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PCSK9 and inflammation: in vitro study on hepatocytes and macrophages

Supervisore: **Chiar.mo Prof Alberto CORSINI**

Coordinatore: **Chiar.mo Prof. Alberico L. CATAPANO**

Chiara RICCI

Matr. N. R11011

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ABSTRACT

English version

The present study aims to investigate two different hypothesis:

1) TNF-α induces proprotein convertase subtilisin kexin type 9 (PCSK9) expression in hepatic HepG2 cell line, through the activation of suppressor of cytokine signaling 3 (SOCS3)

Background. The suppressor of cytokine signaling (SOCS) proteins are negative regulators of the JAK/STAT pathway activated by pro-inflammatory cytokines, including the tumor necrosis factor-α (TNF-α). SOCS3 is also implicated in hypertriglyceridemia associated to insulin-resistance (IR). Proprotein Convertase Subtilisin Kexin Type 9 (PCSK9) levels are frequently found to be positively correlated to IR and plasma very low-density lipoprotein-triglycerides (VLDL-TG) concentrations. Aim. The present study aimed to investigate the possible role of TNF-α and JAK/STAT pathway on de novo lipogenesis and PCSK9 expression in HepG2 cells. Methods and results. TNF-α induced both SOCS3 and PCSK9 in a concentration-dependent manner. This effect was inhibited by transfection with siRNA anti-STAT3, suggesting the involvement of the JAK/STAT pathway. Retroviral overexpression of SOCS3 in HepG2 cells ($HepG2^{SOCS3}$) strongly inhibited STAT3 phosphorylation and induced PCSK9 mRNA and protein levels, with no effect on its promoter activity. Consistently, siRNA anti-SOCS3 reduced PCSK9 mRNA levels while an opposite effect was observed with siRNA anti-STAT3. In addition, $HepG2^{SOCS3}$ express higher mRNA levels of key enzymes involved in the de novo lipogenesis, such as fatty-acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD-1), and apo-B. These responses were associated with significant increase of SCD-1 protein, activation of SREBP-1, accumulation of cellular TG and secretion of apoB. $HepG2^{SOCS3}$ show lower phosphorylation levels of IRS-1 Tyr896 and Akt Ser473 in response to insulin. Finally, insulin stimulation produced an additive effect with SOCS3 overexpression, further inducing PCSK9, SREBP-1, FAS and apoB mRNA. Conclusions. Our data candidate PCSK9 as a gene involved in lipid metabolism regulated by pro-inflammatory cytokine TNF-α, in a SOCS3 dependent manner.

2) Proprotein subtilisin/kexin type 9 (PCSK9) induces pro-inflammatory response in macrophages

Background. Intraplaque release of inflammatory cytokines and chemokines from macrophages is directly implicated in atherogenesis, by inducing the proliferation and migration of media

smooth muscle cells (SMCs) to the neointima. PCSK9 is present and released by SMCs within the atherosclerotic plaque but its role within the vascular wall is still unknown. Aim. In the present study, we tested the hypothesis of a pro-inflammatory effect of PCSK9 on macrophages. Methods and results. The pro-inflammatory effect of PCSK9 was assessed on THP-1-derived macrophages, exposed to different concentrations (0.250 ÷ 2.5 μg/ml) of human recombinant PCSK9 (hPCSK9). After exposure for 24h to 2.5 μg/ml PCSK9, a significant induction of IL-1β (8.17±2.88 fold), IL-6 (36.4±19.3 fold), TNF-α (67.4±25.9 fold), CXCL2 (42.6±0.0 fold), and MCP1 (17.0±6.8 fold), were observed. Importantly, physiological concentration of PCSK9 (0.250 μg/ml) also elicited a significant pro-inflammatory effect. Similar results were observed in human primary macrophages, where 2.5 μg/ml of hPCSK9 increased IL-1β (14.61±2.47 fold), IL-6 (4.17±0.86 fold), TNF-α (4.51±2.08 fold), CXCL2 (2.58±0.26 fold), and MCP-1 (1.76±0.07 fold) gene expression and the released of TNF-α (+82.3%) and IL-6 (+41.8%) in cultured media, as determined by ELISA assay. Co-culture experiments of HepG2 overexpressing hPCSK9 and THP1 macrophages also showed the induction of mRNA TNF-α (1.89±0.35 fold), IL-1β (2.03±0.29 fold), MCP-1 (4.82±1.26 fold) and CXCL2 (5.40±0.61 fold). Finally, the effect of hPCSK9 on TNF-α mRNA in murine LDLR-/- bone marrow macrophages (BMM) was significantly impaired as compared to wild-type BMM (5.44±0.28 fold vs 35.4±2.7 fold for LDLR-/- and wild-type, respectively). Conclusions. The present study provided evidences of a pro-inflammatory action of PCSK9 on macrophages, mainly dependent by the LDLR. The pathophysiological relevance of this effect still needs to be determined.

Italian version:

Il presente studio ha come scopo quello di verificare due diverse ipotesi:

1) Il trattamento con TNF-α induce l'espressione della proproteina convertase subtilisina kexina di tipo 9 (PCSK9) nella linea cellulare epatica HepG2, attraverso l'attivazione del soppressore del segnale delle citochine 3 (SOCS3)

Introduzione. I soppressori del segnale delle citochine (SOCSs) sono proteine in grado di regolare negativamente la via del segnale JAK/STAT, a sua volta attivata dale citochine proinfiammatorie, tra cui il tumor necrosis factor-α (TNF-α). SOCS3, in particolare, risulta coinvolta nell'ipertrigliceridemia associata ad insulino resistenza (IR). Elevati livelli della Proproteina Convertasi Subtilisina Kexina di tipo 9 (PCSK9) sono stati frequentemente associati ad insulino resistenza e ad un'elevata concentrazione plasmatica di trigliceridi VLDL. Scopo: nel

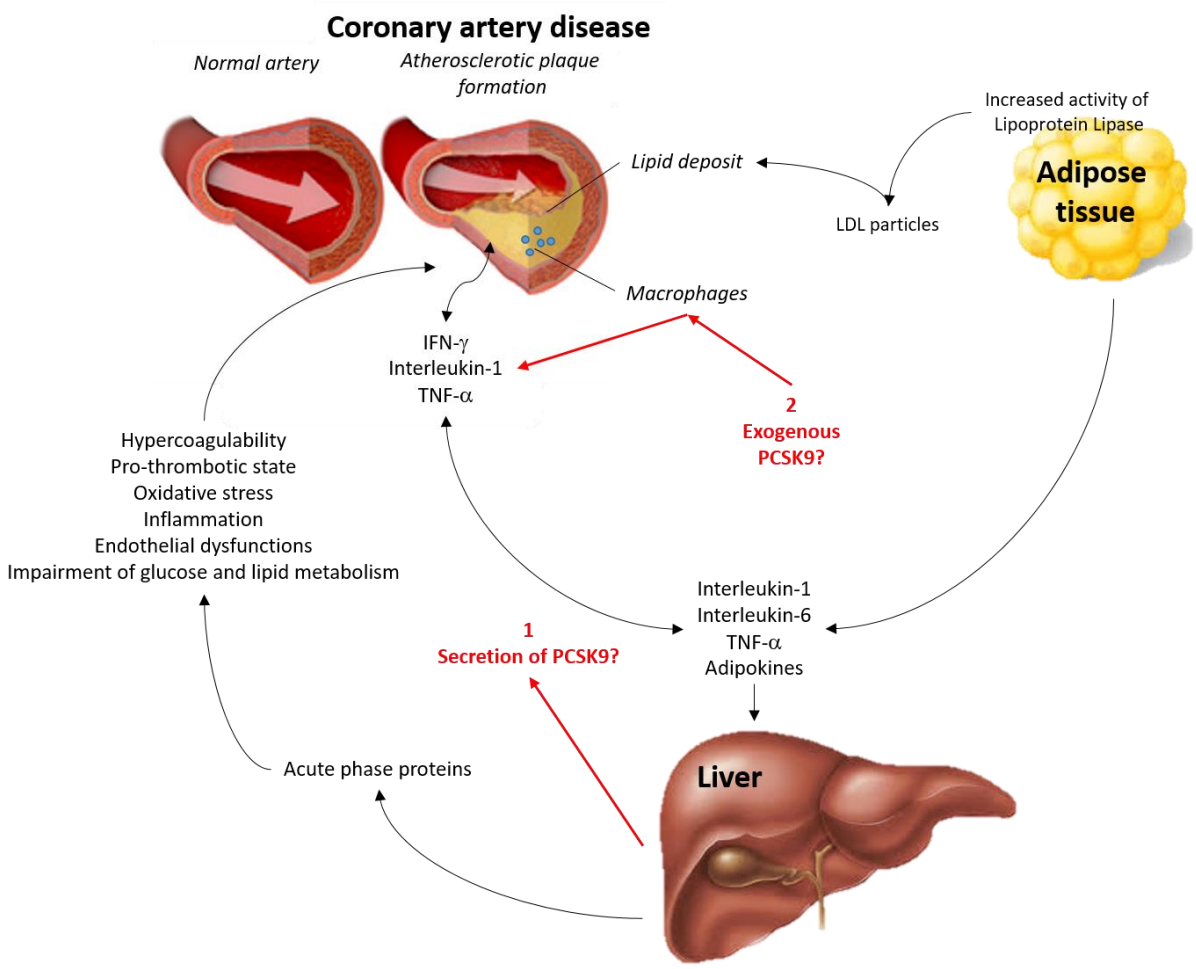
presente studio è stato valutato il possibile ruolo del TNF-α e della via del segnale JAK/STAT sulla de novo lipogenesi e sull'espressione di PCSK9, in cellule di epatocaricinoma umano HepG2. Metodi e risultati. Il trattamento con TNF-α ha provocato un'induzione sia di SOCS3 che di PCSK9 in modo concentrazione-dipendente. Tale effetto risulta inibito in seguito a trasfezione con siRNA anti-STAT3, suggerendo il coinvolgimento della via del segnale JAK/STAT. La sovraespressione retrovirale di SOCS3 nelle cellule HepG2 ($HepG2^{SOCS3}$) ha causato, come atteso, una forte inibizione della fosforilazione di STAT3 ed ha indotto l'espressione genica e proteica di PCSK9, senza alcun effetto sull'attività del suo promotore. Al contrario, la trasfezione con un siRNA anti-SOCS3 ha ridotto i livelli di RNA messaggero di PCSK9. Inoltre, le cellule $HepG2^{SOCS3}$ presentano livelli più elevati dei principali enzimi coinvolti nella de novo lipogenesi, quali sintasi degli acidi grassi (FAS), stearoil-CoA desaturasi 1 (SCD-1) ed apo-B. Queste risposte risultano associate ad un significativo aumento anche dell'espressione proteica di SCD-1, nonché all'attivazione di SREBP-1, all'accumulo di trigliceridi all'interno della cellula ed alla secrezione di apoB. Le $HepG2^{SOCS3}$ mostrano una minor fosforilazione del residuo tirosinico 896 di IRS-1 e del residuo serinico 473 di AKT, in risposta all'insulina. Infine, lo stimolo con insulina produce un effetto additivo alla sovraespressione di SOCS3, inducendo ulteriormente l'espressione genica di PCSK9, SREBP-1, FAS e apoB. Conclusioni. Tali evidenze candidano PCSK9 come gene coinvolto nel metabolism lipidico regolato dalla citochina proinfiammatoria TNF-α, in maniera SOCS3 dipendente.

2) La proproteina subtilisina/kexina di tipo 9 (PCSK9) induce una risposta pro-infiammatoria nei macrofagi

Introduzione. Il rilascio di citochine e chemochine infiammatorie all'interno della placca aterosclerotica da parte dei macrofagi è direttamente implicata nell'aterogenesi, inducendo proliferazione e migrazione delle cellule muscolari lisce della tonaca media verso la neointima. PCSK9 viene secreto dalle cellule muscolari lisce all'interno della placca ma il suo ruolo sulle pareti del vaso è ancora sconosciuto. Scopo. Nel presente studio, abbiamo testato l'ipotesi di un possibile effetto pro-infiammatorio di PCSK9 sui macrofagi. Metodi e risultati. L'effetto pro-infoammatorio di PCSK9 è stato testato in vitro su macrofagi THP-1-derivati, che sono stati esposti a diverse concentrazioni (0.250 ÷ 2.5 μg/ml) di PCSK9 umana ricombinante (hPCSK9). Dopo un'esposizione di 24h alla concentrazione più elevata (2.5 μg/ml) di hPCSK9, è stata osservata un'induzione significativa dell'espressione genica di IL-1β (8.17±2.88 volte), IL-6 (36.4±19.3 volte), TNF-α (67.4±25.9 volte), CXCL2 (42.6±0.0 volte) ed MCP1 (17.0±6.8 volte). Un'osservazione molto importante riguarda anche la concentrazione più fisiologica di PCSK9 (0.250 μg/ml), in quanto anch'essa ha presentato un'attività pro-infiammatoria significativa. Simili risultati sono stati osservati anche in cellule macrofagiche umane primarie, dove la concentrazione 2.5 μg/ml di hPCSK9 ha portato ad un aumento dell'espressione genica di IL-1β (14.61±2.47 volte), IL-6 (4.17±0.86 volte), TNF-α (4.51±2.08 volte), CXCL2 (2.58±0.26 volte) ed

MCP-1 (1.76±0.07 volte), oltre ad un aumentato rilascio di TNF-α (+82.3%) ed IL-6 (+41.8%) nel medium di coltura, come determinato attraverso saggio ELISA. Ponendo i macrofagi THP-1-derivati in co-coltura con cellule HepG2 sovraesprimenti PCSK9, si è osservata, ancora una volta, un'induzione dell'espressione genica di TNF-α (1.89±0.35 volte), IL-1β (2.03±0.29 volte), MCP-1 (4.82±1.26 volte) e CXCL2 (5.40±0.61 volte). Infine, l'effetto di hPCSK9 sull'espressione genica di TNF-α in macrofagi di midollo osseo di topi LDL-/- è risultata significativamente ridotta se comparata a quella di macrofagi derivanti da topi wild-type (5.44±0.28 volte vs 35.4±2.7 volte per i macrofagi di topi LDLR-/- e wild-type, rispettivamente). Conclusioni. Il presente studio fornisce evidenze di un'azione pro-infiammatoria di PCSK9 sui macrofagi, principalmente dipendente dalla presenza del LDLR. La rilevanza fisiopatologica di questo effetto dovrà essere ulteriormente chiarita.

Abstract at a glance:



Modified from: The new england journal of medicine "Mechanisms of disease Inflammation, Atherosclerosis, and Coronary Artery Disease" Göran K. et al.

TNF-α INDUCES PROPROTEIN CONVERTASE SUBTILISIN KEXIN TYPE 9 (PCSK9) EXPRESSION IN HEPATIC HEPG2 CELL LINE, THROUGH THE ACTIVATION OF SUPPRESSOR OF CYTOKINE SIGNALING 3 (SOCS3)

INTRODUCTION (I)

Role of inflammation in atherosclerosis and metabolic disorders:

Development of atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the vascular wall and it is the first cause of death in the Western World. The atherogenesis consists in a slow multifactorial process that results in development of atheromatous plaques within the arterial wall. The trigger factor is represented by a modification of cholesterol-containing low density lipoproteins (LDL) within the tunica intima. In particular, LDL particles undergo biochemical modification, like enzymatic attack or oxidation, with a consequent release of phospholipids, which promote a change in endothelial permeability and extracellular matrix composition beneath endothelium, leading to an increased penetration and retention of LDL particles in the artery wall [1,2]. Endothelial cells increase their adhesion molecules, like vascular-cell adhesion molecule-1 (VCAM-1)[3], and inflammatory genes expression, while modified LDL particles become able to induce leukocytes adhesion and macrophages endocytosis, causing intracellular cholesterol accumulation. Leukocytes migrate inside the tunica intima, the innermost layer of artery, following the chemoattractant mediators action. Once inside the arterial wall, monocytes (the most present white blood cells in the plaque) differentiate into macrophages, that phagocytes oxidized LDL, leading to the formation of foam cells, term given by microscope evidences of lipid-laden cells. Plaque macrophages also exert other inflammatory activities, characteristic of M1 macrophages, by producing high amount of pro-inflammatory mediators, like interleukin-1β (IL-1β) and tumor-necrosis factor-α (TNF-α). Atherosclerotic plaque formation also involves smooth muscle cells (SMCs), recruited from the tunica media, the middle layer of arterial wall. SMCs migrate to the tunica intima and proliferate in response to diverse stimuli, producing also elastin and collagen, the main components of extracellular matrix, and forming the fibrous cap that covers the plaque. Under the fibrous cap, lie macrophages-derived foam cells. Some of them are apoptotic cells that release their lipids, causing an extracellular accumulation. The impairment of efferocytosis, the clearance of dead cells, promotes accumulation of lipids and debris, forming the lipid-rich necrotic core of the plaque [4]. The first clinical evidences of the presence of an atherosclerotic plaque are tissue ischemia, caused by flow-limiting stenosis, and detachment of thrombi after a physical disruption of the most thin, collagen-poor fibrous cap.

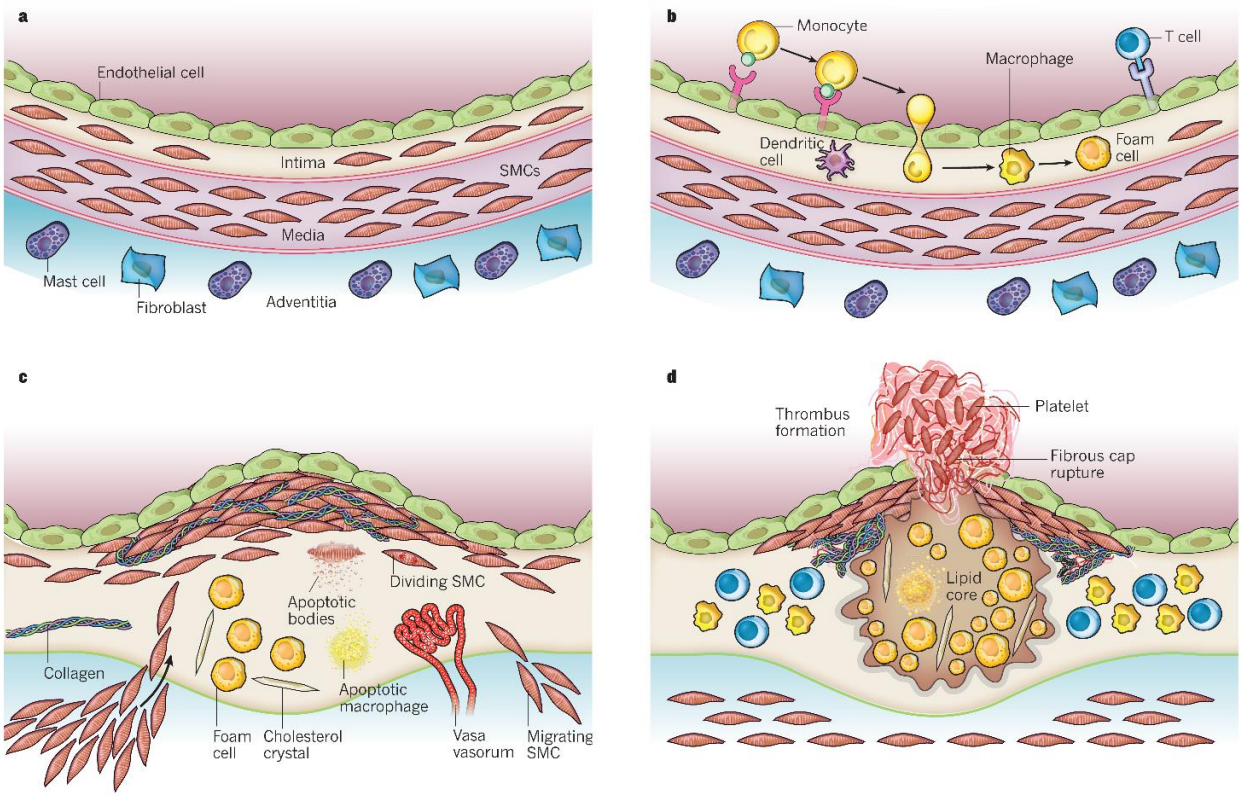


Figure A: Stages of atherosclerotic lesion development. a, the artery is composed by three layers. The inner one, called tunica intima, is lined by a monolayer of endothelial cells, in contact with blood, and contains resident SMCs. The middle one, or tunica media, is composed by a complex extracellular matrix of SMCs. The outer one, tunica adventitia, contains mast cells, nerve endings and microvessels. Physiologically, arteries are elastic, with demarcated laminae. b, the first atherosclerosis steps consist in blood leukocytes (mainly monocytes) adhesion to the activated endothelium and migration into the intima. Then occurs the maturation of monocytes in macrophages and their consequent accumulation of lipids, with the formation of foam cells. c, with the progression of the lesion, SMCs migrates from the tunica media from the intima, where they proliferate together with resident SMCs. Heightened synthesis of extracellular matrix components (collagen, elastin and proteoglycans) improves macrophages and SMCs death and consequent accumulation, forming the lipid core, or necrotic core. Cholesterol crystals and microvessels are typical of advanced plaques. d, the ultimate complication of atherosclerotic plaque is represented by thrombosis, a physical disruption of the fibrotic core of the plaque. Blood coagulation component come into contact with tissue factors inside the plaque, extending the thrombosis into the vessel lumen and blocking blood flow [5].

Future perspectives on atherosclerosis treatment: focus on inflammation

During the last ten-twelve years, the evidences of the role of inflammation in atherosclerosis development and progression have grown and expanded. Many basic and clinical studies have underlined cellular and molecular mechanisms involved in the development of atherosclerosis [6]. Several cell types derived from monocytes/macrophages, as several chemokines, cytokines and adhesion molecules, are implicated in atherogenesis, leading to the conclusion that atherosclerosis is not only a disorder caused by lipid accumulation, but it also has major inflammatory influences, that interact with atherogenic lipoproteins to accelerate disease progression, leading to plaque rupture and clinical events [5]. In several animal models of atherosclerosis, indeed, inflammation evolves together with lipid accumulation in the artery wall. It is well known that modified lipids (like lipid hydroperoxides, lysophospholipids, carbonyl compounds and other active moieties localized in the lipid fraction of atheroma) can induce the expression of adhesion molecules, chemokines, proinflammatory cytokines and other mediators of inflammation in macrophages and vascular wall[7-9]. Elevated values of circulating inflammatory markers usually accompany acute coronary syndrome and correlate with adverse prognosis and hospitalization, due to myocardial necrosis, ischemia-reperfusion damage, severe coronary atherosclerosis and primary inflammation[10-17]. Inflammation not only has a pivotal role in the acute coronary syndrome, but also contributes to the development of atherosclerotic disease already in the earliest steps. Understanding that atherosclerosis is a disorder characterized by a low-grade vascular inflammation, can help us to predict future cardiovascular risk. Indeed, in large scale, population-based studies, it is shown that elevated levels of inflammatory mediators in apparently healthy people can have a predictive value for future vascular events. In particular, increased basal levels of proinflammatory cytokines, like IL-6 and TNF-α, are associated with an increased cardiovascular risk[18-21]. Within the CANTOS Clinical Trial, a randomized, double-blind trial promoted by Novartis, it has been tested the efficacy of canakinumab, a monoclonal antibody targeting interleukin-1β, in the reduction of cardiovascular disease. Canakinumab, without affecting lipid levels, at a dose of 150 mg every 3 months, led to a significantly lower rate of recurrent cardiovascular events, compared with placebo. This evidence demonstrates that reducing inflammation without affecting lipids levels, can reduce cardiovascular disease risk [22]. Statins, pharmacological inhibitors of hydroxymethyl glutaryl coenzyme A reductase (HMG-CoA reductase), regulate LDL cholesterol pathway and are

still the first choice in the hypocholesterolemic therapy [23]. They are characterized by a dual action: the decrease in LDL cholesterol levels is indeed accompanied with the inhibition of C-reactive protein in human, and in large studies of these drugs it appears a clinical benefit due to an anti-inflammatory action, distinct from LDL cholesterol lowering [24,25]. Despite the efficacy of statins, it remains a certain percentage of cardiovascular risk even in patients treated with them. It is estimated that the 20% of patients treated with the higher dose of statin will get into recurrent cardiovascular event within 2 or 3 years after an acute coronary syndrome[26,27]. For this reason, new pharmacological target are emerging, considering not only cholesterol pathways, but also inflammatory pathways.

