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Title: LEPTIN, RESISTIN, AND PCSK9 – THE ROLE OF STAT3

Short title: STAT3 regulates PCSK9

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Abstract

In a condition of dysfunctional visceral fat depots, as in the case of obesity, alterations in adipokines levels may be detrimental for the cardiovascular system. The proinflammatory leptin and resistin adipokines have been described as possible links between obesity and atherosclerosis. The present study was aimed at evaluating whether proprotein convertase subtilisin/kexin type 9 (PCSK9), a key regulator of low-density lipoprotein metabolism, is induced by leptin and resistin through the involvement of the inflammatory pathway of signal transducer and activator of transcript 3 (STAT3). In HepG2 cells, leptin and resistin upregulated PCSK9 gene and protein expression as well as the phosphorylation of STAT3. Upon STAT3 silencing, leptin and resistin lost their ability to activate PCSK9. The knock-down of STAT3 did not affect the expression of leptin and resistin receptors as well as that of PCSK9. The analysis of human *PCSK9* promoter region showed that the two adipokines raise PCSK9 promoter activity via the involvement of sterol regulatory element motif. In healthy male, a positive association between circulating leptin and PCSK9 levels was found only when BMI was < 25 kg/m^2. In conclusion, our study identified STAT3 as one of the molecular regulators of leptin- and resistin-mediated transcriptional induction of PCSK9.

Keywords: PCSK9, leptin, resistin, STAT3

Introduction

Adipose tissue is an endocrine organ able to secrete active molecules, namely, adipokines which contribute to regulate appetite and satiety, fat distribution, and energy expenditure [1]. When the metabolic milieu is deranged, as in the case of obesity and adipose tissue metainflammation, adipokines may play a role in cardiovascular disease (CVD), by promoting atherogenesis, plaque progression and thrombosis [2]. Roughly 80% of patients with coronary heart disease are overweight or obese [3]. Among the huge variety of adipokines produced by adipose tissue, the proinflammatory leptin and resistin have been described as possible links between obesity and atherosclerosis [4, 5].

Leptin, a 16 kDa cytokine produced predominantly by adipose tissue, regulates feeding and promotes energy expenditure.This hormone is implicated also in the regulation of the immune system, autonomic and cardiovascular regulation [6]. Leptin exerts its biological actions by binding to a class I cytokine receptor encoded by *LEPR* in humans. Among the six LepR isoforms (LepRa to LepRf), the LepRb is the only one with a full-lengh intracellular domain which allows the activation of a cell-signalling pathway. In response to leptin, Janus tyrosine kinase 2 (JAK2) phosphorylates LepR triggering the recruitment of the Signal Transducer and Activator of Transcript 3 (STAT3) through its SH2 domain. STAT3 is subsequently phosphorylated by JAK2, resulting in its dimerization and nuclear translocation in order to regulate the transcription of STAT3-target genes [7]. Among STAT proteins (STAT 1, 2, 3, 4, 5a, 5b, and 6), STAT3 contributes to various metabolic processes like hyperphagia and obesity [8], being leptin one of the major players in these events [9].

Resistin is a 12.5 kDa sized C-terminal cysteine-rich signaling peptide secreted predominantly by adipose tissue and macrophages in humans and rodents. In humans, resistin adipose tissue expression and circulating levels are increased in overweight and obese subjects, strongly supporting the association with a raised CV risk in obese individuals [10]. Among the designated receptors mediating resistin effects, *e.g.* the inflammatory cascade, the adenylyl cyclase associated protein 1 (CAP1) has been recently described as a *bona fide* resistin receptor [11].

Proprotein convertase subtilisin/kexin type 9 (PCSK9), mainly secreted by the liver and to a lower extent by the intestine, post-translationally regulates the number of cell-surface LDL receptors (LDLR). At transcriptional level, PCSK9 synthesis is largely controlled by the involvement of transcription factor families sterol regulatory element (SRE)-binding proteins (SREBPs) and by the acute-phase response transcriptional controller hepatocyte nuclear factor-1 (HNF-1) alpha [12]. This last evidence along with the knowledge that PCSK9 expression is also regulated by the pro-

inflammatory cytokine tumor necrosis factors (TNF)-α, in a suppressor of cytokine signaling (SOCS)3-dependent manner, suggests a direct link between inflammation and the regulation of lipid metabolism via PCSK9 [13].

Considering that PCSK9 circulating levels have been associated with atherosclerosis and to support the hypothesis that the derangement in adipokine secretion is involved in obesity-associated CV risk [14], the aim of the present study was to evaluate whether leptin and resistin mediate PCSK9 activation through the inflammatory STAT3 pathway. Indeed, the JAK/STAT signaling pathway, a pillar downstream mediator of a variety of cytokines, is dysregulated in metabolic diseases including obesity [15].

Materials and Methods

Cell cultures – The human hepatocellular carcinoma cell line, HepG2, was routinely cultured in 10% Fetal Bovine Serum (FBS)/DMEM supplemented with penicillin, streptomycin, nonessential amino acids and sodium pyruvate (all from Sigma Aldrich, Milan, Italy). For the experiments, cells were starved overnight and then incubated with DMEM containing 10% of human lipoprotein plasma deprived serum (LPDS).

Reagents and antibodies – DMEM, trypsin EDTA, penicillin, streptomycin, nonessential amino acid solution, FBS, disposable culture flasks and petri dishes were from Merck (Milan, Italy). Molecular weight protein standard, precast polyacrylamide gels (4-12%), BCA assay for determination of protein concentrations, maxima 1strand cDNA and maxima SYBR Green/Fluorescein qPCR were all from Life Technologies (Monza, Italy). Bovine Serum Albumin was from SIGMA-Aldrich (Milan, Italy). Human recombinant leptin (Sigma Aldrich) and resistin (Bio Visoon, CA) were used at 100 ng/mL. Human recombinant PCSK9 was used at 2.5 μg/mL (Cayman Chemicals, item 20631, US) [16].

Transfection of siRNA – ON-TARGET plus SMART pool siRNA directed to STAT3 and PCSK9 or scramble control were from Dharmacon™ (Carlo Erba Reagents, Milan, Italy). Transfections were performed by using SilentFect™ Lipid Reagent (BIO-RAD laboratories, Hercules, CA) according to the manufacturer's protocol. The day before the transfection, HepG2 cells were seeded in

MEM/10%FBS at a density of $6*10^5$/well (6-well tray). Cells were then transfected with 40 nmol/L of siRNA. Fourty-eight hours post transfection, the medium was replaced with DMEM/10%LPDS ± leptin (100 ng/mL) or resistin (100 ng/mL) for an additional 48 h before performing the quantitative (q)PCR analysis.

