ROMANIAN ACADEMY
Advanced Studies School of the Romanian Academy
"N. Simionescu" Institute of Cellular Biology and Pathology

ABSTRACT

CELLULAR AND MOLECULAR MECHANISMS INVOLVED IN THE REMODELING OF THE EXTRACELLULAR MATRIX ASSOCIATED WITH CARDIOVASCULAR DISEASES

PhD SUPERVISOR:
ACAD. ILEANA MÂNDUȚEANU

PhD CANDIDATE:
RĂZVAN DANIEL MACARIE

2023

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Introduction

Cardiovascular diseases stand as the leading cause of mortality in developed countries (Organization, 9 December 2020). Their consistent rank atop these statistics can be attributed to the increasing exposure of modern humans to risk factors associated with cardiovascular diseases. Such risk factors encompass smoking, unhealthy diets, physical inactivity, hyperlipidemia, hypertension, exposure to pollution, alcohol consumption, obesity, and notably, diabetes mellitus. Diabetes mellitus is the most prevalent metabolic syndrome, and its associated complications are primarily macro- and microvascular, leading to nephropathy, retinopathy, and neuropathy as a result of the accelerated atherosclerotic process.

Cardiovascular diseases encompass a wide range of pathologies including heart failure, cardiac valve diseases, arrhythmias, rheumatic heart disease (damage to the myocardium and cardiac valves caused by Streptococcus pyogenes infection), congenital cardiac malformations, deep vein thrombosis with its subsequent complication of pulmonary embolism, and atherosclerosis, which has two major complications: myocardial infarction and ischemic stroke (Thiriet, 2018).

Atherosclerosis is the predominant underlying pathology of coronary artery disease, peripheral artery disease, and cerebrovascular diseases. As a disease of the large and medium arteries, atherosclerosis involves the accumulation of lipoproteins in the subendothelial space, particularly low-density lipoproteins (LDL), immune cells, and smooth muscle cells (SMC). Over time, this accumulation forms obstructive plaques, which subsequently cause arterial stenosis. Throughout the progression of atherosclerosis, SMCs produce various extracellular matrix (ECM) proteins, such as collagen and elastin, as well as proteoglycans and glycosaminoglycans. These contribute to the thickening of the sub-intimal space and the maintenance of a fibrous cap. As the plaque matures, SMCs may undergo metaplasia into foam cells that engulf LDL, undergo apoptosis, and along with the compromised efferocytosis by macrophages, lead to the formation of a necrotic core. All these factors also induce the perpetuation and intensification of inflammation, promoting the recruitment of macrophages that secrete matrix metalloproteinases (MMP), playing a role in ECM degradation. This action thins the fibrous cap, predisposing it to rupture. While the initial LDL accumulation and fibrous cap formation narrow arteries, restricting blood flow and causing tissue ischemia and hypoxia (Wolf and Ley, 2019), is the rupture of the fibrous cap

that leads to thrombus formation, potentially completely blocking arteries. In the case of coronary arteries, this results in myocardial infarction, and in cerebral arteries, a stroke.

Another increasingly prevalent cardiovascular disease is **aortic valve disease (AVD)** (Coffey et al., 2021). The aortic valve is one of the four valves regulating blood flow in the heart, segregating the left ventricle from the aorta. Diseases affecting this valve hamper blood circulation and are separated into two categories: aortic stenosis - a narrowing of the valve opening, impeding blood flow; and aortic insufficiency (or regurgitation) – a condition where the valve doesn't close tightly during diastole. The most common ailment of the aortic valve is aortic stenosis due to valve calcification, hereafter referred to as aortic valve disease (AVD). It is characterized by the thickening, fibrosis, and mineralization of the aortic cusps. Examination of aortic leaflets reveals that dystrophic mineralization and osteogenic transition of valvular interstitial cells (VIC) coincide with neovascularization, microhemorrhages, and abnormal production of extracellular matrix (Moncla et al., 2023). Risk factors for AVD can be classified into non-modifiable ones, like age and congenital bicuspid morphology of the aortic valve, and modifiable ones such as elevated blood pressure, plasma cholesterol levels, and the presence of obesity and diabetes mellitus. Complex interactions between the valve's endothelial and interstitial cells, alongside immune cells, promote the remodeling of the aortic valve and the development of AVD. Since no medical treatment has thus far proven effective in reducing or preventing AVD progression, studies aiming to pinpoint potential therapeutic targets are of paramount importance.

The impact of cardiovascular diseases is increasing, due in part to the rising rates of obesity and diabetes. **Diabetes** is the most common metabolic disease, and its medical and socio-economic burden arises from its associated complications, particularly at the macrovascular and microvascular levels. These lead to retinopathy, neuropathy, and nephropathy, a result of accelerated atherogenesis. It has previously been demonstrated that both types of diabetes mellitus can promote the development of atherosclerosis or further accelerate its progression (Katakami, 2018). Elevated glucose levels, dyslipidemia, and other metabolic changes accompanying diabetes are closely linked to the pathogenesis of atherosclerosis. Moreover, chronic inflammation is present from the very early stages of diabetes onset and is currently considered a key factor in the development of atherosclerosis (Wolf and Ley, 2019).

In the pathology of atherosclerosis and valvular aortic disease (BVA), there are significant transformations of the **extracellular matrix (ECM)**. The ECM provides a vital framework for vascular and valvular tissue, serving as a scaffold that supports cell organization and homeostasis. The ECM is a complex assembly of macromolecules, influencing cell behavior by promoting differentiation, migration, and proliferation through specific interactions between cells and the matrix. Furthermore, the ECM aids in intercellular communication, playing a crucial role in cell motility, development, and tissue repair. It also acts as a storage depot for growth factors and bio-active molecules that can be released (Silva et al., 2020).

In this context, the present study is based on the hypothesis that inflammation and communication between immune cells and resident vascular (SMCs) and valvular (VICs) cells can lead to pathological modifications of the ECM through specific cellular and molecular mechanisms. Moreover, this pathological remodeling is accelerated in atherosclerosis under diabetic conditions.

The work is structured into two main parts: the first section, titled "Current State of Knowledge", explores the existing literature on atherosclerosis, aortic valvular disease, diabetes, and the impact of these pathologies on the extracellular matrix.

The primary objective of the thesis was to investigate the cellular and molecular mechanisms of ECM remodeling in atherosclerosis and valvular aortic disease in the context of diabetes. The original results obtained are presented in the second section of the work, titled "Original Contributions", which is structured into 4 chapters.

Original contributions

Chapter 1 - "Communication between smooth muscle cells and macrophages accelerates the progression of atherosclerotic plaque to vulnerable plaque and plaque rupture through the induction of inflammatory mediators" - aimed to investigate the pathological communication between SMCs and macrophages (MØ) in the context of atherosclerosis.

In the pathological setting of atherosclerosis, there is an interaction between SMCs and macrophages that isn't typically found under vascular homeostatic conditions. SMCs are scarce in the arterial intima and mainly reside in the media of large arteries from where they migrate to areas with LDL infiltration and local inflammation. In these regions, they interact

with macrophages recruited from circulation to the sub-endothelial space, undergoing phenotypic changes that favor atherosclerosis progression. Responding to signals from the vascular microenvironment, macrophages undergo polarization into pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes and induce a pro-atherogenic phenotype in vascular SMCs (Butoi et al., 2011). Moreover, the SMC phenotype can influence the balance between M1 and M2 macrophage populations, thus determining the progression of the atherosclerotic plaque. Within the atherosclerotic plaque, macrophages and SMCs can directly interact - via surface-expressed molecules like ICAM-1, VCAM-1, and CX3CL1 and their receptors - or indirectly through soluble factors (chemokines and cytokines), influencing the plaque's evolution (Manduteanu and Simionescu, 2012).

Previously, our group demonstrated that upon interaction with SMCs, there's an induced expression of numerous cytokines and chemokines in macrophages, with these inflammatory mediators amplifying monocyte chemotaxis (Tucureanu et al., 2016). We also showed that the interaction between macrophages and SMCs affects collagen I and III expression, increases MMP-9 synthesis, and promotes angiogenesis, characteristics that might contribute to plaque vulnerability (Butoi et al., 2016).

In order to investigate the role of communication between macrophages and SMCs in exacerbating atherosclerotic lesions, in this study we examined the inflammatory mediators induced by SMC-macrophage interactions, phenotypic changes in SMCs post-cell interaction, and signaling pathways involved in SMC-macrophage communication.

The experimental model utilized in this chapter was an *in vivo* model using a Boyden double-chamber with differentiated macrophages in the upper chamber on a 4 μm pore insert and SMCs seeded in the bottom chamber. Methods included western blot (WB) techniques, enzyme-linked immunosorbent assays (ELISA), protein arrays, and real-time quantitative polymerase chain reaction (qPCR).

Results from this chapter showed that indirect interaction of SMCs with macrophages led to the secretion of inflammatory mediators such as: G-CSF, GM-CSF, ICAM-1, IL-6, CCL5.

