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Extracellular matrix analysis in vasculature in physiological and pathological conditions

Analisi della matrice extracellulare nel comparto vascolare in condizioni fisiologiche e patologiche

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Abbreviations

ALK1	Activin-like kinase 1
ECM	Extra Cellular Matrix
EXT	Exostosin family
EXT1	Exostosin 1
EXT2	Exostosin 2
EXTL3	Exostosin Like Glycosyltransferase 3
GAGs	Glycosaminoglycans
Glc-NAc	N-acetylglucosamine
Glc-NS	N-sulfo glucosamine
Glc-UA	D-glucuronic acid
HA	Hyaluronan
hAoSMC	human Aortic Smooth Muscle Cells
HAS2	Hyaluronan Synthase 2
HAS3	Hyaluronan Synthase 3
HASs	Hyaluronan Synthases
HE	Heparin
HS	Heparan sulfate
HSPGs	Heparan sulfate proteoglycans
HYAL	Hyaluronidase
HUVEC	Human Umbilical Vein Endothelial Cells
IdoA	Iduronic acid
LOX1	Lectin-like oxidized low-density lipoprotein receptor-1
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
NDST1	N-deacetylase/N-sulfotransferase 1
N-LDL	normal-low density lipoprotein
NO	Nitric oxide
NOS1	Nitric oxide synthase 1
NOS2	Nitric oxide synthase 2
NOS3	Nitric oxide synthase 3
NOSs	Nitric oxide synthases
OX-LDL	oxidised-low density lipoprotein
PCSK9	Proprotein convertase subtilisin/kexin type 9
PGs	Proteoglycans
siRNA	short interference RNA

SDC1	Syndecan 1
SDC2	Syndecan 2
SDC3	Syndecan 3
SDC4	Syndecan 4
SDCs	Syndecans
SMC	Smooth muscle cell (s)
TNFα	Tumor Necrosis Factor alpha

1 – SUMMARY

Since cardiovascular diseases are the first cause of death in developed world, there is an increasing interest in studying the molecular bases of these diseases as well as identifying novel therapies to prevent or control such pathologies.
Atherosclerosis is a chronic inflammatory disease of medium and large size arteries. Endothelial dysfunctions and low-density lipoprotein (LDL) accumulation in the tunica intima are the main factors which contribute to extracellular matrix (ECM) modification during atherosclerotic plaque formation. Nevertheless, the exact mechanisms underlying the impaired vascular structure remain unresolved.

Given these considerations, this project is focused on the glycocalyx and ECM characterization in vascular cells. We want to elucidate the role of hyaluronan (HA) and syndecans, two of the major components of glycocalyx, in endothelial cells after treating with TNFα in order to mimic a pro-inflammatory stimulus. This issue can be useful to understand the prevention potentials of ECM components as well as their negative impact on the onset of the disease. Another aspect examined is the action of PCSK9, a novel therapeutic target for LDL-lowering, on HA metabolism and LDL receptors both in endothelial cells and smooth muscle cells (SMC) because very few information is available about PCSK9 role in the ECM and cholesterol metabolism regulation in vascular cells.

We used human umbilical vein cells (HUVEC) treated with TNFα finding a modulation of HA synthase (HASs) transcripts. In particular, among the three HAS isoenzymes, we found an increase of HAS2 and a decrease of HAS3 expression levels. Accordingly, HA in the pericellular coat slightly increase suggesting that *in vivo* HA could be modified in the glycocalyx altering its adhesive properties controlling immune cells and platelets recruitment.
TNFα stimulation also affects syndecan4 (SDC4) core protein expression as well as its glycosaminoglycans (GAGs) chain composed mainly by heparan sulfate (HS). Moreover, the SDC4 overexpression after the pro-inflammatory stimulation correlates with a reduction in HUVEC permeability measured by FITC-dextran diffusion through a cell coated Transwell system. As many functions of SDC4 depends on its GAGs chain, we found an upregulation of the critical enzymes involved in the synthesis of HS (i.e. EXT1, EXT2 and NDST1). Since NDST1 modifies GAG chain catalyzing both the N-deacetylation and the N-sulfation of glucosamine residue, we measured by HPLC the N-sulfation of HS residues and we found an increased amount. As N-sulfation levels in HS are involved in the inhibition of thrombus formation, we used HS extracted form HUVEC membrane and conditioned medium for platelets assays that revealed a decrease of platelets spreading.
These data suggest that, during endothelial inflammation, the alteration of HA synthesis, SDC4 expression and its GAGs chains chemical composition can influence the platelets recruitments and, further, could promote the LDL accumulation during the onset of atherosclerosis.

In addition, we focused our attention on PCSK9, a critical hematic factor involved in the homeostasis of the cholesterol-LDL regulating LDL receptor (LDLR). This enzyme is mainly synthetized in liver and, to verify whether vascular cells could contribute to PCSK9 expression, we treated HUVEC and human aortic smooth muscle cells (hAoSMC) with several factors finding that only in hAoSMC treated with normal (N-LDL) and oxidized LDL (OX-LDL) PCSK9 mRNA levels and protein secretion were reduced.
As PCSK9 can be present in different plasmatic concentrations in healthy patients and hypercholesterolemic subjects, we examined whether it can have a role in LDL receptors expression in vascular cells. Our results indicated that PCSK9 did not influence neither LDLR nor the ALK1 (that is implicated in N-LDL transcytosis) both in HUVEC and in hAoSMC. Interestingly, LOX1, one of the main OX-LDL scavenger receptor, is not affected by PCSK9 in hAoSMC but is dramatically reduced in HUVEC.
As an alteration in HA metabolism is involved in vessel thickening, we evaluated HA biosynthetic enzymes and receptor after PCSK9 treatments. We found that the expression of HASs and CD44 (the main HA receptor) are differently altered depending on the vascular cell type. In particular, in HUVEC and hAoSMC we measured a decrease of HAS3 whereas HAS2 and CD44 expression decreased specifically in hAoSMC.
These data indicate that PCSK9 could have a vaso-protective role regulating molecules involved in HA homeostasis. Therefore, the therapeutic inhibition of PCSK9 can have a positive function in liver lowering LDL but a different effect on vascular system.

2 – RIASSUNTO

Poiché le malattie cardiovascolari sono la prima causa di morte nel mondo in via di sviluppo, vi è un crescente interesse nello studio delle basi molecolari di queste malattie, nonché nell'identificazione di nuove terapie per prevenire o controllare tali patologie.
L'aterosclerosi è una malattia infiammatoria cronica di arterie di media e grande dimensione. Le disfunzioni endoteliali e l'accumulo di lipoproteine a bassa densità (LDL) nella tunica intima sono i principali fattori che contribuiscono alla modificazione della matrice extracellulare (ECM) durante la formazione della placca aterosclerotica. Tuttavia, i meccanismi esatti alla base della struttura vascolare compromessa rimangono irrisolti.

Date queste considerazioni, questo progetto si focalizza sulla caratterizzazione del glicocalice e della ECM nelle cellule vascolari. Vogliamo chiarire il ruolo dell'acido ialuronico (HA) e dei sindecani, componenti principali del glicocalice, nelle cellule endoteliali dopo il trattamento con TNFα al fine di mimare uno stimolo pro-infiammatorio. Questo punto può essere utile per comprendere i potenziali di prevenzione dei componenti della ECM e il loro impatto negativo sull'insorgenza della malattia. Un ulteriore aspetto esaminato è l'azione di PCSK9, un nuovo bersaglio terapeutico per l'abbassamento dell'LDL. Ci siamo focalizzati sul metabolismo dell'HA e sui recettori per le LDL sia nelle cellule endoteliali che nelle cellule muscolari lisce (SMC) perché sono disponibili pochissime informazioni sul ruolo di PCSK9 nell'ECM e nella regolazione del metabolismo del colesterolo nelle cellule vascolari.

Abbiamo utilizzato cellule umane di vena ombelicale (HUVEC) trattate con TNFα trovando una modulazione delle trascrizioni di HA sintasi (HASs). In particolare, tra i tre isoenzimi di HAS, abbiamo riscontrato un aumento di HAS2 e una diminuzione dei livelli di espressione di HAS3. Di conseguenza, l'HA nel rivestimento pericellulare aumenta leggermente, suggerendo che l'HA *in vivo* potrebbe essere modificato nel glicocalice alterando le sue proprietà adesive e controllando il reclutamento di cellule del sistema immunitario e di piastrine.
La stimolazione del TNFα influenza anche l'espressione del core proteico del syndecan4 (SDC4) e la sua catena di glicosamminoglicani (GAG) composta principalmente da eparan solfato (HS). Inoltre, la sovra-espressione di SDC4 dopo la stimolazione pro-infiammatoria si correla con una riduzione della permeabilità delle HUVEC misurata mediante diffusione di FITC-destrano attraverso un sistema Transwell rivestito di cellule. Molte funzioni di SDC4 dipendono dalla sua catena fatta da GAG e nel nostro studio abbiamo trovato una sovra-regolazione degli enzimi critici coinvolti nella sintesi di HS (cioè EXT1, EXT2 e NDST1). NDST1 modifica la catena di GAG catalizzando sia la N-deacetilazione che la N-solfatazione dei residui di glucosammina. Pertanto, abbiamo misurato mediante HPLC la N-solfatazione dei residui di HS che è risultata aumentata in modo significativo. Poiché i livelli di N-solfatazione in HS sono coinvolti nell'inibizione della formazione di trombi, abbiamo usato HS estratti dalla membrana di HUVEC e dal

loro terreno condizionato per analisi piastriniche che hanno rivelato una diminuzione nello spreading delle piastrine.
Questi dati suggeriscono che, durante l'infiammazione endoteliale, l'alterazione della sintesi di HA, dell'espressione di SDC4 e della composizione chimica della sua catena di GAG possono influenzare il reclutamento di piastrine e, inoltre, potrebbero promuovere l'accumulo di LDL durante l'insorgenza dell'aterosclerosi.

In aggiunta, abbiamo focalizzato la nostra attenzione su PCSK9, un critico fattore ematico coinvolto nell'omeostasi del colesterolo-LDL che regola il recettore per le LDL (LDLR). Questo enzima è principalmente sintetizzato nel fegato e, per verificare se le cellule vascolari potrebbero contribuire all'espressione di PCSK9, abbiamo trattato HUVEC e cellule muscolari lisce dell'aorta umana (hAoSMC) con diversi fattori che ci hanno fatto scoprire che solo in hAoSMC trattate con LDL normali (N-LDL) e ossidate (OX-LDL) i livelli di mRNA e la secrezione proteica di PCSK9 sono ridotti.
Poiché PCSK9 può essere presente in diverse concentrazioni plasmatiche in pazienti sani e in soggetti ipercolesterolemici, abbiamo esaminato se possa avere un ruolo nell'espressione dei recettori per le LDL nelle cellule vascolari. I nostri risultati hanno indicato che PCSK9 non ha influenzato né LDLR né ALK1 (che è implicato nella transcitosi delle N-LDL) sia in HUVEC che in hAoSMC. È interessante notare che LOX1, uno dei principali recettori scavenger per le OX-LDL, non è influenzato da PCSK9 in hAoSMC, ma è drasticamente ridotto in HUVEC.
Poiché un'alterazione del metabolismo dell'HA è coinvolta nell'ispessimento dei vasi, abbiamo valutato gli enzimi e i recettori biosintetici dell'HA dopo i trattamenti con PCSK9. Abbiamo trovato che l'espressione di HAS e CD44 (il principale recettore per l'HA) sono alterati in modo diverso a seconda del tipo di cellule vascolari. In particolare, in HUVEC e hAoSMC abbiamo misurato una diminuzione di HAS3 mentre l'espressione di HAS2 e CD44 è diminuita specificatamente in hAoSMC.
Questi dati indicano che PCSK9 potrebbe avere un ruolo vaso-protettivo che regola le molecole coinvolte nell'omeostasi dell'HA. Pertanto, l'inibizione terapeutica di PCSK9 può avere una funzione positiva nel ridurre il colesterolo LDL ma un diverso effetto sul sistema vascolare.

3 – INTRODUCTION

3.1 Atherosclerosis

Atherosclerosis is considered a chronic inflammation disease of the arterial vessel.
This pathology is characterised by a significative thickening of the intima in which cholesterol and lipids accumulation are covered by a rigid extracellular matrix cap forming the so-called atheromatous plaque that protrudes within the vessel lumen (Boyle et al. 1997).
The lesions occur principally in large and medium-size elastic and muscular arteries (Ross 1999). Inside the arterial tree, branching points and bifurcations are the more susceptible regions because in these vessel portions the blood flow is disturbed. Turbulences and pressure oscillations can lead to the loss of the atheroprotective effect of the endothelial layer (Gimbrone et al. 2000).
The lesion can manifest precociously in human life even during childhood (Lusis 2000). The initial and soft lesion can be reversible, but the formation of a mature and complex plaque in adult age usually lead to thrombosis and the most devastating consequences are myocardial infarction, when the thrombus occurs in the coronary arteries, or stroke, when it occurs in the cerebral arteries (Falk 2006)(E. Karangelis et al. 2012).
Atherosclerosis is the first cause of mortality in the developed world, together with the other cardiovascular diseases. To date, the exact causes of atherosclerosis are unknown. Nevertheless, multiple factors are associated with an increased risk of atherosclerotic cardiovascular disease that are summarized in Table 1. Most of them are modifiable factors indicating that healthy diet, exercising and not smoking are the major preventing factors. Therefore, the treatment of established disease include medical therapies in combination with changes in lifestyle (Rafieian-Kopaei et al. 2014).

Modifiable	Non-modifiable
Hyperlipidaemia	Age
Hypertension	Gender and ethnicity
Diabetes	Genetic disposition
Abdominal obesity	
Smoking	
Unhealthy diet and nutrition	
Physical inactivity	

Table 1. Atherosclerosis risk factors.

3.1.1 Atherosclerosis possible causes

Two hypotheses are emerged about the onset and progression of atherosclerosis.

The ‘‘response of injury’’ hypothesis by Ross proposed that some of atherosclerosis risk factors lead to qualitative changes of endothelium as change in permeability and increase of adhesion molecules expression that attract leucocytes. This endothelial response to injury represents the first phase of an inflammatory response that leads to atherosclerosis onset (Ross 1999). Recent postulates based on experimental studies on atherosclerosis in animals designate that the apparition of endothelial dysfunctions triggered by irritative stimuli as dyslipidemia, hypertension and cigarette smoking, is an early atherogenic event (Maiolino et al. 2013).
On the other hand, Williams and Tabas proposed the ‘‘response to lipoprotein retention’’ hypothesis, which affirms that atherogenic low density lipoproteins (LDL) are retained in the intima by binding to extracellular proteoglycans (Nakashima et al. 2007) that have a high affinity for lipoproteins (Rafieian-Kopaei et al. 2014). In addition, the LDL-proteoglycan complex are more susceptible to oxidation leading to their uptake by macrophages that became foam cells and contribute to plaque formation and inflammatory cells reclutament (Chait and Wight 2000).
From these postulates it is evident that atherosclerosis is a complex disease in which endothelial dysfunction, inflammatory process and lipoprotein retention are in a close relation and contribute to lesion development. The scheme in Figure 1 reports this correlation.

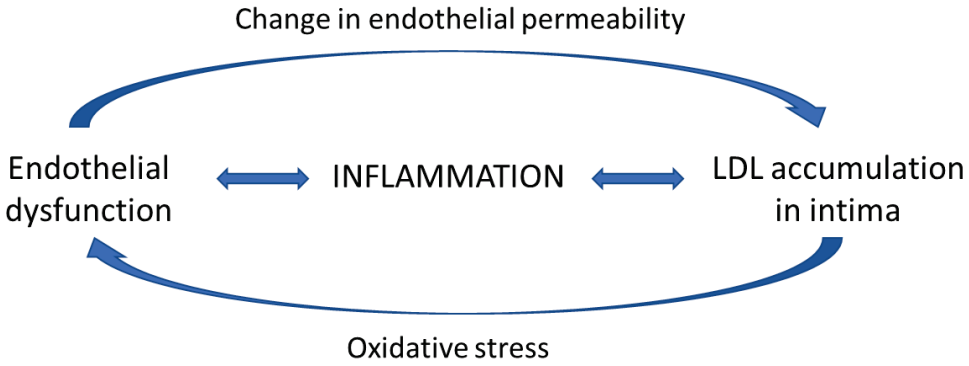

Figure 1 – Relations between the atherosclerosis triggering factors

3.1.2 Role of vascular cells in atherosclerosis process

The vessel wall is composed by the endothelium formed by a single layer of endothelial cells, the tunica intima with smooth muscle cells (SMC), and the tunica adventitia with fibroblasts and SMC. These layers are surrounded by perivascular adipose tissue. Every component is involved in atherosclerosis progression: endothelium dysfunction is considered one of the triggering factors of atherosclerosis development, SMC migration and proliferation are implicated in atherosclerosis progression, and

fibroblasts from adventitia and adipocytes from perivascular adipose tissue contribute to atherosclerosis nourishment (Wang et al. 2017).
The first visible lesions in the development of atherosclerosis plaques are the fatty streaks along the luminal surface of the artery. They appear as an irregular yellow-white discoloration consisting of lipids from lipoprotein of cholesterol and aggregates of foam cells, which are lipoprotein-loaded macrophages located in the intima (Ross 1999).
During atherosclerotic process, an endothelial injury as shear stress or oxidative stress induces in endothelial cell a compensatory response. Endothelial cells start to produce cytokine, chemokines and adhesion molecules (i.e. VCAM-1) causing monocytes and lymphocytes-T migration and adhesion (Ross 1995) (Figure 2).
The injury also induces change in endothelial permeability that increases the passage of lipoproteins to the sub-endothelium and allows the monocytes infiltration in the intima tunica that differentiate into macrophages (Wang et al. 2017). This leads to the early atheroma formation: cytokine stimulated SMC move from media to intima and, after a phenotypically switch to secreting type cells, start to produce extracellular matrix (ECM) components causing a massive deposition of hyaluronan (HA) in the lesion site that enhances the recruitment of myeloid cells as T cells and platelets (Ross 1999)(Viola et al. 2013).
Moreover, in the arterial wall, lipoprotein particles (especially low-density lipoprotein or LDL) undergo oxidation (become OX-LDL) and they are internalized by macrophages and SMC. These cells retain the lipid components of LDL and becoming more lipid-laden form the so called "foam cells" (Chistiakov et al. 2017). The accumulation of OX-LDL can lead to foam cell apoptosis or necrosis (Leiva et al. 2015).
The advanced lesion becomes stable when the SMC production of collagen and elastin lead to the building of a fibrous cap over the lipid core forming by aggregates of lipids and cells debris (Leiva et al. 2015). The consequent increase of intima thickening leads to a lumen diameter reduction.
As showing in Figure 3, the growing plaque can be stabilized, or it can break (unstable plaque) causing the extravasation of the necrotic core of the plaque and the exposition of the sub-endothelium. This causes platelets activation and aggregation leading to a thrombus formation. Usually, the thrombosis can result in stroke or heart attack or sudden cardiac death depending which district is involved (Prathiba et al. 2009).

