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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

The angiogenic potential of leukocyte- and platelet-rich fibrin

Promotor :
dr. Annelies BRONCKAERS

Copromotor :
Prof. dr. Ivo LAMBRICHTS

Kirsten Berkmans

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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Acknowledgments

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List of abbreviations

Ang-1	angiopoietin 1
bFGF	basic fibroblast growth factor
CAM	chorioallantoic membrane
CM	conditioned medium
EC	endothelial cell
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EX	exudate
FBS	fetal bovine serum
FDA	food and drug administration
GRO	growth-regulated oncogene
HMEC	human microvascular endothelial cell
HUVEC	human umbilical vein endothelial cell
IGF	insulin-like growth factor
IL-8	interleukin-8
IL8RB	interleukin-8 receptor beta
LIF	leukemia inhibitory factor
L-PRF	leukocyte- and platelet-rich fibrin
L-PRP	leukocyte- and platelet-rich plasma
MCP-1	monocyte chemoattractant protein-1
MMP	matrix metalloproteinases
NAP-2	neutrophil activating protein-2
NEDB	non-enzymatic dissociation buffer
NO	nitric oxide
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PPP	platelet-poor plasma
PRFM	platelet-rich fibrin matrix
PRP	platelet-rich plasma
RBC	red blood cell
TGF-β1	transforming growth factor beta-1
TNF-α	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor

Abstract

Introduction: Leukocyte- and platelet-rich fibrin (L-PRF) is an autologous platelet concentrate which promotes tissue healing in clinical applications such as dental surgery and chronic wound healing. However, little is known about its biological properties and mechanisms of action. Angiogenesis, the formation of capillaries from pre-existing blood vessels, is a key process in tissue regeneration. Moreover, platelet-derived growth factors, a supportive fibrin matrix and leukocytes, as present in L-PRF, are known to be important in angiogenesis. The goal of this study was to investigate whether L-PRF induces blood vessel formation and to clarify the angiogenic steps and proteins involved.

Materials and methods: L-PRF was produced by immediate centrifugation of whole blood without the use of any additives. The resulting L-PRF clot was compressed to obtain exudate or placed into medium to generate conditioned medium (CM). The angiogenic cytokine profile of L-PRF CM and exudate was determined by an antibody array. Furthermore, the effect of L-PRF on the human umbilical vein endothelial cells (HUVEC) viability, migration and tube formation was examined *in vitro* by MTT assay, transwell migration assay and tube formation assay. To assess whether the effect of L-PRF on HUVEC was mediated by the receptor CXCR2, these *in vitro* assays were performed using the CXCR2 antagonist SB225002. In addition, surface expression of CXCR2 on HUVEC was verified by flow cytometry. The ability of L-PRF to induce blood vessel formation *in ovo* was assessed by a chicken chorioallantoic membrane (CAM) assay.

Results: The analysis of the angiogenic cytokine profile showed a differential expression of vascular endothelial growth factor (VEGF) between L-PRF CM and exudate. An upregulation of other pro-angiogenic factors, such as GRO, IL-8, ENA-78 and NAP-2, that bind to CXCR2 was observed in CM. L-PRF was able to enhance *in vitro* HUVEC viability, migration and tube formation, although the CXCR2 antagonist SB225002 did not attenuate these effects on HUVEC behavior. Flow cytometry showed that only a small percentage of HUVEC expresses CXCR2. The pro-angiogenic effect of L-PRF was confirmed in the CAM assay.

Discussion & conclusions: L-PRF has been shown to exert potent pro-angiogenic effects *in vitro* and *in ovo.* CXCR2 was not found as a main mediator of this enhancement of angiogenesis by L-PRF, which leaves the angiogenic pathways involved to be elucidated. A better insight in the characteristics and mechanisms of action of L-PRF is required to better understand its clinical effects, interpatient variability and its potential as a new strategy for therapeutic angiogenesis.

Samenvatting

Inleiding: Het bloedplaatjesconcentraat L-PRF bevat fibrine, bloedplaatjes en leukocyten. Dit autoloog bloedplaatjesconcentraat wordt toegepast in onder andere de mondheelkunde en heling van chronische wonden om weefselherstel te bevorderen. Nochtans is er weinig geweten over de biologische kenmerken en werkingsmechanismen van dit plaatjesconcentraat. Angiogenese, de vorming van nieuwe bloedvaten vanuit reeds bestaande bloedvaten, zou een essentiële rol spelen in weefselherstel. Daarenboven zijn groeifactoren afkomstig uit bloedplaatjes, fibrine en leukocyten, zoals aanwezig in L-PRF, belangrijk in het angiogene proces. Het doel van deze studie is dan ook te onderzoeken of L-PRF angiogenese induceert en om te identificeren welke proteïnen en stappen hierbij betrokken zijn.