Obesity and low-grade subclinical inflammation

Obesity is commonly associated with severe medical complications, including type 2 diabetes, insulin resistance, peripheral and coronary artery disease, stroke and heart failure. Complications of obesity are due to many events, such as vascular defects, endothelial dysfunctions, arterial stiffening and dysregulated adipose tissue signaling. Overnutrition leads to an increase in adipocytes number and size, resulting in adipose tissue expansion and macrophages infiltration. The presence of obese adipose tissue is frequently associated with a low-grade chronic subclinical inflammatory state [28], characterized by a persistent release of pro-inflammatory cytokines, able to interact with inflammatory and immune pathways and to directly contribute to vascular dysfunction, through the activation of endothelium. In a condition of obesity, adipose tissue can increase the secretion of pro-inflammatory cytokines (TNF-α and IL-6) by resident macrophages and, in addition, the secretion of hormones and chemokines is improved. All those factors are involved in energetic homeostasis and are strongly affected by a condition of metabolic dysregulation[29]. Between 1994 and 1995 additional secreted factors, termed adipokines, were identified in adipocytes[30,31]. Adipokines, such as leptin, adiponectin, retinol binding protein 4 (RBP4) and resistin, interact with both central and peripheral tissues, in particular pancreas, hypothalamus, skeletal muscles, blood vessels, brain and liver, where they affect carbohydrate and lipid metabolism, inflammation, coagulation and blood pressure[32-34]. The dysregulation of adipokines secretion due to the expansion of adipose tissue in obesity is crucial in the development of metabolic diseases such as metabolic syndrome, insulin resistance, type 2 diabetes, vascular and heart diseases.

Focusing on the liver, it has been observed that pro-inflammatory adipokines, released by adipose tissue, can promote the hepatic synthesis of acute phase proteins, leading to hypercoagulability, pro-thrombotic state, oxidative stress, inflammation, endothelial dysfunction and impairment of glucose and lipid metabolism[35].

One of the mechanism behind adipokines activities, and in particular leptin activity, involves the JAK/STAT inflammatory pathway [36,37].

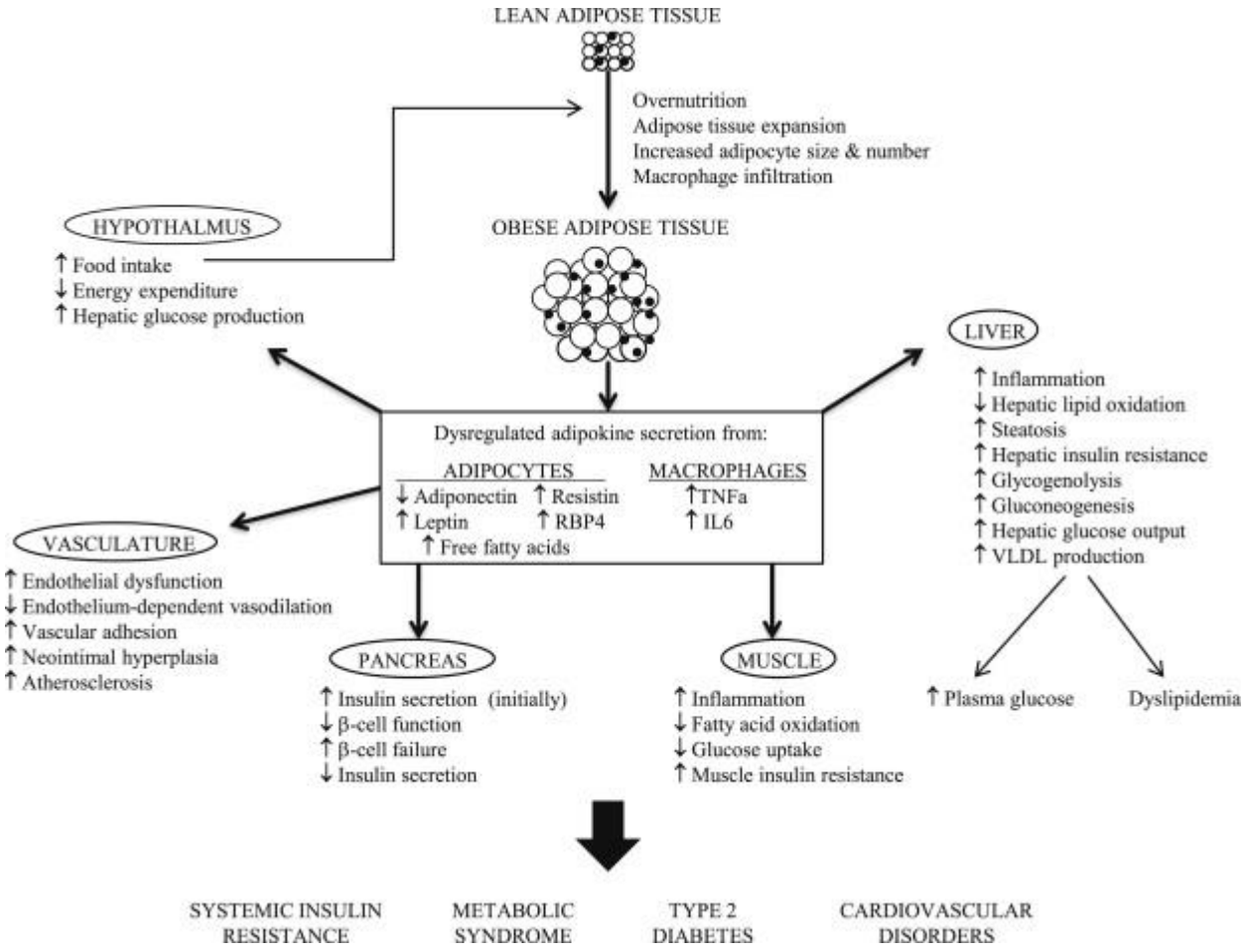


FIGURE B: Effects of obesity-induced changes in adipokines secretion that lead to the development of systemic insulin resistance, metabolic syndrome, type 2 diabetes, and cardiovascular disorders. H.J. Harwood Jr. / Neuropharmacology 63 (2012) 57-75

JAK/STAT pathway: role on inflammation

Signal transducer and activator of transcription (STAT) proteins:

STAT proteins are involved in signal transduction and transcriptional activation and seven members compose the STAT family: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. They present a common structure of five domains: the N-terminal domain, the supercoiled domain, the DNA-binding domain, the sec-homology-2 (SH2) domain and the C-terminal domain. The last one is, involved in transactivation and it contains a tyrosine residue, which promote the dimerization, and a serine residue, that, after phosphorylation, is involved in transcriptional activation[38-40]. STATs are cytoplasmic transcriptional factors, able to mediate cellular response to cytokines, adipokines, growth factors and hormones[38-40], after the interaction with the respective receptor[41].

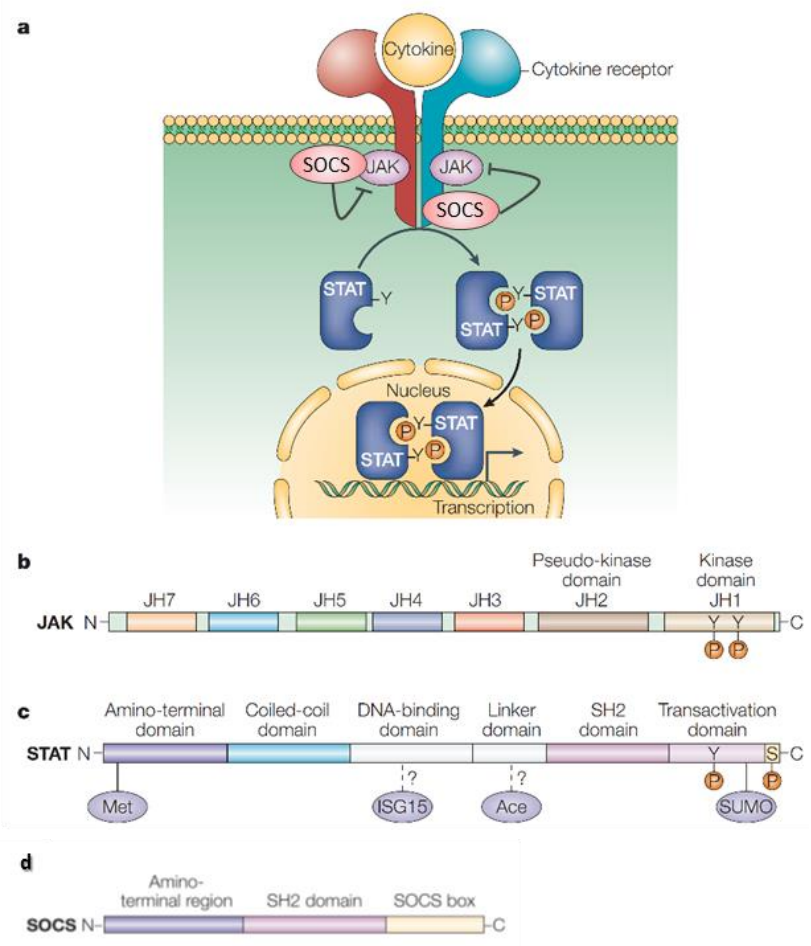


Figure C: The JAK/STAT pathway: a. Schematic representation of the JAK/STAT pathway. b. JAKs domain structure: JH1-7 domains present structural similarity in all the JAKs family, composed

by JAK1-3 and Tyrosine kinase 2 (TYK2); JH1, the kinase domain, contains two tyrosines that undergo phosphorylation after ligand binding to the receptor, while JH6 and 7 mediate JAKs-receptors binding. c. STATs domain structure: the different modification that can occur on STATs structure can modulate these proteins activity. These modifications include tyrosine and serine phosphorylation, methylation (Met), ISGylation (ISG15), acetylation (Ace) and sumoylation (SUMO). d. Domain structure of SOCS family proteins: the SOCS box can bind to elongins B and C, known components of ubiquitin E3 ligase complex[42,43].
Modified from: Ke Shuai and Bin Liu. Nature [44]

The ligand interacts with its receptor on cell surface, causing its dimerization and the consequent activation of the associated JAK tyrosine kinases. The activated JAKs acts through the phosphorylation of specific receptor tyrosine residues, situated on the cytoplasmic domain of the receptor, which become suitable binding site for SH2-containing signaling proteins, like STATs. Once STATs protein interacts with phosphorylated tyrosine residues, in turn, they are phosphorylated by JAKs protein and undergo dimerization, leaving the cytokine receptor and translocating towards the nucleus, where they activate gene transcription, through the activation of the transcriptional factors sterol regulatory element binding proteins (SREBPs)[39,45]. STAT proteins promote fundamental cellular processes, like growth, differentiation, development, apoptosis, immunity response and inflammation [46,47]. In particular, STAT3 represents an important member of the family, since its aberrant expression is linked to cancer formation, transformation and progression. Dysregulated activation of STAT3 is very common in breast cancer, melanoma, prostatic cancer, myeloma, pancreatic, ovarian and cerebral cancers [48,49]. In addition to cancer development, with the regulation of cellular growth and apoptosis, STAT3 is involved in infections, immune disorders and in the development of atherosclerosis.

The regulation of different STATs activities involves the amino-terminal region of these proteins[50,51]. The JAK/STAT pathway is finely regulated through different mechanism, one of which is represented by the negative feedback carried out by suppressor of cytokine signaling (SOCS) proteins.

Suppressor of cytokine signaling (SOCS) proteins

Eight member composes SOCSs family: SOCS1-7 and CIS[52,53]. All the family members contains an SH2 domain, positioned between the amino-terminal and the carboxy-terminal domain, named SOCS box[54]. SOCS1,3 and CIS are the most studied for their activity on the JAK/STAT pathway [55]. Physiologically expressed in all kind of cells, these proteins become rapidly induced by cytokines, through NF-κB and STAT inflammatory pathways and they exert a negative-feedback control on JAK/STAT pathway[56], inhibiting cytokines signaling. The mechanisms behind SOCSs activity are diverse, depending on the type of suppressor. For example, SOCS1 inhibits directly JAKs activity by binding to the activated JAKs, through its SH2 domain, while SOCS3 binds the activated receptor[57-61]. SOCS proteins may also be involved in the ubiquitin-proteasome signaling proteins degradation pathway[42,43]. A further control mechanism of the JAK/STAT/SOCS pathway occurs through SOCS proteins tyrosine phosphorylation by cytokines, at a post-translational level, accelerating its degradation and regulating the JAK/STAT feedback inhibition[62]. SOCS1 and 3 present also a crucial role in immune function regulation. In particular, SOCS1 deficient mice die after few weeks after bird because of a fatal dysregulation of IFN-γ, after showing lymphopenia (caused by increased lymphoid organs apoptosis)[63-66], while SOCS3 knockout mice die before the birth[67-69]. Different studies have also assessed to SOCS3 the important role of IL-6 signaling negative regulation in macrophages. Both SOCS1 and 3 are induced by IL-6, TNF-α [70] and IFN-γ and they can inhibit their responses[71-73]. SOCS proteins play an important role in the regulation of cardiovascular diseases, including heart failure, thrombosis and atherosclerosis. In particular, SOCS1 deficiency in macrophages leads to an increase in TNF-α and IL-12 production, causing intravascular coagulation[73,74]. On the other hand, SOCS3 deficiency in macrophages lead to an enhanced anti-inflammatory activity of STAT3 and a consequent reduction of cytokines production[73]. Moreover, SOCS1 may also prevent atherosclerotic lesion in apoE$^{-/-}$ mice by suppressing IFN-γ signaling[75], while in human atherosclerotic lesions, an increase in STAT3 phosphorylation is associated with a stable plaque phenotype[76]. It is also well known a correlation between SOCS proteins and cardiac dysfunction, where the induction of STAT3 by IL-6 cytokines family promote cardiac hypertrophy, as well as SOCS3 deficiency [77]. SOCS1 and 3 are also involved in insulin signal, by blocking its intracellular signaling through the bind with insulin receptor, in IRS proteins site [78,79] and their high expression in the liver can generate insulin resistance in vivo [80]. Even more important, SOCS3

suppression leads to a significant induction of liver steatosis, through the increase of SREBP-1 expression and STAT3 phosphorylation, as compensatory effects.

Sterol regulatory element binding (SREBPs) proteins

The *sterol regulatory element binding proteins* (SREBPs) are a transcriptional factor family and represent key regulators of cholesterol and lipids biosynthesis. SREBPs precursors are synthetized in the endoplasmic reticulum[81] and their structure is composed by four domains, with two transmembrane regions. The N-terminal domain of 480 amino acids represent the active portion, while the C-terminal domain makes a complex with the WD domain of another protein, called SCAP (SREBP cleavage activating protein), the SREBPs chaperone molecule. SCAP contains a sequence sensible to sterols, like HMG-CoA reductase[82]. SREBP activation is finely regulated by intracellular cholesterol levels. When intracellular cholesterol is high, SREBP is present in the endoplasmic reticulum in its inactive form, complexed with SCAP and with two other proteins, the insulin induced gene 1 and 2 (Insig1 and Insig2), responsible for the inhibition of SREBP translocation. As cholesterol levels decrease, Insig1 and Insig2 leave the SREBP-SCAP complex, which is included in endoplasmic vesicles (that present the COPII protein on the surface, recognized by SCAP), and translocated towards the Golgi apparatus. Here, the complex undergoes proteolytic cleavage by the protein convertase Subtilisin kexin isozyme/Site-1 protease (SKI-1/S1P) and the intramembranous metalloprotease Site-2 protease (S2P). S1P and S2P frees the N-terminal domain of SREBP and the active SREBP goes in the nucleus, where it activates those genes involved in cholesterol and lipids biosynthesis and capitation[82]. While SREBP translocates in the nucleus, SCAP comes back in the endoplasmic reticulum, to create a complex with another SREBP protein. Recent studies demonstrated that SCAP directly binds to the cholesterol, through its sterol sensitive sequence, undergoing some conformational changes[83], which make SCAP unable to interact with vesicles protein COPII[84]. By binding with SCAP, cholesterol also strengthen SCAP and Insig1-2 interaction. These two actions lead to an inhibition of SREBP translocation in the Golgi. Insig1 represent the predominant isoform and its degradation is negatively regulated by sterols[85]. Once SREBP reaches the nucleus, its active portion binds to specific DNA sequences on target genes promotors, modulating their transcription. Most of these genes are involved in the cholesterol and fatty acids biosynthesis, like fatty acid synthase (FAS), Diglyceride acyltransferase (DGAT), HMG-CoA synthase and

reductase, Stearoyl-CoA desaturase (SCD) and LDL receptor and are induced by SREBP[82]. To date, three different isoform of SREBP have been identified: SREBP-1a, SREBP-1c and SREBP-2. SREBP-1a and 1c differs only in their N-terminal domain [81]. SREBP-1a and SREBP-2 are the most expressed in cells, while organs (especially the liver) present higher amount of SREBP-1c and SREBP-2[86]. The three isoforms differ for their in vivo functions. In particular, SREBP-1a strongly activates all those genes regulated by SREBP, including ones mediating cholesterol, triglycerides and fatty acids biosynthesis, while SREBP-1c and SREBP-2 seems to be more selective[87]. SREBP-1c mainly regulates genes involved in fatty acids metabolism, while ones involved in cholesterol metabolism (HMG-CoA synthase and reductase) and uptake (LDL receptor) are regulated by both SREBP-1c and SREBP-2[87,88].

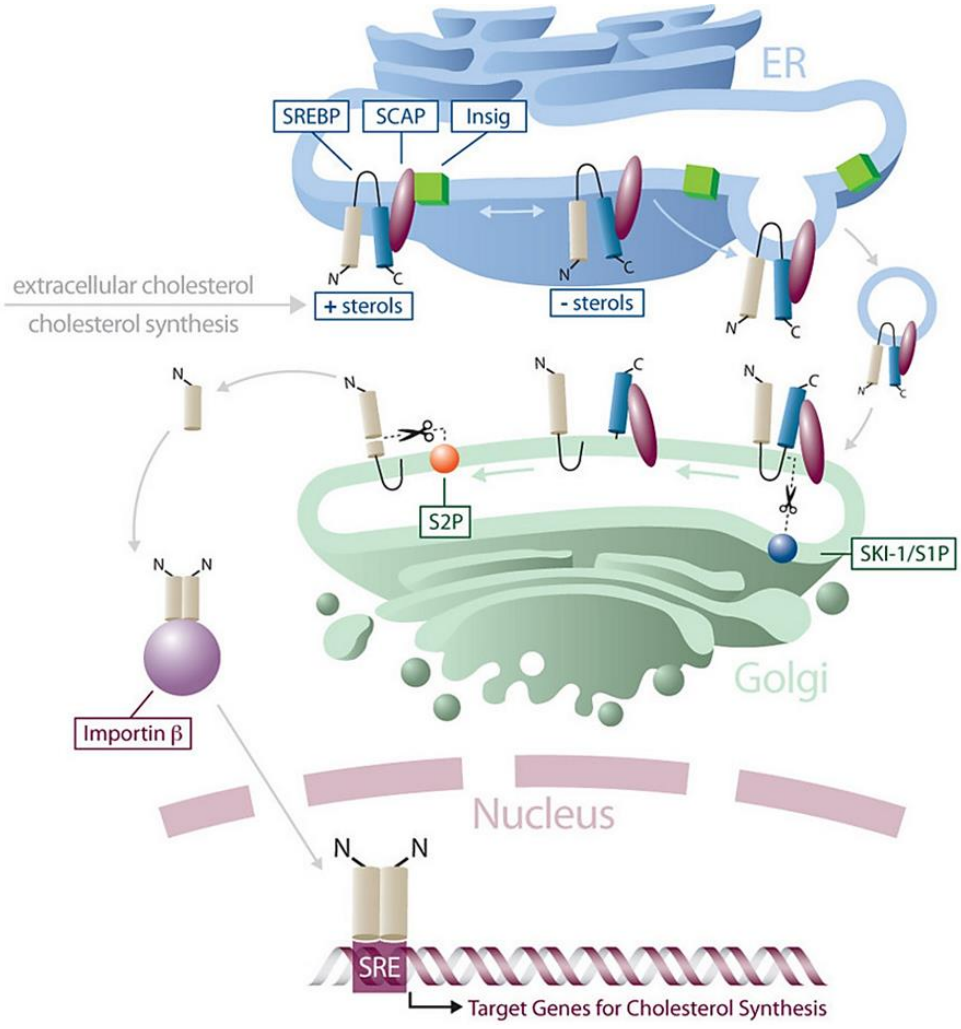


Figure D: SREBP activation process.

Focus on SOCS3 role in obesity and insulin resistance

Elevated levels of pro-inflammatory cytokines have been observed in obesity, infections and inflammation [89,90]. In particular, TNF-α plays a crucial role in insulin resistance in obese animal models [91] and elevated concentrations of IL-6 are associated with the development of type 2 diabetes [92,93]. The mechanism behind these observation includes the inhibited phosphorylation of insulin receptor and insulin receptor substrate 1 (IRS1) by TNF-α, although this stimulus is too rapid to motivate the TNF-α prolonged effect, suggesting the involvement of a transcription-mediated regulation. Pro-inflammatory cytokines increase SOCS protein expression through STAT and NF-κB activation and, in particular, SOCS3 is able to bind to both insulin receptor and insulin-like growth factor 1 receptor [94-97], representing a link between elevated cytokine levels and decreased insulin sensitivity or insulin resistance. In 2004, Ueki et all, demonstrated that SOCS3 levels are increased in a condition of insulin resistance linked to obesity and that SOCS3 binds to insulin receptor of cultured cells, through the interaction with Tyr960, inhibiting IRS1 and IRS2 phosphorylation and, consequently, attenuating insulin signaling, glycogen synthesis and glucose transport, without inhibiting insulin receptor phosphorylation. SOCS3 leads also to a downregulation od PI 3-kinase and Akt [98]. Moreover, SOCS3 mediates the inhibition of IRS phosphorylation, involving TNF-α-mediated inhibition of insulin signaling. Indeed, suppressing SOCS3 in 3T3L1 cells, the inhibition of tyrosine phosphorylation of IRS proteins by TNF-α is partially attenuated and in db/db mice livers, where insulin resistance seems to be strongly mediated by TNF-α , the reduction of SOCS3 partially normalizes IRS phosphorylation and hepatic steatosis [99]. Summarizing, SOCS3 is increased in insulin-sensitive tissue in obesity and it can inhibit IRS1 and IRS2 phosphorylation, leading to insulin resistance.

Proprotein convertase subtilisin kexin type 9 (PCSK9)

Proprotein convertases (PCs) represent important bioactive molecules responsible for inactive proteins and peptides proteolysis in active forms. Since 1990 until 2000 have been identified eight PCs, responsible for many tissue-specific processing of secretory precursors[100,101]. Hormones, growth factors, receptors, metalloproteases, surface glycoproteins and membrane-bound transcription factors are all substrates of PCs, depending on their site of action and protease activity, and undergo activation or inactivation[102-104]. Seven of PCs members belongs

to the kexin subfamily, which has a specific cleavage affinity for basic sites. The proprotein convertases that belong to the kexin family are furin, PC1/3, PC2, PC4, PACE4, PC5/6 and PC7/LPC [104-106], while S1P belongs to the pyrolysin subfamily of subtilase[107]. This one cleaves nonbasic amino acidic residues. The proprotein convertase subtilisin kexin type 9 (PCSK9) is the ninth and last member of mammalian PCs, discovered in 2003 by Dr Nabil G. Seidah and his research group at the Laboratory of Biochemical Neuroendocrinology of the Institute de Recherché Cliniques de Montréal, Canada. First identified as neural apoptosis-regulated convertase 1 (NARC-1) by two different pharmaceutical companies (Millennium Pharmaceuticals, Cambridge, MA, Patent no. WO 01/57081 A2 and Eli Lilly, Patent no. WO 02/14358 A2) in primary cerebellar neurons, PCSK9 was classified in the proteinase K subfamily of subtilase[108]. The structure of this proprotein consist in 692 amino acids organized in a structure similar to others family members: a signal peptide, a prodomain, a subtilisin-like catalytic domain and a C terminal domain, which differs from other family members one[109]. PCSK9 exert its catalytic activity through the catalytic triad Asp186, His226 and Ser386[108,110,111]. PCSK9 is produced as a soluble zymogen (pre-proPCSK9) that undergoes autocatalytic processing in the endoplasmic reticulum [112], to obtain the inactive heterodimer proPCSK9, composed of a prodomain fragment (14-kDa) and a mature fragment (57-kDa), which contains the C-terminal domain and the catalytic one [113]. The prodomain has an important function in the correct secretion and folding of the protein[114-116]. PCSK9 has been discovered in the brain, but almost all the circulating PCSK9 derives from the liver[117]. A significant amount of circulating PCSK9 is associated with LDL, HDL and VLDL [118] and it is present at different concentrations, depending by individual health, sex and age, presenting a minimum of 33ng/ml and a maximum of 2988ng/ml [119]. PCSK9 median levels are higher in women plasma (517ng/ml) then in men (450ng/ml)[119]. Despite its predominant liver derivation, some analysis on adult mice show that PCSK9 is also synthetized and secreted by other tissues, like kidney, cerebellum, small intestine [108], perigonadal fat [120], aorta and pancreatic islets [121]. Recently, our research group demonstrated the presence of PCSK9 also in human atherosclerotic plaque[122]. About cell culture, PCSK9 is highly expressed in human hepatocytes but also in smooth muscle cells, endothelial cells [122,123] and colonic enterocyte-like cells [124], while monocytes and macrophages do not express the protein [122,125].