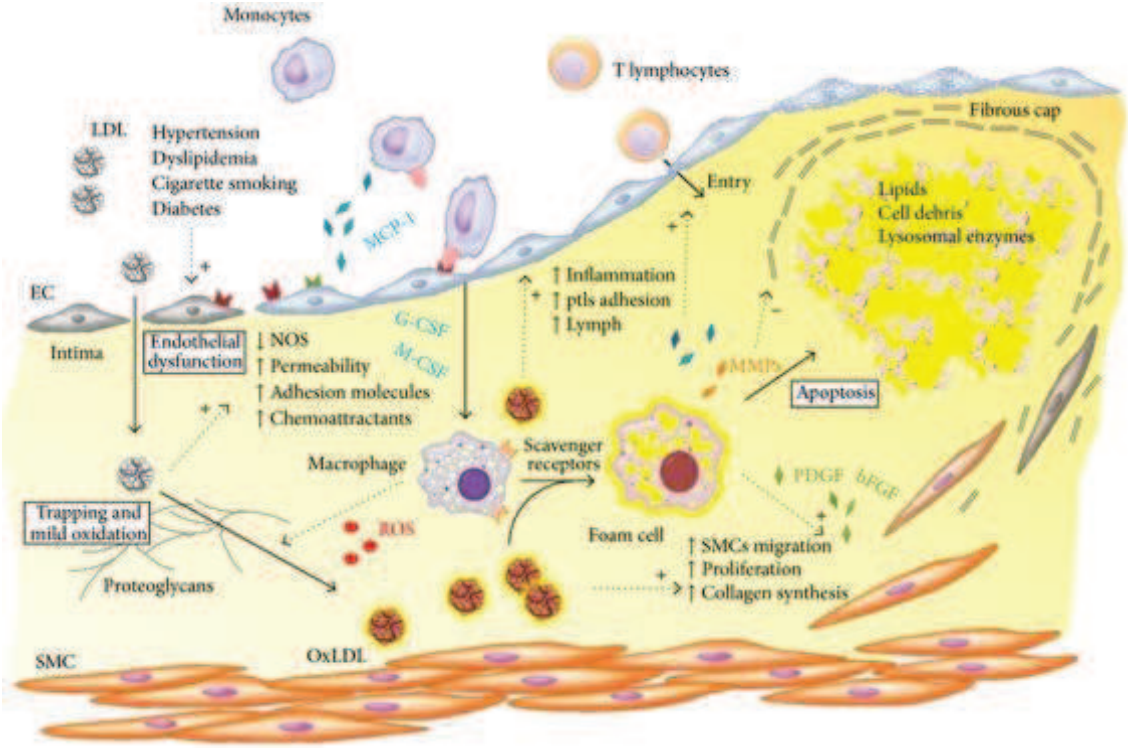

Figure 2– Early phases of atherosclerotic plaque formation.
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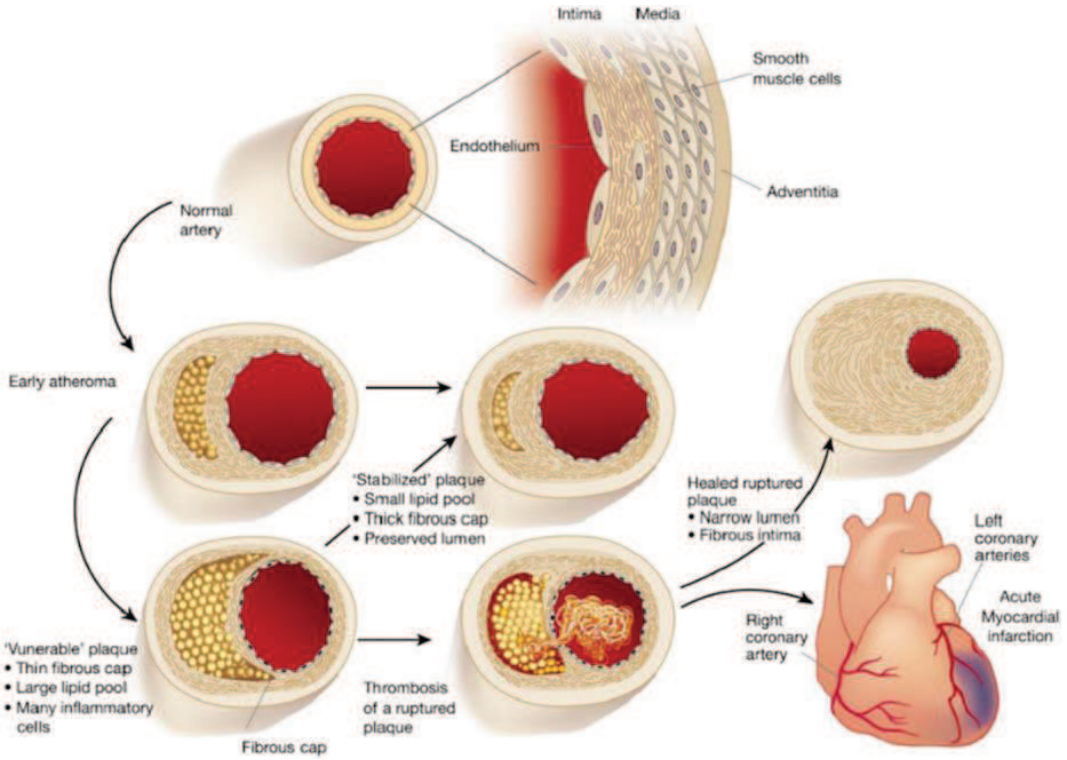

Figure 3 – Scheme of atherothrombosis development.
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3.2 Extracellular matrix: proteoglycans and glycosaminoglycans

As mentioned before, vascular ECM is deeply altered during atherogenesis.
The ECM is composed of macromolecules network that differs in a cell/tissue-specific manner. ECM is not only a scaffold for cell support, but it has also a physiological function because by interaction with growth factors and bioactive molecules, it regulates the signal transduction and the gene expression (Frantz, Stewart, and Weaver 2010). Indeed, ECM is a dynamic structure implicated both in tissue organization, remodelling and homeostasis and in all cell processes such as proliferation, adhesion, migration, differentiation and apoptosis (Yue 2014).
Fundamentally, ECM is composed by water, proteins and polysaccharides. The major components include fibrous protein such as collagen, fibronectin, elastin and laminin and proteoglycans (PGs) with their glycosaminoglycans (GAGs) chains. The PGs and GAGs fill the interstitial space of tissues left free from fibrous protein forming a well-organized network that confers tissue hydration (Iozzo and Schaefer 2015).
In this thesis we focused our attention on syndecans proteoglycans and their GAGs chains.

3.2.1 Heparan sulfate proteoglycans: syndecans

PG are constituted by a linear core protein with GAGs chains covalently attached. In the syndecans proteoglycan, the chains are formed mainly by heparan sulfate (HS).
Syndecans are a family of four transmembrane molecules that are expressed in a cell-type-specific manner (Götte 2003): syndecan1 is present during development and in adult remains express on endothelial and cancers cells; syndecan2 appears in liver, mesenchymal tissue and neuronal cells; syndecan3 is a neuronal type; syndecan4 is ubiquitously distributed (Schaefer and Schaefer 2010). The four syndecans are usually substituted with HS chains but sometimes isoform 1 and 3 possess additional CS chains (Couchman 2010).
All syndecans have an extracellular N-terminal signal peptide, an ectodomain extending out of the cell that bind the GAGs chains, a single hydrophobic transmembrane domain, and a short C-terminal cytoplasmic domain composed of two constant domain (C1 and C2) and one variable domain (V) (Bernfield et al. 1992) (Figure 4).
Syndecans are widely express on the cell surface and they act as co-receptors promoting the signalling of ligands such as growth factors, extracellular matrix components, other cell surface molecules and proteins involved in the regulation of blood coagulation (Reiland and Rapraeger 1993). Many these functions depend on HS chains interaction with the ligand.
The extracellular domains anchored to the membrane can be released from the cell surface by endogenous proteolytic cleavage that happens in a side close to the plasma membrane. This process is known as ectodomain shedding. The shedding permits to

liberate the ectodomains as soluble molecules reducing their concentration at the cell surface (Fitzgerald et al. 2000). An increase of proteoglycans shedding was found in response of inflammatory mediators such as cytokines in various experimental model of inflammation (Kolářová et al. 2014). The massive lack of ectodomain damages the capacity of syndecans to interact with molecules and lets the cell surface less protect.
Syndecans are involved in inflammation-relates events: leucocytes-endothelial interaction, extravasation, cell migration and proliferation as well as coagulation (Götte 2003). Moreover, studies conducted in endothelial cells and fibroblasts suggest a role of syndecan4 (SDC4) in cell adhesion. It co-localizes with integrins into focal adhesion enhanced cell-matrix attachment (Woods and Couchman 1994) and, recently, it seems implicated also in cell-cell junction (Gopal et al. 2017).

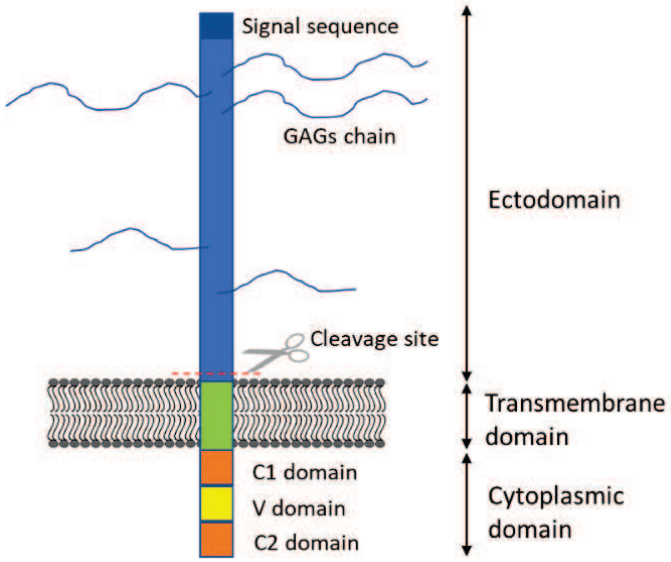

Figure 4 – Domains structure of syndecans.

3.2.2 Heparan sulfate

HS is a linear, negatively charged and sulfated polysaccharide. As all other GAGs [chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparin (HE) and hyaluronan (HA)], HS is composed by a repetition of disaccharides units: uronic acid in a form of D-glucoronic acid (GlcA) or L-iduronic acid (IdoA) and amino sugar like D-galactosamine or D-glucosamine (Kusche-Gullberg and Kjellén 2003).
HS is mainly present as chain of heparan sulfate proteoglycans (HSPGs) as syndecans. To form a mature syndecan is necessary the anchorage and the construction of the polysaccharide chain.
The Figure 5 describes the multistep process of the synthesis of HSPG that starts from the adding of tetrasaccharides linkage region to a specific serine residue of core

protein. This linkage region is composed by the conserved sequence of xylose–galactose–galactose–glucuronic acid. The HS biosynthesis continue thanks to glycosyltransferases action. Among various members of the exostosin (EXT) family, EXT1, EXT2 and EXTL3 are responsible for the chain elongation: EXTL3 catalyzes the addition of the first N-acetylglucosamine (GlcNAc), then an alternating adding of GlcNAc and GlcA is made by EXT1 and EXT2 (Busse-Wicher, Wicher, and Kusche-Gullberg 2014).
Before the chain completion, many modifications occur on the growing polysaccharide. The first is the N-deacetylation and N-sulfation performs by NDST1 (N-deacetylase and N-sulfotransferase) on residues of GlcNAc that becomes N-sulfo glucosamine (GlcNS). This is followed by epimerization of GlcA in IdoA. Some of iduronic acid can be sulfated on residue in 2-O position (2S sulfation) and some glucosamine can be sulfated in 6-O (6S sulfation) or 3-O (3-sulfation) position (Multhaupt and Couchman 2012).

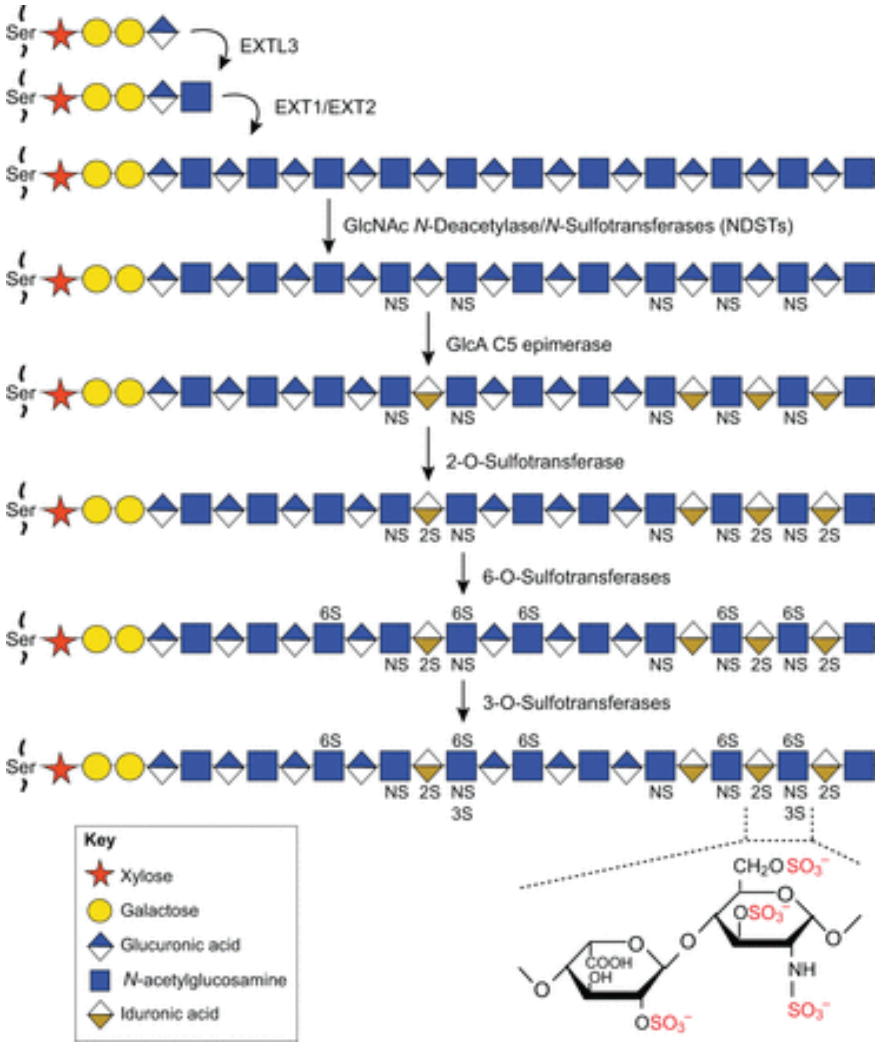

Figure 5 – Synthesis of HS chain.
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The HS synthetic machinery is localized between endoplasmic reticulum and Golgi and the substrates are UDP-monosaccharides and PAPS (3'-phosphoadenosine 5'-phosphosulfate) as a source of free sulfate (John M. Whitelock* and Renato V. Iozzo* 2005).
The complex mechanism of biosynthesis and modification of HS chains generate a variability in N-sulfation levels along the polysaccharides. It produces a region rich in N-acetylated residue (GlcA and GlcNAc) called NA domain, a region rich in N-sufated residue (IdoA and GlcNS derivates) called NS domain and sequences that contain an alteration of both NA and NS units. By the distribution of these domains depends the binding with the ligands (Jackson, Busch, and Cardin 1991).
Two types of binding sequence are known: a) sites for fibroblast growth factor 2 (FGF-2) and FGF receptors that are characterized by high level of N-sulfated residue located in a ectodomain portion far from core protein and b) sites for antithrombin binding that depend to a NA domain near the protein core (Esko and Selleck 2002).
It is evident that the content of charged groups in HS chains is important for electrostatic interaction with proteins. Interestingly, if more than 80% of N-acetylglucosamine become N-sulfo glucosamine and more than 70% of uronic acid is converted L-iduronic acid, HS become similar to heparin and can acquire anticoagulant functions (Schaefer and Schaefer 2010).

3.2.3 Hyaluronan

HA is one of the main components of ECM. It is composed by repetitions of D-glucuronic acid (Glc-UA) and N-acetyl-D-glucosamine (Glc-NAc) units linked with β1-3 and β1-4 bound, respectively. In contrast with all other GAGs, it has not sulphated groups and it is present as a soluble molecule not bound to PG core proteins.
HA is produced at the plasma membrane by three HA synthetases: HAS1, HAS2 and HAS3. HASs are multipass transmembrane enzymes able to produce and extrude the nascent polysaccharide chain by using the UDP sugars present in the cytoplasm: UDP-GlcUA and UDP-Glc-NAc (Figure 6). Interestingly, HASs presents a double catalytic domain and can interact with the two different UDP-sugar substrates (Viola et al. 2015).
HA production depends not only on UDP-sugar precursor availability, but also on HASs regulations. HAS2, that is the mainly responsible for HA synthesis in adult mammal tissues, is not only subject to post traditional modifications as phosphorylation, ubiquitination and O-GlcNAcylation but its expression is modulated at the genetic and epigenetic level by a natural antisense transcript named HAS2-AS1 (Vigetti et al. 2014). This genetic control seems not be present for HAS3 that is regulated by the protein Rab10 modulating the enzyme transport to the membrane (Deen et al. 2014). HAS1 is usually expressed at low level in the adult tissues and it seems to contribute to

inflammation and oncogenesis (Rilla et al. 2013)(Nguyen et al. 2017), but the information about its regulation is very scanty.

