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Post-transcriptional modulators of LDL-R in lipid metabolism and atherosclerosis- animal and human studies

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Post-transcriptional modulators of LDL-R in lipid metabolism and atherosclerosis- animal and human studies

Doctoral Thesis by

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Originality Statement

'I hereby declare that this submission is my own work and solely conducted in our laboratory at University of Milan and at Bassini hospital Milan, Italy. This thesis contains data generated from mice models and on Italian general population, To the best of my knowledge it contains no materials previously published on Italian general population, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at University of Milan and at Bassini hospital, Milan is explicitly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception is also acknowledged.'

Signed……

Ashish Dhyani

Date……..

I certify that I have read this dissertation and that, in my opinion, it is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy.

_____

Prof. Alberico L. Catapano

(Principal Advisor)

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PREFACE

This thesis has begun to be tested the traits associated with Proprotein convertase subtilisin/kexin type 9 (PCSK9) in mice model. During the past decade, studies on mice models and clinical trials have been convincingly established the role of PCSK9 with cholesterol homeostasis, however PCSK9 role in peripheral atherosclerosis development is still under investigation, and there is a contradictory results on glucose homeostasis too. Results from our previous study has demonstrated that vascular smooth muscle cells expresses PCSK9, and also marked its presence in the atherosclerotic plaque. This led us to study the role of locally produced PCSK9 protein on vascular remodelling especially in atherosclerotic plaque composition.

For this purpose, the mice with or without PCSK9 gene were generated of C57/BL6 background. At prima facie, we first studied the role of PCSK9 in peripheral atherosclerosis progression and glucose homeostasis. Secondly, we also intended to observe the replica of results from mice in humans. For this purpose, we chose to study the effect of PCSK9 loss of functioned variants especially R46L (rs11591147), which is more commonly found in Caucasian population, and was previously studied in a large cohort of Dallas heart study (DHS). Together with the screening of PCSK9 R46L gene variants, we also choose to determine the levels of plasma PCSK9 in our studied cohort. This helped us to understand the effect size of gene variants not only on the lipid phenotypes but also on the plasma PCSK9 levels and plasma PCSK9 levels mediated effects on metabolism. Together with the study of PCSK9, we also selected few of the recently identified polymorphism on LDL-R modulating genes discovered in a GWAS based or mono-genic studies, these genes include Inducible degrader of LDL-R (IDOL) and G-protein coupled estrogen receptor (GPER)

Rationality of choosing genetic variants study for PCSK9, IDOL and GPER gene.

The major risk associated with cardiovascular diseases (CVDs) are high blood pressure, high cholesterol level, cigarette smoking, diabetes, obesity, physical inactivity, and unhealthy diets. CVDs may also result from a variety of genetic causes, including single nucleotide

polymorphism (SNPs), heterogenetic changes and other environmental factors. Urbanisation, pollutions, Economic transition, industrialization and western life style also promotes heart diseases. Heart diseases have no social, geographical, gender or ethnic boundaries, and it makes it important cause of worldwide mortality. It has been reported from various studies, where CVDs was found a major health burden and causal risk of highest mortality and morbidity worldwide[1][2][3][4]. Of the estimated 16.6% million deaths attributed to CVD worldwide, 80% deaths were reported from developing countries. In recent years, several awareness programmes and high prognostic techniques/methods have immense impact on reducing CVD risk and further extended our understanding to unmask the complexity of CVD occurrence. With the advancement of various techniques to identify the causal root of CVD occurrence has greatly contributed in understanding the CVD complexity. Especially, recent evidences from Genome-wide association studies (GWAS) and mono-genic population based studies have immensely changed a prospective of risk related to CVDs, many gene to gene interaction and their relation to complex traits are a fascinating thirst area. Recent approaches of GWAS have been identified and characterised several unbiased evidences of the genes contributing to common cardiovascular disease traits, and a powerful tool to identify conditions influences by many genes and environmental factors at once. Maximum data from GWAS has been contributed to the depth of European genetic architecture. A complex genetic architecture and interaction with several endogenous and exogenous factors involving in risk is the primary challenge to solve the depth of network.

Indeed, some recently identified single nucleotide polymorphisms (SNPs) in GWAS and other independent studies and evaluation of their contribution in lipid biology has shifted the paradigm in last decade. Few polymorphisms of lipid related genes have been identified as a potential marker to assess the future CVD risk, therefore, we chose to screen few of them to understand their potential role in Italian general population- enrolled in PLIC study (progression of lesion in intima-media of carotid artery), these genes includes Proprotein convertase subtilisin/kexin type 9 (PCSK9), Inducible degrader of LDL-R (IDOL) and G-protein coupled estrogen receptor (GPER).

Preface

With the surge to identify the penetration of the polymorphisms in these selected genes in our Italian cohort and their potential role in lipid phenotypes and risk of CVDs, We chose to identify the penetration and effect size of PCSK9- R46L(rs11591147), IDOL-N342S (rs9370867) and GPER-P16L(rs11544331) polymorphisms in Italian cohort.

PCSK9 R46L has been identified in Whites subjects in a Dallas-Heart studies (DHS) with the penetration of about 3.2% and had a deep role in reducing the high cholesterol risk (-15%) and CVD risk (-47%) [5] and was later found associated with reduction in plasma PCSK9 levels too (-22.30%) [6]. On the other hand, the GWAS identified SNP- rs9370867 (p.N342S; Asn- Ser substitution) on the IDOL gene had impact on lipid phenotypes in European descends, and was found with the frequency of about 49%-60% while it was only about 2%-8% in African (YRI), Asian (CHB and CHD) and Amerindians (Mayans) ancestries[7][8]. However, the studies of rs9370867 SNP on IDOL gene studied in two different population and their association with lipid phenotypes- emerged with a mixed results, where MAF (25%) of risk allele (Asn) in Mexican dyslipidemic cohort had profound effect on cholesterol levels[9], and on the other hand, MAF (35%) of risk allele (Asn) in Brazilian cohort had no significant association with lipid phenotypes[10]. This variable association of gene polymorphism with specific trait is complex and require more attention, and might be utmost essential in therapeutic implications.

We also chose to study GPER-rs11544331 (p.P16L) polymorphism which was suggested to be linked with high blood pressure and elevated total cholesterol levels in women, probably via elevated PCSK9 expression-shown via series of experiment on HepG2 cell lines. This sex-specific regulation of GPER dragged our attention too for its evaluation in Italian cohort.

The reason of choosing these variants of three different genes (PCSK9, IDOL and GPER) was their direct link on lipid phenotypes via LDL-R modulation as reported by GWAS or Individual population based studies. With the immense literature search, we have not found any information about these SNPs studies (except PCSK9-R46L) on lipid phenotype in Italian population.

Therefore, we for the first time evaluated the penetration and effect size of IDOL-N342S and GPER- P16L variants but also PCSK9-R46L on molecular phenotypes of lipid and peripheral atherosclerosis progression. In addition to the SNP screening, we also performed functional studies of our studied genes by invivo and invitro methods. We use transgenic Mice model to understand the role of PCSK9 in carotid atherosclerosis progression and glucose metabolism. While functional characterisation of IDOL SNP was also evaluated in *invitro* analysis by selecting 5-5 subjects of each respective genotypes of the gene and quantified their scale of LDL uptake and LDL-R expressions and other lipid related gene expression.

With this thesis, we will try to understand the role of PCSK9, IDOL and GPER gene on lipid homeostasis, glucose metabolism and peripheral atherosclerosis progression.

CHAPTER 1. INTRODUCTION

Section [A] LDL-receptor structure and physiologic function

Section [B] Post-transcriptional modulators of LDL-R (extracellular and intracellular modulators)

- [B1] PCSK9 (Proprotein convertase subtilisin/kexin type 9) role in LDL-R modulation
- [B2] GPER (G-protein coupled estrogen receptor) role in cardiovascular system
- [B3] IDOL (Inducible degrader of LDL-R) role in LDL-R modulation

SECTION A. LDL-R STRUCTURE AND PHYSIOLOGICAL FUNCTION

1.1. Introduction and historical perspective

The low-density lipoprotein receptor (LDL-R) is a cell surface glycoprotein and expresses ubiquitously, the major function of LDL-R is to pick up low-density lipoproteins (LDL) circulating in the bloodstream and transport them into the cells, thus play a critical role in the homeostatic control of blood cholesterol[11]. Brown and Goldstein originally hypothesised the role of membrane receptor in 1973 during their search for the molecular basis of Familial hypercholesterolemia (FH)[11][12][13][14], FH is inherited as an autosomal dominant trait and characterised as the exceptional increase in cholesterol levels in blood than normal levels. These high in cholesterol levels of FH patients causes severe clinical phenotypes and eventually one of the major factor causing heart attacks in their early life, therefore cholesterol synthesis was shown as a hallmark of FH,. During late 1960s or early 1970s, Brown and Goldstein hypothesised that FH might not be due to the enzyme deficiency (as was earlier concept of FH), rather due to the defect in metabolic regulation or a defect in protein that participate in cholesterol regulation.

The striking observation came from the classical experiment, where Brown and Goldstein first observed that how fibroblast cells stop endogenous production of cholesterol synthesis (by measuring HMG CoA reductase activity) when exogenous cholesterol supplied via LDL in the serum, while it was not true when fibroblast from FH patients cultured and supplied with exogenous LDL- they suspected that fibroblast have some feedback mechanism of endogenous cholesterol synthesis and addition of LDL in culture depleting the HMG-CoA activity. They further demonstrated that the normal fibroblasts had high-affinity binding sites for LDL particles on their surfaces, while the fibroblast from FH patients had impaired affinity for LDL binding on their surfaces [15]. However, not all lipoproteins could suppress the HMG-CoA activity. A decade later, the gene encoding the LDL-uptake receptor was cloned and sequenced[16][17][18]. Shortly after the discovery of LDL-R as a

membrane receptor responsible to internalise circulating LDL, Brown and Goldstein found that LDL-R itself undergoes to feedback regulation, LDL-R expression is largely relies on intracellular cholesterol trafficking, where increased intracellular cholesterol levels decreases the production of LDL- receptor[19], this was in parallel to the HMG-CoA reductase reduced activity. The underlying mechanism behind this feedback mechanism remained unclear uptil next two decades when Brown and Goldstein further discovered a pair of sterol-regulated, membrane bound transcription factors called SREBPs[20][21].

1.2. Physiologic ligands, endocytosis and receptor recycling pathway

The most virtual physiologic ligand for the LDL-R is LDL, which carries a single copy of apolipoprotein B-100 (ApoB100). In later studies, LDL-R were also identified as a binding mediator for ApoE containing lipoprotein particle. LDL-R binding to LDL take place in extracellular environment, this complex (LDL-R-LDL) further enters the cell by endocytosis via clathrin-coated pits and then delivered to endosomes[22][23]. The acidic environment of endosome allows the dissociation of the complex[24], and LDL-R are subsequently returned to the cell surface in a process called “receptor recycling”- [Figure 1]. The ligand binding to LDL-R requires Ca^{2+}.

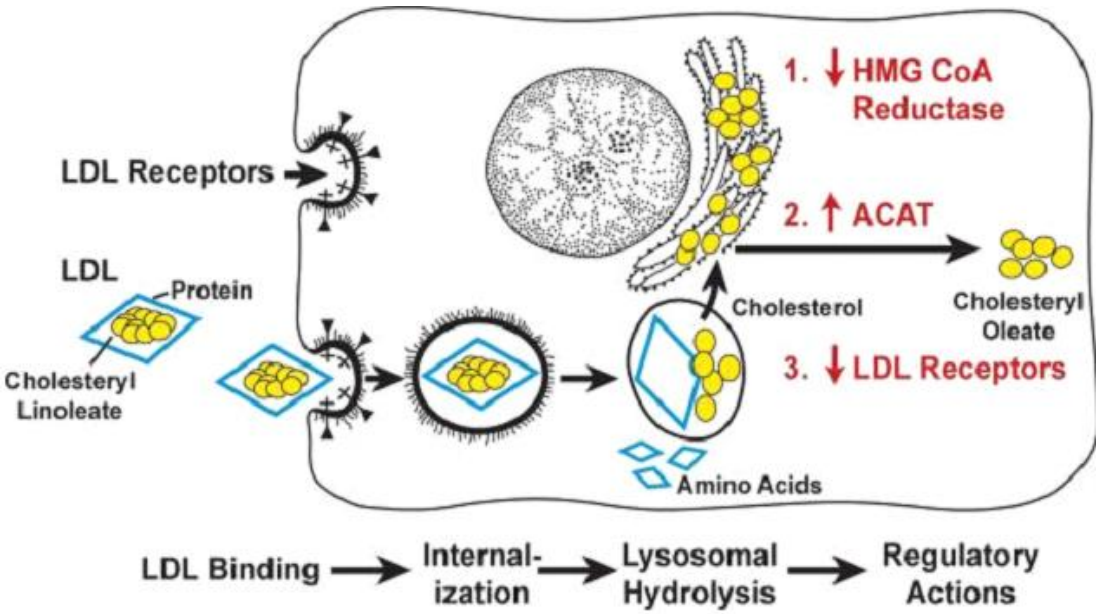


Figure 1. Sequential steps in the LDL receptor pathway of mammalian cells.
A schematic representation of LDLR mediated LDL clearance from circulation. [Figure adapted from Goldstein. J et al; ATVB. 2009 Apr; 29(4): 431-438].

1.3. Structural organisation of LDL-R

The cytogenetic location of human LDL-R is at small arm of chromosome 19 (19p13.2) and contains 18 exons. This human 45kb gene encodes 839 amino acids protein, which carries three types of extracellular modules in its structure[16][17]. The LDL-R is synthesised as a 120-kD glycoprotein precursor that undergoes structural change to a 160-kD mature glycoprotein through the covalent addition of a 40-kD protein[25]**.**

The N-terminal ligand binding domain (LBD) encoded by exons 2 to 6 and consists of 7-contiguous cysteine-rich, complement-type repeats and named LDL receptor type A repeat (LR), each LR module is about 40-residues in length, has six conserved cysteine residues, and contains a conserved acidic region near C-terminus which serves as a calcium-binding site[17]. Adjacent to LBD domain, it also contain three EGF precursor homology domains (comprised of 400 amino acids encoded by exons 7 to 14), this domain of LDL-R is responsible for the dissociation from the lipoproteins in the endocytosis machinery. The another domain in LDL-R is called as O-linked glycosylated domain [17], encoded by exon 15 and comprised with 58 amino acid sequence that is enriched in serine and threonine amino acids, this domain of LDL-R is serve to bind with O-linked sugar chains. A domain which provide support to LDL-R flanking as transmembrane is “membrane-anchoring domain” encoded by exon 16 and 5’ end of exon 17, this domain is essential to the attachment of the receptor to the cell membrane [Figure 2]. The last domain is “cytoplasmic domain or a cytoplasmic tail consists of 50 amino acids encoded by remainder of exon 17 and 18, The cytoplasmic tail contains an NPxY sequence that directs the receptors to clathrin coated pits[26][27], this region is also required for proper sorting of LDL-R [28][29].

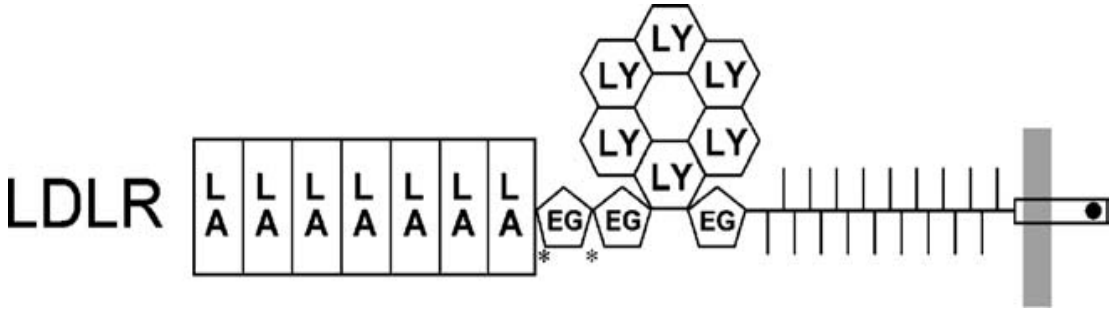


Figure 2. Biological structure of transmembrane LDL-receptor (LDL-R)

Figure depict the domain organisation of the LDLR. "LA" domain- LDL receptor type A also called LBD (Ligand binding domain); "LY"- repeats containing a YWTD consensus motif; "EGF" domain-Epidermal growth factor precursor homology region. [Figure adapted from Jeon H. Blacklow S et al. Annu. Rev. Bichem 2005. 74:535-62].

1.4. Genetic, physiologic modification in LDL-R and role in Familial hypercholesterolemia

Nearly all relevant studies have demonstrated that the major function of LDL-R is to control plasma cholesterol levels, and mutations in the LDL-R gene causes familial hypercholesterolemia (FH)- an autosomal dominantly inherited disease, which is the most common monogenic gene defect in LDL-R found in almost 1:500 people in a heterozygous forms (LDL levels = between 5mmol/L upto 12 mmol/L),. While the homozygote mutation in LDL-R expresses non-functional protein and found with a frequency of about 1:10,000, with a dramatic increases in blood cholesterol levels (LDL levels = >12 mmol/L), which in turns represent severe clinical phenotypes including cutaneous xanthomas and atherosclerotic plaques at a very early age leads to premature death- [*Figure 3*]. The heterozygotes patients carries about half of the normal levels of LDL-R, but still prone to develop xanthomas and atherosclerotic plaques, due to reduced capability of cholesterol clearance. LDL-R mutations, generally classified into five classes viz " Defect in LDL-R synthesis", disturbance in transportation to the cell surfaces", "Binding to ligands", "clustering of the receptor in the endocytic vesicles" and recycling route defect"[11][14]. To date, more than 1400 point mutations and small deletions or insertions associated with FH have been reported in the LDL-R gene.

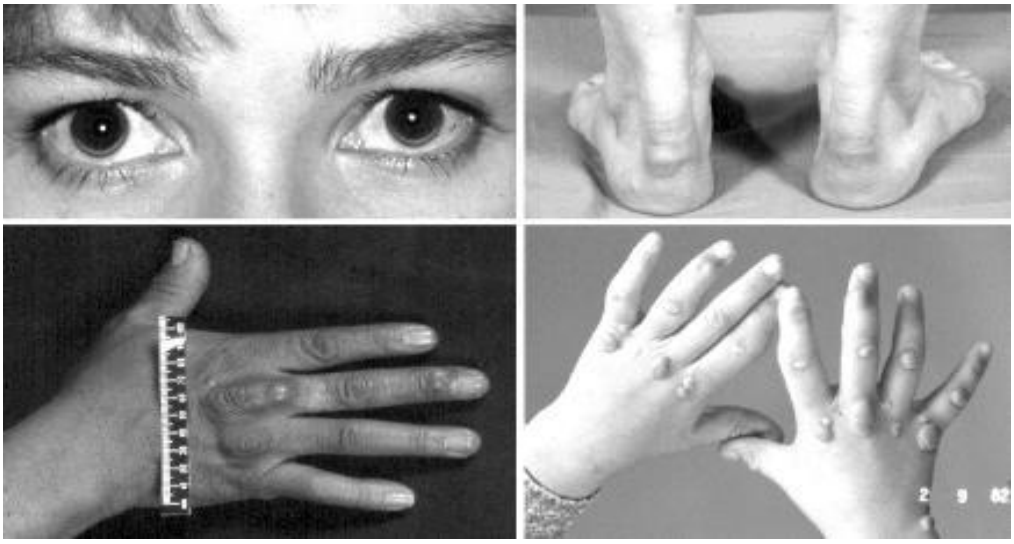


Figure 3. Clinical manifestation of Familial hypercholesterolemia (FH):-
This figure depict the deposition of cholesterol in patients with FH:- Top left panel: a white lining of cholesterol deposition called "arcus conealis" especially seen on the lower part of cornea. Top right panel:- Xanthomas on " Achilles tendons" above the heels. Bottom left panel:- Xanthoma formation in the tendons of the middle and ring fingers. Bottom right panel:- skin xanthomata on the hands of a child with homozygous FH

SECTION B: POST-TRANSCRIPTIONAL MODULATORS OF LDL-R (EXTRACELLULAR AND INTRACELLULAR MODULATORS)

Due to the mounting knowledge of molecular interaction of LDL-R with other proteins [Figure 4] and their role on LDL-R modification elaborated the functional aspect of these proteins in lipid biology. LDL-R major function is to regulate circulating LDL levels, however, LDL-R functionality and its levels also gets regulated by other proteins, these proteins interacts via both extracellular domains or intracellular domains of LDL-R, thereby regulate LDL-R functionality at post-transcriptional level. The two most studied post-transcriptional modulators of LDL-Rs are Proprotein convertase subtilisin/kexin type 9 (PCSK9) and Inducible degrader of LDL-R (IDOL)[30][31]. In this thesis, we will further describe the underlying mechanism of these post-transcriptional modulators in LDL-R viability/functionality.

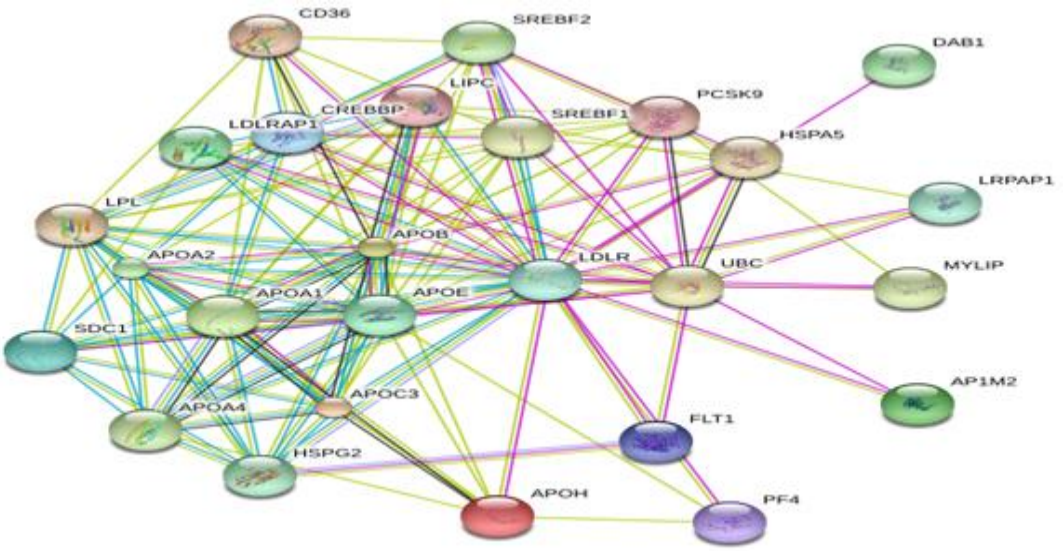


Figure 4. Protein network those interact with LDL-R

Proteins those interact with LDLR in physiological condition. Most of the depicted proteins have been studied extensively, and two proteins on the right hand side (PCSK9 and IDOL-also called MYLIP) was also choosen in our study.

[B1] PCSK9 ROLE IN LDL-R MODULATION

1.5. General Introduction and historical perspective

The first proprotein protease discovered was that of kexin-protease (KEX2 gene) from yeast [*S.cerevisiae],* soon characterised as a Ca^{2+} dependent serine protease[32][33], later homologues proteases in humans were also discovered[34][35]. The mammalian proprotein convertases (PCs) family includes 9 members to date, and well characterised since their respective discoveries, the first seven enzymes (Furin, PC1/3, PC2, PC4, PACE4, PC5/6 and PC7) of PCs families cleaves pro-proteins (precursor proteins) at multi-basic (K/R)-(X)n-(K/R) ! motif, where; X-represent any amino acid besides cysteine, and n= 0,2,4, or 6 [*Figure 5*]. The eighth member is the pyrolysin-like subtilisin kexin isoenzyme-1 (SKI-1)[36], and also known as site-1 protease (S1P)[37] and cleaves substrate at the non-basic motif (R/K)X-(hydrophobic)-X!, where X is variable. The ninth member of PCs widely known as PCSK9 which cleaves the sequence VFAQ152! Within its pro-segment [38][30].

Introduction

A 22kb human Proprotein convertase subtilisin-kexin type 9 (PCSK9) gene located in a region which is linked to ADH in a small arm of chromosome 1p32, and contains 12 exons and 11 introns. The gene encodes a 692-amino acid (aa) protein, which is a 9th member of proprotein convertase family belongs to serine proteases and known to deteriorate the LDL-R activity in liver, eventually causes hypercholesterolemia and hence a suitable target for therapeutic intervention. PCSK9 gene architecture plays a pivotal role and decides the fate of LDL-R viability, GOF mutations in PCSK9 gene causes severe hypercholesterolemia and coronary heart disease (CHD), conversely, LOFs mutations have opposite effect.

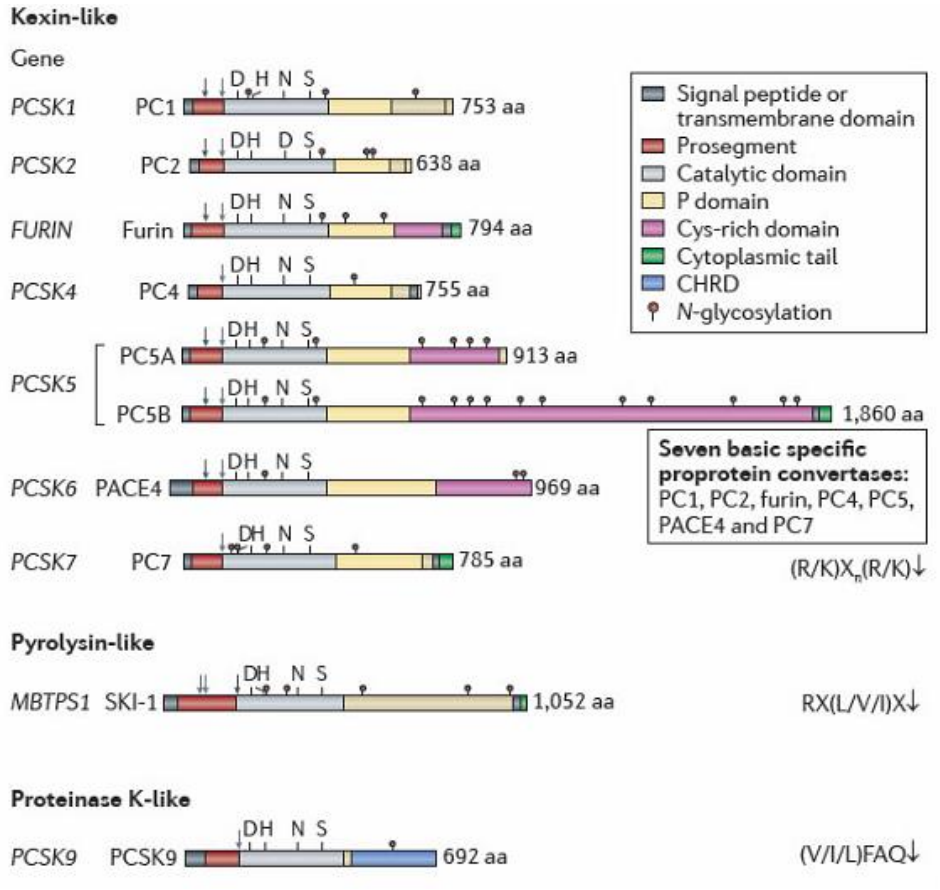


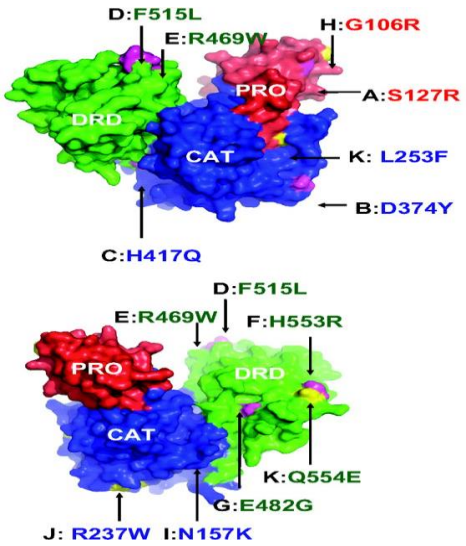
Figure 5. Structure of human Proprotein convertases.

Schematic representation of the human proprotein convertases. Each PC-contains a N-terminal signal domain, Prodomain, Catalytic domain and P-domain responsible for calcium binding and pH dependence. The PCs contain either serine and threonine rich region or cysteine rich regions, and sometime contains transmembrane domain followed by C-terminal cytoplasmic domains. [Figure adapted from Seidah.N et al, Nat. Rev drug discovery. 2012 May; 11, 367-383]

1.6. Benchmark of PCSK9- From discovery to therapeutic application

PCSK9 high expression was initially reported during apoptosis in neuronal cells[30], also called as NARC1 (neural apoptosis regulated convertase-1), it was in 2003, when the first link of PCSK9 in cholesterol metabolism was discovered[39], which opened a new insight into the cardiovascular biology. PCSK9 role in cholesterol metabolism rapidly took into pace when two causative new mutations (S127R and F216R) in two French autosomal dominant hypercholesterolemia (ADH) families were detected between D1S197 and D1S2890 markers in chromosome 1p34.1-p32 (OMIM 63776), that harbouring 41 genes including PCSK9. Later, structural and functional studies revealed that the severe clinical output of these functional variants was due to their enhanced binding activity and degradation capability to LDL-R[40][41]. Although, a pre-mapped region about 17cM between markers D1S2130 and D1S1596 in chromosome 1p32 was already found linked to ADH in Utah population [42] in early 2000s, but remained unidentified for the gene uptil 2004, the region was later found overlapped with previously identified locus causing severe hypercholesterolemia in French and Spanish families[43]. All these discoveries established the role of PCSK9 gene in hypercholesterolemia [*Figure 6*]. Conversely, discovery of two loss-of-functioned (LOFs) mutations in African-Americans (Y142X and C679X) revolutionised the era into therapeutic possibility[44], these mutation had about 40% and 88% reduction in cholesterol and CHD risk respectively. Apart from the PCSK9 genetics, several endogenous and exogenous factors also modulates PCSK9 expressions, including age, gender, dietary habits, life style, drugs etc, in short, Statins are the cornerstone of lipid-lowering treatment, but prevalence of persistent lipid abnormalities was often seen in many patients under Statin treatment, and often remained a challenge for the clinicians. Biochemical studies later discovered the expression of PCSK9 gene goes upregulated upon statin treatment via sterol regulatory element binding protein (SREBP2) pathway. This eventually reducing the efficacy of statins[45][46]. Thus PCSK9 protein was hotcake for pharmaceutical intervention, and various therapeutic alternatives were proposed and tested, including- Monoclonal antibodies, peptides mimics, vaccinations, anti-sense oligonucleotides etc. A meta-analysis of 24 clinical trials of PCSK9 monoclonal antibodies has shown the improved cholesterol profile, cardiac health and all-cause mortality[47]. PCSK9 Monoclonal antibodies

Evolocumab (Amgen), Bococizumad(Pfizer) and alirocumab(Aventis/Regeneron), has been approved.



	Phenotype	Notes
A:S127R	Gain-of-Function	Possible susceptible to proteolysis for removal of auto inhibition. Associated with hypercholesterolemia.
B:D374Y	Gain-of-function	In P' region and may alter specificity of active site regions thus increasing affinity for substrate. Associated with hypercholesterolemia
C:H417Q	Gain-of-function	On helix in region adjacent to another histidine 449. Mode of operation unclear
D:F515L	Gain-of-function	Second beta-turn-beta of putative binding site in SD1 of resistin-like CRD.
E:R469W	Gain-of-function	Putative binding region of resistin-like CRD. Completely conserved in mammals
F:H553R	Gain-of-Function	Situated at bottom of SD1 of resistin-like-CRD
G:E482G	Gain-of-Function	Highly conserved in mammalian proprotein convertases.
H:G106R	Loss-of-function	Exposed loop of pro-domain – likely to restrict conformation and have folding issues
I:N157K	Loss-of-function	Introduction of steric clashes probably hindrance to folding.
J:R237W	Loss-of-function	Exposed loop at opposite side from active site - mode of action for loss-of-function is not clear
K:L253F	Loss-of-function	Active site in S2 pocket, highly conserved across species, increasing the bulk of the side chain will clearly lead to loss of specificity/ability to process.

Figure 6. Mapping of common natural mutants of PCSK9.
A cartoon on left panel depicted the mutation carrying on different domains of PCSK9 while table on the right side depict the phenotype caused by respective mutant. [Figure adapted from Hampton E. et al; PNAS 2007 Sep 11;104 (37): 14604-9].

1.7. Structural and functional activity of PCSK9

PCSK9 is initially synthesized as a zymogen (~74kDa proprotein), from which proteolytic cleavage of the ~14kDa N-terminal prodomain results in ~60kDa mature form consist of Prodomain as a chaperon, catalytic domain and CHRD domain[48][49][50][51]. The inactive form of PCSK9 also make its volume in plasma at certain extent. The furin mediated cleavage at Arg218! in PCSK9 generates ~55kDa protein. The liver is the major site of PCSK9 secretion[30] [52], other site also contribute in plasma PCSK9 volume includes kidneys, small intestine and brain [52][53][54][55][56]. Hepatocytes secreted PCSK9 contributes about 2/3 volume of blood cholesterol [52], due to its exacerbate ability to downregulate LDL-R, PCSK9 binding and internalisation to surface LDL-R thought to be rapid and major pathway of PCSK9 clearance, with 5-minute half-life of the recombinant human protein injected into mice, [55][56], and prolonged stay circulation in LDL-R deficient mice[55]- *[Figure 7].*

PCSK9 shares a structure homology with other proprotein convertases (PCs) and contains four major domain within, namely- signal peptide (1-30 aa), Prodomain (31-152 aa), Catalytic domain (153-425 aa) and c-terminal or Cys-his rich domain (CHRD) (426-692 aa). However, unlike other PCs, PCSK9 undergoes single autocatalytic intramolecular cleavage at non-basic amino-acid residue Gln152Ser at its prodomain[48][49], this cleavage helps PCSK9 in secretion to extracellular environment, however, prodomain remained bounded as chaperon and mask its future proteolytic activity, the chaperon bounded PCSK9 displayed it binding ability to LDL-R and further implicated to the cholesterol homeostasis. The main function of PCSK9 is to bind with transmembrane LDL-R and mediates its degradation [Figure 7]. However, PCSK9 in circulation remained in both mature (Cleaved ~62kDa) and inactivated form (~55kDa), ex-vivo data revealed that furin ability to cleave PCSK9 at Arg(218!)-Gln(219!) reduces plasma PCSK9 levels by ~50% and remained of ~55kDa in size.[57], while 35% increases in PCSK9 mRNA and 26% drop in LDL-R protein levels in mice lacking furin gene was observed. Mass spectrometry analysis revealed that in vivo cleavage of PCSK9 at Arg218! Resulting in pyroglutamic acid formation of the nascent N-terminus amino acid corresponding to Gln!219 of secretory PCSK9.

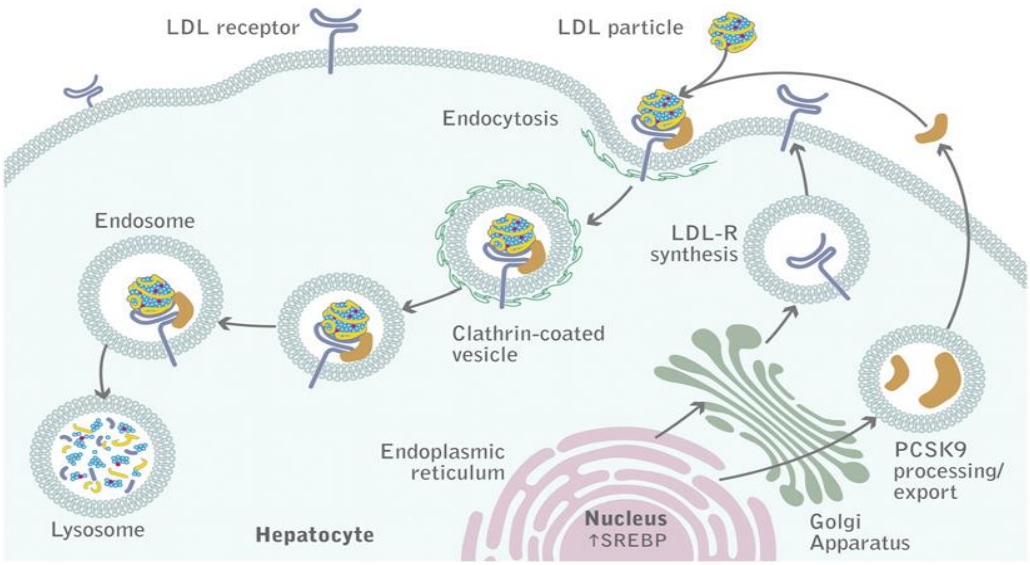


Figure 7. Molecular mechanism of PCSK9 mediated LDLR degradation

A complex of PCSK9-LDL-LDLR formed extracellularly and internalised via clathrin coated pits, fuses into endosome and subsequently undergoes lysosomal degradation. [Figure adapted from Lambert et al, JLR. 2012. 53:2515-2524].