In silico analyses for STAT3 binding motifs on human PCSK9 promoter – Human, gorilla, mouse and rat *PCSK9* promoter sequences (up to 2000 bp upstream the start codon) were retrieved from Ensembl Genome Browser [17] by using the latest released genome assembly (GRCh38.p13, Kamilah_GGO_v0, GRCh38.p6 an Rnor_6.0 for human, gorilla, mouse and rat, respectively). JASPAR, an open-access database of curated, non-redundant transcription factors binding (TFB) profiles modelled as position-specific weight matrices (PSSMs), was used to scan the selected PCSK9 promoter region for any STAT3 binding site [18]. The relative profile score threshold – defined as the minimun relative score required for reporting a match between the TFB model and the imputed sequence – was set at 80%. FIMO tool, from the open-access MEME-Suit 5.1.1, was used to double-check the results obtained from JASPAR [19, 20]. STAT3 PSSMs profile was retrieved from JASPAR and imputed in FIMO and the selected PCSK9 promoter region scanned. Matches with a *p*-value ≤ 0.0001 were took in account. The same analyses were carried out for SRE and HNF-1.

Generation of PCSK9 promoter luciferase reporters with a STAT3 binding site deletion. D1-STAT3-mut plasmid was generated by deleting the STAT3 binding sequence predicted by JASPER in the PCSK9 promoter sequence in plasmid D1. Plasmid D1 is a derivative of the commercial promoter-reporter vector pGL3-Basic, generated by cloning the human PCSK9 promoter fragment spanning -1,711 to -94 upstream of the *luc* reporter gene. Deletion of the STAT3 sequence (CTTCTGGAAAG), spanning - 916 to - 906 in the PCSK9 promoter, was obtained by two-step PCR according to the following protocol. Two fragments flanking the putative STAT3 sequence were PCR amplified using D1 plasmid as template and the following primer pairs: AP679 (5'-CGACGAGGTACCGAGCTCGGATCCACTAGTAAC-3') - AP680 (5'-ATTCAATTTGCAAAGATTC-3'), to generate Fragment UP (upstream), and AP681 (5'-CAAATTGAATCTGAGCTTGTGCCTACCATAG-3') – AP682 (5'- CCCAAGCTTACTGTGCAGGAGCTGAAGTTC-3'), to generate Fragment DOWN (downstream). Primer AP679 and AP682 sequences contain the KpnI and HindIII restriction sites,

respectively. Fragments UP and DOWN were used as template for a second round of PCR amplification using AP679 and AP682 primers. The final PCR product was cloned in the D1 plasmid between KpnI and HindIII restriction sites in place of the PCSK9 promoter region, generating D1-STAT3 plasmid. The deletion of the STAT3 region from the PCSK9 promoter in D1-STAT3 plasmid was confirmed by DNA sequencing.

Transfections of Reporter Constructs – As previously described, HepG2 cells were transfected with the plasmid PCSK9 pGL3-PCSK9-D4 containing the 5' flanking region of the PCSK9 gene from -440 to -94, relative to the ATG start codon in front of the luciferase coding sequence. The promoter constructs contain wild-type, SRE mutated (SRE-mu) and HNF-1 alpha mutated (HNF1-mu) sequences [21]. To measure the human PCSK9 promoter activity, HepG2 cells were seeded in 48-well plates at a density of 4×10^5 cells per well. The day after, cells were transiently transfected with pGL3-PCSK9-D1 (wildtype and STAT3 mutated) and with pGL3-PCSK9-D4 plasmids (wild-type, SRE-mu and HNF-1-mu), with TurboFect™ transfection reagent (Thermo Fisher). Forty-eight hours post transfection, cells were incubated with DMEM/10%LPDS ± leptin (100 ng/mL), resistin (100 ng/mL) and simvastatin (20 μM) for an additional 24 hours. Luciferase activities were measured by using Neolite reagent (Perkin Elmer, Milan, Italy) according to manufacturer's instructions.

RNA preparation and quantitative real time PCR – Total RNA was extracted with the iScript Sample Preparation Buffer cDNA synthesis preparation reagents (BIO-RAD laboratories) according to manufacturer's instructions or by spin column (Qiagen, Milan, Italy). Reverse transcription-polymerase first-strand cDNA synthesis was performed by using maxima 1strand cDNA synthesis kit (Thermo, Life Technologies). qPCR was then performed by using the Kit Thermo SYBR Green/ROX qPCR Master Mix (Thermo, Life Technologies) and specific primers for selected genes. The analyses were performed with the 9600 Biorad Real-Time PCR Detection Systems (Biorad). The primer sequences have been listed in Table 1. PCR cycling conditions were as follows: 94°C for 5 min, 40 cycles at 94°C for 15 s, and 60°C for 30 s. Data were expressed as Ct values and used for the relative quantification of targets with the $2^{-\Delta\Delta Ct}$ calculation.

Western Blot Analysis – Total cytosolic protein extracts of HepG2 were obtained by collecting cells in 70 µl of Mammalian Protein Extraction Reagents (Thermo Fisher Scientific) containing a cocktail of protease and phosphatase inhibitors (Roche Diagnostics). Ten µg of proteins and a molecular mass marker (Novex Sharp Protein Standard, Invitrogen™; Life Technologies) were separated on 4-12% sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE; Novex NuPAGE 4-12% Bis-Tris Mini Gels, Invitrogen; Life Technologies) under denaturing and reducing conditions. Proteins were then transferred to a nitrocellulose membrane by using the iBlotTM Gel Transfer Device (Invitrogen; Life Technologies). The membranes were washed with Tris-Buffered Saline-Tween 20 (TBS-T) and non-specific binding sites were blocked in TBS-T containing 5% BSA (SIGMA-Aldrich) for 90 min at room temperature. The blots were incubated overnight at 4°C with a diluted solution (5% BSA or non-fat dried milk) of the human primary antibodies (listed below). Membranes were washed with TBS-T and then exposed for 90 min at RT to a diluted solution (5% non-fat dried milk) of the secondary antibodies (anti-mouse and anti-rabbit peroxidase-conjugated secondary antibodies (New England Biolabs, MA). Immunoreactive bands were detected by exposing the membranes to Clarity Western ECL chemiluminescent substrates (Bio-Rad Laboratories) for 5 min and images were acquired with a ChemiDoc XRS System (Bio-Rad Laboratories). Densitometric readings were evaluated using the ImageLab software as previously described. The dilution of the human primary antibodies were: PCSK9 (1:1,000 - Genetex, CA); STAT3 (1:1,000 - Cell Signaling); pSTAT3 (1:10,000 - Abcam, UK); actin (1:1,000 – Santa Cruz, CA), tubulin (1:2,000 - Sigma Aldrich) and vinculin (1:1,000 - Genetex).

