UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

Angiogenesis in Atherosclerosis

Expression Analysis of Notch Signaling Components in Human Atherosclerotic Lesions

Constança d'Orey Pereira Coutinho

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Dissertação orientada por:
Doutora Ana Teresa Tavares (FMV)
Prof.ª Doutora Ana Maria Crespo (FCUL)

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The references in this work are written according to Atherosclerosis Journal guidelines.

“When I despair, I remember that all through history the ways of truth and love have always won”.

Mahatma Gandhi

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ABSTRACT

Atherosclerosis is a degenerative disease of large muscular and elastic arteries characterized by endothelial dysfunction, vascular inflammation, and the progressive development of intima plaques (atheroma). As the atherosclerotic plaque develops, the process of angiogenesis occurs, with the growth of new vessels arising from the adventitial *vasa vasorum* into the media and intima layers. This modulates plaque growth and stability. The new microvessels are immature, fragile, leaky and may lead to intraplaque hemorrhages, thrombosis and vessel obstruction. Inhibition of atheroma neovascularization has therefore been proposed as an approach to restrict lesion growth promoting its stabilization.

Delta-like 4 (Dll4)/Notch pathway has been implicated in the regulation of angiogenesis and blood vessel maturation, which introduces DLL4 as potential target for atherosclerosis treatment. In this study, the expression patterns of DLL4/Notch pathway components were analyzed by immunolabeling of histological sections from human atherosclerotic plaque samples. These expression patterns were correlated with the level of *vasa vasorum* maturation and type of atherosclerotic lesion (progression stage).

In summary, the expression results indicated that more advanced lesions present more microvessels but these have less pericyte coverage, which underscores the immaturity of this neovasculature. In parallel, the expression of DLL4 is detected in lower levels in *vasa vasorum* of early lesions and at higher levels in more advanced plaques. These observations indicate that the number of endothelial cells expressing DLL4 increases as immature microvessels proliferate during disease progression. Although it did not present significant correlation with lesion progression, it was also possible to detect the presence of NOTCH1 receptor in endothelial cells of immature microvessels in advanced lesions.

This research contributed to rise our level of knowledge on the molecular regulators of angiogenesis in human atherosclerotic lesions, elucidate the correlation between *vasa vasorum* maturation and plaque progression, and support a potential therapeutic application of Dll4/Notch signaling modulators in the stabilization of atherosclerotic lesions.

Key-Words: angiogenesis, atherosclerosis, Dll4/Notch, human carotid plaques, *vasa vasorum*.

RESUMO

A aterosclerose é uma doença degenerativa das artérias de grande e médio calibre, que se caracteriza pela ocorrência de disfunção endotelial, inflamação vascular, e acumulação de lípidos, cálcio, colesterol e detritos celulares na camada íntima. Estes eventos patológicos levam à formação da placa aterosclerótica (ateroma), remodelação vascular, obstrução luminal, alterações no fluxo sanguíneo, e diminuição do fornecimento de oxigénio a órgãos alvo, o que pode resultar em eventos cardíacos isquémicos e acidente vascular cerebral. Os factores de risco que aceleram significativamente o processo de aterogénese (i.e., formação de placas ateroscleróticas na parede interna do vaso sanguíneo) são a hipertensão, diabetes, tabagismo, obesidade, estilo de vida sedentário, factores hereditários, bem como elevados níveis de lipoproteínas de baixa densidade (LDL) e baixos níveis de lipoproteínas de alta densidade (HDL).

O desenvolvimento de placas ateroscleróticas pode ser classificado em diversas fases. Hoje em dia a classificação recomendada pela *American Heart Association* (AHA) consiste em oito categorias que incluem lesões iniciais (assintomáticas; tipos I-III) e lesões avançadas (sintomáticas; tipos IV-VIII), cada uma com características histológicas específicas. Com o crescimento progressivo da placa aumenta também o grau de estenose luminal. Para pacientes sintomáticos com estenose severa (maior que 70%) é recomendada endarterectomia da carótida, um procedimento cirúrgico que consiste na remoção da placa aterosclerótica. Para casos sintomáticos de estenose entre 50 a 69% os benefícios da cirurgia existem mas são menores. Por outro lado, para pacientes assintomáticos que apresentem grau de estenose maior que 70% a endarterectomia da carótida é também indicada mas com uma eficiência significativamente inferior. As amostras obtidas a partir desta cirurgia são muitas vezes utilizadas em estudos histológicos, tal como foi o caso deste projecto.

Existem três grandes mecanismos de disrupção da placa aterosclerótica que podem levar a eventos trombóticos: ruptura da cápsula fibrosa, erosão superficial e disrupção dos vasos sanguíneos que se formam no interior da placa (com maior importância para este trabalho). O desenvolvimento progressivo da placa aterosclerótica está associado ao processo de angiogénese, isto é, ao crescimento de novos vasos sanguíneos a partir dos *vasa vasorum* da camada adventícia, que se estendem através das camadas média e íntima. Estes microvasos recém-formados são imaturos, frágeis e permeáveis, podendo levar a hemorragias no interior da placa. A consequente acumulação de eritrócitos e inflamação provocam ruptura das placas mais vulneráveis, originando trombose arterial e obstrução vascular.

A grande importância destes microvasos em ateromas tem sido revelada em estudos com modelos animais de ratinhos deficientes na apolipoproteína-E (ApoE-/-), mostrando que a inibição da neovascularização com inibidores de angiogénese leva a uma diminuição acentuada do crescimento da lesão. Em placas ateroscleróticas humanas, onde os microvasos já existem, a estabilização ou "normalização" da microvasculatura imatura tem sido proposta como uma estratégia para prevenir a hemorragia intraplaca e restringir o tamanho da lesão, promovendo assim a sua estabilização. Os vasos normalizados são menos frágeis, dilatados e sinuosos, apresentando uma membrana basal normal e uma maior cobertura de pericitos (indicador de maturidade vascular). Além de prevenir a hemorragia intraplaca, estes vasos normalizados irão também permitir uma melhor perfusão de agentes terapêuticos para o interior da placa.

A grande diversidade de indutores angiogénicos, incluindo factores de crescimento, quimiocinas, enzimas angiogénicos, receptores específicos endoteliais e moléculas de adesão, representa uma grande variedade de potenciais alvos terapêuticos para a restrição da neovascularização patológica. Dentro destes, a via de sinalização Delta-like 4 (Dll4)/Notch merece um foco especial, uma vez que ao apresentar um papel crucial no desenvolvimento vascular embrionário e

na angiogénese fisiológica e patológica, possuí um elevado potencial terapêutico para doenças associadas a proliferação vascular anómala, como é o caso da aterosclerose. Os componentes principais da via de sinalização Notch no sistema vascular incluem três ligandos, DLL4, Jagged1 (JAG1), e Jagged2 (JAG2); três receptores, NOTCH1, 3 e 4; e três genes alvos, *HERP1*/*HEY2*, *HERP2*/*HEY1*, e *HERP3*.

A maioria dos estudos funcionais que incidem sobre os factores reguladores da angiogénese têm sido realizados em tumores pelo facto de representarem um potencial alvo terapêutico. DLL4 é fundamental para a maturação dos vasos recém-formados. Tem sido demonstrado que a inibição da via de sinalização Dll4/Notch bem como o tratamento com anticorpos anti-DLL4 levam a um aumento significativo da densidade vascular, mas simultaneamente restringem o tamanho do tumor, uma vez que os microvasos formados são imaturos e têm baixa capacidade de perfusão. Por outro lado, a sobre-expressão da via de sinalização Dll4/Notch resulta numa diminuição da densidade vascular tumoral, com vasos tumorais menos ramificados mas funcionais. Indo de acordo com o observado na vasculatura tumoral, os nossos trabalhos piloto demonstraram que Dll4 também é expresso nos *vasa vasorum* de placas ateroscleróticas de ratinhos ApoE-/-. Desta forma, a via de sinalização Notch apresenta-se como um potencial regulador da maturação dos *vasa vasorum* em lesões ateroscleróticas.

Torna-se assim importante determinar o papel da via de sinalização Dll4/Notch na angiogénese e progressão de lesões ateroscleróticas humanas. O presente estudo teve como objectivo analisar a expressão de componentes da via de sinalização Dll4/Notch em ateromas humanos, e correlacionar os seus níveis com o grau de maturação dos *vasa vasorum* e com a fase de progressão da lesão. Para tal, um fragmento de cada amostra (i.e., ateroma da carótida) foi avaliado e classificado conforme o tipo de lesão aterosclerótica (fase de progressão) através de coloração com Hematoxilina-Eosina, e posteriormente a expressão de componentes da via de sinalização Dll4/Notch e de marcadores de maturação vascular foi analisada por imunomarcação de cortes histológicos (imunofluorescência). Inicialmente, procedemos à optimização do protocolo de imunofluorescência em amostras de ateroma humano não incluídas nos grupos experimentais, tendo como objectivo minimizar a autofluorescência natural que estes tecidos apresentam. Secções histológicas de placenta foram usadas como controlo positivo para avaliar se determinados anticorpos primários marcavam correctamente as células esperadas, bem como para determinar a concentração óptima de anticorpo a utilizar.

Os nossos resultados mostram uma associação entre a densidade microvascular e a progressão da lesão aterosclerótica, sendo que as lesões mais avançadas apresentam mais microvasos com menor cobertura de pericitos, o que indica imaturidade da neovasculatura. Paralelamente, a expressão de DLL4 é detectada a baixos níveis em lesões precoces e a níveis mais elevados em lesões mais avançadas. Estas observações indicam que o número de células endoteliais que expressam DLL4 aumenta à medida que os microvasos imaturos proliferam durante a progressão da lesão. Apesar de não ter mostrado correlação com o grau de lesão, foi possível verificar também a presença do receptor NOTCH1 em lesões mais avançadas, mostrando ser expresso em células endoteliais de microvasos imaturos.

Tínhamos ainda como objectivo inicial avaliar por PCR em tempo real os níveis de expressão e activação da via de sinalização Notch e outras vias de sinalização envolvidas na angiogénese e/ou inflamação e maturação dos vasos. Contudo, apesar dos diferentes testes utilizados, não foi possível contornar a existência de contaminação genómica no cDNA que nos foi fornecido, entre outros problemas técnicos, e assim este ensaio não pôde ser optimizado nem finalizado.

Em conclusão, os resultados deste projecto contribuiram para elevar o nível de conhecimento em relação aos reguladores moleculares da angiogénese em lesões ateroscleróticas humanas, clarificar a correlação entre a maturação dos *vasa vasorum* e a progressão das placas, e dar suporte à hipótese de utilização de Dll4/Notch como um novo alvo terapêutico na estabilização das lesões ateroscleróticas.

Palavras-Chave: angiogénese, aterosclerose, Dll4-Notch, placas ateroscleróticas de carótidas humanas, *vasa vasorum*.

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LIST OF ABBREVIATIONS

AHA	American Heart Association
ANG2	Angiopoietin 2
ApoE−/−	Apolipoprotein E-deficient mice
BSA	Bovine serum slbumin
cDNA	Complementary DNA
CHLN-HSM	*Centro Hospital Lisboa Norte - Hospital de Santa Maria*
DAPI	4', 6'-diamidino-2-phenylindole, dihydrochloride
DDT	DL-Dithiothreitol
DIT	Diffuse intimal thickening
DLL4	Delta-like 4
dNTP	Deoxyribonucleoside triphosphate
ECs	Endothelial cells
FMV	*Faculdade de Medicina Veterinária*
HDL	High-density lipoprotein
IMM	*Instituto de Medicina Molecular*
JAG	Jagged
LDL	Low-density lipoprotein
NICD	Notch intracellular domain
OCT	Optimal cutting temperature
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
rRNA	Ribosomal RNA
RT	Room temperature
RT-qPCR	Real time – quantitative polymerase chain reaction
SMCs	Smooth muscle cells
SPSS	Statistical package for the social sciences
T_m	Melting temperature
VEGF	Vascular endothelial growth factor
VEGFR1	Vascular endothelial growth factor receptor 1

1 CONTEXT AND BACKGROUND

Atherosclerosis is the main cause of death in the developed world. It's a degenerative disease [1] of large muscular and elastic arteries [1,2] characterized by progressive vascular inflammation, endothelial dysfunction, and accumulation of lipids, calcium, cholesterol, and cellular debris within the tunica intima of the vessel wall. These pathological events lead to plaque (atheroma) formation, vascular remodeling, acute and chronic luminal obstruction, abnormalities on blood flow, and decreased oxygen supply to target organs [1], which may consequently result in ischemic cardiac events and stroke [3–5]. Atherosclerotic lesions preferentially develop in injury-prone areas such as bifurcations [6], as these are regions with small vessel diameter and/or where the blood flow is disrupted [7]. Risk factors that significantly accelerate atherogenesis, the formation of subintimal plaques, are hypertension, diabetes mellitus, serum lipid deficiency, smoking, obesity, a sedentary lifestyle and hereditary factors [5,8]. Moreover, despite not being the main cause, elevated levels of circulating low-density lipoprotein (LDL) and decreased levels of circulating high-density lipoprotein (HDL) strongly predispose the progress of the disease [2,5]. These stimuli have a direct or indirect effect on the damage of the vascular endothelium, triggering an inflammatory cascade through leukocytes that release a variety of mediators. In this way, blood vessel composition, function and integrity remain affected [9].

All large vessels are composed of three concentric layers (*e.g.*, human coronary artery; Figure 1.1): (1) an inner layer adjacent to the vessel lumen, the intima (*tunica intima*, intimal layer), (2) a middle layer, the media (*tunica media*, medial layer), and (3) an outer layer, the adventitia (*tunica adventitia*, adventitial layer) [9,10].

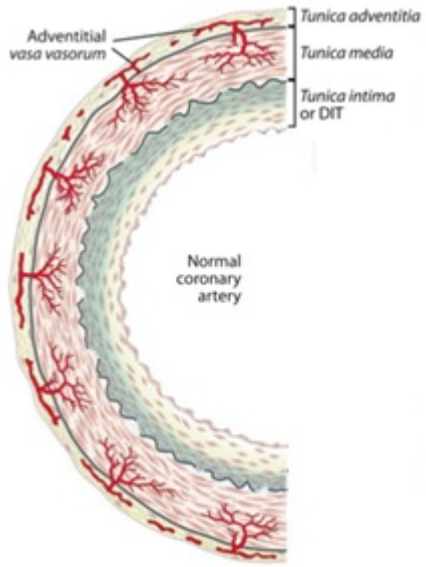

Figure 1.1. Scheme of a normal human coronary artery consisting of three concentric layers: an inner layer, the *tunica intim*a; a middle layer, the *tunica media*, and an outer layer, the *tunica adventitia*. The intima evolves from a single-cell-layer after birth to a multi-layered cell structure known as diffuse intimal thickening (DIT) in adults. The internal elastic membrane acts as a barrier between the intima and the underlying media. The media consists of multiple layers of smooth muscle cells, much more tightly packed than in the diffusely thickened intima, and embedded in a matrix of elastin and collagen. The external elastic membrane separates the media from the adventitia, which consists of fibrous tissue surrounded by nerves, lymphatic vessels, and *vasa vasorum* (adapted from [2]).

The intima is the layer between (and including) the endothelial surface adjacent to the lumen and the luminal border of the media [7], and it evolves from a single-cell-layer after birth to a multi-layered cell structure known as diffuse intimal thickening (DIT) in adults [11]. This layer receives oxygen and nutrients through diffusion from the arterial lumen [2]. The endothelial surface provides a smooth luminal lining and a selective diffusion barrier between the blood and the other layers [10],

and regulates the vascular tone, platelet activation, monocyte adhesion and inflammation, thrombus or blood clot formation, lipid metabolism, cellular growth and vascular remodeling [1]. The intima is separated from the media by the internal elastic membrane (internal elastic lamina, *elastica interna*), a fenestrated sheet of elastic tissue that may be fragmented, duplicated, or focally lost with advancing age or intimal disease [10]. The media consists of tightly packed multiple layers of smooth muscle cells (SMCs), involved in a matrix of elastin, collagen and proteoglycans [12]. Between the media and the adventitia lies the external elastic membrane (external elastic lamina, *elastica externa*), composed of interrupted layers of elastin [10]. Lastly, the adventitia consists of fibrous tissue [10,13] surrounded by nerves, lymphatic vessels, and *vasa vasorum* [10]. The *vasa vasorum*, or "vessels of a vessel", are a fine network of microvessels that stand on the adventitia and enter the outer media of the host vessel wall [14,15]. Their principal functions are nutrient and oxygen delivery as well as removal of systemic waste products [16].

