UNIVERSITÀ DEGLI STUDI DI MILANO

Facoltà di Scienze del Farmaco

Dipartimento di Scienze Farmacologiche e Biomolecolari

PhD program in

Pharmacological Sciences, Experimental and Clinical

PhD cohort XXII

Deciphering the role of adipokines on PCSK9 regulation: experimental evidence

(BIO/14)

PhD candidate
Dott. Margherita Botta
R11541

Supervisor:
Prof. Alberto Corsini

Co-Supervisor:
Dott. Massimiliano Ruscica

PhD program coordinator
Prof. Alberico L.Catapano

A.A. 2018-2019

INDEX

LIST OF ABBREVIATIONS

AKT, RAC-alpha serine/threonine-protein kinase

ALB, albumin

APOB, apolipoprotein B

ApoCIII, apolipoprotein CIII

ApoE2R, apolipoprotein E2R

ASCDV, atherosclerotic cardiovascular disease

BMI, Body Mass Index

CAD, cardiac artery disease

CAP1, adenylyl cyclase associated protein 1

CD36, cluster of differentiation 36

CHD, coronary heart disease

CNX, calxexin

CTR, control

CV, cardiovascular

CVD, cardiovascular disease

EDTA, Ethylenediaminetetraacetic acid

EGF-A, epidermal growth factor A

ELISA, enzyme-linked immunosorbent assay

EV, empty vector

FBS, fetal bovine serum

FCS, familial chylomicronemia syndrome

GOF, gain of function

HeFH, hetrozygote familial hypercholesterolemia

HFD, high fat diet

HMGCR, 3-Hydroxy-3-Methylglutaryl-CoA Reductase

HNF1, hepatic nuclear factor 1

HoFH, homozygote familial hypercholesterolemia

ICAM1, Intercellular Adhesion Molecule 1

IDL, intermediate density lipoprotein

IL-1, interleukin 1

IL-18, interleukin 18

IL-1B, interleukin 1B

IL-6, interleukin 6

IL-8, interleukin 8

IRS1-2, insulin receptor substrate 1-2

JAK, janus kinase

LDL, low density lipoprotein

LDL-C, low density lipoprotein cholesterol

LDLR, low density lipoprotein receptor

LEP, leptin

LOF, loss of function

Lp(a), lipoprotein a

LPDP, lipoprotein deprived plasma

LPL, lipoprotein lipase

MAPK, mitogen-activated protein kinase

MCP1, monocyte chemoattractant protein 1

MEM, minimum essential medium

MetS, metabolic syndrome

M-PER, mammalian protein extraction reagent

MTP, microsomal triglycerides transfer protein

NAFLD, non-alcoholic fatty liver disease

NARC-1, neural apoptosis-regulated convertase 1

NPC1L1, Niemann-Pick C1-Like 1

OB, obese

OxLDL, oxidized low density lipoprotein

PA, pancreatic attack

PBS, phosphate buffer saline

PCR, polymerase chain reaction

PCSK9, proprotein conventase subtilisin/kexin 9

PI3K, phosphatidylinositol 3-kinase

PPAR, peroxisome proliferator-activated receptor

PSSM, position-specific scoring matrix

RELM, resistin-like molecule

RT-qPCR, reverse transcription- quantitative polymerase chain reaction

SEM, standard error of mean

SOCS3, suppressor of cytokine signalling 3

SR-BI, scavenger receptor class B type I

SRE, sterol regulatory element

SREBP, sterol regulatory element binding protein

STAT, signal transducer and activator of transcription

T2DM, type 2 diabetes mellitus

TBST, tris buffered saline with Tween20

TFBS, transcription factor binding sites

TG, triglyceride

TGRL, triglyceride rich lipoproteins

TNFα, tumor necrosis factor α

TLR4, toll-like receptor 4

VLDL, very low density lipoprotein

VSMC, vascular smooth muscle cell

WC, waist circumference

WHO, Word Health Organization

WHR, waist to hip ratio

WHtR, waist to height ratio

WT, wild type

ABSTRACT

Background: Adipose tissue is an endocrine organ secreting active molecules, namely, adipokines. In a condition of dysfunctional visceral fat depots, adipokines may be detrimental for the cardiovascular system. The present study was aimed at evaluating some of the molecular mechanisms linking adipokines and the expression of proprotein convertase subtilisin/kexin 9 (PCSK9), the key regulator of low-density lipoprotein receptor and also involved in triglycerides (TG) metabolism. This latter evidence come from genetic studies reporting high levels of TG in patients with gain of function (GOF) mutations in PCSK9 gene while, in those carrying loss of function (LOF) mutations, LDL-C reduction associates with reduced fasting and post-prandial TG levels. Leptin is a cytokine-like hormone produced mainly by white adipose tissue and playing a role not only in satiety but also in regulating blood pressure, endothelial function, glucose homeostasis, insulin sensitivity. Resistin is an adipokine produced by adipose tissue in mice and by monocytes in human; it is involved in inflammation and insulin resistance. Both leptin and resistin are dysregulated in obesity. In order to study the complex biosystem of TG metabolism we have developed and characterized an *in vitro* tool allowing to study the impact of genetic variants of lipoprotein lipase (LPL), one of the major regulators of TG levels. We believe that this *in vitro* tool could be of interest to study the effect of PCSK9 on TG regulation.

Materials: Human epatocarcinoma cell line (HepG2); silencing RNA (siRNA) anti-PCSK9 and anti-STAT3; qPCR; Western blot; enzyme-linked immunosorbent assay (ELISA); PCSK9 promoter luciferase reporter assay with plasmids pGL3-PCSK9-D4 (wild type), pGL3-PCSK9-SREmut (mutated on SRE element), pGL3-PCSK9-HNFmut (mutated on HNF element), pGL3-PCSK9-D1 (wild type) and pGL3-PCSK9-D1-STAT3mut (mutated on STAT3). C57BL6J mice, ob/ob mice (mice lacking leptin).

Results: In a clinical setting, a positive association between circulating levels of leptin and PCSK9 ($p=0.044$ and $\beta=0.295$) was found in patients with BMI<25 Kg/m^2. In the same cohort and according to BMI strata, no associations were found between resistin and PCSK9. When the link between leptin and PCSK9 was investigate in ob/ob mice lacking leptin, the hepatic expression of PCSK9 was significantly lower compared to the one of wild type mice. In C57BL6 mice a high-fat-diet resulted in raised levels of serum leptin and increased hepatic expression

of PCSK9. In HepG2 cells, leptin and resistin induced (i) *PCSK9* gene expression by +95% and +150%, respectively, and (ii) PCSK9 promoter activity via the involvement of Sterol Regulatory Element motif. Indeed, a mutation in HNF-1 motif did not alter leptin- and resistin-driven luciferase activity. The mandatory role of signal transducer and activator of transcription 3 (STAT3) in the relationship between adipokines and PCSK9 has been confirmed by silencing STAT3. After silencing, leptin- and resistin were not able to regulate the mRNA expression of PCSK9. Although not fully characterized, the involvement of STAT3 in the PCSK9 promoter activity has been tested by inserting a mutation on the STAT3 responsive element. Apolipoprotein (*APOB*) and microsomal triglycerides transfer protein (*MTP*) mRNA were increased by leptin (+57% and +60%, respectively) and resistin (+ 50% for both), an effect dependent on PCSK9, as demonstrated by using siRNA anti-PCSK9. As a further confirmation, a significant increment in APOB was found in response to PCSK9 overexpression.

In order to study the complex biosystem of TG metabolism, which is also a target of PCSK9, we have initially developed and characterized an *in vitro* tool allowing to evaluate the impact of genetic variants of LPL, an enzyme involved in TG metabolism. This technique allowed to distinguished between LPL mutations leading to a reduced secretion or a reduced activity despite a normal synthesis.

Conclusions

This work describes the relationship between PCSK9 expression and the adipokines leptin and resistin. The molecular basis beyond these findings require the involvement of STAT3 pathway and the activation of SRE motif at PCSK9 promoter level. Overexpression of PCSK9 led to an increment of gene expression of *APOB*, whereas silencing PCSK9 corresponded to an opposite effect i.e. a decrement in gene expression. The tool developed to study LPL mutations could be of help in evaluating PCSK9 variants associated with raised TG levels.

ABSTRACT (italiano)

Background: Il tessuto adiposo è un organo endocrino in grado di secernere molecole attive dette adipochine che, in condizioni fisiopatologiche determinanti un aumento di grasso viscerale, possono avere effetti negativi sul sistema cardiovascolare. Lo scopo di questo lavoro è stato quello di valutare alcuni dei meccanismi che legano queste adipochine all'espressione della proproteina convertasi subtilisina kexina di tipo 9 (PCSK9), regolatore chiave del recettore per le lipoproteine a bassa densità (LDL), ma coinvolta anche nel metabolismo dei trigliceridi (TG). Studi genetici hanno infatti riscontrato elevati livelli di TG plasmatici in pazienti portatori di mutazioni a guadagno di funzione (GOF) a carico del gene PCSK9 mentre mutazioni a perdita di funzione (LOF) sono associate a una riduzione della colesterolemia LDL e di TG. La leptina è una citochina prodotta principalmente dal tessuto adipose bianco, che svolge un ruolo regolatorio sull'appetito, la pressione sanguigna, la funzione endoteliale, l'omeostasi del glucosio, la sensibilità e la resistenza all'insulina. La resistina è coinvolta nell'infiammazione e nella resistenza all'insulina, è prodotta nell'uomo dai macrofagi e, nei modelli murini, dal tessuto adiposo. La produzione e secrezione di queste citochine risulta alterata in condizioni di sovrappeso e obesità. PCSK9 è una proteina coinvolta non solo nella regolazione della colesterolemia LDL ma anche nel metabolismo dei TG. Per comprendere meglio i meccanismi alla base del metabolismo dei TG, abbiamo sviluppato e caratterizzato una metodologia che permette di studiare *in vitro* l'impatto di varianti genetiche di uno dei maggiori regolatori dei livelli di TG, la lipoproteina lipasi (LPL). Crediamo che questo modello di studio possa essere applicato anche allo studio degli effetti di PCSK9 sul metabolismo dei TG.

Materiali: Cellule di epatocarcinoma umano (HepG2), silencing RNA (siRNA) anti-PCSK9 e anti-STAT3; qPCR; western blot; ELISA; PCSK9 saggio di attività trascrizionale con luciferasi e plasmidi pGL3-PCSK9-D4 (wild type), pGL3-PCSK9-SREmut (mutato sull'elemento responsivo SRE), pGL3-PCSK9-HNFmut (mutato sull'elemento responsivo HNF), pGL3-PCSK9-D1 (wild type) and pGL3-PCSK9-D1-STAT3mut (mutato sull'elemento responsivo STAT3); topi C57BL6J e ob/ob (topi geneticamente mancanti leptina).

Risultati: Nell'ambito clinico, è stata evidenziata un'associazione positiva tra i livelli circolanti di leptina e di PCSK9 ($p=0.044$ and $\beta=0.295$) solo in pazienti con BMI<25 Kg/m^2. Nella

medesima coorte, non è stata trovata alcuna associazione tra i livelli circolanti di resistina e quelli di PCSK9. La relazione tra leptina e PCSK9 è stata valutata *in vivo* tramite l'utilizzo di topi geneticamente privi di leptina (topi ob/ob) nei quali l'espressione epatica di *PCSK9* era significativamente inferiore rispetto a topi wild type. Modelli C57BL6 messi a dieta ad alto contenuto di grassi hanno confermato livelli sierici di leptina più elevati a cui è corrisposto un aumento dell'espressione epatica di *PCSK9*. In cellule di epatocarcinoma umano della linea HepG2, leptina e resistina inducono (i) l'aumento dell'espressione genica di PCSK9 rispettivamente del +95% e +150%, (ii) un aumento dell'attività del promotore di PCSK9 tramite il coinvolgimento dell'elemento regolare degli steroli (Ganie, Dhingra et al.). L'inserimento nell'elemento responsivo HNF-1 non modifica la capacità di leptina e resistina di indurre l'attività luciferasica. Il ruolo fondamentale di STAT3 nella relazione tra adipochine e PCSK9 è stato confermato tramite silenziamento genico in cui la leptina e la resistina non erano in grado di regolare l'espressione genica di *PCSK9*. Nonostante non sia ancora completamente caratterizzato, il coinvolgimento di STAT3 nell'attività trascrizionale di PCSK9 è stato valutato tramite inserzione di una mutazione nel sito responsivo per STAT3 sul promotore di PCSK9. L'espressione genica dell'apolipoproteina B (*APOB*) e della proteina microsomiale di trasferimento di trigliceridi (*MTP*) è aumentata rispettivamente del 57% e 60% a seguito di trattamenti con leptina e del 50% per entrambe con resistina. Tale effetto, è PCSK9 dipendente, infatti, dopo l'utilizzo di siRNA anti-PCSK9, leptina e resistina non alterano tali geni. Ad ulteriore conferma, un aumento nell'espressione genica di *APOB* è stato osservato dopo sovra-espressione di PCSK9. Per valutare al meglio il complesso sistema del metabolismo dei trigliceridi, anch'esso un target di PCSK9, abbiamo inizialmente sviluppato e caratterizzato un sistema *in vitro,* che permette di valutare l'impatto delle varianti genetiche di LPL, un enzima coinvolto nel metabolismo dei trigliceridi. Questa tecnica ha permesso di distinguere mutazioni che portano ad una ridotta secrezione o ad una ridotta attività dell'enzima, nonostante una normale sintesi.

Conclusioni

Questo lavoro descrive i meccanismi alla base della relazione PCSK9 e le adipochine leptina e resistina. Tali meccanismi coinvolgono la via di segnalazione di STAT3 e l'attivazione dell'elemento SRE a livello del promotore di PCSK9. La sovra-espressione di PCSK9 corrisponde a un aumento dei livelli di espressione genica di *APOB* mentre il suo silenziamento ne provoca

l’effetto contrario i.e. diminuita espressione di *APOB*. Il sistema sviluppato per studiare le mutazioni di LPL potrebbe essere applicato per valutare l’impatto di varianti di PCSK9 che si associano a elevati livelli di trigliceridi.

INTRODUCTION

Obesity

The prevalence of obesity has increased worldwide in the last decades reaching epidemic proportions with approximately 1.9 billion overweight and 650 million obese adults (Ruban, Stoenchev et al. 2019). The World Health Organization (WHO) describes obesity as an excessive accumulation of fat that represents a risk for human health. Body mass index (BMI), which is calculated by using the formula: weight (kg)/height^2 (m^2), is an anthropometric parameter used to classify obesity and, according to this, obesity is categorized as follow: 30.0 < BMI< 34.9 kg/m^2 obese class I; 35.0>BMI<39.9 kg/m^2 obese class II; BMI≥40 kg/m^2 obese class III; BMI≥50 kg/m^2 obese class IV; BMI ≥60 kg/m^2 obese class V (Table 1) (Poirier, Alpert et al. 2009).

Classification	**BMI (kg/m^2)**
Underweight	< 18.5
Normal weight	18.5 – 24.9
Overweight	25.0 – 29.9
Obese class I	30.0 – 34.9
Obese class II	35.0 – 39.9
Obese class III	≥ 40

Table 1 WHO adult body mass index classification.

Body weight gain is the result of a chronic positive energy balance, i.e. energy expenditure is less than energy intake (Alessandra Vecchié and Franco Dallegria 2018). Epidemiologic studies have associated obesity to an increased incidence of cardiovascular diseases (CVD) due to an increase of CV risk factors such as dyslipidemia, increased blood pressure (Reisi, Ghaedamini et al.), insulin resistance and type 2 diabetes mellitus (T2DM) (Piche, Poirier et al. 2018). Although in obese patient, weight loss has been proposed as a possible therapeutic intervention, the long-term effects of this approach in primary prevention failed to improve clinical outcomes, unless a prolonged and large degree of weight loss (Carbone and Lavie 2019). However, in prediabetic patients, weight loss can delay the onset of T2DM by improving

several cardiometabolic risk factors like insulin resistance, raised blood pressure and cholesterol (American Diabetes 2018). This weight loss together with a reduction of sedentary behaviours, i.e. improving exercise training and physical activity, is a way to improve CV risk factors and prevent the incidence of T2DM (Lavie, Ozemek et al. 2019). Besides this evidence, in CVD patients with heart failure or coronary heart disease, obesity does not associate with worse outcomes but instead predicts improved prognosis (Carbone, Lavie et al. 2017). This controversial relationship between obesity and CVD is called "obesity paradox". To better understand the mechanism behind this paradox, several studies have been performed to assess whether or not an excess of adiposity improves the outcome of patients with established CVD. The definition of "obesity paradox" stems on the use of BMI to classify obese patients but this index does not consider body fat distribution and does not discriminate between body lean mass and body fat mass (Nuttall 2015). Indeed, no "obesity paradox" has been observed using other indices to describe obesity, such as central obesity which is a strong risk factor for CVD and mortality in the general population (Pavanello, Zanaboni et al. 2018). The measurement of waist circumference (WC) and waist-to-hip ratio (WHR) are more precise indexes allowing to assess body fat distribution in patients with coronary artery disease (CAD) (Coutinho, Goel et al. 2011). These indices better describe the amount of abdominal adiposity: (i) they independently and linearly associate to the risk of mortality and (ii) predict the CVD outcome in patients with established CVD (Coutinho, Goel et al. 2013). Furthermore, waist to height ratio (WHtR) was the index with the highest sensitivity, but reduced specificity for predicting the development of metabolic syndrome (MetS), defined as a cluster of cardio-metabolic risk factors (Mombelli, Zanaboni et al. 2009, Pavanello, Zanaboni et al. 2018).

Development of atherosclerosis

The triggering factor in the process of atherosclerosis development is the accumulation of low-density lipoproteins (LDL) in the subendothelial space, where they adhere to the extracellular matrix proteins rich in proteoglycans (Raggi, Genest et al. 2018). The accumulation of LDL is due to changes in endothelial permeability and it happens trough active receptor-mediated transcytosis across the cell membrane by SR-BI transporter (Armstrong, Sugiyama et al. 2015). Once in the sub-intimal space, LDL become aggregated and oxidized. Aggregated LDL have a size ranging from 100 nm to 1µm and can undergo phagocytosis by immune cells which are

present in the sub-endothelial space (Kruth 2002). The presence of reactive oxygen species and enzymes like lipoxygenase, modify phospholipids and proteins of LDL, thus making them an optimal substrate for scavenger receptor mediated uptake (Miller, Choi et al. 2011). While the uptake of native LDL has a regulatory feedback depending on intracellular sterol levels, the uptake of oxidized LDL (oxLDL) by scavenger receptor is not subjected to any inhibitory feedback, leading to an unrestricted phagocytosis. Modified lipoproteins are taken up by dendritic cells and macrophages in the arterial intima. Furthermore, non-classical "patrolling" monocytes can phagocyte oxLDL via scavenger receptor CD36 at very early stage of atherosclerosis (Steinberg 2002, Marcovecchio, Thomas et al. 2017). At this point, immune cells induce the expression of endothelial adhesion molecules (*i.e.* ICAM1) to recruit monocytes into the intima. After this step, they differentiate into macrophage and engulf LDL containing lipid droplets with esterified cholesterol becoming foam cells. These induce the recruitment of immune cells by the production and the release of cytokines and chemokines, thus starting the inflammatory response process. The activation of CD36 stimulates the innate immune responses downstream of the toll-like receptor pathway (Stewart, Stuart et al. 2010). Cholesterol crystals, derived from aggregated LDL, activate the inflammasome in the cytoplasm of macrophages in the arterial intima (Sheedy, Grebe et al. 2013). The inflammasome cleaves pro-interleukin-1β (IL-1β) and IL-18 which are then activated and secreted leading to (i) the release of matrix degrading enzymes and reactive oxygen species as well as to (ii) the activation and proliferation of T-cells (Zheng, Gardner et al. 2011). This environment causes the migration of vascular smooth muscle cells (VSMCs) into the luminal side of vessel wall, where the synthesis of extracellular matrix forms the fibrous cap, essential component of the atherosclerotic plaque. This last is composed by a lipid core encapsulated in a fibrous cap which is a layer of connective tissue composed of collagen-rich-fiber tissues, VSMCs, macrophages and T lymphocytes (Rafieian-Kopaei, Setorki et al. 2014). These components separate the lipid core from the arterial lumen. After the plaque formation, the second key step is the calcification. The plaque inflammation causes the phenotypic switch of M1 macrophages to M2 polarized macrophages and this process proceeds in parallel to the VSMCs osteoblastic differentiation and matrix mineralization, contributing to the plaque stabilization (Shioi and Ikari 2018).

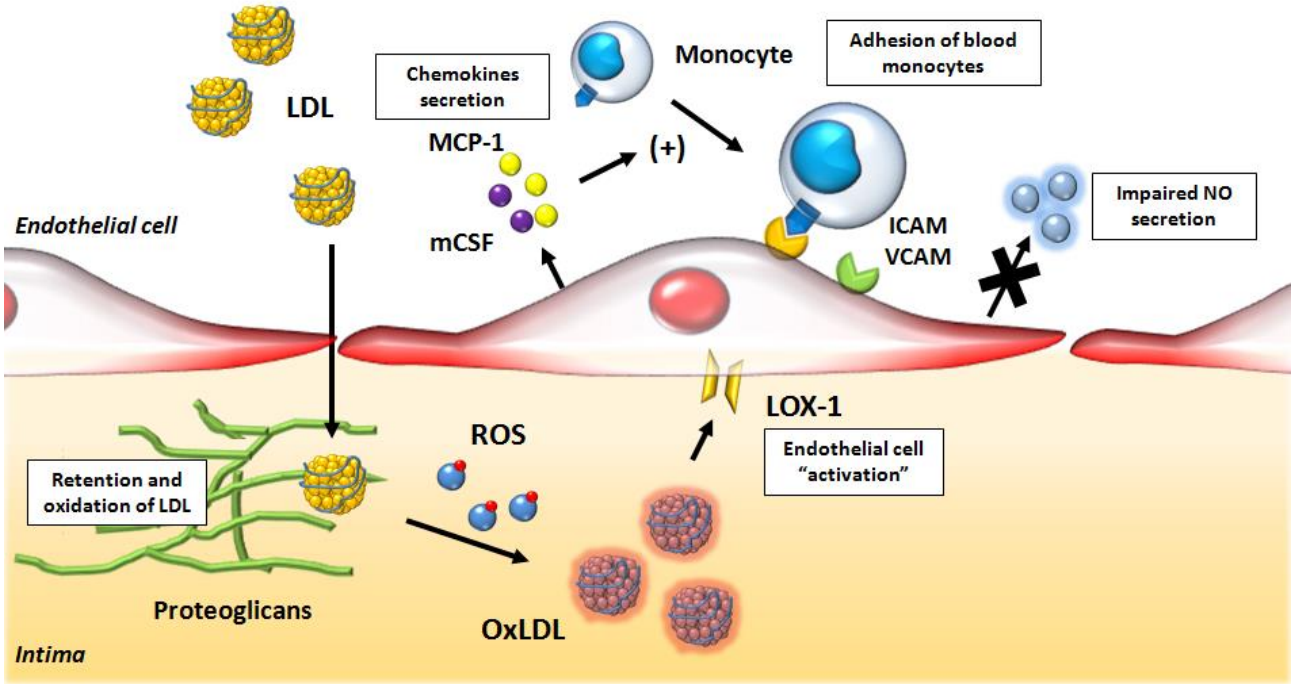

Fig.1 Role of oxidized LDL in early stage atherosclerosis (E. Leiva 2015)

Role of adipose tissue in atherosclerosis

The traditional role of adipose tissue has changed during last 30 years, from being the reservoir for energy storage, to an organ with an active endocrine role. Adipose tissue is involved in the synthesis of bioactive compounds such as adipokines and pro-inflammatory cytokines, which act as autocrine and paracrine signals but also as endocrine signals, at local and systemic level. Adipose tissue expresses different receptors such receptors for traditional endocrine hormones, nuclear hormone, cytokines and catecholamines receptors, by which it responds to different signals from traditional hormones and from central nervous system, undergoing dynamic changes (Kershaw and Flier 2004). Through this network, adipose tissue is actively involved in coordinating different biological processes including energy metabolism, neuroendocrine and immune functions (Weisberg, McCann et al. 2003). Adipose tissue contains not only adipocytes but also connective tissue matrix, stromovascular cells, and immune cells, such as macrophages and lymphocytes, that can alter adipocytes responses to metabolic signals favouring adipose tissue expansion and activation (Kershaw and Flier 2004). It is known that increased body fat mass positively correlates to the production of leptin, an hormone which is one of the key-regulator of energy metabolism and insulin sensitivity (Carbone, La Rocca et al. 2012). This last, conversely, is negatively associated with adiponectin biosynthesis. This adipokine is characterized by insulin sensitizing (Antoniades, Antonopoulos

et al. 2009) and anti-inflammatory properties (Antonopoulos and Tousoulis 2017). In a condition of adipocyte hypertrophy, the secretion of pro-inflammatory cytokines is dysregulated playing a role in atherosclerosis and CVD, influencing lipid and glucose metabolism and affecting vascular function (Van Gaal, Mertens et al. 2006). Dysregulation of adipose tissue is believed to be determinant to the pathogenesis of MetS, a cluster of cardio-metabolic risk factors spanning from insulin resistance to hypertriglyceridemia and to hypertension (Reddy, Lent-Schochet et al. 2019). MetS is diagnosed when 3 out of 5 of the following clinical features are present (see table 2) (Jialal and Devaraj 2018).

High density lipoprotein cholesterol (HDL-c)	< 40mg/dL in males <50 mg/dL in females
Triglycerides (TGs)	>150 mg/dL
Plasma glucose	>100 mg/dL
Elevated waist circumference (WC)	Variable depending on population
Hypertension	<130/85 mmHg

Table 2 Clinical features of MetS

Obesity, especially the abdominal one, measured by WC, is one of the predominant risk factors for MetS. It increases the risk of developing insulin resistance, type 2 diabetes, dyslipidemias, hypertension and non-alcoholic fatty liver disease (NAFLD) (Jung and Choi 2014). Adipose tissue of people with MetS, compared to the one of lean people, releases a higher amount of pro-inflammatory proteins and adipokines (Ruscica, Baragetti et al. 2017). Among them, tumor necrosis factor α (TNF α), interlukin (IL)-1, IL-6, IL-8 and monocyte chemotactic protein (MCP)-1, are known to promote insulin resistance. It is not only a matter of macrophage number but is also important to note that obesity induces a phenotypic switch in these cells, *i.e.* from an anti-inflammatory M2 polarization state to a pro-inflammatory M1 polarization state (Lumeng, Deyoung et al. 2007). This phenotypic change leads macrophages to secrete different pro-inflammatory cytokines and chemokines that potentially contribute to the obesity-related insulin resistance (Moroni, Ammirati et al. 2019).

Leptin

Leptin is a cytokine-like hormone discovered in 1994 as product of the *ob (obese)* gene (Zhang, Proenca et al. 1994). Both human (*OB*) and mouse (*ob)* genes are located on chromosome 7

and 6q31.3, respectively (He, Chen et al. 1995, Isse, Ogawa et al. 1995); moreover, there is a high degree of homology among species, *i.e.* human leptin is 84% identical to the murine one. The *OB* gene product is called leptin, from Greek root *leptos* meaning thin, because of its role in the regulation of satiety and energy expenditure(Halaas, Gajiwala et al. 1995). Leptin is synthesized mainly by white adipose tissue with a structure similar to the one of cytokines (Zhang, Basinski et al. 1997). In humans, leptin plays different functions depending on its form (Sinha, Opentanova et al. 1996): the higher molecular weight form, prevalent in lean subjects, is bound to the soluble leptin receptor and regulates energy expenditure and sympathetic activity (Tank, Jordan et al. 2003); while the monomeric form, also known as free leptin, mediates satiety (Ruscica, Macchi et al. 2016) and it is the major circulating form in obese patient. Six transmembrane leptin receptors are known. They are produced in several alternatively splice forms and are categorized into short isoforms (ObRa, ObRc, ObRd, ObRf) and a long isoform (ObRb) depending on the presence of JAK/STAT binding sites and on the intracellular domain size (Munzberg and Morrison 2015). ObRd is present only in mice (Chua, Koutras et al. 1997) and ObRf in rats (Wang, Zhou et al. 1996), whereas ObRe is the shortest isoform which is secreted only as extracellular domain (Wada, Hirako et al. 2014). Finally ObRa/b and ObRb/c exist as heteromers and are stabilized by the binding of leptin (Ruscica, Baragetti et al. 2017). Upon the binding with its receptor, leptin activates different signal transduction pathways: (i) signal transducer and activators of transcription (STATs), (ii) the Janus kinases (JAKs), (iii) the suppressor of cytokine-signalling-3 (SOCS3) which has a role in switching off cytokine signal transduction by inhibiting JAK activity though the intracellular negative-feedback loop, (iv) the mitogen-activated protein kinase (MAPK) and (v) AKT pathway (Fruhbeck 2006). Leptin signalling plays a role in regulating blood pressure, endothelial function, glucose homeostasis, insulin sensitivity and resistance (Lee, Jo et al. 2012), suggesting that leptin is not only linked to obesity but also to MetS.

