# Inflammatory actions of chemokines and extracellular versicles in pathological tissue remodeling

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Dawid Kaczor

**Inflammatory actions of chemokines and extracellular vesicles in pathological tissue remodeling**

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# Inflammatory actions of chemokines and extracellular vesicles in pathological tissue remodeling

Dissertation

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To my dear parents

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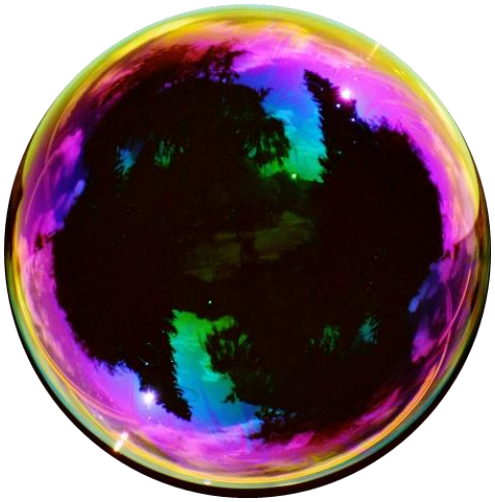
# Chapter 1

## General introduction



## Cardiovascular disease and atherosclerosis

The ageing population continues to suffer from its primary killer, cardiovascular disease (CVD). Most CVD arises from thrombotic rupture of an atherosclerotic plaque, the pathologic thickening of susceptible parts of coronary and carotid arteries and subsequent distal ischemia in heart or brain. The ischemia is mostly caused by rupture, erosion or passive occlusion of the blood vessel. In fact, one-fifth of deaths are directly attributable to thrombotic rupture of a vulnerable plaque. Atherosclerotic lesion formation is caused by a concert of interactions between circulating leukocytes and platelets, interacting with the endothelial barrier, signaling into the arterial wall by the release of cytokines and extracellular vesicles (EVs)[1]. These interactions result in chronic activation of endothelial cells (ECs) that orchestrate the recruitment of inflammatory cells as well as ECs transformation into mesenchymal-like cells (e.g. fibroblast- or smooth muscle cell-like cells)[2]. Furthermore, lipid accumulation and subsequent foam cell formation within the plaque and phenotypic switching of vascular smooth muscle cells (VSMCs) lead to increased migration, proliferation, and secretion of EVs. These processes may also trigger calcification of blood vessels, a consequence of calcium-phosphate crystal formation within the arterial wall[1].

## Extracellular vesicles

Extracellular vesicles (EVs) are highly heterogenous structures, playing an important role in cell-cell communication. They differ in size, biochemical content and type of secretion. Current nomenclature distinguishes three populations of EVs based on size: exosomes, microvesicles and apoptotic bodies[3,4,5]. EVs are small phospholipid membrane-enclosed entities released spontaneously by virtually all cell types into various biological fluids such as blood, saliva and urine[6] in multiple physiological (e.g. cell maturation and aging) and pathological conditions, thus they are attractive diagnostic biomarkers determining the severity of various diseases[7]. In terms of pathological states, cardiovascular disease represents one of the most intensively studied and rapidly developing areas of the extracellular vesicle field[4]. It has been estimated that in the circulation 70–90% of all EVs are derived from platelets and megakaryocytes, 10% from granulocytes and only 5% from endothelial cells, red blood cells and monocytes together[8]. The number of circulating EVs levels has been shown to be augmented in association with various cardiovascular risk factors, including atherosclerosis, diabetes mellitus, dyslipidaemia, hypertension and smoking[9]. Mouse models and human subjects with fatty liver disease also demonstrate increased levels of circulating EVs, released mainly by hepatocytes. These EVs play a role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD), particularly in promoting hepatic cell death, inflammation, pathological angiogenesis and fibrogenesis[6].

## Vascular calcification

Mineralization is a physiological process, sustaining biomechanical and functional properties of bones and teeth. Mineralized tissues execute various functions including internal support and protecting of vital organs. Under normal conditions soft tissues such as blood vessels, kidneys or skin do not mineralize (calcify). However, under pathological conditions, soft tissues as well as some body fluids (i.e. urine, synovial fluid) are prone to calcification, which is generally destructive of compromised tissue[10]. Initially, calcification was considered a passive process – an inevitable consequence of aging and disease, depending solely on extracellular calcium and phosphate levels. Today, we appreciate that vascular calcification is an active, genetically regulated process[10]. Vascular calcification is the accumulation of calcium phosphate salts (mostly in the form of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]) that can occur in the blood vessels, myocardium and cardiac valves[11]. The arterial wall consists of three layers: the

tunica intima (endothelium), the tunica media (vascular smooth muscle cells) and the tunica adventitia (connective tissue)[12]. Intimal calcification is observed in atherosclerosis, whereas medial calcification is common in diabetes, dyslipidaemia and renal disorders[11] (Fig. 1). The morphology of the calcification directly determines the associated cardiovascular risk. Larger, denser calcifications (macrocalcifications) may stabilize atherosclerotic plaques, whereas smaller, ”spotty” calcifications (microcalcifications) seem to contribute to plaque destabilization, plaque rupture and eventually myocardial infarction or stroke[13].

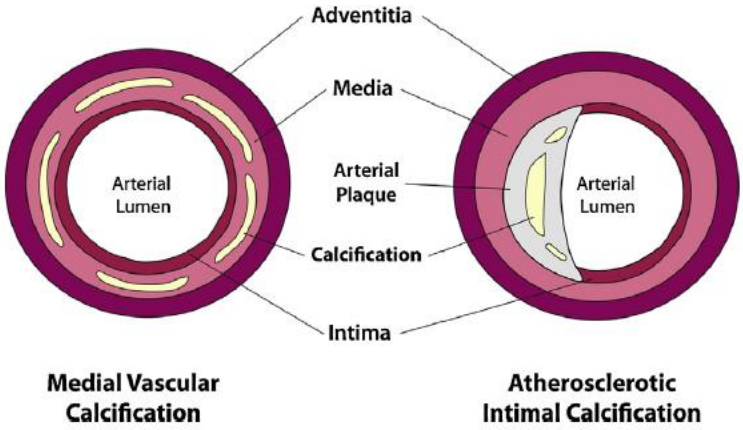



Figure 1. Medial and intimal calcification. Adapted from[14].

### The role of VSMCs phenotypic switch and matrix vesicles in vascular remodeling

A combination of in vitro studies and molecular imaging have formed the concept of an inflammation-dependent calcification, suggesting that macrophage infiltration and inflammation precede calcification[7]. Normally, VSMCs retain in their quiescent, contractile state, but in response to pathological signals such as inflammatory cytokines or a mineral imbalance, VSMCs undergo a phenotypic switch, called osteochondrogenic transformation (Fig. 2). This is characterized by expression of bone-related proteins and the release of matrix vesicles (MVs) that mediate active communication between cells[15]. In vitro, exposure of VSMCs to high phosphate concentrations induces loss of their specific markers and upregulation of osteogenic/chondrogenic markers[16,17,18,19] whereas high calcium induces apoptosis related calcification[20,21]. Electron microscopy revealed that matrix vesicles form the first nidus for mineralization and localize within extracellular matrix[7]. Matrix vesicles are heterogeneous in size (30–300 nm in diameter)[20], contain minerals, and appear to originate from both apoptotic and living VSMCs at sites of medial calcification, as well as from macrophages, endothelial cells, VSMCs, and platelets located in atherosclerotic plaques[15]. Healthy VSMCs efficiently prevent vascular calcification by expression of calcification inhibitors, such as: pyrophosphate [PPi], tissue-specific matrix Gla protein [MGP], osteopontin [OPN] and circulating fetuin A[10]. In case of prolonged calcium-phosphate imbalance in the extracellular space, calcifying VSMCs release matrix vesicles containing lower levels of calcification inhibitors and all enzymatic machinery essential to induce calcification[22]. As a consequence, hydroxyapatite crystal forms and grows inside the vesicles, eventually leading to the loss of their structural integrity. Subsequently, mineral crystals are propagated out onto the extracellular matrix where they can grow even further and form micro- and macro-calcifications[13]. Due to endothelial erosion under irregular flow conditions at the site of the plaque, these calcifications might be exposed to the blood, causing thrombotic complications[23].

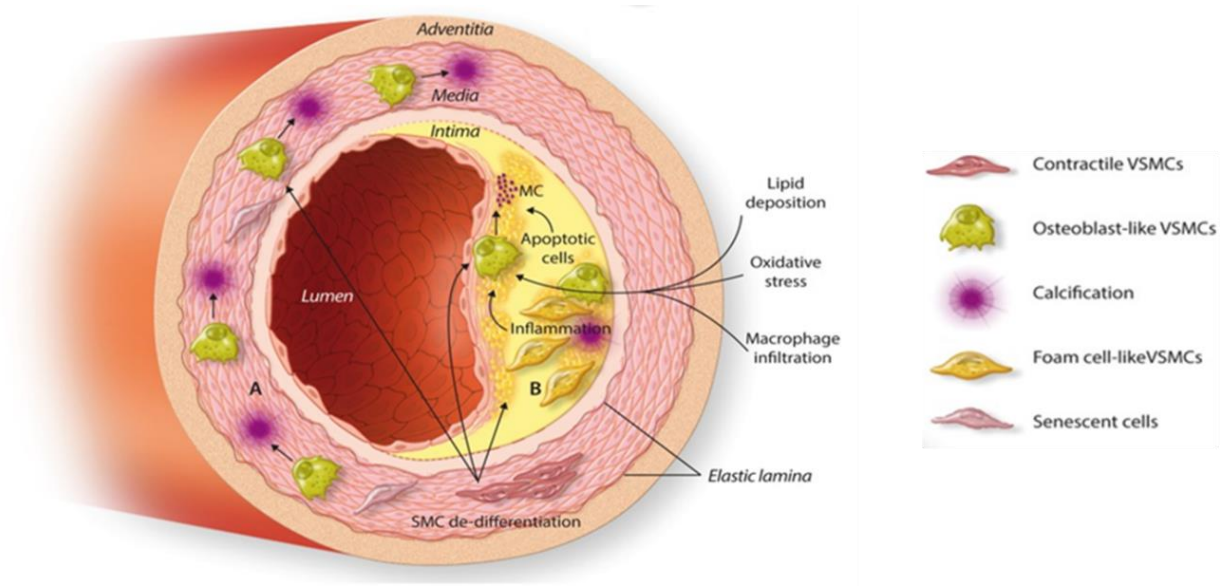



Figure 2. The role of VSMCs in the vessel wall remodeling. Adapted from[19].

## Role of platelet-derived molecules in atherosclerosis

Atherosclerosis is a chronic inflammatory vascular disease involving endothelial cells, vascular smooth muscle cells, mononuclear cells and platelets. Platelets are small, anuclear blood cells derived from bone marrow megakaryocytes and circulate in blood to play a major role in the hemostatic process and in thrombus formation after injury. Platelets have been also recognized to play an important role in inflammation and vessel wall remodeling. Vascular inflammation and smooth muscle cell injury initiate and accelerate a variety of cardiovascular diseases, including atherosclerosis. Under normal conditions, platelets and VSMCs are not in physical contact with each other, but during injury/thrombosis, platelets adhere to the VSMCs and exposed extracellular matrix of the vessel wall and secrete inflammatory molecules that can migrate deep into the vessel wall and affect cells residing in the atherosclerotic niche. Platelet inflammatory mediators include secreted small molecules (ADP, thromboxane A2, serotonin) and surface molecules (CD40 ligand, integrins, P-selectin). Platelet-derived chemokines and cytokines include platelet factor 4 (PF4, CXCL4), RANTES (CCL5), platelet basic protein and its proteolytically processed products β-thromboglobulin and CXCL7, as well as several interleukins (IL-1α, IL-1β, IL-8)[24,25].

## Platelet chemokines

Within their α-granules platelets contain and carry a large variety of bioactive molecules (e.g. chemokines), that can be rapidly released upon activation. Chemokines are small proteins, no larger than 8–10 kDa that primarily control the trafficking of leukocytes by inducing directed cell movement towards the source of chemokine gradients (chemotaxis) during inflammation[26]. Chemokines exert their action through G protein-coupled receptors (GPCR). They are divided into four classes, named according to the type of chemokine (CC, CXC, CX3C or C) with which they interact (Fig. 3). There is apparent redundancy in the system, as many chemokines bind multiple receptors of one class and more than one receptor can interact with each chemokine[27].

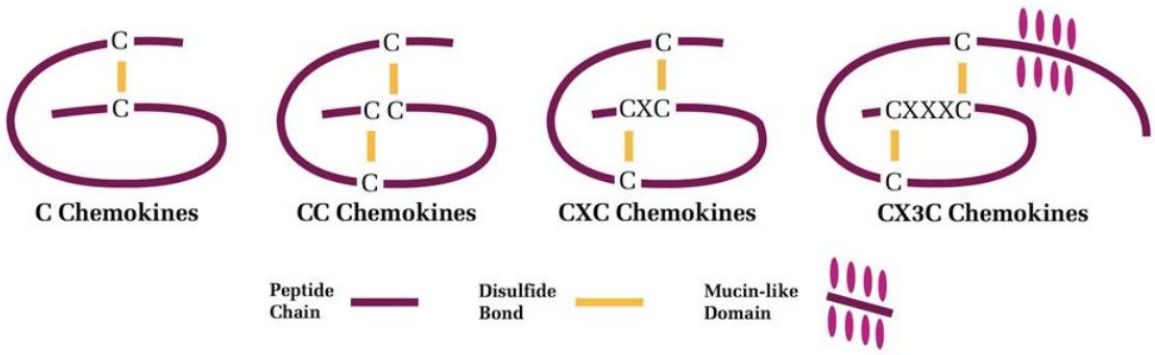



Figure 3. Schematic structure of chemokine classes. Adapted from Peprotech.com.

**Platelet factor 4 (CXCL4) and platelet factor 4 alt (CXL4L1)**

Platelet factor 4 (PF4, CXCL4) is the most abundant platelet protein and have a plethora of biological functions. Even though megakaryocytes and platelets are the major source of CXCL4, its low expression was also found in other cell types like smooth muscle cells, microglia, macrophages, or T cells[26]. Interestingly, plaque macrophages were also shown to prominently express CXCL4 in murine model, which may be of high importance in the development of atherosclerosis[28]. CXCL4 has been shown to interact with many cell types in the vasculature[29]. It binds with exceptionally high affinity to heparin and other negatively charged glycosaminoglycans (GAG), regulating coagulation and thrombosis. It has been also shown to play a role in megakaryocytopoesis, immune modulation, angiogenesis[30] and fibrosis[31,32]. Accordingly, lesional CXCL4 deposition was shown to correlate with the clinical manifestation of atherosclerosis. In human atherosclerotic plaques CXCL4 was detected in the endothelium, in macrophages, and in regions of calcification. It is postulated that CXCL4 is deposited early in the development of atherosclerotic lesions and continues to accumulate in foam cells as the lesion pathology advances[33]. CXCL4 promotes atherogenesis by inducing macrophage polarization towards a proatherogenic phenotype called “M4” which was detected in human atherosclerotic plaques[34]. Moreover, CXCL4 inhibits the catabolism of LDL in vitro in part by competing for binding to LDL-R, by promoting interactions with cell-associated proteoglycans, and by retention of LDL/LDL-R complexes on the cell surface, which predisposes LDL to proatherogenic modifications i.e. oxidation[35]. Simultaneously, CXCL4 mediates oxidized LDL (oxLDL) binding to vascular cells and monocytes via interactions with surface glycosaminoglycans and promotes oxLDL uptake by macrophages as an initial step in early lesion formation[36]. Thus, CXCL4-deficiency reduced lesion formation in two murine models of atherosclerosis and displays a potential target for prevention or treatment[37]. Although CXCL4 has been under intense investigation for more than 40 years, its cellular functions, receptors, and their corresponding signaling pathways are still not fully understood[26].

Platelet factor 4 alt (PF4V1, CXCL4L1) is released by platelets, smooth muscle cells, endothelial cells and is inducible in monocytes by inflammatory mediators[38,39]. CXCL4L1 is a non-allelic variant of CXCL4 which arose from a gene duplication unique to primates. These non-allelic variants show a striking 96% amino acid identity. Only three amino acid substitutions distinguish the mature proteins and yet this subtle difference suffices to alter the chemokine’s three-dimensional structure drastically[40,41]. This region is also critical for CXCL4-heparin interactions, reduction of CXCL4L1’s affinity for GAGs[42] and it makes CXCL4L1 a more potent inhibitor of angiogenesis than CXCL4[30]. In platelets, CXCL4 and CXCL4L1 are likely both released from alpha granules upon activation. In VSMCs, CXCL4 is also released upon activation, but CXCL4L1 appears to be secreted through a constitutive pathway. This constitutive secretion of CXCL4L1 by VSMCs may represent a physiologic regulatory mechanism to maintain vascular homeostasis and to avoid EC proliferation and aberrant

angiogenesis[43]. While CXCL4 plays rather a proinflammatory role in atherogenesis, clinical data suggests that CXCL4L1 might act as a competitive antagonist with a mainly anti-inflammatory and plaque-stabilising effects[29].

### C-C motif ligand 5 (CCL5)

CCL5 is a soluble chemokine secreted by many different cell types, such as ECs, SMCs, activated T cells, macrophages, and platelets[44,45]. It can be deposited on the vessel wall by rolling platelets and its transfer to ECs was shown to facilitate subsequent monocyte arrest[46,47]. Studies in humans and murine models of atherosclerosis have demonstrated a central role of CCL5 in the formation of atherosclerotic plaque and pathological process of late-stage atherosclerosis[48]. Infusion of activated platelets into hyperlipidemic mice resulted in an accelerated development of atherosclerosis, which could be attributed in part to increased immobilization of CCL5 onto the atherosclerotic vessels[49]. Extensive studies have proven that expression of CCL5 was elevated in patients suffering from acute coronary events[50,51,52] and was independently predictive of adverse cardiac outcomes in patients with chronic coronary artery disease[53,54]. The utility of both serum and plaque CCL5 levels as indicators of plaque vulnerability in rabbit model of atherosclerosis was also shown[55]. Interestingly, CCL5 and CXCL4 can interact with each other to form heterodimers, which are particularly potent in the recruitment of monocytes[56] and were shown to modulate the severity of atherosclerosis, stroke, abdominal aneurysm, and myocardial infarction in mice[57,58,59,60,61]. Although the presence of CXCL4 led to increased binding of CCL5 to the surface of monocytic cells, it is unclear whether this explains the synergy between those chemokines[56].

### Aims and outline of the thesis

The research conducted in this thesis aims to unravel the role of chemokines and extracellular vesicles in the pathological remodeling in the development of atherosclerosis and liver fibrosis. In **chapter 1**, a brief introduction about platelets, platelet-derived chemokines, extracellular vesicles (EVs) and vascular smooth muscle cells (VSMCs) will be given. In **chapter 2**, the involvement of EVs derived from platelets and vascular smooth muscle cells (VSMCs) in the formation of calcium-phosphate deposits within the vessel wall will be studied. In **chapter 3**, an extensive review on the initiation of atherosclerosis with the focus on calcification of blood vessels is provided. In **chapter 4**, the effects of platelet-derived chemokines (CXCL4, CXCL4L1) on human vascular smooth muscle cells (VSMCs) will be studied. In **chapter 5**, the interactions of platelet-derived chemokines (CXCL4 and CCL5) with endothelial cells (ECs) will be explored. In **chapter 6**, the role of EVs, isolated from steatotic hepatocytes and their effects on stellate cells will be established. In **chapter 7**, the key findings of the research described in this thesis and their relevance in relation to the current literature are outlined. In **chapter 8** the reader will find the summary of this thesis.

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# Chapter 2

## Initiation and propagation of vascular calcification is regulated by a concert of platelet- and smooth muscle cell-derived extracellular vesicles

Leon J. Schurgers, Asim C. Akbulut, Dawid M. Kaczor, Maurice Halder, Rory R. Koenen, Rafael Kramann

Front. Cardiovasc. Med. 2018; 5(36)

## Abstract

The ageing population continues to suffer from its primary killer, cardiovascular disease (CVD). Despite recent advances in interventional medicinal and surgical therapies towards the end of the 20th century, the epidemic of cardiovascular disease has not been halted. Yet, rather than receding globally, the burden of CVD has risen to become a top cause of morbidity and mortality worldwide. Most CVD arises from thrombotic rupture of an atherosclerotic plaque, the pathologic thickening of coronary and carotid artery segments and subsequent distal ischemia in heart or brain. In fact, one-fifth of deaths is directly attributable to thrombotic rupture of a vulnerable plaque.

Atherosclerotic lesion formation is caused by a concert of interactions between circulating leukocytes and platelets, interacting with the endothelial barrier, signalling into the arterial wall by the release of cytokines and extracellular vesicles (EVs). Both platelet- and cell-derived EVs represent a novel mechanism of cellular communication, particularly by the transport and transfer of cargo and by reprogramming of the recipient cell. These interactions result in phenotypic switching of vascular smooth muscle cells (VSMCs) causing migration and proliferation, and subsequent secretion of EVs. Loss of VSMC attracts perivascular Mesenchymal Stem Cells (MSCs) from the adventitia which are a source of VSMCs and contribute to repair after vascular injury. However, continuous stress stimuli eventually switch phenotype of cells into osteochondrogenic VSMCs facilitating vascular calcification.

Although Virchow's triad is over 100 years old, it is a reality that is accurate today. It can be briefly summarised as changes in the composition of blood (platelet EVs), alterations in the vessel wall (VSMC phenotypic switching, MSC infiltration and EV release) and disruption of blood flow (atherothrombosis). In this paper, we review the latest relevant advances in the identification of extracellular vesicle pathways as well as VSMCs and pericyte/MSC phenotypic switching, underlying vascular calcification.

## Extracellular vesicles

Extracellular vesicles (EVs) are currently considered as important physiological players. Their secretion represents a universally active cellular function in all living organisms from bacteria to humans[1]. EVs are highly heterogeneous structures that differ in size, biochemical content and mode of secretion. Current nomenclature distinguishes three populations of EVs: 1) exosomes (30 – 100 nm), which originate when multivesicular bodies fuse with the plasma membrane, 2) microvesicles/ ectosomes (100 – 1000 nm), which are generated by budding of the plasma membrane and 3) apoptotic bodies (>1000 nm), which are formed in the process of programmed cell death[2,3,4,5]. Each family is composed of small phospholipid membrane-enclosed entities released spontaneously, or, in response to cell activation or apoptosis[6]. EV release is stimulated via multiple physiological and pathological conditions, making them potential diagnostic biomarkers for monitoring various diseases[7]. Their presence has been detected in a number of bodily fluids from healthy individuals, such as peripheral blood, urine, saliva and synovial fluid to name a few[1,8,9]. In terms of pathological states, first breakthroughs in the field of EV research were made in oncology and immunology, yet today cardiovascular disease represents one of the most intensively studied and rapidly developing areas of the extracellular vesicle field[4]. The number of circulating EVs levels has been shown to be associated with various cardiovascular and metabolic disorders, including atherosclerosis and diabetes mellitus[8]. In the vasculature, EVs are released from platelets, endothelial cells, smooth muscle cells, erythrocytes and leukocytes[6,7].

## Platelet-derived extracellular vesicles

EVs from platelets were first described in 1967 by Peter Wolf from human blood samples, where they were originally referred to as "platelet dust"[10]. Further studies demonstrated that EVs are released when platelets attach to the vessel wall[11]. Later it was reported that platelet-EVs are composed of two different types: exosomes and microvesicles[12]. Since then platelet EVs have been shown to be involved in several processes in the human body, such as coagulation and atherosclerosis[13]. Given that EVs express phospholipids on their surface, they are capable of binding (activated) coagulation factors. Interestingly, their coagulation activity is 50 – 100 times higher compared to activated platelets[14]. In fact, a genetic disorder that is associated with deficient EVs formation by platelets leads to bleeding[15]. This suggests that promotion of coagulation by EVs is an important physiological mechanism[6].

On the other hand, platelet EVs are known to accumulate in platelet concentrates[16]. Transfusion of platelet concentrates is associated with adverse reactions in the recipient, more often than any other blood-derived product[17]. This might be explained by the fact that platelet EVs are rich in inflammatory molecules (e.g. CD40L), so they can adhere to leukocytes by CD62P – PSGL1 interactions and transport pro-inflammatory signals. Several studies have demonstrated that platelet EVs isolated from platelet concentrates can modulate the phenotype and activities of leukocytes and vascular cells (see below).

Platelet EVs retain many properties of their parent cells, such as the presence of surface specific antigens (Table 1), the ability to deposit chemokines to the vessel wall and to confer inflammatory signals to distal sites[18]. In human blood, platelet-derived EVs are the most abundant population of EVs, despite the fact that erythrocytes are about 30 times more numerous than platelets[19]. It has been estimated that in circulation 70–90% of all EVs are derived from platelets, 10% originate from granulocytes and only 5% come from endothelial cells, red blood cells and monocytes[1]. The number of circulating platelet EVs is also influenced by cardiovascular medication. Antiplatelet agents, antihypertensive agents and cholesterol-lowering drugs inhibit EV

release from platelets[20,21]. However, in studies on patients treated with statins, consensus on the number of circulating platelet EVs has not been established[20,22]. Of note, it has been proposed that the majority of the circulating CD41-positive EVs actually originate from megakaryocytes, rather than from platelets[23]. In addition, platelet-derived EVs might be distinguished from their megakaryocyte-derived counterparts through the detection of surface molecules such as CD62P, LAMP-1, CLEC-2 and GPVI.

Table 1. Surface markers found on platelet EVs.

| Cluster of differentiation (CD) or abbreviation | Trivial or full name | Reference |
|---|---|---|
| CD9 | Tetraspanin-29 | 24 |
| CD29 | Integrin β1 | 25 |
| CD31 | PECAM-1 | 25 |
| CD36 | Platelet GPIV | 26 |
| CD42a | Platelet GPIX | 27 |
| CD42b | Platelet GP1bα | 25,28 |
| CD63 | Tetraspanin-30 | 25 |
| CD59 | Membrane attack complex inhibition factor | 26 |
| CD61 | Integrin beta 3 | 28 |
| CD154 | CD40 Ligand | 29,30 |
| CD184 | CXCR4 | 29 |
| PAR-1 | Protease-activated receptor-1 | 29 |
| CD321 | Junctional adhesion molecule-A | 31 |
| TSP-1 | Thrombospondin-1 | 27 |
| VN | Vitronectin | 32 |
| VWF | Von Willendbrand Factor | 33 |

## Vascular inflammatory functions of platelet-derived EVs

Besides the involvement of platelet-derived EVs in the coagulation process, evidence also points towards a role in immune- and inflammation-related processes. For example, platelet-derived EVs have been shown to influence vascular cells (endothelial cells and smooth muscle cells) and leukocytes, thereby changing their phenotype and function. EVs are considered to play an important role in cell-cell communication, their membrane-enclosed content, small size and repertoire of surface receptors facilitate long distance transport within bodily fluids[5,8,34]. EVs can influence target cells by providing ligands which augment the secretion of growth factors or cytokines, transfer of cell adhesion molecules or reprogram target cells through their genetic make up[1,18,19]. When isolated platelet EVs are incubated with monocytes, platelet EVs readily bind to monocytes and phagocytic uptake of platelet EVs can be observed over time[35]. In chemotaxis assays, monocytic cells are actively attracted by platelet EVs, an effect that can be blocked by antibodies against CCL5. Prolonged incubation of monocytes with platelet EVs results in a notable change of surface marker expression, indicating a polarisation of the monocytes to M2-type macrophages[35]. Finally, platelet EVs were found to induce the secretion of TNFα from monocytic

cells more strongly than platelets, whereas incubation with platelets led to a robust release of GM-CSF[35]. Similar studies have demonstrated that platelet EVs induce the differentiation of macrophages into dendritic cells[26] and that platelet EVs are even able to "reprogram" the gene expression profile and function of macrophages[36]. Although platelet EVs are present both in diseased patients and healthy subjects, increased levels have been associated with various pathological disorders, such as atherosclerosis and diabetes mellitus (Table 2).

Table 2. Cardiovascular/metabolic diseases associated with increased platelet-EV levels.

| Disorder | Reference |
|---|---|
| Hypercholesterolemia and subclinical atherosclerosis | 37 |
| Coronary calcification | 38 |
| Carotid atherosclerosis | 39 |
| Coronary heart disease | 40 |
| Acute coronary syndrome | 41,42,43,44 |
| Peripheral arterial disease | 45,46,47,48 |
| Hypertension | 49,50 |
| Venous thrombo-embolism | 51,52 |
| Stroke | 53,54,55 |
| Diabetes mellitus | 56,57,58,59 |
| Metabolic syndrome and obesity | 60,61,62,63 |

*Table adapted from Aatonen et al.*[64] *and Ridger et al.*[65]

Pathological remodelling of the vasculature involves an intricate and dynamic interaction between blood cells (platelets, leukocytes), vascular cells (endothelial cells, smooth muscle cells and adventitial cells) and their direct microenvironment[66]. EV-mediated signalling between hematological cells and vascular cells is also of importance in this process. Elevation of platelet EVs in cardiovascular disease appears to be a common process, their interaction with the vascular endothelium has been an area of high interest. However, current knowledge in this field is still very limited and our understanding relies mainly on *in vitro* experiments. The importance of leukocyte-endothelium signalling in pathophysiological conditions of the vasculature is already well known. More recently the role of platelet EVs in these processes has been demonstrated. *In vitro* experimentation has shown that shear stress-activated platelet EVs facilitate the interaction between monocytes and endothelial cells. This is facilitated by increases in inflammatory cytokine levels and cell adhesion molecules on both cell types[67]. Another study indicated that platelet EVs contain bioactive lipids (e.g. arachidonic acid), that stimulate ICAM-1 expression in HUVECs, leading to enhanced monocyte-endothelial interactions[68]. Platelet EVs may also activate endothelial cells and leukocytes, more specifically, neutrophils by surface molecules CD41 and CD62P, further demonstrating their importance in modulating inflammation[69]. It has been also shown that platelet EVs can deposit inflammatory molecules such as CCL5 (RANTES) during rolling interactions over endothelial cell monolayers, facilitating the subsequent recruitment of monocytes[31]. In this study, rolling interactions depended on P-selectin and GPIb, while transfer of CCL5 was dependent on integrin αIIbβ3 and junctional adhesion molecule A[31].

Interestingly, more than 700 miRNAs have been found to be stored in platelets[70]. Platelets also can contain the functional miRNA processing machinery required for the processing of miRNA

precursors[71]. Moreover, evidence is accumulating that platelets and platelet-EVs can horizontally transfer nucleic acids to endothelial cells[72,73].Platelet EVs isolated from activated platelets contain significant amounts of miRNA, further to this, functional transfer of miRNA from platelets to endothelial cells was found to occur through vesicle formation[74,75]. Uptake of platelet EV-associated miRNA results in modulation of endothelial target gene expression demonstrated by a downregulation of ICAM-1[72].

Besides the endothelial lining of the vessel wall, VSMC can also be influenced by platelet EVs. Platelets can adhere directly to VSMCs, facilitated by the interaction of $CX_3CR1$ on platelets with $CX_3CL1$ on VSMCs[76]. Although platelets do not make contact with VSMC under healthy conditions, such encounters might occur after vascular damage, increased endothelial permeability or through intraplaque haemorrhage. Upon endothelial denudation, platelets in a thrombus might release EVs that then come in contact with VSMC. Increased permeability of the endothelial lining migh permit the passage of circulating EVs and during intraplaque haemorrhage, EVs might be formed due to platelet contact with the highly thrombogenic plaque interior. On the other hand, a study investigating EVs isolated from atherosclerotic plaques did not observe a significant fraction of platelet-EVs within plaque[77]. It is possible that platelet-EVs lose their surface markers by enzymatic shedding after activation[78], that platelet-EVs are rapidly phagocytosed by macrophages[35,36], or that platelet-EVs indeed constitute only a minor fraction of the total EV content within a plaque. Nevertheless, a potential influence of platelet EVs on the behaviour and phenotype of VSMCs was investigated in our recent work. Binding studies using CFSE-labelled platelet EVs and VSMCs revealed that the platelet EV–VSMC interaction is metal ion-dependent and that αIIbβ3 on platelet EVs is the primary receptor that mediates interactions with VSMCs[79]. Platelet EVs induce migration and proliferation of VSMCs in a CXCL4-dependent manner. Prolonged incubation of VSMCs with platelet EVs results in an increased adhesiveness for THP1 monocytic cells under flow conditions and an increase in interleukin 6 production, indicating that platelet EVs have pro-inflammatory effects on VSMCs. Interestingly, the incubation of cultured VSMCs with platelet EVs led to a phenotypic switch towards a synthetic phenotype, as evidenced by morphologic changes and a reduced expression of the contractile marker calponin[79]. Although direct contact of VSMCs with platelet EVs leads to changes in proliferation, migration, marker expression and phenotype of VSMCs, the possibility exists that platelet EVs might alter the behavior of surrounding cells e.g. the endothelium, thereby indirectly affecting VSMCs by released factors or the transfer of endothelial EVs, analogous to what has been observed during abnormal shear stress[80].

Taken together, platelet EVs influence both phenotype and behaviour of leukocytes and vascular cells, thus are important initiators and propagators in vascular remodelling and downstream processes, such as calcification.

### Initiation and propagation of vascular calcification is regulated by vascular smooth muscle cell function

Vascular smooth muscle cells (VSMCs) are the most abundant cell source of the vasculature. Unlike most cells, VSMCs arise from several lineages[81]. They are critical to maintaining structure and function of the vascular system[82]. Their role is central to vessel dilation and constriction as well as vessel remodeling. VSMCs produce components of the vascular extracellular matrix (ECM), therefore altering the composition of connective tissue and can increase the number of VSMCs present in the vasculature by proliferating. VSMCs are commonly considered to be heterogeneous, having either contractile or non-contractile (synthetic) properties[83]. This

heterogeneity is present in both developing and adult vasculature and is the most defining feature of VSMCs. It has been hypothesised that the different characteristics and functions of VSMCs originate from early developmental cues, as well as spatiotemporal gene regulation of differentiation markers[84].

While in a contractile state, VSMCs contract and relax to enable blood flow around the body. In this contractile state, they express highly VSMC-specific markers for contractility such as SM-αA, calponin and SM22α. These cells have low motility, hence decreased cellular migration is observed, as well as decreased levels of proliferation and a reduced production of extracellular matrices. This enables the blood vessels to maintain elasticity allowing proper function and delivery of blood supply to the anatomy. When synthetic, VSMCs exhibit a marked decrease in expression for VSMC-specific contractility markers, but express more highly markers for matrix metalloproteinase, collagenase, osteopontin and an increase in production of EVs. Phenotype switching enables VSMCs to maintain blood flow as well as support the vascular niche. During vessel repair, migration and proliferation of VSMCs is necessary. Additionally, increases in expression of growth factors such as PDGF, TGF and VEGF, as well as an increased production of ECM is required to reconstruct vasculature following injury.

## Vascular smooth muscle cell phenotypic switching

Terminal differentiation of VSMCs is not a definitive end and it is possible to switch between phenotypes depending on the demand of the vascular niche. Contractile VSMCs are generally referred to as quiescent differentiated cells, whereas the synthetic state is associated with plasticity and appropriately referred to by some as dedifferentiated VSMCs. Several pathologies of the vasculature are associated with VSMC phenotype switching such as atherosclerosis, restenosis, aneurysm and calcification[85,86,87]. Vascular calcification, the deposition of hydroxyapatite crystals along the vessel, decreases vessel flexibility, impairs proper blood flow and is associated with cardiovascular disease mortality[88].

While culture conditions for in vitro maintenance of human VSMCs in either synthetic or contractile phenotypes have been identified, the precise mechanisms that enable VSMC phenotype switching remains unknown[89]. Literature has sparse number of papers implicating miRNAs and proteins in either maintenance or inducing VSMC phenotype switch[90]. Furthermore, several identified miRNA and proteins have been eluded to modulation of vascular pathologies associated with VSMC phenotype switching or a dysregulation in phenotype switching[90]. During pathological phenotypic switching, VSMCs can adapt an osteogenic, chondrogenic or inflammatory phenotype[91,92,93].

Adding further interference to the quandary of VSMC phenotype switching, wall resident adventitial progenitor cells have been recently implicated as vessel wall regulators[94,95]. Adventitial progenitors have been found to differentiate into osteoblasts, chondrocytes, adipocytes, macrophage as well as VSMCs. This has led to the hypothesis that adventitial progenitor cells are master regulators of the vascular niche. When progenitor dysfunction occurs, differentiation of VSMCs to an osteoblastic, chondrogenic or macrophage-like capacity are formed. This is yet to be fully appreciated.

## VSMC-derived extracellular vesicles

In 1967, Anderson first used the name “matrix vesicles” in cartilage development and calcification[96]. Tanimura and co-workers were the first to report an association between small membrane encapsulated particles, matrix vesicles, and vascular calcification[97]. Vesicular structures

have been found in both intimal and medial layers and are likely derived from VSMCs. The release of EVs from VSMCs was first described as a rescue mechanism against calcium overloading, in an attempt to prevent apoptosis[98].

Today, it is appreciated by many that the role of EVs released by VSMCs is significant in VSMC phenotype switching and calcification. VSMCs have been found to release a variety of EVs when in either synthetic or osteogenic phenotype. A group of VSMC EVs have been identified and somewhat characterised by tetraspanin markers CD9, CD63 and CD81[99]. Furthermore, EVs share similarity to osteoblast EVs, having calcium binding capacity and osteoblast-like ECM production. Interestingly, calcific conditions in vitro increase expression of SMPD3 and subsequent EV genesis. Inhibition of SMPD3 completely ablates generation of EVs and calcification. Additionally, in vivo identification of CD63 with calcification of vessels of chronic kidney disease (CKD) patients implicates that SMPD3 is a potential novel therapeutic target to inhibit EV genesis and thus vascular calcification.

Chen and associates recently identified a novel role between VSMCs and EVs in calcification[100]. Initially they characterised EVs from four sources, two from cellular-derived EVs the others from matrix vesicles. Curiously, they identified that both populations of cellular-derived EVs possessed the capacity to enhance calcification, however matrix vesicles did not possess this ability, even though all four EV types were uniformly endocytosed by VSMCs. They eluded to novel increases in expression for MEK1, Erk1/2, Nox1 and SOD2 alongside increases in intracellular calcium ion content, EV biogenesis and calcification. All EVs were found to express tetraspanins CD9, CD63 and CD81, however, the proportion of expression differed significantly between the media and cellular EVs. Cellular-derived EVs expressed more significantly for CD63 that strikingly coincides with Kapustin et al. in vivo observation of CD63 co-localisation with calcification in calcified CKD vessels[99].

Differences between mineralising and non-mineralising EVs were first revealed by Kirsch et al. within chondrocytes, where they identified high expression of annexin A5 within calcifying EVs[101]. The precise function of annexins in calcification has not been fully unravelled[102]. Regarding in vitro VSMC calcification, annexin A2 has been highlighted in calcium regulation resulting in VSMC EV generation[103]. Annexin A2 may bind to fetuin-A on the cell membrane of VSMCs, this is in turn internalised by endocytosis preventing fetuin-A from blocking mineral formation. Increases of annexin A2, annexin A5 and alkaline phosphatase co-localisation are proportional to decreases in fetuin-A expression within in vitro VSMC calcification models. Macrophage-derived EV calcification is induced via binding of annexins A2 and A5 to phosphatidylserine (PS)[104]. Annexin A5 with S100A9 binding to PS is critical for osteoblast-derived ECM production, interestingly this mechanism also occurs during macrophage production of calcifying EVs. Further interrogation of annexins A2, A5 and A6 in VSMC calcification, phenotype modulation and EV genesis will add to our understanding of the roles of annexins in the cellular context of VSMCs.