Transcriptional regulation of PCSK9:

Similarly to other genes involved in cholesterol homeostasis regulation, PCSK9 is transcriptionally regulated by the SREBPs family[126]. There are many evidences of an induction of PCSK9 after the administration of statins, the HMG-CoA reductase inhibitors, due to the activation of SREBP pathway.

PCSK9 seems to be regulated by both SREBP-1c and 2. The role of SREBP-1c in PCSK9 regulation has been observed in both mice and humans, where PCSK9 is increased in the presence of insulin and is positively correlated with insulin resistance, liver steatosis and VLDL-TG, respectively[126-130]. According to these observations, PCSK9 appears to be not only an important regulator of LDL cholesterol, but also of the triglycerides-rich lipoproteins. Very interesting, it is known that PCSK9 levels are only weakly associated with LDL cholesterol levels, while the correlation is stronger between PCSK9 and triglycerides, glucose and insulin plasma levels[119,127,131,132].

Involvement of PCSK9 in autosomal dominant hypercholesterolemia:

Autosomal dominant hypercholesterolemia (ADH) is a lipid metabolism disorder that induce a strong increase in plasma levels of LDL-cholesterol. This hereditary condition produces different symptoms, ranging from skin xanthomas and arcus corneae to fatal cardiovascular complications[133,134]. There are three important genes involved in ADH: LDL receptor gene [135], APOB gene [136] and a third gene, recognized as PCSK9 [137,138]. In 2003, two different mutations in PCSK9 gene were identified in a French family and associated with the ADH disease. These were the S127R and the F216L, two dominant "gain of function" mutations[112,139]. Subsequently, three "loss of function" mutation of PCSK9, the Y142X, the C679X and the R46L, were discovered in a group of 13,761 patients which presented lower total cholesterol, LDL cholesterol and triglycerides levels, [140]. The mechanism that allows PCSK9 to be linked with ADH is hide in its capability do bind to LDL receptor and, consequently, causing its degradation[141-144]. PCSK9 is rapidly processed from its precursor form of 74-kDa to the mature form of 60-kDa, then secreted from the cell[145]. Plasma PCSK9 interacts directly with LDL receptor, present on cell surface, and undergoes internalization together with the receptor. The internalized complex reach the endocytic-lysosome subcellular compartments[145,146]. LDL receptor can be also degraded through another pathway, the post-ER compartments[125].

PCSK9 – LDL receptor interaction:

Further studies have identified the molecular portion of PCSK9 able to interact with LDL receptor. Through the in vitro deletion of different portion of LDL receptor (epidermal growth factor-like repeat-A, B and C), Zhang and his research group discovered that PCSK9 binds to the EGF-A domain of LDL receptor[147]. Moreover, this bind resulted dependent by pH conditions, becoming significantly tighter at pH 5.2 compared with neutral pH[147]. PCSK9 has a lower affinity to the LDL receptor. It is hypothesized that LDL and VLDL particles can cause a conformational change in LDL receptor, reducing the affinity with PCSK9, compared to LDL particles; moreover PCSK9 serum levels are lower than LDL particles. [148]. In Figure E and F is shown the complex interaction between PCSK9 and LDL receptor and the mechanism behind the receptor degradation, mechanism that does not involve PCSK9 proteolytic activity[113].

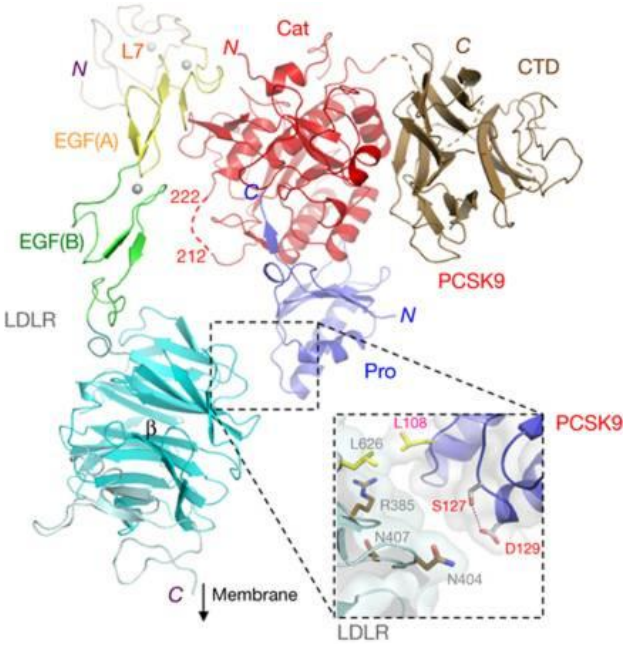


Figure E: PCSK9/LDL receptor complex structure. Pro (blue): PCSK9 prodomain, Cat (red): catalytic domain, CTD (brown): C-terminal domain; LDLR receptor domains are L7 (beige), EGF-A (yellow), EGF-B (green) and EGF-C/β-propeller (cyan). Spheres indicate Ca2+ ions. In the box is represented the interaction between β-propeller of LDL receptor and PCSK9 prodomain[148].

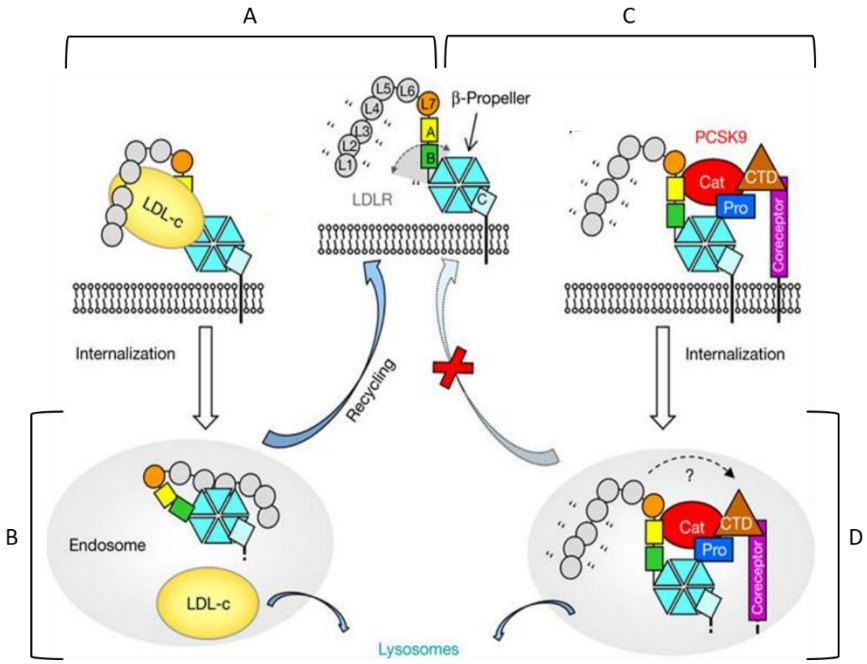


Figure F: Possible mechanism of interaction between PCSK9 and LDL receptor. A. at neutral pH, the L7 domain of LDL receptor on cell surface adopts open extended form and binds to LDL particle through the ligand-binding domain (gray circles). B. inside the endosome, where the pH is lower, LDL receptor closes its structure, releases LDL particle and undergoes recycling. C. PCSK9 binds to LDL receptor through the EGF-A domain through its catalytic domain, while its prodomain restrains EGF-B/β-propeller flexibility. PCSK9 C-terminal domain (CTD) may interact with a co-receptor that promotes or inhibits PCSK9-LDL receptor interaction. D. Inside the endosome, in this case, the lower pH enhances PCSK9/LDL receptor affinity, preventing the complex dissociation and the consequent receptor recycle. LDL receptor undergoes lysosomal degradation[148-151].

Association between PCSK9 mutant variants and LDL cholesterol plasmatic levels:

As hinted before, the first clue of the existence of PCSK9 genetic variants was found in a French family affected by ADH. All the three-generation members affected by the disease showed elevated total cholesterol plasma levels[138]. In particular, LDL cholesterol levels reached concentrations of 236 mg/dl and 312 mg/dl respectively for 17 and 40 years old members[138]. The discovery of the first PCSK9 mutation ($S^{127}R$), through positional cloning analysis, was followed by the observation of a second mutation ($F^{216}L$). Both were characterized as gain of function mutations. The mechanisms behind the correlation between these mutations and ADH disease involves both LDL receptor degradation and PCSK9 structure[152]. PCSK9 affinity with LDL

receptor, is indeed increased in the presence of the $S^{127}R$ mutation [110,150], leading to an improved receptor degradation [153], while for the $F^{216}L$ it has been demonstrated a higher resistance of the $RFHR^{218}$ PCSK9 motif to furin cleavage [110,154,155], leading PCSK9 to an improved stability. However, the strongest gain of function mutation of PCSK9 was identify in Norwegian and Utah subjects with a diagnose of familial hypercholesterolemia (FH) [156,157]. This mutation ($D^{374}Y$) enhances binding affinity with LDL receptor at both neutral and acid pH conditions. There are also some loss of function PCSK9 mutations, associated with low LDL cholesterol serum levels. For example, Dallas Heart Study (performed on Afro-Americans, European-Americans, Hispanics and other ethnicities) [158] identified two different PCSK9 loss of function mutations ($C^{426}G$ and $C^{2037}A$) in Afro-American population [159]. Moreover, diverse healthy persons with PCSK9 null allele homozygosis had been characterized. These people present 14 mg/dl of LDL cholesterol and they do not present any particular disease, like their children[160]. Another PCSK9 loss of function mutation was discovered from Italian patients affected by hypobetalipoproteinemia, which presented very low LDL cholesterol levels[161].

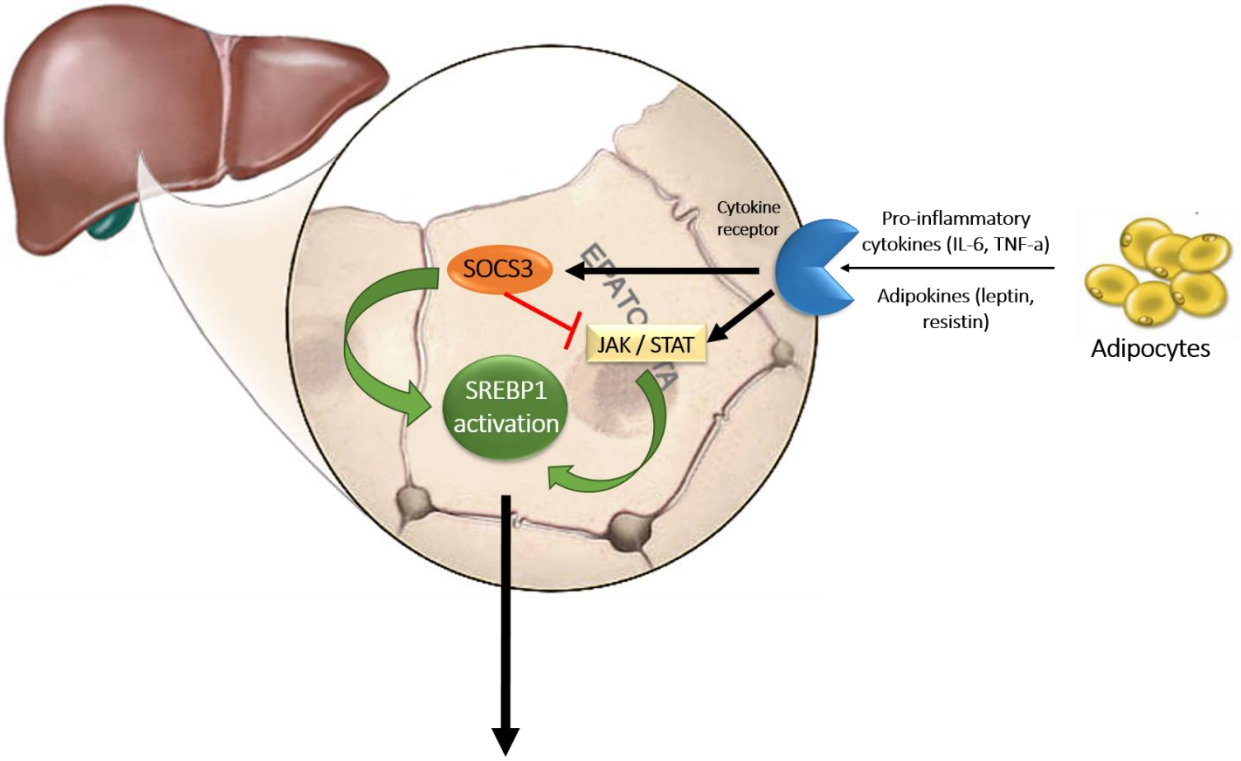
Role of PCSK9 in insulin resistance, VLDL cholesterol and triglycerides metabolism

After the observation of how a PCSK9 loss of function mutation can results in a strong decrease in cardiovascular risk, the next question was if PCSK9 negative effect on LDL cholesterol metabolism was only due to the LDL receptor degradation, or if the VLDL receptor could also be involved. Individuals with gain of function mutation of PCSK9 presented high levels of VLDL, triglycerides and apoB100, ascribable to a higher production of VLDL by the liver[162]. Moreover, fasting PCSK9 levels are positively correlated with fasting serum triglycerides levels[163]. It was also demonstrated that PCSK9 increases VLDL triglycerides and ApoB production rate[163], apparently with a mechanism that involves a direct increase of VLDL secretion rate by the liver, through the degradation of LDL receptor. Indeed, ApoB is a VLDL component, required for their hepatic assembly and secretion, moreover LDL receptor promotes ApoB post-translational degradation in a proteasome-dependent way[164,165], reducing VLDL secretion. In addition, PCSK9 seems to be responsible also for a decreased apoB100 degradation rate[166], mainly in the presence of LDL receptor. An addition mechanism can be the autofagosome/lysosome pathway[166], since lower ApoB levels were observed in hepatocyte autofagosomes of animals with overexpression of PCSK9[166]. It is thought that PCSK9 can deviate ApoB from its

degradation, making it available for VLDL synthesis. In human elevated levels of PCSK9 are also correlated with insulin resistance and it has been observed that the inhibition of insulin receptors expression causes an increase in PCSK9 hepatic expression[130,132,167].

AIM OF THE STUDY (I)

The previous evidences link obesity with a low grade sub-clinical inflammation, responsible for an impaired glucose and lipid metabolism [29, 32, 34]. The secretion of pro-inflammatory proteins from the adipose tissue, indeed, leads to the activation of JAK/STAT/SOCS pro-inflammatory intracellular pathway, involved in inflammation, hypertriglyceridemia and insulin resistance [46, 47]. Recent evidences demonstrated also the involvement of PCSK9 in insulin resistance [130-132, 167] and lipid metabolism [162, 163], suggesting a possible correlation between the JAK/STAT/SOCS pathway activation and PCSK9. The present study aimed to investigate the possible role of the pro-inflammatory protein TNF-α and the JAK/STAT pathway on *de novo* lipogenesis and PCSK9 expression in human HepG2 cell line, through the activation of SOCS3.



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MATERIAL AND METHODS (I)

Cell cultures

The Human hepatocellular liver carcinoma cell line, HepG2, was cultured in 10%FCS/MEM supplemented with penicillin (10,000 U/ml), streptomycin (10 mg/ml), nonessential amino acids and sodium pyruvate. For the experiments, cells were incubated with MEM containing either 10% of lipoprotein plasma deprived serum (LPDS) or 10% Fetal Calf Serum (FCS), as indicated in the figures legend.

Reagents and antibodies

MEM, trypsin EDTA, penicillin, streptomycin, nonessential amino acid solution, FCS, disposable culture flasks and petri dishes were from Euroclone (Pero, Milan, Italy), and filters were from Millipore (Billerica, MA). Molecular weight protein standards were from BIO-RAD Laboratories (Hercules, CA). SDS, TEMED, ammonium persulfate, glycine, and acrylamide solution (30%T, 2.6%C) were obtained from BIO-RAD Laboratories. BCA assay for determination of protein concentrations was purchased from Thermo Fischer Scientific (Waltham, MA). [^{14}C]-Acetate were from Amersham (Cologno Monzese, Milan, Italy). Recombinant insulin, Tumor Necrosis Factor-α (TNF-α) and bovine serum albumin (BSA) were purchased from SIGMA-Aldrich (Milan, Italy). STAT3 inhibitor, MD77, was kindly provided by Prof. Daniela Barlocco (University of Milan, Milan, Italy). The JAK inhibitor JAK1 was purchased from Millipore (Millipore, Milan, Italy). For Western Blot (WB) analysis, the following antibodies were used: anti-PCSK9 (Cayman, Tallinn, Estonia), anti-α-tubulin (SIGMA-Aldrich), anti-pAKT (Millipore); anti-AKT and anti-SOCS3 (Cell Signaling Technology, Denver, MA); anti-pSTAT3, anti-insulin receptor substrate (pIRS-1) and anti- stearoyl-CoA desaturase (SCD-1) (Abcam, Cambridge, UK); anti-STAT3 and SREBP-1 (Santa-Cruz Biotechnology, Santa Cruz, CA); anti-mouse and anti-rabbit peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Lab; Cambridgeshire, UK).

Animals

Four-week-old male ob/ob mice and their lean, wild-type male C57BL/6J controls were purchased from Charles River (Calco, Italy). In compliance with the Principles of Laboratory Animal Care (NIH publication 86-23), mice were housed at constant room temperature (23°C) in a 12-hour light/dark cycle (7 a.m. to 7 p.m.) receiving standard chow and water *ad libitum*. Mice were sacrificed at fourteen weeks of age in the fasted state.

Generation of human SOCS3 expression construct and retroviral infection in HepG2 cells

Full-length human SOCS3 (accession no. 003955) was generated by polymerase chain reaction (PCR) using the following primers: 5'-CGGGATCCATGGTCACCCACAGCAAGTTTCC-3' and 5'-CGCTCGAGTTAAAGCGGG GCATCGTACTGG-3'and the Expand High-Fidelity PCR System (Roche Diagnostics, S.p.A, Monza, Italy). The sequence of the polymerase chain reaction generated construct was confirmed by sequencing (Primm, Milan, Italy). Retroviral expression plasmid was then constructed using the pBM-IRES-PURO [168] expressing the puromycin resistance gene as a selectable second cistron gene, generated from the original pBM-IRES-EGFP, generously provided by Garry P. Nolan (Stanford University, Stanford, CA). Retroviral infections of HepG2 were performed as previously described [168]. A polyclonal population of HepG2 control and SOCS3 overexpressing cells have been than selected with 10 μg/ml of puromycin.

Transfection of siRNA

ON-TARGET plus SMART pool siRNA directed to STAT3 and SOCS3 or scramble control were purchased from DharmaconTM (Carlo Erba Reagents, Milan, Italy). Transfections were performed as previously described using SilentFectTM Lipid Reagent (BIO-RAD laboratories, Hercules, CA) according to the manufacturer's protocol [169,170]. HepG2 cells were seeded at a density of 6×105/well (6 well tray) the day before the transfection in completed medium. Cells were then transfected with 20 nM of siRNA for 48h then the medium replaced with MEM containing 5%LPDS ± TNF-□ for an additional 24h before performing the quantitative (q)PCR or WB analysis.

Synthesis of total cholesterol

Cholesterol biosynthesis was estimated by measuring the incorporation of [14C]-Acetate into cellular cholesterol, as previously described[171].

Evaluation of intracellular triglycerides and cholesterol levels

Total cellular lipids were extracted with hexane/isopropanol 3:2 and TG and Cholesterol contents were determined with enzymatic assays (HORIBA ABX, Montpellier, France).

ELISA assay

Conditioned media was cleared by centrifugation (13,000 rpm for 10 min.) and store at -20°C. The amount of apolipoprotein (Apo) B (Vinci-Biochem, Firenze, Italy) and PCSK9 (R&D System, Minneapolis, MN) was then quantified by using the ELISA assays according to manufacturer's instructions. The values were normalized with total cell protein contents, extracted from the cell monolayer, determined by BCA assay (Thermo Fischer Scientific).

Luciferase reported promoter activities assay

The plasmid pGL3-PCSK9-D4 contains the 5'-flanking region of the PCSK9 gene from -440 to -94, relative to the ATG start codon as previously described[172]. To measure the PCSK9 promoter activity, HepG2 cells were seeded in 48 well plates at a density of 8×105 cells per well. On the next day, cells were transiently transfected with pGL3-PCSK9-D4 plasmids with turbofect reagent (Carlo Erba Reagents) and, 48h post transfection, cells were incubated with serum-free medium in the presence or absence of insulin (10-7M) for an additional 24h. Luciferase activities were measured by using Neolite reagent (Perkin Elmer, Milan, Italy) according to manufacturer's instructions. pCMV-β vector, encoding for β-galactosidase, (Clontech Lab., Mountain View, CA) was cotransfected as internal control. B-galactosidase activity was assayed as described[173]. Luciferase activity was normalized to the β-galactosidase activity of the co-transfected pCMV-β construct.

RNA preparation and quantitative real time PCR

Total RNA was extracted with the iScript Sample Preparation Buffer (BIO-RAD laboratories) cDNA synthesis preparation reagents (BIO-RAD laboratories) according to manufacturer's instructions. Reverse transcription-polymerase first-strand cDNA synthesis was performed by using the iScript cDNA synthesis Kit (BIO-RAD laboratories). Quantitative real time PCR (qPCR) was then performed by using the Kit Thermo SYBR Green/ROX qPCR Master Mix (Carlo Erba Reagents) and specific primers for selected genes. Primer sequences used for qPCR analysis are shown in Table 1. The analyses were performed with the ABI Prism® 7000 Sequence Detection System (Applied Biosystems; Life Technologies Europe BV, Milan, Italy). PCR cycling conditions were as follows: 94°C for 3min, 40 cycles at 94°C for 15s, and 60°C for 1min. Data were

expressed as Ct values and used for the relative quantification of targets with the ΔΔCt calculation.

Table 1. Primer sequence utilized for the qPCR analysis.

Primer	**Forward**	**Reverse**
18S	5'-CGGCTACCACATCCACGGAA-3'	5'-CCTGTATTGTTATTTTTCGTCACTACC-3'
Human		
Fatty Acid synthase	5'-GCAAATTCGACCTTTCTCAGAAC-3'	5'-GGACCCCGTGGAATGTCA-3'
HMG-CoA reductase	5'-CTTGTGTGTCCTTGGTATTAGAGCTT-3'	5'-GCTGAGCTGCCAAATTGGA-3'
SOCS3	5'-GACCAGCGCCACTTCTTCAC-3'	5'-CTGGATGCGCAGGTTCTTG-3'
SREBP-1	5'-CGGAACCATCTTGGCAACA-3'	5'-GCCGGTTGATAGGCAGCTT-3'
SREBP-2	5'- AGCTGGTCTGTGAAG-3'	5'- CGCAATGGGGTCAGC-3'
LDLR	5'-GTGTCACAGCGCCG-3'	5'-CGCACTCTTTGATG-3'
PCSK9	5'-CCTGCGCGTGTCAACT-3'	5'-GCTGGCTTTTCCGAAACTC-3'
SCD-1	5'-CTATACCACCACCACCACCA-3'	5'-GGGCATCGTCTCCAACTTAT-3'
apoB	5'-GCAGACTGAGGCTACCATGA-3'	5'-AGGATTGTTCCGAGGTCAAC-3'
Mouse		
PCSK9	5'- AACCTGGAGCGAATTATCCCA-3'	5'- TTGAAGTCGGTGATGGTGACC-3'

Western Blot Analysis

Total cytosolic protein extracts of HepG2 and HepG2SOCS3, were obtained by collecting cells in 150 µl of Mammalian Protein Extraction Reagents (Thermo Fisher Scientific) containing a cocktail of protease and phosphatase inhibitors (Roche Diagnostics). Twenty µg of proteins and a molecular mass marker (Novex® Sharp Protein Standard, InvitrogenTM; Life Technologies Europe BV) were separated on 4-12% sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE; Novex® NuPAGE® 4-12% Bis-Tris Mini Gels, InvitrogenTM; Life Technologies) under denaturing and reducing conditions. Proteins were then transferred to a nitrocellulose membrane by using the iBlotTM Gel Transfer Device (InvitrogenTM; 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 RT. The blots were incubated overnight at 4°C with a diluted solution (5% BSA or non-fat dried milk) of the following human primary antibodies: anti-PCSK9 (1:100) anti-pAKT, (1:100); anti-AKT (1:1,000); anti-pSTAT3

(1:10,000); anti-pIRS-1 (1:5,000); anti-SCD-1 (1:500); anti-STAT3 (1:1,000); anti-SREBP-1a (1:500); anti-SOCS3 (1:100); anti-HMG-CoA reductase (1:500); and anti-α-tubulin (1:2,000). 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. Immunoreactive bands were detected by exposing the membranes to ClarityTM Western ECL chemiluminescent substrates (Bio-Rad Laboratories) for 5 min and images were acquired with a ChemiDocTM XRS System (Bio-Rad Laboratories). Densitometric readings were evaluated using the ImageLabTM software as previously described[174]. The values of the phosphorylated proteins were normalized to those of the corresponding constitutive forms to express arbitrary units of relative expression.

