CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Leptin, Resistin, and Proprotein Convertase Subtilisin/Kexin Type 9

The Role of STAT3

Chiara Macchi,^{*} Maria Francesca Greco,^{*} Margherita Botta,^{*} Paola Sperandio,^{*} Paola Dongiovanni,[†] Luca Valenti,^{‡§} Arrigo F.G. Cicero,[¶] Claudio Borghi,[¶] Maria Giovanna Lupo,^{||} Stefano Romeo,^{**††‡‡} Alberto Corsini,^{*§§} Paolo Magni,^{*§§} Nicola Ferri,^{||} and Massimiliano Ruscica^{*}

From the Departments of Pharmacological and Biomolecular Sciences^{*} and Pathophysiology and Transplantation,[†] Università degli Studi di Milano, Milan, Italy; General Medicine and Metabolic Diseases[‡] and Translational Medicine, Transfusion Medicine and Hematology,[§] Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; the Department of Medicine and Surgery Sciences,[¶] Alma Mater Studiorum University of Bologna, Bologna, Italy; the Department of Pharmaceutical and Pharmacological Sciences,^{||} Università degli Studi di Padova, Padova, Italy; the Departments of Molecular and Clinical Medicine^{**} and Cardiology,^{††} Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; the Clinical Nutrition Unit,^{‡‡} Department of Medical and Surgical Science, Magna Graecia University, Catanzaro, Italy; and the IRCCS MultiMedica,^{§§} Sesto S. Giovanni, Milan, Italy

Accepted for publication
July 30, 2020.

Address correspondence to
Massimiliano Ruscica, Ph.D.,
or Chiara Macchi, Ph.D.,
Department of Pharmacological
and Biomolecular Sciences,
Università degli Studi di
Milano, Via Balzaretti 9,
20133, Milan, Italy. E-mail:
chiara.macchi@unimi.it or
massimiliano.ruscica@unimi.it.

In a condition of dysfunctional visceral fat depots, as in the case of obesity, alterations in adipokine 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 STAT3. In HepG2 cells, leptin and resistin up-regulated *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 knockdown of STAT3 did not affect the expression of leptin and resistin receptors or that of PCSK9. The analysis of the human *PCSK9* promoter region showed that the two adipokines raised PCSK9 promoter activity via the involvement of a sterol regulatory element motif. In healthy males, a positive association between circulating leptin and PCSK9 levels was found only when the body mass index was <25 kg/m². In conclusion, this study identified STAT3 as one of the molecular regulators of leptin- and resistin-mediated transcriptional induction of PCSK9. (*Am J Pathol* 2020, 190: 2226–2236; <https://doi.org/10.1016/j.ajpath.2020.07.016>)

Adipose tissue is an endocrine organ able to secrete active molecules, namely, adipokines, that contribute to regulating appetite and satiety, fat distribution, and energy expenditure.¹ When the metabolic milieu is deranged, as in the case of obesity and adipose tissue meta-inflammation, adipokines may play a role in cardiovascular (CV) disease, 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 adipokines leptin and resistin have been described as possible links between obesity and atherosclerosis.^{4,5}

Supported by Cariplo Foundation grants 2015-0552 and 2018-0511 (M.R.), a MIUR Progetto Eccellenza grant (A.C.), Ricerca Finalizzata Ministero della Salute grant RF-2016-02364358 (L.V.), a Ricerca corrente Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico grant (L.V.), and European Union (EU) Programme Horizon 2020 grant agreement 777377 for the project LITMUS "Liver Investigation: Testing Marker Utility in Steatohepatitis" (L.V.).

Disclosures: S.R. has been a consultant for, and received lecture fees and research grants from Amgen, Sanofi, and AstraZeneca; A.C. has received honoraria from AstraZeneca, Amgen, Sanofi, Recordati, Novartis, MSD, Mediolanum, DOC, Mylan, and Pfizer; L.V. has been a consultant for Gilead, Pfizer, AstraZeneca, Novo Nordisk, Intercept Pharmaceuticals, Diatech Pharmacogenetics, and IONIS.