Interaction between SMCs and macrophages altered the typical phenotype of SMCs (Figure 1) by reducing α-SMA, which might lead to diminished contractile capacity. Yet, the SMC phenotype is not pro-osteoblastic, as the level of osteopontin is also decreased upon interaction.

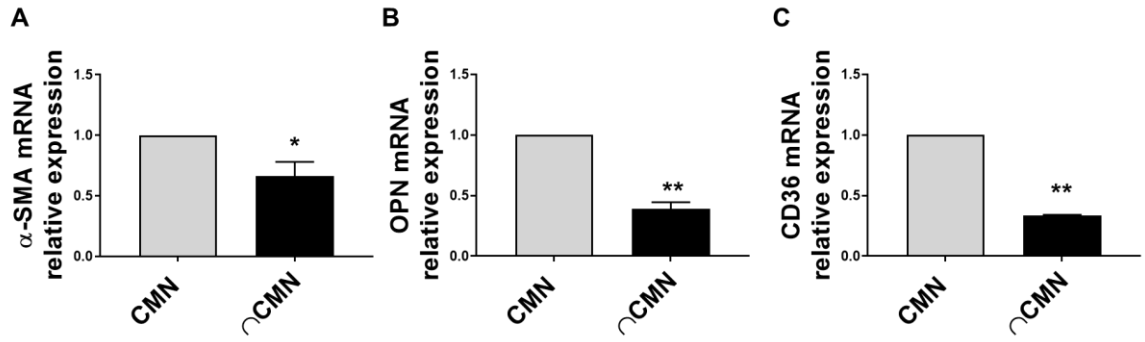

Figure 1 *The gene expression of phenotypic markers of smooth muscle cells is diminished following interaction with macrophages (MØ). The gene expression of α-smooth muscle actin (αSMA) (A), osteopontin (OPN) (B), and CD36 (C) was determined through Real-Time PCR in SMCs interacted with macrophages (ΩCMN) or control SMCs (CMN control).*

The interaction between SMCs and macrophages (MØ) induces the expression of cytokine IL-6 (Figure 2) and chemokine CCL5 in SMCs (Figure 3), molecules that play a crucial role in the progression of atherosclerotic plaque.

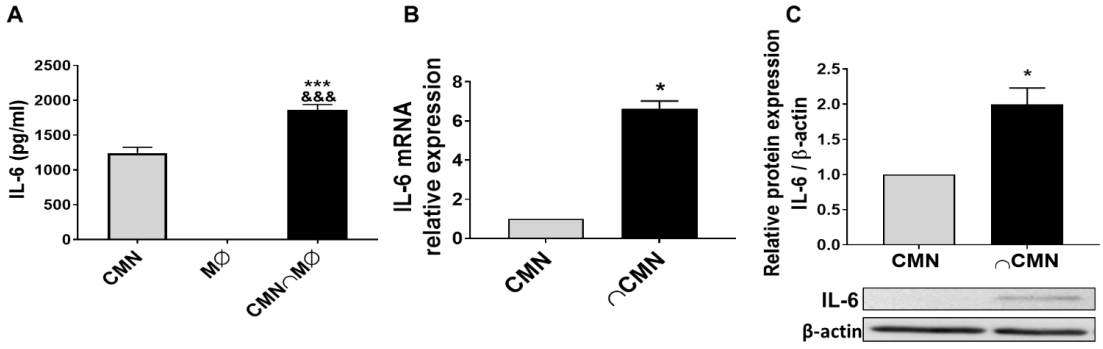

Figure 2 *The secretion and expression of IL-6 is augmented in smooth muscle cells (CMN) following the interaction with macrophages (MØ).*

Migration of SMCs into the vascular wall's intima is known to be directed by chemokines secreted by immune and resident cells within the atherosclerotic plaque (Tedgui and Mallat, 2006). CCL5 (RANTES) is a chemokine essential to the inflammatory process by recruiting immune cells to the inflammation site. CCL5 expression is increased in vascular wall cells, and blocking the binding of CCL5 to its receptor (CCR5) has shown a reduction in atherosclerotic plaque formation and prevention of atherosclerotic lesion progression in mice (Liu et al., 2012).

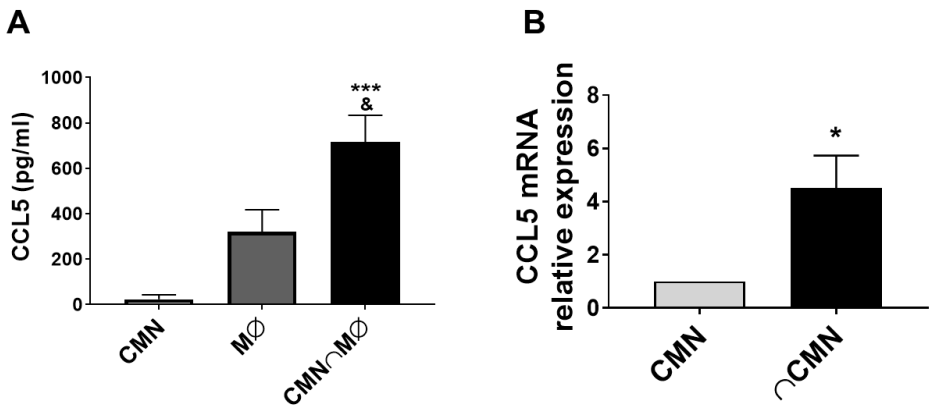

Figure 3 *The secretion and gene expression of CCL5 is enhanced in smooth muscle cells (CMN) following the interaction with macrophages (MØ).*

The activation of MAPK (mitogen-activated protein kinase) signaling pathways has been shown in atherosclerosis. It's known that ERK1/2, JNK, and p38 MAPK are activated in SMCs involved in the pathological process (Pan et al., 2023). Thus, in SMCs interacting with macrophages, we analyzed the MAPK pathway activation by detecting the phosphorylated forms of ERK1/2, JNK, and p38 using the Western Blot technique. Our data showed that the SMC-macrophage interaction activates the ERK1/2 pathway in SMCs after 6 hours of interaction, evidenced by a significant increase (2.7 times) in phospho-ERK1/2 protein compared to total ERK1/2 protein; after 24 hours of interaction with macrophages, the ERK pathway is no longer activated in SMCs.

Caspase-1 is the effector protein of the NLRP3 inflammasome, which cleaves the proform of inflammatory cytokines, generating their active form, which is then released. Under our experimental conditions, NLRP3 is not expressed in control or macrophage-interacted SMCs, though the protein expression of IL-1β is significantly increased, confirming our previous data (Butoi et al., 2016). In contrast, the protein expression of caspase-1 is significantly increased in SMCs after 24 hours of interaction with macrophages.

In macrophages, the interaction with SMCs increases the expression of MIP-1β (Figure 4), which is known to play an essential role in the progression of atherosclerotic disease and plaque vulnerability (Chang and Chen, 2016).

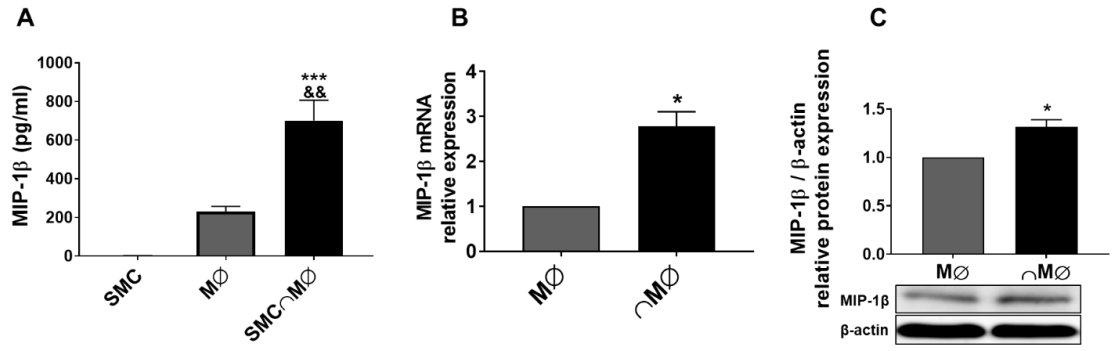

Figure 4 *The secretion and expression of MIP-1β is increased in macrophages (MØ) following the interaction with smooth muscle cells (CMN).*

Considering that MIP-1β is found at increased levels in the serum of patients with advanced atherosclerotic plaques and is an independent predictor of major cardiovascular events (Chang and Chen, 2016), our data suggest the significant contribution of macrophage and SMC communication in aggravating the lesion by increasing the expression and secretion of MIP-1β.

Signaling pathways activated in macrophages following communication with SMCs are represented by the ERK1/2 pathway. Still, activation of p38 and JNK MAPK is not observed. Additionally, the activation of the NLRP3 inflammasome was observed.

These findings enhance the understanding of how communication between SMCs and macrophages amplifies the inflammatory process in the atherosclerotic plaque.

Chapter Two - Diabetes exacerbates extracellular matrix remodeling induced by communication between smooth muscle cells and macrophages - highlights the modulation of ECM remodeling enzymes – MMP – induced by the communication between SMC and MØ under elevated glucose conditions.