Analysis of 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 (I)

TNF-α induces SOCS3 and PCSK9 in HepG2 cells

Release of pro-inflammatory cytokines, such as TNF-α and IL-6, from adipocytes of obese subjects with IR, has been shown to activate the JAK/STAT pathway at the hepatic levels and inducing, either directly or indirectly, the transcription of different target genes including SOCS proteins (6,7). In agreement with these observations, we found that the incubation of hepatic cell line HepG2 with TNF-α induced the expression of SOCS3 mRNA in a time- and concentration-dependent manner (Figure 1A), reaching the maximal induction after 24h incubation at the concentration of 10 ng/ml. This latter resulted in three-fold induction of SOCS3 protein, evaluated by WB analysis (Figure 1B). Under the same experimental conditions, TNF-α induced PCSK9, although to lower extent (Figure 1C). Transfection of HepG2 cells with specific siRNA significantly down-regulated protein expression of STAT3 and SOCS3 (Figure 1D). siRNA STAT3 completely blocked the induction of SOCS3 by TNF-α (Figure 1E). A similar effect was observed on the expression levels of PCSK9, where siRNA to either SOCS3 or STAT3 blocked the effect of TNF-α (Figure 1F). These results suggest the possibility that TNF-α induces PCSK9 by inducing the SOCS3 expression.

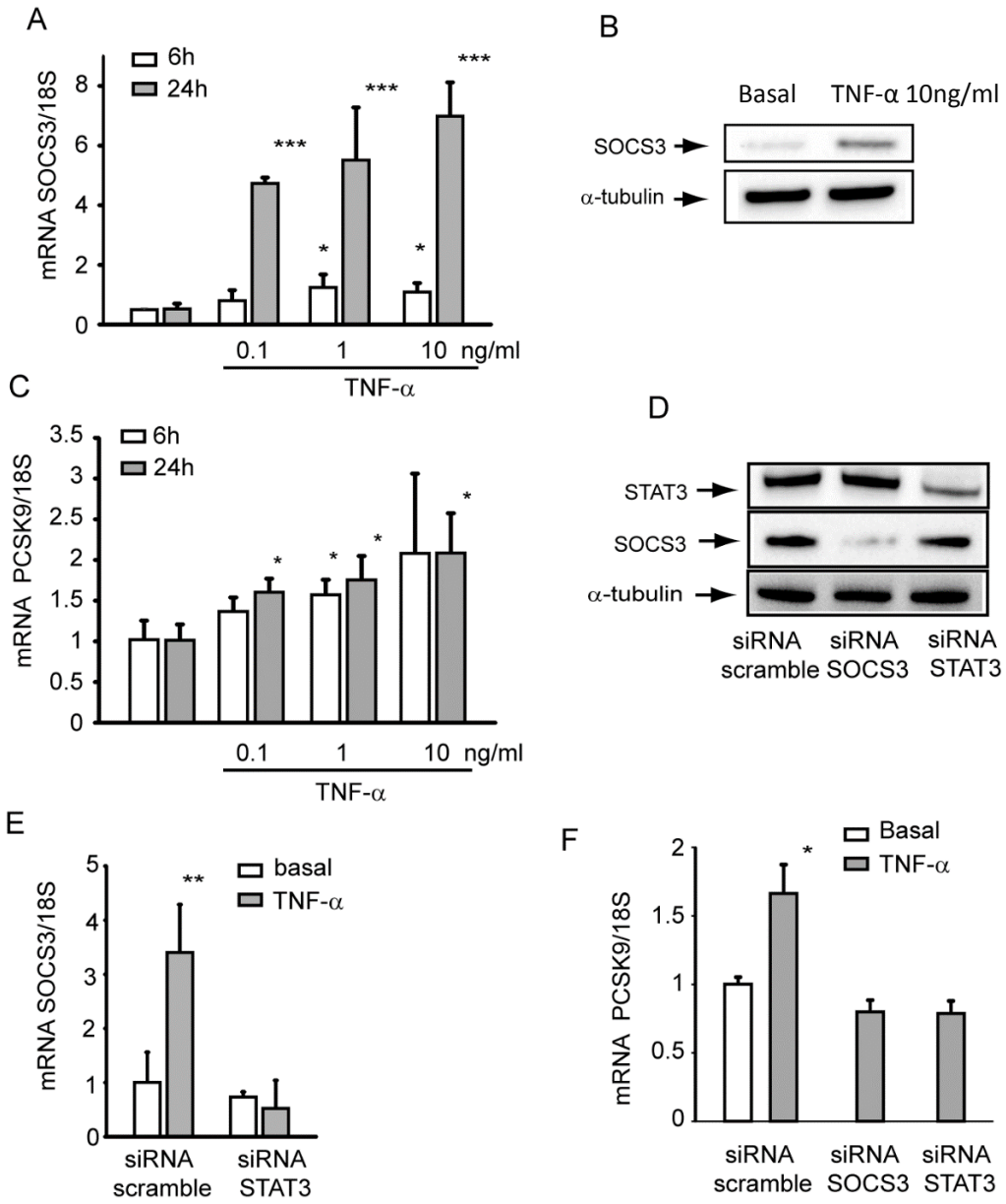


FIGURE 1. TNF-α induces SOCS3 and PCSK9 in HepG2 cells. A and C) HepG2 cells were seeded in MEM/10% FCS and the day after incubated with MEM supplemented with 10% LPDS for 6h and 24h in the presence or absence of different concentrations of TNF-α (0.1, 1 and 10 ng/ml). At the end of the incubation, the total RNA was extracted and mRNA levels of SOCS3 and PCSK9 were determined by qPCR. B) SOCS3 protein expression was evaluated by WB analysis from total protein extracts of HepG2 cells incubated for 24h with 10 ng/ml of TNF-α. α-tubulin was used as loading control. D) HepG2 cells were seeded in MEM/10% FCS and the day after transfected with siRNA scramble, STAT3 and SOCS3. After 48h, the medium was replaced with MEM with 10% LPDS. After an additional 24h, the protein expression of STAT3 and SOCS3 and α-tubulin were evaluated by WB analysis. E and F) The cells were incubated under the same experimental conditions described for panels A-C, in the presence or absence of 10 ng/ml TNF-α

during the last 24h of incubation in MEM with 10% LPDS. At the end of the incubation, the total RNA was extracted and mRNA levels of SOCS3 and PCSK9 were determined by qPCR. Differences between treatments were assessed by Student's t-test (A, B, C) or 1-way ANOVA (D, E, F), *p<0.05; **p<0.01; ***p<0.001.

SOCS3 overexpression induces PCSK9 in HepG2

To further investigate the role of SOCS3 on PCSK9 expression, we generated a retrovirally transduced HepG2 cell line with a plasmid encoding human SOCS3 ($HepG2^{SOCS3}$). After puromycin selection, the mRNA and protein overexpression of exogenous human SOCS3, were evaluated by qPCR and WB analyses, respectively. $HepG2^{SOCS3}$ cells were compared with HepG2 transduced with empty retroviral vector encoding only the puromycin resistance gene. As shown in Figure 2A, retroviral transduction determined approximately 90-fold induction of mRNA of SOCS3 that translated into a 2~5 fold increase of protein SOCS3 (Figure 2B). The overexpression of SOCS3 abrogated basal STAT3 phosphorylation state (Figure 2C). $HepG2^{SOCS3}$ cells show higher levels of PCSK9 mRNA (3.48±0.35 fold, Figure 2D) and increased amount of cellular and secreted PCSK9 (1.53 fold and 2.18±0.38 fold respectively; Figure 2E and 2F). Since SOCS3 has been shown to be up regulated in animal models of obesity (6), we then evaluated the mRNA levels of PCSK9 and SOCS3 in the liver of ob/ob and wild type C57BL/6 control mice. The ob/ob mice expressed higher levels of SOCS3 (6.46±4.8 fold) and this condition was associated with a significant induction of PCSK9 (2.03±1.54 fold) (Figure 2G and 2H). The inhibition of SOCS3 and STAT3 by siRNA resulted in an opposite effect on PCSK9 mRNA levels, with a significant reduction after transfection with siRNA SOCS3 and an induction with siRNA STAT3 (Figure 2I). We then investigated the effect of SOCS3 on PCSK9 promoter activity in HepG2 and $HepG2^{SOCS3}$ transfected with the luciferase reporter construct pGL3-PCSK9-D4 (32). This plasmid contains the 5' flanking region of the PCSK9 gene, from nt -440 to -94 (relative to the ATG start codon), in front of the luciferase coding sequence. The relative luciferase activity of the PCSK9 promoter was not affected by the expression of SOCS3 in HepG2 cells (Figure 2L). A similar response was observed by using the D4 construct containing the mutation of sterol regulatory element (SRE), while the mutation of hepatocyte nuclear factor (HNF)-1 site determined a significant increase of the promoter activity in $HepG2^{SOCS3}$ cells, although the overall transcriptional activity was almost abolished (Figure 2L).

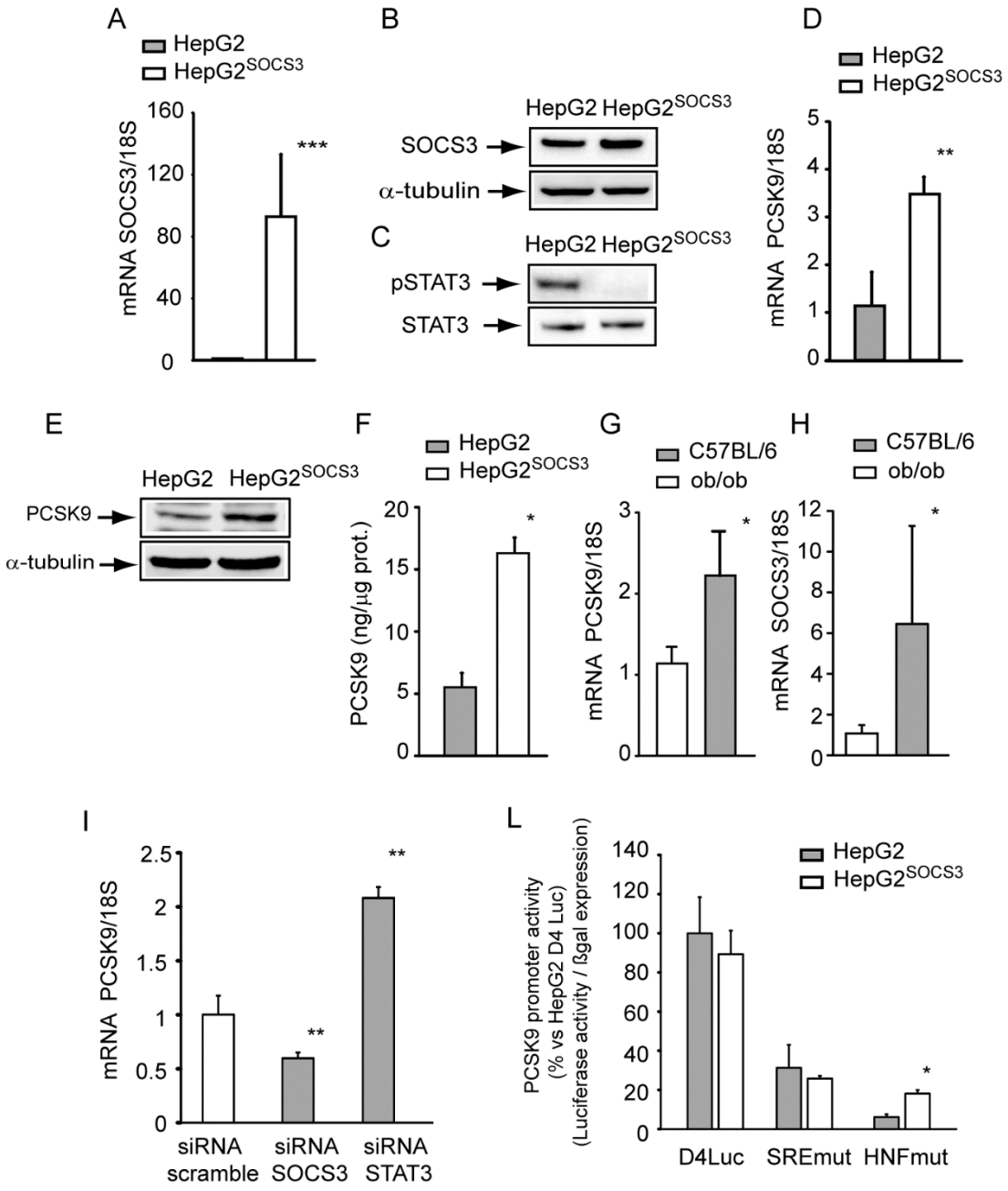


FIGURE 2. SOCS3 overexpression induces PCSK9 in HepG2 cells. A) HepG2 cells were retrovirally transduced with empty retroviral vector or encoding for human SOCS3. After puromycin selection, the expression of SOCS3 mRNA was evaluated by qPCR in HepG2 and HepG2SOCS3 cultured in MEM supplemented with 10% LPDS for 24h. B) Under the same experimental conditions, SOCS3 expression was determined by WB analysis from total cell lysates using an anti-SOCS3 antibody. The membrane was stripped and re-probed with anti-α-tubulin antibody as loading control. C) WB analyses with anti-phospho-STAT3 and anti-STAT3 were performed from the same samples described for panel B. D-F) HepG2 and HepG2SOCS3

cells were seeded in MEM/10%FCS and the day after incubated with MEM containing 10%LPDS for 24h. D) PCSK9 mRNA was determined by qPCR. Intracellular (E) and secreted (F) PCSK9 levels were evaluated by WB analysis and ELISA assay, respectively. For the determination of PCSK9 levels with WB analysis, the cells were incubated for 24h under serum-free condition. G and H) PCSK9 and SOCS3 mRNA expression levels (qPCR) were determined from total hepatic RNA of male ob/ob (n=5) and C57BL/6 control mice (n=5). I) HepG2 cells were seeded in MEM/10% FCS and the day after transfected with siRNA scramble, SOCS3 and STAT3. After 48h, the medium was replaced with MEM with 10% LPDS. After an additional 24h, the total RNA was extracted and mRNA levels of PCSK9 were determined by qPCR. L) HepG2 and HepG2SOCS3 cells were transfected with pGL3-PCSK9-D4 or pGL3-PCSK9-SREmut or pGL3-PCSK9-HNFmut. The day after the transfection the medium was replaced with MEM containing 10%LPDS and, after an additional 24h, luciferase activities were determined by Neolite reagent. Luciferase activities were normalized to the β-galactosidase activity of the cotransfected pCMV-β construct. Differences between groups were assessed by Student's t-test (A-H) or 1-way ANOVA (I, L) *p<0.05; **p<0.01.

Pharmacological inhibition of JAK/STAT pathway induces PCSK9

To further investigate the effect of STAT3 inhibition on PCSK9 expression, HepG2 cells were incubated with non-toxic concentration of STAT3-inhibitor MD77 (Figure 3) (35). Incubation of HepG2 cells for 48h with 10-7M MD77, determined a very similar cellular responses than those observed with SOCS3 overexpression. In particular, incubation with MD77 induced fatty-acid synthase (FAS; 2.93±1.28 fold, Figure 3A), PCSK9 (2.06±0.7 fold, Figure 3B), and only marginally HMG-CoA reductase (1.58±0.2fold, Figure 3C), with a not statistically significant reduction of LDLR (-45.3±24.7%, Figure 3D). The JAK inhibitor, JAK1, significantly increased FAS (1.56±0.05 fold, Figure 3E), PCSK9 (3.30±0.32 fold, Figure 3F) and HMG-CoA reductase (1.49±0.09 fold, Figure 3G), and marginally reduced the LDLR (-43.2±24.2%, Figure 3H). Consistently with the results with HepG2SOCS3, no effect on PCSK9 promoter activity was observed after 24h incubation with JAK1 (Figure 3I). Taken together, these results demonstrate that the inhibition of the JAK/STAT pathway induces PCSK9 in HepG2 cells.

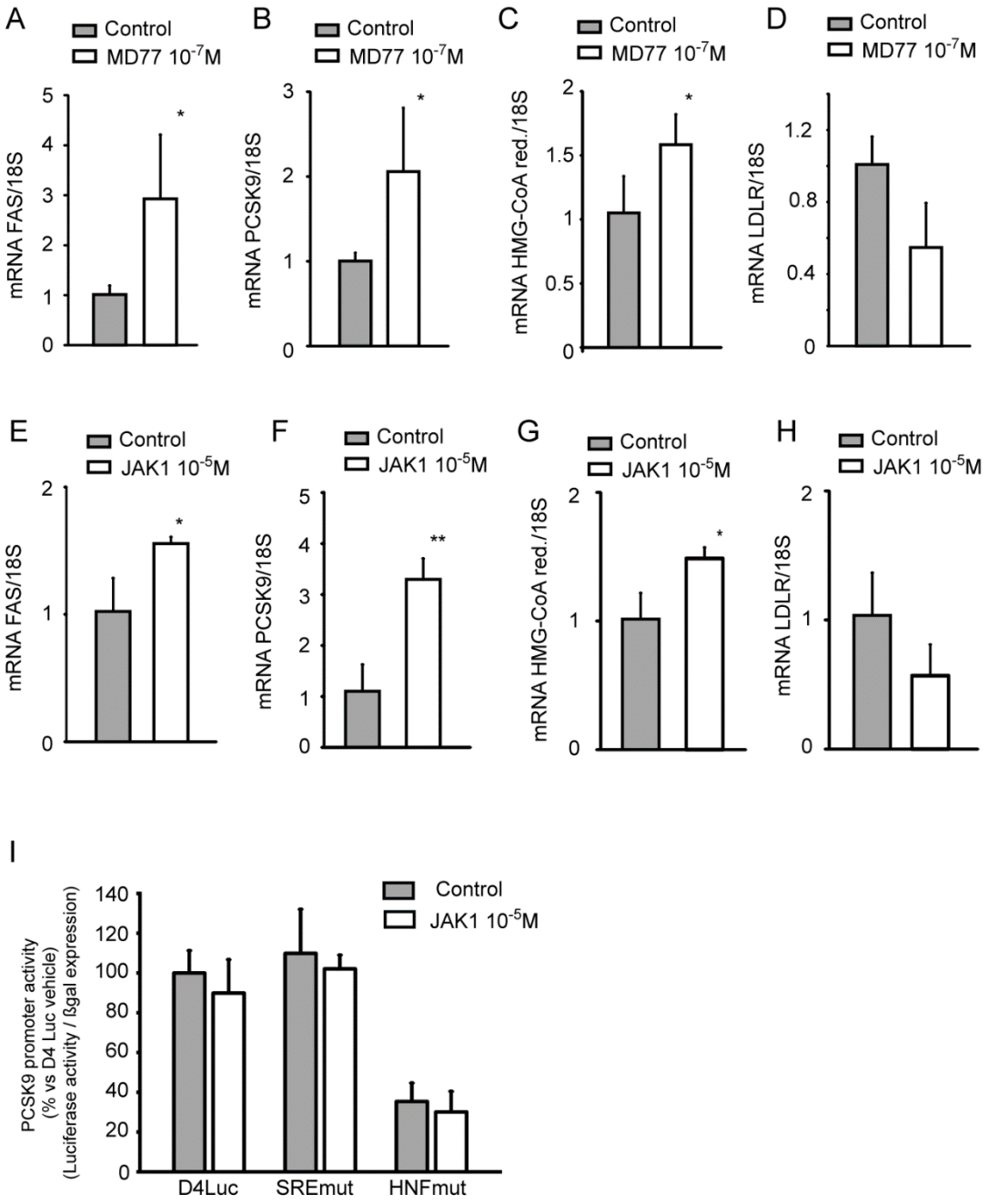


FIGURE 3. Pharmacological inhibition of JAK/STAT pathway induces PCSK9 in HepG2 cells. A-D) HepG2 cells were seeded in MEM/10%FCS and the day after incubated with MEM containing 10%LPDS for 24h in the presence or absence of the STAT3 inhibitor MD77 (10-7M) or E-H) JAK inhibitor JAK1 (10-5M). mRNA levels of FAS (A and E), PCSK9 (B and F), HMG-CoA reductase (C and G), and LDLR (D and H) were determined by qPCR. I) HepG2 cells were transfected with pGL3-PCSK9-D4 or pGL3-PCSK9-SREmut or pGL3-PCSK9-HNFmut. The day after the transfection the medium was replaced with MEM containing 10%LPDS with or without JAK1 (10-5M) and, after an additional 24h, luciferase activities were determined by Neolite reagent. Luciferase activities were normalized to the β-galactosidase activity of the cotransfected pRSV-

galactosidase construct. Differences between groups were assessed by Student's t-test (A-H) or 1-way ANOVA (I). *p<0.05.

SOCS3 overexpression induces de novo lipogenesis in HepG2 cells

Several in vivo evidence demonstrated the pivotal role of SOCS3 and STAT3 on lipid metabolism and SREBP-1 transcriptional activity (6,7,36). To verify that a similar response also occurs in our experimental model, we determined the mRNA levels of SREBP-1 target genes, such as FAS and SCD-1 and the levels of ApoB. HepG2SOCS3 cells show higher mRNA levels of FAS (3.59±0.40 fold, Figure 4A) as well as that of SCD-1 gene (1.92±0.12 fold, Figure 4B) and protein (3.81±0.75 fold; Figure 4C). Together with these changes, we observed a significant increment of ApoB mRNA (4.08±0.41 fold, Figure 4D), and protein in the conditioned media (3.47±0.09 fold, Figure 4E). Accumulation of intracellular TG levels (22.1±7.1µg/mg protein vs. 38.3±9.1µg/mg protein; Figure 4F) was seen in HepG2SOCS3, with no significant changes in total cholesterol content (Figure 4G). To investigate whether the observed induction of the above reported genes was associated to SREBP-1 activation, we evaluated the processing and expression of SREBP-1. WB analysis demonstrated that SOCS3 overexpression increased the active form of SREBP-1 at 68 kDa with a significant reduction of the pro-form at 125 kDa (Figure 4H). The addition of insulin slightly reduced the ApoB levels in the conditioned media both in HepG2 and HepG2SOCS3 cells (Figure 4E). In response to TNF-α we observed a significant induction of SCD-1, ApoB and FAS mRNA levels (2.11±0.43, 1.60±0.33 and 1.39±0.21 fold, respectively; Figure 4I-M). These responses were reversed in HepG2 cells transfected with siRNA STAT3 (Figure 4I-M). Taken together, those results indicate that the overexpression of SOCS3 induces the de novo lipogenesis and increased ApoB production in HepG2 cells, effects dependent by STAT3.