Propagation of calcification via vitamin K-antagonist (VKA) treatment led to the identification of matrix Gla-protein (MGP) as a potent calcification inhibitor[105]. Twenty years have now passed since MGP inhibitory action was identified, ten years since the mechanism of MGP activation was eluded to by means of serine phosphorylation and gamma-glutamyl carboxylation[91,106,107]. More recently it has been demonstrated that VSMC-derived EVs contain copious amounts of Gla-proteins from circulation[108]. Prothrombin (PT), like MGP, contains a Gla domain. It has been recently demonstrated that production of calcifying VSMC-derived EVs can be inhibited by PT interaction. The Gla domain of PT interacts with the surface of EVs, preventing nucleation sites

for calcification[108]. This finding has been substantiated by the localisation of PT in calcified regions of the iliac/ femoral arteries from a cohort of patients with CKD. Furthermore, circulating levels of PT are reduced in patients with vascular calcification. Accordingly, in absence of MGP, VSMC-derived EVs act to induce calcification, suggesting a potential novel role for inhibition of calcification via PT-EV interactions.

Recent findings have implicated a key role for Sortilin in VSMC calcifying EV biogenesis and release. Originally, Sortilin 1 was identified as driving factor in EV-facilitated calcification through Rab11 interaction[109]. More recently, it was shown that Sortilin 1 localises in human calcifying vessels[110]. In addition, Sortilin 1 plays a role in VSMC calcification in vitro. Sortilin 1 contributes to vascular calcification via tissue nonspecific alkaline phosphatase (TNAP) although regulation of TNAP expression within VSMC calcification remains poorly understood. Notably, Sortilin 1 has been identified as a driver in generation of osteoblasts, while TNAP expression was unaffected. This eludes to a potential novel mechanism in the production of VSMC-derived calcifying EVs, which might be a unique mechanism in vascular calcification (Figure 1).

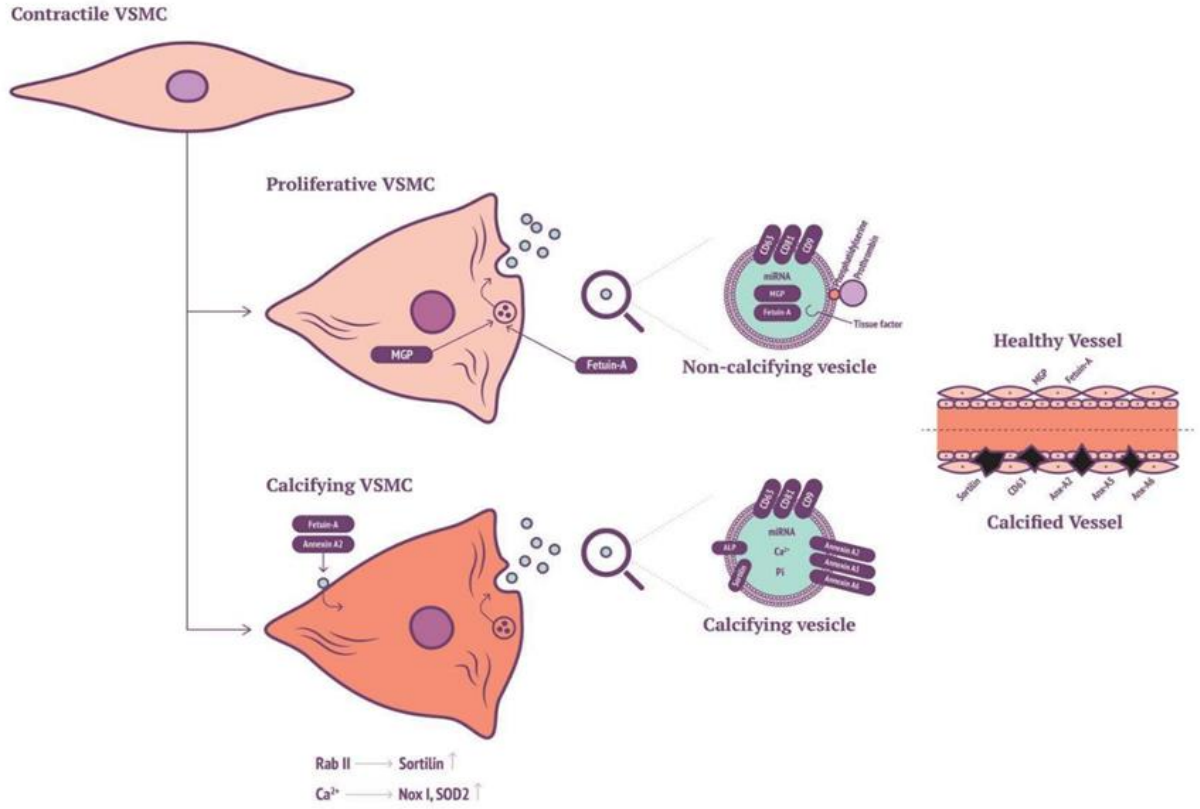


Figure 1. VSMC with Sortilin, MGP and annexin production of calcifying EVs.

Characterisation of EV cargo has been standardised to falling into either protein, RNA or lipid[111]. The composition of lipid content in circulating EVs might provide novel insights into mechanisms for VSMC calcification. RNA content of VSMC-derived EVs may be mRNA, miRNA, lncRNA or circRNA, with miRNA being the most characterised in VSMC phenotype modulation and calcification[112]. RNA lie within the unique position of being both genotype and phenotype which gives them a role in cellular regulation and pathology[113]. Potently a wide array

of miRNAs have been implicated by a variety of mechanisms contributing to, or inhibiting the development of vascular calcification (Figure 2 and Table 3).

Table 3. MiRNA associated with phenotypic switching of VSMC.

| Ref. | Calcifying miRNA | MiRNA Inhibiting Calcification | Paper |
|---|---|---|---|
| 114 | 29b | 133b, 211 | MicroRNAs 29b, 133b, and 211 regulate vascular smooth muscle calcification mediated by high phosphorus. |
| 115 | 32 | | MicroRNA-32 promotes calcification in vascular smooth muscle cells: Implications as a novel marker for coronary artery calcification. |
| 116 | 3960, 2861 | | Runx2/miR-3960/miR- 2861 positive feedback loop is responsible for osteogenic transdifferentiation of vascular smooth muscle cells. |
| 117 | | 29 | MiR-29-mediated elastin down-regulation contributes to inorganic phosphorus-induced osteoblastic differentiation in vascular smooth muscle cells. |
| 118 | | 125b | miR-125b regulates calcification of vascular smooth muscle cells. |
| 119 | | 135a | MiR-135a Suppresses Calcification in Senescent VSMCs by Regulating KLF4/STAT3 Pathway |
| 120 | | 204 | MicroRNA-204 regulates vascular smooth muscle cell calcification in vitro and in vivo. |
| 121 | 221, 222 | | miRNA-221 and miRNA-222 synergistically function to promote vascular calcification. |
| 122 | | 125b, 145, 155 | Decreased microRNA is involved in the vascular remodeling abnormalities in chronic kidney disease (CKD). |
| 123 | 762, 714, 712 | | MicroRNAs that target Ca(2+) transporters are involved in vascular smooth muscle cell calcification. |
| 124 | | 30b, 30c | Bone morphogenetic protein-2 decreases microRNA-30b and microRNA-30c to promote vascular smooth muscle cell calcification. |
| 125 | | 96 | Down-regulation of miR-96 by bone morphogenetic protein signaling is critical for vascular smooth muscle cell phenotype modulation. |
| 126 | | 205 | MicroRNA-205 regulates the calcification and osteoblastic differentiation of vascular smooth muscle cells. |
| 127 | | 133a | MiR-133a modulates osteogenic differentiation of vascular smooth muscle cells. |
| 128 | | 4530, 133b | Differential expression of microRNAs in severely calcified carotid plaques. |
| 129 | | 26a | MiR-26a regulates vascular smooth muscle cell calcification in vitro through targeting CTGF. |
| 130 | | 297a | MicroRNA-297a regulates vascular calcification by targeting fibroblast growth factor 23. |
| 131 | 223 | | Inorganic phosphate accelerates the migration of vascular smooth muscle cells: evidence for the involvement of miR-223. |
| 132 | | 34b, 34c | MicroRNA-34b/c inhibits aldosterone- induced vascular smooth muscle cell calcification via a SATB2/Runx2 pathway. |

MiRNAs which either induce VSMC to an osteoblast-like pro-calcific phenotype or inhibit associated phenotype and calcification. The specific mechanism of which has been reviewed elsewhere[133,134]. Although the role of miRNAs within vascular calcification is being unravelled, many miRNAs have been identified via RNA screening between normal and calcified VSMCs. The origin of miRNA source in a pathological context remains elusive and requires further investigation.

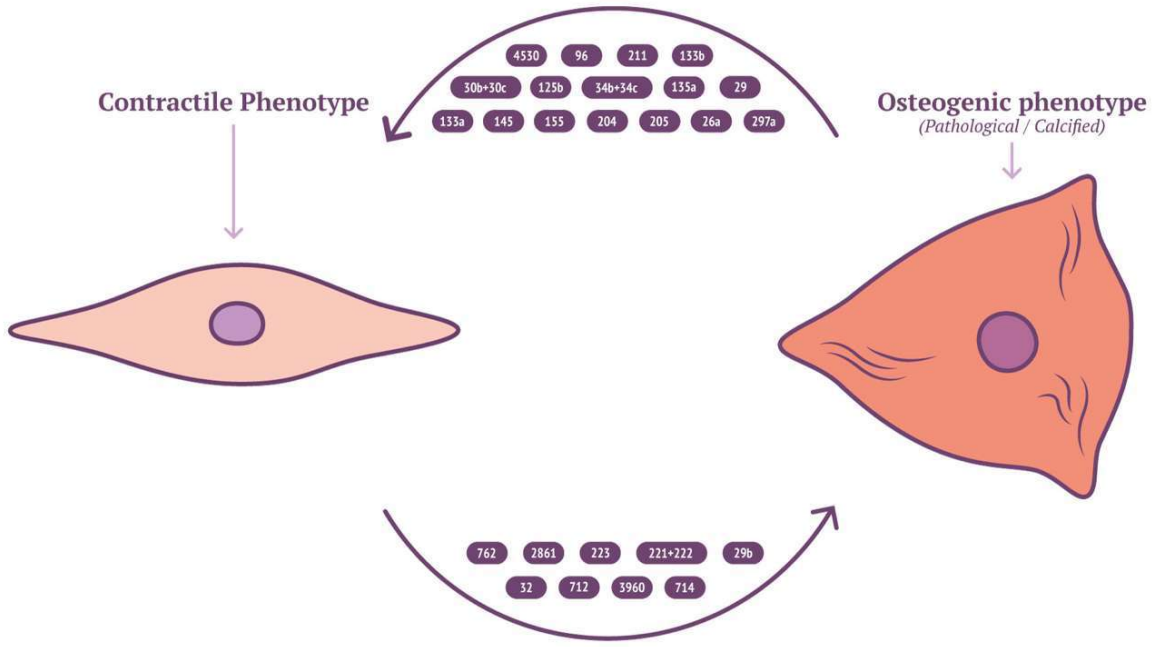


Figure 2. Calcifying miRNAs in VSMC.

Whether there is cross talk of EVs from different facets of the vascular niche or if there is a dysregulation in VSMC maintenance remains an important question. Understanding the cross talk of EVs between vascular cells might be significant to appreciating the precise mechanisms for vascular calcification. MiR-206, ARF6 & NCX1 have been identified as endothelial cell-released EV content capable of regulating VSMC contractile phenotype[135]. Furthermore, given the observed increase of endothelial EV release following injury, it has been noted that endothelial cell EVs from human pulmonary artery not only interacted with pulmonary VSMCs, but interaction induced proliferation and had a seemingly anti-apoptotic effect on VSMCs[136]. This suggests a direct pathological consequence of endothelial EV to VSMC crosstalk during and following intimal vessel injury.

**Mesenchymal Stem Cells as Perivascular Progenitors**

As described above, VSMCs are known to exhibit elaborate phenotypical plasticity and diversity during normal development, disease, and repair of vascular injury. The current theory of vascular calcification is that upon injury VSMCs dedifferentiate. VSMCs become synthetically active and under stress factors, like elevated calcium and phosphate levels or uremic toxins (e.g. during CKD), switch to a dedifferentiated synthetic phenotype and later become osteoblast like cells[99,137]. Genetic fate tracing strongly indicated mature VSMCs to be a major contributor to the development of atherosclerotic plaque remodeling[138]. However, not all elements of plaque development and progression may be indicative as a result of VSMC phenotype switching. The fact, that atherosclerotic human vessels can contain complete trabecular bone with fully formed bone marrow sinusoids even containing hematopoietic cells is difficult to explain without new

concepts, such as vascular stem cells. Indeed, the presence of a specialised progenitor population of VSMCs localised in the adventitia of muscular arteries has been suggested by several groups.

The perivascular niche houses pericytes, which are present at intervals along microcapilaries and pericyte-like cells are also located in the adventitia of large arteries. Peault and coworkers were the first to show that many pericytes are MSCs[139,140]. However, it remains elusive whether all pericytes are MSCs. A pericyte is defined as a cell that is completely or partially embedded in the endothelial basement membrane[141]. The fact that MSCs are also located in the adventitia of large arteries distant from vasa vasorum and in the endosteal niche of the bone marrow indicates that not all MSCs are pericytes[94,142]. The recent evidence that the perivasculature represents the MSC niche explains why MSCs can be isolated from virtually all organs and tissues.

It was first described over 25 years ago that pericytes have an osteogenic potential[143] and subsequently pericyte-like cells with osteogenic capacity were isolated from VSMCs nodules of human aorta[144]. A decade ago it has been reported that Sca1$^+$/ CD34$^+$/ PDGFRβ$^+$ cells that reside in the adventitia of arteries possess a differentiation capacity towards smooth muscle cells and osteoblasts in vitro[145]. Interestingly, these progenitors express the Hedgehog receptor Ptc1 as well as most other hedgehog pathway members including Gli1-3[145]. Other groups have reported that perivascular MSCs were also found to be progenitors of white adipocytes[146], follicular dendritic cells[147] and skeletal muscle[148], while also playing a major role in fibrotic response[149,150,151]. Thus, various studies suggest the presence of adventitial MSC-like cells with osteogenic and myogenic potential. However, until recently the involvement of these MSC-like pericytes in cardiovascular disease development remained unclear.

**Role of perivascular MSC-like cells in vascular calcification**

Using genetic labeling and in vivo fate tracing experiments it was recently revealed that the Hedgehog transcriptional activator Gli1 specifically labels perivascular MSC-like cells[94,142,151,152]. Gli1$^+$ cells reside in the pericyte niche with direct contact to endothelial cells of the microvasculature and in the adventitia of large arteries[94,151]. Gli1$^+$ cells possess all criteria that have been used to define a MSCs including surface marker expression, tri-lineage differentiation and plastic adherence[94,151]. Inducible genetic fate tracing, the gold standard technique to dissect cellular hierarchies, indicated that Gli1$^+$ cells are a major cellular source of myofibroblast in fibrosis of all major organs such as lung, kidney, liver, heart and bone marrow[142,151].

The question is however, whether adventitial Gli1$^+$ MSC-like cells are involved in acute injury repair and chronic vascular disease progression. Using in vivo genetic fate tracing, it was demonstrated that after wire injury of the femoral artery about 50% of newly formed VSMCs were derived from adventitial Gli1$^+$ cells[94]. Furthermore, FACS isolated adventitial Gli1$^+$ MSC could be differentiated into calponin$^+$, αSMA$^+$, smoothelin$^+$ VSMCs in vitro[94]. This data indicates that adventitial Gli1$^+$ MSC are indeed progenitors of VSMCs.

The role of adventitial Gli1$^+$ MSC in vascular calcification was studied in triple transgenic Gli1CreER;tdTomato,ApoEKO mice. Mice were pulsed with tamoxifen in order to genetically tag Gli1$^+$ cells by expression of the bright red fluorochrome tdTomato. Mice were subjected to either subtotal nephrectomy and western diet or sham surgery with standard chow. Interestingly, a continuous low frequency migration of Gli1$^+$ cells into the media during aging in the sham group was observed. Gli1$^+$ cells acquired markers of VSMCs such as α-SMA and calponin suggesting that progenitor cells continuously replace lost VSMCs during aging[94]. Importantly, these data indicate a great migration of Gli1$^+$ cells into the media and neointima during chronic injury and atherosclerosis. Multiple co-staining experiments indicated that adventitial Gli1$^+$ cells first

differentiated into contractile VSMCs (α-SMA$^+$, calponin$^+$) and then underwent a phenotypic switching with loss of contractile VSMC markers and acquisition of synthetic VSMC markers such as Tropomyosin alpha 4 (TPM4) and non-muscle myosin heave chain 2b (nmMHC2b)[94]. Importantly, during vascular calcification a high percentage of Gli1$^+$-derived cells acquired nuclear expression of the transcription factor Runx2 indicating differentiation into osteoblast-like cells. Imaging experiments after injection of a fluorochrome conjugated bisphosphonate (Osteosense) indicated that calcified areas showed intense accumulation of tdTomato$^+$ cells. Importantly, genetic ablation of Gli1$^+$ cells in triple transgenic Gli1CreER;iDTR;ApoEKO mice before onset of CKD completely abolished vascular calcification in both the intima and media. Thus, clearly demonstrating that adventitial Gli1$^+$ cells are important progenitors of synthetic VSMCs and osteoblast-like cells in the vascular wall. Adventitial Gli1$^+$ cells can be considered an important therapeutic target in vascular calcification. Interestingly, we observed a Shh domain in the adventitia of human arteries where endothelial cells of vasa vasorum stained positive for Shh, whereas surrounding cells showed Gli1 expression. Further staining experiments in calcified human arteries showed intense Gli1 expression around calcified intima and media areas.

While these data clearly demonstrate an important role of adventitial MSC-like cells in vascular calcification there are still various open questions that need to be answered (Figure 3).

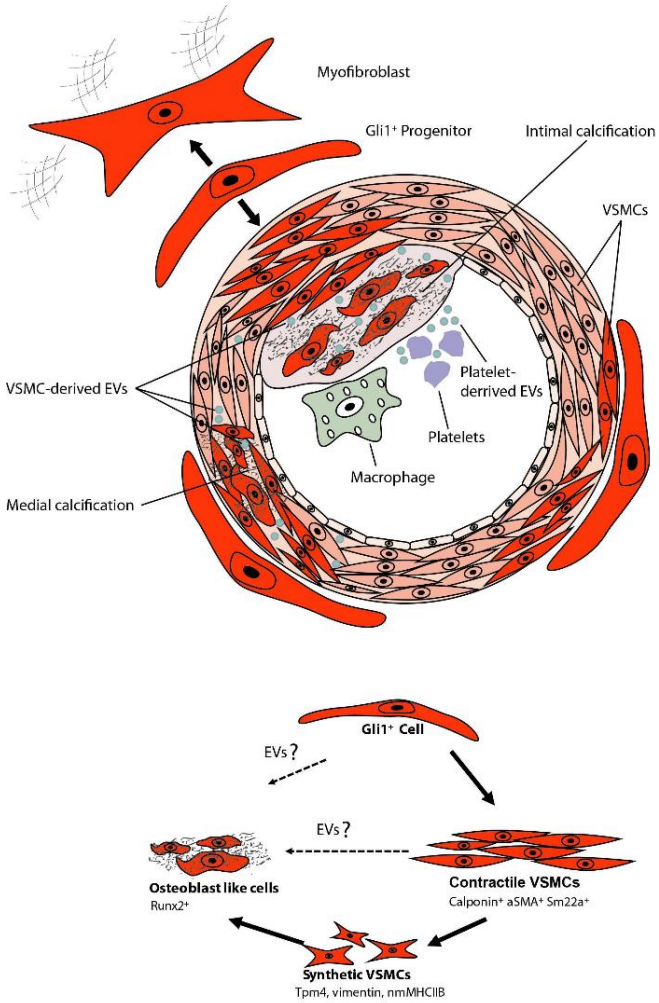


Figure 3. Calcifying EVs and Gli1+ Progentior Cells.

It is unclear whether the Gli1$^+$ population is a homogenous progenitor population or whether several subpopulations exist. Single-cell qPCR analysis of sorted Gli1$^+$ cells for reported markers of adventitial progenitors indicates heterogeneity with three distinct subpopulations (unpublished data). Thus, it will be important to differentiate between these subpopulations. Next, it remains elusive as to whether Gli1-expressing cells of human arteries may also secrete EVs and thereby contribute to vascular calcification. Finally, while our data clearly indicates migration and differentiation of Gli1$^+$ cells during acute injury repair and chronic disease progression, the underlying molecular pathways that activate migration and differentiation of the progenitor population remain obscure. It is currently not known whether EVs are involved in migration and differentiation of MSCs. Further, we must await as to whether adventitial Gli1$^+$ cells produce EVs or interact with EVs from other cells such as platelets, immune cells and VSMCs. Experiments in myelofibrosis demonstrated that a malignant hematopoietic clone in particular megakaryocytes can activate Gli1$^+$ cells to leave their niche partly by CXCL4 release[142]. Thus, involvement of platelet and cellular EVs might be an explanation for activation and migration of Gli1+ cells. Further studies are needed to answer whether EVs are involved in Gli1$^+$ cell recruitment and differentiation.

## Conclusion

The biology of vascular disease and calcification is complex and still poorly understood with respect to cause and consequence of players and pathways, and thus complexity increases continuously by ongoing elucidation of novel players and pathways. A subset of EVs act as mediators of cell-induced extracellular matrix calcification in the pathogenesis of cardiovascular disease. On the other hand, it has become evident that platelet and cellular vesicles play an important role in cellular communication. Whether such communication from platelet EVs can transpire through endothelial cells to the media and adventitia remains unknown, and so a role for EV crosstalk in calcification remains open with promise.

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Chapter 3

## Role of vascular calcification in the initiation of atherosclerosis

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## Introduction

The classical view of atherosclerosis development is the activation of vascular endothelial cells with lipid retention and oxidation in the intima of the artery wall. This in turn attracts smooth muscle cells from the vascular media to the vulnerable regions, playing a significant role in all phases of lesion development. Ultimately, the atherosclerosis progression results in stenosis leading to vascular obstructions or even rupture that may cause a myocardial infarction or stroke. Especially the branching sites in the aorta, coronary arteries, and carotid arteries are mostly affected in atherosclerosis, as irregular flow conditions may occur in these vessels. Atherosclerosis is often understood as a slowly progressing disease and a common feature of atherosclerotic plaques is the deposition of calcium/phosphate crystals in the vessel wall. In the modern era, the risk of developing vascular calcification rises from around 5% per year (for people under the age of 50) to about 12% per year (for people over the age of 80), according to the American Heart Association[1,2]. Of note, a meta-analysis of 218 000 participants indicated a 3.9-fold increased risk of cardiovascular mortality and a 3.4-fold higher risk of any cardiovascular event when vascular calcification was present, what clearly demonstrates the clinical relevance of calcification[3]. Indeed vascular calcification, may be quantified in the coronary arteries using computed tomography (CT) to generate a coronary artery calcium (CAC) score that is used in conjunction with the Framingham Risk Score to improve prognostic value of coronary events[4,5].

## Atherosclerosis; an age- and lifestyle-related disorder?

In the past 100 years, due to increases in life expectancy, numerous traditional lifestyle-associated CVD risk factors were identified, leading many to believe that atherosclerosis is an exclusively lifestyle-dependent modern disease. However, recent reports have challenged this view[6]. For example in the HORUS study, CT scans of Egyptian mummies[7] revealed various degrees of atherosclerosis and arterial calcification as well as the strong correlation between age and presence of calcified vessels. Similar observations were made after the examination of mummified individuals from Peru, United States and Aleutian Islands[8]. These reports confirmed that ancient populations suffered from atherosclerosis, even though they were supposedly lacking exposure to modern risk factors for the development of atherosclerosis. The above findings have stimulated interest in the underlying biological processes that might be involved in an inherent predisposition to the development of atherosclerosis in humans, beyond ageing and lifestyle[9].

Although aging is indisputably the dominant risk factor for clinically significant atherosclerotic lesion formation[6], atherosclerosis does not exclusively occur in elderly people. It has been found in adolescents and newborns as well[6,10,11]. It is thought that the onset of atherosclerosis occurs early in the development and progresses rapidly with age[12]. Arteries of fetuses and infants typically regulate vascular tone to keep an equal balance of blood pressure at all points along the artery to maintain optimal flow. Consequently, an increase in thickness occurs when a smaller group of local intimal SMCs is activated. Early in fetal development, thick segments can be observed in all individuals[13] and are considered as side effects of growth. The monitoring of carotid intima-media thickness (CIMT) has been used extensively in children and young adults with risk factors for CVD. Several large studies have shown that increased CIMT correlated well with traditional cardiovascular risk factors that include excess weight, dyslipidemia, and hypertension. In addition, increased CIMT was found in adolescents with familial hyperlipidemia and in children with a parent with a history of premature myocardial infarction. Young adults with increased CIMT had an increased likelihood of a cardiovascular events that include myocardial infarction and stroke[11]. Interestingly, vascular calcifications were found in pediatric patients with Kawasaki disease, an autoimmune disorder characterized

by systemic vascular inflammation causing necrosis and aneurysms[14,15]. This suggests that vascular calcification is an active and inflammation-driven disorder.

## The contribution of VSMCs to disease initiation

Vascular calcification, once considered a passive precipitative process, is now recognized as an active and tightly regulated cell-mediated process resembling osteogenesis, principally driven by the vascular smooth muscle cells (VSMCs)[16]. Calcification is defined as the deposition of hydroxyapatite mineral in the vessel wall, occurring in the intimal and/or medial layers of the arteries. Both intimal and medial calcifications are triggered through distinct pathways but often occur in the same patient and even at the same arterial site[17]. Intimal calcification is primarily associated with atherosclerosis. Patients with intimal calcification often have high serum pro-inflammatory cytokines, display hyperlipidemia or metabolic syndrome, while calcium phosphate homeostasis is well-maintained[18]. Inflammation, apoptosis, and oxidative stress lead to VSMC differentiation which, in turn, leads to development of microcalcifications (<15 μm) within the fibrous cap of atherosclerotic lesions that promotes local stress. These calcium deposits weaken the structure of the wall, increasing the risk of plaque rupture[19] that may lead to myocardial infarction[20,21] or stroke[22]. In contrast, macrocalcifications (>200 μm) often accumulate in the deep intima or necrotic core in organized structures and may promote plaque stability[23]. Medial calcification however, may occur in a range of conditions, including genetic disorders, ageing, chronic kidney disease, diabetes mellitus, dyslipidemia, systemic lupus erythematosus and vitamin D-deficiency[18]. VSMCs in the media also de-differentiate in response to stimuli that are different from the ones present within atherosclerotic plaques, such as prolonged uremia, elevated calcium and phosphate levels[16]. This de-differentiation may occur in the absence of inducers of intimal calcification, e.g. lipid accumulation and inflammatory cell infiltration[18]. VSMCs exposed to above mentioned stimuli display an intracellular calcium overload that may result in micro- and eventually macro-calcifications, thereby contributing to vascular stiffness[24] and increased pulse wave velocity, a risk factor for the development of heart failure[25]. The diseases associated with medial calcification are diverse and often independent of atherosclerosis suggesting different processes driving VSMC change[18]. Some controversy remains with respect to whether vascular calcification is a cause or consequence of cardiovascular disease. From one viewpoint it may be considered as a consequence, because atherosclerosis induces VSMCs de-differentiation, and from another as a cause, because vascular calcification leads to aortic stiffening which ultimately affects plaque stability[17]. Given the association between calcification morphology and cardiovascular outcomes, it is important to understand the involvement of VSMCs and their release of extracellular vesicles in the deposition and growth of calcium crystals in the vasculature from the earliest stages.

## VSMCs as main drivers of vascular calcification

Lineage-tracing studies have demonstrated that VSMCs generate 30–70% of all plaque cells in murine models of atherosclerosis[26,27,28] by clonal expansion of only few cells[29,30,31]. VSMCs have a fully functional, differentiated phenotype in healthy vessels, yet retain remarkable plasticity. In atherosclerotic plaque, VSMCs can give rise to many cell types, including foam cells, macrophage-like, adipocyte-like, endothelial cell-like, osteochondrogenic, synthetic, mesenchymal stem-cell-like and myofibroblast-like cells (given in order of increasing plaque stabilizing properties)[32]. Native, quiescent VSMCs, express high levels of contractile proteins and are often referred to as ‘contractile’, whereas de-differentiated VSMCs (‘synthetic’) proliferate and migrate more, synthesize more extracellular matrix (ECM) components (i.e. 25 – 46 times more collagen)[33,34], matrix metalloproteinases (MMPs), cytokines (e.g. TNF-α, IL-1β, IL-6)[35] and express more receptors allowing lipid uptake and

foam cell formation (e.g. (very) low density lipoprotein (LDL) and scavenger receptors)[36,37]. Synthetic VSMCs also express a range of adhesion molecules and receptors (e.g. Toll-like-receptors) that promote monocyte recruitment and regulate intracellular inflammatory signaling, respectively. Although these synthetic cells generally drive fibrous cap formation, they may also promote inflammation, calcification, cell senescence, and eventually can lead to plaque instability, depending on the local environment and the disease stage[38]. Therefore, transition to the "synthetic" phenotype facilitates many of the pathogenic roles of VSMCs. Studies in vitro showed that rat and mouse VSMCs can switch between "contractile" and "synthetic" phenotypic states in response to a variety of atherogenic stimuli including extracellular matrix, cytokines, shear stress, reactive oxygen species and lipids[39]. Importantly this process appears to be at least partly reversible and thus therapeutically modifiable. This was elegantly shown in a recent study, in which SMC-derived intermediate cells, termed "SEM" cells (stem cell, endothelial cell, monocyte), were multipotent and could differentiate into macrophage-like and fibrochondrocyte-like cells, as well as return toward the SMC phenotype[40]. Another study showed that rat neointimal VSMCs re-acquired their contractile markers after balloon injury[41] and synthetic VSMCs cultured in vitro could switch back to the contractile phenotype, driven by factors such as heparin or laminin[42]. By contrast, VSMCs retain their synthetic status during atherosclerosis because of the continuous exposure to atherogenic stimuli, although as mentioned above, they may maintain their ability to revert back to their contractile phenotype. Therefore new therapeutic strategies should focus on stimulating the differentiation and accumulation of 'plaque stabilizing' VSMC phenotypes, eliminating the subpopulation of harmful VSMC phenotypes (e.g. pro-inflammatory, macrophage-like VSMCs), or switching detrimental to beneficial phenotypes, as recently shown by activation of retinoic acid signaling pathways[40].

### Osteochondrogenic VSMCs and matrix vesicle (MV) release

Osteochondrogenic VSMCs lose their contractile markers (SM22a and SM a-actin) and show an increased expression of several regulators of bone development, such as RUNX2, SOX9, osteopontin, BMP2, osterix, Msx2, and alkaline phosphatase and deposit calcification-prone matrix (e.g. collagen type II and X)[18,43,44,45]. Of note, some of these markers have been detected in calcified atherosclerotic lesions in people[46]. Cells displaying an osteochondrogenic phenotype are the major contributors to atherosclerotic plaque development. Lineage-tracing studies in mice have shown that 98% of all osteochondrogenic cells in plaques are VSMC-derived[47] and commonly co-localize with calcium–phosphate deposits[48,45,49]. Furthermore, VSMCs spontaneously shed different types of extracellular vesicles (EVs), which can be categorized based on their increasing size: from exosomes to microvesicles and apoptotic bodies. These EVs deliver biomolecular cargo (e.g DNA, RNA, proteins) to the recipient cells that may result in a phenotype change[50,51]. Under normal conditions, these VSMC-derived vesicles do not promote calcification, because they are loaded with mineralization blockers such as matrix Gla protein (MGP) and fetuin-A[52]. However, in the presence of high calcium and phosphate in the extracellular space, calcifying VSMCs release matrix vesicles containing low levels of calcification inhibitors[53] and all machinery essential to induce calcification. Matrix vesicles are preloaded with a nucleational core, composed of annexins A2, A5 and A6, as well as a complex of PS, $Ca^{2+}$ and Pi. Calcium and phosphate ions form calcium-phosphate mineral within the vesicle lumen via multiple routes: intravesicular Pi release from PChol and PEA via PHOSPHO1; Pi release from ATP in the extravesicular space via TNAP and NPP1/3, and import via Pit1/2; and calcium import via annexins A2, A5 and A6. Over time, mineral crystals propagate through the vesicle membrane, where their continued growth is regulated by the ratio of PPi, an inhibitor of crystal growth, to its derivative Pi [54,55,56]. As a result, EV membrane loses

structural integrity, and mineral crystals are propagated out onto the collagen-enriched matrix, where they can grow even further and form micro-calcifications[19].

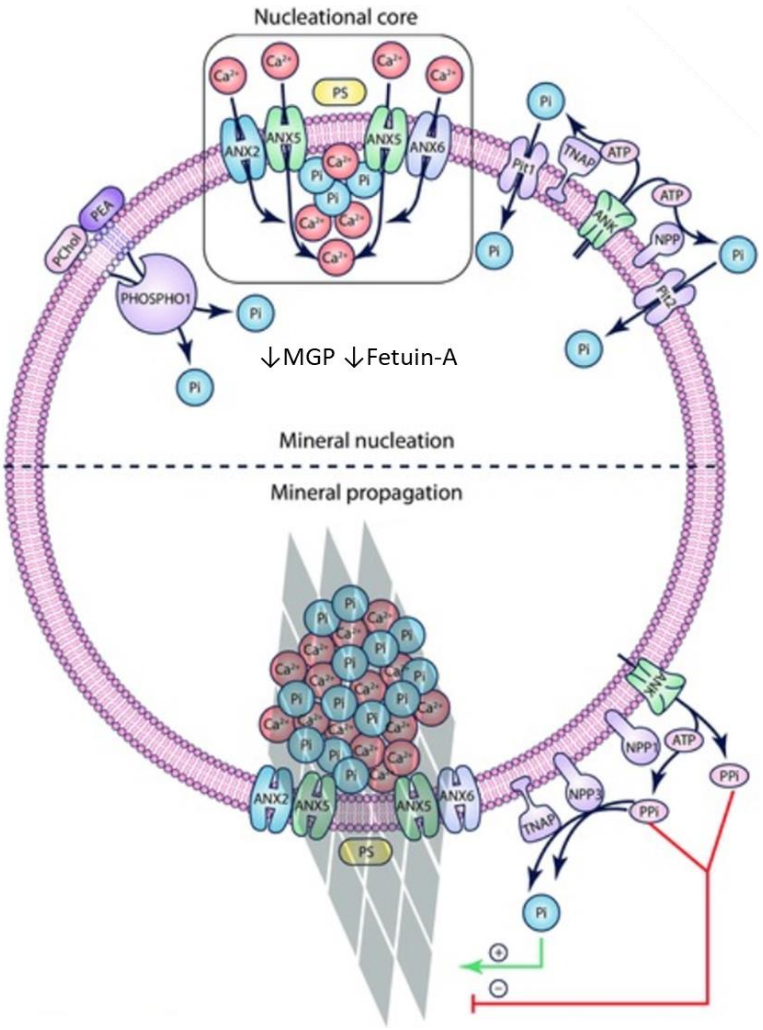


Figure 1. Proposed mechanism of vesicle-mediated mineral formation. Adapted from[56].

**Inflammation as a driver of calcification**

Inflammation also favors the osteochondrogenic transition of VSMCs and the subsequent release of EVs release that serve as hydroxyapatite nucleation points[57]. Hydroxyapatite is in turn a strong inducer of VSMC osteochondrogenic conversion and stimulates resident macrophages to produce pro-inflammatory cytokines (e.g. TNF-α, IL-1β or IL-6)[58,59], which further amplifies the calcification process. Therefore, inflammation can be both a stimulus and a result of calcification, highlighting the existence of a vicious circle by which inflammation promotes vascular calcification and vascular calcification itself promotes inflammation[60]. Inflammatory cytokines such as TNF-α[61], IL-1β[62] and IL-6[63] are related to osteochondrogenic differentiation and progression of intimal calcification. Findings indicate that TNF-α and IL-6 promote calcification directly in vascular cells, and indirectly by decreasing fetuin-A expression in the liver and lowering α-Klotho expression in the kidney[57]. TNF-α, primarily released by macrophages, was found to activate the osteogenic program of VSMCs by Msx2–Wnt–a-catenin signaling[64] and anti-TNF-α treatment was associated with reduced vascular calcification in patients with rheumatoid arthritis[65]. Neutralization of IL-6 reduced RANKL- induced OPN, runx2 and BMP, which led to inhibition of calcification in vitro[63] and might be the treatment of choice to prevent the development or progression of vascular calcification in patients with

chronic kidney disease (CKD)[60]. Recently published CANTOS trial showed that administration of canakinumab (targeting IL-1β) led to a promising improvement in high-risk patients, reducing plasma levels of both C-reactive protein and IL-6 which might be also considered in the treatment of vascular calcification[66]. The results in patients were supported by observations that high levels of IL-1β in arterial medial smooth muscle layers play a role in inducing senescence-associated calcification[67]. Recently, the use of 5-lipoxygenase (5-LO), P-selectin, leukotriene inhibitors as well as intravenous HDL, serpins and colchicine have all been proposed as therapeutic options to impede the initiation of vascular calcification by targeting inflammation[68].

## Cellular sources of VSMCs in the vasculature

Beyond VSMCs, other cell types within the vessel wall contribute to intimal hyperplasia and vascular remodeling, including adventitial mesenchymal stem cells (MSCs)[69], bone marrow mesenchymal stem cells (BMSC)[70], pericytes[71] and endothelial cells[72]. It has been suggested that MSCs derive primarily from pericytes in capillaries and microvessels and from adventitial cells in larger vessels[73]. The contribution of MSCs *in vivo* was unclear so far due to the absence of a MSC-specific marker. The recent demonstration of the zinc finger protein glioma-associated oncogene 1 (Gli1) as a specific MSC marker in adult tissues[74,75,76] has allowed fate-tracing studies in mice, showing that MSC-like cells migrate into the media and neointima during atherosclerosis. The cells carrying the Gli1+ marker were a major source of osteoblasts-like cells, significantly contributing to the process of vascular calcification in both vascular media and intima in a mouse model of femoral artery injury. The genetic ablation of these Gli1+ cells before the infliction of vascular injury dramatically reduced the severity of vascular calcification[77]. Importantly, adventitial cells expressing MSC markers, such as stem cell activating antigen 1 (Sca1) can differentiate into VSMCs *in vitro* and in vein grafts[69]. Furthermore, several models of atherosclerosis have demonstrated that bone marrow-derived/blood-borne progenitors to neointimal lesion formation give rise to a substantial proportion of neointimal cells in line with the severity of vascular injury[78,79]. Bone marrow mesenchymal stem cells (BMSC) can also differentiate into various cell types, including adipogenic, osteogenic, chondrogenic and myogenic lineages. There are large amounts of accumulated BMSC in atherosclerotic vessels that can differentiate into VSMCs. Yan et al. showed that BMSC differentiated into VSMCs after treatment with transforming growth factor β (TGF-β) and that BMSC-derived SMC became foam cells after treatment with oxidized LDL in vitro. Another study revealed that in apoE$^{-/-}$ mice a major portion of α-SMA$^{+}$ SMCs recruited to the neointima after wire-injury originated from the bone marrow[80]. These results suggest that VSMCs may also originate from BMSC and can contribute to vessel wall remodeling, promoting the initiation of atherosclerosis[70]. It is well established that some plaque VSMCs derive from ECs following endothelial-to-mesenchymal transition (EndMT). EndMT, driven by transforming growth factor beta (TGF-β) signaling, oscillatory shear stress, oxidative stress, and hypoxia, promotes progression of experimental atherosclerosis and correlates with an unstable plaque phenotype in humans[72,81]. The remarkable plasticity of various cell types residing in vessel wall and circulating stem cells makes it challenging to explain the initial steps of atherogenesis and shows the complexity of the process. At the same time it gives the researchers possible opportunities to target the onset of atherosclerosis from different angles.