The first evidence of PCSK9 activity in reducing hepatic LDL-R protein emerged from the mice models, and led the discovery of the molecular mechanism of PCSK9. Briefly, the PCSK9-LDL-R complex [Catalytic domain of PCSK9- EGF-a domain of LDL-R] initiates at extracellular surface and subsequently internalises via clathrin coated pathway with the help of ARH-adaptor protein activity. The complex further fuses into endosome, where secondary folding of PCSK9 to LDL-R requires to sustain in the late acidic endosome, CHRD domain of PCSK9 and LBD-domain of LDL-R binding prevent the escape of PCSK9-LDL-R complex from acidic endosomic environment [*Figure 8*]. This complex eventually routed for lysosomal degradation, however, with unknown mechanism.

Intracellular binding of PCSK9 to LDL-R is also studied, it is evident from few studies that PCSK9 mediates LDL-R degradation in ARH-knockout mice too, suggesting that PCSK9 also promotes intracellular degradation of LDL-R before reaching into basolateral surface of the hepatocytes.

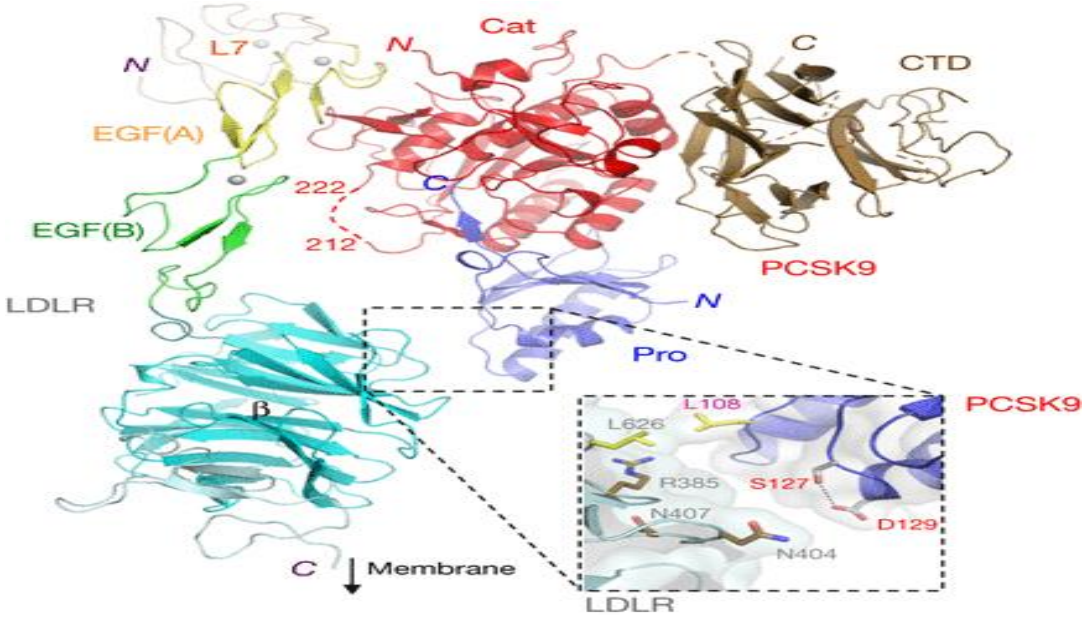


Figure 8. Ribbon diagram of PCSK9-LDLR complex.
The prodomain and catalytic domain of human PCSK9 are shown in purple and light brown color, respectively; the extracellular domain of LDL-R is shown in light green.[Figure adapted from Surdo Lo et al; EMBO Rep. 2011 Dec 1;12(12): 1300-5].

PCSK9 also downregulates LDL-R family members like VLDLR, LRP1, ApoER2.

1.8. Genetic architecture of PCSK9 and role in cholesterol homeostasis.

Genetic mapping of small arm of chromosome-1 identified a 22kb PCSK9 gene which contains 12 exons and 11 introns, this gene encodes a protein comprised of 692aa -which belongs to proprotein convertase family. The PCSK9 gene is located in the locus which was found linked with ADH in French families. The first two gain of function discovered on PCSK9 was S127R and F216L from French ADH families, which led PCSK9 as the 3rd gene causing autosomal dominant hypercholesterolemia (ADH). Gain of function (GOF) mutations on PCSK9 gene causes severe cholesterolemic phenotypes and a risk of CVD. The another most potent mutation was D374Y, which has been shown to increase the interaction between PCSK9 and LDL-R by 5-30 folds, firstly found on Utah and Norwegian population. D374 is a surface amino acid and makes no contacts with other amino acids in the PCSK9 structure. Structure analysis revealed that D374Y mutation causes an increase in affinity for the LDL-R by allowing a hydrogen bond or pi stacking interaction to form between PCSK9 and H306 of the LDL-R-EGF domain. Few gain-of-function (GOFs) and loss-of-function (LOFs) depicted in [Figure 9]

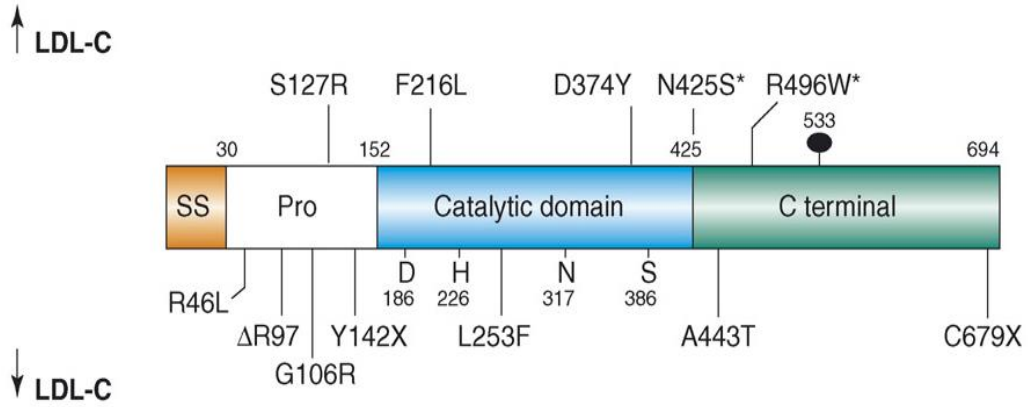


Figure 9.The structure of proprotein convertase subtilisin kexin type 9 (PCSK9)

Structure of PCSK9 contains 3 major domain (Pro, Catalytic and CHRD or C-terminus) after detaching from signal peptide or signal sequence (SS). Several gain-of-function (GOFs) and loss-of-functions (LOFs) identified in PCSK9 protein causing hyper and hypocholesterolaemia respectively. [Figure adapted from Horton.J et al- Trends in biochemical sciences 2007 Feb 32(2): 71-7].

Several other single nucleotide polymorphisms has been detected and characterised on PCSK9 gene. Two Loss of function (LOF) mutations Y142X and C679X results in truncated versions of PCSK9 that disrupt proper folding and secretion while L82X (another LOF) prematurely terminate protein translation within the prodomain. Mutation at 253 aa position (L253F) on catalytic domain results in defect in autoprocessing of the protein. R46L is the most studied LOF variant in Caucasian subjects, also impaired hepatic secretion of PCSK9 and reduces the protein volume in plasma. Several additional PCSK9 variants are associated with reduced plasma LDL levels and reduced cardiovascular risk but their mode of action has not been elucidated yet.

1.9. Population, gender and age disparity in PCSK9 expression.

Inter-individual genetic makeup, age, gender and population are the major factors influencing PCSK9 levels and its functionality. Fluctuation on plasma PCSK9 levels has been advised in many studies, low in early age and increasing in concentration in later ages especially in women. In general, women plasma carries more load of PCSK9 protein than their age-matched men, and this goes further highest in post-menopausal state. Data from recent study encompasses the instability of plasma PCSK9 levels exist right from the birth, a difference of 2 fold was there when analysed the fetus serum for PCSK9 levels than mother [58]. Further age and sex biasness also emerged from cross-sectional studies on 1739 youth, where plasma PCSK9 levels were higher in 9 years of boys group than their age matched girls (+10.78% at 5^{th} percentile and +6.94% at 95^{th} percentile), while girls of 13 years (+13.12% at 5^{th} percentile and +8.28% at 95^{th} percentile), and 16 years (+37.68% at 5^{th} percentile and +40.97% at 95^{th} percentile) age groups had increased concentrations of plasma PCSK9 protein than boys of similar age group, which indicates the strong intervention of hormonal regulation[59].

Plasma PCSK9 levels has been found varies among population to population too, and stressing for additional dependency factors like ethnicity and demography[6][60].

1.10. Transcriptional, hormonal and dietary regulation of PCSK9 expression

PCSK9 gene carries the functional sterol regulatory element (SRE) at its proximal region and is the most conserved transcriptional motif targeted by SRE-binding proteins (SREBPs)- A master transcriptional factors in cholesterol regulatory pathway. SREBP2 majorly plays a key role in regulating or co-regulating PCSK9 and LDL-R receptor expressions to maintain intracellular sterol levels.

The unbiased SREBPs dependent mechanism was described shortly after the discovery of PCSK9 in 2003, where mice liver had less PCSK9 mRNA when mice were stressed on cholesterol rich diet, while had higher PCSK9 mRNA in SREBP1a and SREBP2 overexpressing mice [61]**.** In addition certain lipid lowering drugs especially Statins also had impact on PCSK9 expression via SREBP2 pathway[62]. SREBP-1c was also implicated in postprandial insulin upregulation of PCSK9 gene expression in hepatocytes [63]**.** Similarly, activation of HNF1α also exert in PCSK9 transcription, which binds to an element located 28 nucleotides (nts) upstream of the PCSK9-SRE region, this site is conserved and not present in LDL-R promoter, hence unlike SREBPs, HNF1α only target PCSK9 and not LDL-R [64]**.**

Certain other repressor of PCSK9 transcription were recently identified. Berberine and Curcumin suppresses HNF1 α activation hence negatively affect in PCSK9 transcription too, similarly insulin also exert dual effect on PCSK9 expression, where insulin mediated IRS1 activation leads the activation of mammalian target of rapamycin complex 1 (mTORC1) resulting in profound reduction of HNF1 α and eventually less PCSK9 transcription [65]. PCSK9 expression were also found higher in a gender specific manner, women generally carries higher PCSK9 levels than age-matched men, and this goes further goes higher in their post-menopausal state. Hormonal intervention were long been suspected for the cause, recent evidences also support the role of Estrogen in controlling PCSK9 expression, however the true mechanism is still need to evaluate.

1.11. PCSK9 role in extrahepatic tissues (adipose tissue, small intestine, pancreatic β-cells, kidney).

PCSK9 role in adipose tissue:-

However, adipocytes do not expresses PCSK9, but it appears from recent findings that circulating PCSK9 can modulates the adipocytes functionality, and this physiological modulation is mediated due to its ability to downregulate VLDL-R. Results from both mice[66] and human models[67] exhibited that PCSK9 binding with VLDL-R initiate with the similar fashion as with LDL-R and eventually leads its downregulation. Mice lacking PCSK9 genes (pcsk9 -/-) shows intact surface VLDL-R expression and enhanced uptake of triglycerides resulting in adipocyte hypertrophy[66]. It would be further interesting to see the effect of some LOF variants of PCSK9 role in fat metabolism. Human PCSK9 p.R46L is a strong loss of function variant and decreases about 53% plasma PCSK9 levels, however might be a strong indicator of further risk of central obesity, requires further attention.

Role in Small intestine

All vertebrates except some species of bovine expresses PCSK9 protein in various tissues including small intestine. PCSK9 expression in human, rodents and mice small intestine was also observed especially in the ileum region[52], and suspected for its localized physiological changes in addition to its secretory circulatory effects. Recent in vitro studies on human Intestinal epithelial cell line (parental Caco2 or cloned Caco 2/15) indicated PCSK9 role in intestinal lipid metabolism[68][69][70]. PCSK9-WT and p.D374Y treated Intestinal epithelial cell line had profound increase in apical cholesterol transporter proteins (NPC1L1, CD36 and SRB-1) and exhibited diminished LDL-R activity at the basolateral membrane of intestinal epithelial cells while restoration was observed in PCSK9 deficient cells [69]. Exogenous treatment of PCSK9 were also related with decreased activity of HMG-CoA reductase and Acyl-CoA-cholesterol transferase (ACAT) and with increased Microsomal

transfer protein (MTTP) expression. Rashid et al [70] also recently demonstrated that PCSK9 treated Caco2/15 cells also promotes intestinal overproduction of triglyceride-rich apoB lipoproteins and increased expression of lipid generating enzymes (FAS, SCD and DGAT2) and enhanced activity of MTTP protein providing stability to apoB [70]. This activity of PCSK9 might enhances the clinical risk of postprandial hypertriglyceridemia

Conclusively, these recent findings suggests that reducing PCSK9 activity results in reduction of apoB expression, less triglyceride rich lipoprotein packaging and secretion due to the decrease levels of MTTP and ACAT proteins- eventually leads to better lipid profile. PCSK9 monoclonal antibody treatment seems not only as an additive therapy to improve statin efficacy but might also reduces the risk of post-prandial hypertriglyceridemia.

Role in Pancreatic beta cells

Data from clinical phase trials did not present any significant relationship between plasma PCSK9 levels and glucose metabolism. However, pancreatic beta cells fully expresses PCSK9 and LDL-R. Recent data from PCSK9-/- mice models indicating hypoinsulinemic, hyperglycemic and glucose intolerant phenotypes with increased surface LDL-R expression[71], which might overloading the beta cells with circulating LDL and impairing in the secretion of insulin. Hypercholesterolemia treatment with PCSK9 monoclonal antibodies might require tight surveillance in a longer run.

Role in Kidney

Nephrotic syndrome patients exhibits higher serum total and LDL cholesterol levels, one factor underlying in this due to the acquired LDL-R deficiency[72]. PCSK9 activity was later suspected for the cause due to its endogenous production in kidney [52] [30]. This might be explained by the results produced from transgenic mice expressing human PCSK9

(hPCSK9) especially in the kidney, these mice displayed high plasma PCSK9 levels but with its tissue specificity for LDL-R degradation. hPCSK9 originating from kidney of transgenic mice virtually contributing in hepatic LDL-R degradation, but no change in adrenal and minimal change in its own LDL-R expressions[53]. The reason behind this multifarious role of PCSK9 is unknown, however one possibility might be due to the absence of intracellular unknown protein(s) targeting or sorting the PCSK9-LDL-R complex to lysosome in kidney and adipose tissue. This due observations can be better hypothesized from a recent study on PCSK9-mediated LDL-R downregulation in SV-589 human skin fibroblasts, where PCSK9-LDL-R complex followed the similar pathway of internalization endocytosis but presented a loose association in early endosome which leads in recycling of LDL-R[73]. However, PCSK9 endogeneous role in extra-hepatic tissues cannot be neglected and requires further scrutiny.

In humans, plasma from chronic kidney disease (CKD) patients had about 55.52% higher PCSK9 than controls[74], which further dropped at normal levels after hemodialysis and kidney transplantation. These observations suggests that extra-hepatic expression of PCSK9 also contributes in serum cholesterol levels by downregulating hepatic LDL-R, which is in line with Zaid et al finding in mice[52].

1.12 PCSK9 and Atherosclerosis mice models

PCSK9 role in hypercholesterolemia is very well documented, and this might explained more strongly due to hepatic PCSK9 contributions. On the other hand, studies on transgenic mice models suggest that PCSK9 gene deletion from mice is not lethal to the mice and present normal reproductive ability, they further shows reduction in total plasma cholesterol levels (-48%), HDL (-30%) with no change in plasma and hepatic triglycerides and hepatic cholesterol levels than WT mice[75]. Conversely transgenic mice overexpressing PCSK9 exhibits about 5-15 folds higher circulating LDL-c[67].

Adenoviral-mediated expression of human PCSK9 (hPCSK9) in mice results in an intermediate LDL-R-knockout phenotype [76]. Parabiosis experiments on mice with and without PCSK9 exhibited that PCSK9 act mostly in a paracrine/endocrine fashion [54].

Mice cholesterol transportation system is somehow different from humans, largely carried onto HDL, thus reduction in HDL levels in PCSK9-KO mice is due to the effect of highly expressed LDL-R mediated clearance of ApoE containing particles.

PCSK9 also likely to bound with circulating lipoproteins in both WT and LDL-R-KO mice models[77], PCSK9 binding with LDL-c was also seen in human normolipidemic subjects[78].

Interestingly, since PCSK9 predominantly secreted by hepatocytes, Liver specific conditional PCSK9-KO mice was generated and studied for the lipid metabolism, these transgenic mice exhibited about 35% less reduction in total plasma cholesterol levels than complete PCSK9-KO mice[67], grounding for the role of extra-hepatic PCSK9.

1.13. PCSK9 role in cardiovascular diseases (CVDs)

Plasma PCSK9 levels as a risk factor for future stroke and other CVDs were also assessed in few studies, high levels of plasma PCSK9 was found positively associated with increased intima-media thickness of carotid artery (cIMT) in normotensive subjects[79] and also in hypertensive cohort after adjusting co-variables in multivariable regression analysis[80] -. A recent study on relationship of higher plasma PCSK9 levels in patients with stable coronary artery disease was observed and further found associated with increased cardiovascular events (CVE), hypertension, total cholesterol, fasting triglycerides, hypolipidemics drug (Statins), HbA1c, Insulin and C-reactive protein (CRP), however no association was found with LDLc or HDL- cholesterol. To be noted, the association between plasma PCSK9 and risk of CVE was reduced in a model adjusting for fasting TGs[81]

SECTION [B2]. G-PROTEIN COUPLED ESTROGEN RECEPTOR (GPER) ROLE IN CARDIOVASCULAR SYSTEM

1.14. ESTROGENs (Introduction and Biosynthesis)

Estrogens are class of endocrine hormones, secreted from ovaries and in lesser amounts by the adrenal cortex, placenta and male testes, however often referred as a female sex hormone, a small amount of estrogens are also found in men. The term "estrogens" refers to all of the chemically similar hormones like estrone (also called "E1" - most abundant after Menopause), estradiol (also called "E2"- primarily in women's reproductive age) and estriol (also called "E3"-predominant during pregnancy)- [**Figure 10**]. Estrogens are important for sexual and reproduction, mammary gland development, bone turnover, metabolism, in addition estrogen role in cardiovascular function is also emerging. As from recent reports, endogenous estrogens in pre-menopausal women largely prevent the development of coronary artery disease (CAD), peripheral artery disease (PAD),Myocardium infarction (MI) and Stroke, partly due to the beneficial effects on blood pressure, lipid metabolism and glucose homeostasis while these benefits reverses in post-menopausal state and in experimental overiectomized animal models [82][83][84][85][86][87]. Indeed about 95% of women develop cardiovascular disease after menopause when endogenous estrogens are at their lowest levels[88][89][90]. Thus ovarian dysfunction (hypogonadism), polycystic ovarian syndrome or surgical menopause before natural menopause or even in overiectomized female mice (those mimic the similar menopause), all these conditions have been recognized as a major risk factor for accelerated atherosclerotic vascular damage. Similarly, genetic mutations in estrogen receptors also leads the similar pathophysiological characteristics as represents by estrogen impairment. According to current data, postmenopausal women numbers is expected to rise by 1 billion worldwide within the next 40 years, and might impose a burden on women's cardiovascular health[91]**.** In case of estrogen deficiency, natural and synthetic estrogen preparations may be prescribed. Estrogen also a component of many oral contraceptives.

Biosynthesis of Estrogens :-

Luteinizing hormone (LH) signaling is the hallmark of estrogen synthesis in ovaries, In females, synthesis of estrogens starts in “theca interna” cells on the ovary, by the synthesis of androstenedione from cholesterol. Androstenedione is a substance of weak androgenic activity which serves predominantly as a precursor for the more potent androgens such as testosterone as well as estrogen. This compound crosses the “basal membrane” into the surrounding granulosa cells, where it is converted either immediately into estrone or into testosterone and then estradiol in an additional step. The conversion of androstenedione to testosterone is catalyzed by 17β-hydroxysteroid dehydrogenase (17 β-HSD), whereas the conversion of androstenedione and testosterone into estrone and estradiol, respectively is catalyzed by aromatase enzyme which are both expressed in granulose cells.

Estrogen levels vary throughout the menstrual cycle, with highest at ovulatory phase.

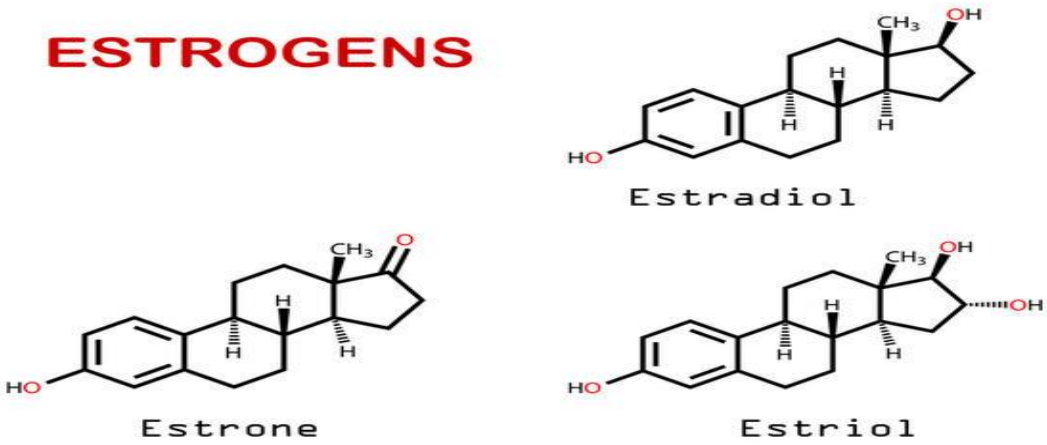


Figure 10. Chemical structure of Estrogens
This figure depict the three major forms of physiological Estrogens.
[Figure adapted from https://www.kingsrxandwellness.com/know-your-estrogens-and-your-body]

1.15. Molecular mechanisms of Estrogen mediated cellular signalling (ERα, ERβ- Classical pathway and non-classical pathway)

Once released from ovaries, estrogen like all steroid hormones, readily diffuses across cell membrane and binds and activate estrogen receptors (ERs) which in turn modulates the expression of many genes. Estrogen binding to its receptors generally fall into two broad categories, genomic or classical pathway and non-genomic or non-classical pathways. Classical pathways includes the functions or activity of two cytoplasmic estrogen receptors viz ERα and ERβ and they act as a ligand-activated transcription factors that reside in the cytosol and translocate into the nucleus upon ligand binding., while G-protein coupled estrogen receptor (GPER 30) which is a membrane receptor located onto the endoplasmic reticulum and mediates rapid estrogen signalling constitute non-genomic pathway.

(a) Classical pathway and cardiovascular system:-

The ERα and ERβ receptors constitutes estrogen mediated classical or genomic pathway. Estrogen binding to ERα and ERβ results in their translocation into the nucleus, where they act as a transcription factors leading to altered gene expressions. ERα and ERβ shares extensive structure homology, DNA binding domain of the receptors (ERα and ERβ) are the most identical (about 96-97% homologous) and ligand binding domain (LBD) sequence also constitutes about 53%- 60% homology between the two's[92]. However, a structure difference is utmost exist between ERα and ERβ in the amino terminal transcription control domain, AF-1 through which regulatory binding partners interacts [**Figure 11**].

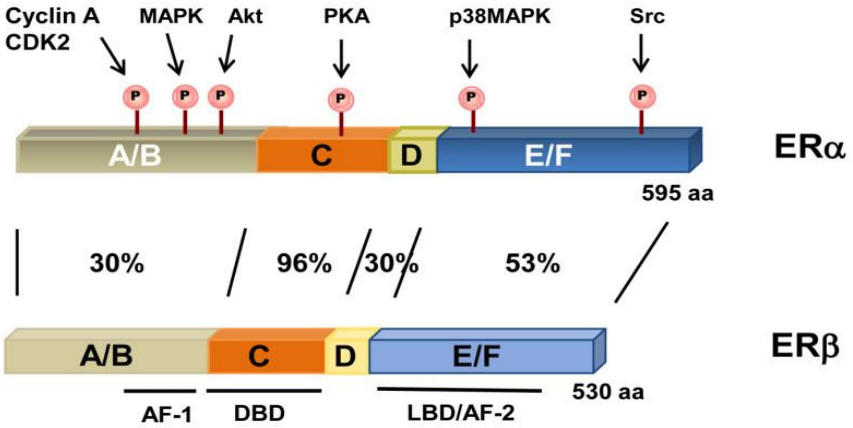


Figure 11. Structural homology of estrogen receptor ERα and ERβ.
ER-α and ER-β shares a structural homology as depicted in this figure and can go homodimerization or heterodimeration upon activation.[Figure adapted from Roman-Blas et al. Arthritis research & therapy 2009. 11:241].

ERα and ERβ carries four functional domains- A DNA binding domain (DBD), a ligand binding domain (LBD) and two transcriptional activation functional domain (AF-1 and AF-2). The percentage of sharing homology between twos, sites of phosphorylation are given in [**Figure 11**]

ERα and ERβ targeted by estrogen and mediates several estrogen responsive element (ERE) containing genes expression, estrogen binding to ERs initiates at LBD/AF-2 domain results in ERs conformational change leading to ERs dimerization and translocation to the nucleus where binding to consensus estrogen response element (ERE) sites to the DNA begins to modulates the ERE-containing genes[93]. The consensus ERE sequence is 5' GGTCAnnnTGACC 3'.

ERs also binds to non-ERE carrying genes, ERs binding to transcription factors like AP1 and Sp1 mediates ERs binding to DNA, which is commonly known as a "transcriptional cross talk" mechanism. ERs can also function in a ligand-independent manner, the phosphorylation of ER can facilitate ERs binding to ERE in the absence of estrogen[94].

Both ERα and ERβ regulates distinct genes expressions in a tissue dependent manner [95][96][97][98] largely relies on the differences in co-activators and corepressors in different tissues and different levels of the ERα and ERβ.

In addition to their differ activity in the tissue dependent manner, both ERα and ERβ can differentially behave in the same tissue too[98], in vascular smooth muscle cells, ERβ enhances nitric oxide synthase gene expression while it is repressed by ERα[99]. There could also be a time-dependent difference in gene expression (few hours until 1 week), as suggested by many studies. In vascular tissue, estrogen recruits in a temporal manner specific transcription factors that propagate distinct estrogen signalling[100][101]. These ERα mediated versus ERβ-mediated differences in gene regulation are likely attributable to differential recruitment of corepressor and coactivators. A candidate gene approach studies revealed that many estrogen mediated gene expressions (directly or indirectly) involved in cardio-protection [102][103], including mitochondrial complex IV[104], GLUT-4[105], Connexin 43[106][107], PPAR-gamma1α[108], adenine nucleotide translocator[109], Heat shock proteins[110].

However there are also studies suggesting for gender difference and in pre vs post-menopausal state[111]. In cardiac myocytes, ERβ is often expresses in a similar fashion irrespective to gender, while ERα shows gender specificity[112]. Different stages if menstrual cycle in pre-menopausal state also constitute different receptor expressions, ERα expression in vascular endothelial cells has been found 30% less during early follicular phase when compared with late follicular phase[113]

(b) Non-Classical pathway and cardiovascular system:-

GPER (alias GPR30) or G-protein coupled estrogen receptor is a seven transmembrane-domain G-protein coupled receptor (GPCR), was first cloned at Lund University during mid 1990s and nominated as “orphan receptor” due to unidentified ligand[114], however for the first time GPR30 de-orphanized in 2005[115], and officially designated as GPER by the “International Union of Pharmacology” in 2007[116]. With certain contradictory reports[117][118][119] GPER still remained an significant contributors of estrogen mediated signalling. In the year 2000, Filardo et al. demonstrated MAP kinase (ERK1/2) activation by estrogen in breast cancer cell lines expressing GPR30 but not in cell

lines lacking GPR30[120]. Subsequent studies revealed a second phase of GPR30-dependent signalling via adenylyl cyclase that resulted in the eventual attenuation of ERK activation[121]. During initial years, the localization of GPER was controversial, Thomas and colleagues identified GPER as a plasma membrane bound G-protein receptor[122], while Revankar et al identified GPER in the endoplasmic reticulum of COS7 monkey kidney cells[115]. GPER expression is found in almost all cell types including endothelial cells, vascular smooth muscle cells (VSMCs)[123], Nervous system[124], Reproductive system[125] and in immune cells[126]-[**Figure 12**].

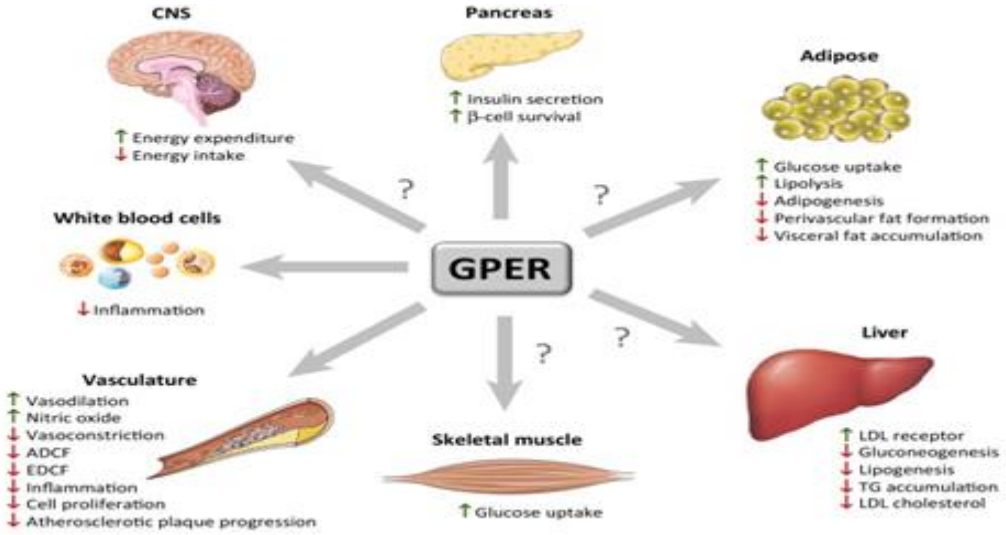


Figure 12. Different sites expression of GPER

GPER protein expresses in several tissues types and triggered signal cascading upon ligand binding.[Figure adapted from Barton. M et al, Trends in endocrinology & metabolism. 2015; Vol 26; issue 4; 185-192].

1.16. G-protein coupled estrogen receptor (GPER) signalling

The G-protein coupled estrogen receptor is a seven transmembrane receptor located onto the endoplasmic membrane and triggers rapid and transient activation of numerous intracellular signalling upon ligand binding. GPER mediated signalling has attracted increasing interest in estrogen biology. GPER like other GPCRs, are coupled to heterotrimeric G-proteins (α,β and γ subunits) that in turns regulate a plethora of downstream signal cascading[127]. As observed for the GPCR mediated responses, the activation of epidermal growth factor receptor (EGFR) is a fundamental integration point in the biological action triggered by GPER, which in-turn leads to the activation of extracellular signal-regulated kinase ½ (ERK1/2) and phosphoinositide 3-kinase (PI3K)[115][120][128] and regulates calcium mobilization[129][130][115] and potassium channels[131]. The activated GPER and its downstream signalling pathways mediated pleotropic functions among others in the cardiovascular, reproductive, central nervous system, endocrine, and immune systems.

Therefore, GPER is likely to play significant roles in mediating many estrogen-associated role in body. The role of GPER deficiency revealed from mice model studies, where GPER-KO mice exhibited advanced thymic atrophy[132], visceral obesity[129], insulin resistance, dyslipidemia[133][134], vasoconstriction and increased vascular smooth muscle cell growth[129]-[**Figure 13**].

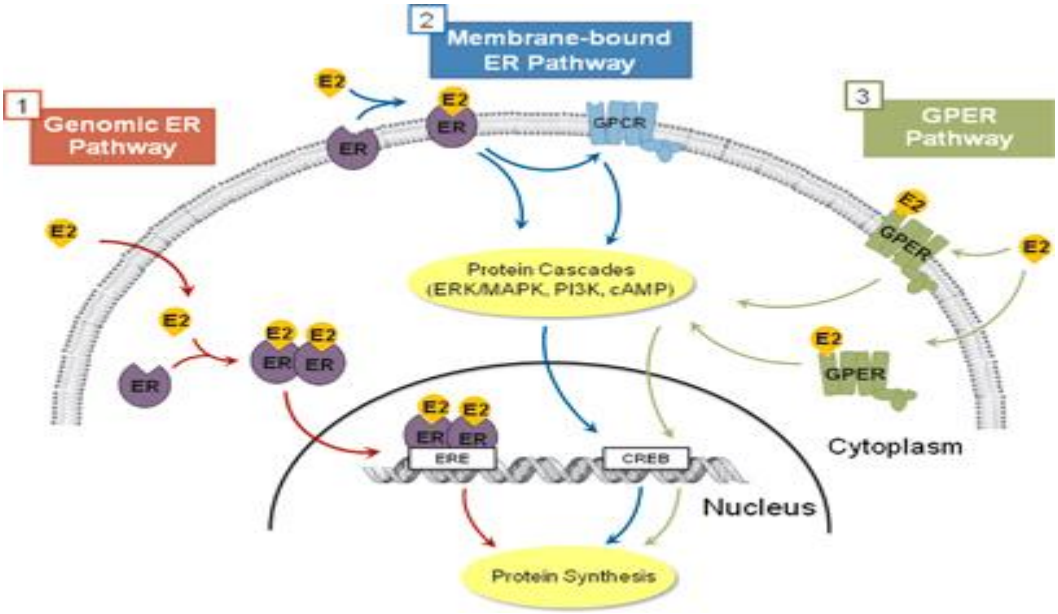


Figure 13. Classical and non-classical signalling pathway

Estrogen binding to receptors of (1) classical or genomic pathway located in the cytoplasm and nucleus, activated Estrogen receptors binds to the estrogen response element (ERE) of the genes and initiate their transcription.(2) Estrogen also activate membrane bound ER-pathway leads to activate intracellular cascading and neighbouring GPCRs (G-protein coupled receptors) and promotes CREB-modulated protein transcription. (3) The non-genomic or non-classical pathway majorly defines for GPER pathway resulting in gene transcription. [Figure adopted from Cover KK et al; translational Psychiatry.2014 August e.422].

1.17. GPER activation in vasculature and heart or Cardiovascular system

Results from several studies indicates for the GPER expression exist in the cardiovascular system-A system which was long suspected for the estrogen mediated beneficial effect in women in their premenopausal phase, but with unclear mechanism. GPER expression in cardiovascular system includes human mammary artery and saphenous vein[123]---, human endothelial cells[135]**,** mouse mesenteric resistance arteries[136] and bovine pericytes [137]**.** In mice, a large proportion of cardiovascular GPER expresses in small arterial vessels, while in CNS, the receptor is expressed primarily in pericytes and smooth muscle cells[138]

GPER activity has been recently studied in vasculature by using mice models of GPER-KO, or GPER agonist (G1) and antagonist (G15). G1 Is most potent and commonly used agonist in routine lab research, which provide a similar affinity and specificity to the

receptor as does by 17β-estradiol (E2) to the GPER[139] G1 treated mice display vasodilation and blunting of endothelin-1-mediated vasoconstriction[140] of epicardial coronary arteries, and vasodilation and decreased oxidative stress in carotid arteries[141],and intracellular calcium in human aortic VSMC exposed to serotonin. A study by Martensson et al demonstrated that 9 month old GPER-KO mice exhibits increased mean arterial blood pressure (MAP); this is associated with the increased media to lumen ratio of second-order mesenteric resistance arteries[136]. Hass et al on the other hand, showed that G1 lowers MAP and dilates pre-constricted mesenteric aretries in rats, and that GPER deletion abolishes G1 dilation of mouse carotid artery rings, pointing to possible direct vascular role of GPER[129]**,** this same group also observed that G1 inhibits proliferation of human umbilical vein smooth muscle cells[129] consistent with a growth-inhibitory effect via GPER in some cell types[142][143][144], but a growth promoting effect in others[145]**.**

1.18. GPER activity on lipid metabolism, Atherosclerosis and Inflammation

Estrogen brings several beneficial effect in early life of females, while post-menopausal phase loses estrogen benefits, similarly overiectomized mice mimics the similar clinical outputs. Several studies are undergoing to know the biological mechanism behind the estrogen mediated effects, a recent discovered estrogen mediated GPER pathway is now exploring by scientific community. GPER-KO female mice presents impaired glucose tolerance, increased blood pressure, decreased HDL and increased LDL and triglycerides levels [133] [136], increased activity of the vasoconstrictor endothelin-1 [130] and the increased adipose-derived contracting factor (ADCF) and excessive formation of perivascular adipose [146] –all these GPER deficient outcomes are the major risk factor associated with coronary atherosclerosis and is a clinical phenotypes of patients with obesity, insulin resistance or diabetes, these female mice also showed a symptoms of hepatic steatosis[147]. Similarly GPER-KO mice under high fat diet also develop atherosclerotic plaques with increased plasma LDL levels in both ovary-intact and surgically postmenopausal mice[148]. Conversely, activation of GPER inhibits the proliferation of

human vascular smooth muscle cells in vitro-an essential protective phenomenon while atherosclerosis [129][149], and reduced vascular inflammation involving reduced macrophage and T-cell dependent mechanisms [148], treatment with highly selective GPER agonist (G1) was effective in reducing atherosclerosis in postmenopausal mice[148], this protection was also seen in mice lacking ERα [134], suggesting a powerful contribution of GPER in cardiovascular system and a promising therapeutic target.

