoregulatory functions, restoring immune defense system homeostasis (145). Lactoferrin has been demonstrated to interact with LPS (146), avoiding the LPS-LBP complex formation (145). Lactoferrin also interacts with CD14, preventing the LPS-

LBP complex interactions with toll like receptor which amplify the inflammatory signal (147). Thus, high lactoferrin concentration lead to decreased free LPS which down-regulates LBP expression (147). We tested the buffering effect of lactoferrin in the LPS-stimulated THP-1 cell line, and we corroborated the anti-inflammatory effect of lactoferrin by decreasing NF-κβ activation and IL-6, IL-8 and MCP-1 expression under LPS challenge.

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 non-diabetic healthy subjects (148). 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 (148). Our results in whole blood experiment support these findings, because we have 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 (42-46). IL-6 co-incubation possibly affected neutrophil function in NGT subjects (acting through impaired cytoskeleton action or inducing insulin resistance), leading to decreased lactoferrin secretion in response to LPS. In subjects with type 2 diabetes subjects 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 (71). 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 (68). Thus, improved insulin action (by the administration of insulin (71) 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 PKB (a major downstream PI3K effector) activity was found to promote neutrophil and monocyte development (148). The anti-inflammatory activities of insulin and rosiglitazone have been extensively reported (87, 149, 150). 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-today 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 (71,72).

In addition, circulating lactoferrin and lactoferrin gene polymorphisms were linked to plasma lipid profile. Virtually all body fluids contain lactoferrin, but it is especially abundant in milk. Ingestion of protein from milk especially affects plasma lipid concentrations, and whey protein has also showed to lower plasma lipids (117). In 2004, Takeuchi *et al.* were the first to demonstrate that milk-derived bovine lactoferrin, mixed with a standard commercial diet, reduced plasma and hepatic cholesterol and triglyceride concentrations, with an increase in plasma HDL-cholesterol in mice fed with chow diet. However, lactoferrin had no significant effects on lipids in mice fed a high fat diet (118). It has been previously described that lactoferrin inhibits selective uptake of HDL-cholesteryl esters by 35%-50% in human primary adipocytes and SW872 liposarcoma cells (151). This action would be mediated by interaction with low density lipoprotein-related protein (LRP), which contributes physiologically to HDL-

cholesteryl ester selective uptake in adipocytes. Lactoferrin would also inhibit the interaction of lipoprotein lipase with LRP. A striking feature of lactoferrin is its ability to inhibit, both in vivo and in vitro, the binding and uptake of apolipoprotein E-(apo-E) bearing lipoproteins by parenchymal liver cells. Arginine residues of lactoferrin are crucial for its recognition by parenchymal liver cells and its capacity to inhibit hepatic uptake of apo-E bearing lipoproteins. This conclusion was based on experiments in which the arginine residues of lactoferrin were selectively modified by 1,2-cyclohexanedione. This modification resulted in a marked reduction of the liver uptake of lactoferrin. We here describe how healthy subjects carrying a SNP associated with an arginine change at position 47 of the lactoferrin molecule (an A5440G non-synonymous gene polymorphism) had significantly higher HDL-cholesterol and significantly lower fasting triglycerides than those subjects carrying a lysine. We hypothesise that this change may lead to differential interactions with apo-E and LRP-1, leading to changes in plasma lipid concentrations. In the G5385A non-synonymous gene polymorphism, AG healthy subjects (associated with a alanine to threonine change at position 29) had significantly and markedly lower fasting triglycerides than GG subjects. Unfortunately, we did not find homozygotes for this polymorphism. The aminoacidic change is found within the sequence of interaction with LRP-1) (119,111,152). Thus, it is possible that the threonine and the arginine at these positions favor a stronger interaction with LRP-1.

Other mechanisms through which lactoferrin concentrations are associated with the lipid profile can not be excluded. In fact, bovine lactoferrin reduced the accumulation of cholesteryl esters in macrophages incubated with acetylated LDL by more than 80% compared with the control value. It has also been reported that treatment with bovine lactoferrin led to decreased triacylglycerol intestinal absorption through lymphatic pathways (118,153).

To gain insight in the relationship between circulating lactoferrin and lipid profile, we also evaluated the associations among circulating endogenous lactoferrin, and lipid changes and oxidative stress after a fat overload. Circulating triglycerides (TGs), free fatty acids (FFA), catalase, glutathione reductase, glutathione-S-transferase activities, and serum uric acid concentration significantly increased alter fat overload, whereas plasma antioxidant capacity significantly decreased, as previously described (154). Subjects with higher than the median value of HOMA-IS had significantly increased lactoferrin concentration after fat load. The increase of lactoferrin was favorably associated with lipid levels and parameters of oxidative stress and inflammation.

The change in circulating lactoferrin after fat overload was significantly and inversely associated with FFA concentration after fat overload. In addition, HDL-cholesterol decreased after fat overload to a lesser extent in those subjects with the highest increase in lactoferrin.

Experimental studies in cells demonstrated that lactoferrin significantly inhibited/decreased intracellular reactive oxygen species levels in a dose-dependent manner and protected from oxidative stress (155,107,108). As FFA produce an increase in oxidative stress (156), the putative decrease of FFA induced by lactoferrin could also contribute. In fact, different protective functions against inflammation have been attributed to lactoferrin (103-105), the preservation of adipose tissue integrity (as shown by lactate dehydrogenase activity), and the associations with lactoferrin with CRP and antioxidant enzymes may be interpreted in this scenario. The induction of peripheral blood mononuclear cell NF-κB activation by a high-fat meal can be reduced by the concurrent ingestion of red wine or olive oil (157,158). An antioxidant mechanism has been put forward to explain this effect. It is interesting to note that both olive oil and resveratrol are potent inhibitors of lipopolysaccharide signaling and the detrimental

effects of experimental endotoxin challenge (159,160). Circulating lactoferrin, a well-known inhibitor of lipopolysaccharide signaling (105,146,155), may contribute to the armamentarium of endogenous antioxidant defenses.