The role of SMC in the progression of atherosclerosis is complex, yet it is primarily considered beneficial, through the secretion of matrix elements, especially type I collagen, which can stabilize the atherosclerotic plaque by forming a fibrous cap. On the other hand, macrophages have an opposing effect, secreting a series of matrix remodeling enzymes that have a pro-thrombotic effect by thinning this fibrous cap.

Data obtained on animal experimental models through histological analysis of the regions prone to rupture of the atherosclerotic plaque, have shown elevated levels of MMP-1, MMP-3 and MMP-9 (Newby, 2005). The loss of collagen fibers and other ECM

components occurs in regions with increased inflammation in the fibrous cap. Recent studies have suggested that atherosclerotic plaques of patients with Type II diabetes are more prone to rupture due to increased vascular inflammation and inefficient reparative response (van Diepen et al., 2016, Edsfeldt et al., 2014). However, the key molecules involved in the progression of the plaque to a vulnerable plaque in diabetic patients are unknown.

The data in this work continues the previous studies of our group, where we demonstrated that the interaction between SMC and MØ produces a series of inflammatory molecules such as MCP-1 and IL-1β, molecules that trigger macrophage activation and a phenotypic alteration of SMC characterized by increased expression of MMP-1, -2 and -9 and decreased expression of Type I and III collagen (Butoi et al., 2016). Silencing CX3CR1, the receptor for fractalkine, led to a decrease in MMP-9 levels, suggesting its involvement in pathological cellular communication (Butoi et al., 2011).

Utilizing the *in vivo* co-culture model established previously, we employed classical WB methods, protein array, qPCR, a method for visualizing collagen assembly, as well as pharmacological inhibitors or ribonucleic acid (RNA) interference to determine the contribution of communication between macrophages and SMC in the progression of atherosclerotic disease towards a vulnerable plaque predisposed to rupture.

Thus, the results presented in this chapter showed that the interaction between SMC and MØ induces the release in the conditioned medium of proteases such as: ADAMTS1, MMP-1, MMP-3, MMP-9 and urokinase. The protein expression of MMP-1 and -9 is significantly increased in SMC after co-culture with human monocyte-derived macrophages (hMDM) from freshly isolated diabetic patients (ScDM) compared to control macrophages (ScCM). To determine the effect of co-culturing SMC with diabetic macrophages or the increased concentrations of glucose (HG) on MMPs produced by SMC following interaction, both types of macrophages derived from healthy donors (CM) or diabetic patients (DM), were co-cultured under normal (NG) or HG conditions with SMC. It is observed that MMP-9 was expressed in SMC interacted with CM in HG compared to NG and also in SMC co-cultured with DM in HG compared to NG, suggesting the effect of elevated glucose on MMP-9 expression (Figure 20A). Moreover, MMP-9 expression is higher in SMC that were co-cultured in HG with DM compared to CM, indicating that diabetic macrophages are responsible for the increase in MMP-9 expression. In the case of MMP-1, the main cause of increased expression is the elevated glucose concentration, as no significant differences were obtained between MMP-1 expression in SMC co-cultured with CM or DM when exposed to HG conditions (Figure 5).

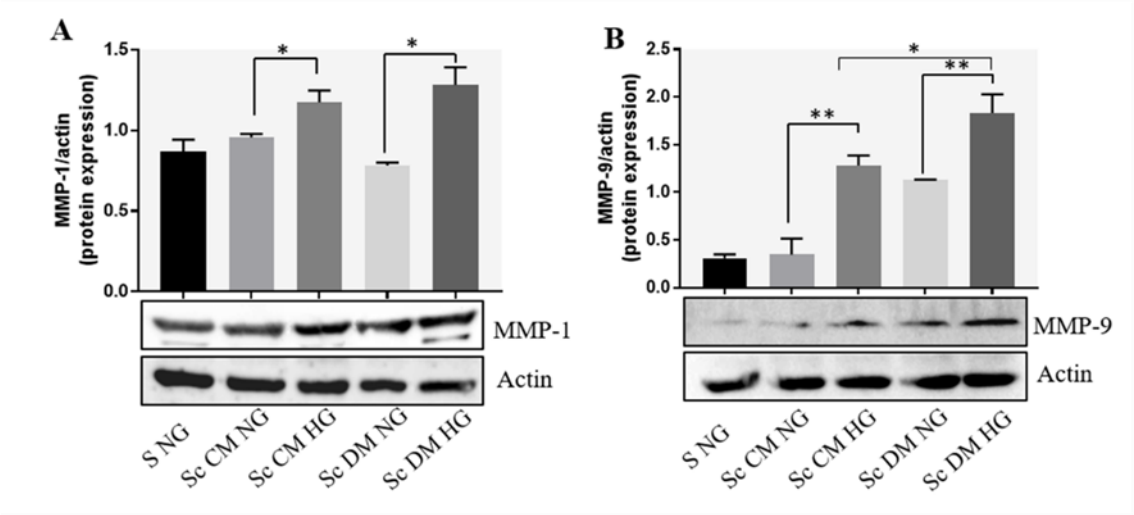

Figure 5 *The protein expression of MMP-1* ***(A)*** *and MMP-9* ***(B)*** *in smooth muscle cells (CMN) in medium with normal (NG) or elevated (HG) glucose concentrations, uninteracted (S) or interacted (Sc) with macrophages differentiated from monocytes isolated from control patients (CM) or diabetic patients (DM). The protein expression of MMP-1 and MMP-9 was normalized to the expression of β-actin, (Macarie et al., 2018, J.Cel.Mol.Med).*

To elucidate the signaling pathways involved in this increase of ECM remodeling enzymes, we performed silencing of the receptor for MCP-1 – CCR2 and the sub-unit p65 of NF-kB by transfecting SMC with interference RNA prior to the co-culture with MØ. These experiments (Figure 6) showed that the protein expression of MMP-1 and MMP-9 was significantly reduced in SMC where CCR2 and p65 were silenced. No effect on MMP expression was detected in SMC transfected with nonspecific siRNA (negative control).

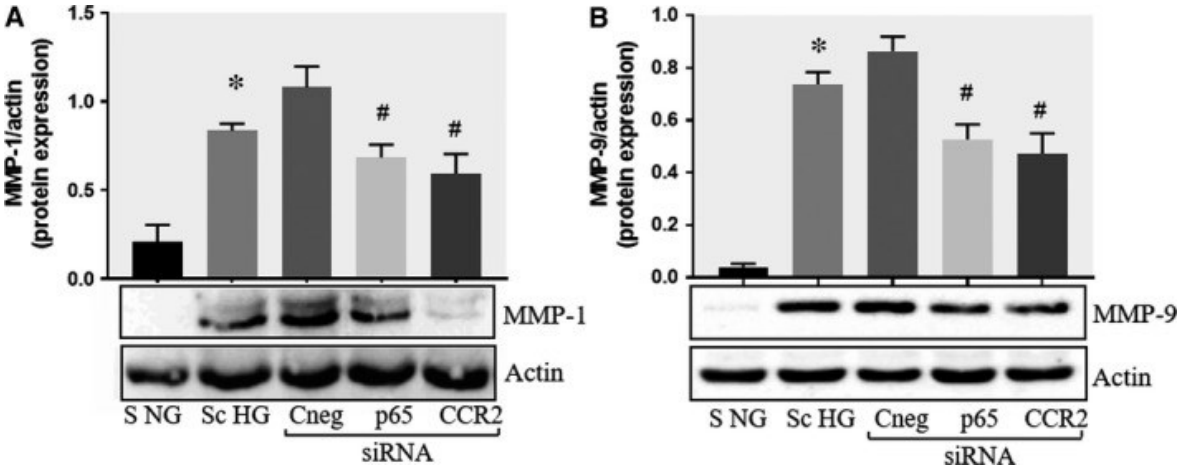

Figure 6 *The effect of silencing CCR2 and p65 in regulating the expression of MMP-1 and MMP-9 in smooth muscle cells (CMN). The expression of MMP-1* ***(A)*** *and MMP-9* ***(B)*** *was determined through Western Blot in control smooth muscle cells (S NG), smooth muscle cells co-cultured with macrophages (MØ) in elevated glucose conditions (Sc HG), and smooth muscle cells silenced for CCR2 and p65, interacted with macrophages (MØ) in elevated glucose conditions (Cneg, p65, CCR2 siRNA), (Macarie et al., 2018, J.Cel.Mol.Med).*

The activation of protein kinase C (PKC) is one of the main signaling events induced by elevated glucose concentration. To examine the possible involvement of PKC in the increase in expression of MMP-1 and MMP-9, we assessed the activation of PKCα in SMC. As evidenced by Western Blot experiments, the SMC-MØ co-culture under elevated glucose conditions induced a significant increase in PKCα phosphorylation in SMC. Interestingly, silencing p65 or CCR2 with specific siRNA prevented PKCα activation.

To evaluate whether PKCα is directly involved in the expression of MMP-1 and MMP-9, the co-culture between SMC-MØ was conducted in the presence of the PKCα inhibitor, Go 6976 (1 μmol/L). In this case, the protein expression of both proteases, MMP-1 and MMP-9, was significantly reduced, suggesting that PKCα activation is involved in the overexpression of MMP-1 and MMP-9 (Figure 7).