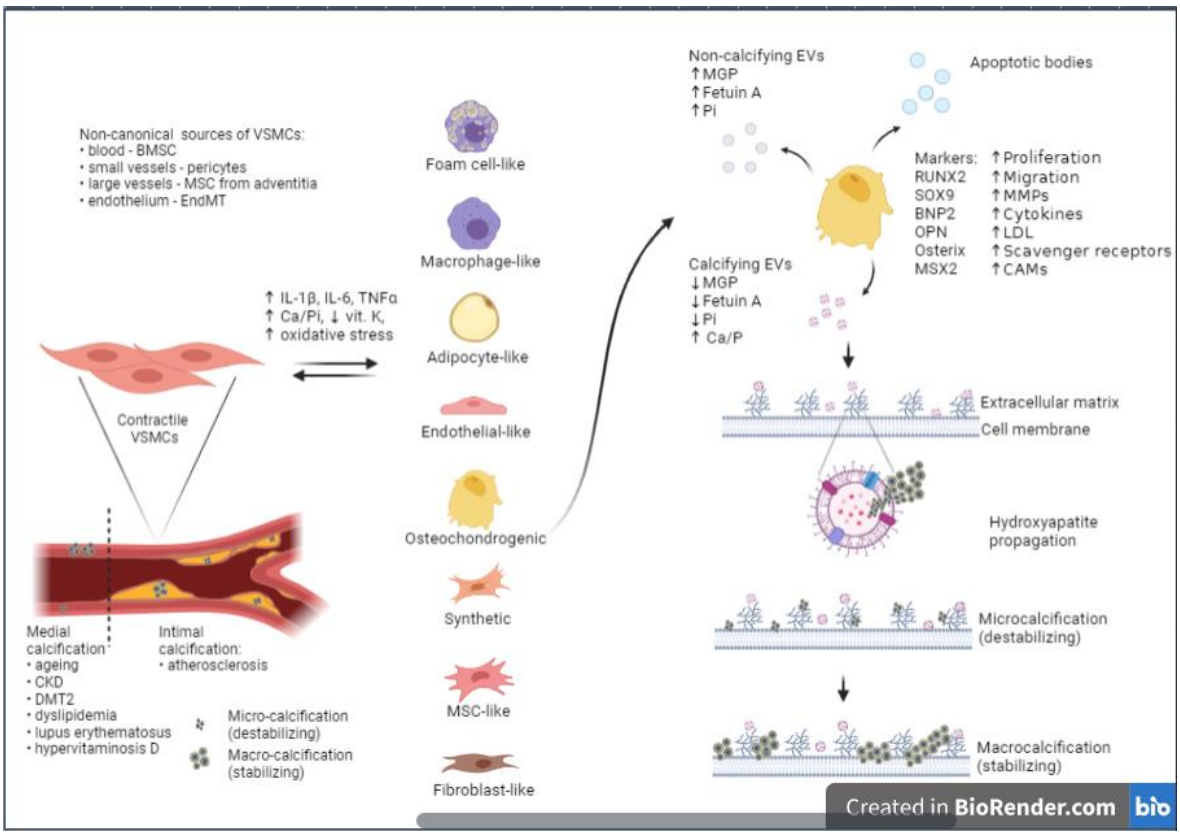


Figure 2. The contribution of VSMCs to the disease initiation with the focus on calcification.

## Reversal of vascular calcification

Although many molecular mechanisms involved in vascular calcification (VC) were described in the past decades, the characterization of its chemical composition and crystalline structure remains poorly understood. Most researchers agree that the calcification present in vascular beds is composed of varying amounts of calcium phosphate minerals like amorphous calcium phosphate, apatite, and whitlockite, with their bulk ratios highly affected by aging and underlying pathologies, such as CKD[82,83,84,85]. Amorphous calcium phosphate is more water-soluble, and under endogenous calcification inhibitors such as magnesium, fetuin-A, inorganic pyrophosphate (PPi), and matrix Gla protein (MGP) prevent its deposition in the vessel wall[86,87]. The preventive effect of these inhibitors is described in many studies, but reversal of VC was rarely observed[86]. Magnesium, for example, was described to reverse calcification in uremic mice and osteogenic transdifferentiation of VSMC incubated with high phosphate[88,89].

Bisphosphonates are analogs of PPi used to treat osteoporosis, and their use to combat VC was discussed in detail previously[90]. Some studies found that etidronate, a first-generation bisphosphonate, partly reversed VC in CKD patients undergoing hemodialysis[91,92].

On the other hand, second-generation bisphosphonates do not show these effects[90]. Moreover, in a rat model of warfarin-induced calcification, high doses of vitamin K were described to reverse arterial calcification, which was accompanied by an increased MGP serum concentration[93]. This evidence suggests that limited spontaneous reversal of calcifications may be possible by increasing the concentration of endogenous calcification inhibitors but, despite promising research, no clear evidence was demonstrated so far.

The inorganic minerals apatite and whitlockite are insoluble under physiological conditions. These minerals require an acidic environment to dissolve, which could be created

by osteoclasts (OC) or VSMC through the secretion of carbonic anhydrases to induce a bone resorption-like state within the vasculature[87,94,95]. In a warfarin-treated rat model, the endothelin-receptor antagonist darusentan regressed aorta mineralization, which was accompanied by up-regulation of carbonic anhydrase II in VSMC. Furthermore, blockade of the enzyme blunted the observed mineral loss. Macrophage-like cells were absent in the resorbed mineralization sites after darusentan treatment[96]. In contrast, the presence of osteoclast-like cells in human arteries was found occasionally and mostly present in heavily calcified arteries[91,97]. In uremic rats, the calcimimetic AMG 641 was able to regress established medial calcification, which was accompanied by the presence of mono- and multinuclear phagocytic cells in the wall of the calcified arteries[98]. Macrophage and monocyte-like cells were also described in the tunica media, in proximity to the calcified foci, after reversibility of calcitriol-induced medial calcification upon withdrawal of calcitriol administration[99]. However, the functionality of osteoclast-like cells in the vasculature is questionable, since atherosclerotic osteoclast-like cells were shown to lack critical cathepsin K activity[100]. Consequently, evidence that OCs are directly involved in the reabsorption of minerals in human arteries remains to be established. Still, using OC cell-therapy might be a promising strategy to reverse VC. Indeed, it has been recently reported that human monocytes or isolated peripheral blood mononuclear cells were able not only to prevent calcium deposition, but also to resorb already formed deposits in rat carotid arteries in vitro[101]. Other authors hypothesized that elastin-targeted delivery of OCs might reduce VC[102]. Nevertheless, neither directly injected nor collagen encapsulated OCs remained at the target site, collagen/alginate microbeads delivery was successful, but due to slow microbead degradation OCs were hindered from migrating[102].

VC regression in humans is rarely described, e.g. in a report, tumorous calcification of the thighs of a CKD patient partially regressed after kidney transplantation[103]. Moreover, in a retrospective case-control study using mammograms to assess the effect of warfarin treatment on women breast arterial calcification, discontinuation of warfarin seemed to reverse the calcification[104].

Nonetheless, the question of the state of the extracellular matrix and functionality of the vessel wall after the reabsorption of the calcium-phosphate deposits remains to be investigated. So far, it appears that with the currently available drugs and therapies, we cannot reverse matrix-associated VC.

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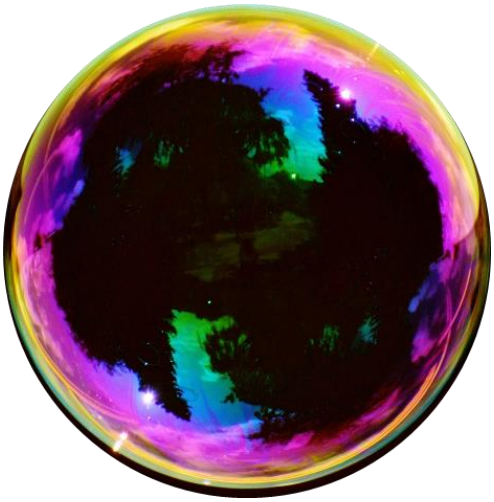
# Chapter 4

## Differential effects of platelet factor 4 (CXCL4) and its non-allelic variant (CXCL4L1) on cultured human vascular smooth muscle cells



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## Chapter 4


### Abstract

Platelet factor 4 (CXCL4) is a chemokine abundantly stored in platelets. Upon injury and during atherosclerosis, CXCL4 is transported through the vessel wall where it modulates the function of vascular smooth muscle cells (VSMCs) by affecting proliferation, migration, gene expression and cytokine release. Variant CXCL4L1 is distinct from CXCL4 in function and expression pattern, despite a minor three-amino acid difference. Here, the effects of CXCL4 and CXCL4L1 on the phenotype and function of human VSMCs were compared in vitro. VSMCs were found to constitutively express CXCL4L1 and only exogenously added CXCL4 was internalized by VSMCs. Pre-treatment with heparin completely blocked CXCL4 uptake. A role of the putative CXCL4 receptors CXCR3 and DARC in endocytosis was excluded, but LDL receptor family members appeared to be involved in the uptake of CXCL4. Incubation of VSMCs with both CXCL4 and CXCL4L1 resulted in decreased expression of contractile marker genes and increased mRNA levels of KLF4 and NLRP3 transcription factors, yet only CXCL4 stimulated proliferation and calcification of VSMCs. In conclusion, CXCL4 and CXCL4L1 both modulate gene expression, yet only CXCL4 increases the division rate and formation of calcium-phosphate crystals in VSMCs. CXCL4 and CXCL4L1 may play distinct roles during vascular remodeling in which CXCL4 induces proliferation and calcification while endogenously expressed CXCL4L1 governs cellular homeostasis. The latter notion remains a subject for future investigation.

## 1. Introduction

Platelet factor 4 (PF4 or CXCL4) is a 7.8 kDa chemokine, synthesized mainly by megakaryocytes and stored in the a-granules of platelets. Unlike most chemokines, there is no single receptor that mediates the actions of CXCL4. Instead, CXCL4 can bind to different receptors, initiating various downstream signaling pathways on distinct cell types[1]. Despite the identification of several putative receptors e.g., glycosaminoglycans (GAGs)[2,3], CXCR3[4,5,6], CCR1[7], and low-density lipoprotein (LDL) family receptors[8,9], the molecular mechanisms behind the biological functions of CXCL4 remain incompletely clarified[10].

Upon injury or during the formation of an atherosclerotic plaque, CXCL4 was found to be transported from the blood into deeper layers of the vessel[11]. Over the past 40 years, studies in mice and humans have confirmed the presence of CXCL4 (and not platelets) in the atherosclerotic plaques[12,13,14].

Recently, CXCL4 has received major attention due to its involvement in the pathophysiology of vaccine-induced thrombocytopenia, a rare and severe complication after vaccination against SARS-CoV-2[15,16].

Vascular smooth muscle cells (VSMCs) in the arterial vessel wall are considered heterogeneous in phenotype and display a high degree of plasticity. In the vascular media, the contractile VSMC phenotype is important in maintaining tissue elasticity, wall homeostasis and vascular tone[17,18]. During pathologic vascular remodeling processes, VSMCs migrate into the intima of the vessel wall and undergo a phenotypic switch towards a synthetic state, hallmarked by dedifferentiation with reduced or absent expression of VSMC-specific genes, increased production of extracellular matrix components and inflammatory cytokines[19]. Interestingly, CXCL4 also contributes to the phenotypic switch of VSMCs towards synthetic states[20,21]. Previous work showed that CXCL4 treatment of VSMCs increased migration and proliferation and decreased the level of contractile markers (calponin, α-actin), consistent with a synthetic phenotype[20]. CXCL4 also co-localizes with calcified regions of human carotid atherosclerotic plaques[12]. Moreover, during plaque development, CXCL4 contributes to the formation of VSMC-derived foam cells, by enhancing the binding of oxidized low-density lipoprotein to VSMCs[22].

CXCL4L1 is a non-allelic variant of CXCL4 that has a different leader peptide and 3 amino acid substitutions at the C-terminus. It is also stored in platelets and released after activation[23,24,25]. Until now, the knowledge of CXCL4 biology is mainly based on studies on native CXCL4, whereas CXCL4L1 has not been studied to this extent. Despite small differences in the primary structure, CXCL4L1 is highly distinct from CXCL4 with regards to 3D-structure and function[26,27]. The two chemokines share several properties such as anti-angiogenic and anti-tumor effects in vivo, when exogenously added as isolated proteins[28], but major differences also exist, such as in binding to GAGs, export, and interaction with receptors or oligomerization[27]. The expression and release of CXCL4 and CXCL4L1 is different in various cell types. In contrast to CXCL4L1, which is continuously synthesized and secreted through a constitutive pathway, CXCL4 is stored in secretory granules and released upon activation (i.e., in platelets)[24]. While CXCL4 mainly has proinflammatory effects in atherogenesis, CXCL4L1 may rather exert homeostatic roles[1,29]. This suggests that the roles of CXCL4 and CXCL4L1 in homeostatic and inflammatory processes are different[24]. Given the versatile role of platelets in atherosclerosis, vascular remodeling and immune regulation, we aimed at elucidating possible functional differences between CXCL4 and CXCL4L1 more precisely, with particular focus on their effects on VSMC function in relation to vascular wall remodeling.

## 2. Results

### *2.1. Platelet Factor 4 (CXCL4), but Not CXCL4L1 Is Internalized by Vascular Smooth Muscle Cells (VSMCs)*

Previous work has demonstrated rapid internalization of CXCL4 by endothelial cells[30]. To investigate whether CXCL4 and its variant CXCL4L1 are taken up by VSMCs, cells were treated with CXCL4 or CXCL4L1, stained with fluorescent antibodies and the measured fluorescence was quantified. The analysis revealed that the internalization of CXCL4 by VSMCs is significantly increased in comparison with control cells at 37 °C (Figure 1A). Interestingly, a baseline level of intracellular CXCL4L1 expression appeared to be present in VSMC, as visualized by staining using a CXCL4L1-specific antibody (Figure 1B). Intracellular CXCL4 levels were further increased by the addition of CXCL4, but not by CXCL4L1 at 37 °C (Figure 1A,C). After addition, CXCL4 staining appeared as a speckled pattern, suggesting endosomal localization. This was visible also without permeabilization, after removal of surface-bound CXCL4 using heparin. At 4 °C, no CXCL4 uptake was observed (Figure S1).

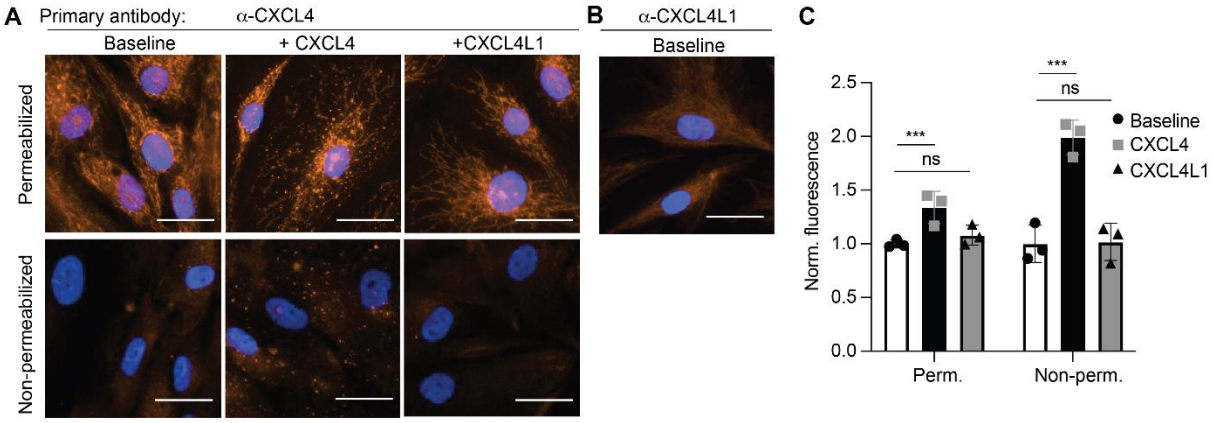


**Figure 1. Uptake of platelet factor 4 (CXCL4) into vascular smooth muscle cells (VSMCs).** CXCL4, CXCL4L1 (at 0.5 µg/mL) or buffer (baseline) was added to VSMC for 1 h at 37 °C, washed with heparin and stained for CXCL4 (**A**) or CXCL4L1 (**B**) with or without prior permeabilization. Representative micrographs showing CXCL4 (**A**) or CXCL4L1 (**B**) staining (orange) and nuclei (blue). Scale bar: 50 µm. (**C**) Quantification of fluorescence expressed normalized to baseline fluorescence, of 4 images per well (3 wells per experiment of 3 separate experiments) divided by cell count, measured using Cytation™. *** $p < 0.001$, n = 3, two-way ANOVA with Dunnett's post-test.

The results obtained suggest that CXCL4L1 is constitutively expressed and that CXCL4 is internalized by VSMCs, which occurs by an energy-requiring process at physiological temperatures.

### *2.2. The Uptake of CXCL4 into VSMCs Is Mediated by the Low-Density Lipoprotein (LDL) Receptor Family*

To investigate possible surface receptors that might be involved in a CXCL4 internalization by VSMCs, the candidates CXCR3, DARC (atypical chemokine receptor 1, ACKR1) and the LDL-receptor family were tested. After confirming the presence of CXCR3 and DARC on VSMCs (Figure S2A,B), their possible role in the uptake of CXCL4 and CXCL4L1 by VSMCs was investigated in the presence of blocking CXCR3 or DARC antibodies. Although a significantly increased uptake of CXCL4 was demonstrated, this was not affected by a blockade of CXCR3 or DARC (Figure 2A). This suggests that these receptors do not play a role in CXCL4 endocytosis by VSMCs.

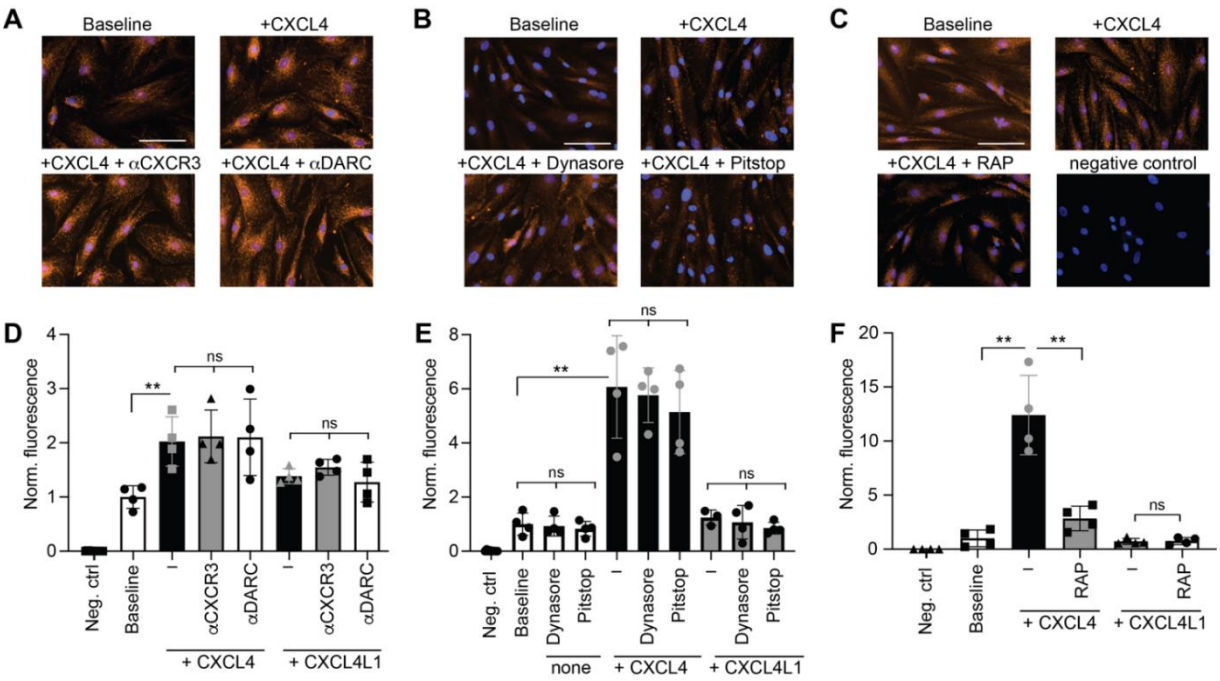


**Figure 2. Molecular determinants of CXCL4 uptake.**
CXCL4, CXCL4L1 (at 0.5 µg/mL) or buffer (baseline) was added to VSMCs in the presence of indicated compounds for 1 h at 37 °C, washed with heparin and stained for CXCL4 with prior permeabilization. (**A**,**D**) Representative images and quantitation of chemokines, co-incubated with antibodies against CXCR3 or DARC and resulting intracellular CXCL4 was expressed normalized to baseline fluorescence, measured using Cytation™. (**B**,**E**) Representative images and quantitation of chemokines, co-incubated with Dynasore (80 µM) or PitStop2 (15 µM) and resulting intracellular CXCL4 was expressed normalized to baseline fluorescence, measured using Cytation™. (**C**,**F**) Representative images and quantitation of chemokines, co-incubated with RAP (200 nM) and resulting intracellular CXCL4 was expressed normalized to baseline fluorescence, measured using ImageJ. Scale bar: 100 µm. Non-significant: ns, ** $p < 0.01$, n = 4, one-way analysis of variance (ANOVA) with Tukey's post-test (**D**,**E**) or with Dunnett's post-test (**F**). Representative figures of CXCL4L1 treatment are shown in Figure S3.

Next, endocytosis was blocked by the clathrin- and dynamin inhibitors Pitstop2 and Dynasore, respectively, to investigate whether the uptake of CXCL4 occurred by endocytosis. While the uptake of fluorescently labelled native LDL particles (DiI-nLDL) was effectively blocked by both Pitstop2 and Dynasore (Figure S2C), no effect of these endocytosis inhibitors on the internalization of CXCL4 was observed (Figure 2B). These data suggest that receptor-mediated endocytosis is not involved in CXCL4 uptake to VSMCs.

To further explore the mechanism of CXCL4 uptake, LDL receptor family members were investigated[20]. The addition of RAP, a well-established blocker of these receptors, led to a significantly decreased endocytosis of DiI-nLDL particles by VSMCs (Figure S2D) and significantly diminished CXCL4 uptake by VSMCs (Figure 2C). Similar to the other results, there was virtually no CXCL4L1 uptake by VSMCs under any condition (Figures 2 and S3). In previous studies, CXCL4 was found to interact with oxidized LDL (oxLDL)[22]. However, co-incubation of CXCL4 or CXCL4L1with DiI-oxLDL did not affect the internalization of DiI-oxLDL by VSMCs, compared to control (Figure S2E).

### *2.3. CXCL4 Is Not Internalized by Heparin Pre-Treated Quiescent VSMCs*

Human VSMCs are characterized by high phenotypic plasticity. To address the question of whether uptake of CXCL4 and CXCL4L1 differs by phenotype of VSMCs, contractile or synthetic phenotypes were induced by pre-treatment with heparin or PDGF, respectively. There

was a significant uptake of CXCL4 and not CXCL4L1 in control cells and PDGF-pretreated cells (synthetic phenotype) (Figure 3A,B). Markedly, while induction of a synthetic phenotype with PDGF did not further enhance CXCL4 uptake, pre-treatment with heparin, followed by washing and overnight starvation completely blocked the internalization of CXCL4 by VSMCs (Figure 3A,B). The effect of heparin appeared to be specific since induction of a contractile phenotype by culturing the cells in low-serum medium did not reduce CXCL4 uptake (Figure S2F). The data suggest that in this experimental setup, CXCL4 internalization is specifically blocked by heparin pre-treatment and might not be a general feature of contractile VSMCs. Of note, since heparin can neutralize CXCL4, it was removed >12 h before addition of the chemokines.

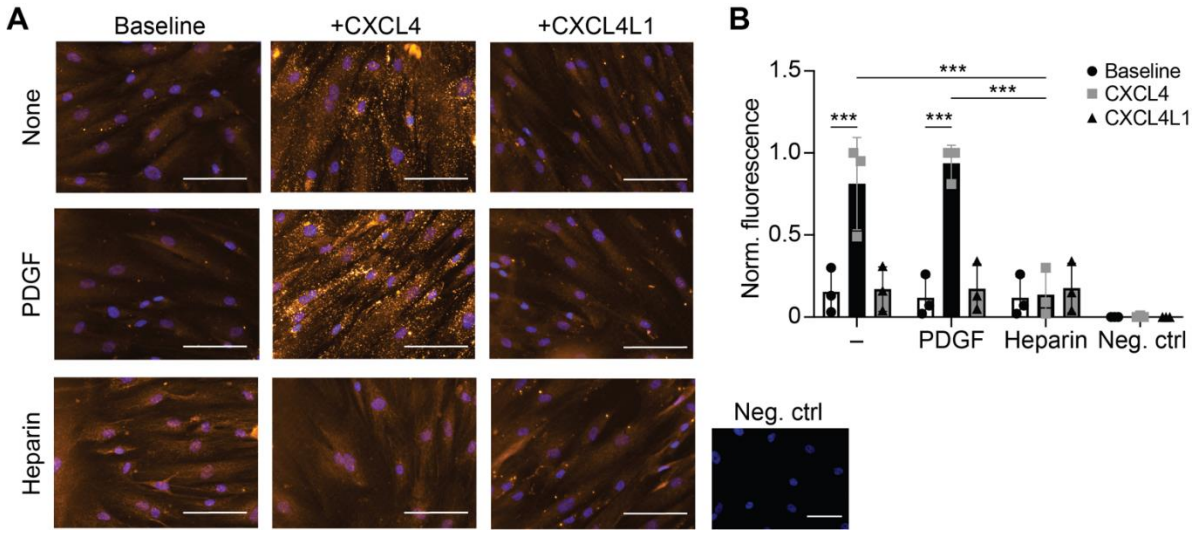


**Figure 3. Influence of VSMC phenotype on chemokine uptake.**
Prior to the addition of CXCL4, CXCL4L1 (at 0.5 µg/mL) or buffer, VSMCs were cultured in the presence of PDGF-BB (20 ng/mL, 3 days) or heparin (200 U/mL, 5 days). After incubation with chemokines for 1 h at 37 °C, cells were washed with heparin and stained for CXCL4 with permeabilization. The negative control is without anti-CXCL4 antibody. (**A**) Representative micrographs showing CXCL4 staining. Scale bar: 100 µm. (**B**) Quantification of fluorescence normalized by minimum and maximum, four images per well (three wells per experiment of three separate experiments). *** $p < 0.001$, n = 3, two-way ANOVA with Dunnett's post-test.

### *2.4. CXCL4 Modifies Expression of Phenotype Switch-Specific Genes in VSMCs*

To investigate whether CXCL4 and CXCL4L1 treatment affect the phenotype of VSMCs on a molecular level, qPCR analysis was performed. We made a selection of contractile markers (calponin—CNN1, alpha smooth muscle actin—αSMA) and transcription factors (Krüppel-like factor 4—KLF4, NLR family pyrin domain containing 3—NLRP3). The cells were incubated with the chemokines for 24 or 72 h, and cDNA was subjected to RT-qPCR. The data show that mRNA levels of CNN1 and α-SMA were significantly lower after 24 h treatment with CXCL4 and CXCL4L1 in comparison to control (Figure 4A,B). On the other hand, although accompanied by a notable experimental variation, mRNA levels of the transcription factors KLF4 and NLRP3 were significantly higher after 24-h incubation with CXCL4 and CXCL4L1 (Figure 4C,D). Interestingly, after 72 h of incubation with CXCL4 or CXCL4L1 all markers remained unchanged and comparable to controls (Figure S4), indicating a transient influence on gene expression.

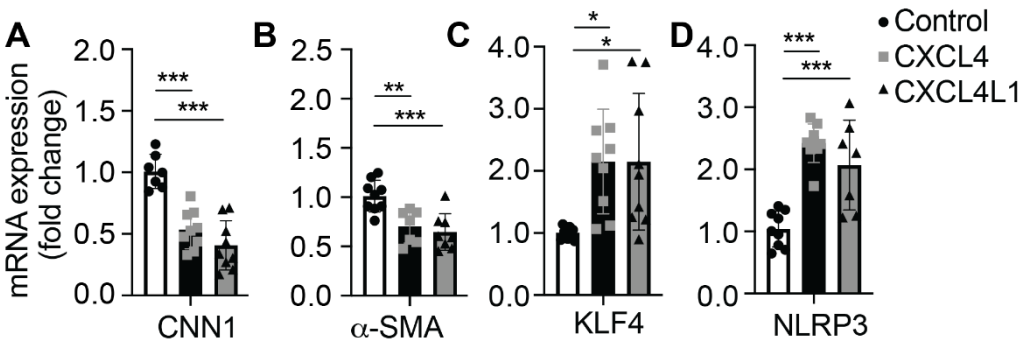


**Figure 4. Expression of mRNA by quantitative real-time PCR.**
Primary VSMCs were cultured at 120,000 cells/well in Dulbecco's modified Eagle medium (DMEM) with 20% FBS prior to treatment with or without CXCL4 or CXCL4L1 at 1 µg/mL in DMEM with 2.5% FBS for 24 h. Relative gene expression was expressed as fold change of (**A**) calponin (CNN1), (**B**) α-smooth muscle actin (α-SMA), (**C**) KLF4, (**D**) NLRP3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n = 7–9 independent experiments, one-way ANOVA with Dunnett's post-test.

*2.5. Effects of CXCL4 and CXCL4L1 on Inflammatory and Calcification Potential of VSMCs*

Consistent with previous studies[20,21], a stimulatory and dose-dependent effect of CXCL4 on proliferation of VSMCs was observed, when compared to control (Figure 5A,B). Interestingly, CXCL4L1 did not affect proliferation of VSMCs at any concentration (Figure 5A,B).

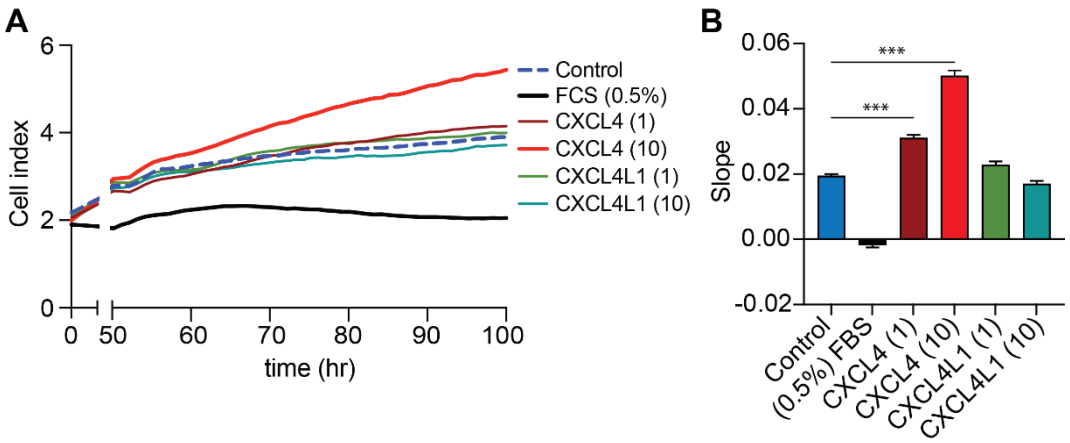


**Figure 5. Influence of CXCL4 and CXCL4L1 on proliferation of VSMCs.**
Cell growth was monitored in 96-well electronic microtiter plates for 9 days and (**A**) plotted as mean cell index over time (n = 5). (**B**) The rate of cell growth expressed as slopes between 50 and 100 h of proliferation from the curves from (**A**), mean ± SD. *** $p < 0.001$, n = 5, one-way ANOVA with Dunnett's post-test.

To examine whether the incubation of VSMCs with CXCL4 or CXCL4L1 stimulates ROS formation, a fluorogenic dye-based ROS assay was performed. Although ROS formation was observed after treatment with hydrogen peroxide (positive control), treatment with CXCL4 or CXCL4L1 did not result in increased ROS production after 6 h of incubation (Figure 6A). Interestingly, there was virtually no change in ROS levels after the treatment with CXCL4 or CXCL4L1 with or without calcium-phosphate supplementation (Figure 6A).

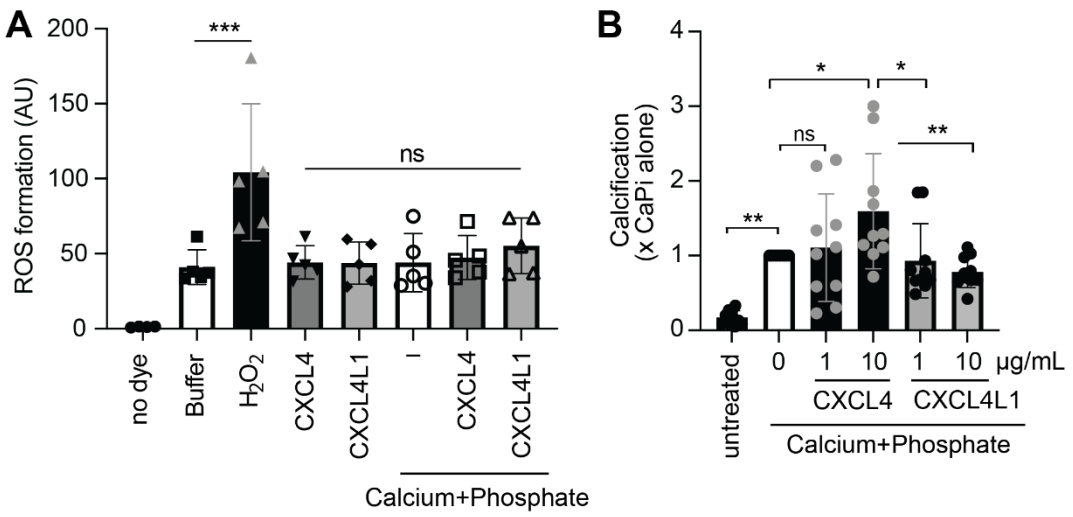


**Figure 6. Formation of reactive oxygen species (ROS) and calcium mineral crystals in VSMCs.** (**A**) Cultured VSMCs in DMEM/2.5% FCS overnight, loaded with or without DCFDA dye and stimulated with vehicle, CXCL4 or CXCL4L1 (10 µg/mL) in the presence or absence of calcium (2.7 mM) and phosphate (2.5 mM). ROS formation was expressed as arbitrary fluorescence units (AU). Non-significant: ns, *** $p < 0.001$, n = 4, one-way ANOVA with Sidak post-test. (**B**) Cultured VSMCs in DMEM/20% FCS treated with or without calcium (2.7 mM) and phosphate (2.5 mM) in the presence of vehicle, CXCL4 or CXCL4L1 (1 and 10 µg/mL). Calcification was expressed as fold control in the absence of chemokines (white bar). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n = 10 independent experiments, one-way ANOVA with Sidak post-test.

To investigate whether CXCL4 and CXCL4L1 play a role in the formation of calcium-phosphate deposits on the cell surface, a calcification assay was performed. The cells were treated with calcium-phosphate alone or combined with CXCL4 or CXCL4L1 at 1 or 10 µg/mL. The addition of CXCL4 at 1 µg/mL did not significantly alter calcification but CXCL4 at 10 µg/mL increased calcification of cultured VSMCs by approximately 50% (Figure 6B). Notably, CXCL4L1 at lower and higher concentration did not significantly affect the formation of calcium-phosphate deposits on the cell surface, with a tendency to lower calcification (Figure 6B).

## 3. Discussion

In this study, we demonstrated that CXCL4L1 and CXCL4 play distinct roles in the phenotypic modulation of VSMCs. Smooth muscle cells have been shown to play an important role in vascular remodeling, atherosclerosis and vascular calcification[19,31]. Previous studies have implicated CXCL4 in the induction of inflammation and phenotype change in VSMCs[20,21], but the role of its non-allelic variant (CXCL4L1) has been unclear. Messenger RNAs of both variants of platelet factor 4 were found in VSMCs and other cell types, but VSMCs were the only cell type showing a predominance of CXCL4L1 expression over the native CXCL4[24]. By staining with a specific antibody, we confirmed these observations on the cellular level, showing that non-treated VSMCs have a baseline content of endogenous CXCL4L1 protein. Since CXCL4 is internalized by endothelial cells[30], we investigated whether CXCL4 is endocytosed by VSMCs or rather binds to the cell surface, resulting in the intracellular signaling without concurrent endocytosis. Our data clearly indicate that unlike its variant, exogenously added, native CXCL4 was internalized by VSMCs and appeared to be enclosed within endosome-like structures.

It is well known that chemokines and chemokine receptors form a redundant system. One chemokine can bind to different receptors and vice versa. Recent evidence suggests that CXCR3 (CXCR3A, -B and -alt) could serve as a functional receptor for both CXCL4 and

CXCL4L1[5,6,32,33,34] and DARC for native CXCL4[35]. Although we found the presence of both receptors in cultured VSMCs, their blockade by specific antibodies did not affect the internalization of CXCL4. This is contrary to the aforementioned studies[4,6,33,35], but in accordance with our previous observation that CXCR3 was not involved in CXCL4 uptake in endothelial cells[30]. In another report, a CXCR3 blocking antibody did not affect CXCL4-induced cytokine production in the human carotid artery SMCs[20].

The most common route of endocytosis of chemokine receptors involves clathrin and during membrane budding and scission also requires the enzyme dynamin[36]. Here, we proved that the endocytosis of CXCL4 by VSMCs was clathrin- and dynamin-independent. This contrasts with our previous work showing an involvement of both proteins during endocytosis of CXCL4 by endothelial cells[30] suggesting distinct uptake mechanisms in different cell types. Alternatively, chemokines were shown to be internalized through clathrin-independent mechanisms involving lipid-rafts and caveolae[37]. The involvement of caveolae-mediated endocytosis in CXCL4 internalization is less likely since this process also requires dynamin for membrane scission[38]. On the other hand, endocytosis of rafts, characterized by cholesterol sensitivity may include caveolin- and dynamin-dependent or independent pathways[39]. Therefore, the exact CXCL4 endocytosis route in VSMCs needs to be investigated more precisely.