Materiaal en methoden: L-PRF werd vervaardigd door vers afgenomen bloed onmiddellijk te centrifugeren zonder toevoeging van additieven. De resulterende L-PRF plug kan enerzijds uitgeperst worden om exsudaat te bekomen of anderzijds in medium gebracht worden om, na incubatie, geconditioneerd medium (CM) te verkrijgen. Het angiogeen cytokineprofiel van L-PRF CM en exsudaat werd bepaald. Verder werd het effect van L-PRF op de proliferatie, migratie en tubuli vorming van endotheelcellen afkomstig van navelstrengbloedvaten (HUVEC) *in vitro* onderzocht. Om na te gaan of het effect van L-PRF op deze endotheelcellen door de receptor CXCR2 gemedieerd werd, werden de *in vitro* experimenten herhaald met het gebruik van de CXCR2 antagonist SB225002. De oppervlakte-expressie van CXCR2 op HUVEC werd gecontroleerd aan de hand van flowcytometrie. Ten slotte werd met behulp van een chorioallantoic membrane assay, kortweg CAM-assay, nagegaan of L-PRF in staat is om bloedvatvorming te induceren *in ovo*.

Resultaten: De analyse van het angiogeen cytokineprofiel toonde aan dat er een verschillende expressie van vasculaire endotheliale groeifactor (VEGF) is tussen het CM en exsudaat. Bovendien werd een upregulatie van cytokines die binden aan CXCR2 waargenomen in het CM. De proliferatie, migratie en tubuli vorming van HUVEC *in vitro* werden positief beïnvloed door L-PRF, hoewel de CXCR2 antagonist SB225002 niet in staat was om dit effect op het gedrag van de endotheelcellen af te zwakken. Flowcytometrie toonde aan dat slechts een klein percentage van de endotheelcellen CXCR2 tot expressie brengt. Het pro-angiogeen effect van L-PRF werd bevestigd in de CAM assay.

Discussie en conclusie: De resultaten tonen aan dat L-PRF pro-angiogene eigenschappen vertoond, zowel *in vitro* als *in ovo*. De invloed van L-PRF op angiogenese werd niet hoofdzakelijk gemedieerd door CXCR2. In verdere experimenten moeten de betrokken mechanismen nader onderzocht worden. Een beter inzicht in de werkingsmechanismen van L-PRF zal leiden tot een beter begrip van de klinische effecten, de variabiliteit ervan tussen patiënten en de mogelijkheid om L-PRF als nieuwe aanpak voor therapeutische angiogenese aan te wenden.

1 Introduction

Angiogenesis, the formation of new blood capillaries out of pre-existing blood vessels, is an important biological process that occurs both in health and disease. Blood vessels supply the body's tissues with oxygen and nutrients, remove metabolic waste and provide gateways for immune surveillance. Endothelial cells (ECs), constituting the inner cellular lining of the blood vessels, and surrounding pericytes form the main components of blood vessels. These cells are both embedded in the same basement membrane and play an important role in the angiogenic process (1).

1.1 Physiological and pathological angiogenesis

Angiogenesis occurs in specific physiological conditions, such as in wound healing, in the female reproductive system, namely during ovulation, menstruation and placenta formation, and in the embryo during embryonal organ development. The angiogenic process is strictly regulated by the counteracting actions of many pro- and anti-angiogenic molecules. The angiogenic mediators influence angiogenesis on its multiple, interdependent steps, namely vasodilatation, degradation of the basement membrane and extracellular matrix (ECM), EC activation, proliferation and migration, tube formation (tubulogenesis), blood vessel stabilization and maturation (1, 2).

When angiogenic activators become more abundant than inhibitors, a transient switch towards a pro-angiogenic state occurs. The angiogenic process then proceeds as follows; an angiogenic stimulus, such as hypoxia, will cause the release of pro-angiogenic molecules, such as vascular endothelial growth factor (VEGF), released by cells subjected to hypoxia, and nitric oxide (NO), secreted by ECs (Figure 1). These factors respectively increase the vascular permeability and initiate vasodilatation. This is followed by the detachment of pericytes and proteolytic degradation of the basement membrane and ECM, which is mainly mediated by matrix metalloproteinases (MMP). The matrix degradation leads to the release of more pro-angiogenic growth factors, such as VEGF and basic fibroblast growth factor (bFGF), which were entrapped in the ECM. These factors activate the ECs by binding to their receptors (3). The EC activation is followed by subsequent EC proliferation and migration. During the previously occurred basement membrane degradation and the increased vascular permeability, the extravasation of plasma proteins was enabled. These proteins have formed an ECM scaffold on which ECs can migrate towards their stimulus. The three major stimuli for endothelial migration are VEGF, bFGF, and angiopoietins (4). Attracted by these stimuli, ECs migrate towards the pro-angiogenic signal and form the vascular lumen (5). In this way, a new blood vessel is formed. In the last phase of the process, the maturation and stabilization of the newly formed blood vessel take place. During this phase, ECs return to their resting state and become surrounded by pericytes, which will lead to the deposition of a basement membrane. Angiopoietin 1 (Ang-1), produced by pericytes, will stabilize the blood vessel and tighten the endothelial junctions to decrease the permeability. In order to obtain functional vessels, the maturation involves EC retraction to create a hollow vascular lumen and the blood vessels will

remodel in a branched, functional network (2, 6). In physiological conditions, the balance between angiogenic activators and inhibitors is then re-established (1).