Currently, the accepted cause of atherosclerosis is the increased hyperplasia of the *tunica intima* [2], which can be triggered by a variety of nonspecific signals [17]. The progression of the disease can be generally explained by maladaptive local inflammatory responses [18]. With the recruitment of inflammatory cells from de the blood [8,19,20], the monocyte enter to the subendothelium, differentiate into macrophage and ingest retained and modified lipoproteins to become cholesterol-laden foam cells [8,21,12] (both of them, macrophages and foam cells, usually marked with antibody against CD68 [22–25]), the hallmark of the earliest lesion of atherosclerosis [26]. As lesions progress, medial SMCs proliferate and migrate into the intima. The consequent accumulation of SMCs promotes the formation of a collagenous fibrous cap [18,19], a distinct layer of SMCs in a collagenous-proteoglycan matrix [3,27] completely covering the lipid core [27,28]. In advanced lesions, as a result of macrophages apoptosis, a necrotic core develops underneath the fibrous cap, being filled with lipids, extracellular debris, cholesterol crystals, proteases, and procoagulant/thrombotic material [18,29] (Figure 1.2).

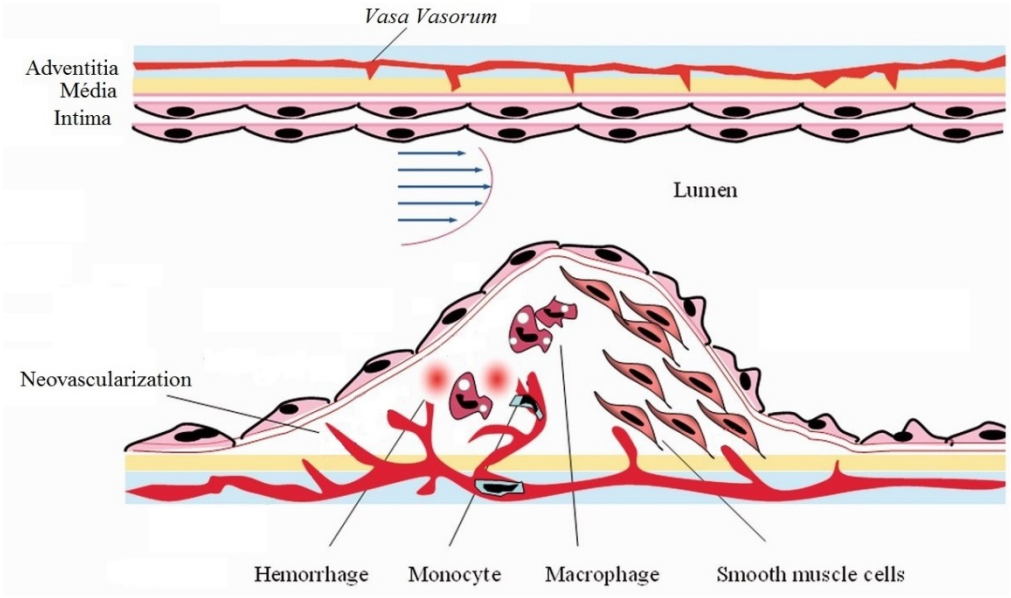

Figure 1.2. Atherosclerotic plaque formation. Inflammation leads to recruitment of inflammatory cells from the blood, with monocytes traversing the microvascular endothelium and differentiating into macrophages and these into foam cells. With lesion progression, smooth muscle cells proliferate and accumulate in the intima, promoting the formation of a collagenous fibrous cap (adapted from [30]).

According to the main stages of the disease, and based on histologic analysis, atherosclerotic plaques can be classified in different categories [7,31,32] (Figure 1.3). The classification of atherosclerotic plaques recommended by the American Heart Association (AHA) was developed by Stary and his colleagues, and consists of eight numeric types that include early (type I to III) and advanced lesions (type IV to VIII) [31,32]. The initial (type I) lesion is characterized by an increase in macrophages and subsequent formation of lipid-laden foam cells. Type II lesions consist of multiple layers of SMCs and macrophage foam cells, and also include fatty streaks, yellow-colored streaks on the intimal surface of arteries [32]. Type III lesions contain an amount of extracellular lipid droplets and particles that affect the coherence of intimal SMCs. This extracellular lipid accumulation becomes larger, confluent, and more disruptive, the so-called lipid core, characterizing type IV lesions. These lesions may also contain thick layers of fibrous connective tissue - type V lesion, and/or fissure, hematoma, and thrombus - type VI lesion [31]. Some type VI lesions may be largely calcified representing type VII and some consist mainly of fibrous tissue with accumulations of lipid or calcium – type VIII [33].

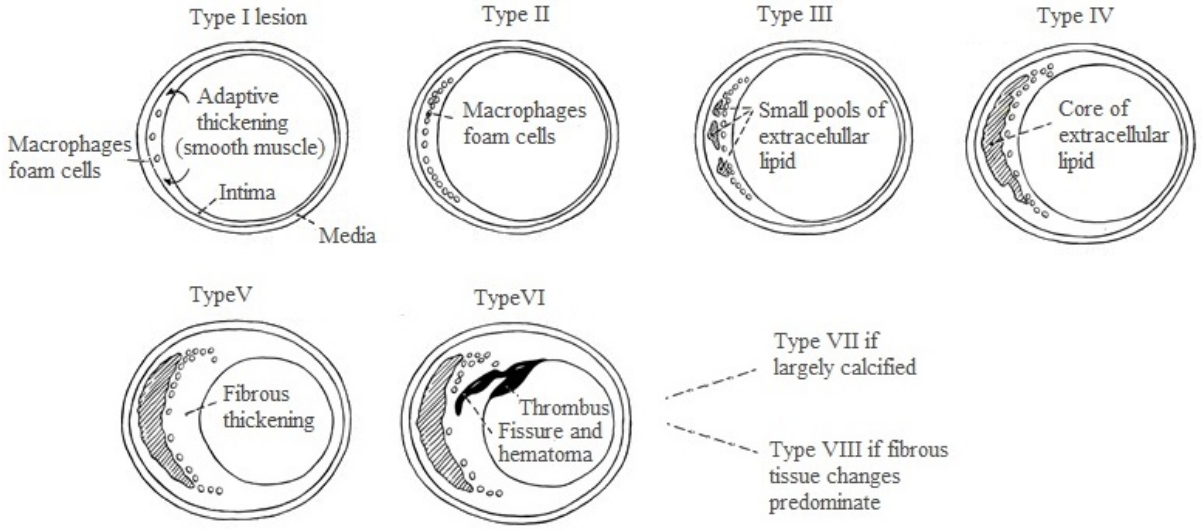

Figure 1.3. Atherosclerotic lesions progression. The main histological characteristics of each lesion type (from I to VIII) are indicated (adapted from [31]).

With progressive plaque growth the luminal stenosis increases [9], and its degree can be ascertained through conventional imaging techniques such as coronary angiography, intravascular ultrasound and optical coherence tomography [34]. For symptomatic patients with severe stenosis (greater than 70%), carotid endarterectomy, a surgical procedure to remove the atherosclerotic plaque, is indicated with clear benefit, and for symptomatic patients with stenosis greater than 50-69% the benefit is lower but still exists. For asymptomatic patients with a degree of stenosis greater than 70% carotid endarterectomy is also indicated but with a significant lower efficiency [35]. Samples obtained from this surgical procedure are often used in histology studies [36].

Two mechanisms of plaque disruption are the rupture of the fibrous cap and superficial erosion [26,27], which promote the expansion of the lesions and may lead to thrombosis [27]. The vulnerability of the plaque to ruptur depends on the integrity of the fibrous cap, which, in turn, is influenced by inflammatory processes within the vessel wall that trigger decreased matrix synthesis, increased matrix degradation, and cell death [26]. Fissure of the fibrous cap allows the coagulation factors contact with tissue factor [12,26], the main pro-thrombotic stimulus found in the lesion's lipid core [37], leading to thrombus formation [26]. In contrast, local denudation of endothelial cells (ECs, usually marked with PECAM/CD31 and CD34 [34]. PECAM marks fully differentiated vascular ECs

and CD34 both progenitor and mature ECs [38]) occurs due ECs apoptosis or mechanical injury [12,28,39], and provokes superficial erosion of the arterial intima, which may also lead to thrombosis [39].

The other extremely important mechanism of plaque disruption is the process of angiogenesis with disruption of the intraplaque microvessels [26]**.** Blood vessels are formed through three main processes: vasculogenesis, angiogenesis and arteriogenesis [40]. *De novo* embryonic blood vessel formation occurs via vasculogenesis, in which ECs differentiate and proliferate within a previously avascular tissue, and then coalesce to form a primary tubular network. By a different process, angiogenesis, this initial network can be expanded and modified through pruning and vessel enlargement into arteries and veins, thus forming the connecting branching patterns characteristic of the functional vasculature [41]. Angiogenesis is responsible for the vascularization of specific structures during normal development, and for major new vessel formation in the adult [42]. Arteriogenesis finally occurs with the tightly integration of ECs with surrounding matrix and supporting mural cells [41], which give stability and control to the perfusion [43]. In general, large vessels are surrounded by SMCs (marked with α-SMA and calponin [44]), whereas small ones are covered by pericytes [41,45] (commonly marked with NG2 [34,45]).

During the formation of atherosclerotic lesions, the early phase of intima thickening is angiogenesis-independent [46]. However, the development of the atherosclerotic plaque is associated with the process of angiogenesis [47,15], mainly in the shoulder regions of the plaque [48] (Figure 1.4). This process involves sprouting, branching, and differential growth of new vessels [41,49] that arise from the adventitial *vasa vasorum* [47,15] and extend through the media into the base of the plaque, where neovascularization is most evident [22,15,50]. These new vessels are morphologically different from adventitia *vasa vasorum* once they have a location closer to the lumen, displaying thicker endothelial walls, a more prominent degree of branching and the presence of dilatations similar to microaneurysms [51].

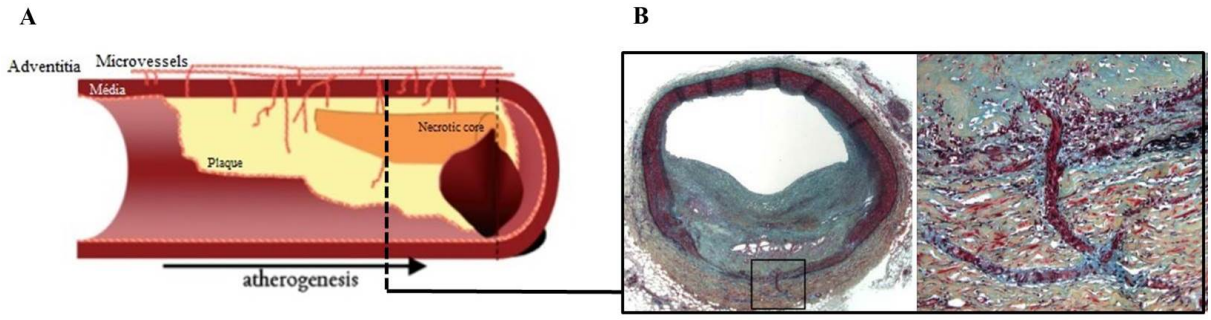

Figure 1.4. Adventitial and intraplaque microvessels. The development of atherosclerotic plaque is associated with the process of angiogenesis, where new vessels arise from the adventitial *vasa vasorum* and extend through the media into the base of the plaque. **A**. Diagram showing longitudinal section (adapted from [36]), **B.** Human atherosclerotic plaque with evidence of *vasa vasorum* sprouting into the plaque (inset; adapted from [52]).

During microvessel development, ECs go through an angiogenic switch from a quiescent, non-proliferative state, to an active and proliferative one. In physiological conditions, endothelial and mural cells are separated from each other by a basement membrane comprised of extracellular matrix proteins. In response to pro-angiogenic signals such as vascular endothelial growth factor (VEGF) stimulation, ECs lose their cellular junctions, activate proteases that degrade the adjacent basement membrane becoming invasive and motile [43], and project filopodial extensions [53]. Detachment of mural cells is stimulated by angiopoietin-2 (ANG2), a growth factor that destabilizes the interaction between ECs and their mural cells [54].

VEGF is an important factor for migration and proliferation of ECs [55], and its expression is highly regulated by hypoxia, thus providing a physiological feedback mechanism to promote tissue oxygenation through blood vessel formation [56]. The negative feedback between VEGF and Notch signaling pathways controls the specification of ECs into tip or stalk cells [55]. On one hand, tip cells spearhead new sprouts that sense and respond to guidance signals within the environment. On the other hand, stalk cells proliferate to support sprout elongation and establish the lumen of the new vessel [53]. Under VEGF stimulation (Figure 1.5), the expression of the ligand Delta-like 4 (DLL4) is upregulated in tip cells via VEGF receptor 2 (VEGFR2). In turn, DLL4 activates Notch signalling in the neighboring stalk cells and induces VEGFR1 expression in ECs [55,57]. VEGFR2 internalization and activation is also regulated by Ephrin-B2, a transmembrane ligand for Eph receptors required for early angiogenic remodeling and vascular sprouting [58].

Tie1/2 receptors bind angiopoietin and regulate mature blood vessel stabilization and the interaction between ECs and their supporting cells [59]. The activation of Tie1/2 by ANG2 secreted from the tip cells lead to stalk cell proliferation and survival [60]. Unlike ANG2, ANG1 acts on normal remodeling, stabilization and maturation of the developing vasculature [41,49,61]. In addition to angiopoietins, platelet derived growth factor (PDGF) and its receptor PDGFRβ are also involved in the stabilization of the new connections by the recruitment of mural cells [62]. The interaction of recruited pericytes with the ligand Jagged 1 (JAG1) induces Notch3 receptor signaling and promotes cell survival and vascular branching [60] as well as the expression of SMC genes, such as α-SMA and CALPONIN [63]. The quiescence, non-proliferative state is re-established when pro-angiogenic signals decrease and the sprouting process stops [53].

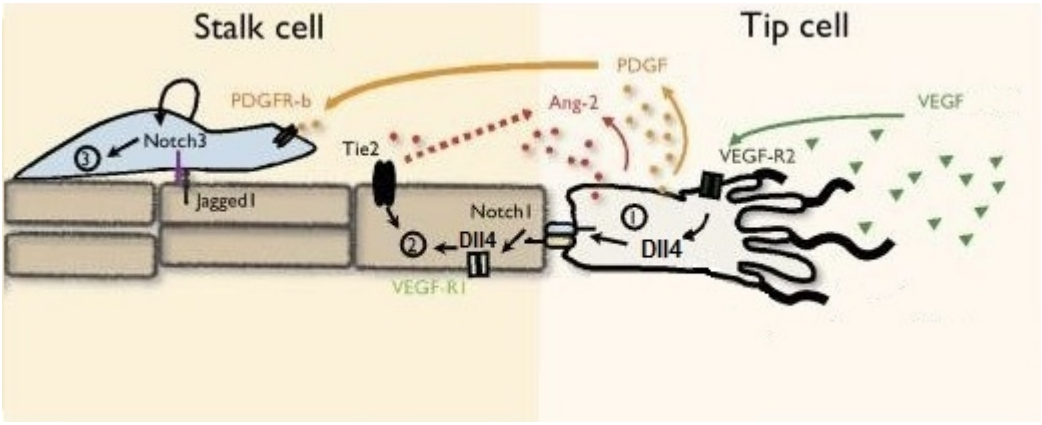

Figure 1.5. Concept of physiological angiogenesis. 1) In tip cells, under VEGF stimulation, via VEGFR2 the expression of the ligand Delta-like 4 (DLL4) is upregulated, inducing VEGFR1 expression in ECs downstream. 2) In stalk cells, predominance of VEGFR1 and activation of Tie2 by Ang2 secreted from the tip cell lead to proliferation and survival. 3) Platelet-derived growth factor receptor (PDGFR)-β enable pericyte to be attracted to the growing sprout by PDGF, released from tip cells. Interaction of recruited pericyte with endothelial cell–derived Jagged 1 induces the expression of Notch3 and activation of an autoregulatory loop that further enhances Notch3 activation, thereby promoting pericyte survival, investment, vascular branching, and induction of smooth muscle cell genes (adapted from [60]).

Angiogenesis has a dual role in atherosclerosis. On the one hand it is beneficial, as a primary response to hypoxia of the inner layers of the vascular wall, thus promoting collateral growth, decreased residual ischemia and protection of the affected tissue from further damage [64]. On the other hand, the new microvessels are fragile and leaky, leading to local extravasation of plasma proteins, erythrocytes (hemorrhage) and inflammatory cells, and contributing to necrotic core enlargement. Furthermore, most of the intraplaque *vasa vasorum* have poorly formed EC junctions and few mural cells (SMCs and pericytes), which underlie their leakiness [22,50].