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 CV 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-length intracellular domain that allows the activation of a cell-signaling pathway. In response to leptin, Janus tyrosine kinase 2 (JAK2) phosphorylates LepR, triggering the recruitment of 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 the STAT proteins (STAT1, -2, -3, -4, -5a, -5b, and -6), STAT3 contributes to various metabolic processes such as hyperphagia and obesity,⁸ leptin being 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, for example, the inflammatory cascade, 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 low-density lipoprotein (LDL) receptors. At the 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 and the knowledge that PCSK9 expression is also regulated by the proinflammatory cytokine tumor necrosis factor (TNF)- α , in a suppressor of cytokine signaling (SOCS) 3—dependent manner, suggest 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)/Dulbecco's modified Eagle's medium (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% 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% to 12%), bicinchoninic acid assay for determination of protein concentrations, Maxima First Strand cDNA, and Maxima SYBR Green/Fluorescein qPCR Master Mix were all from Life Technologies (Monza, Italy). Bovine Serum Albumin was from Sigma-Aldrich. Human recombinant leptin (Sigma-Aldrich) and resistin (BioVision, Milpitas, CA) were used at 100 ng/mL. Human recombinant PCSK9 was used at 2.5 μ g/mL (item 20631; Cayman Chemicals, Ann Arbor, MI).¹⁶

Transfection of siRNA

ON-TARGETplus SMART pools 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 DMEM/10% FBS at a density of 6×10^5 /well (6-well tray). Cells were then transfected with 40 nmol/L siRNA. Forty-eight hours after the transfection, the medium was replaced with DMEM/10% LPDS $\pm$ leptin (100 ng/mL) or resistin (100 ng/mL) for an additional 24 hours before performing quantitative 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 from the start codon) were retrieved from Ensembl Genome Browser¹⁷ by using the latest released genome assembly (GRCh38.p13, Kamilah_GGO_v0, GRCm38.p6, and Rnor_6.0 for human, gorilla, mouse, and rat, respectively). JASPAR (<http://jaspar.genereg.net>, last accessed July 2020), an open-access database of curated, nonredundant transcription factor binding profiles modelled as position-specific weight matrices, was used to scan the selected *PCSK9* promoter region for any STAT3 binding site.¹⁸ The relative profile score threshold—defined as the minimum

relative score required for reporting a match between the transcription factor binding model and the imputed sequence—was set at 80%. The FIMO tool, from the open-access MEME Suite 5.1.1 (<http://meme-suite.org>, last accessed July 2020), was used to double-check the results obtained from JASPAR.^{19,20} The STAT3 position-specific weight matrix profile was retrieved from JASPAR and imputed in FIMO, and the selected *PCSK9* promoter region was scanned. Matches with $P \leq 0.0001$ were taken into account. The same analyses were performed 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 –1711 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 a template and the following primer pairs: AP679 (5'-CGAC-GAGGTACCGAGCTCGGATCCACTAGTAAC-3') and AP680 (5'-ATTCAATTGCAAAGATTC-3') and generate the upstream fragment, and AP681 (5'-CAAATTGAATCT-GAGCTTGTGCCTACCATAG-3') and AP682 (5'-CCCAAGCTTACTGTGCAGGAGCTGAAGTTC-3') to generate the downstream fragment. Primer AP679 and AP682 sequences contain the KpnI and HindIII restriction sites, respectively. The upstream and downstream fragments were used as templates 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 the D1-STAT3 plasmid. The deletion of the STAT3 region from the *PCSK9* promoter in the D1-STAT3 plasmid was confirmed by DNA sequencing.