However, when the angiogenic process becomes dysregulated, known as pathological angiogenesis, it contributes to a variety of diseases. Myocardial infarctions, stroke, neurodegeneration and chronic wounds are just a few of the disorders that are characterized by insufficient angiogenesis (1, 7). Efficient angiogenesis also plays a key role in tissue engineering, since the re-establishment of an adequate vascular network is indispensable for the long-term function of an engineered tissue construct (8). Although, nowadays, various approaches appear to be successful in fighting excessive angiogenesis, as occurs in cancer or macular degeneration, this is not the case for promoting new vessel growth (1, 9). To date, no therapeutic angiogenesis treatment has been approved by the Food and Drug administration (FDA). Yet, several approaches are currently under investigation, such as gene and protein delivery (10, 11). However, several drawbacks are associated with the use of gene therapy, and especially with viral vectors, which are often used as delivery systems in gene therapy. Safety concerns, such as immunogenicity and insertional mutagenesis, and ethical considerations form the main disadvantages. In addition, viral vectors also set a limit to the gene size that can be incorporated. Gene therapy therefore mainly focuses on the administration of a single gene or a limited number of genes (12). However, angiogenesis is, as described above, a complex cascade of events controlled by a multitude of pro- and anti-angiogenic molecules. This natural complexity cannot be mimicked adequately by administering only certain genes (11, 13). This last-mentioned issue can be referred to as well when looking into protein-based therapies administering an exogenous angiogenic growth factor, such as bFGF or VEGF.

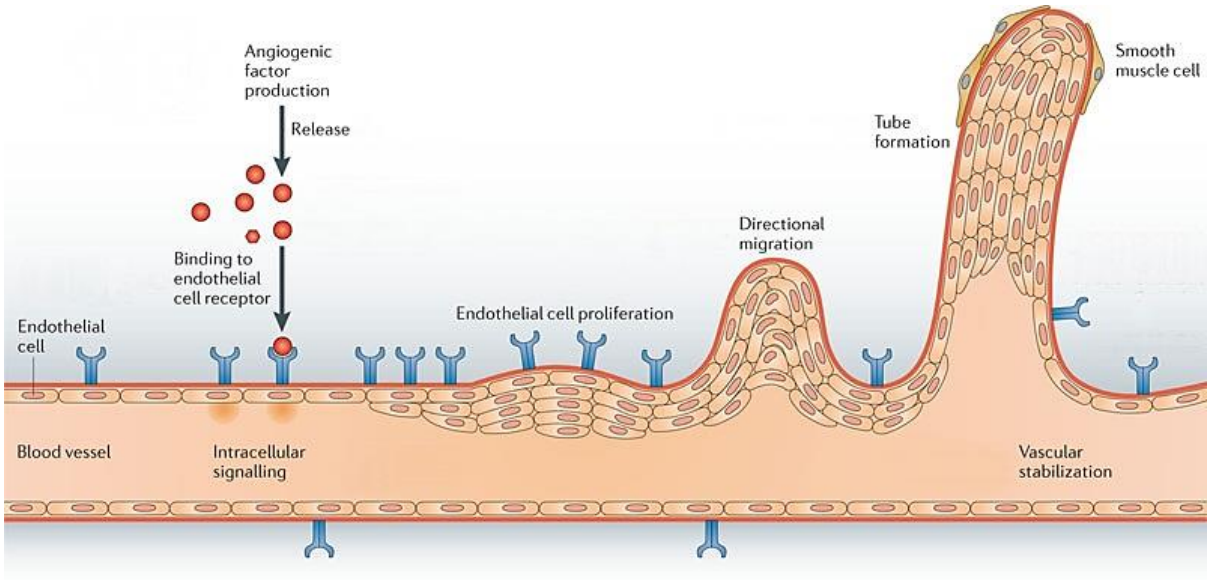

Figure 1: Process of angiogenesis. Angiogenesis is a multistep process, which involves an angiogenic stimulus, the degradation of extracellular matrix, the activation, proliferation and migration of endothelial cells, tube formation, and stabilization and maturation of the newly formed blood vessel. Adapted from Zhang K. *Nature Reviews Drug Discovery* 2012, 11:541-59.

Cell-based therapies could provide a more extensive effect than gene and protein therapy. Stem cells are often used in this field to combine the secretion of pro-angiogenic factors with the capability of differentiating into blood vessel-associated cells. However, viral vectors are, also in this therapy, often used and this to achieve pluripotency of the stem cells (10). The various drawbacks of these therapies establish the need for a new approach to therapeutic angiogenesis. Considering the importance of growth factors, of which many are secreted by platelets (14), autologous platelet concentrates could provide a novel strategy to obtain effective therapeutic angiogenesis.

1.2 Platelet concentrates

Platelet concentrates are blood-derived products that are currently used as bioactive surgical additives in different clinical applications, especially to promote tissue healing. The medical fields in which these concentrates are being applied include chronic ulcer treatment (15), sports medicine and orthopaedics (16), oral or maxillofacial surgery (17) and plastic surgery (18) amongst others. The main advantages related to the use of platelet concentrates are the point-of-care availability, the autologous origin and the related absence of rejection risks (17). However, a variety of platelet concentrates exists. Fibrin glue, the precursor of the platelet concentrates, was already described in 1970 (19). Since then, a range of production techniques to obtain platelet concentrates have been described, all leading to different end products. Consequently, these platelet concentrates differ from one another in their composition, namely the leukocyte content, fibrin architecture and released growth factor, and in their potential applications. However, a classification can be made based on two main categories, namely the first and second generation platelet concentrates (17).