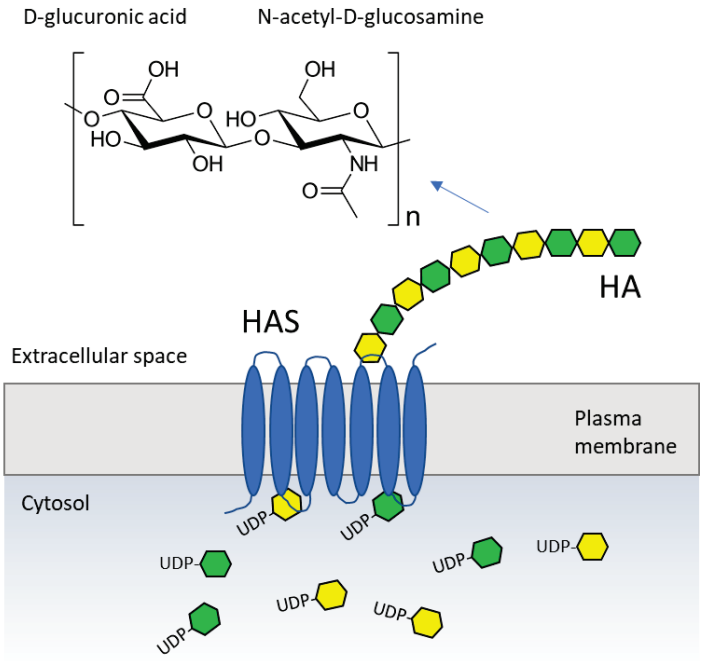


Figure 6 – Hyaluronan synthesis and structure

Since HA is a very hydrated polymer, it controls the water content and the lubrication of all tissues that are critical for cell migration and proliferation. Moreover, HA can interact with proteins and PGs which are important in the assembly of ECM and pericellular glycocalyx. In fact, depending on the extracellular environment HA explicates the activity of inflammation mediator and/or modulator of immune response (Petrey and de la Motte 2014). In addition, HA is important for tissue repair and in wound healing process (Aya and Stern 2014).

HA is a size-dependent mediator of inflammation. In human tissue, HA can have variable molecular weight ranging from $1{,}4 \times 10^7$ Da (high molecular weight or HMW-HA) to fragments of 5×10^3 Da (low molecular weight or LMW-HA) (Moretto et al. 2015). In case of inflammation and tissue injury, HA fragments are more present than in normal conditions. Generally, LMW-HA is considered a pro-inflammatory molecule while HMW-HA an anti-inflammatory polymer (Petrey and de la Motte 2014). Moreover, HMW-HA involved in EMC organization because its presence correlates with tissue integrity and cell quiescence. On the other hand, HA fragments are produced in presence of stress signals (mechanical or oxidative) or through enzymatic degradation of HMW-HA by hyaluronidases (Bollyky et al. 2012b).

Hyaluronidases (HYAL) are responsible for hydrolysis of β 1-4 bounds inside the HA molecule. HA degradation happens both on the cell surface and inside the cell: HAYL2

on cell membrane digest HA in fragments that can be internalized by interaction with CD44 cell receptor and degraded in lysosome by HYAL1 (Stern and Jedrzejas 2006).
Most of the biological functions attributed to HA are associated with specific HA cell receptors. The main HA receptors are: CD44 the ubiquitous receptor, RHAMM that mediates cell mobility, HARE the HA receptor for endocytosis and LYVE1 express on lymphatics endothelial cells involved in HA tournover (Viola et al. 2015). CD44 is a type I transmembrane glycoprotein implicated in cell activation, survival and migration, indicating that CD44 is important in the resolution, rather than the propagation of inflammation (Bollyky et al. 2012a).

3.3 Glycocalyx in atherosclerosis onset

The endothelial glycocalyx is a carbohydrate-rich layer covering the vascular endothelium. It is composed by a network of membrane-bound proteoglycans and glycoproteins in which hyaluronic acid and soluble molecules, either plasma- or endothelium-derived, are incorporated (Figure 7).

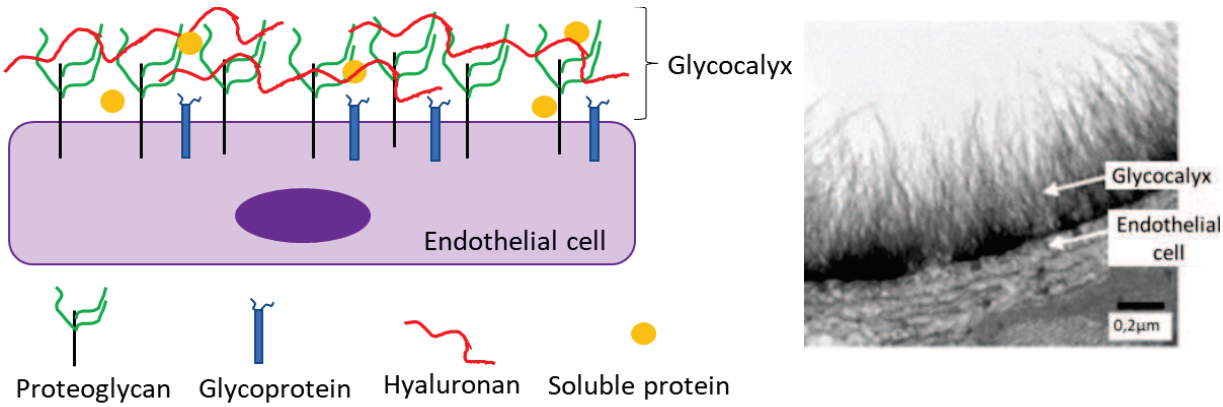


Figure 7 – Left: schematic composition of glycocalyx. Right: electron microscopic overview of goat capillaries glycocalyx. Modified image from Gouverneur et al. (2006). Copyright Clearance Center licence number 4403580245443, 7 Aug 2018

Glycocalyx acts as a barrier between blood plasma and the endothelium explaining different functions.

- Glycocalyx regulates the endothelial permeability restricting the entrance of certain molecules, repulsing red blood cells from endothelium and inhibiting the adhesion of platelets and leukocytes to the vascular wall (Reitsma et al. 2007).
- It has a role in mechanotransduction because its components after hemodynamic changes traduce the mechanical forces in biochemical signals

responsible to endothelial cells responses. One of these reaction involved the nitric oxide production able to regulate the vascular tone (Drake-Holland and Noble 2012).

- Since one of the soluble molecules interacting with glycocalyx is the extracellular superoxide dismutase (SOD) that is involved in reduction of reactive oxygen species (ecROS) (Qin et al. 2008), glycocalyx seems important for the endothelial protection against oxidative stress.

Glycocalyx is continuously subject to a dynamic balance between biosynthesis and degradation. Many factors influence its composition and thickness. One of them is the alteration of shear stress promoting by local turbulences that can affect the integrity of the glycocalyx causing the endothelial denudation (Ballermann et al. 1998).
Interestingly, recent studies indicate a pathological role of the endothelial glycocalyx in atherosclerosis plaque formation where the hydrodynamic force of blood induces the remodeling of the major component of glycocalyx including proteoglycans and hyaluronan (Zeng 2017). Since the functions of glycocalyx depend on its structure and chemically composition, an irregularly arrangement of glycocalyx reduce its endothelial vasculo-protective proprieties (Stancu, Toma, and Sima 2012) leading to an impaired endothelial permeability and increased recruitment and infiltration of inflammatory cell (Zeng 2017).
In addition, since the flux of lipoprotein into the arterial wall depends of both the plasma concentration and the vessel wall permeability(Cancel et al. 2016), an alteration in glycocalyx may allow the LDL passage through dysfunctional endothelial layer and their accumulation in the sub-endothelium increasing the sensitivity of the vessel wall to this pro-atherogenic stimuli (Kolářová et al. 2014).

3.4 Nitric oxide influence on vascular cells

In the vascular system, nitric oxide (NO) plays an important role in regulation of vascular tone, cardiac contractility and vascular remodelling because it causes vasodilatation and inhibits endothelium contracting factor (Matthys and Bult 1997).
Since NO is released from endothelium, an endothelial damage can induce changes in NO levels favouring atherosclerosis.
In physiological condition, NO is produced from L-arginine by nitric oxide synthase (NOS). This process needs the reduced cofactors nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6R-)5,6,7,8-tetrahydrobiopterin (BH4), and the presence of Ca^{2+}/calmodulin (Palmer, Ashton, and Moncada 1988)(Förstermann and Sessa 2012). Three different isoforms of NOSs are known: neuronal (nNOS or NOS1), inducible (iNOS or NOS2) and endothelial (eNOS or NOS3).
As potent vasodilatation factor, NO protects from thrombosis by inhibition of platelets aggregation and adhesion to vessel lamina. This prevents the release of platelet-

derived growth factors that stimulate SMC proliferation and its production of matrix molecules (Förstermann and Sessa 2012).
Pro-inflammatory cytokines as TNFα suppress the expression and function of eNOS in endothelial cells. The diminished synthesis of NO lead to an increase of reactive nitrogen species (RNS) and reactive oxygen species (ROS) which can promote LDL oxidation (Picchi et al. 2006) (Xu et al. 2008). Moreover, the lack of NO inhibition on SMC promotes cells stimulation and migration to the site of inflammation (Zhang et al. 2009) (Figure 8).

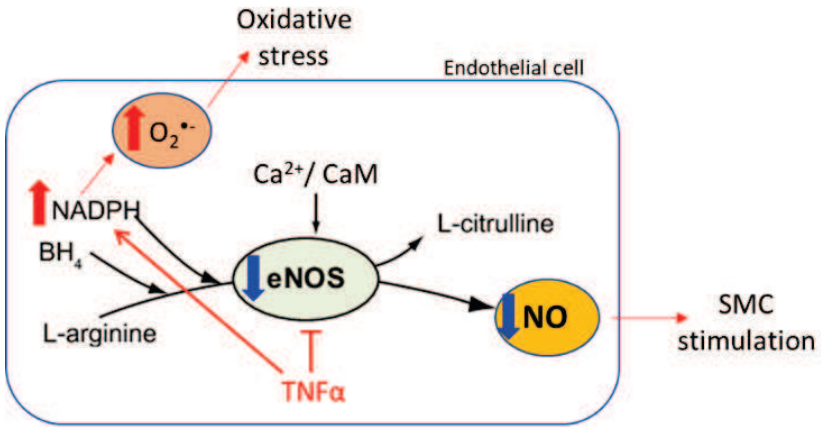


Figure 8 – Effect of TNFα on endothelial NO synthesis.

3.5 LDL oxidation in atherosclerosis development

LDL can be physiologically found in the bloodstream where they contribute to cholesterol transport. They are retained in ECM by the interaction with negative charge GAGs and moving to the subendothelium space they can be acetylated and modified by oxidation.
Oxidized LDL (OX-LDL) is one of the factors that contribute to atherosclerosis progression. Despite the presence of OX-LDL in the atherosclerosis plaque are confirmed by several in vivo studies (Maiolino et al. 2013), it is not clear if the oxidative stress is cause or consequence of the atherosclerosis process and which is the site of oxidation. Possible localizations are the tunica intima because here lipoproteins accumulate or the blood circulation in case of high cholesterol levels accompanied by an oxidative stress condition.
In the atherosclerotic lesion, OX-LDL stimulate monocytes-macrophages, SMC and endothelial cells. An oxidative stimulus in endothelial cells increase the expression of adhesion molecules, cytokines and pro-inflammatory factors. These conditions attract monocytes-macrophages and SMC. SMC from the media migrate into the intima in which they proliferate and contribute to neointimal lesions formation. Here they have a phenotypical change: 1) they acquire the capacity to phagocytize OX-LDL and,

together with macrophages, form foam cells and create the lipid core of the plaque (Lao, Zeng, and Xu 2015); 2) they start to secrete an excess of hyaluronan and collagen (Viola et al. 2013)(Bennett, Sinha, and Owens 2016), at first modifying the extracellular space of intima, and then promoting the formation of fibrous cap over the lipid core. Moreover, OX-LDL present in blood flow can induce a hyperactive state in platelets promoting local platelets aggregation (Leiva et al. 2015).

3.5.1 LDL receptors

LDL can cross the endothelium by two pathways: vesicles and leaky junctions. Vesicles take up LDL from extracellular fluid by receptor-mediated endocytosis, whereas leaky junctions are associated with endothelial cells in a state of turnover or death (apoptosis) (Dabagh, Jalali, and Tarbell 2009). Although LDL are transported mainly by transcytosis (Kuzmenko et al. 2004), hypertension induces apoptosis and increases the damage of endothelial junctions, promoting LDL transendothelial transport and accelerating atherogenesis (Dabagh, Jalali, and Tarbell 2009).
Many receptors are involved in LDL uptake. LDL receptor (LDLR) is the principal receptor involved in native or normal LDL (N-LDL) interaction, while for OX-LDL several scavenger receptors have been identified and the Table 2 resumes the principal ones.

Scavenger receptor	OX-LDL	N-LDL
SR-A I and II	+	-
MACRO	+	-
SRCL	+	-
CD36	+	+
SR-BI	+	+
LOX1	+	-
SREC1	+	-
SP-PSOX	+	-

Table 2 – Native and oxidative LDL as ligand for various scavenger receptors.
Data from Goyal et al. (2012).

Certain scavenger receptors were widely studied for their involvement in atherogenesis.
Lectin-type oxidized LDL receptor 1 or LOX1 mediates the OX-LDL uptake in endothelial cells, macrophages, platelets and SMC. Interestingly, OX-LDL internalization by LOX1 in SMC is related with HA synthesis increase that influence cell adhesion and migration (Viola et al. 2013). Moreover, LOX1 plays an important role in foam cell formation by

binding, internalizing and proteolytically degrading OX-LDL. Since LOX1 is present in large amounts in atherosclerotic lesion in vivo and it is associated with oxidative markers, it reflect the instability of the plaque (Goyal et al. 2012).
Another receptor was recently discovered in endothelial cells called ALK1. It is involved in LDL transcytosis thought endothelium during hypercholesterolemia (Kraehling et al. 2016). ALK1 direct binds N-LDL in a different site respect LDLR and, after the internalization, promotes a non-degradative pathway that permits the passage of intact N-LDL particles from blood to vessel wall. Since ALK1 expression is increased in human coronary atherosclerotic lesion (Yao et al. 2007), ALK1 may have a role during atherogenesis.
LDLR, LOX1 and ALK1 are the main receptors implicated in LDL (normal or oxidized) uptake responsive to the blood cholesterol concentration.

3.6 Therapeutic strategies for atherosclerosis

Atherosclerotic vascular disease is the first cause of death in the world and it is responsible for the majority of cases of cardiovascular disease (CDV) including coronary heart disease, cerebrovascular disease, and peripheral arterial disease (Badimon and Vilahur 2012).
Over the last years it has become clear that atherosclerosis is a multifactorial disease in which many processes contribute to lesion development. Atherosclerosis is not only a lipid-driven disease, but also several immune and inflammatory mediators have a role in the disorder initiation and progression (Weber et al. 2017).
Despite the various mechanism involved in atherosclerosis, the cholesterol-lipid abnormality level is the major modifiable risk factors for atherosclerosis and plasma lipoproteins represent the principal target of drug therapies (Jamkhande et al. 2014).
Within the blood, there are several lipoproteins that each has a different function in lipid transportation. The main lipoproteins are: LDL and HDL (high density lipoprotein). LDL-cholesterol is called "bad cholesterol" because it is implicated in cholesterol transport from liver to extrahepatic tissue and an increase of its plasma concentration contribute to LDL accumulation and oxidation in arterial wall promoting the atherosclerotic plaque formation. On the other hand, HDL-cholesterol is known as ‘‘good cholesterol’’ because it has an important role in reverse cholesterol transport moving the excess cholesterol from peripheral tissue to liver. Moreover, HDL can inhibit LDL oxidation (Navab et al. 2000) and, in addition, they have anti-atherosclerotic, anti-inflammatory and endothelial protective functions (Elshourbagy, Meyers, and Abdel-Meguid 2014).
Given that high LDL-cholesterol and low HDL-cholesterol levels are atherosclerosis risk factors and they are present in established disease, current available strategies for treating atherosclerosis aim to lowering LDL plasma concentration, increasing HDL plasma concentration and reducing LDL oxidation (Moss and Ramji 2016).

List of atherosclerosis therapeutic approaches, summarizing the information given by Toth el at. (2018) and Weber et al. (2018).

- *Statins*: the inhibition of HMG-CoA reductase, essential for cholesterol synthesis, leads to LDL lowering and HDL increase
- *Ezetimibe*: the inhibition of NPC1L1 receptor located in luminal surface of enterocytes responsible for cholesterol up-take, leads to LDL lowering
- *PCSK9 inhibitors*: inhibition of LDLR degradation by PCSK9 to favour LDL uptake and decrease of LDL plasma concentration
- *Microsomal transfer proteins (MTP) inhibitors*: inhibition of lipoproteins synthesis in intestines and liver
- *Oligonucleotide against ApoB-100* (the major component of LDL): inhibition of LDL particles synthesis
- *Increase of HDL presence in blood* by:
    - cholesterol ester transfer protein CETP inhibitors (CETP transfer cholesterol ester from HDL to LDL leading to a reduction of HDL)
    - use of HDL mimetics
    - endogenous stimulation of ApoA-I synthesis (ApoA-I is the main component of HDL
    - direct infusion of ApoA-I (ApoA-I Milano)
- *Bempedoic acid*: the inhibition of adenosine triphosphate-citrate lyase, important for generate cytosolic acetyl-CoA (cholesterol precursor), leads to LDL lowering
- *Anti-platelets aggregation therapy*:
    - aspirin + clopidogrel
    - new oral anti-coagulants (NOACs)
- *Immunomodulation*: inhibition of IL-1beta with monoclonal antibody
- *Anti-inflammatory therapy*: antibodies against P-selectin (P-selectin increase during atherogenesis and is involved in leukocytes recruitment)
- *Nutraceutical therapy*:
    - monacolins such as bergamot or red yeast rice extract: the inhibition of HMG-CoA reductase leads to LDL lowering
    - chokeberry flavonoid extract (anthocyanins, polymeric procyanidins and phenolic acids): reduces OX-LDL levels and chemokine attractive for monocytes
    - spirulina microalga: reduces total cholesterol levels
    - curcumin: anti-oxidant, anti-inflammatory, analgesic

3.6.1 Statins: advantages and disadvantages

Statins are the gold standard pharmacotherapy for managing patients with dyslipidemia and high risk of CVD such as full-blown atherosclerosis (Rosenson 2004). They inhibit the 3-hydroxy-3-methylglutaril coenzyme A (HMG-CoA) reductase which plays a central role in the biosynthesis of cholesterol.
The effect of statins on lipid metabolism drive to a reduction of LDL levels and an increase of HDL levels. Moreover, several studies have shown that statins decrease the susceptibility of LDL to oxidation influencing the oxidative stress and atherosclerosis (Rosenson 2004). Clinical trials with stains reported that they reduce the risk of cardiovascular events decreasing mortality incidence (Taylor et al. 2013).
Despite statins are the first-choice agents, the therapy can be associated with various adverse events as myalgia, myotoxicity and new-onset diabetes (Toth et al. 2018). Intolerance symptoms appears within the first 3 months of statins therapy and sometimes are demonstrable with laboratories analysis that show abnormal levels of muscle creatine kinase (Parker et al. 2013) and hepatic transaminase (Stulc et al. 2015). Since an elevated number of statin-treated patients (about 20-30%) are statins intolerant or do not answer to stains therapy (Mancini et al. 2011) (Arca and Pigna 2011), new drugs targets are developing to lowering LDL-cholesterol and to control the process of atherosclerosis.