Resistin

Resistin is a member of the family of cysteine-rich proteins called “resistin-like molecules”(RELMs) discovered in 2001 by the group of Lazar (Steppan, Bailey et al. 2001). The first studies, conducted in rodents, have shown that resistin is synthesized mainly by adipose tissue; it is up-regulated in obesity and it is involved in the pathogenesis of insulin resistance (Sun, Wu et al. 2010). However, mouse and human resistin carry some different features, i.e.

(i) mouse resistin gene is located on chromosome 8 while the human counterpart is on chromosome 19, (ii) at the mRNA level they share 64.4% sequence homology and (iii) at the amino acid level they exhibit 59% identity (Ghosh, Singh et al. 2003). Human resistin is produced in different isoforms from the trimeric to oligomeric ones. Human resistin is secreted by peripheral blood monocytes and stromovascular portion of adipose tissue, in different isoforms, and has a plasmatic concentration of 50 ng/mL (Banerjee, Rangwala et al. 2004). It is still debated whether a specific resistin receptor exists, indeed Toll-like receptor 4 (TLR4) (Tarkowski, Bjersing et al. 2010) and adenylyl cyclase-associated protein 1 (CAP1) (Lee, Lee et al. 2014) were proposed as *bona fide* functional receptors for human resistin. Through TLR4, resistin activates the phosphatidylinositol 3-kinase (PI3K) in human vascular smooth muscle cells, leading to vascular dysfunction. In human THP-1 monocytes, resistin binds CAP-1 activating cAMP/protein kinase A (PKA) and NF-kB and drives the inflammation through increasing mRNA and protein levels of inflammatory cytokines such as monocyte chemoattractant protein, TNF-alpha, IL-6 and IL-12 (Park and Ahima 2013). Several clinical studies have been conducted to clarify the role of resistin in inflammation and now it is well established that inflammation plays a major role in the formation of atherosclerotic plaque (Bentzon, Otsuka et al. 2014). Resistin levels have been associated with inflammatory markers and indicated as predictive of coronary atherosclerosis (Reilly, Lehrke et al. 2005). As a pro-inflammatory mediator, resistin can lead to insulin resistance by increasing expression of the suppressor of cytokine signaling-3 (SOCS-3) (Qatanani and Lazar 2007) and by reducing the expression and phosphorylation of insulin receptor (IRS1,2) in pre-adipocytes (Palanivel, Maida et al. 2006).

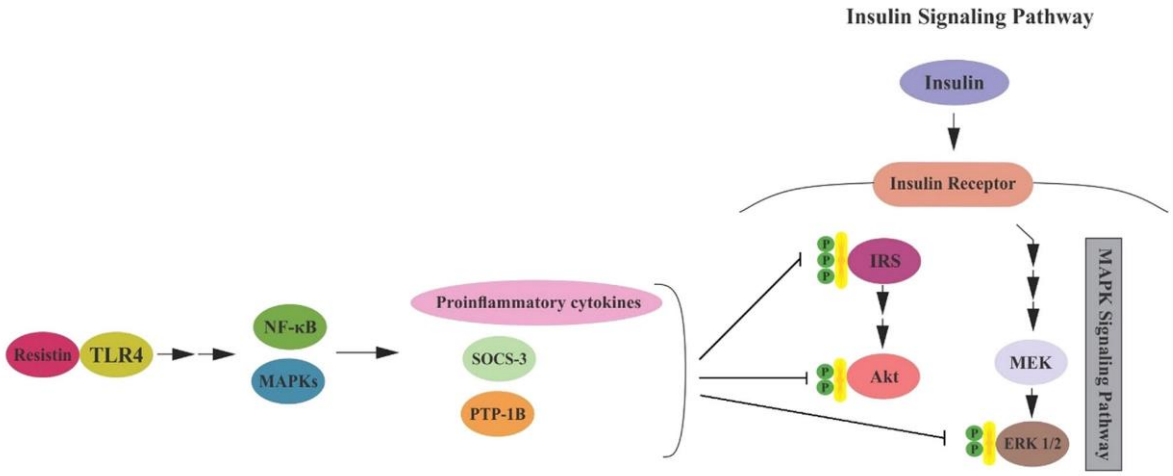

Fig.2 Mechanism of action of resistin on insulin signalling. Resistin binds TLR4 activating NF -kB and MAPKs cascade, during this process, an impairment in insulin signalling with inhibition of IRS, Akt and ERK1/2 phosphorylation cause insulin resistance. ERK 1/2, Extracellular signal -regulated protein kinases 1 and 2; IRS, Insulin receptor substrate; MAPK, mitogen -activated protein kinase; NF -kB, nuclear factor -kappa B; TLR4, toll -like receptor 4. (Emamalipour, Seidi et al. 2019)

Proprotein convertase subtilisin kexin type 9 (PCSK9)

Structure and function

Proprotein convertase subtilisin kexin type 9 (PCSK9) is a member of the proprotein convertase family. It is a serine protease encoded by a gene located on chromosome 1p32.3. It is synthesized as a 692-amino acid soluble zymogen, also called pro-PCSK9 (75 kDa) constituted by a signal peptide (residues 1-30), a pro-domain (residues 31–152), a catalytic domain (residues 153–451) and a C-terminal domain (residues 452–692) rich in Cys and His (CHRD) (Benjannet, Rhainds et al. 2004). Pro-PCSK9 undergoes autocatalytic cleavage at position 152 in the endoplasmic reticulum which is required for PCSK9 maturation and secretion (Norata, Tibolla et al. 2014). In contrast with other proprotein convertase of its family, in the autocatalytic cleavage, the PCSK9 pro-domain remains associated with the catalytic site, leading the formation of PCSK9/pro-domain complex that inhibits the PCSK9 protease activity by preventing the access of protein substrate to the catalytic site (Cunningham, Danley et al. 2007). After the secretion, extracellular PCSK9 plays a key role in the regulation of hepatic low-density lipoprotein receptor (LDLR) function. In particular, it binds the first EGF-like repeat (EGF-A) of the LDLR on the plasma membrane of hepatocytes, fostering its lysosomal degradation, leading to reduced LDLR expression on the cell membrane and increasing plasma levels of LDL-cholesterol (LDL-C) (Norata, Tavori et al. 2016). Human PCSK9 acts also as a courier, facilitating the exit of LDLR from the endoplasmic reticulum (Strom, Tveten et al. 2014). Furthermore, PCSK9 has been shown to play a role in degradation of apolipoprotein (Apo) E receptor 2 and very low density lipoprotein (VLDL) receptor (Canuel, Sun et al. 2013). PCSK9 was firstly discovered in the brain as neural apoptosis-regulated convertase 1 (NARC-1) (Seidah, Benjannet et al. 2003) but the interest about this protein grew after the description of PCSK9 mutations associated with autosomal dominant hypercholesterolemia (Abifadel, Varret et al. 2003).

PCSK9 transcriptional regulation

PCSK9 synthesis is controlled at the transcriptional level by the transcription factors family of sterol regulatory element (Ganie, Dhingra et al.)-binding proteins (SREBPs) (Jeong, Lee et al. 2008). SREBPs are members of basic-helix-loop-helix-leucine zipper family of transcription factors regulating the expression of target genes. Upon the binding to the SRE in their promoter region, SREBPs activate a cascade of enzymes required for endogenous cholesterol, TG, phospholipids and fatty acid synthesis (Eberle, Hegarty et al. 2004). Precisely, in the liver they regulate the production of lipids for the assembly and export of lipoprotein into the plasma and micelles into the bile (Horton, Goldstein et al. 2002). The SREBP family includes three members: SREBP1a, SREBP1c and SREBP2. SREBP-1a is a potent activator of all SREBP-responsive genes, including those that mediate the synthesis of cholesterol, fatty acids, and triglycerides. SREBP-1c enhances transcription of genes required for fatty acid synthesis. SREBP2 preferentially activates the cholesterol synthesis (Brown and Goldstein 1997). *In vivo*, the overexpression of nSREBP-1c in the liver of transgenic mice produces a triglyceride-enrich fatty liver without increasing cholesterol levels (Shimano, Horton et al. 1997) while the overexpression of nSREBP2 in the liver increases the mRNA encoding for enzymes involved in the cholesterol synthesis (Horton, Shimomura et al. 1998). SREBP-1a is constitutively expressed in liver and other adult animal tissues whereas SREBP-1c and SREBP2 transcription involves a feed-forward regulation mediated by SREs present in the enhancer/promoter of these genes(Sato, Inoue et al. 1996, Amemiya-Kudo, Shimano et al. 2000). The SREBP-dependent regulation of PCSK9 was explained by the identification of the SRE in PCSK9 promoter region. PCSK9 promoter activity is induced in response to cell cholesterol depletion or to pharmacological inhibition of intracellular synthesis (i.e. statins), determining increased transcription and higher circulating levels (Dubuc, Chamberland et al. 2004). PCSK9 transcription is regulated also by the hepatocyte nuclear factor 1 α (HNF1 α) (Shende, Wu et al. 2015) which has a binding site in close proximity to SRE in PCSK9 promoter region (Li, Dong et al. 2009). HNF1 α is a member of HNF1 subfamily of transcriptional factors which is composed by two members HNF1 α and HNF1 β, both expressed in many tissues like liver, kidney, pancreas and intestine, where they have a role in tissue development and differentiation (Mendel and Crabtree 1991). The germline deletion of HNF1 α results in

hepatic, renal and pancreatic defects, while the loss of HNF1 β is developmentally lethal (Pontoglio, Barra et al. 1996). *In vivo*, liver-specific knockdown of HNF1 α decreases PCSK9 circulating levels and increases hepatic expression of LDLR protein (Shende, Wu et al. 2015). Moreover, it has been shown that lipid lowering drugs (i.e. berberine) partially exert their effect in repressing PCSK9 transcription through reduction of HNF1 α (Li, Dong et al. 2009).

PCSK9 expression regulators

Upon maturation and secretion, PCSK9 expression is physiologically regulated by many factors such as diet, insulin, hormones and adipokines. There are several findings underlining how the nutritional status is a bias that needs to be addressed. A 35% reduction in PCSK9 plasma levels has been demonstrated in 12 healthy subjects after 18 hours of fasting. In the same subjects, a prolonged fasting showed a more severe reduction in plasmatic PCSK9 (- 50%) (Persson, Cao et al. 2010). These data were confirmed by another study in which a fasting prolonged up to 48 hours resulted in reduced PCSK9 levels which reached a maximum of -58% after 36h (Browning and Horton 2010). Moreover, it has been demonstrated that diets enriched in oleic acid resulted in lower PCSK9 levels (Rodriguez-Perez, Ramprasath et al. 2016); following the Mediterranean diet, even in absence of weight loss, plasma PCSK9 is reduced by -11.7% and LDL-C by -9.9% (Richard, Couture et al. 2012). Another physiological factor that modulates PCSK9 expression is insulin. In mouse primary hepatocytes exposed to insulin, PCSK9 and SREBP-1c mRNA levels were 4-fold increased. This effect was reduced when a dominant negative SREBP-1c was present (Costet, Cariou et al. 2006). The same effect was shown in primary rat hepatocytes, in which treatment with insulin enhanced by 3-fold both PCSK9 mRNA and secreted protein, at the same time SREBP-1c and SREBP1a were increased by 12- and 2-fold, respectively (Miao, Manthena et al. 2015). Furthermore, PCSK9 expression can be modified by hormones; data from the Dallas Heart Study showed that (i) plasma PCSK9 levels were higher in women than in men, and (ii) circulating PSCK9 and LDL-C levels were reduced in postmenopausal group (Lakoski, Lagace et al. 2009). PCSK9 expression can be regulated also by adipokines. Among them, in HepG2 cells, resistin increased PCSK9 mRNA and protein expressions by 40% and 30%, respectively (Melone, Wilsie et al. 2012).

Role of PCSK9 in cardiovascular diseases

The role of PCSK9 in CVDs has been confirmed by several genetic studies demonstrating a reduction in coronary artery diseases (CAD) events in presence of certain PCSK9 genetic polymorphisms. The PCSK9 polymorphism loss-of-function *R46L* has been associated with a reduction on LDL-C, thus lowering the risk of ischemic heart disease in carriers versus non carriers (Benn, Nordestgaard et al. 2010). Furthermore, two nonsense polymorphisms Y142X and C679X are associated with a 40 percent reduction in mean LDL cholesterol (Cohen, Boerwinkle et al. 2006). This evidence led to the study of PCSK9 as a new pharmacological target and the development of numbers of PCSK9 antagonists. PCSK9 is present in a variety of studies as a pharmacological target for treating dyslipidaemia and several strategies are being examined to reduce or to inhibit either PCSK9 protein synthesis or binding to LDLR, by using vaccines, small protein inhibitors, siRNA, antisense oligonucleotides and mAbs. As far as mAbs is concerned, two fully human mAbs, alirocumab and evolocumab, have been approved in the European Union and in the USA, while a humanised mAbs, bococizumab was withdrawn from the market in 2016 (Ferri, Corsini et al. 2017, Ferri, Corsini et al. 2017). Alirocumab is prescribed in adjunct to diet and maximally tolerated statin therapy to treat adults with heterozygous familial hypercholesterolemia (HeFH) or clinical atherosclerotic CVD, requiring additional lowering of LDL-C; while evolocumab is authorised in patients with homozygous familial hypercholesterolemia (HoFH) requiring an additional lowering of LDL-C, i.e. in adults with CVD in order to reduce the risk of myocardial infarction, stroke, and coronary revascularization. PCSK9 inhibitors are to be considered in patients with severe HeFH without atherosclerotic cardiovascular diseases (ASCVD) with LDL-C≥200 mg/dl or LDL-C≥175 mg/dl in the presence of comorbidities, i.e. diabetes mellitus or hypertension. Whereas, patients with HoFH, excluding those with null LDLR mutations, should receive a lipid-lowering therapy which includes lipoprotein apheresis plus a PCSK9 inhibitor, like evolocumab (Pecin, Hartgers et al. 2017). Besides being the key regulator of LDL-C, PCSK9 plays also a role in atherosclerosis and CV risk. PCSK9 is also expressed in endothelial cells, VSMC and, at low level, in macrophages (Shapiro and Fazio 2017). VSMC produce more PCSK9 than endothelial cells do, especially in response to shear stress (Ding, Liu et al. 2015). $PCSK9^{-/-}$ mice are partially protected from neointimal formation, further supporting the positive effect of PCSK9 on intimal thickening (Ferri, Marchiano et al. 2016). In humans, serum PCSK9 levels are linearly associated with a

higher necrotic core fraction in coronary atherosclerosis (Cheng, Oemrawsingh et al. 2016) and significantly associated with arterial stiffness (Ruscica, Ferri et al. 2017). Recently, we have added circulating platelets to the list of targets of PCSK9 (Camera, Rossetti et al. 2018), together with a direct pro-inflammatory effect on macrophages (Ricci, Ruscica et al. 2018). PCSK9 was suggested to be secreted from visceral adipose fat into the portal vein to affect hepatic LDL receptor degradation, LDL plasma levels, and risk for CVD (Franzen, Ermel et al. 2016).

Lipoproteins, triglycerides and atherosclerosis

Multiple lines of evidence have established that cholesterol-rich LDL and other apolipoprotein B (apoB)-containing lipoproteins, including VLDL and their remnants, intermediate density lipoproteins (IDL), and lipoprotein(a) [Lp(a)], are involved in the development of ASCVD (Goldstein and Brown 2015). Exogenous and endogenous cholesterol are transported to peripheral cells mostly by the apoB-containing lipoproteins in plasma. It is known that LDL-C concentration and LDL particle number are correlated, thus plasma LDL-C is a good surrogate for the indirect measurement of LDL particle concentration. Plasma LDL levels are generally not measured directly but instead they are estimated from its cholesterol concentration. For this reason, the calculated plasma LDL-C has become the focus for assessing CV risk and for evaluating therapeutic benefit in randomized clinical trials. It was demonstrated that, if the concentration of LDL-C increases above 20–40 mg/dL, the probability of the initiation and progressive development of atherosclerotic plaque dramatically increases in a dose dependent manner (Goldstein and Brown 2015). Familial hypercholesterolaemia (FH) is an autosomal co-dominant disorder usually linked to a loss-of-function (LOF) mutation in the LDL receptor (LDLR) gene, or in the *APOB* gene reducing the binding of apoB-containing lipoproteins to the LDL receptor or a gain-of-function (GOF) mutation in the PCSK9 gene. FH is characterized by high concentration of circulating LDL particles and LDL-C (Cuchel, Bruckert et al. 2014). There are several large meta-analyses of prospective observational and epidemiologic studies in which is reported a log-linear association between the absolute magnitude of exposure to plasma LDL-C levels and the risk of ASCVD. Mendelian randomization studies have been conducted to introduce a randomization scheme into an observational study and to confirm the causality of the observed association between an exposure and an outcome (Lawlor, Harbord et al. 2008). Numerous variants in multiple genes

(i.e. LDLR, APOB, PCSK9) have been associated with lower LDL-C levels (Teslovich, Musunuru et al. 2010, Willer, Schmidt et al. 2013). These variants are inherited approximately randomly at the time of conception in a process similar to Mendelian randomization, hence, inheriting the mutated allele which lowers LDL-C is analogous to being randomly allocated to treatment with an LDL-C-lowering pharmacological treatment. Whereas inheriting the wild type allele is analogous to being randomly allocated to placebo. For this reason, adjusting for a standard decrement in LDL-C, every genetic variant associated with LDL-C has a similar effect on the risk of CHD per unit of LDL-C lowering. This finding is valid also for variants in the genes of pharmacological targets for LDL-C lower treatment (i.e. PCSK9, the target of alirocumab and evolocumab; 3-hydroxy3-methyl-glutaryl-coenzyme A reductase (HMGCR), the target of statins and Niemann-Pick C1-like 1 (NPC1L1), the target of ezetimibe) (Ference, Yoo et al. 2012, Ference, Majeed et al. 2015). Meta-analyses of Mendelian randomization studies demonstrated that LDL is associated with the risk of ASCVD independently by the mechanism by which LDL is 'lowered'. (see figure 3).

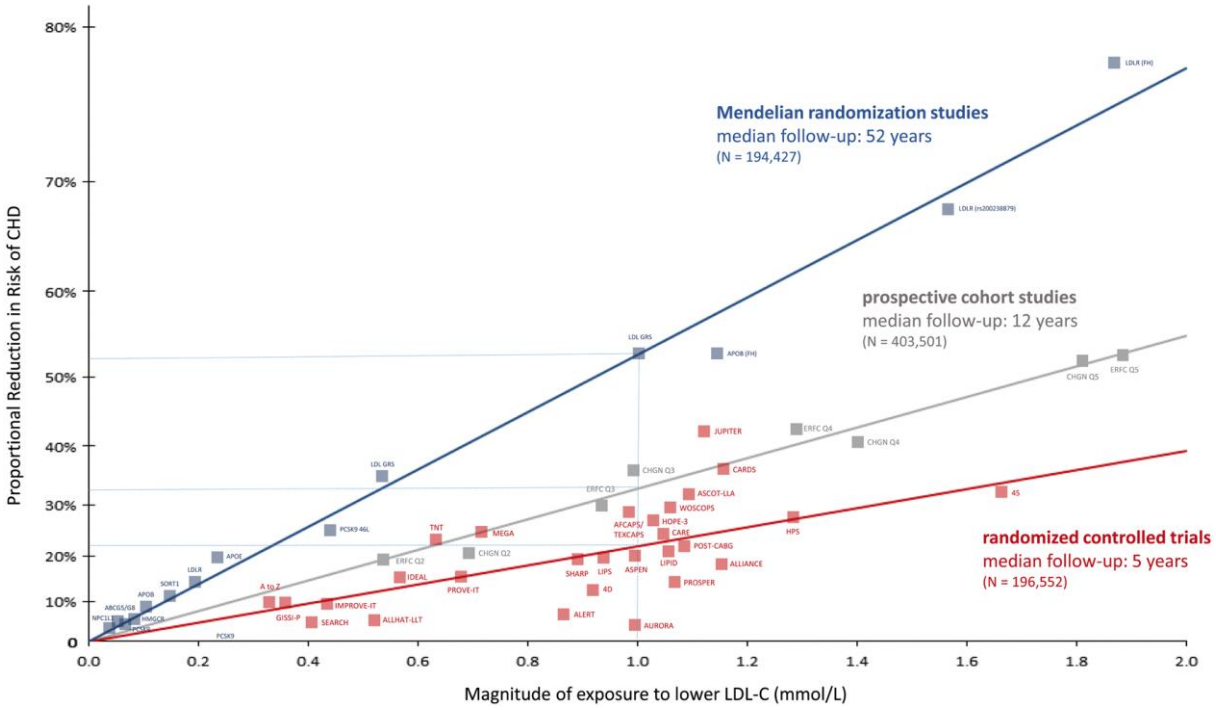

Fig. 3 Log-linear association per unit change in low-density lipoprotein cholesterol (LDL-C) and the risk of cardiovascular disease as reported in meta-analyses of Mendelian randomization studies, prospective epidemiologic cohort studies, and randomized trials. The proportional risk reduction (y axis) is calculated as 1-relative risk on the log scale, then exponentiated and converted to a percentage. (Ference, Ginsberg et al. 2017)

Similarly, lipoprotein lipase (LPL) variants lowering TG were associated with lower risk of CHD per unit lowering level of ApoB-containing lipoproteins and this association is independent and proportional to the absolute change in ApoB (Ference, Kastelein et al. 2019). All ApoB – containing lipoproteins, such as VLDL rich in TG, their metabolic remnants and LDL, have similar effects on the risk of CVD per particle. TG and cholesterol are transported in plasma by ApoB- containing lipoprotein particles which are secreted by the liver as VLDLs. Then LPL acts on these lipoproteins converting the TG-rich VLDL particles in triglyceride-depleted LDL carrying cholesterol, which is then removed from plasma via LDLR. PCSK9 might play a role in the modulation of triglyceride-rich lipoprotein (TGRL) metabolism, mainly VLDL and their remnants. *In vitro*, PCSK9 affects TGRL production by intestinal cells as well as the catabolism of LDL receptor homologous and non-homologous targets such as VLDL receptor, CD36 and ApoE2R (Baragetti, Grejtakova et al. 2018). Thus, a different mechanism has been reported if compared to that mediated by LPL. LPL gain of function is associated with lower plasma TG and remnants cholesterol content, while reduction in circulating PCSK9 associates with lower plasma of ApoC-III, therefore supporting their differential contribution in TGRLs metabolism (Baragetti, K. et al. 2017) Moreover, the pharmacological inhibition of PCSK9 by alirocumab did not alter the mean levels of post-heparin HDL (0.96±0.6 vs 1.05±0.62 µmol ffa/mL plasma/hr) or LPL activities (Reyes-Soffer, Pavlyha et al. 2017), as was for evolocumab (Watts, Chan et al. 2017).

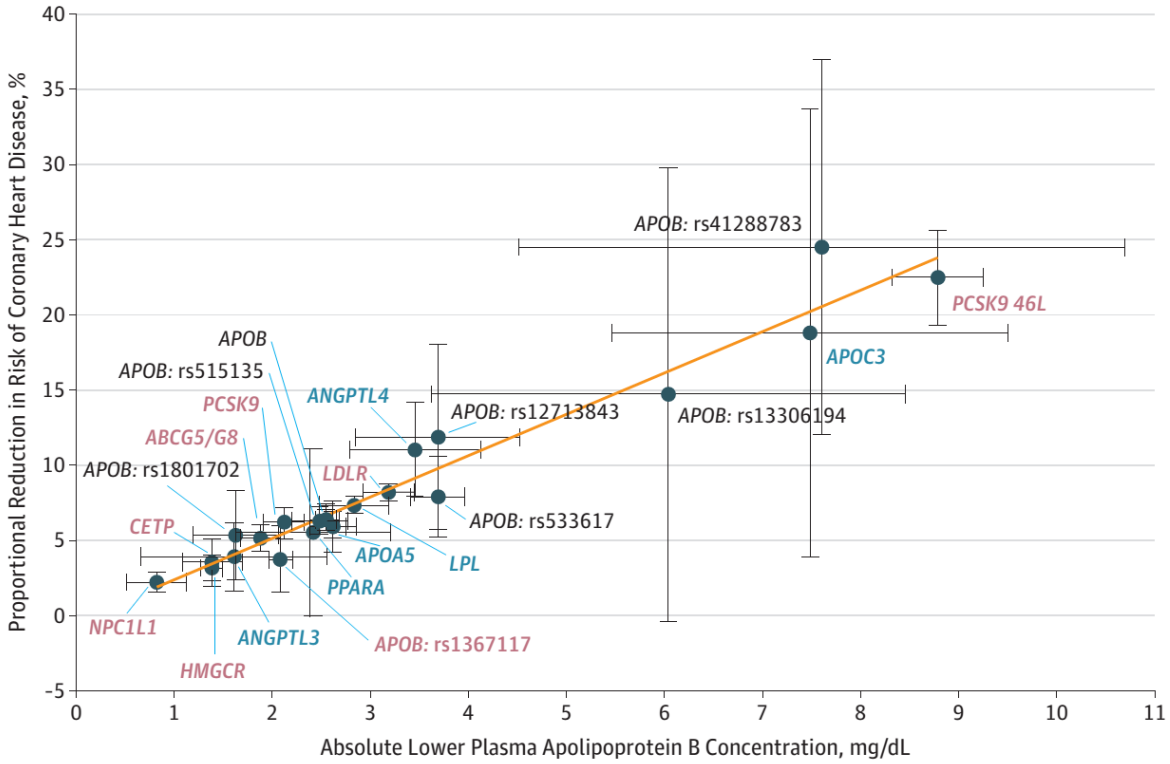

Fig.4 Log-Linear Association Between Absolute Difference s in Apolipoprotein B (ApoB) and Lower Risk of Coronary Heart Disease (CHD). The associations of each gene tic variant with ApoB concentration is plotted against its unadjusted association with CHD, expressed as a proportional lower risk. Variants in the genes that encode the targets of therapies that lower triglycerides through the LPL pathway are marked with blue labels, and variants in the genes that encode the targets of therapies that lower LDL-C through upregulation of the LDL receptor are marked by red labels. Circles represent the associated absolute change in ApoB and corresponding proportional lower risk of CHD for each variant. The horizontal lines through each circle represents ±1 standard error for the associated absolute change in ApoB for each variant; and the vertical line through each circle represents ±1 standard errors for the associated proportional lower risk of CHD. Associations with CHD were measured in all 654 783 participants included in the study; associations with ApoB were measured in a meta-analysis of 14 studies including up to 84 324 participants (Ference, Kastelein et al. 2019)

LPL is a key enzyme in TGs catabolism; it catalyses the hydrolysis of TGs in TGRL such as chylomicrons and VLDL (Rodrigues, Artieda et al. 2016). LPL belongs to the mammalian lipase family (Rader and Jaye 2000) and it is primarily synthesized in adipocytes, macrophages,

muscle cells where the dimer formation is the key step for LPL activity.(Li, He et al. 2014). More than 200 mutations are known in LPL gene but, in particular, the LOF mutations lead to the onset of type I hyperlipoproteinemia, also known as familial lipoprotein lipase deficiency or familial chylomicronemia syndrome (FCS). FCS is characterized by a defective TG metabolism which causes the accumulation of chylomicrons and extremely high levels of TGs in the plasma (880mg/dL or 10mmol/L) (Santamarina-Fojo 1998). Among many symptoms of FCS, the most debilitating symptom is the recurrence of severe potentially life-threatening acute pancreatitis attacks (PAs) occurring in 30% of patients (Pingitore, Lepore et al. 2016). Since TG-lowering drugs, such as niacin and fibrates, are not effective in these patients, the main therapeutic approach consists of diet treatment to reduce TGs levels; acute plasmapheresis may also help during an episode of acute pancreatitis. For the treatment of adults with genetically confirmed type I hyperlipoproteinemia and with an LPL protein mass >5% of normal, who suffer from severe/multiple pancreatitis attacks, the first gene therapy treatment has been approved by the European Medicines Agency but unfortunately has been also withdrawn due to cost-effectiveness (Botta, Maurer et al. 2019).

