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Tissue expression of functional and silent receptors of CCL2 (Chemokine (C-C motif) ligand 2) in coronary arteries: a necropsy study

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ABSTRACT

BACKGROUND: Atherosclerosis (and its manifestation in coronary arteries) is a chronic disease that remains as one of the leading cause of morbidity and mortality worldwide. It is considered a chronic inflammatory disease mainly mediated by chemokines. Chemokines, especially CCL2, play a critical role in the migration of inflammatory cells. CCL2 (chemokine (C-C motif) ligand 2) is a monocyte chemoattractant protein that has an important role in the progression of atherosclerotic plaque. The biological function of CCL2 is possible by its union to CCR2 receptor, but its chemotactic function can be attenuated by "silent" or atypical receptors (DARC and D6). They regulate CCL2 levels in order to balance the inflammatory process.

OBJECTIVE: This study aimed to characterize and compare coronary arteries from individuals that died due to cardiovascular reasons (coronary artery disease, CAD) and the same type of arteries from patients that died from other reasons (unaffected individuals) in order to understand the interactions between CCL2 and its receptors in the development of atherosclerosis. Moreover, the trial aimed to propose a biomarker able to distinguish between affected and non-affected individuals

METHODS: We evaluated the histological changes between the arteries in both groups. We also examined the localization and the expression of different proteins (CCL2, CCR2, DARC, D6 and CD68) in a total 87 subjects, including 19 non-affected patients and 68 patients with CAD by immunochemical staining analysis**.**

RESULTS: There was a significant increase in the expression of CCL2, CCR2, CD68, D6 and DARC in subjects with CAD compared to the non-affected individuals. Results obtained indicate a significative association between CCL2 and progression of atherosclerosis in patients with CAD.

CONCLUSIONS: The presence of CCL2 and its receptors are a key factor in the initiation and progression of atherosclerosis and CCL2 has been proposed as an attractive biomarker for coronary artery disease.

1. INTRODUCTION

Cardiovascular disease (CVD) is the largest single cause of premature mortality in the developed and now developing world. It is the primary cause of death in the Spanish population over 30 years; over these, coronary artery disease (CAD) causes the most significant impact. U.S. data show a reduction in mortality from coronary artery disease in recent years, as a result of a better control of smoking, hypertension and dyslipidemia. However, there is a progressive increase in mortality due to a raise in obesity and diabetes mellitus. [1-3].

1.1 Cardiovascular system

The cardiovascular system comprises heart and blood vessels (arteries and veins) conveying blood to and from body organs and tissues. It is responsible for transporting oxygen, nutrients, hormones and cellular waste products throughout the body so it consists of vessels intended for conductance, resistance, exchange and capacitance [4].

All blood vessels, except capillaries and venules, have walls made up of three main layers (or tunica): intima, media and adventitia (Figure 1) [5].

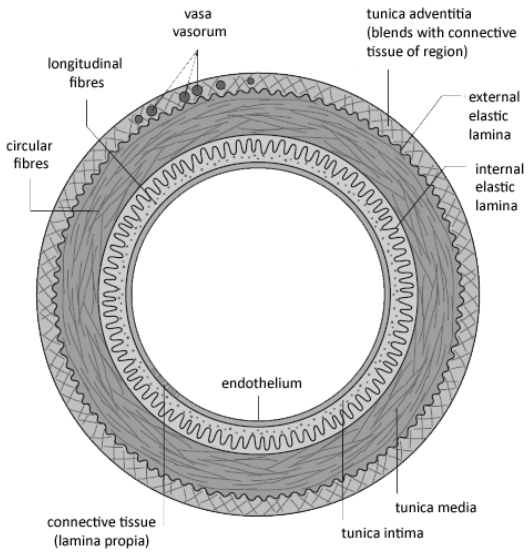


Figure 1. Cross-section of a generic vessel showing the distinction between layers. Picture adapted from Evans *et al*. [2012].

The intima is the innermost layer of the vessel and it is in contact with the blood flow and its components. It is composed of a single layer of highly specialized endothelial cells that sit on a basement membrane and a very thin layer of connective tissue. This structure allows the development of important anti-inflammatory functions and contributes to the homeostatic process of vasodilation and vasoconstriction [5, 6].

The media, most prominent in arteries, is composed primarily of smooth muscle cells and elastic tissue. There is a layer of elastic tissue on both sides of the muscular part: the internal elastic lamina and the external elastic lamina. The elastic component is more prominent in elastic arteries meanwhile vascular smooth muscle predominates in muscular arteries and arterioles [5, 6].

The adventitia is the most external layer in vessels. It composed of connective tissue such as collagen. This layer contains the autonomic nerves that innervate the vascular smooth muscle. There are also small blood vessels called *vasa vasorum,* which send penetrating branches into the media to supply smooth muscle cells. The adventitia is the most prominent layer in veins [5, 6].

The structure of blood vessels can be organized anatomically. The vessels are divided into elastic arteries, muscular arteries, arterioles, capillaries, postcapillary venules, muscular venules and veins. An elastic artery consists of concentric layers of elastic smooth muscle; on the other hand, a muscular artery has a large tunica media and a thin tunica intima besides a prominent internal elastic lamina. In contrast, a vein has a thin middle layer [5, 6].

1.2 Arteriosclerosis

Arteriosclerosis is a term used to describe thickening and loss of elasticity of the arteries, regardless of size [7].

The vessel thickening reduces lumen diameter, which may compromise organ perfusion. The loss of elasticity increases the likelihood of vessel rupture if exposed to increased mechanical stress [8].

There are three important types of arteriosclerosis [5]:

- Arteriolosclerosis: thickening of the wall in small arteries and arterioles.
- Atherosclerosis: thickening of the wall in medium and large arteries.
- Mönckeberg's arteriosclerosis o medial calcific sclerosis: calcifications are found in the middle layer of the arteries of small and medium size.

1.3 Atherosclerosis

Atherosclerosis remains as one of the leading causes of morbidity and mortality worldwide. This disease, clinically manifested as coronary artery disease (CAD), stroke, transient ischemic attack (TIA), and peripheral artery disease (PAD), is estimate to account for around 50% of deaths in Western societies [5]. In Spain, atherosclerosis is responsible for 124,000 deaths each year [9].

Atherosclerosis is a systemic disease that affects arteries at different locations simultaneously but with different degrees of progression. Atherosclerotic lesions usually occur in the arteries supplying the heart (coronary arteries), brain (carotid, vertebral, and brain arteries) and lower extremities (iliac and femoral arteries) [10].

Risk factors for atherosclerosis, and therefore risk factors for its associated conditions, can be broken down into “non-modifiable” (age, sex and genetics) and “modifiable” (smoking, diabetes mellitus, hypertension and hyperlipidemia). Other important factors involved in the development of atherosclerosis are a sedentary lifestyle, a high-fat diet and obesity [10].

1.3.1 Atheromatous plaque formation

Arteriosclerosis is a silent disease characterized by the formation of an atheromatous plaque. In the formation of the atheromatous plaque there are involved inflammatory cells, lipids and extracellular matrix. They can cause the occlusion of the arterial lumen or a thrombi, generated when there is a rupture of atherosclerotic plaque [8].

The progression of atherosclerosis is initiated in response to endothelial dysfunction and vascular inflammation, followed by the progressive accumulation of cholesterol, calcium and cellular debris in the vascular intima wall of arteries [8].

An injury in the endothelium is the first step in the formation of the atheromatous plaque. Endothelial dysfunction may occur as a result of cigarette smoking, hypertension and high cholesterol level (hypercholesterolemia), especially low-density lipoproteins (LDL), leading to metabolic dysfunction and structural changes. This results in an increased permeability of the intima, promoting the entrance of lipids and LDL in the artery wall (Figure 2a) [8, 11, 12].

The immune response induces monocytes and platelets adhere to the injured area. So, in the next step, (Figure 2b) there is a formation of a fatty streak by the adhesion of these platelets and monocytes in the intima. There is a chain reaction: monocytes migrate and adhere into the intima and then get transformed into macrophages, which produce cytokines and growth factors such as CCL2 (Chemokine (C-C motif) ligand 2) and can then attract even more monocytes. These monocytes also produce free radicals, which promote LDL oxidation and cause endothelial damage. Platelets and activated macrophages stimulate smooth muscle cell migration from the media to the intima [8, 10-12].

The third stage is the development of lipid plaque. There is a proliferation of smooth muscle cells and an increase of extracellular matrix in the intima. Additional cytokines and growth factors produced by activated macrophages and platelets promote additional monocyte and smooth muscle cells infiltration. Smooth muscle cells capture LDL and form foam cells (Figure 2c) [8, 11].

Finally, there is a generation of an advanced plaque or a complicated lesion (Figure 2d). An advanced atheromatous plaque consists of a lipid-rich core and a fibrous layer. The core of the lesion (which can become necrotic) is full of free lipids, macrophages, smooth muscle cells and cellular debris. The fibrous layer is composed primarily of muscle, replaced by

collagenous. This advanced plaque may produce the formation of a thrombus if there is a plaque rupture [8, 11, 12]. In addition, the atheromatous plaque can become even more complex with calcification processes, ulceration at the luminal surface and hemorrhagic lesions. Although advanced lesions can cause symptoms of ischemia as a result of the progressive vascular occlusion of the lumen, the most important clinical complication is the rupture of the atheromatous plaque and the formation of a thrombus or blood clot, leading to a myocardial infarction or stroke [9, 11, 13].