Study population – The association between PCSK9 and leptin and resistin plasma levels was evaluated in 149 healthy male subjects from the Brisighella Heart Study. This study represents a longitudinal population study on a randomized sample representative of the entire population of Brisighella, a rural Northern Italian village. The study has been active since 1972 and is carried out in agreement with the Declaration of Helsinki. The protocol was approved by the institutional ethics board of the University Hospital of Bologna [22].

ELISA assays – Plasma PCSK9 concentrations were measured by a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). The minimum detectable PCSK9 concentration was 0.219 ng/mL [23]. Concerning the evaluation of PCSK9 in the conditioned

medium, HepG2 cells were cultured in 12-well plates ($6*10^5$ cells/well). Medium (200 µL) was collected after 24-h treatment with leptin, resistin and simvastatin. Samples were not diluted and results normalized for the total amount of proteins.

Plasma leptin concentrations were measured by a commercial ELISA kit (R&D Systems). The minimum detectable dose of human leptin is typically less than 7.8 pg/mL [24].

Analysis of the data – Statistical analysis was performed using the Prism statistical analysis package version 6.0 (GraphPad Software, San Diego, CA). Data are given as mean ± SD of three independent experiments. When possible, p-values were determined by Student's t-test. Otherwise, differences between treatment groups were evaluated by 1-way ANOVA. A probability value of $p<0.05$ was considered statistically significant.

Results

Leptin and resistin induce PCSK9. First of all, a time course experiment (4-, 8-, 16- and 24 hours) was conducted to evaluate the gene expression of *PCSK9* upon leptin (100 ng/mL) and resistin (100 ng/mL) treatment (Supplemental Figure S1). Incubation of HepG2 cells with human recombinant leptin and resistin resulted in a significant rise in PCSK9 gene expression of roughly +50% (Fig. 1A) after 24 hours. Compared to medium alone, protein expression, evaluated by Western blot analysis, was increased roughly by 2- and 1.7-fold, upon treatment with leptin and resistin, respectively (Fig. 1B). Simvastatin (20 µM) - used as a positive control - raised both PCSK9 gene and protein expression (Fig. 1D and E). To corroborate Western blot analyses, the release of PCSK9 was measured. In the HepG2 conditioned-medium, the levels of PCSK9 were increased by 42.6% ($p< 0.01$) and 36.3% ($p< 0.05$) following leptin and resistin tretments, respectively (Fig. 1C). Simvastatin (20 µM) was used as a positive control (Fig. 1F).

STAT3 mediates leptin- and resistin-driven PCSK9 activation. In our cell-based model, 24-h treatment with leptin and resistin phosphorylated STAT3 ($pSTAT3^{Y705}$) without affecting that of total STAT3 (Fig. 2A). To follow-up the hypothesis that STAT3 was involved in leptin- and resistin-driven PCSK9 activation, HepG2 cells were knock-down for STAT3. Transfection with specific siRNA

anti-STAT3 fully abolished the gene and protein expression of STAT3 (Fig. 2B-C). Under these experimental conditions, leptin and resistin did not upregulate PCSK9 expression (gene and protein), while basal PCSK9 gene and protein expression was not affected (Fig. 2D-F). These data suggest that PCSK9 upregulation by leptin and resistin in hepatocytes requires STAT3. To exclude any effect of PCSK9 on STAT3 expression, we showed that silencing PCSK9 did not result in any change in STAT3 levels (Fig. 2C). Moreover, application of siSTAT3 did not change the gene expression of leptin (*LEPR*) and resistin (*CAP1*) receptors (Supplemental Figure S2).

STAT3 and PCSK9 transcriptional activity. The involvement of STAT3 in the PCSK9 pathway was further investigated at the transcriptional level. First of all, an *in silico* search of STAT3 binding sites on proximal PCSK9 promoter region was performed by using JASPAR database and FIMO tool from MEME-Suit 5.1.1. The analyses revealed a strong STAT3 binding site (JASPAR score: 96%; FIMO threshold: p= 2.07E-05) spanning the region -916 to -906 in the human PCSK9 promoter (Table 2). This region is upstream the Sp1/HNF1-α/SRE triad already published by Li *et al.* [21] (Fig. 3A). To corroborate this finding, a similar analysis was performed on gorilla, mouse, and rat PCSK9 promoters: a multiple alignment of the four sequences was generated thanks to Nucleotide BLAST Alignment tool by NCBI (Fig. 3A). The mild degeneration in the STAT3 consensus sequence has been already reported in literature [25]. After transfection with D1 and D1-STAT3-mut plasmids (Fig. 3B), both containing the human *PCSK9* promoter region spanning -1,711 to -94 nucleotides upstream of the gene coding for the luciferase enzyme, HepG2 cells were treated with leptin, resistin and simvastatin. Twenty-four-hour-treatment enhanced transcriptional activity, an effect which was not counteracted when STAT3 mutation was inserted (Fig. 3C). Simvastatin was used as a positive control (Fig. 3D). These data suggest that PCSK9 upregulation by leptin and resistin in hepatocytes is not directly mediated by binding of STAT3 to the *PCSK9* promoter.

Leptin and resistin affect the transcriptional activity of PCSK9. To further explore the mechanism whereby leptin and resistin upregulate PCSK9 transcript levels, HepG2 cells were transiently transfected with constructs containing the proximal human *PCSK9* promoter region (-440 to -94). As shown in Fig. 4A, leptin and resistin significantly incremented the luciferase activity by 54% and 15%, respectively. Similar results were obtained upon treatment with simvastatin (+141%). Being SREBPs and HNF-1 the major transcription factors regulating PCSK9 [26], we investigated luciferase activity upon the insertion of SRE and HNF-1 mutations in *PCSK9* promoter sequence. The

presence of mutation in the SRE sequence completely abolished the PCSK9 transcriptional activity driven by leptin and resistin as well as that of simvastatin (Fig. 4B). Conversely, mutation in HNF-1 did not alter leptin- and resistin-driven luciferase activities, showing an increment of 43% and 58%, respectively (Fig 4C).