It has been shown that the distribution of *vasa vasorum*, related to lesion formation and vessel lumen diameter, may have a critical role in the development of atherosclerotic lesions [47]. Microvessel density is increased in ruptured plaques, as well as in lipid-rich plaques and in lesions with a thin fibrous cap [23,48]. Moreover, microvessel density is higher in lesions with moderate and severe inflammation, and lower in lesions with mild inflammation and large fibrocalcific plaques [23]. Angiogenesis and intraplaque hemorrhage are unlikely to lead directly to rupture, but will trigger a cascade of events instead, such as necrotic core expansion and enhanced inflammation, thus promoting lesion enlargement and fibrous cap thinning with eventual disruption [65]. Under these circumstances, angiogenesis and inflammation often coexist and can mediate rapid plaque progression [22,23,50], converting a chronic process into an acute disorder with a succeeding thromboembolism [64].

The great importance of neovessels in the growth of atherosclerotic plaques has been revealed in studies with apolipoprotein E-deficient (ApoE -/-) mouse models, showing that the inhibition of plaque neovascularization with angiostatin causes a marked suppression of lesion growth. In humans lesions, where microvessels already exist, the stabilization or "normalization" of the immature microvasculature (Figure 1.6) has been proposed as an approach to prevent intraplaque hemorrhage and restrict lesion growth [66,46]. Normalized vessels are less fragile, dilated and winding than abnormal vessels, and have a normal basement membrane and greater coverage by pericytes [67]. In addition to preventing intraplaque hemorrhage and consequent necrotic core enlargement, normalized vessels would allow for better perfusion of therapeutic agents into the plaque [65].

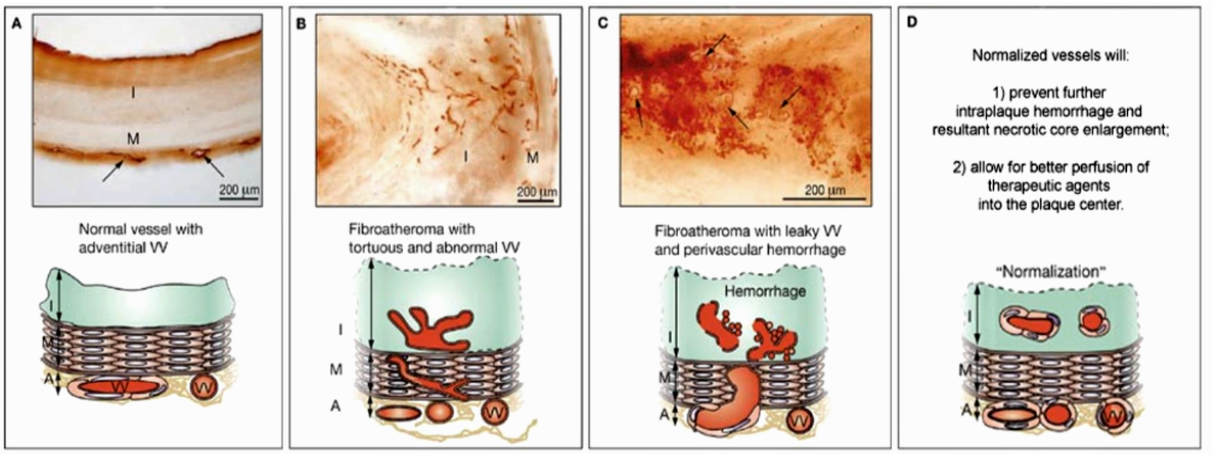

Figure 1.6. Proposed "normalization" of immature vessels in the plaque and its implications in atherosclerotic angiogenesis. **A.** Normal human coronary artery, and **(B)** fibroatheroma without and **(C)** fibroatheroma with intraplaque hemorrhage. The endothelium was visualized using of *Ulex europaeus* immunohistochemical staining. **A.** Adventitial *vasa vasorum* can be seen (arrows). **B.** In nonhemorrhagic fibroatheroma the *vasa vasorum* are intimal and show tortuosity and branching. **C.** In fibroatheromas with intraplaque hemorrhage the *vasa vasorum* are disrupted (arrows) with surrounding hemorrhage. It has been shown that intraplaque hemorrhage contributes to necrotic core enlargement and vulnerability to rupture. The specimens A to C are illustrated in corresponding schematics below them. **D.** Jain *et al*. hypothesize that normalization of the *vasa vasorum* with "muscularization" of the capillaries promote vessel stabilization that will prevent plaque hemorrhages and consequently plaque progression. Abbreviations: **A.** adventitia; **I.** intima; **M.** media; **VV.** *vasa vasorum* (adapted from [65]).

The great diversity of angiogenic inducers, comprising growth factors, chemokines, angiogenic enzymes, endothelial-specific receptors, and adhesion molecules represents a wide range of potential therapeutic targets for the elimination of pathological neovascularization [64]. Among these it is worth highlighting the Notch signaling pathway already mentioned, a key regulator of cell fate with a crucial role in blood vessel formation. It regulates vascular development, especially angiogenesis, as well as differentiation of SMCs [68] and ECs (into tip or stalk cells; [69]). The major components of the pathway in the vascular system include three ligands, DLL4, JAG1, and Jagged2 (JAG2); three receptors, NOTCH1, 3, and 4; and three target genes, *HERP1*/*HEY2*, *HERP2*/*HEY1*,

and *HERP3* [68], all involved in multiple aspects of vascular development including smooth muscle differentiation, angiogenic processes, arterial-venous cell specification and vascular morphogenesis. Both receptors and ligands are transmembrane proteins containing an extracellular domain that facilitates the communication between cells. After ligand binding to Notch receptors present on adjacent cells (Figure 1.7), sequential proteolytic cleavages take place, releasing the Notch intracellular domain (NICD). NICD migrates to the nucleus [70,71] where it regulates the expression of the primary target genes of Notch signaling [70,72].

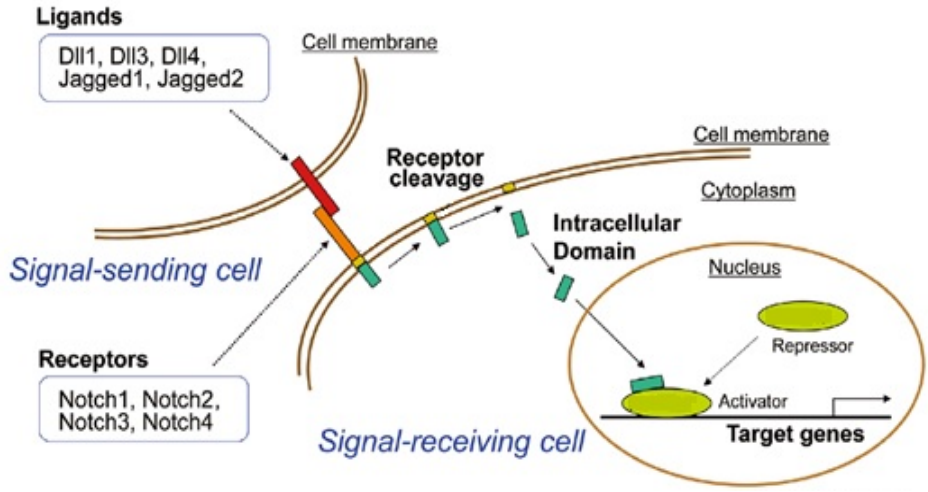

Figure 1.7. Notch signaling pathway. Interaction of Notch receptors with their ligands triggers a cascade of enzymatic cleavages of the receptor allowing the Notch intracellular domain (NICD) to migrate into the nucleus where it regulates the expression of target genes of the pathway (adapted from [71]).

Most of the functional studies on the regulatory factors of angiogenesis are conducted in tumors, for they represent potential therapeutic targets. Blocking Dll4/Notch activity as well as the treatment with anti-Dll4 antibodies significantly increases vessel density, but simultaneously restrains tumour growth [73–76]. This occurs due to the low perfusion capacity of microvessels and to their immaturity, for they lack pericytes and smooth muscle cell coverage [73,75]. On the other hand, increased Dll4/Notch activity results in decreased tumor vascular density [73], with straighter, less branched but functional tumor vessels [76]. Concurrently with these findings, our pilot experiments demonstrated that Dll4 is also expressed in plaque *vasa vasorum* of atherosclerosis-affected mice. Taken together, these observations suggest that the Dll4/Notch signaling pathway may be a potential regulator of *vasa vasorum* maturation in atherosclerotic lesions.

The current project aimed at investigating the role of DLL4/Notch signaling in the angiogenesis of human atherosclerotic lesions. To this end, the expression DLL4/Notch pathway components was analyzed in human atherosclerotic plaques at different stages of lesion progression.

2 AIMS

Over the last decade, accumulating evidence has implicated atheroma microvessels (*vasa vasorum*) in the regulation of plaque growth and stability. In line with these evidence, the induction of atheroma *vasa vasorum* maturation has recently been proposed as a therapeutic strategy for atherosclerosis. Our team and others have implicated the Dll4/Notch signaling pathway in the regulation of blood vessel maturation, which introduces Dll4 as a potential target for atherosclerosis treatment.

The general goal of our research line is to ascertain the role of the Dll4/Notch pathway in plaque angiogenesis and atherosclerosis progression, and evaluate its function as a potential drug target. In this particular project, our aim was to analyze the expression patterns of DLL4 and other components of the NOTCH signaling pathway in human atherosclerotic lesions, and correlate these patterns with the level of *vasa vasorum* maturation and phase of lesion progression.

In order to achieve this main purpose, we aimed at evaluating the atheroma structure and type of atherosclerotic lesion (progression stage), as well as analyzing the expression of DLL4/Notch pathway components in human atheroma samples, both by immunolabeling and by real time quantitative polymerase chain reaction (RT-qPCR). In addition to DLL4/Notch components, markers for vessel maturation and atheroma inflammation were also selected.

Our findings not only contribute to unravel the molecular mechanisms of atheroma angiogenesis, but also help to elucidate the interplay between plaque vessel maturation and atherosclerosis progression. Moreover, this study introduces Dll4/Notch signaling modulators as drug candidates for the treatment of atherosclerosis.

3 MATERIALS AND METHODS

3.1 Biological samples

3.1.1 Origin of human samples

Human carotid endarterectomy samples from 122 patients with advanced atherosclerosis were provided by collaborators at the Vascular Surgery Unit of the "Centro Hospital Lisboa Norte - Hospital de Santa Maria" (CHLN-HSM) and Rheumatology Research Unit at "Instituto de Medicina Molecular" (IMM). Of these 122 samples, 20 were chosen for further analysis (see Section 4.1). Human biological samples of this type were shown to be excellent materials for the study of atheroma-associated *vasa vasorum* [61,77]. This study was approved by the ethics committee of the CHLN-HSM, and was established in collaboration with Helena Canhão and João Eurico da Fonseca from the Rheumatology Research Unit at IMM, as well as with Luis Mendes Pedro and José Fernandes e Fernandes from the Vascular Surgery Unit of CHLN-HSM. The informed consent form (see Annex I) was signed prior to any protocol-specific procedure, and patient data were collected through survey (see Annex II). All proceedures were conducted in compliance with the regulations governing clinical trials and with the Declaration of Helsinki, as amended in Fortaleza, Brazil (2013) [78].

Placental tissue samples were provided by our collaborator Dusan Djokovic at "Hospital de São Francisco Xavier" as part of his clinical research studies. The placenta was elected to be used as a positive control (in both RT-qPCR and immunofluorescence analyses) since it is a vascularized tissue with high expression levels of *DLL4* [79].

3.1.2 Tissue Preparation

After surgery, a segment of the whole carotid lesion was dissected into smaller pieces, immediately snap-frozen in liquid nitrogen and stored at -80ºC until further use for RNA extraction. The remaining sample was embedded in optimal cutting temperature (OCT®) compound (Sakura, Netherlands), immediately frozen in cooled isopentane (Merck, Germany) and stored at -80ºC. These frozen samples were sectioned at 10µm with a tungsten carbide blade in a cryostat Leica model CM3050S at -20ºC, and mounted on Superfrost™ Plus glass slides (Thermo Scientific, USA). The aforementioned procedures were performed by our collaborators at IMM.

Placental tissue was collected from a term pregnant woman following vaginal delivering by our collaborator Doutor Dusan Djokovic. Part of the tissue was immediately placed in RNAlater® Solution (Ambion, USA) for later RNA extraction and reverse transcription (see section 3.3.1). The remaining tissue was fixed with 4% paraformaldehyde (PFA) solution at 4°C for one hour, cryoprotected in 15% sucrose, embedded in 7.5% gelatin, frozen in cooled isopentane (Merck, Germany) and stored at -80ºC. These frozen samples were sectioned at 20µm in a cryostat Leica model CM3050S at -25ºC and mounted on Superfrost™ Plus glass slides (Thermo Scientific, USA).

3.2 Histology and plaque characterization

To assess the type of atherosclerotic lesion in each sample, histological classification by Hematoxylin–Eosin staining was performed as described by Stary and colleagues [31–33]. Hematoxylin stains nucleic acids in blue color, whereas eosin stains proteins from the cytoplasm and extracellular matrix in varying degrees of pink [80].

Following a standard protocol, sections of human carotid samples were air-dried for 30 min at room temperature (RT) and washed in phosphate-buffered saline (PBS) for 10 min to remove OCT® compound. After this, two washes in PBS for five min were conducted, followed by one wash in distilled water for two min. Sections were stained with filtered 0.1% Mayers Hematoxylin (Sigma-Aldrich, USA) for 10 min, washed in running water to remove the excess, placed in distilled water for one min and in 70% ethanol (VWR chemicals, USA) for three min. Slides were then counterstained with 0,5% Eosin Y (Sigma-Aldrich, USA, 1.5g dissolved in 300ml of 95% ethanol) for five min, washed in running water once again to remove the excess and placed in distilled water for one min. Finally, slides were dehydrate by graded ethanol series (70%, 85%, 95%, 100% ethanol), one min each, xylol for five min and mounted in Entellan® mounting medium (Merck, Germany).

After Hematoxylin-Eosin staining, samples were observed under an Olympus BX51 microscope with Olympus 10X/0.30 NA and 40X/0.75 NA dry objectives, and imaged with Olympus DP21 photographic equipment (Olympus®, Japan). Each sample was characterized according to the American Heart Association (AHA) histological classification [31–33], and four groups of five samples each were established for further analysis: group 1 included type II and III, group 2 included the type IV, group 3 included the type V and group 4 included the type VI lesions.

3.3 Gene expression

3.3.1 RNA extraction and Complementary DNA (cDNA) synthesis

RNA from human carotid samples was supplied by our collaborators, having been extracted with RNeasy® Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. For the extraction of placental RNA, small tissue fragments were placed in two ml microcentrifuge tubes with a stainless steel bead and lysis buffer (buffer RLT, Qiagen, Germany) and DL-Dithiothreitol (DTT, Promega, USA). Tubes were placed in the TissueLyser II (Qiagen, Germany) twice for two minutes at 30 Hz. RNA was then extracted with RNeasy® Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. RNA quantification and quality were assessed using a NanoDrop™ 2000c spectrophotometer (Thermo Scientific, USA). High RNA purity is indicated by a ratio of absorbance at 260 nm vs. 280 nm ~2.0, which was the case for all samples tested.

400 ng of RNA of each sample were reverse transcribed into complementary DNA (cDNA) using SuperScript® III First-Strand Synthesis SuperMix kit for real time-quantitative polymerase chain reaction (RT-qPCR; Invitrogen, USA) according to manufacturer’s instructions. The reverse transcription reactions (total volume of 30 µl) were performed in a thermal cycler (VWR, USA) using the following protocol: 25ºC for 10 min, 50ºC for 30 min, at 80ºC for five min and 4ºC for five min. After adding 1 µl of *E. coli* RNase H, reverse transcription reactions were incubated at 37ºC for 20 min. The resulting cDNA samples were then either used straightway for RT-qPCR or stored at -20ºC for later analysis.

3.3.2 Real time-quantitative polymerase chain reaction (RT-qPCR)

The expression of 18 genes associated with the Notch signaling pathway and with the inflammatory response (*ANG1, ANG2, α-SMA, CALP, CD31, CD34, CD68, DLL4, EPHB2, HEY1, HEY2, JAG1, NOTCH1, PDGFRβ, TIE2, VEGF, VEGFR1, VEGFR2*) was analyzed by RT-qPCR in atherosclerotic plaques samples. Two negative controls were performed: a "no template control" without cDNA in the reaction mixture, and a "no amplification control", a minus-reverse transcriptase reverse control which is a mock reverse transcription containing all the RT reagents, except the reverse transcriptase. Placental samples were used for positive control of *DLL4* expression.