1.19. Genetic study of GPER and role in blood pressure & cholesterol homeostasis

Studies of GPER deficiency in human is limited, a recent report on the regulation of LDL-c due to the hypofunctional variant (rs1154331; p.P16L) on GPER gene showed higher circulating plasma cholesterol levels and high blood pressure in women[150][151]*;* furthermore, experiments performed on HepG2 cells, demonstrated an inhibitory role for GPER variant in the regulation of LDL receptor expression[150]. In agreement to the results from GPER-KO mice models and the information from GPER variant (rs1154331) studies indicates the beneficial role of GPER. In this thesis, we will further see the role of GPER rs11544331 variants role in PLIC subjects.

SECTION [B3] IDOL (INDUCIBLE DEGRADER OF LDL-R) ROLE IN LDL-R MODULATION

1.20. Introduction and biological structure of IDOL

The IDOL gene originally cloned as MYLIP (myosin regulatory light chain interacting protein][152] encodes 445 amino acid protein. Structurally IDOL carries two distinct domains: an N-terminal FERM (4.1, Ezrin, radixin and Moesin domain) and C-terminal Ring domain (Really interesting new gene)- [**Figure 14**]. This makes IDOL a unique protein as IDOL is the only protein in the human genome that carries both FERM and RING domain in its structure. The FERM domain is the member of the ERM (Ezrin, radixin and Moesin) family of proteins contributing in interaction between membranes and membrane proteins[153]**.**

The RING domain of IDOL shares structure homology as found in other RING-containing E3-ubiquitin ligases[154]**.**

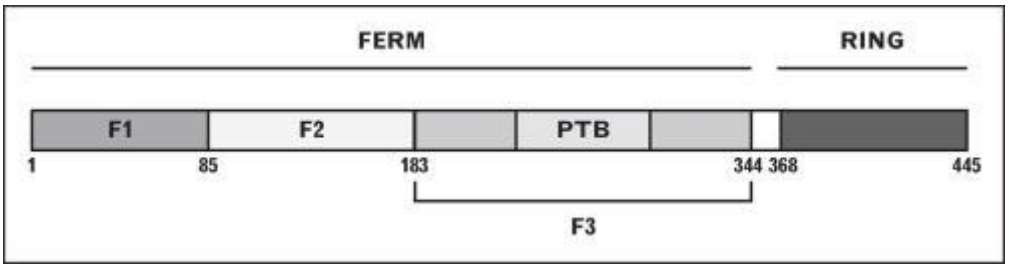


Figure 14. Linear structure of IDOL- protein
The protein carries N-terminal FERM domain and C-terminal RING domain, both domains are separated by a short linker region (from 344 aa uptil 368aa).[Figure adopted from Sorrentino et al. Current opinion in lipidology. June 2012, p 213-219].

1.21. Molecular mechanism of IDOL mediated degradation of LDL-R

The expression of IDOL protein is mediated via activation of Liver –X receptor (LXR). The LXRs are nuclear receptors and their activation is largely relies on the intracellular cholesterol levels, higher intracellular cholesterol trafficking forms oxysterol- which act as a ligands for LXRs [155]and their activation leads the expression of genes encoding cholesterol efflux from cells (like ABCA1 and ABCG1)[156][157], later LXRs was found in regulating LDL-R activity via IDOL protein which ubiquitinises the receptor at cytoplasmic region and direct it to intracellular degradation, hence influences on the cellular uptake of cholesterol **[Figure 15].** Overexpression of IDOL dramatically enhance LDL-R ubiquitination and eventually its degradation, while cells (embryonic stem cells) lacking IDOL expressions had more profound LDL-R levels[158]**.**

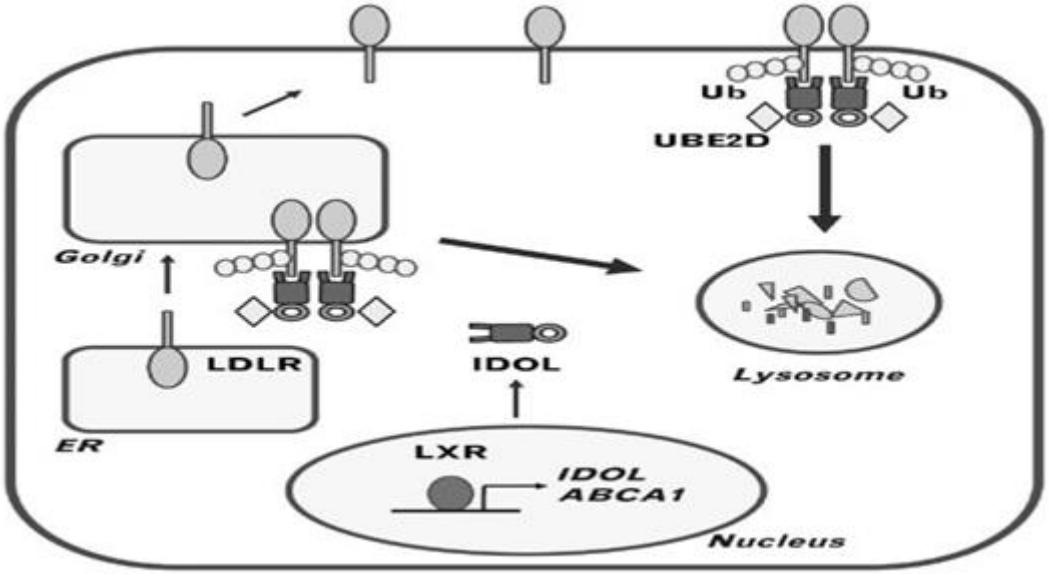


Figure 15. Molecular mechanism of Inducible degrader of LDLR (IDOL) protein
IDOL mediated intracellular ubiquitylation of LDL-R leads to lysosomal degradation.
[Figure adapted from Sorrentino.V et al, Current opinion in lipidology, vol 23(3), June 2012,p213-219].

1.22. GWAS and Population based study on IDOL

Until recently, the genetic mutation profiling of IDOL gene and their impact on cholesterol level was unknown. In last few years, genome wide association studies (GWAS) were conducted and have demonstrated various functional point mutations in the region of IDOL gene- several of these point mutations were linked with total cholesterol and LDL cholesterol levels in different population[159][160]**.** One of the recent identified SNP of IDOL gene which was found associated with high cholesterol levels is rs9370867 (p.N342S). This SNP was found in the coding region and allows a non-synonymous change at position 342aa of the FERM domain in protein chain, the FERM region is essential in order to interact with cytoplasmic tail of LDL-R. The enhanced functionality of GWAS identified SNP was further validated in Mexican cohort too[9]. In this thesis, we will further see the role of IDOL N342S in PLIC subjects.

CHAPTER 2. AIMs OF THE STUDY

In mice

(i) To understand the role of PCSK9 protein in carotid atherosclerosis development by using "Carotid artery injury model"

(ii) PCSK9 role in glucose homeostasis

In Humans

(i) Genetic variant study of LDL-R modulators (PCSK9, IDOL and GPER) and their effect size on lipid variability, glucose homeostasis and atherosclerosis progression.

(ii) Determination of plasma PCSK9 levels in PLIC subjects and association studies.

(iii) Ecstatic effects of genes over plasma PCSK9 levels.

CHAPTER 3. MATERIALS and METHODS

SECTION [A]- STUDIES IN MICE

SECTION [A1] CAROTID PROGRESSION STUDY IN MICE

3.1. Genetically modified PCSK9 mice model

All animal care and experimental procedures were in accordance with the institutional guidelines for animal research.

Mice carrying the PCSK9-wild type and null allele were confirmed by PCR of tail DNA. Animals used in our studies were age-matched (littermates), WT (PCSK9+/+) and PCSK9 (-/-) male offspring produced by 4 consecutive backcross onto a C57BL6/J strain background. Male mice were preferred for the experimental studies to reduce the confounding effect of female predominant hormones. We use a range of 22-30 grams body weight, and all mice were kept in normal chow diet and Diet induced obesity (DIO) diet for specific experiments.

Mice were housed under controlled light (12 hours light/12 hours' dark) and temperature conditions, and had free access to food and water.

3.2. Aseptic and sterile practices

As per institutional guidelines, all surgeries were performed under sterile condition in a germ-free area of surgical room in animal house facility. All sterile instruments, pre-disinfected tables and solutions and a sterile surgical field and surgical attire (like, scrubs, gloves, surgical gown, head and hair covering cap, facemask, shoe cover etc) have been used for surgery.

3.3. DNA isolation, primer sequences and Genotyping

DNA isolation were performed from tail of WT and PCSK9-KO mice, a small piece of about 2-3mm above from the tail tip were cut and placed in 1.5ml Eppendorf tube and allow to digest with 0.5ml DNA digestion buffer (50mM Tris Hcl pH 8.0; 4mM EDTA pH 8.0; 0.2M Nacl and 0.5% SDS), with proteinase K (of 10mg/ml) and placed the tubes for overnight incubation at 56°C. next day, tubes were vortex and centrifuged at 13,000rpm for 10 min, supernant were recovered and 0.8ml neutralised phenol/chloroform/iso-amyl alcohol (25:24:1) were added under the laminar hood, tubes were vortexed for a while and spin at 13000rpm for 10 minutes, and the top white layer were collected in fresh 1.5ml Eppendorf tube. 0.8ml of 95% ethanol were added for DNA precipitation at room temperature, a final centrifugation at 13,000rpm for 10 minutes were given and supernant were discarded. The pellet were allowed to dry at room temperature for about 3-4 hours. At the end, 0.1ml of de-ionized sterilised water were added to elute the DNA followed by 5 min incubation in water bath at 56°C.

PCR were performed for PCSK9 gene to confirm the genotypes of the WT and PCSK9-KO mice. PCSK9 primer details are as follow- (P1- 5' GAT TGG GAA GAC AAT AGC AGG CAT GC 3'; P2-5' ATT GTT GGA GGG AGA AGT ACA GGG GT 3' & P3- 5' GGG CGA GCA TCA GCT CTT CAT AAT CT 3'). WT (653 bp) WT/K0 (653 bp and 320bp) and PCSK9KO (320 bp).

3.4. Lipid measurement by FPLC

Blood was collected from mice fasted for 4 h. Total cholesterol levels in plasma or lipoproteins were assayed by enzymatic methods (Horiba ABX Pentra). Lipoprotein profiles were analysed by fast protein liquid chromatography (FPLC- BIORAD- NGC chromatography system). Pooled mouse plasma (200 μl) from six mice was loaded onto tandem Superose 6 columns *(Pharmacia LKB Biotechnology, Piscataway, NJ*) and eluted with lipoprotein separation buffer (154 mM NaCl, 1 mM EDTA and 0.02% NaN_3) as described[161].

3.5. Serum cholesterol assay

Blood samples were taken under general anaesthesia by retro-orbital puncture at the time of collar surgery and at sacrifice. Serum total cholesterol levels were measured by an enzymatic colorimetric assay (Horiba ABX Pentra).

3.6. Perivascular Manipulation- Collar placement in right carotid artery

Perivascular manipulation were done by placing collar on the right carotid artery of 22-30gm of WT and PCSK9-KO mice, Left carotid use as a "SHAM" or controls, however left carotid artery presents a longer section for intervention and is a choice of control.

Male C57BL/6 mice and PCSK9−/− genotypes (16-week-old, Charles River Laboratories) were used in accordance with the "Guide for the Care and Use of Laboratory animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The schematic diagram of the study design is presented in **Figure 17**. Mice (n=16 C57BL/6 and 16 PCSK9−/−) were maintained on a normal chow diet (Mucedola, Milan, Italy) for 9 weeks post-surgery. Food and water were provided ad libitum.

Briefly, after reaching at 4 weeks age, mice were anaesthetized by the intraperitoneal injection of avertin (*12µg/gr body weight of 2.5% solution*- mother stock:- 1gm of tribromoethanol + 1ml iso-amyl alcohol; working stock:- 1:40 dilution of stock(eg 300µl stock + 11.7ml water)) and subjected to perivascular carotid collar placement. Collars (length: 3 mm, internal diameter: 0.38 mm, external diameter: 2.2 mm) were made from Tygon® tubing and were positioned around the right carotid artery. The contralateral left carotid artery was sham-operated to serve as intra-animal control. Following carotid injury, the animals were maintained on their normal chow diet for 9 weeks, after which they were sacrificed with an overdose of Avertin solution to harvest tissue specimens. Body weights

were recorded at the time of collar surgery and at sacrifice. Weight increases were similar among the groups (data not shown).

3.7. Histological examination

After 9 weeks on chow diet post-surgery, mice were anaesthetised intraperitoneally by avertin, Tissue samples collected at sacrifice (the collared segment of the right carotid artery and the corresponding segment of the contralateral sham-operated artery), both sections were fixed overnight in 4% paraformaldehyde at 4˚C, tissues were embedded in paraffin blocks as per laboratory protocol, and sections were cut transversally into 5µm size in Microtome. Every 200 m ≈, along the entire length of the tissue specimen (not including portions outside – proximal or distal – of the collar), a cross-section was stained with hematoxylin and eosin (H and E). Images of H and E-stained sections were obtained with a Zeiss Microscope mounted with a digital camera (Nikon Coolpix990) and morphometrically analyzed by using the OPTIMAS 6.2 image software (Media Cybernetics, Silverspring, MD, USA). The cross-sectional areas of the intima and the media were measured directly; the intima/media area ratio (I/M) was determined by dividing the intimal area by the medial area. The perimeters of the lumen and the external elastic lamina were recorded and used to derive the lumen area and the area surrounded by the external elastic lamina (EEL), respectively, using the formula $\text{perimeter}^2\ (4\pi)^{-1}$, to normalize for artefacts due to fixation or other steps of sample processing, including occasional deformation and potential tissue shrinkage; for both parameters, calculated values were higher compared to the original measurements (particularly in the case of the thin-walled sham-operated carotids), but were significantly correlated (Pearson's $r = 0.712$ for lumen area and 0.701 for EEL area; $P < 0.0001$ for both correlations). The extent of vascular remodeling was expressed as the remodeling index (RI), calculated as the ratio between the EEL area of collared carotid arteries and that of the corresponding sham-operated arteries, i.e., RI = EEL area collar/EEL area sham. Linear regression analysis using intimal area as the predictor (independent variable) and EEL area as the dependent variable was performed for all specimens from collared carotid arteries.

3.8. Reagents and Antibodies

DMEM, trypsin EDTA, penicillin, streptomycin, nonessential amino acid solution, FBS were from Euroclone (Pero, Milan, Italy). Plates and petri dishes were from Corning (Oneonta, New York, USA). PDGF-BB were from SIGMA. Molecular weight protein standards were from BIO-RAD Laboratories (Hercules, CA). SDS, TEMED, ammonium persulfate, glycine, and acrylamide solution (30%T, 2.6%C) were obtained from BIO-RAD Laboratories. BCA assay for determination of protein concentrations was purchased from Thermo Fischer Scientific (Waltham, MA). For Western Blot (WB) analysis, the following antibodies were used: anti-PCSK9 (Cayman, Tallinn, Estonia), anti-α-tubulin (SIGMA-Aldrich), anti-LDLR, anti-PDGFR, anti-LRP1 (all from Abcam, Cambridge, UK), and secondary IRDye800 Goat anti-mouse and anti rabbit (Carlo Erba reagents, Cornaredo Milan, Italy).

3.9. Immunostaining

Immunostaining with cell type specific primary antibodies and Picrosirius red stain were performed on serial sections adjacent to those selected for morphometry. To detect -smooth muscle actin (-SMA)-positive vascular SMCs, sections were incubated with an anti--SMA mouse monoclonal antibody (1:200, Sigma, clone 1A4) followed by the MOM kit (Vector Labs) to minimize nonspecific staining, and extrAvidin FITC (1:100, Sigma). For detection of monocytes/macrophages and polymorphonuclear leukocytes, sections were blocked for endogenous peroxidase activity and for non-specific staining and incubated with Mac3 rat mAb (1:10; PharMingen) or MCA771GA rat mAb (1:200, Serotec), respectively; sections were then incubated with an avidin-biotin-peroxidase kit (Vectastain ABC Elite, Vector) and visualized with 3,3'-diaminobenzidine (Sigma), followed by counterstaining with 10% Mayer's hematoxylin. To detect intimal collagen, sections were stained with Picrosirius red (Direct red 80; Sigma) and analyzed under white light. Positive areas were

determined by computer assisted image analysis (OPTIMAS 6.2), and related to total intimal surface area.

3.10. Real time Primers (mice)

SREBP-1a-fwd (5'-TAG TCC GAA GCC GGG TGG GCG CCG GCG CCA T-3'), SREBP-1a-rev (5'-GAT GTC GTT CAA AAC CGC TGT GTG TCC AGT TC-3'), IRS1- Fwd (5' CCA GCC TGG CTA TTT AGC TG – 3'), IRS1- rev (5' CCC AAC TCA ACT CCA CCA CT-3'), IRS 2- Fwd(5' GTA GTT CAG GTC GCC TCT GC-3'), IRS-2 rev (5' CAG CTA TTG GGA CCA CCA CT-3'), GLUT4 Fwd(5' CAT GGC TGT CGC TGG TTT CT-3'), GLUT4 rev(5' GCA TCC GCA ACA TAC TGG AA-3'), RESISTIN Fwd (5' CTT TCA TTT CCC CTC CTT TTC CTT-3'), RESISTIN rev(5' AGT CTT GTT TGA TCT TCT TGT C-3').

3.11. Tissue Explants and Processing

After 9 weeks on chow diet post-surgery, mice were anaesthetised intraperitoneally by avertin (12 μl/gr body weight of 2.5% solution), Tissue samples collected at sacrifice (the collared segment of the right carotid artery and the corresponding segment of the contralateral sham-operated artery), both sections were fixed overnight in 4% paraformaldehyde at 4˚C, tissues were embedded in paraffin blocks as per laboratory protocol, and sections were cut transversally into 5μm size in Microtome for morphometric examinations.

3.12. VSMC isolation, proliferation and migration assay

Aorta from WT and PCSK9-KO mice operated collar surgery were explanted and cultured to grow VSMC in DMEM high glucose medium with glutamine and antibiotics (Penicilin and Streptomycin) and 10% FBS. 6-14 passages primary cells were used for studying the VSMCs phenotypic characteristics. Proliferation assay were performed by seeding 10,000 cells in each well of slide provided by company and cells were counted in iCelligence machine (ACEA Biosciences Inc, San Diego CA, USA). VSMCs migration assay were performed in Boyden chamber and using polycarbonate membrane [Biomap, Milan, Italy]. The polycarbonate membrane coated with a 0.1mg/ml of type-I collagen solution (Purecol, Nutacon BV, Leimuiden, The Netheroland) in 0.1M acetic acid at 37°C, and lower compartment of Boyden chamber were filled with DMEM media in the absence or presence of PDGF-BB and upper compartment were used for VSMCs suspension of PCSK9-WT and KO (10^6 cells/ml). The chamber was incubated at 37°C for 6 hours, membrane was stained with Diff-Quik staining set (Biomap, Milan, Italy) and transmigrated cells were counted in four random high-power field (HPFs) under high magnification (20X).

3.13. Western blot analysis

The cultured VSMCs were washed twice with sterilized PBS and lysed with lysis buffer (50mM Tris pH 7.5, 150mM Nacl, 0.5% Nonidet-P40, containing a protease and phosphatase inhibitor cocktails (Sigma Aldrich, Milan, Italy)). The cells were collected in 1.5mL Eppendorf tube and sonicated. Tubes were placed in water bath for 5 minutes and samples were loaded onto polyacrylamide gene for protein separation, the separated protein from gel were transferred to nitrocellulose membrane (GE- Healthcare little Chalfont, Buckinghamshire, UK) and subsequently immunoblotted by primary antibody followed by secondary fluorescently labelled antibody and bands were acquired with the Odissey FC system (LI-COR).

SECTION [A2] TO STUDY PCSK9 ROLE ON GLUCOSE HOMEOSTASIS:-

3.14. Glucose tolerance test (GTT)

Our initial goal to know the PCSK9 role in obesity and insulin resistance, for that purpose, mice were stressed by DIO diet (diet induced obesity) to get the obese and insulin resistance phenotype in both WT and PCSK9-KO mice. 2 months old mice of both the groups were kept on DIO diet for 12th and 20th week. At the end of 12th week and 20th week post-inception- Glucose tolerance test (GTT) were performed by intraperitoneal injection of glucose. The procedure was followed as given below:-

Mice were kept in overnight fast of about 12-16 hours, only water were supplied before GTT. Next day each mice weight were measured and recorded, 20% glucose solution in sterile PBS were prepared (2g of glucose/Kg body mass) for intraperitoneal (IP) injection [volume of IP glucose injection (μl) = 10 x body weight (gm)]. Blood (≈5μl) from mice tail were collected at time 0 (pre-glucose injection) and 15, 30, 60, 90 and 120 minutes (post glucose injection) and placed on the glucose test strip of the blood glucose and reading were recorded. At the end of experiment, mice were kept back on fresh cages with the food and water.

3.15. Insulin tolerance test (ITT)

Mice were kept overnight fast, and weight were recorded prior to insulin injection. The 1mU/10μl solution were prepared from stock of 100 IU insulin solution in sterile PBS and injected 30mU insulin in mice. Blood glucose were measured as stated before at time 0 (t0- prior to insulin injection), and 15, 30, 60, 90 and 120 minutes (post glucose injection) and placed on the glucose test strip of the blood glucose meter (Accuchek Inc.) and reading were recorded. At the end of experiment, mice were kept back on fresh cages with the food and water.

3.16. Hepatic- Gene Expression study

Total RNA was extracted from PCSK9 WT and KO mice of DIO and SFD dietary group. RNA was extracted by using Nucleospin RNA columns (Macherey-Nagel GmbH & Co.). 1 µg of RNA were used to get cDNA using the IScript cDNA Synthesis kit (BioRad, Milan, Italy). 3 µL of cDNA were amplified by real-time quantitative PCR with 2X MAXIMA SYBR Green/Fluorescein qPCR mastermix (Carlo Erba Reagents, Cornaredo, Italy). The specificity of the Sybr green fluorescence was tested by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon. Each sample was analyzed in duplicate using the CFX Connect Real Time detection system (BioRad, Milan, Italy). The primers used are the following:

SREBP-1a-Fwd (5'-TAG TCC GAA GCC GGG TGG GCG CCG GCG CCA T-3'), *SREBP-1a-rev* (5'-GAT GTC GTT CAA AAC CGC TGT GTG TCC AGT TC-3'), *IRS1- Fwd* (5' CCA GCC TGG CTA TTT AGC TG – 3'), *IRS1- rev* (5' CCC AAC TCA ACT CCA CCA CT-3'), *IRS 2- Fwd* (5' GTA GTT CAG GTC GCC TCT GC-3'), *IRS-2 rev* (5' CAG CTA TTG GGA CCA CCA CT-3'), *GLUT4 Fwd* (5' CAT GGC TGT CGC TGG TTT CT-3'), *GLUT4 rev* (5' GCA TCC GCA ACA TAC TGG AA-3'), *RESISTIN Fwd* (5' CTT TCA TTT CCC CTC CTT TTC CTT-3'), *RESISTIN rev* (5' AGT CTT GTT TGA TCT TCT TGT C-3'), *Adiponectin Fwd* 5'(AAC GTC ATC TTC GGC ATG ACT 3'), *Adiponectin Rw* 5' (GCT CAG GAT GCT ACT GTT GCA A 3'), *PCSK9 Fwd* 5'-(AGG TGG AGG TGT ATC TCT TAG ATA CCA 3'), *PCSK9 Rw5'*(CGC TGT TGA AGT CGG TGA TG3').

3.17. Statistical Analysis

Data are presented as mean ± SD, statistical analysis was performed using student t-test or ANOVA when appropriate to analyse statistically significant differences between groups. For this study $P<0.05$ was considered as significant.

SECTION B. IN HUMANS:-

3.18. PLIC COHORT (Progression of lesion in intima-media of carotid artery) and selection criteria.

A cohort of 2141 subjects attending the Atherosclerosis Centre in Bassini Hospital, Department of Pharmacological and Biomolecular Sciences (University of Milan, Italy), were recruited for the PLIC study. The PLIC study was approved by the Ethics Committee of the University of Study of Milan (approved on 06-02-2001 SEFAP protocollo n°0003/2001), all participants signed a written informed consent. The investigation was performed in accordance with the principles of the Declaration of Helsinki. This project is a study designed to investigate the presence and progression of atherosclerotic lesions and intima media thickness (IMT) in the carotid artery of a large local cohort in relation to the major cardiovascular disease risk factors. Exclusion criteria were use of hypolipidemic drugs (esp in PCSK9 studies), presence of liver or kidney disease, thyroid dysfunction. A total of 1110 subjects for PCSK9, 1384 subjects for IDOL and 1405 subjects for GPER studies were selected for the study.

3.19. Blood collection, DNA isolation and Lipid profiling

Blood samples were collected after an overnight fast from all subjects enrolled in the PLIC study. Genomic DNA was extracted from buffy coat samples using the Flexigene DNA kit (Qiagen, Milan, Italy) according to the manufacturer's instructions and stored at -80°C until use. Lipid profiling (LDL, total cholesterol, HDL, Triglycerides, ApoA1, ApoB, non-HDL, remnants etc) were assessed by kit

3.20. Genotyping by allelic discrimination assay (PCSK9 R46L, IDOL N342S, and GPER P16L)

Genotyping for the PCSK9-R46L (rs11591147; c.G5428; p.R46L)), IDOL (rs9370867; c.G1025A, p.N342S) and GPER (rs11544331; c.C47T; p.P16L) was performed on 5 µL of DNA (10–200 ng of DNA), using a TaqMan allelic discrimination test (Applied Biosystem) to evaluate or validate the role of these respective SNPs in lipid profile and atherosclerosis progression.

3.21. Measurement of Carotid Intima media thickness by Ultrasonography

High resolution B-mode ultrasonography of carotid Intima-Media Thickness (c-IMT) with a linear ultrasound probe (4.0–13.0MHz frequency, 14X48 mm footprint, 38 mm field of view) was performed (Vivid S5 GE Healthcare1, Wauwatosa, WI, USA). The determination were performed by a single sonographer, blinded to the subject's identity (intra-class correlation = 0.812, n = 30). All the measurements were done off-line using the software provided by the instrument. The protocol involved the common carotid artery (CCA) (30 mm proximal to the carotid bulb), the carotid bulb and internal carotid artery (ICA) at both sides. The intima-media thickness (IMT) was assessed at the far wall as the distance between the interface of the lumen and intima, and the interface between the media and adventitia in a standardized number of points. The maximal IMT was recorded and averaged for the left and right sides of the CCA (30 mm proximal to the carotid bulb), the carotid bulb, the ICA. Presence of extra-cardiac atherosclerotic vascular involvement was determined with presence of focal plaques (> 1.3 mm in longitudinal resolution, lateral or medial angle) and/or diffusive mean IMT > 1.3 mm (in longitudinal resolution, lateral or medial angle). The protocol involved the determination of the Left Ventricular Mass (LVM) assessment. The measurements were performed by two dimensional guided M-mode echocardiography from the parasternal window using an M-mode color-Doppler (Vivid S5 GE Healthcare1, Wauwatosa, WI, USA) (1.4–4.0 MHz frequency, 19.3X27.6 mm footprint wide-band phased array transducer). Left Ventricular Mass (LVM) was calculated with Devereux's formula, according to guidelines. The definition of cardiovascular events (CVEs) included coronary heart disease (CHD), such as acute myocardial infarction (AMI), acute coronary syndromes,

acute and chronic heart failure (New York Heart Association class II and III); peripheral artery disease (PAD) and cerebrovascular events, such as stroke, transient cerebral ischemic attack in the previous 6 months, and other vascular complications (including diabetic foot ulcers). In addition having undergone major surgery was included in this definition (i.e.: carotid thromboarterectomy, percutaneous coronary angioplasty, arterial angioplasty or arterial by-pass of the lower limbs, coronary by-pass and amputations). Cardiovascular risk was defined in accordance to the Progetto Cuore individual risk score, for the Italian population as described.

3.22. Plasma PCSK9 levels measurement

Blood samples were collected after an overnight fast from all PLIC subjects and plasma was separated and stored at -80 until use. Plasma PCSK9 levels were measured by using AlphaLISA kit commercially available from PerkinElmer Company (AL270C-500 Assay points).

A lyophilized hPCSK9 standard (0.3 µg) reconstituted in 100µL of distilled water-as per company instructions. Further serial dilutions for the standard curve were made in FBS ranging from 3 x 10^5uptil 1pg/ml and were made freshly for each run. All kit components were stored in the dark at +4°C, and reconstituted analyte (hPCSK9) were stored at -20°C. 1:30 dilutions of human plasma in FBS were made to analyze the plasma PCSK9 levels, readings were taken from alphaELISA reader (Perkin Elmer-Envision 2101) for each intra- and inter-assay run.

In an AplhaLISA assay, Donor beads conjugated streptavidin binds with a biotinylated anti-analyte antibody while AlphaLISA acceptor beads conjugated with another anti-analyte antibody. In the presence of the analyte, the beads come into close proximity leads the excitation of the donor beads. Upon excitation, donor beads releases a singlet oxygen

molecules which brings a cascade of energy transfer in the acceptor beads. All readings were taken at 615nm.

3.23. Statistical Analysis

Statistical analysis of data was performed using SPSS® v.21.0 for Windows® (IBM Corporation®, Chicago Illinois, USA) program. Values are shown in mean ± SEM and statistically relevant differences were considered for p values < 0.05.

SECTION [C] EXPERIMENTS FOR *IN-VITRO* ANALYSIS

3.24. Isolation of human peripheral blood mononuclear cells (PBMC)

About 15ml blood from median cubital vein of PLIC subjects (for IDOL- GG and AA carriers ; for GPER- CC and TT carriers)were withdrawn and collected in sterile blood collection tube containing EDTA. Mixing were done immediately to avoid blood coagulation. Blood was diluted 1:3 in phosphate buffer saline (PBS) (15ml) then layered onto 4ml of Ficoll Hipaque (Amersham) and centrifuged at 1500rpm for 35 min. PBMC were removed from the interface and washed twice in PBS before being re-suspended in RPMI-1640 medium supplemented with penicillin (50U/ml), streptomycin (50µg/ml), L-glutamine (mM) and 10% FBS. PBMC were counted and $2x10^6$ cells were plated in 6 well plates and incubated for 2 hours in a humified atmosphere (37˚C, 5% CO2). Non-adherent cells were removed with four rinses of PBS and attached monocytes cultured for 7 days to obtain monocyte-derived macrophages.

3.25. LDL preparation and macrophage LDL uptake assay

LDL (density 1.019–1.063 g/ml) were obtained from freshly isolated human plasma from healthy volunteers by preparative ultracentrifugation in KBr gradient, dialyzed versus PBS containing 0.01% EDTA and sterilized by filtration. Protein content was determined by the colorimetric Lowry assay, using BSA as a standard. We then used a flow-cytometry assay to evaluate LDL uptake by using fluorescently labelled LDL, as widely and robust assay currently used for the investigation of LDLR function.1mg of LDL was labelled overnight at 4°C in 1 mL PBS containing 0,5mg/mL of 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) (stock solution 5mg/mL in dimethylformamide) (Sigma Aldrich, Milan, Italy). DiO-LDL were then isolated again by ultracentrifugation in KBr gradient and the excess of fluorescent probe and KBr salt was removed by dialysis versus PBS containing 0.01% EDTA. LDL were sterilized and the protein content determined as previously described. Monocytes-derived macrophages were washed with PBS then incubated with 10µg/mL of DiO-LDL in RPMI without serum. After 2 hours cells were washed three times with PBS, gently scraped off and collected in tubes for flow citometry analysis. Cells were immediately analyzed using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest software**.**

3.26. Gene expression analysis (Real time PCR)

Total RNA was extracted from monocytes-derived macrophages and 1 µg of RNA underwent reverse transcription using the IScript cDNA Synthesis kit (BioRad, Milan, Italy). 3 µL of cDNA were amplified by real-time quantitative PCR with 2X MAXIMA SYBR Green/Fluorescein qPCR mastermix (Carlo Erba Reagents, Cornaredo, Italy). The specificity of the Sybr green fluorescence was tested by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon. Each sample was analyzed in duplicate using the CFX Connect Real Time detection system (BioRad, Milan, Italy). The primers used are the following:

18S: Fw 5'-CGCAGCTAGGAATAATGGAATAGG-3', *Rw5'*- CATGGCCTCAGTTCCGAAA-3'; *LDLR: Fw* 5'- GTGTCACAGCGGCGAATG -3', *LDLR Rw 5'*- CGCACTCTTTGATGGGTTCA -3'. *IDOL: Fw* 5'- GATAACAGAGACGCACGCATTC -3', *IDOL Rw 5'*- CCCTTCAAGTCACGGCTATACTG -3', *PCSK9: Fwd* 5'-CCT GCG CGT GCT CAA CT3'. *PCSK9 Rw* 5' GCT GGC TTT TCC GAA TAA ACT C 3'.

Chapter 4. RESULTS

SECTION [A]. STUDIES IN MICE MODEL

SECTION [B]. STUDIES IN HUMAN

SECTION [A] STUDIES IN MICE MODEL

SECTION-A1 STUDY OF PCSK9 ROLE IN NEO-INTIMA FORMATION IN CAROTID ARTERY INJURY MODEL (CAIM)

4.1. Serum cholesterol levels, LDLR expression and lipoprotein fractionation profiling

We first examined whether the secreted form of PCSK9 can reduces the levels of LDLRs, as shown in [**Figure 16**] the number of cell-surface LDLRs declined by approximately 60% in PCSK9-WT cells than PCSK9-KO cells- [**Figure 16**-A]. Not only LDLR but PCSK9 has ability to reduce the expression of LDLRs like family members like LRP1, VLDLRs and ApoER2 (Data not shown).

We further analysed cholesterol levels (by enzymatic kit) and the lipoprotein profiles by fast protein liquid chromatography (FPLC), in mice without collar surgery and with surgery. In both the models, the lipoprotein fractionation profile was similar, comparison to PCSK9-WT mice, PCSK9- KO mice had about 71% less plasma cholesterol levels and about 50% less HDL (as measured by FPLC) particles, likely by enhanced clearance of ApoE containing HDL particles due to reduced degradation of surface LDLR –[**Figure 16**-B].

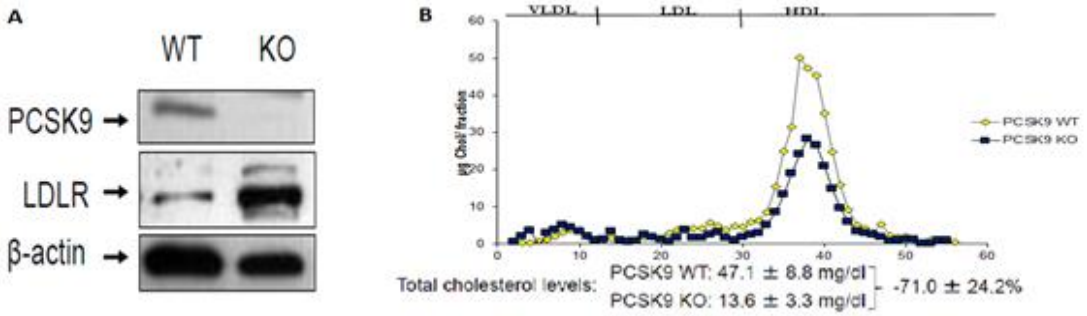


Figure 16. LDLR expression and Lipoprotein levels in PCSK9 WT and KO mice
(A) LDLR expression levels in the presence and absence of PCSK9 in primary hepatocytes
(B) Lipoprotein fractionation in PCSK9 WT and KO mice

4.2. PCSK9 mice presents accelerated neo-intimal hyperplasia in carotid artery injury model

We next aimed to investigate the pleotropic role of PCSK9 in carotid atherosclerosis progression, we used the pre-established settings of our in-house developed collar-technique, based on the perivascular placement of a plastic cuff around the right common carotid artery, and atherosclerosis progression is studied by evaluating the neo-intimal hyperplasia in response to vascular injury. 16 week old littermates of PCSK9 WT and KO were subjected to perivascular carotid collar placement and fed with normal chow diet upto 9 months post-surgery-[**Figure 17**]. Upon sacrifice, morphometric analysis were performed on the right carotid specimens (n=16 animals per group).

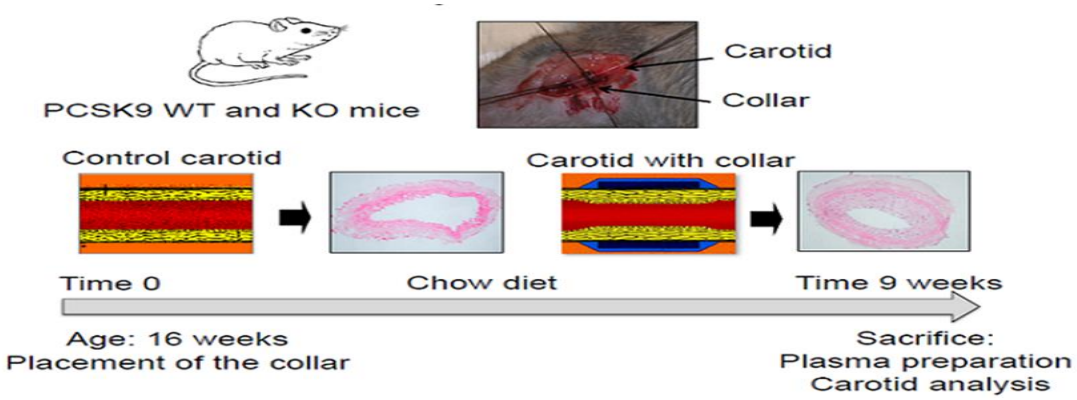


Figure 17. Schematic planning of carotid injury model :-
Perivascular manipulation was initiated by placing collar on right carotid artery of 16 week old mice (n=15 per group) and followed for 9 week post-surgery.