1.2.1 First generation platelet concentrates – Platelet-rich plasma

Platelet-rich plasma (PRP) is considered as the first generation platelet concentrate. However, as stated earlier, the origin of using blood derivatives as bioactive healing agents lies in the fabrication of fibrin sealants. Fibrin sealants, or fibrin glues, are formed as a result of the polymerization of fibrinogen together with thrombin and calcium. Fibrin sealants have been used in clinical applications such as the treatment of burns and oral surgery, for example in sinus floor augmentation and treatment of extraction wounds. However, these fibrin glues were often produced using donor plasma, their stability is low and they do not contain any platelets (19). This has led to the development of the PRP procedure, which is an autologous blood product that combines the positive fibrin properties of fibrin sealants with the presence of platelets. The latter are of interest since they are an important source of growth factors, such as platelet-derived growth factor (PDGF) transforming growth factor beta-1 (TGFβ-1) and VEGF. These growth factors are known to play a role in the stimulation of cell proliferation and angiogenesis (17). The clinical applications of PRP range from the use in oral and maxillofacial surgery, comprising sinus lift procedures, socket preservation and jaw reconstruction surgeries, to the treatment of sports related injuries (19, 20). Although PRP is used in a broad range of clinical applications, there are some drawbacks related to the use of this platelet concentrate. The preparation of PRP consists of a two-step process in which the use of an anticoagulant, bovine thrombin and calcium chloride is

essential. Moreover, numerous different protocols have been described to obtain this platelet concentrate. This leads to various end products that, although prepared in a different way, go under the same name. Furthermore, the obtained PRP gel is weakly polymerized and will, consequently, dissolve quickly (17). To overcome these hurdles, in particular the biochemical handling of the blood product, new preparation methods were described, resulting in the so-called second generation platelet concentrates.

1.2.2 Second generation platelet concentrates - Platelet-rich fibrin

The platelet-rich fibrin concentrates can be divided into two categories, namely pure platelet-rich fibrin (P-PRF), also called leukocyte-poor platelet-rich fibrin, and leukocyte-and platelet-rich fibrin (L-PRF). Due to the absence of bovine thrombin, P-PRF could be considered as a natural platelet concentrate. However, to obtain P-PRF, the use of an anticoagulant (trisodium citrate), a separation gel and calcium chloride are still essential. Therefore, P-PRF does not fully belong to second generation platelet concentrates, characterized by the absence of any biochemical handling (17). Leukocyte- and platelet-rich fibrin (L-PRF) is considered as a second generation platelet concentrate due to its simplified processing and the absence of any biochemical handling (17), the latter making it a completely natural material. L-PRF is obtained by the collection of whole blood in 9 mL dry glass or glass-coated plastic tubes and centrifugation at a low speed (12 minutes at 400g) (21). Platelet activation and fibrin polymerization occur by natural clotting, due to the absence of anticoagulants. This causes the formation of three layers, namely a red blood cell base at the bottom, an acellular plasma as a supernatant and L-PRF, as a dense fibrin clot in the middle of the tube (Figure 2) (22). The L-PRF clot contains the majority of leukocytes and platelets present in the blood sample, which release many growth factors, such as VEGF, TGF-β1, insulin-like growth factor 1 (IGF-1) and PDGF (21), in the fibrin matrix due to the platelet activation. The natural fibrin polymerization leads to a strong but flexible fibrin matrix in which growth factors are embedded and slowly released over more than seven days (17).

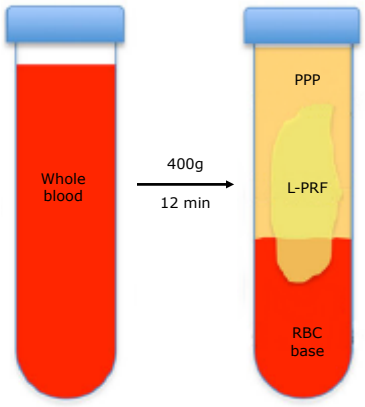

Figure 2: Schematic overview of the L-PRF preparation protocol. Blood is centrifuged at 400g for 12 minutes immediately after collection without addition of anticoagulants. Three layers are formed, namely PPP at the top, L-PRF in the middle and a RBC base at the bottom. L-PRF = leukocyte- and platelet-rich fibrin; PPP = platelet-poor plasma; RBC = red blood cell.

Comparing L-PRF to other platelet concentrates, L-PRF procedure is the most simple, fastest, least expensive protocol developed so far. Furthermore, it is the only completely natural platelet concentrate. The absence of any biochemical handling, however, creates the need to handle immediately and fast after blood collection to avoid diffuse polymerization (17).