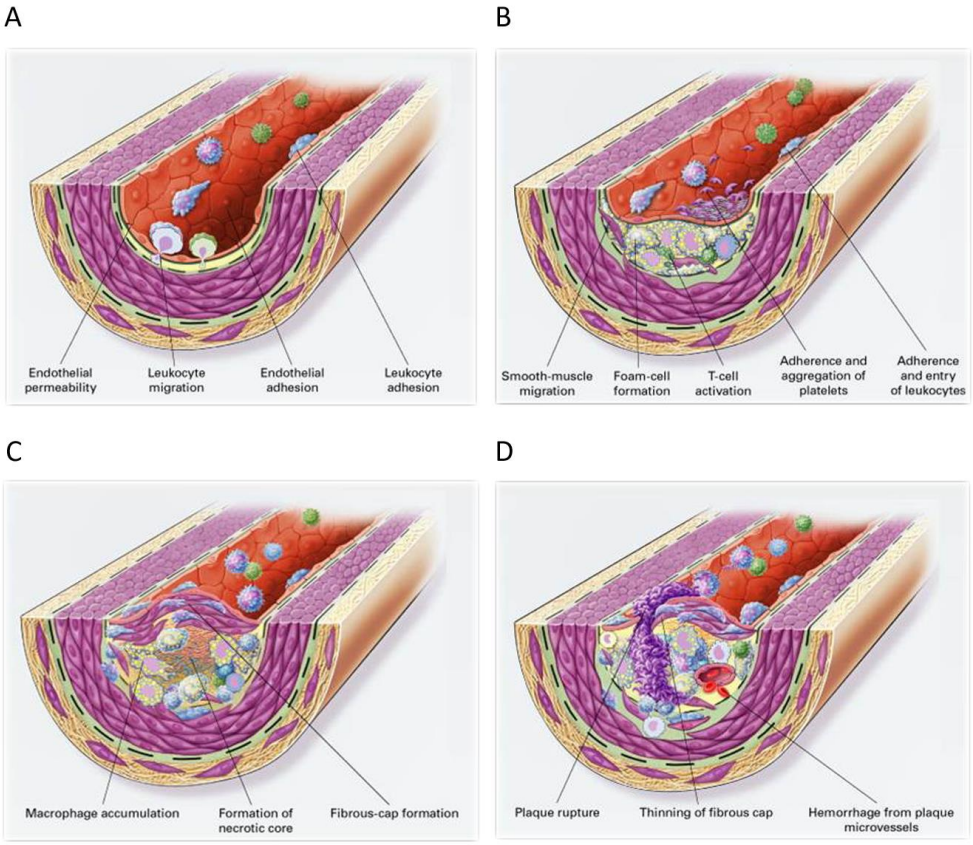


Figure 2. Stages in the development of an atheromatous plaque, from the initial endothelial damage to an advanced plaque. (A) Endothelial dysfunction. (B) Formation of the fatty streak. (C) Development of the lipid plaque. (D) Advanced atherosclerotic plaque [13]. Picture adapted from Ross *et al.* [1999].

Coronary artery disease (CAD) is one of the cardiovascular diseases. The formation of the atheromatous plaque is the main feature for the progression of this disease because there is a severe disorganisation of the intima, the presence of foam cells, macrophages and an extracellular lipid core which entail the formation a thrombus or blood clot [11].

1.4 Relationship between atherosclerosis and inflammation

Inflammation is the immune system repair response to damage in cells and tissues, either by bacterial pathogens or another kind of aggression (physical, chemical or biological). The function of inflammation is to reduce or limit the area of infection. However, the exposure

of the organism to a prolonged inflammation state produces an entirely opposite effect as well as an increasing morbidity and mortality due to cardiovascular disease [14].

Atherosclerosis is considered a chronic inflammatory disease mainly mediated by the infiltration of macrophages and T-cells into the vascular wall. Macrophages participate by mechanisms involving pattern-recognition receptors and scavenger receptors whilst various T-cell subtypes respond to modified lipid or protein antigens to promote inflammatory cascades [2, 15].

Once the endothelial is damaged, monocytes penetrate the tissue and then they are transformed into scavenger cells. Upon stimulation of Th1 cells, a group of cytokines is released and contributes to the inflammatory response of atherosclerotic tissue. When macrophages proliferate they amplify inflammatory response through the secretion of growth factors and cytokines such as TNF and IL-1. In addition, activated monocytes produce more chemokines [15].

Chemokines play a critical role in the migration of inflammatory cells to atherosclerotic lesions, especially, CCL2 (chemokine (C-C motif) ligand 2), CX_3CL1 (chemokine (C-X3-C motif) ligand 1) and CCL5 (chemokine (C-C motif) ligand 5). For this, the study of the function and role of these chemokines in the development of atherosclerosis is a key point for a possible therapeutic target or future biomarkers [2].

1.5 Chemokines

Chemokines are a large group of proinflammatory cytokines. They are inflammatory proteins that act on immune cells and chemokine group with activities related to homeostatic processes rather than inflammatory. The homeostatic chemokines control the immune system; these chemokines are produced and secreted without any need to stimulate their source cells. The inflammatory chemokines are released from a wide variety of cells in response to bacterial infection, viruses and agents that cause physical damage. They are involved in a wide range of important diseases with inflammatory processes, including atherosclerosis [16-18].

Chemokines are cytokines whose function is to lead the migration of circulating leukocytes to the sites of inflammation. Chemokines exert their cellular effects through activation of specific receptors, initiating a signal transduction cascade that promotes changes in shape and cell movement [19].

About 40 chemokines have been identified to date in humans. They are small polypeptides, composed of about 70-125 amino acids with a molecular mass between 8-14 kDa [16, 17].

There are four main families of chemokines, classified depending on the number of amino acids that are among the first cysteine residues at the N-terminal site (Figure 3) [17, 20]. The largest family is divided in CXC (or α chemokines) and CC (or β chemokines). Both differ in their function, sequence, and chromosomal localization. The α chemokines (CXC) have two cysteines separated by one amino acid. They are located on chromosome 4q13 and its action takes place on neutrophils, monocytes and T cells. The β chemokines have two cysteines linked

together and are located on chromosomes 17q11 and 12. These inflammatory proteins are related to homeostatic processes rather than inflammatory processes. [17, 18].

In contrast, there are few families of C chemokine (chemokine γ) and CX3C (δ chemokines). Their function is to stimulate chemotaxis and to act on endothelial cells [17-19].

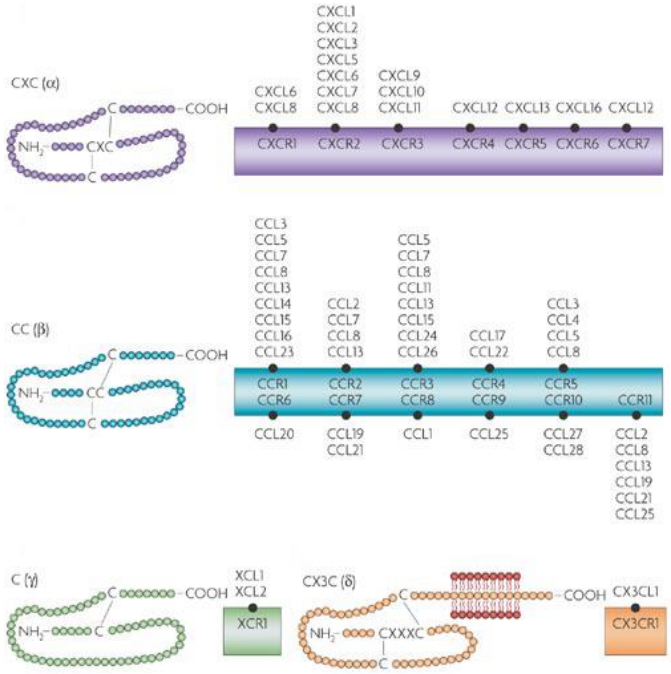


Figure 3. Families of chemokines and their receptors. Picture extracted from Rostene *et al*. [2007]

Monocyte chemoattractant proteins, also known as CCLs are part of the β subfamily. As suggested by the name, they are proteins involved in the recruitment of monocytes at different sites of inflammation [19].

CCLs are produced and secreted by several cell types including leukocytes and tumor cells. In general, the main inductors for the expression of CCLs are pro-inflammatory cytokines such as IL-1, TNF-α or IFN-γ. There are four CCLs identified (CCL1 - CCL4); between them CCL2 is the most studied and characterized chemokine [18, 21].

1.6 CCL2 (Chemokine (C-C motif) ligand 2)

Chemokine (CC-motif) ligand 2 (CCL2), also known as monocyte chemoattractant protein 1 (MCP-1), is a chemoattractant molecule that lead monocytes/macrophages to the inflammatory sites, being a key molecule in the initiation of inflammatory processes [22].

This protein is encoded by 8927bp gene located on the long arm of chromosome 17q11 and, depending on containing or not glycosylation, it submits a molecular weight of 9 or 13 KDa. It is synthesized in two main ways depending on the type of glycosylation. One of the isoforms contains the galactose-β-N-acetyl-galactosamine glycosylation, while the other does not. Glycosylation affects the stability of the molecule but not its function [23].

CCL2 is secreted by all cells involved in the vascular lesion (macrophages, smooth muscle cells and endothelium cells) [22]. This induces monocyte adhesion to vascular endothelium, facilitating and promoting the differentiation of monocytes to foam cells [24].

1.6.1 Regulation

Numerous studies show that blood levels of CCL2 are modulated by different factors. CCL2 expression is regulated at the transcriptional level by stimulating agents like tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), platelet-derived growth factor (PDGF) and stress factors. In contrast, estrogen, retinoic acid and glucocorticoids have been shown to inhibit CCL2 expression. The pro-inflammatory nuclear factor kappa B (NF-κB) transcription factor is a key mediator (Figure 4) [23, 25]. All of these regulatory features are connected with the structure and composition of the promoter region of the CCL2 gene. The overexpression of CCL2 is involved in many pathogenesis for example: atherosclerosis, angiogenesis and cancer progression, autoimmune diseases and ischemia [23, 24].

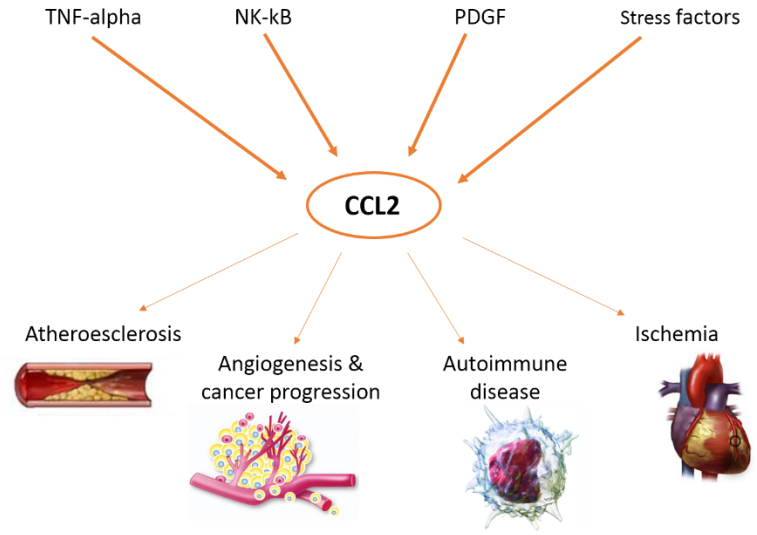


Figure 4. Factors that stimulate CCL2 transcription and some of the pathologies associated with CCL2 overexpression. Picture adapted from Melgarejo *et al.* [2009].