Association studies. The circulating levels of leptin and PCSK9 were measured by ELISA in a clinical setting of 149 males (56 ± 4 years) free of cardiovascular disease at enrolment and belonging to the cohort of Brisighella Heart Study [22]. A positive association between circulating leptin and PCSK9 levels (β= 0.352, p= 0.014) was found only in subjects with BMI <25 kg/m^2 (Fig 5A). When BMI rose, *i.e.* between 25 and 30 kg/m^2 (β= 0.147, p= 0.295; Fig 5B) or > 30 kg/m^2 (β= -0.030, p= 0.840; Fig 5C), the association was lost. Concerning resistin, the circulating levels did not associate with PCSK9 (data not shown). The main characteristics of the selected participants have been reported in the Supplemental Table S1.

Discussion

Obesity and associated metabolic disorders are becoming major health care concerns worldwide. Obesity is highly associated with chronic low-grade inflammation and it is believed that this obesity-linked inflammatory state is due to changes in the expression of cytokines released by adipose tissue. Adipose tissue is a source of secreted adipokines that act as modulators of metabolic and cardiovascular processes [27]. Among adipokines, the expression of LDLR has been described to be modulated by leptin and resistin, the last being able to stabilize the cellular expression of PCSK9 [5, 28]. Adiponectin has been also described to affect PCSK9 expression/production, although via the activation of PPARγ and AMPK pathways [29].

In the tight liaison among adipokines, inflammation and atherosclerosis, the main finding of this study relies on the evidence that leptin and resistin, two adipokines associated to regulation of atherogenesis and inflammation, induce the expression of PCSK9 in hepatocytes, which are the main source of circulating protein, through the Janus kinase (JAK)/STAT3 inflammatory pathway. A growing body of evidence suggests that this pathway is dysregulated in the context of obesity and metabolic disease [15]. The interplay between inflammation and PCSK9 has been investigated in the last years leading to the hypothesis that it represents a fed-forward loop in which inflammation activates PCSK9 and *vice versa* PCSK9 impacts on the inflammatory burden associated to atheroma formation [30].

The cytokine responsive JAK/ STAT pathway is involved in adipokine-mediated crosstalk between adipocytes and liver or skeletal muscle [31, 32]. The involvement of STAT3 signaling upon leptin receptor activation represents the primary mechanism by which leptin regulates energy balance [33] and resistin has also been reported to activate this pathway [34]. In particular, STAT3, a transcription factor expressed in multiple metabolic tissues, is activated in response to cytokines, growth factors, and nutrients [35]. STAT3 may account for the constitutive activation of NF-κB during chronic inflammation. This activation contributes to the transcriptional regulation of inflammatory cytokines, such as ICAM-1, VCAM-1, MCP-1 and IL-6, as well as matrix metalloproteinases [36]. Moreover, it has been showed that the impairment of oncostatin M signaling, a secreted cytokine mainly involved in chronic inflammatory and cardiovascular diseases, has a protective effect on atherosclerosis partially due to the inhibition of the JAK2/STAT3 activation in macrophages [37]. Thus, overall, by silencing STAT3, we have demonstrated that this inflammatory pathway may be at the crossroad between adipose tissue and PCSK9 activation. Furthermore, STAT3 phosphorylation is markedly increased in atherosclerotic lesions, and interfering with STAT3 pathway prevents atherosclerotic lesion formation *in vivo* [38]. In line with this evidence, the direct role of PCSK9 in atheroma formation should be considered. The Atherosclerosis Risk in Communities (ARIC) epidemiological study demonstrated that genetically reduced levels of PCSK9 were associated with a reduction in myocardial infarction, fatal coronary heart diseases or coronary revascularization, despite a significant prevalence of other nonlipid-related CV-risk factors [39]. Higher serum PCSK9 levels were linearly associated with a higher necrotic core fraction in coronary atherosclerosis, regardless of serum LDL-C, confirming data in PCSK9 knock-out mice that were partially protected from neointimal formation [40]. In humans, PCSK9 has been also described to be associated with other components of atheroma formation, *i.e.* platelet aggregation [16], hypertension and inflammation [30].

We have found that leptin and resistin activate the transcription of PCSK9 through the involvement of the SRE motif, apparently, without the involvement of HNF-1 [41]. The involvement of STAT3 pathway at the transcriptional level was negligible. However, this latter analysis requires further investigation since the promoter region of human *PCSK9* contains other STAT3 responsible elements (Table 2). Since PCSK9 transcription is controlled through cis regulatory elements imbedded in the proximal promoter region of *PCSK9* gene where the Sp1 sites, SRE and HNF-1 are located, we utilized a PCSK9 promoter luciferase reporter plasmid containing mutations for both SRE and HNF responsive elements. We found that exposure of HepG2 hepatocytes to leptin and

resistin increases the transcriptional activity of the PCSK9 promoter. Furthermore, while a construct variant containing a mutation in HNF-1 motif did not affect the ability of adipokines to upregulate luciferase activity, no induction was detected after introducing a mutation in the SRE motif. Relative to this latter evidence, our data are in line with findings demonstrating that, at transcriptional level PCSK9 synthesis is largely controlled by SREBPs [42]. Our findings are in agreement with the observation that leptin induces SREBP1 activation in porcine granulosa cells [43].

When looking at the correlation between circulating levels of PCSK9 and leptin, we found that this exists only in the group with BMI < 25 Kg/m^2, an evidence in line with that previous published by Kwakernaak et al. [44] in 30 women. This becomes relevant considering that both PCSK9 and leptin levels follow a gender difference, being higher in female subjects than in male[45-47]. Overall, leptin may be a determinant of PCSK9 levels predominantly before a state of leptin resistance occurs and, most of all, in obese patients other factors may impact on the regulation of PCSK9 levels. Leptin resistance, proposed to take place during the onset and progression of obesity [48], could be the result of an impaired leptin signaling pathway, including reduced access of the hormone to its receptor or due to epigenetic modifications that could contribute to leptin expression and signaling disturbances [49]. This complex scenario has been also highlighted in ob/ob mice lacking leptin in which the effects of leptin replacement on PCSK9 mRNA expression and protein levels were sex-dependent [50]. In male mice, leptin treatment reduced plasma levels of PCSK9, whereas no changes were observed in females [50]. On the other hand, although it was suggested that resistin may be involved in the interplay between inflammation and atherosclerosis, whether circulating resistin is a determinant of higher PCSK9 levels needs to be further explored [44]. Indeed, our data obtained in healthy male subjects did not fit with a previous report by Li et al. [51], who found that in patients with cardiovascular artery disease resistin positively correlate with PCSK9 levels. Some of these discrepancies may also rely on the tight liaison surrounding resistin and PCSK9, *i.e.* the C-terminal domain of PCSK9 has a structural homology with the human resistin C-terminal domain, and CAP1 represents a binding partner of PCSK9 in mediating LDLR degradation [52]. On this matter, we have previously demonstrated that PCSK9 induces a pro-inflammatory response in macrophages [53], an effect common to resistin, *i.e.* a rise in IL6, TNF-α, MCP-1 (data not published). Conversely, a possible *vicious circle* in the liver has been excluded based on the observation that treatment with human recombinant PCSK9 did not increase the gene expression of *IL6* in HepG2 cells (Supplemental Figure S3). This is in line with

recent findings showing that PCSK9 is not induced in artificial human inflammation and is not correlated with inflammatory response [54].

