CX$_3$CR1/CX$_3$CL1 Axis Mediates Platelet–Leukocyte Adhesion to Arterial Endothelium in Younger Patients with a History of Idiopathic Deep Vein Thrombosis

Elena Furio[1,2,*]   Maria Jose García-Fuster[1,2,*]   Josep Redon[1,2]   Patrice Marques[1,3]   Rebeca Ortega[1,3]
Maria Jesus Sanz[1,3]   Laura Piqueras[1,3]

[1] Department of Pharmacology, Institute of Health Research-INCLIVA, Valencia, Spain
[2] Medicine Unit, University Clinic Hospital of Valencia, Valencia, Spain
[3] Department of Pharmacology, Faculty of Medicine, University of Valencia, Valencia, Spain

Address for correspondence Laura Piqueras, Institute of Health Research–INCLIVA, Department of Pharmacology, Av. Menéndez Pelayo 4, 46010 Valencia, Spain (e-mail: piqueras_lau@gva.es).

Maria-Jesus Sanz, Institute of Health Research INCLIVA, Department Pharmacology, Av. Menéndez Pelayo 4, 46010 Valencia, Spain (e-mail: maria.j.sanz@uv.es).

Abstract

Mechanisms linking deep vein thrombosis (DVT) and subclinical atherosclerosis and risk of cardiovascular events are poorly understood. The aim of this study was to investigate the potential impact of CX$_3$CR1/CX$_3$CL1 axis in DVT-associated endothelial dysfunction. The study included 22 patients (age: 37.5 ± 8.2 years) with a history of idiopathic DVT and without known cardiovascular risk factors and 23 aged-matched control subjects (age: 34 ± 7.8 years). Flow cytometry was used to evaluate peripheral markers of platelet activation, leukocyte immunophenotypes and CX$_3$CR1/CX$_3$CL1 expression in both groups. A flow chamber assay was employed to measure leukocyte arrest under dynamic conditions. Platelet activation and the percentage of circulating CX$_3$CR1-expressing platelets, CX$_3$CR1-expressing platelet-bound monocytes and CD8$^+$ lymphocytes were higher in patients with DVT than in controls. Additionally, patients with DVT had increased plasma levels of CX$_3$CL1, soluble P-selectin and platelet factor 4/CXCL4. Interestingly, this correlated with enhanced platelet–leukocyte interaction and leukocyte adhesion to TNFα-stimulated arterial endothelial cells, which was partly dependent on endothelial CX$_3$CL1 upregulation and increased CX$_3$CR1 expression on platelets, monocytes and lymphocytes. In conclusion, increased CX$_3$CR1 expression on circulating platelets may constitute a prognostic marker for long-term adverse cardiovascular events in patients with DVT. Blockade of CX$_3$CL1/CX$_3$CR1 axis may represent a new therapeutic strategy for the prevention of cardiovascular comorbidities associated with DVT.

Keywords
► deep vein thrombosis
► platelets
► leukocyte recruitment
► chemokines
► endothelial dysfunction

Introduction

Venous and arterial thrombotic disorders have traditionally been viewed as separate pathophysiological entities, in part because of their differential anatomical localization as well as their distinct clinical manifestations.[1,2] This view has been recently challenged by the demonstration of a link between deep vein thrombosis (DVT) and the presence of subclinical atherosclerosis and higher risk of subsequent cardiovascular (CV) events,[3,4] raising the hypothesis that arterial and venous thrombosis share common mechanisms that contribute to endothelial dysfunction, a key pathogenic step in atherogenesis.[5,6]

---

* Both authors contributed equally to this study.

Copyright © 2018 Schattauer

Endothelial dysfunction occurs early in the process of atherogenesis and triggers a proinflammatory and prothrombotic phenotype in the endothelium,[7] provoking the binding and subsequent migration of leukocytes. Indeed, adhesive interactions between leukocytes and endothelium precede leukocyte infiltration into the subendothelial space during early athersclerosis.[7–9] The migration of leukocytes from circulation to sites of extravascular injury is mediated through a sequential cascade of leukocyte–endothelial cell adhesive interactions that involve an array of cell adhesion molecules (CAMs) present on leukocytes and endothelial cells.[10] In addition to CAMs, chemoattractant molecules such as chemokines have the potential to recruit specific cell types and are involved in regulation of leukocyte trafficking.[11] Among them, fractalkine (CX$_3$CL1) is a unique member of the CX$_3$C subfamily that exists both as a soluble form, inducing chemotaxis, and as a membrane-bound form on the surface of inflamed endothelium, promoting cell–cell adhesion; in both cases, this involves activation of its cognate receptor CX$_3$CR1.[12] Interestingly, circulating CX$_3$CL1 has been recently associated with both atherosclerosis[13,14] and diabetes;[15] however, its potential involvement in DVT is unknown.

Platelets are known to play critical roles in DVT and atherosclerosis.[16,17] Platelet tethering and activation results in the secretion of inflammatory cytokines and in the expression of platelet surface P-selectin and other CAMs, which collectively contribute to the inflammatory state of endothelial cells in the vascular wall.[18,19] These local cellular changes lead to the activation of coagulation pathways and the development of DVT.[20] However, studies investigating whether platelet activation is present in the stable phase of DVT in young patients are limited.

In the present study, we tested the hypothesis that platelet activation, circulating levels of fractalkine/CX$_3$CL1 and inflammatory markers of endothelial damage are elevated long term in younger patients with a single episode of idiopathic DVT. The objective of the study was therefore to explore the functional role of CX$_3$CL1/CX$_3$CR1 axis in those patients.

Methods

Study Population

The study conformed to the principles outlined in the Declaration of Helsinki for the use of human subjects. The study protocol was approved by the Ethics Committee of the University Clinic Hospital of Valencia. Written informed consent was obtained from all participants. Reporting of the study conformed to the STROBE statement along with references to STROBE and the broader EQUATOR guidelines.[21]

Consecutive patients (18–50 years of age) admitted to the Internal Medicine Unit at the University Clinic Hospital of Valencia (Spain) between January 2010 and January 2015 with a symptomatic first episode of idiopathic DVT of the lower limbs were potentially eligible for the study. The diagnosis of DVT was confirmed by echo Doppler examination. All patients were treated in the acute phase with low-molecular-weight heparin at a therapeutic dose and after with the vitamin K antagonist dicumarol for 6 months. Exclusion criteria for cases

and controls were the following: (1) transitory risk factors for DVT, for example, <3 months after trauma or surgery, immobilization, hormonal treatment or pregnancy; (2) congenital thrombophilia, or other hypercoagulability states such as antiphospholipid antibody syndrome, hyperhomocysteinaemia, chronic inflammatory diseases or cancer; (3) previous CV events (acute myocardial infarction, coronary heart disease, ischaemic or haemorrhagic stroke, and peripheral artery disease) or CV risk factors such as smoking, arterial hypertension (systolic blood pressure >140 mm Hg and/or diastolic blood pressure >90 mm Hg), diabetes mellitus (plasma glucose level of >126 mg/dL after an overnight fast, or use of antidiabetic drugs), obesity (body mass index >30), hypercholesterolaemia (plasma cholesterol level >200 mg/dL after an overnight fast or use of lipid-lowering drugs); (4) renal disease or advanced renal dysfunction (creatinine clearance <60 mL/min); (5) hepatic insufficiency (Child-Pugh class B or C) or (6) psychiatric disorders that impairs adherence to treatment. The final study group comprised 22 patients and the experimental procedures was performed 1 year after the acute DVT event. A control group of 23 healthy volunteers, age- and sex-matched with the study group, were recruited. The demographic, clinical and laboratory characteristics of patients and controls are shown in ►Table 1.