AIM

The rationale for performing this study lay on the following assumptions: (i) obesity is associated with an increased risk of CVD, (ii) obese patients are characterized by increased levels of inflammatory adipokines, (iii) PCSK9, plays a role in CVD, (iv) there is a lack of molecular mechanisms linking adipokines and the risk of CVD. Obesity is characterized by an excessive accumulation of adipose tissue which is an endocrine organ secreting active molecules, namely, adipokines. In a condition of dysfunctional visceral fat depots, adipokines exert different effects which may be detrimental for the CV system. In this context, PCSK9 plays also a role on atheroma formation (Ruscica, Ferri et al. 2017). It has been demonstrated that serum PCSK9 levels are linearly associated with a higher necrotic core fraction (Cheng, Oemrawsingh et al. 2016) making circulating platelets a further target. Together with a direct pro-inflammatory effect on macrophages (Ricci, Ruscica et al. 2018), PCSK9 was suggested to be secreted from visceral adipose fat into the portal vein to affect hepatic LDLR degradation, LDL plasma levels, and risk for CVD (Franzen, Ermel et al. 2016). Since no clear mechanisms have been so far described relative to the relationship between elevated adipose tissue content and an increased risk of CVD, the study of adipokines could be of interest. It has been demonstrated that the activation of adiponectin receptors induced PCSK9 expression through the involvement of PPARγ pathway (Sun, Yang et al. 2017). Relative to resistin, Rashid et al. demonstrated that both resistin and PCSK9 play a role in atherogenic dyslipidemia (Rashid and Kastelein 2013) whereas leptin decreases LDLR expression through PCSK9 (Du, Li et al. 2016). In particular, by using *in vitro* tools, the present work aimed to demonstrate some of the possible mechanisms by which leptin and resistin activate PCSK9, in parallel testing the involvement of STAT3 inflammatory pathway and trying to decipher the role of SRE and HNF-1 motifs in PCSK9 transcription. Since obese patients have raised levels of TG and apolipoprotein B (apoB)-containing lipoproteins a feature associated with an increased CV risk (Lavie, Milani et al. 2009), this issue has been also addressed. PCSK9 is not only involved in LDL-C regulation but also in TG metabolism and elevated plasmatic levels of TGs have been reported in those with gain of function (GOF) mutations in PCSK9 gene while, in patients carrying loss of function (LOF) mutations, LDL-C reduction associates with reduced fasting and post-prandial TG levels. Thus, in order to study the complex biosystem of TG metabolism, we have developed and characterized an *in vitro* tool allowing to evaluate the impact of genetic

variants of LPL, an enzyme involved in TG metabolism (Roubtsova, Munkonda et al. 2011). We believe that this model could be of interest to study the effect of PCSK9 on TG regulation. Finally, to study the effect of leptin on PCSK9 expression, animal models have been used, *i.e.* wild type C57BL6J mice and ob/ob mice.

MATERIALS AND METHODS

Population studied.

To investigate if there was a link between BMI and PCSK9 expression, we selected 80 healthy subjects from Brisighella Heart Study cohort. The Brisighella Heart Study, a prospective, population-based longitudinal epidemiological investigation involving 2939 randomly selected patients, free of cardiovascular disease at enrolment, resident in the northern Italian rural town of Brisighella (Cicero, D'Addato et al. 2012).

Enzyme-linked immunosorbent assay (ELISA)

Blood plasma samples were collected and stored at -80°C until assayed. PCSK9 concentrations were measured by using Quantikine® ELISA Human Proprotein Convertase 9/PCSK9 Immunoassay kit (R&D system, Space Import-Export Srl, Milan, Italy). In a microplate, pre-coated with monoclonal antibody specific for human PCSK9 or human leptin, the assay diluent RD1-9 was pipetted into each well and 50 µL of standards, prepared by dilution series from a stock solution of human PCSK9 (40 ng/mL), control and 20-fold diluted samples were added to each well. The microplate was incubated for two hours, protected from light, at room temperature, to allow the binding between PCSK9 and the primary antibody. After two hours, the unbound was washed away using the washing buffer included in the kit and 200 µL of human PCSK9 conjugate was pipetted into each well. Two hours later, the plate was washed three times and 200 µL of substrate solution, prepared by mixing in equal part a stabilized hydrogen peroxide solution and a stabilized chromogen (tetramethylbenzidine) was added to the plate followed by an incubation of 30 minutes at room temperature, protected from light. The enzyme reaction resulted in a blue product which turned yellow when the reaction was stopped using 50 µL of Stop Solution. The optical density (O.D.) of each well was analysed within 30 minutes using a microplate reader (EnSpire Multimode Plate Reader, Perkin Elmer, Milan, Italy) set to 450 nm with a wavelength correction at 570 nm. Data were linearized by plotting the log of the human PCSK9 concentrations versus the log of the O.D. on a linear scale, and best fit line was determined by regression analysis. The assay has a range of detection from 0.030 ng/mL to 0.219 ng/mL.

Similarly, leptin concentration was evaluated using Quantikine® ELISA Human Leptin Immunoassay kit (R&D system, Space Import-Export Srl, Milan, Italy)

Serum of HFD mice was used to quantify circulating leptin using the Mouse/Rat Quantikine® enzyme-linked immunosorbent assay (ELISA) kit (R&D system, Space Import-Export Srl, Milan, Italy) following the instruction of manufacturer.

Animals

All the *in vivo* experiments were conformed to the European Commission Directive 2010/63/EU and were authorized by the Italian Ministry of Health. For this project, four-week-old male ob/ob mice (n=5) and their lean (n=5), wild-type male C57BL/6J controls were purchased from Charles River (Calco, Italy). In compliance with the Principles of Laboratory Animal Care (National Institutes of Health publication 86-23), mice were housed at constant room temperature (23 °C) in a 12-h light/dark cycle (7 a.m. to 7 p.m.) receiving standard chow diet and water ad libitum. Mice were sacrificed at 14 weeks of age in the fasted state. Liver was dissected, flash frozen in liquid nitrogen and stored at -80°C for RNA extraction.

Wild-type male C57BL/6J mice (n=10) followed a 6 weeks high fat diet and wild-type male C57BL/6J mice (n=10) followed 6 weeks chow diet scheme. The kcal per each diet are reported in table 3.

After 6 weeks, mice were sacrificed. Blood was collected and stored at -80°C and liver was dissected, flash frozen in liquid nitrogen and stored at -80°C for RNA extraction.

	Chow	**HFD**
Protein	20	20
Carbohydrates	70	20
Fat	10	60

Table 3 *Chow and high fat diet composition; values are expressed as percentage of kcal.*

Cell cultures

The human hepatocellular carcinoma cell line, HepG2, were used to study the effect of adipokines on PCSK9. These cells were grown in monolayer in a humified incubator at 37°C with 5% of CO_2. The culture medium used was the Minimum Essential Medium (MEM, Sigma-Aldrich, Milan, Italy) supplemented with sodium pyruvate (1mM; Sigma-Aldrich, Milan ,Italy), nonessential amino acid (1mM; Sigma Aldrich, Milan, Italy), penicillin (100 U/mL; Sigma-

Aldrich, Milan, Italy) and streptomycin (100 µg /mL; Sigma-Aldrich, Milan, Italy) and 10% Fetal Bovine Serum (FBS; Sigma-Aldrich, Milan, Italy). Medium was replaced every 3 days and, once confluent, cells were harvested with 0.05% Trypsin/0.02% ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, Milan, Italy) and seeded in 100mm Petri dish with 8mL of medium at density of 1 $x10^6$ cells. For all the experiments the day after the seeding, the medium was changed with fresh MEM containing 10% LPDS (serum deprived of lipoproteins). $HepG2^{PCSK9}$, a model stably overexpressing PCSK9, were used to better characterize the *in vitro* findings. In particular, these cells were generated by transfecting HepG2 cells with pBM-IRES-PURO retrovirus encoding control vector (PURO) or human PCSK9. After puromycin selection, the amount of PCSK9 released in the cultured media was measured by ELISA assay and while control HepG2 cells released 1.7 ng/ml of PCSK9, in the $HepG2^{PCSK9}$ values were approximately 10 ng/ml (9.0 ± 1.0 ng/ml) (Ricci, Ruscica et al. 2018).

Cellular RNA extraction and RT

Total RNA was extracted with the iScript™ RT-qPCR Sample Preparation Buffer (BIO-RAD laboratories) according to manufacturer's instructions. Briefly, cells seeded in a 48 well plate were washed once with 500 µL of Phosphate Saline Buffer containing Mg^{2+}/K^{2+} (PBS, Sigma-Aldrich, Milan, Italy) and lysed directly in 50 µL of iScript™ RT-qPCR Sample Preparation Buffer.

One µg of total RNA was retro-transcribed into firststrand complementary DNA (cDNA) using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (ThermoFisher Scientific) in a final volume of 10 µL containing:

COMPONENT	**FINAL CONCENTRATION**
Maxima Enzyme Mix	2 µL
5x Reaction Mix	1 µL
Nuclease free water	6 µL
RNA template	1 µL

Table 4 Components for Maxima First Strand cDNA synthesis kit for RT-qPCR

The mixture was then incubated 10' at 25°C, 15' at 50°C and 5' at 85°C. The product of the first strand cDNA synthesis was used directly in qPCR or stored at -20°C. Maxima Enzyme Mix

contains Maxima Reverse Transcriptase and RiboLock™ RNase Inhibitor which protects RNA template from degradation by RNases A, B and C at temperatures up to 55°C; the 5X Reaction Mix is composed by: reaction buffer, dNTPs, oligo $(dT)_{18}$ and random hexamer primers.

Real Time quantitative PCR (qRT-PCR)

qPCR was carried out by using 2 µL of cDNA following a dye-based method (SYBR Green), a technique that utilizes a fluorescent signal to quantify the amount of DNA in a sample. All the reactions were performed in duplicate in 96-well PCR plates utilizing the CFX96 C1000 Touch™ Real-Time detection system (Bio-Rad Laboratories). The reactions were carried out in 14 µL final volume containing:

COMPONENT	FINAL CONCENTRATION
Maxima SYBR Green/Fluorescein qPCR Master Mix	2x
Sense primer (Sigma-Aldrich)	500 nM
Antisense primer (Sigma-Aldrich)	500 nM
cDNA template	50 ng
Nuclease Free water	

Table 5 Components for Maxima SYBR Green /Fluorescein qPCR Master Mix

The maxima SYBR Green/Fluorescein qPCR Master mix contains the Maxima® Hot Start Taq DNA polymerase, dNTPs, SYBR® Green I dye and fluorescein passive reference dye.

For each primer set, no-template controls, obtained replacing cDNA with water, and RT controls were included to detect possible contaminations. The expression of β-actin gene was used as internal control. Table 7 indicates the sequences of primers used. Relative differences in target mRNA levels between control and treated samples were analyzed using the $^{\Delta\Delta}$Ct method.

The thermal cycling protocol was:

STEP	TEMPERATURE	TIME	CYCLES
Polymerase Activation, DNA Denaturation	95°C	10 minutes	1

Denaturation	95°C	15 seconds	40
Annealing and extension	55°C	1 minutes	

Table 6 Conditions for SYBR Green detection method.

Gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
PCSK9	CCTGCGCGTGTCAACT	GCTGGCTTTTCCGAAACTC
B- ACTIN	TTCTACAATGAGCTGCGTGTG	GGGGTGTTGAAGGTCTCAAA
STAT3	CAGCAGCTTGACACACGGTA	AAACACCAAAGTGGCATGT
APOB	GCAGACTGAGGCTACCATGA	AGGATTGTTCCGAGGTCAAC
CAP1	ACTGGCCTGGAGCAAAACG	CGGCAGAGGGTCCAGATG
OBRb	TACTTTGGAAGCCCCTGATG	AAGCACTGAGTGACTGCACG
18S	CTCGCTCCTCTCCTACTTGG	CCATCGAAAGTTGATAGGGC

Table 7 Primers sequences

Protein extraction

To evaluate the possible effect of adipokines on PCSK9 and STAT3 inflammatory pathway, HepG2 cells were seeded in a 6-well plate at the density of 0.9 million/well in MEM containing 10% FBS and, once confluent, were starved overnight (MEM without FBS). The medium was then changed with MEM containing 10% LPDS (serum deprived in lipoproteins) and added of leptin or resistin both at the dose of 50 ng/mL. After 24 and 48 hours of treatment, before harvesting, the cells were washed once with warm (37°C) PBS containing Ca^{2+} and Mg^{2+} and lysed with 80 µL of Mammalian Protein Extraction Reagent (M-PER ™, ThermoScientific) containing 1% protease (Complete Mini EDTA-free, Protease Inhibitor Cocktail Tablets; Roche Diagnostic, Mannheim, Germany) and phosphatase (PhosSTOP, Phosphatase Inhibitor Cocktail Tablets, Roche Diagnostic) inhibitors. The lysis procedure was performed on a shaker for 10 minutes at room temperature, then the lysate was collected and centrifuged at 14000 rcf for 10 minutes at 4°C. The supernatant, containing cytoplasmatic and nuclear protein extract, was transferred into a new tube for dosage analysis by Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). Two mg/mL of albumin were used to set a curve of standards in M-PER Buffer and the absorbance of the standards and the samples was measured at 550nm on a plate reader (EnSpire® Multimode Plate Reader, Perkin Elmer, Milan, Italy). Sample

concentrations were interpolated according to a quadratic polynomial equation ($y= a + bx + cx^2$).

Transfection of reported constructs

To explore if leptin and resistin had a direct effect on PCSK9 transcription activity, we evaluated this phenomenon by transiently transfected HepG2 cells with constructs containing a luciferase sequence in the proximal human PCSK9 promoter region (-440 to -94). HepG2 cells were transfected with the plasmid PCSK9 pGL3-PCSK9-D4 containing the 5' flanking region of the PCSK9 gene from -440 to -94, relative to the ATG start codon in front of the luciferase coding sequence. Three different plasmid variants were used: the wild type, PCSK9 pGL3-PCSK9-D4 with a mutation on SRE responsive element (SRE-mu) and PCSK9 pGL3-PCSK9-D4 with a mutation on HNF-1 site (HNF-1-mu). HepG2 cells were seeded in 6mm Petri dish at the density of $9x10^7$cells. The day after, cells were transiently transfected with pGL3-PCSK9-D4 plasmids (wild-type, SRE-mu and HNF-1-mu), using the TurboFect™ transfection reagent (Thermo Fisher). Briefly, in every 6mm Petri dish the medium was changed with 4mL of MEM with 10% FBS, in a tube 400 µL of MEM, 16 µL of TurboFect™ and 4 µg of plasmidic DNA were mixed and incubate for 20 minutes at room temperature. After incubation time, the mixture was added to the plates. The day after cells were harvested and seeded in a 48 well plate and 48 hours post transfection, cells were incubated with MEM/5% LPDS ± leptin (50 ng/mL) and resistin (50 ng/mL) for an additional 24- and 48 hours. At the end of treatment times, in each well, cell culture medium was replaced by 50 µL of medium without phenol red, to not interfere with luciferase detection, and 50 µL of Neolite reagent (Perkin Elmer, Milan, Italy) and incubated on a shaker for 5 minutes. 80 µL of culture medium were then transferred in a 96 well plate and the luciferase activity was detected using a luminescence counter (Wallac 1450 MicroBeta®TriLux, Perkin Elmer, Milan, Italy). The same procedure was used for the transfection with pGL3-PCSK9-D1 or pGL3-PCSK9 with a mutation on STAT3 responsive element. Briefly, HepG2 cells were transfected with the construct pGL3-PCSK9-D1 containing the 5' flanking region of PCSK9 gene (from -1711 to -94) in front of the luciferase coding sequence or pGL3-PCSK9-D1-STAT3mut and after 48h the luciferase activity was detected and analyzed. To identify where to insert the mutation on STAT3 responsive element, the 2000 bp upstream the start codon of PCSK9 promoter sequence were analysed with JASPAR database, a collection of transcription factor DNA-binding sites (TFBS), modelled as position-specific

weight matrices (PSSMs). The sequence was scanned for human STAT3 sequence binding profile, setting at 80% the relative profile score threshold, defined as the minimum relative score required for reporting a match between a TFBS model and a sequence. The TFBS with the highest relative score was used to create a plasmid containing the pGL3-PCSK9-STAT3 mutation (pGL3-PCSK9-STAT3mut)

Transfection of siRNA

HepG2 cells were seeded in a 6 well plate at the density of $2x10^6$ cells per well in MEM with 10% FBS. The day after they were transfected with ON-TARGET plus SMART pool siRNA directed to STAT3 and PCSK9 or scramble control (Dharmacon™, Carlo Erba Reagents, Milan, Italy). Transfections were performed by using SilentFect™ Lipid Reagent (BIO-RAD laboratories, Hercules, CA). In detail, one hour before transfection, medium was changed in each well using MEM with 10% FBS. For every transfection two solution were prepared:

- Reagent Solution, containing 121 µL of MEM and 4 µL of SilentFect™
- siRNA Solution, containing 123 µL of MEM and 2 µL of siRNA of interest (20nM)

After mixing the two solutions, 250 µL were added to the well to treat. Fourty-eight hours post transfection, the medium was replaced with MEM/5%LPDS ± leptin (50 ng/mL) or resistin (50 ng/mL) for an additional 48 hours. Depending on the experiment, after treatments proteins or RNA were extracted and stored at -20°C until assayed.

Western Blot analysis

Sample preparation and protein electrophoresis were performed following the Protein Electrophoresis workflow NuPAGE® (Life Technologies ™). To 20 µg of protein sample were added: NuPAGE® Sample Reducing Agent (10X) (Novex®, Life Technologies ™) containing 500nM dithiothreitol (DTT), NuPAGE® LDS (lithium dodecyl sulfate) Sample Buffer (4X) (Novex®, Life Technologies ™) and deionized water; then the samples were heated at 70°C for 12 minutes. Following this protocol, the electrophoresis was conducted in denaturing and reducing conditions allowing the separation of proteins by their molecular mass. Samples and 10 µL of Protein Standard (Novex®, Sharp Pre-Stained Protein Standard; Life Technologies ™) were loaded in a precast polyacrylamide del (NuPAGE® 10% Bis-Tris Protein Gels,1.5 mm, 10 well, Life Technologies ™). The gel runs using a specific running buffer, the NuPAGE® MES SDS Running Buffer (20X) (Life Technologies ™) added with an antioxidant (NuPAGE® Antioxidant;

Life Technologies™) to maintain the reduced state of proteins while running through the gel. The run was performed at constant voltage (200V) for 35 minutes. Proteins were then transferred to a nitrocellulose membrane (0.45 µm pore size) (ThermoScientific, Rockford, USA) using a sandwich method. Briefly, the sandwich is assembled with nitrocellulose membrane, filter paper and sponges using a gel holder cassette. This cassette, together with a sealed ice block is placed into a transfer tank filled of transfer buffer. The transfer buffer is composed of 700mL of bi-distillated water, 200 mL of methanol and 100 mL of Tris-Glycine Buffer (10X, Bio-Rad). The transfer run was performed at constant amperage (200 mA) for 2 hours, in ice. Subsequently to verify the correct transfer of proteins, the nitrocellulose membrane was stained with Red Ponceu 1X, then washed 3 times with TBST (tris-buffered saline, 0.1% Tween20) and blocked in a solution of 5% BSA in TBST for 1 hour and 30 minutes. The primary antibody was diluted in 5% BSA or 5% milk solution and the membrane was incubated overnight on a shaker, at 4°C. The day after, the membrane was washed 3 times with TBST and incubated 1 hour and 30 minutes at room temperature with the secondary antibody, diluted in 5% milk solution. After further 3 washes, the membrane was exposed to chemiluminescence substrates (Clarity™ Western ECL Substrates, Bio-Rad) for 5 minutes. The protein bands were revealed using the ChemiDoc™ XRS System (Bio-Rad) and analysed through the Image Lab™ 3.0 Software (Bio-Rad). To evaluate the expression of different proteins on the same membrane, the Thermo Scientific™ Restore™ Plus Western Blot Stripping Buffer was used before re-incubating the membrane with a different primary antibody.

All the antibodies used in this project are reported in Table 8.

NAME	**kDa**	**PRIMARY ANTIBODY**	**SECONDARY ANTIBODY**
PCSK9	74	1:1000	1:10000 (anti - rabbit)
p-STAT3	88	1:10000	1:10000 (anti - rabbit)
STAT3	88	1:10000	1:10000 (anti - rabbit)
Tubulin	55	1:2000	1:10000 (anti-mouse)
B-actin	44	1:1000	1:10000 (anti-mouse)
ApoB		1:250	1:2000 (anti-mouse)
Albumin	69	1:2500	1:2000 (anti-mouse)

Table 8 Primary antibody used with indication of dilutions and molecular weight. In the last column is indicated the species and dilution of the secondary antibody used.

The following protocol has been used for the experiments in paragraph "Molecular characterization of mutations causing LPL deficiency". Post-heparin plasma samples were diluted 1:50 in 0.5-M Tris-HCl (pH 7). The diluted plasma samples, cell lysates, and concentrated media samples were mixed with Laemmli buffer 5X (SDS 10%, Tris HCl 62.5mM pH 6.8, glycerol 50%, bromophenol blue 0.01% and β-mercaptoethanol 25%) and boiled for 5minat 95°C. Samples were loaded into a 10% acrylamide gel containing SDS 0.1%. The gel ran at 100V for 90min using a running buffer containing 0.1% SDS. The gel was then transferred onto a nitrocellulose membrane (400mA, 60min). Membranes were incubated for 1h with primary antibodies, washed 2 times for 10min with 0.2% tris-buffered saline containing 0.2% tween (TBST), incubated 1h with HRP-conjugated secondary antibodies and washed 3 times for 10min with TBST. After an incubation of 5 minutes with chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA) the bands were detected and visualized by Chemidoc XRS System (Biorad, Hercules, CA). The protein expression was quantified using Image Lab Software (Biorad). The antibodies used are: mouse anti-LPL (Sigma-Aldrich) (1:1000), mouse antiAPOA1 (AbD Serotec, Oxford, UK) (1:500), mouse anti-V5 (Invitrogen) (1:5000), rabbit anti-Calnexin (Sigma-Aldrich) (1:2000), mouse antiAlbumin (Sigma-Aldrich) (1:1000).

Patients

Two patients from the Lipid Clinic in Vienna (Austria) with excessive primary hypertriglyceridemia, with no other apparent cause and not carriers of well characterized LPL mutations, underwent routine clinical determination of serum lipid levels performed by enzymatic methods using Roche reagents, run on a CobasC111 analyser. Chylomicrons were analyzed by floating in microliter tubes for 10minat 16.000g (maximal) at 4°C prior to triglyceride determinations. Chylomicron triglyceride concentrations were determined by subtracting the cleared infranatant from original serum.

DNA sequencing

For DNA sequencing, using a customized gene panel (Nextera Rapid Capture Custom Enrichment; Illumina, San Diego, CA) genes linked to hyperlipidaemias were enriched from isolated genomic DNA and sequenced on a MiSeq sequencing platform (Illumina, USA) using

2×150-bp paired-end chemistry following the manufacturer instructions. Sequenced reads were aligned to the GRCh37/ hg19 human reference. The SeqNext® software (JSI Medical Systems GmbH, Germany) was utilized to perform the data analysis including copy number variation (CNV) analysis. The region of interest was the coding region ± 30 bp intronic flanking regions of the *LPL* gene (NM_000237.2). In addition to LPL, also *APOC2, APOA5, LMF1* and *GPIHBP1* genes have been sequenced, without highlight any other mutation.

Post-heparin plasma samples collection

Post-heparin blood was collected at a fasting state, 10 minutes after an intravenous bolus injection of heparin (60 IU/kg body weight) from the contralateral arm. Blood samples were centrifuged at 3000 rpm at 4°C for 10min and supernatants were stored at −80°C until analysed.

Site-direct mutagenesis, cloning and transfection

Wild type LPL cDNA was synthesized and cloned in pcDNA3.1 vector with a V5 epitope tag at the C-terminus by GeneArt Gene Synthesis (Thermo Fisher Scientific, Rockford, IL). A single base-pair change from valine to alanine at nucleotide 200 was introduced using in situ mutagenesis technique to obtain the LPL V200A mutation (primer forward: CAG AAA CCA GCT GGG CAT GTT and primer reverse: AAC ATG CCC AGC TGG TTT CTG). While to obtain the LPL N291S substitution (from asparagine to serine at nucleotide 291) the following primers were used: primer forward GCT ATG AGA TCA CTA AAGTCA GAGC, primer reverse GCT CTGACT TTACTG ATCTCA TAG G. All the primers were purchased from Sigma Aldrich (St. Louis, MO). The presence of the LPL mutation and fidelity of each construct were verified by DNA sequencing (Eurofins Genomic, Germany). Human embryonic kidney 293T/17 (HEK 293T/17) cells were purchased from American Tissue Culture Collection (Manassas, VA) and cultured in high glucose Dulbecco's Modified Eagle's Medium containing 10% Fetal Bovine Serum (FBS), 5% penicillin-streptomycin and 2mM L-glutamine. Cells were transiently transfected with one of the two plasmids, the first one containing the human wild type *LPL* cDNA or the second one, carrying the other variants (3 μg/mL). TurboFect transfection reagent (Thermo Fisher Scientific) was used following the manufacturer's instructions. After 48h cells were collected and lysed using mammalian protein extraction reagent (M-PER, ThermoFisherScientific) containing a complete protease inhibitor cocktail (Sigma Aldrich). Moreover, media were

concentrated 10 times by centrifuging using VIVASPIN tubes (Sartorius Stedim Biotech, Göttingen, Germany). HEK 293T/17 lysates and media were used to analyze protein synthesis and secretion by Western blot; media fractions were additionally used to measure LPL activity.

Lipoprotein lipase enzymatic assay

The LPL enzymatic activity was measured by an assay which take advantage of a stable, radioactive substrate emulsion. LPL activity was measured in pre-heparin and post-heparin human plasma samples and in media fractions of HEK293T/17cells transfected with LPL wild type plasmid and the two plasmids containing LPL mutants. Plasma samples were first diluted: 7.5µL of each plasma sample was mixed with 42.5µL of 0.2-M Tris HCl buffer (pH 8.0). The diluted plasma samples and 50µL of each concentrated medium sample were incubated for 15 minutes at 37°C with a reaction mix containing radio-labeled substrate [9,10-3H(N)]-triolein (Perkin Elmer, Waltman, MA), cold triolein and phosphatidylcholine (Sigma-Aldrich, St Louis, MO), heat-inactivated FBS and bovine serum albumin (SigmaAldrich, St Louis, MO) in presence or absence of 1M NaCl. The reaction was stopped and lipids were extracted using a solution of heptane/chloroform/methanol (1:1.25:1.41). Samples were then centrifuged at 3000g for 15 minutes and the amount of [3H]-oleic acid released in the upper phase was analysed by scintillation counting. A mouse (C57BL/6) post-heparin plasma was used as a positive control, while 1M NaCl was used for LPL specificity.

Analysis of LPL dimerization

To analyse the dimerization of LPL, a western blot in non-denaturing conditions was performed. Equal volumes of post-heparin plasma samples diluted 1:50 and conditioned media were mixed with non-denaturing loading buffer 5X (Tris HCl 62.5mM pH 6.8, glycerol 50%, bromophenol blue 0.01%, β-mercaptoethanol 5% and Coomassie blue G250 0.05%). Samples were not boiled. Proteins were separated by non-denaturing PAGE (4–15% gradient acrylamide gel without SDS, 100V, 90min), and transferred onto a nitrocellulose membrane (400mA, 60min). Then, western blotting was performed as described before.

Homology modelling of LPL

The homology model of human LPL was created with the protein fold recognition server Swiss-Model. As template was used the 3D structure of the human pancreatic lipase-related protein 1 and the calculated models were visualized by Swiss PDB Viewer 4.1.0. On the computer the

mutations were inserted into LPL model by substituting valine (V), a non-polar aliphatic residue, for alanine (A), another non-polar aliphatic residue at position 200. The same procedure was applied to N291S mutation, substituting asparagine(N), an uncharged polar residue, at position 291for serine (S), another uncharged polar residue.