For its use in clinical applications, the L-PRF clot can be compressed to obtain a membrane and exudate. L-PRF is nowadays applied in different fields such as oral and maxillofacial surgery (23), ear-nose-throat surgery, implant dentistry and the treatment of chronic diabetic ulcers (24). In these applications, L-PRF is used to enhance hard and soft tissue healing (25). Other potential applications of L-PRF are currently tested, such as the use in esthetic plastic surgery, orthopedic surgery and as a scaffold in tissue engineering (26-28). Although L-PRF is already used in clinical settings, little is known about the mechanisms of action and the intrinsic biology of L-PRF. This more in-depth insight is of importance to better understand its effects on the healing process and its interpatient variability and to broaden its possible applications. Considering future applications of L-PRF, it should be kept in mind that the autologous origin of L-PRF limits its application to the donor himself and cannot be broadened towards an allogenic use. Setting up a L-PRF tissue bank is not a possibility since the fibrin matrix concentrates immune cells and highly antigenic plasmatic molecules (29). However, the simple and fast chairside protocol to produce L-PRF eliminates the need for this kind of storage.

1.3 L-PRF and angiogenesis

Since platelets, leukocytes and fibrin all influence the angiogenic process, the relation between angiogenesis and these different components of L-PRF needs to be considered. Schär *et al.* (2015) already demonstrated that L-PRF and its released growth factors can induce EC migration. Besides these findings, the effect of L-PRF on angiogenesis is not yet further described in literature.

1.3.1 Leukocytes and angiogenesis

Leukocytes have a dual role in the angiogenic process since they can release both angiogenic stimulators and inhibitors. Whether they will initiate or terminate angiogenesis is based on the balance between their released molecules. This balance is influenced by the chemokines present in the environment. Angiogenic chemokines can namely exert a direct role on ECs or an indirect role on angiogenic factor-expressing leukocytes (30). Angiogenic factors such as growth-related oncogene (GRO), interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1) can cause recruitment of leukocytes and PDGF and FGFs are capable of modulating the function of leukocytes. Different types of leukocytes can be involved in the regulation of angiogenesis. Neutrophils contribute to angiogenesis due to their VEGF-A production in physiological conditions (31, 32), while monocytes can mimic endothelial progenitor cells and differentiate in endothelial-like cells (33). Macrophages release PDGF, bFGF and tumor necrosis factor (TNF-α), which are known to stimulate angiogenesis (34, 35).

1.3.2 Platelets and angiogenesis

Platelets play an important role in tissue repair. They are able to secrete over 30 growth factors from their granules which will influence the cells at the site of injury. This effect will be even more pronounced by concentrating the platelets in a platelet-rich fibrin matrix. The platelet-derived growth factors, which are released after platelet activation, become incorporated into the fibrin matrix and will progressively be released from the matrix. Platelets contain, and release, pro-angiogenic factors such as VEGF, PDGF, bFGF, epidermal growth factor (EGF), TGF-β, IGF-1, Ang-1 and MMPs (20). The regulatory role of platelets in the angiogenic process *in vivo* is not yet elucidated, but *in vitro* studies showed that platelets are capable of stimulating EC proliferation and tube formation (36).

1.3.3 Fibrin and angiogenesis

Fibrin, the activated form of fibrinogen, is a soluble molecule that is present in plasma. The formation of a fibrin network is the result of the transformation of fibrinogen into fibrin (37). The architecture of this network is one of the important characteristics of platelet concentrates since it influences the strength of the matrix and therefore its ability to trap growth factors. The absence of bovine thrombin in the preparation of L-PRF leads to the establishment of a fine and flexible fibrin matrix. Such a fibrin matrix allows a slow and progressive release of growth factors (38, 39). Furthermore, the fibrin matrix provides a scaffold for migrating EC and leukocytes and can stimulate the expression of the pro-angiogenic αvβ3 integrin, which functions as an adhesion molecule in the angiogenic process. As a consequence, the fibrin matrix exerts an influence on different steps of angiogenesis (40).

1.3.4 Angiogenic effects of L-PRF

The promising results obtained with L-PRF in clinical applications make the examination of its mechanisms of action an interesting research topic in order to better understand its clinical effects and interpatient variability, which can lead to broadening of its clinical applications. Therefore, this study aims to investigate the angiogenic potential of L-PRF and to clarify the angiogenic steps and proteins involved. The first objective is to identify the secretion profile of L-PRF. Secreted, angiogenic factors will be identified by using both an antibody array that simultaneously detects the levels of 80 human cytokines and an enzyme-linked immunosorbent assay (ELISA). The second objective of the study is to examine the *in vitro* effect of L-PRF on the different steps of the angiogenic process, namely the EC viability, migration and tube formation. The third objective is to determine whether L-PRF is capable of inducing blood vessel formation in an *in ovo* setting. Therefore, the influence of L-PRF on the formation of fully functional blood vessels will be assessed in a chicken chorioallantoic membrane (CAM) assay. The possible angiogenic potential of L-PRF and its earlier mentioned advantages make L-PRF a very promising treatment strategy with a broad range of future applications. In addition to its current applications, its use could be broadened towards other clinical applications in which therapeutic angiogenesis is desirable.