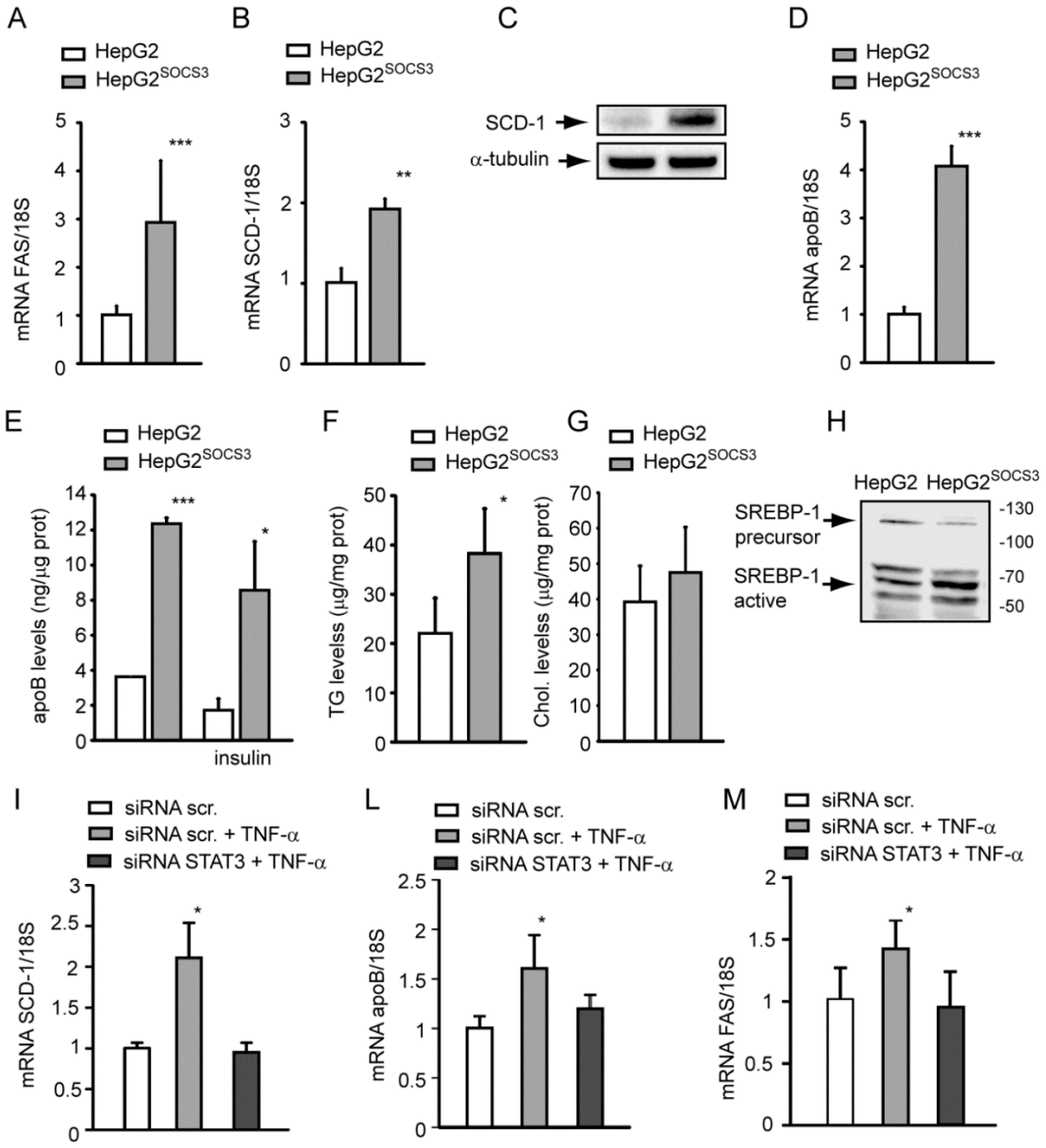


FIGURE 4. SOCS3 overexpression induces de novo lipogenesis in HepG2 cells. A, B and D) HepG2 and HepG2SOCS3 cells were seeded in MEM/10%FCS and the day after incubated with MEM supplemented with 10% LPDS for 24h. mRNA levels of FAS, SCD-1, and ApoB were evaluated by qPCR analysis from total RNA. C) Under the same experimental conditions, the SCD-1 expression was determined by WB analysis from total protein extracts. E) HepG2 and HepG2SOCS3 were cultured in serum free medium for 24h in the presence or absence of 10-7M insulin, conditioned media was than collected and ApoB concentrations determined by ELISA assay. F and G). Under the same experimental conditions described for panel A, the intracellular TG and cholesterol levels were determined in HepG2 and HepG2SOCS3 by using an enzymatic assay. The values were normalized for total protein content. H) WB analysis of SREBP-1. The

active form shows a molecular weight of 68 kDa and the respective pro-form was detected at 125 kDa. I-M) HepG2 cells were seeded in MEM/10% FCS and the day after transfected with siRNA scramble and STAT3. After 48h, the medium was replaced with MEM with 10% LPDS in the presence or absence of 10 ng/ml TNF-α. After an additional 24h, the total RNA were extracted and mRNA levels of SCD-1, ApoB and FAS were determined by qPCR. Differences between HepG2 and HepG2SOCS3 were assessed by Student's t-test (A-H) or 1-way ANOVA (I-M), *p<0.05; **p<0.01; ***p<0.001.

SOCS3 overexpression does not affect the cholesterol biosynthesis in HepG2

A series of experiments were performed to determine the effect of SOCS3 on genes under the control of SREBP-2, such as HMG-CoA reductase and LDLR. HMG-CoA reductase mRNA was induced in HepG2SOCS3 cells (2.10±0.66 fold, Figure 5A); conversely, the LDLR mRNA levels were reduced in response to SOCS3 overexpression (-56%±21.6%, Figure 5B). These changes were not associated with significant variation of HMG-CoA reductase protein level (Figure 5C). The lack of significant changes of HMG-CoA reductase was also confirmed by the fact that the cholesterol biosynthesis was not altered in HepG2SOCS3 cells compared to HepG2 cells (Figure 5D).

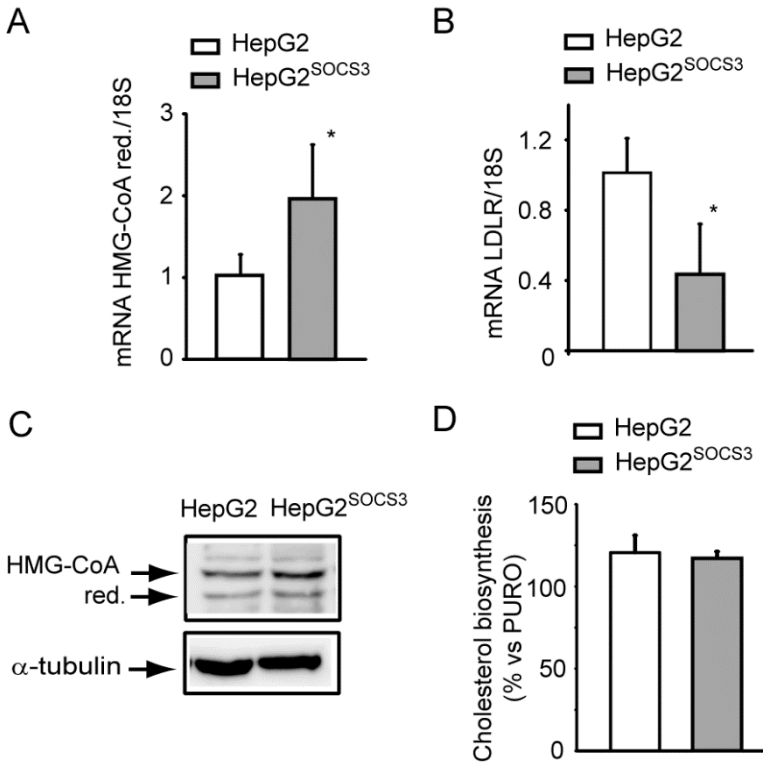


FIGURE 5. Cholesterol biosynthesis is not influenced by SOCS3 in HepG2 cells. A and B) HepG2 and HepG2SOCS3 cells were seeded in MEM/10%FCS and the day after incubated with MEM containing 10%LPDS for 24h and HMG-CoA reductase and LDLR mRNA determined by qPCR (A and B, respectively). C) Under the same experimental conditions, HMG-CoA reductase expression was assessed by WB analysis from total protein extracts. D) HepG2 and HepG2SOCS3 were cultured in MEM containing 10%LPDS in the presence of [14C]-Acetate. After 48 hours [14C]-Acetate incorporation into cellular cholesterol was evaluated. Each bar represents the mean±SD of triplicate dishes. Differences between HepG2 and HepG2SOCS3 were assessed by Student's t-test, *p<0.05.

SOCS3 overexpression reduces insulin-dependent activation of IRS1 and AKT

To corroborate the pathophysiological relevance of our observations, we then investigated the effect of SOCS3 overexpression on insulin signaling. As shown in Figure 6A, in response to insulin (10-7 M), a significant induction of IRS-1 Tyr896 and Akt Ser473 phosphorylations were observed in HepG2. Overexpression of SOCS3 (HepG2SOCS3 cells) resulted in a reduced activation of both IRS-1 (Figure 6A) and AKT (Figure 6B) in response to insulin. In accordance with previous studies [175], ectopic expression of SOCS-3 appeared to elevate basal autophosphorylation, although this increase was not statistically significant.

Moreover, STAT3 phosphorylation was induced in response to insulin in control cells (HepG2), while SOCS3 overexpression ($HepG2^{SOCS3}$) determined an abrogation of this response (Figure 6C). As previously reported by Costet et al [128], the incubation of HepG2 cells with insulin (10-7 M), significantly induced both PCSK9 secretion and mRNA (Figure 7A and 7B), and the presence of SOCS3 further up regulated the response to insulin at the mRNA levels. However, this effect did not translate to a further induction of PCSK9 secretion in the cultured media, as determined by ELISA assay (Figure 7A). Similarly, we observed an additive effect of insulin and SOCS3 on the FAS and SREBP-1 mRNA levels (Figure 7C and D) with no effect on SREBP-2 and HMG-CoA reductase levels (Figure 7E and F), further supporting a selective activation of SREBP-1 transcriptional activity rather than SREBP-2.

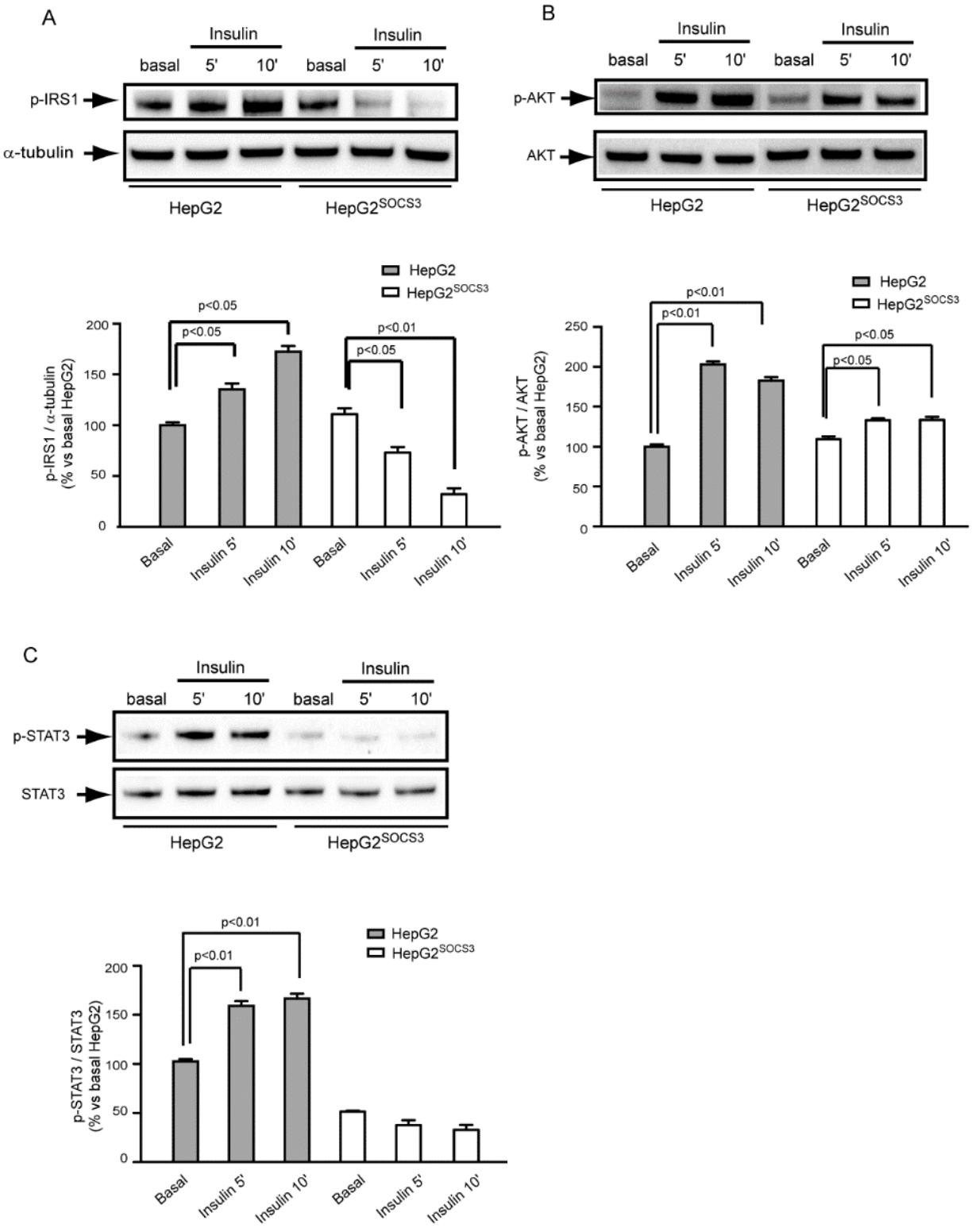


FIGURE 6. Effect of SOCS3 on insulin-induced IRS-1, Akt and STAT3 phosphorylation. A-C) HepG2 and HepG2SOCS3 were seeded in MEM/10%FCS and starved overnight with serum free medium before stimulation with insulin (10-7M) for 5 and 10 min. WB analysis was then performed from total protein extracts by using anti-pIRS-1, anti-pAkt, anti-pSTAT3, anti-STAT3

and anti-α-tubulin antibodies. Densitometric analysis was than evaluated using the ImageLabTM software. Differences between groups were assessed by 1-way ANOVA.

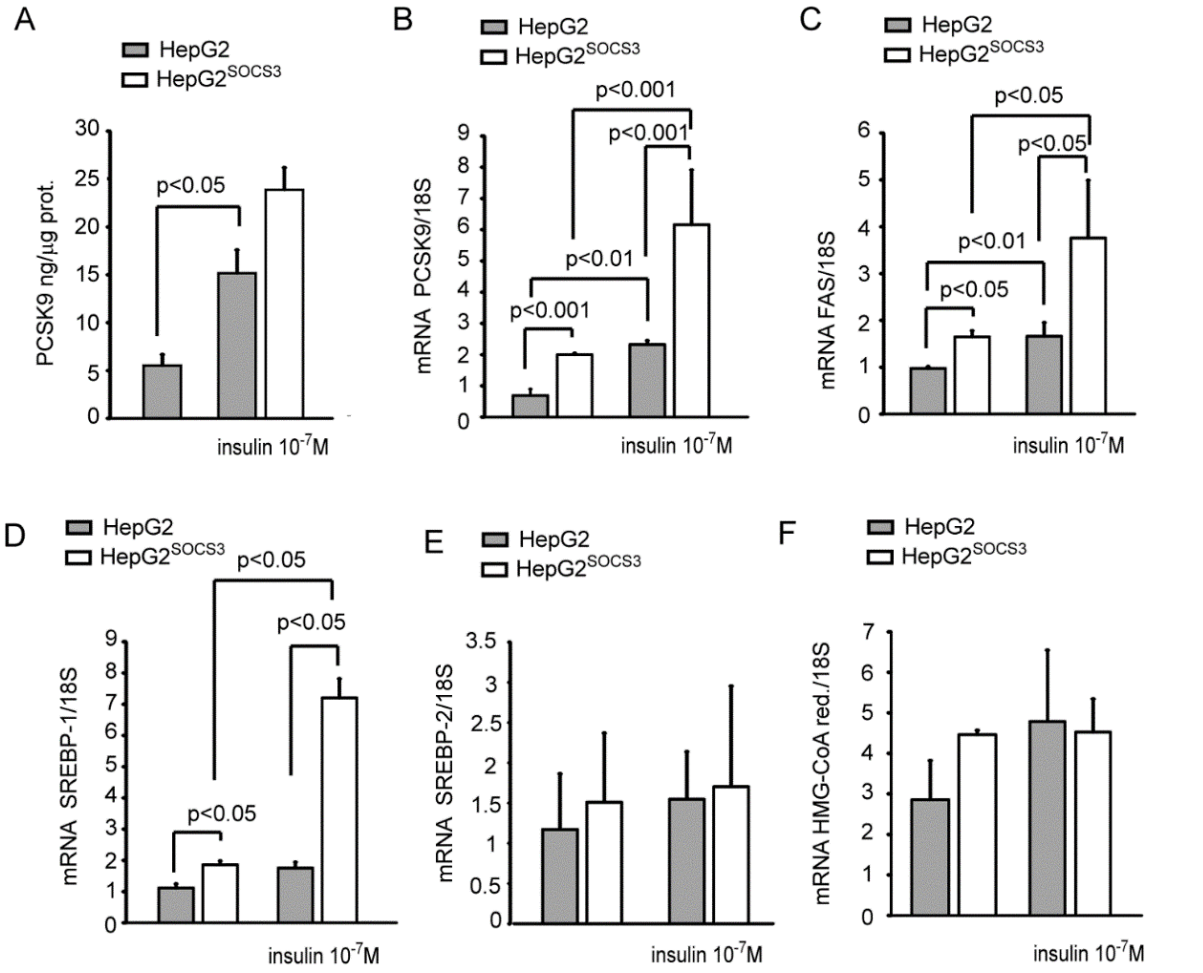


FIGURE 7. Effect of insulin on PCSK9 expression in HepG2^{SOCS3}. A-F) HepG2 and HepG2^{SOCS3} cells were seeded in MEM/10%FCS and the day after incubated in serum free medium for 24h in the presence or absence of insulin (10-7M). A) At the end of the incubation, the PCSK9 levels in the conditioned media were evaluated by ELISA assay, and mRNA levels of PCSK9, FAS, SREBP-1, SREBP-2 and HMG-CoA reductase were determined by qPCR (B-F). Differences between groups were assessed by 1-way ANOVA.

DISCUSSION (I)

Obesity leads to the development of a low grade sub-clinical inflammation, with the release of pro-inflammatory proteins (adipokines and cytokines) by the adipose tissue. Pro-inflammatory proteins, released in the systemic circulation, interact with a variety of receptor in different organs. In the present work, we decided to focus our attention on the liver, where cytokines activate the JAK/STAT3 pathway through the interaction with cytokine receptor, expressed on hepatocytes surface. As a consequence of JAK/STAT3 pathway activation by pro-inflammatory cytokines, the protein SOCS3 is released inside the cell, with the aim of down-regulate the JAK/STAT3 pathway with a negative feedback mechanism. The increase in SOCS3 levels leads to a further release of acute phase proteins, responsible for an impaired lipid and glucose metabolism [29, 32-35]. We know that the adipokine resistin and insulin induce both SOCS3 and PCSK9 expression in cultured cell lines and animal models [176-179] and that, in clinical settings, a positive relationships of apoB-containing lipoproteins and PCSK9 has been observed [119,130-132]. Moreover, SOCS3 overexpression, in mice, induces SREBP-1 transcriptional activation and de novo lipogenesis [98,99] and PCSK9 has been shown to be transcriptionally regulated by SREBP-1 and SREBP-2 [128,129]. For these reasons, in the present study, we decided to investigate the possible correlation between SOCS3 and PCSK9 and, in particular, to understand the effects of an overexpression of SOCS3 on *de novo* lipogenesis, insulin resistance and PCSK9 expression. The first novel aspect that we observed was the induction of PCSK9 in response to the pro-inflammatory cytokine TNF-α, the same factor that efficiently induces SOCS3 (Figure 1A, B, C) and has been implicated in chronic-inflammation associated with IR [80,91]. To study the possible link between SOCS3 and PCSK9, we first suppressed the expression of SOCS3 by siRNA in HepG2. By using this approach, we demonstrated that SOCS3 is required for the TNF-α-driven induction of PCSK9. In addition, the silencing of STAT3 blocked the induction of SOCS3, PCSK9, ApoB, FAS and SCD-1 in response to TNF-α (Figures 1D, E, F and Figure 4I, L). Although the effect of pro-inflammatory cytokines on genes regulating the de novo lipogenesis has been previously described [91,180], our data, candidates PCSK9 as a gene involved in lipid metabolism regulated by pro-inflammatory cytokine TNF-α, in a SOCS3 dependent manner. We then established an hepatic cell line stably overexpressing SOCS3 (Figure2A, B), the endogenous inhibitor of STAT proteins, and consistently found to be up-regulated in the liver of genetically- and diet-induced obese animal [98,99]. By WB analysis, we demonstrated that SOCS3 overexpression abrogated STAT3 phosphorylation and thus, the JAK/STAT pathway (Figure 2C). By using this cellular model, we observed a significant induction of both PCSK9 mRNA and protein (Figure 2D, E). The

involvement of both JAK and STAT proteins was then confirmed by the use of selective pharmacological inhibitors, JAK-I and MD77 (Figure 3). The suppression of STAT3 by siRNA induced PCSK9, while the opposite effect was seen after the downregulation of SOCS3 (Figure 2I). In combination with the induction of PCSK9, we also observed increased SREBP-1 processing (Figure 4H) and a significant up-regulation of the key genes involved in the *de novo* lipogenesis, such as FAS and the SCD-1, together with the ApoB mRNA, ApoB secretion and intracellular TG (Figure 4A, B, C, D, E, F). Importantly, PCSK9 was up regulated in the liver of ob/ob mice, together with SOCS3 (Figure 2G, H), further supporting the link between the two genes observed in cultured system. The consistent and very significant induction of ApoB secretion, confirmed the pivotal role of SOCS3 and the related inflammatory pathway, on hypertriglyceridemia. In addition, our observation suggested a possible involvement of PCSK9 on ApoB production by hepatic cells and/or by the liver of animal models previously described [155,166,181,182]. For instance, the physical interaction between PCSK9 and ApoB has been shown to increase ApoB production, possibly through the inhibition of intracellular ApoB degradation [166]. Our evidence further delineate the potential link between PCSK9 and ApoB by demonstrating that the inhibition of the JAK/STAT pathway by SOCS3 is a common determinant of their increased production and secretion. Notably, recent findings suggest that PCSK9 may directly induce ApoB mRNA levels in enterocytes [182]. It is, therefore, possible to speculate that SOCS3 could activate a positive feedback loop by increasing the expression levels of PCSK9, which, in turn, determines the induction of ApoB secretion. Previous studies have established the SREBP-dependent transcriptional activation of PCSK9. Indeed, similarly to the LDLR and other cholesterol-regulated genes, the proximal region of the PCSK9 promoter contains an SRE-1 motif [128,183]. In addition, two independent studies have demonstrated that insulin, by inducing SREBP-1c activity, induces PCSK9 both *in vitro* and *in vivo* [128,179]. We confirmed this evidence at both mRNA and protein levels (Figure 7A, B), and the combination of SOCS3 and insulin determined a further induction of *de novo* lipogenesis genes (Figure 7C), SREBP-1 (Figure 7D), and PCSK9 (Figure 7B), with no additive effect on PCSK9 secretion (Figure 7A). These results are in agreement with previous studies showing that the inhibition of STAT proteins by SOCS3 suppresses the transcription of SREBP-1 [99,184]. In our study, the transcriptional activation of SREBP-1 was demonstrated by the induction of key genes involved in the lipid synthesis, such as FAS and SCD-1 (Figure 4A, B, C), while SREBP-2 appears to be unaffected by SOCS3 since the cholesterol biosynthesis is not altered and an opposite regulation was observed for the HMG-

CoA reductase and the LDLR mRNA (Figure 5). To better define the effect of SOCS3 on PCSK9 transcription, we analyzed PCSK9 promoter activities in HepG2 and HepG2^{SOCS3} transiently expressing the PCSK9 promoter-driven luciferase reporters PCSK9-D4 [172]. Unexpectedly, we found that PCSK9-D4, the functional promoter reporter (nt 2455 to 294), was unresponsive to SOCS3 overexpression (Figure 2L) and incubation with JAK1 (Figure 3I), while its activity was induced by simvastatin in the same experimental settings (data not shown). These results suggest that the SOCS3 response sequences could possibly locate upstream of the promoter region considered. On this regard, it is important to mention that the link between JAK/STAT pathway and PCSK9 has been previously investigated in the same cell line by the use of the cytokine oncostatin M [185]. Similar to our study, oncostatin M did not affect the PCSK9 promoter activity excluding also an epigenetic modification of the PCSK9 gene [185]. Since previous studies have shown that PCSK9 transcription is controlled through cis regulatory elements located in the proximal promoter region of the PCSK9 gene where the Sp1 sites, HNF1, and SRE-1 are located [129,172,186]. We utilized also a PCSK9-D4 containing a mutation for the HNF responsive element. Under this condition, we detected a significant increase of the promoter activity in HepG2^{SOCS3} cells (Figure 2L). These observations potentially related to the SREBP pathway, activated in response to STAT inhibition, although the overall activity was very low and potentially not responsible for the induction of PCSK9 mRNA levels [99]. This effect was also not confirmed with the incubation of the JAK inhibitor (Figure 3I), most likely because of the minor inhibition of the pathway in comparison to the complete block after SOCS3 overexpression. Additional analysis are thus required to better define the effect of SOCS3 on the transcription of PCSK9 as well as the possible effect on mRNA stability. On this regard, it is important to point out that also an additional regulator of the JAK/STAT pathway, oncostatin M, was shown to reduce PCSK9 mRNA levels without affecting its stability[185]. In addition, berberine, which up-regulates PCSK9 mRNA [187], has been shown to modulate LDLR mRNA stability by regulating the activity of AU-rich (AREs) elements binding proteins [172]. Thus, it would be interesting to study the involvement of AREs proteins on PCSK9 mRNA stability. Several studies have described the effect of SOCS proteins on insulin signaling [70,80,188]. Although the observations may differ by the cell type and by the time considered, the majority of the studies suggests that SOCS proteins control insulin action by reducing the expression of IRS proteins [70,80,188]. Indeed, in our experimental conditions, SOCS3 overexpression reduced both IRS-1 and Akt phosphorylation in response to insulin (Figure 6A, B). Moreover, insulin stimulation of HepG2^{SOCS3} determined a

further induction of genes related to *de novo* lipogenesis (FAS and SREBP1), as well as PCSK9 (Figure 7). Although this finding has been obtained in *in vitro*, it is conceivable to hypothesize a functional contribution of PCSK9 on the hypertriglyceridemic condition observed in type 2 diabetes mellitus and obesity. Indeed, PCSK9 directly affects the VLDR expression at the adipose tissue determining the accumulation of visceral adipose tissue in mice [120]. For such effect, PCSK9 deficient mice show adipocyte hypertrophy, enhanced *in vivo* fatty acid uptake, and ex vivo triglyceride synthesis [120]. In conclusion, in the present study, we provided evidence for the JAK/STAT dependent expression of PCSK9 in hepatic cell line, suggesting the potential molecular basis of the direct relationship between PCSK9 and TG levels observed in clinical trials [119,130-132].