Transfections of Reporter Constructs

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 (wild-type and STAT3 mutated) and with pGL3-*PCSK9*-D4 plasmids (wild-type, SRE-mu, and HNF-1-mu), with TurboFect transfection reagent (Thermo Fisher Scientific, Waltham, MA). Forty-eight hours after the transfection, cells were incubated with DMEM/10% LPDS $\pm$ leptin (100 ng/mL), resistin (100 ng/mL), or simvastatin (20 μ mol/L) for an additional 24 hours. Luciferase activity was measured by using Neolite reagent (Perkin Elmer, Milan, Italy) according to the 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 First Strand cDNA synthesis kit (Thermo Fisher Scientific). Quantitative PCR was then performed by using the Thermo SYBR Green/ROX qPCR Master Mix kit (Thermo Fisher Scientific) and specific primers for selected genes. The analyses were performed with the 9600 Bio-Rad Real-Time PCR Detection Systems (Bio-Rad Laboratories). The primer sequences are listed in Table 1. PCR cycling conditions were as follows: 94°C for 5 minutes, 40 cycles at 94°C for 15 seconds, and 60°C for 30 seconds. Data are 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 μ L of Mammalian Protein Extraction Reagents (Thermo Fisher Scientific) containing a cocktail of protease and phosphatase inhibitors (Roche Diagnostics, Basel, Switzerland). Ten micrograms of proteins and a

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'-GGGGTGTGAAGGTCTCAAA-3'
LEPR	5'-TACTTTGGAAGCCCTGATG-3'	5'-AAGCACTGAGTGACTGCACG-3'
CAP1	5'-ACTGGCCTGGAGCAAAACG-3'	5'-CGGCAGAGGGTCCAGATG-3'
IL6	5'-ACCCCAAGGAGAAGATTCCA-3'	5'-GGTTGTTTCTGCCAGTGCC-3'

ACTB, β -actin; CAP1, adenyl cyclase associated protein 1; LEPR, leptin receptor; PCSK9, proprotein convertase subtilisin/kexin type 9.

Figure 1 HepG2 cells were seeded in DMEM/10% fetal bovine serum. Cells were incubated with leptin (100 ng/mL) or resistin (100 ng/mL) in DMEM supplemented with 10% LPDS for 24 hours. Simvastatin (20 μmol/L) has been used as a positive control. At the end of the incubation, total RNA and proteins were extracted. β-Actin was used as housekeeping for both gene (*ACTB*) and protein expression. **A** and **D**: *PCSK9* gene expression (qPCR). **B** and **E**: *PCSK9* protein expression (WB). **C** and **F**: *PCSK9* levels assayed by ELISA (data were normalized for the total amount of proteins). The values above the blots represent the densitometric readings. $n \geq 3$ independent experiments (**A–F**). Differences between groups have been assessed by *t*-test (**D** and **F**) or one-way analysis of variance (**A** and **C**). * $P < 0.05$, ** $P < 0.01$ versus control. *ACTB*, β-actin gene; CTR, control (medium alone); DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; LPDS, human lipoprotein plasma deprived serum; *PCSK9*, Proprotein convertase subtilisin/kexin type 9; qPCR, quantitative real-time PCR; WB, Western blot.

molecular mass marker (Novex Sharp Protein Standard, Invitrogen; Thermo Fisher Scientific) were separated on 4% to 12% SDS-PAGE (Novex NuPAGE 4% to 12% Bis-Tris MiniGels; Invitrogen; Thermo Fisher Scientific) under denaturing and reducing conditions. Proteins were then transferred to a nitrocellulose membrane by using the iBlot™ Gel Transfer Device (Invitrogen; Thermo Fisher Scientific). The membranes were washed with Tris-buffered saline-Tween 20, and nonspecific binding sites were blocked in Tris-buffered saline-Tween 20 containing 5% bovine serum albumin (Sigma-Aldrich) for 90 minutes at room temperature. The blots were incubated overnight at 4°C with a diluted solution (5% bovine serum albumin or nonfat dry milk) of the human primary antibodies (listed below). Membranes were washed with Tris-buffered saline-Tween 2 and then exposed for 90 minutes at room temperature to a diluted solution (5% nonfat dry milk) of the secondary antibodies (anti-mouse and anti-rabbit peroxidase-conjugated secondary antibodies; New England Biolabs, Ipswich, MA). Immunoreactive bands were detected by exposing the membranes to Clarity Western ECL chemiluminescent substrates (Bio-Rad Laboratories) for 5 minutes, and images were acquired with a ChemiDoc XRS System (Bio-Rad Laboratories). Densitometric readings were evaluated using the ImageLab software version 6.0.1 (Bio-Rad Laboratories). The dilution of the human primary antibodies were: *PCSK9* (1:1000; Genetex, Irvine, CA), *STAT3* (1:1000; Cell Signaling Technology, Danvers, MA), *pSTAT3* (1:10,000;