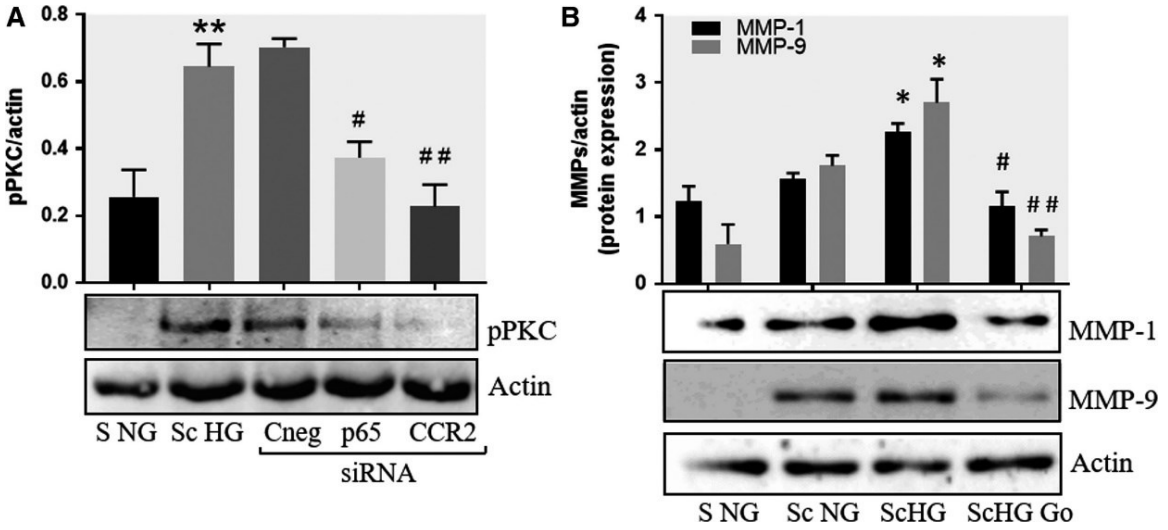

***Figure** 7 The inhibition of PKCα decreases the protein expression of MMP-1 and MMP-9 induced by the interaction of smooth muscle cells (CMN) with macrophages (MØ). **(A)** Evaluation of PKCα activation in control smooth muscle cells (S NG) compared with smooth muscle cells co-cultured with macrophages (MØ) in elevated glucose conditions (Sc HG). The phosphorylated form of PKCα is significantly increased in Sc HG. Silencing CCR2 or p65 reduces PKCα activation to the control level. **(B)** The effect of the PKCα inhibitor (Go 6976) on the protein expression of MMP-1 and MMP-9 induced by the communication between smooth muscle cells (CMN) and macrophages (MØ), (Macarie et al., 2018, J.Cel.Mol.Med).*

Furthermore, we investigated whether SMC activated by interaction with macrophages retain their ability to form collagen fibrils, an important feature in vascular stability (Li et al., 2003). For this, solubilized collagen labeled with Texas Red was added to the culture of control SMC or SMC after their interaction with macrophages under normal glucose (NG) or elevated glucose (HG) conditions. The assembly of collagen fibrils was monitored by fluorescence microscopy.

The results showed (Figure 8) that after interaction with MØ under normal or elevated glucose conditions, SMC decrease their ability to assemble collagen monomers into fibers, compared to non-interacted SMC maintained in medium with normal glucose concentration. When SMC interacted with MØ under elevated glucose conditions were pre-incubated for 3 minutes with blocking antibodies anti-MMP-1 or anti-MMP-9, they regained their ability to assemble collagen monomers.

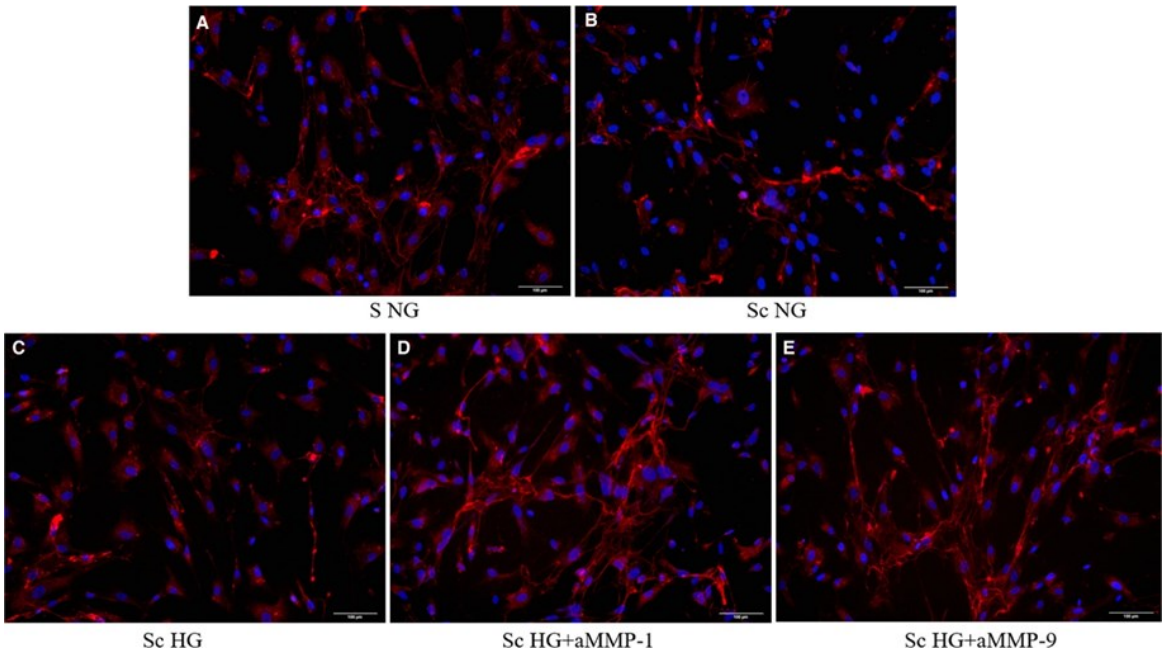

Figure 8 *The formation of collagen fibers by smooth muscle cells (CMN), from soluble collagen marked with Texas Red. (Macarie et al., 2018, J.Cel.Mol.Med).*

The findings of these two chapters primarily targeted atherosclerosis and underscored the relevance of intercellular communication in biological processes, particularly under pathological conditions where the equilibrium of these interactions can be completely deregulated. Hence, we demonstrated that macrophages have a pro-inflammatory effect and that SMC can, in turn, become mediators of local inflammation leading to the chronicity of inflammation. Additionally, elevated glucose concentrations modulate this interaction, causing an increased secretion of matrix proteases and anomalies in the collagen assembly process, changes that may favor the development of an atherosclerotic plaque prone to rupture.

Chapter 3 - Early diabetes induces matrix remodeling and functional changes in the aortic valve in a murine model of atherosclerosis and in a three-dimensional cell culture model – was aimed primarily at studying aortic valve disease and the ECM alterations induced by early diabetes.

The structural integrity of the ECM is crucial for the normal function of the aortic valve, and changes in the ECM have been observed in all stages of aortic valve disease (AVD) pathogenesis. Diabetes accelerates the progression of AVD and may induce changes in the ECM. However, diabetes-induced ECM alterations in the early stages of AVD progression have not been studied.

This chapter approached two experimental study models, one *in vivo*, represented by an apolipoprotein E (ApoE) deficient mouse, in which diabetes was induced via streptozotocin injections, and which was then maintained on a cholesterol-rich diet to induce atherosclerosis and changes at the level of the aortic valve; and one *in vitro* model, represented by a three-dimensional co-culture model based on a methacrylated gelatin hydrogel incorporating human valvular cells and exposed to elevated glucose concentrations. Methods employed in this chapter included: immunofluorescence, isolation of primary human valvular cells, Western Blot (WB), and qPCR.

The findings of this chapter showed that *in vivo*, experimental diabetes induces alterations of matrix elements and remodeling enzymes such as collagen 3, fibronectin, MMP-2, and MMP-9 (Figure 9).