Overall, we provided evidence that PCSK9 may be one of the players at crossroad of the interplay between obesity, adipose tissue dysfunction and CVD risk. Such effect appears to be dependent on the inflammatory JAK/STAT pathway.

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Figure legend

Figure 1. HepG2 cells were seeded in DMEM/10% FBS. Cells were incubated with leptin (100 ng/mL) or resistin (100 ng/mL) in DMEM supplemented with 10%LPDS for 24 hours. Simvastatin (20 μM) has been used as a positive control. At the end of the incubation, total RNA and proteins were extracted. β-actin was used as a housekeeping for both gene (*ACTB*) and protein expression. Panels A and D represent PCSK9 gene expression (qPCR); panels B and E represent PCSK9 protein expression (WB); panels C and F represent PCSK9 levels assayed by ELISA (data were normalized for the total amount of proteins). The values above the blot represent the densitometric readings. Differences between groups have been assessed by Student's t-test or one-way ANOVA; *p<0.05, **p<0.01. CTR, medium alone. At least three independent experiments were conducted. qPCR, quantitative real-time PCR; WB, Western blot; *ACTB*, β-actin gene; ELISA, enzyme-linked immunosorbent assay; LPDS, human lipoprotein plasma deprived serum.

Figure 2. Panel A) HepG2 cells were seeded in DMEM/10% FBS. After 48h, the medium was replaced with DMEM with 10%LPDS supplemented with leptin (100 ng/mL) and resistin (100 ng/mL). After 24 h, the expression of $pSTAT3^{Y705}$ and STAT3 was evaluated. β-actin and vinculin were used as housekeeping proteins. Panel B-C) HepG2 cells were seeded in DMEM/10% FBS and the day after transfected with siRNA scramble and siRNA anti-STAT3 or siRNA anti-PCSK9. After 48h, the expression of STAT3 was evaluated. β-actin and tubulin were used as housekeeping to normalize the data. Panel D-E) HepG2 transfected with siRNA scramble and siRNA anti-STAT3 were or were not treated with leptin (100 ng/mL) and resistin (100 ng/mL) and the gene expression of PCSK9 was evaluated by qPCR. Actin was used as a housekeeping gene. Panel F) HepG2 transfected with siRNA scramble and siRNA anti-STAT3 were or were not treated with leptin (100 ng/mL) and resistin (100 ng/mL) and the protein expression of PCSK9 was evaluated by Western blot. Vinculin was used as housekeeping to normalize the data. In panels A, C and F (WB), the values above the blots represent the densitometric readings evaluated by using the ImageLab software. Differences between groups were assessed by Student's t-test and one-way ANOVA; *p<0.05, **p<0.01, † p<0.05 refers to Leptin treatment vs Leptin treatment plus siSTAT3. CTR, medium alone. At least three independent experiments were conducted. qPCR, quantitative real-time PCR; WB, Western blot.

Figure 3. Panel A) Sequence comparison of proximal regions of PCSK9 promoter of human, gorilla, mouse, and rat species. Panel B) Schematic representation of the human PCSK9 promoter-luciferase reporter plasmid D1. Positions are referred to the nucleotide preceding the ATG start codon of human PCSK9 gene, arbitrarily designated as - 1. Positions - 94 and - 1,711 indicate the 3' and the 5' ends of PCSK9 promoter insert, respectively. Positions of the 5' and 3' ends of the STAT3 binding sequence are indicated. Panel C-D) HepG2 cells were transfected with pGL3-PCSK9-D1 and pGL3-PCSK9-D1-STAT3-mutated. The day after the transfection, the medium was replaced with DMEM containing 10%LPDS supplemented with leptin (100 ng/mL), resistin (100 ng/mL) or simvastatin (20 μM). After an additional 24 hours, luciferase activities have been determined by Neolite reagent. Differences between groups were assessed by Student's t-test or one-way ANOVA; *p<0.05, **p<0.01. CTR, medium alone. At least three independent experiments were conducted. Numbers inside the bars represent the percentage of increment. Nucleotides in red refer to STAT3 sequence in PCSK9 promoter region; Nucleotides in blue refer to Sp1 sequence in PCSK9 promoter region; Nucleotides in green refer to HNF-1 sequence in PCSK9 promoter region; Nucleotides in purple refer to SRE sequence in PCSK9 promoter region; ** refer to bp spanning between the inter-region sequences of PCSK9 promoter region. STAT3, signal transducer and activator of transcript 3; Sp1, Proximal specificity protein 1; HNF1, hepatocyte nuclear factor-1; SRE, sterol regulatory element.

Figure 4. HepG2 cells were transfected with pGL3-PCSK9-D4 (*panel A*), pGL3-PCSK9-SREmut (*panel B*) and pGL3-PCSK9-HNFmut (*panels C*). The day after the transfection, the medium was replaced with DMEM containing 10%LPDS supplemented with leptin (100 ng/mL), resistin (100 ng/mL) or simvastatin (20 μM). After an additional 24 hours, luciferase activities have been determined by Neolite reagent. Differences between groups were assessed by Student's t-test; *p<0.05. CTR, medium alone. At least three independent experiments were conducted.

Figure 5. Plasma levels of leptin and PCSK9 were measured by commercial ELISA assays. Subjects have been stratified according to BMI, i.e. <25 Kg/m^2 (n=48; panel A), between 25 and 30 Kg/m^2 (n= 53; panel B) and > 30 Kg/m^2 (n= 48; panel C).