Laboratory Analyses

Blood was drawn by venipuncture after a fasting period of at least 12 hours. For analyses, whole blood samples were either heparinized or drawn in EDTA or sodium citrate. Serum was centrifuged at 4,000 × g for 10 minutes and frozen at −80°C until analysis. Lipid serum levels (total, HDL and LDL cholesterol, triglycerides), blood glucose, HbA1c, fibrinogen and cellular counts were measured immediately. Soluble P-selectin (sP-selectin) CX$_3$CL1, platelet factor 4 (PF4)/CXCL4 and vascular cell adhesion molecule-1 (VCAM-1) were quantified in plasma by ELISA using DuoSet kits from R&D Systems (Wiesbaden, Germany).

Cell Culture

Human umbilical arterial endothelial cells (HUAECs) and human umbilical vein endothelial cells (HUVECs) were isolated by collagenase treatment as described[22,23] and maintained in human endothelial cell–specific medium (EBM-2) supplemented with endothelial growth medium (EGM-2) and 10% foetal bovine serum (FBS). Cells up to passage 1 were grown to confluence to preserve endothelial features. Cells were incubated for 16 hours in medium containing 1% FBS prior to every experiment.

Flow Cytometry

To assess endothelial expression of CX$_3$CL1, HUAEC and HUVEC were grown to confluence and stimulated with TNFα (20 ng/mL) for 24 hours. Cells were then detached from culture flasks by scraping in ice-cold phosphate buffered saline (PBS) containing 2 mM EDTA and recovered by centrifugation. Washed cells (2 × 10^6 cells/mL) were incubated with a phycoerythrin (PE)-conjugated monoclonal antibody (Ab) against human CX$_3$CL1 (R&D Systems) diluted

Table 1 Demographic and laboratory characteristics in young patients with a previous episode of idiopathic venous thrombosis (DVT) and controls

	Control group ($n = 23$)	DTV subjects ($n = 22$)	p-Value
Age (y)	34 ± 7.8	37.5 ± 8.2	1.41
Gender M/F (%)	7/16 (30.4/69.5%)	6/16 (27.3/72.7%)	0.13
BMI (kg/cm^2)	23.7 ± 3.5	25.41 ± 3.9	0.09
Systolic BP (mm Hg)	106.6 ± 10.3	111.5 ± 10.7	0.12
Diastolic BP (mm Hg)	73.1 ± 7.0	70.18 ± 8.3	0.13
Glucose, mg/dL	90.8 ± 11.2	93 ± 10.1	0.55
Urea, mg/dL	34 ± 8.4	30.5 ± 7.1	0.19
Creatinine, mg/dL	0.73 ± 0.1	0.7 ± 0.2	0.58
Uric acid, mg/dL	4.83 ± 1.5	5.01 ± 1.5	0.71
Cholesterol, mg/dL	187.4 ± 28.8	202 ± 28.5	0.13
Triglycerides, mg/dL	76.2 ± 44.8	114.1 ± 113.3	0.23
Ferritin, ng/mL	63.6 ± 60.0	63.5 ± 68.4	0.99
Microalbuminuria, mg/L	9.11 ± 9.6	27.7 ± 72.7	0.38
Leukocytes count, per mL	6,460 ± 1,371.5	8,185.9 ± 9,815	0.50
Neutrophils (%)	56.5 ± 9.03	59.2 ± 11.5	0.43
Lymphocytes (%)	32.6 ± 7.8	29.06 ± 9.9	0.24
Monocytes (%)	5.8 ± 1.9	5.33 ± 1.9	0.45
Haemoglobin, g/dL	13.6 ± 0.8	13.64 ± 1.4	0.92
Platelet count, × 10^9/L	257 ± 49.8	221 ± 36.4	0.15
ESR, mm	9.13 ± 7.6	11.85 ± 10.6	0.40
PT, s	10.5 ± 0.7	10.7 ± 0.8	0.47
QI, %	99.2 ± 3.0	99.23 ± 2.4	0.96
Fibrinogen, g/L	3.4 ± 0.3	3.58 ± 0.4	0.40
D Dimer, ng/mL	172.7 ± 75.3	189.73 ± 164.7	0.71
HbA1c, %	5.30 ± 0.3	5.2 ± 0.2	0.83
Homocysteine, μmol/L	10.6 ± 5.8	9.9 ± 2.0	0.59
ANA, ratio/quotient	0.78 ± 1.0	0.9 ± 2.0	0.78
TSH, μU/mL	2.51 ± 1.5	2.2 ± 1.2	0.56

Abbreviations: ANA, antinuclear antibodies; BMI, body mass index; BP, blood pressure mean; ESR, erythrocyte sedimentation rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PT, prothrombin time; QI, quick index; TSH, thyroid-stimulating hormone.
Notes: Data are expressed as mean ± SEM. $p < 0.05$ relative to values in the control group.

1:25 in PBS with 3% BSA, for 1 hour at 4°C in the dark. Samples were run in a FACSVerse flow cytometer (BD Biosciences, San Jose, California, United States) and the expression of CX$_3$CL1 (PE fluorescence) was calculated as the mean of fluorescence intensity (MFI).

Platelet activation was measured by P-selectin (CD62P) surface expression. Duplicate samples (6.25 μL) of citrated blood, diluted 1:10 in glucose buffer (1 mg/mL glucose in PBS/0.35% BSA), were incubated for 30 minutes in the dark with a 5-carboxyfluorescein-conjugated Ab against human CD41 (1.25 μL; Immunostep, Salamanca, Spain) and an allophycocyanin-conjugated Ab against human P-selectin (1.25 μL, Immunostep). The CD41$^+$ population (platelets) was selected according to the gating strategy illustrated

in ►**Supplementary Fig. S1** [available online only], and expressed as the percentage of positive platelets.