Circulating lactoferrin was also positively associated with endothelium-dependent arterial vasodilatation ($r=0.54$, $p=0.04$) among the sub-sample of obese men with altered glucose tolerance. There is evidence that lactoferrin increases nitric oxide production (161). In fact, lactoferrin has shown to affect peripheral opioid-mediated antinociception via nitric oxide (162,103).

To gain insight in the mechanisms that could be involved in these clinical observations, we evaluated *in vitro* the effects of lactoferrin in insulin action and adipocyte differentiation in several cell lines (HepG2 and 3T3-L1) and in primary cell culture (subcutaneous and visceral human preadipocytes).

Lactoferrin increased insulin-induced ^{473Ser}AKT phosphorylation in non-differentiated and pre-differentiated 3T3-L1 cells and in HepG2 cells. At the dose of 1 μM and higher, lactoferrin increased the response to insulin in all cell lines tested. This effect was especially observed in those conditions where insulin action was blunted, such as pre-differentiated (after INS-DEX-IBMX mixture) 3T3-L1 cells and in cells under treatment with proinflammatory media. The differentiation cocktail, with high insulin and dexamethasone concentration, decreased insulin-induced ^{473Ser}AKT phosphorylation in the first phase of differentiation process (163), thus explaining the relative "insulin resistance" of pre-differentiated (after INS-DEX-IBMX treatment) 3T3-L1 cells. This effect was reversible, because during the second phase of the differentiation process (in which only insulin is present) insulin-induced ^{473Ser}AKT phosphorylation was recovered. Impaired insulin action in these cells was also rescued by increasing lactoferrin concentration. Under inflammatory conditions, lactoferrin dose-dependently

rescued the low response to insulin in both HepG2 and 3T3-L1 cells. Lactoferrin could have an insulinotropic activity, increasing AKT phosphorylation on serine 473 in absence of insulin. Antioxidant and antinflammatory action of lactoferrin also could be involved in the improvement of insulin action under inflammatory conditions (164).

Recently, Yagi et al. reported that lactoferrin suppressed the adipogenic differentiation of MC3T3-G2/PA6 cells. Lactoferrin co-treatment decreased lipid droplets and the mRNA expression of several adipogenesis markers (C/EBPalpha, PPAR-γ, aP2, and adiponectin) (165,166), while inducing osteogenesis (increasing expression Runx2, osteocalcin, and Sox9). These findings fit with existing hypotheses that a reciprocal or inverse relationship exists between adipogenesis and osteogenesis in the marrow microenvironment (167,168). According to our data, lactoferrin also inhibited 3T3-L1 differentiation, decreasing lipid droplets accumulation. Red oil density was negatively associated with lactoferrin concentration. Lactoferrin also decreased lipogenic proteins (FASN, ACC, PPAR-γ) dose-dependently. In parallel, there was an increase in GLUT-4 levels and AMPK activation (^{p172Thr}AMPK). This latter observation could be related with the lactoferrin interference of adipogenic differentiation. In fact, AMPK activation lead to increased fat oxidation by the upregulation of the transcription factor NRF-1, which stimulates mitochondrial biogenesis, and inhibits both lipolysis and lipogenesis, affecting directly the enzymes engaged in lipid metabolism and downregulating PPAR-γ expression (169).

Hyperphosphorylation of Rb (inhibition) is necessary to promote clonal expansion and adipocyte differentiation of 3T3-L1 cells (170,171). When Rb activity and protein expression were tested, we found decreased Rb activity and protein levels in parallel to the differentiation process of 3T3-L1 cells. Lactoferrin increased Rb activity (hypophosphorylation and Rb expression) dose-dependently in parallel with the

inhibition of adipogenesis. Thus, lactoferrin could inhibit adipogenic differentiation through the increase of hypophosphorylated Rb (Rb activity) levels, inducing cell cycle arrest (135). In MTT-based cell viability assays to evaluate lactoferrin cytotoxicity, we found that lactoferrin (10 μM) decreased cell viability of 3T3-L1 by only 15 %. Cell counting corroborated the negative effects of lactoferrin on 3T3-L1 expansion during the differentiation process.

In human preadipocytes, lactoferrin promotes adipogenesis by enhancing insulin signaling (increasing insulin-induced ^{pSer473}Akt) and inhibiting Rb and AMPK activities. Stimulation of Akt activity was in parallel to adipogenic gene expression and enhanced preadipocyte differentiation (172,173). Thus, the insulin sensitizing effects of lactoferrin might explain the adipogenic effects in human preadipocytes. In fact, a significant reduction of AMPK and Rb activities is well known during adipocyte differentiation (169-171).

The divergent lactoferrin effects in human preadipocytes and 3T3-L1 cell line could be explained antitumoral activity of lactoferrin (enhancing Rb activity) (135). Lactoferrin antiadipogenic effects on 3T3-L1 cell line might be mediated by the induction of cell cycle arrest, blunting clonal expansion, the first step in the differentiation process. These divergent effects have been previously described for other factors. For instance, Tomlinson and collaborators showed that exposure of human primary preadipocytes to glucocorticoids increased their sensitivity to insulin (improving insulin-mediated activation of Akt) and enhanced their subsequent response to stimuli that promote adipogenesis. This effect was observed in primary human preadipocytes but not in immortalized 3T3-L1 murine preadipocytes (174).

In parallel with these adipogenic effects, lactoferrin displayed antinflammatory activity on adipocytes, reducing *IL-8* and *MCP-1* gene expression on visceral adipocytes and *IL-6* gene expression on both visceral and subcutaneous adipocytes.

Wellen and collaborators has shown that STAMP2 (a transmembrane protein found on adipocytes membrane) had an important role in adipocyte physiology (175). *STAMP2* gene KO showed a deterioration of insulin sensitivity and adipogenesis, and increased the nutrient– induced inflammatory responses (175). We, here, confirmed that *STAMP2* gene expression increased during differentiation of adipocytes. Another recent study confirmed that STAMP2 has an important role mediating insulin sensitivity in adipocytes (176). Lactoferrin treatment increased significantly *STAMP2* gene expression in comparison with differentiated control adipocytes. We might speculate that lactoferrin effects on *STAMP2* gene expression could be behind its adipogenic and insulin sensitizing effects.