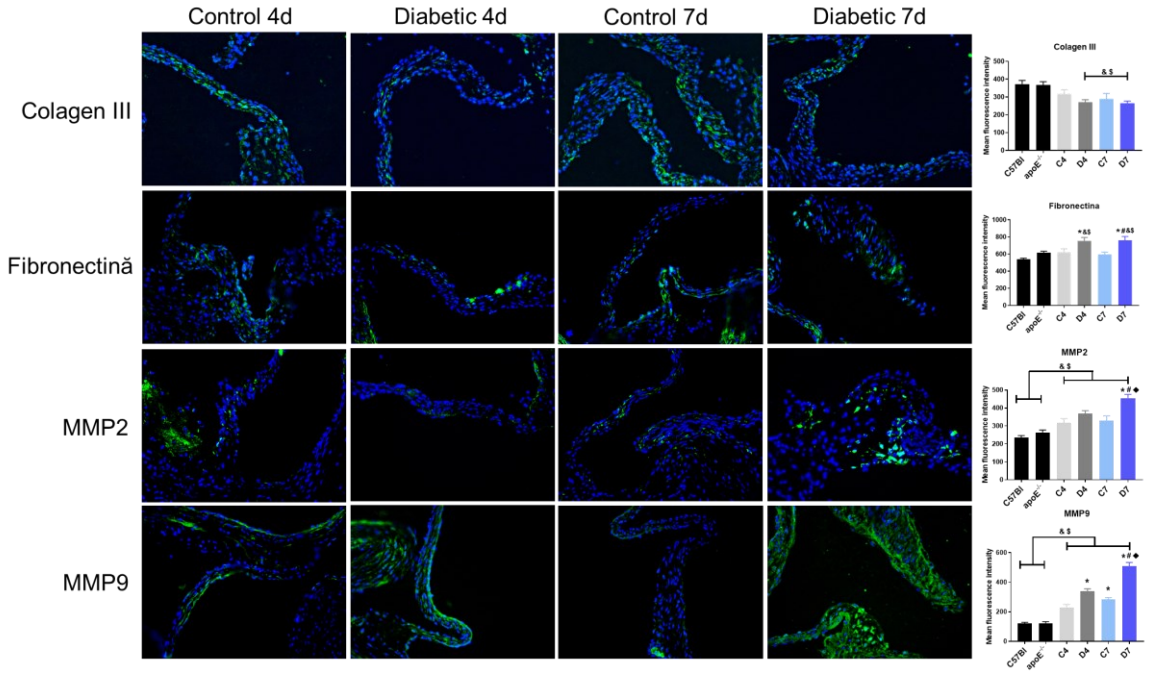

Figure 9 *The protein expression of extracellular matrix proteins: collagen III and fibronectin, and the gelatinases MMP-2 and MMP-9. To the right of the immunofluorescence images is the statistical analysis of the expression of MMP-2, -9, collagen III, and fibronectin in control and diabetic mouse groups.*

These alterations are correlated with the plasmatic and functional parameters of the aortic valve (Figure 10) as follows: the elevated level of MMP-9 strongly positively correlates with plasmatic parameters such as fetuin, triglycerides, total cholesterol and LDL, with the expression of myofibroblast marker – αSMA, with the expression of another matrix metalloprotease - MMP2, as well as with the functional parameters of the valve - velocity

and VTI; fibronectin is positively associated with MMP-2 and MMP-9 and negatively with collagen III, while type III collagen is negatively correlated with αSMA, vimentin, MMP9, LDL, and total cholesterol.

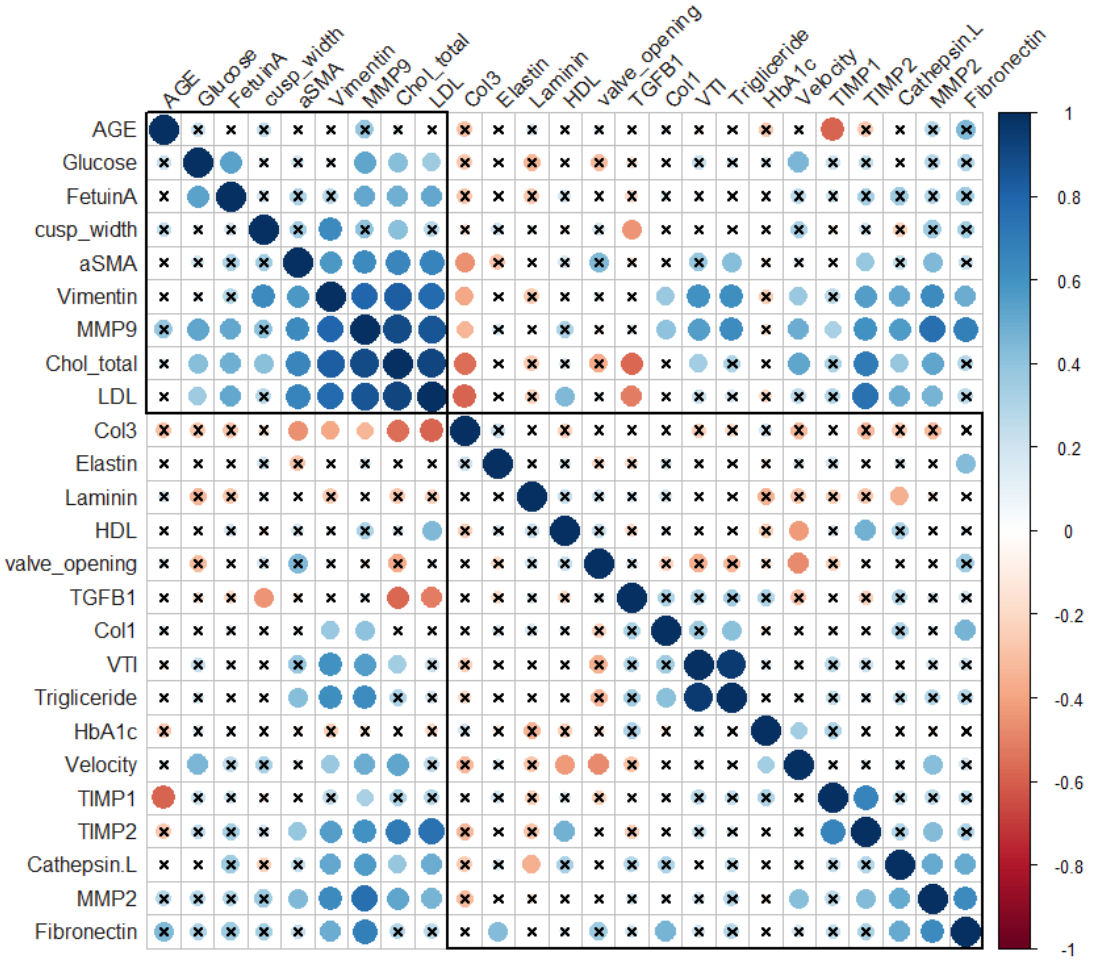

Figure 10 *Pearson correlation matrix among extracellular matrix proteins, remodeling markers, and functional and plasmatic parameters of the aortic valve. The mean values measured for each experimental group were used for Pearson correlations. Positive correlations are represented by the color blue, negative correlations by the color red, and the legend color palette indicates the Pearson coefficient (r). Correlations that do not reach the threshold of statistical significance ($p > 0.05$) are marked with an x (modified after Țucureanu, et al., 2019).*

To identify the cell type affected by hyperglycemic conditions and the one responsible for the observed alterations on molecules associated with the remodeling process, the study continued with the analysis of glucose effects on VEC (Valvular Endothelial Cells) and VIC (Valvular Interstitial Cells) in a 3D valve leaflet model developed in our laboratory (Vadana et al., 2020) and submitted for patenting. The 3D model

is obtained from hydrogel (methacrylated gelatin) and has the following advantages: i) it respects the structure of the native valve leaflet having VEC on the exterior and encapsulated VIC within, and ii) the valvular cells grown in this model are human, which confers significant relevance regarding the results obtained with its help.

The findings obtained using this model highlighted specific cellular alterations of ECM remodeling enzymes (Figure 11A) and transmembrane receptors that establish the connection between cells and ECM (Figure 11B).