Primers were either designed using qPrimerDepot [81], Probefinder, or selected from the literature [82,83] (Annex IV). All RT-qPCR reaction mixtures were made in duplicate to a final volume of 12.5µl, using 0.54 µl of cDNA solution. RT-qPCR reactions were performed in 7500 Fast Real-Time PCR System (Applied Biosystems™, USA), with the DyNAmo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. SYBR Green I is specific for double-stranded DNA and becomes fluorescent when bound to the amplified double-stranded PCR product [84]. Briefly, reactions started with a hot-start, followed by an initial denaturation to activate the DNA polymerase, and cycles of denaturation, annealing and extension. Different numbers of cycles and annealing and extension periods were tested in order to improve the specificity of the amplified products (Table 3.1). For all reactions, the housekeeping gene ribosomal RNA 18S (rRNA18S) was used as an internal control of expression, and the presence of a single amplification product was evaluated.

Table 3.1. RT-qPCR reaction conditions.

Hot-start	**Initial denaturation**	**Denaturation**	**Annealing and extension**	**Melting curve**
50ºC	**95ºC**	**95ºC**	**66ºC, 68ºC** or **70ºC**	**50-95ºC**
2 min	7 min	10 sec	30 sec or 45 sec	1ºC/Step
		48 or 50 cycles		

3.3.3 Polymerase chain reaction (PCR)

PCR was performed in a thermal cycler (VWR, USA), with DreamTaq™ DNA Polymerase (Thermo Fisher Scientific, USA). PCR mix was prepared with 2 µl cDNA solution, 10X DreamTaq Buffer (containing 20 mm $MgCl_2$), 5 µl of deoxyribonucleoside triphosphate (dNTP) mix (with 2mM each), 1.0 µM of each primer, and 0.2 µl of DreamTaq DNA Polymerase, in a final reaction volume of 25 µl. Briefly, reactions started with an initial denaturation step at 95ºC for three min, followed by 40 cycles of denaturation at 95º for 30 sec, annealing at 66ºC for 30 sec, and extension at 72ºC for one minute. In the end, a final extension step occurred at 72ºC for 5 minutes.

PCR reactions were run on a 2% agarose gel for electrophoresis (Hi Res Standard Agarose, GTC Bioproducts, UK) prepared in 1X TAE with 2.5 ng/100 ml of GreenSafe Premium (NZYTech, Portugal). DNA was visualized under UV light on a ChemiDoc™ XRS^+ System with Image Lab™ Software (BioRad, USA). NZYDNA ladder VI (NZYTech, Portugal) was used as a molecular weight marker.

3.4 Immunofluorescence

Sections of carotid and placental samples were air-dried for 30 min and washed in PBS at 37ºC for 10-15 min to remove OCT® (carotid samples) or the gelatin (placental samples), followed by two washes at RT in PBS for five min and a post-fixation step in 4% PFA in PBS at 4ºC for five min. For tissue permeabilization, slides were washed twice in PBS for five min each, incubated in methanol AnalaR NORMAPUR® (VWR chemicals, USA) with 3% hydrogen peroxide (Scharlab, Spain) for 30 min in the dark at RT, and washed two times in PBS for five min each. For cellular permeabilization, two 10 min washes in 0.1% PBS Triton® X-100 (Applichem, Germany) were performed. In order to reduce fixative-induced fluorescence, slides were washed six times for 10 min each in Sodium Borohydride ReagentPlus® (Sigma-Aldrich, USA) (1mg/ml) on ice. To prevent unspecific binding of the antibodies to non-target structures and minimize false-positive signals, slides were incubated for one hour in a dark wet chamber at RT with blocking solution: 5% Bovine Serum Albumin (BSA) in PBSW (PBS with 0,1% Tween® 20; VWR chemicals, USA).

Subsequently, slides were incubated for one hour with primary antibodies diluted in blocking solution (Table 3.2) in a dark wet chamber at RT. After incubation, slides were washed in PBSW five times for 10 min, and incubated with secondary antibodies diluted at 1:300 in blocking solution (Table 3.2). After one hour of incubation in wet chamber at RT, slides were washed in the dark twice in PBSW and once with PBS (10 min each). Nuclei were counterstained with DAPI® (4', 6'-diamidino-2-phenylindole, dihydrochloride, Sigma-Aldrich, USA). For this, slides were incubated with a 0.15% DAPI® solution and washed twice in PBS. These five-min incubations were performed in the dark. To reduce tissue natural autofluorescence slides were incubated with 0.3% Sudan Black B (Merck, Germany) in 70% EtOH (VWR chemicals, USA) for 10 min in the dark. Slides were then washed eight times in PBS. Finally, slides were mounted with Mowiol® (Calbiochem, USA), sealed with nail polish and stored at 4°C (protected from light) until further examination.

Fluorescent immunostained samples were examined under a Leica DMR fluorescence microscope with Leica PL Fluotar 10x, HC PL Fluotar 20X and HCX PL Fluotar 40X/0.75 NA dry objectives (Leica, Heidelberg, Germany) available at *Faculdade de Medicina Veterinária (FMV)*. Images were processed using Adobe Photoshop. For microvascular density quantification, the percentage of PECAM fluorescence area was averaged from the analysis of 6-18 plaque image fields (20X magnification) per group. Pixel intensity measurements were determined by the percentage of black pixels per field (after transforming the RGB images into binary files) using NIH ImageJ 1.50b program (NIH, Bethesda, USA, [85]).

Autofluorescence is largely due to natural tissue fluorescence or to fixative-induced fluorescence [86,87]. The natural autofluorescence found in the atherosclerotic plaque samples results mainly from the presence of elastin and collagen in blood vessel walls, and lipofuscin in red blood cells [87]. In order to reduce tissue autofluorescence, five different protocols were tested before the election of the one described above.

Protocol 1: Blocking for one hour at RT with 5% BSA + 0.3M Glycine in PBSW, before primary antibody incubation (adapted from [87])

Protocol 2: 0.25% ammonia (Merck, Germany) in 70% ethanol (VWR chemicals, USA), one hour at RT, followed by three washes of five minutes each in PBS solution with 0.02% Tween® 20, before primary antibody incubation [86].

Protocol 3: 0.1% Sudan Black B (Merck, Germany) in 70% ethanol (VWR chemicals, USA) for 20 min at RT and in a dark chamber, followed by three washes in PBSW for five min, after secondary antibody incubation [86].

Protocol 4: 1 mg/ml solution of Sodium Borohydride ReagentPlus® (Sigma-Aldrich, USA) in PBS (prepared on ice immediately before use) six times for 10 min each, before blocking [87,88].

Table 3.2. Antibodies used in immunofluorescence assay**.**

Primary Antibodies	Dilution	Staining	Secondary Antibodies	Dilution
Mouse anti-human CD31 antibody (Dako, USA)	1:40	Fully differentiated vascular endothelial cells [38]	Alexa Fluor® 594 Goat anti-mouse (Thermo Scientific, USA)	1:300
Goat anti-human DLL4 (Dako, USA)	1:10	DLL4	Alexa Fluor® 594 Chicken anti-goat (Thermo Scientific, USA)	1:300
Mouse anti-human CD34 (Dako, USA)	1:50	Both progenitor and mature endothelial cells [38]	Alexa Fluor® 488 Donkey anti-mouse (Thermo Scientific, USA)	1:300
Rabbit anti-human NG2 (Dako, USA)	1:200	Pericytes [34,45]	Alexa Fluor® 488 Goat anti-rabbit (Thermo Scientific, USA)	1:300
Rabbit anti-human NOTCH1 (Abcam, UK)	1:50	Endothelial cells [89]	Alexa Fluor® 488 Goat anti-rabbit (Thermo Scientific, USA)	1:300

3.5 Statistical analysis

Data processing was carried out using the Statistical Package for the Social Sciences software, version 22.0 (SPSS v 22.0; Chicago, IL). Statistical analyses were performed using Mann-Whitney-Wilcoxon test. All results are presented as mean ± SEM, P-values < 0.05 were considered significant (indicated in the figures with *).

4 RESULTS

4.1 Classification of atherosclerotic lesions

Histological specimens of carotid samples were analyzed on the basis of the classification scheme of the AHA previously described (Fig. 1.3). From the 122 samples, two samples were classified as lesion type II, four as type III, five as type IV, 21 as type V and 90 as type VI. Type I, VII and VIII lesions were not found. In order to have equal number of sample (n=5) in each group reflecting the progression of the disease, four groups were established: group 1 with type II and type III lesions, group 2 with type IV lesions, group 3 with type V lesions, and group 4 with type VI lesions. Some features of atherosclerotic plaques of type III and VI lesions are shown in Figure 4.1, according to previous observations [90].

All the images shown in Figures 4.6, 4.8-4.10 were taken in regions similar to Fig. 4.1H.

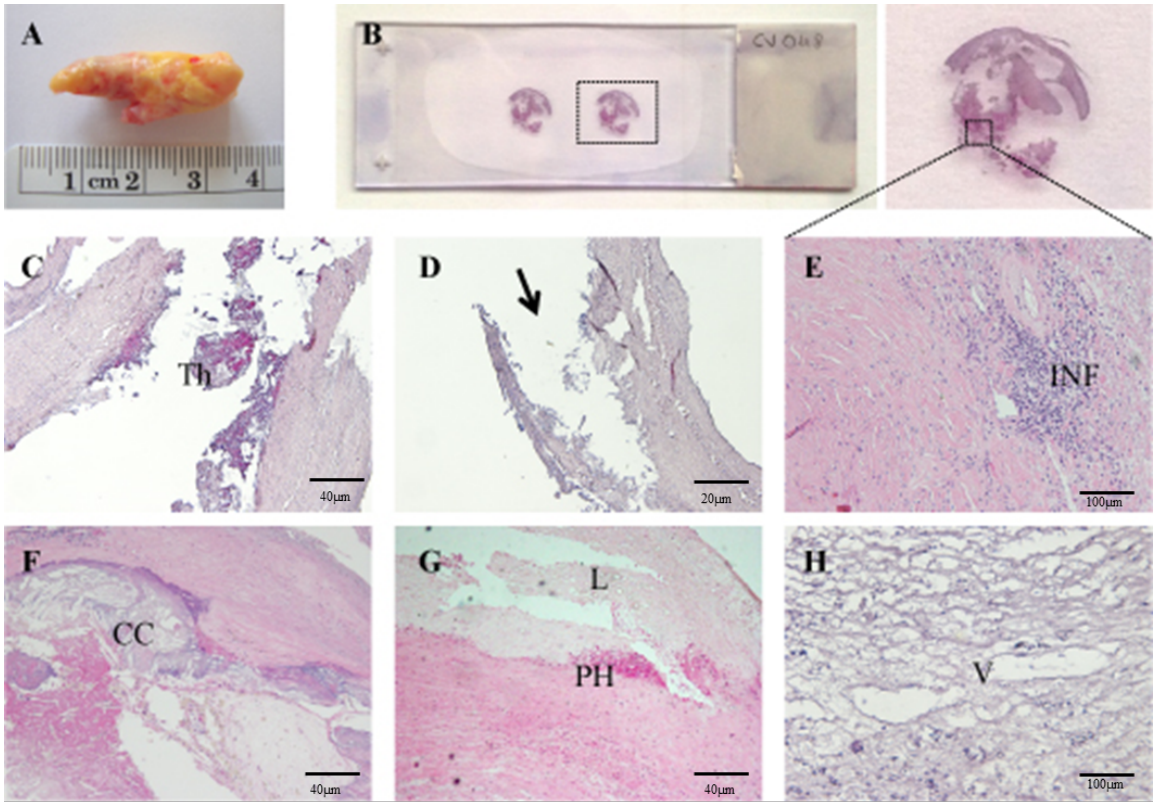

Figure 4.1. A. Atherosclerotic plaques removed from carotid artery at 95% stenosis; **B.** Section of human carotid sample; **C-H**. Hematoxilin and Eosin staining of atherosclerotic plaques of type VI (**C-F**) and type III (**G,H**). **Th**. Thrombus; **INF**. Inflammatory infiltrate; **CC**. Cholesterol crystals; **PH**. Plaque hemorrhage; **L**. extracellular lipids; **V**. Vessels. Arrow in **D** - plaque fissure.

4.2 Immunofluorescence method optimization

4.2.1 Protocol testing to reduce autofluorescence

In order to optimize the immunofluorescence protocol (adapted from [91]) for human atherosclerotic tissue sections, slides from samples not included in the four experimental groups were used and tested. The presence of nonspecific fluorescent signal both in negative control slides incubated only with secondary antibody and in unstained slides (Figure 4.2) lead us to test different protocols known to eliminate or minimize tissue autofluorescence (Figure 4.3).

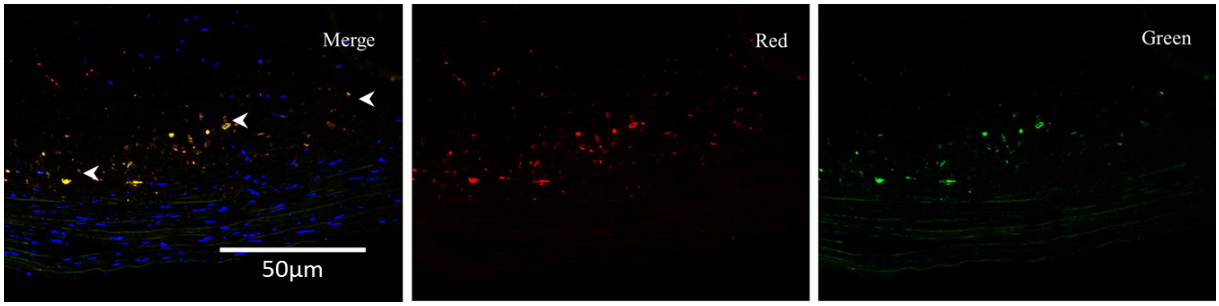

Figure 4.2. Autofluorescence of unstained atheroma sections (white arrowhead) in both red and green channels. Nuclei were stained with DAPI (blue).

Protocol 1 (blocking with 5% BSA + 0.3M Glycine in PBSW) and protocol 2 (ammonia-ethanol) did not significantly decrease autofluorescence (Fig. 4.3B and C). In addition, using protocol 3 (Sudan Black) and protocol 4 (sodium borohydride), autofluorescence was reduced but was still visible (Fig. 4.3D and E).

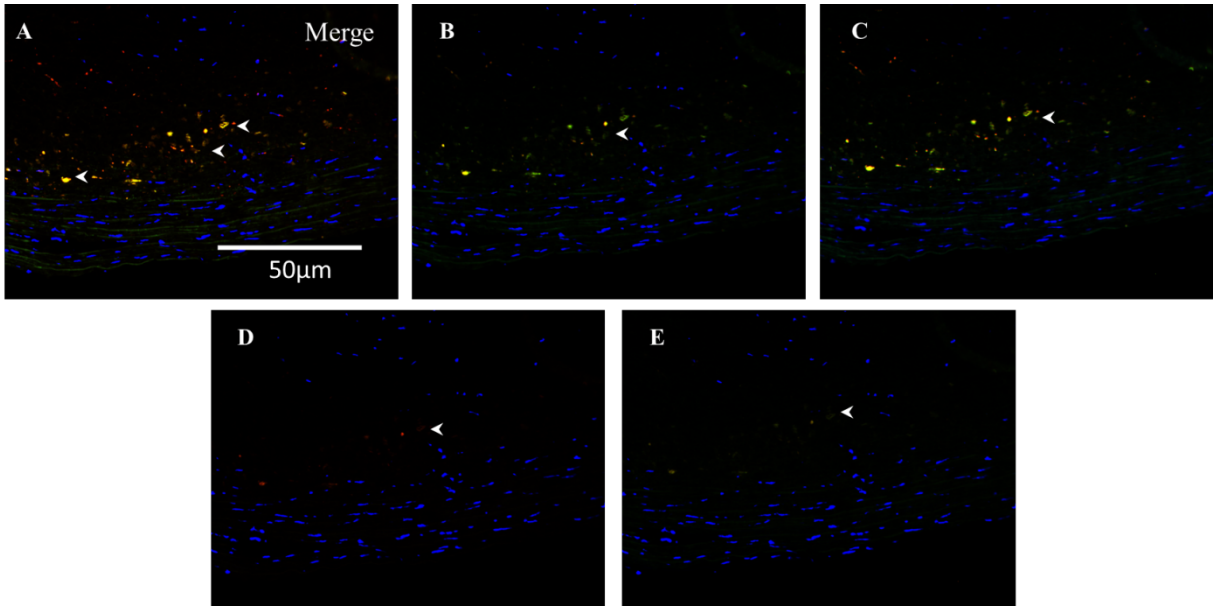

Figure 4.3. Atheroma tissue sections subject to different protocols to remove autofluorescence. **A**. Unstained section; **B**. Blocking with 5% BSA + 0.3M Glycine in PBSW (Protocol 1); **C.** Ammonia-ethanol (Protocol 2); **D**. Sudan Black (Protocol 3); **E**. Sodium borohydride (Protocol 4). Arrowhead - autofluorescence regions. Nuclei were stained with DAPI (blue).