Initially, it was believed that its expression was only in certain types of cells and tissues. However, subsequent studies have shown that this protein is expressed and secreted ubiquitously in most tissues. Therefore, this discovery provided new information about the ability of the tissues themselves to respond to an attack or damage by an inflammatory response [26].

1.6.2 Biological Function

CCL2 is a proinflammatory molecule that attracts monocytes to the damaged tissue and it has an important role in the inflammatory process. CCL2 is also important for memory T-cells and Natural Killer (NK) cells. Furthermore, along with interleukin-8 (IL-8), CCL2 has been

shown to trigger the firm adhesion of monocytes to vascular endothelium under flow conditions [23, 26].

CCL2 has been shown both to upregulate the expression of β2 integrin on monocyte cell surfaces and to activate this molecule for adhesion of monocytes into extracellular matrix proteins. CCL2 is also involved in allergic inflammation [23, 26].

Chemokines exert their effects through binding to G-protein coupled receptors on the surfaces of cells targeted for activation and migration. The action of CCL2 is possible by its union with CCR2 receptor [26].

The signaling axis of CCR2 once activated triggers the synthesis of inositol triphosphate, causing intracellular calcium release and protein kinase C activation. The latter activates NF-KB, charge factor that stimulates the transcription of many genes related with cell mobilizing monocytes [23].

1.6.3 Functional CCL2 receptor: CCR2 (chemokine (C-C motif) receptor 2)

The cellular receptor for CCL2 is CCR2 (Chemokine C-C motif receptor 2). This receptor is found primarily on monocytes, T-cells, B-cells and basophils. It is a G protein-coupled receptor (GPCRs) with seven transmembrane domains. It is encoded by a gene located on the short arm of chromosome 3, which has two isoforms: 2a and 2b [23, 27].

The difference between the isoforms resides in the primary structure of the protein. Each isoform contains a different amino acid sequence in the cytoplasmic region originated by alternative splicing process. The change in the sequence alters the location of the protein in the cell, that's why the CCR2B isoform is mainly found on the cell surface whereas the CCR2A isoform is mainly located in the cytoplasm (Figure 5) [28].

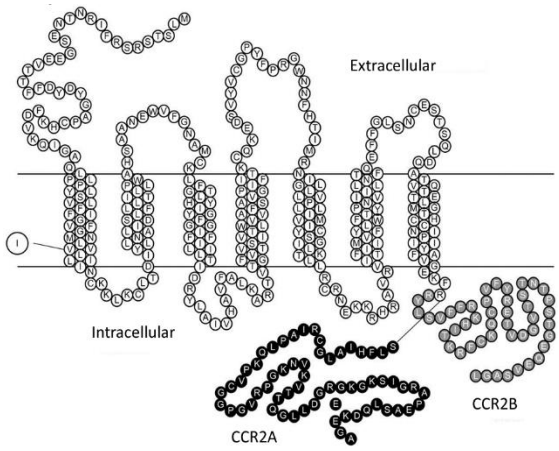


Figure 5. Structure of the CCR2A and CCR2B receptors. Picture adapted from Bartoli *et al.* [2001].

It is believed that the main cause for which most of the CCR2A isoform remains in the cytoplasm is that it is retained in the Golgi apparatus due to some interactions. However, in some cases CCR2A is anchored to the cell surface and then it presents the same activity as the CCR2B isoform [28].

An important feature is that chemokine receptors responsible for controlling the homeostatic response of cell migration have only one ligand described (receivers), while recipients apparently associated with pathological phenotypes have multiple ligand(s) (shared receptors). The CCL2/CCR2 complex is clearly within the latter [29].

1.6.4 Silent or atypical Chemokine Receptors (ACRs)

The biological functions of chemokines are mediated by G-protein coupled chemokine receptors (GPCRs), which sense extracellular chemokines and transmit signals to change cell behavior. However, there is a group of receptors among the chemokine system that, although binding to its ligand, do not transmit any signal and, hence, do not exert cellular response. These receptors are Atypical Chemokine Receptors (ACRs), which although having a similar structure to GPCRs, are not coupled to a protein G [30-33].

They are believed to exert control over the amount of chemokines present in a particular place, optimizing the response pro- or anti-inflammatory. Thereby, these receptors regulate the concentrations of the inflammatory chemokines and endothelial transport [33, 34].

Atypical Chemokine Receptors are also named as *non-signaling chemokine-binding proteins*, *promiscuous decoy receptors*, *silent receptors*, *interceptors* (internalizing receptors) and *chemokine 'decoy' receptors*. It is known that these receptors are internalized but do not transmit a signal and, therefore, do not emit a cellular response [31].

Within this group, the most known receptors are Duffy antigen receptor for chemokines (DARC), Chemokine binding protein 2 (D6), CCX-CKR (CCR11) and CXCR7 (ACKR3). The first two are the ones that are most studied [30, 31].

DARC and D6 capable of binding chemokines with high affinity and without signaling function. Therefore, some chemokines are subjected to modulation of their concentrations by proteins to which they bind without any signaling response [31].

1.6.4.1 DARC (Duffy antigen receptor for chemokines)

Duffy antigen receptor for chemokines (DARC) is a member of atypical receptor subfamily that binds a broad range of inflammatory C-C and C-X-C chemokines. DARC is a transmembrane glycoprotein that is widely expressed in postcapillary venular endothelial cells, which are the primary site of leukocyte transmigration in most tissues. Besides, it is also expressed in red blood cells and in neuronal cells. This receptor acts as a cytokine receptor, especially in CCL2, IL-8 and CCL5 [32, 35].

The current theory suggests that DARC receptor acts as a regulator of chemokine activity. Although its function is not yet fully understood, it is known to be involved in the uptake of cytokines regulating the circulatory system and blood levels of these cytokines [32].

DARC expressed on the endothelial blood vessels can act as transporter of chemokines. It is believed that it could have a pro-inflammatory role by concentrating inflammatory chemokines on the endothelium [33, 35].

The biological action of receptor DARC on venular endothelium consists in the mediation of chemokine internalization at the *ab*-luminal surface followed by transcytosis and transport of the chemokine load onto the luminal surface (Figure 6a). DARC receptor expressed on the erythrocyte membrane binds plasma chemokines resulting in neutralization of chemokines in the blood (Figure 6b) [35].

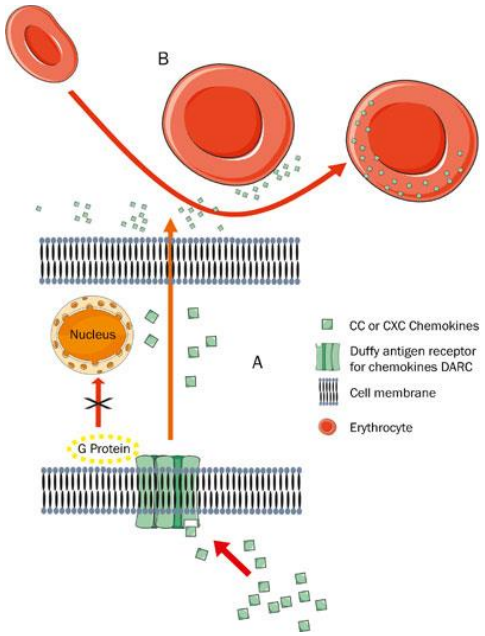


Figure 6. Schematic representation of the biological action of DARC receptor. Picture extracted from *Apostolakis et al.* [2011].

Some studies indicate that DARC receptor is involved in the regulation of CCL2 because it is apparently able to regulate its blood concentration and thereby regulate the amount of free cytokine that can perform its function [31, 32].

1.6.4.2 D6 (atypical chemokine receptor 2)

D6, also named as chemokine binding protein 2, chemokine binding receptor 2, CC chemokine receptor 9 (CCR-9) and CCR10, is another receptor without signaling activity. Despite that, it does not mean that it has no function [30].

D6 molecule is an atypical chemokine receptor and, as DARC, it lacks sequence motif that are critical for G protein coupling and signaling functions of chemokine receptors. D6 receptor is characterized by its binding affinity for inflammatory CC chemokines such as CCL2, but also its selectivity, as it does not recognize homeostatic CC chemokines or CXC, XC or CX3C chemokines [30, 34, 36].

D6 is selectively expressed at high levels by endothelial cells of lymphatic vessels in the skin, the gut, the lungs and may be found on trophoblasts, leukocytes, macrophages and dendritic cells [30, 33, 36]. During the inflammation process, D6 is expressed on the leukocytes, especially in those that invade the inflamed tissues. In contrast to DARC, D6 does not promote chemokine transcytosis, but contributes to their degradation, by directing the complex receptor-ligand toward the endosomes [33].

The main physiological role of D6 is acting as a negative regulator, helping regulate chemokines during inflammation processes (in order to prevent an excessive response) and finally finish the inflammatory response [30, 33].

In the presence of ligand, D6 binds to chemokines; they rapidly enter the cell through endosomal compartments and the chemokine dissociates quickly from the receptor in order to be targeted to degradation. At the same time, D6 is recycled back to the cell surface for further chemokine capture. One property of this system is that it is not influenced by chemokine exposure so chemokine-induced signaling is not needed [30, 33].

Therefore, DARC and D6 are crucial receptors in inflammation as well as in chemokine-associated diseases, for instance coronary artery disease, since they can capture chemokines and block their signaling function. In the context of atherosclerosis, the chemokine-blocking function of this receptors has been widely described [35].

2. HYPOTHESIS AND OBJECTIVES

Atherosclerosis is an inflammatory disease, which is now the leading cause of death in the world. Within this inflammatory process, the role of CCL2 is to attract monocytes/ macrophages to sites of inflammation.

However the assessment of the actual role of this chemokine is difficult to interpret. This is due to the fact that CCL2 has a functional receptor (CCR2) but also can be bound to “silent” receptors (DARC, D6).

We hypothesised that the expression of these silent receptors may modify the histologic features of coronary arteries with atherosclerosis and consequently be important in the fate of the patients. Moreover, we think that we should keep CCL2 and its receptors in mind for a future implementation as biomarkers for coronary artery disease.

This study aims to characterize and compare arteries the sources of which were routine necropsies from patients who died either by cardiovascular problems or other non-cardiovascular reasons.