PROPROTEIN SUBTILISIN/KEXIN TYPE 9 (PCSK9) INDUCES PRO-INFLAMMATORY RESPONSE IN MACROPHAGES

INTRODUCTION (II)

Role of macrophages in plaque development:

One of the critical steps of atherosclerotic plaque development is the entering of monocytes inside the plaque[189]. These white blood cells, attracted by cytokine or growth factor produced within tunica intima during the inflammatory process, differentiate in macrophages and contribute to the increased expression of inflammatory receptors, such as toll-like receptors [190,191], able to activate macrophages and recognize particles and molecules expressing typical pathogen-like pattern. In particular, the atherosclerotic process starts with the retention of apolipoprotein B (apoB)-containing lipoproteins, mainly the low-density lipoproteins (LDL) within the artery wall [192], which undergo chemical modification, in particular oxidation, and are recognized and capitated by scavenger receptors upon infiltrated macrophages membrane. In physiological conditions, this pathway removes and destroys oxidized LDL, bacterial endotoxins and fragments of apoptotic cells, but if the amount of oxidized LDL is too high, cholesterol crystals accumulate inside macrophages cytosol, leading to foam cells formation [190], and promoting endothelium dysfunction and activation, with a further recruitment of white blood cells. Activated macrophages are responsible for the production of pro-inflammatory cytokines, chemokines, coagulation factors, vasoactive molecules (such as adhesion molecules), oxygen and nitrogen radicals and proteases. The accumulation of LDL contributes not only to the formation of foam cells, but also to the amplification of a chronic inflammatory state. With the production of many pro-inflammatory molecules, activated macrophages are responsible for the initiation of thrombus formation and for the destabilization of the lesion and the inhibition of a resistant and stable fibrous cap formation[193-196]. Taken together, these action lead to plaque rupture, a consequent thrombosis and ischemia. It appears quite clear the central role of macrophages in atherogenesis and several studies have clarified the existence of two different macrophages population, that, following *in vitro* criteria, can be divided into pro-inflammatory (M1 cells) and anti-inflammatory (M2 cells) [197]. The polarization toward the M1 state is obtained through several stimuli, such as lipopolysaccharide (LPS) and interferon-γ, two of the Toll-like receptor (TLR) most important ligands, while the polarization toward M2 state can be obtain with the treatment with interleukine-4 and -13. M1 cells represent the main source of pro-inflammatory cytokines and have been observed in both mouse and human atherosclerotic plaque [197]. They are responsible for the secretion of many pro-inflammatory mediators, such as nitric oxide synthase, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-12 and

proteolytic enzymes, all involved in the amplification of the inflammatory process and in the extracellular matrix components degradation.

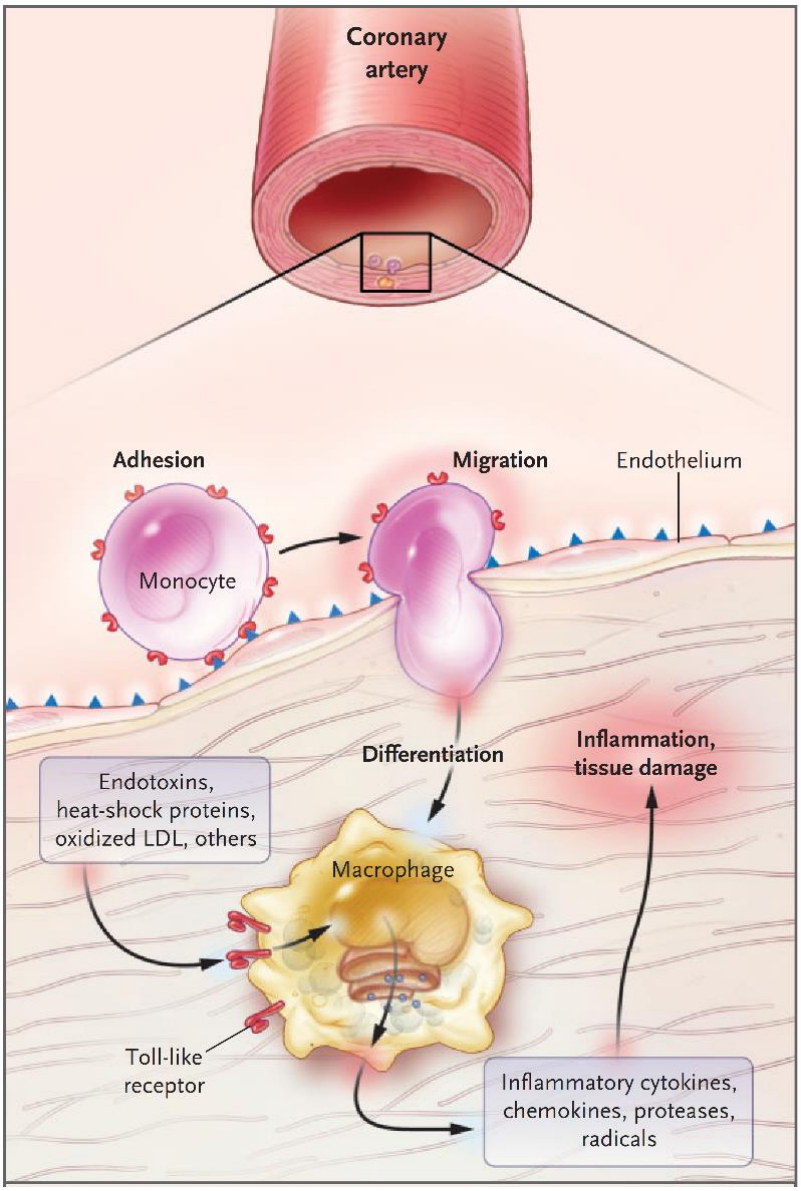


Figure G: Role of macrophages inflammation on the artery. Monocytes are recruited inside the tunica intima and differentiate in macrophages, able to bind to diverse microbial and endogenous particles through the toll-like receptor. Activated macrophages release pro-inflammatory cytokines, chemokines and radicals, exacerbating inflammatory process [198].

Pro-inflammatory cytokines - markers of systemic inflammation:

Pro-inflammatory cytokines are secreted from different tissues and cells in all those situation of infection, inflammation or trauma. Some of them are secreted locally and are responsible for the activation of cell surface receptor pathways that regulates inflammatory response. Atherosclerosis is not only a local inflammation. The development of the disease is accompanied with increased levels of pro-inflammatory cytokines in the bloodstream. For example, high levels of C-reactive protein and IL-6 can predict worse prognosis in patients affected by angina or myocardial infarction [11,199,200]. Elevated inflammatory markers levels are present in patients with acute disease, but also an inactive atherosclerotic plaque can release in the bloodstream some markers, like C-reactive protein, erythrocyte sedimentation rate, soluble intracellular VCAM-1, P-selectin and fibrinogen [201,202]. This observation leaded to consider those proteins markers of inflammatory process in the artery and predictive for cardiovascular risk development, also in healthy population[18,203].

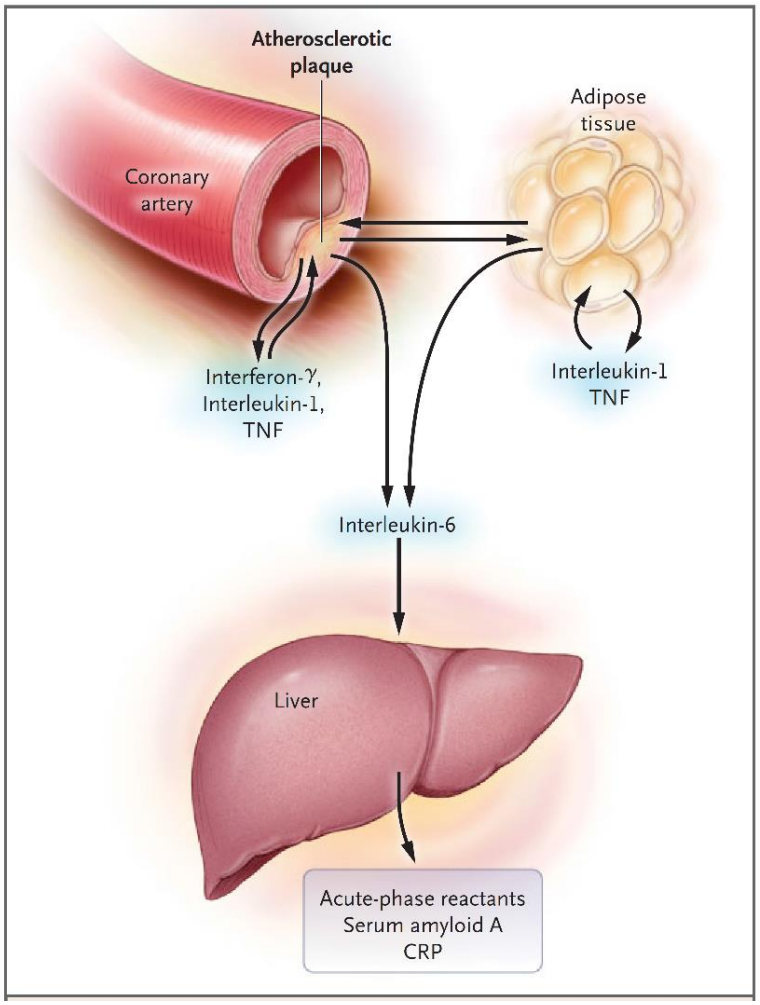


Figure H: The cytokine cascade: pro-inflammatory cytokines produced by activated macrophages within the plaque, reach the bloodstream, causing a further release of other cytokines from other tissues, like adipose tissue of patients with metabolic syndrome. In particular, interferon-γ, interleukin-1 and TNF-α, produced by plaque macrophages and other immune cells of the plaque, induce IL-6 production, which, in turn, is responsible for the liver production of high amount of acute-phase reactants, like C-reactive protein, serum amyloid A and fibrinogen. The continuous amplification of cytokines cascade make possible clinical diagnosis [198].

Tumor Necrosis Factor-α (TNF-α) and coronary events risk:

TNF-α is a circulating multifunctional pro-inflammatory cytokine, produced and secreted by endothelial cells, smooth muscle cells and macrophages within the atherosclerotic lesion[204-206]. The name of this cytokine derives from its first discovered action in the promotion of hemorrhagic necrosis in transplanted tumors [207], but TNF-α is also involved in several cardiovascular processes, in particular, contributing to the development of atherosclerosis by inducing endothelial dysfunction. Elevated TNF-α levels are observed in advanced heart failure [208,209] and myocardial ischemia and reperfusion [210-213] and, vice-versa, coronary ischemia causes acute increase in TNF-α, while myocardial infarction causes TNF-α increase several months after the event [214,215]. Moreover, in experimental models, TNF-α is able to induce left ventricular dysfunction [216], pulmonary edema [217,218] and cardiomyopathy [219]. In 2000, Ridker and his research group demonstrated that post-myocardial infarction patients, recruited in the Cholesterol and Recurrent Events (CARE) trial[220], presented persistently elevated plasma concentration of TNF-α, suggesting that a persistent inflammatory instability is present in stable patients. Moreover, those patients presenting highest TNF-α levels presented also the highest cardiovascular risk. By decreasing the secretion of apolipoproteins, modifying sphingolipid content and reducing cholesterol excretion and catabolism, TNF-α can interfere with cholesterol metabolism and can promote the formation of pro-atherogenic small dense LDL and oxidized LDL [221]. Thus, elevated levels of TNF-α can be considered as a long-term prognostic value among apparently stable patients. The source of this persistent TNF-α elevated levels is still unknown, but it is possible to hypothesize that its origin derives from an increased infiltration of inflammatory cells in the peri-infarct zone[222]. From these evidences, it appears

quite clear that novel antiinflammatory therapies might represent a valid tool in the treatment of myocardial infarction[20,223,224].

Vascular inflammation and low-density lipoproteins:

As described before, LDL-cholesterol represents the most important and validated risk factor for atherosclerosis. Several studies tried to clarify the possible link between cholesterol and inflammation, speculating different mechanisms. In 2013, Grebe and Latz, hypothesized the involvement of NLRP3 inflammasome, that can be able to promote the amplification of the immune-inflammatory response. Moreover, elevated LDL-cholesterol levels are associated with single nucleotide polymorphisms in the CELSR2/PSRC1/SORT1 locus and in the APOE/APOC1/TOMM40 locus, recently assessed as inflammatory-related phenotypes [225]. There are also evidences of a possible link between cholesterol and proinflammatory cytokines. For example, is has been shown that modified LDL up regulate both IL-6, whose levels are predicting for future cardiovascular events [226] and correlate with carotid media-intima thickness [19] and IL-1, the last upregulated after the activation of NLRP3 inflammasome [227,228]. Among the different mechanisms postulated, one points toward the increase of plasma membrane cholesterol, through the involvement of the TLRs [229,230].

Extrahepatic function of PCSK9:

Despite PCSK9 is mainly expressed in the liver and exerts its principal function on hepatic LDL receptor, it is well known that not only the proprotein convertase is present also in other tissues, but it can exert diverse activities also in those tissue that are not involved in PCSK9 secretion [108]. For example, circulating hepatic PCSK9 interacts with pancreatic islets LDL receptor, degrading it [121,231]. PCSK9 null mice present abdominal, perigonadal and perineal fat accumulation, suggesting that the proprotein may be involved in adipogenesis. PCSK9 is also expressed in the brain and, after ischemic stroke, it has a relevant impact on LDL receptor expression in the lesion. Recently, Zimetti et al have investigated the role of PCSK9 on Alzheimer's disease, highlighting an increased PCSK9 concentration in cerebrospinal fluid of patients affected by the disease, even if the clinical relevance of this link needs to be more deepened Increased[232]. As said before, our research group has recently demonstrated the presence of PCSK9 also in the atherosclerotic lesion in vivo and it has evaluated the effect of

PCSK9 on macrophages LDL receptor in vitro, observing a significant degradation of the receptor[122].

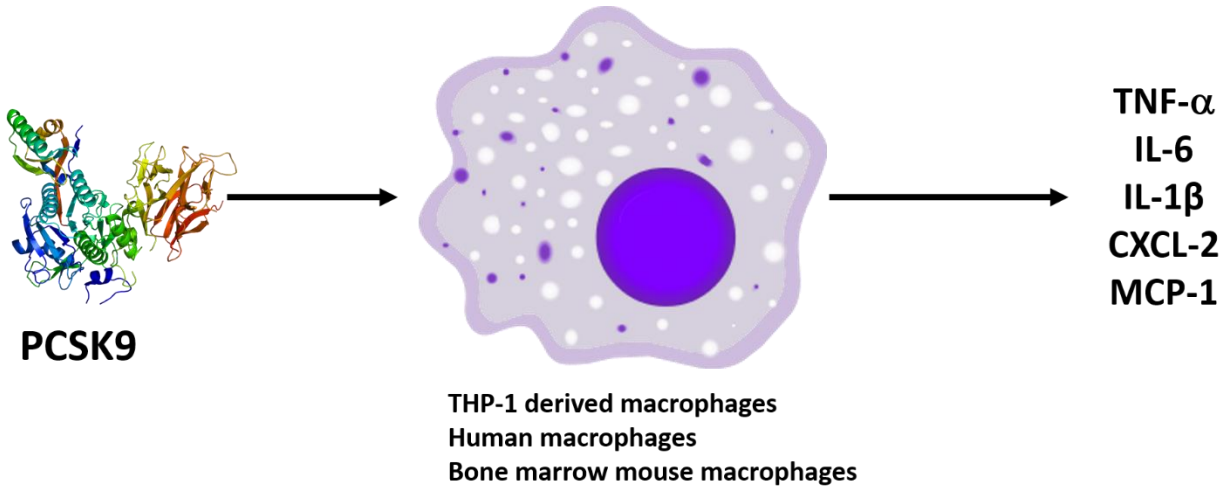
PCSK9 and inflammation

In the last few years, most of the research groups involved in the study of PCSK9 are moving their attention toward the possible involvement of this protein in inflammation, performing contrasting opinions and evidences. For example, in the Atheroremo IVUS Study[233,234], it is shown a positive correlation between PCSK9 and the atherosclerotic plaque necrotic core fraction and, consequently, between PCSK9 and plaque inflammation. PCSK9 knock-down macrophages show attenuate induction of pro-inflammatory cytokines in response to oxLDL[235]. Moreover, Dwivedi and his research group demonstrated how PCSK9 deficiency confers protection against tissue inflammation in mice, while its overexpression exacerbates proinflammatory states in early sepsis conditions [236]. Giunzioni *et.al*, showed an increased proinflammatory cytokine pattern in ApoE$^{-/-}$ transgenic mice, which express human PCSK9, effect that disappears in LDL receptor$^{-/-}$ transgenic mice [237] and Nozue *et.al*, demonstrated that PCSK9 positively correlates with oxidized LDL in patients with coronary artery disease [238]. Exogenous overexpresson of PCSK9 in macrophages enhanced the response to LPS, increasing IL-1β and TNF-α expression and decreasing anti-inflammatory markers such as Arg1 and IL-10, while PCSK9 null mice show blunted systemic response to LPS treatment[239]. Lastly, LDL receptor increased expression is associated with protection from severe sepsis[240]. After the authorization of the use of monoclonal antibodies anti PCSK9 in therapy, the first evidences of their possible effect on inflammation are coming out. In particular, by lowering PCSK9 and, consequently, lowering circulating LDL cholesterol, monoclonal antibodies showed a significant reduction in the pro-inflammatory monocytes phenotype, typical of patients affected by familial hypercholesterolemia[241]. In contrast, in patients with established coronary heart disease or in patients presenting high cardiovascular risk, the use of RG7652 (one of those monoclonal antibodies) did not affect neither systemic circulating pro-inflammatory cytokines, nor C-reactive protein[242]. In the first part of this work, we demonstrated that PCSK9 present a strong correlation with the proinflammatory JAK/STAT[243], so we decided to deepen this part of our research.

AIM OF THE STUDY (II)

From the previous part of this work, we know that PCSK9 hepatic expression is regulated by pro-inflammatory cytokines activation of the JAK/STAT pathway. The second part of the present study aimed to test the hypothesis of a direct pro-inflammatory effect of PCSK9 on THP-1 derived macrophages, human macrophages and bone marrow mouse macrophages.

Moreover, it has been observed that PCSK9 activities are dependent by its interaction with LDL receptor. We wanted to understand if the possible pro-inflammatory activity of PCSK9 was LDL receptor-dependent too, or if other receptors may be involved.



MATERIAL AND METHODS (II)

Reagents and antibodies:

RPMI media was obtained by Sigma, penicillin, streptomycin, nonessential amino acid solution, FCS, disposable culture flasks and petri dishes were from Euroclone. Molecular weight protein standards, SDS, TEMED, ammonium persulfate, glycine, and acrylamide solution (30%T, 2.6%C) were from BIO-RAD Laboratories. BCA assay for determination of protein concentrations was purchased from Thermo Scientific. Recombinant Tumor Necrosis Factor-α (TNF-α) was purchased from Sigma. Recombinant PCSK9 was obtained by Vinci Biochem. The JAK inhibitor JAK1 was purchased from Millipore and fatostatin hydrobromide from Sigma.

Cell culture:

THP1-derived macrophages: THP-1 monocyte cells were cultured in RPMI media supplemented with penicillin (10,000 U/mL), streptomycin (10 mg/mL), nonessential amino acid, 10% Fetal Calf Serum (FCS) and β-mercaptoethanol 0.05mM. In order to obtain THP-1 macrophages derived cells, THP-1 monocytes were seeded in appropriate multiwell plate and additioned with PMA (3.2 x 10-7M) for 72h.

Preparation of Human Macrophages: Human monocytes were obtained from healthy donors from Centro Cardiologico Monzino who gave their informed consent to participate in the study. *Blood sampling* - Venous blood samples were obtained by venipuncture of the antecubital vein with a 19G needle without venous stasis. After discarding the first 4 ml, blood was drawn into 4 ACD-containing vacutainers (Becton Dickinson, CA, USA) for monocyte isolation and into one Z-vacutainer (Becton Dickinson, Ca, USA) for serum preparation. Haemocrome was assessed by Sysmex XS-1000i hematologic analyzer (Kobe, Japan). *Serum preparation* - Serum was obtained by incubating blood at 37°C for 1 hour followed by centrifugation at 1700 g, 10 minutes at 4°C, transferred, under sterile conditions, into a new tube and stored at 4°C until use. *Monocytes isolation* - Immediately after withdrawal, ACD-anticoagulated blood was centrifuged at 100g, 10 minutes at room temperature with no brake. The platelet rich-plasma was completely removed and the remaining blood was mixed with RPMI (Lonza, 1640, Swiss) containing 0,1% L-glutamine (Thermo Fisher, USA), 0.5% Pen-strep (Thermo Fisher, USA), 20% Fetal Calf Serum (FCS, Euroclone, Italy) and 0.38% sodium citrate (Sigma, Germany) to restore the initial volume of blood in the tubes. Mononuclear cells were isolated by Ficoll-Paque Plus (GE Healthcare, vWR Int., Milan, Italy) density centrifugation at 600g, 20 minutes at room temperature with no brake.

Cells were washed with PBS (Thermo Fisher, USA) containing 0,1% glucose (Sigma, Germany), 0,5% BSA (Sigma, Germany) and 5 mM EDTA and centrifuged at 830g for 10 minutes at 4°C with brake. Cells were further washed twice in PBS without EDTA and centrifuged at 350g for 10 minutes at 4°C with brake. Mononuclear cells resuspended in RPMI supplemented with 10% autologous serum, 0,1% L-glutamine and 0.5% Pen-strep were then plated (100.000 monocytes/100 µl) in a 48 Well Cell Culture Plate (Corning Incorporated, USA) and incubated at 37°C. After 2 hours, cells were washed 3 times with PBS in order to remove non-adherent lymphocytes; adherent monocytes were then cultured in the same medium over 7 days at 37°C (5% CO2). Medium was not replaced throughout the culture period.

Murine bone marrow macrophages (BMM) isolation:

Bone marrow macrophages were collected from the femur and tibia of C57BL/6 or LDLR-/- mice. All experiments were conducted in conformity with the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and performed with the approval of the Ethical Committee for Animal Experiments of the University of Parma. After isolation, cells were seeded in 24 well plates in high glucose DMEM containing 30% L-929 cells conditioned medium and 10% FBS and maintained at 37°C, 5% CO2. After 4 days, non-adherent cells were removed and fresh medium was added. After additional 3 days, cells were differentiated and treated according to the protocol.

Generation of human PCSK9 expression construct and retroviral infection in HepG2 cells:

The retroviral expression plasmid encoding PCSK9-FLAG tag was constructed using the pBM-IRES-PURO plasmid. Human PCSK9-FLAG tag cDNA was kindly provided by Prof. P. Tarugi (University of Modena) and subcloned into retroviral expression plasmid by blunt-end ligation. Retroviral infections of HepG2 were performed as previously described (Material and methods I). A polyclonal population of HepG2 control and PCSK9 overexpressing cells have been then selected with 10µg/ml of puromycin.

HepG2^{PCSK9} and THP-1 co-culture: HepG2 and HepG2^{PCSK9} were cultured together with THP-1 derived macrophages in a transmembrane system (Transwell, Corning, Lowell, MA, USA) using polycarbonate membrane with 0.4 µm pores. HepG2 and HepG2PCSK9 cells were seeded in 6-well plates at a density of 6x105 cells/well in MEM with 10%FCS. After 3 days, HepG2 medium was discarded and THP-1 cells, suspended in fresh medium containing 3.2 x 10-7M of PMA, in

order to differentiate them in macrophages, were added in the transmembrane system to each well seeded with HepG2 and HepG2PCSK9 cells, at a density of 2x105 cells/well.