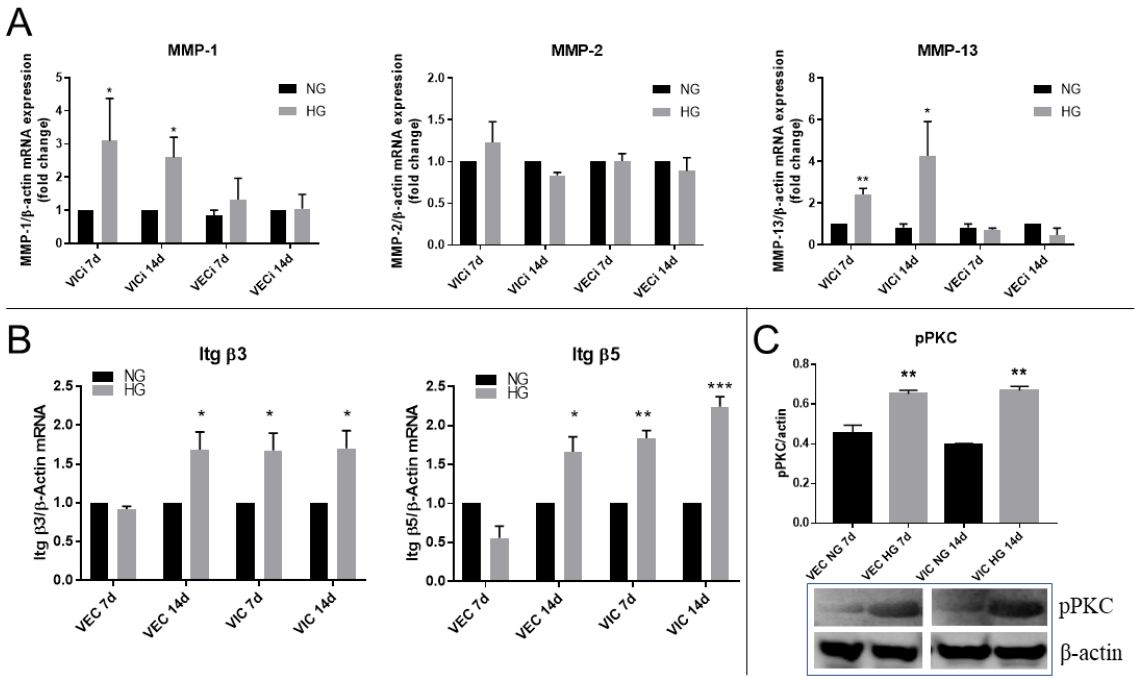

Figure 11 A. *Gene expression of MMP-1, -2, and -13 in valvular cells (VEC and VIC) in the 3D model exposed to normal (NG) or elevated (HG) glucose concentrations for 7 and 14 days.* ***B.*** *Gene expression of integrin subunits β3 and β5 in VEC and VIC from the 3D model exposed to normal (NG) or elevated (HG) glucose concentrations for 7 and 14 days. Cells were isolated from 3D constructs after 7 and 14 days and investigated through qPCR reaction.* ***C.*** *Expression of phosphorylated forms of PKC in VEC and VIC isolated from the 3D model of the aortic valve. Western blot was performed on valvular cell lysates isolated from the 3D construct after 7 and 14 days of glucose exposure. In comparison with NG (black columns), cells from HG (grey columns) exhibit an increased expression of pPKC-α. N = 3, ** $p < 0.01$.*

Previous studies have shown that PKC regulates the expression of MMP-1 and here we have shown that its active, phosphorylated form is increased in VIC and VEC under hyperglycemic conditions (Figure 11C).

The results from this chapter contribute to understanding valvular pathologies under early diabetes conditions and may open research avenues for the development of targeted therapies for the prevention or attenuation of AVD progression.

Chapter 4 of this work named −**Bioinformatic analysis of valvular cell communication in an Apoe deficient mouse model**− was aimed primarily at determining mediators of intercellular communication that can induce the modification of ECM elements observed in valvular interstitial cells, the predominant cells in valvular tissue, in an $ApoE^{-/-}$ mouse model.

This chapter addresses methods of bioinformatic analysis of some next-generation sequencing data at the single-cell level (scRNA-seq) obtained and published in 2021 (Lee et al., 2022). This type of sequencing allows the observation of cellular heterogeneity and also enables the exploration of functional interactions between sub-populations of cells in the valve.

The bioinformatic methods involved the processing and analysis of sc-RNAseq data using the software package Seurat v4 (Hao et al., 2021) in the R programming language. This was followed by a differential gene expression analysis, for the identified cell sub-populations, between control populations and those from $ApoE^{-/-}$ mice.

The scRNA-seq analysis demonstrated the existence of 14 populations of valvular cells (Figure 12), of which three sub-populations represent valvular interstitial cells.

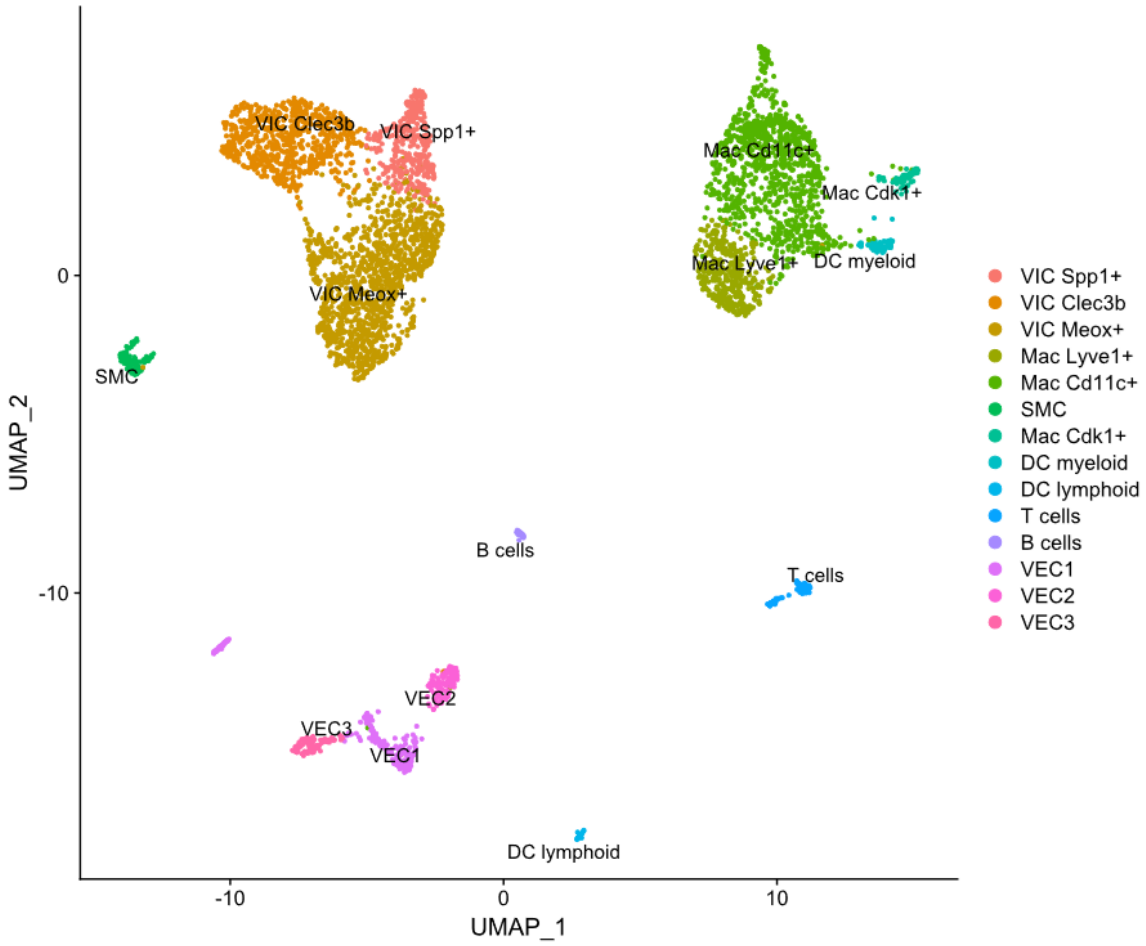

Figure 12 *UMAP projection capturing the structure of sc-RNAseq data and the relationship between the 14 groups of valvular cells.*

Among these VIC groups, a sub-population characterized by increased osteopontin expression exhibits significant alterations in ECM genes under hyperlipidemia, with observed increases in the genes Col1a1, Col2a1, Col3a1, Fn1, Spp1, and the MMP inhibitor – Timp1 (Figure 13A). A GSEA (Gene Set Enrichment Analysis) of all significantly altered genes in this group (Figure 13B) revealed that most genes modified under hyperlipidemia, are genes present in the extracellular space and are involved in collagen fiber organization or extracellular matrix arrangements.