Table 1. List and sequence of primers used in this study

Primers	Forward	Reverse
PCSK9	5'-CCTGCGCGTGTCAACT-3'	5'-GCTGGCTTTTCCGAAACTC-3'
STAT3	5'-CAGCAGCTTGACACACGGTA -3'	5'-AAACACCAAAGTGGCATGT-3'
ACTB	5'-TTCTACAATGAGCTGCGTGTG-3'	5'-GGGGTGTTGAAGGTCTCAAA-3'
LEPR	5'-TACTTTGGAAGCCCCTGATG-3'	5'-AAGCACTGAGTGACTGCACG-3'
CAP1	5'-ACTGGCCTGGAGCAAAACG-3'	5'-CGGCAGAGGGTCCAGATG-3'
IL6	5'-ACCCCCAGGAGAAGATTCCA-3'	5'-GGTTGTTTTCTGCCAGTGCC-3'

PCSK9, proprotein convertase subtilisin/kexin type 9; STAT3, Signal Transducer and Activator of Transcript 3; ACTB, β-actin; LEPR, leptin receptor; CAP1, adenylyl cyclase associated protein 1; IL6, interleukin 6

Table 2. *In silico* search of STAT3 binding sites on proximal PCSK9 promoter region.

Predicted Site Sequence	***Start***	***End***	***Strand***	***JASPAR Score (%)***	***FIMO Threshold (p-value)***
CTTCTGGAAAG	-916	-906	+	95.92	2.07×10^{-5}
CTTCCAGAAAG	-1082	-1072	+	93.06	7.44×10^{-4}
CTTTCTGGAAG	-1072	-1082	-	92.42	1.01×10^{-4}
ATACTGGGAAG	-1091	-1101	-	89.88	2.93×10^{-4}
TTGCCTGTAAT	-1385	-1395	-	88.45	4.46×10^{-4}
CAGCAGGGAAA	-1485	-1475	+	87.79	4.61×10^{-4}
GTTCAAGAAAT	-950	-960	-	87.27	7.13×10^{-4}
GTGAAGGGAAA	-2184	-2174	+	87.08	6.22×10^{-4}

PCSK9, proprotein convertase subtilisin kexin type 9; STAT3, Signal Transducer and Activator of Transcript 3.