RNA preparation and quantitative real time PCR (qRT-PCR) assay:

THP-1 derived macrophages and human macrophages: THP1-derived and human macrophages (differentiated respectively from THP-1 monocytes and human monocytes as previously described), were seeded in 48 well plates (500.000 cells/well) and treated for 24h with PCSK9 0.25, 0.5, 1 and 2.5 □g/ml and TNF-α 10ng/ml as positive control. Total mRNA was extracted from the cells, after 24h of treatments, using iScript Sample Preparation Buffer (BIO-RAD laboratories, Hercules, CA, USA), according to manufacturer's instructions. Reverse transcription-polymerase first-strand cDNA synthesis was performed by using the iScript cDNA synthesis Kit (BIO-RAD laboratories, Hercules, CA, USA) and for Real time PCR were used the Kit Thermo Sybr Green/ROX qPCR Master Mix (Carlo Erba Reagents S.r.l. Cornaredo, Milan, Italy) and specific primers of the genes of interest (described below).

Murine bone marrow derived macrophages: The analyses were performed with the ABI Prism® 7000 Sequence Detection System (Applied Biosystems). PCR cycling conditions were as follows: 94°C for 3min, 40 cycles at 94°C for 15s, and 60°C for 1min. Data were expressed as Ct values and used for the relative quantification of targets with the ΔΔCt calculation. We evaluated gene expression of:

Table 2. Primer sequence utilized for the qPCR analysis.

Primer	**Forward**	**Reverse**
18S	5'-CGGCTACCACATCCACGGAA-3'	5'-CCTGTATTGTTATTTTTCGTCACTACC-3'
Human		
IL-6	5-'GGTACATCCTCGACGGCATCT-3'	5'-GTGCCTCTTTGCTGCTTTCAC
IL-1β	5'-ATGCACCTGTACGATCACTG-3'	5'-ACAAAGGACATGGAGAACACC-3'
TNF-α	5'-ACTTTGGAGTGATCGGCC-3'	5'-GCTTGAGGGTTTGCTACAAC-3'
CXCL-2	5'-CGCCCATGGTTAAGAAAATCA-3'	5'-CCTTCTGGTCAGTTGGATTTGC-3'
MCP1	5'-CGCCTCCAGCATGAAAGTCT-3'	5'-GGAATGAAGGTGGCTGCTATG-3'
LDLR	5'-GTGTCACAGCGCCG-3'	5'-CGCACTCTTTGATG-3'
CAP1	5'-ACTGGCCTGGAGCAAAACG-3'	5'-CGGCAGAGGGTCCAGATG-3'
Mouse		
TNF-α	5'-CCCTCACACTCAGATCATCTTCT-3'	5'-GCTACGACGTGGGCTACAG-3'

IL-6	5’-GAGGATACCACTCCCAACAGACC-3’	5’-AAGTGCATCATCGTTGTTCATACA-3’
IL-1β	5’-CAACCAACAAGTGATATTCTCCATG-3’	5’-GATCCACACTCTCCAGCTGCA-3’
CXCL-2	5’-CCAAGGGTTGACTTCAAGAAC-3’	5’-AGCGAGGCACATCAGGTACG-3’
MCP-1	5’-ACCACAGTCCATGCCATCAC-3’	5’-TTGAGGTGGTTGTGGAAAAG-3’
LDLR	5’-GTGTGACCGTGAACATGACTGC-3’	5’-CACTCCCCACTGTGACACTTGA-3’

ELISA assay:

THP-1 derived macrophages and human macrophages (differentiated respectively from THP-1 monocytes and human monocytes as previously described) were seeded in 6 well plates (3.000.000 cells/well) in RPMI 10%FCS and then treated with for 24h with PCSK9 0.25, 0.5, 1 and 2.5 μg/ml and TNF-α 10ng/ml as positive control. Conditioned media from treated cells was collected and used for the protein quantification of IL-6 and TNF-α, through ELISA kit (Human IL-6 Quantikine ELISA Kit and Human TNF-alpha Quantikine ELISA Kit, R&D Systems respectively).

Western Blot analysis:

THP-1 macrophages and human macrophages (differentiated respectively from THP-1 monocytes and human monocytes as previously described) were seeded in 6well plate, in a concentration of 3.000.000 cells/well. Cells were treated for 24h with PCSK9 0.25, 0.5, 1 and 2.5 μg/ml and TNF-α 10ng/ml as positive control and, the day after, total cytosolic protein extracts were obtained by collecting cells in 200μl of Mammalian Protein Extraction Reagents (Thermo Fisher Scientific, MA, USA) containing a cocktail of protease and phosphatase inhibitors (Roche Diagnostics S.p.A., Monza, Italy). Molecular mass marker (Novex® Sharp Protein Standard, InvitrogenTM; Life Technologies Europe BV, Milan, Italy) and proteins were separated through 10-12% SDS-PAGE gel. Proteins were then transferred to a nitrocellulose membrane and blocked with albumin buffer containing 0.05% of TWEEN. Incubation with primary antibodies occurred overnight at 4°C with the following antibodies: LDL Receptor rabbit Polyclonal Antibody 1:200 (Cayman Chemical, 1180 East Ellsworth RD Ann Arbor, MI 48108 – USA), and α-tubulin (Sigma-Aldrich) 1:5000. Membrane was incubated with anti-mouse and anti-rabbit peroxidase-conjugated secondary antibodies (1:5000) (Jackson ImmunoResearch Lab; Cambridgeshire, UK). Immunoreactive bands were detected by acquiring images with Odyssey®, LI-COR Biosciences, Carlo Erba, Milan, Italy. Densitometric readings were evaluated using the Image Studio Software. Results were normalized with α-tubulin.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde at room temperature for 10 min, permeabilized in 0.1% Triton X-100 in PBS for 5 min, and incubated for 1 h with 1% bovine serum albumin (BSA). Cells were then incubated with primary antibody anti NF-κB p65 (Rabbit polyclona GeneTex) for 1 h at room temperature, followed by three washes with PBS and subsequent incubation with Alexafluor®-568 anti-rabbit antibody. Cells were then washed four times with PBS, incubated with DAPI solution for 5 min, mount and coversliped with Vectashield. Immunostaining of cells was analyzed by fluorescent confocal microscope (Zeiss LSM 800).

Subjects

Study participants were patients enrolled in the Brisighella Heart Study. All the involved subjects have signed an informed consent form. The Brisighella Heart Study protocol and its substudies have been evaluated and approved by the Ethical Board of the S. Orsola-Malpighi University Hospital (Bologna, Italy). We selected a sample of overall healthy adult subjects (M: 533, F: 537), after exclusion of active smokers, those subjects affected by chronic inflammatory disorders (including atopic diseases) and subjects chronically assuming non-steroidal antinflammatory drugs, systemic corticosteroids, or immunosuppressants 16. A Pearson's bivariate correlation followed by a multiple linear regression have been carried out to evaluate the eventual relationship between TNF-α and PCSK9 plasma levels in humans.

Analysis of data

Statistical analysis. Statistical analysis was performed using the Prism statistical analysis package version 5.01 (GraphPad Software). 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 (II)

PCSK9 exerts a pro-inflammatory activity on THP-1 derived macrophages:

To test the hypothesis that PCSK9 could have a pro-inflammatory effect on macrophages, we performed a series of experiments with macrophages derived from human monocyte cell line THP-1 differentiated with PMA, incubated with increasing concentrations of human recombinant PCSK9. The incubation for 24h induced the mRNA levels of proinflammatory cytokines, markers of M1 phenotype, IL-1β, IL-6 and TNF-α (Figure 1A, B and C). In particular, the effect of hPCSK9 on IL-1β appears to be more concentration-dependent, with a significant induction from 250 ng/ml up to 2.5 µg/ml (Figure 1A). The mRNA levels of TNF-α also increased in a concentration-dependent manner, although a significant difference was observed between 1 and 2.5 µg/ml (5.1±1.6 fold vs 67.4±25.9 fold, respectively) (Figure 1B). Similar effect was also observed for the mRNA of IL-6, where hPCSK9 strongly induced its levels (36.4±19.3Fold) exclusively at 2.5 µg/ml with no effect from 250 ng/ml and 1 µg/ml (Figure 1C). The determination of mRNA levels of monocyte chemokines, such as MCP-1 and CXCL-2 also revealed a positive effect of hPCSK9 (Figure 1 D and E), with a concentration dependent response for MCP-1 (similar to IL1-β) and a strong induction at 2.5 µg/ml for CXCL-2 (similar to TNF-α). THP1 macrophages were also incubated with TNF-α (10 ng/ml) as positive control. Importantly, the effect of 250 ng/ml hPCSK9, thus within its physiological serum concentrations (median 270 ng/mL, ranging from 91 to 804 ng/mL[234]), exerted a similar pro-inflammatory effect than TNF-α.

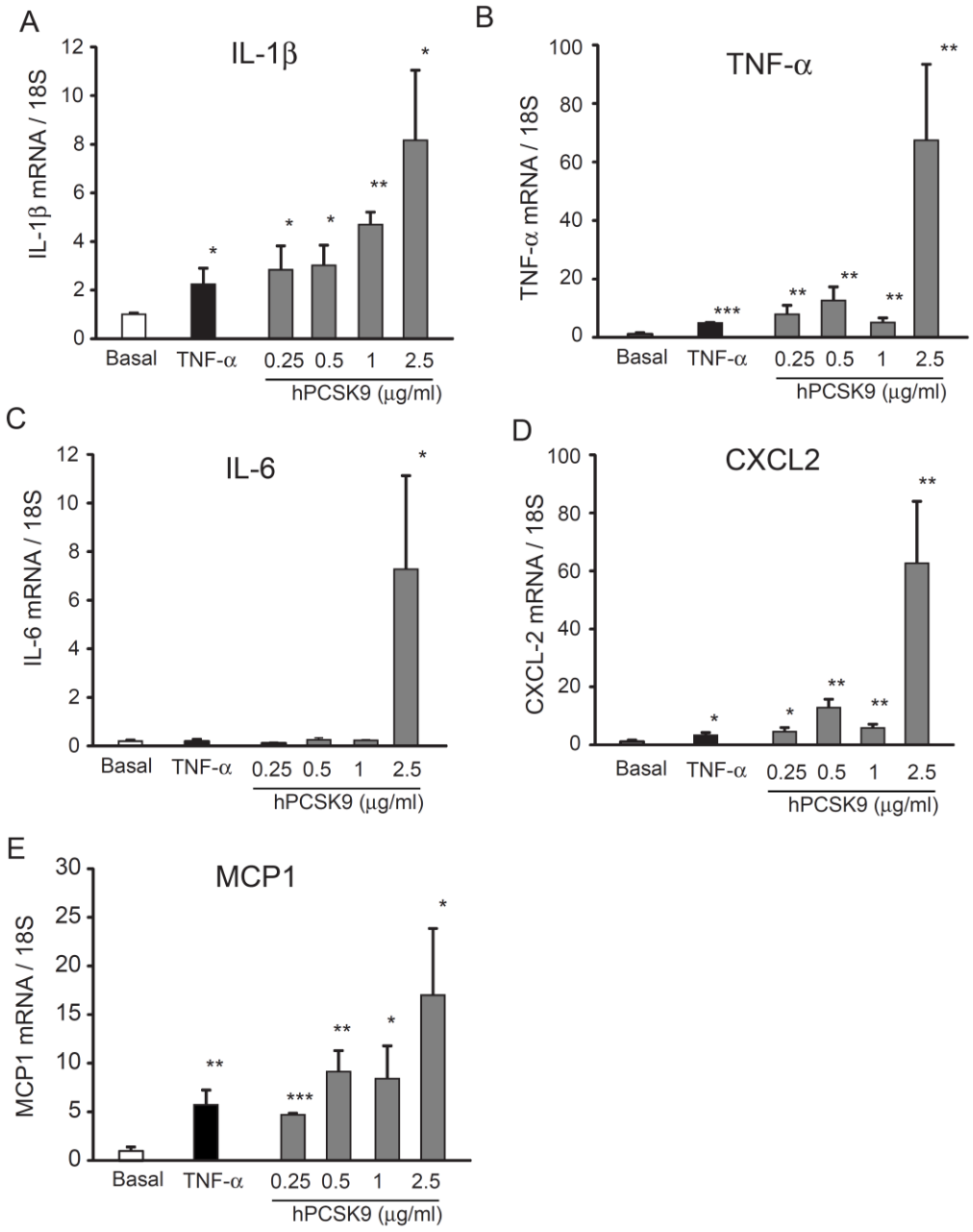


Figure 1: Increased gene expression of different proinflammatory cytokines in THP-1 derived macrophages, in response to hPCSK9. THP-1 monocytes were seeded in 48 well plate (500.000 cells/well) in RPMI 10%FCS and differentiated in macrophages with PMA 3.2 x10-7M for 72h. THP-1 derived macrophages were then incubated for 24h with TNF-α 10ng/ml (as positive control) and different concentration of recombinant hPCSK9 (0.25, 0.5, 1 and 2.5 μg/ml). At the end of the incubation, total RNA was extracted and (A) IL-1β, (B) IL-6, (C) TNF-α, (D) CXCL-2 and (E) MCP-1 gene expression was evaluated through qRT-PCR. Differences between basal and treated samples were assessed by Student's t-test: *p<0.05; **p<0.01; ***p<0.001.

The equimolar concentration of albumin did not induce pro-inflammatory response on THP-1 macrophages:

In order to assess if the results obtained were due to the incubation of THP-1 derived macrophages with an high amount of protein, we treated cells with the equimolar concentration of albumin (BSA), following the same experimental condition used for the incubation with PCSK9. We did not observe any pro-inflammatory response in cells after 24h of incubation.

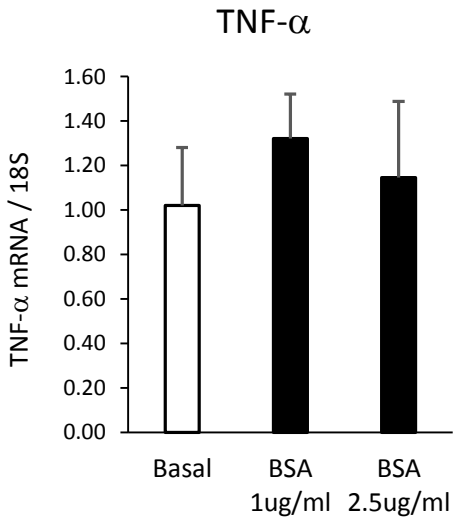


Figure 2: The treatment with an equimolar concentration of BSA does not induce pro-inflammatory response in THP-1 derived macrophages. THP-1 monocytes were seeded in 48 well plate (500.000 cells/well) in RPMI 10%FCS and differentiated in macrophages with PMA 3.2 x10-7M for 72h. THP-1 derived macrophages were then incubated for 24h with two concentration of BSA (1 and 2.5 μg/ml). At the end of the incubation, total RNA was extracted and TNF-α gene expression was evaluated through qRT-PCR. Differences between basal and treated samples were assessed by Student's t-test: *p<0.05; **p<0.01; ***p<0.001.

The incubation with human recombinant PCSK9 activates NF-κB transcription factor

Since transcription of the majority of proinflammatory cytokines are under the control of nuclear factor-κB (NF-κB) transcription factor, we investigated the effect of PCSK9 on the nuclear localization of p65 subunit in THP-1 derived macrophages. As expected, the incubation with TNF-α induces a marked response of NF-κB activation with positive nuclear staining of p65

cells NF-κB in approximately 30% of the cells, while no nuclear staining was observed under basal condition. Interestingly, the incubation with 2.5µg/ml of PCSK9 induced a significant NF-κB activation, with 16% of the cells with positive nuclear staining for p65 (Figure 3).

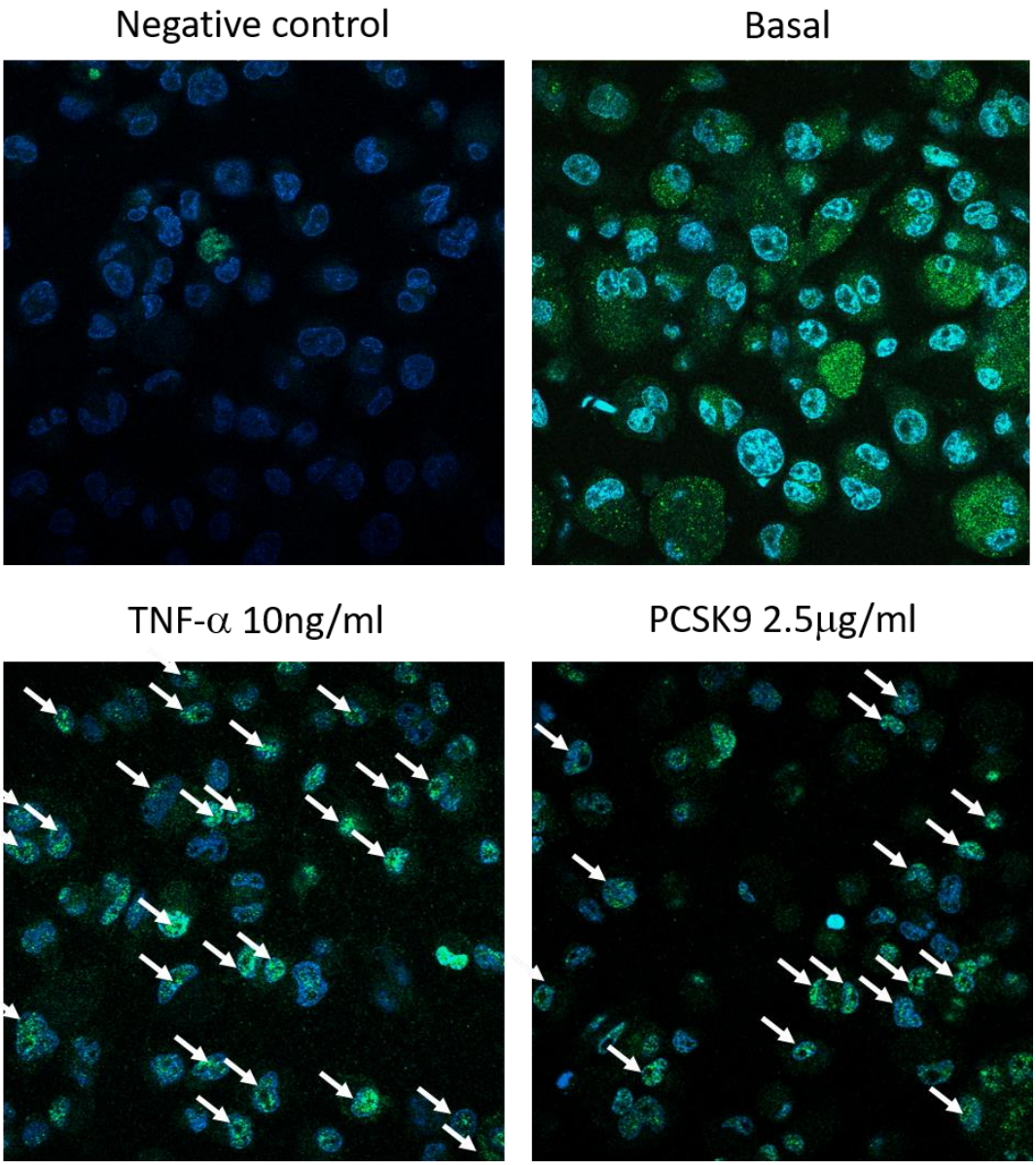


***Figure 3: PCSK9 increases NF*-κB nuclear activation.** THP-1 macrophages were incubated with TNF-α 10ng/ml (as positive control) and recombinant hPCSK9 (2.5 µg/ml). After 24h cells were fixed and immunostaining performed for NF-κB p65 (blue: nuclei; green: p65 NF-κB). Arrows indicate cells with positive nuclear staining of p65.

PCSK9 exerts a pro-inflammatory activity on human macrophages from healthy volunteers:

To extend our observation, we performed the same analysis on human macrophages derived from plasma of healthy volunteers. A concentration-dependent effect of hPCSK9 on mRNA levels of IL-1β, IL-6 and TNF-α was observed, with a significant induction at 1 μg/ml and 2.5 μg/ml for all genes. As observed with THP-1, hPCSK9 induced IL-1β also at 250 ng/ml. A significant increase of MCP1 and CXCL2 mRNA levels were observed after incubation with 2.5 μg/ml of hPCSK9 (Figure 4).

To further validate our observation, we determined, by ELISA assay, the effect of hPCSK9 on IL1-β and IL-6 released by human macrophage in the cultured media. As shown in Figure 5, 24h incubation with 2.5 μg/ml hPCSK9 significantly increased both IL-6 and TNF-α concentration (3.6±1.3 fold and 4.5±0.2 fold, respectively).

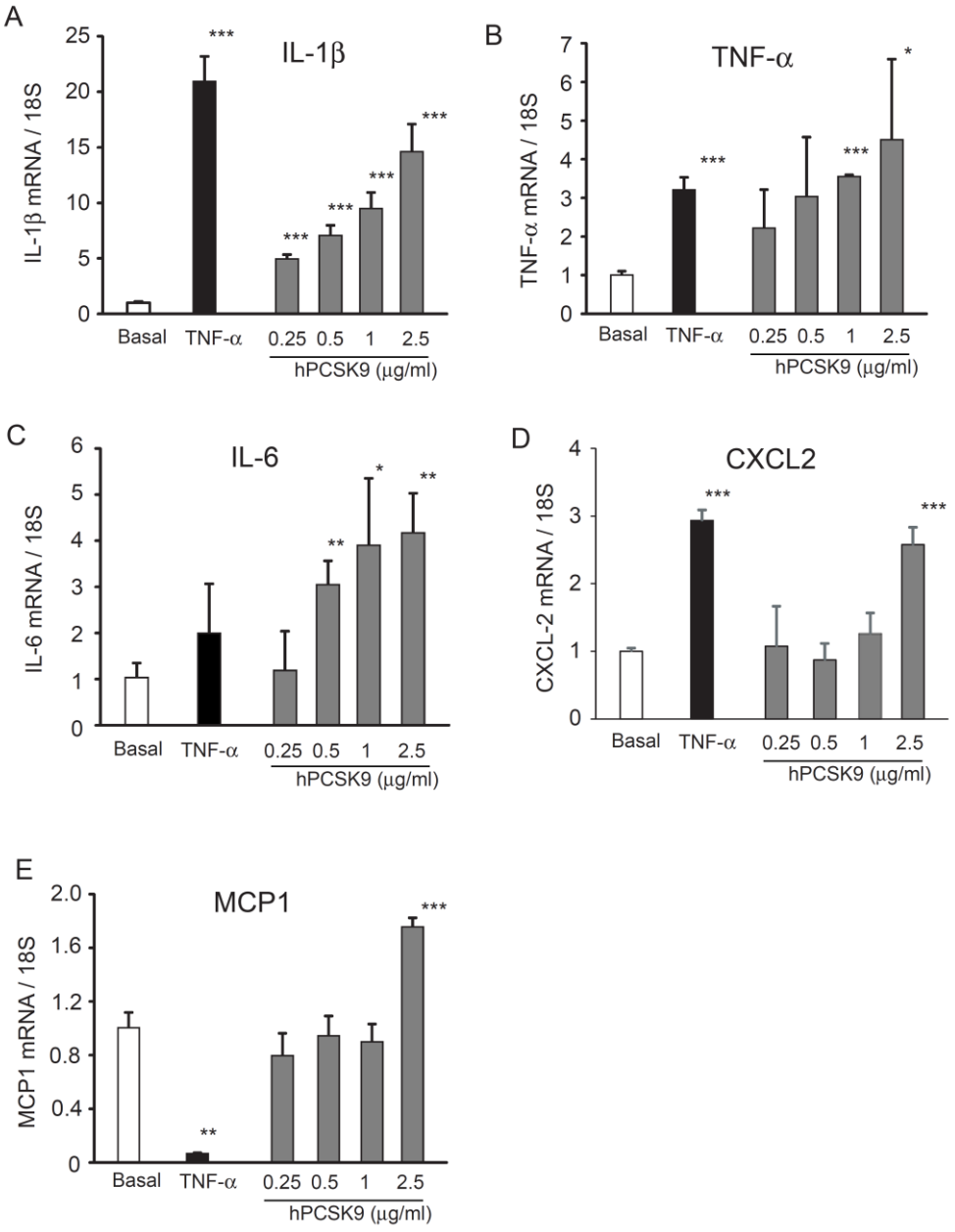


Figure 4: Increased gene expression of different proinflammatory cytokines in human macrophages, in response to hPCSK9. Human monocytes were seeded in 48 well plate (500.000 cells/well) in RPMI additioned with 10% of the volunteer autologous serum for 1 week, in order to differentiate them in macrophages. After incubation for 24h with TNF-α 10ng/ml (as positive control) and different concentrations of recombinant hPCSK9 (0.25, 0.5, 1 and 2.5 g/ml), quantitative (A) IL-1β, (B) IL-6, (C) TNF-α, (D) CXCL-2 and (E) MCP-1 gene expression analysis were performed from total RNA extracted from cells. Differences between basal and treated samples were assessed by Student's t-test: *p<0.05; **p<0.01; ***p<0.001.