Analysis of data

Statistical analysis was performed by using the Prism statistical package (Graph Pad Software, San Diego,CA). Data were expressed as mean ± standard error of the mean (SEM). Differences between treatment groups were evaluated by Mann Witney t-test when the comparison was made between two groups, and by *non parametric one-way ANOVA and Dunn's post hoc test;* for multiple comparisons for more groups. Differences were considered significant at $p<0.05$. Results are representative of three independent experiments.

RESULTS

In vivo results

Association between leptin, resistin and PCSK9 in humans

The circulating levels of leptin and PCSK9 were measured by ELISA in a clinical setting of 80 males (56±4 y) belonging to the Brisighella Heart Study, a prospective, population-based longitudinal epidemiological investigation involving 2939 randomly selected patients, free of cardiovascular disease at enrolment, resident in the northern Italian rural town of Brisighella (Cicero, D'Addato et al. 2012) . A positive association between circulating leptin and PCSK9 levels (β= 0.295, p= 0.044) was found only in those with BMI<25 Kg/m^2 and is lost if the data are considered across the all BMI strata, *i.e.* those with BMI between >25 and 30 Kg/m2 (β= 0.104, p= 0.452) or > 30 Kg/m2 (β= -0.040, p= 0.787) (fig.5). This lack of correlation may be due to the fact that higher BMI is associated to obesity and metabolic disturbances, often characterized by a lack of a proper hormone response following the engagement by the specific receptor, a condition defined “adipokine-resistance”. Resistin did not associate with PCSK9 (data not shown).

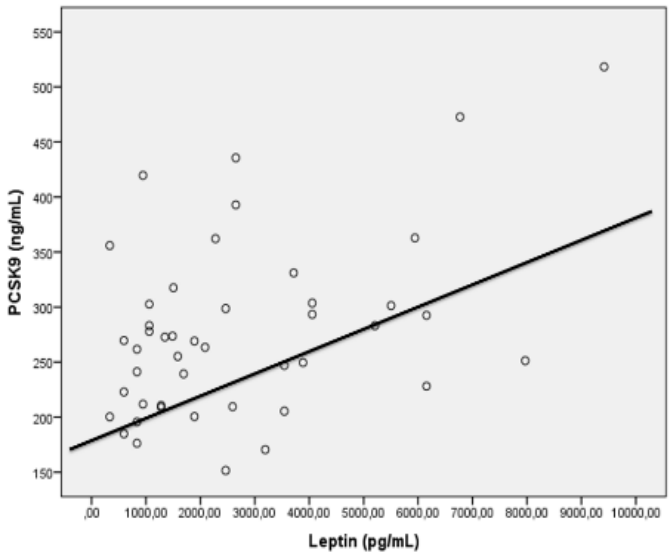

Fig.5 Association between circulating levels of leptin and PCSK9.

A positive association between circulating leptin and PCSK9 levels has been found only in patient with BMI<25 Kg/m^2 (β = 0.295, p= 0.044)

Correlation between leptin and PCSK9 in mice

To further investigate the linkbetween leptin and PCSK9, *ob/ob* mice, a genetic model lacking leptin, were used to evaluate mRNA expression of hepatic PCSK9. *Ob/ob* mice showed significant lower hepatic PCSK9 gene expression compared to wildtype mice (fig.6)

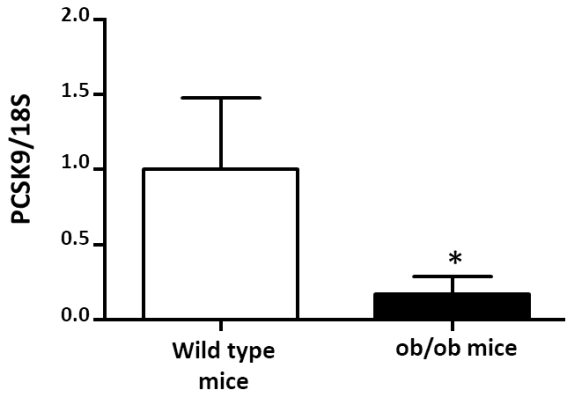

*Fig. 6 Hepatic expression of PCSK9 in mice lacking leptin. P value was calculated by Mann Whitney T-test *p<0.05*

To confirm this evidence, wild type C57BL6J mice were divided in two group: the first fed 12-weeks high fat diet (60% fat) and the controls fed chow diet. As depicted in fig. 7, an increment of serum leptin (A) corresponded to a 3-fold rise in PCSK9 liver expression in high fat diet-fed mice (B).

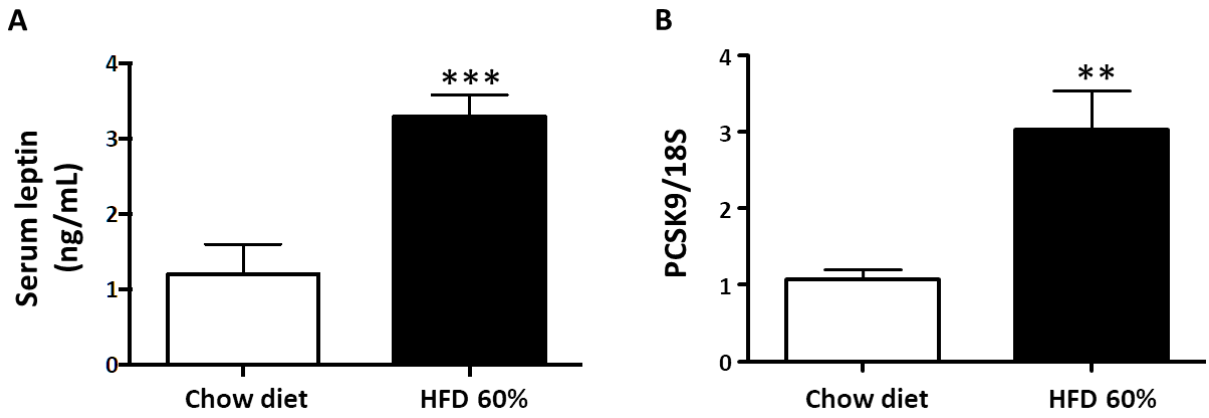

*Fig.7 Differences in serum leptin levels and PCSK9 hepatic expression in wild type mice C57BL6J fed chow diet or 60% high fat diet. P values were assessed by Mann Whitney T-test **p<0.01;***p<0.001*

***In vitro* results**

Characterization of *in vitro* model: HepG2

Although HepG2 cells have been demonstrated to express resistin receptor (CAP1) and leptin receptor, OBRb (Zhou, Lei et al. 2008, Liu, Cui et al. 2014), in order to validate the presence of these receptors in our cell strains, a qPCR was performed. Table 9 reports the Ct values of CAP1 and OBRb compared to Ct values of the housekeeping gene.

	Ct value gene of interest	Ct value of housekeeping (18S)
OBRb	23,74	9,69
CAP1	22,21	9,69

Table 9 Ct values of OBRb and CAP1. The total RNA was extracted from HepG2 cell, retrotranscribed and a qPCR was performed in order to verify the presence of leptin receptor (OBRb) and resistin receptor (CAP1)

To clarify whether the expression of OBRb and CAP1 could have been influenced by the levels of PCSK9, a real time PCR was performed on HepG2^{PCSK9} cells, a cell line stably overexpressing PCSK9. The expression of OBRb and CAP1 resulted to be not influenced by PCSK9.

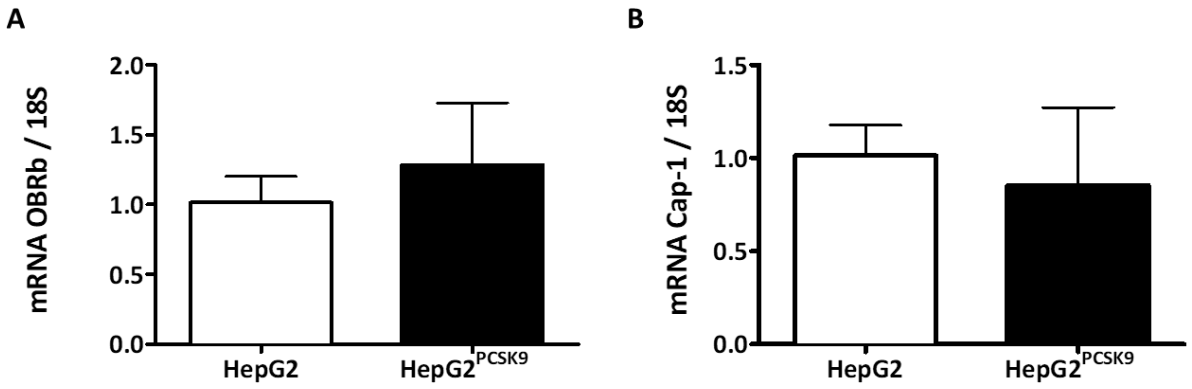

Fig. 8 OBRb and CAP1 expression in HepG2^{PCSK9} cells.
Cells were seeded in MEM supplemented with 10% FBS and the day after a Real Time PCR has been performed to evaluate the expression of OBRb (A) and CAP1 (B).

Three different serum conditions were tested to find the most suitable HepG2 culture condition to perform the experiments. HepG2 cells were cultured in: i) 1% FBS, ii) 10% FBS iii)

10% lipoprotein deficient serum (LPDS) and treated with simvastatin 20 µM, as a positive control of PCSK9 activation. Data on PCSK9 mRNA expression have been below reported.

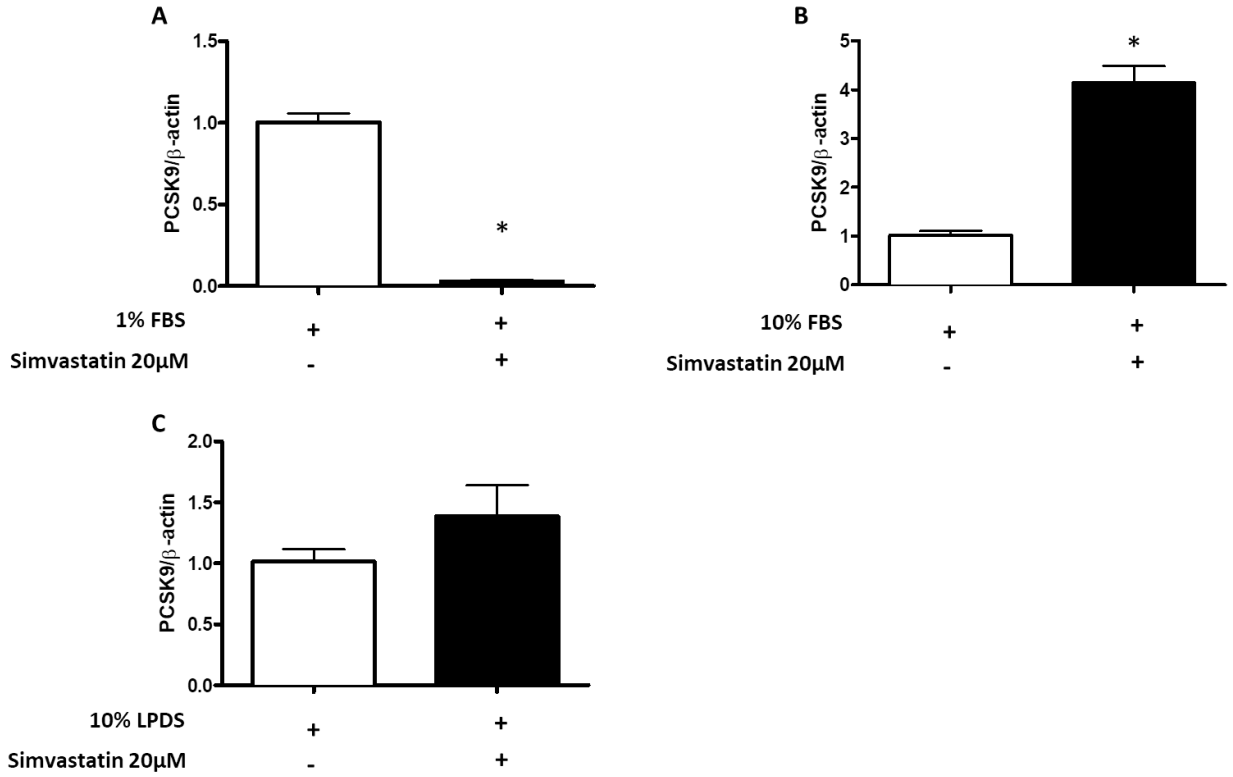

Fig. 9 PCSK9 gene expression in HepG2 cells cultured by different serum concentrations.
*HepG2 cells were seeded in MEM/10% FBS and the day after incubated with MEM supplemented with 1% FBS (A), or 10% FBS (B), or 10% LPDS (C). After 24h, the total RNA was extracted, retrotranscribed and mRNA levels of PCSK9 were determined by qPCR. Simvastatin was used as positive control. P value was calculated by Mann Whitney T-test *p<0.05*

Although simvastatin, the positive control, activated PCSK9 expression in both 10% FBS or LPDS supplementation, the last condition was used. Indeed, as reported by Pramfalk et al (Pramfalk, Jiang et al. 2010), the use of LPDS in HepG2 cells leads to a phenotype similar to the one observed by using human primary hepatocytes.

Effects of leptin on PCSK9 expression in HepG2 cells

First of all, to select the lowest dose of leptin able to modulate PCSK9 expression, the two different doses were tested: 50 ng/mL and 100 ng/mL. Although both doses increased PCSK9 mRNA expression, the maximal stimulatory effect was found after treating cells with the 50 ng/ml that was the chosen concentration for the subsequent experiments.

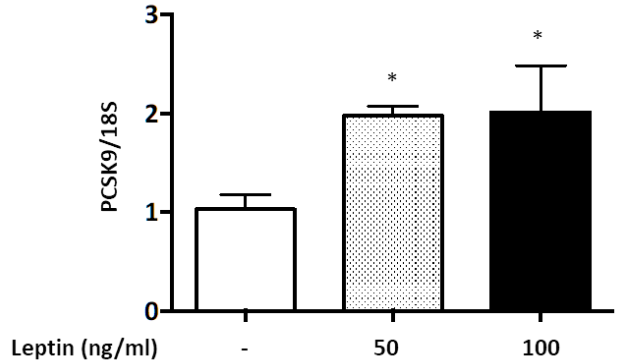

Fig.10 *Leptin induces PCSK9 mRNA expression in HepG2 cells.*
*These were seeded in MEM/10% FBS and the day after incubated with MEM supplemented with 10% LPDS for 48h at different doses of leptin. The mRNA levels of PCSK9 were determined by qPCR. Differences between groups were assessed by non parametric one-way ANOVA and Dunn's post hoc test; *p<0.05*

A 48-h incubation of HepG2 cells with 50 ng/mL leptin doubled PCSK9 gene expression, as was upon simvastatin (20 µM) treatment, the positive control.

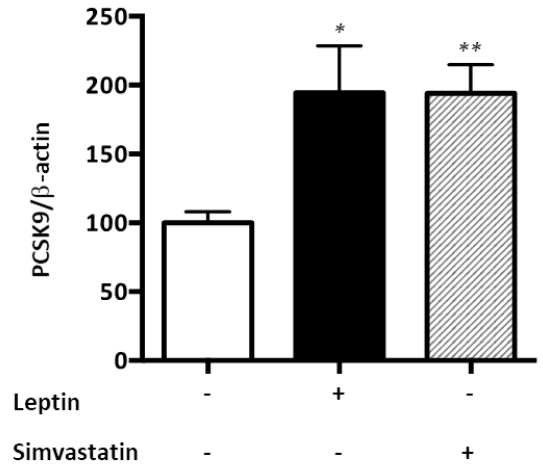

Fig.11 Leptin induces PCSK9 mRNA expression in HepG2 cells.

*These were seeded in MEM/10% FBS and the day after incubated with MEM supplemented with 10% LPDS for 48h in the presence or absence of leptin (50 ng/ml). Simvastatin (20 µM) was used as a positive control for PCSK9 activation. At the end of the incubation, the total RNA was extracted and mRNA levels of PCSK9 were determined by qPCR. Differences between groups were assessed by non parametric one-way ANOVA and Dunn's post hoc test; *p<0.05, **p<0.01.*

A preliminary analysis of PCSK9 protein expression has been done to confirm the rise in gene expression. A Western blot (WB) analysis on protein extracted from HepG2 cells treated 48h with leptin 50 ng/mL showed a trend to significance.

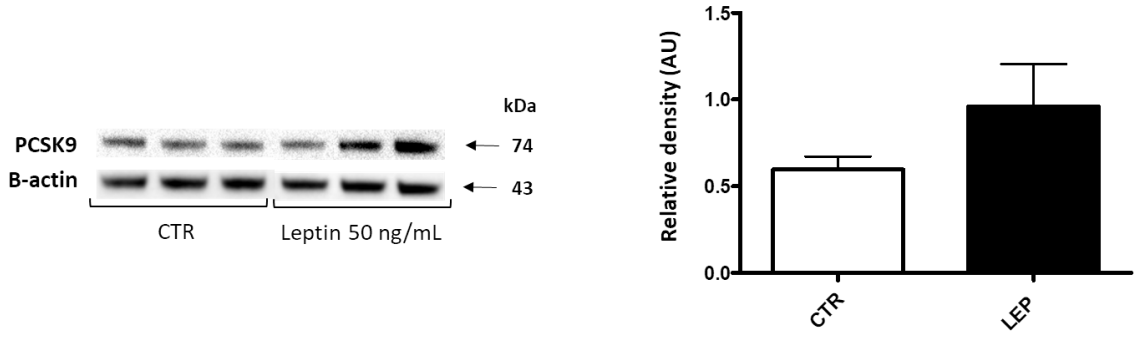

Fig. 12 Leptin induces PCSK9 protein expression in HepG2 cells.
HepG2 cells were seeded in MEM/10% FBS and the day after incubated with MEM supplemented with 10% LPDS for 48h in the presence or absence of leptin (50 ng/ml). At the end of the incubation, proteins were extracted and PCSK9 expression was evaluated using WB analysis.

Effects of leptin on STAT3 pathway

STAT3 is a well-known inflammatory pathway activated by leptin. A 48-h incubation with leptin (50ng/mL) increased STAT3 mRNA expression in HepG2 cells (fig.13 A). The activation of pSTAT3 was confirmed by WB analysis (fig.13 B). To further investigate the involvement of STAT3 in leptin-driven PCSK9 activation, HepG2 cells were transfected with siRNA scramble and siRNA anti-STAT3. To verify that the condition used to silence STAT3 were effective, we tested HepG2 for both STAT3 gene and protein expressions. These were significantly reduced (fig 13 C, D).

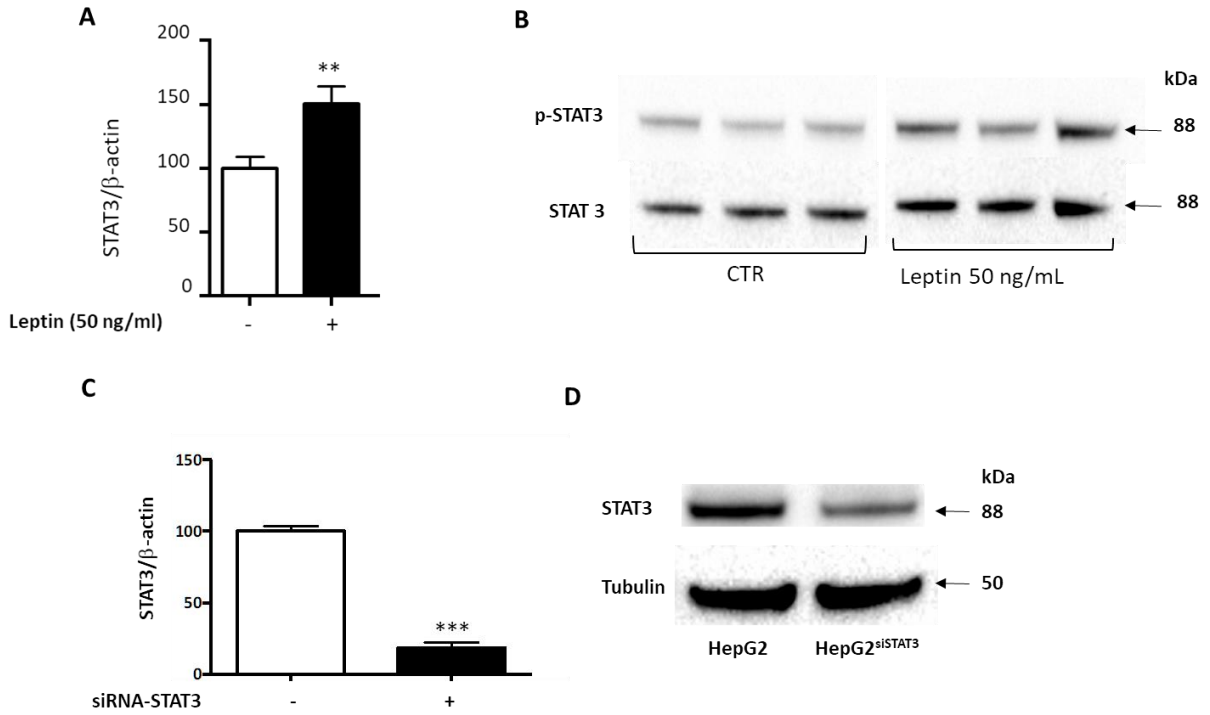

Fig.13 Leptin induces STAT3 expression.

*HepG2 cells were seeded in MEM/10% FBS and treated 48h with leptin 50 ng/mL, after the incubation time, the gene expression of STAT3 was evaluated by qPCR (A) and the activation of pSTAT3 was evaluated by western blot analysis (B). HepG2 cells were seeded in MEM 10% FBS and the day after transfected with siRNA scramble (HepG2) and siRNA anti-STAT3 (HepG2^{siSTAT3}). After 48h, the medium was replaced with MEM with 10% LPDS and 48h later, the gene and protein expression of STAT3 and PCSK9 was evaluated by qPCR and WB analysis (C and D). Differences between groups were assessed by Mann Withney t-test; **p<0.01, ***p<0.001*

Under the same experimental conditions, siRNA anti-STAT3 abolished the gene expression of STAT3 (fig.14 A) and PCSK9 (fig.14 B) induced by leptin. These results indicate the pivotal role of STAT3 in promoting gene PCSK9 expression.

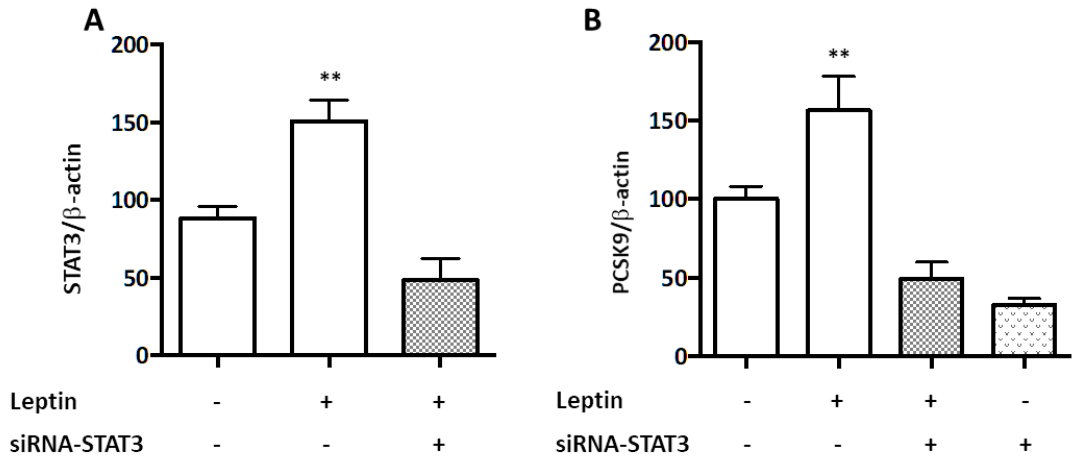

Fig. 14 Leptin regulates PCSK9 gene expression through the involvement of STAT3.
*HepG2 transfected with siRNA anti-STAT3 were or were not treated for 48h with leptin (50 ng/mL) and the gene expression of STAT3 and PCSK9 was evaluated by qPCR. Differences between groups were assessed by non parametric one-way ANOVA and Dunn's post hoc test; **p<0.01.*

Effects of leptin on PCSK9 transcriptional activity

To explore the effect of leptin on PCSK9 transcription activity, HepG2 cells were transfected with the PCSK9 pGL3-PCSK9-D4 containing the 5' flanking region of the PCSK9 gene from -440 to -94, relative to the ATG start codon in front of the luciferase coding sequence and treated with leptin. After 24h and 48h the luciferase activity was detected and analyzed. Leptin incremented the luciferase activity after 24 and 48 hours of about +54% and +20%, respectively (see fig. 15 A). Being SREBPs and HNF-1 well-known activators of PCSK9 (Jeong, Lee et al. 2008, Li, Dong et al. 2009, Ruscica, Ricci et al. 2016), the luciferase activity has been investigated upon the insertion of SRE and HNF-1 mutations in the human PCSK9 promoter sequence. In presence of mutation in the SRE sequence, the PCSK9 transcriptional activity induced by leptin was completely abolished (fig. 15 B); while, mutating the HNF-1 alpha sequence did not alter the leptin-driven luciferase activity (fig.15 C).

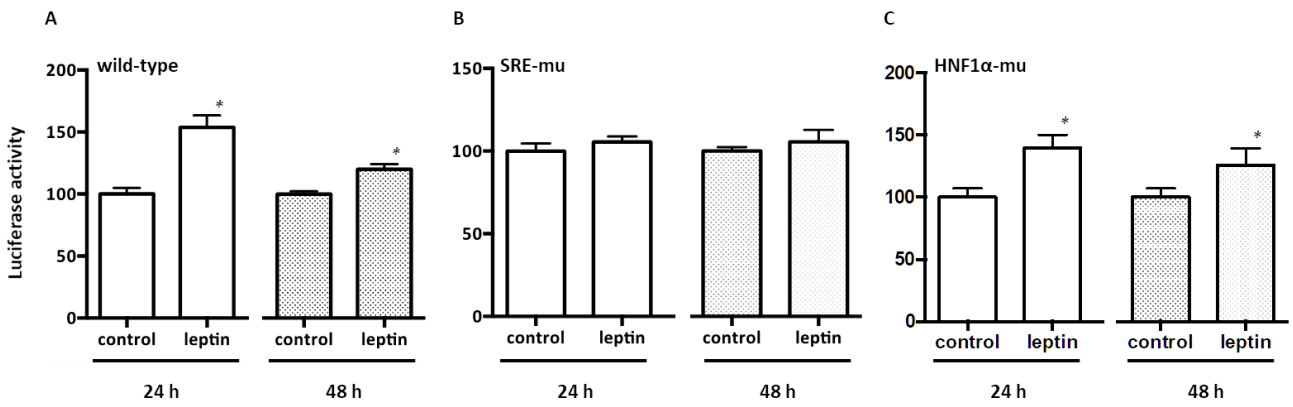

Fig.15 Luciferase activity in HepG2 cells.

*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 and, after an additional 24h and 48h, luciferase activities were determined by Neolite reagent. Differences between groups were assessed by non parametric one-way ANOVA and Dunn's post hoc test;; *p<0.05.*

Role of STAT3 in PCSK9 transcriptional activity

To investigate the role of STAT3 in PCSK9 transcriptional activity, the PCSK9 promoter sequence was analysed in order to identify transcription factor DNA-binding sites (TFBS) for STAT3. Briefly, the human PCSK9 promoter sequence was retrieved from Ensembl Genome Browser, using *Homo Sapiens* genome assembly GRCh38.p12 and 2,000 bp sequence upstream the start codon has been analysed with JASPAR database, a collection of transcription factor DNA-binding sites (TFBS), modelled as position-specific weight matrices (PSSMs). The sequence was scanned for human STAT3 sequence binding profile, setting at 80% the relative profile score threshold, defined as the minimum relative score required for reporting a match between a TFBS model and a sequence. The TFBSs obtained are reported in table 2. The TFBS with the highest relative score (13.3) was used to generate a plasmid with the pGL3-PCSK9-STAT3 mutation.