To determine CX$_3$CR1 expression on platelets, circulating monocytes and lymphocytes, duplicate samples (20 μL) of heparinized whole blood were incubated in the dark for 30 minutes with saturated amounts of a PE-conjugated Ab against human CX$_3$CR1 (R&D Systems) or an PE-conjugated isotype control Ab (BD Biosciences). In some experiments, blood samples were incubated with 10 mM EDTA (15 minutes at 37°C) to promote platelet dissociation, as described.[24] Red blood cells were lysed and leukocytes were fixed using a commercial lysis buffer (BD Pharm Lyse, BD Biosciences). Expression of CX$_3$CR1 (PE fluorescence) was measured on CD41$^+$ (platelets), CD14$^+$ (monocytes) and CD3$^+$ (lymphocytes)

populations according to the gating strategy illustrated in ►**Supplementary Figs. S2** and **S3** [available online only].

All samples were run in a FACSVerse flow cytometer (BD Biosciences) and cell surface expression was calculated as the mean of MFI. All flow cytometry data were analysed with FlowJo v10.0.7 software (FlowJo, Ashland, Oregon, United States).

Leukocyte–Endothelial Cell Interactions under Flow Conditions

A dynamic flow chamber assay was used to examine leukocyte–endothelial cell interactions in vitro using whole blood diluted 1:10 in Hank's buffered salt solution, as described.[25,26] Cells were grown to confluence and stimulated with TNFα (20 ng/mL) for 24 hours. The flow chamber (GlycoTech, Rockville, Maryland, United States) was assembled and placed onto an inverted microscope stage. Diluted blood was then perfused across the endothelial cell monolayers and cell interactions were determined after 5-minute perfusion at 0.5 dyn/cm. Cells interacting on the surface of the endothelium were visualized and recorded in phase contrast (×20 objective, ×10 eyepiece) using a Zeis Axio Observer A1 microscope (Zeis, Thornwood, New York, United States).

In parallel, some plates were incubated with a monoclonal neutralizing antibody against human CX₃CL1 (5 μg/mL) or with an isotype-matched control antibody (MOPC-21, 5 μg/mL) added 10 minutes before blood perfusion. To evaluate the contribution of platelets to leukocyte adhesion, the experiments were performed in heparinized blood incubated or not with 10 mM EDTA (15 minutes at 37°C).

Immunofluorescence Microscopy

To visualize adherent platelet–mononuclear cell complexes to endothelial cells, we performed immunofluorescence analysis after flow chamber assays. Cells were fixed with 4% paraformaldehyde and blocked in PBS containing 1% BSA. Then, cells were incubated at 4°C overnight with a FITC-conjugated antibody against human CD45 (1:50 dilution) and an APC-conjugated antibody against human CD41 (1:50 dilution).

In additional experiments, confluent endothelial cells were grown on glass coverslips and stimulated with TNFα (20 ng/mL) for 24 hours. Cells were then fixed with 4% paraformaldehyde and blocked in PBS containing 1% BSA. Subsequently, cells were incubated at 4°C overnight with a primary goat monoclonal Ab against human CX₃CL1 (1:200 dilution) in 0.1% BSA/PBS, followed by incubation with a fluorescein isothiocyanate-conjugated secondary rabbit anti-goat Ab (1:1,000 dilution) at room temperature for 45 minutes. Cell nuclei were counterstained with 4'-6-diamidino-2-phenylindole. Images were captured with Zeis Axio Observer A1 fluorescence microscope.

Statistical Analysis

Normality of data was analysed using the Shapiro–Wilk test. Normally distributed values are expressed as percentage or mean ± SEM, when appropriate. Non-parametric variables are expressed as median and interquartile range (25th–75th). For comparisons of two groups, Student's t-test was used in data that passed both normality and variance tests; otherwise, the non-parametric Mann–Whitney U-test was performed. For comparisons between multiple groups, one-way analysis of variance followed by *post hoc* analysis (Bonferroni's test) was used in data that passed both normality and variance tests; otherwise, the nonparametric Kruskal–Wallis test followed by Dunn's *post hoc* analysis was used. Data were considered statistically significant at a *p*-value less than 0.05.

Results

Twenty-two patients with idiopathic DVT of the lower limbs (6 males and 16 females, aged 37.5 ± 8.2 years) and 23 healthy control subjects (7 males and 16 females, aged 34 ± 7.8 years) were enrolled in the study. The demographic, clinical and laboratory characteristics of patients and controls are shown in ►**Table 1**. There were no statistically significant differences between the two groups with regards to age, sex and laboratory parameters (►**Table 1**).

Platelet Activation and CX₃CR1 Expression Is Increased in Patients with DVT

We compared platelet activation between the two groups by flow cytometry. We found that platelet P-selectin expression and the percentage of platelets expressing P-selectin was significantly higher in young DVT patients than in controls (►**Fig. 1A**, **B**, $p < 0.05$). Upon cell activation, P-selectin translocates to the cell surface where it can be cleaved and released into blood as sP-selectin. We thus determined circulating
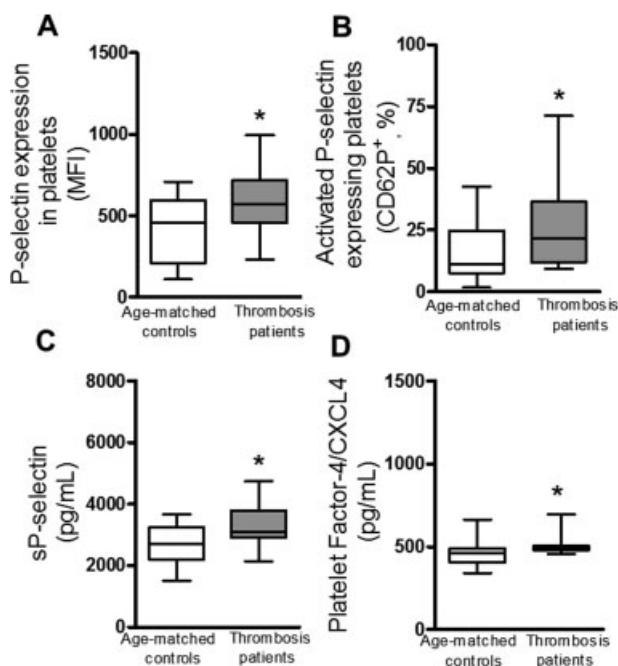

Fig. 1 P-selectin expression in platelets from DVT patients and age-matched controls by flow cytometry. Platelets were stained with conjugated antibodies against CD41 and P-selectin. Results are expressed as (**A**) mean fluorescence intensity and (**B**) percentage of positive cells ($n = 23$ age-matched controls, $n = 22$ DVT patients).*$p < 0.05$ relative to values in the control group. (**C**) Soluble P-selectin and (**D**) PF4/CXCL4 plasma levels were determined by ELISA. Values are expressed as mean ± SEM. *$p < 0.05$ relative to values in the control group. ($n = 23$ age-matched controls, $n = 22$ DVT patients).

levels of sP-selectin in plasma by ELISA, finding that the levels were significantly higher in the DVT group than in the control group ($\blacktriangleright$**Fig. 1C**, $p < 0.05$). Similarly, circulating plasma levels of PF4/CXCL4, a platelet chemokine released upon platelet activation, were significantly higher in young DVT patients than in controls ($\blacktriangleright$**Fig. 1D**, $p < 0.05$). Flow cytometry enabled us to identify platelets using the gating strategy depicted in $\blacktriangleright$**Supplementary Fig S1** [available online only], and we additionally found that the percentage of circulating platelets expressing CX$_3$CR1 receptor was significantly higher in DVT patients than in controls ($\blacktriangleright$**Fig. 2A, B**, $p < 0.05$), which was accompanied by significantly higher plasma-soluble levels of CX$_3$CL1 ($\blacktriangleright$**Fig. 2C**, $p < 0.05$). By contrast, no differences were detected in the plasma levels of VCAM-1 between the two groups ($\blacktriangleright$**Fig. 2D**, $p > 0.05$).