Recently, in agreement with these findings, Ono and collaborators have reported that the ingestion of enteric-coated LF tablets for an 8-week period can reduce visceral fat accumulation in Japanese men and women with abdominal obesity without the need for any lifestyle change (177).

In summary, lactoferrin might mediate an important role in the control of obesity-induced metabolic disturbances, impacting on liver and adipose tissue physiology.

CONCLUSIONS

1- Plasma lactoferrin and non-synonymous lactoferrin gene polymorphisms (modifying aminoacid composition within the sequence of interaction with LRP-1) were found to be associated with plasma lipid profile and endothelium-dependent vasodilation. The decreased values of lactoferrin found in subjects with obesity, high waist-to-hip ratio and altered glucose tolerance might contribute to dyslipidemia in these subjects.

2- Decreased circulating lactoferrin is associated 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 to type 2 diabetes.

3- The increase of lactoferrin after acute-fat overload was favorably associated with lipid levels, parameters of oxidative stress and inflammation.

4- Lactoferrin improved insulin action (^{p473Ser}AKT) even inflammatory and dexamethasone conditions in 3T3-L1 cell line and in human predipocytes. However, lactoferrin blunted adipogenesis during 3T3-L1 cell line differentiation through AMPK and Rb activation, increasing ^{p172Thr}AMPK and hypophosphorylation and Rb expression, respectively.

5- Inversely, lactoferrin increased adipogenesis during human subcutaneous and visceral preadipocyte differentiation decreasing AMPK and Rb activity on human cells.

FINAL CONCLUSION

Lactoferrin might mediate an important role in the control of obesity-induced metabolic disturbances, impacting on adipose tissue physiology. Further studies will be necessary to clarify this hypothesis.

REFERENCES

1- Hotamisligil GS. Inflammation and metabolic disorders. Nature 2006; 444:860-867.

2- Janssen I, Katzmarzyk PT, Ross R. Body mass index, waist circumference, and health risk: evidence in support of current National Institutes of Health guidelines. Arch Intern Med 2002; 162:2074-2079.

3- Kurth T, Gaziano JM, Berger K *et al.* Body mass index and the risk of stroke in men. Arch Intern Med 2002; 162:2557-2562.

4- Calle EE, Rodriguez C, Walker-Thurmond K, *et al.* Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. N Engl J Med 2003; 348:1625-1638.

5- Behn A, Ur E. The obesity epidemic and its cardiovascular consequences. Curr Opin Cardiol 2006; 21:353-360.

6- Ritchie SA, Connell JM. The link between abdominal obesity, metabolic syndrome and cardiovascular disease. Nutr Metab Cardiovasc Dis. 2007; 17:319-326.

7- Björntorp P. Body fat distribution, insulin resistance, and metbolic diseases. Nutrition 1997;13:795-803.

8- Tran TT, Kahn CR. Transplantation of adipose tissue and stem cells: role in metabolism and disease. Nat Rev Endocrinol 2010;6:195-213.

9- Lebovitz HE. Insulin resistance a common link between type 2 diabetes and cardiovascular disease. Diabetes Obes Metab 2006; 8:237-249.

10- Savage DB, Petersen KF, Shulman GI. Disordered lipid metabolism and the pathogenesis of insulin resistance. Physiol Rev 2007; 87:507–520.

11- Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest 2005; 115; 1111–1119.

12- Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006; 444: 840-846.

13- Rahbar S. The discovery of glycated hemoglobin: a major event in the study of nonenzymatic chemistry in biological systems. Ann N Y Acad Sci 2005; 1043: 9-19.

14- Tziomalos K, Athyros VG, Karagiannis A, *et al.* Endothelial dysfunction in metabolic syndrome: Prevalence, pathogenesis and management. Nutr Metab Cardiovasc Dis 2010;20:140-146.

15- Meyers MR, Gokce N. Endothelial dysfunction in obesity: etiological role in atherosclerosis. Curr Opin Endocrinol Diabetes Obes 2007;14:365-369.

16- Steinberg HO, Baron AD. Vascular function, insulin resistance and fatty acids. Diabetologia 2002; 45:623-634.

17- Natali A, Toschi E, Baldeweg S, *et al.* Clustering of insulin resistance with vascular dysfunction and low-grade inflammation in Type 2 Diabetes. Diabetes 2006; 55:1133-1140.

18- Esteve E, Castro A, López-Bermejo A, *et al.* Serum interleukin-6 correlates with endothelial dysfunction in healthy men independently of insulin sensitivity. Diabetes Care 2007; 30:939-945.

19- Sondergaard L. Homology between the mammalian liver and the Drosophila fat body. Trends Genet 1993; 9: 193.

20- Song M.J, Kim K.H, Yoon J.M, *et al.* Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes. Biochem. Biophys. Res. Commun 2006; 346, 739–745.

21- Shi H, Kokoeva MV, Inouye K, *et al.* TLR4 links innate immunity and fatty acid-induced insulin resistance. J. Clin. Invest 2006; 116, 3015–3025.

22- Kopp A, Buechler C, Neumeier M, *et al.* Innate immunity and adipocyte function: ligand-specific activation of multiple Toll-like receptors modulates cytokine, adipokine, and chemokine secretion in adipocytes. Obesity (Silver Spring) 2009; 17:648-56.

23- Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest 2005; 115, 1111–1119.

24- Lumeng CN, Deyoung SM, Saltiel AR. Macrophages block insulin action in adipocytes by altering expression of signaling and glucose transport proteins. Am J Physiol Endocrinol Metab 2007; 292: E166-E174.

25- Constant VA, Gagnon A, Landry A. Macrophage-conditioned medium inhibits the differentiation of 3T3-L1 and human abdominal preadipocytes. Diabetologia 2006; 49: 1402-1411.

26- Zhou L, Sell H, Eckardt K. Conditioned medium obtained from in vitro differentiated adipocytes and resistin induce insulin resistance in human hepatocytes. FEBS Lett 2007; 581: 4303-4308.

27- Yarmo MN, Landry A, Molgat AS. Macrophage-conditioned medium inhibits differentiation-induced Rb phosphorylation in 3T3-L1 preadipocytes. Exp Cell Res 2009; 315: 411-418.