Previously CXCL4 was reported to signal through low-density lipoprotein receptor-related protein-1 (LRP1)[9] and LDL receptor (LDLR)[8] in endothelial cells. Here, we showed that the uptake of CXCL4 was abrogated after treatment with a universal inhibitor of the LDL receptor family (RAP). Since RAP blocks all members of the LDLR family, further studies using specific blocking antibodies or RNA interference are required to pinpoint a specific receptor for CXCL4. Taken together, our findings indicate that CXCL4 uptake in VSMCs is mediated by the LDL receptor family and may signal mainly through LRP1, which was previously reported by Shi et al.[20]. In this respect it might appear paradoxical that, while sharing the receptors, LDL enters the cell by receptor-mediated endocytosis, whereas CXCL4 is taken up independently of clathrin or dynamin. This also contrasts with our previous observations of CXCL4 uptake in endothelial cells, which did appear to occur by clathrin- and dynamin-dependent endocytosis. The LDL-receptor family members might serve as a functional docking site for CXCL4, while entry into the cell is mediated by a different process.

The interactions of CXCL4 and oxidized LDL (oxLDL) was also investigated. Oxidized LDL can bind to the LOX-1 receptor on VSMCs and upregulate expression of several cytokines (e.g., IL-1β and TNF-α), which may result in a proinflammatory phenotype[40]. Former observations suggested that CXCL4 promoted binding of oxLDL to VSMCs and enhanced the internalization of oxLDL[22]. In this study we showed that the co-addition of oxLDL with CXCL4 and CXCL4L1 did not affect endocytosis of oxLDL.

Since the phenotype might influence the uptake of CXCL4, VSMCs were treated with PDGF-BB or heparin to direct the cells towards synthetic or contractile phenotypes, respectively[41,42,43]. We hypothesized that the different composition of extracellular matrix proteins and GAGs (CXCL4 co-receptors) on various VSMCs phenotypes can affect the internalization of investigated chemokines. Unlike PDGF-induced synthetic cells, heparin-induced contractile cells did not take up CXCL4. This effect was not observed when low-serum was used to induce a contractile phenotype. An explanation of the action of heparin on vascular cells is the removal of various growth factors attached to GAGs, decrease of ERK signaling or inhibition of proliferation and transcription factor activity in VSMCs[44]. Taking this into account, we conclude that the blockade of CXCL4 internalization was caused by a heparin-specific phenotypic switch of VSMCs and might not be a general feature of contractile VSMCs.

Treatment of VSMCs with CXCL4 and CXCL4L1 resulted in decreased expression of both CNN-1 and ACTA-2, indicating a phenotype switch from contractile to synthetic. These data

confirmed the previous findings of Shi et al.[20]. The addition of CXCL4 and CXCL4L1 also elevated the expression of Krüppel-like factor 4 (KLF4), a transcription factor that directs cell fate, differentiation and proinflammatory response of VSMCs[45,46] and was previously found to be upregulated in VSMCs during atherogenesis[47] and upon vascular injury[46]. This is also in line with previous observations that CXCL4 accelerated VSMCs inflammatory responses to injury, in part by stimulating the expression of KLF4[20]. The activation of NLRP3 inflammasome has been previously shown to contribute to VSMCs phenotypic transformation and proliferation in rats and primary VSMCs[48]. To the best of our knowledge, the effect of CXCL4 and CXCL4L1 on the NLRP3 mRNA stimulation has not been previously described. In a recent publication, soluble platelet factors were found to enhance NLRP3 transcription and boost IL-1 expression in macrophages[49]. Physiological concentrations of CXCL4 were shown to directly stimulate human VSMCs inflammatory responses, with partly KLF4-dependent increase of IL-6 and CXCL8 secretion[20]. CXCL4 has also been shown to boost IL-6 and TNF-α production in monocytes[50,51] and bone marrow stroma[52]. In contrast, intrahepatic mRNA IL-6 expression was significantly increased in CXCL4 knockout mice compared to wild-type mice[53]. It is interesting that both CXCL4 and CXCL4L1 induced a change in the expression of the above markers in VSMCs. This indicates that internalization is not strictly required for these effects, since only CXCL4 was shown to be taken up and both chemokines might activate VSMCs through their cognate surface receptors.

Already in the 1970s it was suggested that platelets have a causative role in VSMCs proliferation and CXCL4 was implied among the mitogens carried by platelets. In this study, we showed that CXCL4 stimulated the proliferation of VSMCs, which is in line with previous observations[20,21,54]. These results indicate a role for CXCL4 in the induction of intimal thickening following vascular injury through direct or indirect pathways and subsequent induction of medial SMCs proliferation and migration. Interestingly, it was previously reported that both variants inhibited the proliferation of endothelial cells[34,55] and in this study CXCL4L1 did not affect VSMC proliferation. Therefore, the baseline expression of CXCL4L1 in VSMCs might represent a physiologic regulatory mechanism to maintain vascular homeostasis and to avoid endothelial cell proliferation and aberrant angiogenesis[24].

Oxidative stress also participates in pathophysiological processes of vascular damage. It has been shown that CXCL4 induced ROS production in various cell types[56,57] and that the CXCL4L1 did not affect ROS generation in rat retinas[58]. Recently it was also demonstrated that ROS production in VSMCs is required for the release of extracellular vesicles that promote calcification[59]. However, in our experimental setup neither CXCL4 nor CXCL4L1 was able to stimulate ROS production in VSMCs.

We also hypothesized that CXCL4 may facilitate the formation of microcalcifications that leads to plaque destabilization and rupture. Previous studies in mouse and human revealed that CXCL4 was present in atherosclerotic plaques as well as regions of calcification[12,14] and its elimination from platelets reduced atherosclerosis[14,60]. Here, we showed that although the experimental variation was large, CXCL4 appeared to increase calcification at higher concentrations while the addition of CXCL4L1 showed a non-significant trend towards a reduction. Unlike CXCL4, CXCL4L1 did not bind to CCL5 and did not enhance atherosclerosis in transgenic mice with CXCL4 modified to CXCL4L1, indicating that CXCL4L1 has reduced inflammatory potential, at least during plaque development[29]. On the other hand, a recent study showed that CXCL4L1 induced a different palette of cytokines and chemokine receptors in monocytes than CXCL4, skewing them towards a pro-inflammatory phenotype[61]. Thus, the question of whether CXCL4L1 exerts a homeostatic rather than an inflammatory function can likely only be answered within a defined cellular context. To further study the role of endogenous CXCL4L1, its expression can be downregulated by RNA interference. However, this might be accompanied by phenotypic and expression changes induced by liposome- or

viral-mediated transfection methods. Alternatively, the CXCL4L1 gene can be excised using CRISPR/CAS9 technology in induced pluripotent stem cells, prior to phenotypic induction to VSMCs.

Taken together, CXCL4 is taken up into VSMCs by the action of the LRP receptor (or LDL-receptor family). The receptors CXCR3 and LRP might mediate the signaling by CXCL4. CXCL4L1, on the other hand, is not internalized by VSMCs, but expressed endogenously. Previous studies have suggested CXCR3 as a functional receptor for CXCL4L1[6]. This is schematically summarized in Figure 7. This study provides further evidence for the pro-inflammatory actions of CXCL4 on vascular cells and highlights the distinct properties of the highly homologous chemokines CXCL4 and CXCL4L1.

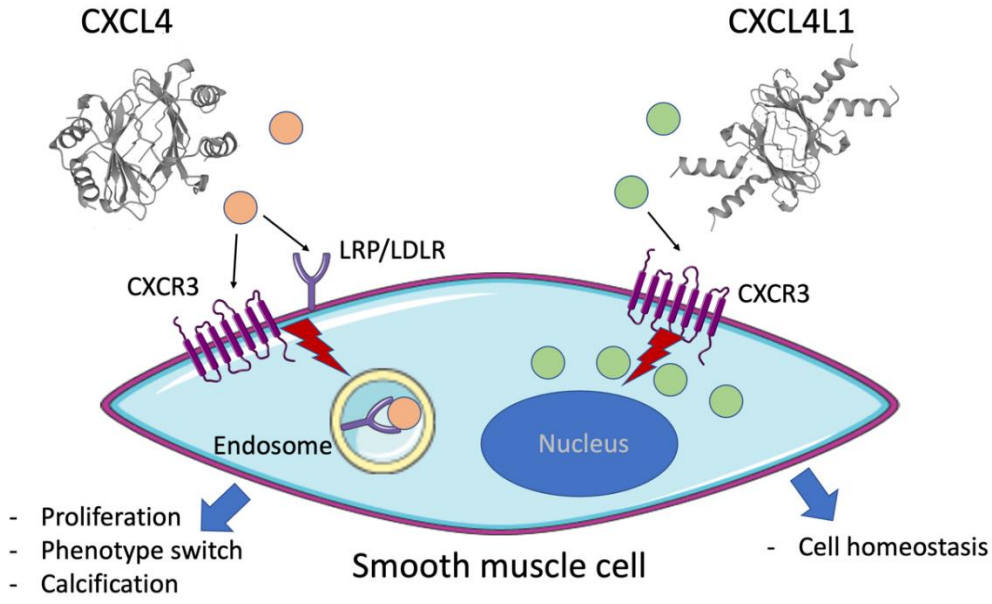


**Figure 7. Schematic summary of the proposed action of CXCL4 and CXCL4L1 on smooth muscle cells.**
CXCL4 (orange dot and ribbon structure pdb: 1PF9Q) is taken into the cells by the action of the low-density lipoprotein (LDL) receptor family proteins or lipoprotein receptor-related protein (LRP) into endosomes. CXCL4-induced signals might be transmitted through both CXCR3[4] and LRP[20], leading to proliferation, phenotypic alterations and calcification. CXCL4L1 (green dot and ribbon structure pdb: 4HSV) is present endogenously and is not taken up by VSMCs, but may induce cell signaling through CXCR3[6]. It is postulated that CXCL4L1 might be involved in cell homeostatic processes.

## 4. Materials and Methods

### *4.1. Reagents*

CXCL4 was isolated from anonymous expired platelet packs as described elsewhere[23]. Recombinant CXCL4L1 was expressed in *E. coli* and purified in our laboratory as described previously[26,55]. Products were analyzed by Xevo® UPLC-MS (Waters Corporation, Milford, MA, USA) as described and results are shown in Figure S5[62]. Receptor-associated protein (RAP) was purchased from Enzo Life Sciences (Lörrach, Germany). Native human LDL (SAE0053), Dynasore (CAS: 304448-55-3) and PitStop2 (CAS: 1419093-54-1) and TRI reagent were purchased from Merck Millipore (Darmstadt, Germany). Interleukin 6 (IL) ELISA kits, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled oxLDL, 2’,7’-dichlorofluorescin diacetate (DCFDA), and attachment factor (AF), anti-DARC clone 2C3, polyclonal anti-CXCL4L1 antibody, and goat anti-rabbit antibodies conjugated with

AF532 were purchased from ThermoFisher Scientific (Waltham, MA, USA). Cytokines and rabbit anti-human CXCL4 were from Peprotech (Rocky Hill, NJ, USA). Monoclonal mouse anti-CXCR3 clone #49801 and monoclonal mouse anti-DARC, clone #358307 were from R&D systems (Minneapolis, MN, USA). FITC-conjugated goat anti-mouse from Jackson ImmunoResearch (Ely, UK). MicroBCA kit and iScript™ Reverse Transcription Supermix were from Bio-Rad (Hercules, CA, USA). Takyon™ No Rox SYBR® MasterMix dTTP blue was from Eurogentec (Seraing, Belgium). O-cresolphthalein assay was purchased from Randox (Crumlin, UK).

*4.2. Cell Culture*

Human vascular smooth muscle cells (VSMCs) were isolated from a single patient in our laboratory from tissue explants (human thoracic aorta) as described previously and cultured between passage 4 and 10[59,63]. Collection, storage, and use of tissue and human aortic samples were performed in agreement with the Dutch Code for Proper Secondary Use of Human Tissue.

*4.3. CXCL4/CXCL4L1 Internalization*

VSMCs were seeded in attachment factor-coated black 96-well plate at a density of 3–5 × $10^3$ cells/well for 16–20 h and subsequently starved in DMEM with 0.5% FCS overnight. After that, cells were incubated with the vehicle (50 mM Na-Acetate pH 5.5, 600 mM NaCl and 0.5 mM EDTA), 0.5 µg/mL CXCL4 or CXCL4L1 in DMEM containing 0.5% FCS or 0.3% BSA (for oxLDL-experiments) for 1 h, either at 37 °C or 4 °C.

In some experiments, blocking antibodies (10 µg/mL of anti-CXCR3 or anti-DARC), Dynasore (80 µM) or PitStop2 (15 µM), (DiI)-labeled oxLDL (5 µg/mL), RAP (200 nM) or the vehicle, were added for up to 30 min, and then 0.5 µg/mL of the chemokines (CXCL4 or CXCL4L1) were added to the cells and the culture plates were incubated for additional 1 h at 37 °C.

For experiments on VSMCs phenotype, cells were pre-incubated without or with PDGF (20 ng/mL, 3 days) or heparin (200 U/mL, 5 days) in DMEM with 20% FCS, or cultured in low serum DMEM (0.5% FCS). After up to 5 days, cells were starved in DMEM with 0.5% FCS overnight before addition of the chemokines.

Then, the cells were washed (200 U/mL heparin in PBS) to remove remaining surface chemokines. After that, cells were fixed with 4% paraformaldehyde and blocked with PBS containing 2% BSA with or without 0.1% Triton X-100 for 1 h at room temperature. The cells were stained as described below.

*4.4. LDL Receptor Family Blockade by RAP or Endocytosis Inhibitors*

VSMCs were cultured as above, starved in DMEM/0.3% BSA overnight, and the cells were pre-treated with vehicle or RAP (200 nM), Dynasore (80 µM) or PitStop2 (15 µM) for 15–30 min. Subsequently, the cells were incubated with the vehicle, DiI-nLDL (5 µg/mL) alone or RAP and DiI-nLDL together for an additional 1 h at 37 °C. Afterwards, the cells were incubated with 200 U/mL heparin for 5 min. Subsequently, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, washed with PBS, stained with Hoechst and analyzed with Cytation™ (BioTek, Agilent, Santa Clara, CA, USA).

*4.5. Immunocytochemistry*

The primary antibodies (rabbit anti-human CXCL4(L1), monoclonal mouse anti-CXCR3, monoclonal mouse anti-ACKR1/DARC) were added to VSMCs at a final concentration of 2 µg/mL or 10 µg/mL (CXCR3, DARC) and incubated overnight at 4 °C. After washing, the secondary antibody was added (goat anti-rabbit AF532, goat anti-mouse-FITC) at a final concentration of 5 µg/mL for 1 h. Next, cells were washed, and nuclei were stained with

Hoechst solution. Then, the cell count and the fluorescence were analyzed with Cytation™. Four pictures containing 20–30 cells per field of view were taken for each well, and each condition was represented by at least three internal replicates. Per experiment, three independent culture plates were analyzed. In negative control wells, the addition of primary antibody was omitted. Micrographs were taken with Cytation™. Cell counting and fluorescence analysis were performed with ImageJ software 1.5[64]. The obtained relative fluorescent units (RFU) per field were divided by the number of cells. In some experiments, the fluorescence of exogenously added CXCL4(L1) was normalized against the baseline fluorescence.

### *4.6. Quantitative Real-Time PCR (qPCR)*

VSMCs were seeded in triplicate in 6-well plates at a density of 120,000 cells/well in DMEM with 20% FCS. The next day cells were treated without or with CXCL4 or CXCL4L1 at (1 µg/mL) in DMEM, supplemented with 2.5% FCS and kept in the incubator for 24 or 72 h. After that, VSMCs were lysed in TRI reagent and total RNA extracted, quantified at 260 nm and reverse transcribed using iScript for RT-qPCR. Gene expression levels were analyzed by real-time quantitative PCR (qPCR) on a LightCycler 480 (Roche Applied Science, Basel, Switzerland). Amplification reactions were carried out in a volume of 10 µL including 45 ng of total cDNA, 5.5 µL of SYBR-dTTP mix and 62.5 nM of each primer. Amplification specificity was confirmed by melting curves and curves were analyzed with LinRegPCR software (11.0) [65]. Relative quantification of expression was achieved by plotting Cq ratios.

### *4.7. Proliferation Assay Using the xCELLigence System*

Cells were seeded into 96-well electronic microtiter plates (E-plate®) at 3000 cells/well in DMEM and observed for 9 days. Cell growth (impedance value) was plotted as a unitless parameter (Cell Index) over time. The rate of cell growth was determined by calculating the slope of the curve between the end of the lag phase (50 h) and the start of the stationary confluent phase (100 h).

### *4.8. Reactive Oxygen Species (ROS) Assay*

VSMCs were cultured in DMEM, supplemented with 2.5% FCS overnight, incubated with the DCFDA dye for 30 min at 37 °C and stimulated with the vehicle and stimuli (see above). ROS production was measured for 6 h by Cytation™ under at 5% $CO_2$ and 37 °C. The data were analyzed by calculating area under the curve (AUC) and normalized to the cell count.

### *4.9. Calcification Assay*

VSMCs were seeded in the 48-well plates (10,000 cells/well), cultured in DMEM/20% FCS and treated with or without increased calcium (2.7 mM) and phosphate (2.5 mM) or calcium/phosphate in combination with CXCL4 or CXCL4L1 (1 µg/mL or 10 µg/mL). To accelerate calcification, serum was reduced to 0.5% FCS in all conditions and incubated for 24 h. Calcification was quantified using a commercial o-cresolphthalein assay and normalized to protein content.

### *4.10. Statistical Analysis*

Statistical analysis was performed using Graphpad Prism 9.0.0 (Graphpad Software, San Diego, CA, USA). Data are presented as means ± SD and were compared by (non-)parametric 1-way or 2-way ANOVA. Appropriate correction for multiple comparisons was achieved depending on the statistical test. Differences with $p < 0.05$ were considered as statistically significant. Each experiment was independently repeated at least three times, as indicated for each experiment in the figure legends.

**Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1.

**Author Contributions:** Conceptualization, R.K., L.J.S. and R.R.K.; methodology, D.M.K.; formal analysis, D.M.K.; investigation, D.M.K., R.R.K.; resources, R.K., L.J.S., T.M.H.; writing—original draft preparation, D.M.K., R.R.K.; writing—review and editing, R.K., T.M.H., L.J.S. and R.R.K.; visualization, D.M.K., R.R.K.; supervision, R.K., L.J.S., R.R.K.; project administration, R.R.K.; funding acquisition, R.K., R.R.K. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** Collection, storage, and use of tissue and human aortic samples were performed in agreement with the Dutch Code for Proper Secondary Use of Human Tissue. No experiments on human or animal material requiring ethical approval were performed in this study.

**Informed Consent Statement:** Collection, storage, and use of tissue and human aortic samples were performed in agreement with the Dutch Code for Proper Secondary Use of Human Tissue.

**Data Availability Statement:** Data are available upon reasonable request from the corresponding author. Reagents and detailed methods of all procedures are provided in the Supplementary Materials.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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**Differential effects of platelet factor 4 (CXCL4) and its non-allelic variant (CXCL4L1) on cultured human vascular smooth muscle cells**

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Supplementary material

## Chapter 4

### Detailed methods

*Reagents*

CXCL4 was isolated from expired platelet packs using a method described elsewhere[1]. Recombinant CXCL4L1 was expressed in E. coli and purified in our laboratory similar as described previously[2,3] and analyzed by Xevo® UPLC-MS (Waters Corporation, Milford, MA) as described[4]. Receptor-associated protein (RAP) was purchased from Enzo Life Sciences (Lörrach, Germany). Native human LDL (SAE0053), Dynasore (CAS: 304448-55-3) and PitStop2 (CAS: 1419093-54-1) and TRI reagent were purchased from Merck Millipore (Darmstadt, Germany). Interleukin 6 (IL) ELISA kits, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled oxLDL, 2',7'-dichlorofluorescin diacetate (DCFDA), and attachment factor (AF), anti-DARC clone 2C3, polyclonal anti-CXCL4L1 antibody, and goat anti-rabbit antibodies conjugated with AF532 were purchased from ThermoFisher Scientific (Waltham, MA). Native human LDL was labeled with DiI as described[5]. Cytokines and rabbit anti-human CXCL4 were from Peprotech (Rocky Hill, NJ). Monoclonal mouse anti-CXCR3 clone # 49801 and monoclonal mouse anti-DARC, clone #358307 were from R&D systems (Minneapolis, MN). FITC-conjugated goat anti-mouse from Jackson ImmunoResearch (Ely, UK). MicroBCA kit and iScript™ Reverse Transcription Supermix were from Bio-Rad (Hercules, CA). Takyon™ No Rox SYBR® MasterMix dTTP blue was from Eurogentec (Seraing, Belgium). O-cresolphthalein assay was purchased from Randox (Crumlin, UK).

*Cell culture*

Human vascular smooth muscle cells (VSMCs) were isolated in our laboratory from tissue explants (human thoracic aorta) as described previously[6,7]. Collection, storage, and use of tissue and human aortic samples were performed in agreement with the Dutch Code for Proper Secondary Use of Human Tissue. Human VSMCs were cultured in DMEM medium supplemented with 20% FCS, 2 mM L-glutamine, 1% penicillin/streptomycin and incubated in a humidified atmosphere with 5% $CO_2$ at 37°C. Importantly, in all experiments VSMCs (between passage 4 and 10) isolated from a single patient were used. Before the start of experiments cells were cultured to at least 80% confluence.

*CXCL4/CXCL4L1 internalization*

Before the experiment, all culture plates were coated with AF. Human VSMC were seeded in a black 96-well plate at a density of $3\text{-}5\times10^3$ cells/well and incubated in DMEM, supplemented with 20% FCS overnight. The next day, cells were starved in DMEM with 0.5% FCS overnight. After that, cells were incubated with vehicle (50 mM Na-Acetate pH 5.5, 600 mM NaCl and 0.5 mM EDTA), 0.5 µg/ml CXCL4 or CXCL4L1 in DMEM containing 0.5% FCS for 1 hour, either at 37°C or 4°C.

In chemokine receptor blocking experiments, 10 µg/ml of blocking antibodies (anti-CXCR3 or anti-DARC clones #358307 and 2C3) for up to 30 minutes, and then 0.5 µg/ml of the chemokines (CXCL4 or CXCL4L1) were added to the cells and the culture plates were incubated for additional 1 hour at 37°C.

In LDL receptor family blocking experiments, the cells were starved in DMEM supplemented with 0.3% BSA overnight (to prevent the interference of lipids from FCS). The following day, the cells were pre-treated with vehicle or RAP (200 nM) for 30 minutes. Subsequently, the cells were incubated with vehicle, DiI-nLDL (5 µg/ml) alone or RAP and DiI-nLDL together for additional 1 hour at 37°C.

In endocytosis blocking experiments, the cells were pre-treated with Dynasore (80 µM) or PitStop2 (15 µM) for 15-30 minutes. Then, the fresh medium (DMEM + 0.3% BSA) containing

Dynasore (80 µM) or PitStop2 (15 µM) together with CXCL4 or CXCL4L1 (0.5 µg/ml) was added to the cells and the culture plates were incubated for additional 1 hour at 37°C.

For oxidized LDL coincubation experiments, the cells were simultaneously treated with (DiI)-labeled oxLDL (5 µg/ml) and vehicle, CXCL4 (0.5 µg/ml) or CXCL4L1 (0.5 µg/ml) in DMEM containing 0.3% BSA for 1 hour at 37°C.

In experiments exploring the influence of VSMCs phenotype, cells were pre-incubated with nothing (control), PDGF (20 ng/ml, 3 days) or heparin (200 U/ml, 5 days) in DMEM with 20% FCS or cultured in low serum DMEM (0.5% FCS). After up to 5 days, cells were starved in DMEM with 0.5% FCS overnight before addition of the chemokines (0.5 µg/ml).

Then, the cells were washed with 200 U/ml heparin to remove the remaining chemokines from the cell surface. After that, cells were fixed with 4% paraformaldehyde and blocked with blocking buffer (PBS containing 2% BSA) with or without 0.1% Triton X-100 for 1 hour at room temperature. The cells were stained as described below.

*LDL receptor family blockade by RAP or endocytosis inhibitors*

Before the experiment, all culture plates were coated with the AF. Human VSMCs were seeded in a black 96-well plate at a density of 5.000 cells/well and incubated in DMEM, supplemented with 20% FCS for 24 hours. The next day, the cells were starved in DMEM supplemented with 0.3% BSA overnight (to prevent the interference of lipids from FCS). The following day, the cells were pre-treated with vehicle or RAP (200 nM) for 30 minutes. Subsequently, the cells were incubated with vehicle, DiI-nLDL (5 µg/ml) alone or RAP and DiI-nLDL together for additional 1 hour at 37°C.

In endocytosis blocking experiments, the cells were pre-treated with Dynasore (CAS: 304448-55-3) (80 µM) or PitStop2 (CAS: 1419093-54-1) (15 µM) for 15-30 minutes. Then, the fresh medium (DMEM + 0.3% BSA) containing Dynasore (80 µM) or PitStop2 (15 µM) together with DiI-nLDL (5 µg/ml) was added to the cells and the culture plates were incubated for additional 1 hour at 37°C.

Afterwards, the cells were incubated with 200 U/ml heparin for 5 minutes. Subsequently, cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Next, cells were washed with PBS and nuclei were stained with Hoechst solution. Then, the cell count and the fluorescence were analysed with Cytation™ (BioTek, Agilent, Santa Clara, Ca)

*Immunocytochemistry*

The primary antibody (rabbit anti-human CXCL4 from Peprotech or anti-CXCL4L1 from ThermoFischer) was added to VSMCs at a final concentration of 2 µg/ml and incubated overnight at 4°C. After washing, the secondary antibody was added (goat anti-rabbit, conjugated with AF532 from Life Technologies) at a final concentration of 5 µg/ml for 1 hour. Next, cells were washed and nuclei were stained with Hoechst solution. Then, the cell count and the fluorescence was analysed with Cytation™. In negative control wells, the addition of primary antibody was omitted. The microscopic pictures were taken with Cytation™. Cell counting and fluorescence analysis were performed with ImageJ software.

Chemokine receptor staining - Then, the primary antibody (anti-CXCR3 or anti-DARC clone #358307) was added at a final concentration of 10 µg/ml and incubated overnight at 4°C. After washing, the secondary antibody was added (FITC-conjugated goat anti-mouse from Jackson) at a final concentration of 6 µg/ml for 1 hour. Next, cells were washed, and nuclei were stained with Hoechst solution. Then, the cell count and the fluorescence were analyzed with Cytation™. In negative control wells, the addition of primary antibody was omitted.

*Quantitative real-time PCR (qPCR)*

Primary VSMCs were seeded in 6-well plates at a density of 120.000 cells/well in DMEM with 20% FBS. The next day cells were treated with vehicle, CXCL4 (1 µg/ml) or CXCL4L1

(1 μg/ml) in DMEM, supplemented with 2.5% FBS and kept in the incubator for 24 or 72 hours. After that, VSMCs were directly lysed in TRI reagent (1 ml/well) and total RNA was extracted using a manufacturer's protocol. RNA concentration was quantified spectrophotometrically at 260 nm using NanoDrop™. Reverse Transcription was performed using iScript™ Reverse Transcription Supermix for RT-qPCR, following manufacturer's recommendations. Gene expression levels were quantified by real-time quantitative PCR (qPCR) on a LightCycler 480 Real-Time PCR instrument (Roche Applied Science, Basel, Switzerland). Amplification reactions were carried out in a volume of 10 μl including 45 ng of total cDNA, 5.5 μl of Takyon™ No Rox SYBR® MasterMix dTTP blue and 62.5 nM of each primer. Relative quantification was calculated by plotting Cq ratios. Experiments were performed in triplicate, with internal triplicate determinations.

Table S1. Primer sequences

| Target | Sequence |
|---|---|
| **GAPDH** | Forward: AAC-GGA-TTT-GGT-CGT-ATT-GGG-C |
| | Reverse: CTT-GAC-GGT-GCC-ATG-GAA-TTT-G |
| **CNN1** | Forward: GCT-GGA-GAA-CAT-CGG-CAA-CTT-CAT-CAA-G |
| | Reverse: GCT-CCT-GCT-TCT-CTG-CGT-ACT-TCA-CTC |
| **α-SMA** | Forward: CCT-GAC-TGA-GCG-TGG-CTA-TT |
| | Reverse: GCC-CAT-CAG-GCA-ACT-CGT-AA |
| **KLF4** | Forward: AGA-GGA-GCC-CAA-GCC-AAA |
| | Reverse: AGC-CGT-CCC-AGT-CAC-AGT |
| **NLRP3** | Forward: GGG-ACT-GAA-GCA-CCT-GTT-GT |
| | Reverse: GAG-TCT-GGT-CAG-GGA-ATG-GC |

*Proliferation assay using the xCELLigence system*

To measure the proliferation rate of VSMCs, an in vitro xCELLigence RTCA (real-time cell analysis, Agilent) platform was used. Cells were seeded into 96-well electronic microtiter plates (E-plate®) at 3000 cells/well in DMEM and observed for 9 days. Adhesion of cells to the gold microelectrodes in the wells impedes the flow of electric current between electrodes and impedance value is plotted as a unitless parameter (Cell Index) over course of time. The rate of cell growth was determined by calculating the slope of the curve line between the end of the lag phase (0-50 hours) and the start of the stationary confluent phase (after 100 hours).

*Calcification assay*

Primary VSMCs were seeded in the 48-well plates (10.000 cells/well). After overnight incubation in DMEM (supplemented with 20% FCS) cells were treated with control medium (DMEM), increased calcium (2.7 mM) and phosphate (2.5 mM) or calcium/phosphate in combination with CXCL4 or CXCL4L1 (1 μg/ml or 10 μg/ml). To accelerate calcification, serum was reduced to 0.5% FCS in all conditions. Purified, plasma CXCL4 or recombinant CXCL4L1 was added to calcium/phosphate enriched cell culture media and incubated for 24 hours. After the experiment, calcification was quantified. Briefly, calcified material from each well was extracted from non-fixed cell layers with 0.1 mol/l HCl, incubated for 30 minutes at room temperature and analysed using o-cresolphthalein assay according to manufacturer's

recommendations. Hereafter, the remaining solution was neutralized with 0.1 M NaOH and 0.2% SDS. Subsequently, protein determination was performed by MicroBCA kit. At the end the level of calcification was normalized to protein content.

*Reactive oxygen species (ROS) assay*

Human VSMCs were seeded in a black 96-well plate at a density of 8.000 cells/well and incubated in DMEM, supplemented with 2.5% FCS overnight. The next day, cells were pre-incubated with the dye for 30 min at 37°C and stimulated with vehicle and various test media at times of measurement. Hydrogen peroxide ($H_2O_2$) was used as a positive control. The combination of calcium (2.7 mM) and phosphate (2.5 mM) alone or combined with CXCL4 or CXCL4L1 [10 µg/ml] was used. All mastermixes were prepared in the KRPG medium, supplemented with Hoechst solution to visualize nuclei. Intracellular reactive oxygen species (ROS) were measured using cell permeable reagent DCFDA, a fluorogenic dye that measures hydroxyl, peroxyl and other ROS activity in the cell. The fluorescence intensity was detected with Cytation$^{TM}$ for 6 hours in controlled conditions (5% $CO_2$, 37°C). The data was analyzed by calculating area under the curve (AUC) and normalized to the cell count.

*Statistical analysis*

Statistical analysis was performed using Graphpad Prism 9.0.0. Data are presented as means ± SD and were compared by (non-)parametric 1-way or 2-way ANOVA. Appropriate correction for multiple comparisons was achieved depending on the statistical test. Differences with $P<0.05$ were considered as statistically significant. Each experiment was independently repeated at least 3 times, as indicated for each experiment in the figure legends.

## Supplementary figures

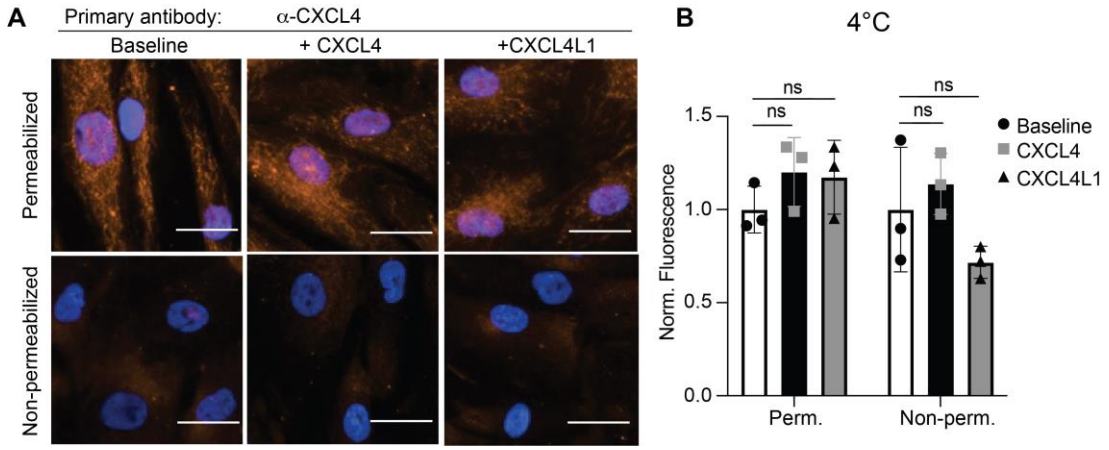


**Figure S1. Uptake of CXCL4 into VSMCs.**

CXCL4, CXCL4L1 (at 0.5µg/mL) or buffer (baseline) was added to VSMCs for 1 hour at 4°C, washed with heparin and stained for CXCL4 without or with prior permeabilization. **(A)** Representative micrographs showing CXCL4 staining (orange) and nuclei (blue). Scale bar: 50 µm. **(B)** Quantification of fluorescence expressed as normalized fluorescence relative to baseline, measure using Cytation™. ***P<0.001, n=3, two-way ANOVA with Dunnett's post-test.

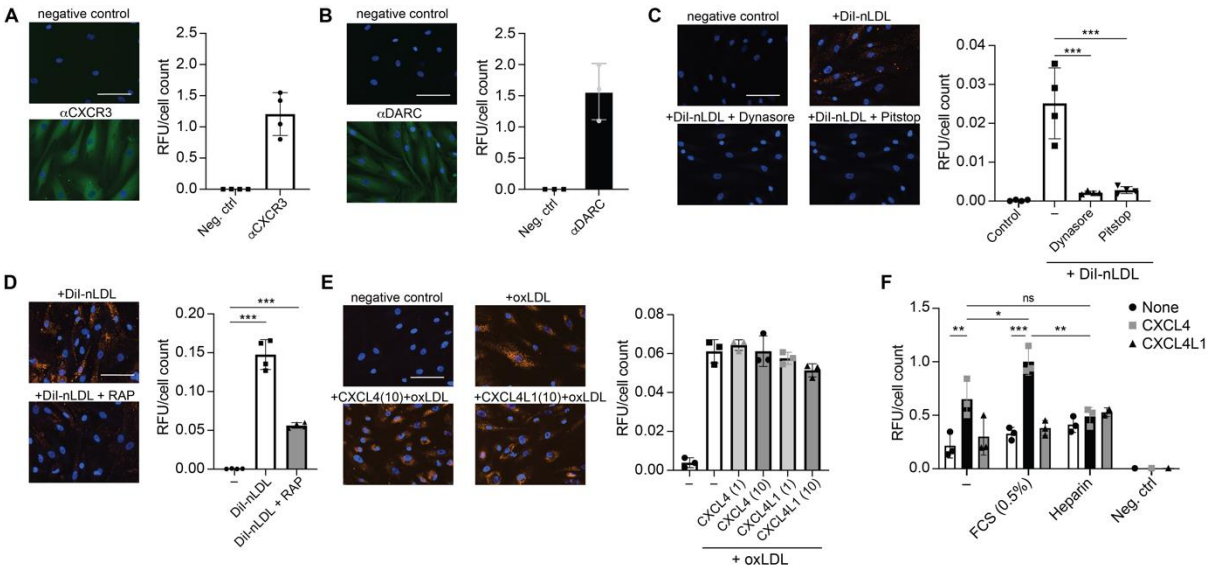


**Figure S2. Chemokine receptor staining and molecular determinants of LDL and CXCL4 uptake.**

Cells were stained with indicated antibodies or labeled proteins and representative images **(A-E)** are shown (scale bar: 100µm). Fluorescence was quantified and expressed as relative fluorescent units (RFU) divided by cell count **(A-F)**.

Cells were stained with antibodies against CXCR3 **(A)** or DARC **(B)**. DiI-labeled LDL was co-incubated with Dynasore (80 µM) or PitStop2 (15 µM) **(C)** or RAP (200 nM) **(D)**. **(E)** Cells were incubated with (DiI)-labeled oxLDL (5 µg/ml) with or without CXCL4 or CXCL4L1 at 1 and 10 µg/mL. **(F)** Prior to the addition of CXCL4, CXCL4L1 (at 0.5µg/mL) or buffer, VSMCs were cultured in the presence of heparin (200 U/ml) or in DMEM/0.5% FCS for 5 days. After incubation with chemokines for 1 hour at 37°C, cells were washed with heparin and stained for CXCL4 with permeabilization. *P<0.05, **P<0.01, ***P<0.001, n=3-4 independent experiments, one or two-way ANOVA with Dunnett's or Tukey's post-test.

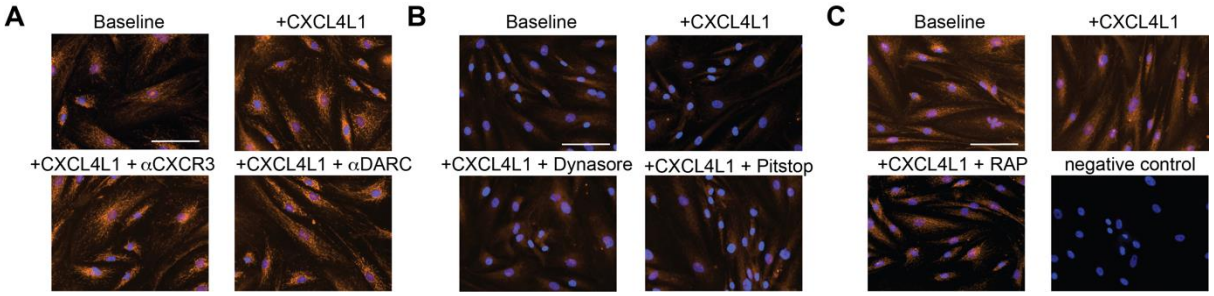


**Figure S3. Molecular determinants of CXCL4L uptake.**

CXCL4L1 (at 0.5 µg/mL) or buffer was added to VSMCs in the presence of indicated compounds for 1 hour at 37°C, washed with heparin and stained for CXCL4 with prior permeabilization. (A) Representative images of CXCL4L1, co-incubated with antibodies against CXCR3 or DARC and resulting intracellular CXCL4(L1) was visualized using Cytation™. (B) Representative images of CXCL4L1, co-incubated with Dynasore (80 µM) or PitStop2 (15 µM) and resulting intracellular CXCL4(L1) was visualized using Cytation™. (C) Representative images of CXCL4L1, co-incubated with RAP (200 nM) and resulting intracellular CXCL4(L1) was visualized using Cytation™.