Since the two later procedures lead to better results, a protocol that included both steps was tested. The results showed an almost complete absence of tissue autofluorescence (Figure 4.4). Therefore, our final immunofluorescence protocol included incubation with Sudan Black and washes with sodium borohydride.

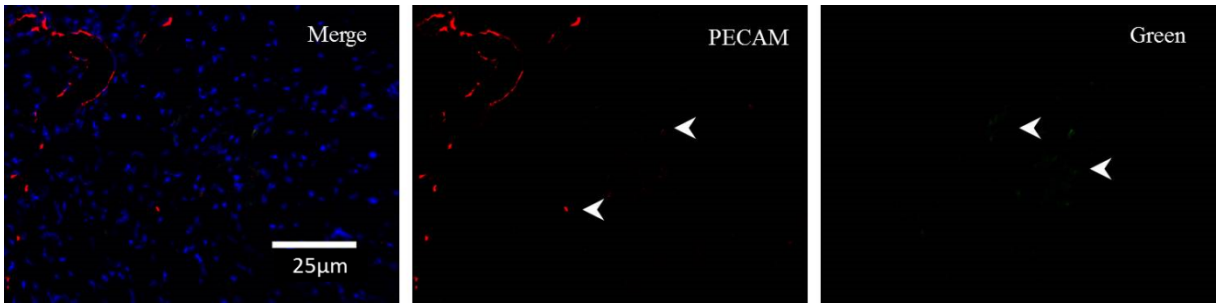

Figure 4.4. Immunofluorescence staining for PECAM/CD31 (red; endothelial cells) in atheroma sections using protocols 3 and 4. Autofluorescence (white arrowhead) was almost completely absent. Nuclei were stained with DAPI (blue).

4.2.2 Positive control for expression analysis (human placenta)

Placental tissue sections were used as positive controls for the detection of our epitopes of interest. Immunofluorescence tests were performed in order to evaluate whether a particular primary antibody was correctly labeling the expected cells as well as for determining the optimal antibody concentration. Double staining for DLL4 with CD34 (Figure 4.5A), NG2 (Fig.4.5B) or NOTCH1 (Fig.4.5C) were tested. As expected, the ligand DLL4 and the receptor NOTCH1 are detected in the placenta endothelial cells, among other cell types [92].

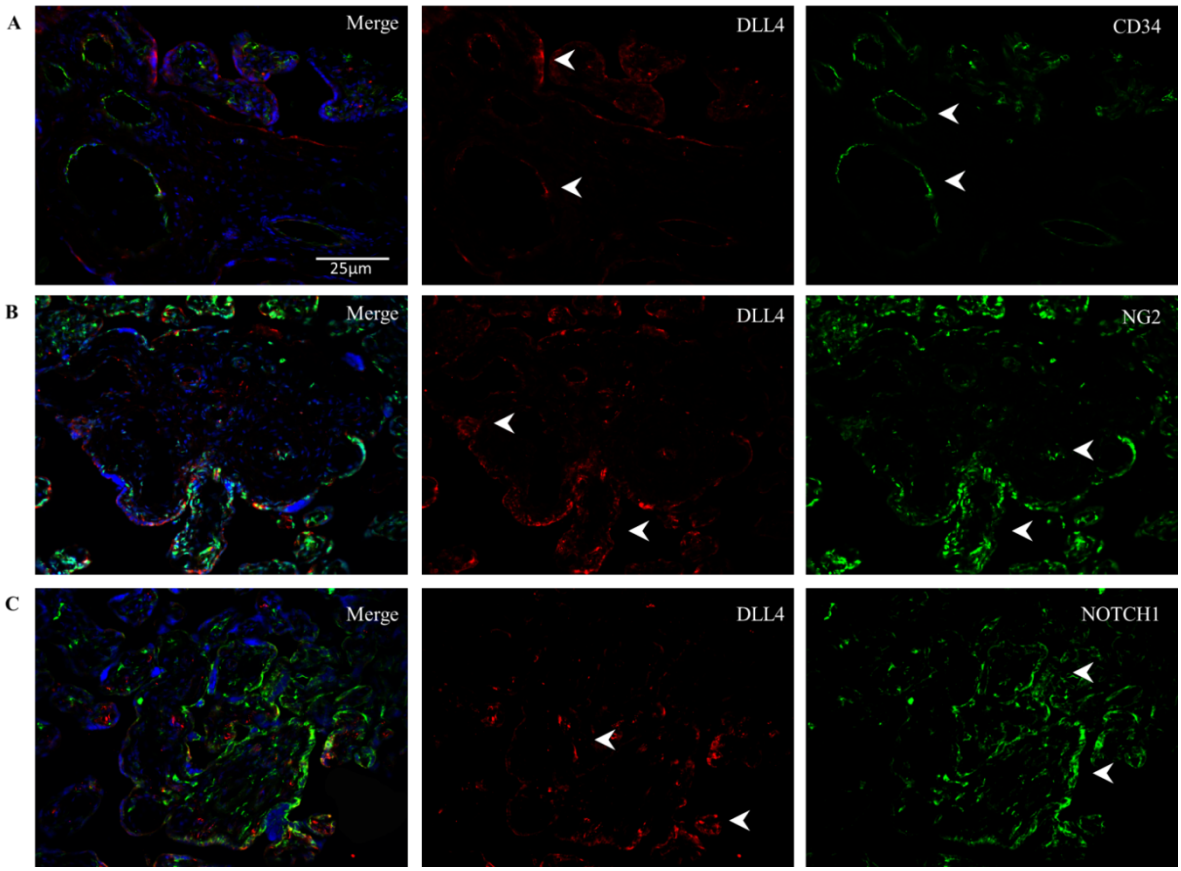

Figure 4.5. Immunofluorescent staining of placental tissue sections. **A**. DLL4 (red) and CD34 (green); **B.** DLL4 (red) and NG2 (green); **C**. DLL4 (red) and NOTCH1 (green). CD34 and Notch 1 are present in endothelial cells, NG2 in pericytes, and CD68 in macrophages and foam cells. Nuclei were stained with DAPI (blue).

4.3 Expression analysis of atheroma sections by immunofluorescence

4.3.1 Microvessel density evaluation

To characterize and quantify the microvascular network in human lesions, the expression of the vascular marker PECAM was analyzed [34]. In sections from all lesion stages analyzed, PECAM expression was detected in endothelial cells of the plaque microvasculature (Figure 4.6). Microvessel density (MVD) was defined as the percentage of PECAM-positive area per image field (see Materials and Methods section 3.4). Groups 2, 3 and 4 showed higher MVD when compared to group 1 (Figure 4.7; group 1=1.7%±1.2, group 2=2.5%±1, group 3 =2.3%±1.8, group 4 =2.5%±1.4;). Therefore, it is possible to conclude that lesions from more advanced stages of the disease have higher neovascularization, as previously shown [23,93,94].

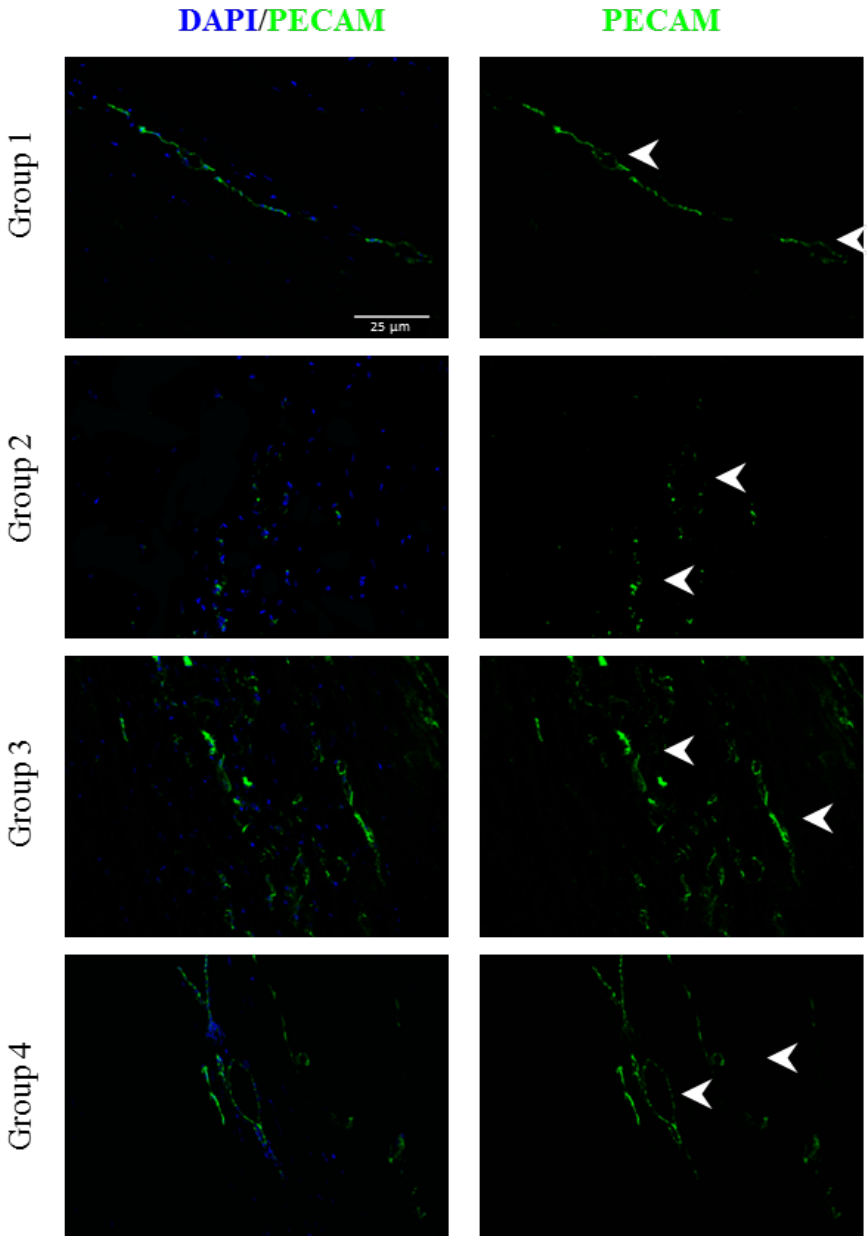

Figure 4.6. Immunostaining for the vascular marker PECAM (green) in atherosclerotic plaque sections. Images are representative of each group of lesion stages. Nuclei were stained with DAPI (blue).

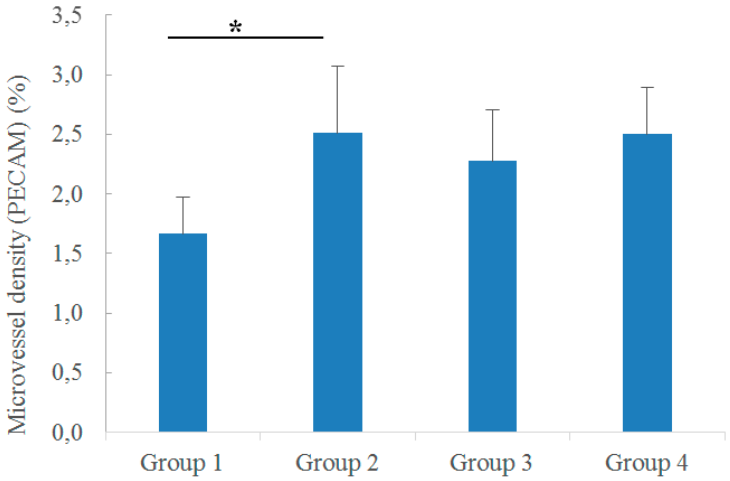

Figure 4.7. Microvessel density (MVD) in each group of lesion stages. MVD was higher in groups 2, 3 and 4 when compared to group 1. For the quantification, the percentage of PECAM area *per* plaque image field (20x magnification) was analyzed in sections from each group of lesions.

4.3.2 Pericyte coverage

In order to evaluate the vascular maturity of atherosclerotic lesions, pericyte coverage of microvessels was analyzed in sections immunolabeled for PECAM (endothelial marker) and NG2 (pericyte marker) [76]. As shown in Figure 4.8, group 1 microvessels exhibited a high maturation state, as indicated by the abundance of pericyte present, whereas group 4 showed vessel immaturity with complete absence of NG2 labeling. Previous studies have shown that microvessels in human coronary atherosclerotic samples have incomplete mural cell coverage [95,96], but none of them examined different stages of lesion progression.

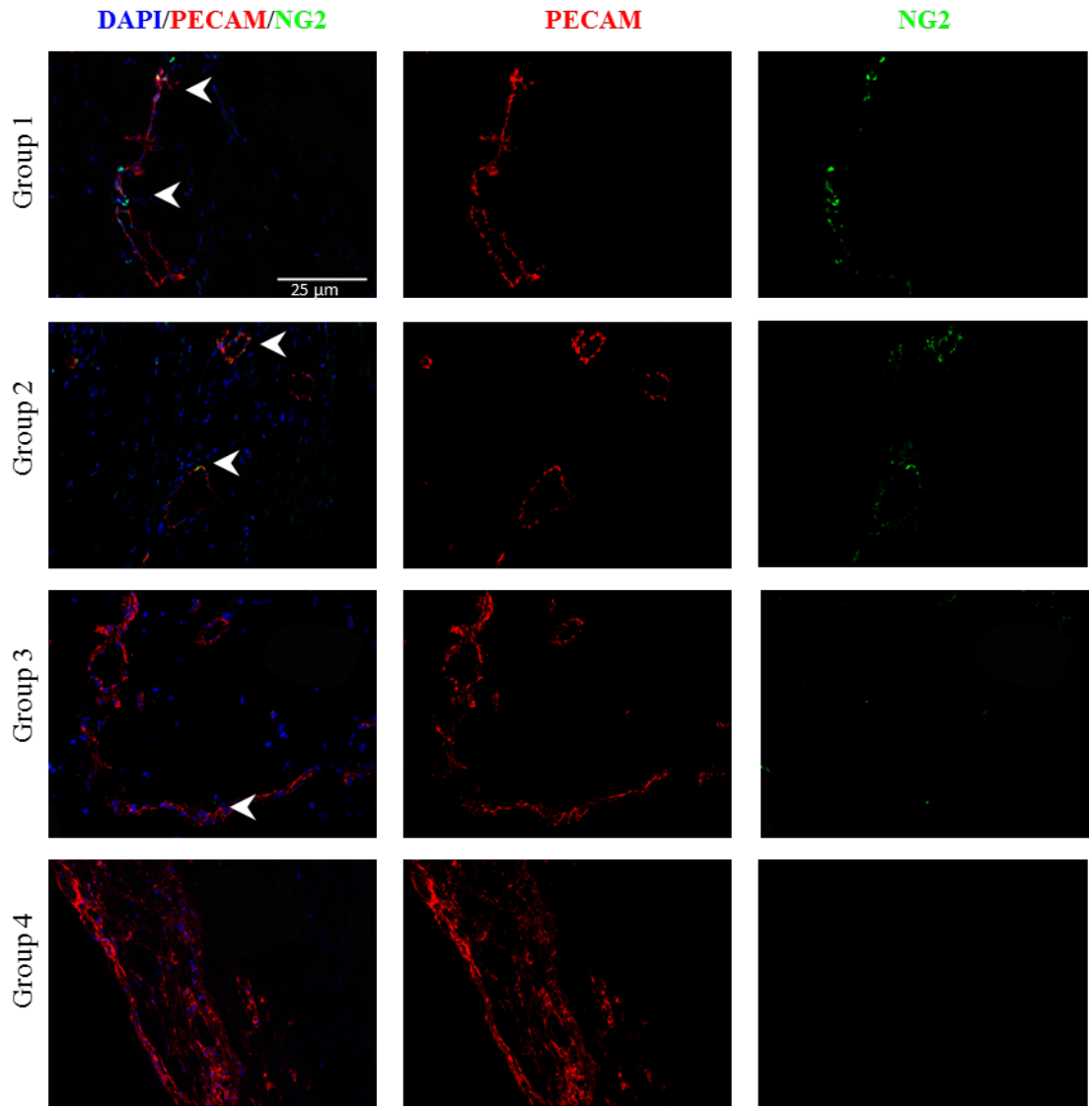

Figure 4.8. Immunostaining of atherosclerotic plaque sections labeled for PECAM (red) and NG2 (green) to evaluate pericyte coverage. Images are representative of each group of lesion stages. Nuclei were stained with DAPI (blue).