Model ID	Model name	Score	Relative score	Start	End	Strand	Predicted site sequence
MA0144.2	STAT3	1.469	0.815351011114919	82	92	1	TGGCTAGAAAA
MA0144.2	STAT3	6.048	0.870823554605476	156	166	1	GTGAAGGGAAA
MA0144.2	STAT3	3.115	0.835291567259373	290	300	-1	CTTCAGGGCAG
MA0144.2	STAT3	3.556	0.840634085643155	452	462	1	CATTTGAGAAG
MA0144.2	STAT3	2.776	0.831184733399732	494	504	1	CTTATGGGAGG
MA0144.2	STAT3	0.649	0.805417076705166	681	691	1	ATGCTGGACAG
MA0144.2	STAT3	6.633	0.877910568788044	806	816	1	CAGCAGGGAAA
MA0144.2	STAT3	3.802	0.843614265966081	890	900	-1	GTAATTGGAAT
MA0144.2	STAT3	7.180	0.884537229912598	896	906	-1	TTGCCTGTAAT
MA0144.2	STAT3	1.098	0.810856511522214	1070	1080	-1	TTGTTTGCAAA
MA0144.2	STAT3	8.358	0.898808174710999	1190	1200	-1	ATACTGGGAAG
MA0144.2	STAT3	3.916	0.84499532514012	1208	1218	-1	TTTCTGGAAGG
MA0144.2	**STAT3**	**10.451**	**0.924163936564185**	**1209**	**1219**	**-1**	**CTTTCTGGAAG**
MA0144.2	**STAT3**	**10.985**	**0.930633108484682**	**1209**	**1219**	**1**	**CTTCCAGAAAG**
MA0144.2	STAT3	1.354	0.813957837386722	1287	1297	1	CTGCCTGGTAC
MA0144.2	STAT3	4.120	0.8474666941884	1308	1318	-1	CATCCAGTAAA
MA0144.2	STAT3	0.989	0.809536025118966	1308	1318	1	TTTACTGGATG
MA0144.2	STAT3	6.202	0.872689195945845	1331	1341	-1	GTTCAAGAAAT
MA0144.2	STAT3	3.594	0.841094438701168	1332	1342	1	TTTCTTGAACA
MA0144.2	STAT3	2.053	0.822425910743328	1374	1384	-1	TTTCCAGAAGA
MA0144.2	STAT3	1.568	0.816550351976584	1374	1384	1	TCTTCTGGAAA
MA0144.2	**STAT3**	**13.347**	**0.959247685406433**	**1375**	**1385**	**1**	**CTTCTGGAAAG**
MA0144.2	STAT3	0.802	0.807270603491376	1468	1478	-1	TTTCAAGGGAT
MA0144.2	STAT3	3.057	0.834588923118196	1696	1706	1	GACCCAGGAAA
MA0144.2	STAT3	0.887	0.808300340594826	1982	1992	1	CACCCTAGAAG

Table 10 Putative sites predicted using JASPAR database, setting at 80% the relative profile score in sequence named hPCSK9. In bold with the higher score. The one with the highest score was used to create the plasmid.

HepG2 cells were transfected with the constructs pGL3-PCSK9-D1 containing the 5' flanking region of PCSK9 gene (from -1711 to -94) in front of the luciferase coding sequence and treated with leptin (50ng/mL) and simvastatin (20µM) as positive control. After 48h the luciferase activity was detected and analyzed (fig.16 A). The same conditions were used to repeat the experiment using the pGL3-PCSK9-D1. STAT3mut to investigate the effect of the STAT3 mutation on PCSK9 promoter activity (fig.16 B). No differences have been detected in the two constructs.

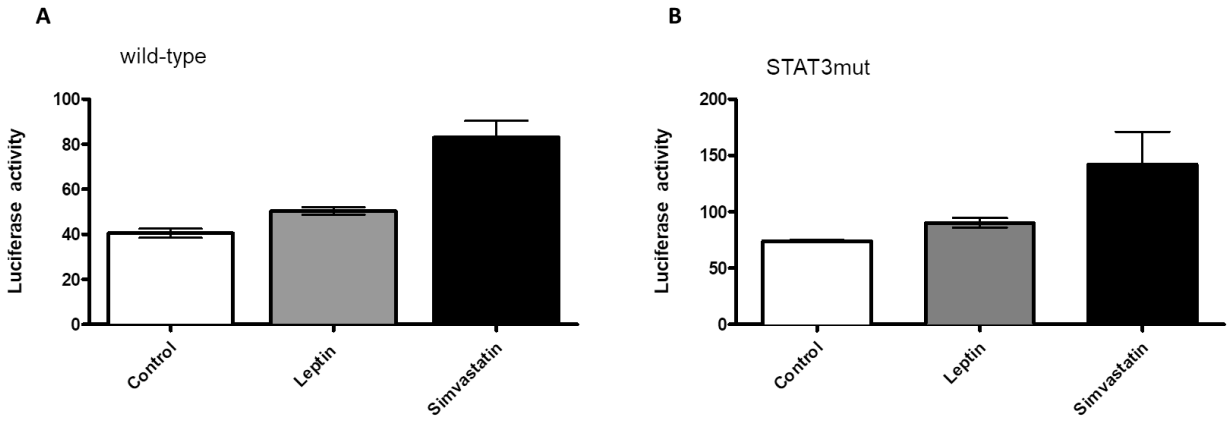

Fig.16 Luciferase activity in HepG2 cells transfected with pGL3-PCSK9-D1 wild type or STAT3 mutated.

HepG2 cells were transfected with pGL3-PCSK9-D1 or pGL3-PCSK9-D1-STAT3mut. The day after the transfection, the medium was replaced with MEM containing 10%LPDS and, after an additional 48h, luciferase activities were determined by Neolite reagent. Differences between groups were assessed by non parametric one-way ANOVA.

Effects of resistin on PCSK9 expression in HepG2 cells

To evaluate the effect of resistin on PCSK9 expression we took advantage of previous findings showing that, in HepG2 cells, incubation with resistin (50 ng/mL) stimulates PCSK9 activation (Costandi, Melone et al. 2011). The same dose has been used in our experiments. HepG2 cells were incubated for 48h demonstrating that resistin (50ng/mL) enhances PCSK9 gene expression (Figure 17); as for leptin, simvastatin (20 µM) has been used as a positive control.

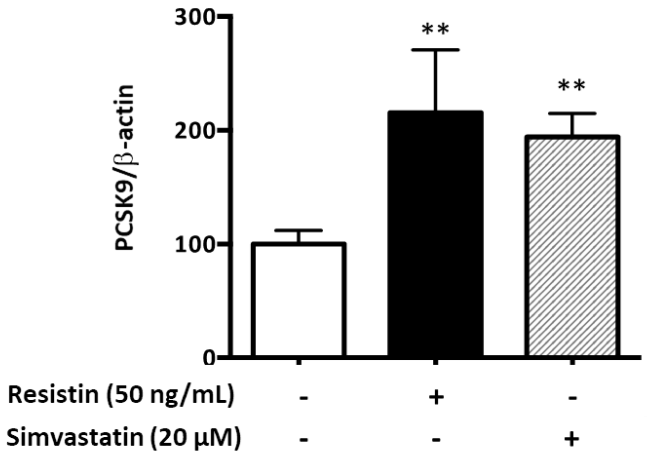

Fig. 17 Resistin induces PCSK9 in HepG2 cells.

*HepG2 cells were seeded in MEM/10% FBS and the day after incubated with MEM supplemented with 10% LPDS for 48h in the presence or absence of resistin (50 ng/ml). Simvastatin (20 µM) was used as a positive control for PCSK9 activation. At the end of the incubation, the total RNA was extracted and mRNA levels of PCSK9 were determined by qPCR. Differences between groups were assessed by non parametric one-way ANOVA and Dunn's post hoc test;; **p<0.01.*

Effects of resistin on STAT3 inflammatory pathway

Wondering whether STAT3 inflammatory pathway was involved in PCSK9 resistin-driven activation, HepG2 cells were incubated with resistin (50ng/mL) for 48h. qPCR analysis showed an increment in STAT3 gene expression (fig. 18 A). Thus, HepG2 cells were transfected with siRNA anti-STAT3 and the PCSK9 mRNA expression was significantly reduced.

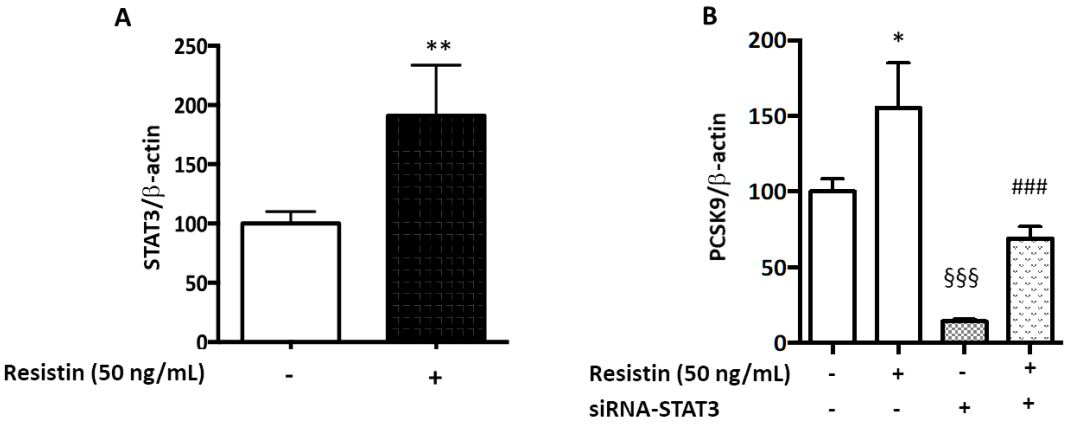

*Fig. 18 Incubation with resistin (50ng/mL) enhances STAT3 gene expression in HepG2 cells. HepG2 cells were seeded in MEM/10% FBS and the day after transfected with siRNA anti-STAT3. After 48h, the medium was replaced with MEM with 10% LPDS with or without resistin (50 ng/ml). After an additional 48h, the PCSK9 gene expression by qPCR analysis. A) shows STAT3 gene expression upon resistin treatment; differences between group were assessed by Mann Withney T test. **p<0.001. Panel B) shows the effect of resistin treatment on PCSK9 gene expression (*p<0.05); the use of a specific siRNA against STAT3 led to a 80% decrement in the expression of PCSK9 (§§§ vs control p<0.001). Treatment with resistin and siRNA against STAT3 corresponded to a decrement in resistin-driven PCSK9 activation (### p<0.00). Differences between groups were assessed by non parametric one-way ANOVA and Dunn's post hoc test.*

Effects of resistin on PCSK9 transcriptional activity

HepG2 cells were transfected with the construct pGL3-PCSK9-D4 containing the 5 ′ flanking region of the PCSK9 gene from nucleotide 2440 to 294. After 24h and 48h the luciferase activity was detected and analyzed. Similar to leptin, resistin (50ng/mL) increases PCSK9 transcriptional activity (fig.19 A). To investigate the involvement of SRE and HNF-1 in this mechanism the luciferase activity has been investigated upon the insertion of SRE and HNF-1 mutations. This effect was maintained despite the presence of HNF-1 mutation (fig.16 C). Conversely, the effects on luciferase activity were lost when cells were transfected with a plasmid carrying the SRE mutation (fig.19 B).

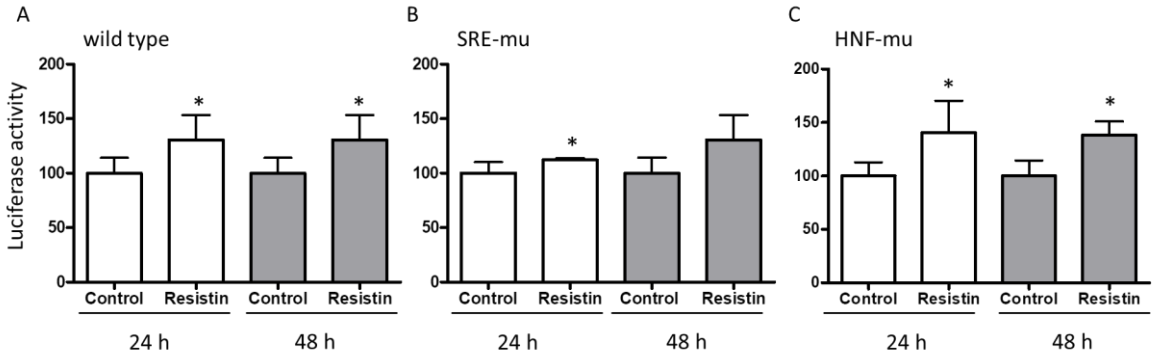

Fig. 19 Luciferase activity in HepG2 cells transfected with pGL3-PCSK9-D4 wild type or mutated on SRE or HNF1.

*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 and, after an additional 24h and 48-h, luciferase activities were determined by Neolite reagent. Differences between groups were assessed by Mann Withney t test ;*p<0.05.*

Role of STAT3 in PCSK9 transcriptional activity

The role of STAT3 in PCSK9 transcriptional activity, has been investigated by the transfection of HepG2 cells with pGL3-PCSK9-D1 or pGL3-PCSK9-STAT3mut. Briefly, HepG2 cells were transfected with the construct pGL3-PCSK9-D1 containing the 5’ flanking region of PCSK9 gene (from -1711 to -94) in front of the luciferase coding sequence or pGL3-PCSK9-D1-STAT3mut. After 48h the luciferase activity was detected and analyzed. HepG2 cells transfected with the pGL3-PCSK9-D1 showed an increase of luciferase activity after treatments with resistin and simvastatin while the same treatments in cells transfected with pGL3-PCSK9-STAT3mut did

not increase the transcriptional activity. To confirm the role of STAT3 in PCSK9, further experiments are required.

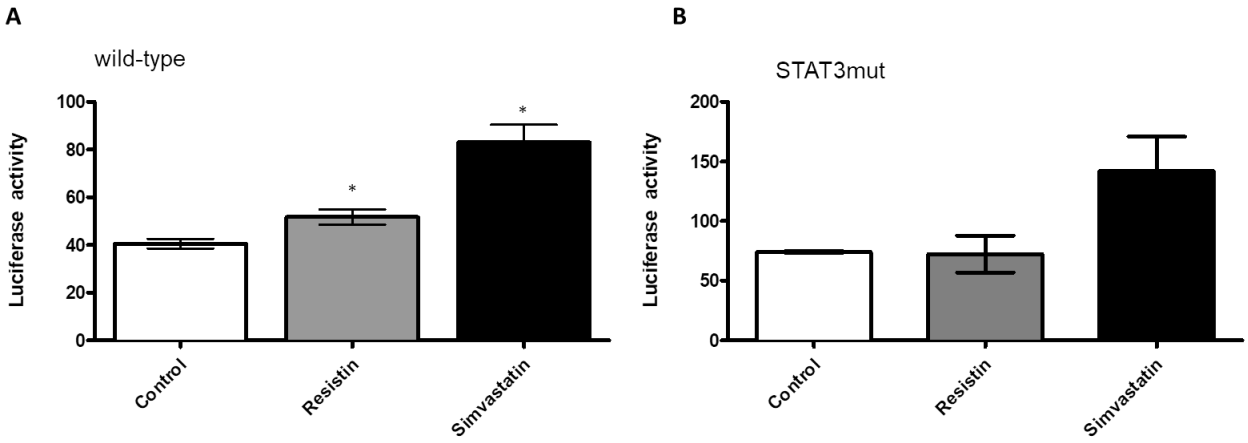

Fig.20 Luciferase activity in HepG2 cells transfected with pGL3-PCSK9-D1 wild type or STAT3 mutated.

HepG2 cells were transfected with pGL3-PCSK9-D1 or pGL3-PCSK9-D1-STAT3mu. The day after the transfection, the medium was replaced with MEM containing 10%LPDS in presence or absence of resitin (50ng/mL) or simvastatin (20µM), after an additional 48h, luciferase activities were determined by Neolite reagent.

Effect of leptin and resistin on apoB release

It has been reported that PCSK9 promotes the production of triglyceride-rich-apolipoprotein-B lipoproteins (Rashid, Tavori et al. 2014). Thus, we first tested this evidence in our cell-based models. A qPCR analysis has been conducted on HepG2 and HepG2^{PCSK9} cells in order to evaluate *APOB* mRNA expression (fig.21 A) which resulted enhanced in HepG2 ^{PCSK9}. Thus, PCSK9 gene has been silenced with siRNA anti-PCSK9 and this effect has been abolished (fig.21 B).

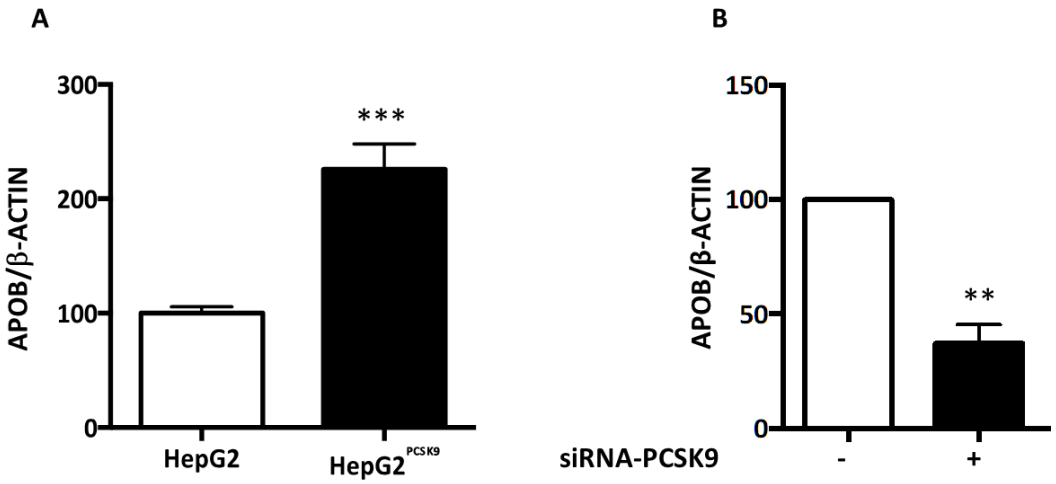

Fig.21 APOB expression in HepG2 and HepG2^{PCSK9} cells.
*Cells were seeded in MEM/10% FBS and the day after incubated with MEM supplemented with 10% LPDS. After an additional 48h the APOB gene expression was evaluated by qPCR analysis. Differences between groups were assessed by Mann withney t test; **p<0.01***p<0.001;*

Wondering whether leptin and resistin play a role in this mechanism, HepG2 cells were silenced with siRNA anti-PCSK9 or anti-STAT3 and treated for 48h with leptin (50ng/mL) or resistin (50ng/mL). We tested the effect of leptin and resistin treatment upon either siRNA anti-PCSK9 or anti-STAT3on the gene expression of APOB and microsomal triglyceride transfer protein (MTP), a protein which shuttles TG, CE, and phospholipids from the ER membrane to the primordial apoB particle. Without MTP function, underlipidated apoB is subjected to proteasomal degradation. Defective or missing MTP function corresponds to a defect in assembly and secretion of hepatic and intestinal apoB-containing lipoproteins, VLDL, and chylomicrons, leading to fat malabsorption, sub-sequent steatorrhea, and fat-filled enterocytes and hepatocytes(Biterova, Isupov et al. 2019). . The 48-h treatment with 50 ng/ml leptin significantly increased the APOB and MTP mRNA (fig.22 A-B). Upon silencing of PCSK9 or STAT3, leptin lost its ability to mediate APOB and MTP gene expression. Similar effects were observed with a 48-h treatment with 50 ng/mL resistin, the resistin-driven APOB and MTP gene expressions were abolished by silencing of PCSK9 or STAT3 (fig.22 C-D).

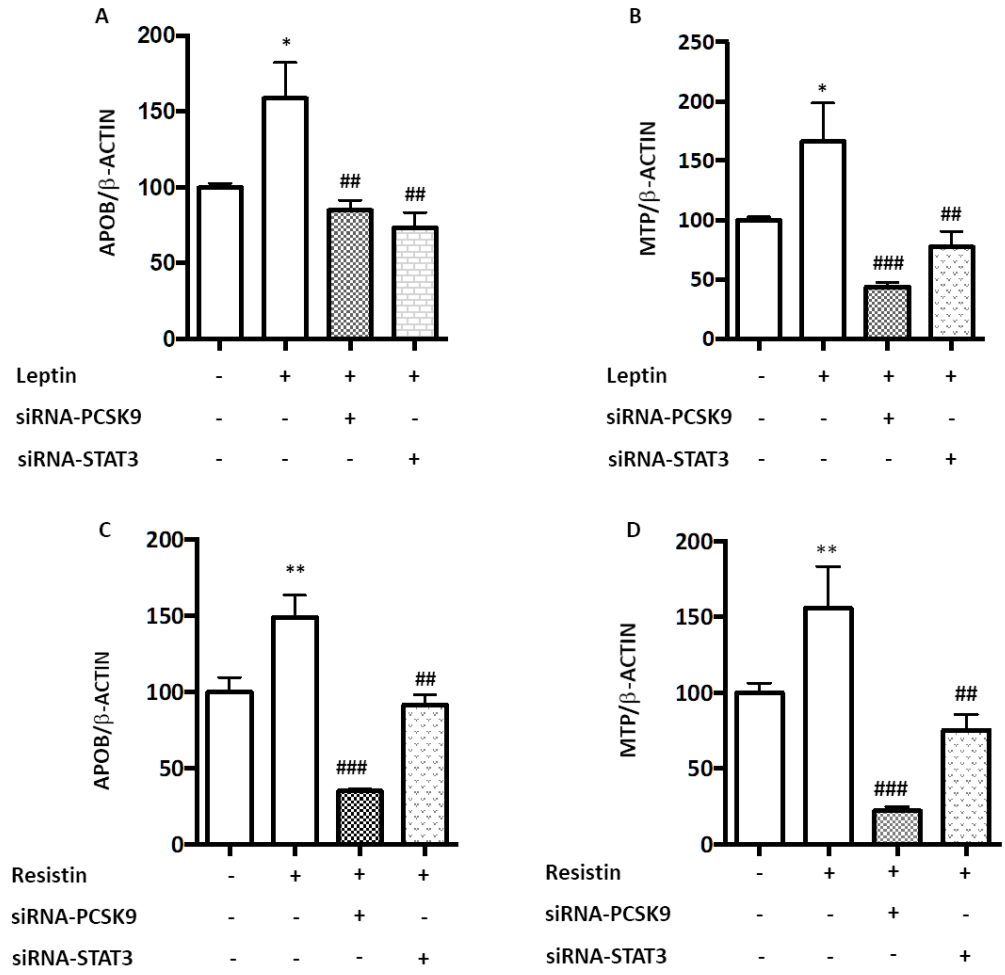

Fig.22 Leptin and resistin enhance APOB and MTP gene expression.

*A) HepG2 transfected with siRNA anti-STAT3 or siRNA anti-PCSK9 were or were not treated for 48h with leptin (50 ng/mL) and the APOB gene expression was evaluated by qPCR analysis.B) MTP gene expression was evaluated upon transfection with siRNA anti-STAT3 or siRNA anti PCSK9 and treatment or not with leptin (50 ng/mL). C) Cells transfected with siRNA anti-STAT3 or siRNA anti-PCSK9 were or were not treated for 48h with resistin (50 ng/mL) and the APOB gene expression was evaluated by qPCR analysis. D) MTP gene expression was evaluated upon transfection with siRNA anti-STAT3 or siRNA anti PCSK9 and treatment or not with resistin (50 ng/mL)Differences between groups were assessed by Kruskal-Wallis non parametric one-way ANOVA and Dunn's post hoc test; *p<0.05 (vs control, namely no leptin or resistin treatment); $^{##}$p<0.01 and $^{###}$p<0.001 (vs leptin or resistin treatment).*

A preliminary analysis of ApoB protein expression has been performed on HepG2 cells, incubated 48h in MEM 10%LPDS in presence or absence of leptin (50 ng/mL) or resisitin (50 ng/mL). Cells were seeded in MEM 10%FBS, after 24h the medium was replaced with MEM

10% LPDS and leptin (50ng/mL) or resistin (50 ng/mL). After 48h the proteins were extracted, and a WB analysis was performed.

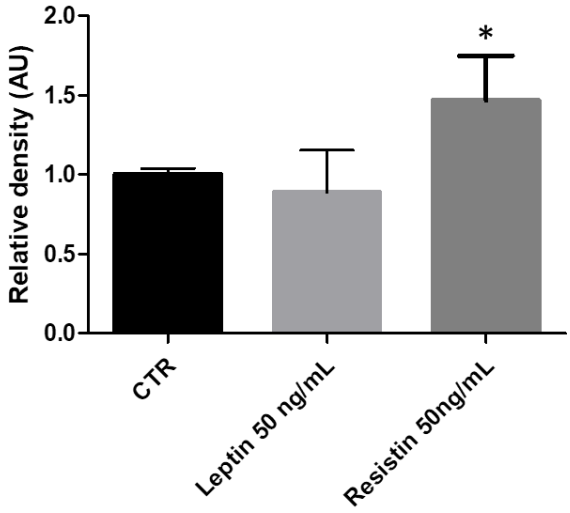

Fig.23 Resistin enhance ApoB protein expression.

*HepG2 cells were seeded in MEM/10% FBS and the day after incubated with MEM supplemented with 10% LPDS. After an additional 48h in the presence or absence of leptin (50 ng/mL) or resistin (50 ng/ml) a western blot analysis was performed to evaluate ApoB protein expression. Differences between groups were assessed by non parametric one-way ANOVA and Dunn's post hoc test; *p<0.05*

Molecular characterization of mutations causing LPL deficiency

*P*ost-heparin plasma samples of two patients from the lipid clinic of Vienna were used for genetic sequencing and hey found two mutations on LPL gene in each patient. The first patient was compound heterozygous for c.680T>C (p.V200A) and c.1139+1G>A (Fig. 24 A). The second patient was compound heterozygous for c.953A>G (p.N291S) and c.1019-3C>A (fig. 24 B).

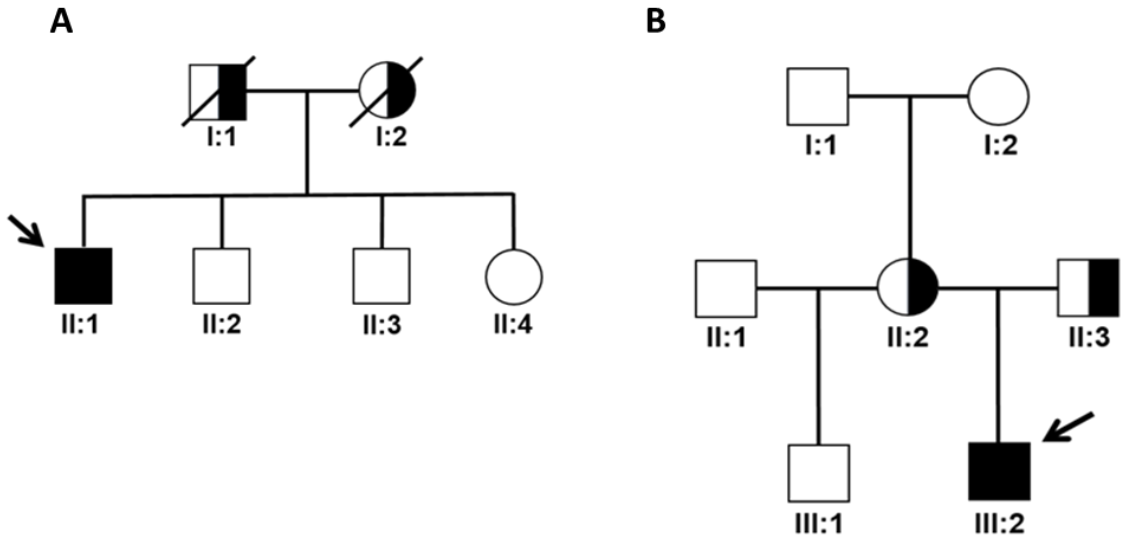

Fig.24 Pedigree of 1 (II:1) carrying two heterozygous mutations: c.680T > C (p.V200A) and c.1139+1G > A (intron 7 splice site). (B) Family tree of patient 2 (III:2) carrying two heterozygous mutations: c.953A > G (p.N291S) and c.1019-3C > A (intron 6 splice site). Open symbols indicate no known hyperlipidemia. The half-filled symbols are only by inference. Squares and circles represent males and females respectively and filled symbols plus arrows indicate the affected individuals.

