

# Leptin, Anti-HSP60, Anti-HSP65 and Anti-oxLDL in Autoimmune Rheumatic Diseases

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To investigate whether leptin, heat shock proteins (HSP), oxidized low-density lipoprotein (oxLDL) are involved in the autoimmune rheumatic diseases (AIRD). We studied sera of 47 patients with vasculitis, 38 patients with Sjögren's syndrome (SS), 44 patients with systemic sclerosis (SSc) and 154 sex-matched healthy subjects (HS). Serum leptin, autoantibody against (anti-) HSP60, HSP65 and oxLDL were determined by EIA. The level of leptin was increased in male patients with vasculitis ( $p < 0.05$ ), anti-HSP60 in all patient groups was lower ( $p < 0.05$ ) and anti-oxLDL was higher in SSc-patients ( $p < 0.05$ ) than HS. We conclude that levels of serum leptin, anti-HSP60, anti-HSP65 and anti-oxLDL multiply varied in vasculitis-patients, SS-patients and SSc-patients. These non-traditional factors are probably involved in the process of AIRD.

## Introduction

Pathological and clinical studies show that many non-traditional factors may play an important role in the developing process of autoimmune rheumatic diseases (AIRD). Leptin possesses a cytokine-like ability to modulate the immuno-inflammatory response. Heat shock proteins (HSP) are generated when cells are exposed to elevated temperatures or other environmental stress. Low-density lipoprotein (LDL) undergoes oxidative modification *in vivo* and it is immunogenic. All these factors are involved in immune-inflammatory response. Leptin is a nonglycosylated multiple functional protein which may possess a hormonal feature to regulate the balance between food intake, energy expenditure, and a role of a cytokine to promote or reduce an immune reaction (1). Leptin has been suggested

(2-4) to be a modulator of pre-inflammatory and immune response by virtue of its interaction with leptin receptors expressed on peripheral blood mononuclear cells, vascular endothelial cells, smooth muscle cells and osteoblasts.

HSP, a putative intracellular autoantigens, constitute a group of highly conserved intracellular proteins that are produced by prokaryotic and eukaryotic cells in response to perturbations of the cell's environment, such as those induced by high temperature, infection, or mechanical stress. However, when over expressed in response to stress, HSP could translocate to and reside on the cell surface, where these cryptic antigens could be recognized by the immune system, thereby triggering an immune response. Under certain conditions, they could be released from cells (5,6). Anti-HSP cause cytotoxic effects on heat-stressed human endothelial cells through complement activation or antibody dependent cellular cytotoxicity (7).

OxLDL frequently presents in the sera of patients with autoimmune conditions (8). OxLDL has been associated with both subclinical atherosclerosis and inflammatory variables. OxLDL is immunogenic and stimulates the induction of anti-oxLDL. Anti-oxLDL is involved in the progression of autoimmune disease, i.e. rheumatoid arthritis, systemic lupus erythematosus and antiphospholipid syndrome, and in diabetes mellitus, uremia, chronic periaortitis, hepatocellular carcinoma, beta-thalassemia, psoriasis, preeclampsia and eclampsia (8).

During the last decade evidence has accumulated to indicate that leptin, oxLDL and HSP often are involved in modulation and initiation of autoimmune diseases. In order to investigate the role of these factors, we have determined the concentrations of serum leptin, anti-HSP60, anti-HSP65 and anti-oxLDL in several patient groups with AIRD and 135 healthy subjects.

## **Patients and Methods**

Thirty-eight female patients with Sjögren's syndrome (SS) with an average age 48.7 years and a mean disease duration of 7.27 years were included in this group. The diagnosis of SS was performed according to the American-European classification criteria for SS (9). Forty-seven patients with vasculitis, some of which had either Wegener's granulomatosis, microscopic polyangiitis or renal-limited vasculitis (20 females and 27 males with an average age of 43.9 years), were enrolled in the study. Forty-four women patients with systemic sclerosis (SSc) with an average age 45.6 years were included in the study. One hundred and fifty-four healthy subjects (HS) (64 females and 90 males with an average age of 44.2 years) were used as control. All the patients and the HS had a body mass index ranging between 18 and 25.