Intended tasks:

- Histologically assess of the state of arteries in both groups of study: evaluation of the state of the layers intima, media and adventitia.
- Qualitatively assess the presence of calcium deposits in all biological samples.
- Structurally observe the composition of the arteries of both groups.
- Perform immunohistochemical analysis to determine the presence of CCL2, CCR2, CD68, DARC and D6 in affected and non-affected coronary arteries.
- Consider the election of a possible biomarker.

3. MATERIALS AND METHODS

3.1 Participants

A descriptive study design was used to test the hypothesis. Biological samples (coronary arteries) from patients with coronary artery disease (CAD) and with cardiovascular cause of death (n=68) were obtained from necropsies at Hospital Universitari Joan XXIII in Tarragona between 2003-2004. This trial included samples from patients older than 38 years old and younger than 93 years old, with a diagnosis of CAD confirmed by Pathological Anatomy's Department.

Biological samples from 19 individuals with other causes of death were also obtained from necropsies at Hospital Universitari Joan XXIII in Tarragona between 1995-2011 and were selected as a less affected/unaffected population (Figure 7).

The exclusion criteria were age (excluding patients younger than 18 years old), analytical or clinical evidence of infection, kidney problems, liver disease, neoplasm and autoimmune diseases.

Clinical characteristics (e.g. age, grade of atherosclerosis, hypertension…) and biochemical variables (e.g. glucose, total cholesterol, triglycerides…) were obtained from the clinical histories by the Pathological Anatomy's Department.

The stages of atherosclerosis development according to injuries in coronary arteries were classified into: mild (atherosclerosis type I), moderate (atherosclerosis type II) and severe (atherosclerosis type III). These analysis were made by pathologists of Pathological Anatomy's Department and used the system proposed by Stary, H.C. *et al.* [37].

Local Ethics Committee of the Hospital Universitari Joan XXIII approved the study. The anonymity of the patients was guaranteed at all times.

Samples were collected at the moment of the histological study and all tissues were washed with PBS and were fixed in 4% neutral buffered formalin during 18-24 hours. Then the biopsies were embedded in paraffin according to the Pathological Anatomy's standard procedure and stored at Hospital Universitari Joan XXIII for later usage.

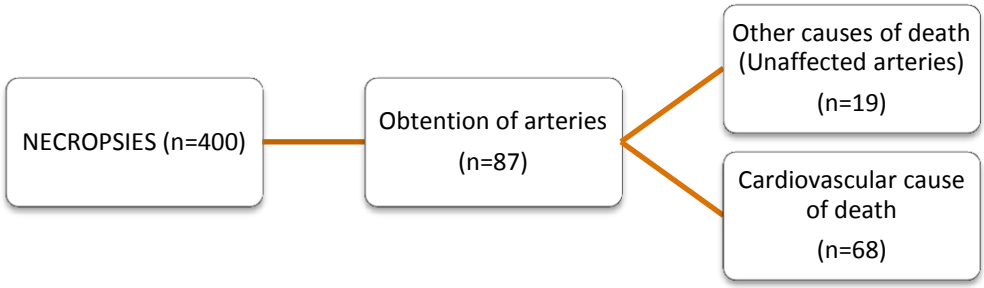


Figure 7. Schematic representation of the obtention of the biological samples.

3.2 Histological examination

Sections of 4µm thick were stained with haematoxylin and eosin to evaluate the histology of the arteries [38]. Using an optical microscope (Nikon, Eclipse E600, Madrid, Spain) and an image analysis system (Soft Imaging System, Münster, Germany), the intima-media thickness (IMT) was measured in these sections as the thickness between the intima and the media. The relationship intima/media (I/M) was also obtained.

Masson's trichrome staining (Masson's Trichrome Stain Kit Artisan, Dako, Glostrup, Denmark) was used to assess structure and the extent of fibrosis in the arteries. Briefly, this staining is useful to differentiate distinct molecules: in our work, black colour for nuclei, red colour for cytoplasm, keratin muscle fibbers and acidophil granules and blue colour for collagen, mucus and basophil granules.

Alizarin Red staining (Alizarin Red S, Sigma–Aldrich Inc., Steinheim, Germany) was used to assess the localization of micro crystalline or non-crystalline calcium phosphate salts. This procedure is based on the staining of calcium present in histological sections of biological sample from patients with atherosclerosis in coronary arteries. Alizarin Red is also used in biochemical assays for determining quantitatively the presence of calcium deposits colorimetrically. Calcium forms a complex with Alizarin Red S in a chelation process.

3.3 Immunohistochemical analyses

Immunohistochemical technique is employed to detect the presence of an antigen in histological sections of tissue by using an antibody that is specific for the antigen. A secondary antibody is used which is coupled to an enzyme. The enzyme converts a colourless substrate into a colored insoluble substance that precipitates at the point where the antibody is situated and consequently, reveals the position of the antigen [39].

3.3.1 Deparaffinised and hydration

Formalin-fixed, paraffin-embedded sections from human coronary arteries were subjected to immunohistochemical staining for CCL2, CCR2, DARC, D6 and CD68 (human macrophage-associated antigen).

The tissue included in paraffin was sectioned with a microtome at a 4µm thickness and was collected on slides coated with poly-L-Lysine. The tissues on slides were heated for 30 minutes at 56 degrees in a stove and then they were deparaffinized and rehydrated with xylene and a descending ethanol concentration series to distilled water.

3.3.2 Immunohistochemistry

The first step is antigen retrieval, that was carried out by placing the slides in 0.15mol/L sodium citrate buffer at pH=6 (DakoCytomation, Dako, Glostrup, Denmark) in a microwave oven until reaching 90ºC.

After waiting 30 minutes for tempering, the slides were washed using phosphate-buffered saline (PBS) solution, followed by the blockade of the endogenous peroxidase activity with 3% hydrogen peroxide for 25 minutes.

The third step was the incubation of sections in 2% bovine serum albumin to block non-specific binding sites. Then the sections were incubated overnight at 4ºC with the corresponding primary polyclonal antibody (Table 1).

Primary antibody's signal was amplified for 90 minutes with the corresponding biotinylated secondary antibody (Table 1) followed by incubation for 45 minutes with avidin – biotin conjugated with a peroxidase enzyme (ABC Complex, Vectastain ABC Kit Standard, Vector, Burlingame, California). It binds to the biotin attached to the secondary antibody.

The display of the specific binding of the primary antibody with the secondary antibody is given by the oxidation of the soluble chromogen D.A.B (3,3´_diaminobenzedine, Dako, Glostrup, Denmark), which reacts with the peroxidase yielding the brown precipitate observed in the microscope.

The sections were counterstained with Mayer's haematoxylin (Sigma–Aldrich Inc., Steinheim, Germany) for 2 minutes and then dehydrated through ethanol and xylene series. Finally, they were fixed in D.P.X. mounting medium (VWR International, Llinars del Vallès, Spain).

Table 1. Antibodies and dilutions used in the immunohistochemical assay.

Antigen	Primary antibody	Dilution	Secondary antibody	Dilution
CCL2	Rabbit anti-human MCP-1 (Abcam, Cambridge, United Kingdom, ab9669)	1:100	Goat anti-rabbit IgG (Vector, Burlingame, CA, USA, BA-1000)	1:200
CCR2	Rabbit anti-human CCR2 (Abcam, Cambridge, United Kingdom, ab21667)	1:100	Goat anti-rabbit IgG (Vector, Burlingame, CA, USA, BA-1000)	1:200
DARC	Rabbit anti-human DARC (Abnova, Taipei, Taiwan, PAB13254)	1:200	Goat anti-rabbit IgG (Vector, Burlingame, CA, USA, BA-1000)	1:200
D6	Goat anti-human D6 (Abcam, Cambridge, United Kingdom, ab1658)	1:200	Rabbit anti-goat IgG (Vector, Burlingame, CA, USA, BA-5000)	1:200
CD68	Mouse anti-human CD68 (Dako, Glostrup, Denmark, MCA497R)	Ready to use	Horse anti-mouse IgG (Vector, Burlingame, CA, USA, BA-2000)	1:200

3.3.3 Evaluation of immunostaining

Sections were evaluated with an optical microscope (Nikon, Eclipse E600, Madrid, Spain) by using objectives with x20 magnification. Section's area and colour intensity were measured by using the software AnaliSYS (Soft Imaging System, Münster, Germany).

3.4 Statistical analysis.

In order to evaluate our hypothesis different statistical tests were applied. Kolmogorov-Smirnov test was used to assess the normal distribution of our variables.

Differences between groups were evaluated by Student's t-test (parametric variables), with the U test of Mann-Whitney (nonparametric variables) and using chi-square test for categorical variables.

To describing and evaluate sensitivity/specificity, ROC (Receiver Operating Characteristic) curves were carried out.

Results are expressed as median and SD (Standard Deviation) for parametric variables and median and interquartile range for nonparametric if not otherwise indicated. The qualitative variables were expressed as n (%) of the total participants.

All statistical analyses were carried out by using the SPSS 19.0 package (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.). P values <0.05 were considered statistically significant in all of the analysis.

4. RESULTS

Biological samples from 19 patients with less affected/unaffected arteries and 68 patients with CAD have been used to carry out this study. Immunohistochemical analyses were done to assess the inflammatory state of the arteries.

4.1 Clinical and biochemical characteristics of patients

This study was conducted with samples from two types of population: (1) a group of non-affected individuals that died for other cause (n = 19), 71% of whom were males with a median age of 45 years old; and (2) a group of individuals with coronary artery disease (CAD) that died for cardiovascular reasons (n = 68), 73.5% of whom were men with a median age of 71 years old.

The main clinical and demographic characteristics of both populations and also biochemical parameters are shown in Table 2.

Table 2. Main clinical and biochemical parameters of less affected group and patients with CAD.