28- Fernandez-Real JM, Ricart W. Insulin resistance and chronic cardiovascular inflammatory syndrome. Endocr Rev 2003; 24:278-301.

29- Hotamisligil GS. Inflammatory pathways and insulin action. Int J Obes Relat Metab Disord 2003; 27 Suppl 3: S53-55.

30- Dandona P, Aljada A, Bandyopadhyay A. Inflammation: the link between insulin resistance, obesity and diabetes. Trends Immunol 2004; 25: 4-7.

31- He J, Usui I, Ishizuka K, *et al.* Interleukin-1alpha inhibits insulin signaling with phosphorylating insulin receptor substrate-1 on serine residues in 3T3-L1 adipocytes. Mol Endocrinol 2006; 20: 114-124.

32- Rotter V, Nagaev I, Smith U. Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. J Biol Chem 2003; 278: 45777-45784.

33- Senn JJ, Klover PJ, Nowak IA, *et al.* Interleukin-6 induces cellular insulin resistance in hepatocytes. Diabetes 2002; 51: 3391-3399.

34- Stephens JM, Lee J, Pilch PF. Tumor necrosis factor-alpha-induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. J Biol Chem 1997; 272: 971-976.

35- Hotamisligil GS, Peraldi P, Budavari A, *et al.* IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-α- and obesity-induced insulin resistance. Science 1996; 271: 665–668.

36- Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. Nature Rev. Mol. Cell Biol 2006; 7: 85–96.

37- Aguirre V, Uchida T, Yenush L, *et al.* The c-Jun NH_2-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser^{307}. J. Biol. Chem 2000; 275: 9047–9054.

38- Paz K, Hemi R, LeRoith D. *et al.* Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. J Biol Chem 1997; 272: 29911–29918.

39- Hotamisligil G S, Shargill N S, Spiegelman B M. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 1993; 259: 87–91.

40- Uysal K T, Wiesbrock S M, Marino M W, *et al.* Protection from obesity-induced insulin resistance in mice lacking TNF-α function. Nature 1997; 389: 610–614.

41- Ventre J, Doebber T, Wu M, *et al.* Targeted disruption of the tumor necrosis factor-alpha gene metabolic consequences in obese and nonobese mice. Diabetes 1997; 46: 1526–1531.

42- Krogh-Madsen R, Plomgaard P, Moller K, *et al.* Influence of TNF-α and IL-6 infusions on insulin sensitivity and expression of IL-18 in humans. Am J Physiol Endocrinol Metab 2006; 291: E108–E114.

43- Ofei F, Hurel S, Newkirk J, *et al.* Effects of an engineered human anti-TNF-α antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM. Diabetes 1996; 45: 881–885.

44- Lagathu C, Bastard JP, Auclair M, *et al.* Chronic interleukin-6 (IL-6) treatment increased IL-6 secretion and induced insulin resistance in adipocyte: prevention by rosiglitazone. Biochem Biophys Res Commun 2003; 14;311:372-379.

45- Kim HJ, Higashimori T, Park SY, *et al.* Differential effects of interleukin-6 and -10 on skeletal muscle and liver insulin action in vivo. Diabetes 2004; 53:1060-1067.

46- Al-Khalili L, Bouzakri K, Glund S, *et al.* Signaling specificity of interleukin-6 action on glucose and lipid metabolism in skeletal muscle. Mol Endocrinol 2006;20:3364-3375.

47- Nieto-Vazquez I, Fernández-Veledo S, de Alvaro C, *et al.* Dual role of interleukin-6 in regulating insulin sensitivity in murine skeletal muscle. Diabetes. 2008: 57:3211-3221.

48- Kim JH, Kim JE, Liu HY, *et al.* Regulation of interleukin-6-induced hepatic insulin resistance by mammalian target of rapamycin through the STAT3-SOCS3 pathway. J Biol Chem 2008;283:708-715.

49- Jager J, Grémeaux T, Cormont M, *et al.* Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression.. Endocrinology 2007;148:241-251.

50- Lagathu C, Yvan-Charvet L, Bastard JP, *et al.* Long-term treatment with interleukin-1beta induces insulin resistance in murine and human adipocytes. Diabetologia 2006;49:2162-2173.

51- Bruun JM, Pedersen SB, Richelsen B. Regulation of interleukin 8 production and gene expression in human adipose tissue in vitro. J Clin Endocrinol Metab 2001;86:1267–1273.

52- Kobashi C, Asamizu S, Ishiki M, *et al.* Inhibitory effect of IL-8 on insulin action in human adipocytes via MAP kinase pathway. J Inflamm (Lond) 2009;6:25.

53- Kjeldsen L, Johnsen AH, Sengeløv H, *et al.* Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J Biol Chem 1993; 268:10425-10432.

54- Flo TH, Smith KD, Sato S, *et al.* Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. Nature 2004; 432:917-921.

55- Bu DX, Hemdahl AL, Gabrielsen A, *et al.* Induction of neutrophil gelatinase-associated lipocalin in vascular injury via activation of nuclear factor-kappaB. Am J Pathol 2006; 169:2245-2253.

56- Wang Y, Lam KS, Kraegen EW, *et al.* Lipocalin-2 is an inflammatory marker closely associated with obesity, insulin resistance, and hyperglycemia in humans. Clin Chem 2007; 53:34-41.

57- Yan QW, Yang Q, Mody N, *et al.* The adipokine lipocalin 2 is regulated by obesity and promotes insulin resistance.. Diabetes 2007;56:2533-2540.

58- Catalán V, Gómez-Ambrosi J, Rodríguez A, *et al.* Increased adipose tissue expression of lipocalin-2 in obesity is related to inflammation and matrix metalloproteinase-2 and metalloproteinase-9 activities in humans. J Mol Med 2009; 87:803-813.

59- Moreno-Navarrete JM, Manco M, Ibáñez J, *et al.* Metabolic endotoxemia and saturated fat contribute to circulating NGAL concentrations in subjects with insulin resistance. Int J Obes (Lond) 2010;34:240-249.

60- Law IK, Xu A, Lam KS, *et al.* Lipocalin-2 deficiency attenuates insulin resistance associated with ageing and obesity. Diabetes 2010;59:872-882.