3.6.2 PCSK9 inhibitors as a novel therapeutic approach

In clinical practice, the main indication for statins intolerance is PCSK9 inhibition therapy. PCSK9 (proprotein convertase subtilisin/kexin type 9) is a member of proprotein convertase, enzymes that acts as molecular scissors for the tissue-specific processing of multiple precursor proteins (Davignon, Dubuc, and Seidah 2010). PCSK9 is expressed in a limited number of tissues including liver, kidney, cerebellum and small intestine (Seidah et al. 2003). The synthesis starts in endoplasmic reticulum and the mature PCSK9 is release in the extracellular space where it can interfere with matrix components as HSPGs (Gustafsen et al. 2017).
In adults, liver is the primary organ that products PCSK9 and releases it into the circulation (Davignon, Dubuc, and Seidah 2010). The secreted form of PCSK9 can be inactivated by cleavage from proprotein convertase (i.e. furin or PC5/6) (Chaudhary et al. 2017).
The best characterized function of PCSK9 relates to the binding to LDLR in hepatocites (Schulz, Schlüter, and Laufs 2015) where PCSK9 can regulate LDLR both in an extracellular and in intracellular way. Inside the hepatocytes, PCSK9 acts as a chaperone because it binds LDLR precursor in the endoplasmic reticulum and regulates the expression of mature LDLR by inducing its degradation before receptor transport to the membrane (Schulz, Schlüter, and Laufs 2015). While outside the cell PCSK9 binds to the

LDLR, they are internalized through clathrin coated vesicle and LDLR is degraded in endosomal/lysosomal way (Horton, Cohen, and Hobbs 2007) (Nassoury et al. 2007) (Figure 9).
The bound of PCSK9 to LDLR regulates the plasma concentration of LDL. Generally, LDL binds to the LDLR, they are endocytosed and the receptor is recycled to the cell surface (Elshourbagy, Meyers, and Abdel-Meguid 2014). This mechanism favours the presence of LDLR in membrane that enhance the LDL intake. PCSK9 reduces the levels of LDLR on the cell surface and decreases the uptake of circulating LDL (Schulz, Schlüter, and Laufs 2015).
A gain-of-function mutation of PCSK9 are associated with familial hypercholesterolemia leading to higher levels of LDL-cholesterol and increased risk of cardiovascular disease. Moreover, a loss-of-function mutation in enzyme cause hypocholesterolemia. It is clear that plasma levels of LDL-cholesterol are closely related to hepatic expression of PCSK9 and LDLR (Chaudhary et al. 2017).

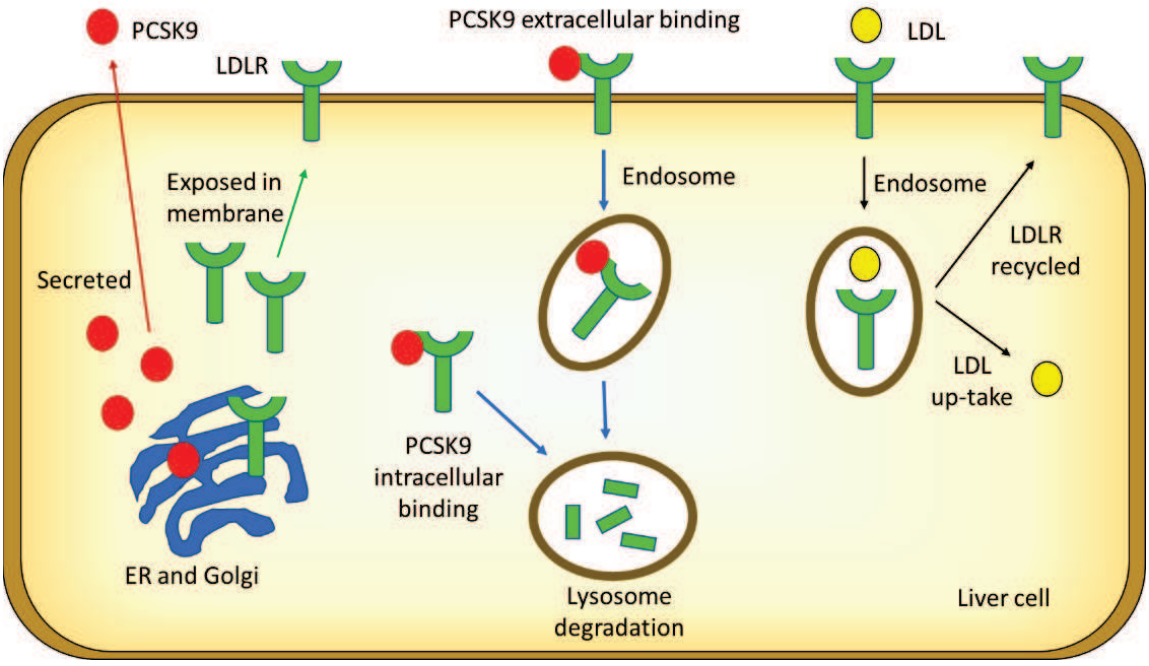


Figure 9 – PCSK9 and LDLR interaction in hepatocytes

In the last decade, a new pharmacological therapy for lowering cholesterol-LDL is proposed antagonizing PCSK9. Inhibition of PCSK9 results in an increased LDLR presence on cell surface capable to increase the up-take of LDL-cholesterol (Weber et al. 2017) (Figure 10).
The main approaches used to reduce PCSK9 synthesis and PCSK9/LDLR interaction consist of a) inhibition of PCSK9 expression using anti-sense oligonucleotides and short interference RNA, b) disrupting PCSK9 binding with LDLR using a mimic peptide and c)

inhibition of PCSK9 activity on LDLR using neutralizing antibodies (Davignon, Dubuc, and Seidah 2010) (X. L. Lin et al. 2018).
The most advanced approach is the administration of fully human monoclonal IgG antibodies against PCSK9 (Glerup et al. 2017). Evolocumab and alirocumab have been approved in USA and Europe in 2015. They show positive results by decreasing LDL-cholesterol of about 50-60% and by increasing HDL (more than 4%) and recent meta-analysis calculated a 50% reduction of cardiovascular events (Stulc et al. 2015) (Solanki, Bhatt, and Johnston 2018).
In addition to lowering LDL-cholesterol, evolocumab in combination with statins demonstrated regression of atherosclerotic lesions in terms of the percent atheroma volume and total atheroma volume (Solanki, Bhatt, and Johnston 2018).

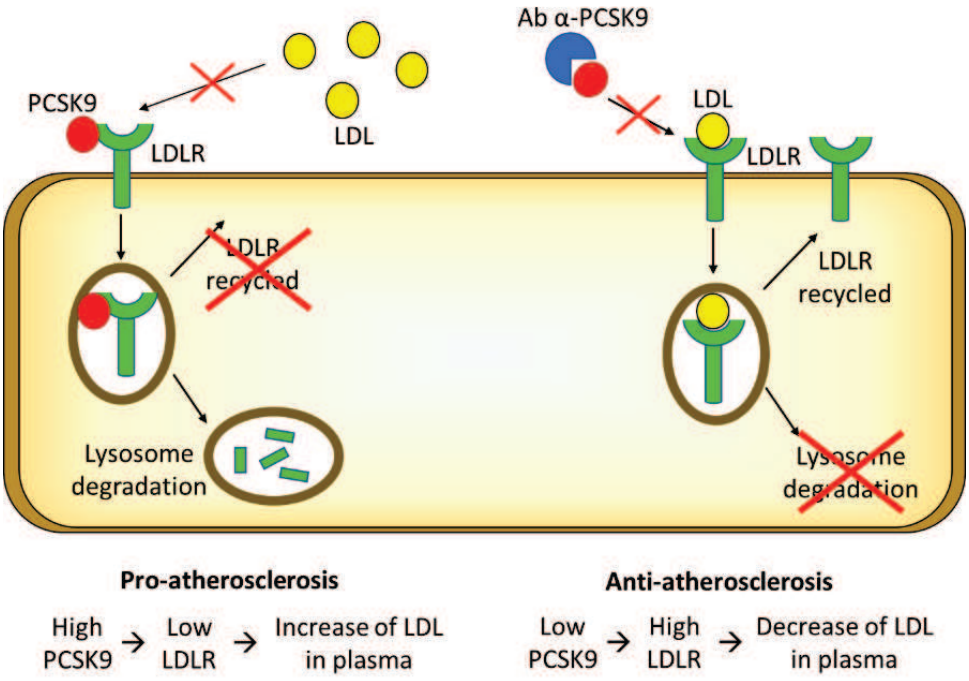


Figure 10 – LDL plasmatic concentration is regulated by PCSK9 and LDLR levels

4 – AIM OF THE WORK

Atherosclerosis is a chronic inflammatory disease where an endothelial dysfunction and the LDL accumulation in vascular wall lead to ECM modification and atherosclerotic plaque formation.
Given that glycocalyx alterations can cause changes in endothelial permeability and this may promote LDL passage inside vessel wall, the first aim of this study was to clarify the role of hyaluronan and syndecans, two of the major component of glycocalyx, in endothelial cells after inflammatory stimulus.
After a validation of the model consisting in HUVEC treated with TNFα, it was evaluated the expression of enzymes involved in hyaluronan synthesis and its presence in pericellular coat. In addition, protein core and GAGs chains composition of syndecans were studied in order to evaluate: a) the core protein expression and its implication in endothelial permeability, b) the quality of HS chains and their influence on platelets stimulation.
Another part of the work is dedicated to PCSK9, a novel therapeutic target for cholesterol lowering and atherosclerosis.
Although PCSK9 is mainly synthetized by the liver, such protein is also present in blood. Therefore, the second aim of my work is to study whether PCSK9 influence the expression of receptors involved in LDL intake in vascular cells and in which way the extracellular matrix is implicated in that process.
PCSK9 expression and production was assessed in HUVEC and hAoSMC after stimulation with LDL (normal and oxidized) to clarify PCSK9 behaviour in extrahepatic cells. Subsequently, HUVEC and hAoSMC stimulated with PCSK9 were used to evaluate the effect of PCSK9 on expression of some LDL receptors and on hyaluronan metabolism.

5 - MATERIALS AND METHODS

5.1 Cell cultures

Human umbilical vein endothelial cells (HUVEC) obtained from Gibco, were grown for 4-8 passages in Medium 200 culture medium (Gibco) supplemented with 2% fetal bovine serum. The cultures were maintained in an atmosphere of humidified 95% air, 5% CO_2 at 37 °C. Twenty-four hours before treatments, subconfluent HUVEC were cultured in DMEM with 0.5% fetal bovine serum. The medium was then changed to Medium 200 with 0.1 μg/ml of TNF-alfa (Sino Biological Inc.) or with 20 μg/ml of normal or oxidized LDL or with 80-100 ng/ml of PSCK9 (R&D Systems) and incubated for 24 or 48 hours.

Human aortic smooth muscle cells (hAoSMCs) obtained from Gibco were cultured at 37°C in an atmosphere of 5% CO_2 relative humidity between passages 4-8 in Medum 231 culture medium (Gibco). After 24h starvation in DMEM with 0.2% fetal bovine serum, cells were treated with 20 μg/ml of normal or oxidized LDL or with 80-100 ng/ml of PSCK9 for 24 hours.

5.2 Quantitative RT-PCR

Total RNA samples were extracted from untreated or treated cells with Absolutely RNA Microprep Kit (Agilent Thechologies). cDNAs were generated by using the High Capacity cDNA synthesis kit (Applied Biosystems) and were amplified on an Abi Prism 7000 instrument (Applied Biosystems) using the Taqman Universal PCR Master Mix (Applied Biosystems) following the manufacturer's instructions. The following human TaqMan gene expression assays were used: HAS2 (Hs00193435_m1), HAS3 (Hs00193436_m1), NOS1 (Hs00167223_m1), NOS2 (Hs01075529_m1), NOS3 (Hs01574659_m1), SYND1 (Hs00174579_m1), SYND2 (Hs00299807_m1), SYND3 (Hs00206320_m1), SYND4 (Hs00161617_m1), NDST1 (Hs00155454_m1), EXT1 (Hs00609162_m1), EXT2 (Hs00181158_m1) and β-actin (Hs99999903_m1). For some genes the expression was measured using SYBR Green Master Mix (Applied Biosystems). In this case we used the following primers: ALK1 designed on the basis of NM_000020.2 sequence (forward nt 981-1001; 5'-CGAGGGATGAACAGTCCTGG-3' and reverse nt 1057-1078; 5'-GTCATGTCTGAGGCGATGAAG-3'), LOX1 designed on the basis of NM_001172632.1 sequence (forward nt 948-969; 5'-GCCTGGCACCTTTATGTCAAC-3' and reverse nt 990-1016; 5'-CTTGGGACAAGCTAGGTGAAATAATA-3'), PCSK9 designed on the basis of NM_174936.3 sequence (forward nt 1534-1550; 5'-ACGTGGCTGGATTGCA-3' and reverse nt 1593-1616; 5'-AAGTGGATCAGTCTCTGCCTCAA-3'), LDLR designed on the basis of NM_000527.4 sequence (forward nt 492-510; 5'-AAGGCTGTCCCCCCAAGA-3' and reverse nt 548-567; 5'-CGAACTGCCGAGAGATGCA-3'), and housekeeping β-Actin designed on the basis of NM_001101.4 sequence (forward nt 1172-1195; 5'-TCAAGATCATTGCTCCTCCTGAG-3' and reverse nt 1238-1259; 5'-ACATCTGCTGGAAGGTGGACA-3'). The relative quantification of gene expression levels was determined by comparing $2^{-\Delta\Delta Ct}$ (Viola et al. 2013).

5.3 Western blotting

RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% TRITON X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing Protease Inhibitor Cocktail (Sigma) was used to prepare cell lysates. Proteins were quantified, separated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. After incubation in blocking solution (5% BSA in 1X Tris-Buffered Saline Tween-20), membrane was incubated overnight with primary antibody at 4°C. Antibodies used were rabbit polyclonal antibody against Syndecan4 (ABT157, Merck Millipore, dilution 1:250) and goat polyclonal antibody against β-actin (#J1805, Santa Cruz Biotechnology, dilution 1:1000). The membrane was washed with 1x TBS-T and incubated 1 hour with secondary antibody. Bands visualization was carried out by the chemiluminescence system LiteAblot TURBO (Euro Clone). The relative intensities of the protein bands were analysed with ImageJ software. β-actin levels were used as controls for protein loading.

5.4 LDL isolation and oxidation

Native LDL was isolated from plasma of normolipidemic healthy volunteers kindly provided by the "Ospedale di Circolo" of Varese. After ultracentrifugation at 4°C, LDL were collected, freed of EDTA by gel filtration using PD-10 column (GE Healtcare) and concentrated with Centriprep Centrifugal Filters (10 KDa, Merck). LDL was oxidized with 1mM $CuSO_4$ overnight at 37°C. At the end of incubation time, the effective oxidation of LDL was monitored by recording the increase of absorbance at 234nm comparted with normal non-oxidized LDL (Albertini et al. 1999) (Viola et al. 2013).

5.5 ELISA for PCSK9 quantification

Conditioned media from treated cells were collected and used for the protein quantification of PCSK9 by the Quantikine ELISA kit (R&D Systems) following the protocol provided by the manufacturer.

5.6 Cell transfection

HUVEC were transfected with siRNA against syndecans4 (S12639, Ambion) using a nucleofector apparatus (Amaxa) and the Amaxa HUVEC Nucleofactor kit (Lonza) following manufacturer's instructions. Briefly, $5x10^5$ cells were resuspended in 100 µl HUVEC Nucleofector solution and transfected with 40nM siRNA against syndecans4. Cells were treated 24 hours after the transfection. The control siRNA used was Silencer Negative Control siRNA #1 (AM4611, Ambion). The syndecans4 silencing efficiency was determined by qRT-PCR.

5.7 Cell viability assay

HUVEC metabolic activity was evaluated with the MTT assay. Cells were seeded at a density of $6x10^3$ cells/well in a 96-well plate. The following day, HUVEC were treated with 0.1 µg/ml of TNFα. After 4, 16, 24 or 48 hours, the cells were washed with PBS and

MTT solution (50 µl of 5 mg/ml) was added to each well for 4 hours al 37°C. Subsequently, the medium was removed and dimethyl sulfoxide (Sigma-Aldrich) was added (200 µl/well) to solubilize the formazan crystals. Optical density was measured at 570 nm with Tecan microplate reader (Thermo Scientific).

5.8 Wound healing assay

HUVECs seeded in 6-weel plates were cultured until confluence. Three parallel scratches were introduced to the HUVEC monolayer with a 200 µl sterile pipette tip. Cells were washed to remove debris and incubated in fresh medium with or without TNFα. Images from three different scratch areas in each culture well were obtained using Olimpus IX51 microscope after 2, 4, 6 and 8 hours.