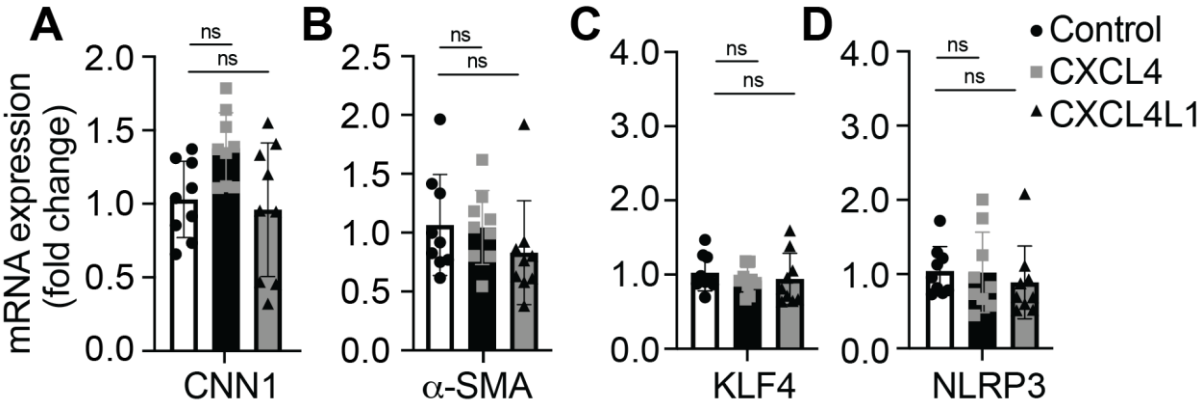


**Figure S4. Expression of mRNA by quantitative real time PCR.**

Primary VSMCs were cultured at 120.000 cells/well in DMEM with 20% FBS prior to treatment without or with CXCL4 or CXCL4L1 at (1 µg/ml) in DMEM with 2.5% FCS for 72 hours. Relative gene expression was expressed as fold change of **(A)** calponin (CNN1), **(B)** a-smooth muscle actin (a-SMA), **(C)** KLF4 and **(D)** NLRP3. Non-significant, n=7-9 independent experiments, one-way ANOVA with Dunnett's post-test.

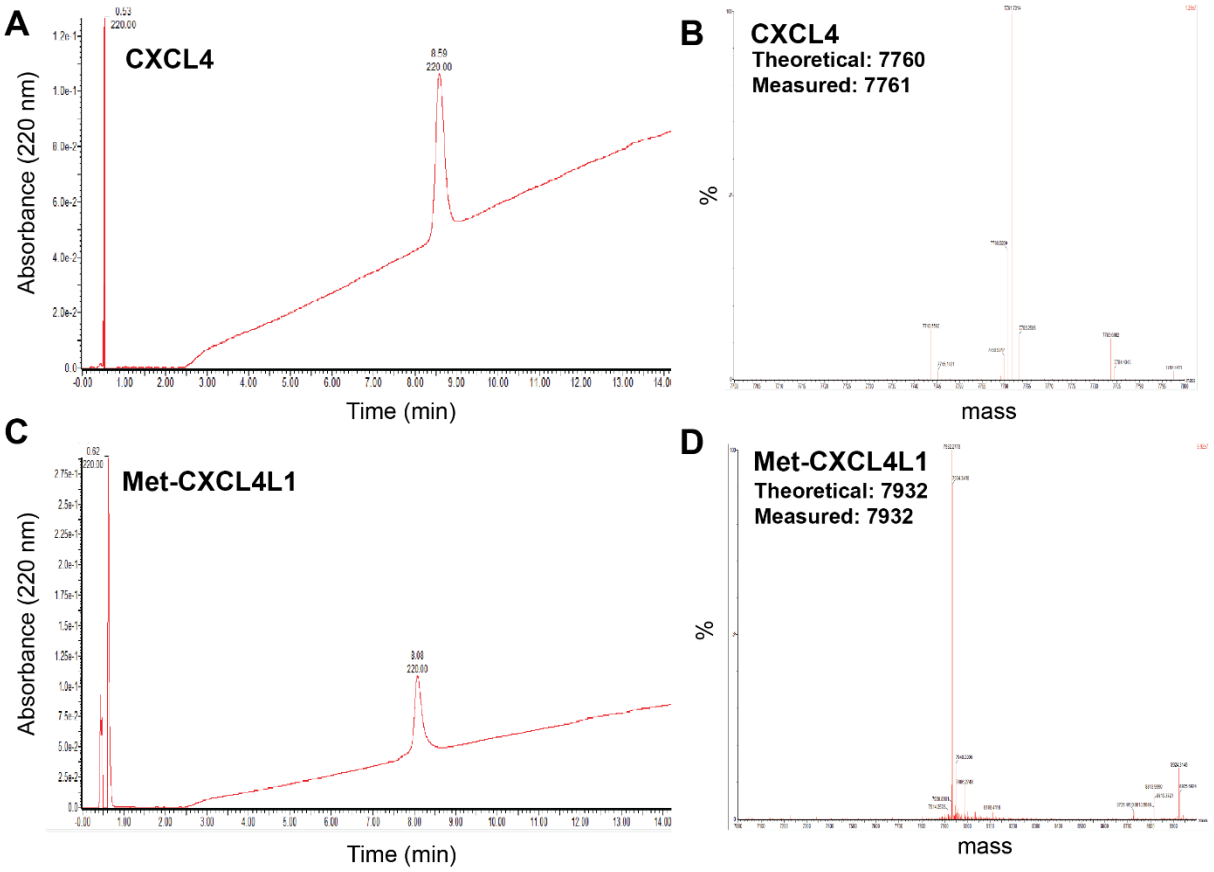


**Figure S5. Liquid chromatography–mass spectrometry (LC-MS) analysis of CXCL4 and CXCL4L1.**

The final pooled CXCL4 and CXCL4L1 fractions from reverse-phase HPLC were analyzed for purity by UPLC-MS. Chromatograms from a C18 column monitored at 220 nm of CXCL4 (A) and CXCL4L1 (B) and deconvoluted mass spectra from CXCL4 (C) and CXCL4L1 (D) with indicated (monoisotopic) theoretical and measured masses. Note: recombinant CXCL4L1 retains its N-terminal methionine, which does not affect its function.

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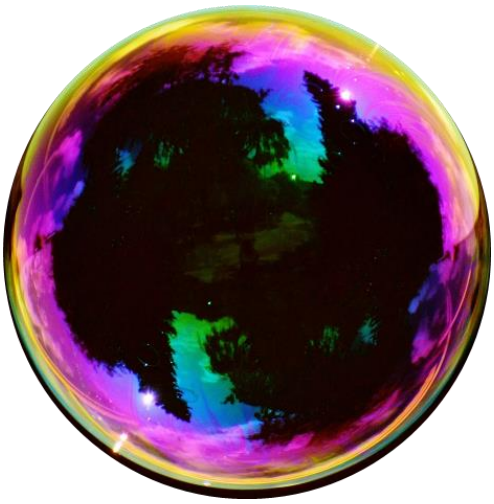
# Chapter 5

## Rapid internalization and nuclear translocation of CCL5 and CXCL4 in endothelial cells



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## Abstract

The chemokines CCL5 and CXCL4 are deposited by platelets onto endothelial cells, inducing monocyte arrest. Here, the fate of CCL5 and CXCL4 after endothelial deposition was investigated. Human umbilical vein endothelial cells (HUVECs) and EA.hy926 cells were incubated with CCL5 or CXCL4 for up to 120 min, and chemokine uptake was analyzed by microscopy and by ELISA. Intracellular calcium signaling was visualized upon chemokine treatment, and monocyte arrest was evaluated under laminar flow. Whereas CXCL4 remained partly on the cell surface, all of the CCL5 was internalized into endothelial cells. Endocytosis of CCL5 and CXCL4 was shown as a rapid and active process that primarily depended on dynamin, clathrin, and G protein-coupled receptors (GPCRs), but not on surface proteoglycans. Intracellular calcium signals were increased after chemokine treatment. Confocal microscopy and ELISA measurements in cell organelle fractions indicated that both chemokines accumulated in the nucleus. Internalization did not affect leukocyte arrest, as pretreatment of chemokines and subsequent washing did not alter monocyte adhesion to endothelial cells. Endothelial cells rapidly and actively internalize CCL5 and CXCL4 by clathrin and dynamin-dependent endocytosis, where the chemokines appear to be directed to the nucleus. These findings expand our knowledge of how chemokines attract leukocytes to sites of inflammation.

## 1. Introduction

Chemokines are small chemotactic cytokines that have an important role in regulating leukocyte trafficking during health and disease[1,2]. Through binding and activation of their cognate G protein-coupled receptors, they can rapidly induce leukocyte responses e.g., integrin activation, flow-resistant arrest, cell polarization, and transendothelial migration to sites of inflammation or infection. On a structural level, chemokines are hallmarked by a disordered N-terminus, a 3-strand antiparallel β-sheet, and a C-terminal α-helix. In addition, stretches of basic amino acids mediate binding to glycosaminoglycans (GAGs), e.g., heparin, heparan sulfate, and similar sulfated polysaccharides that constitute the cellular glycocalyx[3,4]. This warrants immobilization of the chemokines to the cell surface, e.g., of endothelial cells (EC) of the vessel wall, allowing them to be visible by rolling leukocytes. Besides this concept of chemokine presentation on the endothelial surface, constituting a message for leukocytes, some chemokines might be produced by the EC themselves and stored in small vesicles below the apical cell surface, which can be located by adherent monocytes prior to diapedesis[5]. In addition, chemokines on the vessel wall might originate from the subendothelial tissue and move to the vascular surface by transcytosis[6,7], yet they can also be deposited on the vessel wall by rolling platelets, as was shown for CCL5 (RANTES)[8]. This chemokine transfer to EC by activated platelets was shown to facilitate subsequent monocyte arrest[8,9]. Infusion of activated platelets into hyperlipidemic mice resulted in an accelerated development of atherosclerosis, which could be attributed in part to increased immobilization of CCL5 onto the atherosclerotic vessels[10]. Interestingly, CCL5 and CXCL4 (platelet factor 4), one of the most abundant chemokines in platelets, can interact with each other to form heterodimers, which are particularly potent in the recruitment of monocytes[11] and were shown to modulate the severity of atherosclerosis, stroke, abdominal aneurysm, and myocardial infarction in mice[12,13,14,15,16]. Although the interaction of CCL5 with GAGs has been postulated as essential for function in vitro and in vivo[17], the exact mechanism of CCL5 presentation to the cell surface and recognition by immune cells is incompletely characterized. Although the presence of CXCL4 led to increased binding of CCL5 to the surface of monocytic cells, it is unclear whether this explains the synergy between those chemokines[11]. A previous study has indicated that CCL5 is immobilized on the surface of human umbilical vein endothelial cells (HUVEC) in filamentous flow-resistant polymers, which might form a scaffold for leukocyte recruitment[18]. Interestingly, part of the CCL5 was observed intracellularly. To elaborate on the previous findings and to further investigate the mechanisms that underlie chemokine-induced leukocyte recruitment, we investigated the fate of exogenously added CCL5 and CXCL4 to EC. We found that incubation of EC with CCL5 and CXCL4 under static conditions led to rapid internalization of the chemokines, where CXCL4 remained partly presented on the cell surface. Internalization was an active process and dependent on G protein-coupled receptor (GPCR) signaling and classic endocytosis and resulted in calcium signaling within endothelial cells. Remarkably, internalized CCL5 and CXCL4 were targeted to the nucleus. Leukocyte arrest was not altered upon pretreatment with chemokines.

## 2. Results

### *2.1. Surface Presentation of the Chemokines CCL5 and CXCL4 on EC*

To initially investigate the interaction of chemokines with endothelial cells, cells from the line EA.hy926 (EAHy) were incubated without or with CCL5 and CXCL4, for a prolonged time of 60 min at 37 °C. The cells were subsequently stained using specific fluorescent antibodies without prior permeabilization. Thus, only the extracellular fraction of CCL5 and CXCL4 would be visible. Absence of exogenous chemokines before staining did not result in a notable fluorescent signal for either CCL5 or CXCL4 (Figure 1A,F). Likewise, the fluorescent intensity did not notably increase after 60 min treatment of EAHy cells with CCL5 (Figure 1B).

However, incubation of EAHy with CXCL4 led to a robust fluorescent signal (Figure 1G). Washing the EAHy cells with heparin after incubation with chemokines, but prior to antibody staining, led to loss of fluorescent signal (Figure 1C,H). Co-staining of confluent EAHy cells with CD31 and CCL5 or CXCL4, respectively, revealed a cytoplasmic staining pattern of the chemokines that was distinct from the typical accumulation of the CD31 signal at the cell-cell contacts (Figure 1D,E,I,J). Imaging along the *z*-axis implied faint CCL5 at the luminal aspect of the EAHy cells and increased staining intensities toward the basolateral side (Figure S1B).

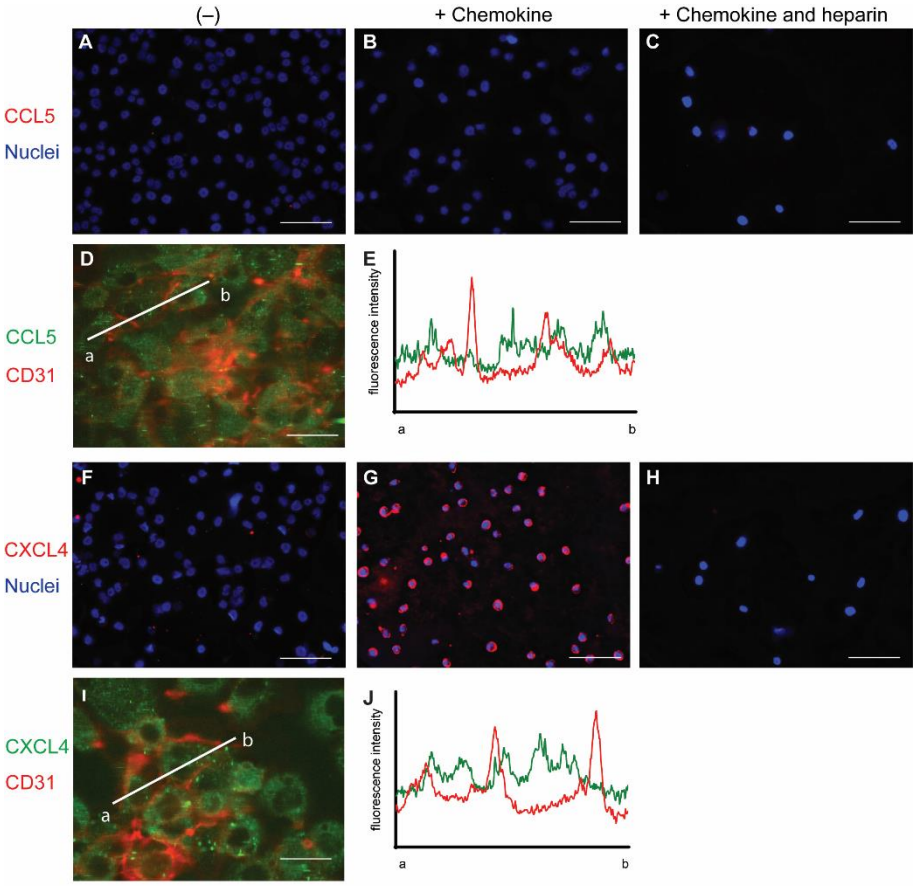


**Figure 1. Staining of chemokines after addition to endothelial cells.**
EAHy were grown on a cell culture slide, mock-treated (A, F), or incubated with the chemokines CCL5 (**B**–**D**,) or CXCL4 (**G**–**I**) for 60 min at 37 °C, and cells were washed with PBS alone or PBS with 1 mg/mL heparin (**C**,**H**). External chemokines on living cells were then stained with the respective primary antibodies and an Alexa Fluor 647-coupled secondary antibody, and nuclei were visualized with Hoechst 33342. (**A**–**C**,**F**–**H**). Internalized chemokines were stained after fixation and permeabilization of cells using a FITC-coupled secondary antibody (green), and cell membrane was visualized with APC-coupled CD31 (red), using confocal microscopy (**D**,**I**). Intensity profile through adjacent endothelial cells indicated by line a-b in (**D**,**I**) respectively, showing cell membrane (red) and CCL5 (**E**) or CXCL4 (**J**) resp. (green). Scale bar: 100 µm (**A**–**C**,**F**–**H**) or 50 µm (**D**,**I**); ($n$ = 4).

### *2.2. Permeabilization of EAHy Increases the CCL5 and CXCL4 Antigen Signal*

Because chemokines are known to be retained by EC, the EAHy cells were permeabilized in order to investigate an intracellular presence. After permeabilization and addition of the fluorescent antibodies, minimal staining of CCL5 and CXCL4 was observed in the absence of exogenous chemokines (Figure 2A). This signal might reflect low levels of endogenous CCL5

or CXCL4 (or variant CXCL4L1[19]) present in EAHy. Interestingly, incubation of EAHy with exogenously added CCL5 and CXCL4 at 37 °C for 60 min, followed by permeabilization and staining, resulted in a high signal intensity of the respective chemokine (Figure 2B). Incubation of EAHy with chemokines at 4 °C did not lead to an increase in fluorescent signal (Figure 2C), suggesting that the intracellular accumulation of CCL5 and CXCL4 is an active and energy-requiring cellular process.

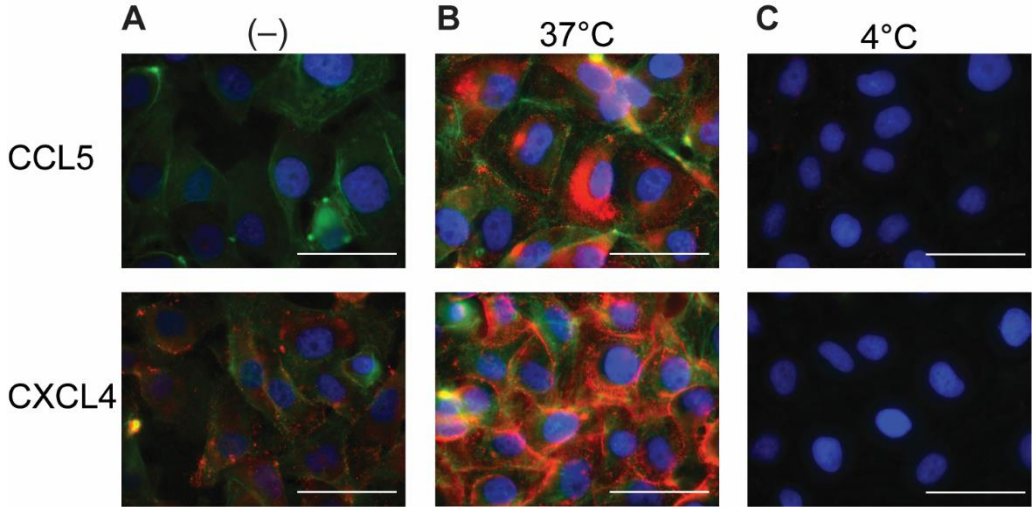


**Figure 2. Addition of CCL5 and CXCL4 to EC leads to internalization.**
EAHy incubated with buffer (**A**) or the chemokines CCL5 (top row) or CXCL4 (bottom row) at 37 or 4 °C (**B**,**C**, respectively) for 60 min and washed with heparin (1 mg/mL) prior to fixation, permeabilization, and staining. CCL5 (red, upper row), CXCL4 (red, lower row), F-actin (green), and nuclei (blue). Scale bar: 50 µm. ($n$ = 4).

### *2.3. Intracellular Accumulation of CCL5 and CXCL4 Is Time-Dependent*

In further experiments, the uptake of CCL5 and CXCL4 was followed in time. The chemokines were added and remained present at various increasing time points at 37 °C. Then, surface-bound and excess chemokines were removed by washing with heparin, and cells were fixed, permeabilized, and stained with specific fluorescent-labeled antibodies. Subsequently, the presence of the chemokines was visualized using fluorescent microscopy. In addition, the chemokine-treated cells were lysed after washing, and intracellular chemokine concentrations were measured by ELISA.

Both CCL5 and CXCL4 appeared to be taken up in a time-dependent manner (Figure 3). An increase in subcellular fluorescent signal was already observed after 5 min of incubation and increased over the 120-min duration of the experiment (Figure 3A,C). This was paralleled by an increase of intracellular CCL5 and CXCL4 antigen as observed with ELISA, with an apparent maximal uptake of CXCL4 after 60 min (Figure 3B,D). This supported the idea that EAHy cells actively and rapidly take up the chemokines CCL5 and CXCL4. For clarity, images with separate color channels are shown in Figure S1. Bovine chemokines from FCS did not cross-react with either antibodies used for fluorescence or ELISA (data not shown).

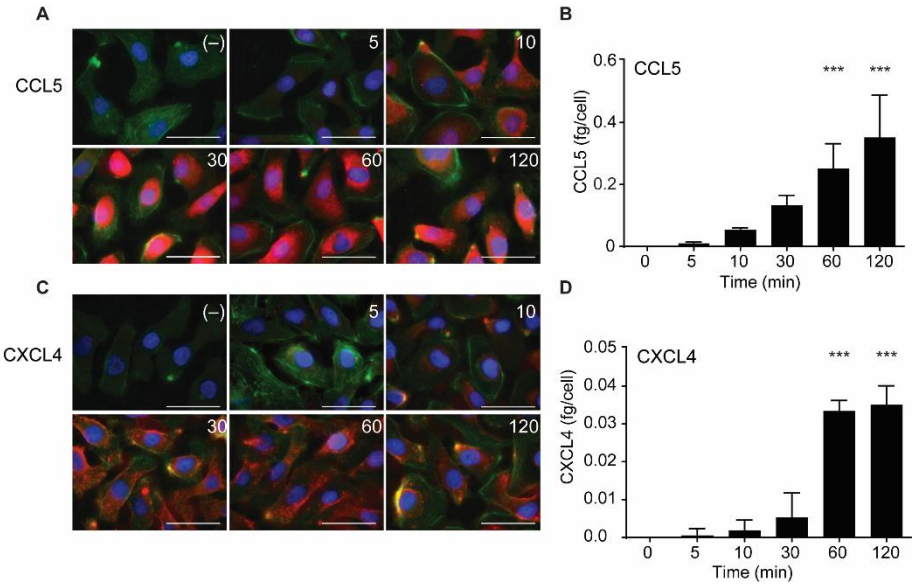


**Figure 3. Time-dependent uptake of CCL5 and CXCL4 in EC.**
CCL5 and CXCL4 were added to EAHy for indicated times at 37 °C. After washing with heparin, cells were fixed, permeabilized, and stained. Fluorescent signals of CCL5 (**A**, red) and CXCL4 (**C**, red), actin (green), and nuclei (blue) ($n = 3$). Quantification of intracellular chemokine concentrations of CCL5 (**B**) and CXCL4 (**D**) by ELISA. *** $p < 0.001$ ($n = 3$), ANOVA with Sidak's post-test.

### *2.4. Internalization of CCL5 and CXCL4 Is Dependent on Dynamin- and Clathrin-Mediated Endocytosis*

In order to investigate the manner of chemokine uptake, the EAHy cells were pre-treated with inhibitors of clathrin- (Pitstop2) or dynamin-mediated endocytosis (Dynasore) for 15 min, after which the cells were incubated with the chemokines in the presence of the inhibitors. Then, the same procedure for fixation, staining, and imaging as described above was followed. Of note, the pre-treatment with endocytosis inhibitors led to some detachment of the EAHy cells and loss of monolayer properties. Both inhibitors abolished the intracellular uptake of CCL5 and CXCL4 (Figure 4A–H). In addition, blockade of chemokine receptor- and other GPCR-induced signaling by pertussis toxin also led to a reduced internal presence of CCL5 and CXCL4. Effective inhibition of clathrin- and dynamin-dependent endocytosis by the above inhibitors was demonstrated by measurement of DiI-labeled nLDL uptake (Figure 4I). Surprisingly, enzymatic removal of GAGs from the cell surface resulted in an increased uptake of both CCL5 and CXCL4 into EAHy cells (Figure S1). Although chemokine uptake was abolished by pertussis toxin, antagonists of the CCL5 receptor and of the putative CXCL4 receptor CXCR3 prevented neither CCL5 nor CXCL4 internalization (Figure S2). Interestingly, pre-incubation of EAHy cells with CXCL4 prior to the addition of CCL5 led to a significant reduction of subsequent CCL5 uptake (Figure 5A). By contrast, pre-incubation of the cells with CCL5 prior to CXCL4 addition did not affect CXCL4 uptake. This indicates that CXCL4 can desensitize EAHy cells for the uptake of CCL5 (Figure 5B).

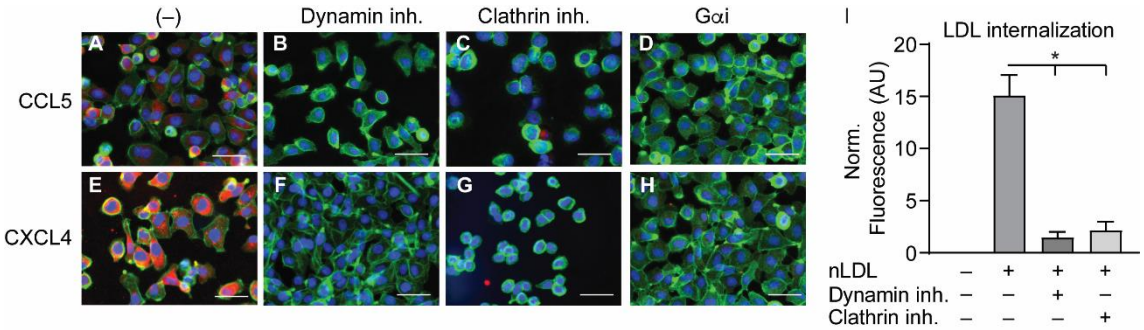


**Figure 4. Internalization of CCL5 and CXCL4 is dependent on dynamin- and clathrin-mediated endocytosis, and on G protein-coupled receptor signaling.**
EAHy were pre-incubated without (**A**,**E**) or with inhibitors to dynamin (**B**,**F**), clathrin (**C**,**G**), or GPCR (**D**,**H**) prior to incubation with CCL5 (top row) or CXCL4 (bottom row) at 37 °C. Cells were then washed with heparin prior to fixation and permeabilization and stained for the chemokines (red), F-actin (green), and nuclei (blue). Scale bar: 100 µm. Panel (**I**) shows the internalization of LDL in EAHy, and the blocking thereof by the inhibitors to dynamin or clathrin, as a positive control. * $p < 0.05$, ANOVA with Dunn's post-hoc test ($n = 6$).

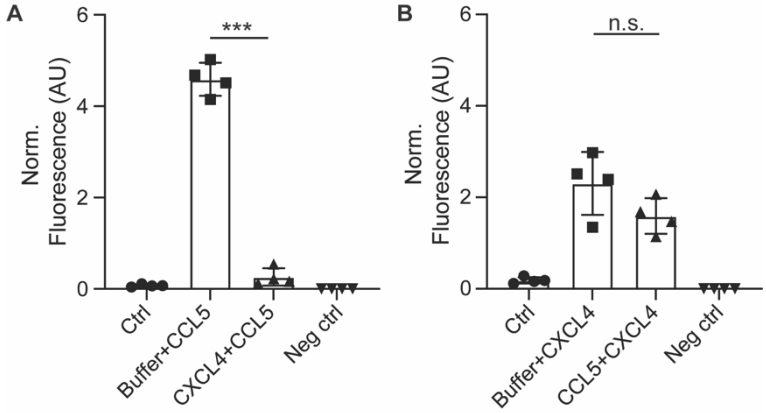


**Figure 5. Pre-incubation with CXCL4 inhibits subsequent uptake of CCL5.**
EAHy cells were pre-incubated with CXCL4 for 30 min prior to incubation with CCL5 for 120 min and its uptake measured by antibody staining after heparin washing (**A**), or vice versa (**B**). *** $p < 0.001$ ($n = 4$), ANOVA with Sidak's post-test.

To investigate whether EC showed evidence of intracellular signaling upon treatment with CCL5 or CXCL4, HUVECs were loaded with a calcium-sensitive dye and treated with buffer, thrombin as a positive control, or chemokines (Figure 6A–D). Upon stimulation with CCL5 and CXCL4, the HUVECs showed a notable and lasting increase in intracellular calcium levels, which was comparable to that induced by thrombin, as evidenced by classification of the individual cellular calcium mobilization profiles (Figure 6E).

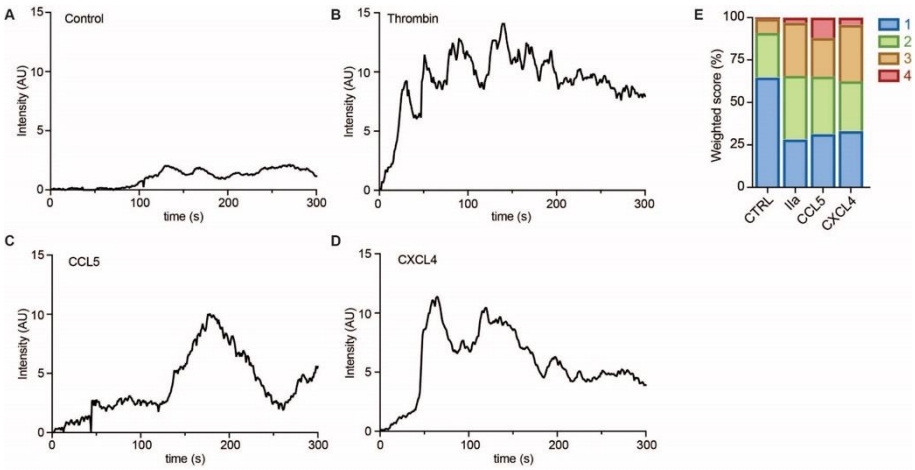


**Figure 6. Chemokines induce calcium signaling in EC.**
HUVECs loaded with Fluo-4 were stimulated with PBS (control, **A**), thrombin (IIa, **B**), CCL5 (**C**), and CXCL4 (**D**), and intensity profiles (in arbitrary units, AU) were recorded of >30 cells in over 3 independent experiments. Representative traces are shown in (**A**–**D)** and traces were classified as described in the methods section and summarized in (**E**).

### *2.5. CCL5 and CXCL4 Are Targeted to the Nucleus after Endothelial Uptake*

Because uptake of chemokines appeared to be an active process accompanied by cell signaling events, the intracellular fate of CCL5 and CXCL4 was investigated further. Confocal microscopy indicated that the chemokines accumulated in the nucleus 60 min after addition (Figure 7A). This is supported by a high optical resolution Z-stack movie that suggests accumulation of CXCL4 in the nucleoli (Supplementary movie 1). In order to further elucidate the subcellular localization of CCL5 and CXCL4, EAHy were lysed after 30 and 120 min of treatment. Subcellular fractions were isolated and measured. Interestingly, both CCL5 and CXCL4 showed an association with the cytoskeleton after 30 min, with a slight further increase at 120 min. However, the nuclear content was only barely increased after 30 min, but strongly increased after 120 min (Figure 7B,C). These data suggest that both CCL5 and CXCL4 are transported to the nucleus through cytoskeletal associations. To investigate whether inflammatory conditions could affect the uptake of chemokines, EAHy were activated using TNFa for 4 or 18 h, prior to addition of CCL5 or CXCL4. However, no difference in chemokine uptake was observed compared with resting cells (Figure S3).

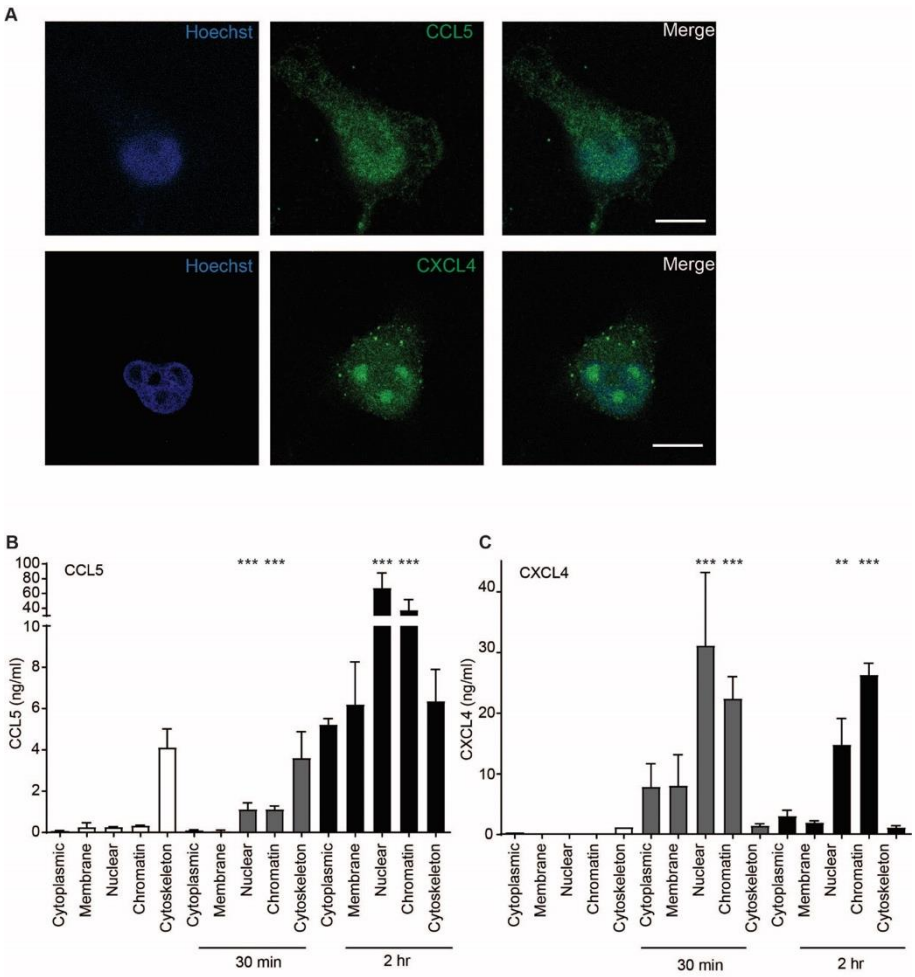


**Figure 7. CCL5 and CXCL4 are targeted to the nucleus.**
EAHy were incubated with CCL5 or CXCL4 for 60 min at 37 °C, fixed, and stained for nuclei (blue), chemokine CCL5, and CXCL4 (green). Shown are confocal micrographs. Scalebar = 10 µm. ($n$ = 3) (**A**). EAHy were incubated without (white bars) or with CCL5 (**B**) or CXCL4 (**C**) for 30 or 120 min (gray and black bars, respectively), lysed, and chemokines were determined in subcellular fractions ($n$ = 3, ** $p < 0.01$, *** $p < 0.001$, ANOVA with Dunn's post-test).

### *2.6. CCL5 and CXCL4 Internalization Does Not Affect Leukocyte Arrest*

Previous findings suggested that chemokines e.g., CCL2, stored in subluminal vesicles, could guide lymphocyte tracking on EC[5]. In order to investigate whether a similar mechanism could regulate CCL5- and CXCL4-induced monocyte arrest, HUVECs were incubated with these chemokines for 120 min, treated without or with heparin to remove residual surface chemokines, and subsequently perfused with monocytic MonoMac6 cells. Addition of chemokines for 120 min did not affect MonoMac6 adhesion to HUVECs (Figure 8), indicating that internalized chemokines neither make HUVECs competent for leukocyte interactions nor directly induce leukocyte arrest. Similar results were observed after activation of the HUVECs with TNFa for 4 h, prior to the addition of chemokines (Figure 8). The combined addition of CCL5 and CXCL4, which is a potent stimulus for monocyte arrest, did not increase MonoMac6 adhesion, which indicates that TNFa activation might already support maximal monocyte arrest. These results suggest that at least CCL5 and CXCL4 require surface presentation for leukocyte recruitment.

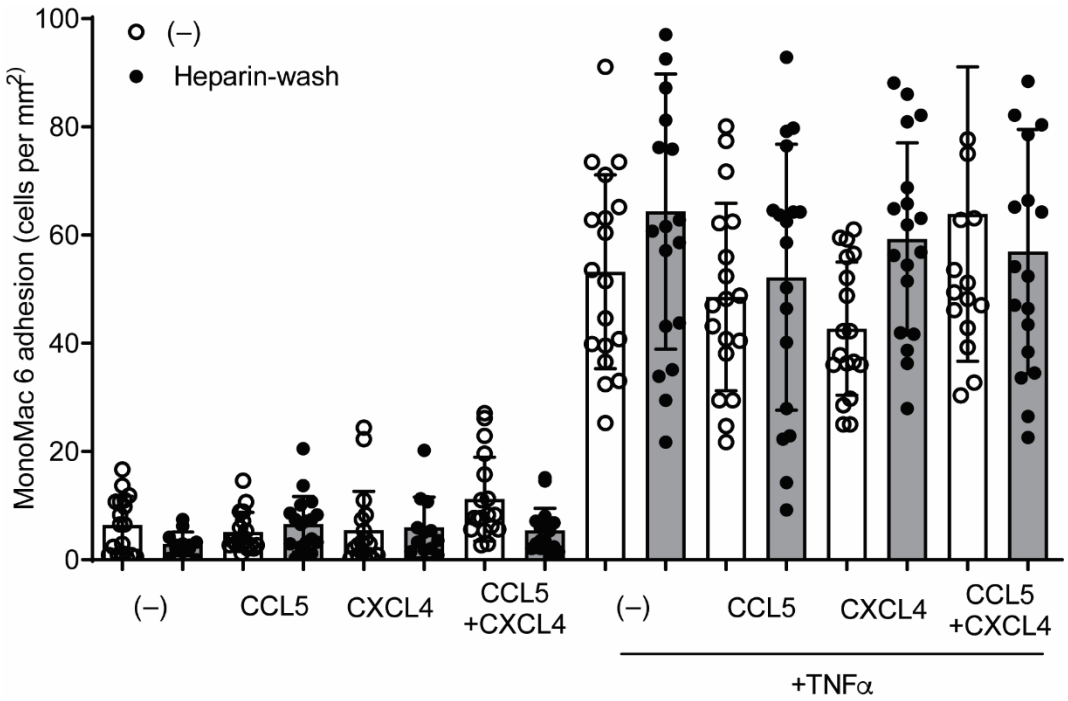


**Figure 8. CCL5 and CXCL4 internalization does not affect monocytic cell arrest to endothelial cells.**
HUVECs were stimulated with 10 ng/mL TNFa for 4 h and/or CCL5 or CXCL4 for 1 h and washed without (black bars) or with heparin (white bars) prior to perfusion of Syto 13-labeled MonoMac-6 cells at 3 dynes/cm$^2$. Arrested cells were counted in 6 different fields ($n$ = 4).