CX$_3$CR1 Expression on Monocytes and Lymphocytes Is Increased in Patients with DVT

Monocytes were identified by flow cytometry using the gating strategy shown in $\blacktriangleright$**Supplementary Fig S2** [available online only]. The percentage of CX$_3$CR1-expressing monocytes was significantly higher in heparinized whole blood from young DVT patients than from age-matched controls ($\blacktriangleright$**Fig. 3A, B**;

$p < 0.05$); however, CX$_3$CR1 expression was lost when platelets were dissociated from this leukocyte subset ($\blacktriangleright$**Fig. 3C, D**). Lymphocytes were also identified using the gating strategy depicted in $\blacktriangleright$**Supplementary Fig. S3** [available online only], and we observed that CX$_3$CR1 expression was also greater in lymphocytes from DVT patients than from control subjects ($\blacktriangleright$**Fig. 4A, B**; $p < 0.05$), and was comparable to that found in monocytes when platelets were dissociated ($\blacktriangleright$**Fig. 4C, D**).

Circulating Leukocytes of DVT Patients Show Increased Adhesiveness to TNFα-Stimulated HUAEC

To explore the functional consequences of these observations, we examined the involvement of platelet CX$_3$CR1 and CX$_3$CL1-dependent leukocyte–endothelial cell interactions under dynamic flow conditions. First, we evaluated CX$_3$CL1 expression in arterial (HUAEC) and venous (HUVEC) endothelial cells. To do this, HUAEC and HUVEC were stimulated with TNFα (20 ng/mL) for 24 hours to mimic dysfunctional endothelium. Flow cytometry analysis revealed a clear upregulation of CX$_3$CL1 expression in TNF-stimulated HUAEC and HUVEC as compared with vehicle-treated cells ($\blacktriangleright$**Fig. 5A**). These observations were confirmed by immunofluorescence studies ($\blacktriangleright$**Fig. 5B**).

We then perfused heparinized blood from DVT patients and controls across HUAEC and HUVEC monolayers that were stimulated or not with TNFα, and evaluated leukocyte–endothelial cell interactions. When whole blood from DVT patients and their respective controls was perfused
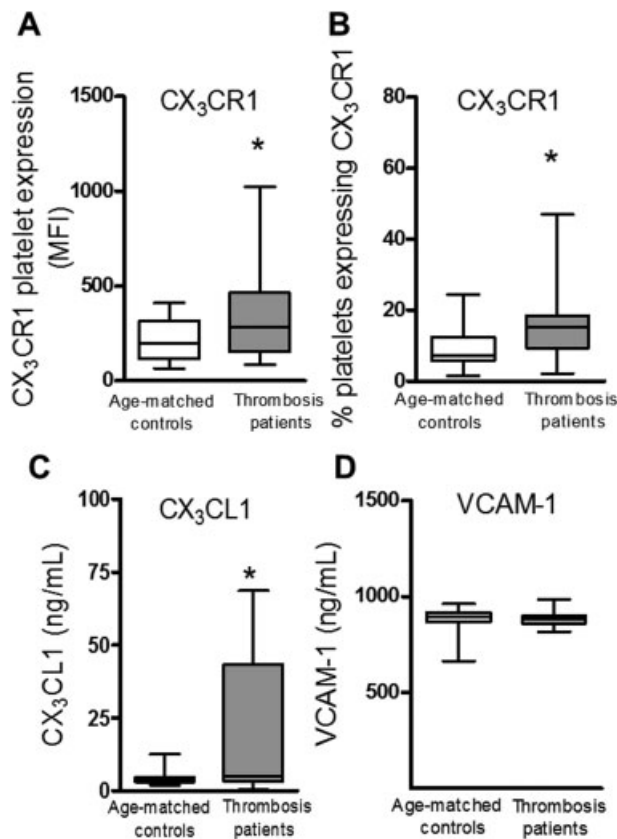

Fig. 2 CX$_3$CL1 receptor expression (CX$_3$CR1) in platelets from DVT patients and age-matched controls by flow cytometry. Heparinized whole blood was co-stained with specific markers for CD41 and CX$_3$CR1. Results are expressed as (**A**) mean fluorescence intensity and (**B**) percentage of positive cells ($n = 23$ age-matched controls, $n = 22$ DVT patients). (**C**) CX$_3$CL1 and (**D**) VCAM-1 plasma levels were measured by ELISA ($n = 23$ age-matched controls, $n = 22$ DVT patients). $^*p < 0.05$ relative to values in the control group.
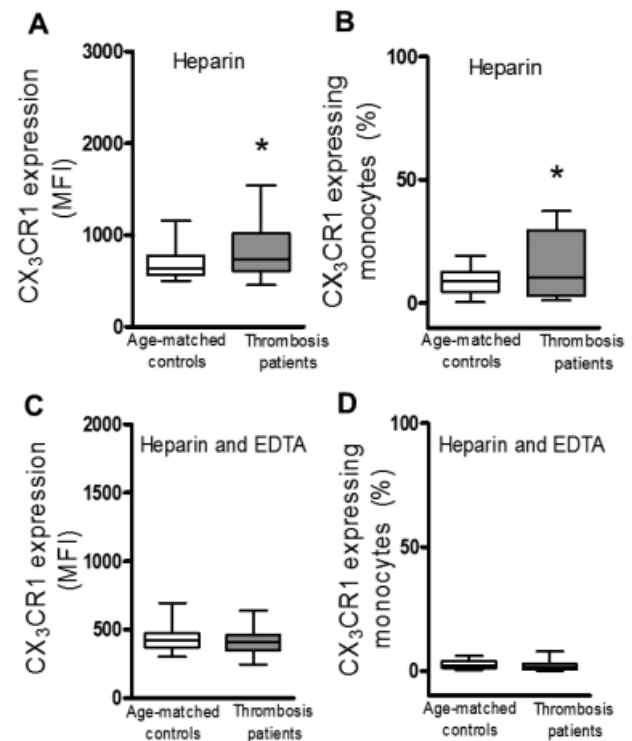