2 Materials and methods

2.1 Culturing of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVEC) (HUVEC-2, Catalog Number 354151, BD, Erembodegem, Belgium) were cultured in endothelial cell growth medium (EBM-2, Lonza, Walkersville, MD, USA) supplemented with growth factors (EGM-2 SingleQuots™ Kit Supplements and Growth Factors, Catalog Number CC-4176, Lonza) and 10% fetal bovine serum (FBS, Gibco, ThermoFisher Scientific, Erembodegem, Belgium) and maintained at 37°C, 5% CO_2. The culture flasks were coated with 1 ng/ml fibronectin (R&D Systems, Oxon, UK) in Milli-Q. The culture medium was changed every three days. When confluent, HUVEC were retrieved from the culture flasks using 0.05% trypsin/EDTA (Sigma, St-Louis, Mo, USA) and sub-cultured for further experiments. For all experiments, HUVEC from passage 6-10 were used.

2.2 Preparation of L-PRF

Blood samples were obtained from 10 healthy male and female volunteers with oral informed consent. The study was conducted in accordance with the Belgian law on human bodily materials of 19 December 2008 and approved by the Medical Ethics Committees Hasselt University and KU Leuven. By means of a venipuncture, blood samples (for each volunteer 8 tubes of 9 ml each) were collected in glass-coated plastic tubes (VACUETTE® 9 ml Z Serum Clot Activator Tubes, Greiner Bio-One, Kremsmünster, Austria) and immediately centrifuged (IntraSpin™ Centrifuge, Intra-Lock, Boca Raton, Florida, USA) for 12 minutes at 2700 rpm (400g) without adding any additives. After centrifugation, the L-PRF clot was removed from the tube using sterile tweezers and separated from the red blood cell base using a straight iris spatula (Iris spatula, Article number 10094-13, Fine Science Tools (FST), Heidelberg, Germany).

2.2.1 Collection of exudate and conditioned medium

To obtain conditioned medium (CM), L-PRF clots were placed in a 6-well plate, each well filled with 6 ml αMEM (Sigma) 0% FBS supplemented with 2 mM L-glutamine (Sigma), 100 U/ml Penicillin (Sigma) and 100 µg/ml Streptomycin (Sigma). After 48h, 96h or 144h the medium was collected, centrifuged for 6 minutes at 300g and filtered (Filtropur S 0.2, Sarstedt, Nümbecht, Germany). To collect exudate, L-PRF clots were transferred to a sterile box (Xpression™ Fabrication Box, Intra-Lock). The weighted press of the box transformed the L -PRF clot into a thin membrane and the exudate was released from the clot during this process. The exudate was collected and filtered (Filtropur S 0.2).

2.3 Enzyme-Linked Immunosorbent Assay (ELISA)

An ELISA was performed on L-PRF CM (48h, 96h and 144h) and exudate of four different donors to determine the concentration of VEGF (RayBio® Human VEGF-A ELISA, Catalog Number ELH-VEGF, Raybiotech, Boechout, Belgium). The ELISA kit was used according to the manufacturers'

guidelines. The absorbance was measured at a wavelength of 450 nm using a Benchmark microplate reader (Bio-Rad Laboratories, Nazareth Eke, Belgium).

2.4 Human Cytokine Antibody Array

To simultaneously detect the relative expressions of factors secreted by L-PRF, a Human Cytokine Antibody Array (ab133998, Abcam, Cambridge,UK) was performed. L-PRF CM and exudate of two different donors were used at a protein concentration of 10 mg/ml, previously determined by the BCA method (Pierce™ BCA Protein Assay Kit, Catalog number 23225, Thermo Scientific, Erembodegem, Belgium). The BCA kit was used following the manufacturers' instructions to determine the total protein concentration in each CM and exudate. The cytokine antibody array was performed in accordance with the manufacturers' guidelines. Briefly, membranes were blocked with 1X blocking buffer for 30 minutes at room temperature and afterwards incubated overnight at 4°C with CM or exudate. Following several wash steps, an 1X biotin-conjugated anti-cytokines solution was added to the membranes the next day for one hour at room temperature. After repeated washing, membranes were incubated for two hours with HRP-conjugated streptavidin at room temperature. Afterwards, a mixture of detection buffers was added to the membranes for two minutes, preceded by multiple wash steps. Immediately afterwards, pictures were taken (Image Quant LAS 4000 mini, GE Healthcare Life Sciences, Diegem, Belgium) with exposure times between four and ten seconds. Quantification was performed using ImageQuant™ TL software (GE Healthcare Life Sciences).