4.3.3 DLL4/NOTCH expression

In order to study the expression of DLL4/Notch signaling components in human atherosclerotic lesions, immunolabeling of DLL4 and NOTCH1 was analyzed in atheroma sections. DLL4 expression was detected in low levels in group 1 lesions and at higher levels in those of groups 2, 3 and 4 (Figure 4.9). NOTCH1 expression was observed to be relatively uniform in all groups (representative image in Figure 4.10). These observations indicate that the number of ECs expressing the ligand DLL4 increases with the angiogenesis observed during disease progression, and that the expression of the receptor NOTCH1 is also detected in ECs of immature microvessels of atheroma lesions.

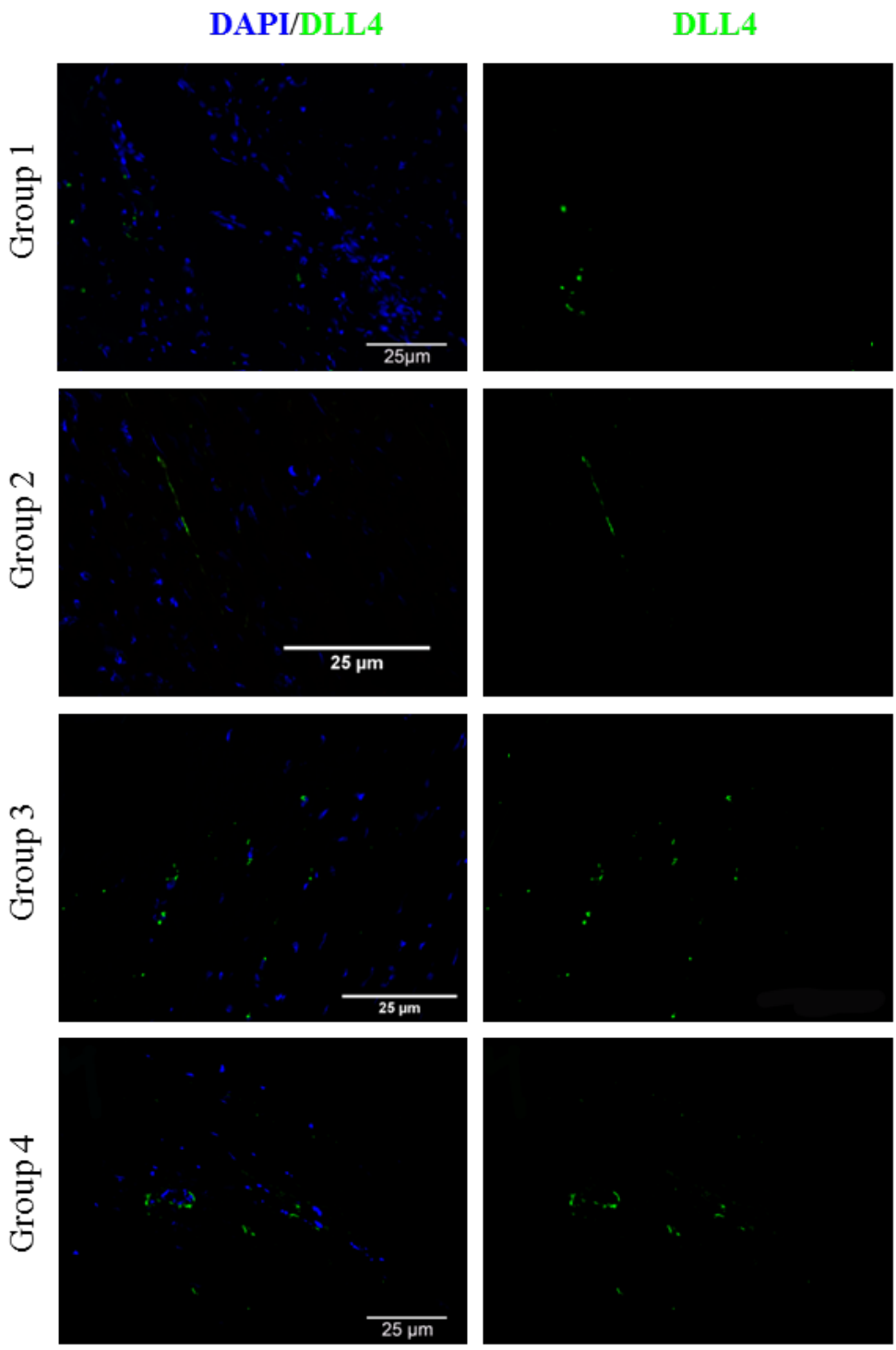

Figure 4.9. Immunostaining for DLL4 (green) in sections from atherosclerotic plaques of each group. Images are representative of each group of lesions stages. Nuclei were stained with DAPI (blue).

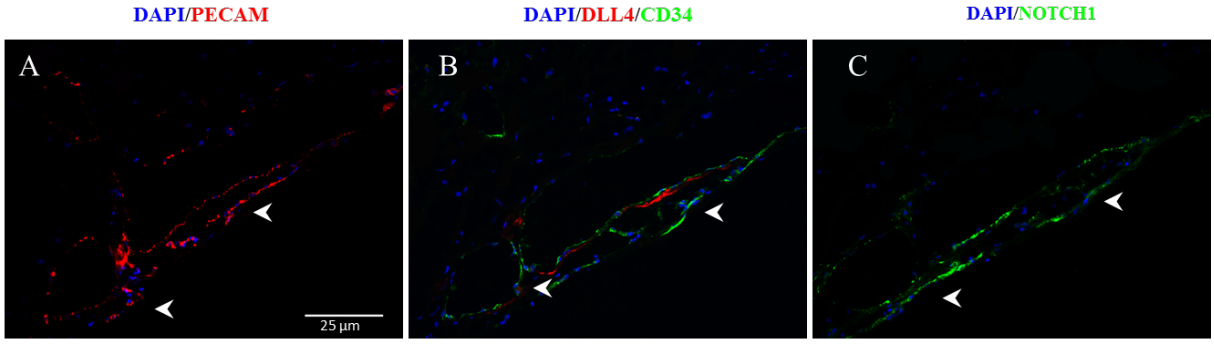

Figure 4.10. Immunostaining of twin sections from an atherosclerotic plaque of group 3, marked for PECAM (**A**), DLL4 (**B**, red), CD34 (**B, green**) and NOTCH1 (**C**). Nuclei were stained with DAPI (blue).

4.4 RT-qPCR protocol optimization

As complement to the immunolabeling results, we aimed at analyzing the mRNA expression levels of Dll4/Notch pathway components and vessel maturation markers in human atherosclerotic plaques by semi-quantitative real time polymerase chain reaction (RT-qPCR). In particular, we addressed the expression of 18 genes involved in the Notch signalling pathway and inflammatory response *DLL4, JAG1, NOTCH1, HEY1* and *HEY2* (ligands, receptor and target genes of the NOTCH signaling pathway [68]), *VEGF, VEGFR1* and *VEGFR2* (components of the VEGF signaling pathway [55]), *ANG1, ANG2* and *TIE2* (components of the angiopoietin signaling pathway [59]), PDGFRβ (receptor of the PDGF signaling pathway [62]), *CD31* (fully differentiated vascular ECs [38]) and *CD34* (both progenitor and mature ECs [38]), *α-SMA* and *CALP* (perivascular cells [34,44]), and *CD68* (macrophages and foam cells [22]), and the housekeeping gene *18S* [97].

In the first RT-qPCR assays, the amplification of some of the genes (*VEGF, HEY2, VEGFR2, ANG1, ANG2* and *TIE2*) showed normal fluorescence signal and melting curves (only one peak; Figure 4.11), whereas the reaction for other genes (*18S, PECAM, DLL4, α-SMA, NOTCH1, JAG1, VEGFR2, CALPONIN, EPHB2, HEY1, PDGFRβ, CD34* and *CD68*) displayed melting curves that indicate the presence of nonspecific products and primer-dimers (more than one peak; Figure 4.12).

The total fluorescence generated by SYBR Green binding to double-stranded DNA can be plotted in real time as a function of temperature. The change in fluorescence accompanies the melting of double-stranded PCR products. The melting temperature (T_m) of each product is defined as the temperature at which the corresponding peak maximum occurs, and then a sharp decrease in SYBR Green fluorescence is observed as the product undergoes denaturation. This analysis can confirm the specificity of the primers as well as reveal the presence of primer-dimers. Because of their small size, primer-dimers usually melt at lower temperatures than the desired product. Furthermore, nonspecific amplification may result in PCR products that melt at temperatures above or below the desire product [98].

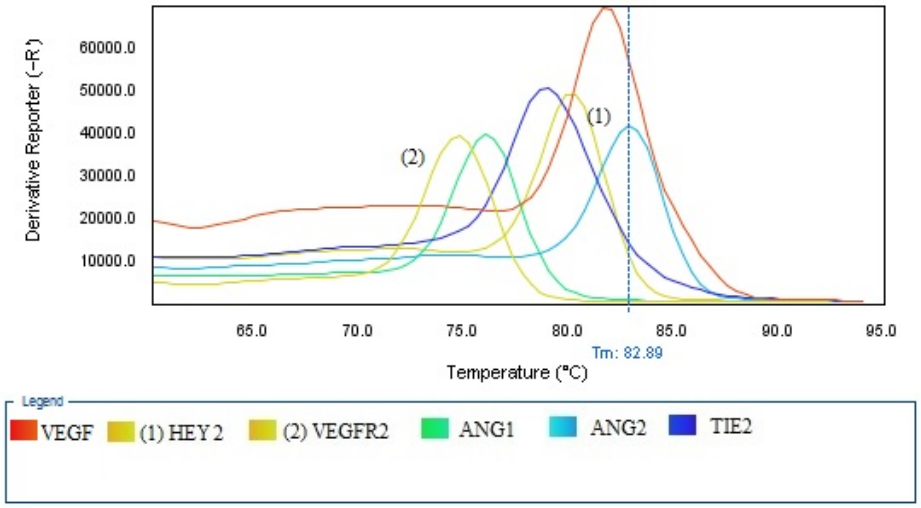

Figure 4.11. RT-qPCR melting curves for the amplification of *VEGF, HEY2, VEGFR2, ANG1*, *ANG2* and *TIE2* showing specific product peaks.

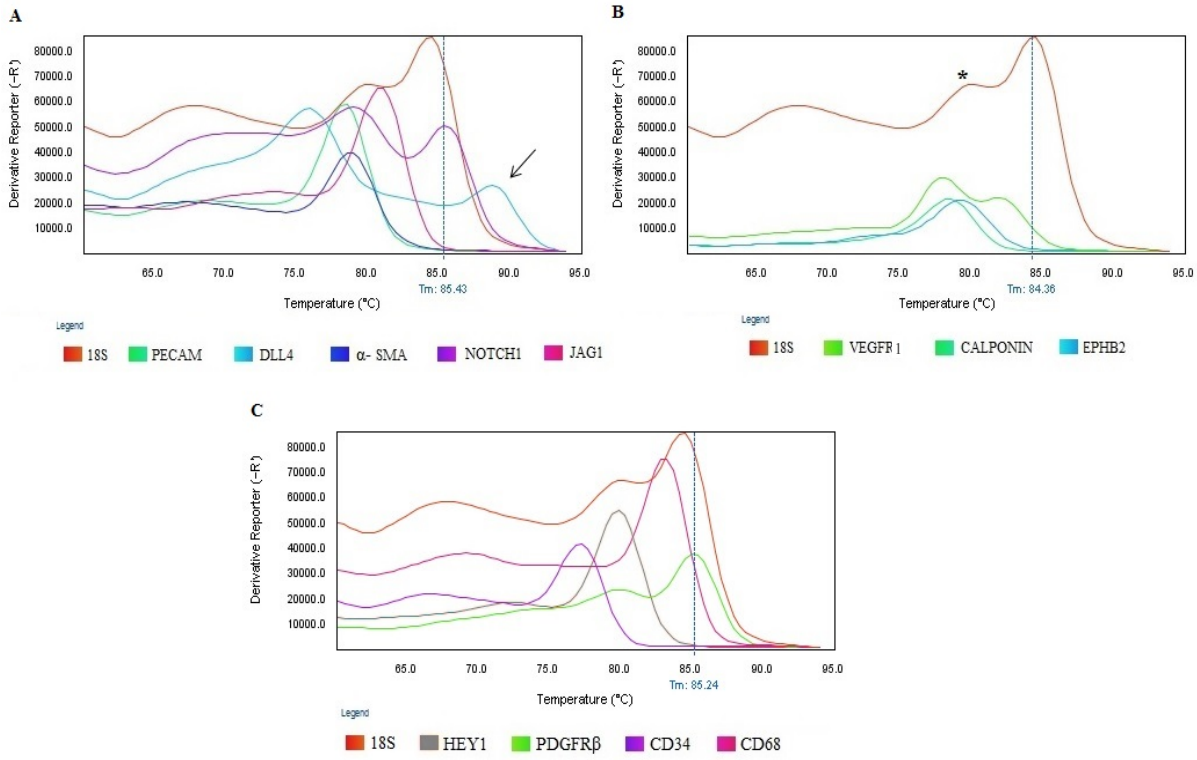

Figure 4.12. RT-qPCR melting curves for the amplification of *PECAM*, *DLL4*, *α-SMA*, *NOTCH1*, *JAG1* (**A**), *VEGFR2*, *CALPONIN, EPHB2* (**B**), *HEY1*, *PDGFRβ*, *CD34* and *CD68* showing primer-dimers or nonspecific product peaks. All the graphs show the melting curve for the amplification of the housekeeping gene 18S for comparison. Asterisk (B) – Primer-dimer; arrow (C) – nonspecific product peak.

Since one of the main goals of the project was to analyze the expression of *DLL4*, an attempt has been made to optimize the RT-qPCR conditions for the amplification of this gene product (and for the housekeeping gene *18S*), prior to the assay optimization for all the other genes. The RT-qPCR

melting curve for *DLL4* amplification indicated the presence of primer-dimer and/or nonspecific products (Fig. 4.12A; light blue), which may be due to several different factors [98]. Therefore, the following conditions were tested: (i) use new and fresh reagents to eliminate the possibility of contamination with human DNA, (ii) test two other pairs of primers for *DLL4* amplification (DLL4new and DLL4 160bp; Figure 4.13A), (iii) increase template concentration, (iv) optimize primer concentration, (v) increase the annealing temperature and decrease the number of cycles. Several tests with both placental and atheroma cDNA were conducted.

In order to investigate these potential causes for the RT-qPCR results, placenta and atheroma cDNA samples were tested by PCR using the different Dll4 primers (Fig. 4.13). Since the PCR reactions using Dll4 and Dll4new primers were not successful, only the gel electrophoresis of the PCR using Dll4 160bp primers is shown (Fig. 4.13B). The presence of primer-dimers was identified in the PCR reactions of both placenta and atheroma cDNA samples. In addition to the expected 160bp product, a genomic DNA product (1270bp) was detected in placenta PCR reactions. However, no *DLL4* amplification products were observed in atheroma reactions, suggesting that the amount of *DLL4* cDNA may be too low in these atheroma samples.

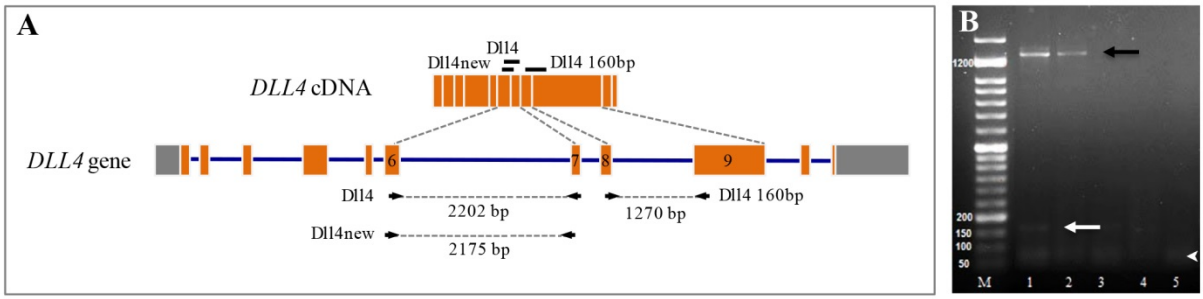

Figure 4.13. A. Schematic representation of human DLL4 cDNA and genomic structure, indicating the localization of the three primer pairs tested in RT-qPCR and PCR (Dll4, Dll4new and Dll4 160bp). **B.** Gel electrophoresis of PCR products for DLL4 using the Dll4 160bp primers. The expected cDNA amplification product is detected in placenta samples (lanes 1 and 2; 160 bp; white arrow), but not in atheroma samples (lanes 3 and 4). A genomic DLL4 fragment was also amplified in the placenta samples (1270bp; black arrow). Possible primer-dimer complexes are observed in all PCR reactions (arrowhead). M – DNA Ladder (50-1500 bp); lane 5 – no template control.