5.9 Red blood cell exclusion assay

Pericellular area (coat) around HUVEC was visualized using a particle exclusion assay involving human erythrocytes fixed with formaldehyde. Cells were seeded at a density of $6x10^3$ cells/well in 12-well plate and treated or untreated (control) with TNFα. After 24 hours, 500 µl of a suspension of fixed and washed human erythrocytes ($15x10^6$ erythrocytes/ml) was added to the cells and allowed to settle for 20 minutes at 37°C. Images of pericellular coat were obtained using phase contrast microscope Olimpus IX51. The dependence of the pericellular matrix on HA was shown by treating the cultures with 2 U/ml of HA-specific Hyaluronate Lyase from Streptomyces hyalurolyticus (Sigma) in medium for 1 hours at 37°C before visualization with the particle exclusion assay. Representative cells were photographed at a magnification of ×40. ImageJ software was used to quantify area delimited by red blood cells and the area delimited by the cell membrane to give a coat-to-cell ratio (Vigetti et al. 2009).

5.10 Transwell permeability assay

FITC labelled dextrans were used as the representative of hydrophilic molecules to measure the permeability of endothelial cell monolayer (Lal et al. 2001) (Simoneau, Houle, and Huot 2012). HUVEC were plated in the upper part of a Transwell filter (0.4 µm, 6.5 mm diameter, Corning) at a density of $8x10^3$ cell per well and were cultured until the formation of a tight monolayer checked with microscope. Cells were treated with TNFα (0.1 µg/ml) and FITC-dextran (Mw~250000 Sigma) was added to the top chamber of the Transwell for a final concentration of 1 mg/ml. The culture medium in the upper and in the lower chamber was collected 24 hours post-treatment and fluorescence was measured by fluorimeter (Tecan, Thermo Scientific) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. To evaluate the FITC-dextran passage thought cells monolayer, we calculated the total fluorescence of each well adding upper fluorescence value and lower fluorescence value. Total fluorescence was used to find the percentage of fluorescence of the lower chamber. Lower percentage value of control has been set as 1 to normalize the others.

5.11 Glycosaminoglycans determinations

Glycosaminoglycan released into the culture medium or binding on cells membrane were extracted using the protocol of Viola et al. (Viola et al. 2008). Briefly, after the stimulation of samples, conditioned media were collected as well as trypsin supernatants after cells harvesting (membrane GAGs extraction). Samples were subjected to digestion with proteinase K (20 U/ml, Finnzymes) and precipitation with ethanol (9:1/ethanol:water) in order to recover GAG in the pellets. HE/HS disaccharides were obtained digesting the GAG with a mix of heparinases I–II–III (form F. heparinum, Seikagaku) and AMAC (Sigma) derivatization. HE/HS disaccharides were used for HPLC relative quantification and characterization. As standards we used a mix of non-sulfated (0S), mono-sulfated (6S, NS, S2), di-sulfated (S1) and tri-sulfated (Tri-S) AMAC-derived HE/HS disaccharides at the concentration of 5 nmol (Seikagaku). While, HS GAGs were purified through a digestion with chondroitinase ABC (from Proteus vulgaris, Seikagaku) for 5 hours at 37°C. After the uronic acid quantification, HS were used for platelets adhesion and spreading assay.

5.12 Glucuronic acid quantification

HS GAGs content (membrane and medium) was measured by using uronic acid quantification following the van den Hoogen et al. method (van den Hoogen et al. 1998). Brefily, to each well of a 96 plate was applied 40 μl of HS sample and 200 μl of concentrated sulfuric acid (80% w/w) was added. The plate was placed in incubator for 1 hour at 80°C and, after cooling to room temperature, the background absorbance of samples was measured at 540nm on a microplate reader (Tecan, Thermo Scientific). Then, 40 μl of 3-hydroxybiphenyl solution (100 μl of 3-hydroxybiphenyl (Fluka) in DMSO, 100 mg/ml mixed with 4.9 ml 80% (v/v) sulfuric acid) was added. After an overnight incubation the absorbance of the pink-colored samples was read again at 540nm. A commercial D-Glucuronic acid (Sigma) was included as standard series. The background absorbance was subtracted from the second reading and the uronic acid content was interpolated from the corresponding reference curve.

5.13 Platelet-rich Plasma (PRP) preparation

As described in Posner et al. (Posner et al. 2015), human blood was drawn from healthy volunteers by median cubital vein puncture under local ethics committee approval. Sodium citrate was used as anticoagulant (0.5% w/v for platelet isolation, 0.1% w/v for whole blood experiments). PRP was separated from whole blood by centrifugation (200 g, 15 min), and platelets were separated from PRP by a second centrifugation step (400 g, 10 min), in the presence of prostaglandin E1 (PGE1, 40 ng/ml) and indomethacin (10 M). Washed platelets were then resuspended in a modified Tyrode's-HEPES buffer (10 mM HEPES, 145 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, pH 7.3) at a density of $4x10^8$ /ml.

5.14 Platelet adhesion and spreading assay

Following Canobbio et al. (Canobbio et al. 2016) protocol, glass coverslips were coated overnight at room temperature with 100 μg/ml fibrinogen, 0.32 μg HS GAGs from membrane or medium and then blocked with 1% BSA for 2 hours at room temperature. Washed platelets (0.5 ml; 4×10^7 cells/ml) were added to dishes coated with fibrinogen and GAGs in the presence of 1 mM $CaCl_2$. No-adherent cells were discharged, and adherent platelets were fixed, permeabilized with Triton X-100, and actin filaments were stained by TRITC-conjugated phalloidin. Platelets were viewed on a fluorescence microscope (Olympus BX51), and digital images (400×) were acquired. The number of adherent cells was determined using the ImageJ Version 1.42 software.

5.15 Data analysis

Data are presented as mean ± S.D. Statistical significance was determined using unpaired Student's t test. Statistical significance was established at $P < 0.05$ for *, $P < 0.01$ for ** and $P < 0.001$ for ***.

6 – RESULTS

6.1 HUVEC treated with TNFα as in vitro model of endothelial inflammation

Atherosclerosis is a chronic inflammation of arterial vessel wall. One of the atherosclerosis triggering factor is the endothelial dysfunction in response to inflammation stimuli. Since the development of atherosclerosis is coupled to dramatic alteration of extracellular matrix components, we used HUVEC treated with TNFα as a model of endothelium inflammation to clarify the role of glycocalyx components as HA and HS PGs in the onset of atherosclerosis.

The experiments were carried out by using HUVEC left untreated (control) or treated with TNFα 0.1 μg/ml for 24 hours and ECM composition studied in conditioned media or in the cells extracts. To validate our *in vitro* model, nitric oxide synthases (NOSs) expression levels were analysed after the cytokine stimulation. Nitric oxide (NO) is important to maintain normal vascular functions and endothelial integrity. As expected, the endothelial isoform NOS3 was the most expressed form in HUVEC (Figure 11A). Moreover, the expression levels of NOS3 and NOS1 were significant decreased after TNFα stimulation while NOS2 showed a non-significant tendency to decrease (Figure 11B). These data agree with the literature where *in vitro* studies confirm the defect in the NO production in isolated atherosclerotic blood vessels (Matthys and Bult 1997).

Since a reduction in NO synthesis and/or enzymatic activity may contribute to the initiation and progression of atherosclerosis (Napoli and Ignarro 2018), we can consider HUVEC treated with TNFα a good *in vitro* model of endothelial inflammation of atherosclerosis early stage.

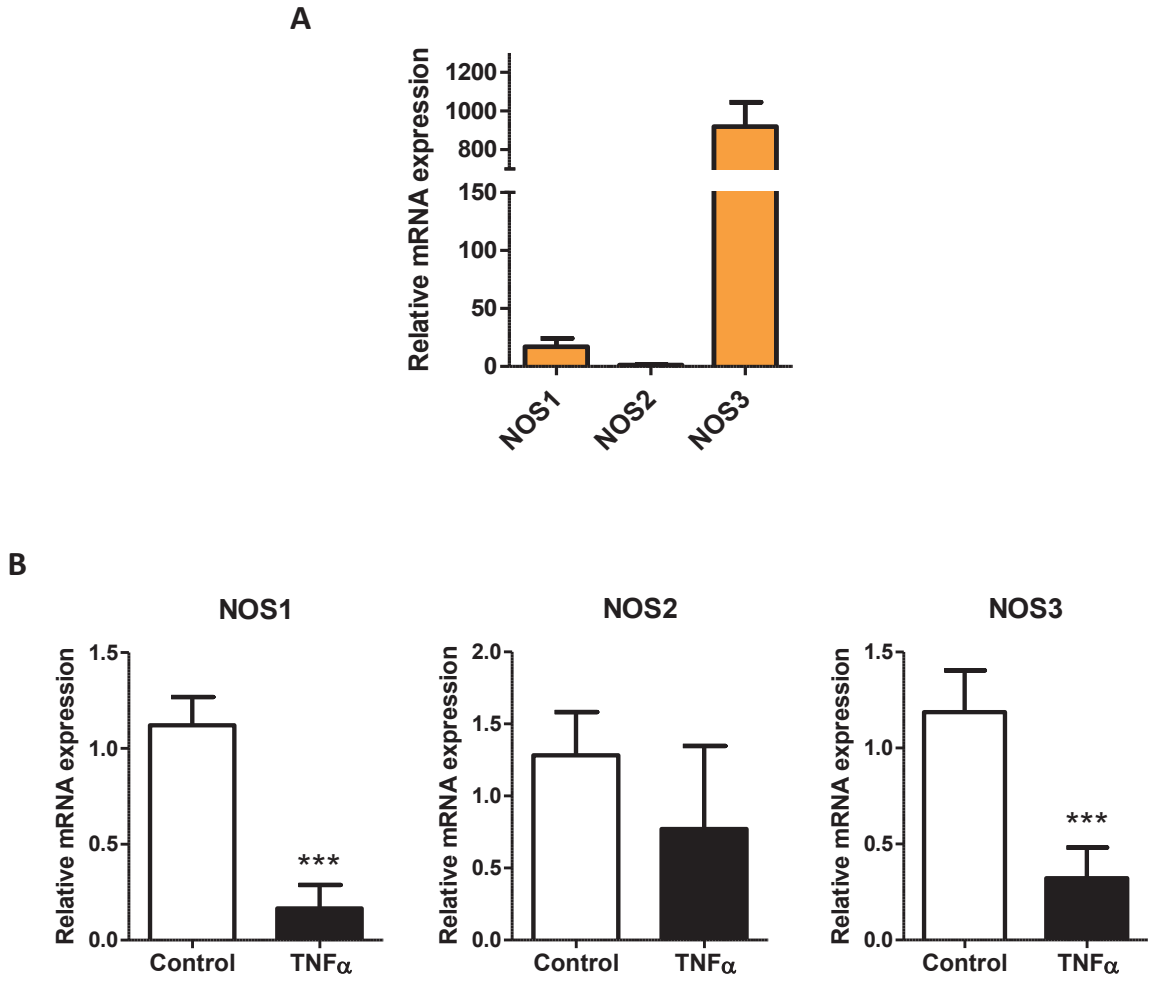


Figure 11 - Effect of TNFα on NO synthesis enzymes in HUVEC. A, relative expression of NOSs (neuronal NOS1, inducible NOS2 and endothelial NOS3) in HUVEC. B, NOSs expression in HUVEC untreated (control) and treated with TNFα (0.1 μg/ml) for 24 hours. Data are mean ± S.D. of three independent experiment, *** $p<0.001$.

6.2 TNFα regulates HA synthesis enzymes and increases the HA presence in the endothelial coat area

Hyaluronan (HA) is the principal component of extracellular matrix and it is produced by HAS1, HAS2 and HAS3. HA can have a pro or anti-inflammatory role depending on its molecular mass. Since HA molecular mass can depend on which HASs is expressed, we measured the level of the HASs in cells control. In untreated HUVEC cells, HAS3 mRNA was the most abundant whereas HAS1 messenger was not detected (Figure 12A). Interestingly, after TNFα stimulation, HAS2 and HAS3 increased and decreased expression, respectively (Figure 12B).

HASs are transmembrane enzymes able to extrude out of the cell the nascent polysaccharides chain as well as able to retain the nascent HA bond to the plasma membrane. Therefore, HA is not only present in the conditioned medium, but also present around endothelial cells surface as a component of glycocalyx. To evaluate the pericellular space (i.e. glycocalyx) as well as amount of HA, we performed the particle exclusion assay. The pericellular space was significant increased after TNFα stimulation. Moreover, the coat disappeared after specific HA lyase (Hyaluronidase) treatment and remain increased when HUVEC were treated with both TNFα and Hyaluronidase inactivated with high temperature. These results indicated that the modulation of HASs expression by TNFα altered the HA presence in the endothelial pericoat.

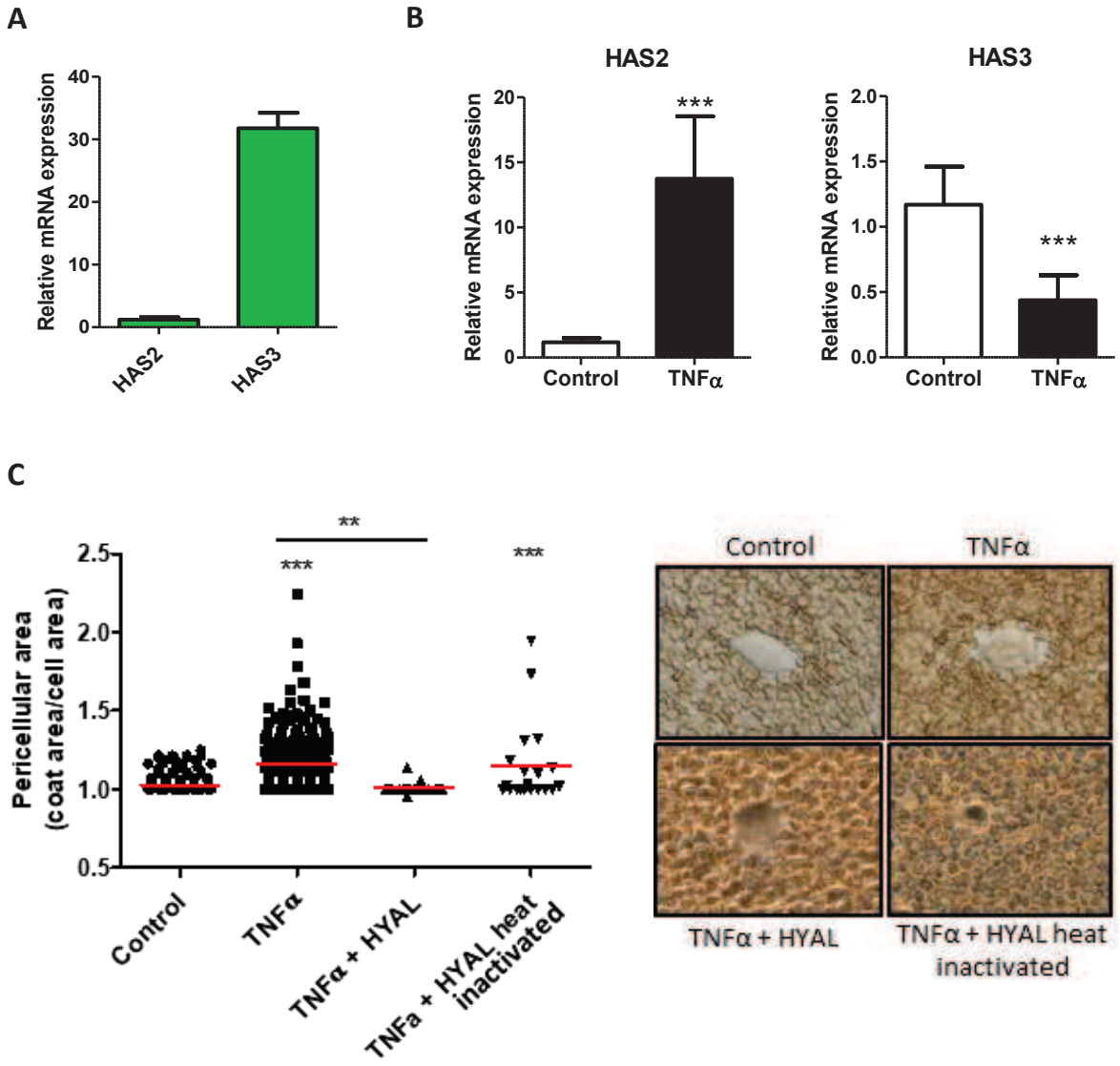


Figure 12 - Effect of TNFα on Hyaluronan synthesis in HUVEC. A, HASs expression profile in HUVEC. B, relative expression of HAS2 and HAS3 in HUVEC untreated (control) and treated with TNFα (0.1 μg/ml) for 24 hours. Data are mean ± S.D. of four independent experiment, *** $p<0.001$. C, particle exclusion assay performed on HUVEC control and under TNFα stimulation (0.1 μg/ml) for 24 hours. To clarify the HA composition of the pericellular matrix, we digested HA with 2 U/ml of Hyaluronate Lyase from Streptomyces hyalurolyticus (HYAL) before addition of erythrocytes. Original magnification 40 x. Values represent the measure of single cell pericellular area (control n=70, TNFα n=118, TNFα+HYAL n=21 and TNFα+HYAL heat inactivated n=19) and the red bars are the mean of three independent experiments, *** $p<0.001$ and ** $p<0.01$

6.3 HUVEC vitality and migration are not influenced by TNFα

In the vascular system HA is important to maintain the vascular homeostasis and it has a crucial role in glycocalyx as well as in the regulation of angiogenesis during the normal wound healing (Slevin et al. 2007). Moreover, its viscoelastic propriety is important for the vascular remodelling during atherosclerosis development.
To verify if the increase of HA presence in HUVEC coat after TNFα stimulation influence the vitality and migration of endothelial cells, we performed an MTT assay and a wound healing assay. The data in Figures 13 and 14 proved that endothelial metabolic activity and migration were not affected by TNFα treatment.