Figure 1

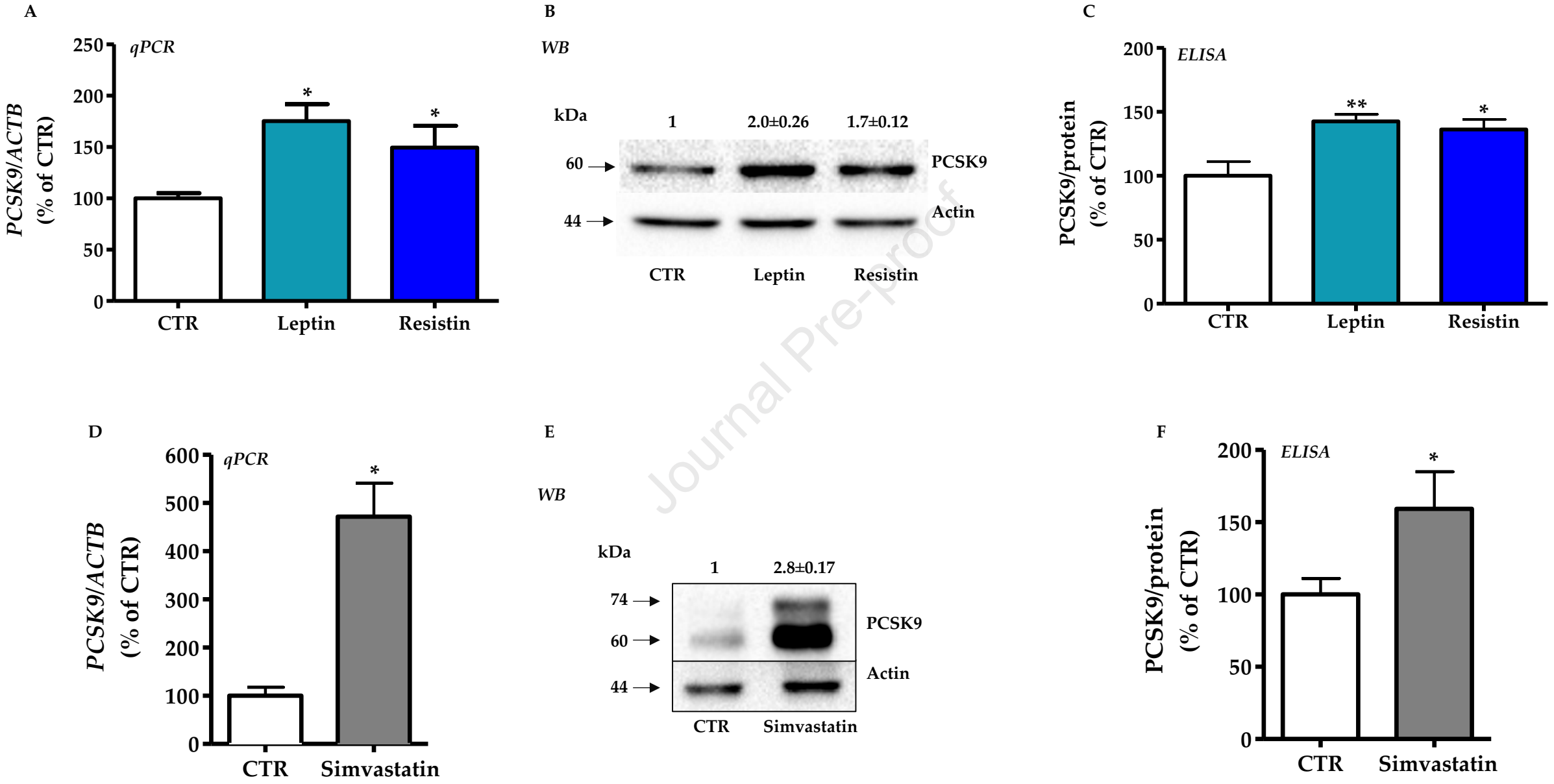


Figure 2

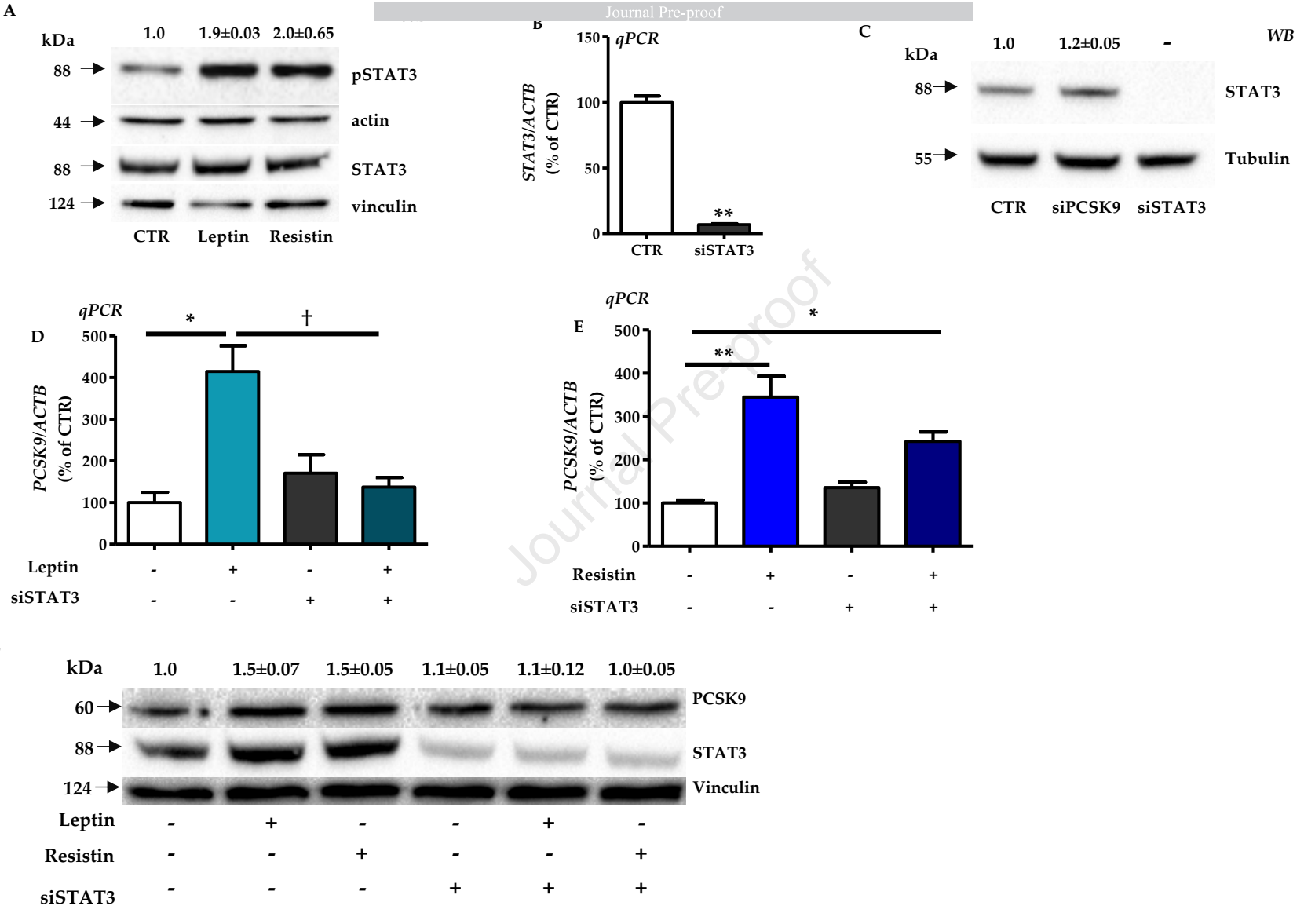
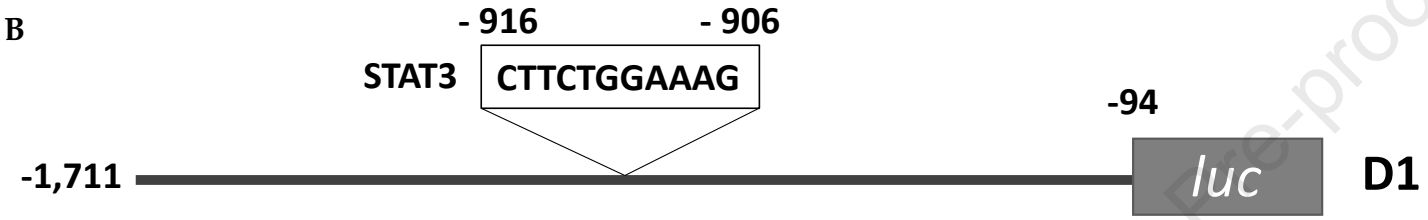