Abcam, Cambridge, UK), *actin* (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), *tubulin* (1:2000; Sigma-Aldrich), and *vinculin* (1:1000; 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 is 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 has been performed 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). The medium (200 μL) was collected after 24-hour treatment with leptin, resistin, and simvastatin. The samples were not

Figure 2 **A:** HepG2 cells were seeded in DMEM/10% FBS. After 48 hours, the medium was replaced with DMEM with 10% LPDS supplemented with leptin (100 ng/mL) and resistin (100 ng/mL). After 24 hours, the expression of pSTAT3^{Y705} and STAT3 was evaluated. β -Actin and vinculin were used as housekeeping proteins. **B and 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 48 hours, the expression of STAT3 was evaluated. To normalize the data, *ACTB* was used as housekeeping gene and tubulin as housekeeping protein. **D and 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. **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 WB. Vinculin was used as a housekeeping protein to normalize the data. In **A**, **C**, and **F**, the values above the blots represent the densitometric readings evaluated by using ImageLab software version 6.0.1. At least three independent experiments were conducted. Differences between groups were assessed by *t*-test (**B**) and one-way analysis of variance (**D** and **E**). * $P < 0.05$, ** $P < 0.01$. *ACTB*, β -Actin; CTR, control (medium alone); DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LPDS, human lipoprotein plasma deprived serum; PCSK9, proprotein convertase subtilisin/kexin type 9; qPCR, quantitative real-time PCR; siRNA, silencing RNA; WB, Western blot.

diluted, and results were 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 <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 means $\pm$ SD of three independent experiments. When possible, *P* values were determined by *t*-test. Otherwise, differences between treatment groups were evaluated by one-way analysis of variance. A probability value of $P < 0.05$ was considered statistically significant.

Results

Leptin and Resistin Induce PCSK9

First, 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% (Figure 1A) after 24 hours. Compared with 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 (Figure 1B). Simvastatin (20 μ mol/L)—used as a positive control—raised both *PCSK9* gene and protein expression (Figure 1, D 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 treatments, respectively (Figure 1C). Simvastatin (20 μ mol/L) was used as a positive control (Figure 1F).

STAT3 Mediates Leptin- and Resistin-Driven PCSK9 Activation

In this cell-based model, 24-hour treatment with leptin and resistin phosphorylated STAT3 (pSTAT3^{Y705}) without affecting the protein expression of total STAT3 (Figure 2A). To follow up the hypothesis that STAT3 was involved in leptin- and resistin-driven PCSK9 activation, HepG2 cells were knocked down for STAT3. Transfection with specific siRNA anti-STAT3 fully abolished the gene and protein expression of STAT3 (Figure 2, B and C) without affecting that of PCSK9 (Figure 2, D–F). Under these experimental conditions, leptin and resistin did not up-regulate PCSK9 expression (gene and protein). These data suggest that PCSK9 up-regulation by leptin and resistin in hepatocytes requires STAT3. To exclude any effect of PCSK9 on STAT3 expression, it was shown that silencing PCSK9 did not result in any change in STAT3 levels (Figure 2C).

Table 2 *In Silico* Search of STAT3 Binding Sites on the Proximal *PCSK9* Promoter Region

Predicted site sequence	Start	End	Strand	JASPAR score, %	FIMO threshold, <i>P</i> 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}
GTTCAGAAAT	−950	−960	−	87.27	7.13×10^{-4}
GTGAAGGGAAA	−2184	−2174	+	87.08	6.22×10^{-4}

PCSK9, proprotein convertase subtilisin kexin type 9.