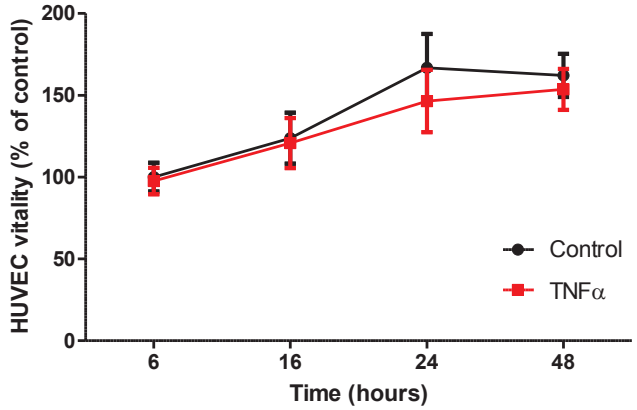


Figure 13 - MTT vitality assay. HUVEC were treated with TNFα (0.1 µg/ml) and after 6, 16, 24, and 48 hours cell viability was assessed by MTT assay. Values represent mean ± S.D. (n=2).

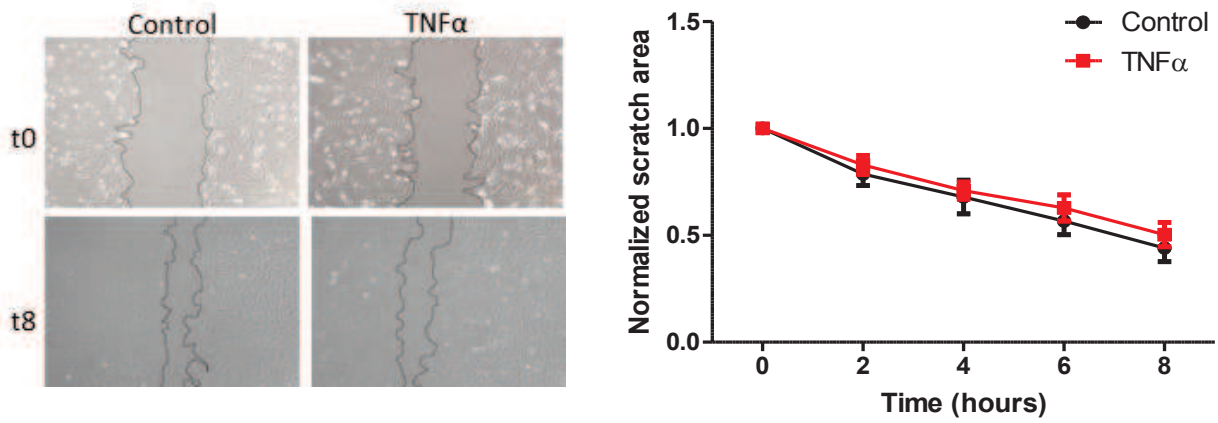


Figure 14 - Wound healing assay of HUVEC. The assay was conducted in HUVEC treated with TNFα (0.1 µg/ml) and control cells. Representative photographs were taken at 0 h and 8 h post-scratch (× 40) and the scratch closure was quantified at 0, 2, 4, 6 and 8 hours post-wound by measuring the remaining unmigrated area using ImageJ. Values represent mean ± S.D. (n=3).

6.4 SDC4 increases after TNFα stimulation in HUVEC

Syndecans are a family of four transmembrane proteoglycans acting as co-receptors interacting with different molecules including growth factors, matrix components and cytokines that are present in glycocalyx (Götte 2003). To test their involvement in endothelial inflammation, we evaluated the expression of the different isoforms (SDC1, SDC2, SDC3, SDC4) in HUVEC treated or untreated (control) with TNFα for 24 and 48 hours.

Despite SDC3 and SDC4 were the most expressed isoform in HUVEC, only SDC4 was upregulated by TNFα and the increase of mRNA levels remained high also after 48 hours of treatment. Data were confirmed by western blot analysis (Figures 15 A, B and C). SDC4 seemed the major isoform implicated in inflammation response of endothelial cells.

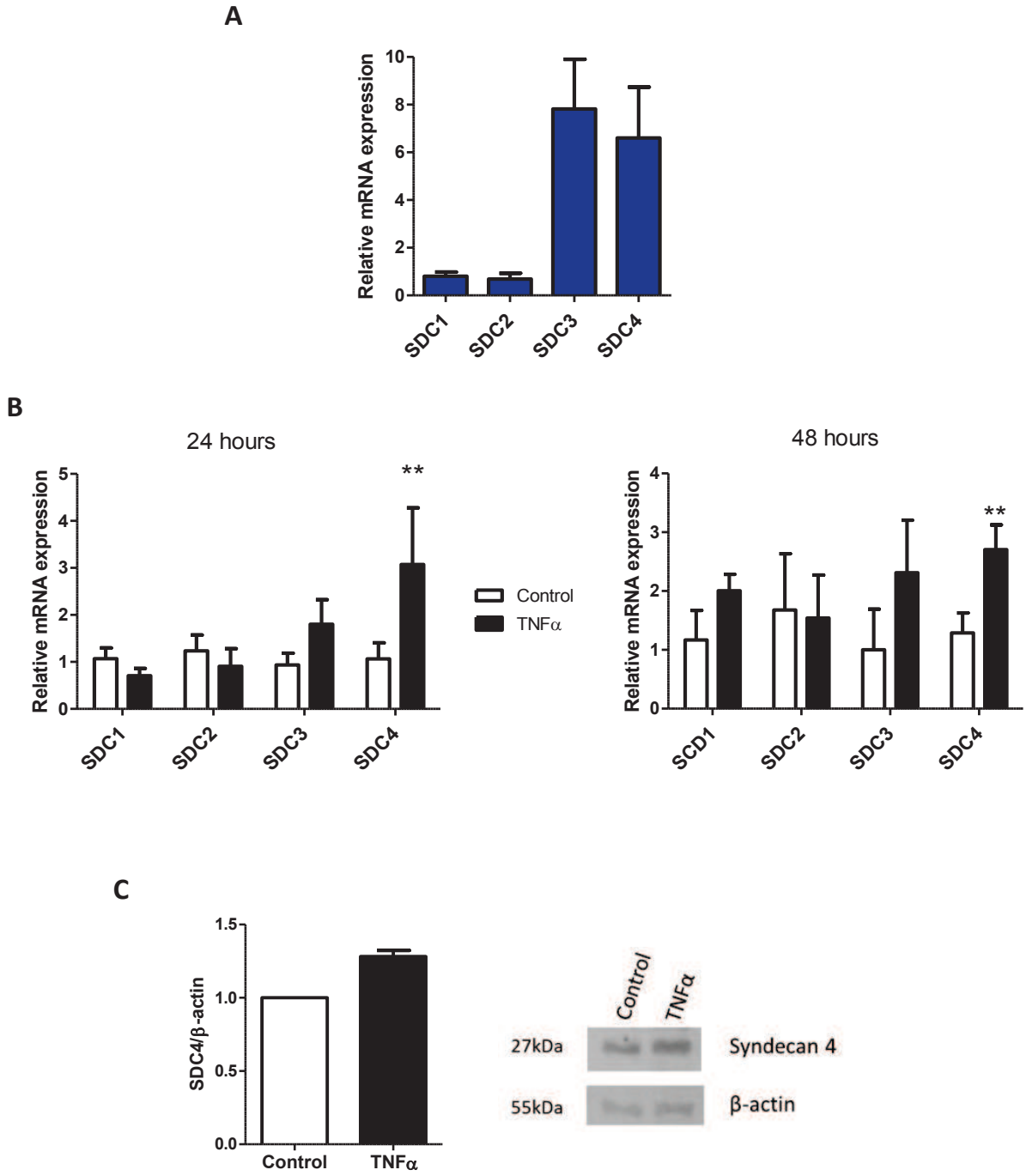


Figure 15 - TNFα influence on syndecans core protein. A, Syndecans expression profile in HUVEC. B, expression of syndecan isoforms in HUVEC control and after 24 and 48 hours TNFα stimulation (0.1 µg/ml). Values represent mean ± S.D. (n=3), ** p<0.01. C, western blot analysis of SDC4 protein in HUVEC untreated and treated 24 hours with TNFα (0.1 µg/ml). Bar chart represents normalized mean ± S.D. of two independent experiment and the figure is a representative SDS-PAGE.

6.5 SDC4 silencing influences the endothelial permeability in HUVEC

Atherosclerosis onset is driven by a massive flow of LDL that pass though the damaged endothelium and lead to a LDL accumulation in the sub-endothelium; in fact, is well known that one of the major atherosclerosis trigger factor is the accumulation of LDL (normal and modified) in the tunica intima (Leiva et al. 2015). To test whether the endothelial permeability is altered under inflammatory condition, we cultured HUVEC in a transwell system over a permeable membrane (0.4 μm pore size) and we measured FITC-dextran passage through the endothelial monolayer. As reported in Figure 16A, the presence of the HUVEC layer (control) over the membrane leaded to the fluorescence decreasing in the lower compartment in comparison with membrane alone (no cells), showing that dextran passage was prevented by cells covering the membrane pore. When HUVEC were treated with TNFα there was a significative reduction of FITC-dextran passage compared with the control.
Since SDC4 seemed to have an important role in cell adhesion, we investigated the effect of SDC4 on endothelial permeability. We transfected the cells with siRNA against SDC4 and the SDC4 silencing increased the FITC-dextran passage compared with siControl (siRNA scrambled) (Figure 16B) indicating a role of SDC4 in endothelial permeability.

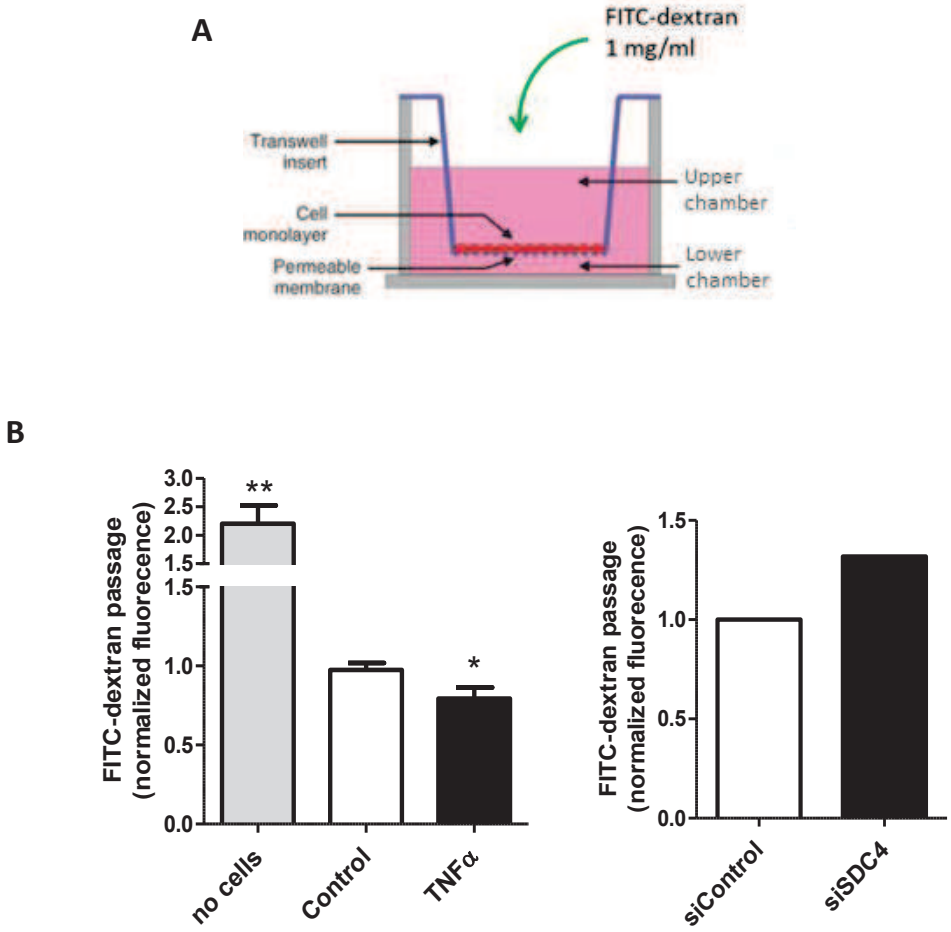


Figure 16 - Transwell permeability assay. A, scheme of FITC-dextran flow through HUVEC monolayer. B, the experiment was performed using HUVEC control and treated with TNFα (0.1 µg/ml) for 24 hours or transfected with a scrambled siRNA (siControl) or with siRNA against SDC4 (siSDC4). Confluent cells were treated (or not) with TNFα (0.1 µg/ml) and 1 mg/ml of dextran conjugated with FITC was added to the upper chamber. After 24 hours the medium in the lower chamber was collected and FITC fluorescence was measured. Data in the left graph are mean ± S.D. of two experiments conducted in duplicate, * $p<0.05$ and ** $p<0.01$, while in the right graph they are preliminary data of a single experiment.

6.6 TNFα alters syndecans HS chains in HUVEC: both enzymes of chain biosynthesis and N-sulfated levels are affected

As mentioned in the introduction, syndecans are composed of core protein and lateral chains formed by GAGs present in the ectodomain. Since the main GAG is HS and many critics functions are played by HS, we measured HS content in conditioned medium and in plasma membrane compartment of HUVEC treated and untreated (control) with TNFα. We have distinguished medium GAG from membrane ones because syndecan ectodomain can be present in association with plasmatic membrane as whole transmembrane protein or in the medium as soluble molecule after protein shedding (Couchman 2010). Although there were no changes in the amount of HS (Figure 17), we analysed the expression levels of the main enzymes involved in HS chains biosynthesis; in particular EXT1 and EXT2 that are important for chain elongation and NDST1 for chain maturation. TNFα increased the expression levels of all chain biosynthetic enzymes take in consideration (Figure 18).

Importantly, NDST1 catalyses the N-deacetylation and N-sulfation of N-acetylglucosamine (GlcNAc) that become N-sulfo glucosamine (GlcNS). Since NDST1 transcription was altered in inflammation response (see Figure 18), we analysed the composition in HS/HE disaccharides of HS extracted from medium and membrane of HUVEC, both control and TNFα treated, using HPLC analysis. As shown in Table 3, TNFα altered the level of GlcNAc sulfation both in membrane and in the medium HS/HE disaccharides: di-sulfation (S1) on GlcNAc and on C6 of GlcNS, and mono-sulfation level (NS) on GlcNAc were increased. These increases of GlcNAc sulfation levels after TNFα stimulation seemed to agree with the increase of NDTS1 expression. On the other hand, there was a reduction of mono-sulfation level (S6) on C6 of GlcNAc in the GAGs membrane and of non-sulfation (0S) in GAGs medium.

TNFα treatment drived to an alteration in sulfation level of HS both in membrane and in medium. It seemed that TNFα affected not only the protein but all the syndecan molecules, HS chains included.

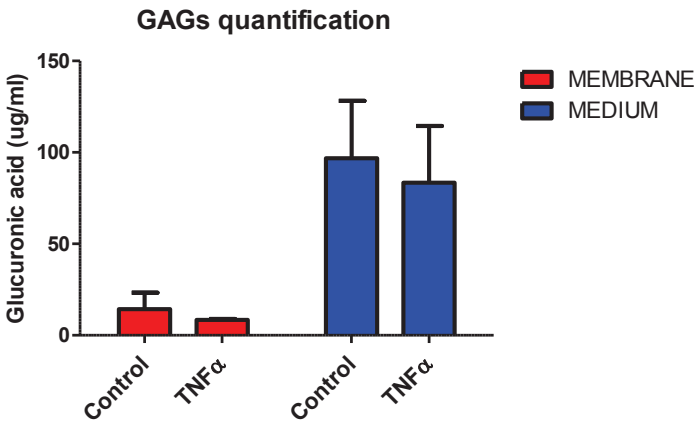


Figure 17 – GAGs quantification. HS GAGs extracted from conditioned medium and plasma membrane of HUVEC untreated and treated 24 hours with TNFα (0.1 µg/ml), were used to measure the concentration of glucuronic acid as detector of HS quantity. Data are mean ± S.D. of three independents experiments.

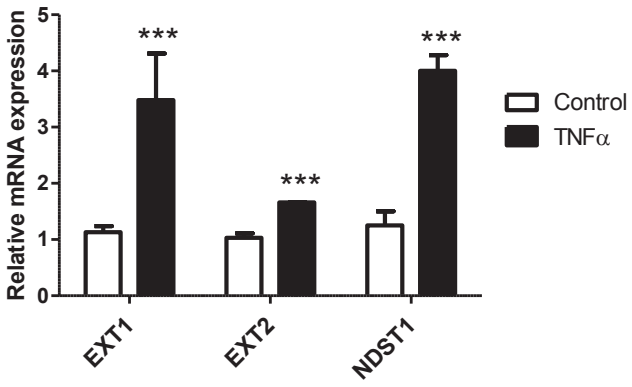
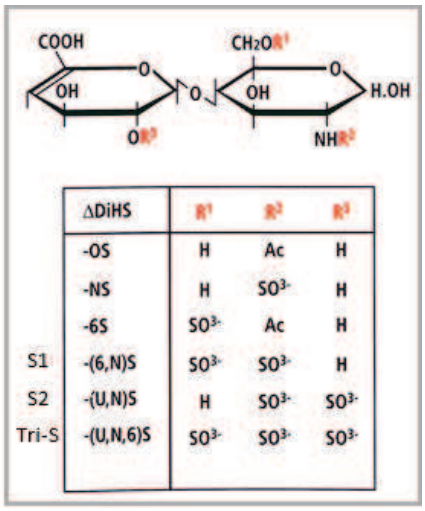


Figure 18 - Expression levels of enzymes involved in HS chains biosynthesis. EXT1 and EXT2 are involved in chains elongation while NDST1 acts in chains modification and maturation. Using RT-PCR analysis the transcription levels of EXT1, EXT2 and NDST1 were examined in HUVEC control and stimulated with TNFα (0.1 µg/ml) for 24 hours. Values represent mean ± S.D. (n=3), *** p<0,001.