Fig. 3 CX$_3$CL1 receptor expression (CX$_3$CR1) in circulating monocytes from DVT patients and age-matched controls. Heparinized whole blood was co-stained with specific markers for monocytes and CX$_3$CR1. Blood samples were incubated or not with EDTA. Results are expressed as (**A, C**) mean fluorescence intensity (MFI) and (**B, D**) percentage of positive cells ($n = 23$ age–matched controls, $n = 22$ DVT patients). $^*p < 0.05$ relative to values in the control group.
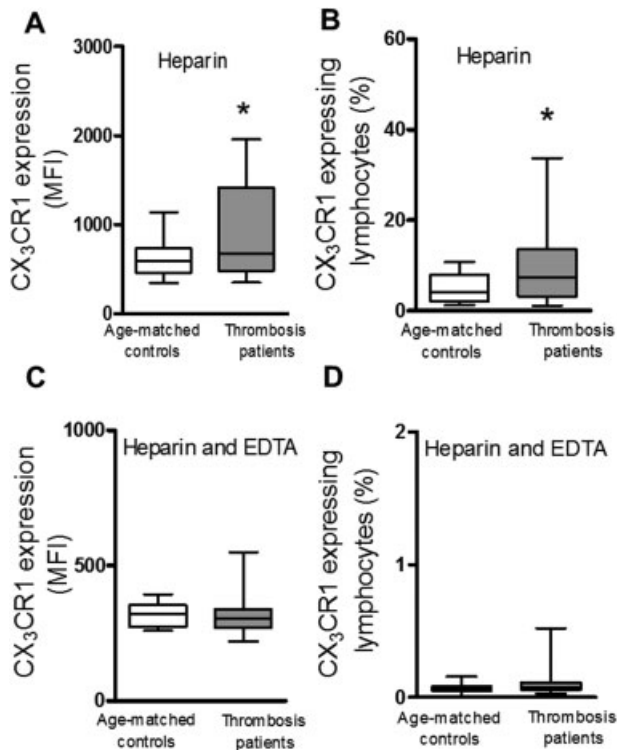
Fig. 4 CX₃CL1 receptor expression (CX₃CR1) in circulating lymphocytes from DVT patients and age-matched controls. Heparinized whole blood was co-stained with specific markers for lymphocytes and CX₃CR1. Blood samples were incubated or not with EDTA. Results are expressed as (**A**, **C**) mean fluorescence intensity (MFI) and (**B**, **D**) percentage of positive cells ($n = 23$ age-matched controls, $n = 22$ DVT patients). $^*p < 0.05$ relative to values in the control group.

across TNFα-stimulated HUAEC, we found a significant increase in leukocyte adhesion in both groups, which was more marked using blood from the DVT group (►**Fig. 6A**, $p < 0.05$). By contrast, no difference between the two groups was found when the experiment was repeated using HUVEC (►**Fig. 6B**). Neutralization of CX₃CL1 activity on endothelial cell surface resulted in a significant reduction of TNFα-induced leukocyte adhesion to HUAEC (45%), but not to HUVEC in the DVT group (►**Fig. 6A**, **B**). Of note, when platelets were disaggregated from leukocytes with EDTA, leukocyte adhesion to both HUAEC and HUVEC significantly diminished in the DVT group (►**Fig. 6C**, **D**). In this case, neutralization of CX₃CL1 activity failed to affect leukocyte adhesion to the dysfunctional endothelium (►**Fig. 6C**, **D**). To corroborate these findings, we investigated adherent platelet–mononuclear cell complexes with endothelial cells by immunofluorescence using platelet marker staining. Confirming our previous observations, when platelets were disaggregated from leukocytes with EDTA, leukocyte adhesion to HUAEC was notably diminished (►**Supplementary Fig. S4** [available online only]).

Discussion

Several studies have reported an association between atherosclerosis and venous thromboembolism. Patients with idiopathic venous thromboembolism have a higher risk of developing symptomatic atherosclerosis than do patients with the secondary form of the disease[3,24] or the general population.[4] There is increasing evidence that inflammatory processes and DVT are intimately linked, and recent data have provided new insights into the inflammatory mechanisms involved in the acute stage of DVT.[27] However, less is known about the inflammatory status in the chronic phase after the primary event of DVT in young patients and in the absence of CV risk factors. Here, we provide the first evidence that young patients with a previous idiopathic DVT have a low-grade systemic inflammation even in the absence of CV risk factors. Accordingly, patients had greater numbers of activated circulating platelets with increased surface expression of P-selectin than controls, and this was accompanied by a marked increase in plasma levels of sP-selectin, PF4/CXCL4 and CX₃CL1. This activated status correlated with increased platelet–leukocyte and leukocyte adhesion to TNFα-stimulated human arterial endothelium, which was partly dependent on arterial CX₃CL1 upregulation and increased CX₃CR1 expression on platelet–leukocyte aggregates in blood from patients with DVT.

Platelets are implicated as key mediators in the pathogenesis of DVT.[28–30] Correspondingly, activated platelets can mediate the endothelial adhesion of circulating leukocytes, a feature typical of dysfunctional endothelium that precedes atherogenesis.[7] Upon activation, platelets express specific CAMs such as P-selectin, and release several inflammatory chemokines including PF4/CXCL4.[31] P-selectin interacts with its receptor P-selectin glycoprotein ligand-1 present on leukocytes and endothelial cells, and mediates leukocyte recruitment to sites of inflammation. In turn, leukocytes become activated and can release other factors that stimulate platelets to secrete more inflammatory mediators.[31] Dysfunctional endothelium can also express P-selectin, which is preformed and stored in the Weibel-Palade bodies of endothelial cells.[31] An increase in the expression of P-selectin and platelet activation in patients with DVT in the acute phase has been reported previously.[32] The present study thus indicates that residual platelet activation persists in patients with previous DVT.