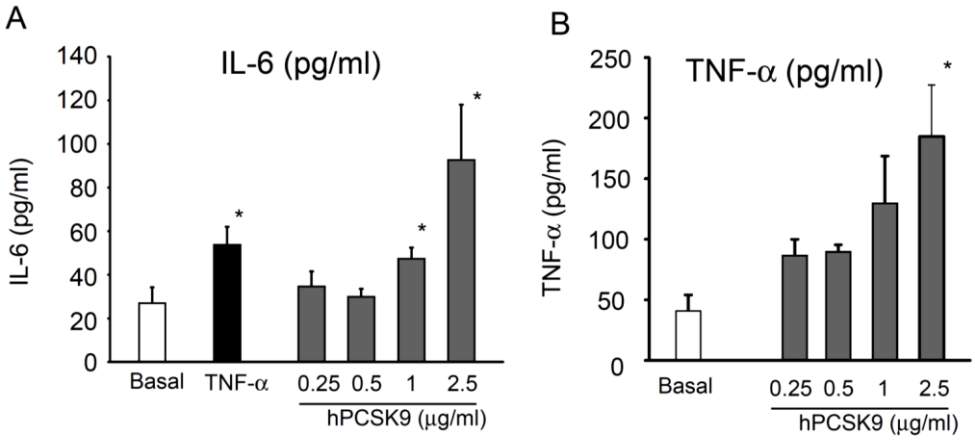


Figure 5: Increased IL-6 and TNF-α protein expression in human macrophages media, in response to hPCSK9. Human monocytes were seeded in 6 well plates in RPMI containing 10% of the volunteer autologous serum and incubated for 1 week in order to differentiate them in human macrophages. Macrophages were then incubated for 24h with TNF-α 10ng/ml (as positive control) and different concentrations of recombinant hPCSK9 (0.25, 0.5, 1 and 2.5 μg/ml). IL-6 and TNF-α protein expression was then assessed through ELISA analysis on collected media. Results were expressed as pg of protein for ml of media. Differences between basal and treated samples were assessed by Student's t-test: *p<0.05; **p<0.01; ***p<0.001.

Taken together, recombinant hPCSK9 induced a pro-inflammatory response in both THP1 and human macrophages, showing a robust induction of mRNA of IL-1β, IL-6, TNF-α, MCP1, CXCL2, and an increased IL-1β and TNF-α released from human macrophages.

PCSK9 levels positively correlate with TNF-α plasma concentration in Brisighella Heart Study population

To further corroborate our results, we investigated a possible correlation between PCSK9 and TNF-α plasma levels from a selected overall healthy adult subjects (males 533, females 537) enrolled in the Brisighella Heart Study[244]. The main characteristics of the selected subjects are resumed in Table 3. The Pearson's bivariate correlation between TNF-α and PCSK9 showed a correlation coefficient of 0.388 with a p<0.001 (Figure 6). After adjustment for age, gender, and BMI, in the multiple linear analysis, TNF-α and PCSK9 plasma levels were significantly related

(B= 8.73, 95%CI 7.54÷9.93, p<0.001 (Figure 6). This evidence further support a relationship between chronic inflammation and PCSK9 levels.

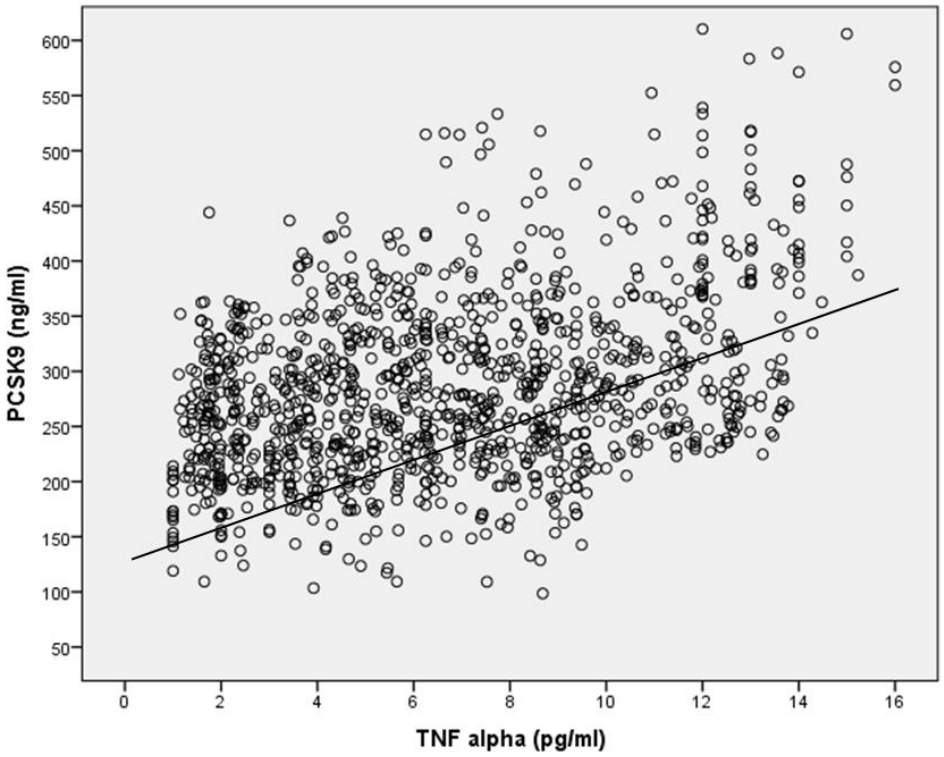


Figure 6: Relationship between TNF-α and PCSK9 serum levels. Results were determined by ELISA assay from human samples.

	Mean ± SD
AGE	57.66 ± 11.77
BMI	23.33 ± 3.46
SBP	130.02 ± 11.37
DBP	67.78 ± 7.12
TC	218.12 ± 19.38
TG	118.29 ± 69.35
HDL-C	51.97 ± 5.54
LDL-C	141.58 ± 18.31
FPG	93.24 ± 5.61
SUA	5.215 ± 1.29
GOT	23.17 ± 7.59
GPT	24.13 ± 9.32
gGT	25.67 ± 13.47
Creatinine	1.03 ± 0.19
eGFR (CKD-EPI)	81.30 ± 15.41
PCSK9 (ng/ml)	286.25 ± 81.14

TNF alpha (pg/ml)	6.69 ± 3.60

Table 3: main characteristics of the selected subjects of the Brisighella Heart Study

PCSK9 secreted from HepG2^{PCSK9} induces a pro-inflammatory response in THP-1 derived macrophages

To exclude a possible effect of endotoxins present in recombinant hPCSK9 and to further establish the pro-inflammatory effect of PCSK9, we performed co-cultured experiments of THP-1 macrophages and HepG2 control or overexpressing PCSK9. HepG2 were transduced with pBM-IRES-PURO retrovirus encoding control vector (PURO) or human PCSK9. After puromycin selection, we measured the amount of PCSK9 released in the cultured media by ELISA assay. While control HepG2 cells released 1.7 ng/ml of PCSK9, the HepG2PCSK9 reached approximately 100 ng/ml (90±0.1 ng/ml) (Figure 7A). The co-culture with THP1 macrophage showed that the exposure to conditioned media from HepG2^{PCSK9} significantly induced the pro-inflammatory genes TNF-α and IL-1β, by 2.4±0.5 fold and 8.6±1.8 fold, respectively, as compared to HepG2 (Figure 6B and C).

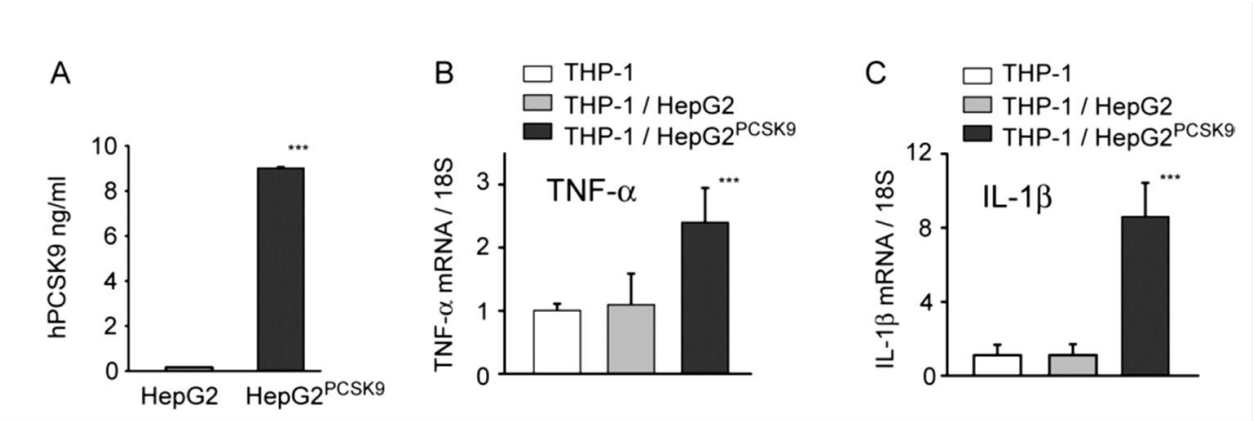


Figure 7: PCSK9 released from HepG2 increased TNF-α and IL-1β gene expression in THP-1 derived macrophages. THP-1 macrophages were co-cultured with HepG2 or HepG2PCSK9 seeded on top of the transwell system. After 24h, total RNA was extracted from THP-1 macrophages and TNF-α and IL-1β gene expression was evaluated by qRT-PCR. Differences were assessed by Student's t-test (A) or one-way ANOVA (B, C), *p<0.05; **p<0.01; ***p<0.001.

PCSK9 pro-inflammatory effect is mainly, but not entirely, due to LDL receptor interaction

Since the main molecular target of PCSK9 is the LDL receptor, we performed the same analysis on bone marrow macrophages (BMM) isolated from C57BL/6 and $LDLR^{-/-}$ mice. The incubation of BMM macrophage $LDLR^{+/+}$ with increasing concentrations of hPCSK9 determined a significant induction of the LDL receptor mRNA (Figure 8A), and a downregulation of LDL receptor (-33% at 2.5 μg/ml), as assessed by western blot analysis (Figure 8B). Under the same experimental conditions, BMM $LDLR^{+/+}$ respond to 2.5 μg/ml hPCSK9 by increasing the expression of TNF-α (31.1±6.1 fold), while no significant effect was observed at lowest PCSK9 concentrations (Figure 8C). Interestingly, the BMM $LDLR^{-/-}$ showed only a marginal increase of TNF-α expression (4.3±1.6 fold), which is significantly lower than that observed in BMM $LDLR^{+/+}$. The different response to PCSK9 is further validated by the fact that both macrophage types respond at similar extent to TNF-α (5.7±1.5 vs 4.9±0.3 fold for BMM $LDLR^{+/+}$ and BMM $LDLR^{-/-}$, respectively) (Figure 8C). Taken together, the present results suggest that the pro-inflammatory response of PCSK9 on macrophages is mainly, but not exclusively, dependent by the presence of the LDL receptor.

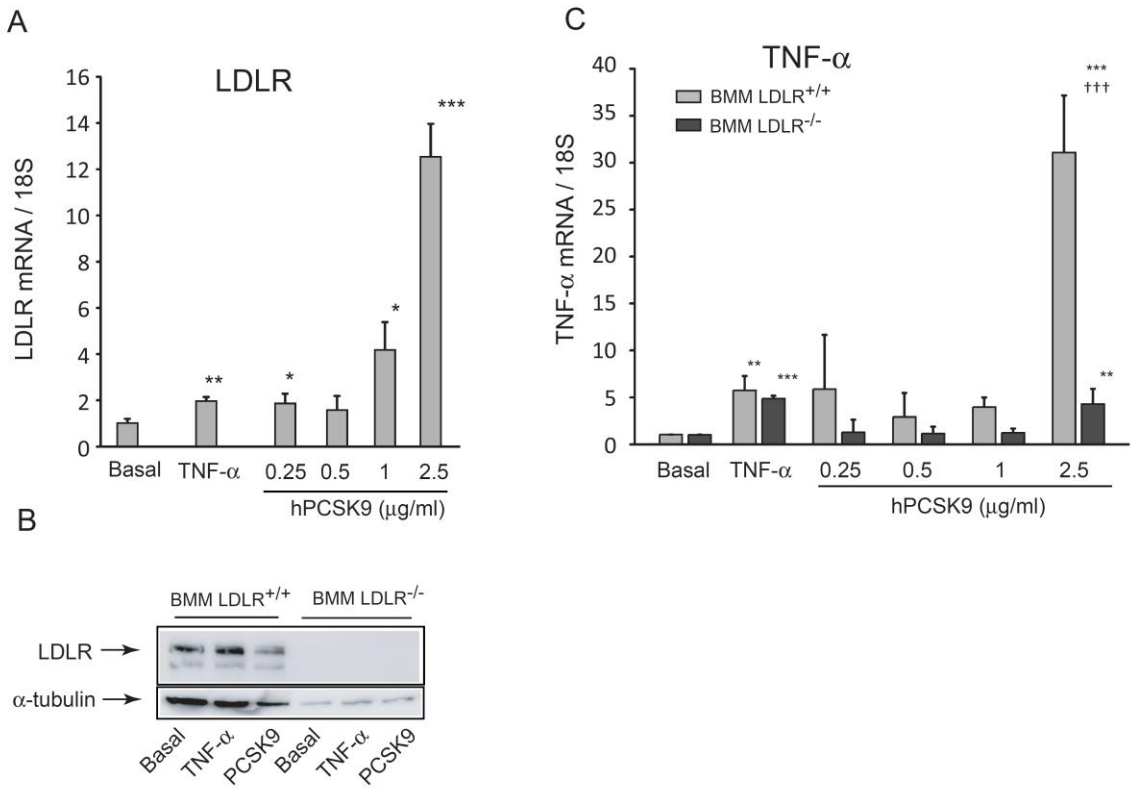


Figure 8: Lower increase of TNF-α gene expression in murine LDLR-/- BMM after treatment with recombinant hPCSK9. Both WT and LDLR-/- murine BMM were incubated for 24h with TNF-α 10ng/ml (as positive control) and different concentrations of recombinant hPCSK9 (0.25, 0.5, 1 and 2.5 μg/ml). Total RNA and total proteins were extracted and (A) TNF-α gene expression was evaluated. (B) The absence of LDLR in LDLR-/- murine bone marrow macrophages was confirmed by qt-PCR from total RNA and (C) by western blot analysis from total protein extracts. Differences between basal and treated samples (*) and between BMM LDLR+/+ and BMM LDLR-/- (†) were assessed by Student's t-test (A) or one-way ANOVA (B, C), *p<0.05; **p<0.01; ***p<0.001. †p<0.05; ††p<0.01; †††p<0.001.

The pharmacological inhibition of JAK/STAT pathway and SREBP totally abrogates pro-inflammatory response after the treatment with PCSK9 in THP-1 macrophages

Interestingly, the pharmacological inhibition of Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) signaling, by a JAK inhibitor, and of SREBP pathway, by fatostatin, completely prevented the induction of the TNF-α mRNA by PCSK9.

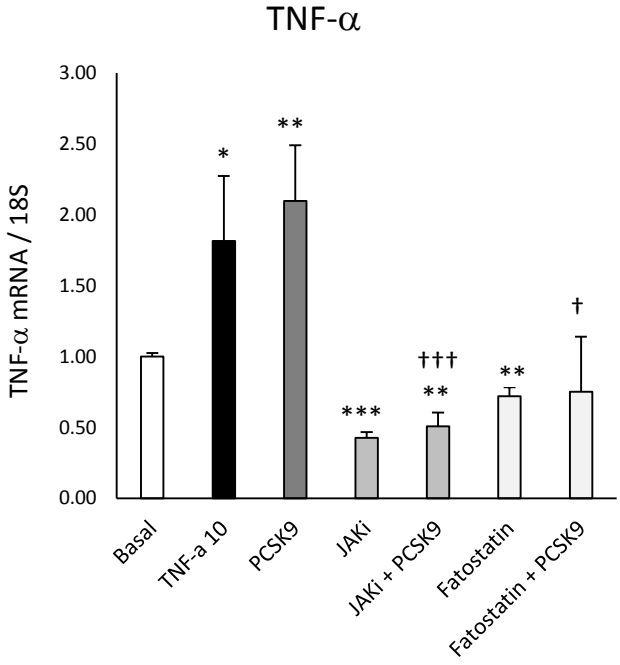


Figure 9: The pharmacological inhibition of both JAK/STAT and SREBP pathways prevents the pro-inflammatory response to PCSK9 treatment in THP-1 macrophages. THP-1 monocytes were seeded in 48 well plate (500.000 cells/well) in RPMI 10%FCS and differentiated in macrophages with PMA 3.2 x10^{-7}M for 72h. THP-1 derived macrophages were then incubated for 24h with TNF-α 10ng/ml (as positive control), PCSK9 2.5μg/ml, the pharmacological inhibitor of the JAK/STAT pathway, JAKi, at a concentration of 10^{-5}M and the pharmacological inhibitor of SREBP, fatostatin, at a concentration of 100μM. We incubated cells also with JAKi 10^{-5}M + PCSK9 2.5μg/ml and fatostatin 100μM + PCSK9 2.5μg/ml combinations. At the end of the incubation, total RNA was extracted and TNF-α gene expression was evaluated through qRT-PCR. Differences between basal and treatments (*) and between PCSK9 2.5μg/ml treatment and JAKi 10^{-5}M + PCSK9 2.5μg/ml and fatostatin 100μM + PCSK9 2.5μg/ml treatments (†) were assessed by one-way ANOVA, *p<0.05; **p<0.01; ***p<0.001. †p<0.05; ††p<0.01; †††p<0.001.

DISCUSSION (II)

In the present study, we demonstrated that human recombinant PCSK9 drives an inflammatory response on macrophages by inducing the pro-inflammatory cytokines TNF-α, IL-1 and IL-6, and the chemokines MCP-1 and CXCL2. This effect was observed in THP-1 (Figure 1), human primary (Figure 4), and bone marrow derived macrophages (Figure 8B). In addition, the inflammatory response was observed when THP-1 macrophages were co-cultured with HepG2 cells overexpressing PCSK9 (Figure 7). This data excludes the possibility that endotoxin contamination of human recombinant PCSK9 can be responsible for the proinflammatory effect observed. Finally, we found a positive correlation between plasma levels of PCSK9 and TNF-α, in a population of overall healthy subjects [245] (Figure 6 – Brisighella Hearth Study), an observation that further support the role of PCSK9 on inflammation. Macrophages present in the atherosclerotic plaque are derived, mainly but not exclusively, from circulating monocytes that differentiated into macrophages within the arterial wall [246]. The main function of macrophages is to scavenge lipoprotein particles, and eventually to become foam cells [247], which then contribute to a local release of inflammatory molecules and factors that further promote lipoprotein retention and extracellular matrix degradation [248]. Macrophages are very heterogeneous cells that can polarized to different phenotypes in response to the environmental cues encountered [249]. Local cytokines, as well as oxidized LDL, have a very potent effect on macrophage polarization and our data show that PCSK9 is also capable to influence the pro-inflammatory status of macrophages [250]. The discovery that PCSK9 is present in the atherosclerotic plaque argues a possible local effect on SMCs, macrophages and endothelial cells [122]. PCSK9 can reach the plaque from the bloodstream, as it binds to lipoproteins [251], or been synthesized within the arterial wall from SMCs [122,252]. Both sources can be theoretically possible since both protein and mRNA PCSK9 have been detected in the human atherosclerotic plaques [122,252]. Although the intraplaque concentration of PCSK9 is not known, it is important to underlying that we observed a pro-inflammatory effect on macrophages at relatively low concentrations, starting from 250 ng/ml, thus even below the mean plasma levels found in observational studies [245]. The translation of our findings to experimental atherosclerosis and human pathology still needs to be determined. However, alirocumab, a monoclonal antibody anti PCSK9, has shown to influence the atherosclerotic plaque development towards a more stable characteristic with reduced macrophage content and higher deposition of extracellular matrix [253]. In addition, the treatment with 14 monoclonal antibodies anti PCSK9 reduces the CCR2 expression and the migratory capacity of circulating monocytes in familial

hypercholesterolaemia (FH) patients. This effect seems to be due to a reduction of both LDL-C circulating levels and intracellular lipid accumulation. This evidence suggest a possible association between LDL-C lowering and anti-inflammatory effect on circulating monocytes [241]. In addition, the ATHEROREMO-IVUS study demonstrated a linear relationship between fraction and amount of necrotic core tissue in coronary atherosclerosis and PCSK9 plasma levels, independently of serum LDL cholesterol levels [234]. In the attempt to define the molecular mechanism of the pro-inflammatory effect of PCSK9, we have utilized macrophages derived from LDL receptor null mice. From these analysis, we observed that the presence of the LDL receptor is required for the effect of PCSK9, although a significant induction of TNF-α was still present in BMM LDLR-/- (Figure 8). Thus, additional receptors targeted by PCSK9, such as CD36 [254], VLDLR, LRP-1 [255] or ApoER2 [256], could participate in the pro-inflammatory response. Our data are in agreement with in vitro [237] and in vivo experimental settings [257]. By using chemical inhibitors, we also demonstrated that the proinflammatory effect of PCSK9 is dependent by the activation of JAK and SREBP pathways (Figure 9). Although the regulation of the intracellular molecular pathways by PCSK9 requires further investigations, it is relevant to note that THP-1 derived macrophages showed a nuclear localization of p65 NF-κB proinflammatory transcription factor, although at lower extent than the TNF-α (Figure 3). This evidence further support a prof-inflammatory function of PCSK9 as potential cytokine. Native LDL, a natural LDL receptor ligand, has recently shown to promote the differentiation of monocytes to macrophages [258]. In particular, LDL facilitated the M1 polarization, blocked the ability of monocytes to polarize into M2 macrophages, and enhanced the inflammatory M1 response [258]. From this evidence, it is conceivable to hypothesize that LDL particles and PCSK9 activate similar intracellular signaling pathways by interacting with the LDL receptor, which induce a pro-inflammatory effect on macrophages. By using a bone marrow transplantation model, Fazio et al, have investigated the effect of specific PCSK9 overexpression in macrophages on experimental atherosclerosis [237]. Despite no differences on lipid profile, the lesions of mice with macrophages expressing the PCSK9 transgene, showed LDL receptor-dependent increase of pro-inflammatory monocytes [237]. In line with our finding, the expression of PCSK9 increased the presence of CD11b- and Ly6Chi-positive cells in spleens of 15 apoE−/− mice [237]. Lastly, the presence of PCSK9 significantly improves the pro-inflammatory response to lipopolysaccharide (LPS) [237]. Thus, our findings are in agreement with this study and further extend the pro-inflammatory effect of PCSK9 in the absence of a co-stimulus, such as LPS. On this matter, it is important to mention that

lipoproteins and their receptors play a key role during sepsis as they favor the hepatic clearance of endotoxins, such as LPS. Indeed, PCSK9 null mice show higher hepatic LDL receptor expression and improved clearance of bacterial endotoxin via the LDLR pathway, and a higher resistance to LPS-induced septic shock [239]. Together with pro-inflammatory cytokines, PCSK9 significantly induced two chemokines relevant for monocyte recruitment in the atherosclerotic plaque, such as MCP-1 [259] and CXCL2 [260] (Figure 1D, E and Figure 2D, E). From this in vitro observation, it is tempting to speculate that PCSK9 could promote a local inflammatory response by reinforcing the recruitment of circulating monocytes and neutrophils in the atherosclerotic plaque. Nevertheless, additional and more specific experiments needs to be performed in order to demonstrate this action. In the attempt to translate our findings to a clinical setting, we performed a correlation analysis between the plasma levels of PCSK9 and TNF-α in a population of healthy subjects recruited in the Brisighella Heart Study [244]. For the analysis, we excluded those subjects affected by chronic inflammatory disorders, and those chronically assuming non-steroidal antinflammatory drugs, systemic corticosteroids, or immunosuppressants. Interestingly, a positive association between the two factors was found, supporting the possibility that either TNF-α drives the expression of PCSK9 [243], or vice versa PCSK9 induces TNF-α (Figure 6). In conclusion, in the present study we provided evidence for a direct pro-inflammatory effect of PCSK9 on macrophages, a pathophysiological relevance of this in vitro observation required further investigations.

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