Plasma samples were then sent to Gothenbung University where they have been used for LPL functional tests. In particular the presence of LPL in post-heparin plasmas of the two patients was verified through immunoblotting (fig.25 A), then the activity of the enzyme was tested trough the LPL enzymatic assay. In both experiments post-heparin mouse plasma was used as positive control as mouse and human LPL amino acidic sequences show 90% homology.

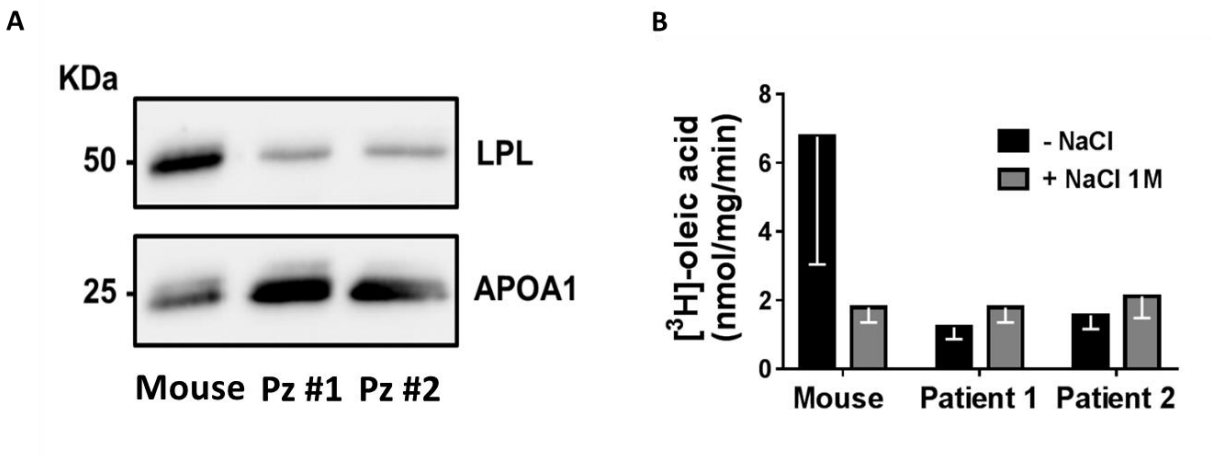

Fig 25 Circulating LPL levels and LPL activity in post-heparin plasma from patient 1 and 2. (A) Western blotting showing circulating levels of LPL in post-heparin plasma samples from patient 1 compound heterozygote forc.680T > C (p.V200A) and c.1139+1G > A(intron 7 splice site) and patient 2 compound het-erozygote for c.953A > G (p.N291S) andc.1019-3C > A (intron 6 splice site). Mouse post-heparin plasma was used as a positive control. Anti-human APOA1 antibody was used as a loading control. Post-h, post-heparin; kDa, kilodalton; LPL, lipoprotein lipase; APOA1, apolipoprotein A1. (B) LPL induced release of radio-labeled oleic acid from triolein was measured in post-heparin plasma of patient 1 and 2. Mouse post-heparin plasma was used as a positive control. Sodium chloride 1 M (NaCl) was used to inhibit specifically LPL activity.

To dissect the molecular mechanism of these two mutations in the LPL gene, wild type LPL cDNA was cloned into pcDNA3.1 expression vector and the two mutants were obtained by in situ mutagenesis. Moreover, to ensure the specificity for LPL, at the C-terminus of the protein a V5 tag was added. HEK293T/17 cells were transiently transfected with LPL V200A and N291S constructs. After 48 h cell culture media were collected and concentrated 10 times, cells were harvested and lysate. Cell lysate was used to load a western blot and quantify LPL synthesis (fig.26 A). The concentrated media were used to analyse the LPL secretion (fig. 26 A). LPL activity was evaluated in concentrated media of cells transfected with wild type or mutant plasmids, by LPL enzymatic assay. In the media of cells transfected with LPL V200A was not detected any LPL activity, while a strong reduction was found with LPL N291S compared to LPL wild type (Fig. 26 C).

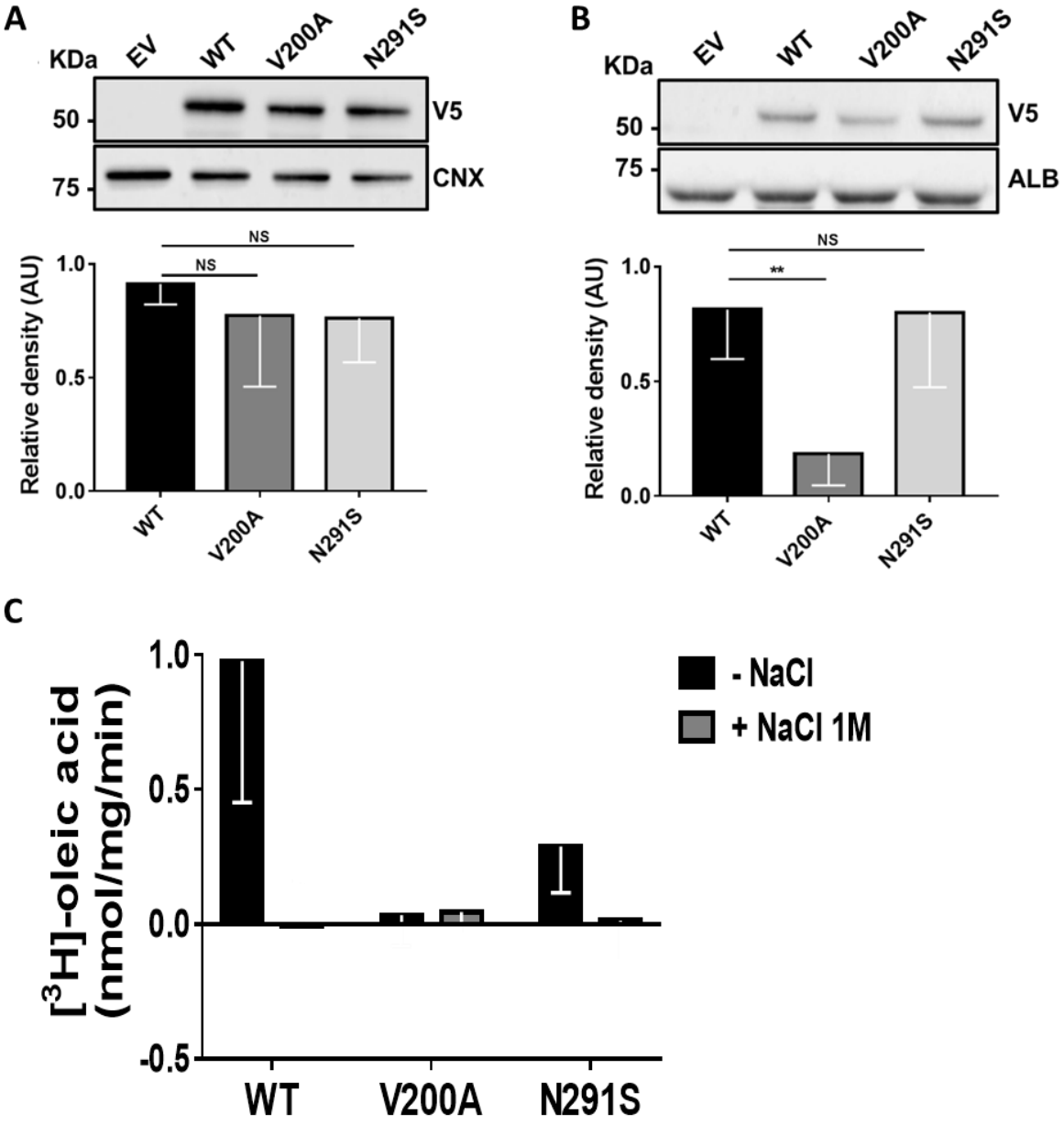

Fig 26 HEK 293T/17 cells transiently transfected with wild type or mutants LPL (V200Aand N291S).

(A) Western blotting analysis of cell lysates (N = 5). Calnexin was used as loading control. (B) Western blotting analysis of cell media (N = 8). Albumin was used as loading control. EV: empty vector; WT: wild type; CNX: calnexin; ALB: albumin. P-value was calculated by Mann Whitney test, (p= 0.008). (C) LPL activity is absent in the media of HEK 293T/17 cells transfected with V200A and N291S LPL. LPL induced release of radiolabeled oleic acid from triolein was measured in cell media of HEK 293T/17 cells transiently transfected with wild type, V200A or

N291S LPL. Wild-type (WT) LPL medium was used as a positive control. Sodium chloride 1 M (NaCl) was used to inhibit specifically LPL activity

To study whether V200A and N291S mutations influence the dimerization of LPL, post-heparin plasma and conditioned media were analysed by western blot in a non-denaturing condition. Both mutations did not influence LPL dimerization *ex vivo* (fig.27 A) and *in vitro* (fig.27 B). Mouse plasma was used as positive control.

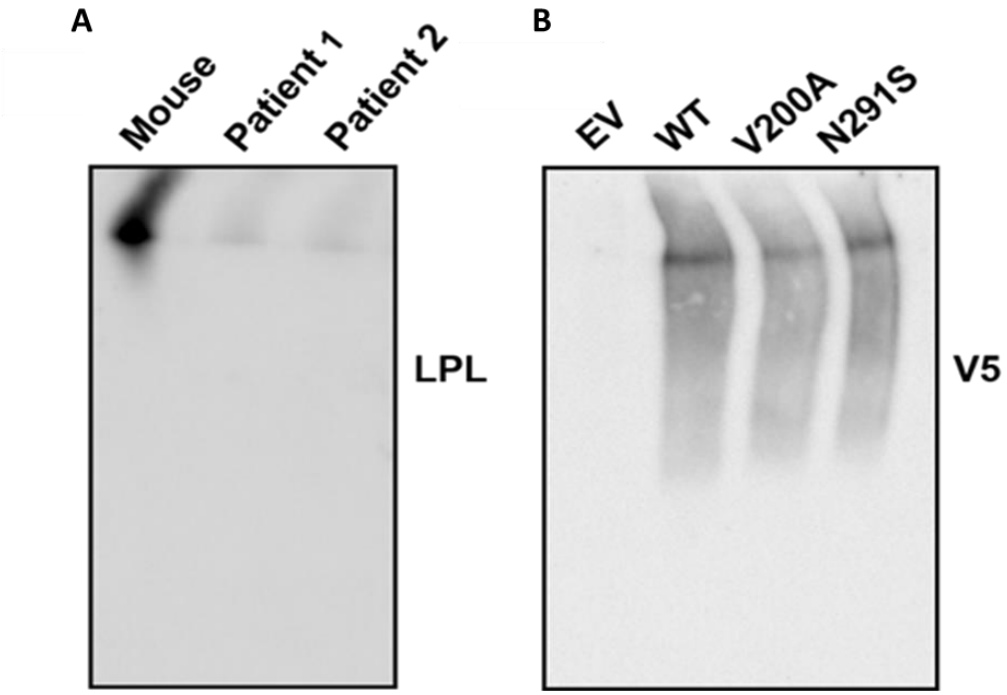

Fig.27 A non-denaturing Western blot analysis of LPL dimerization in post heparin plasma and HEK293 T/17 conditioned media.

(A) Non-denaturing Western blotting of post-heparin plasma samples from patient 1 compound heterozygote for c.680T > C (p.V200A) and c.1139+1G > A (intron 7 splice site), patient 2 compound heterozygote for c.953A > G (p.N291S) and c.1019-3C > A (intron 6 splice site). Mouse post-heparin plasma was used as a positive control. (B) Non-denaturing Western blotting of media from cells LPL-V5 tagged mutant compared to the wild type form. EV: empty vector; WT: wild type. Ponceau was used as loading control (data not shown).

DISCUSSION

Eighty percent of patients with coronary heart disease are overweight or obese (Ades and Savage 2017), but clear determinants of a direct effect of obesity on CV risk has not been demonstrated, yet. To address some of these controversies (Lavie, Milani et al. 2009, Carbone, Lavie et al. 2017, Carbone and Lavie 2019, Lavie, Ozemek et al. 2019), we have studied molecular determinants linking adipokines to CVD. The rationale of performing this study is based on four previously reported observations: 1) leptin (Du, Li et al. 2016) and resistin (Melone, Wilsie et al. 2012) induce PCSK9 in HepG2 cells; 2) TNF-α induces PCSK9 expression in hepatocytes, through the involvement of SREBP-1 and HNF-1 alpha via SOCS3 (Ruscica, Ricci et al. 2016); 3) in clinical settings, a positive relationship between apoB-containing lipoproteins and PCSK9 has been observed (Cariou, Langhi et al. 2013, Ferri and Ruscica 2016); 4) in humans, circulating PCSK9 levels and hepatic PCSK9 expression correlate with the gene expression of APOB (Ruscica, Ferri et al. 2016). Following our hypothesis relative to a link between adipose tissue-derived hormones and CVD (Ruscica, Baragetti et al. 2016), we studied this possible link in a cohort of healthy subjects from the Brisighella Heart Study. A positive association between circulating levels of PCSK9 and of leptin was found in patients with BMI <25 kg/m^2. When the analysis was extended across different degrees of BMI (between 25 and 30 Kg/m^2 or $\geq$ 30 Kg/m^2) the association was lost. This effect may owe to the leptin-driven PCSK9 activation which could blunt the association. On the other hand, it is not surprisingly the lack of association between resistin and PCSK9; in humans, resistin is produced by macrophages and not by adipocytes. However, other studies are required to assess whether circulating resistin is a physiologically important determinant of higher PCSK9 (Kwakernaak, Lambert et al. 2014, Li, Xu et al. 2015). To better understand the effect of leptin on PCSK9, we took advantage of a mouse genetic model lacking leptin, the *ob/ob* mice, in which we found a significant lower hepatic PCSK9 gene expression compared to wildtype mice, thus suggesting that leptin may play a role in PCSK9 hepatic expression. However, it has been reported that *ob/ob* mice treated with leptin through a subcutaneous osmotic pump, showed a decrease in hepatic PCSK9 as well as on the activity of SREBPs. The authors reported a sexual dichotomy, with a decrement in SREBPs activity but not in PCSK9 expression. These controversial results may be due to differences in the experimental paradigm *i.e* male mice. Further experiments are required to understand the link between leptin and PCSK9 *in vivo*. To

dissect out the molecular mechanism of our *in vivo* findings, HepG2 cells were used as *in vitro* model. First finding is that leptin (50ng/mL) and resistin (50ng/mL) increased PCSK9 mRNA expression of about 95% and 115% respectively, with a trend to significance in promoting PCSK9 protein expression in line with literature data (Melone, Wilsie et al. 2012, Du, Li et al. 2016). This evidence corroborates the finding that inflammation regulates PCSK9 expression, as in the same cell model, TNFα through SOCS3 increases PCSK9 gene and protein expression (Ruscica, Ricci et al. 2016). Wondering to dissect out the mechanisms involved in this adipokine-driven PCSK9 activation, the transcriptional activity of PCSK9 promoter has been studied. Since PCSK9 transcription is controlled through *cis*-regulatory elements embedded in the proximal promoter region of PCSK9 gene where the Sp1 sites, SRE and HNF-1 are located (Ferri, Corsini et al. 2016), we utilized a PCSK9 promoter luciferase reporter plasmid containing mutations for both SRE and HNF responsive elements. Upon leptin and resistin treatment, in HepG2 cell lines transfected with a plasmid carrying the PCSK9 promoter containing a mutation in HNF-1 motif, a significant increment in luciferase activity was found. Conversely, no changes were found when a mutation in SRE motif was inserted. Relative to this latter evidence, our data are in line with findings demonstrating that, at gene transcriptional level, PCSK9 synthesis is largely controlled by transcription factor families sterol regulatory element (Ganie, Dhingra et al.)-binding proteins (SREBPs) (Careskey, Davis et al. 2008, Jeong, Lee et al. 2008). When the involvement of HNF-1 is considered, our findings are in contrast with evidence reporting how HNF-1 alpha plays a key role in the control of PCSK9 gene transcription in liver tissue of hamster species (Dong, Singh et al. 2017), C57Bl/6 mice (Shende, Wu et al. 2015) or in hepatic cells (Li, Dong et al. 2009). However, since it is demonstrated that, in the same cell-based model, the PCSK9 transcriptional activity happens also in the presence of a mutation in HNF-1 motif (Ruscica, Ricci et al. 2016), such a difference might be related to the experimental conditions. As described above, PCSK9 is regulated through the involvement of the inflammatory pathway (Ruscica, Ricci et al. 2016), thus, the present project explored whether proinflammatory adipokines, e.g. leptin and resistin, activate PCSK9 through the involvement of the inflammatory pathway STAT3. After confirming that leptin or resistin increased STAT3 expression, STAT3 gene was transiently silenced and PCSK9 expression was re-evaluated we found that STAT3 was mandatory for PCSK9 activation. The subsequent step was to investigate the role of STAT3 in PCSK9 transcriptional activity. Using JASPAR database, 2,000 bp upstream the start codon of PCSK9 promoter have been analysed and the sequence

was scanned for human STAT3 sequence binding profile. Even though the *in silico* data are promising, no differences in promoting PCSK9 transcriptional activity were found between the two constructs. Because the analysis of PCSK9 promoter sequence with JASPAR database identified other two putative STAT3 DNA-binding sites, further experiments are required to better investigate the role of STAT3 in PCSK9 transcription. Indeed, the understanding of the physiological regulation of PCSK9 expression at transcriptional level remains incomplete. PCSK9 is not only involved in LDL-C regulation but also in TG metabolism (Roubtsova, Munkonda et al. 2011). Elevated plasmatic levels of TG are reported in patients with gain of function (GOF) mutations in PCSK9 gene (Qiu, Zeng et al. 2017). By contrast, in patients carrying loss of function (LOF) mutations, LDL-C reduction associates with reduced fasting and post-prandial TG levels (Verbeek, Boyer et al. 2017). In addition, it has also been reported that PCSK9 increases the production of apoB, possibly through the inhibition of intracellular apoB degradation via the autophagosome/lysosome pathway(Rashid, Tavori et al. 2014). In obese subjects, with higher levels of proinflammatory adipokines, most often, the rise of TG and apoB-containing lipoproteins has been associated to an increment in CVD risk (Lavie, Milani et al. 2009). This evidence has been tested in our cellular models (HepG2 and HepG2^{PCSK9}) showing that cells overexpressing PCSK9 have increased *APOB* mRNA levels. HepG2 cells were incubated with leptin (50ng/mL) or resistin (50ng/mL) and *APOB* and *MTP* mRNA expressions were raised. s These effects were abolished by siRNA anti-STAT3 and siRNA anti-PCSK9 highlighting the concept that STAT3-driven PCSK9 expression is required for a proper *APOB* and *MTP* expression. Indeed,defective or missing MTP function corresponds to a defect in assembly and secretion of hepatic and intestinal apoB-containing lipoproteins, VLDL, and chylomicrons, leading to fat malabsorption, sub-sequent steatorrhea, and fat-filled enterocytes and hepatocyte However, a preliminary analysis of apoB protein expression after leptin and resistin treatments revealed significance only after 48h of treatment with resistin (50ng/mL). Further experiments are required to verify the effect of leptin.

To further dissect out the role of PCSK9 on TG metabolism, I took the opportunity to work with DNA of two carriers of LOF mutations in LPL gene which strongly affect TG metabolism. We believe that the model used could be of interest to study the effect of mutation on PCSK9 gene since LOF mutation in PCSK9 gene are characterized by a -26% decrease in PCSK9 secretion whereas GOF mutations although responsible for an altered lipid profile, do not change PCSK9 circulating levels. Concerning the characterization of LPL mutations, the model

we used allowed to discriminate between hypertriglyceridemia associated to a loss of functionality or an impaired synthesis.

CONCLUSION

This project provided evidence of a direct relationship between leptin and resistin and PCSK9. A positive correlation between PCSK9 and leptin circulating levels was found, a result in line with previous evidence of a direct association between these two parameters (Kwakernaak, Lambert et al. 2014); conversely, the hypothesis of circulating resistin being a determinant of PCSK9 levels needs to be further explored (Kwakernaak, Lambert et al. 2014, Li, Xu et al. 2015). In *ob/ob* mice, genetically lacking leptin, there is a strong hepatic suppression of PCSK9. Conversely, in C57BL6J mice fed 12 weeks high fat diet there is a rise in serum leptin and hepatic expression of PCSK9. The interplay between leptin and resistin and PCSK9 appears to be dependent on the activation of the inflammatory pathway JAK/STAT and provides a basic molecular mechanism for the link between low-grade inflammation and dyslipidemia observed in obese subjects. Studies on the PCSK9 promoter activity demonstrated that SRE motif plays a pivotal role in PCSK9 promoter activity. The absolute requirement of PCSK9 for the leptin and resistin-driven induction of APOB and MTP genes, involved in hepatic lipogenesis, is in agreement with the recent observation that the PCSK9 inhibitors decreased the production rate of LDL apoB by 23.9% (Reyes-Soffer, Pavlyha et al. 2017).

FUTURE PERSPECTIVES

All the findings obtained with gene expression experiments need to be confirmed with protein expression and further experiments are required to better investigate the role of STAT3 in PCSK9 transcription. Although we found that the induction of APOB and MTP driven by adipokines requires PCSK9 is needed, this mechanism still needs to be fully addressed in an *in vivo* setting. In the context of metabolic dysregulation, peroxisome proliferator-activated receptors (PPARs) are other important players that can be of interest; it has been demonstrated that in HepG2 cells, adiponectin through Adipo R1 increases PCSK9 expression (Sun, Yang et al. 2017) with the involvement of AMPK and PPAR-γ pathways. Thus it would be of interest to study whether leptin and resistin could act through PPAR-γ since it is mainly expressed in adipocytes (brown and white) and plays a major role in cell differentiation and energy metabolism (Chawla, Schwarz et al. 1994) (Botta, Audano et al. 2018).

RESEARCH AVCTIVITIES

Academic year 2016-2017

Attended congresses

1. 30 th congresso nazionale SISA, 20-22 November 2016, Rome, Italy
   Ruscica M, Botta M, Garzone M, Macchi C, Marchiano S, Corsini A, Magni P, Ferri N, Pro-inflammatory cytokines and adipokines regulate pcsk9 expression in hepg2 cells.
2. Giornata della ricerca del Centro E.Grossi Paoletti, 9 June 2017, Milano Italy
   POSTER SIMONELLI
3. EAS congress -23-27 April 2017, Prague, Czech Republic
   Ruscica M, Simonelli S, Botta M, Ossoli A, Magni P, Corsini A, Arca M, Pisciotta L, Veglia F, Franceschini G, Ferri N, Calabresi L, Plasma pcsk9 levels and lipoprotein distribution are preserved in carriers of hypoalphalipoproteinemia.

   Ruscica M, Botta M, Macchi C, Garzone M, Giorgio E, Marchianò S, Corsini A, Magni P, Ferri N, Leptin and resistin: a crossroad between adipose tissue and pcsk9 activation.

Attended congresses with oral presentations

1. Convegno Regionale SISA Lombardia XV giornata di studio. 20 0ctober 2016, Milano Italia
   M. Botta, N. Ferri, E. Giorgio, C. Macchi, A. Corsini, P. Magni, M. Ruscica,
   Leptin and resistin adipokines regulate PCSK9 expression in HepG2 cells

2. Spring Meeting dei Gruppi Giovani Ricercatori di SIIA, SIMI e SISA, 7-8 April 2017, Rome, Italy
   M. Botta, C. Macchi, S. Marchianò, E. Giorgio, P. Magni, A. Corsini, N. Ferri, M. Ruscica, Leptin and resistin affect PCSK9 and *de novo* lipogenesis through the involvement of STAT3

Attended congresses with poster presentations

1. Young scientist meeting SIPMeT, 15-16 September 2017
   M. Botta, C. Macchi, R. Bosisio, C. Pavanello, C. M. Toldo, G. Mombelli, L. Calabresi, M. Ruscica, P. Magni

Efficacy and safety of a nutraceutical with probiotic and red yeast rice extract in patients with moderate hypercholesterolemia: a randomized, double-blind, placebo-controlled study.

C. Macchi, M. Botta, S. Marchianò, A. Corsini, N. Ferri, P. Magni, M. Ruscica
Leptin and resistin induce PCSK9 through the involvement of STAT pathway

Attended courses

1. Corso di formazione speciale e aggiornamento professionale "Prevenzione dei rischi chimico e biologico in laboratorio" January 2017, Milan, Italy
2. PhD courses in epigenetic mechanisms and their relevance for human pathology, 21-22 February 2017, Segrate, Italy
3. Corso introduttivo alla Sperimentazione Animale, 29,30,31 March, Milan, Italy

Publications

Ruscica M, Ferri N, Fogacci F, Rosticci M, Botta M, Marchiano S, Magni P, D'Addato S, Giovannini M, Borghi C, Cicero AFG; Brisighella Heart Study Group. Circulating Levels of Proprotein Convertase Subtilisin/Kexin Type 9 and Arterial Stiffness in a Large Population Sample: Data From the Brisighella Heart Study. J Am Heart Assoc. 2017 May 3;6(5).

Academic year 2017-2018
Attended congresses

1. Convegno Regionale SISA Lombardia XV giornata di studio. 19 0ctober 2017, Milano Italia
2. XXXI congress nazionale SISA, 19-21 November 2017, Palermo, Italy

Attended congresses with oral presentations

1. Atheroma Club 2018, 19-21 September 2018, Ystad Saltjöbad, Sweden
   M. Botta, C. Macchi, A. Corsini, P. Magni, P.Dongiovanni, L.Valenti, J.Liu, A.Cicero, N. Ferri, M. Ruscica, Adipose tissue and pcsk9: do leptin and resistin play a role?
2. Spring Meeting dei Gruppi dei Giovani Ricercatori di SIIA, SIMI, SISA. 28th February-2nd March, Rimini, Italy

M. Botta; E. Maurer; M. Ruscica; S. Romeo; T. M. Stulnig; P. Pingitore. Deciphering the role of V200A and N291S mutations leading to LPL deficiency

Attended congresses with poster presentations

1. 24th Annual Scandinavian Atherosclerosis Conference, 11-14 april 2018, Humlebaek, Denmark
   M. Botta, C. Macchi, D.Dall'Orto, A. Corsini, P. Magni, P.Dongiovanni, L.Valenti, J.Liu, A.Cicero, N. Ferri, M. Ruscica, Adipokines affect PCSK9 expression: *in vitro* and *in vivo* evidence
2. Atheroma Club 2018, 19-21 September 2018, Ystad Saltjöbad, Sweden
   M. Botta, C. Macchi, A. Corsini, P. Magni, P.Dongiovanni, L.Valenti, J.Liu, A.Cicero, N. Ferri, M. Ruscica, Adipose tissue and pcsk9: do leptin and resistin play a role?