Fractalkine/CX₃CL1 is a proinflammatory chemokine expressed in several cell types including immune and endothelial cells, and both experimental models and human studies suggest that it may contribute to atherogenesis by affecting leukocyte adhesion and migration. Proteolytic cleavage of the CX₃CL1 mucin stalk close to the junction with the transmembrane domain produces a soluble form of CX₃CL1 that is a potent chemoattractant for monocytes and T cells, but not for neutrophils,[33] and which can interact with leukocytes expressing its receptor CX₃CR1. The ability of fractalkine/CX₃CL1 to both attract and arrest blood monocytes and lymphocytes, as well as its presence in vascular wall cells, makes this chemokine an obvious candidate to play a pivotal role in atherosclerotic lesion formation. Against this background, some studies using CX₃CR1⁻/⁻apoE⁻/⁻ or CX₃CR1⁻/⁻LDLr⁻/⁻ mice have associated the substantial decrease in macrophage infiltration within the arterial wall with the marked reduction in atherosclerosis development,
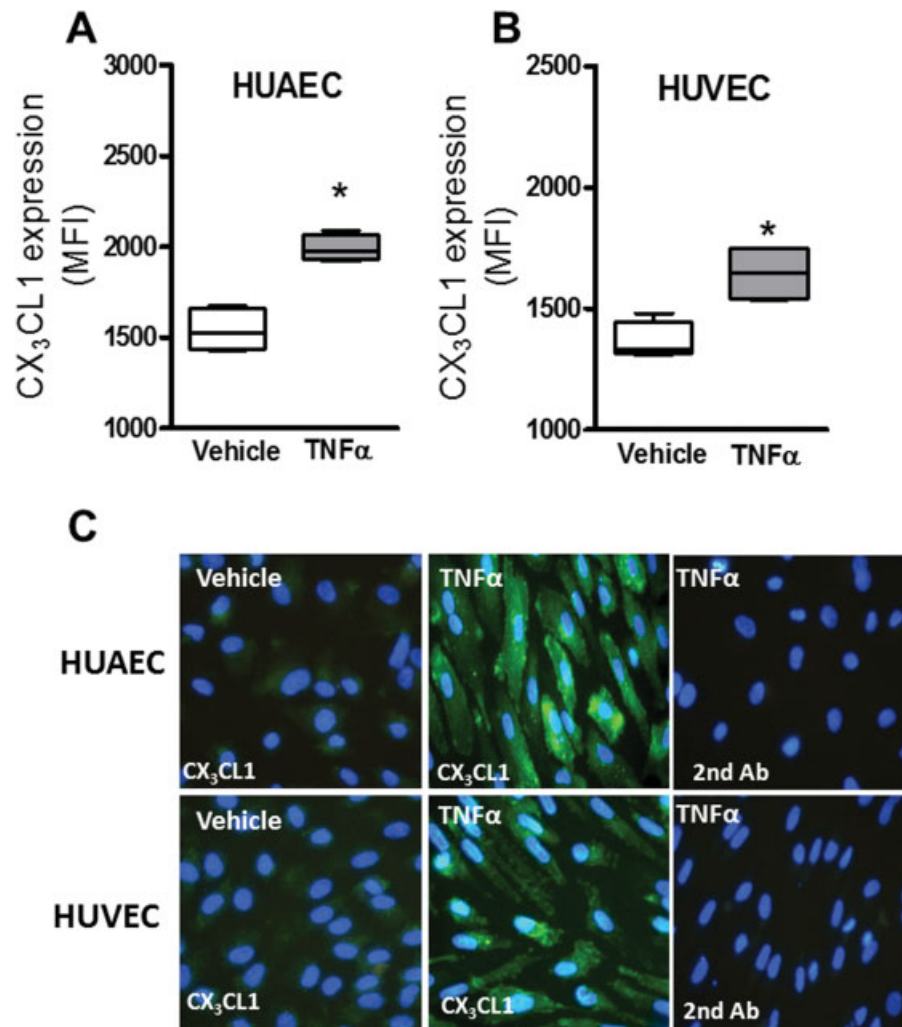
Fig. 5 TNFα induces CX₃CL1 expression in human umbilical arterial endothelial cell (HUAEC) and human umbilical vein endothelial cell (HUVEC). (A, B) Protein expression was determined by flow cytometry. HUAEC and HUVEC were stimulated with TNFα (20 ng/mL) for 24 hours. Results (mean ± SEM of $n = 4$ independent experiments) are expressed as mean fluorescence intensity (MFI). $^*p < 0.05$ relative to values in the vehicle-treated group. (C) CX₃CL1 expression was visualized in HUAEC and HUVEC by immunofluorescence. Nuclei were counterstained with DAPI. Results are representative of three independent experiments.

suggesting a relevant role for the CX₃CL1/CX₃CR1 axis in this process.[34,35] Consequently, CX₃CL1/CX₃CR1 axis has emerged as a potential CV biomarker and a target for therapeutic intervention. In line with our findings, upregulation of CX₃CL1 and CX₃CR1 has been associated with plaque rupture in patients with unstable angina pectoris.[36] More recently, in a large cohort of patients from the Chronic Renal Insufficiency Cohort (CRIC) study, Shah et al[15] have showed that circulating CX₃CL1 levels may contribute to both atherosclerosis and diabetes.[15] To our knowledge, our study is the first report showing an association between circulating CX₃CL1 levels in idiopathic DVT in humans and its potential involvement in subsequent CV events.

A clinically relevant finding of our study was that, under dynamic flow conditions, platelet–leukocyte adhesion to TNFα-stimulated HUAEC was significantly more pronounced in the DVT group than in controls, whereas no differences were observed between the two groups when HUVECs were used as endothelium. Our findings also suggest that platelets

are critical for leukocyte adhesion to dysfunctional arterial endothelium, because in their absence no significant adhesion differences were found between the DVT and the control groups. In this regard, platelets induce leukocyte recruitment in multiple inflammatory disorders, a property somehow dissociated from their role in haemostasis.[37] Therefore, in DVT patients, platelet–leukocyte aggregates are likely responsible for the arterial interactions detected since TNFα-induced increased adhesiveness is partly dependent on endothelial CX₃CL1.[38] Although monocytes and lymphocytes express CX₃CR1,[39] in the absence of platelets, CX₃CL1/CX₃CR1 interaction had no functional role. Indeed, previous studies performed by Schäfer et al demonstrated for the first time the expression of CX₃CR1 on platelets and provided experimental evidence for a functional role of the chemokine CX₃CL1 in platelet activation and adhesion.[40] Subsequently, Schulz and colleagues showed that CX₃CL1 expressed on inflamed endothelial cells triggers the adhesion of leukocytes under arterial shear conditions both in vitro and in
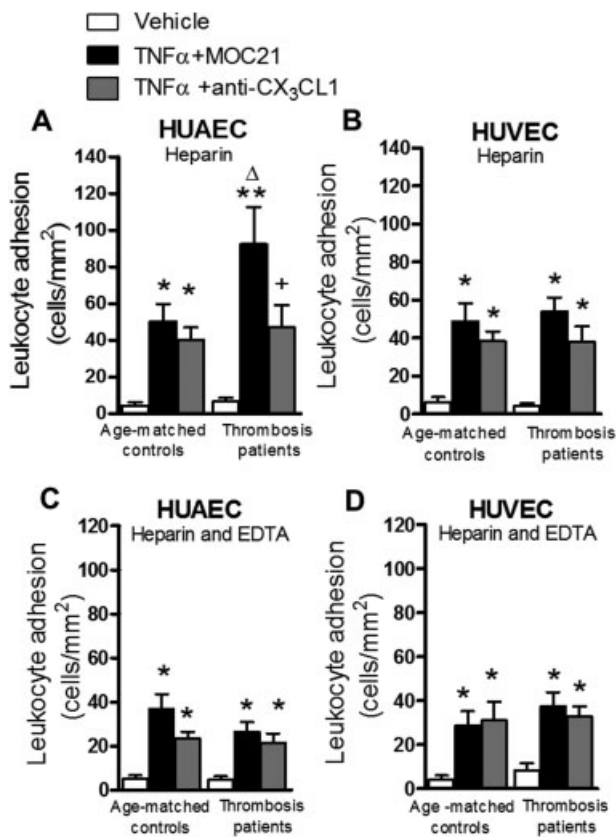
Fig. 6 A neutralizing antibody against CX$_3$CL1 function inhibits the recruitment of leukocytes to TNFα-stimulated human umbilical arterial endothelial cell (HUAEC) but not human umbilical vein endothelial cell (HUVEC). Leukocyte recruitment by TNFα-stimulated HUAEC and HUVEC. HUAEC and HUVEC were stimulated with TNFα (20 ng/mL) for 24 hours. Some cells were incubated with a neutralizing antibody against CX$_3$CL1 function (5 μg/mL) or with the same concentration of an irrelevant isotype-matched monoclonal antibody (MOPC21). Subsequently, whole blood from patients with DVT and healthy age-matched controls incubated (**A**, **B**) without, or (**C**, **D**) with EDTA, was perfused over endothelial monolayers for 5 minutes at 0.5 dyn/cm and leukocyte adhesion quantified ($n = 23$ age-matched controls, $n = 22$ DVT patients). Values are expressed as the mean ± SEM. *$p < 0.05$ or **$p < 0.01$ relative to values in the vehicle-treated cells; $^{+}p < 0.05$ TNFα-stimulated cells in DVT group; $p < 0.05$ relative to values of TNFα-stimulated cells in age-matched control group.