	Unaffected (n=19)	CAD (n=68)	p-value
Clinical characteristics			
Age, years	45 (33 - 69)	71 (60.50 - 77)	**<0.001**
Males, n (%)	13 (71.7)	50 (73.5)	0.853
Non smokers, n (%)	15 (78.4)	42 (61.8)	0.164
Heart weight, g	380 (300-450)	480 (400-600)	**0.002**
Infarction, n (%)	-	13 (19.2)	0.234
Ventricular thickness, mm	16 (14-18)	19 (15-20)	**0.005**
Fibrosis, n (%)	-	10 (15.9)	0.110
Affected valves, n (%)	2 (10.5)	31 (45.6)	**0.005**
Hypertension, n (%)	2 (10.5)	35 (51.5)	**<0.001**
Diabetes mellitus, n (%)	2 (10.5)	19 (27.9)	0.117
Biochemical variables			
Glucose, mg/dL	86 (76-96)	88 (82.5-110.5)	0.296
Total cholesterol, mg/dL	142 (114.9-169)	164.70 (122.7-212.9)	0.223
HDL - cholesterol, mg/dL	43.50 (31.8-51)	39.50 (21-63)	0.641
LDL - cholesterol, mg/dL	76.75 (70.8-95.1)	78 (66-130)	0.658
Triglycerides, mg/dL	69 (48.8-90.3)	86 (71.3-161.8)	**0.045**

Results are expressed as medium and interquartile range (25-75%) for nonparametric variables. Qualitative variables were expressed as number of patients who have these characteristics in percentage (%). n: number of individuals; HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein.

Both groups had the same men and women percentage (approximately 30% were women and 70% were men). However, we did not observe the same in the age, which was higher in individuals with CAD.

In patients with CAD, we could observe a significant increase in heart weight, in the number of affected valves, in ventricular thickness and in the presence of hypertension. Nevertheless, regarding the risk of infarction, although it is higher in the CAD group, no significant difference was observed between groups.

Biochemical variables showed that glucose levels were not significantly higher in patients with CAD compared to less affected/unaffected population. Regarding lipid profile, serum concentration of total cholesterol, HDL-cholesterol and LDL-cholesterol were not significantly different among CAD patients and non-affected patients. However, the concentration of total cholesterol and LDL-cholesterol tended to be higher whereas HDL-concentration tended to decrease in patients with CAD. Moreover, there was a significant increase of triglycerides concentration in CAD patients.

4.2 Evaluation of the histology of coronary arteries.

Different stainings were performed in coronary artery tissue to determine and analyze the histology of the arteries and their components.

Haematoxylin / eosin staining was used in coronary arteries in order to differentiate its different layers. For this, the thickness of the media and intima of the arteries of both groups was measured in order to determine IMT (intima-media thickness) and I/M (intima/media ratio). As seen in the Figure 8, the thickness of the IMT significantly increased ($p=0.0002$) in CAD patients compared to the less affected group. We obtained the same results in I/M measurements ($p=0.0069$).

To analyze qualitatively the presence of calcium deposits in the arteries, it was performed Alizarin Red Staining. There was a greater presence of calcium deposits in the intima and media of the arteries with CAD (Figure 9A).

The Masson's trichrome staining was used in order to visualize the structure of arteries and besides being able to differentiate distinct molecules. As shown in Figure 9B, black color stained nuclei, red color stained cytoplasm, keratin muscle fibers and acidophil granules and blue color stained collagen, mucus and basophil granules.

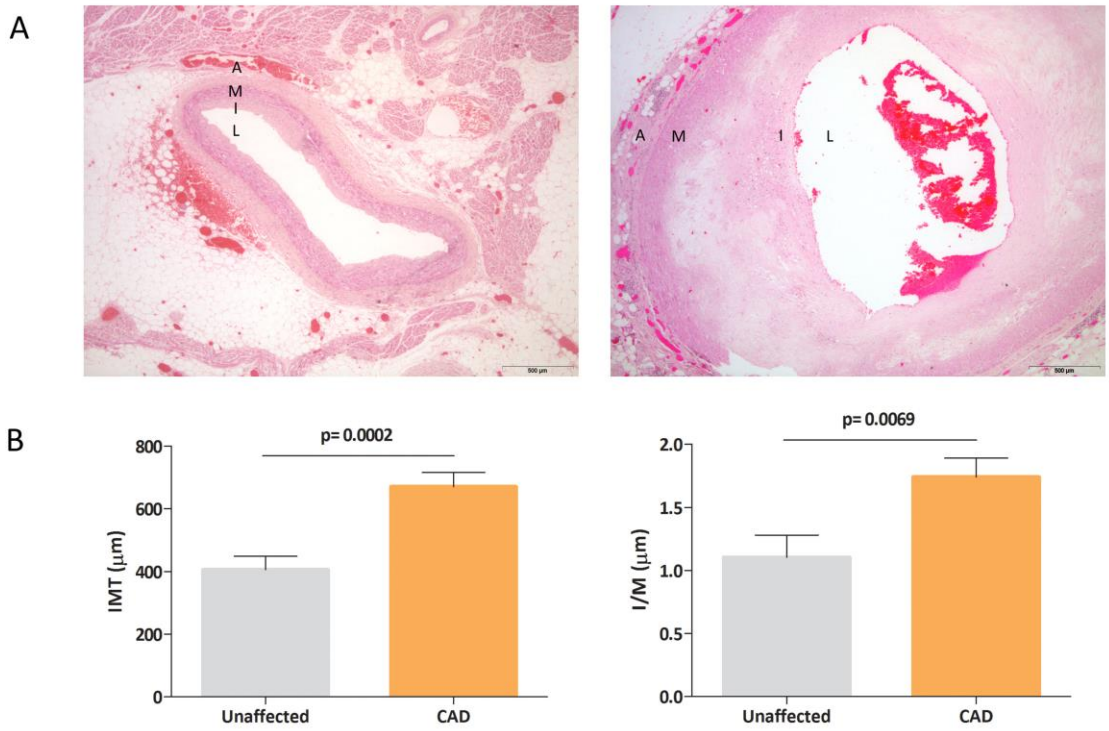


Figure 8. (A) Representative micrography of non-affected or minimally-affected coronary arteries and affected coronary arteries (right) (x20). (B) Graphical display of the manual quantification of IMT (µm) and I/M (µm). Mann-Whitney U test was used to compare unaffected vs. CAD. L (Lumen); I (Intima); M (Media); A (Adventitia).

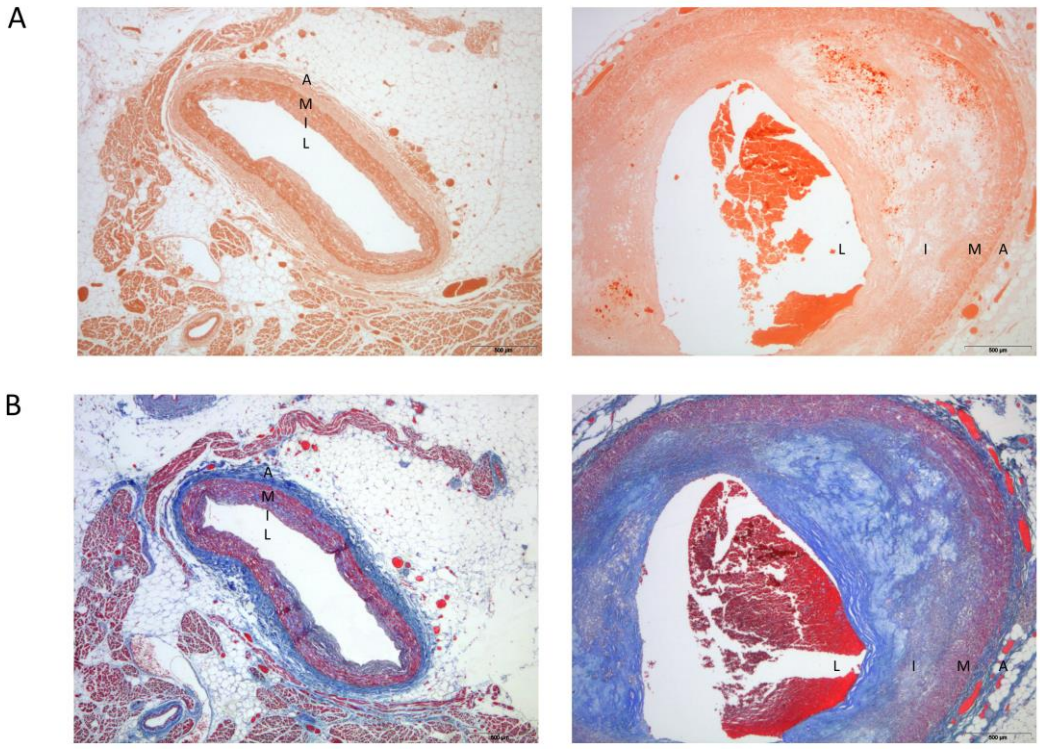


Figure 9. Representative micrography of coronary arteries (x20). (A) Comparative image between arteries from unaffected group (left) and affected arteries (right) using Alizarin Red staining. The presence of calcium in affected arteries is limited to the intima.(B) Comparative image between arteries from unaffected group (left) and affected arteries (right) using Masson's trichrome staining. L (Lumen); I (Intima); M (Media); A (Adventitia).

4.3 Immunohistochemical differences between unaffected patients and CAD patients

Using immunohistochemical techniques, it was possible to evaluate the inflammatory process in coronary artery tissue from less affected/unaffected individuals and patients with coronary artery disease (CAD).

By the detection of CCL2, which is a monocyte chemoattractant protein, we could evaluate inflammatory processes in tissue of non-affected coronary arteries and pathological coronary arteries.

Figure 10 shows the obtained results from the immunohistochemical staining of CCL2. In unaffected arteries (A), there was not a high expression of CCL2, whereas in individuals with CAD (B), there were a further increase of CCL2 in media and adventitia due to an increase of atherosclerosis. Statistically significant differences ($p<0.0001$) were observed between non-affected arteries and CAD arteries, as shown in graph (C).

In figure 11, we could observe the immunohistochemical detection of CCR2 receptor (the specific receptor of CCL2) in non-affected and CAD arteries. CCR2 was located in the media in a greater proportion than in the adventitia and intima in both non-affected (A) and affected (B) arteries. In affected arteries (B) we could determine that when there is a thickening in the intima, the presence of CCR2 is greater in endothelial cells and in the boundary between layers, data not shown in the figure. So, there are significant differences ($p=0.0306$) between control and CAD (C).

As shown in the Figure 12, the presence of macrophages (CD68) in non-affected arteries (A) was significantly lower ($p=0.0025$) than in samples from patients with CAD (B). The accumulation of CD68 in these patients was mainly found in the intima and in less intensity in the adventitia. That is because atherosclerotic lesions were in advanced stages, with presence of smooth muscle cells, an atherosclerotic plaque and an increased concentration of lipids. Consequently, as we could see, this leaded to an increase in the presence of monocytes in those arteries.