## 3. Discussion

In this study, we observed that chemokines are taken up by EC in an active manner because uptake did not occur at 4 °C. At 37 °C, intracellular chemokines were detectable as soon as 10 min after addition, and internalized levels approached a steady state after 60 min. This indicates that internalization of the chemokines CCL5 and CXCL4 is a rapid and actively triggered process. Co-staining with CD31 indicated that the chemokines did not co-localize with the cell-cell contacts, and z-stack imaging suggested intracellular rather than luminal localization. The active character of chemokine uptake is further supported by the observed increase of intracellular calcium upon chemokine addition and the blockade of chemokine uptake by the G protein inhibitor pertussis toxin. Together with the observation that chemokine internalization was blocked by inhibitors of clathrin-mediated endocytosis, these findings imply that specific (G protein-coupled) receptors for the uptake of chemokines are involved. However, blockade of CCR5, a receptor for CCL5, by small molecular antagonists did not influence CCL5 uptake, implying that CCR5 does not play a role. In addition, enzymatic removal of GAGs from EC even increased the uptake of CCL5 and did not affect the uptake of CXCL4. Thus, these findings suggest either that binding to GAGs is not important for the uptake of chemokines, that the GAGs involved are not targeted by the enzymatic treatment, or that the remainder of the glycocalyx is sufficient to absorb the chemokines prior to their uptake[3]. Given the highly positive charge of both CCL5 and CXCL4 at physiologic pH, there might be additional molecules that mediate binding of chemokines and support uptake through electrostatic interactions, e.g., negatively charged phospholipids or membrane proteins. In addition, because the endothelial cells were cultured under static conditions in this study, it cannot be excluded that flow-dependent adaptations of the glycocalyx[20] support chemokine binding in a physiologic setting involving blood flow. The absence of flow can be considered a limitation of this study, as well as the 2-dimensional culture conditions under which the endothelial cells were grown. The development

of innovative blood vessels-on-a-chip, e.g., to study blood–brain barrier physiology[21] has opened the possibility to investigate chemokine uptake and subsequent monocyte arrest in a system of continuous flow. In addition, molds and tissue supports now enable the culturing of cells in 3 dimensions, which is a further approximation toward physiology[22,23]. Future studies will take these aspects into account, as well as the possible influence of membrane water flux in endothelial cells on chemokine uptake, as can be measured by a recently established calcein quenching-based method[24].

In EC, an alternatively spliced variant of CXCR3, termed CXCR3B, was suggested to serve as a receptor for CXCL4[25]. However, whether CXCR3 acts as a bona fide receptor for CXCL4 and other angiostatic chemokines is still unclear[26]. In addition, the expression of CXCR3 in EC depends on the cell cycle[27], and CXCR3B signaling is not inhibited by pertussis toxin[25]. Thus, these findings and our observations in this study speak against the involvement of CXCR3B in the internalization of CXCL4. Alternatively, galectins might be involved in chemokine uptake into EC. Galectins are a family of b-galactoside-binding lectins that are involved in various physiologic processes and are established mediators of endocytosis of plasma membrane proteins and glycolipids[28,29]. Recent studies by us and others have shown an interplay between galectins and chemokines[30,31]. For example, there is an interplay between galectin-1 and CXCL4 in platelet activation[30], and CCL5 was shown to undergo physical interactions with galectin-3[31]. However, whether galectins play a role in the uptake of CCL5 and CXCL4 remains a subject for future investigations.

A somewhat surprising observation is that pre-incubation with CXCL4 can apparently inhibit subsequent CCL5 internalization, but not vice versa. These results show that there is an interdependency between the uptake of CXCL4 and CCL5. We do not have a conclusive mechanistic explanation, but we speculate that a potent internalization induced by CXCL4 may exhaust the uptake machinery for CCL5, or that a CCL5 receptor is co-internalized along with CXCL4, making it less available for CCL5. Future studies are needed to further unravel the mechanisms of chemokine uptake by endothelial cells.

Interestingly, CCL5 was no longer detectable on the surface of EC 60 min after its addition. This appears to be somewhat at odds with the common notion that chemokine presentation onto the luminal surface of the vessel wall directs leukocyte recruitment[32,33]. For CCL5 in particular, binding to GAGs is essential for its leukocyte-recruiting functions[17], and a GAG-binding mutant of CCL5 was shown to act as a dominant-negative antagonist[34]. However, other studies have demonstrated that subluminal presentation of chemokines also can affect leukocyte trafficking. After stimulation with cytokines, the chemokines CCL2 and CXCL10 are stored in vesicles underneath the endothelial membrane in HUVECs[5]. Being localized below the membrane associated with actin, these chemokines are no longer accessible to blocking antibodies yet are still able to trigger the transmigration of lymphocytes by localized release in the tight immunologic synapse[5]. This possibility was also investigated by determining monocytic cell arrest, a well-established function of CCL5, on endothelial cells after incubation with chemokines, without or with removal of residual surface chemokine. After 60 min, no CCL5 was detectable on the surface of EC, implying complete uptake. Possible residual chemokine was removed. Under these conditions, our findings suggest that internalized CCL5 does not induce arrest of MonoMac6 cells on EC, both under resting conditions and after cytokine stimulation. In addition, neither CXCL4 nor CCL5 increase the adhesiveness of EC for monocytic cells under resting or inflammatory conditions.

Internalization of chemokines has been observed in several studies and is considered a physiologic mechanism for their transport from inflamed tissues toward the endothelial lining of the vasculature[6,7]. In polarized Madin–Darby canine kidney cells, rapid internalization within 30 min of the chemokine CCL2 was demonstrated, and CCL2 was transported from the basolateral side to the apical side within 120 min[7]. The atypical chemokine receptor ACKR1, which

is also known as Duffy antigen receptor for chemokines (DARC), was shown to mediate the cellular transport of CCL2. Because ACKR1 can both bind CCL5 and CXCL4[35,36], an involvement of this atypical receptor appears plausible. A previous study has indeed demonstrated an involvement of ACKR1 in the uptake of CXCL1, which is structurally related to CXCL4, in an immortalized HUVEC cell line, yet only when these cells were stably transfected with ACKR1[37]. Interestingly, the endothelial internalization of CXCL1 was found not to depend on clathrin and only partially on dynamin. Thus, although ACKR1 appears to be a plausible candidate receptor for CCL5 and CXCL4 uptake into EC, it is unclear whether basal expression levels support internalization.

An interesting observation is the nuclear accumulation of the chemokines after uptake into EC. Previous studies have observed a nuclear targeting of the chemokine CXCL12 gamma[38], a splice variant of CXCL12 with a nuclear translocation signal in its C-terminus, and an alternatively spliced form of CCL27, which is targeted to the nucleus by an endogenous nuclear targeting signal[39]. Interestingly, in the latter study by Gortz et al., CCL5 was not targeted to nucleus when expressed in Chinese hamster ovary cells, indicating an absence of an endogenous nuclear translocation signal. Apart from the different cell type, the mechanisms of nuclear targeting might be different in the experimental setting of the two studies and ours. Endogenous expression from a cDNA might sort the newly produced chemokine (precursors) in the endoplasmic reticulum for secretion or for transport to the nucleus, whereas exogenously added chemokine enters the cell through an endocytic pathway. Of note, the association of CCL5 and CXCL4 with the cytoskeletal fractions is indicative of a transport mechanism toward the nucleus. Association of chemokine-loaded vesicles with actin has been observed before[5], yet our experiments yielded no evidence of a packing of CCL5 or CXCL4 into vesicles. Within the nucleus, CXCL4 appeared to accumulate in the nucleolus. The significance of these findings is currently unknown, but CXCL4 is known to have a high affinity for nucleic acids[40], and CXCL4/DNA complexes were recently shown to serve as a ligand for toll-like receptor 9 in the inflammatory activation of dendritic cells[41]. It is tempting to speculate on an active involvement of CXCL4 in transcriptional processes. In monocytes/macrophages, CXCL4 is able to induce considerable changes in the transcriptional landscape, leading to pro-inflammatory and profibrotic phenotypes[42,43]. It is worth noting that unlike other chemokines, no single receptor has been identified that explains all CXCL4 functions. Other cytokine (-like) or danger-associated molecules have intra- and extracellular actions (reviewed in[44]), and it is conceivable that CXCL4 might act in a similar fashion. Alternatively, chemokine uptake by EC might serve to maintain or regulate local chemokine levels in a fashion similar to the ACKRs[44,45] and might be targeted to the nucleus for proteasomal degradation[46].

In conclusion, the results in this study suggest an active and directed uptake of the chemokines CCL5 and CXCL4, resulting in an accumulation toward the nucleus. Although the (patho)physiologic relevance needs to be further characterized, these findings add a further dimension to the cell regulating activities of the chemokine system in health and disease.

## 4. Materials and Methods

### *4.1. Cells*

EA.hy926 cells ("EAHy", ATCC® CRL-292) were cultured in Dulbecco modified Eagle medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 1× HAT (5 mmol/L sodium hypoxanthine, 20 μM aminopterin, and 0.8 mmol/L thymidine) and 100 U/mL penicillin-streptomycin (Thermo Fisher Scientific). Cells were used between passage 7–25. Human Umbilical Vein Endothelial Cells (HUVEC) (Promocell GmbH) were grown in endothelial cell growth medium (Promocell, Heidelberg, Germany) supplemented with 100 U/mL penicillin-streptomycin. Cells were used

between passage 4 and 7. MonoMac-6 cells (DSMZ, ACC124) were cultured in RPMI supplemented with 10% FBS, 2 mmol/L L-glutamine, non-essential amino acids, 1 mmol/L sodium pyruvate, and 10 μg/mL human insulin. Cells were used between passage 7 and 25. Bimonthly samples were measured for the absence of mycoplasma using the MycoAlert Mycoplasma Detection Kit as per manufacturer's protocol (Lonza, Walkersville, MD). All cells were maintained in a humidified incubator at 37 °C and 5% $CO_2$.

*4.2. Chemokine Internalization*

Cells were cultured on 8-well Falcon Chambered Cell Culture slides or in 6-well cell culture plates, and incubated with 500 ng/mL recombinant human CCL5/RANTES (R&D Systems, Minneapolis, MN, USA) or recombinant human CXCL4/PF4 (Peprotech, Rocky Hill, NJ, USA), at 4 °C or 37 °C for indicated times, specific per experiment. The chemokine concentration of 500 ng/mL (63 nmol/L was found optimal for triggering monocyte arrest on endothelial cells in previous studies[9,11,14,15]. Native low-density lipoprotein (nLDL) labeled with 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine (Dil-nLDL, 5μg/mL-Sigma SAE0053) was used as a positive control and to assess the functionality of the endocytosis inhibitors.

In some experiments, it was investigated whether pre-incubation with chemokines could influence internalization. Before the experiment, all culture plates were coated with Attachment Factor (AF, from Gibco). Cells were seeded in a black 96-well plate at a density of 15,000 cells/well and incubated in DMEM, supplemented with 10% FCS for 24 h. The next day, the cells were starved in DMEM supplemented with 0.5% FCS overnight. The following day, the cells were pre-treated with vehicle or 500 ng/mL of the first chemokine (CXCL4 or CCL5) for 30 min. Then, fresh medium (DMEM + 0.5% FCS) containing 500 ng/mL of the other chemokine (CXCL4 or CCL5) was added to the cells, and the culture plates were incubated for an additional 2 h at 37 °C. Then, the cells were incubated with 200 U/mL heparin for 5 min, in order to remove the remaining chemokines from the cell surface. Subsequently, cells were fixed with 4% paraformaldehyde for 10 min and blocked with a blocking buffer (PBS containing 2% BSA and 0.1% Triton X-100) for 1 h at room temperature. Then, the primary antibody (rabbit anti-human PF4 from Peprotech or rabbit anti-human CCL5 from Abcam) was added at a final concentration of 2 μg/mL and incubated overnight at 4 °C. After washing, the secondary antibody was added (goat anti-rabbit, conjugated with AF532 from Life Technologies) at a final concentration of 5 μg/mL for 1 h. Next, cells were washed and nuclei were stained with Hoechst solution. Then, the cell count and the fluorescence were analyzed using a Cytation™ imager. In negative control wells, the addition of primary antibody was omitted.

*4.3. Inhibitors*

Cells were incubated with Dynasore (324410 Merck, Darmstadt, Germany), PitStop2 (ab120687, Abcam, Cambridge, UK), Bordetella pertussis toxin blocking G proteins ($Ga_i$, $Ga_o$, and $Ga_t$, BML-G101-0050 Enzo Life Sciences, Farmingdale, NY, USA), and DAPTA (2423 R&D Systems). TAK-779 (SML0911), blocking anti-CXCR3[25] (clone 49801, R&D systems), heparinase III from Flavobacterium heparinum (H8891), chondroitinase ABC from Proteus vulgaris (C2905), and hyaluronidase Type VI-S (H3631) were all obtained from Merck. Neuraminidase (C. perfringens) (P5289) was from Abnova (Taipei City, Taiwan).

*4.4. Localization*

Cells were incubated with recombinant human CCL5/RANTES or recombinant human PF-4/CXCL4, as described above, and fractionated using the subcellular protein fractionation kit for cultured cells (78840-Thermo) as per manufacturer's protocol. Chemokine quantification in the different subcellular fractions was performed using chemokine-specific sandwich ELISA.

*4.5. Immunocytochemistry*

EA.hy926 cells were cultured on 8-well Falcon Chambered Cell Culture slides (Thermo) and fixed and permeabilized using BD Cytofix/Cytoperm™ as per manufacturer's instructions. Cells were blocked for 30 min at room temperature using PBS supplemented with 5% FCS. CCL5 was detected using a rabbit polyclonal antibody against RANTES (Abcam ab9679), and CXCL4 was detected using a rabbit polyclonal antibody against platelet factor 4 (Peprotech) at 2 µg/mL. CXCR3 was detected using a monoclonal mouse anti-CXCR3 (R&D systems) at 10 µg/mL. Visualization was performed using donkey-anti-rabbit Alexa Fluor 647 (Thermo) at 5 µg/mL, or goat-anti-mouse FITC (Jackson) at 6 µg/mL. F-actin was visualized using phalloidin Alexa Fluor 488 (Thermo). Antibodies were diluted in PBS supplemented with 5% FCS. Finally, cells were mounted using Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA), or stained with Hoechst 33342 (Thermo) and mounted with a glycerol-based mounting medium containing Mowiol 4-88 (Merck). Cells were then imaged using an EVOS FL Cell imaging system, using an Olympus 60× oil objective (1.42NA) and standard filter cubes for DAPI (ex/em 357/447 nm), GFP (ex/em 470/525 nm), and Cy5 (ex/em 628/692 nm). Image overlays and cross-sections were made using Fiji V1.52k[47]. Fluorescence quantification per cell count was analyzed with a Cytation 3 Cell Imaging Multi-Mode Reader (Biotek Instruments Inc., Winooski, VT, USA) using donkey-anti-rabbit Alexa Fluor 532 (Thermo) and Hoechst 33342.

*4.6. Live Cell Imaging*

EA.hy926 cells were grown on 8-well Falcon Chambered Cell Culture slides (Thermo) and incubated for 60 min with CCL5 or CXCL4. Briefly, cells were washed with PBS alone or PBS with 1 mg/mL Heparin to wash away membrane-bound chemokines. Cells were then stained with the respective primary antibodies, and Alexa Fluor 647-coupled secondary antibody and nuclei were visualized with Hoechst 33342 as described before, at 37 °C and 5% $CO_2$. Cells were then imaged using an EVOS FL Cell imaging system, using the 20× objective (0.45 NA) and standard filter cubes for DAPI (ex/em 357/447 nm) and Cy5 (ex/em 628/692 nm). Image overlays and cross-sections were made using Fiji V1.52k[20]. All antibodies used against CCL5 and CXCL4 did not detect the bovine chemokine orthologs.

*4.7. Confocal Imaging*

EAhy cells were fixed and stained for CCL5 and CXCL4 as described above. Visualization was performed using Goat-anti-Rabbit FITC (Thermo). Cells were imaged on a Leica TCS SP8 Confocal microscope with a 100× oil immersion/1.4 NA objective and 2× optical zoom, with excitation at 405 or 488 nm, and emissions were collected at 413–480 nm and 498–580 nm, respectively. Images of 512 × 512 pixels were obtained with a pixel size of 0.09 µm, standard pinhole size, and scan speed of 400 Hz. Z-stacks were made with a step size of 0.219 µm (Movie S1) or 0.3 µm (Figure S1B).

*4.8. Calcium Influx*

For the influence of chemokines on intracellular calcium concentrations $[Ca^{2+}]i$, HUVECs were cultured in 8-well glass-bottomed ibidi culture slides, coated with 30 µg/mL collagen. After reaching confluency, cells were incubated with 8 µmol/L Fluo-4 acetoxymethyl ester in the presence of 0.4 mg/mL pluronic for 40 min at 37 °C and 5% $CO_2$. Changes in $[Ca^{2+}]i$ were recorded for 10 min, using a Zeiss LSM 510 confocal microscope (488 nm excitation). At the start of the recording, PBS, chemokines (500 ng/mL each), or thrombin (10 nmol/L) were added to the wells. Changes in fluorescence intensity indicated a spike in cytosolic $[Ca^{2+}]i$ and were represented as false-color images (blue: low, green: high). Fluorescence images were analyzed with ImageJ/Fiji software. $[Ca^{2+}]i$ spikes were classified based on the type of oscillatory signal

in cytosolic-free calcium concentration. Score 1 indicated no to minimal rise of $[Ca^{2+}]i$, Score 2 was indicative of short and low amplitude rises of $[Ca^{2+}]i$, a score of 3 was a single, high rise of $[Ca^{2+}]i$, and a score of 4 was a repetitive and high rise of $[Ca^{2+}]i$.

*4.9. Quantification of Internalized Chemokines*

EA.hy926 cells were cultured in 6-well cell culture plates (Corning, Glendale, AZ, USA), treated with chemokines and/or inhibitors according to the experiment, and washed with 1 mg/mL heparin (180 U/mL) to remove membrane-bound chemokines prior to cell lysis using 400 µL CytoBuster protein extraction reagent (71009-Merck). Cell lysates were centrifuged for 5 min at 16.000× *g* and stored at −20 °C for ELISA. Sandwich ELISA was performed using human CCL5/RANTES (DY278) or human CXCL4/PF4 (DY795) DuoSet ELISA protocols (R&D Systems), respectively, as described[30,48]. Both ELISAs were found to detect neither CCL5 nor CXCL4 in samples of fetal bovine serum.

*4.10. Laminar Flow-Based Leukocyte Adhesion Assay*

Laminar flow-based leukocyte adhesion assay was performed as described in detail previously[49]. Briefly, HUVEC cells were cultured in 35 mm TC-treated cell culture dishes (Thermo) with a density of $1 \times 10^5$ cells/cm$^2$ for 48 h before stimulating with TNFα (10 ng/mL) for 4 h. Chemokines CCL5 or CXCL4 (both 500 ng/mL) were added for 1 h. MonoMac6 cells were stained with Syto 13 (Thermo) for 30 min at 37 °C and washed and perfused in Hank's buffer pH 7.45 containing 10 mmol/L Hepes, 3 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$, and 0.2% human serum albumin for 3–6 min at 3 dynes/cm$^2$. Adherent cells were counted in 6 view fields and expressed in cells/mm$^2$.

*4.11. Statistical Analysis*

Data are presented as mean ± SD unless stated otherwise. Statistical analysis was performed using Graphpad Prism 9 (San Diego, CA, USA), using one-way analysis of variance ANOVA and Sidak's (parametric) or Dunn's (non-parametric) post hoc analysis, as indicated. Differences were considered statistically significant at $p < 0.05$ (*).

**Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1.

**Author Contributions:** A.D. performed the main experiments, analyzed data, and co-wrote the paper; D.M.K. performed experiments; A.C.A.H. and S.L.N.B. performed and analyzed calcium mobilization experiments; J.W.M.H. and M.A.M.J.v.Z. provided intellectual input and infrastructure; R.R.K. supervised study, obtained funding, and wrote the paper. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

# Chapter 5

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**Rapid internalization and nuclear translocation of CCL5 and CXCL4 in endothelial cells**

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Supplementary material

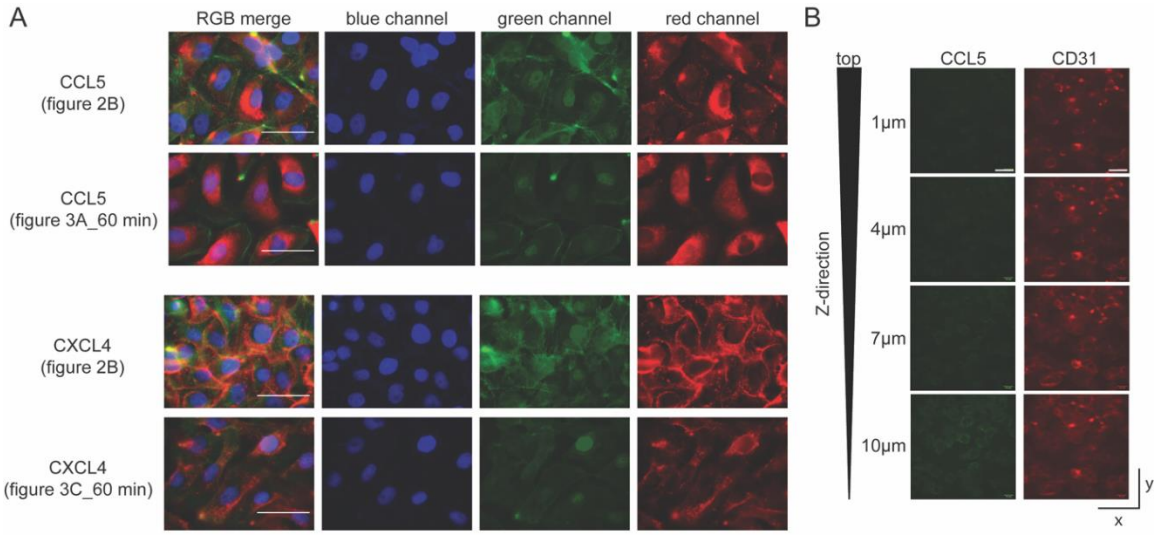



**Figure S1. Images of EAHy and chemokines split into individual colour channels**.
(A) Indicated images from main figures 2 and 3 split into red, green and blue channels. Blue: nuclei (DAPI), green: actin (phalloidin-AF488), red: chemokine (CCL5 or CXCL4). Scale bar 50µm. (B) Fluorescence of cellular CCL5 (green) and CD31 (red) along the z-axis in EAHy at indicated relative distance, recorded by confocal microscopy after 60 minutes of incubation. Scale bar 50µm.

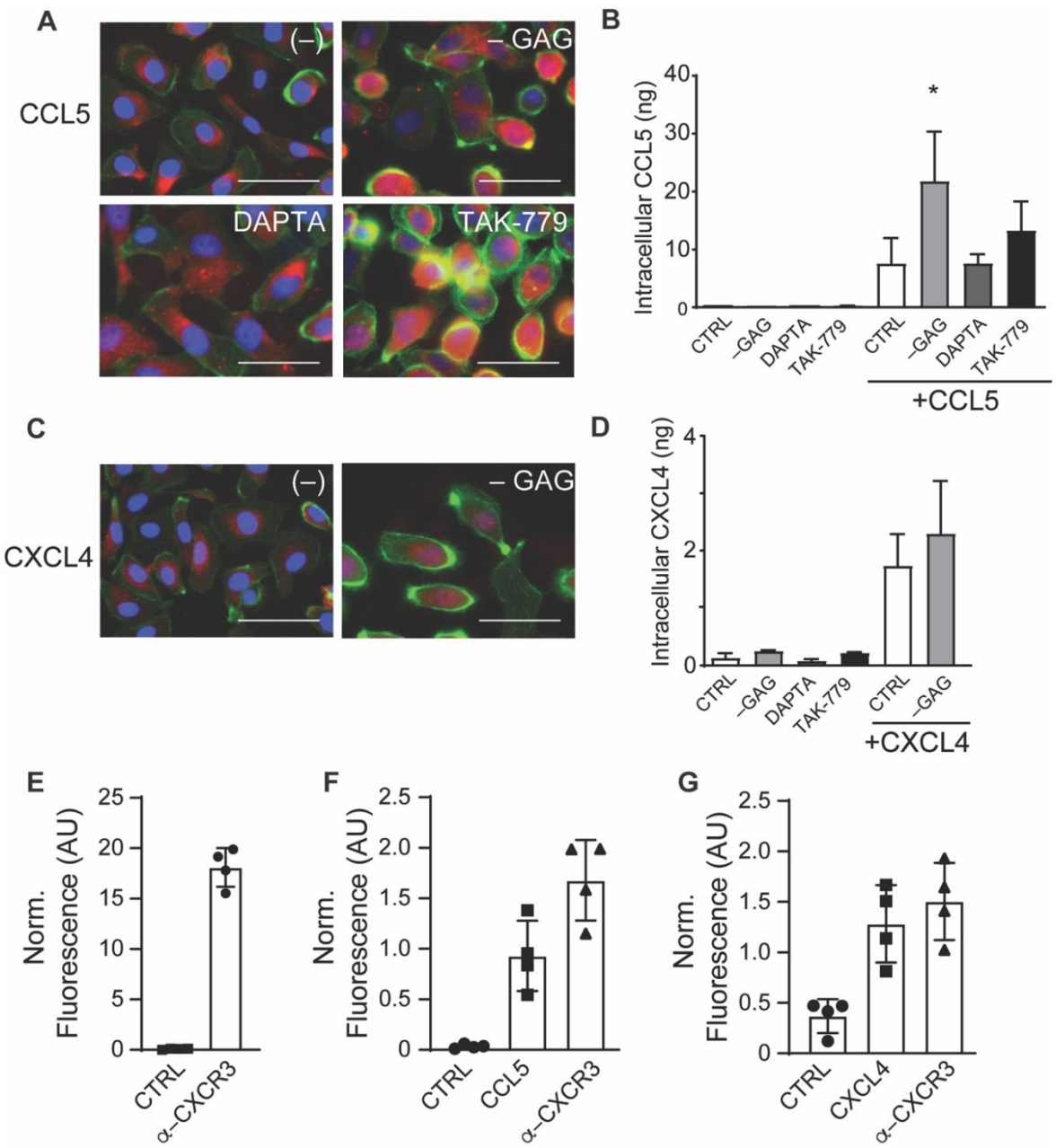



**Figure S2. Internalization does not depend on GAGs or CXCR3 and is increased by an inhibitor of CCR5.**

EAHy were grown on a coverslip (A, C), in a 35 mm dish (B, D), or in a 96-well plate (E-F) and incubated with the enzymes heparinase III, chondroitinase ABC, hyaluronidase, and neuraminidase to cleave glycosaminoglycans (GAG), or DAPTA or TAK-779, inhibitors of CCR5 for 3 hours at 37°C. Blocking of CXCR3 was accomplished with antibodies for 15 minutes at 37°C. Cells were then incubated in presence of the inhibitors with CCL5 or CXCL4 for 60 minutes at 37 °C. Cells were washed with heparin prior to fixation and permeabilization (A, C, E, F, G) or lysis (B, D). Cells were stained for the respective chemokine (red), F-actin (green), and nuclei (blue) (A, C). Scale bar: 50 µm. Quantification of CCL5 and CXCL4 levels using ELISA (B, D). Cells were stained for the receptors, CCL5 or CXCL4 respectively, using Alexa Fluor 532, and nuclei (Hoechst). The cell count and fluorescence was analyzed with Cytation™ (E, F, G). ($n$=6, $p<0.05$, Kruskal Wallis test).

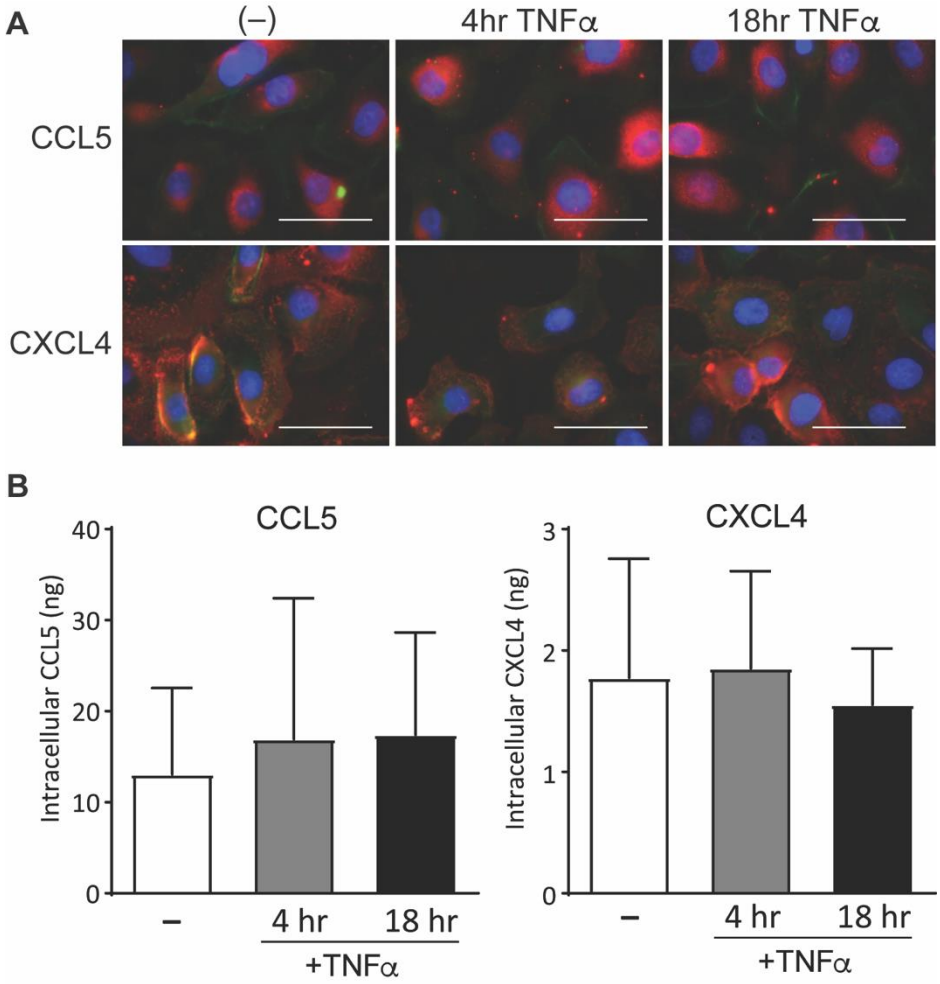
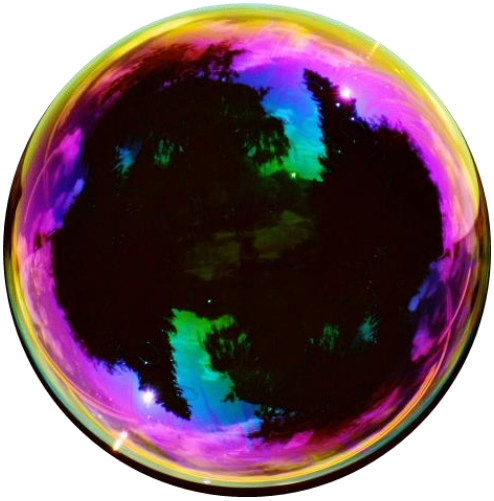



**Figure S3. TNFa-treatment does not alter internalization of chemokines**.
EAHy cells were incubated with 10 ng/ml TNFa for 4 h or 18 h and were subsequently treated with the chemokines CCL5 (top row) or CXCL4 (bottom row) for 60 min 37 °C. (A) Cells were then washed with heparin prior to fixation and permeabilization and stained against CCL5 (top-red), CXCL4 (bottom-red), F-actin (green), and nuclei (blue). Scale bar: 50 µm. (B) Cells were washed with heparin. Subsequently, CCL5 and CXCL4 levels in cell lysates were determined using ELISA. (*n*=6, ns, Kruskal Wallis).

# Chapter 6

## Extracellular vesicles from steatotic hepatocytes provoke pro-fibrotic responses in stellate cells



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## Abstract

*Background and aims* – Hepatic steatosis and chronic hepatocyte damage ultimately lead to liver fibrosis. Key pathophysiologic steps are the activation and transdifferentiation of hepatic stellate cells. Here, we assessed the interplay between hepatocytes and hepatic stellate cells under normal and steatotic conditions. We hypothesized that hepatocyte-derived extracellular vesicles (EV) modify the phenotype of stellate cells.

*Methods* - By high speed centrifugation, EV were isolated from conditioned media of the hepatocellular carcinoma cell line HepG2, under baseline conditions (C-EV) or after induction of steatosis by linoleic and oleic acid for 24 hours (FA-EV). Migration of the stellate cell line TWNT4 and of primary human stellate cells towards respective EV as well as sera of MAFLD patients was investigated using Boyden chambers. Phenotype alterations after incubation with EV were determined by qPCR, western blotting and immunofluorescence staining.

*Results* - HepG2 cells released more EV after treatment with fatty acids. Chemotactic migration of TWNT4 and of primary stellate cells was increased specifically towards FA-EV. Prolonged incubation of TWNT4 cells with FA-EV induced expression of proliferation markers and a myofibroblast-like phenotype. Whereas the expression of the collagen type 1 α1 gene did not change after FA-EV-treatment, expression of the myofibroblast markers e.g. α-smooth muscle cell actin and TIMP1 were significantly increased.

*Conclusion* - We conclude that EV from steatotic HepG2 cells can influence the behaviour and phenotype as well as the expression of remodeling markers of stellate cells and guides their directed migration. These findings imply EV as operational, intercellular communicators in the pathophysiology of steatosis-associated liver fibrosis.

## 1. Introduction

Non-alcoholic fatty liver hepatitis (NASH), recently renamed to Metabolic Associated Fatty Liver Disease (MAFLD), is the leading cause of liver disease in the western world[1,2]. Obesity, dyslipidemia and type 2 diabetes mellitus are the main pathogenic triggers[3]. MAFLD is clinically defined by atypical aspects during imaging and an increase in transaminases and cholestasis parameters. It leads to liver fibrosis and with long-term progression to cirrhosis[4,5], which is associated with numerous potentially lethal complications such as gastrointestinal bleeding, coagulation disorders and ultimately, the development of hepatocellular carcinoma[6].

The emergence and progression of MAFLD at the cellular level is the subject of many current research projects. Although the diverse aspects of the pathophysiology of MAFLD remain to be completely characterized, it becomes increasingly clear that inflammation plays a pivotal role[7]. For example, altered microbiota and gut permeability during obesity may result in low grade systemic inflammation contributing to the development of MAFLD[8,9,10]. In addition, dyslipidemia and adipocytokines from visceral adipose tissue are considered to initiate and propagate liver inflammation[4]. Subsequent drivers are fatty acid-, cytokine- and cholesterol-induced stress responses in Kupffer cells and hepatocytes, leading to the release of fibrogenic factors e.g. transforming growth factor beta (TGF-β)[11], platelet-derived growth factor (PDGF)[12,13] and chemokines e.g. CCL2 and CCL5. These factors consecutively promote activation and differentiation of hepatic stellate cells into myofibroblasts and the recruitment of additional inflammatory cells e.g. classical monocytes into the liver[14,15,16,17,18].

Hepatic stellate cells are central in the pathophysiology of liver fibrosis by responding to the fibrogenic and morphogenic signals mainly derived from infiltrated bone marrow-derived macrophages and resident Kupffer cells[5,19]. Transdifferentiation of stellate cells is associated with the expression of myofibroblast markers e.g. α-smooth muscle cell actin (α-SMA), matrix metalloproteinases (MMPs), vimentin and glial fibrillary acidic protein (GFAP) and extracellular matrix components e.g. collagen types I and III[20,21]. Excessive extracellular matrix production along with ineffective resorption are the key processes in organ fibrosis[22].

Extracellular vesicles (EV) are small cell fragments measuring up to 1000nm encased by fragments of the endosomal or surface membranes of their parent cells[23,24]. EV are released into the immediate environment of the parent cells and may be transported through the bloodstream to distal areas of the organism. Their content is composed of proteins, lipids and nucleic acid sections of the parent cell. EV can be incorporated by target cells and may thereby influence the behavior of the recipient cell. Thus, EV can be considered as a physiologic mechanism for cell-cell communication not requiring direct spatial interaction. Previous work by us and by others has demonstrated that EV can alter the behavior of target cells[25,26]. Also, hepatocytes have been found to release increased amounts of EV after treatment with fatty acids and these EV were able to activate macrophages[27] and the release of EV by hepatocytes was implied to activate hepatic stellate cells and promote fibrosis in a mouse model of CCL4 liver injury[28].

In the present work, the influence of EV derived from fatty HepG2 cells on hepatic stellate cells is evaluated. The hypothesis is that hepatocytes stressed by fatty acid excess can transmit signals through EVs to hepatic stellate cells, which may facilitate the transformation of resting stellate cells to active myofibroblasts and thus promote initiation and progression of the fibrogenic process during MAFLD.

## Results

*2.1 HepG2 cells release increased amounts of EV under steatogenic conditions.*

The hepatocellular carcinoma cell line HepG2 was treated without or with a mixture of linoleic and oleic acid (fatty acids, FA) and the release of EV by HepG2 cells under baseline conditions (C-EV) and after treatment with FA (FA-EV) was assessed in conditioned culture media collected over 24 hours. Characterization of the EV derived from those HepG2 cells by NTA showed an average size of 0.17 µm (Fig. 1A,C), which is also supported by cryo-TEM, revealing spherical, membrane enclosed, mainly unilamellar EV (Fig. 1B,D). The EV were morphologically comparable between EV isolated after either treatment. Western blot analysis revealed the presence of exosome markers Alix, TSG101, and synthenin[29] in C-EV and FA-EV, similar to control EV isolated from platelets or normal pooled plasma (Figure 1E). This suggests that the EV preparations contain both larger microvesicles and exosomes. The HepG2 cells were found to constitutively release extracellular vesicles (EV) in the culture media (Figure 1F). Interestingly and in line with previous studies, the number of EV released per cell was significantly pronounced after treatment with FA (Figure 1E). The steatotic phenotype was confirmed by enhanced staining with Oil-red-O and increased cellular triglyceride content (Supplementary Figure S1A). In addition, the expression of the lipid droplet-associated perilipin (PLIN-1) and fatty acid binding protein 1 (FABP1) were increased (Supplementary Figure S1B-D).

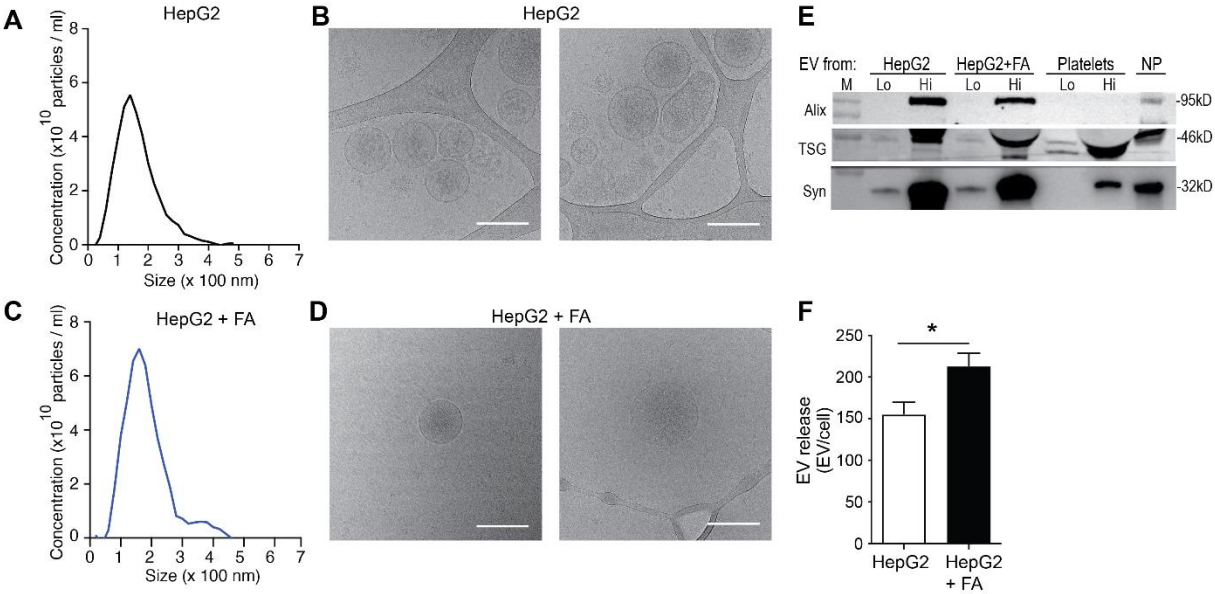


**Figure 1. Treatment with fatty acids induces steatosis in HepG2 cells.**
NTA analysis (A,C) and representative electron micrographs (B,D at 53000x magnification) of EV in media of HepG2 cells collected under resting conditions (A,B) or after treatment with fatty acids (FA) for 24 hours (C,D). Scale bars: 200 nm. (E) Western blot of the EV markers Alix, TSG101, and Syntenin in EV isolated from HepG2 without or with FA-treatment and from platelets (Lo: $10^7$, Hi: $10^8$ EV) or normal pooled plasma (NP, $6*10^7$ EV). (F) Expression of EV release as number of EV per cell. *$p<0.05$ with 2-tailed t-test, mean ± SEM (n=3).