In both PCSK9-WT and KO mice, collared carotid arteries showed the formation of a intimal lesions that invaded the lumen region with different extent, in contrast, no neo-intima thickening was apparent in sham-operated carotid arteries and no anatomical differences was apparent between the two strains [**Figure 18**-A] . Morphometric analysis revealed about 50% reduction of neo-intima media thickness in PCSK9-KO mice when compared with WT strain (23.955±1.832 μm^2 *vs* 12.344±1.668 μm^2 for PCSK9-WT and PCSK9-KO respectively; P=0.049) or intima/media ratio (0.473±0.073 *vs* 1.380±0.175; for PCSK9-WT and PCSK9-KO respectively; P=0.024 (**Figure 18**-**Figure 18**B]. These differences

were associated to a tendency of a higher lumen area in PCSK9-KO mice vs PCSK9-WT mice (30.194±4.324 µm^2 *vs* 18.283±1.912 µm^2 respectively), however, this difference were found statistically insignificant**.**

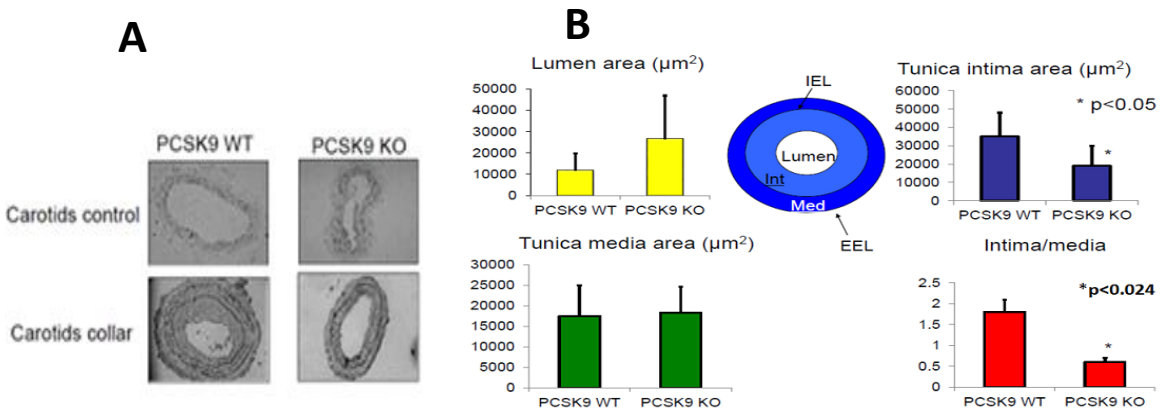


Figure 18. Morphometric analysis of Carotid arteries of WT and PCSK9-KO mice post-surgery

(A) Histologic appearances of carotid artery of control vs experimental mice of PCSK9 WT and KO mice. (B) Quantitative analysis of lumen area and intima-media ratio of carotid artery

4.3. Molecular characterization of carotids of CAIMs

We further examined the contractile ability of VSMCs cultured from respective carotids of PCSK9-WT and KO mice, Immunostaining with anti α-smooth muscle actin (α-SMA) antibody showed that carotid lesions of PCSK9-WT mice had higher relative SMCs accumulation than PCSK9-KO (21.0±7.56 % *vs.* 10.7±1.97 % respectively; P<0.05) [**Figure 19**-A]. Analysis under polarized light of picrosirius red stained specimens, showed a higher relative collagen accumulation in PCSK9-WT mice (18.38±7.90 % vs. 10.45±9.11 % respectively; P<0.01) [**Figure 19**-B], while no difference in macrophage content was detected, by immunostaining with anti F4/80 antibody, between the two groups.

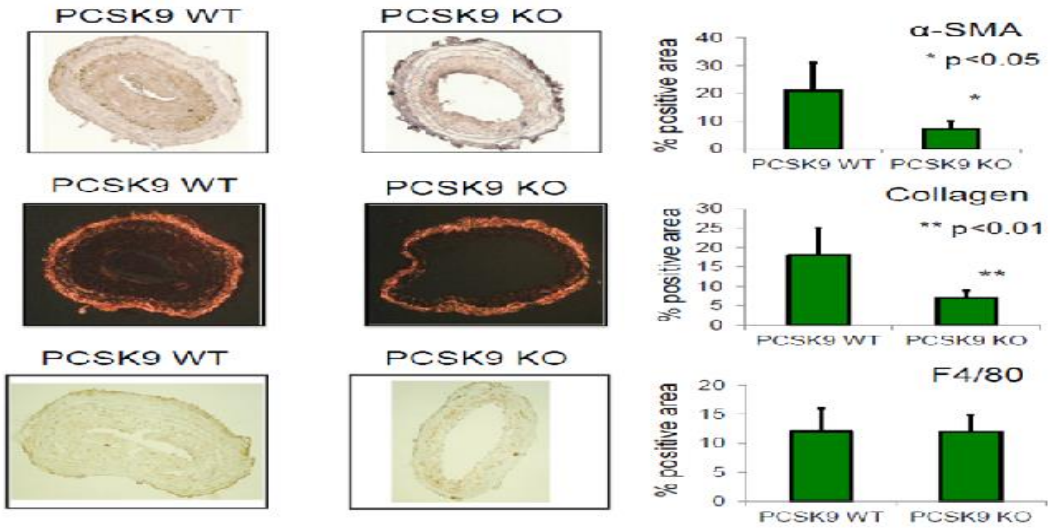


Figure 19. Immuno-fluorescence staining to study VSMCs phenotypes in controls and operated carotids of PCSK9 WT and KO mice.

4.4. Role of PCSK9 in vascular smooth muscle cells (VSMCs) phenotypes

We cultured aortic SMCs from PCSK9-WT and KO mice to study the role of PCSK9 on neo-intimal hyperplasia. No PCSK9 protein were detected in VSMCs of PCSK9-KO mice, as expected, while SMC from PCSK9-WT mice had visible PCSK9 occurrence, this difference was associated with increased levels of the LDLR in knock-out cells. At passage one after isolation, the expression of phenotypic markers were determined by quantitative RT-PCR reaction. SMCs from PCSK9-KO line expresses higher levels of α-smooth muscle actin (α-SMA) and lower levels of both caldesmon and collagen type-Ia1. In contrast, no differences were found on SM22α expression [**Figure 20**].

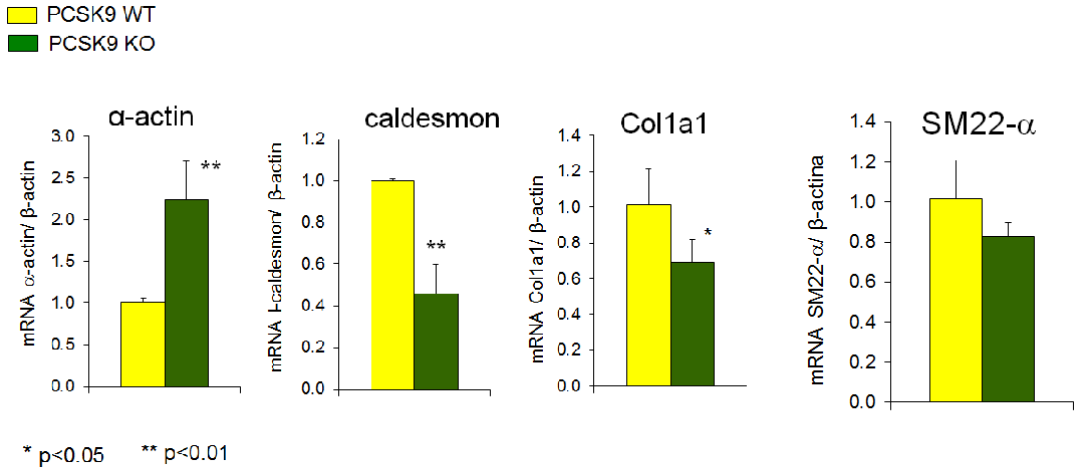


Figure 20. Vascular smooth cells phenotypic markers:- Gene expression profiling of vascular smooth muscles cell cultured from PCSK9 WT and PCSK9-KO mice.
All abovementioned proteins are normalized with β-actin and p-value were considered significant if found <0.05.

We next examined the proliferation pattern among the mice strains, Cell proliferation rate were assessed by using iCelligence real time monitoring instrument [**Figure 21**-A] , VSMCs were seeded (about 10,000 no. of cells/well) with 10% FBS and proliferation were detected in each 3rd and 6th day post-inception. PCSK9-KO mice had about 46% less proliferation rate than the WT mice (57.3 ± 2.1 hr vs 106.3 ± 4.5 hr respectively)- [**Figure 21**-B], while the SMCs from PCSK9-WT continuously grown up to 120 h after stimulation (**Figure 21**-C).

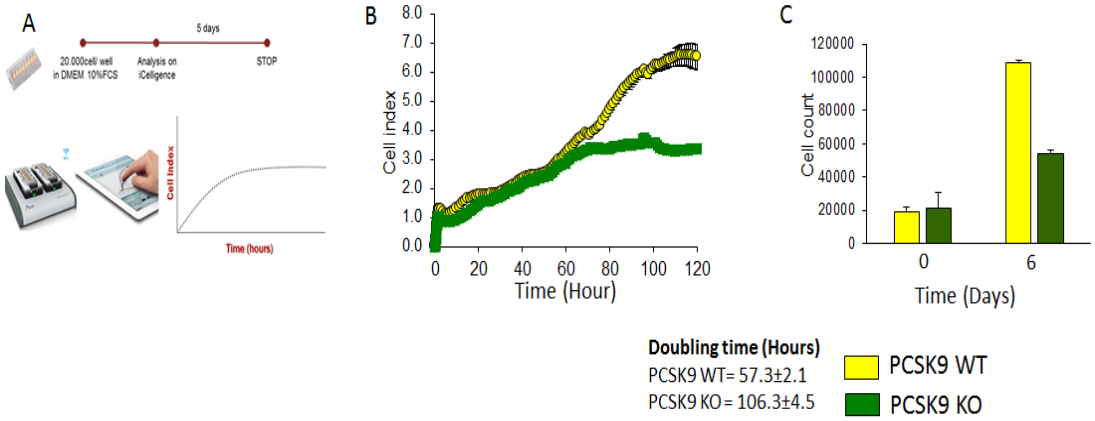


Figure 21. Vascular smooth muscle cell proliferation profile.
(A)-iCeilligence instrument with the graphical scheme of experiment set on the machine. (B) Equal amount of VSMCs (n=10,000) from PCSK9 WT and KO were seeded with 10% FBS and followed the growth uptil 120 hours. (C) Quantitative analysis of cell growth at Day 0 and Day 6 from both the group.

Similarly, when studied the migration profile of VSMCs derived from PCSK9-WT and KO mice by using by using Boyden chamber chemotaxis assay and the PDGF-BB as a chemotactic agent, revealed that VSMCs of PCSK9-WT had higher migration towards PDGF-BB, and the migration pattern were increased as increases the PDGF-BB concentration [**Figure 22**], while VSMCs from PCSK9-KO mice expresses lower migratory profile.

Taken together, our data suggest that PCSK9 deficient mice shows less neo-intimal hyperplasia in response to perivascular carotid injury, probably might be due to the less efficient dedifferentiating phenotype switch of vascular smooth cells.

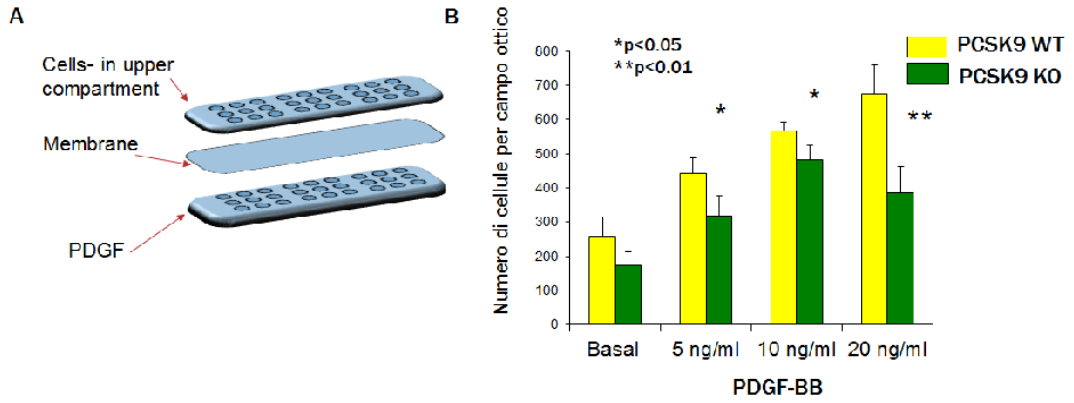


Figure 22. VSMCs migration profiling.
(A) A Boyden's chamber consist of upper and lower compartment and middle space for polycarbonate membrane. (B) The difference in migration profile between PCSK9-WT and KO derived VSMCs.

[SECTION A2] PCSK9 ROLE IN GLUCOSE HOMEOSTASIS:- RESULTS FROM GLUCOSE TOLERANCE TEST (GTT):-

The objective of this study was to determine the role of PCSK9 in glucose homeostasis by using or comparing the glucose absorbance ability in WT and PCSK9-KO mice upon intra-peritoneal glucose challenge (2mg/kg, diluted in sterilised PBS). These mice were nutritionally modified and supplied with a high fat rich diet [or DIO-diet (Diet induced obesity)] to induce obese phenotype [DIO diet- 24% Protein (gm), 41% Carbohydrate (gm) and 24% Corn oil (gm); or 20% protein (kcal), 35% Carbohydrate (kcal) and 45% corn oil

(kcal)] and subsequently assessed for glucose tolerance test and insulin induced glucose absorbance at different time points during the following 2 hours.

4.5. Body weight of PCSK9 strains in nutrition modified model (DIO-diet vs SFD diet)

All mice of more than 21-22gm in weight at Day- 0 were used for diet induced modification uptil week 20th. We first evaluate whether PCSK9 deficiency exacerbate weight gain and/or obesity in response to high fat diet.

Weight were measured at time 0 (week-0) prior keeping on diets and followed their weight in every week uptil the end of diet-cycle of 20 week. No significant weight gain were observed between the mice strains on DIO-diet [**Figure *23***].

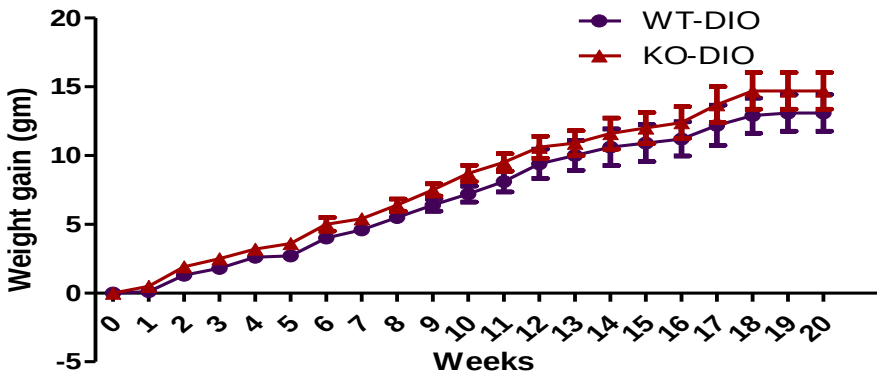


Figure 23. Weight Gain of PCSK9 WT vs KO mice of DIO diet

Weight of mice from each dietary group were measure since inception. This figure represents the weight gain status between the genotypes per DIO diet. Values are in Mean ± SEM.

4.6. Lipid levels in nutrition modified model

8 weeks old mice of WT and PCSK9-KO were stressed with DIO (diet induced diet) diet to generate the obese phenotype, and compared with the normal fed control SFD diet

(standard fat diet) for 20 week. Prior to sacrifice, plasma cholesterol and triglycerides levels were measured in both the strains. PCSK9-KO mice had about 25.33% less cholesterol than WT mice from DIO-diet group (values in Mean ± SEM ; 92.16 ± 4.77 vs 123.43 ± 5.29 respectively; p=0.001), while there was no difference found for triglycerides levels between the PCSK9 WT and KO groups (values in Mean ± SEM ; 62.81 ± 7.46 vs 66.81 ± 3.81 respectively; p=0.66)-[**Figure 24**].

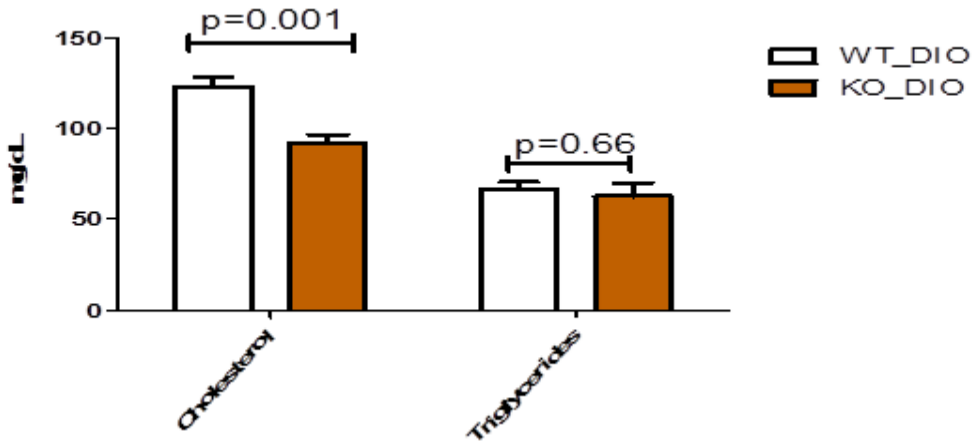


Figure 24. Plasma total cholesterol and triglyceride levels determination between the PCSK9 genotypes kept on DIO diet

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4.7. PCSK9 deficiency leads to hyperglycemic and hypoinsulinemic phenotypes

Mice were fed with SFD (11% energy by fat) and DIO diet (45% energy by fat) for 20 week and IPGTT (intraperitoneal glucose tolerance test- 2mg/kg glucose diluted in dterilised PBS) and IPITT (intraperitoneal insulin tolerance test-1mU/10μl) were performed at two checkpoints of 12th week and 20th week of post-inception. Mice were kept overnight fasting before GTT, intra-peritoneal glucose challenge to both the dietary group (DIO and SFD) revealed a moderate but significant glucose resistance in PCSK9-KO mice than PCSK9-WT mice at/after 20 min of glucose challenge-[**Figure 25**-A and B], at 12th week and also after 20th week post-inception of respective diets [**Figure 25**-**Figure 25**C & D]. We further choose

to determine the glucose intolerance between the genotypes with same dietary group (esp for DIO diet) and analysed for the glucose clearance at different time points (0, 20, 40, 60 and 120 min) of PCSK9-WT and KO mice from DIO diet group, the phenotypes of PCSK9-KO mice resembles with the significant elevation of plasma glucose levels immediately after 20 min of glucose challenge than PCSK9 WT of DIO diet group [**Figure 25**-**Figure 25**E & F], and the comparable elevation remain statistical significant upto 120 min post glucose challenge. However, the cause of this difference in glucose absorbance was not clear, therefore, we further examine the insulin sensitivity test to know whether this difference is due to the lack of adequate studied model or beta-cell impaired function, or any other confounding factors.

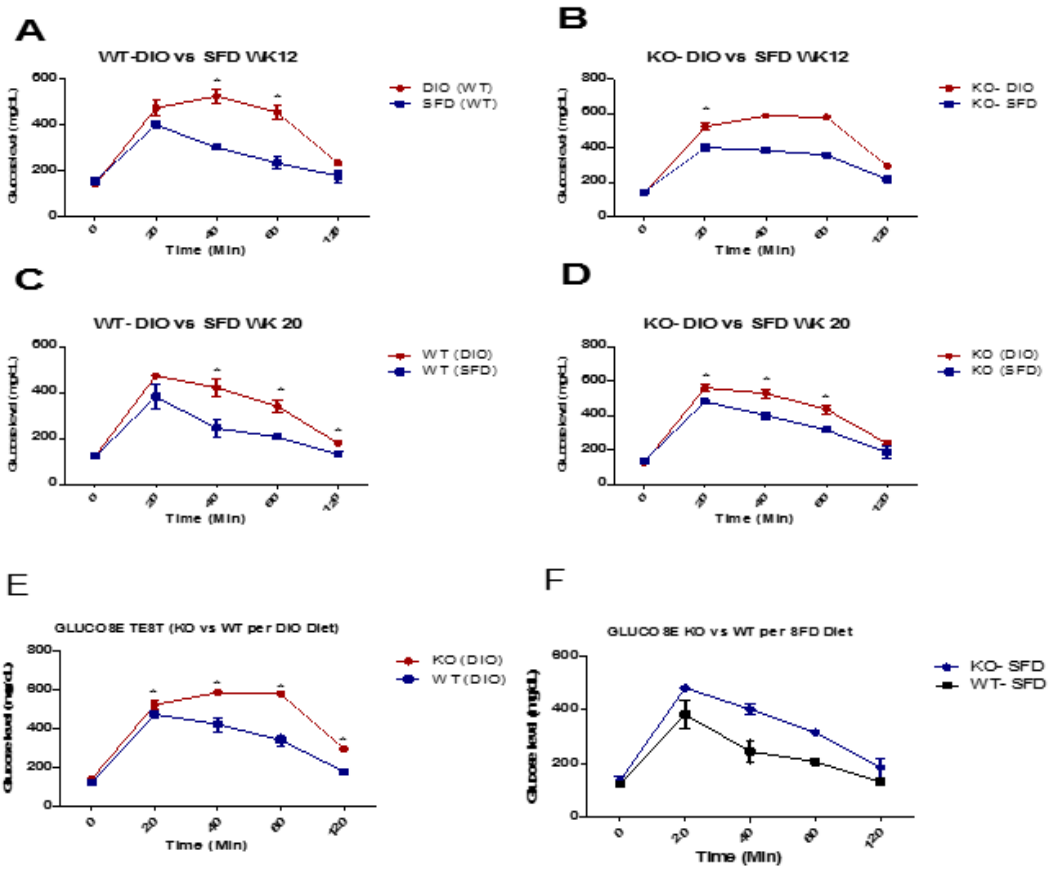

Figure 25. Glucose tolerance test

Blood glucose levels after intraperitoneal injection of glucose (2g/kg) in WT and PCSK9-KO mice following check points at time (0, 20,40 60 and 120 min) in WT and PCSK9-KO mice fed a SFD(standard fat diet) and DIO (Diet induced obesity) diets for 20 weeks. Results are shown in Means ± SEM. *p<0.05 considered as statistical significant for SFD vs DIO diet in WT mice and KO mice as well as between KO vs WT for DIO and KO vs DIO for SFD diets.

Therefore, we challenged the mice (9 hr fasting) of both groups (DIO and SFD diets) with insulin (injected 30mU from intra-peritoneal route), and glucose levels were checked at different time points (0, 20, 40, 60 and 120 min). No difference in glucose absorbance upon insulin injection were observed. It appears that pancreatic beta cells presents impaired section of insulin in PCSK9-KO mice [**Figure 26**].

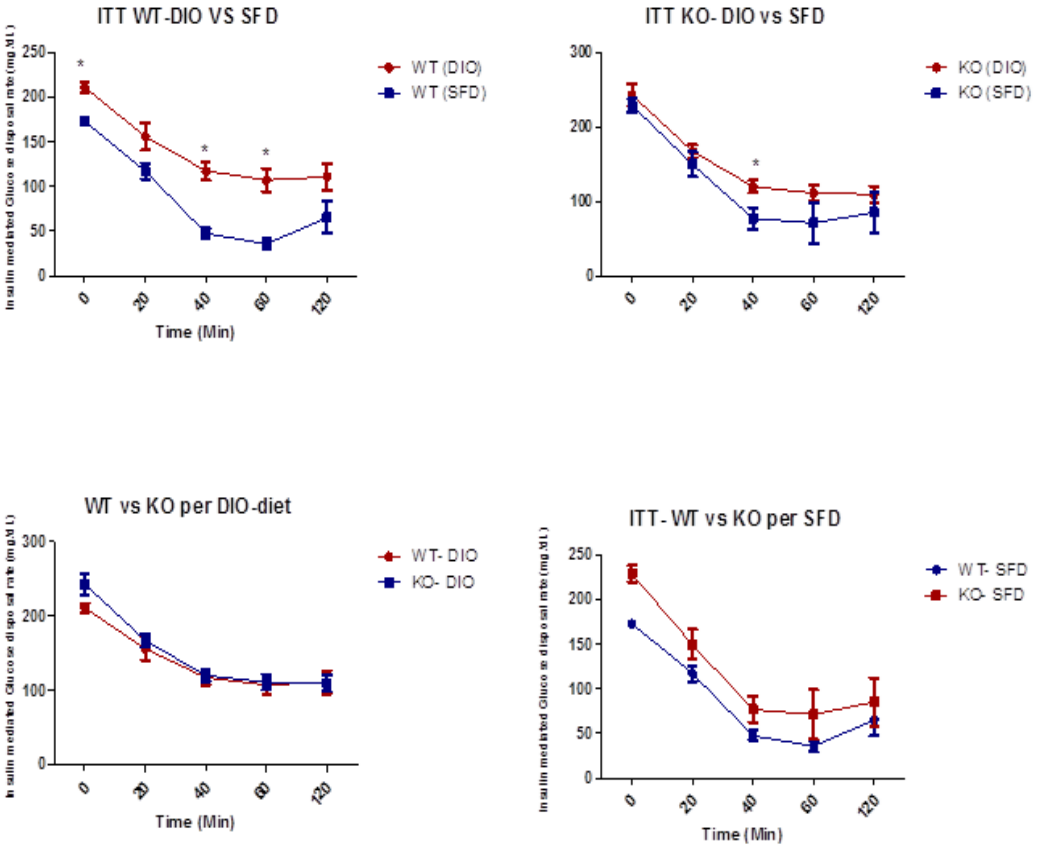

Figure 26. Insulin stimulated glucose disposal rate test.
Glucose disposal rate were measured between the genotypes of different dietary sets

4.8. Hepatic gene expression profiling of PCSK9 mice (DIO and SFD diets)

We further investigated the possibility of insulin resistance between the strains of both the dietary groups. The ex-vivo liver gene expression studies were performed in both the group, and as expected, PCSK9-KO mice of either of the dietary group(DIO and SFD) did not show any difference in hepatic insulin receptors (IRS1 and IRS2) expressions than Wild-type mice [**Figure 27**]. The glucose disposal rate between the genotypes (WT vs KO) upon

insulin injection was similar, while it was not true when only checked for glucose intolerance by performing only Glucose tolerance test (GTT), our results indicate that the difference in glucose disposal between the WT and KO mice upon dietary stress might be due to the impaired insulin secretion from pancreatic beta-cells in PCSK9-KO mice.

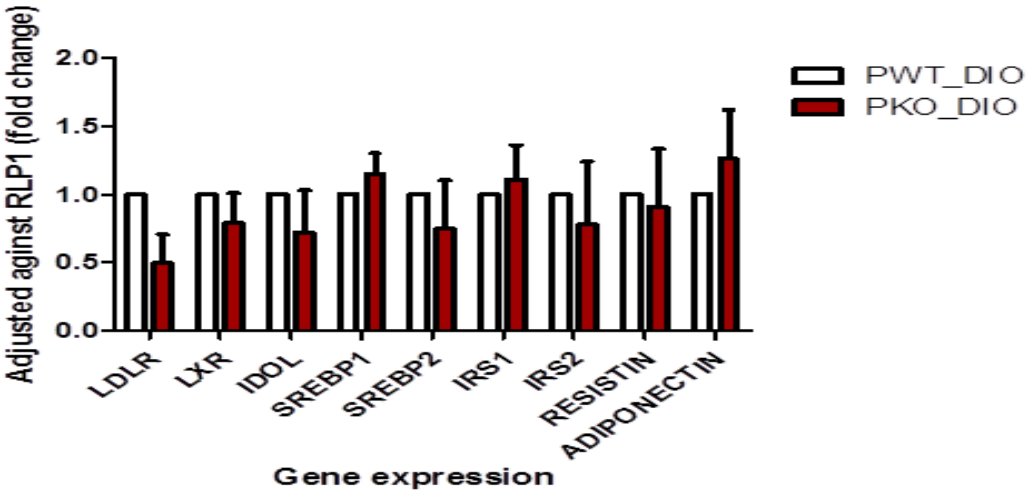

Figure 27. Dietary effect on hepatic gene expression.
Hepatic lipid related genes were evaluated in both PCSK9-WT and KO mice of DIO-diet group

[SECTION B] STUDIES IN HUMAN

[SECTION-B1] PCSK9 ACTIVITY ON LIPOPROTEIN AND ATHEROSCLEROSIS PROGRESSION

4.9. Background of selecting PCSK9 R46L and plasma PCSK9 levels study

Proprotein convertase subtilisin/kexin type 9 (PCSK9) protein has been emerged as a promising therapeutic target to treat dyslipidemia in last decade. Several genetic polymorphisms has been detected and characterised. PCSK9 gain-of-function (GOF) causes the severe hypercholesterolemia, while loss-of-functioned (LOF) mutations maintains low cholesterol profile and reduced risk of cardiovascular diseases. Discoveries of many GOF and LOF variants on PCSK9 gene were also found related to ethnicity. In Caucasian population, the R46L is known as one of the most potent LOF variant of PCSK9, lying at prodomain segment of PCSK9 protein, which reproducibly associated with significant

reductions in plasma levels of LDL-C (-15%) and reduction in CVD risk (-47%) by attenuating the autocatalytic cleavage of PCSK9 protein and hence reduction in plasma PCSK9 volume. The effect of PCSK9 mutations on plasma levels of LDL-C and coronary heart disease suggest that PCSK9 is a major determinant of plasma levels of LDL-C and hence an attractive target of cholesterol-lowering therapy.

PCSK9 R46L has been identified in Whites subjects in a Dallas-Heart studies (DHS) with the penetration of about 3.2%, and reduces the plasma cholesterol levels (-15%)[6][5] [162]probably via its ability to reduces plasma PCSK9 levels (-22.30%)[6] and hence contribute in reduction in CVD risk too (-47%)[5]**.**

4.10. Genotype and allelic frequencies of PCSK9 rs11591147 (p.R46L)

Table 1**Error! Reference source not found.** summarizes the genotype and allelic distribution of PCSK9 rs11591147 (c.5428 G>T; p.R46L), in the general population, the frequencies of the PCSK9 rs11591147 "G" variant allele and of the homozygous genotype (GG) is 98.90% and 97.81% respectively. In our population, we have not observed the homozygous genotype of recessive allele "TT" in our studied population. However, considering the 50% distribution and effect size over population, it comprises with about 1.10% distribution in our PLIC cohort.

Table 1. Genotype and allelic distribution of PCSK9 rs11591147(p.R46L)

		GENOTYPES		**ALLELES**	
	PLIC subjects (n)	GG (n; %)	GT (n;%)	G (%)	T (%)
Total	1919	1877; 97.81%	42; 2.19%	98.90	1.10
Men	813	794; 42.30%	19; 41.87%	98.83	1.17
Women	1106	1083;57.70%	23; 58.13%	98.96	1.04

4.11. General and clinical characteristics of PLIC subjects according to PCSK9 rs11591147 (p.R46L).

Table 2 summarizes general and clinical data of total population and subjects without hypolipidemics drugs. To observe the direct effect of R46L variant on plasma PCSK9 levels, we only choose the subjects those were free of hypolipidemics drugs to avoid the confounding effects of drugs over plasma PCSK9 levels.

In total population, the lipid phenotypes association with PCSK9 R46L genotypes appeared in various degree, where [values in Mean ± SEM] HDL (55.54 ± 0.33 vs 50.24 ± 1.96; p=0.022), and ApoA1(149.35 ± 0.61 vs 139.88 ± 3.84; p=0.024) remained higher among R46R carriers than R46L carriers respectively, although, a trend for total cholesterol (9 mg/dL difference) and LDL-c (10 mg/dL difference) were also appeared between the genotypes (R46R vs R46L) but it could not touch the statistical significance. However, when adjusted for age, gender and BMI, a significant trend appeared for LDL-c between the genotypes (p=0.007).

On the other hand; the mean levels of BMI in carriers of R46L were higher than R46R (values in Mean ± SEM; 26.48 ± 0.09 vs 28.15 ± 0.61 respectively; p=0.011). In addition, R46L carriers were observed with higher in Triglycerides (108.60 ± 1.44 vs 137.17 ± 14.0; p=0.004), and glucose (92.83 ± 0.36 vs 100.0 ± 4.18; p=0.004) levels than for R46R- All values were in Mean ± SEM.

Similarly, when subjects selected without hypolipidemics drugs, the pharmacogenomics effect on lipid phenotypes between carriers of R46R and R46L was neutral for HDL (58.37 ± 0.51 vs 48.82 ± 2.56; p=0.010), and ApoA1 (151.29 ± 0.87 vs 134.29 ± 6.27; p=0.007) as observed in total population-[**Table 2 A**], however a statistical significance for total cholesterol (237.04 ± 1.34 vs 207.23 ± 10.32; p=0.002) and LDL-c (158.35 ± 1.26 vs 136.70 ± 8.93; p=0.017) between the genotypes (R46R vs R46L) were appeared **[Table 2 B**]. This variation of association with total cholesterol and LDL levels prompted us to determine the plasma PCSK9 levels, as recent studies revealed the effect of R46L variants over PCSK9 secretion from hepatocytes.

Table 2. Anthropometric and clinical parameters according to PCSK9 rs11591147 (p.R46L)

Table 2(A) Of total population											
	Age	**BMI**	**TC**	**LDLc**	**HDLc**	**TG**	**Apo1**	**ApoB**	**SBP**	**DBP**	**GLUCOSE**
R46R	54.77 ± 0.26	26.48 ± 0.09	221.85 ± 0.90	144.58 ± 0.83	55.52 ± 0.33	108.60 ± 1.44	149.35 ±0.61	113.61 ±0.60	131.40 ± 0.40	81.65 ± 0.22	92.83 ± 0.362
R46L	54.17 ± 1.69	28.14 ± 0.61	212.10 ± 6.62	134.42 ± 6.35	50.24 ± 1.96	137.17 ± 14.0	139.88 ± 3.84	108.95 ± 4.18	134.39 ± 2.50	83.54 ± 1.37	100.0 ± 4.18
p-value	0.75	**0.011**	0.113	0.075	**0.022**	**0.004**	**0.024**	0.262	0.275	***0.53***	**0.004**
Women											
R46R	55.29 ± 0.336	26.04 ± 0.14	223.69 ± 1.16	144.48 ± 1.09	59.78 ± 0.44	97.34 ± 1.52	154.90 ± 0.78	112.61 ±0.76	129.28 ±0.55	80.42 ± 0.29	89.42 ± 0.37
R46L	54.18 ± 2.39	28.26 ± 0.91	224.18 ± 9.52	146.12 ± 8.83	51.95 ± 2.54	130.50 ± 16.12	144.95 ± 5.92	109.59 ± 6.0	134.77 ± 3.92	84.32 ± 2.31	100.77 ± 7.63
p-value	0.62	0.049	0.54	0.54	0.01	0.002	0.034	0.32	0.23	0.43	0.03
MEN											
R46R	54.07 ± 0.41	27.07 ± 0.122	219.34 ± 1.40	144.76 ± 1.30	49.79 ± 0.44	123.90 ± 2.61	141.82 ± 0.90	114.96 ± 0.97	134.30 ± 0.58	83.31 ± 0.32	97.45 ± 0.65
R46L	54.16 ± 2.46	28.01 ± 0.83	198.11 ± 8.23	120.86 ± 8.32	48.26 ±3.04	144.89 ± 24.15	134.0 ± 4.45	108.21 ± 5.93	133.95 ± 3.02	82.63 ± 1.34	99.11 ± 2.21
p-value	0.53	0.053	0.72	0.04	0.18	0.002	0.074	0.05	0.43	0.37	0.05

2(b) Subjects free of hypolipidemics drugs

	Age	BMI	TC	LDLc	HDLc	TG	Apo1	ApoB	SBP	DBP	GLUCOSE	PCSK9
R46R	60.47 ± 0.37	26.83 ± 0.16	237.04 ± 1.34	158.35 ± 1.26	58.37 ± 0.51	100.99 ± 1.82	151.29 ± 0.87	124.24 ± 1.09	131.22 ± 0.59	78.35 ± 0.29	98.89 ± 0.53	294.33 ± 6.34
R46L	63.29 ± 1.99	28.84 ± 0.78	207.23 ± 10.32	136.70 ± 8.93	48.82 ± 2.56	108.52 ± 10.24	134.29 ± 6.27	100.29 ± 6.36	128.82 ± 3.72	76.17 ± 1.51	99.23 ± 3.48	136.97 ± 23.10
p-value	0.275	0.071	**0.002**	0.017	0.010	**0.654**	**0.007**	**0.002**	0.598	0.201	0.984	**<0.001**
Women												
R46R	60.66 ± 0.54	26.66 ± 0.25	240.48 ± 2.01	159.30 ± 1.89	62.14 ± 0.79	95.20 ± 2.68	157.36 ± 1.22	126.26 ± 1.65	128.69 ± 0.94	77.13 ± 0.46	95.84 ± 0.72	312.0 ± 10.12
R46L	65.63 ± 2.16	28.92 ± 1.30	215.25 ± 14.09	142.87 ± 13.48	52.0 ± 4.41	101.75 ± 12.78	140.25 ± 10.37	107.75 ± 8.06	126.25 ± 6.03	73.75 ± 2.63	94.12 ± 2.22	132.98 ± 33.68
MEN												
R46R	59.06 ± 0.72	27.07 ± 0.20	228.52 ± 2.35	153.64 ± 2.25	52.83 ± 083	108.23 ± 3.47	141.70 ± 1.63	118.66 ± 1.80	132.73 ± 10.7	79.37 ± 0.49	102.67 ± 1.08	276.90 ± 10.91
R46L	57.33 ± 3.91	28.04 ± 0.65	205.50 ±19.87	133.66 ± 15.64	49.16 ± 3.10	113.16 ±24.78	137.66 ± 8.49	98.50 ± 12.47	130.83 ± 5.83	77.50 ± 1.70	98.83 ± 3.11	163.57 ± 41.53

4.12. Common carotid intima-media thickness according to PCSK9 rs11591147 (p.R46L genotype)- longitudinal study.

The role of the increase in the common carotid artery (CCA) intima-media wall thickness (IMT) in the atherosclerotic plaque is still debatable. Similarly, plasma PCSK9 levels or local production of PCSK9 from vascular cells especially vascular smooth muscle cells (VSMCs) role in cIMT progression is not very well understood. We measured the intima-media thickness of carotid artery of our all subjects enrolled in the PLIC study and also performed longitudinal study examining the predictive value of CCA-IMT measured at baseline and after 10 year follow ups. According to the PCSK9 R46L genotypes, the mean levels of cIMT at baseline (values in Mean ± SEM; 0.65 ± 0.003 vs 0.66 ± 0.017 mm; p= 0.142) and later 10 years follow ups (0.78 ± 0.005 vs 0.76 ± 0.23 mm; p= 0.116) did not reach to the statistical significance- [**Table 3].**

Table 3 :- PCSK9 R46L variants and status of intima-media thickness of carotid artery during follow up study

	R46R [n; (mean ± SEM)	**R46L [n; (mean ± SEM)**	**p- value**
Baseline	1848; (0.65 ± 0.003)	41; (0.66 ± 0.017)	0.142
10 year later	1067; (0.78 ± 0.005)	25; (0.76 ± 0.23)	0.116

4.13. PCSK9 R46L genotypes and plasma PCSK9 levels

To evaluate the role of PCSK9 R46L variants with plasma PCSK9 levels, we first choose subjects those were free of lipid lowering drugs to avoid the confounding effect of drugs on plasma PCSK9 levels. Some drugs especially statins, elevates the PCSK9 expression, as reported in many studies. As expected, we have observed a difference of about 157.36 ng/ml (about 53.46% difference) of mean levels in plasma PCSK9 between R46L genotypes, where the mean levels of plasma PCSK9 in R46R carrier was about 294.33 ± 6.34 ng/ml while

it was 136.97 ± 23.10 ng/ml in R46L carriers (values in Mean ± SEM). The reduction in plasma PCSK9 levels for R46L genotypes was irrespective to gender. Men of R46L carrier had about 40.93% reduction in mean plasma PCSK9 levels than men R46R carrier (163.57 ± 41.53 vs 276.90 ± 10.91 respectively; p <0.05), similarly, but highest reduction in plasma PCSK9 levels of about 57.38% (mean value) was seen in case of women carrying heterozygous R46L than women of R46R carrier- **[*Table 4*].**

Table 4. Difference of plasma PCSK9 levels and associated reduction in LDL and total cholesterol as per the PCSK9 R46L genotypes.