In Figure 13, we could see the immunohistochemical results for the atypical receptor DARC. DARC receptor was not located in a specific artery layer. In unaffected arteries (A) the expression of DARC was mild in all of the layers although, we could observe a high concentration of this receptor in the smooth muscle that surrounded the artery. Nevertheless, in affected arteries (B) DARC receptor expression was higher and it was located in the intima, media and adventitia at different ratios. These differences between non-affected individuals and CAD individuals were significantly different ($p=0.0059$) (C).

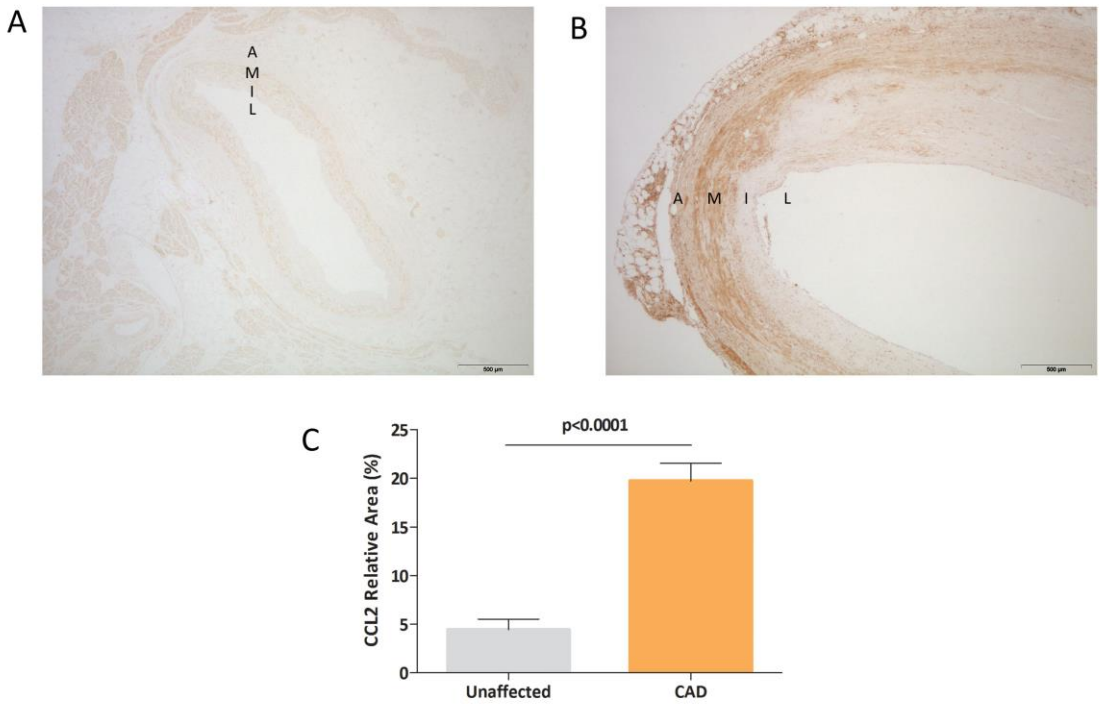


Figure 10. Representative micrography of immunohistochemical staining of CCL2 (x20) in non-affected or minimally affected coronary arteries (A) and in affected coronary arteries (B). CCL2 was mildly expressed in non-affected arteries, whereas the expression was more intense in the media and adventitia of affected arteries. (C) Graphical display of positively stained area on total area (% relative area). Mann-Whitney U test was used to compare unaffected vs. CAD. L (Lumen); I (Intima); M (Media); A (Adventitia).

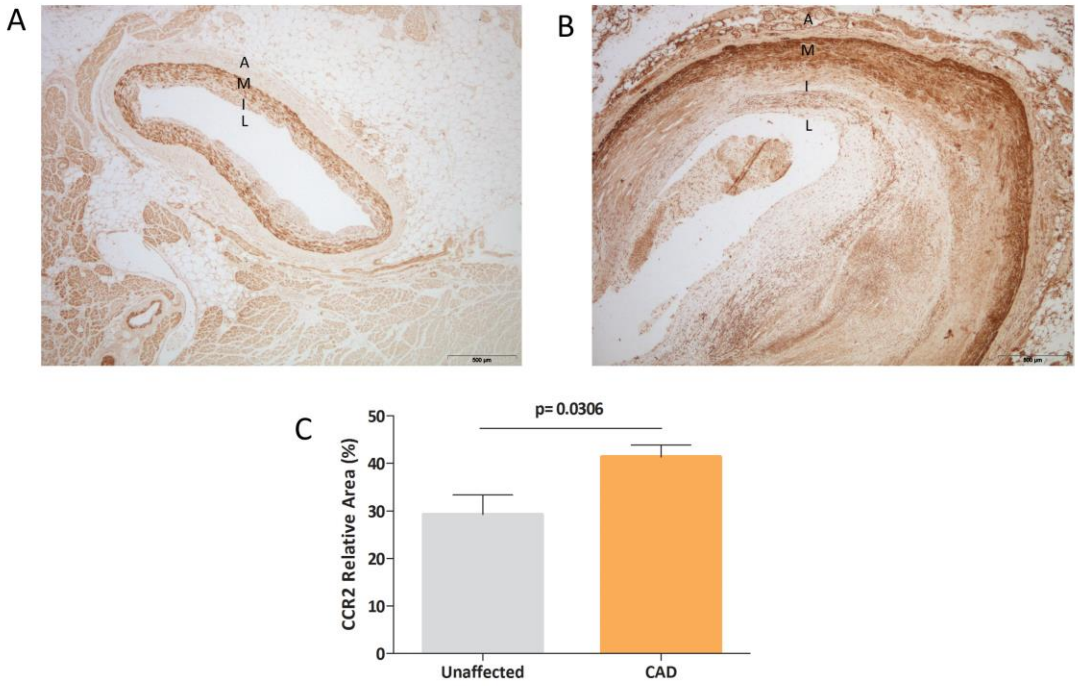


Figure 11. Representative micrography of immunohistochemical staining of CCR2 (x20) in non-affected or minimally affected coronary arteries (A) and in affected coronary arteries (B). CCR2 was higher expressed in affected coronary arteries in the media. (C) Graphical display of the manual quantification of positively stained area on total area (% relative area). Mann-Whitney U test was used to compare unaffected vs. CAD. L (Lumen); I (Intima); M (Media); A (Adventitia).

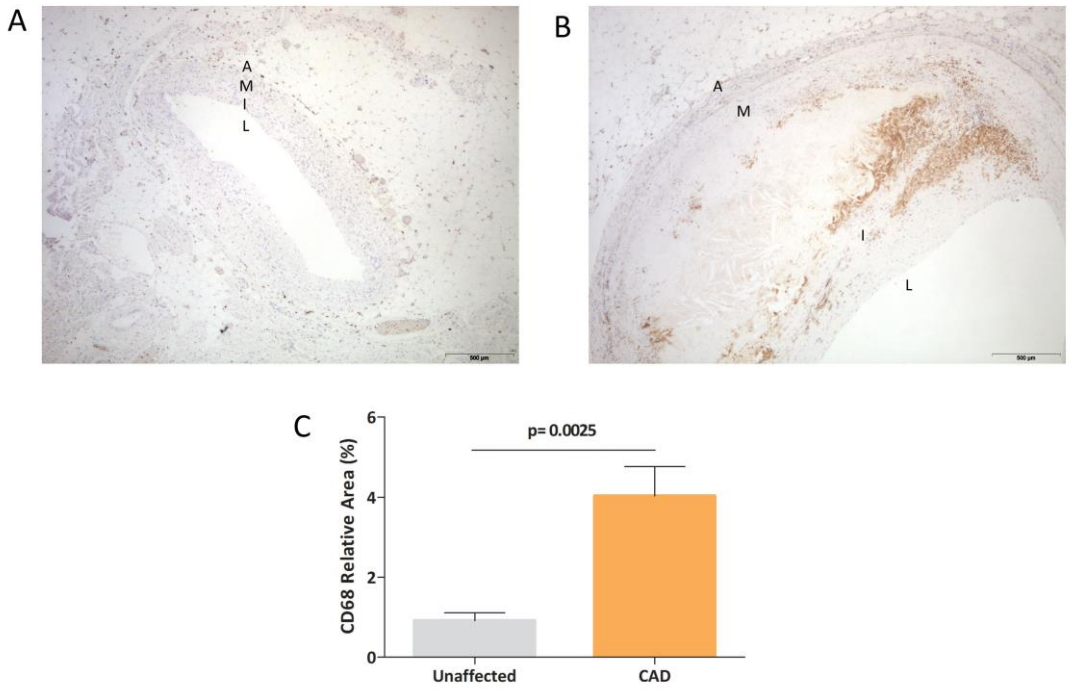


Figure 12. Representative micrography of immunohistochemical staining of macrophages (CD68) (x20) in non-affected or minimally affected coronary arteries (A) and in affected coronary arteries (B). Macrophages were not detected in less affected coronary arteries. However, in affected coronary arteries, the expression was more intense in the intima, but could also be located in the adventitia (B). (C) Graphical display of the manual quantification of positively stained area on total area (% relative area). Mann-Whitney U test was used to compare unaffected vs. CAD. L (Lumen); I (Intima); M (Media); A (Adventitia).