61- Fasshauer M, Klein J, Kralisch S, *et al.* Monocyte chemoattractant protein 1 expression is stimulated by growth hormone and interleukin-6 in 3T3-L1 adipocytes. Biochem Biophys Res Commun 2004;317:598-604.

62- Kanda H, Tateya S, Tamori Y, *et al.* MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. J Clin Invest 2006;116:1494-1505.

63- Sell H, Dietze-Schroeder D, Kaiser U, *et al.* Monocyte chemotactic protein-1 is a potential player in the negative cross-talk between adipose tissue and skeletal muscle. Endocrinology 2006; 147:2458-2467.

64- Moutschen MP, Scheen AJ, Lefebvre PJ. Impaired immune responses in diabetes mellitus: analysis of the factors and mechanisms involved. Relevance to the increased susceptibility of diabetic patients to specific infections. Diabetes Metab 1992;18:187–201.

65- Wang H, Meng QH, Gordon JR, *et al.* Proinflammatory and proapoptotic effects of methylglyoxal on neutrophils from patients with type 2 diabetes mellitus. Clin Biochem 2007; 40:1232-1239.

66- Hatanaka E, Monteagudo PT, Marrocos MS, *et al.* Neutrophils and monocytes as potentially important sources of proinflammatory cytokines in diabetes. Clin Exp Immunol. 2006;146:443-447.

67- Advani A, Marshall SM, Thomas TH. Impaired neutrophil actin assembly causes persistent CD11b expression and reduced primary granule exocytosis in Type II diabetes. Diabetologia. 2002 ;45:719-727.

68- Walrand S, Guillet C, Boirie Y, *et al.* In vivo evidences that insulin regulates human polymorphonuclear neutrophil functions. J Leukoc Biol 2004; 76:1104–1110.

69- Okouchi M, Okayama N, Shimizu M, *et al.* High insulin exacerbates neutrophil-endothelial cell adhesion through endothelial surface expression of intercellular adhesion molecule-1 via activation of protein kinase C and mitogen-activated protein kinase. Diabetologia 2002; 45:556–555.

70- Walrand S, Moreau K, Caldefie F, *et al.* Specific and nonspecific immune responses to fasting and refeeding differ in healthy young adult and elderly persons. Am J Clin Nutr 2001;74:670–678.

71- Walrand S, Guillet C, Boirie Y, *et al.* Insulin differentially regulates monocyte and polymorphonuclear neutrophil functions in healthy young and elderly humans. J Clin Endocrinol Metab 2006;91:2738-2748.

72- Stegenga ME, van der Crabben SN, Blumer RM, *et al.* Hyperglycemia enhances coagulation and reduces neutrophil degranulation, whereas hyperinsulinemia inhibits fibrinolysis during human endotoxemia. Blood 2008;112:82-89.

73- Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. J Clin Invest 2006;116:1793-801.

74- Menzaghi C, Trischitta V, Doria A. Genetic influences of adiponectin on insulin resistance, type 2 diabetes, and cardiovascular disease. Diabetes 2007; 56:1198-1209.

75- Peake PW, Shen Y, Campbell LV, *et al.* Human adiponectin binds to bacterial lipopolysaccharide. Biochem Biophys Res Commun 2006; 341:108-115.

76- Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. Endocrinol Rev. 2005; 26:439-451.

77- Yamauchi T, Kamon J, Waki H, *et al.* The fatderived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nat Med 2001;7:941-946.

78- Tomas E, Tsao TS, Saha AK, *et al.* Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. Proc Natl Acad Sci USA 2002; 99:16309-16313.

79- Wu X, Motoshima H, Mahadev K, *et al.* Involvement of AMP-activated protein kinase in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes. Diabetes 2003; 52:1355-1363.

80- Kim HJ, Higashimori T, Park SY, *et al.* Differential effects of interleukin-6 and -10 on skeletal muscle and liver insulin action in vivo. Diabetes 2004; 53:1060-1067.

81- Hong EG, Ko HJ, Cho YR, *et al.* Interleukin-10 prevents diet-induced insulin resistance by attenuating macrophage and cytokine response in skeletal muscle. Diabetes 2009; 58:2525-2535.

82- Gao Z, Zuberi A, Quon MJ, *et al.* Aspirin inhibits serine phosphorylation of insulin receptor substrate 1 in tumor necrosis factor-treated cells through targeting multiple serine kinases. J Biol Chem 2003;278:24944-24950.

83- Möhlig M, Freudenberg M, Bobbert T, *et al.* Acetylsalicylic acid improves lipid-induced insulin resistance in healthy men. J Clin Endocrinol Metab 2006; 91:964-967.

84- An Y, Liu K, Zhou Y, *et al.* Salicylate inhibits macrophage-secreted factors induced adipocyte inflammation and changes of adipokines in 3T3-L1 adipocytes. Inflammation 2009; 32:296-303.

85- Fernández-Real JM, López-Bermejo A, Ropero AB, *et al.* Salicylates increase insulin secretion in healthy obese subjects. J Clin Endocrinol Metab 2008; 93:2523-2530.

86- Leffler M, Hrach T, Stuerzl M, *et al.* Insulin attenuates apoptosis and exerts anti-inflammatory effects in endotoxemic human macrophages. J Surg Res 2007; 143:398-406.

87- Ghanim H, Dhindsa S, Aljada A, *et al.* Low-dose rosiglitazone exerts an antiinflammatory effect with an increase in adiponectin independently of free fatty acid

fall and insulin sensitization in obese type 2 diabetics. J Clin Endocrinol Metab 2006; 91:3553-3558.

88- Mohanty P, Aljada A, Ghanim H, *et al.* Evidence for a potent antiinflammatory effect of rosiglitazone. J Clin Endocrinol Metab 2004;89:2728-2735.

89- Baker EN, Baker HM. A structural framework for understanding the multifunctional character of lactoferrin. Biochimie 2009;91:3-10.

90- Iijima H, Tomizawa Y, Iwasaki Y *et al.* Genetic and epigenetic inactivation of LTF gene at 3p21.3 in lung cancers. Int J Cancer 2006; 118:797-801.