2.5 Cell viability assay

The effect of L-PRF CM and exudate on the EC viability was determined by a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HUVEC were seeded in a flat bottom 96-well plate at a cell density of 29412 cells per cm^2 in endothelial cell growth medium supplemented with growth factors and 10% FBS. The next day, cells were rinsed with phosphate buffered saline (PBS) and different conditions were added, namely 100% L-PRF CM, 50% L-PRF CM, 1%, 3% or 10% exudate. All dilutions were made using αMEM 0% FBS. As a negative and positive control, respectively, αMEM 0% FBS and αMEM supplemented with 10% FBS were used. To examine whether the effect of L-PRF on EC viability was mediated by the CXCR2 pathway, 100 nM of the selective CXCR2 antagonist SB225002 (Selleckchem, Munich, Germany) was added to different L-PRF conditions (50% CM, 100% CM and 3% exudate) and HUVEC were pre-incubated with 100 nM SB225002 for 15 minutes to ensure effective blocking. CM and exudate of 10 different donors in total were used. After 48h of incubation at 37°C, media were removed and 500 µg/ml MTT (Sigma) in αMEM 0% FBS was added to each well. After 4h of incubation at 37°C, the MTT solution was removed and a mixture of 0,1 M glycine and DMSO (Sigma) was added to each well. The absorbance was measured at a wavelength of 570 nm with a Benchmark microplate reader (Bio-Rad Laboratories).

To test the efficacy of the blocker, the same experiment was performed with IL-8. HUVEC were incubated for 48h with αMEM 0% FBS containing different concentrations of IL-8 (1, 10 and 100

ng/ml), whether or not supplemented with SB225002 (100 nM). For the SB225002 conditions, HUVEC were pre-incubated with 100 nM SB225002 for 15 minutes.

2.6 Transwell migration assay

To evaluate the effect of L-PRF on HUVEC migration, a transwell migration assay was performed. Therefore, HUVEC were seeded on Thincert™ tissue culture inserts (24 well, pore size 8 µm; Greiner Bio-One) in αMEM 0% FBS at a density of 223214 cells per cm² (upper compartment). The inserts were placed in wells, containing 100% L-PRF CM, 50% L-PRF CM, 10%, 3% or 1% L-PRF exudate in αMEM 0% FBS (lower compartment). Wells filled with αMEM 0% FBS or αMEM 10% FBS were used as a negative or positive control, respectively. To test whether or not the effect of L-PRF CM on HUVEC migratory activity was mediated by CXCR2, HUVEC were pre-incubated with 100 nM SB225002 for 15 minutes and the mixture was then seeded in inserts, while wells were filled with CM 50% or CM 100%. CM and exudate of 10 donors in total were tested. After 24h of incubation at 37°C and 5% CO_2, wells and inserts were emptied and washed with PBS. Migration was stopped by removing non-migrated cells on the top of the insert with a cotton swab. The transmigrated cells, attached to the lower surface of the upper compartment, were detached and stained by, respectively, non-enzymatic dissociation buffer (NEDB, Sigma) containing 1,667 mM calcein-acetoxymethyl (calcein-AM) (BD). After one hour of incubation at 37°C, the ThinCert™ cell culture inserts were discarded and the buffer containing the transmigrated cells was transferred to a flat-bottom black 96 well plate. Next, fluorescence was measured using a fluorescence plate reader (Fluostar Optima, BMG Labtech, Germany) at an excitation wavelength of 495 nm and an emission wavelength of 515 nm.

In a second experiment, it was examined whether IL-8 influences HUVEC migration and whether SB225002 can change this effect. The transwell migration assay as described above was performed with IL-8. Wells were filled with αMEM 0% FBS containing different concentrations of IL-8 (1, 10 and 100 ng/ml). HUVEC were seeded in inserts in αMEM 0% FBS without or with SB225002 (100 nM), the latter preceded by 15 minutes incubation of HUVEC with 100 nM SB225002.

2.7 Tube formation assay assay

In order to examine whether L-PRF influences tubulogenesis, a tube formation assay was performed. Therefore, a growth factor-reduced, Phenol Red-free Matrigel™ Basement Membrane Matrix (Corning, Bedford, MA, USA) was added to wells of an Angiogenesis µ-slide (Ibidi GmbH, Planegg/Martinsried, Germany). 80,000 HUVEC per cm² were seeded on the solidified matrix in 100% L-PRF CM, αMEM 0% FBS containing 50% L-PRF CM or 1% L-PRF exudate, αMEM 0% FBS (negative control) or αMEM 10% FBS (positive control). To test the involvement of the CXCR2 pathway, HUVEC were pre-incubated with 100 nM SB225002 for 15 minutes and 100 nM SB225002 was added to the L-PRF CM conditions (50% and 100% CM). CM and exudate derived from 10 different donors in total were tested and each condition was measured in duplicate. After 6h of incubation at 37°C and 5% CO_2, 2 pictures per well were taken at a magnification of 4x with a Nikon eclipse TS100 inverted microscope with a relay lens (Nikon Microscope DXM Relay Lens

MQD42070) and equipped with a Jenoptik ProgRes C3 camera. ImageJ Angiogenesis Analyzer software was used to determine the number of nodes and total branching length in order to quantify the tube formation.

The effect of IL-8 and SB225002 on tube formation was examined. HUVEC were seeded on Matrigel in αMEM 0% FBS containing IL-8 (1, 10 or 100 ng/ml) with or without SB225002 (100 nM). For the SB225002 conditions, HUVEC were pre-incubated for 15 minutes with SB225002 (100 nM). As a negative and positive control, respectively, αMEM 0% FBS and αMEM 10% FBS were used.