atherosclerotic murine arteries in vivo.[41] In agreement with these observations, it seems that CX$_3$CR1 expressing platelets were strictly required for CX$_3$CL1-induced leukocyte adhesion in our experimental setting. It is also likely that endothelial CX$_3$CL1 may be involved in the activation of surface-adherent platelets, the subsequent platelet degranulation and P-selectin surface expression.

TNFα has several actions linking thrombosis to inflammation, including downregulation of thrombomodulin and stimulation of tissue factor expression.[42,43] Experimental studies have demonstrated that combined neutralization of TNFα and P-selectin reduces inflammation associated with venous thrombosis.[44] Additionally, TNFα is one of the main inducers of CX$_3$CL1 expression.[12] In agreement with these earlier observations, we found an increase in CX$_3$CL1 protein expression in primary arterial and venous endothelial cells stimulated with

TNFα. Moreover, CX$_3$CL1 neutralization reduced the adhesion of CX$_3$CR1+ platelet–leukocyte aggregates to the arterial endothelium, and this effect was more marked in whole blood from DVT patients. Two explanations may be offered for the potential clinical impact of these results. First, an identified mutant form of the CX$_3$CL1 receptor, termed CX$_3$CR1-M280, is defective in mediating adhesive and chemotactic activity,[45,46] and is linked to a lower risk of atherosclerosis, acute coronary events and coronary artery endothelial cell dysfunction.[45,46] Second, CX$_3$CR1 upregulation has been detected in circulating monocytes of patients with coronary artery disease.[39] Given these data, it is feasible that increased CX$_3$CR1 expression/function in circulating mononuclear cells attached to platelets may establish a direct link between DVT and the development of subsequent CV disorders.

In conclusion, young patients with a previous history of idiopathic DVT have increased platelet activation and circulating numbers of CX$_3$CR1+ platelets, monocytes and lymphocyte bound to platelets, a mechanism that can contribute to the higher risk to develop atherosclerosis reported in some studies. Blockade of CX$_3$CR1/CX$_3$CL1 axis in blood from DVT patients reduced both platelet and leukocyte attachment to dysfunctional arterial endothelium. Accordingly, the CX$_3$CL1/CX$_3$CR1 axis may become a powerful tool in the control of the arterial infiltration of mononuclear cells that occur in thrombosis disorders.

What is known about this topic?

- Inflammatory mechanisms are involved in the acute stage of DVT; however, considerably less is known about the inflammatory status in the chronic phase of the condition after the primary event in younger patients.
- The role of CX3CL1/CX3CR1 axis in DVT has not been previously investigated.

What does this paper add?

- CX3CL1 levels and CX3CR1 expression on platelets and mononuclear cells is increased in younger patients with a previous history of idiopathic DVT.
- Platelet–leukocyte and leukocyte adhesion to dysfunctional endothelium depends on endothelial CX3CL1 upregulation.

Authors' Contributions
L.P. and M.J.S. conceived and designed the study. E.F., M.J.G-F. and J.R. recruited and collected the information of patients. E.F., P.M. and R.O. performed the in vitro and ex vivo assays, flow cytometry and ELISA experiments. L.P. and M.J.S. wrote the manuscript. All authors reviewed the manuscript.

Conflict of Interests
None.

References

1 Fahrni J, Husmann M, Gretener SB, Keo HH. Assessing the risk of recurrent venous thromboembolism–a practical approach. Vasc Health Risk Manag 2015;11:451–459

2 Myers DD Jr. Pathophysiology of venous thrombosis. Phlebology 2015;30(1, Suppl):7–13

3 Bova C, Marchiori A, Noto A, et al. Incidence of arterial cardiovascular events in patients with idiopathic venous thromboembolism. A retrospective cohort study. Thromb Haemost 2006;96 (02):132–136

4 Prandoni P, Ghirarduzzi A, Prins MH, et al. Venous thromboembolism and the risk of subsequent symptomatic atherosclerosis. J Thromb Haemost 2006;4(09):1891–1896

5 Rival Y, Bénéteau N, Taillandier T, et al. PPARalpha and PPARdelta activators inhibit cytokine-induced nuclear translocation of NF-kappaB and expression of VCAM-1 in EAhy926 endothelial cells. Eur J Pharmacol 2002;435(2-3):143–151

6 Poredos P, Jezovnik MK. In patients with idiopathic venous thrombosis, interleukin-10 is decreased and related to endothelial dysfunction. Heart Vessels 2011;26(06):596–602

7 Landmesser U, Hornig B, Drexler H. Endothelial function: a critical determinant in atherosclerosis? Circulation 2004;109(21, Suppl 1): II27–II33

8 Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993;362(6423):801–809

9 Galkina E, Ley K. Immune and inflammatory mechanisms of atherosclerosis (*). Annu Rev Immunol 2009;27:165–197

10 Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol 2007;7(09):678–689

11 Baggiolini M. Chemokines in pathology and medicine. J Intern Med 2001;250(02):91–104

12 Ludwig A, Weber C. Transmembrane chemokines: versatile 'special agents' in vascular inflammation. Thromb Haemost 2007;97 (05):694–703

13 Edsfeldt A, Grufman H, Asciutto G, et al. Circulating cytokines reflect the expression of pro-inflammatory cytokines in atherosclerotic plaques. Atherosclerosis 2015;241(02):443–449