The concentration of serum leptin was determined by a commercial active® human leptin enzyme linked-immunosorbent assay (EIA) kit (Di-

agnostic Systems Laboratories, Inc. Webster, Texas). The separation and copper-oxidization of LDL were performed as described previously (10). To assess the extent of oxLDL in terms of the modified surface charge on the apolipoprotein B100, lipoprotein electrophoresis was performed using a Beckman Paragon® electrophoresis system (11). The concentration of LOOHs following oxidation was  $481 \pm 38$  nmol/mg of LDL protein ( $n=3$ ) as determined using the FOX assay (11), and the concentration of conjugated dienes was  $219 \pm 32$  nmol/mg of LDL protein ( $n=3$ ) as measured spectrophotometrically. The alteration of the surface charge of apolipoprotein B100, as assessed by measuring the electrophoretic mobility relative to BSA, was  $0.27 \pm 0.04$  ( $n=3$ ) and  $0.45 \pm 0.03$  ( $n=3$ ) for native LDL and oxLDL, respectively.

IgG and IgM anti-oxLDL were determined by EIA essentially as described (10). OxLDL was diluted to 2  $\mu\text{g}/\text{mL}$  in coating buffer (carbonate-bicarbonate buffer 50 mmol/L, pH 9.7), and 100 mL/well was used to coat ELISA plates (Costar 2581). The plates were kept at 4°C overnight, washed 4 times with phosphate-buffered saline (PBS) containing 0.05% Tween-20, and then blocked with 20% adult bovine serum in PBS (20% ABS-PBS) for 2 hours at room temperature. They were then incubated with 100  $\mu\text{L}$  serum diluted 1:100 in 20% ABS-PBS at 4°C overnight. After 4 washings with PBS, the plates were incubated with 100 mL of alkaline phosphatase-conjugated goat anti-human IgG (Sigma A-3150; Sigma Chemical, St Louis, MO) diluted 1:9000, or IgM (Sigma A-3275) diluted 1:7000 with PBS at 37°C for 1 hour. After 4 washings, 100  $\mu\text{L}$  of substrate (phosphatase substrate tablets, Sigma 104) was added. The plates were incubated at a room temperature for 30 minutes and read in an ELISA Multiskan Plus spectrophotometer at 405 nm. Each determination was done in triplicate. All samples were measured in a single assay, and the coefficient of variation was 10% to 15%.

Anti-HSP60 and anti-HSP65 were determined (12) on EIA plates coated with 100  $\mu\text{L}/\text{well}$  of 1 mg/mL HSP60 (StressGen, Victoria, BC Canada) or HSP65 (Lionex Diagnostics, Braunschweig, Germany) in a coating buffer over night. Plates were washed, and nonspecific binding sites were blocked. Serum samples were diluted in 1:100 in PBS containing 1% of BSA, then added into each well, and bound antibodies were detected by alkaline phosphatase conjugated polyclonal goat anti-human IgG/M followed by *p*-nitrophenyl phosphate substrate. Antibody concentrations were determined by comparison with a standard curve that was generated with samples of predetermined high levels that had been assigned a concentration of 1000 arbitrary units/mL.

## Statistical Analysis

Conventional methods were used to calculate the means and standard deviations. For skewed variables, non-parametric tests were employed to determine for comparisons between the groups (Mann-Whitney U-test),

whereas simple regression was used to analyze the correlations between the concentrations of leptin and the titers of antibodies. The significance levels was put at a probability of  $< 0.05$ .

## Results

The concentrations of serum leptin in patients and healthy subject groups are shown in Table 1. Elevated levels of leptin were found only in male-patients with vasculitis ( $p < 0.01$ ; Table 1 and Fig. 1) with a decreased trend of leptin levels in female patients with SS and SSc (Table 1).

Anti-HSP60, anti-HSP65 and anti-oxLDL titers were measured. The

Table 1. The concentrations of serum leptin in patients with autoimmune rheumatic diseases and healthy subjects

Group	Sex	Leptin	<i>p</i> value
		Mean $\pm$ SD (ng/mL)	
Healthy Subject	Male	13.1 $\pm$ 6.50	
	Female	45.5 $\pm$ 46.5	
Vasculitis	Male	27.7 $\pm$ 24.3	0.0042*
	Female	50.7 $\pm$ 52.3	0.8831*
Sjogren's Syndrome	Female	25.4 $\pm$ 22.1	0.083*
Systemic Sclerosis	Female	29.0 $\pm$ 37.2	0.092*