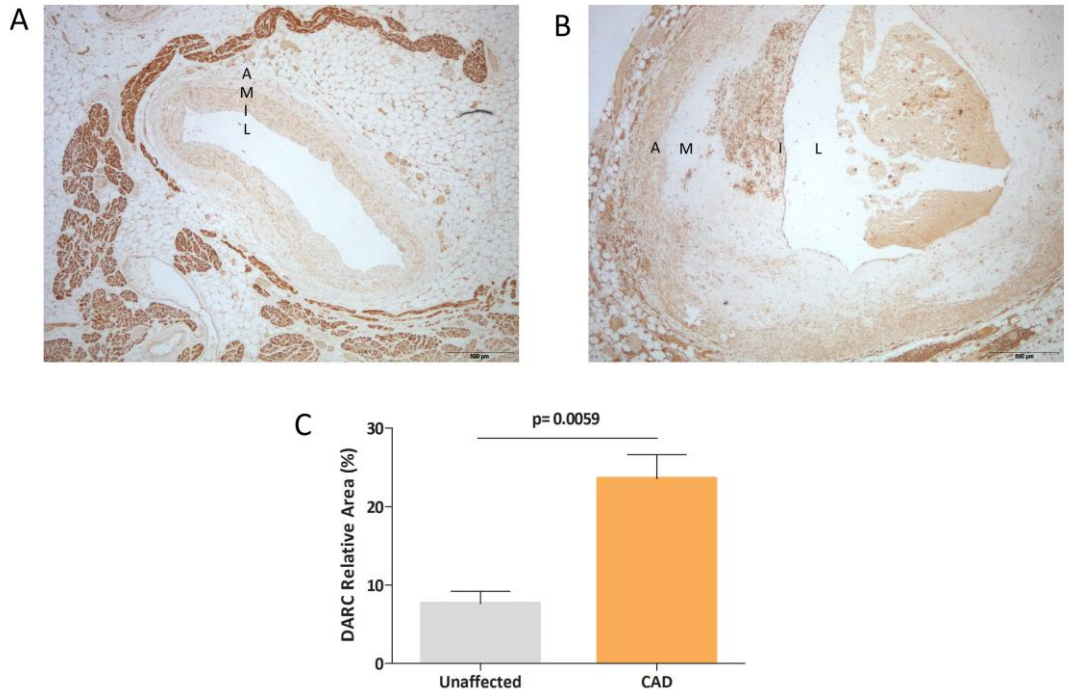


Figure 13. Representative micrography of immunohistochemical staining of DARC (x20) in non-affected or minimally affected coronary arteries (A) and in affected coronary arteries (B). DARC receptor was not expressed in a specific layer of the arteries; although it was highly expressed in the muscle that surrounded the vase. (C) Graphical display of the manual quantification of positively stained area on total area (% relative area). Mann-Whitney U test was used to compare unaffected vs. CAD. L (Lumen); I (Intima); M (Media); A (Adventitia).

As we can observe in figure 14, the expression of D6, an atypical receptor of CCL2, was mainly located in the adventitia in normal or minimally affected coronary arteries (A). In affected arteries, the expression of this receptor was significantly higher (p=0.0010) compared to the less affected/unaffected group. D6 was mainly located in the adventitia but we could observe an increase in its expression in the intima and media because of the advanced and severe stage of atherosclerosis. In these cases, the intima had a large and undifferentiated size.

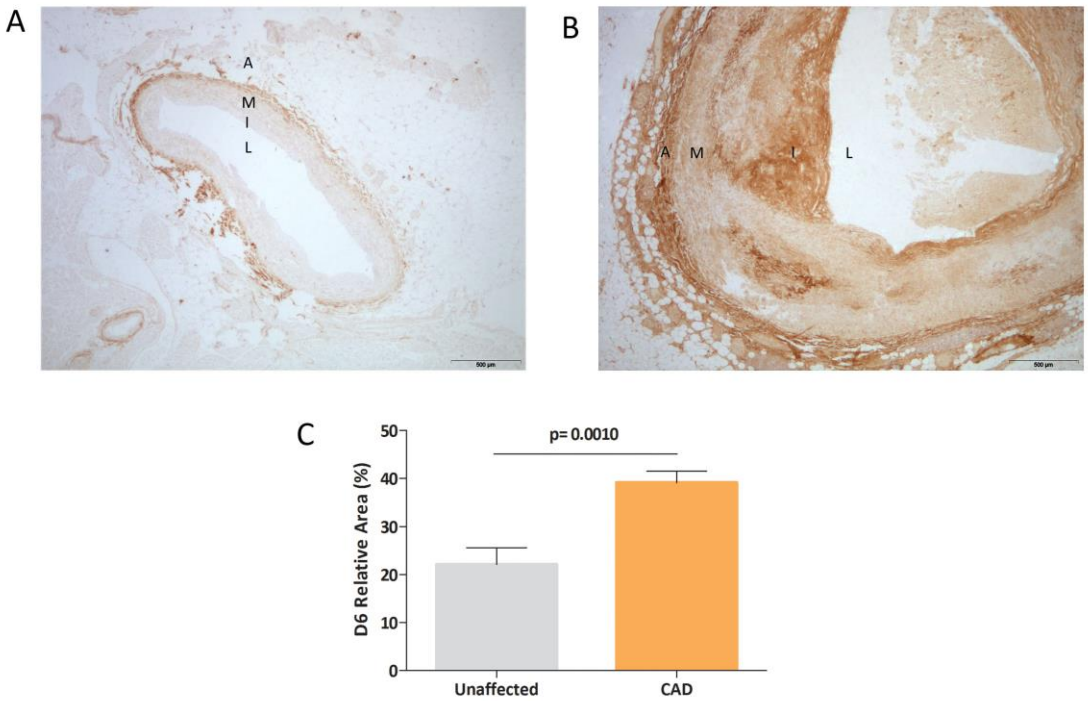
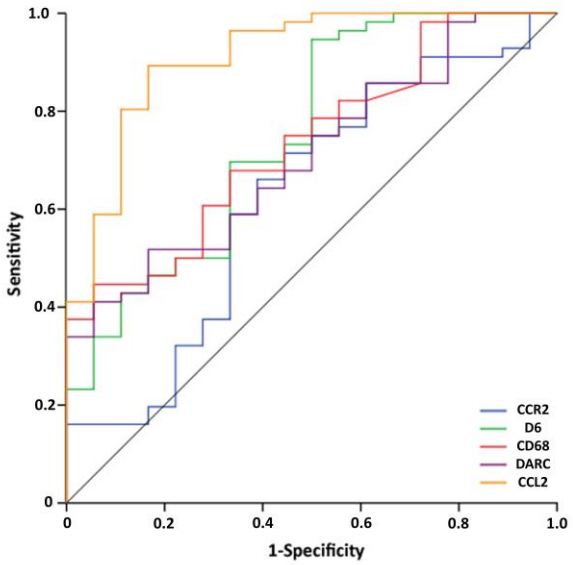


Figure 14. Representative micrography of immunohistochemical staining of D6 (x20) in non-affected or minimally affected coronary arteries (A) and in affected coronary arteries (B). D6 were almost absent in less affected arteries except a mild presence in the adventitia (A). In affected arteries, D6 was more intense in the adventitia and also in the media and intima. (C) Graphical display of the manual quantification of positively stained area on total area (% relative area). Mann-Whitney U test was used to compare unaffected vs. CAD. L (Lumen); I (Intima); M (Media); A (Adventitia).

4.4 CCL2 and its relationship to coronary artery disease (CAD)

The presence of CCL2 and their receptors in affected coronary arteries (CAD patients), as observed in the immunohistochemical results, is greater than in non-affected coronary arteries. In order to validate the results, a statistical study was conducted to determine if any of the proteins studied, besides being significantly different between groups, could be a potential biomarker of coronary artery disease.



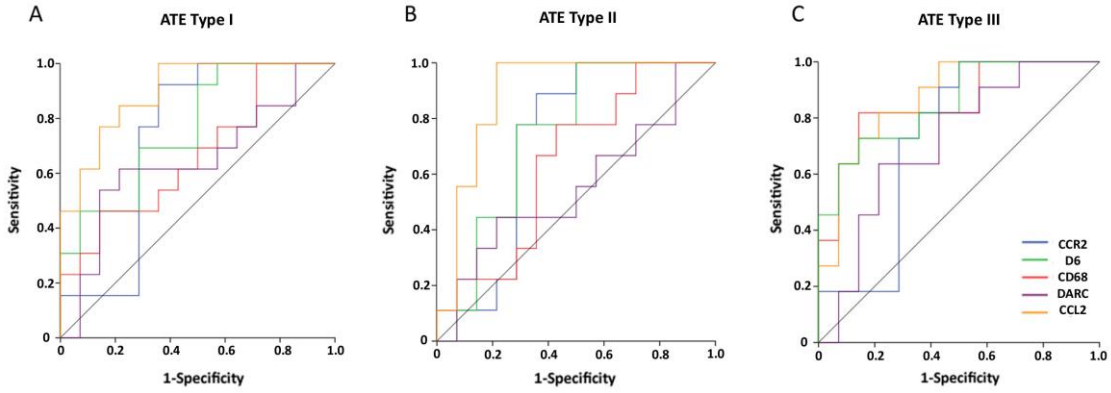
Variable	Area	Error	P-value	95% CI		Sensitivity (%)	Specificity (%)
				Lower	Upper		
CCR2	0.624	0.079	0.115	0.469	0.779	80.0	38.9
D6	0.749	0.069	0.002	0.614	0.884	80.0	50.0
CD68	0.734	0.063	0.003	0.611	0.857	80.0	44.4
DARC	0.707	0.064	0.008	0.581	0.834	80.0	38.9
CCL2	0.911	0.041	<0.001	0.831	0.991	80.0	89.0

Figure 15. Graphical representation of ROC curves for the different proinflammatory molecules of the study. CCL2, CD68 and atypical receptors were the better predictors of CAD. Among them, CCL2 had the biggest area under the curve and the biggest specificity.

A ROC curve was done in order to evaluate which protein could better distinguish the individuals between unaffected and CAD groups. As seen in Figure 15, CCL2, the atypical receptors of CCL2 and CD68 were good predictors of atherosclerosis. Among them, CCL2 was the molecule with the best sensitivity and specificity, because the area under the curve was significantly higher compared to the other molecules.

Taking these results into account, we wondered whether CCL2 would be able to distinguish individuals in different stages of coronary atherosclerosis from non-affected population.

As shown in the figure 16, CCL2 was able to differentiate the populations in all the stages of atherosclerosis development: mild (ATE Type I), moderate (ATE Type II) or severe (ATE Type III). It was also the protein with the best area under the curve in all stages, and moreover, it had a high sensitivity (80%) and specificity (78.6 %). It is important to note that CCR2 and the atypical receptor D6 may be important during the first stage of atherosclerosis (ATE type I) whereas CD68 and the atypical receptor D6 when atherosclerosis has evolved into a serious and a problematic disease (advanced stages).



D

	CCL2	Area	Error	P-value	95% CI Lower	95% CI Upper	Sensitivity (%)	Specificity (%)
Control vs	ATE Type I	0.896	0.059	<0.001	0.780	1.000	80.0	78.6
	ATE Type II	0.889	0.072	0.002	0.748	1.000	80.0	78.6
	ATE Type III	0.870	0.071	0.002	0.731	1.000	80.0	78.6

Figure 16. Representations of ROC curves for different proinflammatory molecules of the study. (A) Unaffected patients vs patients with mild atherosclerosis. (B) Unaffected patients vs. patients with moderate atherosclerosis. (C) Unaffected patients vs patients with severe atherosclerosis. (D) CCL2 had the best values compared to the other inflammatory molecules in the different stages of atherosclerosis. ATE Type I (mild atherosclerosis); ATE Type II (moderate atherosclerosis); ATE Type III (severe atherosclerosis).