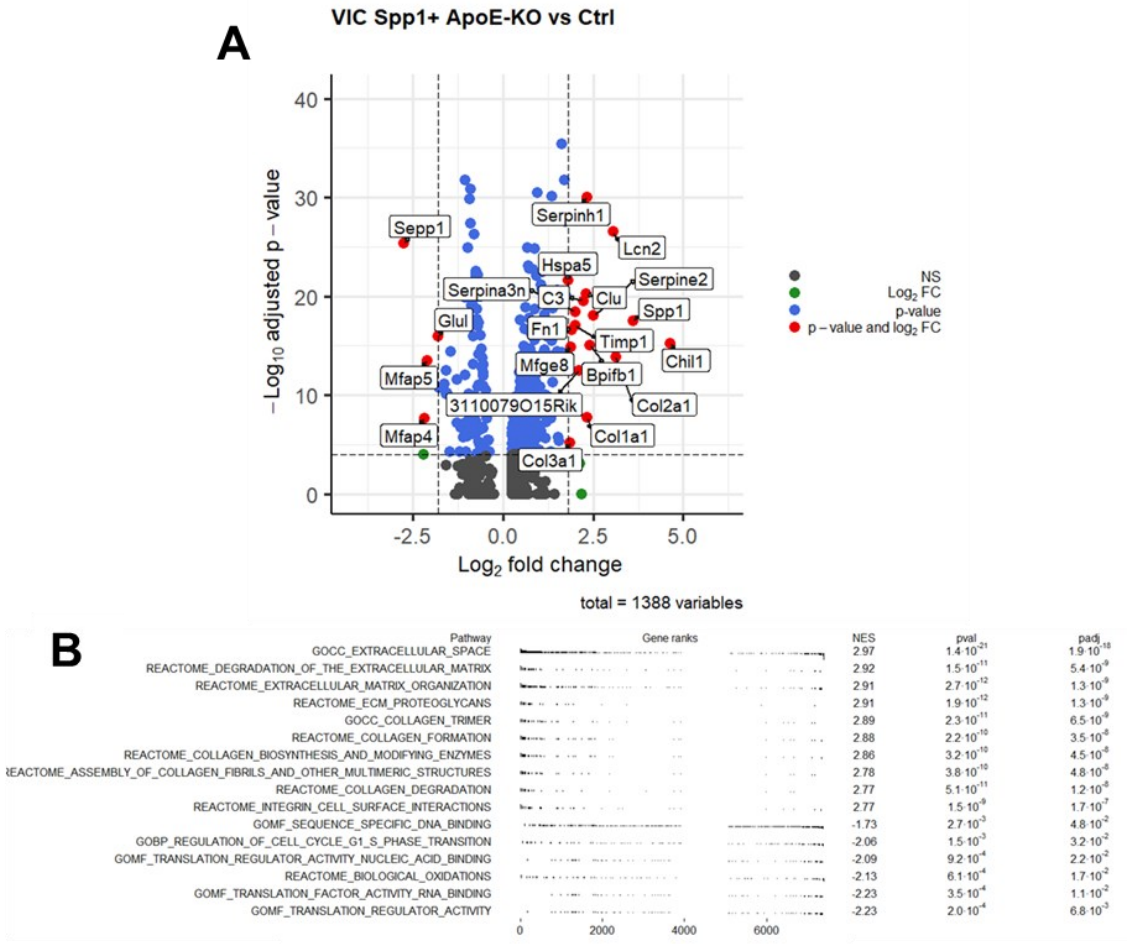

Figure 13 A. *Differential gene expression analysis of Spp1+ VIC between the Apoe−/− condition and control. In the volcano plot, grey defines unmodified genes; blue - genes that pass the p-value threshold; green – genes that pass the Log2FC threshold but not the p-value threshold; and red genes are significantly modified genes that meet both conditions. The threshold of significance was p≤10^-5 and Log2FC= 1.8; and the statistical test used was Wilcoxon rank sum.* ***B.*** *The most enriched gene sets for significantly modified Spp1+ VIC in the Apoe−/− condition, ordered by normalized enrichment score (NES).*

To discern which mediators of valvular intercellular communication might drive the observed changes in the Spp1+ VIC group, we employed a computational method for modeling intercellular communication using the NicheNet software package (Browaeys et al., 2020). This package integrates the experimental sc-RNAseq information with databases containing data about ligand-receptor interactions, intracellular signaling, and transcription regulation, thereby enabling the identification of new signaling mediators and inter-cellular communication in pathological processes.

This analysis, summarized in Figure 14, established a series of potential regulatory ligands for this Spp1+ VIC cluster.

Figure 14 ***The summarization of intercellular communication bioinformatics analysis carried out using the NicheNet package.*** ***A)*** *The top 40 ligands resulted from ligand activity analysis ordered by the area under the precision-recall curve (AUPR).* ***B)*** *The relative expression and the percentage of cells expressing the 40 ligands calculated for each group of valvular cells. The size of the dots is directly proportional to the percentage of cells expressing the respective ligand in each group, with red defining a higher expression compared to the average expression of that ligand, while blue defines a relatively lower expression.* ***C)*** *The alteration of gene expression of ligands in the* $Apoe^{-/-}$ *mouse. Red represents an increase in gene expression in* $Apoe^{-/-}$ *compared to the control, and blue represents a decrease in gene expression.* ***D)*** *Target genes expressed in* $Spp1^{+}$ *VIC and modified in* $Apoe^{-/-}$ *that each ligand can alter. Purple is used to calculate a regulatory potential based on ligand-target information present in the NicheNet model.*

The ligands with the highest regulatory potential are represented by IL-1β, FGF-2, TGF- β1, BMP-4, and CSF2 (Figure 15). Among the identified ligands, TGF- β1 is primarily expressed by VIC but also by CD11c+ macrophages, BMP-4 is mainly expressed by valvular endothelial cells, and IL-1β is expressed by dendritic cells and Cd11c+ macrophages. Moreover, proinflammatory macrophages can communicate and induce changes in Spp1+ VIC through the secretion of Il1b, Lgals3, Tgf-β1. Among these, both Lgals3 and Il1b exhibit an expression increase under hyperlipidemia.

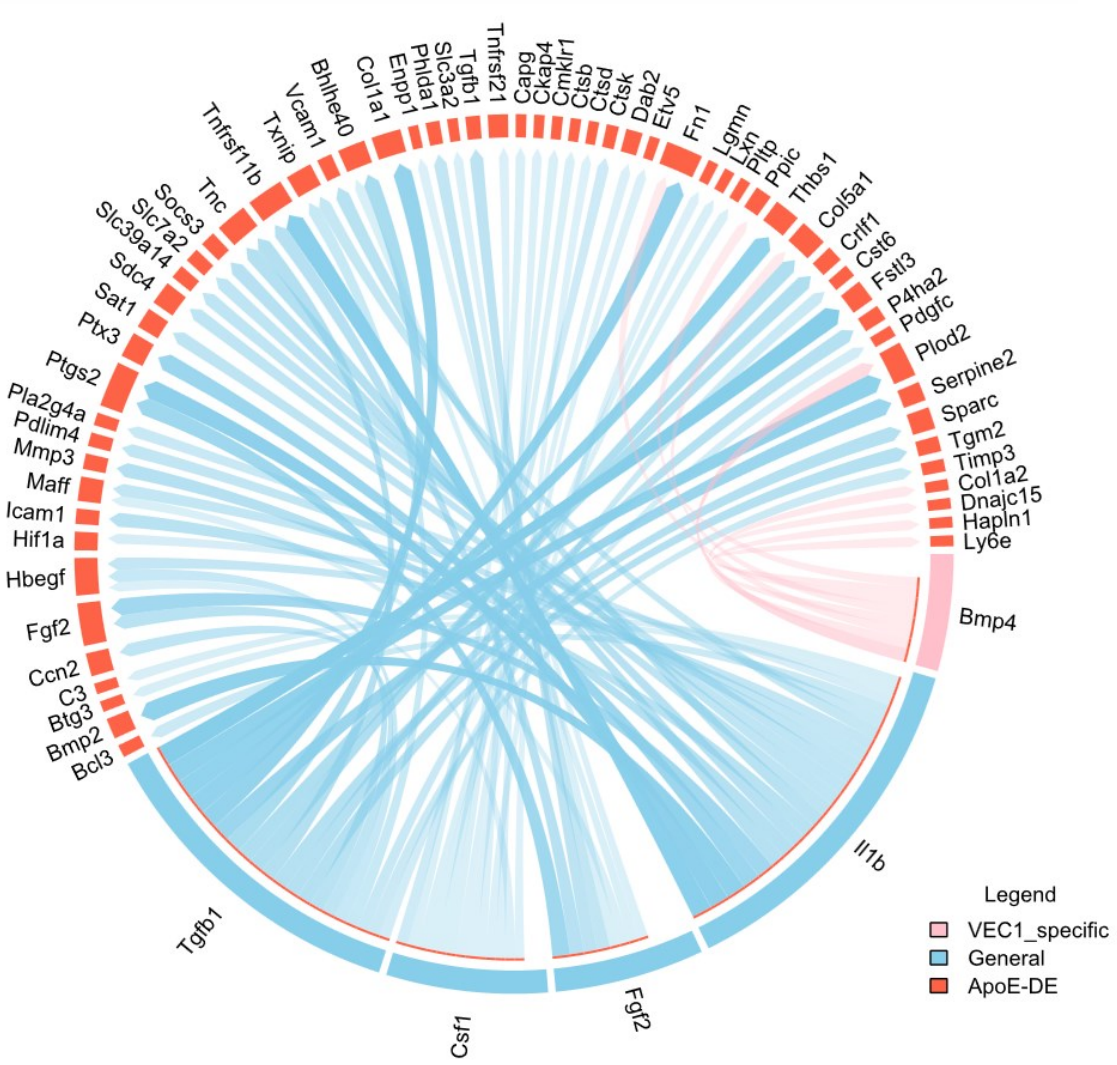

Figure 15 *The circular graphic representation of ligands with the strongest regulatory potential and their target genes. At the bottom marked in blue are ligands (Tgfb1, Csf1, Fgf2, and Il1b) which are not expressed by only a single specific cell type; pink marks Bmp4 which is expressed only in the VEC group; and at the top of the graphic and marked in orange are represented the target genes for each ligand.*

In conclusion, this work renders substantial contributions towards understanding the pathological alterations of the extracellular matrix, occurring within the context of atherosclerosis and BAV (Bicuspid Aortic Valve), employing complex research techniques.

In the first chapter, novel mechanisms were underscored by which communication between smooth muscle cells and macrophages might influence the atheroma's evolution towards a vulnerable plaque by augmenting inflammation driven by the elevation of inflammatory mediators: G-CSF, GM-CSF, ICAM-1, IL-6, CCL5, and MIP-1β. It was also demonstrated that this inflammation is contingent upon the activation of the NLRP3 inflammasome and the ERK/MAPK signaling pathway.