\*Compared with sex matched healthy subject group

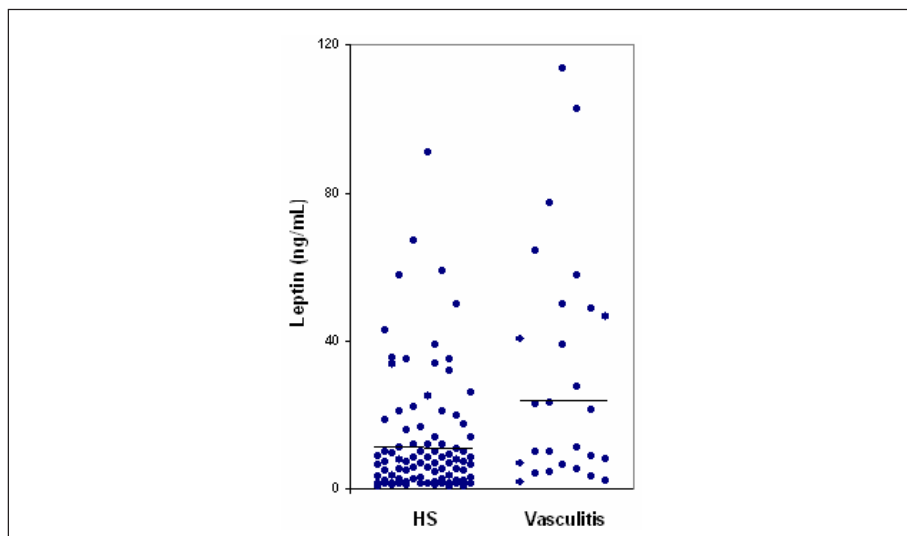


Figure 1. Individual concentration of serum leptin in male healthy subjects and male vasculitis-patients. The narrow lines show the mean levels in each group.

Table 2. The levels of autoantibodies to HSP60, HSP65 and oxLDL in patients with autoimmune rheumatic diseases and healthy subjects

Antibody Specificity		HSP60	HSP60	HSP65	HSP65	OxLDL	OxLDL
Antibody Class		IgG	IgM	IgG	IgM	IgG	IgM
Group	Sex	Mean±SD (unit/mL)					
Healthy Subject	Mixed	162.3±55	82±36	98.6±108	111.6±33	96.6±9	113.5±78
	Male	104.3±42*	58.7±22*	115.2±134	100.7±17	103.7±42	110.9±39
Vasculitis	Female	125.8±151*	64.4±33*	112.5±204	97.2±12	94.2±10	108.6±25
	Female	112.3±30*	67.9±17*	61.7±40*	96.1±9	96.8±19	94.8±19
Sjogren's Syndrome	Female	137.6±70*	86.8±31	109±104	114.5±38	110.3±25#	126±32#

\* Significant lower than healthy subjects,  $p < 0.05$

#Significant higher than healthy subjects,  $p < 0.05$

results of the patients and healthy subject groups are depicted in Table 2. Both IgG and IgM levels of anti-HSP60 were lower in all patient groups except IgM in patients with SSc. The IgG level of anti-HSP65 was lower in the group with SS. Both IgG and IgM levels of anti-oxLDL were higher in the group with SSc.

We analyzed the relationship between the serum leptin and the autoantibodies titers. There were a significantly inverse correlations between the levels of leptin and IgM anti-HSP65 ( $R=0.391$ ,  $p<0.05$ ) or IgM anti-oxLDL ( $R=0.382$ ,  $p<0.05$ ) in patients with SS.

## Discussion

We determined the bioactive molecular leptin concentration, anti-oxLDL, anti-HSP60 and anti-HSP65 titers in several patient groups with AIRD. Our results showed significantly increased levels of serum leptin in males with vasculitis and a decreased trend of leptin levels in patients with SS and SSc; a significantly decreased IgG and IgM anti-HSP60 in all patient groups, except IgM in SSc, and increased IgG and IgM anti-oxLDL in patients with SSc compared with sex and age matched healthy subjects.

Leptin possesses several functions including an adipose tissue-derived messenger, physiological hormone-modulating action and immune cytokine-regulating role (13,14). Serum leptin levels were found to vary in a variety of AIRD conditions, such as systemic lupus erythematosus (SLE), Behcet's syndrome and rheumatoid arthritis (RA) (15-21). The levels of serum leptin are elevated in patients with Behcet's syndrome and RA (15,16). Thus, the leptin could be specifically related to degree or activity of the disease (15,16). The recent investigations indicate that serum levels of leptin correlated negatively with androstenedione concentrations and directly influenced chronic inflammation in SLE and RA-patients, not HS (17). Leptin production is increased in the circulation and consumed within synovial fluid in patients with RA to suggest that leptin has a protective role against the destructive

course of RA (18). Another study showed although concentrations of leptin were higher in SLE-patients than controls, but the concentrations of leptin did not relate with disease activity index (19). Other studies have, however, failed to show any relationship between RA activity and levels of leptin. The leptin levels were nearly the same between RA-patients and HS (20,21).