91- Kajikawa M, Ohta T, Takase M, *et al.* Lactoferrin inhibits cholesterol accumulation in macrophages mediated by acetylated or oxidized low-density lipoproteins. Biochim Biophys Acta 1994; 1213: 82-90.

92- Bellamy W, Takase M, Yamauchi K, *et al.* Identification of the bactericidal domain of lactoferrin. Biochim Biophys Acta 1992; 1121:130-136.

93- Baveye S, Elass E, Mazurier J, *et al.* A Multifunctional Glycoprotein Involved in the Modulation of the Inflammatory Process. Clin Chem Lab Med 1999; 37:281–286.

94- Ward PP, Mendoza MM, Saucedo-Cardenas O, *et al.* Restricted spatiotemporal expression of lactoferrin during murine embryogenesis. Adv Exp Med Biol. 1998; 443:91-100.

95- Bullen JJ, Rogers HJ, Spalding PB, *et al.* Iron and infection: the heart of the matter. FEMS Immunol Med. Microbiol. 2005; 43: 325-330.

96- Valenti P, Antonini G. Lactoferrin: an important host defence against microbial and viral attack. Cell Mol Life Sci. 2005;62:2576-2587.

97- Paesano R, Torcia F, Berlutti F, *et al.* Oral administration of lactoferrin increases hemoglobin and total serum iron in pregnant women, Biochem Cell Biol. 2006; 84:377-380.

98- Paesano R, Pietropaoli M, Gessani S, *et al.* The influence of lactoferrin, orally administered, on systemic iron homeostasis in pregnant women suffering of iron deficiency and iron deficiency anaemia. Biochimie 2009; 91:44-51.

99- Bellamy W, Takase M, Yamauchi K, *et al.* Identification of the bactericidal domain of lactoferrin. Biochim Biophys Acta 1992; 1121:130-136.

100- Conneely OM. Antiinflammatory activities of lactoferrin J Am Coll Nutr 2001; 20:389S–397S.

101- Ward PP, Paz E, Conneely OM. Multifunctional roles of lactoferrin: a critical overview. Cell Mol Life Sci 2005;62: 2540-2548.

102- Otsuki K, Yakuwa K, Sawada M, *et al.* Recombinant human lactoferrin has preventive effects on lipopolysaccharide-induced preterm delivery and production of inflammatory cytokines in mice. J Perinat Med 2005; 33: 320-323.

103- Hayashida K, Kaneko T, Takeuchi T, *et al.* Oral administration of lactoferrin inhibits inflammation and nociception in rat adjuvant-induced arthritis. J Vet Med Sci 2004; 66: 149-154.

104- Kruzel ML, Harari Y, Chen CY, *et al.* The gut. A key metabolic organ protected by lactoferrin during experimental systemic inflammation in mice. Adv Exp Med Biol 1998; 443: 167-173.

105- Zimecki M, Artym J, Chodaczek G, *et al.* Protective effects of lactoferrin in Escherichia coli-induced bacteremia in mice: relationship to reduced serum TNF alpha level and increased turnover of neutrophils. Inflamm Res 2004; 53: 92-96.

106- Haversen L, Ohlsson BG, Hahn-Zoric M, *et al.* Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-kappa B. Cell Immunol 2002; 220: 83-95.

107- Konishi M, Iwasa M, Yamauchi K, *et al.* Lactoferrin inhibits lipid peroxidation in patients with chronic hepatitis C. Hepatol Res 2006;36:27

108- Tsubota A, Yoshikawa T, Nariai K, *et al.* Bovine lactoferrin potently inhibits liver mitochondrial 8-OHdG levels and retrieves hepatic OGG1 activities in Long-Evans Cinnamon rats. J Hepatol 2008;48:486-493.

109- Naot D, Grey A, Reid IR, *et al.* Lactoferrin--a novel bone growth factor.Clin Med Res 2005; 3: 93-101.

110- Cornish J, Callon KE, Naot D, *et al.* Lactoferrin is a potent regulator of bone cell activity and increases bone formation in vivo. Endocrinology 2004; 145: 4366-4374.

111- Grey A, Banovic T, Zhu Q, *et al.* The low-density lipoprotein receptor-related protein 1 is a mitogenic receptor for lactoferrin in osteoblastic cells. Mol Endocrinol 2004; 18: 2268–2278

112- Hagiwara T, Shinoda I, Fukuwatari Y, *et al.* Effects of lactoferrin and its peptides on proliferation of rat intestinal epithelial cell line, IEC-18, in the presence of epidermal growth factor. Biosci Biotechnol Biochem 1995;59: 1875–1881.

113- Nichols BL, McKee KS, Huebers HA. Iron is not required in the lactoferrin stimulation of thymidine incorporation into the DNA of rat crypt enterocytes. Pediatr Res 1990;27:525–528.

114- Oguchi S, Walker WA, Sanderson IR. Iron saturation alters the effect of lactoferrin on the proliferation and differentiation of human enterocytes (Caco-2 cells). Biol Neonate 1995; 67:330–339.

115- Hashizume S, Kuroda K, Murakami H. Identification of lactoferrin as an essential growth factor for human lymphocytic cell lines in serum-free medium. Biochim Biophys Acta 1983; 763: 377–382.

116- Oria R, Alvarez-Hernandez X, Liceaga J, *et al.* Uptake and handling of iron from transferrin, lactoferrin and immune complexes by a macrophage cell line. Biochem J 1988; 252: 221–225.

117- Thang X, Beynen AC. Lowering effect of dietary milk-whey protein v. casein on plasma and liver cholesterol concentrations in rats. Br J Nutr 1993;70: 139-146.

118- Takeuchi T, Shimizu H, Ando K, *et al.* Bovine lactoferrin reduces plasma triacylglycerol and NEFA accompanied by decreased hepatic cholesterol and triacylglycerol contents in rodents. Br J Nutr 2004;91:533-538.

119- Huettinger M, Retzek H, Eder M, *et al.* Characteristics of chylomicron remnant uptake into rat liver. Clin Biochem 1988;21: 87-92.