14 Zhang X, Feng X, Cai W, et al. Chemokine CX3CL1 and its receptor CX3CR1 are associated with human atherosclerotic lesion vulnerability. Thromb Res 2015;135(06):1147–1153

15 Shah R, Matthews GJ, Shah RY, et al; CRIC Study Investigators. Serum fractalkine (CX3CL1) and cardiovascular outcomes and diabetes: findings from the Chronic Renal Insufficiency Cohort (CRIC) Study. Am J Kidney Dis 2015;66(02):266–273

16 Wolberg AS, Rosendaal FR, Weitz JI, et al. Venous thrombosis. Nat Rev Dis Primers 2015;1:15006

17 Schulz C, Engelmann B, Massberg S. Crossroads of coagulation and innate immunity: the case of deep vein thrombosis. J Thromb Haemost 2013;11(Suppl 1):233–241

18 Bombeli T, Schwartz BR, Harlan JM. Adhesion of activated platelets to endothelial cells: evidence for a GPIIbIIIa-dependent bridging mechanism and novel roles for endothelial intercellular adhesion molecule 1 (ICAM-1), alphavbeta3 integrin, and GPIbalpha. J Exp Med 1998;187(03):329–339

19 Tabuchi A, Kuebler WM. Endothelium-platelet interactions in inflammatory lung disease. Vascul Pharmacol 2008;49(4-6):141–150

20 Xu XR, Zhang D, Oswald BE, et al. Platelets are versatile cells: new discoveries in hemostasis, thrombosis, immune responses, tumor metastasis and beyond. Crit Rev Clin Lab Sci 2016;53(06):409–430

21 Simera I, Moher D, Hoey J, Schulz KF, Altman DG. A catalogue of reporting guidelines for health research. Eur J Clin Invest 2010;40 (01):35–53

22 Martorell S, Hueso L, Gonzalez-Navarro H, Collado A, Sanz MJ, Piqueras L. Vitamin D receptor activation reduces angiotensin-II-induced dissecting abdominal aortic aneurysm in apolipoprotein E-knockout mice. Arterioscler Thromb Vasc Biol 2016;36(08): 1587–1597

23 Escudero P, Navarro A, Ferrando C, et al. Combined treatment with bexarotene and rosuvastatin reduces angiotensin-II-induced abdominal aortic aneurysm in apoE(-/-) mice and angiogenesis. Br J Pharmacol 2015;172(12):2946–2960

24 Postea O, Vasina EM, Cauwenberghs S, et al. Contribution of platelet CX(3)CR1 to platelet-monocyte complex formation and vascular recruitment during hyperlipidemia. Arterioscler Thromb Vasc Biol 2012;32(05):1186–1193

25 Rius C, Piqueras L, González-Navarro H, et al. Arterial and venous endothelia display differential functional fractalkine (CX3CL1) expression by angiotensin-II. Arterioscler Thromb Vasc Biol 2013; 33(01):96–104

26 Sanz MJ, Albertos F, Otero E, Juez M, Morcillo EJ, Piqueras L. Retinoid X receptor agonists impair arterial mononuclear cell recruitment through peroxisome proliferator-activated receptor-γ activation. J Immunol 2012;189(01):411–424

27 Fox EA, Kahn SR. The relationship between inflammation and venous thrombosis. A systematic review of clinical studies. Thromb Haemost 2005;94(02):362–365

28 Brill A, Fuchs TA, Chauhan AK, et al. von Willebrand factor-mediated platelet adhesion is critical for deep vein thrombosis in mouse models. Blood 2011;117(04):1400–1407

29 von Brühl ML, Stark K, Steinhart A, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. J Exp Med 2012;209(04):819–835

30 Key NS. Bench to bedside: new developments in our understanding of the pathophysiology of thrombosis. J Thromb Thrombolysis 2013;35(03):342–345

31 von Hundelshausen P, Weber C. Platelets as immune cells: bridging inflammation and cardiovascular disease. Circ Res 2007;100 (01):27–40

32 Cay N, Ipek A, Gumus M, Birkan Z, Ozmen E. Platelet activity indices in patients with deep vein thrombosis. Clin Appl Thromb Hemost 2012;18(02):206–210

33 Imai T, Hieshima K, Haskell C, et al. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. Cell 1997;91(04): 521–530

34 Lesnik P, Haskell CA, Charo IF. Decreased atherosclerosis in CX3CR1-/- mice reveals a role for fractalkine in atherogenesis. J Clin Invest 2003;111(03):333–340

35 Combadière C, Potteaux S, Gao JL, et al. Decreased atherosclerotic lesion formation in CX3CR1/apolipoprotein E double knockout mice. Circulation 2003;107(07):1009–1016

36 Ikejima H, Imanishi T, Tsujioka H, et al. Upregulation of fractalkine and its receptor, CX3CR1, is associated with coronary plaque rupture in patients with unstable angina pectoris. Circ J 2010;74 (02):337–345

37 Page C, Pitchford S. Neutrophil and platelet complexes and their relevance to neutrophil recruitment and activation. Int Immunopharmacol 2013;17(04):1176–1184

38 Rius C, Company C, Piqueras L, et al. Critical role of fractalkine (CX3CL1) in cigarette smoke-induced mononuclear cell adhesion to the arterial endothelium. Thorax 2013;68(02):177–186

39 Apostolakis S, Krambovitis E, Vlata Z, Kochiadakis GE, Baritaki S, Spandidos DA. CX3CR1 receptor is up-regulated in monocytes of coronary artery diseased patients: impact of pre-inflammatory

stimuli and renin-angiotensin system modulators. Thromb Res 2007;121(03):387–395

40 Schäfer A, Schulz C, Eigenthaler M, et al. Novel role of the membrane-bound chemokine fractalkine in platelet activation and adhesion. Blood 2004;103(02):407–412

41 Schulz C, Schäfer A, Stolla M, et al. Chemokine fractalkine mediates leukocyte recruitment to inflammatory endothelial cells in flowing whole blood: a critical role for P-selectin expressed on activated platelets. Circulation 2007;116(07):764–773

42 Nawroth PP, Stern DM. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. J Exp Med 1986;163(03): 740–745

43 Bevilacqua MP, Pober JS, Majeau GR, Fiers W, Cotran RS, Gimbrone MA Jr. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. Proc Natl Acad Sci U S A 1986;83(12):4533–4537

44 Wakefield TW, Strieter RM, Downing LJ, et al. P-selectin and TNF inhibition reduce venous thrombosis inflammation. J Surg Res 1996;64(01):26–31

45 McDermott DH, Fong AM, Yang Q, et al. Chemokine receptor mutant CX3CR1-M280 has impaired adhesive function and correlates with protection from cardiovascular disease in humans. J Clin Invest 2003;111(08):1241–1250

46 McDermott DH, Halcox JP, Schenke WH, et al. Association between polymorphism in the chemokine receptor CX3CR1 and coronary vascular endothelial dysfunction and atherosclerosis. Circ Res 2001;89(05):401–407