	R46R	R46L	p	% Reduction in PCSK9	% Reduction in LDL [refer table1]	% Reduction in Total cholesterol [refer table1]
All	294.33 ± 6.34	136.97 ± 23.10	<0.001	53.46 %	13.49%	12.39%
Men	276.90 ± 10.91	163.57 ± 41.53	***<0.002***	40.93 %	13.0%	10.07%
Women	312.00 ± 10.12	132.98 ± 33.68	**<0.001**	57.38%	10.31%	10.49%

4.14. Plasma PCSK9 levels in total PLIC cohort

Plasma PCSK9 levels were determined for all PLIC subjects of visit 3 (n= 1517) attending at Bassini hospital, Milan, Italy. All general and biochemical analysis were performed for the study and recorded for Age, BMI, lipid profiles, blood pressure, PCSK9 level, CRP and cIMT progression- can be seen in ***Table 5*.**

Plasma PCSK9 levels were determined for all 1517 subjects and correlation studies were performed, we excluded the subjects those were under lipid lowering drugs (like Statins, fibrates etc) to avoid the confounding effect over plasma PCSK9 levels. Thereby the final selected 1110 subjects were studied (free of lipid lowering drugs), At prima facie, the distribution of plasma PCSK9 levels (Mean ± SD) were not distributed normally among the population, where the mean plasma PCSK9 levels in total 1110 subjects was 285 ± 173.7

ng/ml (range 8.18 ng/ml to 1432.20 ng/ml) and was found increased in women than their age-matched man (mean ± SD; 296.35 ± 176.52 vs 267.84 ±168 ng/ml respectively; p=0.008) - **[*Table 5*]** [**Figure 28** A-D] and was highest in post-menopausal women (n=618) than pre-menopausal(n=63) (Mean ± SD; 332.69 ± 207.08 vs 259.36 ± 152.11 ng/ml; p= 0.007)-[**Figure 28**- E]. When stratified for hormone replacement therapy (HRT) vs non-hormone replacement therapy (non-HRT) effect over plasma PCSK9 levels among post-menopausal women, no dose effect of HRT on plasma PCSK9 levels were observed among HRT-taker versus non-HRT-takers (p=0.480)-[**Figure 28**-F] .

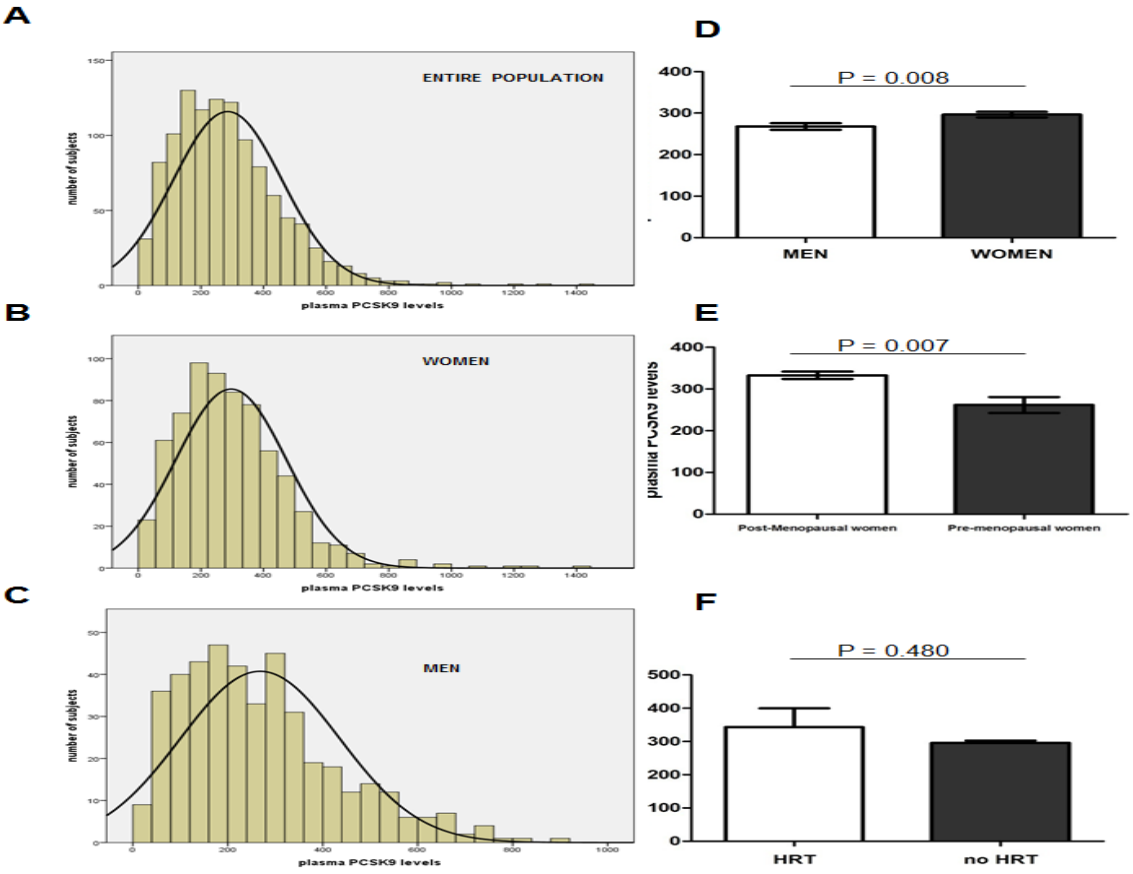

Figure 28. Plasma PCSK9 levels in PLIC cohort

A)Plasma PCSK9 levels in the entire studied cohort (n= 1.110); B) Plasma PCSK9 levels in the women cohort (n= 681); C) Plasma PCSK9 levels in the men cohort (n= 429); D) gender difference in plasma PCSK9 levels. E) difference in plasma PCSK9 levels according to menopausal status. E) difference in plasma PCSK9 levels according to use of hormone replacement therapy (HRT). P is derived from Analysis of Co-Variances (ANCOVA) using an age-adjusted model.

The another difference in plasma PCSK9 levels were observed between lipid lowering treatment vs non-lipid lowering treatment thus was the main excluding criteria for our further analysis. Statins treated subjects (n-337) had about 36% high plasma PCSK9 levels than subjects free of hypolipidemics drugs (p<0.001)- [**Figure 29**].

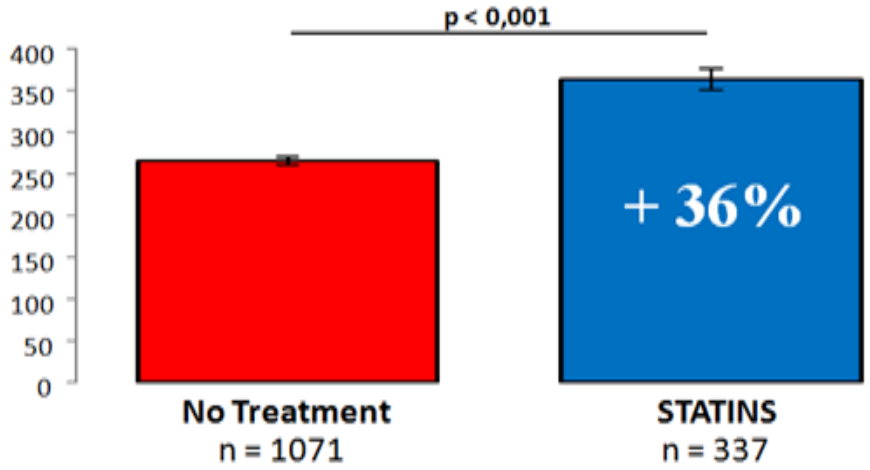

Figure 29. Statin induces plasma PCSK9 levels:-
Statins treated subjects had about 36% higher plasma PCSK9 levels than non-treated subjects in our studied cohort.

Table 5. General characteristics and plasma lipid levels of PLIC subjects

	All (n= 1110) Mean±S.D	Men (n= 429; 38.60%)	Women (n=681; 61.40%)	P value (Men vs Women)
Age (years)	59.6 ± 11.5	59.9 ± 1.5	59.4 ± 11.5	0.491
BMI (kg/m²)	26.7 ± 4.2	27.1 ± 3.4	26.4 ± 4.7	0.013
Waist (cm)	90.99 ± 11.8	97.6 ± 9.1	86.8 ± 11.5	<0.001
Total Cholesterol (mg/dL)	234.61 ± 42.14	226.9 ± 43.37	239.47 ± 40.63	<0.001
LDL-C (mg/dL)	156.18 ± 38.89	153.05 ± 40.46	158.15 ± 37.78	0.034
HDL-C (mg/dL)	58.52 ± 14.89	52.55 ± 13.0	62.28 ± 14.78	<0.001
Triglycerides(mg/dL)	99.84 ± 54.69	107.24 ± 58.46	95.19 ± 51.68	<0.001
Fasting glucose (mg/dL)	98.85 ± 15.94	103.38 ± 17.12	96.0 ± 14.45	<0.001
SBP (mmHg)	130.0 ± 18	133.0 ± 17.43	128.0 ± 18	<0.001
DBP (mmHg)	78.0 ± 9	79.0 ± 8	77.0 ± 9	<0.001
ApoAI(mg/mL)	151.44 ± 29.30	141.12 ± 25.45	157.93 ± 22.96	<0.001
ApoB (mg/mL)	123.01 ± 33.09	117.76 ± 32.44	126.31 ± 33.10	<0.001
CRP(mg/L)	2.82 ± 3.17	2.64 ± 3.25	2.93 ± 3.11	0.146
cIMT (mm)	0.74 ± 0.13	0.75 ± 0.13	0.73 ± 0.13	0.003
PCSK9 (ng/mL)	285.3 ± 173.7	267.84 ± 168.0	296.35 ± 176.52	**0.008**

4.15. Clinical and Anthropometric parameters according to PCSK9 levels

We further performed Univariate analysis to evaluate high order interaction between plasma PCSK9 levels of PLIC cohort and clinical and general variables. As stated in **Table 6** plasma PCSK9 levels were found associated with various metabolic parameters viz BMI (r=0.075, p= 0.024), LDL-c (r=0.094; p=0.002), ApoB (r=0.152, p<0.001); Total cholesterol (r=0.151, p<0.001); HDL (r=0.124, p<0.001) triglycerides (r=0.114, p<0.001) and ApoA1 (r=0.128, p<0.001), although all associated parameter with PCSK9 levels was not

more than 16% in a spearmen coefficient correlation analysis- [**Table 6**]. To know whether this degree of variability or association with metabolic parameters also exist between sexes- as PCSK9 expression also varies due to gender. We found that despite of higher PCSK9 in women plasma, Men cohort had presented modest association between plasma PCSK9 levels and metabolic determinants than women. The reason of this low association in women despite of higher in plasma PCSK9 levels is not fully understood, but hormonal intervention might be one of the primary reason.

Table 6. Clinical and Anthropometric parameters according to plasma PCSK9 levels

	All		**Men**		**Women**	
	ρ	p	ρ	p	ρ	p
Age (years)	-0.019	0.520	-0.061	0.205	0.015	0.687
BMI (kg/m²)	0.075	0.024	0.088	0.097	0.080	0.060
Waist (cm)	0.056	0.070	0.086	0.084	0.106	0.007
Total Chol (mg/dL)	**0.151**	**<0.001**	0.196	<0.001	0.105	0.006
LDL-c (mg/dL)	**0.094**	**0.002**	0.126	0.009	0.064	0.098
HDL (mg/dL)	**0.124**	**<0.001**	0.161	<0.001	0.061	0.112
Triglycerides(mg/dL)	**0.114**	**<0.001**	0.146	0.002	0.113	0.003
Fasting Glucose (mg/dL)	0.019	0.524	0.106	0.028	0.008	0.844
SBP (mmHg)	0.005	0.870	-0.001	0.986	0.024	0.527
DBP (mmHg)	0.037	0.217	0.053	0.273	0.040	0.294
ApoAI (mg/mL)	0.128	<0.001	0.166	0.001	0.062	0.106
ApoB (mg/mL)	0.152	<0.001	0.181	<0.001	0.120	0.002
CRP (mg/L)	0.031	0.308	0.019	0.703	0.030	0.438
cIMT (mm)	0.010	0.747	0.002	0.967	0.028	0.47

4.16. Increased levels of Plasma PCSK9 levels observed in Men with Metabolic syndrome phenotypes

Since most of the association between plasma PCSK9 levels and clinical variables were modestly stronger in men than women cohort, we further aim to understand the role or levels of plasma PCSK9 in metabolic syndrome (MetS) phenotypes, which is a common cluster of multiple risk factors (like dyslipidemia, hypertension and hyperglycemia) of CVDs, as defined by ATP-III guidelines.

At prima facie, the PCSK9 levels were found higher in MetS cohort , therefore, we also looked for genderwise difference in plasma PCSK9 levels in subjects with MetS phenotype, Men (n=134) and women (n=193) of MetS group compared with non-MetS of men (n=295) and women (n=488) for plasma PCSK9 levels distribution-[**Table 7**] [**Figure 30**]. A difference in mean plasma levels of about 31ng/ml (or increased by 10.47%) was existed between men of MetS and men of non-MetS phenotypes with statistical significant p-value of 0.04. While difference in plasma PCSK9 levels between MetS and non-MetS cohort of women did not reach to the statistical significance (p= 0.252).

Table 7. Plasma PCSK9 levels in subjects with Metabolic syndrome vs non-Metabolic syndrome

	Plasma PCSK9 (mean ± S.D ng/ml)			
	All (n; mean ± S.D)	**Men (n; mean ± S.D)**	**Women (n; mean ± S.D)**	**P Value**
MS Group	327; 301.04 ± 187.54	134; 288.63 ± 167.95	193; 309.66 ± 200	0.319
Non-MS Group	783; 278.77 ± 167.37	295; 258.40 ± 167.51	488; 291.08 ± 166.25	0.008
P value	0.016	**0.04**	0.236	

Our data suggest that despite of high in plasma PCSK9 levels, women generally present protection against various metabolic disturbances during their pre-menopausal status- as stated in various other studies. We further try to understand the robustness of increased plasma PCSK9 levels in MetS, we choose to perform longitudinal analysis on the

subjects those had increased plasma PCSK9 levels but did not have MetS phenotype at baseline.

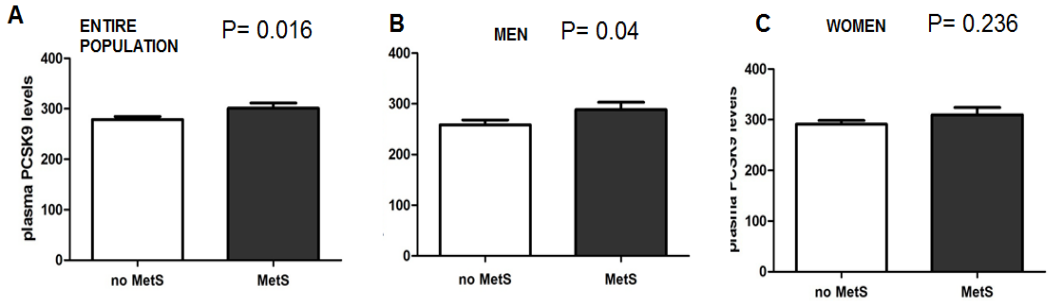

Figure 30. Plasma PCSK9 levels in Metabolic syndrome in Total population and genderwise manner.

(A) Plasma PCSK9 levels in total population of MetS Vs Non MetS group (B) Plasma PCSK9 levels difference in Men Vs Men in MetS and non MetS group (C) Plasma PCSK9 levels difference in Women Vs Women in MetS and Non MetS group

4.17. PCSK9 levels are independent prognostic marker of Metabolic Syndrome

To determine whether to consider plasma PCSK9 levels as independent prognostic marker of MetS incidence. Subjects with high plasma PCSK9 levels (>Mean levels) but free of MetS phenotypes were followed for 4 years, 1.6% subjects of studied group had developed MetS during a median of 4 years of follow up time. Cox regression model, adjusting for age, gender, lifestyle habits and ongoing treatments, showed that high plasma PCSK9 levels were predictive of an increased risk of MetS in men (HR= 1.616 [1.112-1.918] HR 95% C.I., P= 0.043), but not in women (HR= 1.007 [0.987-1.617] HR 95% C.I., P= 0.515). Our data suggest that PCSK9 levels might be considered as a gender-specific prognostic marker for the incidences of MetS in a longer run.

SECTION [B2]. GPER P.P16L GENOTYPES AND ROLE IN LIPID HOMEOSTASIS AND ATHEROSCLEROSIS PROGRESSION

We next investigated the role of GPER rs11544331 variants in lipid metabolism and peripheral atherosclerosis progression in our PLIC cohort. The reason of choosing this variant for the study, is due to the mounting hypothesis of estrogen mediated regulation of PCSK9 expression, GPER rs11544331 (p.P16L) variants were recently reported to be associated with sex-specific increase in blood pressure and cholesterol levels in women, further, analysis on HepG2 cells also revealed that GPER activity downregulates PCSK9 expression and thus enhances the expression of LDLRs and increased LDL uptake.

These findings prompted us to evaluate this GPER rs11544331 SNP in our population, as we have already studied the plasma PCSK9 levels difference in women and men, however, we were also interested to know the molecular mechanism behind the cause.

4.18. Genotype and allelic distribution frequency of GPER rs11544331 genotypes

The genotype distribution of the GPER rs11544331 polymorphism was within Hardy-Weinberg equilibrium. To understand better association of GPER rs11544331 variants role, we have done group-wise stratification, Group-I was consist of total population (n-= 1405) and Group-II was consist of subjects free of hypolipidemics drugs (n=898) to avoid the lipid lowering drugs effect over plasma PCSK9 levels.

The frequencies of the GPER rs11544331 ‘C’ variant allele and of the homozygous genotype (CC) was higher in total population (Group-I) of 1405 subjects (69.68 % and 47.97% respectively) in comparison to the ‘T’ variant allele and of the homozygous genotype (TT) (30.32%, 8.61% respectively). Further, no significant differences in TT-genotype distributions between men and women (8.87 % vs 8.44% respectively) were observed **– [Table 8].** A similar trend were observed in subjects without lipid lowering drugs(n=898;

Group-II), where, 'C' variant allele and of the homozygous genotype (CC) was higher in total population (70.38 % and 49.44% respectively) in comparison to the 'T' variant allele and of the homozygous genotype (TT) (29.62%, 8.69% respectively). And, no significant differences in TT-genotype distribution was observed among men and women (8.48 % vs 8.83% respectively).

Table 8. Penetration of Genotype [n (%)] and allelic frequency of the rs11544331 variants of GPER gene in Italian general population.

		GENOTYPE			ALLELES	
	PLIC subjects	**CC (n; %)**	**CT (n; %)**	**TT (n; %)**	**C (%)**	**T (%)**
GROUP-I	1405	674; (47.97%)	610 (43.42%)	121 (8.61%)	69.68%	30.32%
MEN	575	276; (48%)	248 (43.13%)	51 (8.87%)	69.57%	30.43%
WOMEN	830	398; (47.95%)	362 (43.61%)	70 (8.44%)	69.76%	30.24%
χ^2	0.093					
p-value	0.954					
GROUP-II	898	444; (49.44%)	376; (41.87%)	78; (8.69%)	632; 70.38%	266; 29.62%
MEN	354	182; (51.41%)	142; (40.11 %)	30; (8.48%)	253; 71.47%	101; 28.53%
WOMEN	544	262; (48.16%)	234; (43.01%)	48; (8.83%)	379; 69.67%	165; 30.33%
χ^2	0.92					
p-value	0.63					

4.19. General and biochemical characteristics of PLIC subjects according to GPER rs11544331 gene variants.

We studied GPER variants role in total cohort (n=1405, henceforth called Group- I) **[Table 9**] and in subjects free of lipid lowering drugs (n=898; henceforth called Group- II) **[Table 10].**

Univariate analysis were performed in both the studied groups to evaluate high order interaction among GPER genotypes with general and biochemical profiles. No significant association were found among carriers of different GPER genotypes in either of the studied group (Group-I & II) for anthropometric and clinical parameters such as BMI, Waist, LDL, TC, Tg, HDL, Glucose, SBP, DBP etc. in either of the groups [**Table 9 & Table 10].** Previous studies on the role of GPER P16L genotype on total cholesterol by Hussain et al and

on blood pressure by Feldman et al was reported as sex-specific activity of GPER, where only women was affected due to the non-synonymous change(rs11544331; C>T; 16L) in GPER gene. We also did gender-wise stratification in both the groups to evaluate the sex-specific role of GPER P16L genotype and their influence on lipids and blood pressures. No significant association of 16L genotype with total cholesterol were seen among sexes (men vs Women; Mean ± SEM [mg/dL]) in group-I (216.86. ± 5.0 vs 231.59 ± 4.10; p>0.05)-[**Table 11],** and in group II (218.63 ± 5.95 vs 243.29 ± 6.02; p>0.05)-[Table **12**]. Similarly, no association was there for blood pressure among GPER 16L genotype and sexes of either group (men vs Women; Mean ± SEM [mg/dL]) in group-I (134.12. ± 2.35 vs 133.29 ± 2.4; p>0.005**)-[Table 11**], and in group II (132.83 ± 2.84 vs 131.77 ± 3.20; p>0.005) -[**Table 12]**

Table 9. General and clinical characteristics of the **GROUP-I** cohort according to the rs11544331 SNP of GPER gene.

	Total (n= 1405)	**CC (n=674)**	**CT (n=610)**	**TT (121)**	**p value**	**CC Vs CT**	**CC vs TT**	**CT VS TT**
Age	54.90 ± 0.29	54.81 ± 0.43	54.96 ± 0.45	55.10 ± 0.99	0.952	0.811	0.795	0.902
BMI (Kg/m²)	26.56 ± 0.11	26.69 ± 0.17	26.37 ± 0.17	26.82 ± 0.33	0.326	0.186	0.749	0.290
Waist	90.26 ± 0.35	90.50 ± 0.48	89.77 ± 0.56	91.38 ± 1.0	0.377	0.318	0.498	0.219
Tg (mg/dL)	108.59 ± 1.71	108.01 ± 2.3	108.33 ± 2.6	113.12 ± 6.3	0.716	0.929	0.420	0.453
TC (mg/dL)	221.69 ± 1.0	220.15 ± 1.48	222.66 ± 1.55	225.38 ± 3.2	0.271	0.240	0.166	0.474
LDL (mg/dL)	145.24 ± 0.95	144.66 ± 1.37	145.29 ± 1.46	148.18 ± 3.11	0.608	0.751	0.319	0.418
HDL (mg/dL)	54.73 ± 0.38	53.88 ± 0.54	55.69 ± 0.59	54.57 ± 1.34	0.079	0.025	0.628	0.434
ApoA1 (mg/dL)	146.96 ± 0.68	145.78 ± 0.99	148.24 ± 1.0	147.04 ± 2.5	0.235	0.089	0.622	0.641
ApoB (mg/dL)	113.19 ± 0.68	112.97 ± 0.97	112.83 ± 1.0	116.25 ± 2.22	0.389	0.919	0.196	0.181
Glycemia (mg/dL)	91.15 ± 0.43	90.08 ± 0.49	92.06 ± 0.79	92.51 ± 1.3	0.060	0.030	0.131	0.780
SBP (mmHg)	132.94 ± 0.45	132.79 ± 0.64	132.96 ± 0.68	133.64 ± 1.7	0.878	0.856	0.612	0.689
DBP (mmHg)	82.96 ± 0.24	83.09 ± 0.33	82.65 ± 0.37	83.88 ± 0.83	0.345	0.385	0.370	0.168
Non-HDL (mg/dL)	166.96 ± 1.05	166.26 ± 1.52	166.96 ± 1.61	170.80 ± 3.31	0.507	0.752	0.244	0.328
Remnants (mg/dL)	21.71 ± 0.34	21.60 ± 0.47	21.66 ± 0.52	22.62 ± 1.27	0.716	0.929	0.420	0.453

Note:- Values are indicated in Mean ± SEM, and p-value considered significant if less than 0.05.

Table 10. General and clinical characteristics of the PLIC cohort (**Group-II**) according to the rs11544331 SNP of GPER gene

	Total (n= 898)	CC (n=444)	CT (n=376)	TT (78)	p value	CC Vs CT	CC vs TT	CT VS TT
Age	60.07 ± 0.37	59.94 ± 0.53	60.30 ± 0.57	59.68 ± 1.23	0.854	0.646	0.848	0.655
BMI (Kg/m²)	26.83 ± 0.15	26.95 ± 0.21	26.64 ± 0.25	27.02 ± 0.49	0.599	0.346	0.911	0.524
Waist	91.52 ± 0.39	92.42 ± 0.53	90.30 ± 0.64	91.73 ± 1.36	0.051	0.015	0.631	0.367
Tg (mg/dL)	100.76 ± 1.85	103.02 ± 2.71	98.13 ± 2.76	100.52 ± 6.46	0.457	0.211	0.715	0.73
TC (mg/dL)	233.92 ± 1.30	231.86 ± 1.95	236.39± 1.86	233.80 ± 4.54	0.254	0.098	0.685	0.595
LDL(mg/dL)	155.62 ± 1.21	154.68 ± 1.81	156.59± 1.76	156.35 ± 4.19	0.743	0.454	0.708	0.958
HDL (mg/dL)	58.22 ± 0.49	56.57 ± 0.66	60.37 ± 0.81	57.34 ± 1.64	0.001	0.001	0.672	0.101
ApoAI (mg/dL)	151 ± 0.84	148.65 ± 1.16	154.19± 1.33	149.12 ± 2.91	0.006	0.002	0.877	0.106
ApoB (mg/dL)	122.27 ± 1.03	121.51 ± 1.52	123.41± 1.52	121.14 ± 3.57	0.646	0.383	0.922	0.557
Glycemia (mg/dL)	98.63 ± 0.51	97.88 ± 0.68	99.31 ± 0.83	99.65 ± 1.96	0.352	0.19	0.353	0.86
SBP (mmHg)	130.51 ± 0.61	130.54 ± 0.91	130.12± 0.90	132.18 ± 2.24	0.67	0.746	0.472	0.372
DBP (mmHg)	78.15 ± 0.29	78.25 ± 0.42	78.19 ± 0.44	77.37 ± 1.15	0.716	0.919	0.419	0.459

Note:- Values are indicated in Mean ± SEM, and p-value considered significant if less than 0.05.

Table 11. Sex-specific impact of rs11544331 variants of GPER gene on general and biochemical parameters **of Group-I.**

	Total ; %	Age	BMI	Waist	Tg	TC	LDL	HDL	ApoAI	ApoB	GLYCEMIA	SBP	DBP	Non-HDL	Remnants
In Men	575; 40.8%	54.60 ± 0.46	27.06 ± 0.14	96.31 ± 0.40	123.56 ± 3.14	218.67 ± 1.59	145.35 ± 1.49	48.60 ± 0.49	137.49 ± 0.96	114.91 ± 1.0	95.38 ± 0.69	135.69 ± 0.66	84.50 ± 0.36	170.06 ± 1.62	24.71 ± 0.62
CC	276; 48.00%	54.47 ± 0.67	26.99 ± 0.21	96.13 ± 0.58	120.37 ± 4.27	216.50 ± 2.18	143.95 ± 2.06	48.47 ± 0.67	137.02 ± 1.38	113.72 ± 1.46	93.74 ± 0.83	135.60 ± 0.97	84.64 ± 0.50	168.03 ± 2.25	24.07 ± 0.85
CT	248; 43.14%	54.87 ± 0.70	27.0 ± 0.21	96.57 ± 0.63	125.80 ± 5.01	221.45 ± 2.5	147.26 ± 2.3	49.02 ± 0.74	138.54 ± 1.42	116.06 ± 1.78	97.25 ± 1.21	136.11 ± 1.0	84.58 ± 0.58	172.42 ± 2.61	25.16 ± 1.0
TT	51; 8.86%	54.04 ± 1.61	27.38 ± 0.37	96.11 ± 1.10	129.92 ± 11.3	216.86 ± 5.0	143.58 ± 4.80	47.29 ± 2.0	134.90 ± 3.82	115.76 ± 3.64	95.24 ± 2.3	134.12 ± 2.35	83.43 ± 1.21	169.56 ± 4.80	25.98 ± 2.27
p value		0.858	0.763	0.862	0.585	0.316	0.537	0.613	0.534	0.577	0.054	0.715	0.657	0.433	0.585
CC VS CT		0.686	0.80	0.60	0.41	0.14	0.29	0.59	0.45	0.30	0.01	0.71	0.93	0.19	0.41
CC vs TT		0.80	0.46	0.98	0.40	0.95	0.94	0.51	0.54	0.60	0.55	0.54	0.36	0.79	0.40
CT vs TT		0.63	0.55	0.74	0.72	0.43	0.50	0.34	0.30	0.94	0.43	0.41	0.39	0.63	0.72
In Women	830; 59.07%	55.11 ± 0.38	26.22 ± 0.16	86.26 ± 0.45	98.22 ± 1.82	223.78 ± 1.32	145.16 ± 1.24	58.97 ± 0.50	153.52 ± 0.88	112.00 ± 0.87	88.22 ± 0.53	131.02 ± 0.60	82.01 ± 0.44	164.81 ± 1.38	19.64 ± 0.36
CC	398; 47.95%	55.05 ±0.55	26.48 ± 0.24	86.79 ± 0.62	99.44 ± 2.68	222.67 ± 2.0	145.15 ± 1.83	57.63 ± 0.74	151.86 ± 1.29	112.46 ± 1.30	87.55 ± 0.56	130.82 ± 0.84	81.32 ± 0.47	165.04 ± 2.05	19.88 ± 0.53
CT	362; 43.62%	55.03 ± 0.59	25.89 ± 0.25	85.43 ± 0.73	96.36 ± 2.65	223.48 ± 1.92	143.95 ± 1.83	60.26 ± 0.77	154.89 ± 1.33	110.62 ± 1.29	88.50 ± 1.0	130.79 ± 0.91	84.21 ± 1.14	163.22 ± 2.02	19.27 ± 0.53

TT	48; 8.43%	55.87 ± 1.24	26.43 ± 0.49	87.56 ± 1.58	100.89 ± 6.92	231.59 ± 4.10	151.53 ± 4.08	59.87 ± 1.46	155.89 ± 2.91	116.60 ± 2.79	90.53 ± 1.67	133.29 ± 2.4	81.89 ± 0.31	171.71 ± 4.55	20.17 ± 1.38
p value		0.83	0.22	0.25	0.65	0.19	0.26	**0.04**	0.19	0.17	0.29	0.52	**0.046**	0.26	0.65
CC VS CT		0.97	0.09	0.15	0.420	0.77	0.64	**0.013**	0.103	0.316	0.396	0.97	0.29	0.530	0.420
CC vs TT		0.57	0.93	0.66	0.832	0.07	0.16	0.235	0.225	0.206	0.136	0.274	0.059	0.196	0.832
CT vs TT		0.56	0.39	0.23	0.510	0.10	0.10	0.838	0.765	0.070	0.314	0.271	0.014	0.102	0.510

Note:- Values are indicated in Mean ± SEM, and p-value considered significant if less than 0.05.

Table 12. Sex-specific impact of rs11544331 variants of GPER gene on general and biochemical parameters **(Group-II).**

	Total; %	**Age**	**BMI**	**Waist**	**Tg**	**TC**	**LDL**	**HDL**	**ApoAI**	**ApoB**	**Glycemia**	**SBP**	**DBP**	**PCSK9**
In Men	354; 39.42%	59.80 ± 0.59	27.07 ± 0.18	97.50 ± 0.46	108.20 ± 3.13	226.67 ± 1.98	152.67 ± 1.84	52.54 ± 0.66	141.57 ±1.32	117.59 ± 1.53	102.42 ± 0.88	132.68 ± 0.93	79.25 ± 0.43	269.49 ± 9.30
CC	182; 51.41%	59.47 ± 0.82	27.05 ± 0.24	97.67 ± 0.62	108.77 ± 4.38	224.91 ± 2.94	151.63 ± 2.72	51.52 ± 0.86	140.26 ± 1.78	116.92 ± 2.30	100.47 ± 0.99	132.80 ± 1.44	79.53 ± 0.62	258.42 ± 12.56
CT	142; 40.12%	60.44 ± 0.92	26.96 ± 0.32	97.08 ± 0.78	106.53± 4.63	230.6 ± 2.92	155.55± 2.69	54.24 ± 1.18	143.75± 2.20	119.88± 2.30	105.19± 1.70	132.50± 1.31	78.98 ± 0.67	287.39 ± 14.99
TT	30; 8.47%	58.77 ± 2.22	27.68 ± 0.66	98.50 ± 1.45	112.60 ± 13.7	218.63 ± 5.95	145.41 ± 6.21	50.70 ± 1.79	139.16 ± 4.16	110.80 ± 3.82	101.13 ± 2.45	132.83 ± 2.84	78.82 ± 1.26	254.09 ± 35.12

p value		0.645	0.609	0.674	0.863	0.185	0.296	0.11	0.394	0.269	0.036	0.987	0.797	0.298
CC VS CT		0.439	0.810	0.545	0.735	0.173	0.315	0.054	0.212	0.363	0.011	0.879	0.544	0.141
CC vs TT		0.751	0.379	0.633	0.743	0.393	0.364	0.737	0.822	0.283	0.839	0.993	0.663	0.901
CT vs TT		0.458	0.321	0.421	0.610	0.110	0.147	0.160	0.359	0.119	0.221	0.925	0.929	0.351
In Women		60.25 ± 0.47	26.67 ± 0.22	87.61 ± 0.51	95.91 ± 2.26	238.65 ± 1.69	157.54 ± 1.60	61.92 ± 0.65	157.14 ± 1.01	125.32± 1.37	96.17 ± 0.60	129.09 ± 0.81	77.44 ± 0.39	307.51 ± 8.51
CC	262; 48.16%	60.27 ± 0.69	26.88 ± 0.33	88.78 ± 0.71	99.02 ± 3.43	236.68± 2.56	156.79± 2.41	60.08 ± 0.89	154.47± 1.42	124.69± 2.01	96.09 ± 0.92	128.97± 1.16	77.37± 0.56	291.25 ± 11.90
CT	234; 43.02%	60.22 ± 0.73	26.45 ± 0.35	86.31 ± 0.81	93.01 ± 3.39	239.90± 2.38	157.22± 2.31	64.08 ± 1.02	160.51± 1.53	125.57± 2.01	95.72 ± 0.76	128.68 ± 1.20	77.71 ± 0.57	313.27 ± 13.65
TT	48; 8.82%	60.25 ± 1.44	26.61 ± 0.69	87.50 ± 1.77	92.97 ± 5.91	243.29± 6.02	163.19± 5.42	61.50 ± 2.23	155.35± 3.70	127.60± 5.10	98.72 ± 2.80	131.77± 3.20	76.46 ± 1.69	366.92± 24.13
p value		0.99	0.66	0.73	0.42	0.46	0.54	0.013	0.015	0.837	0.408	0.583	0.686	**0.043**
CC VS CT		0.958	0.371	0.022	0.207	0.364	0.899	0.003	0.005	0.763	0.774	0.863	0.678	0.217
CC vs TT		0.990	0.751	0.495	0.467	0.286	0.276	0.550	0.812	0.564	0.237	0.348	0.534	**0.015**
CT vs TT		0.986	0.847	0.531	0.997	0.588	0.314	0.281	0.167	0.689	0.182	0.304	0.394	0.086

Note:- Values are indicated in Mean ± SEM, and p-value considered significant if less than 0.05.