*2.2 EV from HepG2 modulate migration of stellate cells.*

Steatotic hepatocytes have been shown to secrete several factors, including distinct cytokines and EVs, which could be measured in the sera of individuals with fatty liver disease[25,30]. These factors might directly impact biological features of hepatic stellate cells such as their migration to sites of release and thereby modulate the distribution of hepatic stellate cells and fibrotic response in the tissue. In a Boyden chamber setup, TWNT-4 cells, a human

hepatic stellate cell line, and primary human hepatic stellate cells (HSC) were allowed to migrate towards the established chemoattractant CCL5, serum from healthy individuals and those with clinically diagnosed MAFLD. Interestingly, the sera from healthy individuals did not induce migration of TWNT-4 cells, whereas TWNT-4 cells showed pronounced migration towards sera from MAFLD patients (Figure 2A). However, of course, a myriad of factors within the sera might contribute to the enhancement of hepatic stellate cells migration to a distinct extent. To specifically address the impact of HepG2-derived EV on hepatic stellate cell function, we went back to the controlled *in vitro* setting and assess EV associated modulation of distinct hallmarks of hepatic stellate cell biology. We started with analyzing C-EV and FA-EV induced TWNT-4 migration. Stellate cells are activated by and migrate to various molecular cues. For example, the chemokine CCL5 was found to trigger migration of TWNT-4 stellate cells and to be involved in the development of experimental liver fibrosis[17]. In line with the findings in the patient sera setting, FA-EV triggered chemotactic migration of TWNT-4 cells to the same extent as CCL5, whereas C-EV did not trigger chemotaxis (Figure 2B). In this regard, further analyses were mainly focused on FA-EV. Interestingly, when the TWNT-4 were co-cultured with FA-EV prior to chemotaxis, the cells no longer migrated towards CCL5 (Figure 2C). Of note, primary HSC were also found to migrate towards FA-EV to a similar extent as CCL5, further supporting the chemoattractant potential of steatotic HepG2-derived FA-EV (Figure 2D). To rule out that the impaired migration was due to the loss of expression of the CCL5 receptor CCR5 on the surface of the cells, we performed flow cytometry based analysis of the surface expression levels of CCR5 on the TWNT-4 cells, which remained unaltered after co-culture with EV of either origin (Supplementary Figure 1E,F).

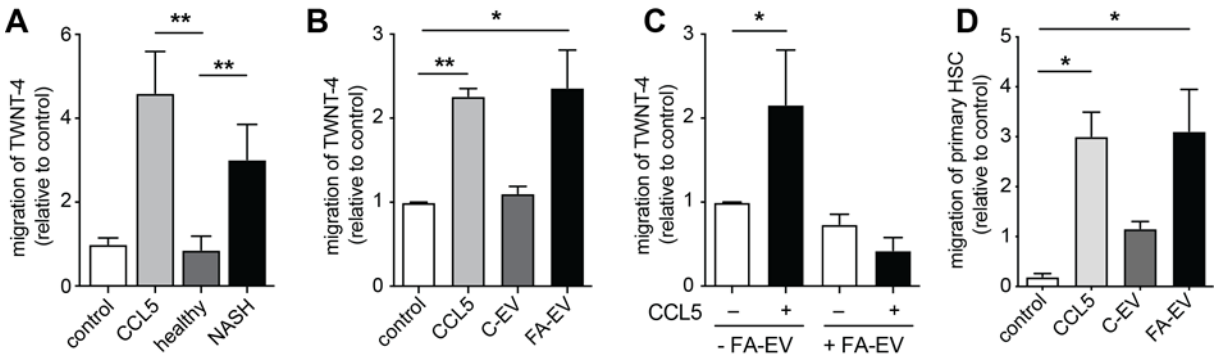


**Figure 2. Migration of TWNT4 and primary stellate cells towards patient sera and EV released from HepG2 cells.**
Chemotaxis of TWNT4 cells towards CCL5 or (A) sera from individuals without or with MAFLD, or (B) towards EV harvested from HepG2 under resting conditions (C-EV) or after fatty acid treatment for 24 hours (FA-EV). (C) Chemotaxis of TWNT4 cells towards CCL5 without or with pre-treatment with FA-EV for 24 hours. (D) Chemotaxis of primary human hepatic stellate cells (HSC) towards C-EV or FA-EV. Five view fields were counted per filter and normalized to control. *p<0.05, **p<0.01 with one-way ANOVA and Bonferroni post-hoc test, mean ± SEM (n=3).

*2.3 EV from HepG2 increase the proliferation of TWNT-4 stellate cells.*

Activation of hepatic stellate cells leads to mitogenic responses and increased proliferation[4,5]. We therefore next assessed the impact of HepG2-derived EV on hepatic stellate cells proliferation. TWNT-4 cells were stimulated with CCL5 and with FA-EV for 24 hours, and proliferation was determined by BrdU incorporation as well as by the mRNA expression of the proliferation marker Ki-67 and PCNA. Expression of Ki67 and PCNA were increased in TWNT-4 cells after treatment with FA-EV. The BrdU incorporation was also significantly enhanced (Figure 3A-C). The viability of the TWNT-4 cells was not reduced after treatment

with CCL5 or FA-EV, although the combination of CCL5 and FA-EV led to a notable reduction of cell viability (Figure 3D). In contrast to the TWNT-4 cell line, the treatment of primary HSC with FA-EV did not increase the expression of the proliferation marker PCNA (Supplementary Figure 2A).

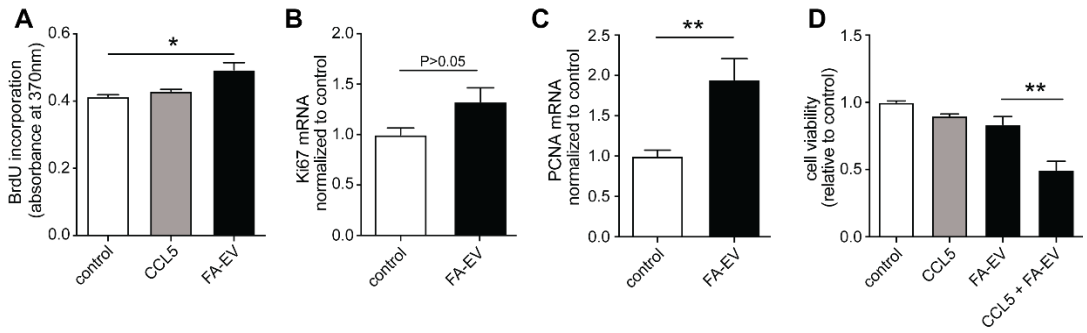


**Figure 3. Proliferation and cell viability of TWNT4 cells after EV treatment.**
(A) Proliferation of TWNT4 cells measured by BrdU-incorporation after treatment with CCL5 or FA-EV. (B) Expression of the proliferation markers Ki67 (B) and PCNA (C) after treatment of TWNT4 with FA-EV. (D) Viability of TWNT4 cells after treatment with CCL5, FA-EV or a combination of CCL5 and FA-EV. *P<0.05, **P<0.01 with ANOVA and Bonferroni test or 2-tailed t-test, mean ± SEM (n=3-4).

*2.4 EV from steatotic HepG2 modulate the expression of matrix myofibroblast- and remodeling-markers in stellate cells.*

Stellate cells are crucially involved in fibrotic liver remodeling and are considered as the central effectors of liver fibrosis[4,5]. Once hepatic stellates are activated, they undergo a process of transdifferentiation and gain a myofibroblast-like phenotype associated with the synthesis and release of collagen and matrix remodeling factors such as TIMP-1. Since hepatic stellate cells are in direct proximity to hepatocytes, a possible influence of EV derived from HepG2 cells under steatotic conditions on transdifferentiation and collagen production was investigated in TWNT4 cells. After treatment of TWNT4 cells with CCL5 and FA-EV for 24 hours, the fraction of elongated cells that have assumed a myofibroblast-like morphology has significantly increased almost 1.5-fold (Figure 4A-D). In addition, expression levels of myofibroblast markers α-SMA, GFAP and vimentin were significantly increased after treatment with FA-EV (Figure 4E-G). An increased expression of α-SMA was also observed in primary HSC treated with FA-EV (Supplementary Figure 2B).

In contrast, collagen production visualized by fluorescence microscopy and mRNA expression levels of the collagen type 1 α1 gene (COL1A1) as well as TGF-β did not significantly change after FA-EV-treatment of TWNT4 cells (Figure 5A-E) or primary HSC (Supplementary Figure 2C). However, expression of the matrix remodeling markers TIMP1 and MMP2 increased significantly after incubation with FA-EV (Figure 5F-H).

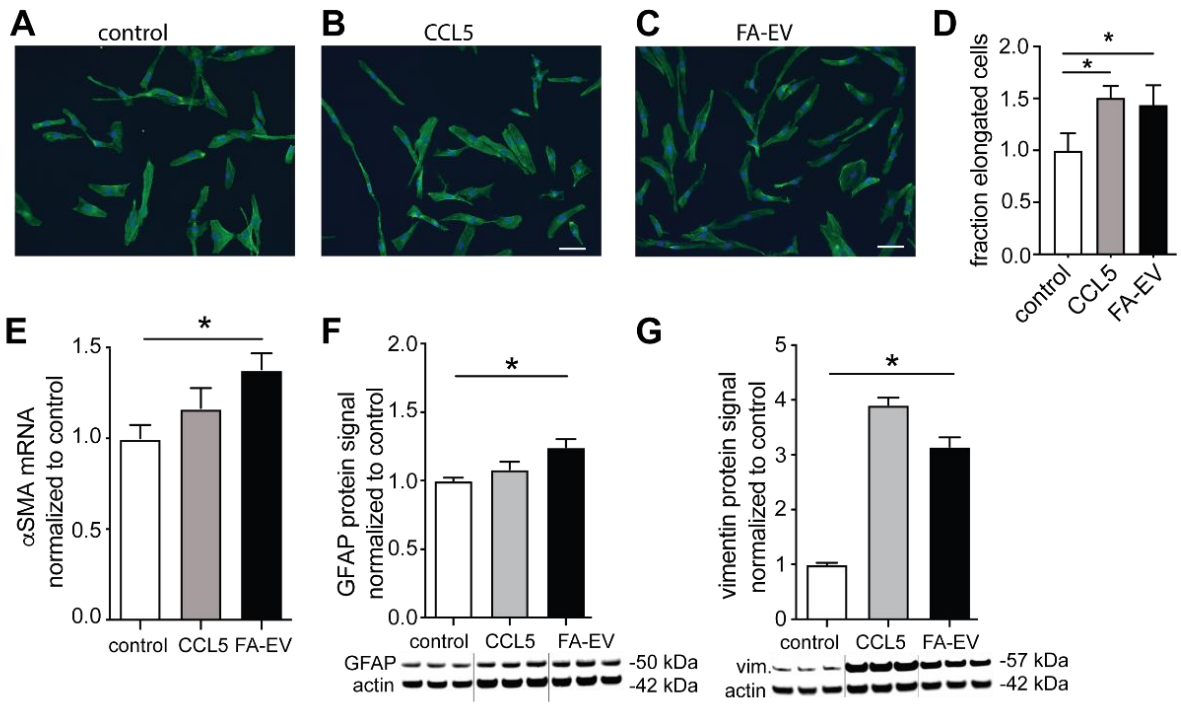


**Figure 4. Morphology and collagen production of FA-EV–treated TWNT4 cells.**
Representative phalloidin staining after treatment with vehicle (A), CCL5 (B) or FA-EV (C). Scale bar: 100 µm. (D) Quantitation of elongated cells from the micrographs (n=3). mRNA expression of a-SMA (E), and antigen levels GFAP (F) and vimentin (G) after treatment with vehicle, CCL5 or FA-EV. *P<0.05 with ANOVA and Bonferroni test, mean ± SEM (n=3-5).

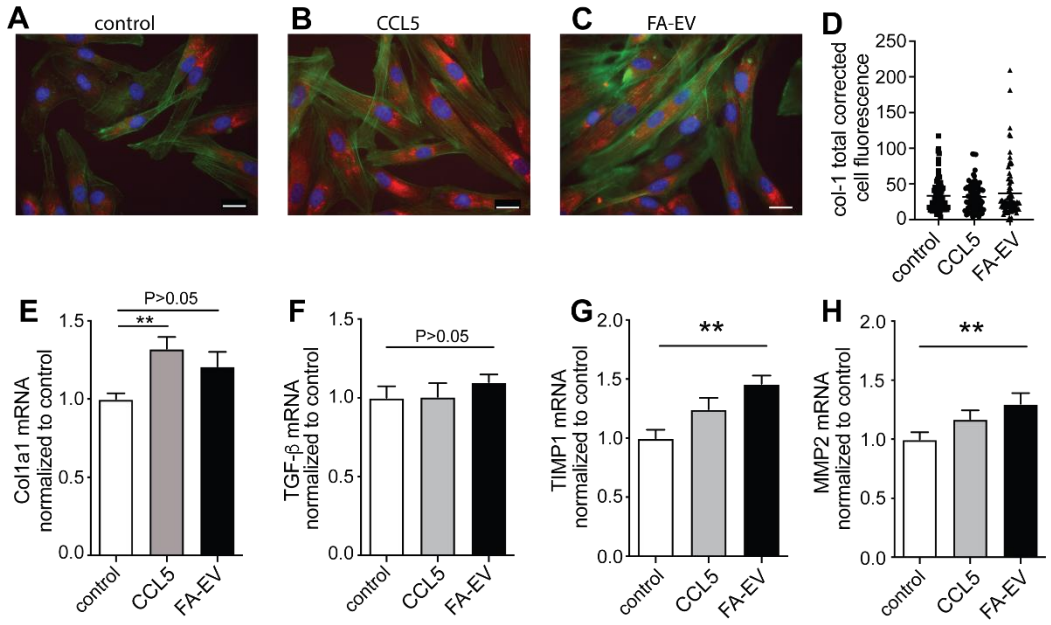


**Figure 5. Expression of fibrotic markers in FA-EV–treated TWNT4 cells.**
Representative phalloidin staining and collagen staining after treatment with vehicle (A), CCL5 (B) or FA-EV (C), scale bar: 20 µm. (D) Quantitation of red (collagen) fluorescence. mRNA expression levels of COL1A1 (E), TGF-b (F), TIMP1 (G), MMP2 (H), after treatment with vehicle, CCL5 or FA-EV. **P<0.01 with ANOVA and Bonferroni test, mean ± SEM (n=3-5).

## 2. Discussion

In this study, the effects of EV harvested from FA-treated HepG2 cells (FA-EV) on stellate TWNT4 cells were investigated. In accordance with previous observations, treatment of HepG2 cells with lipotoxic compounds e.g. lysophosphatidylcholine (LPC) or its precursors palmitic and oleic acid leads to an increase of EV formation[27,30,31,32]. Given the important function of hepatic stellate cells in liver inflammation and the progression from steatosis to fibrosis, human TWNT4 cells and primary HSC were used as a model system to investigate mechanisms behind the transition of hepatic steatosis to fibrosis.

In chemotaxis experiments, TWNT4 cells showed migration solely towards sera of individuals with MAFLD, whereas control sera did not induce any effects. Thus, MAFLD sera may contain increased amounts of inflammatory factors that induce responses in target cells, e.g. hepatic stellate cells. As previous studies have identified increased amounts of cytokines[27] or EV[30] in sera from MAFLD patients, it was hypothesized that EV derived from steatotic HepG2 cells might induce these effects in TWNT4 cells. Interestingly, both TWNT4 and primary HSC cells were found to migrate only towards FA-EV and CCL5 and not towards control EV from HepG2 cells. A possible explanation is that the lipotoxic effects of the FA-treatment results in an altered packaging of the EV cargo, with an enrichment of components involved in inflammation or cell proliferation. For example, Hirsova and colleagues observed an enrichment of the cell death- and inflammation-related Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) in EV isolated from LPC-treated hepatocellular carcinoma Huh7 cells[27]. In other studies, it was observed that EV from LPC-treated Huh7 cells were enriched in integrin β1[30] or CXCL10[32]. In a mouse model of liver fibrosis, exosomes were shown to activate toll-like receptor 3 in HSC[28]. In addition to proteins, also bioactive lipids e.g. ceramides might be enriched in EV released from hepatocytes after treatment with fatty acids[31] and recent studies have also provided evidence of the transfer of micro RNAs, carried by hepatocyte-derived exosomes to stellate cells[33],[34].

Interestingly, although TWNT4 cells actively migrated towards a gradient of FA-EV, culture of TWNT4 with FA-EV prior to the chemotaxis experiment abolished the migration of these cells towards CCL5, an established chemoattractant for these cells[17]. This was not due to alterations in the surface expression of CCR5, which was found to be equal on the cells between all treatments. It might be surprising that the FA-EV induce chemotaxis on the short term and inhibit this process on the longer term. However, once the stellate cells are at the site of inflammation, they may be kept in place by migration-inhibiting signals contained in the FA-EV. In addition to local effects of fatty acid-induced EV release, EVs from damaged hepatocytes might also attract the stellate cells over a longer distance to the focus of developing disease. It is tempting to speculate that this might be among the causes of the typical histological fibrosis image in MAFLD cirrhosis, which presents itself as sinusoidal fibrosis. Thus, a chemotactic effect of the EVs, as suggested by our experiments, would cause the stellate cells to migrate along the liver sinusoids (Figure 6). The EVs are incorporated by the stellate cells once in contact at higher concentrations, which might lead to arrest of migration and production of extracellular matrix to cause the specific image of the “chicken wire” fibrosis – collagen fibers along the sinusoids lined by steatotic hepatocytes[35].

Once hepatic stellate cells had arrived at the site of inflammation, proliferation and production of extracellular matrix are further processes finally inducing the progression to liver fibrosis. Treatment of TWNT4 cells with FA-EV led to an increase in BrdU incorporation and of proliferation markers. Cell viability was not significantly affected by FA-EV, although the combination of CCL5 and FA-EV led to a notable reduction in cell viability. Interestingly, whereas the production of collagen by FA-EV-treated TWNT4 cells did not significantly increase, the cells appeared to change phenotype to an elongated appearance typical of myofibroblasts[20,36]. In addition, the expression of several markers of a myofibroblast phenotype

were found to be increased both in TWNT4 and primary HSC cells after FA-EV-treatment. Thus, FA-EV have the potential to modulate the responses of recipient cells, such as HSC, and these observations are in line with recent studies[33,34], supporting the concept of EV as mediators of cell-to-cell communication[24,37].

Two recent studies implementing next generation sequencing have yielded novel mechanistic insights in the cellular mechanisms governing the development of MAFLD[38] and liver fibrosis[39]. Interestingly, unsupervised clustering of single-cell sequencing data revealed the involvement of a variety of non-parenchymal cell types and their subpopulations. A surprising involvement of endothelial cells, which exist in distinct subpopulations in MAFLD and fibrotic livers and might control liver metabolism and populate the fibrotic niche. In addition, a special type of macrophages expressing Triggering Receptors Expressed on Myeloid cells 2 (TREM2) was identified. Although no study has focused on the role of EV on the development of this macrophage subset has been published so far, a role for EV-mediated signaling in this process can be envisioned. The study investigating cell populations in human and mouse MAFLD has taken effort to unravel intercellular signaling between the identified cell types[38]. Here, hepatic stellate cells were found to be an important cellular hub, both in receiving and transmitting signals from and to hepatocytes, macrophages and endothelial cells. Although a role of EV in the trafficking of these cellular signals has not been explicitly taken into account, the numerous recent studies that highlighted EV as mediators make a strong case for their integral role in intercellular signaling leading to MAFLD and liver fibrosis.

In this study, an EV-mediated signal transmission from FA-treated hepatocytes to hepatic stellate cells was investigated. Although other cell types are instrumental in the development of liver disease, hepatic stellate cells are central in the orchestration of the pathologic responses of non-parenchymal cells in the liver. An excessive flow of hepatocyte-derived EV to macrophages and stellate cells might facilitate the development of MAFLD and liver fibrosis through their potential to modulate cellular phenotype and behavior. Thus, future investigation of this process might not only lead to potentially novel options for therapeutic intervention, but also to improved diagnostic insights through analysis of plasma EV numbers and content.

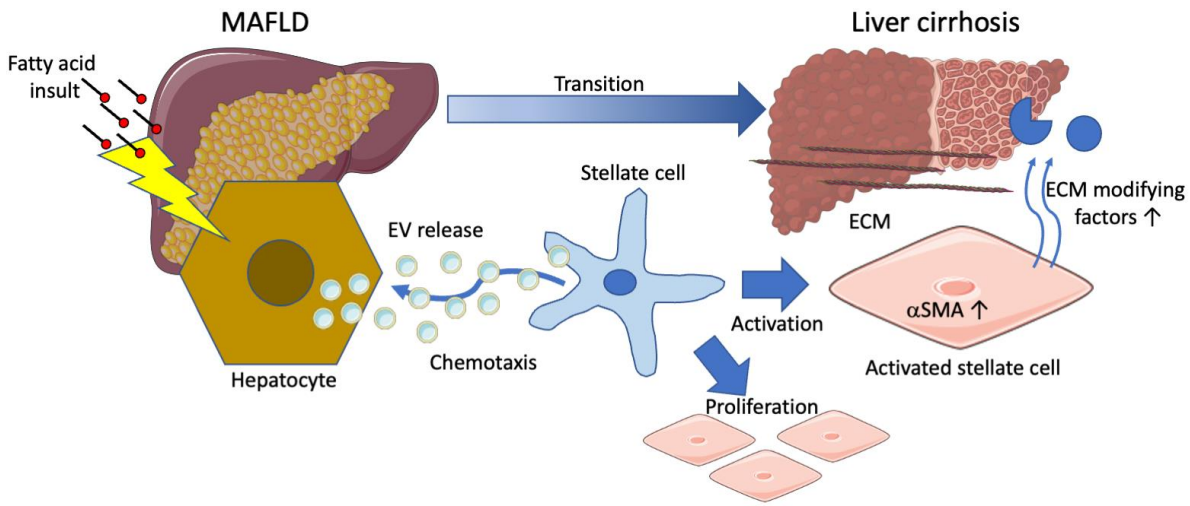


**Figure 6. Schematic overview of the effects of EV on HSC during liver fibrosis pathogenesis.** During MAFLD, a chronic insult of fatty acids may lead to increased EV release by hepatocytes, which causes chemotaxis of HSC along the liver sinusoids and activation and proliferation of HSC, leading to a myofibroblast-like phenotype with increased a-SMA as marker and an increased release of extracellular matrix (ECM) modifying factors.

## 3. Material and methods

*4.1 Patient Samples, Reagents and Cell Lines*

TWNT-4, immortalized human stellate cells, kindly provided by Professor Scott Friedman, Icahn School of Medicine at Mount Sinai, NY, USA[40], and HepG2, human hepatoma cells, were cultured in filtered Dulbecco`s Modified Eagle`s Medium (DMEM) with 2% or 10% fetal bovine serum (Pan-Biotech GmbH, Aidenbach, Germany) and 1% penicillin and streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at passages 3-8 in 37°C and 5% $CO_2$ in a humidified incubator. For induction of a steatotic phenotype, the cells were treated with 10% linoleic-oleic-acid (Sigma-Aldrich, USA) for 24h. Primary human stellate cells were obtained from Lonza (Basel, CH) and cultured in human stellate cell growth media (Lonza) according to manufacturer's instructions. Serum was taken from female and male patients with diagnosed MAFLD (n=5, m:f 3:2, age 43-77 years, mean 52.6 years) and controls (n=5, m:f 3:2, age 33-56 years, mean 44.8 years) by venipuncture after informed consent was obtained. The study has been approved by the University Hospital RWTH Aachen ethics board. Normal pooled plasma was obtained from healthy volunteers as described[41].

*4.2 Isolation of EV*

EV were isolated from expired platelet packs, normal pooled plasma, or conditioned cell culture supernatants collected up to 300 ml as previously described[26] according to ISEV recommendations[42,43]. The medium was filtered through 0.8 µm filters (Sartorius, Göttingen, Germany) by gravity flow and subsequently centrifuged at 20,000g for 1h at 16°C. The pellet was resuspended in Hepes buffer pH 6.6 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mM glucose and 0.1% BSA).

*4.3 Quantification of EV*

Concentrations and vesicle size distributions were determined by NTA (nanoparticle tracking analysis, Malvern NanoSight NS300, Malvern Technologies, Malvern, UK or ZetaView, Particle Metrix, Wildmoos, Germany) equipped with a 488nm laser at 1:100 or 1:1,000 dilutions. Every sample was counted 5 times for 1 minute at 20°C. The camera level was set at 16 and traces were analyzed using NTA 3.1.54 or ZetaView 8.05.11 software with a detection threshold of 5 as described[26]. To compare the cellular release of EV between treatments, the absolute counts of EVs in the cell media samples were normalized to the cell count in the respective culture vessels.

*4.4 Cryo-Transmission Electron Microscopy (cryo-TEM)*

The preparations of platelet EVs were visualised by the cryo-TEM method. A thin aqueous film was formed by applying a 5 µl droplet of the suspension to a bare specimen grid. Glow-discharged holey carbon grids were used. After the application of the suspension the grid was blotted against filter paper, leaving thin sample film spanning the grid holes. These films were vitrified by plunging the grid into ethane, which was kept at its melting point by liquid nitrogen, using a Vitrobot (Thermo Fisher Scientific / FEI Company, Eindhoven, Netherlands) and keeping the sample before freezing at 95% humidity. The vitreous sample films were transferred to a microscope Tecnai T12 Spirit (FEI Company) using a Gatan cryotransfer. The images were taken at 200 kV and 53000x magnification with a 4096×4096 pixel CCD Eagle camera (FEI Company) at a temperature between -170°C and -175°C and using low-dose imaging conditions.

*4.5 Cell Migration by Boyden Chamber*

Home-made filter chambers were assembled with Whatman Nuclepore™ Track-Etched filters (Merck / Sigma Aldrich, St. Louis, MO, USA), coated with gelatine prior to use. The lower chamber was filled with DMEM/2% FBS and the chemoattractant or undiluted patient and control sera (800 µl). As a positive control for the primary stellate cells and TWNT4 cells, CCL5 at 10 ng/ml was used (PeproTech, Hamburg, Germany). The upper chamber was filled with $2x10^5$ TWNT4 or primary stellate cells in 250 µl DMEM/2% FBS. After 4h at 37°C and 5% $CO_2$, membranes were stained using the hemacolor quick stain kit (Merck) and cells quantified under a microscope. Five view fields were counted per filter. For every probe three filters were evaluated.

*4.6 Qualitative and Quantitative DNA Analysis by RT-PCR*

For RNA isolation, peqGOLD TriFast™ was used according the manufacturer's instructions (VWR international, Darmstadt, Germany). RNA-containing pellets were resuspended in nuclease free water and concentrations were determined using a NanoDrop spectrometer (Thermo Fisher). cDNAs were generated using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher) as described[44]. Quantitative RT-PCR analysis was performed using GoTaq®, qPCR Mastermix (Promega, Madison, WI, USA) with primers for PLIN2, KI67, PCNA, ASMA, COL1A1, TIMP1, TGFB1, 18S, and MMP2. The cycler program was 2 minutes at 50°C, 10 minutes at 95°C, and 1 min at 60°C for 40 cycles using a qPCR system Applied Biosystems 7300 (Thermo Fisher). Delta-deltaCT values were calculated relative to the housekeeping gene 18S and results were normalized to controls.

*4.7 Protein Determination by Bicinchoninic Acid Assay (BCA)*

Protein concentrations were determined using the BCA method (Bio-Rad, Hercules, CA, USA). Cells were cultured in 6-well plates and washed for 2 times with PBS, lysed in 300µl RIPA-buffer, incubated with reagents and absorbance was measured at 560nm in a microplate reader. Concentrations were calculated using a standard curve prepared with BSA.

*4.8 SDS-PAGE and Western Blot*

For protein analysis, NuPAGE 4-12 % Bis-Tris Gel SDS-PAGE was performed in MOPS-SDS running buffer (Thermo Fisher) for 60 minutes at 160 V and proteins were subsequently transferred to nitrocellulose (Whatman, Cytiva, Marlborough, MA, USA) for 1 hour at 100V. The membranes were blocked using 5% BSA and incubated with primary and peroxidase-conjugated secondary antibodies prior to detection using enhanced chemiluminescence.

*4.9 Cell Viability*

TWNT4 cells were added at 50.000 per well in a 96-well microplate and cultured overnight under the conditions described above. Media were replaced with fresh medium containing EVs at 300 per cell and cultured for further 12h. Then, 25ng CCL5 was added and culture was continued for 24h. As positive control, cells were treated with 30% $H_2O_2$. Cell-Titer-Blue-reagent (Promega, Madison, WI, USA) was added for 2h followed by measurement of the fluorescent signal at 560 em/ 590ex nm.

*4.10 Oil-red-O and Phalloidin Staining*

Cells were seeded in 4-well chamber slides at 50,000 cells per well. After 24h, the cells were stimulated overnight with EVs (300 per cell) and CCL5 (10 ng/mL).

To investigate lipid uptake, linoleic-oleic acid (300 µM) was added to HepG2-cells and culture was continued for further 24h. Medium was removed, cells were washed with PBS, fixed with 4% formaldehyde for 5 minutes and washed again. Intracellular lipid vacuoles were

stained in filtered 0.12% Oil-red-O in 20% isopropanol for 1h. Cells were washed with water. After staining of nuclei for 25 seconds in Mayer's hematoxylin solution (Sigma Aldrich), cells were washed again and covered with a cover slip treated with glycerine-gelatine. Evaluation of Oil-red-O uptake was performed by light microscopy.

To visualize the cytoskeleton in TWNT4-cells, medium was removed, and cells were washed with PBS, fixed in 4% formaldehyde and washed again with PBS prior to permeabilization by 0.1% Triton-X100 in PBS for 15 minutes. After washing with PBS, cells were blocked in 1% BSA-PBS for 1h. Anti-collagen antibody (Thermo Fisher) was given to the cells for one hour. Fluorescein-conjugated phalloidin (1U in 1% BSA/PBS) was added for 30 minutes. The cells were washed with PBS and nuclei were colored by DAPI. Evaluation of cytoskeletal staining was performed by fluorescence microscopy at 490/530 nm. Corrected total cell fluorescence (CTCF) was calculated using Image J 1.5[45] as described in[46].

*4.11 Cell Proliferation Measurement*

The 5-bromo-2'-deoxyuridine (BrdU) assay (Thermo Fisher) was used for quantification of proliferating cells according to the manufacturer's instructions.

*4.12 Flow Cytometry Analysis of CCR5 Surface Expression*

TWNT-4 were seeded at 100,000 cells per well in a 96-well plate and were allowed to rest overnight. Cells were treated with 10 ng/ml CCL5 or 300 EV/cell for 24h. Cells were detached, filtered using a cell strainer, washed in PBS and stained in the dark with rat anti-human/mouse CCR5 FITC-conjugated antibody (clone HEK/1/85a from Thermo Fisher) for 1h at 4°C. The cells were subsequently stained with Fixable Viability Dye eFluor450 (Thermo Fisher, eBioscience, cat. 65-0863-18) for a further 30 min. A negative control only contained viability dye. Cells were gated according to FSC and SSC parameter and live cells using a FACSCanto II (Becton Dickinson, Franklin Lakes, NJ). Data were processed and analyzed using FlowJo 7.6 software (Becton Dickinson).

*4.13 Statistical Analysis*

All authors had access to the study data and have reviewed and approved the final manuscript. All data generated or analysed during this study are included in this published article. Experiments were performed at least 3 independent times. Experimental data were represented as mean ± SEM. Statistical analysis of the data were performed by two-tailed t-test or ANOVA with Bonferroni post-hoc test as indicated in the figure legends. A p value below 0.05 was considered significant. Statistical analysis was performed with Graphpad Prism 9 software (San Diego, CA, USA).

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**Conflict of interest statement:** The authors confirm that there are no conflicts of interest.

**Author contributions:** MTK: obtained funding, analyzed data, coordinated study, wrote manuscript; TC, DMK, SG, WJD, ACAH, PF, DH, EB: performed experiments; CT: obtained funding and intellectual input; RRK: obtained funding and provided intellectual input, wrote the manuscript; MLB: obtained funding, provided intellectual input, critically edited the manuscript, coordinated study.

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**Extracellular vesicles from steatotic hepatocytes provoke pro-fibrotic responses in stellate cells**

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Supplementary material

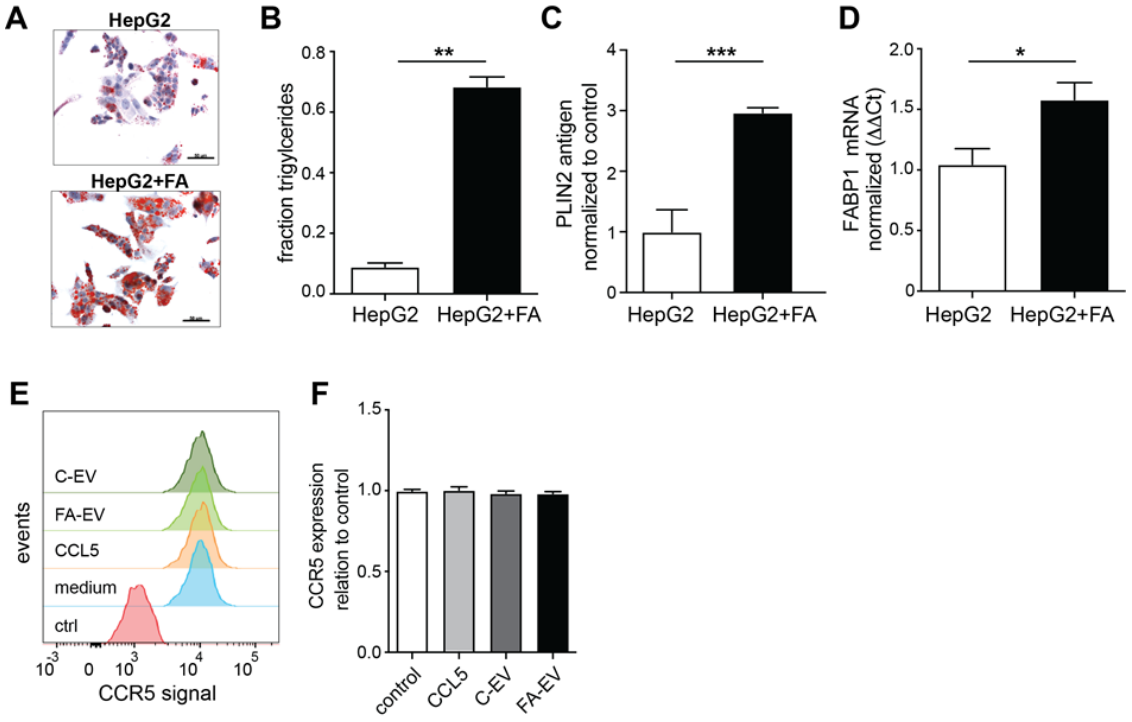



**Figure S1. Markers of steatosis in HepG2 cells and effects of EV on CCR5 expression in TWNT4 cells.**
(A) Representative oil-red-o staining after treatment with linoleic-oleic-acid for 24 hours. Scale bar: 50 µm. Fraction of triglycerides in cell lysates (B), antigen levels of PLIN2 (C) and mRNA expression levels of FABP1 (D) after treatment with linoleic-oleic-acid for 24 hours. Representative histograms (E) and quantitation (F) of CCR5 expression of TWNT4 cells after pretreatment with CCL5, C-EV or FA-EV. *p<0.05, **p<0.01, ***p<0.001 with 2-tailed t-test, mean ± SEM (n=3).

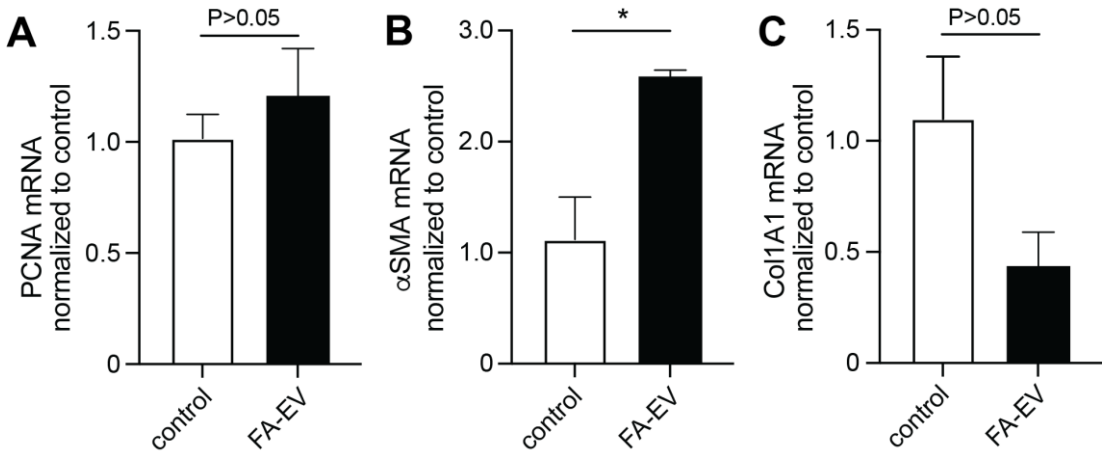
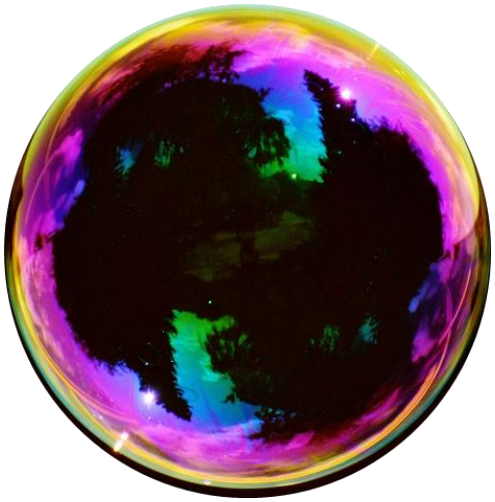



**Figure S2. Effects of EV from steatotic HepG2 on proliferation, phenotype and matrix production markers in primary human hepatic stellate cells.**
Primary human hepatic stellate cells were treated with vehicle or FA-EV and mRNA expression levels of PCNA (A), α-SMA (B) and COL1A1 (C) were determined by qPCR. *p<0.05 with 2-tailed t-test, mean ± SEM (n=3).