A



	ΔDiHS	R1	R2	R3
	-0S	H	Ac	H
	-NS	H	SO^{3-}	H
	-6S	SO^{3-}	Ac	H
S1	-(6,N)S	SO^{3-}	SO^{3-}	H
S2	-(U,N)S	H	SO^{3-}	SO^{3-}
Tri-S	-(U,N,6)S	SO^{3-}	SO^{3-}	SO^{3-}

B

		GAG medium		GAG membrane	
		Control	TNFα	Control	TNFα
Tri-S	ΔUA-2S-β[1→4]-GlcNS-6S	0.7±1.3	0.4±0.7	4.9±6.9	5.5±7.8
S1	ΔUA-β[1→4]-GlcNS-6S	1.2±1.0	2.9±1.0	0	91±6 **
S2	ΔUA-2S-β[1→4]-GlcNS	0.4±0.7	2.5±4.3	0.9±1.3	0.7±0.5
NS	ΔUA-β [1→4]-GlcNS	3.8±5.0	34±21	34±23	0.2±0.2
6S	ΔUA-β[1→4]-GlcNAc-6S	66±2	48±36	55±21	0
0S	ΔUA-β[1→4]-GlcNAc	50±39	13±19	5.3±6.6	2.3±0.7

Table 3 - HPLC analysis of main HS/HE disaccharides. A, scheme of main modifications of HS disaccharides. B, GAGs were isolated from plasma membrane and from culture medium of HUVEC control and TNFα treated (0.1 μg/ml for 24 hours). To obtain HS/HE disaccharides we digested GAGs with heparinases. After AMAC derivatization, the disaccharides were analyzed by means of HPLC. Data are express as % area of HS / % area total. Values are mean ± SD of three independent experiment, ** $p<0.01$.

6.7 Alteration in N-sulfation levels of syndecans HS chains affects platelets stimulation

Sulfated GAGs chains present in the glycocalyx provide negative charge for the endothelial surface layer and contribute to repulsion of platelets and leukocytes to the vascular wall in normal condition (Kolářová et al. 2014). Since syndecan GAGs chains interact with circulating blood cells and we found altered N-sulfated levels in HS from medium and membrane, we wanted to test the behaviour of platelets in presence of HS GAGs. In collaboration with and Dr. Jessica Canino (PhD student of University of Pavia supervised by Dr. Ilaria Canobbio from Department of Biology and Biotechnology), we performed a platelets adhesion and spreading assay. We used fibrinogen as positive control (100% of platelets adhesion and spread) and intact extracted HS GAGs (long chains) from HUVEC membrane and medium with or without TNFα treatment. HSs extracted were used to coat a glass slide and isolated platelets were added. Adherent platelets were visualized in immunofluorescence after staining of actin filaments with phalloidin-TRITC as described in methods. As shown in Figures 19 A and B, HSs extracted from the medium of HUVEC TNFα stimulated leaded to a tendency to increase in platelets adhesion and a significant decrease in spreading index. While membrane HSs did not influence platelets conduct.

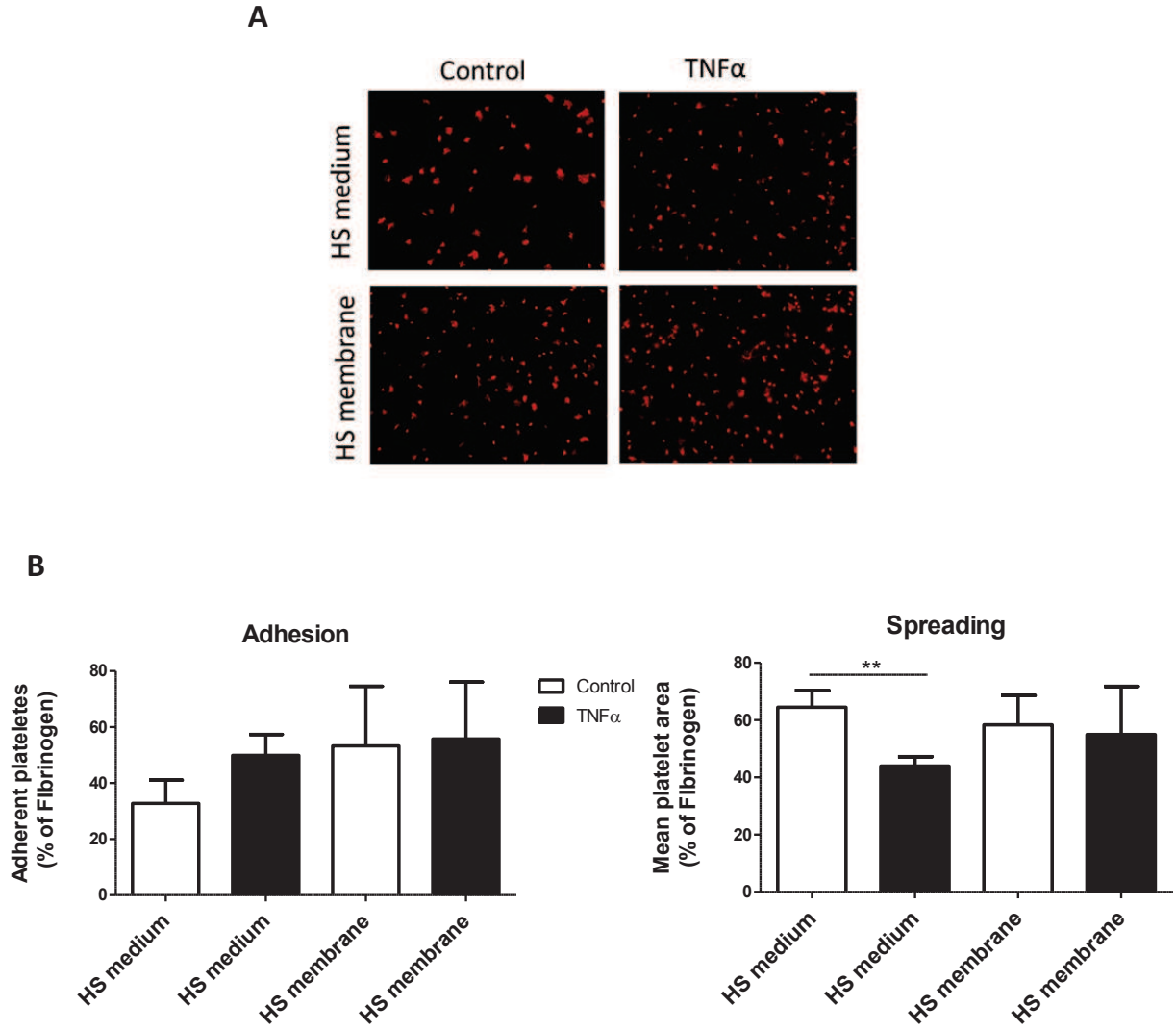


Figure 19 - Platelets adhesion and spreading assays. HS purified from plasma membrane or from culture medium of HUVEC control or TNFα treated (0.1 µg/ml for 24 hours) were used for platelets assay. Platelets were let to adhere on glass coverslips coated with 0.32µg of HS GAGs, as described in methods. A, representative images at 400× magnification of adherent platelets are reported. B, quantification of platelets adhesion and spreading is reported as % of fibrinogen used as positive control for these assays. Values represent mean ± S.D. of five independent experiments, ** $p<0.01$. Data are made in collaboration with Dr. Ilaria Canobbio and Dr. Jessica Canino of Pavia University.

6.8 LDL influence LDLR both in HUVEC and hAoSMC, but PCSK9 is expressed and influenced by LDL only in hAoSMC

Despite the exact causes of atherosclerosis are still unknown, high levels of cholesterol in blood is associated with an elevated risk of cardiovascular diseases. Cholesterol in LDL or in OX-LDL, seems to be involved in atherosclerosis plaque formation (Rafieian-Kopaei et al. 2014). For lowering cholesterol levels (total and LDL-associated), statins are the first-line choice for patients with established disease (Rosenson 2004). However, many patients are intolerant or do not answer to stains therapy (Chaudhary et al. 2017) (Arca and Pigna 2011). In the last decade, a new pharmacological therapy is proposed antagonizing PCSK9. In hepatocyte, PCSK9 reduces LDL intake from circulation by enhancing LDLR degradation and preventing LDLR recycling to the cell surface (X.-L. Lin et al. 2018). Therefore, PCSK9 inhibition decreases the cholesterol-LDL levels in blood.

Although PCSK9 performs its principal action on liver, the tissue target of atherosclerosis process is the vessel wall. Therefore, we investigated the possible role of PCSK9 in endothelial cells and smooth muscle cells.

To develop a system as similar as possible to atherosclerosis situation characterized by high level of cholesterol, we treated HUVEC and hAoSMC with N-LDL and OX-LDL for 24 hours to assess if PCSK9 can be produced locally in vascular cells. The expression of LDLR and PCSK9 was evaluated by RT-PCR, and PCSK9 concentration in conditioned medium was quantified by using an ELISA commercial kit.

Results indicated that both HUVEC and hAoSMC replied to LDL (N and OX) downregulating the LDLR (Figures 20 and 21 A and B), adopting a feedback negative mechanism.

While PCSK9 was not transcript in HUVEC treated and untreated and it was not present in HUVEC conditioned medium, PCSK9 expression decreased after LDL (both N and OX) stimulation in hAoSMC (Figure 21A). Moreover, OX-LDL leaded a diminished release of PCSK9 in hAoSMC medium (Figure 21B). Interestingly, PCSK9 and LDLR show the same regulation after LDL stimulation in smooth muscle cells.

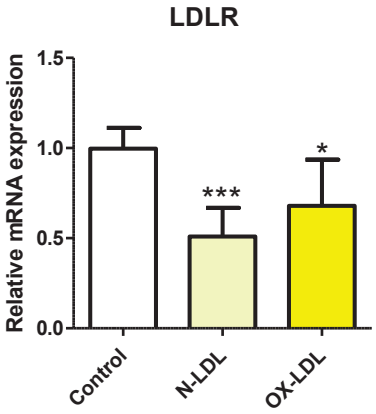


Figure 20 – LDL regulation on LDLR expression in HUVEC. RT-PCR analysis of LDLR in HUVEC control and treated with N and OX LDL (20 µg/ml) for 24 hours. Values represent mean ± S.D. of three independent experiments, *** p<0.001 and * p<0.05.

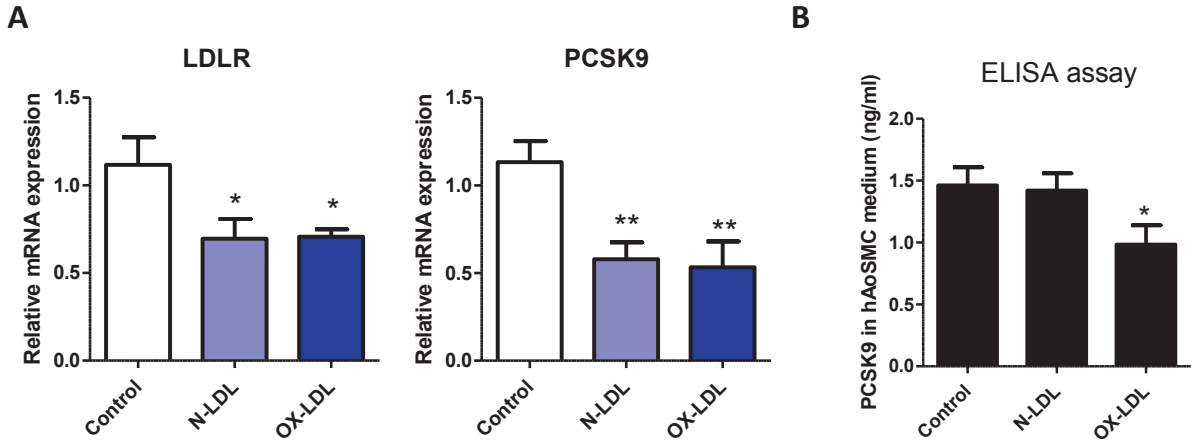


Figure 21 – LDL influence on LDLR expression and PCSK9 production in hAoSMC. A, after 24 hours of LDL (N and OX, 20 µg/ml) treatment, hAoSMC mRNA was used for quantify LDLR and PCSK9 expression. Values represent mean ± S.D. of three independent experiments, ** p<0.01 and * p<0.05. B, PSCK9 quantification using ELISA commercial kit. Values represent mean ± S.D. (n=3), * p<0.05.

6.9 Effect of PCSK9 stimulation on LDL receptors in vascular cells

Although PCSK9 inhibitory effect on LDLR in liver is well known, its role in vascular cell remains cryptic. As PCSK9 is produced primary from liver and it is present in blood as circulating factor, we tested if PCSK9 has an influence on the main LDL receptors in vascular cell.

HUVEC and hAoSMC were treated with two different concentration of PCSK9: 80 ng/ml and 100 ng/ml. These values were respectively quantified in healthy patients and in hypercholesterolemic subjects by Dubuc et al (Dubuc et al. 2010). Since LDL can enter the cells by different mechanisms, we focused our attention on LDLR, the receptors for N-LDL, LOX1 the scavenger receptor for OX-LDL and ALK1 that is implicated in LDL transcytosis in endothelial cells.

In HUVEC, LDLR and LOX1 transcription tended to increase, while LOX1 showed a dramatic reduction after stimulation with both PCSK9 concentrations (Figure 22A). In addition, to evaluate the regulation of LDLR by PCSK9 in a hypercholesterolemic situation, HUVEC were co-treated with PCSK9 and N-LDL. LDLR transcription after N-LDL treatment was downregulated (confirmed as shown before) and it remained low after co-stimulation with PCSK9 and N-LDL (Figure 22B). It suggests a primary role of N-LDL to LDLR regulation despite of PCSK9.

LDLR and ALK1 mRNA levels were not affected by PCSK9 stimulation in hAoSMC, while LOX1 tended to decrease with the lower concentration of PCSK9 (Figure 23).

These data sudgest that in vascular cells, both endothelial and smooth muscle, PCSK9 has more influence on LOX1 scavenger receptor in comparison with LDLR that is regulated by LDL.

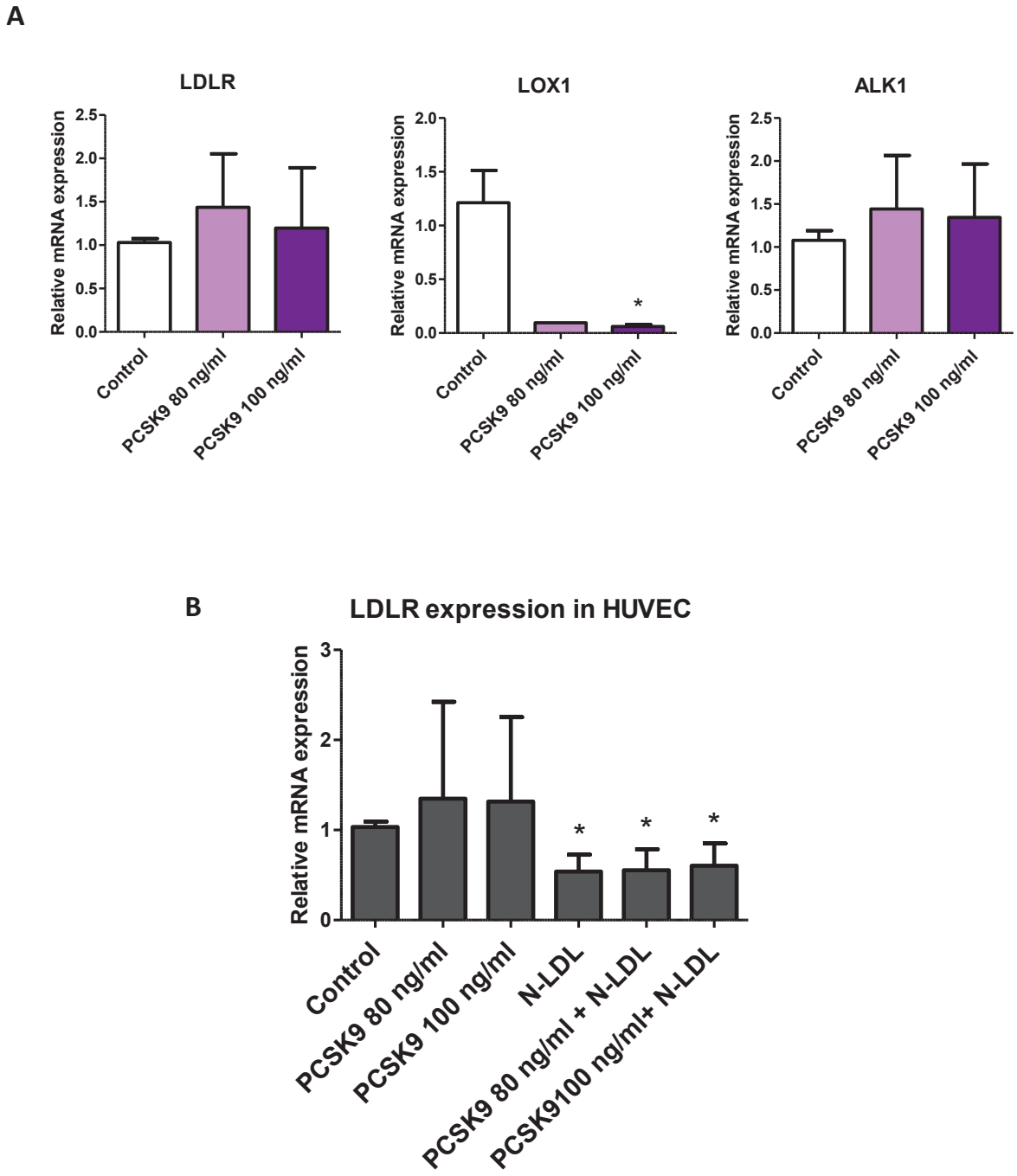


Figure 22 – PCSK9 effect on LDL receptors in HUVEC. A, expression of LDLR, LOX1 and ALK1 in HUVEC control and after 24 hours of PCSK9 treatment (80 and 100 ng/ml). B, LDLR transcription levels in hAoSMC control and co-treated with PCSK9 (both concentrations) and N-LDL (20 µg/ml) for 24 hours. Values represent mean ± S.D. (n=3), * $p<0.05$.