In the second chapter, the effect of elevated glucose levels in amplifying matrix remodeling was scrutinized, and it was shown that the expression and activity of extracellular matrix remodeling enzymes: MMP-3, -8, -1, and -9 are elevated under heightened glucose conditions. These alterations are modulated by signaling through PKCα and the CCR2 receptor.

In the third chapter, alterations of the valvular extracellular matrix were identified in a diabetic $Apoe^{-/-}$ mouse model at short intervals of 4 and 7 days post-diabetes onset, prior to valve fibrosis and calcification. Utilizing this experimental model, it was illustrated that the expression of fibronectin and metalloproteases MMP-2 and -9 is elevated in these mice, and these alterations positively correlate with the plasma and functional parameters of the valve. Aiming to investigate the diabetes-induced cellular level alterations, the experiments in Chapter 3 continued on a three-dimensional *in vivo* study model with VIC internally and VEC externally, which was maintained in elevated glucose for 7 and 14 days. The results garnered from this model demonstrated that increased glucose causes alterations in the gene expression of laminin gamma, type III collagen, MMP-1 and MMP-13 enzymes, and β3 and β5 integrin subunits in VIC, while in VEC, the gene expression of α4, αV, and β integrin subunits is elevated.

The fourth chapter entailed a complex analysis of single-cell RNA sequencing data from the aortic valve sourced from C57BL/6J and $Apoe^{-/-}$ mice. Through this analysis, the existence of a VIC sub-population exhibiting significant ECM gene alterations under hyperlipidemia was identified. Bioinformatic modeling of intercellular communication in the valve revealed that these alterations could be mediated by: IL-1β, FGF-2, TGF- β1, BMP-4, and CSF2.

The investigation of ECM alterations and the effect of diabetes uncovers a research domain where scientific contributions are yet sparse. The data from the current study

signifies a significant advancement in this domain, providing new insights into how ECM remodeling affects BAV progression and atherosclerotic lesions. Moreover, these data may aid in developing novel therapeutic approaches, focused on targeting intercellular communication and ameliorating the pathological remodeling of the ECM, thereby potentiating a vanguard in the therapeutic domain of cardiovascular pathologies.

Bibliography

BROWAEYS, R., SAELENS, W. & SAEYS, Y. 2020. NicheNet: modeling intercellular communication by linking ligands to target genes. *Nat Methods,* 17**,** 159-162.

BUTOI, E., GAN, A. M., TUCUREANU, M. M., STAN, D., MACARIE, R. D., CONSTANTINESCU, C., CALIN, M., SIMIONESCU, M. & MANDUTEANU, I. 2016. Cross-talk between macrophages and smooth muscle cells impairs collagen and metalloprotease synthesis and promotes angiogenesis. *Biochim Biophys Acta,* 1863**,** 1568-78.

BUTOI, E. D., GAN, A. M., MANDUTEANU, I., STAN, D., CALIN, M., PIRVULESCU, M., KOENEN, R. R., WEBER, C. & SIMIONESCU, M. 2011. Cross talk between smooth muscle cells and monocytes/activated monocytes via CX3CL1/CX3CR1 axis augments expression of pro-atherogenic molecules. *Biochim Biophys Acta,* 1813**,** 2026-35.

CHANG, T. T. & CHEN, J. W. 2016. Emerging role of chemokine CC motif ligand 4 related mechanisms in diabetes mellitus and cardiovascular disease: friends or foes? *Cardiovasc Diabetol,* 15**,** 117.

COFFEY, S., ROBERTS-THOMSON, R., BROWN, A., CARAPETIS, J., CHEN, M., ENRIQUEZ-SARANO, M., ZUHLKE, L. & PRENDERGAST, B. D. 2021. Global epidemiology of valvular heart disease. *Nat Rev Cardiol,* 18**,** 853-864.

EDSFELDT, A., GONCALVES, I., GRUFMAN, H., NITULESCU, M., DUNER, P., BENGTSSON, E., MOLLET, I. G., PERSSON, A., NILSSON, M., ORHO-MELANDER, M., MELANDER, O., BJORKBACKA, H. & NILSSON, J. 2014. Impaired fibrous repair: a possible contributor to atherosclerotic plaque vulnerability in patients with type II diabetes. *Arterioscler Thromb Vasc Biol,* 34**,** 2143-50.

HAO, Y., HAO, S., ANDERSEN-NISSEN, E., MAUCK, W. M., 3RD, ZHENG, S., BUTLER, A., LEE, M. J., WILK, A. J., DARBY, C., ZAGER, M., HOFFMAN, P., STOECKIUS, M., PAPALEXI, E., MIMITOU, E. P., JAIN, J., SRIVASTAVA, A., STUART, T., FLEMING, L. M., YEUNG, B., ROGERS, A. J., MCELRATH, J. M., BLISH, C. A., GOTTARDO, R., SMIBERT, P. & SATIJA, R. 2021. Integrated analysis of multimodal single-cell data. *Cell,* 184**,** 3573-3587 e29.

KATAKAMI, N. 2018. Mechanism of Development of Atherosclerosis and Cardiovascular Disease in Diabetes Mellitus. *Journal of Atherosclerosis and Thrombosis,* 25**,** 27-39.

LEE, S. H., KIM, N., KIM, M., WOO, S.-H., HAN, I., PARK, J., KIM, K., PARK, K. S., KIM, K., SHIM, D., PARK, S.-E., ZHANG, J. Y., GO, D.-M., KIM, D.-Y., YOON, W. K., LEE, S.-P., CHUNG, J., KIM, K.-W., PARK, J. H., LEE, S. H., LEE, S., ANN, S.-J., LEE, S.-H., AHN, H.-S., JEONG, S. C., KIM, T. K., OH, G. T., PARK, W.-Y., LEE, H.-O. & CHOI, J.-H. 2022. Single-cell transcriptomics reveal cellular diversity of aortic valve and the immunomodulation by PPARγ during hyperlipidemia. *Nature Communications,* 13**,** 5461.

LI, S., VAN DEN DIEPSTRATEN, C., D'SOUZA, S. J., CHAN, B. M. & PICKERING, J. G. 2003. Vascular smooth muscle cells orchestrate the assembly of type I collagen via alpha2beta1 integrin, RhoA, and fibronectin polymerization. *Am J Pathol,* 163**,** 1045-56.

LIU, H., NING, H., MEN, H., HOU, R., FU, M., ZHANG, H. & LIU, J. 2012. Regulation of CCL5 expression in smooth muscle cells following arterial injury. *PLoS One,* 7**,** e30873.

MANDUTEANU, I. & SIMIONESCU, M. 2012. Inflammation in atherosclerosis: a cause or a result of vascular disorders? *J Cell Mol Med,* 16**,** 1978-90.

MONCLA, L.-H. M., BRIEND, M., BOSSÉ, Y. & MATHIEU, P. 2023. Calcific aortic valve disease: mechanisms, prevention and treatment. *Nature Reviews Cardiology,* 20**,** 546-559.

NEWBY, A. C. 2005. Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. *Physiol Rev,* 85**,** 1-31.

ORGANIZATION, W. H. 9 December 2020. *The top 10 causes of death* [Online]. https://www.who.int/en/news-room/fact-sheets/detail/the-top-10-causes-of-death: WHO. Available: https://www.who.int/en/news-room/fact-sheets/detail/the-top-10-causes-of-death [Accessed 2022].

PAN, H., HO, S. E., XUE, C., CUI, J., ROSS, L. S., LI, F., SOLOMON, R. A., CONNOLLY, E. S. & REILLY, M. P. 2023. Atherosclerosis is a smooth muscle cell-driven tumor-like disease. *bioRxiv*.

SILVA, A. C., PEREIRA, C., FONSECA, A., PINTO-DO, O. P. & NASCIMENTO, D. S. 2020. Bearing My Heart: The Role of Extracellular Matrix on Cardiac Development, Homeostasis, and Injury Response. *Front Cell Dev Biol,* 8**,** 621644.

TEDGUI, A. & MALLAT, Z. 2006. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev,* 86**,** 515-81.

THIRIET, M. 2018. Cardiovascular Disease: An Introduction. *In:* THIRIET, M. (ed.) *Vasculopathies: Behavioral, Chemical, Environmental, and Genetic Factors.* Cham: Springer International Publishing.

TUCUREANU, M. M., BUTOI, E., GAN, A. M., STAN, D., CONSTANTINESCU, C. A., CALIN, M., SIMIONESCU, M. & MANDUTEANU, I. 2016. Amendment of the cytokine profile in macrophages subsequent to their interaction with smooth muscle cells: Differential modulation by fractalkine and resistin. *Cytokine,* 83**,** 250-261.

VAN DIEPEN, J. A., THIEM, K., STIENSTRA, R., RIKSEN, N. P., TACK, C. J. & NETEA, M. G. 2016. Diabetes propels the risk for cardiovascular disease: sweet monocytes becoming aggressive? *Cell Mol Life Sci,* 73**,** 4675-4684.

WOLF, D. & LEY, K. 2019. Immunity and Inflammation in Atherosclerosis. *Circ Res,* 124**,** 315-327.