Moreover, application of siSTAT3 did not change the gene expression of leptin (*LEPR*) and resistin (*CAPI*) 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, an *in silico* search of STAT3 binding sites on proximal *PCSK9* promoter region was performed by using JASPAR database and FIMO tool from MEME Suite 5.1.1. The analyses revealed a strong

STAT3 binding site (JASPAR score: 96%; FIMO threshold: $P = 2.07 \times 10^{-5}$) spanning the region −916 to −906 in the human *PCSK9* promoter (Table 2). This region is upstream from the Sp1/HNF1- α /SRE triad already published by Li et al²¹ (Figure 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 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Figure 3A). The mild degeneration in the STAT3 consensus sequence has been already reported in the literature.²⁵ After transfection with

Figure 3 **A:** Sequence comparison of proximal regions of PCSK9 promoter of human, gorilla, mouse, and rat species. **B:** Schematic representation of the human PCSK9 promoter-luciferase reporter plasmid D1. Positions refer to the nucleotide preceding the ATG start codon of the human *PCSK9* gene, arbitrarily designated as −1. Positions −94 and −1711 indicate the 3' and the 5' ends of the PCSK9 promoter insert, respectively. Positions of the 5' and 3' ends of the STAT3 binding sequence are indicated. **C** and **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 Dulbecco's modified Eagle's medium containing 10% LPDS supplemented with leptin (100 ng/mL), resistin (100 ng/mL), or simvastatin (20 μ M/L). After an additional 24 hours, luciferase activities were determined by Neolite reagent. Differences between groups were assessed by *t*-test (**D**) or one-way analysis of variance (**C**). Numbers inside the bars represent the percentage of increment. Nucleotides in red refer to the STAT3 sequence in the PCSK9 promoter region; nucleotides in blue refer to the Sp1 sequence in the PCSK9 promoter region; nucleotides in green refer to the HNF-1 sequence in the PCSK9 promoter region; nucleotides in purple refer to the SRE sequence in the PCSK9 promoter region (**A**); double asterisks in **A** refer to bp spanning between the interregion sequences of the PCSK9 promoter region. $n \geq 3$ independent experiments (**C** and **D**). * $P < 0.05$, ** $P < 0.01$ versus control. CTR, control (medium alone); HNF1, hepatocyte nuclear factor-1; LPDS, human lipoprotein plasma deprived serum; PCSK9, proprotein convertase subtilisin/kexin type 9; Sp1, proximal specificity protein 1; SRE, sterol regulatory element.

Figure 4 HepG2 cells were transfected with pGL3-PCSK9-D4 (A), pGL3-PCSK9-SREmut (B), and pGL3-PCSK9-HNF1mut (C). The day after the transfection, the medium was replaced with Dulbecco's modified Eagle's medium containing 10% LPDS supplemented with leptin (100 ng/mL), resistin (100 ng/mL), or simvastatin (20 μ mol/L). After an additional 24 hours, luciferase activities are determined by Neolite reagent. At least three independent experiments were conducted. * $P < 0.05$ versus control (t -test). CTR, control (medium alone). LPDS, human lipoprotein plasma deprived serum; HNF1, hepatocyte nuclear factor-1; PCSK9, proprotein convertase subtilisin/kexin type 9; SRE, sterol regulatory element.

D1 and D1-STAT3-mut plasmids (Figure 3B), both containing the human *PCSK9* promoter region spanning -1711 to -94 nucleotides upstream of the gene coding for the luciferase enzyme, HepG2 cells were treated with leptin, resistin, and simvastatin. A twenty-four-hour treatment enhanced transcriptional activity, an effect that was not counteracted when STAT3 mutation was inserted (Figure 3C). Simvastatin was used as a positive control (Figure 3D). These data suggest that *PCSK9* up-regulation 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 up-regulate *PCSK9* transcript levels, HepG2 cells were transiently transfected with constructs containing the proximal human *PCSK9* promoter region (-440 to -94). Leptin and resistin significantly incremented the luciferase activity by 54% and 15%, respectively (Figure 4A). Similar results were obtained upon treatment with simvastatin (+141%). Because SREBPs and HNF-1 are the major transcription factors regulating *PCSK9*,²⁶ luciferase activity was investigated upon the insertion of SRE and HNF-1 mutations in the *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 (Figure 4B). Conversely, mutation in HNF-1 did not alter leptin- and resistin-driven luciferase activities, showing an increment of 43% and 58%, respectively (Figure 4C).