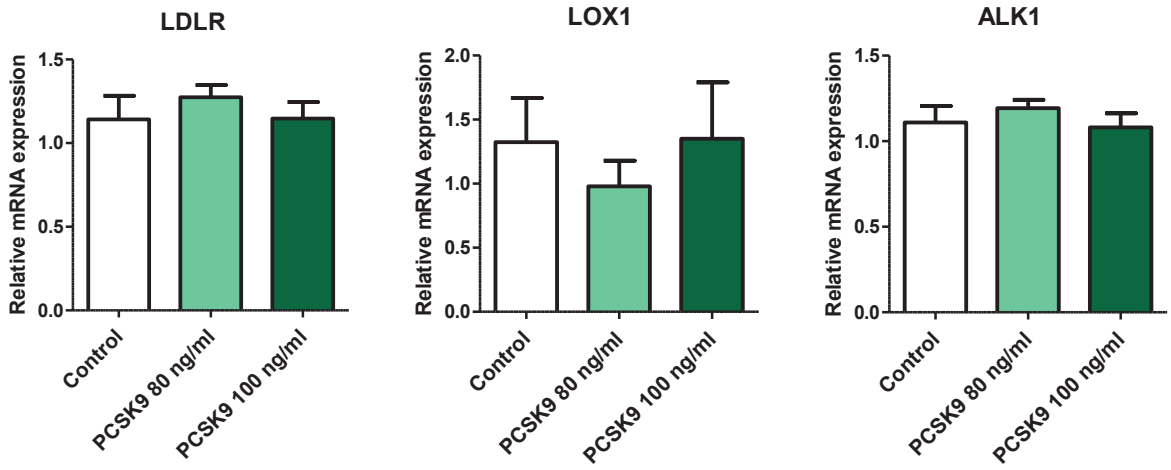


Figure 23 – PCSK9 influence on LDL receptors in hAoSMC. Expression of LDLR, LOX1 and ALK1 in hAoSMC control and after 24 hours of PCSK9 treatment (80 and 100 ng/ml). Values represent mean ± S.D. (n=3), * $p<0.05$.

6.10 HA synthesis is sensible to PCSK9 treatment in HUVEC and hAoSMC

Since OX-LDL accumulated in the vascular intima can adhere to extracellular matrix components, we evaluated the effect of PCSK9 on HA biosynthesis in vascular cells. HUVEC and hAoSMC were treated with both PCSK9 concentrations and HAS2, HAS3 and CD44 expression was quantified with RT-PCR analysis.
HAS2 and CD44 transcription tended to decrease but without significance in HUVEC, while HAS3 was significantly reduced by PCSK9 treatment (both concentrations) (Figure 24A). HAS3 was downregulated also in hAoSMC after PCSK9 stimulation, while HAS2 mRNA levels were decreased after stimulation with the higher concentration of PCSK9 (Figure 24B). Moreover, the expression of CD44 decreased in relation with HASs decrease in hAoSMC. These data suggest a protective role of PCSK9 in vascular wall decreasing the expression of HA synthesis enzyme and its specific receptor.

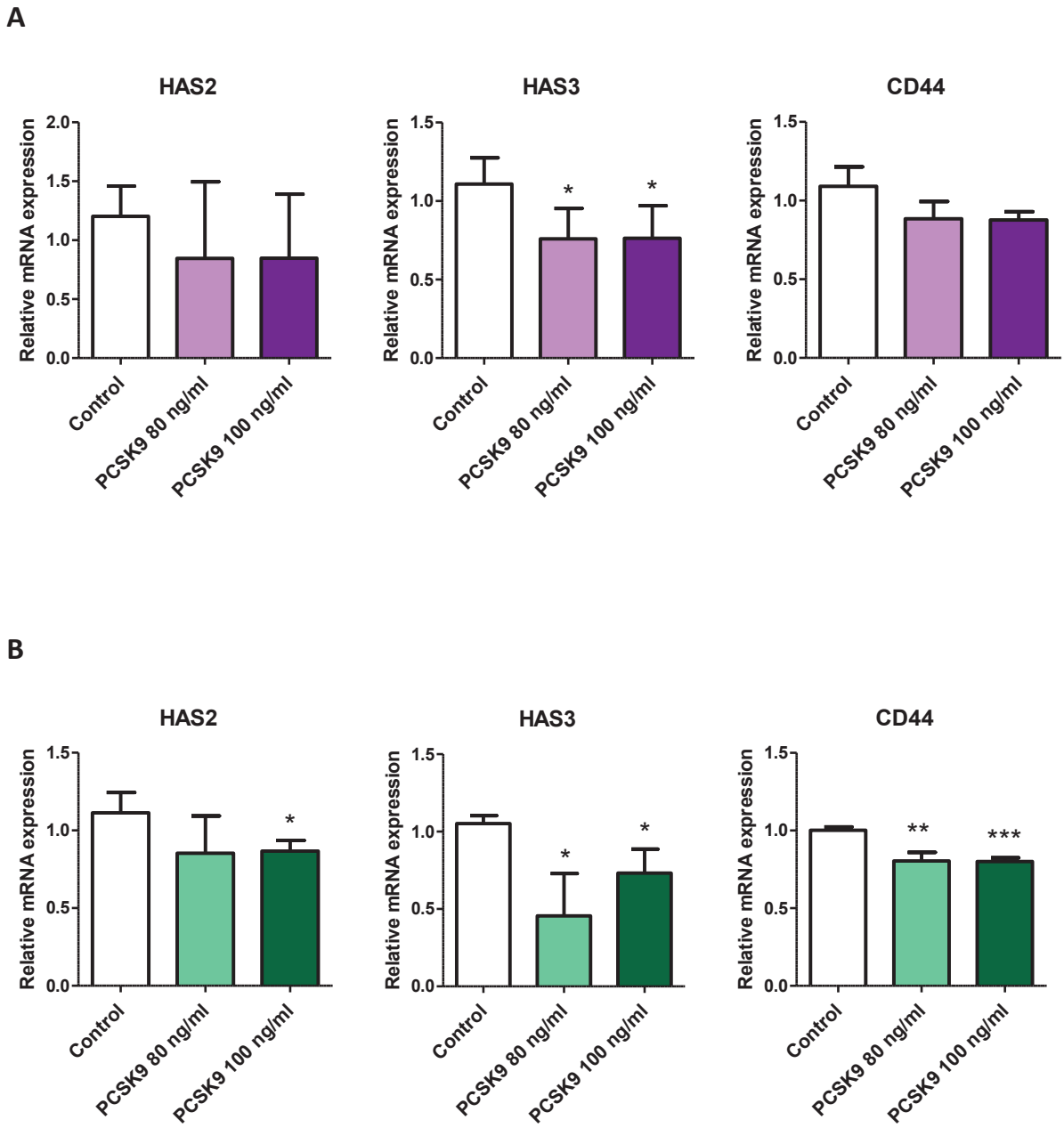


Figure 24 – HA metabolism in vascular cells after PCSK9 stimulation. Expression of HAS2, HAS3 and CD44 in HUVEC (A) and hAoSMC (B) control and treated with PCSK9 (80 and 100 ng/ml) for 24 hours. Values represent mean ± S.D. (n=3), *** p<0.001, ** p<0.01 and * p<0.05.

7 – DISCUSSION

In this study we investigated the role of hyaluronan and syndecans in HUVEC after TNFα stimulus in order to understand *in vitro* their impact on the endothelial inflammation, one of starting point of atherogenesis. In addition to this, we examined the action of PCSK9 in endothelial and smooth muscle cells to expand knowledge about its role in vascular district.

After TNFα treatment, our model showed changes in hyaluronan metabolism including the decrease of HAS3 expression, the increase of HAS2 and the augment of HA presence in pericelllular space. We used particle exclusion assay to measure HA only in the pericellular coat and not in the medium in order to evaluate HA in the glycocalyx *in vivo*. HA is a crucial regulator of endothelial inflammation. Since HAS3 is associated with LMW-HA synthesis that has pro-inflammatory proprieties, the decrease of HAS3 seems to have an endothelial protective role. In fact, the inhibition of HAS3-dependent synthesis of HA decrease the inflammation in atherosclerotic plaque in *has3/Apoe* deficient mice (Homann et al. 2018). On the other hand, the increase of HAS2 related with a HA-rich pericellular space suggests its pro-inflammatory role promoting the interaction between endothelial cell and leukocytes or platelets that express CD44, the principal HA receptor. In agreement with that, Vigetti et al demonstrated that HA synthesis due to HAS2 up-regulation promotes monocytes adhesion of endothelial cells via NF-kB pathway (Vigetti et al. 2010).
Further, despite HAS2 produces the anti-inflammatory HMW-HA, it can be subjected to fragmentation by digestive enzymes as hyaluronidases (HYALs) or oxidative stress. HYALs not only from the pro-inflammatory HA oligosaccharides but also, they degrade HA on the apical surface of endothelial cells and lead to a change in the glycocalyx layer (Kong et al. 2016). In fact, *in vivo* study demonstrate that a systemic inhibition of HA synthesis by 4-MU interferes with the protective function of the endothelial glycocalyx, facilitating inflammation and progression of atherosclerosis (Nagy et al. 2010). HMW-HA can be cleaved into fragments also by platelets that up-regulate leukocyte production of chemokines and cytokines (Sadowitz et al. 2012).
Another glycocalyx component studied were syndecans which were influenced by TNFα both in core protein expression and in N-sulfation levels of HS chains.
Despite SDC4 and SDC3 were the most represented core protein isoform in HUVEC, only SDC4 showed an altered expression. Its up-regulation due by TNFα seems to be related to NF-kB (Okuyama et al. 2013) that is implicated in inflammatory responses. An increase of SDC4 core protein can activate PKCα pathway involved in antithrombin-III modulation and endothelial prostacyclin release (Götte 2003) which are important factors for the regulation of platelets aggregation and vascular tone. Since syndecans are subjected to shedding, an increase of soluble ectodomain of SDC4 can favour HS chains interaction with a multitude cell types as leucocytes and platelets as well as with soluble factors as FGF-2. The interaction of HS with FGF-2 can promote the repair of endothelial damage (Melrose 2016).

Moreover, we demonstrated that the increase expression of SDC4 affects the endothelial cell permeability. Since SDC4 is present in adherent junctions (Gopal et al. 2017) (Cavalheiro et al. 2017), we speculate that the decrease of FITC-dextran passage through HUVEC monolayer can be involved in the compensatory mechanism of endothelial cell in order to prevent the LDL passage to the subendothelium.
In order to examine the importance of the levels of N-sulfated residues in syndecans interaction, we found an increase of N-sulfation on the GlcNS-6S residue that was directly related with the increase of NDST1 expression, the specific enzyme that catalyzes this modification. Moreover, the N-sulfated alteration involved all HS residues analysed. Change in HS quality (not in total amount of HS but in N-sulfation) induced platelets spreading reduction that is correlated with anti-coagulant proprieties. Alternatively, platelets express CD44 and can interact with HA present in the glycocalyx. Therefore, after TNFα treatment, a HA-rich pericellular space increases the platelets recruitment favouring platelets aggregation.
As represented in the Figure 25, an inflammatory stimulus as TNFα can altered ECM characteristics (i.e. HS N-sulfation and HA) which alter the endothelial glycocalyx composition modulating immune cell recruitment (monocytes and platelets) and affecting the endothelial permeability. These aspects highlight the contribution of inflammation to regulate the endothelial behaviour that has a critical role in atherosclerosis development.

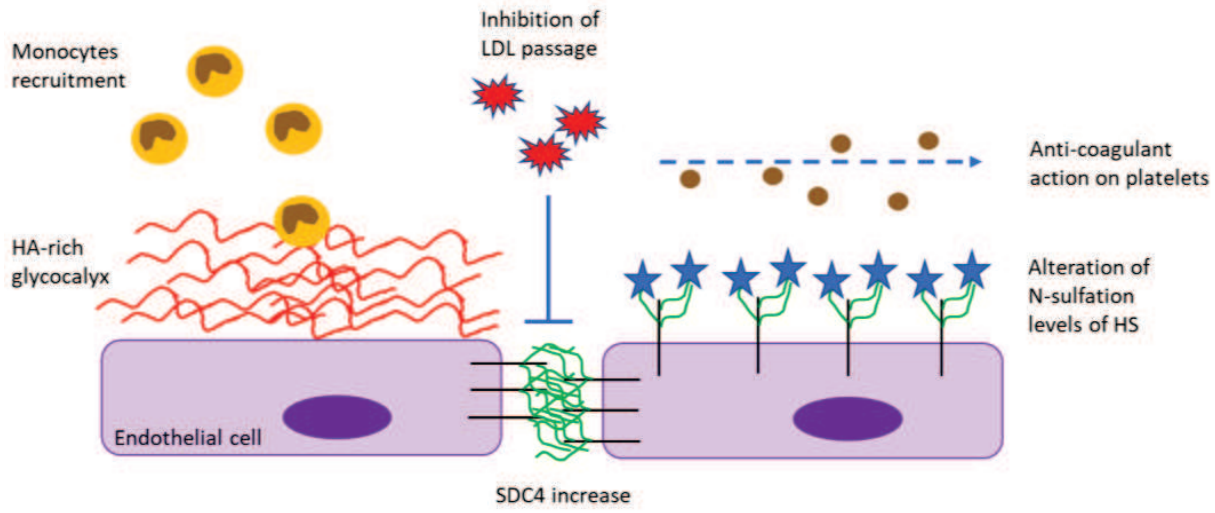


Figure 25 – Mechanisms of action proposed for hyaluronan and syndecans, the major endothelial glycocalyx components, in response to an inflammatory stimulus in the early phase of atherosclerosis onset. HA-rich glycocalyx can stimulate the monocytes recruitment and favour an inflammation reaction, while syndecan4 localized in adherent junction can close the space between cells and prevent the LDL passage. Moreover, an alteration of N-sulfation levels of HS chains seems have an anti-coagulant action in confront to platelets stimulation.

Another aspect we have focused on is PCSK9. Although the role of PCSK9 in liver is well known, no information is available on PCSK9 role in vascular district. In fact, PCSK9 is known to be released in the bloodstream and can differently affect cholesterol metabolism in several tissues (Gustafsen et al. 2017). Our results indicated that PCSK9 has similar but not equal action on LDL receptors and HASs expression in endothelial cells and in SMC.

We demonstrated that PCSK9 is not only express and secreted by liver but also by SMC. Moreover, in these cells PCSK9 production is regulated by both N-LDL and OX-LDL. Since OX-LDL induce apoptosis increasing Bax and decreasing Blc-2 (Kataoka et al. 2001), PCSK9 in SMC can be implicated in cell death and this could be an important target for preventing and delaying the atherosclerosis in the early stages (Li et al. 2017). On the other hand, endothelial cells do not express PCSK9 neither in N-LDL nor in OX-LDL. Our data are in agreement with several studies demonstrating that PCSK9 expression in endothelial cells is less than in smooth muscle cells. However, in another study PCSK9 can be regulated in concentration-dependent manner by LPS (Ding et al. 2015).

It is known that healthy patient and hypercholesterolemic subjects have a PCSK9 circulating concentration of 80 and 100 ng/ml, respectively (Dubuc et al. 2010). Therefore, we treated vascular cells with such amounts of PCSK9 to verify a dose dependent effect.

LDL receptors did not show alteration in their expression in both cells lines. Interestingly, LOX1, the main OX-LDL scavenger receptor in endothelial cells, was dramatically reduced after PCSK9 treatments. Since LDLR seems to be regulated mainly by N-LDL in HUVEC, it could be that PCSK9 acts not on N-LDL receptors but only on scavenger receptors. Consequently, the decrease of LOX1 expression could inhibit the uptake of the pro-inflammatory factor OX-LDL that could also cause an oxidative stress and damage the endothelial layer. Interestingly, also HA synthesis is known to be regulated by OX-LDL as well as (R)-hydrocholesterol in vascular cells (Viola et al. 2013). As HA has a critical role in atherosclerosis onset (Viola et al. 2013) ,we verify whether PCSK9 modulates HA metabolism. In HUVEC we found that HAS3 decreases after PCSK9 treatments. This regulation could lead to diminished immune cells recruitment correlating to an anti-inflammatory response. This finding is intriguing because it will permit to specifically study the critical role of HAS3 in vascular endothelial cells.

On the other hand, in SMC we found a reduction of HAS2 and HAS3 after PCSK9 treatments. As in SMC HA is primary involved in proliferation and migration (Cuff et al. 2001), the effect of PCSK9 of these cells could be positive reducing HASs expression that could correlate with an increase vascular protection.

To further confirm this new protective role of PCSK9 in vessels, we studied CD44. This molecule is known to be a critical HA receptor required for atherosclerosis development mediating immune cells infiltrated in subendothelium, SMC differentiation and signalling (Cuff et al. 2001). After PCSK9 treatments, we clearly showed a CD44 decrement in SMC highlighting that PCSK9 could impair different mechanisms involved in atherosclerosis onset.

Another important mechanism that could be involved with HA metabolism is platelets stimulation. In fact, the observed reduction of HASs expression could diminish the HA content in the glycocalyx preventing platelets adhesion, activation and eventually aggregation reducing thrombus formation.
Very recently, it has been discovered that PCSK9 can be interact with HS in N-sulfation rich domain (Gustafsen et al. 2017). Since we found the TNFα alters HS chemical composition in N-sulfation, we can speculate that HS with an increase of such modification could bind PCSK9 and leave LDLR free to interact with LDL (Figure 26). Therefore, HS proteoglycans could be use as biological PCSK9 inhibitor and, consequently, as a new therapeutic approach. This point is crucial because in recent years there is an increasing interest in glycobiology. As HS and other GAGs are able to interact with different molecules and are involved in practically every area of biology (i.e. coagulation process, helping cells to communicate or to recognize pathogens, cancer metastasis, ecc), they are emerging as important factors in the most programmes of biological drug.
In conclusion, despite PCSK9 inhibition has a pivotal role in LDL-cholesterol lowering in hepatocytes, we find that the PCSK9 in vascular district could have protective effects inhibiting critical mechanism regulating LDL scavenger receptors expression and HA metabolic enzymes that are involved in vessel thickening.

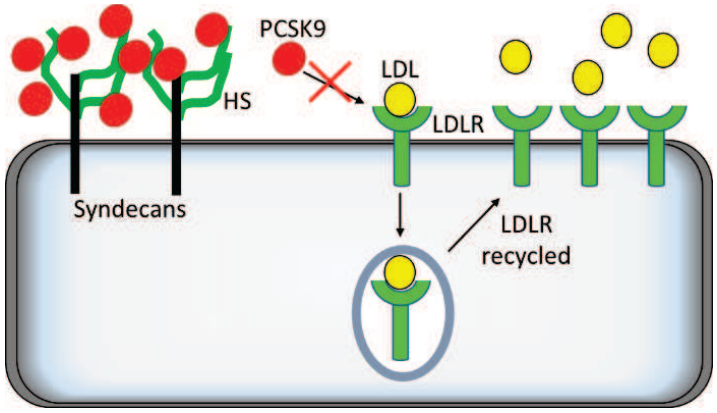


Figure 26- Proposed attractive action of HS proteoglycans with high levels of N-sulfation on PCSK9.

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