5. DISCUSSION

In recent years, there has been an increased consumption of high fat diets, increased consumption of tobacco and sedentary lifestyles, consequently an increase of obesity, dyslipidemia, hypertension and diabetes mellitus. That is why atherosclerosis (and its manifestation in coronary arteries) has become one of the leading causes of death in the world according to the World Health Organization (WHO) [40]. So, the search for new biomarkers to improve both the prevention and treatment of this disease is essential.

The main processes in atherosclerosis are atherogenesis and destabilization of atherosclerotic plaque. Both factors are important for the study of the pathogenic mechanisms of this disease.

Atherosclerosis is considered an inflammatory disease; several studies suggest that chemokines are important factors in this process [40-42]. It is currently accepted that CCL2 is involved in this pathology as a cardiovascular risk marker [43, 44].

Taking a look to the population studied in this trial, patients with coronary artery disease (CAD) die mainly due to this disease when they are old, about 65 years old. It may be because this disease is linked to aging problems, so the older the population, the worse the prognosis. It causes more deaths in men (about 73%) than in women. This population tends to be smoker and suffer from hypertension. These factors are characteristic in people developing atherosclerosis, and consequently people with a high cardiovascular risk [11, 40].

Compared to non-affected group, there was a significant increase in hypertension and in problems related to heart in the affected individuals. For example, patients with CAD have an increased heart weight, a higher ventricular thickness and affected valves. These features were corroborated in studies carried out by William C. *et al*. [1990]. They observed that heart weight in patients with CAD was higher, principally in patients with a severe degree of atherosclerosis in their coronary arteries [45]. Moreover, the lesions in the valves and the left ventricle were significantly higher in patients with CAD [46, 47].

The pathophysiology of atherosclerosis is accompanied by significant lipid metabolic disturbances. With regard to the biochemical results, it has only been detected a significant increase in triglycerides concentration. However, it can be noted that total cholesterol and LDL cholesterol tend to be higher in affected individuals whereas HDL cholesterol level was lower in these patients. [2, 13].

In the histological observations of non-affected or minimally affected arteries (control population), we observed normal histology without any important alteration in the intima and media. On the other hand, most of the individuals with affected coronary arteries had serious intimal disorganization caused by the presence of smooth muscle cells, an atherosclerotic plaque and an extracellular lipid core (in some cases, including cholesterol crystals) [11]. Therefore, these arteries showed a significant increase in IMT and in I/M.

Deo *et al*. [2004], observed while measuring the levels of CCL2 in plasma in patients with CAD, that coronary artery calcification (CAC) could be associated with levels of CCL2, as a possible marker of subclinical atherosclerosis [40]. These data coincide with our results in tissue, where there was a significant calcium load (mainly in the intima) in coronary arteries of affected patients, and it could be clinically important.

The performed study has shown that coronary arteries of individuals suffering from CAD have an expression of monocyte chemoattractant protein-1 (CCL2) significantly higher compared to arteries of less affected/unaffected population. The results are related to various studies, both in mice and humans, [40, 48-50] which have shown that CCL2 plays an important role in the progression of atherosclerosis in coronary arteries.

The role of CCL2 in recruiting monocytes and subsequent differentiation is involved in the initiation and progression of atherosclerosis [48]. It has also been determined that CCL2 is located primarily in the middle layer in both non-affected and CAD patients. Moreover, in affected arteries, it is also expressed in the adventitia and this location may be important for the role in the migration of monocytes to the media.

The availability of CCL2 may vary depending on the effects induced by the expression of functional receptors (CCR2) and the presence of atypical chemokine receptors (DARC and D6). The last ones are thought to have an unknown function in the regulatory network, but it is known that they modulate leukocyte migration by capturing CCL2 molecules [51].

As noted, CCR2 expression was significantly higher in individuals with CAD compared to non-affected group. These high levels of CCL2 and CCR2 reflect the relationship between ligand and receptor to mediate signal transduction. CCL2/CCR2 not only mediates recruitment of monocytes/macrophages in inflammatory processes, but also induces the transcriptional regulation of genes that lead to differentiation and cell death in cardiovascular disease [50]. The fact that CCR2 and CCL2 are expressed mainly in the media could be a predictor of their role in inflammation and the formation of atherosclerotic plaque.

Some studies have shown that CCL2 is a critical molecule for the recruitment of monocytes expressing CCR2 [2, 19, 50]. In fact, CCL2 is continuously recruiting monocytes during atherosclerotic plaque formation, and then they get differentiate into macrophages [49].

Macrophages can be detected with anti-CD68 antigen, so CD68 could be established as possible molecular marker of atherosclerosis in a near future [52]. Thereby, CCL2 expression could be related to CD68 expression. So, it is not strange that arteries of CAD individuals had a higher expression of CD68 compared to unaffected patients. These increased macrophages in patients with CAD are a clear factor related with inflammation and atherosclerotic plaque formation [53]. They are mainly located in the intima because of the accumulation of cholesterol-laden macrophages in fatty plaque formation process in atherosclerosis [53].

The atypical receptors DARC and D6 have not any role in cell migration process. They are believed to play an important role in modulating the inflammatory response, so preventing atherosclerosis formation [33, 35]. These receptors do not elicit a signal transduction and exhibits poor selectivity towards chemokines [33].

The current theory on atypical receptor DARC suggests that it acts as a regulator of chemokines signalling [33, 35]. In this study, DARC levels in affected coronary arteries are significantly higher than in unaffected patients. It has been demonstrated both in mice and humans that DARC is considered a decoy receptor for CCL2. Reports suggested that the Duffy antigen absorbs chemokines and stimulates the disappearance of chemokines from the plasma. Studies carried out by Fukuma *et al*. [2003] demonstrated, using gene knockout mice, that the Duffy antigen delays the disappearance of chemokines from the plasma. They proposed that the Duffy antigen acts as a reservoir of chemokines: it can release chemokines when plasma concentrations decrease. Therefore, it is believed that Duffy antigen may have two functions: it acts as a regulator of cytokines levels in the circulation and it acts as a reservoir of chemokines in the plasma. [32, 54]. Moreover, studies carried out by Jilma – Stohlawetz *et al*. [2001] showed that Duffy–negative humans were noted to have significantly lower basal CCL2 levels tan Duffy-positive ones [32] and these results were corroborated by other studies [31, 32, 35]. Although DARC seems to have a key role in the formation and progression of atherosclerotic plaque, is not sufficient evidence to show the relationship between the expression of DARC in inflammatory processes and the progression of atherosclerotic disease. Therefore, more studies are advisable to determine the role of DARC protein in atherosclerosis [32].

In this study, it was observed that D6 levels in affected arteries were significantly higher, mainly in the adventitia compared to media and intima. D6 is thought to act as a probable regulator of inflammation [33, 35]. It is expressed in leukocytes, especially in those that invade inflamed tissues. In contrast to DARC, D6 does not promote chemokine transcytosis, but rather contributes to their degradation [34, 55]. Torre *et al.* [2005] and other authors have seen that D6 Knock-out mice show high levels of inflammatory chemokines. Nibbs *et al.* [2007], determined that the over expression of D6 reduce leukocyte response in inflammation models [33]. Taking this into account, the most likely physiological role of D6 is to clear tissues from remaining chemokines in order to prevent an excessive response, and eventually terminate the inflammatory response [33, 34].

DARC and D6 have not been shown to present any relationship between each other. It may be because these chemokine scavengers mediate two different mechanisms by which the concentration of proinflammatory chemokines is controlled [31, 32]. We think that the expression of these receptors in affected arteries may be a result of the failed attempt to control CCL2 concentration. In a chronic inflammatory state, CCL2 is thought to be produced continuously. In this case, atypical receptors would not be able to control its concentration by themselves. That is why, in the end, there is a formation of atherosclerotic process.

Recent results obtained in our laboratory using “healthy” arteries (not coronary arteries but peripheral arteries) from our Biobank show that there isn’t any expression of CCL2, CD68, CCR2 and DARC or it is significantly lower compared to the arteries with atherosclerotic involvement. However, we have observed that expression of D6 can be found in “healthy” arteries; the same result that we have observed in this study in unaffected arteries. Therefore, the expression of these proteins is closely related to the progression of atherosclerosis and consequently with an increased risk of cardiovascular affectation.

CCL2 levels have allowed differentiating unaffected individuals from CAD patients (in any of the stages: mild, moderate or severe). This may be because CCL2 is used to predict patients with cardiovascular risk, as well as being a good inflammatory biomarker. However, it must be note that CD68, DARC and D6 present a good ability to differentiate unaffected patients from CAD patients, but they don’t have a good predictive accuracy.

Finally, preliminary results of ongoing validation studies suggest that the use of CCL2 as a biomarker in tissue requires further assessments. There is some controversy on whether or not it is a good marker of cardiovascular risk [56]. Nonetheless, many authors suggest that CCL2 could be attractive as a surrogate biomarker in these patients and merits further study as a potential therapeutic target [19, 40, 48-50]. However, it may be important to study the role of CCL2 in plasma, because it will be easier than taking a tissue sample.

6. CONCLUSIONS

In recent years, atherosclerosis has been linked to aging and related to an imbalance in inflammatory process. Chemokines seem to have a key role in the regulation of the metabolism and are also important in the formation of atherosclerotic plaque.

The histologic features of coronary arteries with atherosclerosis are modified, as they have an irregular structure of the intima and media, an increase the calcium deposits, presence extracellular lipid core and formation the crystals of cholesterol.

Considering our results, CCL2 and macrophages are expressed in a greater proportion in coronary arteries. The presence of both is a key factor in the initiation and progression of coronary artery disease. CCL2 atypical receptors (DARC and D6) are also expressed in a higher proportion, in a failed attempt to modulate the chemokine concentration and the inflammatory process. So, these silent receptors are linked to a change in the histological features of the arteries and an aggravation of the atherosclerotic process. Whether this is causality important remain to be fully understood.

Finally, CCL2 has been proposed as a biomarker. However, it is needed more research to find a therapeutic strategy to prevent and monitor the disease.

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