120- Ziere GJ, van Dijk MC, Bijsterbosch MK, *et al.* Lactoferrin uptake by the rat liver. Characterization of the recognition site and effect of selective modification of arginine residues. J Biol Chem 1992;267:11229-11235.

121- Ziere GJ, Kruijt JK, Bijsterbosch MK, *et al.* Recognition of lactoferrin and aminopeptidase M-modified lactoferrin by the liver: involvement of the remnant receptor. Z Gastroenterol 1996;34:118-121.

122- Crawford SE, Borensztajn J. Plasma clearance and liver uptake of chylomicron remnants generated by hepatic lipase lipolysis: evidence for a

lactoferrin-sensitive and apolipoprotein E-independent pathway. J Lipid Res 1999;40:797-805.

123- Bezault J, Bhimani R, Wiprovnick J, *et al.* Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. Cancer Res 1994; 54:2310–2312.

124- Wang WP, Iigo M, Sato J, *et al.* Activation of intestinal mucosal immunity in tumor-bearing mice by lactoferrin. Jpn J Cancer Res. 2000; 91: 1022–1027.

125- Shimamura M, Yamamoto Y, Ashino H, *et al.* Bovine lactoferrin inhibits tumorinduced angiogenesis. Int J Cancer 2004;111:111–116.

126- Tsuda H, Sekine K, Fujita K, *et al.* Cancer prevention by bovine lactoferrin and underlying mechanisms – a review of experimental and clinical studies. Biochem Cell Biol 2002; 80:131–136.

127- Varadhachary A, Wolf JS, Petrak K, *et al.* Oral lactoferrin inhibits growth of established tumors and potentiates conventional chemotherapy. Int J Cancer 2004; 111: 398–403.

128- Panella TJ, Liu YH, Huang AT, *et al.* Polymorphism and altered methylation of the lactoferrin gene in normal leukocytes, leukemic cells and breast cancer. Cancer Res 1991;51:3037–3043.

129- Benaissa M, Peyrat JP, Hornez L, *et al.* Expression and prognostic value of lactoferrin mRNA isoforms in human breast cancer. Int J Cancer 2005; 114: 299–306.

130- Siebert PD, Huang BC. Identification of an alternative form of human lactoferrin mRNA that is expressed differentially in normal tissues and tumor-derived cell lines. Proc Natl Acad Sci USA 1997; 94: 2198–2203.

131- Xiao Y, Monitto CL, Minhas KM, *et al.* Lactoferrin down-regulates G1 cyclin-dependent kinases during growth arrest of head and neck cancer cells. Clin Cancer Res 2004; 10: 8683–8686.

132- Horwitz DA, Bakke AC, Abo W, *et al.* Monocyte and NK cell cytotoxic activity in human adherent cell preparations: discriminating effects of interferon and lactoferrin. J Immunol 1984; 132: 2370–2374.

133- Shau H, Kim A, Golub SH. Modulation of natural killer and lymphokine-activated killer cell cytotoxicity by lactoferrin. J Leukoc Biol 1992; 51: 343–349.

134- Damiens E, Mazurier J, el Yazidi I, *et al.* Effects of human lactoferrin on NK cell cytotoxicity against haematopoietic and epithelial tumour cells. Biochim Biophys Acta 1998; 1402: 277–287.

135- Son HJ, Lee SH, Choi SY. Human lactoferrin controls the level of retinoblastoma protein and its activity. Biochem Cell Biol 2006; 84: 345–350.

136- Shurtz-Swirski R, Sela S, Herskovits AT, *et al.* Involvement of peripheral polymorphonuclear leukocytes in oxidative stress and inflammation in type 2 diabetic patients. Diabetes Care 2001;24:104-110.

137- Shim WS, Kim HJ, Kang ES, *et al.* The association of total and differential white blood cell count with metabolic syndrome in type 2 diabetic patients. Diabetes Res Clin Pract 2006;73:284-291.

138- Gubern C, Lopez-Bermejo A, Biarnes J, *et al.* Natural antibiotics and insulin sensitivity: the role of bactericidal/permeability-increasing protein. Diabetes 2006;55:216-224.

139- Cani PD, Amar J, Iglesias MA, *et al.* Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007;56:1761-1772.

140- Koivuranta-Vaara P, Banda D, Goldstein IM. Bacterial-lipopolysaccharide-induced release of lactoferrin from human polymorphonuclear leukocytes: role of monocyte-derived tumor necrosis factor alpha. Infect Immun 1987;55:2956-2961.

141- Srivastava CH, Rado TA, Bauerle D, *et al.* Regulation of human bone marrow lactoferrin and myeloperoxidase gene expression by tumor necrosis factor-alpha. J Immunol 1991;146:1014-1019.

142- Zimecki M, Chodaczek G, Kocieba M, *et al.* Lethality in LPS-induced endotoxemia in C3H/HeCr mice is associated with prevalence of proinflammatory cytokines: lack of protective action of lactoferrin. FEMS Immunol Med Microbiol 2004;42:167-172.

143- Fernández-Real JM, Broch M, Ricart W, *et al.* Plasma levels of the soluble fraction of tumor necrosis factor receptor 2 and insulin resistance. Diabetes 1998;47:1757-1762.

144- Ward PP, Uribe-Luna S, Conneely OM. Lactoferrin and host defense. Biochem Cell Biol 2002;80:95-102.

145- Legrand D, Elass E, Carpentier M, *et al.* Lactoferrin: a modulator of immune and inflammatory responses. Cell Mol Life Sci 2005;62:2549-2559.

146- Japelj B, Pristovsek P, Majerle A, *et al.* Structural origin of endotoxin neutralization and antimicrobial activity of a lactoferrin-based peptide. J Biol Chem 2005;280:16955-16961.

147- Baveye S, Elass E, Fernig DG, *et al.* Human lactoferrin interacts with soluble CD14 and inhibits expression of endothelial adhesion molecules, E-selectin and ICAM-1, induced by the CD14-lipopolysaccharide complex. Infect Immun 2000;68:6519-6525.

148- Buitenhuis M, Verhagen LP, van Deutekom HW, *et al.* Protein kinase B (c-akt) regulates hematopoietic lineage choice decisions during myelopoiesis. Blood 2008;111:112-121.