4.20. GPER rs11544331 variants influences plasma PCSK9 levels in a sex-specific manner

We chose to perform correlation studies among the different carriers of GPER genotypes and plasma PCSK9 levels only in the Group-II subjects (n=898; free of hypolipidemics), to avoid the confounding effect of hypolipidemic drugs over plasma PCSK9 levels, as reported in various clinical based studies. In general, no significant association between clinical variables (nearly all) and rs11544331 genetic variants of GPER gene in a univariate analysis were observed- [**Table 10].** However, a modest association were found among carriers of GPER genotypes (CC, CT and TT) and plasma PCSK9 in total group-II cohort; [277.49 ± 8.71; 303.52 ± 10.22 and 324.34 ± 20.95 respectively; p=0.045]- [**Table 13 and Figure 31A**], which were only remained significant with women cohort (n=544) (291.25 ± 11.90; 313.27 ± 13.65; 366.92 ± 24.13; p=0.043)- [**Table 14** and **Figure 31 B**]. The mean values of plasma PCSK9 levels (mean ± SEM; ng/ml) in total cohort of group-II was 292. ± 6.35 ng/ml, and was higher in women (307.51 ± 8.51 ng/ml) than men (269.29 ± 9.30 ng/ml), was further higher with TT carriers (p.P16L) of women than men of the same genotype (366.92 ± 24.13 and 254.09 ± 35.12 respectively; p=0.029)-[**Figure 31 C**]. However, the genotype frequency for P16L variants was insignificantly different among men and women (8.48% vs 8.83% respectively; p>0.05).

We further investigated the possibility of GPER gene rs11544331variants (p.P16L) induced plasma PCSK9 levels effect on lipid levels and other clinical parameters in women of TT carriers (n=48). However, we failed to see the direct effect of increased plasma PCSK9 association with either of any biochemical parameters (data not shown).

Table 13. Plasma PCSK9 levels (ng/mL) in total population of Group-II

GROUP-II	Total (n= 898)	CC (n=444)	CT (n=376)	TT (78)	p value	CC Vs CT	CC vs TT	CT VS TT
PCSK9 LEVELS	292.39 ± 6.35	277.49± 8.71	303.52± 10.22	324.34± 20.95	**0.045**	0.051	**0.045**	0.38

Table 14. Genderwise plasma PCSK9 distribution (ng/mL) among GPER genotypes of Group-II

GROUP-II	ALL (n=898)	CC	CT	TT	P	CC Vs CT	CC vs TT	CT VS TT
MEN	269.49 ± 9.30	258.42 ± 12.56	287.39 ± 14.99	254.09 ± 35.12	0.298	0.141	0.901	0.351
WOMEN	307.51 ± 8.51	291.25 ± 11.90	313.27 ± 13.65	366.92 ± 24.13	0.043	0.217	**0.015**	0.086

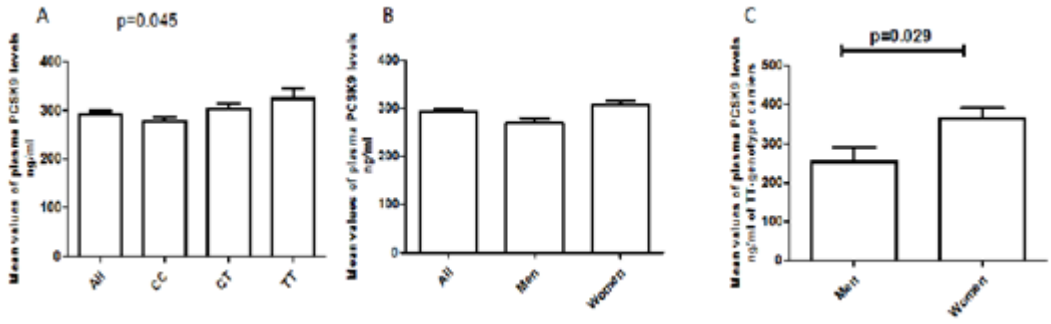

Figure 31. Plasma PCSK9 distribution in PLIC cohort according to rs11544331 variants of GPER gene.

Panel (a) Mean values of circulating plasma PCSK9 levels in total population according to genotypes of rs11544331 variants of GPER gene, carriers of TT-genotypes (p.P16L) had higher circulating plasma PCSK9 levels than their counterparts. Panel (b) Representing the circulatory plasma PCSK9 levels difference between sexes, women carriers presenting higher plasma PCSK9 levels than men in our studied cohort. Panel (c) A difference of mean values of plasma PCSK9 levels between TT-carriers of men and women, where women had about 112.83ng/ml high plasma PCSK9 levels than men of similar genotype (L16)

All values are in Mean ± SEM

4.21. Carotid atherosclerosis progression is not associated with GPER rs11544331 SNP

We further evaluated the GPER variants (rs11544331) role in the progression of carotid atherosclerosis, and to the best of our knowledge, this is the first evaluation performed in relation to GPER activity in Italian general population. GPER P16L genotypes in total population of Group I and II, and Women cohort of group I and II were chosen to screen the association at prima facie. No significant association between GPER genotypes (CC, CT and TT) in total population (0.647 ± 0.005; 0.642 ± 0.006, 0.651 ± 0.012 respectively; p=0.726) and women (0.637 ± 0.006, 0.636 ± 0.007, 0.631 ± 0.014; p=0.944) of group-I and in total population ((0.745 ± 0.006, 0.748 ± 0.007, 0.736 ± 0.013; p=0.764) and women (0.741 ± 0.008, 0.739 ± 0.009, 0.734 ± 0.015; p=0.944) of group-II were observed- [**Table 15**].

To deepen our research, we further sorted our data of the common participants from the 10 year follow up study. A total of 512 subjects (men= 206; women=306) were stratified according to rs11544331 variants of GPER gene and association study were performed to see their influence on the progression of cIMT. No association were observed neither at baseline (p= 0.250) nor after 10 year follow up study (p=0.877) in total cohort. Same were true when analysed for men and women cohort for baseline and in 10 year longitudinal study (p= 0.846 vs p=0.112 respectively; p= 0.942 vs 0.863 respectively)- [**Table *16***].

Table 15. Representation of intima-media thickness of carotid artery according to rs11544331 genotypes of GPER gene in PLIC subjects of **Group I and II**.

		GROUP-I (N=1405; Men=575 ; Women = 830)					**GROUP-II** (n=898; Men=354; Women= 544)			
	All	**CC**	**CT**	**TT**	**p**	**All**	**CC**	**CT**	**TT**	**p**
Total	0.646 ± 0.003	0.647 ± 0.005	0.642 ± 0.006	0.651± 0.01	0.320	0.746 ± 0.004	0.745 ± 0.06	0.748 ± 0.007	0.736 ± 0.013	0.764
Men	0.65 ± 0.005	0.66 ± 0.008	0.651 ± 0.006	0.678 ± 0.021	0.405	0.755 ± 0.007	0.75 ± 0.009	0.76 ± 0.011	0.74 ± 0.025	0.56
Women	0.636 ± 0.004	0.637 ± 0.006	0.636 ± 0.007	0.631± 0.01	0.944	0.740 ± 0.005	0.741 ± 0.008	0.739 ± 0.009	0.734 ± 0.015	0.94

Table 16. Prospective studies of PLIC subjects representing the intima-media thickness of carotid artery according to rs11544331 genotypes of GPER at baseline and 10 years follow up.

	Total (n=512)		**Men (n=206)**		**Women(n=306)**	
	Baseline	After 10 yr	Baseline	After 10 yr	Baseline	After 10 yr
All	0.623 ± 0.005	0.739 ± 0.005	0.637 ± 0.009	0.747 ± 0.008	0.614 ± 0.007	0.733 ± 0.007
CC	0.621 ± 0.007	0.731 ± 0.007	0.632 ± 0.01	0.734 ± 0.01	0.613± 0.009	0.729 ± 0.01
CT	0.628 ± 0.009	0.750 ± 0.009	0.643 ± 0.015	0.771 ± 0.015	0.616 ± 0.01	0.737 ± 0.01
TT	0.618 ± 0.02	0.728 ± 0.01	0.634 ± 0.04	0.723 ± 0.03	0.607 ± 0.02	0.731 ± 0.02
p	0.877	0.250	0.846	0.112	0.942	0.863

Note:- Intima-media thickness was measured in millimetres and Values are indicated in Mean ± SEM, and p-value considered significant if less than 0.05.

SECTION [B3]:- IDOL ACTIVITY ON LIPID METABOLISM AND PERIPHERAL ATHEROSCLEROSIS PROGRESSION

4.22. IDOL genotype and allelic distribution frequency

No deviation from the Hardy-Weinberg equilibrium was observed for the rs9370867 (c.G1025A) SNP. In the PLIC population analyzed (n=1384) the allelic frequencies were 48.01% and 51.99% for the G and A allele respectively, no significant differences in the genotypes distribution between men (n=485) and women (n=899) were observed [**Table 17**].

Table 17. Genotypic [n(%)] and allelic frequencies (%) of the rs9370867 SNP in the PLIC population.

		Genotype				Allele	
	n	**GG**	**GA**	**AA**		**G**	**A**
PLIC	1384	328(23.7%)	673(48.6%)	383(27.7%)		48.01%	51.99%
Men	485	127(26.2%)	230(47.4%)	128(26.4%)		49.9%	50.1%
Women	899	201(22.4%)	443(49.3%)	255(28.4%)		47%	53%
χ2	2.621					2.552	
P-value	0.454					0.110	

4.23. General and clinical characteristics of PLIC subjects according to the rs9370867 SNP.

No major differences were found in the anthropometric and biochemical parameters, such as total cholesterol (TC), LDL-C, high density lipoprotein cholesterol (HDL-C), triglycerides (TG) and glycemia among carriers of the different genotypes. [**Table 18**]. Previous association of the rs9370867 SNP with cholesterol metabolism were observed in a Mexican dyslipidemic population, characterized by high levels of TC and TG. For this reason we stratified the PLIC population according to TG and TC plasma levels and we repeated the

analysis in two sub-groups upper the 75th percentile of TC (mean value 271.2 ± 23.7 mg/dL) and TG (mean value 191 ± 71,1mg/dL). The results were similar compared to the whole population and showed no significant impact of the rs9370867 (c.G1025A) SNP on the lipid profile [**Table 19** A& B].

Table 18. Anthropometric and biochemical characteristics according to the rs9370867 SNP in subjects from the PLIC population

	GG	AG	AA	P-value GG vs AA	P-value GG vs GA	P-value AA vs GA
Age	65.21 ± 9.97	64.36 ± 1.084	64.90 ± 9.88	0.735	0.342	0.515
Body mass index (Kg/m2)	26.8 ± 4.6	26.7 ± 4.3	27.0 ± 4.2	0.756	0.739	0.456
Total cholesterol (mg/dL)	209.9 ± 35.9	206.1 ± 34.6	204.7 ± 34.3	0.089	0.172	0.570
LDL-cholesterol (mg/dL)	125.2 ± 32.8	123.3 ± 31.9	122.6 ± 31.6	0.356	0.449	0.775
HDL-cholesterol (mg/dL)	64.60 ± 18	63.08 ± 16.18	62.64 ± 14.8	0.168	0.249	0.701
Triglycerides (mg/dL)	100.5 ± 42.4	98.91 ± 48.0	97.27 ± 45.6	0.391	0.646	0.634
Apolipoprotein A-I (mg/dL)	158.2 ± 23.4	156.4 ± 21.0	155.3 ± 20.7	0.133	0.311	0.455
Apolipoprotein B (mg/dL)	108.7 ± 22.1	105.6 ± 22.2	107.0 ± 22.2	0.389	0.078	0.380
SBP (mmHg)	128 ± 17	129 ± 18	128 ± 18	0.568	0.352	0.749
DBP (mmHg)	82.03 ± 9.4	82.37 ± 8.7	82.32 ± 8.8	0.983	0.228	0.195
Glucose (mg/dL)	95.38 ±15.89	94.97 ± 17.93	92.94±13.72	0.057	0.762	0.091

Table 19. Clinical characteristics according to the rs9370867 SNP in subjects from the PLIC population included in the upper 75th percentile of total cholesterol (A) and triglycerides (B).- Multivariate Analysis.

19[A] Upper 75th percentile of Total Cholesterol

	GG (n= 74)	AG (n= 130)	AA (n= 72)	P-value GG vs AA	P-value GG vs GA	P-value AA vs GA
Age	61.97 ±10.58	63.06 ± 10.08	64.97 ± 10.22	0.293	0.708	0.207
Body mass index (Kg/m2)	27.2 ± 4.2	25.8 ± 3.9	26.1 ± 3.3	0.198	0.076	0.340
Total cholesterol (mg/dL)	249.57 ± 21.7	249.76 ± 18.58	248.90 ± 21.54	0.866	0.593	0.850
LDL-cholesterol (mg/dL)	158.83 ± 25.0	161.87 ± 22.16	160.20 ± 23.01	0.595	0.488	0.340
HDL-cholesterol (mg/dL)	68.57 ± 19.57	65.08 ± 16.04	65.63 ± 14.18	0.208	0.401	0.078
Triglycerides (mg/dL)	110.86 ± 47.65	114.02 ± 53.71	115.38 ± 53.99	0.601	0.465	0.357
Apolipoprotein A-I (mg/dL)	161.41 ± 25.46	159.54 ± 22.59	158.01 ± 18.38	0.101	0.094	0.060
Apolipoprotein B (mg/dL)	130.88 ± 19.82	132.85 ± 18.16	131.72 ± 21.18	0.525	0.809	0.265
Glucose (mg/dL)	95.08 ± 12.46	93.64 ± 11.78	91.35 ± 8.88	0.201	0.093	0.941
Left Ventricular Mass	184.95 ± 64.78	156.48 ± 66.93	165.74 ± 71.88	0.268	0.109	0.677
cIMT	0.74 ± 0.14	0.75 ± 0.17	0.77 ± 0.15	0.607	0.806	0.424
SBP	125.0 ± 16.0	132.0 ± 20.0	126.0 ± 14.0	0.076	0.036	0.107
DBP	78.0 ± 9.0	80.0 ± 12.0	77.0 ± 7.0	0.205	0.154	0.126

(Table 19B) Upper 75th percentile of Triglycerides

	GG (n=43)	AG (n = 81)	AA (n= 41)	P-value GG vs AA	P-value GG vs GA	P-value AA vs GA
Age	62.42 ± 10.25	64.10 ± 10.55	64.33 ± 8.77	0.288	0.575	0.563

Body mass index (Kg/m2)	28.0 ± 5.5	27.1 ± 4.1	28.4 ± 3.9	0.495	0.663	0.233
Total cholesterol (mg/dL)	220.53 ± 39.86	221.86 ± 35.22	224.95 ± 41.25	0.678	0.476	0.234
LDL-cholesterol (mg/dL)	134.11 ± 38.80	136.27 ± 34.15	137.33 ± 40.20	0.987	0.332	0.322
HDL-cholesterol (mg/dL)	52.81 ± 11.16	49.02 ± 11.34	51.07 ± 13.29	0.503	0.525	0.167
Triglycerides (mg/dL)	168.05 ± 38.67	182.85 ± 55.85	182.76 ± 57.24	0.483	0.161	0.485
Apolipoprotein A-I (mg/dL)	145.84 ± 19.88	142.77 ± 18.69	141.83 ± 18.12	0.221	0.591	0.434
Apolipoprotein B (mg/dL)	118.42 ± 25.87	118.16 ± 24.72	119.07 ± 27.01	0.964	0.338	0.297
Glucose (mg/dL)	96.77 ± 16.90	99.42 ± 26.12	100.17 ± 16.21	0.482	0.651	0.218
Left Ventricular Mass	190.64 ± 58.76	167.56 ± 60.78	200.33 ± 84.51	0.494	0.295	0.069
cIMT	0.79 ± 0.18	0.78 ± 0.19	0.76 ± 0.14	0.297	0.433	0.745
SBP	130.0 ± 17.0	134.0 ± 18.0	129.0 ± 21	0.65	0.193	0.384
DBP	79.0 ± 70	83.0 ± 10.0	78.0 ± 6.0	0.81	0.008	0.003

4.24. Impact of the rs9370867 SNP for IDOL on carotid atherosclerosis and the prevalence of cardiovascular events.

Although no association between the rs9370867 SNP and LDL-C or other lipid variables were observed, this does not exclude the possibility that this functional SNP might affects other pathways associated with cardiovascular disease. Therefore to further investigate the potential role for the rs9370867 SNP, its association with left ventricular mass and intima media thickness of carotid arteries (**Figure 32** A and B) and the incidence of coronary heart disease, peripheral artery disease and cardiovascular events (CHD, PAD and CVE) was investigated (**Figure 33** A to D). The results show that the rs9370867 genotype is not associated with any of the parameters investigated. Also when carriers of the A allele both in homozygosis and in heterozygosis were compared to carriers of the G allele, no

differences in the odd ratio for the incidence of CHD, PAD or CVE were observed. These data limit the impact of rs9370867 SNP for IDOL on cardiovascular outcome.

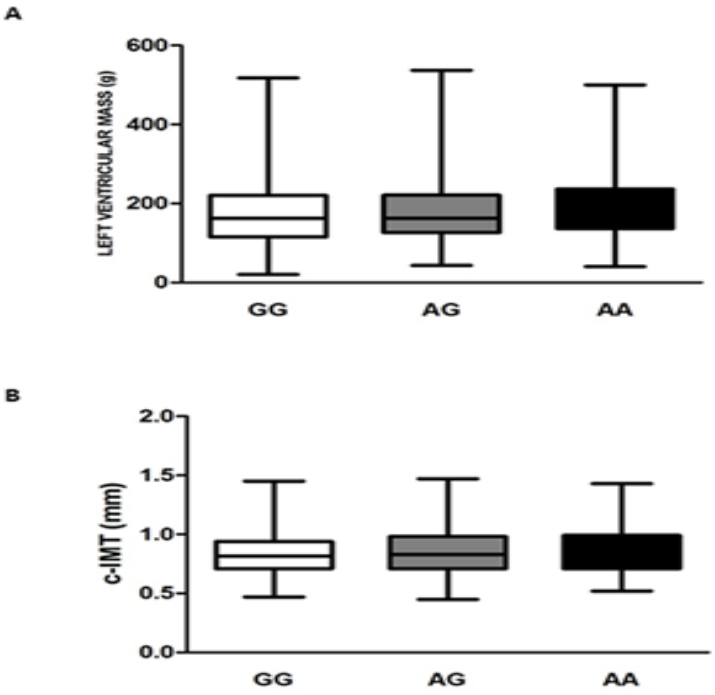

Figure 32. Heart left ventricular mass and carotid intima media thickness of the common carotid arteries in the PLIC population according to the rs9370867 IDOL SNP

Panel A shows the mean ± SD for left ventricular mass for GG, AG or AA carriers. Panel B shows the mean ± SD of carotid intima media thickness of the common carotid arteries (right and left) for GG, G or AA carriers.

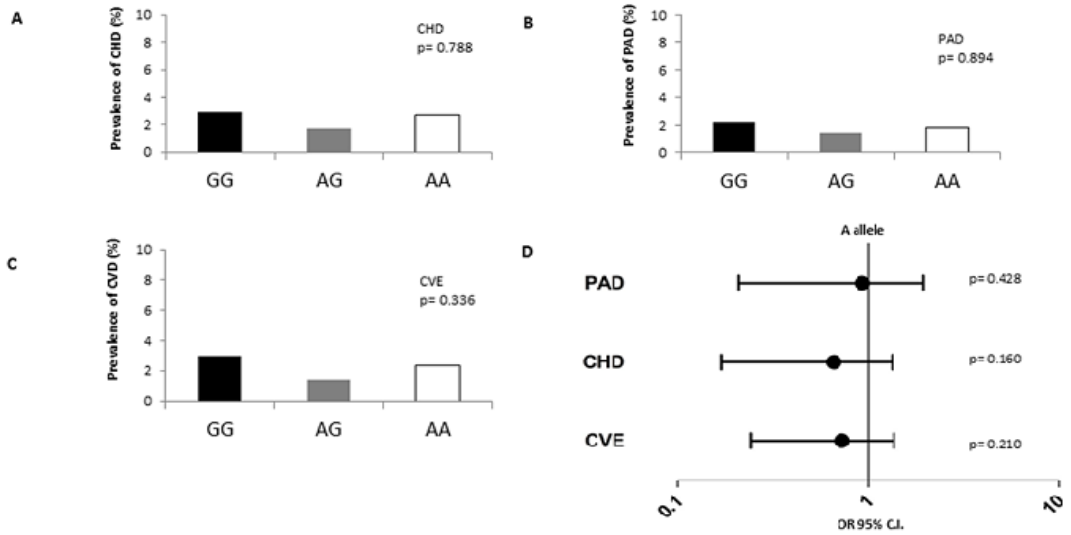

Figure 33. Incidence of coronary heart disease (CHD), peripheral artery disease (PAD) and cardiovascular events (CVE) in the PLIC population according to the rs9370867 SNP.

Panels A, B and C shows the incidence of CHD, PAD and CVE for GG, AG or AA carriers of the rs9370867 IDOL SNP. Panel D reports the OR (Odd Ratio), the 95% Confidence Interval (C.I.) and the P- values for CHD, PAD and CVE for carriers of the A allele of the rs9370867 SNP.

4.25. LDLR expression and activity according to the rs9370867 SNP.

The relevance of the rs9370867 IDOL SNP on LDL-R expression was further investigated in macrophages generated from peripheral blood mononuclear cells from N342 or S342 IDOL carriers. LDL-R expression and the capacity to internalize LDL particle, as a functional readout of the LDL-R activity. LDL-R mRNA expression and IDOL mRNA expression were similar in N342 and S342 macrophages (**Figure *34*** A) and more importantly the genotype was not associated with differences in LDLR function. N342 and S342 macrophages showed indeed the same levels of LDL uptake, as assessed by flow cytometry after incubation of the cells with fluorescently labelled LDL particle (**Figure *34*** B and C).

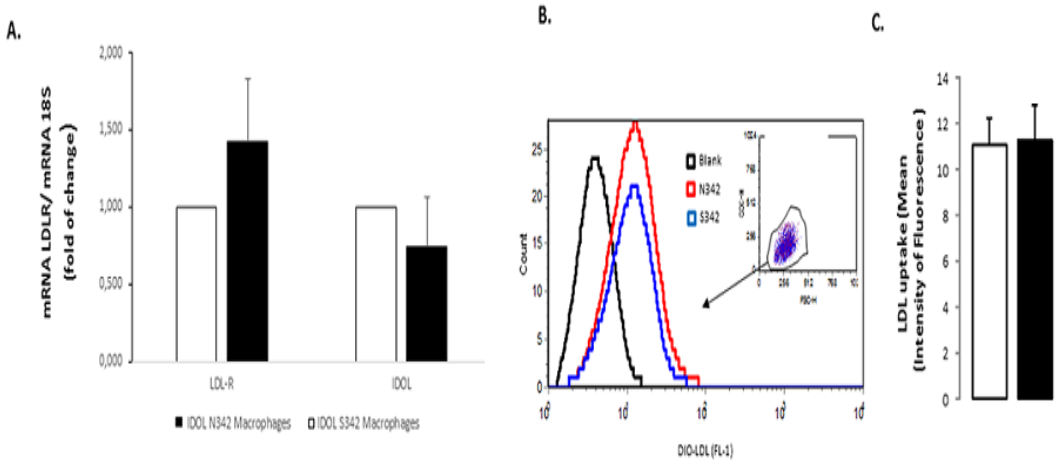

Figure 34. mRNA expression of LDL-R and IDOL and LDL uptake in macrophages from N342 and S342 macrophages.

Panel A shows mRNA expression for LDL-receptor and for IDOL in macrophages obtained from N342 and S342 carriers (n=5 for both genotypes) cultured in complete RPMI medium The results, normalized for the expression of an housekeeping gene (18S), data are shown as mean ± SEM. Next flow cytometry was used to study the LDL uptake in macrophages obtained from N342 or S342 carriers. To determine the LDL uptake, macrophages were incubated with fluorescently labelled LDL (DiO-LDL, 10 µg/mL) for 2 hours at 37°C. Panel B shows representative images for flow cytometry in N342 and S342 macrophages, with the gating strategy of macrophages (inside panel) and the fluorescence intensity for Dio-LDL while panel C shows the results from five N342 and five 342 patients (mean intensity fluorescence is given, mean ± SEM is shown).

CHAPTER 5. DISCUSSION

In my studies I have intended to understand and characterise the role of three genes (known to regulate LDL-R) over lipid metabolism, glucose homeostasis and their role on the progression of peripheral atherosclerosis. For that purpose, the study was conducted on both mice and human models. In addition, certain functional characterisation of selected genetic polymorphism was also conducted in *in-vitro* studies.

The major findings of my study is divided into the direct role of PCSK9 protein in carotid atherosclerosis progression and glucose metabolism in mice models, while the selected genetic variants of three genes (PCSK9-R46L, IDOL-N342S and GPER-P16L) were evaluated for their association with anthropometric parameters, lipid phenotypes and peripheral atherosclerosis progression in human subjects those were enrolled in our PLIC (progression of lesion in intima-media of carotid artery) study.

5.1 MICE STUDY

In mice, I have studied the physiological role of PCSK9 protein on LDLR activity and glucose homeostasis which was further extended to study our hypothesis of PCSK9 role in vascular plaque modulation- derived from our previous findings related to PCSK9 secretion by vascular cells[163].

Proprotein convertase subtilisin/kexin type-9 (PCSK9) binds to LDL receptor (LDLR) and targets it for lysosomal degradation in the cells. Decreased hepatic clearance of plasma LDL-cholesterol is the primary concern of PCSK9 activity. For this reason, we have evaluated the effective role of PCSK9 in our studied mice models, the two line of mice carrying PCSK9 allele (PCSK9 WT) and null allele (PCSK9- KO) were first generated and evaluated for the effective role of PCSK9 over LDLR viability. Our results indicate that, mice carrying PCSK9 allele (WT) causes about 71% reduction in LDLR expression when compared to PCSK9-KO mice. This reduction of cholesterol was further seen in reduction in circulating HDL particle, since HDL is the prominent cholesterol carrier in mice, PCSK9-KO mice also presented the

less HDL particle (about -50%) in lipoprotein fractionation profile (FPLC), might be due to high in LDLR expression leading the rapid clearance of ApoE containing lipoproteins.

The information of PCSK9 role on hypercholesterolemia is well established, although the role of PCSK9 on atherosclerosis progression is still under investigation, however, few recent studies have correlated plasma PCSK9 levels with carotid atherosclerosis as measured by carotid intima-media thickness (cIMT), and was found associated in heterozygous (He) FH subjects[79], in chronic chest pain subjects who underwent angiography[164], in hypertensive cohort [80], and was also seen associated with PCSK9 allelic changes like E670G[165].

These reports prompted us to evaluate the direct role of PCSK9 in peripheral atherosclerosis progression. In virtue, we have previously demonstrated that PCSK9 was locally produced by vascular smooth muscle cells (VSMCs) and might play a leading role in the modulation of atherosclerotic plaque composition[163], for this reason we first aim to evaluate the direct role of PCSK9 with atheroma burden. We utilised the advantage of pre-established perivascular technique, and compared the neo-intimal lesions induced by peri-adventitial carotid placement of a non-occlusive collar in mice of both genotypes (PCSK9 WT and PCSK9-KO). We have observed a remarkable reduction of about 49% in intimal thickening in PCSK9KO mice when compared with PCKS9-WT mice (12.344 ± 1.66 vs 23.95 ± 1.82µm²; p=0.049) **[Figure 18],** this reduction was further characterised where carotid lesions had reduced smooth muscle cells (SMC) content of about 49% in comparison to the carotids of PCSK9WT mice (10.7 ± 1.97 vs 21.0 ± 7.56%; p= <0.05)-**[Figure 19].,**. The characterisation of vascular smooth muscle cells (VSMCs) derived from both genotypes further revealed the dedifferentiated profiling of VSMCs rapidly occurs in PCSK9-WT mice, where PCSK9-KO mice had higher α-actin (VSMCs contractile marker) and lesser caldesmon (VSMCs synthetic marker) mRNA levels in comparison to PCSK9-WT mice**[Figure 20].,**. These Vascular cells from PCSK9-KO mice also had reduced doubling time profile of about 47% in proliferation and low migration profile than PCSK9WT mice **[Figure 21-22].** Therefore we

report that PCSK9 deficiency not only maintain the healthy lipid profile but further reduces the risk of peripheral atherosclerosis, however we have not seen the similar outcomes when evaluated the carotid atherosclerosis progression in human subjects carrying the PCSK9-loss-of-functioned (p.R46L) gene variants, the results from carotid scanning of R46L subjects had the similar progressive profile as of PCSK9 R46R carriers. PCSK9 R46L is a non-synonymous change and leads the impairment of PCSK9 secretion from hepatocytes. The phenotypes of PCSK9 R46L mimics the PCSK9 partial deficient profile at certain extent. However, we have seen a dramatic impact of R46L change over glucose and lipid metabolism-will be discussed further.

We further characterize the role of PCSK9 in glucose homeostasis- which was also existed with admixed results from several studies, we use the high-fat diet (HFD) as a model for impaired glucose tolerance (IGT). All mice of each group (PCSK9 -WT and PCSK9-KO) were fed a high fat diet (45% kcal energy by fat) and a normal control SFD-diet (11 % fat). Intraperitoneal glucose challenge revealed a modest increase in glucose intolerance in PCSK9-KO mice from time 20 min onwards, we further challenged the mice with intraperitoneal insulin and checked the glucose absorbance, no difference were observed in glucose disposal at different check points upon insulin injection, suggesting that PCSK9-KO mice might have some defect in pancreatic beta cells. Our data suggest that the absence of PCSK9 allele causes the slow metabolic rate leads to glucose intolerance phenotypes probably due to pancreatic islet abnormalities, and this was also true in case of humans, where PCSK9 R46L carriers also found with high in plasma glucose levels than R46R carriers ($p<0.004$;**Table 2**). First in mice, the energy intake and weights of mice throughout the intervention was similar between the mice group (PCSK9-KO vs WT) of each dietary set (HFD vs control). However, the difference was observed in growth curve of glucose absorbance measured by glucose tolerance test (GTT), performed at two check points (of week 12^{th} and 20^{th}). The outcomes of glucose absorbance difference between the twos (PCSK9-KO vs WT) of high fat diet groups observed in early intervention phase (week 12^{th} post-inception), which further emerged as a strong phenotype during the late intervention phase (week 20^{th}

post-inception). We have observed the mild phenotypes of glucose intolerance was existed after time 60min post glucose challenge in early week 12 of intervention phase while the phenotypes of glucose intolerance was more stronger when characterised in late intervention phase of week 20th, where low glucose disposal was existed right from the time 20 min and it was remained higher uptil 120min [**Figure 25**]. The support of our supposition of pancreatic islet cell abnormalities arose from the intraperitoneal insulin challenge test and hepatic gene expression profiling, where intraperitoneal challenge of insulin in twos (PCSK9KO and WT) of HFD- groups did not have significant difference in glucose disposal rate [**Figure 26**], neither had any difference in hepatic insulin receptor gene expressions-determined by internally controlled reverse transcriptase polymerase chain reaction (RT-PCR), both insulin receptors IRS-1 and IRS-2 mRNA in PCSK9 KO and WT had similar pattern of expressions in HFD-diet group [**Figure 27**], suggesting that the slow metabolic rate or slow glucose disposal rate in PCSK9KO mice is might be due to the impaired secretion of insulin from pancreatic beta cells and thus causing hypoinsulinemic and hyperglycemic profile. Our this findings also goes in line to the research conducted by Mbikay et al [71].

Similarly, we have observed the same phenotypes when studied in humans, our study on common PCSK9 LOF variant (p.R46L), also displayed the similar results in humans. This non-synonymous change (p.R46L) on PCSK9 gene causes defect in PCSK9 autocatalytic activity and hence impairs its secretion from hepatocytes, leads in causing low in plasma PCSK9 volume. Therefore our study on PCSK9 association with glucose homeostasis on humans subjects also draw the same results as seen in mice, the low levels of plasma PCSK9 was associated with subjects carrying R46L allele and presented about 7% higher in plasma glucose levels than subjects carrying R46R allele [**Table 2**]. Therefore our data from both mice and human suggest that PCSK9 deficiency causes impaired glucose tolerance due to pancreatic islet abnormalities. Although, our results are contradictory to the observatory data accrued from clinical trials of PCSK9 monoclonal antibodies, however require long term follow ups.

5.2 IN HUMANS

In humans, my study was begin with the thirst to observe the PCSK9 related phenotypes as seen in mice study. For that reason, we have opted to dissect the role of PCSK9 polymorphic activity which mimics the PCSK9 partial deficiency in humans. We choose PCSK9 rs11591147 (p.R46L) variants for our study. PCSK9 p.R46L is known as a common variant in Caucasian subjects and represents minimal circulating plasma PCSK9 levels. Similarly, the simultaneous selected polymorphisms of IDOL gene (rs9370867; p.N342S) and GPER gene (rs11544331; p.P16L) were also studied those were identified in a genome-wide association study (GWAS) based studies and found associated with lipid levels. The general information about studied genes mentioned in **Discussion table *1***.

The initial screening of studied variants was their penetration and the effect size in our population [**Discussion table *2***], the frequencies of all the loci (PCSK9 and GPER) were similarly matched as found in other referred population except the causal allele of IDOL gene [**Discussion table *3***]. The studied allelic frequencies of PCSK9 (rs11591147; p.R46L)) and IDOL (rs9370867; p.N342) in different population can be seen in **Discussion table *4***

The spectrum of studied genetic variants of PCSK9 (p.R46L), GPER(p.P16L) and IDOL (p.N342S) gene with clinical variables in our studied cohort was not too strong as seen in various other population based studies, however this disparity might be due to the various dependent variables like demographic change, ethnicity, life styles etc. The magnitude of effect of homozygous mutated loci of each studied gene when evaluated for their association with LDL-c and total cholesterol levels against their respective homozygous wild type allele was maximally associated with increase of 3.52mg/dL and 5.23mg/dL respectively of mean levels in case of GPER p.P16L polymorphisms while it was only 2.6mg/dL and 5.2mg/dL respectively in case of IDOL p.N342S polymorphism. However, the studied polymorphism of PCSK9 gene (p.R46L) is a loss-of-functioned (LOF) variants and presents a better lipid profile, in our case the carriers of 46L (n=42) had about 10.2 mg/dL and 9.75 mg/dL reduced levels of LDL-c and total cholesterol respectively than subjects

carrying homozygous 46R allele [***Discussion table 5***]. In addition, as expected carriers of 46L had about 7.2 mg/dL of increased mean levels of glucose levels (p=<0.004), and 2.43mg/dL in case of GPER 16L carrier but could not reach to the statistical significance (p=0.06), while it was reverse in case of IDOL where supposedly homozygous mutated allele (342S) carriers had increased glucose levels by 2.44mg/dL than 342N carriers (p=0.057). Other variables like BMI, triglycerides, HDLc, ApoA1, ApoB, SBP, DBP and cIMT did not reach to the statistical significance in case of IDOL and GPER gene variants. However, subjects carrying PCSK9 p.46L allele also had reduced HDL (p=0.022) as expected but increased triglycerides (p=0.004) levels ***[Discussion table 6*]**.

Our study suggest that, except PCSK9 p.R46L variants, not other studied loci had profound impact on lipid phenotypes neither on anthropometric parameters and nor on cIMT progression [**Discussion table *7***]. PCSK9 allele R46L was associated with significantly beneficial profile with a range of effects on plasma levels of lipoproteins (esp. LDL-C). However, we observed that PCSK9 deficiency have both beneficial and harmful outcomes (as observed in case of glucose in mice and human too).