Figure 3

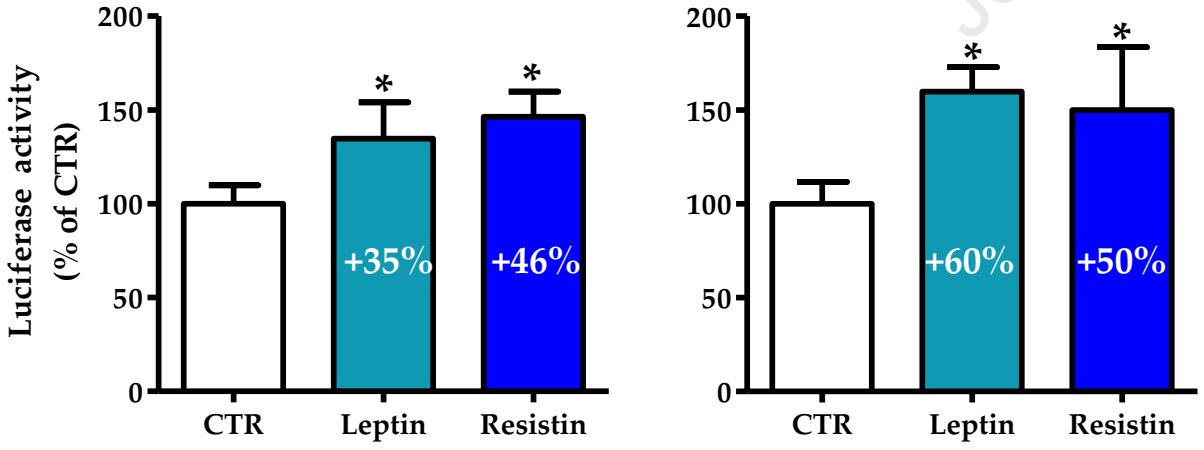
A

	(-916 to -906) STAT3		(-430 to -422) Sp1		(-386 to -374) HNF1		(-345 to -337) SRE
H. sap	AATCTTCTGGAAAG	+++CTG **467bp**	GTTTGGGGAGGGC	GAGGCCGAAACCTGATCCTCCAGTCCGGGGG	+TTCCGTTAATGTTTAATCAG	**21bp**	CTGGTGGCGTGATCTG
G. gor	AATCTTCTGGAACG	+++CTG **469bp**	GTTTGGGGAGGGC	GAGGCCGAAACCTGATCCTCCAGTCCCGGGG	+TTCCGTTAATGTTTAATCAG	**21bp**	CTGGTGGCGTGATCTG
M. mus	AAGCTGCTTGAAT	++++CTG **649bp**	GTCTGGGGAGGGC	GAGGCCGAAACCTGATCCTTTAGTACCGGGG	++CCCGTTAATGTTTAATCAG	**22bp**	GGGGTGGCGTGATCTC
R. nor	TTGCTTCTGAGCAG	AGACTG **791bp**	GTCTGGGGAGGGC	GAGGCCGAAACCCGATCCTTTAGTACCCGGGGCCCCGTTAATGTTTAATCAG		**22bp**	GGGGTGGCGTGATCTC

B



C



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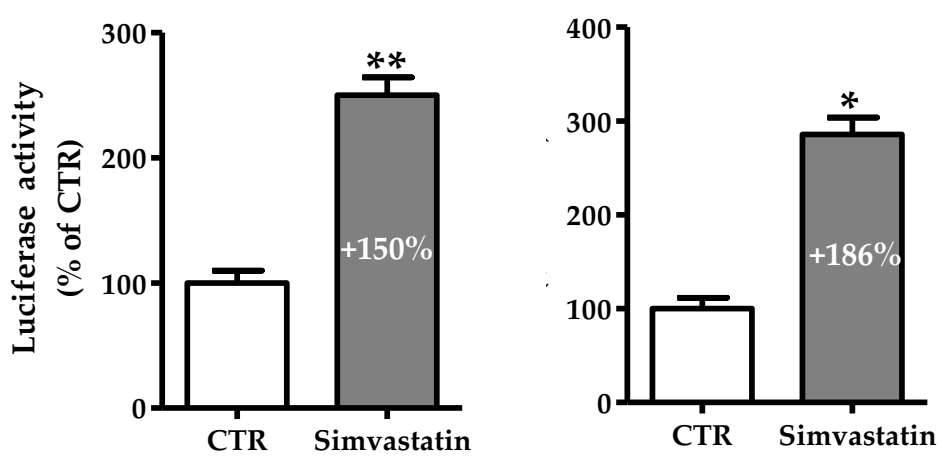

Figure 4

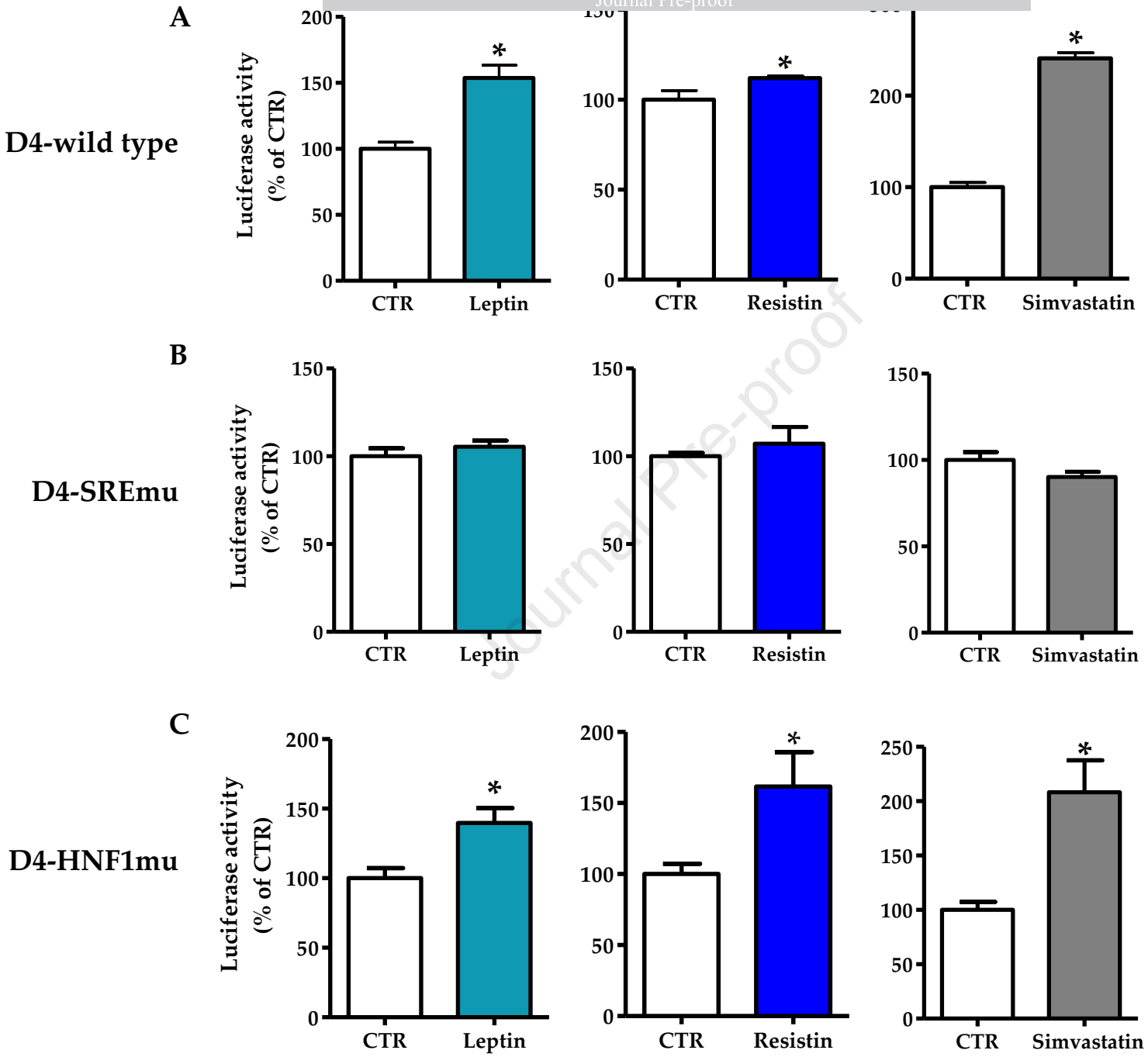

Figure 5