3. XXXII Congresso Nazionale SISA 25-27 November, Bologna, Italy
   M. Botta; E. Maurer; M. Ruscica; S. Romeo; T. M. Stulnig; P. Pingitore. **Deciphering the role of V200A and N291S mutations leading to LPL deficiency**

Publications

Ruscica M, Botta M, Ferri N, Giorgio E, Macchi C, Franceschini G, Magni P, Calabresi L, Gomaraschi M. High Density Lipoproteins Inhibit Oxidative Stress-Induced Prostate Cancer Cell Proliferation. Sci Rep. 2018

Pavanello C, Zanaboni AM, Gaito S, Botta M, Mombelli G, Sirtori CR, Ruscica M**.** Influence of body variables in the development of metabolic syndrome-A long term follow-up study. PLoS One. 2018

Botta M, Audano M, Sahebkar A, Sirtori CR, Mitro N, Ruscica M. PPAR Agonists and Metabolic Syndrome: An Established Role? Int J Mol Sci. 2018

Ruscica M, Simonelli S, Botta M, Ossoli A, Lupo MG, Magni P, Corsini A, Arca M, Pisciotta L, Veglia F, Franceschini G, Ferri N, Calabresi L. Plasma PCSK9 levels and lipoprotein distribution are preserved in carriers of genetic HDL disorders. Biochim Biophys Acta Mol Cell Biol Lipids 2018

Ruscica M, Simonelli S, Botta M, Ossoli A, Lupo MG, Magni P, Corsini A, Arca M, Pisciotta L, Veglia F, Franceschini G, Ferri N, Calabresi L. Plasma PCSK9 levels and lipoprotein distribution are preserved in carriers of genetic HDL disorders**.** Biochim Biophys Acta Mol Cell Biol Lipids

Strollo F, Macchi C, Eberini I, Masini MA, Botta M, Vassilieva G, Nichiporuk I, Monici M, Santucci D, Celotti F, Magni P, Ruscica M. Body composition and metabolic changes during a 520-day mission simulation to Mars. J Endocrinol Invest. 2018

Academic year 2018-2019

10 months at Wallenberg Laboratory, Gothenburg, Sweden under the supervision of Prof. Romeo

Attended congresses

International Symposium "HDL-Beyond atherosclerosis", 1st February 2018, Milan, Italy

Attended congresses with oral presentations

1. Spring Meeting dei Gruppi dei Giovani Ricercatori di SIIA, SIMI, SISA. 28th February-2nd March, Rimini, Italy
   M. Botta; E. Maurer; M. Ruscica; S. Romeo; T. M. Stulnig; P. Pingitore.
   Deciphering the role of V200A and N291S mutations leading to LPL deficiency

Attended congresses with poster presentations

1. XXXII Congresso Nazionale SISA 25-27 November, Bologna, Italy
   M. Botta; E. Maurer; M. Ruscica; S. Romeo; T. M. Stulnig; P. Pingitore.
   Deciphering the role of V200A and N291S mutations leading to LPL deficiency

Publications

Zenti MG, Altomari A, Lupo MG, Botta M, Bonora E, Corsini A, Ruscica M, Ferri N. From lipoprotein apheresis to proprotein convertase subtilisin/kexin type 9 inhibitors: Impact on low-density lipoprotein cholesterol and C-reactive protein levels in cardiovascular disease patients**.** Eur J Prev Cardiol. 2018

Da Dalt L, Ruscica M, Bonacina F, Balzarotti G, Dhyani A, Di Cairano E,Baragetti A, Arnaboldi L, De Metrio S, Pellegatta F, Grigore L, Botta M, Macchi C, Uboldi P, Perego C, Catapano AL,

Norata GD. PCSK9 deficiency reduces insulin secretion and promotes glucose intolerance: the role of the low-density lipoprotein receptor Eur Heart J. 2019

Ruscica M, Pavanello C, Gandini S, Macchi C, Botta M, Dall'Orto D, Del Puppo M, Bertolotti M, Bosisio R, Mombelli G, Sirtori CR, Calabresi L, Magni P. Nutraceutical approach for the management of cardiovascular risk - a combination containing the probiotic Bifidobacterium longum BB536 and red yeast rice extract: results from a randomized, double-blind, placebo-controlled study. Nutr J. 2019

Botta M, Maurer E, Ruscica M, Romeo S, Stulnig TM, Pingitore P. Deciphering the role of V200A and N291S mutations leading to LPL deficiency. Atherosclerosis 2019

Conti E, Grana D, Stefanoni G, Corsini A, Botta M, Magni P, Aliprandi A, Lunetta C, Appollonio I, Ferrarese C, Tremolizzo L. Irisin and BDNF serum levels and behavioral disturbances in Alzheimer's disease. Neurol Sci. 2019

BIBLIOGRAFY

Abifadel, M., M. Varret, J. P. Rabes, D. Allard, K. Ouguerram, M. Devillers, C. Cruaud, S. Benjannet, L. Wickham, D. Erlich, A. Derre, L. Villeger, M. Farnier, I. Beucler, E. Bruckert, J. Chambaz, B. Chanu, J. M. Lecerf, G. Luc, P. Moulin, J. Weissenbach, A. Prat, M. Krempf, C. Junien, N. G. Seidah and C. Boileau (2003). "Mutations in PCSK9 cause autosomal dominant hypercholesterolemia." Nat Genet **34**(2): 154-156.

Ades, P. A. and P. D. Savage (2017). "Obesity in coronary heart disease: An unaddressed behavioral risk factor." Prev Med.

Alessandra Vecchié and F. C. Franco Dallegria, Aldo Bonaventura, Luca Liberale,Piero Portincasa, Gema Frühbecke, Fabrizio Montecucco (2018). "Obesity phenotypes and their paradoxical association with cardiovasculardiseases." European Journal of Internal Medicine **48**: 6-17.

Amemiya-Kudo, M., H. Shimano, T. Yoshikawa, N. Yahagi, A. H. Hasty, H. Okazaki, Y. Tamura, F. Shionoiri, Y. Iizuka, K. Ohashi, J. Osuga, K. Harada, T. Gotoda, R. Sato, S. Kimura, S. Ishibashi and N. Yamada (2000). "Promoter analysis of the mouse sterol regulatory element-binding protein-1c gene." J Biol Chem **275**(40): 31078-31085.

American Diabetes, A. (2018). "5. Prevention or Delay of Type 2 Diabetes: Standards of Medical Care in Diabetes-2018." Diabetes Care **41**(Suppl 1): S51-S54.

Antoniades, C., A. S. Antonopoulos, D. Tousoulis and C. Stefanadis (2009). "Adiponectin: from obesity to cardiovascular disease." Obes Rev **10**(3): 269-279.

Antonopoulos, A. S. and D. Tousoulis (2017). "The molecular mechanisms of obesity paradox." Cardiovasc Res **113**(9): 1074-1086.

Armstrong, S. M., M. G. Sugiyama, K. Y. Fung, Y. Gao, C. Wang, A. S. Levy, P. Azizi, M. Roufaiel, S. N. Zhu, D. Neculai, C. Yin, S. S. Bolz, N. G. Seidah, M. I. Cybulsky, B. Heit and W. L. Lee (2015). "A novel assay uncovers an unexpected role for SR-BI in LDL transcytosis." Cardiovasc Res **108**(2): 268-277.

Banerjee, R. R., S. M. Rangwala, J. S. Shapiro, A. S. Rich, B. Rhoades, Y. Qi, J. Wang, M. W. Rajala, A. Pocai, P. E. Scherer, C. M. Steppan, R. S. Ahima, S. Obici, L. Rossetti and M. A. Lazar (2004). "Regulation of fasted blood glucose by resistin." Science **303**(5661): 1195-1198.

Baragetti, A., D. Grejtakova, M. Casula, E. Olmastroni, G. S. Jotti, G. D. Norata, A. L. Catapano and S. Bellosta (2018). "Proprotein Convertase Subtilisin-Kexin type-9 (PCSK9) and triglyceride-rich lipoprotein metabolism: Facts and gaps." Pharmacol Res **130**: 1-11.

Baragetti, A., G. K., G. L., G. D. Norata and A. L. Catapano (2017). "Differential contribution of PCSK9 and LPL gene variants on lipid profile in the general population." Atherosclerosis **263**: e29ee110.

Benjannet, S., D. Rhainds, R. Essalmani, J. Mayne, L. Wickham, W. Jin, M. C. Asselin, J. Hamelin, M. Varret, D. Allard, M. Trillard, M. Abifadel, A. Tebon, A. D. Attie, D. J. Rader, C. Boileau, L. Brissette, M. Chretien, A. Prat and N. G. Seidah (2004). "NARC-1/PCSK9 and its natural mutants: zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol." J Biol Chem **279**(47): 48865-48875.

Benn, M., B. G. Nordestgaard, P. Grande, P. Schnohr and A. Tybjaerg-Hansen (2010). "PCSK9 R46L, low-density lipoprotein cholesterol levels, and risk of ischemic heart disease: 3 independent studies and meta-analyses." J Am Coll Cardiol **55**(25): 2833-2842.

Bentzon, J. F., F. Otsuka, R. Virmani and E. Falk (2014). "Mechanisms of plaque formation and rupture." Circ Res **114**(12): 1852-1866.

Biterova, E. I., M. N. Isupov, R. M. Keegan, A. A. Lebedev, A. A. Sohail, I. Liaqat, H. I. Alanen and L. W. Ruddock (2019). "The crystal structure of human microsomal triglyceride transfer protein." Proc Natl Acad Sci U S A **116**(35): 17251-17260.

Botta, M., M. Audano, A. Sahebkar, C. R. Sirtori, N. Mitro and M. Ruscica (2018). "PPAR Agonists and Metabolic Syndrome: An Established Role?" Int J Mol Sci **19**(4).

Botta, M., E. Maurer, M. Ruscica, S. Romeo, T. M. Stulnig and P. Pingitore (2019). "Deciphering the role of V200A and N291S mutations leading to LPL deficiency." Atherosclerosis **282**: 45-51.

Brown, M. S. and J. L. Goldstein (1997). "The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor." Cell **89**(3): 331-340.

Browning, J. D. and J. D. Horton (2010). "Fasting reduces plasma proprotein convertase, subtilisin/kexin type 9 and cholesterol biosynthesis in humans." J Lipid Res **51**(11): 3359-3363.
Camera, M., L. Rossetti, S. S. Barbieri, I. Zanotti, B. Canciani, D. Trabattoni, M. Ruscica, E. Tremoli and N. Ferri (2018). "PCSK9 as a Positive Modulator of Platelet Activation." J Am Coll Cardiol **71**(8): 952-954.
Canuel, M., X. Sun, M. C. Asselin, E. Paramithiotis, A. Prat and N. G. Seidah (2013). "Proprotein convertase subtilisin/kexin type 9 (PCSK9) can mediate degradation of the low density lipoprotein receptor-related protein 1 (LRP-1)." PLoS One **8**(5): e64145.
Carbone, F., C. La Rocca and G. Matarese (2012). "Immunological functions of leptin and adiponectin." Biochimie **94**(10): 2082-2088.
Carbone, S. and C. J. Lavie (2019). "An opposing point of view on the obesity paradox." Postgrad Med: 1-2.
Carbone, S., C. J. Lavie and R. Arena (2017). "Obesity and Heart Failure: Focus on the Obesity Paradox." Mayo Clin Proc **92**(2): 266-279.
Careskey, H. E., R. A. Davis, W. E. Alborn, J. S. Troutt, G. Cao and R. J. Konrad (2008). "Atorvastatin increases human serum levels of proprotein convertase subtilisin/kexin type 9." J Lipid Res **49**(2): 394-398.
Cariou, B., C. Langhi, M. Le Bras, M. Bortolotti, K. A. Le, F. Theytaz, C. Le May, B. Guyomarc'h-Delasalle, Y. Zair, R. Kreis, C. Boesch, M. Krempf, L. Tappy and P. Costet (2013). "Plasma PCSK9 concentrations during an oral fat load and after short term high-fat, high-fat high-protein and high-fructose diets." Nutr Metab (Lond) **10**(1): 4.
Chawla, A., E. J. Schwarz, D. D. Dimaculangan and M. A. Lazar (1994). "Peroxisome proliferator-activated receptor (PPAR) gamma: adipose-predominant expression and induction early in adipocyte differentiation." Endocrinology **135**(2): 798-800.
Cheng, J. M., R. M. Oemrawsingh, H. M. Garcia-Garcia, E. Boersma, R. J. van Geuns, P. W. Serruys, I. Kardys and K. M. Akkerhuis (2016). "PCSK9 in relation to coronary plaque inflammation: Results of the ATHEROREMO-IVUS study." Atherosclerosis **248**: 117-122.
Chua, S. C., Jr., I. K. Koutras, L. Han, S. M. Liu, J. Kay, S. J. Young, W. K. Chung and R. L. Leibel (1997). "Fine structure of the murine leptin receptor gene: splice site suppression is required to form two alternatively spliced transcripts." Genomics **45**(2): 264-270.
Cicero, A. F., S. D'Addato, F. Santi, A. Ferroni, C. Borghi and S. Brisighella Heart (2012). "Leisure-time physical activity and cardiovascular disease mortality: the Brisighella Heart Study." J Cardiovasc Med (Hagerstown) **13**(9): 559-564.
Cohen, J. C., E. Boerwinkle, T. H. Mosley, Jr. and H. H. Hobbs (2006). "Sequence variations in PCSK9, low LDL, and protection against coronary heart disease." N Engl J Med **354**(12): 1264-1272.
Costandi, J., M. Melone, A. Zhao and S. Rashid (2011). "Human resistin stimulates hepatic overproduction of atherogenic ApoB-containing lipoprotein particles by enhancing ApoB stability and impairing intracellular insulin signaling." Circ Res **108**(6): 727-742.
Costet, P., B. Cariou, G. Lambert, F. Lalanne, B. Lardeux, A. L. Jarnoux, A. Grefhorst, B. Staels and M. Krempf (2006). "Hepatic PCSK9 expression is regulated by nutritional status via insulin and sterol regulatory element-binding protein 1c." J Biol Chem **281**(10): 6211-6218.
Coutinho, T., K. Goel, D. Correa de Sa, R. E. Carter, D. O. Hodge, C. Kragelund, A. M. Kanaya, M. Zeller, J. S. Park, L. Kober, C. Torp-Pedersen, Y. Cottin, L. Lorgis, S. H. Lee, Y. J. Kim, R. Thomas, V. L. Roger, V. K. Somers and F. Lopez-Jimenez (2013). "Combining body mass index with measures of central obesity in the assessment of mortality in subjects with coronary disease: role of "normal weight central obesity"." J Am Coll Cardiol **61**(5): 553-560.
Coutinho, T., K. Goel, D. Correa de Sa, C. Kragelund, A. M. Kanaya, M. Zeller, J. S. Park, L. Kober, C. Torp-Pedersen, Y. Cottin, L. Lorgis, S. H. Lee, Y. J. Kim, R. Thomas, V. L. Roger, V. K. Somers and F. Lopez-Jimenez (2011). "Central obesity and survival in subjects with coronary artery disease: a systematic review of the literature and collaborative analysis with individual subject data." J Am Coll Cardiol **57**(19): 1877-1886.

Cuchel, M., E. Bruckert, H. N. Ginsberg, F. J. Raal, R. D. Santos, R. A. Hegele, J. A. Kuivenhoven, B. G. Nordestgaard, O. S. Descamps, E. Steinhagen-Thiessen, A. Tybjaerg-Hansen, G. F. Watts, M. Averna, C. Boileau, J. Boren, A. L. Catapano, J. C. Defesche, G. K. Hovingh, S. E. Humphries, P. T. Kovanen, L. Masana, P. Pajukanta, K. G. Parhofer, K. K. Ray, A. F. Stalenhoef, E. Stroes, M. R. Taskinen, A. Wiegman, O. Wiklund, M. J. Chapman and H. European Atherosclerosis Society Consensus Panel on Familial (2014). "Homozygous familial hypercholesterolaemia: new insights and guidance for clinicians to improve detection and clinical management. A position paper from the Consensus Panel on Familial Hypercholesterolaemia of the European Atherosclerosis Society." Eur Heart J **35**(32): 2146-2157.

Cunningham, D., D. E. Danley, K. F. Geoghegan, M. C. Griffor, J. L. Hawkins, T. A. Subashi, A. H. Varghese, M. J. Ammirati, J. S. Culp, L. R. Hoth, M. N. Mansour, K. M. McGrath, A. P. Seddon, S. Shenolikar, K. J. Stutzman-Engwall, L. C. Warren, D. Xia and X. Qiu (2007). "Structural and biophysical studies of PCSK9 and its mutants linked to familial hypercholesterolemia." Nat Struct Mol Biol **14**(5): 413-419.

Ding, Z., S. Liu, X. Wang, X. Deng, Y. Fan, C. Sun, Y. Wang and J. L. Mehta (2015). "Hemodynamic shear stress via ROS modulates PCSK9 expression in human vascular endothelial and smooth muscle cells and along the mouse aorta." Antioxid Redox Signal **22**(9): 760-771.

Dong, B., A. B. Singh, V. R. Shende and J. Liu (2017). "Hepatic HNF1 transcription factors control the induction of PCSK9 mediated by rosuvastatin in normolipidemic hamsters." Int J Mol Med **39**(3): 749-756.

Du, Y., S. Li, C. J. Cui, Y. Zhang, S. H. Yang and J. J. Li (2016). "Leptin decreases the expression of low-density lipoprotein receptor via PCSK9 pathway: linking dyslipidemia with obesity." J Transl Med **14**(1): 276.

Dubuc, G., A. Chamberland, H. Wassef, J. Davignon, N. G. Seidah, L. Bernier and A. Prat (2004). "Statins upregulate PCSK9, the gene encoding the proprotein convertase neural apoptosis-regulated convertase-1 implicated in familial hypercholesterolemia." Arterioscler Thromb Vasc Biol **24**(8): 1454-1459.

E. Leiva, S. W., L. Guzmán and R. Orrego (2015). Role of Oxidized LDL in Atherosclerosis. Hypercholesterolemia, IntechOpen.

Eberle, D., B. Hegarty, P. Bossard, P. Ferre and F. Foufelle (2004). "SREBP transcription factors: master regulators of lipid homeostasis." Biochimie **86**(11): 839-848.

Emamalipour, M., K. Seidi, A. Jahanban-Esfahlan and R. Jahanban-Esfahlan (2019). "Implications of resistin in type 2 diabetes mellitus and coronary artery disease: Impairing insulin function and inducing pro-inflammatory cytokines." J Cell Physiol.

Ference, B. A., H. N. Ginsberg, I. Graham, K. K. Ray, C. J. Packard, E. Bruckert, R. A. Hegele, R. M. Krauss, F. J. Raal, H. Schunkert, G. F. Watts, J. Boren, S. Fazio, J. D. Horton, L. Masana, S. J. Nicholls, B. G. Nordestgaard, B. van de Sluis, M. R. Taskinen, L. Tokgozoglu, U. Landmesser, U. Laufs, O. Wiklund, J. K. Stock, M. J. Chapman and A. L. Catapano (2017). "Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel." Eur Heart J **38**(32): 2459-2472.

Ference, B. A., J. J. P. Kastelein, K. K. Ray, H. N. Ginsberg, M. J. Chapman, C. J. Packard, U. Laufs, C. Oliver-Williams, A. M. Wood, A. S. Butterworth, E. Di Angelantonio, J. Danesh, S. J. Nicholls, D. L. Bhatt, M. S. Sabatine and A. L. Catapano (2019). "Association of Triglyceride-Lowering LPL Variants and LDL-C-Lowering LDLR Variants With Risk of Coronary Heart Disease." JAMA **321**(4): 364-373.

Ference, B. A., F. Majeed, R. Penumetcha, J. M. Flack and R. D. Brook (2015). "Effect of naturally random allocation to lower low-density lipoprotein cholesterol on the risk of coronary heart disease mediated by polymorphisms in NPC1L1, HMGCR, or both: a 2 x 2 factorial Mendelian randomization study." J Am Coll Cardiol **65**(15): 1552-1561.

Ference, B. A., W. Yoo, I. Alesh, N. Mahajan, K. K. Mirowska, A. Mewada, J. Kahn, L. Afonso, K. A. Williams, Sr. and J. M. Flack (2012). "Effect of long-term exposure to lower low-density lipoprotein

cholesterol beginning early in life on the risk of coronary heart disease: a Mendelian randomization analysis." J Am Coll Cardiol **60**(25): 2631-2639.
Ferri, N., A. Corsini, C. Macchi, P. Magni and M. Ruscica (2016). "Proprotein convertase subtilisin kexin type 9 and high-density lipoprotein metabolism: experimental animal models and clinical evidence." Transl Res **173**: 19-29.
Ferri, N., A. Corsini, C. R. Sirtori and M. Ruscica (2017). "Bococizumab for the treatment of hypercholesterolaemia." Expert Opin Biol Ther **17**(7): 909-910.
Ferri, N., A. Corsini, C. R. Sirtori and M. Ruscica (2017). "Bococizumab for the treatment of hypercholesterolaemia." Expert Opin Biol Ther **17**(2): 237-243.
Ferri, N., S. Marchiano, G. Tibolla, R. Baetta, A. Dhyani, M. Ruscica, P. Uboldi, A. L. Catapano and A. Corsini (2016). "PCSK9 knock-out mice are protected from neointimal formation in response to perivascular carotid collar placement." Atherosclerosis **253**: 214-224.
Ferri, N. and M. Ruscica (2016). "Proprotein convertase subtilisin/kexin type 9 (PCSK9) and metabolic syndrome: insights on insulin resistance, inflammation, and atherogenic dyslipidemia." Endocrine **54**(3): 588-601.
Franzen, O., R. Ermel, A. Cohain, N. K. Akers, A. Di Narzo, H. A. Talukdar, H. Foroughi-Asl, C. Giambartolomei, J. F. Fullard, K. Sukhavasi, S. Koks, L. M. Gan, C. Giannarelli, J. C. Kovacic, C. Betsholtz, B. Losic, T. Michoel, K. Hao, P. Roussos, J. Skogsberg, A. Ruusalepp, E. E. Schadt and J. L. Bjorkegren (2016). "Cardiometabolic risk loci share downstream cis- and trans-gene regulation across tissues and diseases." Science **353**(6301): 827-830.
Fruhbeck, G. (2006). "Intracellular signalling pathways activated by leptin." Biochem J **393**(Pt 1): 7-20.
Ganie, M. A., A. Dhingra, S. Nisar, V. Sreenivas, Z. A. Shah, A. Rashid, S. Masoodi and N. Gupta (2016). "Oral glucose tolerance test significantly impacts the prevalence of abnormal glucose tolerance among Indian women with polycystic ovary syndrome: lessons from a large database of two tertiary care centers on the Indian subcontinent." Fertil Steril **105**(1): 194-201 e191-193.
Ghosh, S., A. K. Singh, B. Aruna, S. Mukhopadhyay and N. Z. Ehtesham (2003). "The genomic organization of mouse resistin reveals major differences from the human resistin: functional implications." Gene **305**(1): 27-34.
Goldstein, J. L. and M. S. Brown (2015). "A century of cholesterol and coronaries: from plaques to genes to statins." Cell **161**(1): 161-172.
Halaas, J. L., K. S. Gajiwala, M. Maffei, S. L. Cohen, B. T. Chait, D. Rabinowitz, R. L. Lallone, S. K. Burley and J. M. Friedman (1995). "Weight-reducing effects of the plasma protein encoded by the obese gene." Science **269**(5223): 543-546.
He, Y., H. Chen, M. J. Quon and M. Reitman (1995). "The mouse obese gene. Genomic organization, promoter activity, and activation by CCAAT/enhancer-binding protein alpha." J Biol Chem **270**(48): 28887-28891.
Horton, J. D., J. L. Goldstein and M. S. Brown (2002). "SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver." J Clin Invest **109**(9): 1125-1131.
Horton, J. D., I. Shimomura, M. S. Brown, R. E. Hammer, J. L. Goldstein and H. Shimano (1998). "Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2." J Clin Invest **101**(11): 2331-2339.
Isse, N., Y. Ogawa, N. Tamura, H. Masuzaki, K. Mori, T. Okazaki, N. Satoh, M. Shigemoto, Y. Yoshimasa, S. Nishi and et al. (1995). "Structural organization and chromosomal assignment of the human obese gene." J Biol Chem **270**(46): 27728-27733.
Jeong, H. J., H. S. Lee, K. S. Kim, Y. K. Kim, D. Yoon and S. W. Park (2008). "Sterol-dependent regulation of proprotein convertase subtilisin/kexin type 9 expression by sterol-regulatory element binding protein-2." J Lipid Res **49**(2): 399-409.
Jialal, I. and S. Devaraj (2018). "Subcutaneous adipose tissue biology in metabolic syndrome." Horm Mol Biol Clin Investig **33**(1).

Jung, U. J. and M. S. Choi (2014). "Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease." Int J Mol Sci **15**(4): 6184-6223.
Kershaw, E. E. and J. S. Flier (2004). "Adipose tissue as an endocrine organ." J Clin Endocrinol Metab **89**(6): 2548-2556.
Kruth, H. S. (2002). "Sequestration of aggregated low-density lipoproteins by macrophages." Curr Opin Lipidol **13**(5): 483-488.
Kwakernaak, A. J., G. Lambert and R. P. Dullaart (2014). "Plasma proprotein convertase subtilisin-kexin type 9 is predominantly related to intermediate density lipoproteins." Clin Biochem **47**(7-8): 679-682.
Lakoski, S. G., T. A. Lagace, J. C. Cohen, J. D. Horton and H. H. Hobbs (2009). "Genetic and metabolic determinants of plasma PCSK9 levels." J Clin Endocrinol Metab **94**(7): 2537-2543.
Lavie, C. J., R. V. Milani and H. O. Ventura (2009). "Obesity and cardiovascular disease: risk factor, paradox, and impact of weight loss." J Am Coll Cardiol **53**(21): 1925-1932.
Lavie, C. J., C. Ozemek, S. Carbone, P. T. Katzmarzyk and S. N. Blair (2019). "Sedentary Behavior, Exercise, and Cardiovascular Health." Circ Res **124**(5): 799-815.
Lawlor, D. A., R. M. Harbord, J. A. Sterne, N. Timpson and G. Davey Smith (2008). "Mendelian randomization: using genes as instruments for making causal inferences in epidemiology." Stat Med **27**(8): 1133-1163.
Lee, S., H. C. Lee, Y. W. Kwon, S. E. Lee, Y. Cho, J. Kim, S. Lee, J. Y. Kim, J. Lee, H. M. Yang, I. Mook-Jung, K. Y. Nam, J. Chung, M. A. Lazar and H. S. Kim (2014). "Adenylyl cyclase-associated protein 1 is a receptor for human resistin and mediates inflammatory actions of human monocytes." Cell Metab **19**(3): 484-497.
Lee, S. W., H. H. Jo, M. R. Kim, Y. O. You and J. H. Kim (2012). "Association between metabolic syndrome and serum leptin levels in postmenopausal women." J Obstet Gynaecol **32**(1): 73-77.
Li, H., B. Dong, S. W. Park, H. S. Lee, W. Chen and J. Liu (2009). "Hepatocyte nuclear factor 1alpha plays a critical role in PCSK9 gene transcription and regulation by the natural hypocholesterolemic compound berberine." J Biol Chem **284**(42): 28885-28895.
Li, S., R. X. Xu, Y. Zhang, Y. L. Guo, C. G. Zhu, G. Liu, Q. Dong and J. J. Li (2015). "Relation of resistin to proprotein convertase subtilisin-kexin type 9 levels in coronary artery disease patients with different nutritional status." J Endocrinol Invest **38**(12): 1291-1299.
Li, Y., P. P. He, D. W. Zhang, X. L. Zheng, F. S. Cayabyab, W. D. Yin and C. K. Tang (2014). "Lipoprotein lipase: from gene to atherosclerosis." Atherosclerosis **237**(2): 597-608.
Liu, Y., X. Cui, B. Hu, C. Lu, X. Huang, J. Cai, S. He, L. Lv, X. Cong, G. Liu, Y. Zhang and R. Ni (2014). "Upregulated expression of CAP1 is associated with tumor migration and metastasis in hepatocellular carcinoma." Pathol Res Pract **210**(3): 169-175.
Lumeng, C. N., S. M. Deyoung, J. L. Bodzin and A. R. Saltiel (2007). "Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity." Diabetes **56**(1): 16-23.
Marcovecchio, P. M., G. D. Thomas, Z. Mikulski, E. Ehinger, K. A. L. Mueller, A. Blatchley, R. Wu, Y. I. Miller, A. T. Nguyen, A. M. Taylor, C. A. McNamara, K. Ley and C. C. Hedrick (2017). "Scavenger Receptor CD36 Directs Nonclassical Monocyte Patrolling Along the Endothelium During Early Atherogenesis." Arterioscler Thromb Vasc Biol **37**(11): 2043-2052.
Melone, M., L. Wilsie, O. Palyha, A. Strack and S. Rashid (2012). "Discovery of a new role of human resistin in hepatocyte low-density lipoprotein receptor suppression mediated in part by proprotein convertase subtilisin/kexin type 9." J Am Coll Cardiol **59**(19): 1697-1705.
Mendel, D. B. and G. R. Crabtree (1991). "HNF-1, a member of a novel class of dimerizing homeodomain proteins." J Biol Chem **266**(2): 677-680.
Miao, J., P. V. Manthena, M. E. Haas, A. V. Ling, D. J. Shin, M. J. Graham, R. M. Crooke, J. Liu and S. B. Biddinger (2015). "Role of Insulin in the Regulation of Proprotein Convertase Subtilisin/Kexin Type 9." Arterioscler Thromb Vasc Biol **35**(7): 1589-1596.