The explanation for the phenomenon of the leptin level variation in these AIRD is complicated. It is known that leptin may play a role in inflammation and angiogenesis (14). Leptin stimulates hematopoiesis and enhances the production and phagocytic activity. It looks like that leptin may function as a stress-related hormone, related to immuno-inflammatory responses (22). Our data show elevated concentration of leptin in male vasculitis-patients. A probable explanation is that leptin exerts an effect to up-regulate the inflammatory response in the disease process of vasculitis. In a general inflammatory response, the component events of acute inflammation work together to alleviate the noxious stimulus and promote healing so that the area can be cleared for the repair process. Meanwhile, the acute inflammatory response, as the combination of vascular and cellular events that can give way in time to chronic inflammation, results in tissue damage. Several studies have been reported that infection and inflammation may elevate the production of leptin. It has a systemic effect in regulating the neuroendocrine and immune function (23). The multiple cytokines and inflammatory processes can raise leptin levels and play a significant role in the anorexia and cachexia of inflammatory diseases (24). Leptin enhances Th1-type cytokines production by human T lymphocytes and may cause an effect on anti-CD3 stimulation, which increases the production of proinflammatory cytokine IFN- $\gamma$  and IL-2 (25,26). All these immune factors may be involved in the AIRD process. However, the change in levels of leptin whether playing a stimulatory or an inhibitory effect and the sex dimorphism of leptin in vasculitis is not yet clear.

AIRD disorders are characterized by autoantibodies to self-antigens or alloantigens, non-organ specific antigens, specifically, antigens occurring in nucleated cells or among circulating plasma proteins (27). Tissue injury in autoimmunity may be induced by different stimuli, including mechanical stress and reaction to oxLDL. Injury endothelial cells may induce an increased expression of some cell protein, such as HSP (28). It has been suggested that free radical-induced oxidative stress may alter the immune process; scleroderma antigens are sensitive to reactive oxygen species and result in fragmentation of the autoantigen. The increased LDL oxidation and the reduced circulating levels of anti-oxidants occur in SSc-patients (29,30). The oxidative damage is widespread and multi-aspects. The numbers of oxygen free radicals are increased in SSc-patient, which can play an important role in the development of SSc progression (31,32). Our results indicate elevated levels of anti-oxLDL in SSc-patients. The antigen epitopes of oxLDL also include a copper-oxidized LDL besides the MDA-oxidized LDL (33) and the calcium-oxidized LDL (34). It provides further evidence that oxidative

stress with oxLDL contributes to the pathogenesis of SSc.

The levels of anti-HSP60 were decreased in all three patient groups with AIRD. The underlying cause of the decreased titers is not clear but probably may entail immunological, endocrine and genetic factors. Vasculitis, SS, and SSc with systemic manifestations are diseases reflecting a generalized defect in immune regulation, the tissue injury that is mainly caused by autoantibodies. The occurrence of HSP is an earlier event in tissue damage and has a protecting role to neighboring cells and tissues (5,35). On the other hand, the expression of HSP on the surface of the endothelium can stimulate the lymphocytes to release anti-HSP (6,7). In principle, decreased antibody levels may reflect either an increased consumption or a decreased production of the antibodies. In this case, the most possible explanation for the anti-HSP60 in vasculitis, SS, and SSc-patients is an increased consumption due to an increased binding of HSP60 resulting in immune complex formation and enhanced removal in circulation. Another alternative explanation (36) is that more anti-HSP60 could be trapped in the endothelium or tissues that express HSP on the surface, which might further extend the tissue damage. The third explanation is that a decreased production of certain autoantibodies *in vivo* can be induced by a strong cell mediated responses. T cells are capable of modulating the immune response in a positive sense by providing T-cell help, which is mainly exerted by Th1 and Th2. As Th1 cells mediate the T cell response, there occurs a concomitant inhibition of both Th2 cell-mediated production of antibodies and humoral immune responses. It has been analyzed that T cells are important in the pathogenesis of the certain AIRD. SSc may be a specific antigen-driven T cell disease (37).

Taken together, our investigation indicates increased leptin concentration in male vasculitis-patients, elevated anti-Cu<sup>++</sup> oxLDL in SSc-patients and decreased anti-HSP60 in vasculitis-patients, SS-patients and SSc-patients. The nature and origin of these non-traditional factors in AIRD is not yet clear. The importance of the findings in evaluating these diseases also remains to be further investigated.

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