Our next examination was on the levels of plasma PCSK9 protein. Here we studied the effects of our selected gene polymorphisms of PCSK9 (rs1191147; p.R46L), GPER (rs11544331; p.P16L) and IDOL (rs9370867; p.N342S) on plasm PCSK9 levels. This study not only unravel the effects of PCSK9 R46L variants on plasma PCSK9 but also attributed further in our knowledge about the ecstatic effect of other studied genes like GPER and IDOL **[Discussion table *8*]**. Since, hypolipidemics drugs (like statins) have confounding effect over PCSK9 expression [Figure 27], thus we have only selected subjects those were free of hypolipidemics drugs. GPER studied variant (rs11544331; p.P16L) but not IDOL gene variants (rs9370867; p.N342S) had profound influence on the plasma PCSK9 levels (p=0.045), however was found sex specific (p=0.043). Women carriers of GPER mutated allele (16L carrier) had about 112ng/mL of elevated plasma PCKS9 levels than women carriers of homozygous wild type allele **(p=0.015)**. The difference in plasma PCSK9 were further

statistical relevant when compared with men of similar 16L carrier **(p=0.029) [Discussion table *9*].** The reason behind the regulation of PCSK9 expression via GPER activity is not known, however, the regulatory action of estrogen on PCSK9 levels might be explained by GPER activity. In one study, overexpression of IDOL gene also had a profound effect on PCSK9 protein expression [166], and GWAS based identified polymorphism rs9370867 (p.N342S) on IDOL gene also had increase in lipid profile, might also indicate the co-ordinating activity with PCSK9 protein. However, in our study we found neutral activity of IDOL polymorphism (rs9370867; p.N342S) not only on lipid phenotypes, cIMT but also when studied their effect on plasma PCSK9 levels (p=0.092).

However, when analysed for PCSK9 R46L variants, the dramatic changes in plasma PCSK9 variants (R46L) levels were seen in total population and that was irrespective of gender we found a drop of about 157.36ng/ml of PCSK9 in subjects carrying 46L (mutated allele) when compared with 46R carriers($p<0.001$), and a significant drop in both men (-40%; p=0.04) and women (-57.38%; p=0.001) were found **[Discussion table *8* & Discussion table *9*]**.

Our observation suggest that not only the variants of PCSK9 gene but other gene variants also influences plasma PCSK9 levels.

We further investigated the role of plasma PCSK9 levels irrespective to the effect of gene variants. The distribution of plasma PCSK9 levels in our studied population was not normally distributed, a wide range of PCSK9 levels were found (8ng/ml- 1432ng/ml) in the population, this might be due to the high in number of women and aged people in our studied population. The mean level of plasma PCSK9 in total population was 285.3 ng/ml, while it was higher in women than men cohort (296.35 vs 267.84 ng/ml; p= 0.008). The levels in women was further found maximum in post-menopausal phase than pre-menopausal phase (p=0.007)- **[Figure 28]**. Subjects with lipid lowering drugs also had higher trend of plasma PCSK9 levels than subjects without lipid lowering drugs ($p<0.001$)**[Figure 29]**. When evaluated further, plasma PCSK9 levels were found associated with various lipid

variables in both univariate analysis and after adjustment for age and gender in a multivariate regression analysis, including Total cholesterol ($p<0.001$, $\beta=0.196$), LDL ($p=0.002$, $\beta=0.094$), HDL ($p<0.001$, $\beta=0.124$), triglycerides ($p<0.001$; $\beta=0.114$), however all these association were found modestly stronger in men than women cohort **[Table 6].** Although there was no association of plasma PCSK9 with blood pressure and glucose levels was appeared in a univariate and multivariate analysis, but the associations with lipid parameters prompted us to evaluate the role of PCSK9 in a metabolic syndrome (MetS) phenotype, As expected, a group of MetS subjects had about 8% (22ng/ml) higher plasma PCSK9 levels than subjects without MetS phenotypes ($p=0.016$), this was further found related with men cohort, when performed genderwise analysis. Men cohort of MetS but not women cohort had a significant elevation of plasma PCSK9 levels than men of non-MetS group ($p=0.04$) **[Table 7].**

As stated before there was no association of IDOL gene and GPER gene polymorphisms on the lipid levels were appeared in our population based study. However, a strong association of IDOL polymorphism with lipid levels was described in GWAS study[160] and in Mexican population based study[9], which prompted us to characterise the functionality of IDOL SNP (rs9370867; p.N342S) at in-vitro level too, the selected subjects of the IDOL p.N342S genotypes (AA and GG) were re-called and analysed further for LDLR expression and variability in LDL uptake, as expected, no variation in LDLR expression and LDL uptake were observed among the IDOL genotypes.

Our observation suggest that among the studied genetic variants of three genes (PCSK9, GPER, and IDOL) only PCSK9 variants (R46L) have profound effect on lipids and glucose levels, while no association with lipid and glucose was found when studied for GPER (p.P16L) and IDOL (p.N342S) in Italian population. Neither of three studied loci (PCSK9, GPER and IDOL) have any influence on intima-media thickness of carotid artery (cIMT). Further, PCSK9 p.R46L and GPER p.P16L variants influences plasma PCSK9 levels. These plasma

PCSK9 levels had high impact on lipid variability and found increased in subjects with metabolic syndrome phenotypes.

CHAPTER 6. CONCLUSION

Conclusion

In My study- among the three studied genes, PCSK9 role on glucose homeostasis is clearly demonstrated in both mice and human subjects, however its role on carotid atherosclerosis progression still require more study. Higher plasma PCSK9 levels also marked huge impact on lipoprotein levels and increases in subjects with metabolic syndrome (MetS) phenotypes, all these associations were more strongly appeared in case of men. While GPER and IDOL studied polymorphisms have no influence on lipid metabolism, glucose levels and peripheral atherosclerosis progression in Italian general population. Although, we observed that GPER variants are associated with sex-specific elevation of plasma PCSK9 levels and require further investigation for the underlying molecular mechanism.

CHAPTER 7. KEY FINDINGS

Key Findings

[A] From Mice study

1. Proprotein convertase subtilisin/kexin type 9 (PCSK9) protein induces hepatic LDLR degradation and hence increases plasma cholesterol level.
2. Upon perivascular manipulation- the presence of PCSK9 increases the risk of neo-intimal hyperplasia, probably due to enhanced dedifferentiating phenotypes of underlying vascular smooth muscle cells.
3. PCSK9 deficiency however found linked to glucose intolerance.

[B] From Human study

(i) Findings from Proprotein convertase subtilisin/kexin type 9 (PCSK9) study

1. The role of loss-of-functioned (LOF) variant of PCSK9 rs11591147 (p.R46L) presents favourable lipid phenotype profile.
2. Similar to the mice, PCSK9 p.R46L causes increase in blood glucose levels.
3. PCSK9 p.R46L carriers also presents low plasma PCSK9 levels.
4. This partial deficient phenotype of PCSK9 did not present significant impact on reduction of intima-media thickness of carotid artery (cIMT)-as shown in mice study with PCSK9 deficient phenotype.
5. Plasma PCSK9 levels are highly varied in Italian general population.
6. Plasma PCSK9 levels are age and sex dependent- women carries higher plasma PCSK9 levels than their age-matched men, and PCSK9 levels further goes maximum upon menopause.
7. Hypolipidemic drugs (like Statins) increases plasma PCSK9 levels.
8. Plasma PCSK9 levels found associated with various lipoproteins, including LDL, HDL, Triglycerides, ApoAI, and ApoB.

Key Findings

9. High Plasma PCSK9 levels does not promote carotid atherosclerosis progression neither glucose intolerance, while low levels of PCSK9 affects glucose metabolism.
10. Men with high plasma PCSK9 levels present modest associations with clinical variables while women are generally protected.
11. Plasma PCSK9 levels increases in men with metabolic syndrome phenotypes.

(ii) Findings from G-protein coupled estrogen receptor (GPER) study

1 GPER rs11544331 (p.P16L) variants does not alter lipid levels and neither Influences intima media thickness of carotid artery (cIMT) - in Italian general population.

2 Neither sex-specific influence of GPER p.16L on lipid variables were found as seen in other referred studies.

3 GPER p.P16L variants however increases plasma PCSK9 levels in women.

(iii) Findings from Inducible degrader of LDL-R (IDOL) study

1. IDOL rs9370867 (p.N342S) variants does not alter glucose and lipid variable in Italian general population.
2. Neither IDOL p.N342S variants influences plasma PCSK9 levels nor intima-media thickness of carotid artery in Italian general population.
3. The *in-vitro* functional characterization of IDOL p.N342S variants also did not present the functional difference among the genotypes for LDLR expression and LDL uptake ability.

CHAPTER 8. TABLES FOR DISUCUSSION

Tables for discussion

Discussion table 1. GWAS identified and PLIC studied loci-associated with Lipid phenotypes and CHD

Gene	Chromosome	Chromo-some position	Contig position	rs number	Location	Nucleotide change	Amino acid change	Unicluster ID	Population studied	Reference
PCSK9	1p32	55039974	54453986	rs11591147	Exon	c.5428. G>T	p.R46L	18844	Whites, PLIC	[167]
GPER	7p22.3	1091775	1081775	rs115444331	Exon	c.47. C>T	p.P16L	552573	Ontario, PLIC	[150]
IDOL/MYLIP	6p23	16145325	16085325	rs9370867	Exon	c.1025. A>G	p.N342S	484738	Mexican, Brazilian, PLIC	[9], [10]

Discussion table 2. Genotype and allelic Frequency of studied loci in PLIC cohort

	R46L (n=1919); G>T		P16L (n=1405); C>T		N342S (n=1384); A>G	
Frequencies	**Genotype (n; %)**	**Allelic**	**Genotype (n; %)**	**Allelic**	**Genotype**	**Allelic**
Homozygous (Major allele)	1877; 97.81%	98.90%	674; 47.97%	69.68%	383; 27.7%	51.99%
Heterozygous	42; 2.19%	-	610; 43.42%	-	673; 48.6%	-
Homozygous (Minor allele)	NA	1.10%	121; 8.61%	30.32%	328; 23,7%	48.01%

Tables for discussion

Discussion table 3. Comparative genotype and allelic frequency of studied loci in different population.

	PCSK9 rs11591147 (p.R46L)		GPER rs11544331 (p.P16L)		IDOL rs9370867 (p.N342S)	
	PLIC (Genotype %)	**Ref study (Genotype %) [Reference]**	**PLIC (Genotype %)**	**Ref study (Genotype %) [Reference]**	**PLIC (Genotype%/allelic%)**	**Ref study (allelic frequency%) [Reference]**
Homozygous (wild allele)	97.81%	96.80% **[5]**	47.97%	56.34% **[150]**	27.70%/ 51.99%	25% **[9]**; 35.72 **[10]**
Homozygous mutated allele	2.19% (in heterozygous form)	3.20% (in heterozygous form) **[5]**	8.61%	6.78% **[150]**	23.70%; 48.01%	75% **[9]**, 64.28 **[10]**

Discussion table 4. Common allele frequencies of PCSK9 R46L and IDOL N342S polymorphisms in different population

POPULATION STUDIED FOR PCSK9 (p.R46L; c.5428. G>T; rs11591147)											
	LWK	**YRI**	**CHD**	**ASW**	**TSI**	**CHB**	**CEU**	**JPT**	**MEX**	**BRAZ**	**PLIC**
G	0.991	1	0.995	0.991	0.985	0.993	0.991	0.991	0.966	-	0.989
T	0.009	0	0.005	0.009	0.015	0.007	0.009	0.009	0.034	-	0.011
POPULATION STUDIED FOR IDOL (p.N342S; c.1025. A>G; rs9370867)											
A	0.032	0.021	0.079	0.105	0.49	0.052	0.514	0.013	0.259	0.357	0.519
G	0.968	0.979	0.921	0.895	0.51	0.948	0.486	0.987	0.741	0.643	0.481

ASW- African ancestry in Southwest USA; CEU- Utah residents with Northern and Western European ancestry from the CEPH collection
CHB- Han Chinese in Beijing, China; CHD- Chinese in Metropolitan Denver, Colorado; GIH- Gujarati Indians in Houston, Texas, JPT- Japanese in Tokyo, Japan; LWK- Luhya in Webuye, Kenya, MXL- Mexican ancestry in Los Angeles, California; MKK- Maasai in Kinyawa, Kenya; TSI- Toscani in Italia; YRI- Yoruba in Ibadan, Nigeria,

Tables for discussion

Discussion table 5. Magnitude of effect of the studied Loci (gene variations) reproducibly associated with plasma Lipoprotein levels in PLIC subjects

Effect, Sequence variation and Genotype	No. of individuals	Mean ± SEM LDLc (mg/dL)	Difference in mean LDLc (mg/dL)	Mean ± SEM TC (mg/dL)	Difference in mean TC (mg/dL)	Mean ± SEM Tg (mg/dL)	Difference in mean Tg (mg/dL)	Mean ± SEM Glucose (mg/dL)	Difference in mean Glucose (mg/dL)
PCSK9[R46L]; G>T									
GG	1877	144.58 ± 0.83	10.2	221.85 ± 0.90	9.75	108.61 ± 1.44	28.56	92.83 ± 0.36	7.2
GT	42	134.42 ± 6.35		212.10 ± 6.62		137.17 ± 14.0		100.0 ± 4.18	
P value		0.075		0.113		**0.004**		**0.004**	
IDOL[N342S]; A>G;									
AA	383	122.6 ± 31.6	2.6	204.7 ± 34.3	5.2	97.27 ± 45.6	**3.23**	92.94	2.44
GG	328	125.2 ± 32.8		209.9 ± 35.9		100.5 ± 42.4		95.38	
P value		0.356		0.089		0.391		0.057	
GPER [P16L]; C>T									
CC	674	144.66 ± 1.37	3.52	220.15 ± 1.48	5.23	108.01 ± 2.3	5.11	90.08	2.43
TT	121	148.18 ± 3.11		225.38 ± 3.2		113.12 ± 6.3		92.51	
P value		0.608		0.271		0.716		0.06	

Discussion table 6. General and clinical characteristics of PLIC subjects according to loci studied (PCSK9, IDOL and GPER genes.)

		AGE	BMI	TC	LDLc	HDLc	Tg	ApoA1	ApoB	SBP	DBP	GLUCOSE
R46L	RR (GG)	54.77 ± 0.26	26.48 ± 0.09	221.85 ± 0.90	144.62 ± 0.83	55.52 ± 0.33	108.60 ± 1.44	149.35 ±0.61	113.61 ±0.60	131.40 ± 0.40	81.65 ± 0.22	92.83 ± 0.362
	RL (GT)	54.17 ± 1.69	28.14 ± 0.61	212.10 ± 6.62	134.42 ± 6.35	50.24 ± 1.96	137.17 ± 14.0	139.88 ± 3.84	108.95 ± 4.18	134.39 ± 2.50	83.54 ± 1.37	100.0 ± 4.18
	P-VALUE	0.75	**0.011**	0.113	0.075	**0.022**	**0.004**	**0.024**	0.262	0.275	***0.32***	**0.004**
N342S												
	NN (AA)	64.90 ± 9.8	27.0 ± 4.2	204.7 ± 34.3	122.6 ± 31.6	62.64 ± 14.8	97.27 ± 45.6	155.3 ± 20.7	107.0 ± 22.2	128 ± 18.0	82.32 ± 8.8	92.94 ± 13.72
	NS (AG)	64.36 ± 1.08	26.7± 4.3	206.1 ± 34.6	123.3 ± 31.9	63.08 ± 16.18	98.91 ± 48.0	156.4 ± 21.0	105.6 ± 22.2	129 ± 18.0	82.37 ± 8.70	94.97 ± 17.93
	SS (GG)	65.21 ± 9.97	26.8 ± 4.6	209.9 ± 35.9	125.2 ± 32.8	64.60 ± 18	100.5 ± 42.4	158.2 ± 23.4	108.7 ± 22.1	128 ± 17	82.03 ± 9.4	95.38 ± 15.89
	P-VALUE	0.735	0.756	0.089	0.356	0.168	0.391	0.133	0.389	0.568	0.983	0.057
P16L												
	PP (CC)	54.81 ± 0.43	26.69 ± 0.17	220.15 ± 1.48	144.66 ± 1.37	53.88 ± 0.54	108.01 ± 2.3	145.78 ± 0.99	112.97 ± 0.97	132.79 ± 0.64	83.09 ± 0.33	90.08 ± 0.49
	PL (CT)	54.96 ± 0.45	26.37 ± 0.17	222.66 ± 1.55	145.29 ± 1.46	55.69 ± 0.59	108.33 ± 2.6	148.24 ± 1.0	112.83 ± 1.0	132.96 ± 0.68	82.65 ± 0.37	92.06 ± 0.79
	LL (TT)	55.10 ± 0.99	26.82 ± 0.33	225.38 ± 3.2	148.18 ± 3.11	54.57 ± 1.34	113.12 ± 6.3	147.04 ± 2.5	116.25 ± 2.22	133.64 ± 1.7	83.88 ± 0.83	92.51 ± 1.3
	P-VALUE	0.952	0.326	0.271	0.608	0.079	0.716	0.235	0.389	0.878	0.345	0.06

Discussion table 7. Comparative of loci effect size in cIMT progression and prospective genetic study

Gene	SNP	Baseline (cIMT)	10 yr after (cIMT)
		(mm)	(mm)
PCSK9	R46R	0.652 ± 0.003	0.78 ± 0.005
	R46L	0.661 ± 0.017	0.76 ± 0.23
	P-value	0.142	0.116
IDOL	N342N	0.639 ± 0.006	0.760 ± 0.07
	S342S	0.634 ± 0.006	0.768 ± 0.005
	p-value	0.205	0.066
GPER	P16P	0.64 ± 0.005	0.781 ± 0.007
	L16L	0.65 ± 0.01	0.785 ± 0.171
	p-value	0.726	0.970

Tables for discussion

Discussion table 8. Plasma PCSK9 distribution according to studied loci in PLIC subjects (free of hypolipidemics)

Serial	Frequencies	PCSK9 - R46L (n=1919); G>T	GPER- P16L (n=898); C>T	IDOL- N342S (n=1384); A>G
A	Homozygous (Major allele)	294.33 ng/mL	277.49 ± 8.71 ng/mL	266.92
B	Heterozygous	136.97 ng/mL	303.52 ±10.22 ng/mL	301.77
C	Homozygous (Minor allele)	-	324.34 ± 20.95 ng/mL	273.15
	Difference in mean plasma PCSK9 levels (ng/ml)	-157.36 [A - B]	+46.85 [A - C]	+6.23 [A – C]
	P-value	**<0.001**	**0.045**	0.53

Discussion table 9. Plasma PCSK9 distribution in men and women according to studied loci.

	PCSK9 - R46L (n=1919)			GPER- P16L (n=898); C>T		
Frequencies	**Men**	**Women**	**p-value**	**Men**	**Women**	**p-value**
Homozygous (Major allele)	276.90 ± 10.91	312.00 ± 10.12	***0.32***	258.42 ± 8.71 ng/mL	291.25 ±	***0.15***
Heterozygous	163.57 ± 41.53	132.98 ± 33.68	0.067	287.39 ±14.99 ng/mL	313.27 ± 13.65	***0.23***
Homozygous (Minor allele)	NA	NA		254.09 ± 35.12 ng/mL	366.92 ± 24.13	**0.029**
P-value	**0.04**	**0.001**		0.298	**0.043**	
% of PCSK9 change with mutated allele	**-40%**	**-57.38%**		-1.67%	**+24.15%**	

Note:- values are in Mean ±SEM ; and IDOL gene values are not included as there was no significant association.

CHAPTER 9. ABREVIATIONS

Abbreviations

ABI- Ankle-brachial index

ADH- Autosomal dominant hypercholesterolemia

ApoAI- Apolipoprotein AI

ApoB- Apolipoprotein-B

ApoE- Apolipoprotein-E

ApoER2- Apolipoprotein-E receptor 2

ATP-III- National Cholesterol Education Program's Adult Treatment Panel III report

CAD- Coronary artery disease

CAIP- Carotid artery injury model

CHD- Coronary heart disease

cIMT- intima-media thickness of carotid artery

CVD- Cardiovascular diseases

CVE-Cardiovascular events

DBP- Diastolic blood pressure

DIO- Diet induced obesity

ERα- Estrogen receptor-α

ERβ- Estrogen receptor-β

FBS- Fetal bovine serum

FH- Familial hypercholesterolemia

FPLC- Fast performance liquid chromatography

GOF- Gain of function

GTT- Glucose tolerance test

GPCR- G-protein coupled receptors

GPER- G-protein coupled estrogen receptor

HDL- High density lipoprotein

IDOL- Inducible degrader of LDL-receptor

ITT- Insulin glucose test

LDL- Low density lipoprotein

LDLR- Low density lipoprotein receptor

Abbreviations

LOF- Loss of function

LRP- Low density lipoprotein receptor-related protein 1

MetS- Metabolic syndrome

PAD- Peripheral artery disease

PBMC- Peripheral blood mononuclear cells

PBS- Phosphate buffer saline

PCR- Polymerace chain reaction

PCSK9- Proprotein convertase subtilisin/kexin type 9

PLIC- Progression of lesion in intima-media of carotid artery

RT-PCR- Real time polymerase chain reaction

SBP- Systolic blood pressure

SFD- Standard fat diet

TC- total cholesterol

TG- Triglycerides

VLDL- Very low density lipoprotein

VLDLR- very low density lipoprotein receptor

VSMCs- Vascular smooth muscle cells

CHAPTER 10. REFERENCES

References

[1] W. Rosamond, K. Flegal, K. Furie, A. Go, K. Greenlund, N. Haase, et al., Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee., Circulation. 117 (2008) e25–146. doi:10.1161/CIRCULATIONAHA.107.187998.

[2] C.D. Mathers, A.D. Lopez, C.J.L. Murray, The Burden of Disease and Mortality by Condition: Data, Methods, and Results for 2001, (2006). http://www.ncbi.nlm.nih.gov/books/NBK11808/ (accessed September 28, 2015).

[3] A.M. Miniño, S.L. Murphy, J. Xu, K.D. Kochanek, Deaths: final data for 2008., Natl. Vital Stat. Rep. 59 (2011) 1–126. http://www.ncbi.nlm.nih.gov/pubmed/22808755 (accessed September 16, 2015).

[4] N.J. Pagidipati, T. a. Gaziano, Estimating deaths from cardiovascular disease: A review of global methodologies of mortality measurement, Circulation. 127 (2013) 749–756. doi:10.1161/CIRCULATIONAHA.112.128413.

[5] J.C. Cohen, E. Boerwinkle, T.H. Mosley, H.H. Hobbs, Sequence variations in PCSK9, low LDL, and protection against coronary heart disease., N. Engl. J. Med. 354 (2006) 1264–72. doi:10.1056/NEJMoa054013.

[6] S.G. Lakoski, T.A. Lagace, J.C. Cohen, J.D. Horton, H.H. Hobbs, Genetic and metabolic determinants of plasma PCSK9 levels., J. Clin. Endocrinol. Metab. 94 (2009) 2537–43. doi:10.1210/jc.2009-0141.

[7] A haplotype map of the human genome., Nature. 437 (2005) 1299–320. doi:10.1038/nature04226.

[8] J.K. Pickrell, G. Coop, J. Novembre, S. Kudaravalli, J.Z. Li, D. Absher, et al., Signals of recent positive selection in a worldwide sample of human populations., Genome Res. 19 (2009) 826–37. doi:10.1101/gr.087577.108.

[9] D. Weissglas-Volkov, A.C. Calkin, T. Tusie-Luna, J.S. Sinsheimer, N. Zelcer, L. Riba, et al., The N342S MYLIP polymorphism is associated with high total cholesterol and increased LDL receptor degradation in humans., J. Clin. Invest. 121 (2011) 3062–71. doi:10.1172/JCI45504.

[10] P.C.J.L. Santos, T.G.M. Oliveira, P.A. Lemos, J.G. Mill, J.E. Krieger, A.C. Pereira, MYLIP p.N342S polymorphism is not associated with lipid profile in the Brazilian population., Lipids Health Dis. 11 (2012) 83. doi:10.1186/1476-511X-11-83.

[11] M.S. Brown, J.L. Goldstein, A receptor-mediated pathway for cholesterol homeostasis., Science. 232 (1986) 34–47. http://www.ncbi.nlm.nih.gov/ pubmed/ 3513311 (accessed July 22, 2015).

[12] J.L. Goldstein, M.S. Brown, The low-density lipoprotein pathway and its relation to atherosclerosis., Annu. Rev. Biochem. 46 (1977) 897–930. doi:10.1146/annurev. bi.46.070177. 004341.

[13] J.L. Goldstein, M.S. Brown, R.G. Anderson, D.W. Russell, W.J. Schneider, Receptor-mediated endocytosis: concepts emerging from the LDL receptor system., Annu. Rev. Cell Biol. 1 (1985) 1–39. doi:10.1146/annurev.cb.01.110185.000245.

[14] H.H. Hobbs, D.W. Russell, M.S. Brown, J.L. Goldstein, The LDL Receptor Locus in Familial Hypercholesterolemia: Mutational Analysis of a Membrane Protein, Annu. Rev. Genet. 24 (1990) 133–170. doi:10.1146/annurev.ge.24.120190.001025.

[15] J.L. Goldstein, M.S. Brown, Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia., J. Biol. Chem. 249 (1974) 5153–62. http://www.ncbi.nlm.nih.gov/pubmed/4368448 (accessed October 28, 2015).

[16] D.W. Russell, W.J. Schneider, T. Yamamoto, K.L. Luskey, M.S. Brown, J.L. Goldstein, Domain map of the LDL receptor: sequence homology with the epidermal growth factor precursor., Cell. 37 (1984) 577–85. http://www.ncbi.nlm.nih.gov/ pubmed /6327078 (accessed November 19, 2015).

[17] T. Yamamoto, C.G. Davis, M.S. Brown, W.J. Schneider, M.L. Casey, J.L. Goldstein, et al., The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA., Cell. 39 (1984) 27–38. http://www.ncbi.nlm.nih.gov/pubmed/6091915 (accessed November 19, 2015).

[18] V. Lindgren, K.L. Luskey, D.W. Russell, U. Francke, Human genes involved in cholesterol metabolism: chromosomal mapping of the loci for the low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl-coenzyme A reductase with cDNA probes., Proc. Natl. Acad. Sci. U. S. A. 82 (1985) 8567–71. /pmc/articles/ PMC390958/?report=abstract (accessed November 5, 2015).

[19] M.S. Brown, J.L. Goldstein, Regulation of the activity of the low density lipoprotein receptor in human fibroblasts., Cell. 6 (1975) 307–16. http://www.ncbi.nlm.nih.gov/ pubmed/212203 (accessed September 24, 2015).

[20] M.S. Brown, J.L. Goldstein, A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood., Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 11041–8.

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=34238&tool=pmcentrez&rendertype=abstract (accessed November 19, 2015).

[21] J.L. Goldstein, R.A. DeBose-Boyd, M.S. Brown, Protein sensors for membrane sterols., Cell. 124 (2006) 35–46. doi:10.1016/j.cell.2005.12.022.

[22] R.G. Anderson, J.L. Goldstein, M.S. Brown, Localization of low density lipoprotein receptors on plasma membrane of normal human fibroblasts and their absence in cells from a familial hypercholesterolemia homozygote., Proc. Natl. Acad. Sci. U. S. A. 73 (1976) 2434–8. http://www.pubmedcentral.nih.gov/articlerender. fcgi?artid = 430596 &tool=pmcentrez&rendertype=abstract (accessed November 19, 2015).

[23] R.G. Anderson, M.S. Brown, J.L. Goldstein, Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts., Cell. 10 (1977) 351–64. http://www.ncbi.nlm.nih.gov/pubmed/191195 (accessed November 19, 2015).

[24] C.G. Davis, J.L. Goldstein, T.C. Südhof, R.G. Anderson, D.W. Russell, M.S. Brown, Acid-dependent ligand dissociation and recycling of LDL receptor mediated by growth factor homology region., Nature. 326 760–5. doi:10.1038/326760a0.

[25] H. Tolleshaug, J.L. Goldstein, W.J. Schneider, M.S. Brown, Posttranslational processing of the LDL receptor and its genetic disruption in familial hypercholesterolemia., Cell. 30 (1982) 715–24. http://www.ncbi.nlm.nih.gov/pubmed/6291781 (accessed November 19, 2015).

[26] C.G. Davis, M.A. Lehrman, D.W. Russell, R.G. Anderson, M.S. Brown, J.L. Goldstein, The J.D. mutation in familial hypercholesterolemia: amino acid substitution in cytoplasmic domain impedes internalization of LDL receptors., Cell. 45 (1986) 15–24. http://www.ncbi.nlm.nih.gov/pubmed/3955657 (accessed November 19, 2015).

[27] W.J. Chen, J.L. Goldstein, M.S. Brown, NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor., J. Biol. Chem. 265 (1990) 3116–23. http://www.ncbi.nlm.nih.gov/pubmed/1968060 (accessed November 19, 2015).

[28] M. Yokode, Cytoplasmic sequence required for basolateral targeting of LDL receptor in livers of transgenic mice, J. Cell Biol. 117 (1992) 39–46. doi:10.1083/jcb.117.1.39.

[29] U.M. Koivisto, A.L. Hubbard, I. Mellman, A novel cellular phenotype for familial hypercholesterolemia due to a defect in polarized targeting of LDL receptor., Cell. 105 (2001) 575–85. http://www.ncbi.nlm.nih.gov/pubmed/11389828 (accessed November 19, 2015).

[30] N.G. Seidah, S. Benjannet, L. Wickham, J. Marcinkiewicz, S.B. Jasmin, S. Stifani, et al., The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-

1): liver regeneration and neuronal differentiation., Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 928–33. doi:10.1073/pnas.0335507100.

[31] N. Zelcer, C. Hong, R. Boyadjian, P. Tontonoz, LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor., Science. 325 (2009) 100–4. doi:10.1126/science.1168974.

[32] D. Julius, A. Brake, L. Blair, R. Kunisawa, J. Thorner, Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-alpha-factor., Cell. 37 (1984) 1075–89. http://www.ncbi.nlm.nih.gov/pubmed/6430565 (accessed October 28, 2015).

[33] R.S. Fuller, A.J. Brake, J. Thorner, Intracellular targeting and structural conservation of a prohormone-processing endoprotease., Science. 246 (1989) 482–6. http://www.ncbi.nlm.nih.gov/pubmed/2683070 (accessed October 28, 2015).

[34] S.P. Smeekens, D.F. Steiner, Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2., J. Biol. Chem. 265 (1990) 2997–3000. http://www.ncbi.nlm.nih.gov/ pubmed/2154467 (accessed October 28, 2015).

[35] N.G. Seidah, L. Gaspar, P. Mion, M. Marcinkiewicz, M. Mbikay, M. Chrétien, cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products: tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases., DNA Cell Biol. 9 415–24. http://www.ncbi.nlm.nih.gov/ pubmed/2169760 (accessed October 28, 2015).

[36] N.G. Seidah, S.J. Mowla, J. Hamelin, A.M. Mamarbachi, S. Benjannet, B.B. Toure, et al., Mammalian subtilisin/kexin isozyme SKI-1: A widely expressed proprotein convertase with a unique cleavage specificity and cellular localization, Proc. Natl. Acad. Sci. 96 (1999) 1321–1326. doi:10.1073/pnas.96.4.1321.

[37] J. Sakai, R.B. Rawson, P.J. Espenshade, D. Cheng, A.C. Seegmiller, J.L. Goldstein, et al., Molecular identification of the sterol-regulated luminal protease that cleaves SREBPs and controls lipid composition of animal cells., Mol. Cell. 2 (1998) 505–14. http://www.ncbi.nlm.nih.gov/pubmed/9809072 (accessed September 30, 2015).

[38] P. Pullikotil, S. Benjannet, J. Mayne, N.G. Seidah, The proprotein convertase SKI-1/S1P: alternate translation and subcellular localization., J. Biol. Chem. 282 (2007) 27402–13. doi:10.1074/jbc.M703200200.

[39] M. Abifadel, M. Varret, J.-P. Rabès, D. Allard, K. Ouguerram, M. Devillers, et al., Mutations in PCSK9 cause autosomal dominant hypercholesterolemia., Nat. Genet. 34 (2003) 154–6. doi:10.1038/ng1161.

[40] D. Cunningham, D.E. Danley, K.F. Geoghegan, M.C. Griffor, J.L. Hawkins, T.A. Subashi, et al., Structural and biophysical studies of PCSK9 and its mutants linked to familial hypercholesterolemia., Nat. Struct. Mol. Biol. 14 (2007) 413–9. doi:10.1038/nsmb1235.

[41] M.C. McNutt, H.J. Kwon, C. Chen, J.R. Chen, J.D. Horton, T.A. Lagace, Antagonism of secreted PCSK9 increases low density lipoprotein receptor expression in HepG2 cells., J. Biol. Chem. 284 (2009) 10561–70. doi:10.1074/jbc.M808802200.

[42] S.C. Hunt, P.N. Hopkins, K. Bulka, M.T. McDermott, T.L. Thorne, B.B. Wardell, et al., Genetic Localization to Chromosome 1p32 of the Third Locus for Familial Hypercholesterolemia in a Utah Kindred, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 1089–1093. doi:10.1161/01.ATV.20.4.1089.

[43] M. Varret, J.P. Rabès, B. Saint-Jore, A. Cenarro, J.C. Marinoni, F. Civeira, et al., A third major locus for autosomal dominant hypercholesterolemia maps to 1p34.1-p32., Am. J. Hum. Genet. 64 (1999) 1378–87. doi:10.1086/302370.

[44] J. Cohen, A. Pertsemlidis, I.K. Kotowski, R. Graham, C.K. Garcia, H.H. Hobbs, Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9., Nat. Genet. 37 (2005) 161–5. doi:10.1038/ng1509.

[45] J. Mayne, T. Dewpura, A. Raymond, M. Cousins, A. Chaplin, K.A. Lahey, et al., Plasma PCSK9 levels are significantly modified by statins and fibrates in humans, Lipids Health Dis. 7 (2008) 22. doi:10.1186/1476-511X-7-22.

[46] F. Raal, V. Panz, A. Immelman, G. Pilcher, Elevated PCSK9 Levels in Untreated Patients With Heterozygous or Homozygous Familial Hypercholesterolemia and the Response to High-Dose Statin Therapy, J. Am. Heart Assoc. 2 (2013) e000028–e000028. doi:10.1161/JAHA.112.000028.

[47] E.P. Navarese, M. Kolodziejczak, V. Schulze, P.A. Gurbel, U. Tantry, Y. Lin, et al., Effects of Proprotein Convertase Subtilisin/Kexin Type 9 Antibodies in Adults With Hypercholesterolemia: A Systematic Review and Meta-analysis., Ann. Intern. Med. 163 (2015) 40–51. doi:10.7326/M14-2957.

[48] N.G. Seidah, The proprotein convertases, 20 years later., Methods Mol. Biol. 768 (2011) 23–57. doi:10.1007/978-1-61779-204-5_3.

[49] N.G. Seidah, M.S. Sadr, M. Chrétien, M. Mbikay, The multifaceted proprotein convertases: their unique, redundant, complementary, and opposite functions., J. Biol. Chem. 288 (2013) 21473–81. doi:10.1074/jbc.R113.481549.

[50] N.G. Seidah, A. Prat, The biology and therapeutic targeting of the proprotein convertases, Nat. Rev. Drug Discov. 11 (2012) 367–383. doi:10.1038/nrd3699.

[51] N.G. Seidah, G. Mayer, A. Zaid, E. Rousselet, N. Nassoury, S. Poirier, et al., The activation and physiological functions of the proprotein convertases., Int. J. Biochem. Cell Biol. 40 (2008) 1111–25. doi:10.1016/j.biocel.2008.01.030.

[52] A. Zaid, A. Roubtsova, R. Essalmani, J. Marcinkiewicz, A. Chamberland, J. Hamelin, et al., Proprotein convertase subtilisin/kexin type 9 (PCSK9): hepatocyte-specific low-density lipoprotein receptor degradation and critical role in mouse liver regeneration., Hepatology. 48 (2008) 646–54. doi:10.1002/hep.22354.

[53] Y. Luo, L. Warren, D. Xia, H. Jensen, T. Sand, S. Petras, et al., Function and distribution of circulating human PCSK9 expressed extrahepatically in transgenic mice, J. Lipid Res. 50 (2008) 1581–1588. doi:10.1194/jlr.M800542-JLR200.

[54] T.A. Lagace, D.E. Curtis, R. Garuti, M.C. McNutt, S.W. Park, H.B. Prather, et al., Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and in livers of parabiotic mice., J. Clin. Invest. 116 (2006) 2995–3005. doi:10.1172/JCI29383.

[55] A. Grefhorst, M.C. McNutt, T.A. Lagace, J.D. Horton, Plasma PCSK9 preferentially reduces liver LDL receptors in mice, J. Lipid Res. 49 (2008) 1303–1311. doi:10.1194/jlr.M800027-JLR200.

[56] R.J. Schmidt, T.P. Beyer, W.R. Bensch, Y.-W. Qian, A. Lin, M. Kowala, et al., Secreted proprotein convertase subtilisin/kexin type 9 reduces both hepatic and extrahepatic low-density lipoprotein receptors in vivo, Biochem. Biophys. Res. Commun. 370 (2008) 634–640. doi:10.1016/j.bbrc.2008.04.004.

[57] R. Essalmani, D. Susan-Resiga, A. Chamberland, M. Abifadel, J.W. Creemers, C. Boileau, et al., In vivo evidence that furin from hepatocytes inactivates PCSK9, J. Biol. Chem. 286 (2011) 4257–4263. doi:10.1074/jbc.M110.192104.