Miller, Y. I., S. H. Choi, P. Wiesner, L. Fang, R. Harkewicz, K. Hartvigsen, A. Boullier, A. Gonen, C. J. Diehl, X. Que, E. Montano, P. X. Shaw, S. Tsimikas, C. J. Binder and J. L. Witztum (2011). "Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity." Circ Res **108**(2): 235-248.
Mombelli, G., A. M. Zanaboni, S. Gaito and C. R. Sirtori (2009). "Waist-to-height ratio is a highly sensitive index for the metabolic syndrome in a Mediterranean population." Metab Syndr Relat Disord **7**(5): 477-484.
Moroni, F., E. Ammirati, G. D. Norata, M. Magnoni and P. G. Camici (2019). "The Role of Monocytes and Macrophages in Human Atherosclerosis, Plaque Neoangiogenesis, and Atherothrombosis." Mediators Inflamm **2019**: 7434376.
Munzberg, H. and C. D. Morrison (2015). "Structure, production and signaling of leptin." Metabolism **64**(1): 13-23.
Norata, G. D., H. Tavori, A. Pirillo, S. Fazio and A. L. Catapano (2016). "Biology of proprotein convertase subtilisin kexin 9: beyond low-density lipoprotein cholesterol lowering." Cardiovasc Res **112**(1): 429-442.
Norata, G. D., G. Tibolla and A. L. Catapano (2014). "PCSK9 inhibition for the treatment of hypercholesterolemia: promises and emerging challenges." Vascul Pharmacol **62**(2): 103-111.
Nuttall, F. Q. (2015). "Body Mass Index: Obesity, BMI, and Health: A Critical Review." Nutr Today **50**(3): 117-128.
Palanivel, R., A. Maida, Y. Liu and G. Sweeney (2006). "Regulation of insulin signalling, glucose uptake and metabolism in rat skeletal muscle cells upon prolonged exposure to resistin." Diabetologia **49**(1): 183-190.
Park, H. K. and R. S. Ahima (2013). "Resistin in rodents and humans." Diabetes Metab J **37**(6): 404-414.
Pavanello, C., A. M. Zanaboni, S. Gaito, M. Botta, G. Mombelli, C. R. Sirtori and M. Ruscica (2018). "Influence of body variables in the development of metabolic syndrome-A long term follow-up study." PLoS One **13**(2): e0192751.
Pecin, I., M. L. Hartgers, G. K. Hovingh, R. Dent and Z. Reiner (2017). "Prevention of cardiovascular disease in patients with familial hypercholesterolaemia: The role of PCSK9 inhibitors." Eur J Prev Cardiol **24**(13): 1383-1401.
Persson, L., G. Cao, L. Stahle, B. G. Sjoberg, J. S. Troutt, R. J. Konrad, C. Galman, H. Wallen, M. Eriksson, I. Hafstrom, S. Lind, M. Dahlin, P. Amark, B. Angelin and M. Rudling (2010). "Circulating proprotein convertase subtilisin kexin type 9 has a diurnal rhythm synchronous with cholesterol synthesis and is reduced by fasting in humans." Arterioscler Thromb Vasc Biol **30**(12): 2666-2672.
Piche, M. E., P. Poirier, I. Lemieux and J. P. Despres (2018). "Overview of Epidemiology and Contribution of Obesity and Body Fat Distribution to Cardiovascular Disease: An Update." Prog Cardiovasc Dis **61**(2): 103-113.
Pingitore, P., S. M. Lepore, C. Pirazzi, R. M. Mancina, B. M. Motta, L. Valenti, K. E. Berge, K. Retterstol, T. P. Leren, O. Wiklund and S. Romeo (2016). "Identification and characterization of two novel mutations in the LPL gene causing type I hyperlipoproteinemia." J Clin Lipidol **10**(4): 816-823.
Poirier, P., M. A. Alpert, L. A. Fleisher, P. D. Thompson, H. J. Sugerman, L. E. Burke, P. Marceau, B. A. Franklin, P. A. American Heart Association Obesity Committee of Council on Nutrition, C. o. C. P. Metabolism, C. o. C. S. Critical Care and C. o. C. Anesthesia (2009). "Cardiovascular evaluation and management of severely obese patients undergoing surgery: a science advisory from the American Heart Association." Circulation **120**(1): 86-95.
Pontoglio, M., J. Barra, M. Hadchouel, A. Doyen, C. Kress, J. P. Bach, C. Babinet and M. Yaniv (1996). "Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome." Cell **84**(4): 575-585.
Pramfalk, C., Z. Y. Jiang, Q. Cai, H. Hu, S. D. Zhang, T. Q. Han, M. Eriksson and P. Parini (2010). "HNF1alpha and SREBP2 are important regulators of NPC1L1 in human liver." J Lipid Res **51**(6): 1354-1362.

Qatanani, M. and M. A. Lazar (2007). "Mechanisms of obesity-associated insulin resistance: many choices on the menu." Genes Dev **21**(12): 1443-1455.
Qiu, C., P. Zeng, X. Li, Z. Zhang, B. Pan, Z. Y. F. Peng, Y. Li, Y. Ma, Y. Leng and R. Chen (2017). "What is the impact of PCSK9 rs505151 and rs11591147 polymorphisms on serum lipids level and cardiovascular risk: a meta-analysis." Lipids Health Dis **16**(1): 111.
Rader, D. J. and M. Jaye (2000). "Endothelial lipase: a new member of the triglyceride lipase gene family." Curr Opin Lipidol **11**(2): 141-147.
Rafieian-Kopaei, M., M. Setorki, M. Doudi, A. Baradaran and H. Nasri (2014). "Atherosclerosis: process, indicators, risk factors and new hopes." Int J Prev Med **5**(8): 927-946.
Raggi, P., J. Genest, J. T. Giles, K. J. Rayner, G. Dwivedi, R. S. Beanlands and M. Gupta (2018). "Role of inflammation in the pathogenesis of atherosclerosis and therapeutic interventions." Atherosclerosis **276**: 98-108.
Rashid, S. and J. J. Kastelein (2013). "PCSK9 and resistin at the crossroads of the atherogenic dyslipidemia." Expert Rev Cardiovasc Ther **11**(11): 1567-1577.
Rashid, S., H. Tavori, P. E. Brown, M. F. Linton, J. He, I. Giunzioni and S. Fazio (2014). "Proprotein convertase subtilisin kexin type 9 promotes intestinal overproduction of triglyceride-rich apolipoprotein B lipoproteins through both low-density lipoprotein receptor-dependent and -independent mechanisms." Circulation **130**(5): 431-441.
Reddy, P., D. Lent-Schochet, N. Ramakrishnan, M. McLaughlin and I. Jialal (2019). "Metabolic syndrome is an inflammatory disorder: A conspiracy between adipose tissue and phagocytes." Clin Chim Acta **496**: 35-44.
Reilly, M. P., M. Lehrke, M. L. Wolfe, A. Rohatgi, M. A. Lazar and D. J. Rader (2005). "Resistin is an inflammatory marker of atherosclerosis in humans." Circulation **111**(7): 932-939.
Reisi, P., A. R. Ghaedamini, M. Golbidi, M. Shabrang, Z. Arabpoor and B. Rashidi (2015). "Effect of cholecystokinin on learning and memory, neuronal proliferation and apoptosis in the rat hippocampus." Adv Biomed Res **4**: 227.
Reyes-Soffer, G., M. Pavlyha, C. Ngai, T. Thomas, S. Holleran, R. Ramakrishnan, W. Karmally, R. Nandakumar, N. Fontanez, J. Obunike, S. M. Marcovina, A. H. Lichtenstein, N. R. Matthan, J. Matta, M. Maroccia, F. Becue, F. Poitiers, B. Swanson, L. Cowan, W. J. Sasiela, H. K. Surks and H. N. Ginsberg (2017). "Effects of PCSK9 Inhibition With Alirocumab on Lipoprotein Metabolism in Healthy Humans." Circulation **135**(4): 352-362.
Ricci, C., M. Ruscica, M. Camera, L. Rossetti, C. Macchi, A. Colciago, I. Zanotti, M. G. Lupo, M. P. Adorni, A. F. G. Cicero, F. Fogacci, A. Corsini and N. Ferri (2018). "PCSK9 induces a pro-inflammatory response in macrophages." Sci Rep **8**(1): 2267.
Richard, C., P. Couture, S. Desroches, S. Benjannet, N. G. Seidah, A. H. Lichtenstein and B. Lamarche (2012). "Effect of the Mediterranean diet with and without weight loss on surrogate markers of cholesterol homeostasis in men with the metabolic syndrome." Br J Nutr **107**(5): 705-711.
Rodrigues, R., M. Artieda, D. Tejedor, A. Martinez, P. Konstantinova, H. Petry, C. Meyer, D. Corzo, C. Sundgreen, H. U. Klor, I. Gouni-Berthold, S. Westphal, E. Steinhagen-Thiessen, U. Julius, K. Winkler, E. Stroes, A. Vogt, P. Hardt, H. Prophet, B. Otte, B. G. Nordestgaard, S. S. Deeb and J. D. Brunzell (2016). "Pathogenic classification of LPL gene variants reported to be associated with LPL deficiency." J Clin Lipidol **10**(2): 394-409.
Rodriguez-Perez, C., V. R. Ramprasath, S. Pu, A. Sabra, R. Quirantes-Pine, A. Segura-Carretero and P. J. Jones (2016). "Docosahexaenoic Acid Attenuates Cardiovascular Risk Factors via a Decline in Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) Plasma Levels." Lipids **51**(1): 75-83.
Roubtsova, A., M. N. Munkonda, Z. Awan, J. Marcinkiewicz, A. Chamberland, C. Lazure, K. Cianflone, N. G. Seidah and A. Prat (2011). "Circulating proprotein convertase subtilisin/kexin 9 (PCSK9) regulates VLDLR protein and triglyceride accumulation in visceral adipose tissue." Arterioscler Thromb Vasc Biol **31**(4): 785-791.
Ruban, A., K. Stoenchev, H. Ashrafian and J. Teare (2019). "Current treatments for obesity." Clin Med (Lond) **19**(3): 205-212.

Ruscica, M., A. Baragetti, A. L. Catapano and G. D. Norata (2016). "Translating the biology of adipokines in atherosclerosis and cardiovascular diseases: Gaps and open questions." Nutr Metab Cardiovasc Dis.
Ruscica, M., A. Baragetti, A. L. Catapano and G. D. Norata (2017). "Translating the biology of adipokines in atherosclerosis and cardiovascular diseases: Gaps and open questions." Nutr Metab Cardiovasc Dis **27**(5): 379-395.
Ruscica, M., N. Ferri, F. Fogacci, M. Rosticci, M. Botta, S. Marchiano, P. Magni, S. D'Addato, M. Giovannini, C. Borghi, A. F. G. Cicero and G. Brisighella Heart Study (2017). "Circulating Levels of Proprotein Convertase Subtilisin/Kexin Type 9 and Arterial Stiffness in a Large Population Sample: Data From the Brisighella Heart Study." J Am Heart Assoc **6**(5).
Ruscica, M., N. Ferri, C. Macchi, M. Meroni, C. Lanti, C. Ricci, M. Maggioni, A. L. Fracanzani, S. Badiali, S. Fargion, P. Magni, L. Valenti and P. Dongiovanni (2016). "Liver fat accumulation is associated with circulating PCSK9." Ann Med **48**(5): 384-391.
Ruscica, M., C. Macchi, S. Gandini, B. Morlotti, S. Erzegovesi, L. Bellodi and P. Magni (2016). "Free and bound plasma leptin in anorexia nervosa patients during a refeeding program." Endocrine **51**(2): 380-383.
Ruscica, M., C. Ricci, C. Macchi, P. Magni, R. Cristofani, J. Liu, A. Corsini and N. Ferri (2016). "Suppressor of Cytokine Signaling-3 (SOCS-3) Induces Proprotein Convertase Subtilisin Kexin Type 9 (PCSK9) Expression in Hepatic HepG2 Cell Line." J Biol Chem **291**(7): 3508-3519.
Santamarina-Fojo, S. (1998). "The familial chylomicronemia syndrome." Endocrinol Metab Clin North Am **27**(3): 551-567, viii.
Sato, R., J. Inoue, Y. Kawabe, T. Kodama, T. Takano and M. Maeda (1996). "Sterol-dependent transcriptional regulation of sterol regulatory element-binding protein-2." J Biol Chem **271**(43): 26461-26464.
Seidah, N. G., S. Benjannet, L. Wickham, J. Marcinkiewicz, S. B. Jasmin, S. Stifani, A. Basak, A. Prat and M. Chretien (2003). "The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation." Proc Natl Acad Sci U S A **100**(3): 928-933.
Shapiro, M. D. and S. Fazio (2017). "PCSK9 and Atherosclerosis - Lipids and Beyond." J Atheroscler Thromb **24**(5): 462-472.
Sheedy, F. J., A. Grebe, K. J. Rayner, P. Kalantari, B. Ramkhelawon, S. B. Carpenter, C. E. Becker, H. N. Ediriweera, A. E. Mullick, D. T. Golenbock, L. M. Stuart, E. Latz, K. A. Fitzgerald and K. J. Moore (2013). "CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation." Nat Immunol **14**(8): 812-820.
Shende, V. R., M. Wu, A. B. Singh, B. Dong, C. F. Kan and J. Liu (2015). "Reduction of circulating PCSK9 and LDL-C levels by liver-specific knockdown of HNF1alpha in normolipidemic mice." J Lipid Res **56**(4): 801-809.
Shimano, H., J. D. Horton, I. Shimomura, R. E. Hammer, M. S. Brown and J. L. Goldstein (1997). "Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells." J Clin Invest **99**(5): 846-854.
Shioi, A. and Y. Ikari (2018). "Plaque Calcification During Atherosclerosis Progression and Regression." J Atheroscler Thromb **25**(4): 294-303.
Sinha, M. K., I. Opentanova, J. P. Ohannesian, J. W. Kolaczynski, M. L. Heiman, J. Hale, G. W. Becker, R. R. Bowsher, T. W. Stephens and J. F. Caro (1996). "Evidence of free and bound leptin in human circulation. Studies in lean and obese subjects and during short-term fasting." J Clin Invest **98**(6): 1277-1282.
Steinberg, D. (2002). "Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime." Nat Med **8**(11): 1211-1217.
Steppan, C. M., S. T. Bailey, S. Bhat, E. J. Brown, R. R. Banerjee, C. M. Wright, H. R. Patel, R. S. Ahima and M. A. Lazar (2001). "The hormone resistin links obesity to diabetes." Nature **409**(6818): 307-312.
Stewart, C. R., L. M. Stuart, K. Wilkinson, J. M. van Gils, J. Deng, A. Halle, K. J. Rayner, L. Boyer, R. Zhong, W. A. Frazier, A. Lacy-Hulbert, J. El Khoury, D. T. Golenbock and K. J. Moore (2010). "CD36

ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer." Nat Immunol **11**(2): 155-161.
Strom, T. B., K. Tveten and T. P. Leren (2014). "PCSK9 acts as a chaperone for the LDL receptor in the endoplasmic reticulum." Biochem J **457**(1): 99-105.
Sun, C. A., M. H. Wu, C. H. Chu, Y. C. Chou, G. C. Hsu, T. Yang, W. Y. Chou, C. P. Yu and J. C. Yu (2010). "Adipocytokine resistin and breast cancer risk." Breast Cancer Res Treat **123**(3): 869-876.
Sun, L., X. Yang, Q. Li, P. Zeng, Y. Liu, L. Liu, Y. Chen, M. Yu, C. Ma, X. Li, Y. Li, R. Zhang, Y. Zhu, Q. R. Miao, J. Han and Y. Duan (2017). "Activation of Adiponectin Receptor Regulates Proprotein Convertase Subtilisin/Kexin Type 9 Expression and Inhibits Lesions in ApoE-Deficient Mice." Arterioscler Thromb Vasc Biol **37**(7): 1290-1300.
Tank, J., J. Jordan, A. Diedrich, C. Schroeder, R. Furlan, A. M. Sharma, F. C. Luft and G. Brabant (2003). "Bound leptin and sympathetic outflow in nonobese men." J Clin Endocrinol Metab **88**(10): 4955-4959.
Tarkowski, A., J. Bjersing, A. Shestakov and M. I. Bokarewa (2010). "Resistin competes with lipopolysaccharide for binding to toll-like receptor 4." J Cell Mol Med **14**(6B): 1419-1431.
Teslovich, T. M., K. Musunuru, A. V. Smith, A. C. Edmondson, I. M. Stylianou, M. Koseki, J. P. Pirruccello, S. Ripatti, D. I. Chasman, C. J. Willer, C. T. Johansen, S. W. Fouchier, A. Isaacs, G. M. Peloso, M. Barbalic, S. L. Ricketts, J. C. Bis, Y. S. Aulchenko, G. Thorleifsson, M. F. Feitosa, J. Chambers, M. Orho-Melander, O. Melander, T. Johnson, X. Li, X. Guo, M. Li, Y. Shin Cho, M. Jin Go, Y. Jin Kim, J. Y. Lee, T. Park, K. Kim, X. Sim, R. Twee-Hee Ong, D. C. Croteau-Chonka, L. A. Lange, J. D. Smith, K. Song, J. Hua Zhao, X. Yuan, J. Luan, C. Lamina, A. Ziegler, W. Zhang, R. Y. Zee, A. F. Wright, J. C. Witteman, J. F. Wilson, G. Willemsen, H. E. Wichmann, J. B. Whitfield, D. M. Waterworth, N. J. Wareham, G. Waeber, P. Vollenweider, B. F. Voight, V. Vitart, A. G. Uitterlinden, M. Uda, J. Tuomilehto, J. R. Thompson, T. Tanaka, I. Surakka, H. M. Stringham, T. D. Spector, N. Soranzo, J. H. Smit, J. Sinisalo, K. Silander, E. J. Sijbrands, A. Scuteri, J. Scott, D. Schlessinger, S. Sanna, V. Salomaa, J. Saharinen, C. Sabatti, A. Ruokonen, I. Rudan, L. M. Rose, R. Roberts, M. Rieder, B. M. Psaty, P. P. Pramstaller, I. Pichler, M. Perola, B. W. Penninx, N. L. Pedersen, C. Pattaro, A. N. Parker, G. Pare, B. A. Oostra, C. J. O'Donnell, M. S. Nieminen, D. A. Nickerson, G. W. Montgomery, T. Meitinger, R. McPherson, M. I. McCarthy, W. McArdle, D. Masson, N. G. Martin, F. Marroni, M. Mangino, P. K. Magnusson, G. Lucas, R. Luben, R. J. Loos, M. L. Lokki, G. Lettre, C. Langenberg, L. J. Launer, E. G. Lakatta, R. Laaksonen, K. O. Kyvik, F. Kronenberg, I. R. Konig, K. T. Khaw, J. Kaprio, L. M. Kaplan, A. Johansson, M. R. Jarvelin, A. C. Janssens, E. Ingelsson, W. Igl, G. Kees Hovingh, J. J. Hottenga, A. Hofman, A. A. Hicks, C. Hengstenberg, I. M. Heid, C. Hayward, A. S. Havulinna, N. D. Hastie, T. B. Harris, T. Haritunians, A. S. Hall, U. Gyllensten, C. Guiducci, L. C. Groop, E. Gonzalez, C. Gieger, N. B. Freimer, L. Ferrucci, J. Erdmann, P. Elliott, K. G. Ejebe, A. Doring, A. F. Dominiczak, S. Demissie, P. Deloukas, E. J. de Geus, U. de Faire, G. Crawford, F. S. Collins, Y. D. Chen, M. J. Caulfield, H. Campbell, N. P. Burtt, L. L. Bonnycastle, D. I. Boomsma, S. M. Boekholdt, R. N. Bergman, I. Barroso, S. Bandinelli, C. M. Ballantyne, T. L. Assimes, T. Quertermous, D. Altshuler, M. Seielstad, T. Y. Wong, E. S. Tai, A. B. Feranil, C. W. Kuzawa, L. S. Adair, H. A. Taylor, Jr., I. B. Borecki, S. B. Gabriel, J. G. Wilson, H. Holm, U. Thorsteinsdottir, V. Gudnason, R. M. Krauss, K. L. Mohlke, J. M. Ordovas, P. B. Munroe, J. S. Kooner, A. R. Tall, R. A. Hegele, J. J. Kastelein, E. E. Schadt, J. I. Rotter, E. Boerwinkle, D. P. Strachan, V. Mooser, K. Stefansson, M. P. Reilly, N. J. Samani, H. Schunkert, L. A. Cupples, M. S. Sandhu, P. M. Ridker, D. J. Rader, C. M. van Duijn, L. Peltonen, G. R. Abecasis, M. Boehnke and S. Kathiresan (2010). "Biological, clinical and population relevance of 95 loci for blood lipids." Nature **466**(7307): 707-713.
Van Gaal, L. F., I. L. Mertens and C. E. De Block (2006). "Mechanisms linking obesity with cardiovascular disease." Nature **444**(7121): 875-880.
Verbeek, R., M. Boyer, S. M. Boekholdt, G. K. Hovingh, J. J. Kastelein, N. Wareham, K. T. Khaw and B. J. Arsenault (2017). "Carriers of the PCSK9 R46L Variant Are Characterized by an Antiatherogenic Lipoprotein Profile Assessed by Nuclear Magnetic Resonance Spectroscopy-Brief Report." Arterioscler Thromb Vasc Biol **37**(1): 43-48.
Wada, N., S. Hirako, F. Takenoya, H. Kageyama, M. Okabe and S. Shioda (2014). "Leptin and its receptors." J Chem Neuroanat **61-62**: 191-199.

Wang, M. Y., Y. T. Zhou, C. B. Newgard and R. H. Unger (1996). "A novel leptin receptor isoform in rat." FEBS Lett **392**(2): 87-90.
Watts, G. F., D. C. Chan, R. Dent, R. Somaratne, S. M. Wasserman, R. Scott, S. Burrows and R. B. PH (2017). "Factorial Effects of Evolocumab and Atorvastatin on Lipoprotein Metabolism." Circulation **135**(4): 338-351.
Weisberg, S. P., D. McCann, M. Desai, M. Rosenbaum, R. L. Leibel and A. W. Ferrante, Jr. (2003). "Obesity is associated with macrophage accumulation in adipose tissue." J Clin Invest **112**(12): 1796-1808.
Willer, C. J., E. M. Schmidt, S. Sengupta, G. M. Peloso, S. Gustafsson, S. Kanoni, A. Ganna, J. Chen, M. L. Buchkovich, S. Mora, J. S. Beckmann, J. L. Bragg-Gresham, H. Y. Chang, A. Demirkan, H. M. Den Hertog, R. Do, L. A. Donnelly, G. B. Ehret, T. Esko, M. F. Feitosa, T. Ferreira, K. Fischer, P. Fontanillas, R. M. Fraser, D. F. Freitag, D. Gurdasani, K. Heikkila, E. Hypponen, A. Isaacs, A. U. Jackson, A. Johansson, T. Johnson, M. Kaakinen, J. Kettunen, M. E. Kleber, X. Li, J. Luan, L. P. Lyytikainen, P. K. E. Magnusson, M. Mangino, E. Mihailov, M. E. Montasser, M. Muller-Nurasyid, I. M. Nolte, J. R. O'Connell, C. D. Palmer, M. Perola, A. K. Petersen, S. Sanna, R. Saxena, S. K. Service, S. Shah, D. Shungin, C. Sidore, C. Song, R. J. Strawbridge, I. Surakka, T. Tanaka, T. M. Teslovich, G. Thorleifsson, E. G. Van den Herik, B. F. Voight, K. A. Volcik, L. L. Waite, A. Wong, Y. Wu, W. Zhang, D. Absher, G. Asiki, I. Barroso, L. F. Been, J. L. Bolton, L. L. Bonnycastle, P. Brambilla, M. S. Burnett, G. Cesana, M. Dimitriou, A. S. F. Doney, A. Doring, P. Elliott, S. E. Epstein, G. Ingi Eyjolfsson, B. Gigante, M. O. Goodarzi, H. Grallert, M. L. Gravito, C. J. Groves, G. Hallmans, A. L. Hartikainen, C. Hayward, D. Hernandez, A. A. Hicks, H. Holm, Y. J. Hung, T. Illig, M. R. Jones, P. Kaleebu, J. J. P. Kastelein, K. T. Khaw, E. Kim, N. Klopp, P. Komulainen, M. Kumari, C. Langenberg, T. Lehtimaki, S. Y. Lin, J. Lindstrom, R. J. F. Loos, F. Mach, W. L. McArdle, C. Meisinger, B. D. Mitchell, G. Muller, R. Nagaraja, N. Narisu, T. V. M. Nieminen, R. N. Nsubuga, I. Olafsson, K. K. Ong, A. Palotie, T. Papamarkou, C. Pomilla, A. Pouta, D. J. Rader, M. P. Reilly, P. M. Ridker, F. Rivadeneira, I. Rudan, A. Ruokonen, N. Samani, H. Scharnagl, J. Seeley, K. Silander, A. Stancakova, K. Stirrups, A. J. Swift, L. Tiret, A. G. Uitterlinden, L. J. van Pelt, S. Vedantam, N. Wainwright, C. Wijmenga, S. H. Wild, G. Willemsen, T. Wilsgaard, J. F. Wilson, E. H. Young, J. H. Zhao, L. S. Adair, D. Arveiler, T. L. Assimes, S. Bandinelli, F. Bennett, M. Bochud, B. O. Boehm, D. I. Boomsma, I. B. Borecki, S. R. Bornstein, P. Bovet, M. Burnier, H. Campbell, A. Chakravarti, J. C. Chambers, Y. I. Chen, F. S. Collins, R. S. Cooper, J. Danesh, G. Dedoussis, U. de Faire, A. B. Feranil, J. Ferrieres, L. Ferrucci, N. B. Freimer, C. Gieger, L. C. Groop, V. Gudnason, U. Gyllensten, A. Hamsten, T. B. Harris, A. Hingorani, J. N. Hirschhorn, A. Hofman, G. K. Hovingh, C. A. Hsiung, S. E. Humphries, S. C. Hunt, K. Hveem, C. Iribarren, M. R. Jarvelin, A. Jula, M. Kahonen, J. Kaprio, A. Kesaniemi, M. Kivimaki, J. S. Kooner, P. J. Koudstaal, R. M. Krauss, D. Kuh, J. Kuusisto, K. O. Kyvik, M. Laakso, T. A. Lakka, L. Lind, C. M. Lindgren, N. G. Martin, W. Marz, M. I. McCarthy, C. A. McKenzie, P. Meneton, A. Metspalu, L. Moilanen, A. D. Morris, P. B. Munroe, I. Njolstad, N. L. Pedersen, C. Power, P. P. Pramstaller, J. F. Price, B. M. Psaty, T. Quertermous, R. Rauramaa, D. Saleheen, V. Salomaa, D. K. Sanghera, J. Saramies, P. E. H. Schwarz, W. H. Sheu, A. R. Shuldiner, A. Siegbahn, T. D. Spector, K. Stefansson, D. P. Strachan, B. O. Tayo, E. Tremoli, J. Tuomilehto, M. Uusitupa, C. M. van Duijn, P. Vollenweider, L. Wallentin, N. J. Wareham, J. B. Whitfield, B. H. R. Wolffenbuttel, J. M. Ordovas, E. Boerwinkle, C. N. A. Palmer, U. Thorsteinsdottir, D. I. Chasman, J. I. Rotter, P. W. Franks, S. Ripatti, L. A. Cupples, M. S. Sandhu, S. S. Rich, M. Boehnke, P. Deloukas, S. Kathiresan, K. L. Mohlke, E. Ingelsson, G. R. Abecasis and C. Global Lipids Genetics (2013). "Discovery and refinement of loci associated with lipid levels." Nat Genet **45**(11): 1274-1283.
Zhang, F., M. B. Basinski, J. M. Beals, S. L. Briggs, L. M. Churgay, D. K. Clawson, R. D. DiMarchi, T. C. Furman, J. E. Hale, H. M. Hsiung, B. E. Schoner, D. P. Smith, X. Y. Zhang, J. P. Wery and R. W. Schevitz (1997). "Crystal structure of the obese protein leptin-E100." Nature **387**(6629): 206-209.
Zhang, Y., R. Proenca, M. Maffei, M. Barone, L. Leopold and J. M. Friedman (1994). "Positional cloning of the mouse obese gene and its human homologue." Nature **372**(6505): 425-432.
Zheng, Y., S. E. Gardner and M. C. Clarke (2011). "Cell death, damage-associated molecular patterns, and sterile inflammation in cardiovascular disease." Arterioscler Thromb Vasc Biol **31**(12): 2781-2786.

Zhou, J., W. Lei, L. Shen, H. S. Luo and Z. X. Shen (2008). "Primary study of leptin and human hepatocellular carcinoma in vitro." World J Gastroenterol **14**(18): 2900-2904.