# Chapter 7

## General discussion



## Key findings in perspective

Atherosclerosis is a progressive, chronic inflammatory disease affecting large blood vessels by triggering endothelial cell injury and the build-up of plaque within the vessel wall. Lesion formation is characterized by an accumulation of lipids and fibrous elements. Also present in advanced lesions are new blood vessels and deposits of calcium hydroxyapatite[1]. Pathological remodelling of cells from vasculature involve a dynamic interaction between different cell types residing in the vessel wall (e.g. ECs, VSMCs and macrophages) with blood components (e.g. platelets and leukocytes), and their secretory molecules (e.g. cytokines and chemokines) that trigger cell migration, proliferation, inflammation, and angiogenesis[2]. The intercellular signalling mediated by extracellular vesicles (EVs) is also involved in these processes. EVs serve as carriers of many bioactive molecules, including proteins, lipids and nucleic acids from parent to recipient cell. In atherosclerosis the interplay between hematological cells and vascular cells is of high importance. The concentration of EVs increases in patients with CVD, and large amounts of EVs are found within the atherosclerotic plaque[3]. EVs participate in key steps of atherosclerosis development, including cellular lipid metabolism, endothelial dysfunction and vascular wall inflammation, ultimately resulting in vascular remodelling[4]. In the context of non-alcoholic fatty liver disease (NAFLD), EVs derived from adipocytes are implicated in the development of insulin resistance and fatty liver (steatosis). Excessive fatty acid accumulation can induce the release of hepatocyte-EVs, which can mediate the progression of fibrosis via the activation of nearby macrophages and hepatic stellate cells[5]. In this thesis I have provided an extensive review on the initiation of atherosclerosis with the focus on calcification of blood vessels (Chapter 2). Moreover, I have described the involvement of EVs derived mainly from platelets and vascular smooth muscle cells (VSMCs) in the formation of calcium-phosphate deposits within the vessel wall (Chapter 3). Experimentally, I studied the effect of platelet-derived chemokines (CXCL4, CXCL4L1 and CCL5) on human vascular smooth muscle cells (hVSMCs) (Chapter 4) and endothelial cells (ECs) *in vitro* (Chapter 5). Finally, I investigated the role of EVs, isolated from steatotic hepatocytes and their effects on stellate cells (Chapter 6).

**<u>The key findings of this thesis are</u>**:

1. Two variants of platelet factor 4 (CXCL4 and CXCL4L1) differently affect VSMCs phenotype in vitro (Chapter 4)

2. Endothelial cells internalize CCL5 and CXCL4 by clathrin- and dynamin-dependent endocytosis, where the chemokines appear to be directed to the nucleus (Chapter 5)

3. Extracellular vesicles from steatotic hepatocytes provoke pro-fibrotic responses in stellate cells (Chapter 6)

## 1. Two variants of platelet factor 4 (CXCL4 and CXCL4L1) differently affect VSMCs phenotype in vitro

It is well established that beyond their very important role in coagulation and hemostasis, platelets also play a role in the development of atherosclerosis. The most abundant platelet protein that is stored and released from intracellular granules is platelet factor 4 (PF4/CXCL4). Upon injury[6] or during the formation of an atherosclerotic plaque, CXCL4 is transported from a bloodstream into deeper layers of the vessel via an unknown mechanism. Over the past forty years, both mouse[7] and human[8,9] studies have confirmed the presence of CXCL4 (and not platelets) in the atherosclerotic plaques. Up until now, the knowledge of platelet factor 4 biology mainly comes from the studies on CXCL4, whereas its non-allelic variant (PF4alt/CXCL4L1) has been somewhat neglected[10,11]. Even though both mature proteins are highly homologous, CXCL4L1 is distinct from CXCL4[12]. In this thesis we elucidated the functional differences between two platelet-derived chemokines – CXCL4 and CXCL4L1, especially with relation to their ability to vascular wall remodelling in atherosclerosis. VSMCs are the most abundant cell type in the arterial vessel wall and are pivotal in maintaining vessel structure and function. Here, we provided evidence that CXCL4 (and not CXCL4L1) treatment increases proliferation of VSMCs, what confirms the stimulatory properties of CXCL4. VSMCs are considered heterogeneous and display a high degree of plasticity, being on a phenotypic spectrum between contractile and synthetic. We found that heparin pre-treatment (maintaining the contractile phenotype of VSMCs) completely abrogated the uptake of CXCL4. In contrast, PDGF pre-treatment (stimulating synthetic phenotype of VSMCs) did not influence chemokine endocytosis. Moreover, we proved that both CXCL4 and CXCL4L1 contribute to the phenotypic switch of VSMCs, what was confirmed by the lower mRNA expression of contractile markers (calponin, α-actin), and higher expression of transcription factors involved in phenotype change (KLF4) and inflammation (NLRP3).

Interestingly, our hypothesis that CXCL4/CXCL4L1 stimulates pro-inflammatory responses in VSMCs was not confirmed. Interleukin 6 (IL-6) levels measured in the supernatant after chemokine treatment was not altered. Also, RT-qPCR analysis showed a decrease in gene expression of IL-6 and IL-1β after chemokine treatment. The obtained results are in contrast with other reports, showing CXCL4 potential to stimulate cytokine production or release in various cell types[13,14]. For example, our group previously showed that CXCL4 triggers the production and release of IL-6 by SMC to a similar extent as exogenously added platelet EVs. This may suggest that CXCL4 association with platelet EVs amplifies the effect of cytokine production by VSMCs[15].

In this thesis we also investigated the mechanistic route of chemokine endocytosis. Chemokines and chemokine receptors create a redundant system. This means that CXCL4 and CXCL4L1 can bind to many cell surface receptors. Various CXCL4 receptors on different cell types have been proposed, including low density lipoprotein receptors (LDL-R)[13,16], chemokine receptor CXCR3[17], Duffy antigen receptor for chemokines (DARC)[18,19], C-C chemokine receptor type 1 (CCR1)[20], proteoglycans[21] and others. In this thesis we showed that the family of LDL receptors appears to be an important player in this process and that CXCR3 and DARC receptors are not or only minimally involved. We also investigated the mechanism of chemokine internalization by VSMCs. The most common route of endocytosis of chemokine receptors is clathrin-mediated endocytosis. The last stage of this process involves membrane budding and scission, processes which also require the enzyme dynamin[22]. Here, we showed that CXCL4 endocytosis occurs via clathrin- and dynamin-independent mechanisms in VSMCs. In contrary, our recent work proved that the internalization of CXCL4 and CCL5 (RANTES) by endothelial cells (ECs) is mediated by clathrin and dynamin. The observed discrepancy shows the distinct chemokine endocytosis routes on VSMCs and ECs[23]. Clathrin-independent mechanisms involving for example caveolae and lipid rafts also exist[24]. The

involvement of caveolae-mediated endocytosis in CXCL4 internalization is less likely since this process also requires dynamin for membrane scission[25]. Endocytosis of rafts, characterized by cholesterol sensitivity may include caveolin- and dynamin-dependent or independent pathways[26,27]. Therefore the exact CXCL4 endocytosis route in VSMCs needs to be investigated more precisely. Because CXCL4 also appears to co-localize with calcified regions of human carotid atherosclerotic plaques[8], we hypothesized that CXCL4/CXCL4L1 may stimulate the process of vascular calcification. We provide evidence that CXCL4 and CXCL4L1 differently affect the calcification of VSMCs. In our hands, CXCL4 appears to rather stimulate the process whereas its variant does not have an effect. Calcium-phosphate crystal formation was previously linked to reactive oxygen species (ROS) production[28]. However, in our in vitro model, the cell incubation with the chemokines did not affect the production of ROS in VSMCs. While CXCL4 has mainly detrimental effects in atherogenesis by triggering phenotype switching and subsequent calcification of VSMCs, CXCL4L1 may at the same time act as a competitive antagonist with a potential anti-inflammatory and plaque-stabilising effect[29]. CXCL4 stimulates the proliferation of VSMCs[13] and at the same time inhibits mitosis in ECs[30]. This phenomenon may be explained by CXCL4 binding to different receptors on distinct cell types, that triggers separate downstream signalling pathways. Interestingly, it was shown that CXCL4L1 is a more potent inhibitor of ECs than the CXCL4[11]. Thereby, the primary function of CXCL4L1 released by VSMCs may be to maintain vascular homeostasis and to avoid EC proliferation and aberrant angiogenesis[32]. The evidence that CXCL4L1 can inhibit metastasis by preventing angiogenesis appears to confirm this hypothesis[31]. Taken together, the constitutive production of CXCL4L1 predominantly by VSMCs, and the regulated secretion of CXCL4 by platelets and the immune cells upon activation, suggest that these 2 chemokines display distinct roles in inflammatory and homeostatic processes[32].

## 2. Endothelial cells internalize CCL5 and CXCL4 by clathrin- and dynamin-dependent endocytosis, where the chemokines appear to be directed to the nucleus

Chemokines can be deposited on cellular glycocalyx of ECs by rolling platelets, facilitating monocyte arrest, as was shown for CCL5 (RANTES)[33,34]. They can rapidly induce leukocyte responses e.g., integrin activation, flow-resistant arrest, cell polarization, and transendothelial migration to sites of inflammation or infection[35,36]. Infusion of activated platelets into hyperlipidemic mice resulted in an accelerated development of atherosclerosis, which could be attributed in part to increased immobilization of CCL5 onto the atherosclerotic vessels[37]. Interestingly, CCL5 and CXCL4 can interact with each other to form heterodimers, which are particularly potent in the recruitment of monocytes[38] and were shown to modulate the severity of atherosclerosis, stroke, abdominal aneurysm, and myocardial infarction in mice[39,40,41,42,43]. To elaborate on the previous findings and to further investigate the mechanisms that underlie chemokine-induced leukocyte recruitment, we investigated the fate of exogenously added CCL5 and CXCL4 to ECs. We found that incubation of ECs with CCL5 and CXCL4 under static conditions led to rapid internalization of the chemokines. A cytoplasmic staining pattern of the chemokines was distinct from the typical accumulation of the CD31 signal at the cell-cell contacts, suggesting the presence of the chemokines inside, rather than outside the cells. This intracellular accumulation of CCL5 and CXCL4 was also proven to be an active and energy-requiring cellular process. Chemokine endocytosis can occur via different pathways and the most common route involves proteins such as clathrin and dynamin. Here, we showed that the internalization of CCL5 and CXCL4 by ECs was indeed clathrin- and dynamin-mediated. Interestingly, these findings were in contrast to our results from VSMCs where dynamin and clathrin did not appear to be involved in the endocytosis of CXCL4, what was explained above. The surprising data suggest that the enzymatic removal of glycosaminoglycans (GAGs) from the cell surface results in an increased uptake of both CCL5 and CXCL4 into EAHy cells.

Interestingly, EAHy stimulation with pro-inflammatory TNF-α did not affect the uptake of CCL5 and CXCL4, when compared to resting cells. We also showed that CXCR3 receptor was not involved in their uptake by endothelial cells, in contrast to what was suggested before[44]. Another interesting analysis demonstrated that the pre-incubation of EAHy cells with CXCL4 prior to the addition of CCL5 led to a significant reduction of subsequent CCL5 uptake, but not vice versa. This indicates that CXCL4 can desensitize EAHy cells for the uptake of CCL5 and the mechanism of this process remains unknown. Taking into account the redundancy of the chemokine system as well as the existence of many CXCL4 receptors on different cell types, we can speculate that there are many more cell surface partners/receptors on ECs for CXCL4 than for CCL5. During the pre-incubation step, CXCL4 can occupy the majority of its own receptors as well as potential binding partners for CCL5, which may be subsequently internalized by the cells (following degradation or recycling), resulting in the lower number of available receptors for CCL5 on the cell surface. Alternatively, the process of heterologous desensitization might occur. In this phenomenon the stimulation of one chemokine receptor can affect the responsiveness to other chemokine receptors[45], what has been shown for CCR7 and CCL19[46]. In this case, the responses to CCL5 receptors would be desensitized by previous CXCL4 treatment. In contrary, CCL5 pre-incubation cannot block the uptake of CXCL4 by ECs, which suggests that CXCL4 indeed can enter the cell via other receptors, that were not occupied by CCL5.

Moreover, using confocal microscopy and analysis of subcellular fractions by ELISA, we provided evidence that both CCL5 and CXCL4 accumulated in the nucleus. Particularly, CXCL4 appeared to accumulate in the nucleolus and the significance of these findings are currently unknown. Both chemokines were also associated with the cytoskeleton, what suggests that they are transported to the nucleus through cytoskeletal associations, what was observed before[47]. It is tempting to speculate on an active involvement of CXCL4 in transcriptional processes. Genetic analyses performed in the recent study revealed that CXCL4 dramatically alters the trajectory of monocyte differentiation. These pro-inflammatory and pro-fibrotic monocytes directly trigger a fibrotic cascade by producing extracellular matrix molecules and inducing myofibroblast differentiation[48]. In line with these finding were the results of another group which showed that increased expression of CXCL4 is associated with the progression of bone marrow fibrosis and is a mediator of inflammation[49]. In another study, elevated levels of CXCL4 correlated with lung fibrosis in patient with systemic sclerosis and predicted the risk and progression of the disease[50]. Therefore, targeting CXCL4 might be a promising strategy to reduce inflammation in fibrotic disease. Lastly, we showed that the addition of CCL5 and CXCL4 did not affect MonoMac6 adhesion to HUVECs, indicating that internalized chemokines neither make HUVECs competent for leukocyte interactions nor directly induce leukocyte arrest. These results suggest that CCL5 and CXCL4 require surface presentation for leukocyte recruitment.

## 3. Extracellular vesicles from steatotic hepatocytes provoke pro-fibrotic responses in stellate cells

Obesity, dyslipidemia and type 2 diabetes mellitus are the main pathogenic triggers of non-alcoholic steatohepatitis (NASH) that lead to fibrosis and ultimately cirrhosis[51,52,53]. Fatty acid, cytokine- and cholesterol-induced inflammatory responses in Kupffer cells and hepatocytes lead to differentiation of hepatic stellate cells into myofibroblasts[54,55,56,57,58] and excessive extracellular matrix production[59], which are central in the pathophysiology of liver fibrosis[53,60]. EVs, carrying a specific cargo (proteins, lipids and nucleic acids) are released by parent cells and can be incorporated in recipient cells, changing their phenotype. For instance, hepatocytes

were found to release more EVs after treatment with fatty acids and these EVs were able to activate macrophages[61].

Our hypothesis was that hepatocytes stressed by fatty acid excess can transmit signals through EVs to hepatic stellate cells, which may facilitate the transformation of resting stellate cells to active myofibroblasts and thus promote initiation and progression of the fibrogenic process during NASH. In our study we found that the hepatocellular carcinoma cells (HepG2) released increased amounts of EVs upon treatment with a mixture of linoleic and oleic acid. The steatotic phenotype was confirmed by Oil-red-O staining, high triglyceride content, increased expression of the lipid droplet-associated perilipin (PLIN-1) and fatty acid binding protein 1 (FABP1). We provided evidence that sera isolated from NASH patients induced migration of human hepatic stellate cells (TWNT-4), whereas sera from healthy donors did not. A myriad of factors within sera from NASH patients can contribute to this effect, such as increased levels of biologically active chemokines[62], including CCL5[63] that were extensively investigated in this thesis. However, in this chapter, we aimed to prove the involvement of EVs in this process. We compared control EVs (C-EVs) and fatty acid-induced EVs (FA-EVs) and found that FA-EVs triggered chemotactic migration of TWNT-4 cells to the same extent as positive control (CCL5), whereas C-EVs did not trigger chemotaxis. Interestingly, when the TWNT-4 were co-cultured with FA-EVs prior to chemotaxis, the cells no longer migrated towards CCL5.

We found an increased proliferation of TWNT-4 cells upon FA-EVs stimulation, which was also confirmed by increased expression of Ki67 and PCNA. The viability of the TWNT-4 cells was not reduced after treatment with CCL5 or FA-EVs, although the combination of CCL5 and FA-EVs led to a notable reduction of cell viability. We showed that EVs from steatotic HepG2 modulate the expression of matrix myofibroblast- and remodeling-markers in stellate cells. We found that after treatment of TWNT4 cells with CCL5 and FA-EVs, the fraction of elongated cells and the expression of myofibroblast markers α-SMA, GFAP and vimentin were significantly increased. In contrast, collagen production visualized by fluorescence microscopy and mRNA expression levels of the collagen type 1 α1 gene (COL1A1) as well as TGF-ß did not significantly change after FA-EVs-treatment. However, expression of the matrix remodeling markers TIMP1 and MMP2 increased significantly after incubation with FA-EVs. Today we know that two distinct cell-cell communication systems: chemokines and EVs are not strictly separated, as many cytokines in vitro, ex vivo, and in vivo are released in EV-encapsulated forms and are capable of eliciting biological effects upon contact with sensitive cells[64]. Thus, we must not exclude the possibility that FA-EVs were packed with a composition of chemokines that at least partially contributed to the observed effects.

## Concluding remarks, recommendations and future perspectives

The aim of this thesis was to gain further insight in the role of chemokines and extracellular vesicles in the pathological remodeling, with emphasis on atherosclerosis and liver fibrosis. Here, we provided evidence that CXCL4 and CXCL4L1 are two different proteins, that share some similarities, yet may have different functions in VSMCs and in other cell types. We also emphasized that the properties of CXCL4 and CXCL4L1 must always be explained in a strictly defined cellular context to avoid false conclusions from experiments performed on distinct cell types. Furthermore, we investigated the fate of exogenously added CCL5 and CXCL4 to ECs and the mechanism of their endocytosis. We showed that the internalized chemokines did not induce leukocyte arrest and that both chemokines accumulated in the nucleus where they might be involved in transcriptional processes. Lastly, we showed that hepatocytes stressed by fatty acid excess can transmit signals through EVs to hepatic stellate cells, which may facilitate their transformation to myofibroblasts and thus promote initiation of the fibrogenic process in the liver. We found that the hepatic cells released higher amounts of EVs upon treatment with fatty

acids. These FA-EVs triggered proliferation, chemotactic migration and modulated expression of matrix myofibroblast-markers in stellate cells. Taken together we clearly demonstrated that both chemokines and EVs participate in pathological remodeling of blood vessels in cardiovascular diseases as well as liver diseases such as NASH.

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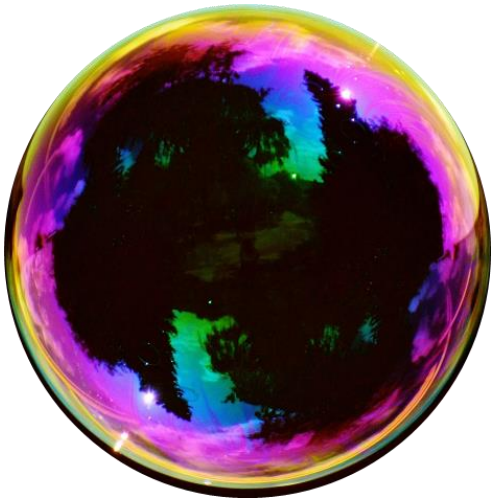
# Chapter 8

## Summary



## Summary

The present thesis describes the initiation of atherogenesis with the focus on calcification of blood vessels. The role of platelet and VSMC-derived extracellular vesicles (EVs), and chemokines in this process was especially emphasized. Moreover, presented work aimed to elucidate the role of platelet-derived chemokines in the vasculature with the focus on vascular smooth muscle cells (VSMCs) and endothelial cells (ECs). Finally, the role of steatotic hepatocyte-derived EVs in modulating stellate cells was investigated.

**Chapter 1** gives the reader the general overview on the importance of extracellular vesicles, platelets and smooth muscle cells in the process of calcification of blood vessels that occurs in various pathological states, including atherosclerosis.

**Chapter 2** addresses the importance of extracellular vesicles derived from platelets and smooth muscle cells in the initiation and propagation of vascular calcification. The involvement of perivascular mesenchymal stem cell (MSC)-like cells in this process is also addressed.

**Chapter 3** explains the importance of calcification in the initiation of atherogenesis. Moreover, the mechanisms of calcification on a cellular level and the concept of reversing calcium/phosphate crystals from the vasculature is discussed.

**Chapter 4** emphasizes the role of platelet-derived chemokines (CXCl4/CXCL4L1) in vascular remodeling and calcification *in vitro*. In this experimental work various cell-based assays were used to elucidate the functional differences between CXCL4 and CXCL4L1 in their ability to modulate human VSMCs. The proliferation, gene expression of selected contractile markers, transcription factors and cytokines were investigated. Also, the role of CXCL4/CXCL4L1 in initiation of calcification of VSMCs as well as their receptors playing a role in endocytosis were examined. Overall, CXCL4 and CXCL4L1 interacted differently with VSMCs and may thus play distinct roles during vascular remodeling.

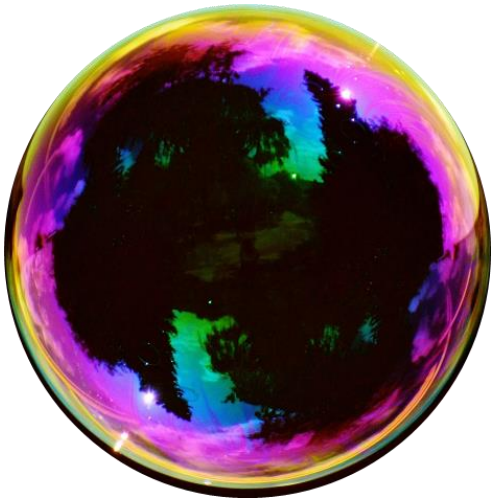
**Chapter 5** describes the fate of CCL5 and CXCL4 after deposition on endothelial cells. Chemokine uptake was analyzed by microscopy and by ELISA. Intracellular calcium signaling and monocyte arrest was evaluated under laminar flow upon chemokine treatment. The mechanism of endocytosis of CCL5 and CXCL4 was investigated as well as the accumulation of chemokines in various cell organelle fractions. Overall, even though CCL5 and CXCL4 treatment does not affect monocyte adhesion to endothelium, they are rapidly and actively internalized by ECs where they are directed to the nucleus.

**Chapter 6** investigates the interplay between hepatocytes and hepatic stellate cells under normal and steatotic conditions and the role of EVs in this process. Migration of the stellate cell line TWNT4 towards control or steatotic EV as well as sera of NASH patients was investigated. TWNT4 phenotype alterations after incubation with EV was determined by qPCR, western blotting and immunofluorescence staining. Overall, EVs from steatotic HepG2 cells can influence the behavior and phenotype of TWNT4 cells as well as the expression of remodeling markers and guide directed migration.

Finally, the results are critically discussed and translated to a clinically relevant context in **Chapter 7.**

# Appendix

Valorisation



## Appendix

### Valorisation

According to the Dutch Research Council 'Valorisation is the process of creating value from knowledge, by making knowledge suitable and available for societal or economic application and by transforming it into products, services, processes and new business.' To put it simply, valorisation is a process in which interaction between theory and practice plays an important role. There is another very valid reason to valorise. The research is financed with public funding. It is therefore logical that researchers share their results with their financers, such as taxpayers (commit-nl.nl). The work presented in this thesis aimed to investigate fundamental basic processes such as cellular communication in the clinically relevant context of cardiovascular diseases (CVDs). It is estimated that 17.9 million people die each year of CVDs what accounts for 32% of all deaths worldwide (WHO; June, 2021). The underlying cause of CVDs is atherosclerosis, a disease that is characterized by a slowly progressing lesion formation of the arterial wall. These slowly growing plaques expand gradually, tend to calcify and are prone to rupture. This may lead to a complete occlusion of the vessel, resulting in myocardial infarction or stroke[1]. The basic research, focused on understanding the mechanisms of CVD is important and can help to develop new treatment strategies and may lead to an earlier detection. Most clinical tools that are currently available diagnose at a late stage i.e. vascular calcification is diagnosed overdue, when only treatment is focused on resolving the symptoms, rather than curing the disease.

Platelets are usually known as the key players in thrombosis and hemostasis and their pro-inflammatory role in the vasculature is understudied. In this thesis the role of three platelet-derived chemokines in the vessel wall remodeling (CXCL4, CXCL4L1 and CCL5) was investigated. During atherosclerosis CXCL4 interacts with VSMCs, endothelial cells and leukocytes. In this thesis we show the functional differences between two structurally similar variants of platelet factor 4, which is often neglected in the literature, therefore expanding our knowledge on how platelet-derived molecules modulate the cells in the vasculature. Also, we hypothesize that CXCL4 neutralization could potentially hamper or even decrease calcification of the blood vessels, as indicated by our results, but this aspect of our research needs to be more thoroughly investigated. However, it is important to remember that such interventions may lead to thrombosis or bleeding, since most probably they would affect platelet function as well. Besides VSMCs, CXCL4 interacts also with studied in this thesis endothelial cells (ECs), which are important players in the development of atherosclerosis. It has been shown that CXCL4 may play a pro-atherogenic role by interacting with endothelial cells and monocytes[2] and its neutralization inhibits neutrophil extracellular traps (NETs) formation in ANCA-associated vasculitis[3].

The other investigated chemokine – CCL5 (RANTES) is a model chemokine of relevance to a myriad of diseases. CCL5 can have detrimental effects via the recruitment of immune cells that enhance inflammatory processes[4]. Studies in humans and murine models have demonstrated a central role of CCL5 in the formation of atherosclerotic plaque[5]. In this thesis we showed that endothelial cells rapidly and actively internalize CXCL4 and CCL5 and observed the interdependency between the uptake of CXCL4 and CCL5, which may imply the existence of overlapping pathways for both chemokines in ECs. The investigation of the mechanism of endocytosis, involvement of receptors and downstream signalling pathways is the first step to understand the onset of the disease.

It is known that CXCL4 and CCL5 tend to form heteromers that facilitate leukocyte recruitment[6,7,8]. Blocking those interactions attenuates vascular remodeling processes e.g. atherosclerosis, aortic aneurysm, lung injury and myocardial infarction[7,8,9,10,11]. Interestingly, our observations imply that after the uptake into ECs, CCL5 and CXCL4 appear to be directed to the nucleus, and CXCL4 seemed to accumulate in the nucleolus. The significance of these findings is currently unknown, but it is tempting to speculate on an active involvement of these

chemokines in transcriptional processes. Even though the relevance of these results needs to be further characterized, this adds a further dimension to the cell regulating activities of the chemokine system.

The knowledge of platelet-derived chemokines can provide valuable directions for the development of novel strategies, such as anti-chemokine therapy for various medical indications (i.e. cancer). CXCL4 displays potent anti-angiogenic activity that results in diminished tumour growth and metastasis in vivo by inhibition of ECs[2]. In contrast to CXCL4, CCL5 is rather associated with the promotion of cancer (e.g., prostate and breast)[4]. However, experiments in mice showed that neutralization of CXCL4[12] and CCL5[13] may be of therapeutic potential in colorectal cancer treatment. The understanding how CXCL4 and CCL5 modulate ECs seems to be of high clinical relevance, because it might have a direct effect on cancer patients as well.

The chemokine signalling network consists of more than 50 chemokines and 20 chemokine receptors. This network is redundant in nature, implying that some chemokines can bind to several receptors and vice versa[14]. Chemokine receptor antagonists represent an extremely fruitful intervention therapy in the treatment of inflammatory and autoimmune diseases[15]. However, despite a great therapeutic potential, the success rate of the clinical trials is low. Currently only three drugs directed against chemokine system reached the market: (i) plerixafor (cancer treatment); (ii) maraviroc (HIV treatment); and (iii) mogamulizumab (lymphoma treatment). Of note, phase 3 trials are ongoing to evaluate many other promising potent candidates[14]. Therefore, a more detailed understanding of the biology of chemokines and their receptors in homeostasis and disease is clearly required to enable their targeting with greater efficacy.

After successful blocking/neutralization experiments in mice, researchers hypothesize that blocking CCL5 may be of therapeutic benefit for Parkinson's disease[16] and colorectal carcinoma[13]. Blocking CXCL4 protects against chronic liver allograft dysfunction (CLAD) by reducing liver fibrosis[17] and has beneficial effect on alopecia[18] as well as colorectal cancer re-growth[12]. On the other hand, CXCL4 and CCL5 can either suppress or enhance HIV replication, which is dependent on the ability to self-aggregate and bind to glycosaminoglycans[19,20].

In the context of a recent COVID-19 pandemic, CXCL4 and CCL5 gained even more attention. It was recently reported that autoantibodies to CXCL4 contribute to thrombotic thrombocytopenia, which occasionally occurs during COVID-19 or after vaccination. Although the reported incidence remains very low, if left untreated, it can be debilitating or even fatal[21,22,23]. The mechanism of autoantibodies production in COVID-19 resembles the process that occurs in well-characterized heparin-induced thrombocytopenia (HIT). In regards to CCL5, it was recently reported that blocking CCL5-CCR5 interactions may reduce inflammation and viremia levels in critical COVID-19 patients[24]. However, in some circumstances, anti-CCL5 treatment might exert unwanted effect i.e. by exacerbating other viral infections[25].

Thus, it becomes more evident that platelets play an important role in various physiological processes, beyond thrombosis and hemostasis and can be considered as the inflammatory/immune cells as well. Taken together, above mentioned reports highlight the importance of platelet-derived chemokines in various CVD pathologies, immunology, cancer as well as virology.

Additionally, the role of extracellular vesicles (EVs) in the development of liver disease was investigated in this thesis. Non-alcoholic fatty liver hepatitis (NASH) is the main cause of the liver disease in the western world[26] that may lead to fibrosis and cirrhosis[27,28]. During the disease development, stress responses in hepatocytes and Kupffer cells promote the differentiation of hepatic stellate cells[29] that are central in the development of liver fibrosis. Here, we described the role of EVs from steatotic hepatocytes in the modulation of stellate cells. EVs are small cell fragments (up to 1000 nm), carrying a specific cargo that may be transported

by the bloodstream to distal areas of the organism and influence the behaviour of the recipient cells. Our results showed that hepatic cells released more EVs after treatment with fatty acids and that the chemotactic migration of stellate cells was increased specifically towards FA-EVs. Also, prolonged incubation of stellate cells with FA-EVs induced the expression of proliferation markers and a myofibroblast-like phenotype. We propose that EVs are operational intercellular communicators that provoke pro-fibrotic responses in stellate cells. Moreover, while most of the studies investigating the effect of EVs derived from FA-treated hepatocytes focused on macrophages[30,31,32], our study characterized for the first time the consequences of FA-treatment on hepatic stellate cells. These results will help to understand the mechanisms of intercellular communication in the development of NASH and liver fibrosis. Thus, future investigation of this process may lead to novel therapeutic intervention as well as improved diagnostics through analysis of plasma EV number and content.

Recently, the EV field gained considerable interest. The possibility of isolating EVs from different biofluids makes EVs valuable biomarkers. The concept of a “liquid biopsy” emerged in the field. In this context, EVs may serve as circulating biomarkers with a potential role in the detection of the early stages of various diseases[33]. The application of EVs as immunotherapeutics for cancer is promising, especially the use of EVs derived from immune cells, however clinical trials have shown that using immune cell-derived EVs alone is often insufficient to induce an effective immune response in vivo[34]. EVs are also novel candidates for drug delivery systems because of their high bioavailability, exceptional biocompatibility, and low immunogenicity[35]. Mesenchymal stem cell-derived EVs (MSC-EVs) as therapeutics were shown to have beneficial outcomes in a variety of chronic liver disease models[36]. For example, MSC-EVs exerted anti-fibrotic and anti-inflammatory effects in a rat model of chronic liver fibrosis[37] and ameliorated cirrhosis in chronic rat liver injury[38].

Even though EVs have a growing future as biomarkers, drug delivery systems, or therapeutic targets, there remain milestones to be achieved on the path to their clinical application. There are also some technical issues concerning standardization to be resolved. The application of the study of EVs in a daily clinical setting requires suitable technologies and quality controls that could be managed by the hospital itself or delegated to specific facilities. For that, close collaboration between clinicians from hospitals, the biotech industry, and basic researchers is necessary to turn what is currently an idea into a product[39].

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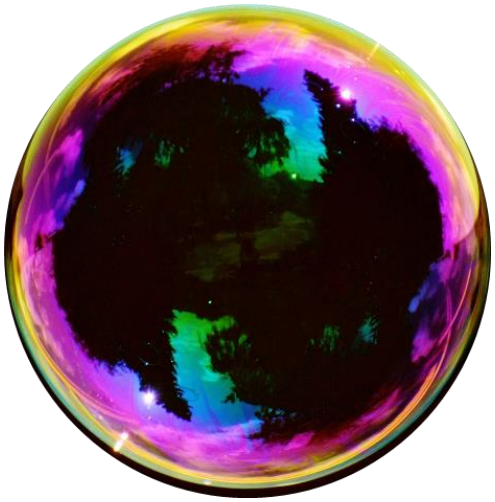
# Appendix

## Curriculum Vitae



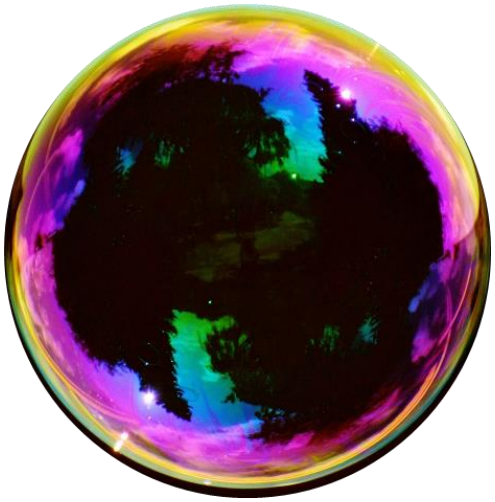
## Appendix

### Curriculum vitae

Dawid Kaczor was born on 13th April 1991 in Krakow, Poland. In 2010 he initiated his Bachelor's degree in Biotechnology at the University of Agriculture in Krakow, Poland. Afterwards he pursued his Master's degree in Applied Biotechnology at the same university and graduated in 2015 with excellent results. During his Master's degree he obtained two Sokrates Erasmus Programme scholarships. One of them resulted in completing one of the semesters at Polytechnic University of Valencia, Spain. The second scholarship resulted in the summer internship at the biotechnological company Bioplis S.L. in Valencia, Spain. After graduation, Dawid was employed at the Jagiellonian Centre for Experimental Therapeutics in Krakow, Poland. His research was focused mainly on interactions of cancer cells with endothelium *in vitro* as well as cancer metastasis *in vivo*. In 2017 he was accepted into a doctoral training programme by the INTRICARE (International Network for Training on Risks of Vascular Intimal Calcification and roads to Regression of Cardiovascular Disease). This Marie-Curie Sklodowska Actions Innovative Training Network PhD position was pursued at the department of Biochemistry at the Cardiovascular Research Institute Maastricht (CARIM) under the supervision of Associate Professor Rory R. Koenen. During the course of his PhD Dawid was a visiting researcher at the Rheinisch-Westfälische Technische Hochschule (RWTH) in Aachen, Germany. He was assigned to the Institute of Experimental Internal Medicine and Systems Biology, where he was supervised by Univ.-Prof. Dr. med. Rafael Kramann. The main aim of his project was to investigate the role of platelet-derived chemokines in vascular remodeling during atherosclerosis.

# Appendix

## Publication list



## Appendix

### Publications with peer review process.

1. L.J Schurgers, A.C. Akbulut, **D.M. Kaczor**, M. Halder, R.R. Koenen, and R. Kramann. 2018. "Initiation and propagation of vascular calcification is regulated by a concert of platelet- and smooth muscle cell-derived extracellular vesicles." Frontiers in Cardiovascular Medicine 5(36). https://doi.org/10.3389/fcvm.2018.00036.

2. Dickhout, **D.M. Kaczor**, A.C.A. Heinzmann, S.L.N. Brouns, J.W.M. Heemskerk, M.A.M. J. van Zandvoort, and R.R. Koenen. 2021. "Rapid internalization and nuclear translocation of CCL5 and CXCL4 in endothelial cells." International Journal of Molecular Sciences 22(14): 7332. https://doi.org/10.3390/IJMS22147332.

3. **D.M. Kaczor**, R. Kramann, T.M. Hackeng, L.J. Schurgers, and R.R. Koenen. 2022. "Differential effects of platelet factor 4 (CXCL4) and its non-allelic variant (CXCL4L1) on cultured human vascular smooth muscle cells." International Journal of Molecular Sciences 23(2): 580. https://doi.org/10.3390/ijms23020580.

4. M.T. Koenen, T. Caspers, A.C.A. Heinzmann, P. Fischer, D. Heinrichs, E.F. Brandt, T. Wirtz, C. Trautwein, R.R. Koenen, **D.M. Kaczor**, and M.L. Berres. "Extracellular vesicles from steatotic hepatocytes provoke pro-fibrotic responses in stellate cells." Biomolecules 2022, 12(5): 698. https://doi.org/10.3390/biom12050698.

### Manuscripts in preparation

1. **D.M. Kaczor**, L. Dai, M.A. Heuschkel, A.C. Akbulut, S. Samal, R. Dzhanaev, S. Reinhold, R.R Koenen, L.J. Schurgers. "Role of vascular calcification in the initiation of atherosclerosis". *In preparation*.

## Published publications not related to this thesis

1. M. Smeda, K. Przyborowski, B. Proniewski, A. Zakrzewska, **D.M. Kaczor**, M. Stojak, E. Buczek, Z. Nieckarz, J.A. Zoladz, J. Wietrzyk, S. Chlopicki. 2017. "Breast cancer pulmonary metastasis is increased in mice undertaking spontaneous physical training in the running wheel; a call for revising beneficial effects of exercise on cancer progression." American Journal of Cancer Research 7(9): 1926–36. http://www.ncbi.nlm.nih.gov/pubmed/28979814.

2. M. Smeda, A. Kieronska, M.G. Adamski, B. Proniewski, M. Sternak, T. Mohaissen, K. Przyborowski, K. Derszniak, **D.M. Kaczor**, M. Stojak, E. Buczek, A. Jasztal, J. Wietrzyk, and S. Chlopicki. 2018. "Nitric oxide deficiency and endothelial-mesenchymal transition of pulmonary endothelium in the progression of 4T1 metastatic breast cancer in mice." Breast Cancer Research 20(1): 86 https://doi.org/10.1186/S13058-018-1013-Z.

3. M. Smeda, A. Kieronska, B. Proniewski, A. Jasztal, A. Selmi, K. Wandzel, A. Zakrzewska, T. Wojcik, K. Przyborowski, K. Derszniak, M. Stojak, **D.M. Kaczor**, E. Buczek, C. Watala, J. Wietrzyk, and S. Chlopicki. 2018. "Dual antiplatelet therapy with clopidogrel and aspirin increases mortality in 4T1 metastatic breast cancer-bearing mice by inducing vascular mimicry in primary tumor." Oncotarget 9(25): 17810–24. https://doi.org/10.18632/ONCOTARGET.24891.

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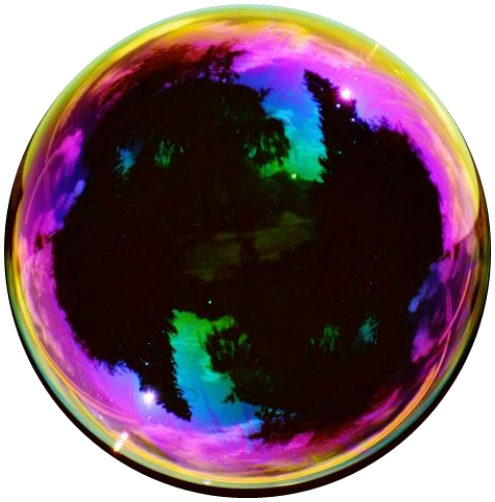
# Appendix

## Acknowledgements



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