[58] P. Peticca, A. Raymond, A. Gruslin, M. Cousins, E. Adetola, H. Abujrad, et al., Human Serum PCSK9 Is Elevated at Parturition in Comparison to Nonpregnant Subjects While Serum PCSK9 from Umbilical Cord Blood is Lower Compared to Maternal Blood., ISRN Endocrinol. 2013 (2013) 341632. doi:10.1155/2013/341632.

[59] A. Baass, G. Dubuc, M. Tremblay, E.E. Delvin, J. O'Loughlin, E. Levy, et al., Plasma PCSK9 is associated with age, sex, and multiple metabolic markers in a population-based sample of children and adolescents., Clin. Chem. 55 (2009) 1637–45. doi:10.1373/clinchem.2009.126987.

[60] Q. Cui, X. Ju, T. Yang, M. Zhang, W. Tang, Q. Chen, et al., Serum PCSK9 is associated with multiple metabolic factors in a large Han Chinese population, Atherosclerosis. 213 (2010) 632–636. doi:10.1016/j.atherosclerosis.2010.09.027.

[61] K.N. Maxwell, R.E. Soccio, E.M. Duncan, E. Sehayek, J.L. Breslow, Novel putative SREBP and LXR target genes identified by microarray analysis in liver of cholesterol-fed mice., J. Lipid Res. 44 (2003) 2109–19. doi:10.1194/jlr.M300203-JLR200.

[62] G. Dubuc, A. Chamberland, H. Wassef, J. Davignon, N.G. Seidah, L. Bernier, et al., Statins upregulate PCSK9, the gene encoding the proprotein convertase neural apoptosis-regulated convertase-1 implicated in familial hypercholesterolemia., Arterioscler. Thromb. Vasc. Biol. 24 (2004) 1454–9. doi:10.1161/01.ATV. 0000134621.14315.43.

[63] P. Costet, B. Cariou, G. Lambert, F. Lalanne, B. Lardeux, A.-L. Jarnoux, et al., Hepatic PCSK9 expression is regulated by nutritional status via insulin and sterol regulatory element-binding protein 1c., J. Biol. Chem. 281 (2006) 6211–8. doi:10.1074/ jbc. M508582200.

[64] H. Li, B. Dong, S.W. Park, H.-S. Lee, W. Chen, J. Liu, Hepatocyte nuclear factor 1alpha plays a critical role in PCSK9 gene transcription and regulation by the natural hypocholesterolemic compound berberine., J. Biol. Chem. 284 (2009) 28885–95. doi:10.1074/jbc.M109.052407.

[65] D. Ai, C. Chen, S. Han, A. Ganda, A.J. Murphy, R. Haeusler, et al., Regulation of hepatic LDL receptors by mTORC1 and PCSK9 in mice., J. Clin. Invest. 122 (2012) 1262–70. doi:10.1172/JCI61919.

[66] A. Roubtsova, M.N. Munkonda, Z. Awan, J. Marcinkiewicz, A. Chamberland, C. Lazure, et al., Circulating proprotein convertase subtilisin/kexin 9 (PCSK9) regulates VLDLR protein and triglyceride accumulation in visceral adipose tissue., Arterioscler. Thromb. Vasc. Biol. 31 (2011) 785–91. doi:10.1161/ATVBAHA.110.220988.

[67] S. Poirier, G. Mayer, S. Benjannet, E. Bergeron, J. Marcinkiewicz, N. Nassoury, et al., The proprotein convertase PCSK9 induces the degradation of low density lipoprotein receptor (LDLR) and its closest family members VLDLR and ApoER2., J. Biol. Chem. 283 (2008) 2363–72. doi:10.1074/jbc.M708098200.

[68] F. Leblond, N.G. Seidah, L.-P. Précourt, E. Delvin, M. Dominguez, E. Levy, Regulation of the proprotein convertase subtilisin/kexin type 9 in intestinal epithelial cells., Am. J. Physiol. Gastrointest. Liver Physiol. 296 (2009) G805–15. doi:10.1152/ajpgi.90424.2008.

[69] E. Levy, A. Ben Djoudi Ouadda, S. Spahis, A.T. Sane, C. Garofalo, É. Grenier, et al., PCSK9 plays a significant role in cholesterol homeostasis and lipid transport in intestinal epithelial cells., Atherosclerosis. 227 (2013) 297–306. doi:10.1016/ j.atherosclerosis.2013.01.023.

[70] S. Rashid, H. Tavori, P.E. Brown, M.F. Linton, J. He, I. Giunzioni, et al., 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 (2014) 431–41. doi:10.1161/CIRCULATIONAHA.113.006720.

[71] M. Mbikay, F. Sirois, J. Mayne, G.-S. Wang, A. Chen, T. Dewpura, et al., PCSK9-deficient mice exhibit impaired glucose tolerance and pancreatic islet abnormalities., FEBS Lett. 584 (2010) 701–6. doi:10.1016/j.febslet.2009.12.018.

[72] N.D. Vaziri, K.H. Liang, Down-regulation of hepatic LDL receptor expression in experimental nephrosis., Kidney Int. 50 (1996) 887–93. http://www.ncbi.nlm.nih.gov/pubmed/8872963 (accessed October 5, 2015).

[73] M.-A. Nguyen, T. Kosenko, T.A. Lagace, Internalized PCSK9 dissociates from recycling LDL receptors in PCSK9-resistant SV-589 fibroblasts., J. Lipid Res. 55 (2014) 266–75. doi:10.1194/jlr.M044156.

[74] M. Konarzewski, M. Szolkiewicz, E. Sucajtys-Szulc, J. Blaszak, S. Lizakowski, J. Swierczynski, et al., Elevated circulating PCSK-9 concentration in renal failure patients is corrected by renal replacement therapy., Am. J. Nephrol. 40 (2014) 157–63. doi:10.1159/000365935.

[75] S. Benjannet, D. Rhainds, J. Hamelin, N. Nassoury, N.G. Seidah, The proprotein convertase (PC) PCSK9 is inactivated by furin and/or PC5/6A: functional consequences of natural mutations and post-translational modifications., J. Biol. Chem. 281 (2006) 30561–72. doi:10.1074/jbc.M606495200.

[76] K.N. Maxwell, J.L. Breslow, Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype, Proc. Natl. Acad. Sci. 101 (2004) 7100–7105. doi:10.1073/pnas.0402133101.

[77] H. Tavori, D. Fan, J.L. Blakemore, P.G. Yancey, L. Ding, M.F. Linton, et al., Serum proprotein convertase subtilisin/kexin type 9 and cell surface low-density lipoprotein receptor: evidence for a reciprocal regulation., Circulation. 127 (2013) 2403–13. doi:10.1161/CIRCULATIONAHA.113.001592.

[78] T. Kosenko, M. Golder, G. Leblond, W. Weng, T.A. Lagace, Low density lipoprotein binds to proprotein convertase subtilisin/kexin type-9 (PCSK9) in human plasma and inhibits PCSK9-mediated low density lipoprotein receptor degradation., J. Biol. Chem. 288 (2013) 8279–88. doi:10.1074/jbc.M112.421370.

[79] R. Huijgen, S.W. Fouchier, M. Denoun, B.A. Hutten, M.N. Vissers, G. Lambert, et al., Plasma levels of PCSK9 and phenotypic variability in familial hypercholesterolemia, J. Lipid Res. 53 (2012) 979–983. doi:10.1194/jlr.P023994.

[80] C.J. Lee, Y.-H. Lee, S.W. Park, K.J. Kim, S. Park, J.-C. Youn, et al., Association of serum proprotein convertase subtilisin/kexin type 9 with carotid intima media thickness in

hypertensive subjects., Metabolism. 62 (2013) 845–50. doi:10.1016/ j.metabol. 2013. 01.005.

[81] C. Werner, M.M. Hoffmann, K. Winkler, M. Böhm, U. Laufs, Risk prediction with proprotein convertase subtilisin/kexin type 9 (PCSK9) in patients with stable coronary disease on statin treatment, Vascul. Pharmacol. 62 (2014) 94–102. doi:10.1016/ j.vph.2014.03.004.

[82] C. Giannattasio, M. Failla, A. Grappiolo, M.L. Stella, A. Del Bo, M. Colombo, et al., Fluctuations of radial artery distensibility throughout the menstrual cycle., Arterioscler. Thromb. Vasc. Biol. 19 (1999) 1925–9. http://www.ncbi.nlm.nih.gov/ pubmed/10446072 (accessed October 21, 2015).

[83] L. Baker, K.K. Meldrum, M. Wang, R. Sankula, R. Vanam, A. Raiesdana, et al., The role of estrogen in cardiovascular disease., J. Surg. Res. 115 (2003) 325–44. http://www.ncbi.nlm.nih.gov/pubmed/14697301 (accessed October 21, 2015).

[84] V.L. Ballard, Harnessing Hormonal Signaling for Cardioprotection, Sci. Aging Knowl. Environ. 2005 (2005) re6–re6. doi:10.1126/sageke.2005.51.re6.

[85] C. Vitale, M.E. Mendelsohn, G.M.C. Rosano, Gender differences in the cardiovascular effect of sex hormones, Nat. Rev. Cardiol. 6 (2009) 532–542. doi:10.1038/ nrcardio. 2009.105.

[86] A.M.R. Billeci, M. Paciaroni, V. Caso, G. Agnelli, Hormone replacement therapy and stroke., Curr. Vasc. Pharmacol. 6 (2008) 112–23. http://www.ncbi.nlm.nih.gov /pubmed/18393913 (accessed October 21, 2015).

[87] L. Zhu, W.C. Brown, Q. Cai, A. Krust, P. Chambon, O.P. McGuinness, et al., Estrogen treatment after ovariectomy protects against fatty liver and may improve pathway-selective insulin resistance. Diabetes. 62 (2013) 424–34. doi:10.2337/db11-1718.

[88] J.C. Witteman, D.E. Grobbee, F.J. Kok, A. Hofman, H.A. Valkenburg, Increased risk of atherosclerosis in women after the menopause., BMJ. 298 (1989) 642–4. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1835855&tool=pmcentrez&rendertype=abstract (accessed November 6, 2015).

[89] E. Barrett-Connor, Sex differences in coronary heart disease. Why are women so superior? The 1995 Ancel Keys Lecture., Circulation. 95 (1997) 252–64. http://www.ncbi.nlm.nih.gov/pubmed/8994444 (accessed November 6, 2015).

[90] R.K. Dubey, B. Imthurn, M. Barton, E.K. Jackson, Vascular consequences of menopause and hormone therapy: importance of timing of treatment and type of estrogen., Cardiovasc. Res. 66 (2005) 295–306. doi:10.1016/j.cardiores.2004.12.012.

[91] M. Barton, M.R. Meyer, Postmenopausal hypertension: mechanisms and therapy., Hypertension. 54 (2009) 11–8. doi:10.1161/HYPERTENSIONAHA.108.120022.

[92] E.M. McInerney, B.S. Katzenellenbogen, Different regions in activation function-1 of the human estrogen receptor required for antiestrogen- and estradiol-dependent transcription activation., J. Biol. Chem. 271 (1996) 24172–8. http://www.ncbi.nlm.nih.gov/pubmed/8798658 (accessed November 6, 2015).

[93] J.M. Hall, J.F. Couse, K.S. Korach, The Multifaceted Mechanisms of Estradiol and Estrogen Receptor Signaling, J. Biol. Chem. 276 (2001) 36869–36872. doi:10.1074/jbc.R100029200.

[94] S.W. Curtis, T. Washburn, C. Sewall, R. DiAugustine, J. Lindzey, J.F. Couse, et al., Physiological coupling of growth factor and steroid receptor signaling pathways: estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor., Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 12626–30. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=38043&tool=pmcentrez&rendertype=abstract (accessed November 6, 2015).

[95] S. Nuedling, S. Kahlert, K. Loebbert, P.A. Doevendans, R. Meyer, H. Vetter, et al., 17 Beta-estradiol stimulates expression of endothelial and inducible NO synthase in rat myocardium in-vitro and in-vivo., Cardiovasc. Res. 43 (1999) 666–74. http://www.ncbi.nlm.nih.gov/pubmed/10690338 (accessed November 7, 2015).

[96] R. O'Lone, K. Knorr, I.Z. Jaffe, M.E. Schaffer, P.G. V Martini, R.H. Karas, et al., Estrogen receptors alpha and beta mediate distinct pathways of vascular gene expression, including genes involved in mitochondrial electron transport and generation of reactive oxygen species., Mol. Endocrinol. 21 (2007) 1281–96. doi:10.1210/me.2006-0497.

[97] I. Nikolic, D. Liu, J.A. Bell, J. Collins, C. Steenbergen, E. Murphy, Treatment with an estrogen receptor-beta-selective agonist is cardioprotective., J. Mol. Cell. Cardiol. 42 (2007) 769–80. doi:10.1016/j.yjmcc.2007.01.014.

[98] M.K. Lindberg, S. Movérare, S. Skrtic, H. Gao, K. Dahlman-Wright, J.-A. Gustafsson, et al., Estrogen receptor (ER)-beta reduces ERalpha-regulated gene transcription, supporting a "ying yang" relationship between ERalpha and ERbeta in mice., Mol. Endocrinol. 17 (2003) 203–8. doi:10.1210/me.2002-0206.

[99] S. Tsutsumi, X. Zhang, K. Takata, K. Takahashi, R.H. Karas, H. Kurachi, et al., Differential regulation of the inducible nitric oxide synthase gene by estrogen receptors 1 and 2., J. Endocrinol. 199 (2008) 267–73. doi:10.1677/JOE-07-0292.

[100] K.K. Schnoes, I.Z. Jaffe, L. Iyer, A. Dabreo, M. Aronovitz, B. Newfell, et al., Rapid recruitment of temporally distinct vascular gene sets by estrogen., Mol. Endocrinol. 22 (2008) 2544–56. doi:10.1210/me.2008-0044.

[101] S.C. Hewitt, B.J. Deroo, K. Hansen, J. Collins, S. Grissom, C.A. Afshari, et al., Estrogen receptor-dependent genomic responses in the uterus mirror the biphasic physiological response to estrogen., Mol. Endocrinol. 17 (2003) 2070–83. doi:10.1210/me.2003-0146.

[102] K. Boengler, R. Schulz, G. Heusch, Connexin 43 signalling and cardioprotection., Heart. 92 (2006) 1724–7. doi:10.1136/hrt.2005.066878.

[103] A. Shamaei-Tousi, J.P. Halcox, B. Henderson, Stressing the obvious? Cell stress and cell stress proteins in cardiovascular disease., Cardiovasc. Res. 74 (2007) 19–28. doi:10.1016/j.cardiores.2006.10.025.

[104] Y.-C. Hsieh, H.-P. Yu, T. Suzuki, M.A. Choudhry, M.G. Schwacha, K.I. Bland, et al., Upregulation of mitochondrial respiratory complex IV by estrogen receptor-beta is critical for inhibiting mitochondrial apoptotic signaling and restoring cardiac functions following trauma-hemorrhage., J. Mol. Cell. Cardiol. 41 (2006) 511–21. doi:10.1016/j.yjmcc.2006.06.001.

[105] R.P.A. Barros, U.F. Machado, M. Warner, J.-A. Gustafsson, Muscle GLUT4 regulation by estrogen receptors ERbeta and ER , Proc. Natl. Acad. Sci. 103 (2006) 1605–1608. doi:10.1073/pnas.0510391103.

[106] T.-H. Chung, S.-M. Wang, J.-C. Wu, 17beta-estradiol reduces the effect of metabolic inhibition on gap junction intercellular communication in rat cardiomyocytes via the estrogen receptor., J. Mol. Cell. Cardiol. 37 (2004) 1013–22. doi:10.1016/j.yjmcc.2004.08.003.

[107] W. Yu, G. Dahl, R. Werner, The connexin43 gene is responsive to oestrogen., Proc. Biol. Sci. 255 (1994) 125–32. doi:10.1098/rspb.1994.0018.

[108] Y.-C. Hsieh, M.A. Choudhry, H.-P. Yu, T. Shimizu, S. Yang, T. Suzuki, et al., Inhibition of cardiac PGC-1alpha expression abolishes ERbeta agonist-mediated cardioprotection following trauma-hemorrhage., FASEB J. 20 (2006) 1109–17. doi:10.1096/fj.05-5549com.

[109] C.K. Too, A. Giles, M. Wilkinson, Estrogen stimulates expression of adenine nucleotide translocator ANT1 messenger RNA in female rat hearts., Mol. Cell. Endocrinol. 150 (1999) 161–7. http://www.ncbi.nlm.nih.gov/pubmed/10411310 (accessed November 7, 2015).

[110] M.R. Voss, J.N. Stallone, M. Li, R.N.M. Cornelussen, P. Knuefermann, A.A. Knowlton, Gender differences in the expression of heat shock proteins: the effect of estrogen., Am. J. Physiol. Heart Circ. Physiol. 285 (2003) H687–92. doi:10.1152/ajpheart.01000.2002.

[111] C.J. Lagranha, A. Deschamps, A. Aponte, C. Steenbergen, E. Murphy, Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females., Circ. Res. 106 (2010) 1681–91. doi:10.1161/CIRCRESAHA.109.213645.

[112] C. Grohé, S. Kahlert, K. Löbbert, H. Vetter, Expression of oestrogen receptor alpha and beta in rat heart: role of local oestrogen synthesis., J. Endocrinol. 156 (1998) R1–7. http://www.ncbi.nlm.nih.gov/pubmed/9518889 (accessed October 9, 2015).

[113] K.M. Gavin, D.R. Seals, A.E. Silver, K.L. Moreau, Vascular endothelial estrogen receptor alpha is modulated by estrogen status and related to endothelial function and endothelial nitric oxide synthase in healthy women., J. Clin. Endocrinol. Metab. 94 (2009) 3513–20. doi:10.1210/jc.2009-0278.

[114] C. Owman, P. Blay, C. Nilsson, S.J. Lolait, Cloning of human cDNA encoding a novel heptahelix receptor expressed in Burkitt's lymphoma and widely distributed in brain and peripheral tissues., Biochem. Biophys. Res. Commun. 228 (1996) 285–92. doi:10.1006/bbrc.1996.1654.

[115] C.M. Revankar, D.F. Cimino, L.A. Sklar, J.B. Arterburn, E.R. Prossnitz, A transmembrane intracellular estrogen receptor mediates rapid cell signaling., Science. 307 (2005) 1625–30. doi:10.1126/science.1106943.

[116] S.P.H. Alexander, A. Mathie, J.A. Peters, Guide to Receptors and Channels (GRAC), 5th edition., Br. J. Pharmacol. 164 Suppl (2011) S1–324. doi:10.1111/j.1476-5381. 2011. 01649_1.x.

[117] E.R. Levin, G protein-coupled receptor 30: estrogen receptor or collaborator?, Endocrinology. 150 (2009) 1563–5. doi:10.1210/en.2008-1759.

[118] C. Otto, B. Rohde-Schulz, G. Schwarz, I. Fuchs, M. Klewer, D. Brittain, et al., G protein-coupled receptor 30 localizes to the endoplasmic reticulum and is not activated by estradiol., Endocrinology. 149 (2008) 4846–56. doi:10.1210/en.2008-0269.

[119] C. Otto, I. Fuchs, G. Kauselmann, H. Kern, B. Zevnik, P. Andreasen, et al., GPR30 does not mediate estrogenic responses in reproductive organs in mice., Biol. Reprod. 80 (2009) 34–41. doi:10.1095/biolreprod.108.071175.

[120] E.J. Filardo, J.A. Quinn, K.I. Bland, A.R. Frackelton, Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF., Mol. Endocrinol. 14 (2000) 1649–60. doi:10.1210/mend.14.10.0532.

[121] E.J. Filardo, J.A. Quinn, A.R. Frackelton, K.I. Bland, Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated

attenuation of the epidermal growth factor receptor-to-MAPK signaling axis., Mol. Endocrinol. 16 (2002) 70–84. doi:10.1210/mend.16.1.0758.

[122] P. Thomas, Y. Pang, E.J. Filardo, J. Dong, Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells., Endocrinology. 146 (2005) 624–32. doi:10.1210/en.2004-1064.

[123] E. Haas, M.R. Meyer, U. Schurr, I. Bhattacharya, R. Minotti, H.H. Nguyen, et al., Differential effects of 17beta-estradiol on function and expression of estrogen receptor alpha, estrogen receptor beta, and GPR30 in arteries and veins of patients with atherosclerosis., Hypertension. 49 (2007) 1358–63. doi:10.1161/HYPERTENSIONAHA.107.089995.

[124] E.J. Filardo, J.A. Quinn, E. Sabo, Association of the membrane estrogen receptor, GPR30, with breast tumor metastasis and transactivation of the epidermal growth factor receptor., Steroids. 73 (2008) 870–3. doi:10.1016/j.steroids.2007.12.025.

[125] B.F. O'Dowd, T. Nguyen, A. Marchese, R. Cheng, K.R. Lynch, H.H. Heng, et al., Discovery of three novel G-protein-coupled receptor genes., Genomics. 47 (1998) 310–3. doi:10.1006/geno.1998.5095.

[126] N.J. Olsen, W.J. Kovacs, Gonadal steroids and immunity., Endocr. Rev. 17 (1996) 369–84. doi:10.1210/edrv-17-4-369.

[127] E. Goupil, S.A. Laporte, T.E. Hébert, Functional selectivity in GPCR signaling: understanding the full spectrum of receptor conformations., Mini Rev. Med. Chem. 12 (2012) 817–30. http://www.ncbi.nlm.nih.gov/pubmed/22681252 (accessed October 8, 2015).

[128] A.L. Scaling, E.R. Prossnitz, H.J. Hathaway, GPER mediates estrogen-induced signaling and proliferation in human breast epithelial cells and normal and malignant breast., Horm. Cancer. 5 (2014) 146–60. doi:10.1007/s12672-014-0174-1.

[129] E. Haas, I. Bhattacharya, E. Brailoiu, M. Damjanović, G.C. Brailoiu, X. Gao, et al., Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity., Circ. Res. 104 (2009) 288–91. doi:10.1161/CIRCRESAHA.108.190892.

[130] M.R. Meyer, A.S. Field, N.L. Kanagy, M. Barton, E.R. Prossnitz, GPER regulates endothelin-dependent vascular tone and intracellular calcium., Life Sci. 91 (2012) 623–7. doi:10.1016/j.lfs.2012.01.007.

[131] W.-H. Dong, J.-C. Chen, Y.-L. He, J.-J. Xu, Y.-A. Mei, Resveratrol inhibits K(v)2.2 currents through the estrogen receptor GPR30-mediated PKC pathway., Am. J. Physiol. Cell Physiol. 305 (2013) C547–57. doi:10.1152/ajpcell.00146.2013.

[132] C. Wang, B. Dehghani, I.J. Magrisso, E.A. Rick, E. Bonhomme, D.B. Cody, et al., GPR30 contributes to estrogen-induced thymic atrophy., Mol. Endocrinol. 22 (2008) 636–48. doi:10.1210/me.2007-0359.

[133] G. Sharma, C. Hu, J.L. Brigman, G. Zhu, H.J. Hathaway, E.R. Prossnitz, GPER Deficiency in Male Mice Results in Insulin Resistance, Dyslipidemia, and a Proinflammatory State, Endocrinology. 154 (2013) 4136–4145. doi:10.1210/en.2013-1357.

[134] A.C. Villablanca, A. Tenwolde, M. Lee, M. Huck, S. Mumenthaler, J.C. Rutledge, 17β-Estradiol Prevents Early-Stage Atherosclerosis in Estrogen Receptor-Alpha Deficient Female Mice, J. Cardiovasc. Transl. Res. 2 (2009) 289–299. doi:10.1007/s12265-009-9103-z.

[135] Y. Takada, C. Kato, S. Kondo, R. Korenaga, J. Ando, Cloning of cDNAs encoding G protein-coupled receptor expressed in human endothelial cells exposed to fluid shear stress., Biochem. Biophys. Res. Commun. 240 (1997) 737–41. doi:10.1006/bbrc.1997.7734.

[136] U.E.A. Mårtensson, S.A. Salehi, S. Windahl, M.F. Gomez, K. Swärd, J. Daszkiewicz-Nilsson, et al., Deletion of the G protein-coupled receptor 30 impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice., Endocrinology. 150 (2009) 687–98. doi:10.1210/en.2008-0623.

[137] E. Kamanga-Sollo, M.E. White, K.Y. Chung, B.J. Johnson, W.R. Dayton, Potential role of G-protein-coupled receptor 30 (GPR30) in estradiol-17beta-stimulated IGF-I mRNA expression in bovine satellite cell cultures., Domest. Anim. Endocrinol. 35 (2008) 254–62. doi:10.1016/j.domaniend.2008.06.001.

[138] J. Isensee, L. Meoli, V. Zazzu, C. Nabzdyk, H. Witt, D. Soewarto, et al., Expression pattern of G protein-coupled receptor 30 in LacZ reporter mice., Endocrinology. 150 (2009) 1722–30. doi:10.1210/en.2008-1488.

[139] C.G. Bologa, C.M. Revankar, S.M. Young, B.S. Edwards, J.B. Arterburn, A.S. Kiselyov, et al., Virtual and biomolecular screening converge on a selective agonist for GPR30, Nat. Chem. Biol. 2 (2006) 207–212. doi:10.1038/nchembio775.

[140] M.R. Meyer, O. Baretella, E.R. Prossnitz, M. Barton, Dilation of epicardial coronary arteries by the G protein-coupled estrogen receptor agonists G-1 and ICI 182,780., Pharmacology. 86 (2010) 58–64. doi:10.1159/000315497.

[141] B.R.S. Broughton, A.A. Miller, C.G. Sobey, Endothelium-dependent relaxation by G protein-coupled receptor 30 agonists in rat carotid arteries., Am. J. Physiol. Heart Circ. Physiol. 298 (2010) H1055–61. doi:10.1152/ajpheart.00878.2009.

[142] T.M. Ahola, T. Manninen, N. Alkio, T. Ylikomi, G protein-coupled receptor 30 is critical for a progestin-induced growth inhibition in MCF-7 breast cancer cells., Endocrinology. 143 (2002) 3376–84. doi:10.1210/en.2001-211445.

[143] J. Teng, Z.-Y. Wang, E.R. Prossnitz, D.E. Bjorling, The G protein-coupled receptor GPR30 inhibits human urothelial cell proliferation., Endocrinology. 149 (2008) 4024–34. doi:10.1210/en.2007-1669.

[144] B. Kleuser, D. Malek, R. Gust, H.H. Pertz, H. Potteck, 17-Beta-estradiol inhibits transforming growth factor-beta signaling and function in breast cancer cells via activation of extracellular signal-regulated kinase through the G protein-coupled receptor 30., Mol. Pharmacol. 74 (2008) 1533–43. doi:10.1124/mol.108.046854.

[145] N. Kanda, S. Watanabe, 17beta-estradiol stimulates the growth of human keratinocytes by inducing cyclin D2 expression., J. Invest. Dermatol. 123 (2004) 319–28. doi:10.1111/j.0022-202X.2004.12645.x.

[146] M.R. Meyer, N.C. Fredette, M. Barton, E.R. Prossnitz, Regulation of vascular smooth muscle tone by adipose-derived contracting factor., PLoS One. 8 (2013) e79245. doi:10.1371/journal.pone.0079245.

[147] L. Meoli, J. Isensee, V. Zazzu, C.S. Nabzdyk, D. Soewarto, H. Witt, et al., Sex- and age-dependent effects of Gpr30 genetic deletion on the metabolic and cardiovascular profiles of diet-induced obese mice., Gene. 540 (2014) 210–6. doi:10.1016/j.gene.2014.02.036.

[148] M.R. Meyer, N.C. Fredette, T.A. Howard, C. Hu, C. Ramesh, C. Daniel, et al., G protein-coupled estrogen receptor protects from atherosclerosis., Sci. Rep. 4 (2014) 7564. doi:10.1038/srep07564.

[149] F. Li, X. Yu, C.K. Szynkarski, C. Meng, B. Zhou, R. Barhoumi, et al., Activation of GPER Induces Differentiation and Inhibition of Coronary Artery Smooth Muscle Cell Proliferation., PLoS One. 8 (2013) e64771. doi:10.1371/journal.pone.0064771.

[150] Y. Hussain, Q. Ding, P.W. Connelly, J.H. Brunt, M.R. Ban, A.D. McIntyre, et al., G-protein estrogen receptor as a regulator of low-density lipoprotein cholesterol metabolism: cellular and population genetic studies., Arterioscler. Thromb. Vasc. Biol. 35 (2015) 213–21. doi:10.1161/ATVBAHA.114.304326.

[151] R.D. Feldman, R. Gros, Q. Ding, Y. Hussain, M.R. Ban, A.D. McIntyre, et al., A common hypofunctional genetic variant of GPER is associated with increased blood pressure in women., Br. J. Clin. Pharmacol. 78 (2014) 1441–52. doi:10.1111/bcp.12471.

[152] P.A. Olsson, L. Korhonen, E.A. Mercer, D. Lindholm, MIR is a novel ERM-like protein that interacts with myosin regulatory light chain and inhibits neurite outgrowth., J.

Biol. Chem. 274 (1999) 36288–92. http://www.ncbi.nlm.nih.gov/pubmed/10593918 (accessed November 19, 2015).

[153] A. Bretscher, K. Edwards, R.G. Fehon, ERM proteins and merlin: integrators at the cell cortex., Nat. Rev. Mol. Cell Biol. 3 (2002) 586–99. doi:10.1038/nrm882.

[154] R.J. Deshaies, C.A.P. Joazeiro, RING domain E3 ubiquitin ligases., Annu. Rev. Biochem. 78 (2009) 399–434. doi:10.1146/annurev.biochem.78.101807.093809.

[155] B.A. Janowski, M.J. Grogan, S.A. Jones, G.B. Wisely, S.A. Kliewer, E.J. Corey, et al., Structural requirements of ligands for the oxysterol liver X receptors LXR and LXR , Proc. Natl. Acad. Sci. 96 (1999) 266–271. doi:10.1073/pnas.96.1.266.

[156] J.J. Repa, S.D. Turley, J.A. Lobaccaro, J. Medina, L. Li, K. Lustig, et al., Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers., Science. 289 (2000) 1524–9. http://www.ncbi.nlm.nih.gov/pubmed/10968783 (accessed November 19, 2015).

[157] A. Venkateswaran, B.A. Laffitte, S.B. Joseph, P.A. Mak, D.C. Wilpitz, P.A. Edwards, et al., Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR alpha., Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 12097–102. doi:10.1073/pnas.200367697.

[158] E. Scotti, C. Hong, Y. Yoshinaga, Y. Tu, Y. Hu, N. Zelcer, et al., Targeted disruption of the idol gene alters cellular regulation of the low-density lipoprotein receptor by sterols and liver x receptor agonists., Mol. Cell. Biol. 31 (2011) 1885–93. doi:10.1128/MCB.01469-10.

[159] D.I. Chasman, G. Paré, S. Mora, J.C. Hopewell, G. Peloso, R. Clarke, et al., Forty-three loci associated with plasma lipoprotein size, concentration, and cholesterol content in genome-wide analysis., PLoS Genet. 5 (2009) e1000730. doi:10.1371/ journal .pgen.1000730.

[160] T.M. Teslovich, K. Musunuru, A. V Smith, A.C. Edmondson, I.M. Stylianou, M. Koseki, et al., Biological, clinical and population relevance of 95 loci for blood lipids., Nature. 466 (2010) 707–13. doi:10.1038/nature09270.

[161] O.L. Francone, M. Haghpassand, J.A. Bennett, L. Royer, J. McNeish, Expression of human lecithin:cholesterol acyltransferase in transgenic mice: effects on cholesterol efflux, esterification, and transport., J. Lipid Res. 38 (1997) 813–22. http://www.ncbi.nlm.nih.gov/pubmed/9144096 (accessed November 30, 2015).

[162] I.K. Kotowski, A. Pertsemlidis, A. Luke, R.S. Cooper, G.L. Vega, J.C. Cohen, et al., A spectrum of PCSK9 alleles contributes to plasma levels of low-density lipoprotein cholesterol., Am. J. Hum. Genet. 78 (2006) 410–22. doi:10.1086/500615.

[163] N. Ferri, G. Tibolla, A. Pirillo, F. Cipollone, A. Mezzetti, S. Pacia, et al., Proprotein convertase subtilisin kexin type 9 (PCSK9) secreted by cultured smooth muscle cells reduces macrophages LDLR levels., Atherosclerosis. 220 (2012) 381–6. doi:10.1016/j.atherosclerosis.2011.11.026.

[164] T.A. Walton, S. Nishtar, P.J. Lumb, M.A. Crook, M.S. Marber, J. Gill, et al., Pro-protein convertase subtilisin/kexin 9 concentrations correlate with coronary artery disease atheroma burden in a Pakistani cohort with chronic chest pain., Int. J. Clin. Pract. 69 (2015) 738–42. doi:10.1111/ijcp.12615.

[165] G.D. Norata, K. Garlaschelli, L. Grigore, S. Raselli, S. Tramontana, F. Meneghetti, et al., Effects of PCSK9 variants on common carotid artery intima media thickness and relation to ApoE alleles., Atherosclerosis. 208 (2010) 177–82. doi:10.1016/j.atherosclerosis.2009.06.023.

[166] M. Sasaki, Y. Terao, M. Ayaori, H. Uto-Kondo, M. Iizuka, M. Yogo, et al., Hepatic overexpression of idol increases circulating protein convertase subtilisin/kexin type 9 in mice and hamsters via dual mechanisms: sterol regulatory element-binding protein 2 and low-density lipoprotein receptor-dependent pathways., Arterioscler. Thromb. Vasc. Biol. 34 (2014) 1171–8. doi:10.1161/ATVBAHA.113.302670.

[167] D.-W. Zhang, R. Garuti, W.-J. Tang, J.C. Cohen, H.H. Hobbs, Structural requirements for PCSK9-mediated degradation of the low-density lipoprotein receptor., Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 13045–50. doi:10.1073/pnas.0806312105.

CHAPTER 11. PUBLICATIONS

Publications/Book Chapters:

(1) **Ashish Dhyani** , Gianpaolo Tibolla , Andrea Baragetti , Katia Garlaschelli , Liliana Grigore , Giuseppe Danilo Norata and Alberico Luigi Catapano. IDOL N342S gene variant and atherosclerosis progression in the Italian general population. **PLoS One** 2015 Apr 30; 10(4): e0122414. Doi: 10.1371/journal.pone.0122414. (PMID:-25927920).

(2) Vivek Krishna Pulakazhi Venu , Patrizia Uboldi, **Ashish Dhyani**, Alessandro Patrini, Andrés F. Muro, Alberico Luigi Catapano, Giuseppe Danilo Norata: Fibronectin Extra domain A improves atherosclerotic plaque phenotype in apolipoprotein E and in LDL-receptor deficient mice. **Thrombosis and Haemostasis 2**015 Jul; 114 (1): 186-97. Doi; 10.1160/TH14-09-0790. Eupb 2015 Apr16.

(3) Tejpal Dhewa, Rishi Saxena, **Ashish Dhyani**. Lactic acid bacteria as probiotics: Inventory Potential Application, *chapter*, *Recent trends in Microbial Biotechnology*, p111-127., ISBN No.:-978-3-8433-9002-6.

(4) **Ashish Dhyani**, Rishi k Saxena, Vijendra Mishra and Pallavi Sharma (2011). Agrobacterium tumefaciens- Natural source for Plant transformation, In Microbial Ecology (eds Pallavi Sharma et al), Lap Lambert Academic Publishing Ag & Co. Kg, Dudweller Landstr, Germany, pp - 100 - 116.

(5) **Ashish Dhyani**, Tejpal Dhewa, Vijendra Mishra, Vivek Bajpai (2011). Detoxification characteristics of Probiotics-preventive role in human cancer, In Microbial Ecology (eds Pallavi Sharma et al), Lap Lambert Academic Publishing Ag & Co. Kg, Dudweller Landstr, Germany, pp - 39 - 52.

Manuscript under Writing/Editing:-

(1) **Ashish Dhyani,** Andrea Baragetti , Gianpaolo Tibolla, Katia Garlaschelli, Liliana Grigore, Giuseppe Danilo Norata and Alberico Luigi Catapano**. Plasma PCSK9 levels are associated with metabolic syndrome in the general population.** [will be submitted soon].

(2) **Ashish Dhyani,** Andrea Baragetti , Katia Garlaschelli, Liliana Grigore, Giuseppe Danilo Norata and Alberico Luigi Catapano. **GPER p.P16L polymorphism is not associated with altered lipid phenotypes and cIMT progression- A PLIC study** [will be submitted soon].

(3) V.K.Pulakazhi Venu*, **Ashish Dhyani***, Annalisa Moregola, Patrizia Uboldi, A.F.Muro, A.L.Catapano, G.D.Norata. **Absence of Fibronectin-EDA contributes to sepsis pathogenesis in mouse endotoxic shock and cecal ligation and puncture model.** [will be submitted soon]- *Sharing 1st Authorship

(4) Nicola Ferri, Gianpaolo Tibolla, **Ashish Dhyani**, Roberta Baetta, Silvia Marchiano, Alberico L. Catapano, Alberto Corsini. **PCSK9 knock out mice are protected from neo-intimal formation in response to perivascular carotid collar placement.** [Will be submitted soon].

(5) G. Balzarotti, G. Tibolla, F.Bonacina, C.D'Alonzo, **A. Dhyani**, M. Falasca, G. D Norata, A. Catapano**. PI2K-C2β plays a key role in the activation and the proliferation of the T-lymphocytes: Impact on vascular diseases** [Will be submitted soon].