149- Dandona P, Aljada A, Mohanty P, *et al.* Insulin inhibits intranuclear nuclear factor kappaB and stimulates IkappaB in mononuclear cells in obese subjects: evidence for an anti-inflammatory effect? J Clin Endocrinol Metab 2001;86:3257-3265.

150- Jeschke MG, Klein D, Thasler WE, *et al.* Insulin decreases inflammatory signal transcription factor expression in primary human liver cells after LPS challenge. Mol Med 2008;14:11-19.

151- Vassiliou G, Benoist F, Lau P, *et al.* The low density lipoprotein receptor-related protein contributes to selective uptake of high density lipoprotein cholesteryl esters by SW872 liposarcoma cells and primary human adipocytes. J Biol Chem 2001;276: 48823-48830.

152- Shakibaei M, Frevert U. Dual interaction of the malaria circumsporozoite protein with the low density lipoprotein receptor-related protein (LRP) and heparan sulfate proteoglycans. J Exp Med 1996;184: 1699-1711.

153- Takeuchi T, Kitagawa H, Harada E. Evidence of lactoferrin transportation into blood circulation from intestine via lymphatic pathway in adult rats. Exp Physiol 2004;89:263-270.

154- Cardona F, Túnez I, Tasset I, *et al.* Circulating antioxidant defences are decreased in healthy people after a high-fat meal. Br J Nutr 2008; 10: 1-5.

155- Steijns JM, van Hooijdonk AC. Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. Br J Nutr 2000; 84: S11-17.

156- Fridlyand LE, Philipson LH. Oxidative reactive species in cell injury: Mechanisms in diabetes mellitus and therapeutic approaches. Ann N Y Acad Sci 2005; 1066: 136-151.

157- van Oostrom AJ, Rabelink TJ, Verseyden C, *et al.* Activation of leukocytes by postprandial lipemia in healthy volunteers. Atherosclerosis 2004; 177: 175–182.

158- Bellido C, López-Miranda J, Blanco-Colio LM, *et al.* Butter and walnuts, but not olive oil, elicit postprandial activation of nuclear transcription factor B in peripheral blood mononuclear cells from healthy men. Am J Clin Nutr 2004; 80:1487–1491.

159- Leite MS, Pacheco P, Gomes RN, *et al.* Mechanisms of increased survival after lipopolysaccharide-induced endotoxic shock in mice consuming olive oilenriched diet. Shock 2005;23:173–178.

160- Youn HS, Lee JY, Fitzgerald KA, *et al.* Specific inhibition of MyD88-independent signaling pathways of TLR3 and TLR4 by resveratrol: molecular targets are TBK1 and RIP1 in TRIF complex. J Immunol 2005; 175: 3339–3346.

161- Hayashida K, Takeuchi T, Ozaki T, *et al.* Bovine lactoferrin has a nitric oxide-dependent hypotensive effect in rats. Am J Physiol Regul Integr Comp Physiol 2004;286: R359-365.

162- Hayashida K, Takeuchi T, Harada E. Lactoferrin enhances peripheral opioid-mediated antinociception via nitric oxide in rats. Eur J Pharmacol 2004;484:175-181.

163- Buren J, Lai YC, Lundgren M, *et al.* Insulin action and signalling in fat and muscle from dexamethasone-treated rats. Arch Biochem Biophys 2008; 474: 91-101.

164- Maneva A, Taleva B, Maneva L. Lactoferrin-protector against oxidative stress and regulator of glycolysis in human erythrocytes. Z Naturforsch [C] 2003; 58: 256-262.

165- Yagi M, Suzuki N, Takayama T, *et al.* Lactoferrin suppress the adipogenic differentiation of MC3T3-G2/PA6 cells. J Oral Sci 2008; 50: 419-425.

166- Yagi M, Suzuki N, Takayama T, *et al.* Effects of lactoferrin on the differentiation of pluripotent mesenchymal cells. Cell Biol Int 2009; 33: 283-289.

167- Rosen CJ, Bouxsein ML. Mechanisms of disease: is osteoporosis the obesity of bone? Nat Clin Pract Rheumatol 2006; 2: 35-43.

168- Gimble JM, Zvonic S, Floyd ZE, *et al.* Playing with bone and fat. J Cell Biochem 2006; 98: 251-266.

169- Rossmeisl M, Flachs P, Brauner P, *et al.* Role of energy charge and AMP-activated protein kinase in adipocytes in the control of body fat stores. Int J Obes Relat Metab Disord 2004; 28: S38-S44.

170- Usui I, Haruta T, Iwata M, *et al.* Retinoblastoma protein phosphorylation via PI 3-kinase and mTOR pathway regulates adipocyte differentiation. Biochem Biophys Res Commun 2000; 275: 115-120.

171- Abella A, Dubus P, Malumbres M, *et al.* Cdk4 promotes adipogenesis through PPARgamma activation. Cell Metab 2005; 2: 239-249.

172- Peng XD, Xu PZ, Chen ML, *et al.* Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. Genes Dev 2003;17:1352-1365.

173- Baudry A, Yang ZZ, Hemmings BA. PKBalpha is required for adipose differentiation of mouse embryonic fibroblasts. J Cell Sci 2006;119:889-897.

174- Tomlinson JJ, Boudreau A, Wu D, *et al.* Insulin sensitization of human preadipocytes through glucocorticoid hormone induction of forkhead transcriptions factors. Mol Endocrinol 2010;24:104-113.

175- Wellen KE, Fucho R, Gregor MF, *et al.* Coordinated regulation of nutrient and inflammatory responses by STAMP2 is essential for metabolic homeostasis. Cell 2007;129:537-548.

176- Chen X, Zhu C, Ji C, *et al.* STEAP4, a gene associated with insulin sensitivity, is regulated by several adipokines in human adipocytes. Int J Mol Med 2010;25:361-367.

177- Ono T, Murakoshi M, Suzuki N, *et al.* Potent anti-obesity effect of enteric-coated lactoferrin: decrease in visceral fat accumulation in Japanese men and women with abdominal obesity after 8-week administration of enteric-coated lactoferrin tablets. Br J Nutr 2010; 9:1-8.