Association Studies

The circulating levels of leptin and *PCSK9* were measured by ELISA in a clinical setting of 149 males (56 ± 4 years of age) free of CV disease at enrollment and belonging to the cohort of Brisighella Heart Study.²² A positive association between circulating leptin and *PCSK9* levels ($\beta = 0.352$; $P = 0.014$) was found only in subjects with a body mass index <25 kg/m² (Figure 5A). When the body mass index rose, that is, between 25 and 30 kg/m² ($\beta = 0.147$; $P = 0.295$) (Figure 5B) or >30 kg/m² ($\beta = -0.030$; $P = 0.840$) (Figure 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 Supplemental Table S1.

Discussion

Obesity and associated metabolic disorders are becoming major health care concerns worldwide. Obesity is highly

Figure 5 Plasma levels of leptin and PCSK9 are measured by commercial ELISA. Subjects are stratified according to BMI, that is, <25 kg/m² (**A**), between 25 and 30 kg/m² (**B**) and >30 kg/m² (**C**). *n* = 48 subjects (**A** and **C**); *n* = 53 subjects (**B**). BMI, body mass index; ELISA, enzyme-linked immunosorbent assay; PCSK9, proprotein convertase subtilisin/kexin type 9.

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 CV processes.²⁷ Among adipokines, the expression of LDL receptors 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 PPAR γ 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 with 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 few years, leading to the hypothesis that it represents a fed-forward loop in which inflammation activates PCSK9, and vice versa, PCSK9 impacts the inflammatory burden associated with 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 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- κ B 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 shown that the impairment of oncostatin M signaling, a secreted cytokine mainly involved in chronic inflammatory and CV 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, it has been 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) epidemiologic 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-cholesterol, confirming data in PCSK9 knockout mice that were partially protected from neointimal formation.⁴⁰ In humans, PCSK9 has been also associated with other components of atheroma formation, that is, platelet aggregation,¹⁶ hypertension, and inflammation.³⁰

It has been 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 the STAT3 pathway at the transcriptional level was negligible. However, this latter analysis requires further investigation because the promoter region of human *PCSK9* contains other STAT3 responsible elements (Table 2). Because *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, a *PCSK9* promoter luciferase reporter plasmid containing mutations for both SRE and HNF responsive elements was used. It was found that exposure of HepG2 hepatocytes to leptin and resistin increases the transcriptional activity of the *PCSK9* promoter. Furthermore, although a construct variant containing a mutation in the HNF-1 motif did not affect the ability of adipokines to up-regulate luciferase activity, no induction was detected after introducing a mutation in the SRE motif. Relative to this latter evidence, the current study data are in line with findings demonstrating that, at the transcriptional level, *PCSK9* synthesis is largely controlled by SREBPs.⁴² These 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, it was found that this exists only in the group with a body mass index $<25 \text{ kg/m}^2$, an evidence in line with that previously described by Kwakernaak et al⁴⁴ 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, this study's data obtained in healthy male subjects did not fit with a previous report by Li et al,⁵¹ who found that in patients with CV artery disease resistin positively correlate with *PCSK9* levels. Some of these discrepancies may also rely on the tight liaison surrounding resistin and *PCSK9*, that is, 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 LDL receptor degradation.⁵² On this matter, the authors have previously demonstrated that *PCSK9* induces a

proinflammatory response in macrophages,⁵³ an effect common to resistin, that is, a rise in IL6, TNF- α , and 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, the study provides evidence that *PCSK9* may be one of the players at the crossroad of the interplay between obesity, adipose tissue dysfunction, and CV disease risk. Such effect appears to be dependent on the inflammatory JAK/STAT pathway.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.07.016>.

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