Even though several protocol modifications for minimizing primer-dimer and nonspecific product amplification were tested (data not shown), the RT-qPCR melting curves for *DLL4* amplification could not be improved. These poor results could be due to low mRNA/cDNA concentration, poor primer design and/or the presence of genomic DNA in the cDNA samples. Unfortunately, because of time and resources restrains, the gene expression analysis by RT-qPCR of the genes involved in the Notch signalling pathway and inflammatory response in human atherosclerotic plaques could not be optimized and concluded.

5 DISCUSSION

Several studies have implicated atheroma microvessels (or *vasa vasorum*) in the regulation of atherosclerotic plaque growth and stability [22,23,46,94,99,100]. In line with this evidence, induction of atheroma *vasa vasorum* maturation has recently been proposed as a therapeutic strategy to prevent intraplaque hemorrhage and restrict lesion growth. Our team and others have implicated the Dll4/Notch signaling pathway in the regulation of microvessel maturation, which introduces Dll4 as a potential target for atherosclerosis treatment. The aim of this study was to analyze the expression patterns of DLL4 and other components of the NOTCH signaling pathway in human atherosclerotic lesions, and to correlate these patterns with the level of *vasa vasorum* maturation and phase of lesion progression.

In order to achieve our goal, we aimed at evaluating atheroma structure and type of atherosclerotic lesion (progression stage), as well as analyzing the expression of DLL4/Notch pathway components in human carotid atheroma samples, both by immunolabeling and by real time polymerase chain reaction (RT-qPCR). We started by optimizing the immunofluorescence protocol for human atherosclerotic tissue sections. By observing unlabeled sections, we were able to detect tissue autofluorescence, which has also been documented by other studies (*e.g.*, [101]). Autofluorescence is mainly due to natural tissue fluorescence or to fixative-induced fluorescence (aldehyde fixatives) [86,87]. The natural autofluorescence found in atherosclerotic plaque samples results mainly from the presence of elastin and collagen in blood vessel walls, and lipofuscin in red blood cells [87]. To minimize this issue, we tested different protocols using sections from samples not included in the experimental groups. We concluded that a protocol comprising incubation with Sudan Black and washes with sodium borohydride lead to a significant reduction of autofluorescence without decreasing the ability to visualize specific labeling. Previous studies demonstrated that sodium borohydride is an aldehyde blocker, decreasing fixative-induced fluorescence [87]. In turn, Sudan Black is thought to act by obscuring lipofuscin [86,102]. Using these two reagents to minimize autofluorescence, we were able to proceed with the experimental immunolabeling assays.

To evaluate atheroma structure and stage of the atherosclerotic lesions, we classified our samples according to the AHA consensus [31–33] and sorted them into four groups that reflect different phases of lesion progression. Some features documented in previous studies [22,23,94,103] were identified in advanced lesions, such as the presence of microvessels, thrombus, fissures and hematomas. Samples from each group were then used to investigate the expression levels of DLL4/Notch components and microvessel maturation markers.

In order characterize and quantify the neovascular network of human atheroma lesions, visualization of newly formed microvessels in human plaque sections was achieved through immunofluorescence staining of histological sections with the commonly used vascular marker PECAM [34]. Regarding the results, it was shown that microvessels are already detected in early lesions, and their density is higher in more advanced lesions, which in accordance with other studies [23,93,94]. This process of angiogenesis occurs as a primary response to hypoxia of the inner layers of the vascular wall, as a consequence of *tunica intima* hyperplasia[64].

In order to evaluate intraplaque neovessels maturation, pericyte coverage of microvessels was analyzed. It was found that the neovascular network of human advanced lesions has less pericyte coverage than early lesions. Previous studies have shown that microvessels in human coronary

atherosclerotic samples have incomplete mural cell coverage [95,96], but none of them examined different stages of lesion progression. Microvessel leakage is determined by microvessel structure and endothelial integrity [95]. Newly formed blood vessels with less pericyte coverage are immature, fragile, leaky and lead to local extravasation of plasma proteins, erythrocytes (hemorrhage) and inflammatory cells, further contributing to plaque progression [22,50]. Our results show that the microvessels of advanced lesions are indeed more immature than those of early-stage lesions, in agreement with a higher risk for plaque hemorrhage and rupture in late stages of lesion progression.

To the best of our knowledge, this is the first study that analyzes the expression of DLL4/NOTCH1 in *vasa vasorum* of human atherosclerotic lesions in relation to lesion progression. The results showed that DLL4 is expressed in *vasa vasorum* ECs of atherosclerotic lesions, being present in low levels in early lesions and in higher levels in more advanced plaques. Although it did not present significant correlation with lesion progression, it was also possible to detect the presence of NOTCH1 receptor in ECs of immature microvessels in advanced lesions. Our observations indicate that the number of endothelial tip cells expressing the ligand DLL4 increases as immature microvessels proliferate with the progression of the disease, and that these cells also express the NOTCH1 receptor, suggesting that the Notch pathway is activated in ECs of atherosclerotic lesions. In concordance, previous studies in mice [104,105] and also in human [105] demonstrated that Notch1 and DLL4 expression is significantly higher in atherosclerotic lesions when compared to non-atherosclerotic sites.

The RT-qPCR analysis would provide information on the levels of the Notch, VEGF and angiopoietin signaling pathways, as well as other components involved in angiogenesis, inflammation and vessel maturation, in each phase of lesion progression. In our RT-qPCR experiments, nonspecific product amplification was observed, even when using several protocol modifications for minimizing primer-dimer and nonspecific product amplification. This was one of the major limitations of our work. The RNA samples from human carotid plaques supplied by our collaborators were not subject to DNAse treatment to remove genomic DNA contamination. The poor results obtaind might also have been due to low mRNA/cDNA concentration and/or to poor primer design. Unfortunately, because of time and resources restrains, the gene expression analysis of human atherosclerotic plaques by RT-qPCR could not be optimized and completed. To try to overcome this limitation, we would have ordered and used new primers for the amplification of housekeeping and experimental genes. In addition, the use of DNAse-treated RNA samples, as well as higher RNA concentrations would have been taken into account.

In conclusion, this study demonstrated that more advanced lesions present more microvessels and these have less pericyte coverage. The stabilization or “normalization” of the immature microvasculature of atherosclerotic lesions has been proposed as an approach to prevent intraplaque hemorrhage and restrict lesion growth [66,46]. In addition, normalized vessels would allow for better perfusion of therapeutic agents into the plaque [65]. Dll4 has been described as the major inducer of Notch activation in ECs [106], being a critical regulator of angiogenesis and vessel maturation [73,106]. Our studies demonstrated that DLL4 and NOTCH proteins are expressed in human plaque *vasa vasorum*. These observations introduce the Dll4/Notch signaling pathway as a potential regulator of *vasa vasorum* maturation in atherosclerotic lesions. Taken together, our research contributed to increase the level of knowledge regarding the molecular regulators of angiogenesis in human atherosclerotic lesions, clarify the relation between *vasa vasorum* maturation and plaque progression, and provide support to the potential therapeutic application of Dll4/Notch signaling modulators for the stabilization of atherosclerotic lesions.

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7 ANNEXES

Annex I - Statement of Informed Consent

FORM

Instituto de Medicina Molecular Lisboa

DECLARAÇÃO DE CONSENTIMENTO INFORMADO

INFORMAÇÃO AO DADOR DE AMOSTRAS BIOLÓGICAS

Título do projecto de investigação
Biobanco do Instituto de Medicina Molecular (IMM) da Faculdade de Medicina da Universidade de Lisboa - Banco de amostras biológicas Humanas para fins de investigação biomédica

Objectivo do Estudo
A criação de um banco de amostras biológicas humanas permitirá o desenvolvimento de ferramentas de diagnóstico e investigação em múltiplas áreas da medicina, com especial impacto em doenças crónicas, como as doenças oncológicas, cardiovasculares, neurológicas, ósseas e imunológicas. Contudo, este objectivo só será cumprido com a colaboração dos doentes e de indivíduos saudáveis, através da doação de amostras biológicas que serão guardadas e preservadas em condições apropriadas de forma a serem utilizadas para futuros estudos. Caso o doente ou indivíduo saudável e/ou o seu representante legal decida participar, terá de fazer apenas os procedimentos habituais de uma consulta.

Procedimentos
No caso de concordar em participar neste projecto, ser-lhe-á colhida uma amostra biológica. A amostra habitualmente solicitada será aproveitada a partir da colheita de sangue e/ou urina que irá efetuar. Para os indivíduos que estejam a realizar exames diagnósticos ou que estejam a ser sujeitos a tratamentos cirúrgicos poderá ser pedida autorização para colheita de uma pequena amostra do material removido durante o procedimento (como por exemplo saliva, liquido cefalo-raquidiano, tecidos removidos para biópsias ou removidos no decurso de cirurgias). Estas colheitas serão efetuadas sem alterar os procedimentos médicos habituais e sem interferir com a rentabilidade diagnóstica do procedimento ou com o sucesso da cirurgia. . Esta amostra será preservada em condições apropriadas e as informações clínicas com ela relacionada serão introduzidas numa base de dados, passando a sua identificação pessoal a estar codificada e não acessível aos utilizadores das amostras.
A doação da amostra é voluntária e revogável, sendo que o dador, ou o seu representante legal, tem o direito de retirar a amostra e/ou interromper a colaboração assim que achar conveniente, sem necessidade de justificação e não podendo ser descriminado por isso. O dador ou o seu representante legal deverá manifestar por escrito a sua vontade em retirar a amostra ou interromper a colaboração e nestas situações a amostra será imediatamente destruída.
O Biobanco do IMM propõe-se armazenar as amostras biológicas e seus possíveis derivados tais como, soro, plasma, DNA, RNA e células. No caso da colheita de sangue ou em qualquer outra circunstância de colheita para a qual seja necessário um acto médico invasivo será adoptada uma técnica de imortalização de células, evitando-se assim nova colheita de amostra.
O biobanco do IMM não divulgará resultados envolvendo o material biológico. No entanto, o dador poderá escolher se quer ser informado dos resultados com potencial relevância para a sua saúde. O pedido de resultados deverá ser feito por escrito para o Biobanco do IMM pelo dador ou representante legal e deve ser expresso no consentimento informado.

Serão cumpridas todas as normas éticas aceites internacionalmente para o uso de matérias biológicos para fins de investigação. Todos os projetos que fizerem uso das amostras depositadas no Biobanco do IMM serão submetidos à Comissão de Ética competente para a sua avaliação.

Identificação das amostras e Confidencialidade
A existência de um biobanco pressupõe a existência de uma base de dados contendo informação clínica referente ao doente ou indivíduo saudável. Após a colheita, as amostras serão identificadas por um código de forma a preservar a privacidade.
Durante o desenvolvimento de um projecto de investigação, a equipa de investigação poderá ter necessidade de recolher informação do processo clínico para a execução do estudo. O anonimato será, contudo mantido, ou seja os dados constantes do seu processo clínico serão fornecidos ao investigador, mas sem qualquer identificação, ou qualquer informação que permita saber a quem pertencem.
A descodificação apenas poderá ser efectuada pelo médico (que será o responsável pela base de dados, de acordo com a informação fornecida à Comissão Nacional de Protecção de Dados - CNPD), em caso de absoluta necessidade, por motivos de saúde do dador e a pedido deste, e sempre de acordo com as disposições legais em vigor.
Os dados serão tratados confidencialmente, de acordo com a Lei, com os regulamentos e de acordo com as normas éticas aprovadas pela Comissão de Ética do CHLN/FMUL e pela CNPD.
Os dados resultantes dos estudos realizados serão alvo de publicação de uma forma anónima e agregada, em termos de percentagens ou de dados numéricos, nunca individualmente.

Tempo de conservação
As amostras serão conservadas por um período de 20 anos no Biobanco do Instituto de Medicina Molecular (IMM) da Faculdade de Medicina da Universidade de Lisboa, sob a responsabilidade da Equipa ligada ao projecto,

enquanto este estiver devidamente credenciado pelas entidades competentes. As coleções de amostras serão avaliadas periodicamente, nomeadamente para aferir da sua qualidade, podendo ser destruídas ou, findo o período da conservação, poder-se-á solicitar a prorrogação da conservação. Nestas condições excecionais o Biobanco do IMM poderá recontactar os dadores.

Comunicação e divulgação de dados

Os dados genéticos e as amostras biológicas colhidas para fins de investigação científica podem ser transferidos para outras organizações ou centros de investigação, para fins de pesquisa e somente em projetos desenvolvidos conjuntamente com o IMM, mediante consentimento do participante expresso na declaração de consentimento informado.

Possíveis Benefícios para os Participantes
Esta é uma doação altruísta, não havendo por isso qualquer compensação para o dador. Não se garante que este estudo envolva quaisquer benefícios directos para o participante. Se algum dos estudos puder ser relevante para a saúde do dador, este será informado, se essa for a sua vontade expressa na declaração de consentimento informado. Contudo, a sua participação proporcionará a aquisição de conhecimentos que poderão vir a beneficiá-lo a si ou a terceiros no futuro.

Riscos físicos previsíveis
Na maioria dos casos, os riscos e o desconforto associados serão mínimos ou inexistentes. Nas colheitas associadas a procedimentos com fins diagnósticos ou terapêuticos, os riscos e o desconforto serão os inerentes ao procedimento em si. Em qualquer dos casos, o dador será sempre antecipadamente informado dos riscos e grau de desconforto associados aos procedimentos.

Participação Voluntária e Direitos de Abandono
O presumível dador terá toda a liberdade para se recusar a participar no estudo ou retirar o seu consentimento, suspendendo a participação em qualquer momento e, consequentemente, as amostras serão destruídas. A participação é voluntária e a sua recusa em participar não envolverá qualquer penalização ou perda de benefícios. A recusa ou abandono não colocarão em risco o direito a receber tratamento ou assistência médica, presentemente ou no futuro.
O dador poderá retirar o seu consentimento nas modalidades **sem contacto futuro** (as amostras poderão ser usadas normalmente até se esgotarem, mas não serão estabelecidos futuros contactos para a obtenção de mais amostras) ou **sem uso futuro** (não serão estabelecidos futuros contactos e as amostras serão imediatamente destruidas e os registos eliminados).

Se tiver qualquer dúvida, em qualquer momento, mesmo após a colheita, sobre este estudo poderá contactar o Director do Biobanco do IMM:
Prof. Doutor Sérgio Dias e Doutor Joaquim Polido Pereira, dirigindo-se a:
Drª Angela Maria Afonso
Biobanco
Instituto de Medicina Molecular
Faculdade de Medicina da Universidade de Lisboa
Telefone: +351 217999437
Ext:47047/92903
Email: immbiobanco@fm.ul.pt

FORM

Instituto de Medicina Molecular Lisboa

DECLARAÇÃO DE CONSENTIMENTO INFORMADO

DECLARAÇÃO DE CONSENTIMENTO INFORMADO

Banco de amostras biológicas para fins de investigação biomédica

Investigador:_____ Hospital: _____
Nome do dador: _____
Número de estudo do dador: _____

Eu, , portador do bilhete de identidade/cartão do cidadão n.º [ ], declaro ter tomado conhecimento e aceitar participar neste projecto, de forma a contribuir para a criação de um banco de amostras biológicas com informação clínica associada, para fins de investigação biomédica.
Aceito que a minha amostra biológica seja utilizada em projectos de investigação de mecanismos das doenças, diagnóstico precoce, fatores de prognóstico e novos alvos terapêuticos em múltiplas áreas da medicina, nomeadamente nas doenças oncológicas, cardiovasculares, neurológicas, ósseas e imunológicas. Poderei revogar a autorização para utilização da minha amostra biológica e informação clínica em qualquer altura.
O objectivo do banco de amostras biológicas foi-me claramente explicado e foi-me dada a oportunidade de colocar questões sobre o seu funcionamento, bem como os procedimentos relativos à colheita e utilização da minha amostra biológica e dados a ela associados.
Declaro que aceito participar, voluntariamente, neste estudo. Especificamente concordo com os seguintes pontos:

- Consinto a colheita de material biológico (sangue / ..... / .....) e autorizo a conservação de amostras no Biobanco, de modo a que possam ser usados para pesquisas futuras, incluindo estudos genéticos e cultura de linhas celulares por investigadores portugueses e estrangeiros, sem fins lucrativos;

Sim ☐ Não ☐

- **Esta opção é para ser respondida apenas por participantes que já cederam amostras biológicas colhidas no âmbito de outros projetos.** Nestas circunstâncias, autorizo a transferência para o Biobanco as minhas amostras biológicas, préviamente colhidas no âmbito de outros projectos, de modo que elas possam ser utilizadas em pesquisas futuras, incluindo estudos genéticos e cultura de linhas celulares por investigadores portugueses e estrangeiros, mas sem fins lucrativos;

Sim ☐ Não ☐

- Estou consciente de que minha participação é voluntária e que posso em qualquer altura solicitar a destruição das minhas amostras biológicas, invalidando assim o consentimento informado prévio, sem justificar, tendo recebido a garantia de que o meu pedido não desenvolverá discriminação;

Sim ☐ Não ☐

- Declaro que quero conhecer resultados que possam ser relevantes para a minha saúde.

Sim ☐ Não ☐

- Autorizo ser contactado novamente pelo Biobanco do IMM para pedido de atualização sobre a minha situação clínica;

Sim ☐ Não ☐

FORM

Instituto de Medicina Molecular Lisboa

DECLARAÇÃO DE CONSENTIMENTO INFORMADO

- Autorizo o contacto do Biobanco do IMM a familiares meus para pedido autorização de colheita de amostras biológicas e/ou informação clínica;

Sim ☐ Não ☐

_____ _____

Data Assinatura do Dador/Representante Legal

Em caso de representante legal, este actua na qualidade de:

□ Titular do poder paternal, quando o dador é menor
□ Tutor, quando o dador foi declarado interdito
□ Herdeiro, quando o dador faleceu

Discuti este estudo de investigação com o participante e/ou o seu representante legal, utilizando uma linguagem compreensível e apropriada. Informei adequadamente o participante sobre a natureza deste estudo e sobre os seus possíveis benefícios e riscos, considerando que o participante compreendeu a minha explicação.

_____ _____ _____

Data Nome do Médico Assinatura do Médico

Foi entregue um duplicado deste documento ao doente/representante legal.

Annex II – Clinical Protocol

INQUÉRITO

ATHEROGENESIS AND OSTEOPOROSIS COMMON UNDERLYING MECHANISMS

CV_____

DADOS EPIDEMIOLÓGICOS DO DOENTE

Nome: _____

NSC: _____ Data de Nascimento: _____/_____/_____

Raça: _____

Sexo: ☐ M ☐ F

Profissão: _____

Escolaridade (nº anos): _____

Contacto: _____

Causa da cirurgia actual: _____

Data da cirurgia actual: _____/_____/_____ **Hora:** ____:____

Local da cirurgia: _____

Cirurgião Vascular assistente: _____

1

RISCO CARDIOVASCULAR

1. Idade: _____

2. PA sistólica (mmHg): _____ PA diastólica (mmHg): _____

3. Tabagismo: ☐ Fumador ☐ Não fumador

4. Colesterol Total (mg/dL): _____

5. HDL (mg/dL): _____

6. LDL (mg/dL): _____

7. Triglicéridos (mg/dL): _____

8. Glicemia em jejum (mg/dL): _____

9. PCR (mg/dL): _____

14. Creatinina (mg/dL): _____

15. Ureia: _____

16. Ácido Úrico: _____

10. Homocisteína: _____

11. Cálcio: _____

12. Vitamina D: _____

13. Coagulação:

APTT (tempo doente / tempo controlo): _____ / _____ seg.

TP (tempo doente / tempo controlo): _____ / _____ seg.

INR: _____

Altura: _____ cm Peso: _____ Kg % massa gorda: _____

IMC: _____ Perímetro abdominal: _____ cm

FRACTURAS PRÉVIAS

Já fez fractura após um traumatismo mínimo ou espontâneas (p.ex queda na rua)?

☐ Não ☐ Sim

☐ Anca ☐ Coluna ☐ Punho ☐ Úmero proximal

Data: _____/_____/_____

Idade: _____ anos

Outras fracturas prévias: ☐ Não ☐ Sim

Idade, ossos que fracturou e descrição da fractura _____

_____

_____

HISTÓRIA FAMILIAR DE OSTEOPOROSE

Algum dos seus pais fracturou o colo do fémur (anca)?

☐ Não sabe ☐ Não ☐ Sim

Qual: ☐ Pai ☐ Mãe

HÁBITOS ALIMENTARES E ESTILO DE VIDA

HÁBITOS ALCOÓLICOS

Consome ou consumiu: ☐ > 3 unidades/dia ☐ < 3 unidades/dia

Entre que idades? _____

HÁBITOS TABÁGICOS

Fuma: ☐ Não ☐ Sim Nº cigarros/dia: _____

Entre que idades? _____

Se ex-fumador: Nº cigarros/dia: _____

Entre que idades? _____

NUTRIÇÃO

Nº copos leite/dia: _____

Nº iogurtes/semana: _____

Nº fatias de queijo/semana: _____

Come regularmente vegetais? ☐ Não ☐ Sim

Come carne ou peixe todos os dias? ☐ Não ☐ Sim

Dieta: ☐ Pouco sal ☐ Sal normal ☐ Salgada

Nº cafés/dia: _____ Nº chávenas chá preto/dia: _____

ACTIVIDADE FÍSICA

Actualmente tem: ☐ Vida sedentária ☐ Vida activa

Prática regular de desporto: ☐ Não ☐ Sim

Nº horas/semana: _____ Tipo de desporto: _____

Já fez actividade física regular? ☐ Não ☐ Sim

Que actividade? _____ Nº horas/semana: _____

Entre que idades? _____

A sua profissão obriga a efectuar esforço físico intenso? ☐ Não ☐ Sim

O doente está ou esteve acamado ou imobilizado por mais de 3 meses?

☐ Não ☐ Sim Qual o motivo? _____

SE MULHER:

Idade menopausa: _____ anos ☐ Espontânea ☐ Cirúrgica

QUEDAS

Queda nos últimos 3 meses ☐ Não ☐ Sim Nº quedas: _____ Como? _____

6 meses ☐ Não ☐ Sim Nº quedas: _____ Como? _____

12 meses ☐ Não ☐ Sim Nº quedas: _____ Como? _____

ANTECEDENTES PESSOAIS

1. Artrite Reumatóide ☐ Não ☐ Sim Idade início: _____ anos

Médico Assistente: _____

2. Lúpus Eritematoso Sistémico ☐ Não ☐ Sim Idade início: _____ anos

Médico Assistente: _____

3. Outras doenças inflamatórias sistémicas crónicas ☐ Não ☐ Sim

Quais? _____ Idade de início: _____ anos

4. HTA ☐ Não ☐ Sim

5. EAM ☐ Não ☐ Sim

6. AVC ☐ Não ☐ Sim

7. Dislipidemia ☐ Não ☐ Sim Qual o tipo? _____

8. Outras doenças cardiovasculares ☐ Não ☐ Sim Qual o tipo? _____

9. Insuficiência renal ☐ Não ☐ Sim Hemodiálise? _____

10. Patologia de tiróide/paratiróide ☐ Não ☐ Sim Qual? _____

11. Outras doenças endócrinas ☐ Não ☐ Sim Qual? _____

12. Má absorção gastro-intestinal (ex: dça Celiaca, fibrose quistica) ☐ Não ☐ Sim

Qual? _____

13. Neoplasias ☐ Não ☐ Sim Qual? _____ Idade início: _____ anos

14. Doença óssea metabólica (osteoporose, doença óssea de Paget) ☐ Não ☐ Sim

Qual? _____ Idade início: _____ anos

15. Outras doenças actuais: ☐ Não ☐ Sim (especificar doença e idade de início)

_____

16. Outras doenças no passado: ☐ Não ☐ Sim (especificar doença e idade de início)

_____

17. Corticoterapia oral por mais de 3 meses: ☐ Não ☐ Sim

Dose máxima diária _____

ANTECEDENTES FAMILIARES

1. Artrite Reumatóide ☐ Não ☐ Não sabe ☐ Sim
2. Lúpus Eritematoso Sistémico ☐ Não ☐ Não sabe ☐ Sim
3. Outra doença reumática ☐ Não ☐ Não sabe ☐ Sim

Qual? _____

5. HTA ☐ Não ☐ Não sabe ☐ Sim
6. EAM ☐ Não ☐ Não sabe ☐ Sim
7. AVC ☐ Não ☐ Não sabe ☐ Sim
8. Dislipidemia ☐ Não ☐ Não sabe ☐ Sim

Qual o tipo? _____

9. Outras doenças cardiovasculares ☐ Não ☐ Não sabe ☐ Sim

Qual? _____

10. Aneurismas ☐ Não ☐ Não sabe ☐ Sim

TERAPÊUTICA ACTUAL

_____

_____

TERAPÊUTICA

TERAPÊUTICA (POR MAIS DE 3 MESES)

	S	N	?	
Glicorticóide				Dose _____ Data Inicio _____ Duração _____ Qual? _____
Anticoagulantes				Dose _____ Data Inicio _____ Duração _____ Qual? _____
Bifosfonato				Dose _____ Data Inicio _____ Duração _____ Qual? _____
SERM				Dose _____ Data Inicio _____ Duração _____
Estrôncio				Dose _____ Data Inicio _____ Duração _____
Teriparatida				Dose _____ Data Inicio _____ Duração _____
Suplemento Ca				Dose _____ Data Inicio _____ Duração _____
Vitamina D				Dose _____ Data Inicio _____ Duração _____
B-bloqueantes				Dose _____ Data Inicio _____ Duração _____ Qual? _____
IECA				Dose _____ Data Inicio _____ Duração _____ Qual? _____
ARA				Dose _____ Data Inicio _____ Duração _____ Qual? _____
Diuréticos				Dose _____ Data Inicio _____ Duração _____ Qual? _____
Vasodilatadores				Dose _____ Data Inicio _____ Duração _____ Qual? _____
Hipolipidemiantes				Dose _____ Data Inicio _____ Duração _____ Qual? _____
Estatinas				Dose _____ Data Inicio _____ Duração _____
AAS				Dose _____ Data Inicio _____ Duração _____
Antidiabéticos orais/insulina				Dose _____ Data Inicio _____ Duração _____
Denosumab				Dose _____ Data Inicio _____ Duração _____

Outras terapêuticas actuais ou anteriores (especificar terapêutica e data de início): _____

Data de preenchimento do protocolo: _____/_____/_____ Assinatura: _____

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Annex III - Solutions and Buffers

TAE (Tris-acetate-EDTA) Buffer: This buffer was used both in the agarose gel and as electrophoresis running buffer. The 1X working solution is made from a 50X stock solution prepared by dissolving 242 g of Trizma® base (Sigma-Aldrich, USA), 57.1 mL of glacial acetic acid (Sigma-Aldrich, USA) and 100 mL of EDTA 0.5M pH8.0 (Sigma-Aldrich, USA) in deionized water to a final volume of 1L.

PBS (Phosphate-Buffered Saline) Buffer: 8 g of NaCl (Sigma-Aldrich, USA) is added to 0.2 g of KCl (Merck, Germany), 1.44 g of $NaHPO_4$ (Sigma-Aldrich, USA) and 0.24 g of KH_2PO_4 (Sigma-Aldrich, USA) and dissolved in 800 mL of deionized water. The pH is adjusted to 7.4 with HCl (Sigma-Aldrich, USA) and deionized water is then added to perform a total volume of 1 L. The buffer is finally sterilized by autoclaving 20 min at 121ºC.

DAPI: 5 mg of DAPI® dilactate (Sigma-Aldrich, USA) is added to 50 mL of double-distilled water and agitated with a magnetic stir bar during 60 min. The solution is then filtered through a 0.22 µm pore filter and stored at -20ºC in 500 µl aliquots.

Mowiol: 6 g of glycerol (Sigma-Aldrich, USA) is mixed with 2.4 g of Mowiol® (Calbiochem, USA), 6 mL of double-distilled water and 12 mL of 0.2M Tris-HCl pH8.5 (Sigma-Aldrich, USA). This mixture is stirring during six hours and left to rest for two hours. To dissolve the Mowiol, the tube with the mixture is incubated at 50ºC for 10 min. After centrifugation (5000g, 15 min) to remove any undissolved solids, the supernatant is aliquoted and stored at -20ºC.

4% Paraformaldehyde Solution (4%PFA): 800 mL of 1X PBS was heated at 60ºC before adding 40 g of paraformaldehyde powder (Sigma-Aldrich, USA). The pH was raised by adding 1M NaOH (Sigma-Aldrich, USA). After the paraformaldehyde was dissolved, the solution was cooled down, filtered and the volume was adjusted to 1 L with 1X PBS. The pH was rechecked and adjusted with small amounts of dilute HCl (Sigma-Aldrich, USA) to approximately 6.9. The solution was aliquoted and stored at -20°C.

Annex IV – Primer Sequences

Table 7.1. Primers sequence used in real time PCR (RT-qPCR).

Primer name	Oligonucleotide sequence (5'-3')	Gene	Source
18S (Fw)	TGTGATGCCCTTAGATGTCC	18S	Probefinder
18S (Rev)	CTTATGACCCGCACTTACTG		
Dll4 (Fw)	AGGCCTGTTTTGTGACCAAG	DLL4	qPrimerDepot
Dll4 (Rev)	GTGCAGGTGTAGCTTCGCT		
Dll4 new (Fw)	CCTGGCAATGTACTTGTGAT	DLL4	[82]
Dll4 new (Rev)	TGGTGGGTGAGTAGTTGAG		
Dll4 160bp (Fw)	GCCAACTATGCTTGTGAATGTCC	DLL4	[83]
Dll4 160bp (Rev)	CAGTAGGTGCCCGTGAATCC		
Pecam (Fw)	CCTTCTGCTCTGTTCAAGCC	PECAM	qPrimerDepot
Pecam (Rev)	GGGTCAGGTTCTTCCCATTT		
Notch1 (Fw)	CCTTCTGCTCTGTTCAAGCC	NOTCH1	qPrimerDepot
Notch1 (Rev)	GTTGGGGTCCTGGCATC		
Tie2 (Fw)	GGAGTCAGCTTGCTCCTTTC	TIE2	qPrimerDepot
Tie2 (Rev)	AGGCAATGCAGGTGAGAGAT		
Ang1 (Fw)	ACCGGATTTCTCTTCCCAGA	ANG1	qPrimerDepot
Ang1 (Rev)	CCGACTTCATGTTTTCCACA		
Ang2 (Fw)	CCTACGTGTCCAATGCTGTG	ANG2	qPrimerDepot
Ang2 (Rev)	GCCACTGAGTGTTGTTTTCC		
VEGF(Fw)	CTACCTCCACCATGCAAGT	VEGF	qPrimerDepot
VEGF (Rev)	AGCTGCGCTGATAGACATCC		
VEGFR1(Fw)	AAGAAGGAAACAGAATCTGCAA	VEGFR1	qPrimerDepot
VEGFR1(Rev)	TCCCTTCCTTCAGTCATGTGT		
VEGFR2 (Fw)	CCTGTATGGAGGAGGAGGAA	VEGFR2	qPrimerDepot
VEGFR2 (Rev)	CGGCTCTTTCGCTTACTGTT		
Jag1 (Fw)	ATCGTGCTGCCTTTCAGTTT	JAG1	qPrimerDepot
Jag1 (Rev)	ACTGTCAGGTTGAACGGTGTC		
Hey1 (Fw)	TGGATCACCTGAAAATGCTG	HEY1	qPrimerDepot
Hey1 (Rev)	CGAAATCCCAAACTCCGATA		
Hey2 (Fw)	TTTGAAGATGCTTCAGGCAA	HEY2	qPrimerDepot
Hey2 (Rev)	GGCACTCTCGGAATCCTATG		
PDGFRβ (Fw)	AACTGTGCCCACACCAGAAG	PDGFRβ	qPrimerDepot
PDGFRβ (Rev)	CAGGAGAGACAGCAACAGCA		
α-SMA (Fw)	CAGCCAAGCACTGTCAGG	α-SMA	qPrimerDepot
α-SMA (Rev)	CCAGAGCCATTGTCACACAC		
Calponin (Fw)	CTGAGAGAGTGGATCGAGGG	CALPONIN	qPrimerDepot
Calponin (Rev)	CTGGCTGCAGCTTATTGATG		
EphB2 (Fw)	CTTCGAGGCCGTTGAGAAT	EPHB2	qPrimerDepot
EphB2 (Rev)	TTGATGGGACAGTGGGTACA		
CD34 (Fw)	TTTGCTTGCTGAGTTTGCTG	CD34	qPrimerDepot
CD34(Rev)	ATTTGAAAATGTTCCCTGGGT		
CD68 (Fw)	TTCACCAGCTGTCCACCTC	CD68	qPrimerDepot
CD68 (Rev)	CACTGGGGCAGGAGAAACT		

Dll4 - Delta-like 4; PECAM - Platelet Endothelial Cell Adhesion Molecule; Ang - Angiopoitin; VEGF - Vascular Endothelial Growth Factor; VEGFR - Vascular Endothelial Growth Factor Receptor; JAG - Jagged; PDGFRβ - Platelet-Derived Growth Factor Receptor β ; α-SMA - alpha smooth muscle actin; qPrimerDepot - https://primerdepot.nci.nih.gov [81]; ProbeFinder - https://qpcr.probefinder.com