2.8 Flow cytometric analysis

The cell surface expression of CXCR2 on HUVEC was analyzed by flow cytometry using a PE-labeled anti-CXCR2 antibody (PE mouse anti-human CXCR2 antibody, Biolegend, Europe BV, Uithoorn, the Netherlands). HUVEC were labeled according to standard protocols and a matched labeled isotype was used as control. Flow cytometric analysis was performed on a BD FACSCalibur™ System (BD), using BD CellQuest Pro™ Software (BD).

2.9 Chick Chorioallantoic Membrane (CAM) assay

Fertilized chicken eggs (*gallus gallus*) from day 3 of the embryonic development (E3) were obtained to examine the angiogenic effect of L-PRF *in ovo*. At E3, a small hole was created into the eggshell where the air sac is located. 3-4 ml albumin was aspirated to detach the CAM from the eggshell. A window of 1 cm² was made in the egg shell to control the viability of the embryo and resealed afterwards with cellophane tape. The eggs, lying horizontally, were incubated for 6 days at 37°C and 40-60% humidity.

At E9, 50% CM or 50% exudate, derived from eight different donors, was mixed with growth factor-reduced Matrigel™ Basement Membrane Matrix in a total volume of 30 µl. Matrigel droplets containing VEGF (16,67 ng/µl, R&D), FGF-2 (16,67 ng/µl, immunotools, Friesoythe, Germany) or 50% αMEM 10% FBS were used as positive controls, while 50% αMEM 0% FBS in matrigel was used as a negative control. SB225002 (100 nM) was added to matrigel containing 50% L-PRF CM to examine the involvement of the CXCR2 pathway in the *in* ovo effect of L-PRF on angiogenesis. The mixtures were applied on small plastic discs and allowed to solidify for 2 hours at 37°C. The cellophane tape was removed to place the solidified droplets on top of the CAM near a pre-existing blood vessel. The window was resealed with cellophane tape and the eggs were returned to the incubator for three days. At E12, the windows were made bigger and the CAM was excised from the eggs. Pictures of each CAM were taken using a digital camera (Sony HDR-XR350VE handycam camera, Sony corporation, Tokyo, Japan). To assess angiogenesis, all vessels intersecting two concentric circles (with a radius of 0.6 and 0.9 mm) digitally positioned around the matrigel droplets were counted. The quantification was performed in a double-blind fashion and independently by two investigators.

2.10 Statistical analysis

Statistical analysis was performed using Graphpad Prism Version 5.01 (GraphPad Software, CA, USA). Normality of the data was controlled by means of a D'Agostino & Pearson omnibus normality test. In case of a Gaussian distribution, one-way ANOVA followed by Dunnett's multiple comparison test was performed. In case of non-parametric data, comparisons were made using a Kruskal-Wallis test followed by a Dunn's multiple comparison post-hoc test. To observe the difference between paired data, a Friedman test followed by a Dunn's multiple comparison post-hoc test was performed for non-parametric data, while normally distributed, paired data were analyzed with Repeated Measures ANOVA with Bonferroni's multiple comparison test. Differences were considered statistically significant at P-values < 0.05. All data are expressed as mean ± standard error of mean (SEM).

3 Results

3.1 Expression of angiogenesis-related factors

To determine the release of angiogenic factors by L-PRF, a VEGF ELISA and a human cytokine antibody array were performed. The VEGF release by L-PRF was assessed in CM and exudate. The results showed the presence of VEGF in the CM of 48h, 96h and 144h, at a concentration of respectively 980.5 pg/ml, 2010.08 pg/ml and 1763.83 pg/ml. The VEGF concentration in the exudate, 43.15 pg/ml, was significantly lower compared to the CM of 96h (Figure 3). Although not significant, CM 96h shows a trend towards a higher VEGF concentration compared to CM 48h and CM 144h.

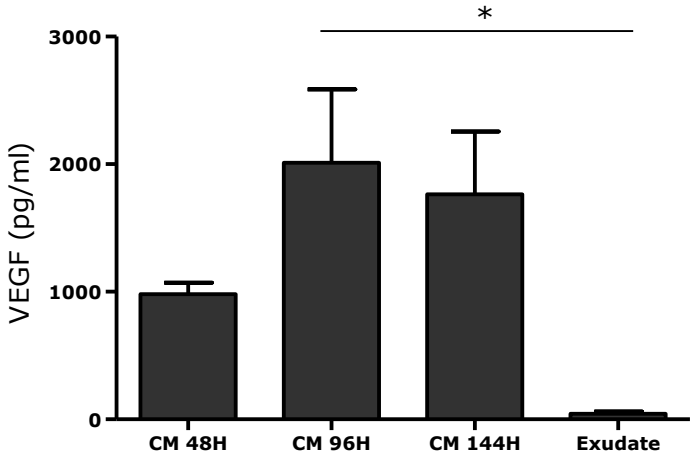

Figure 3: Differential levels of VEGF in L-PRF CM and exudate. The VEGF concentration was measured by ELISA in CM (n=4) harvested after 48h, 96h and 144h of incubation with L-PRF and in exudate (n=4). Data are represented as mean ± SEM. * = p-value < 0.05. CM = conditioned medium; L-PRF = leukocyte- and platelet-rich fibrin; VEGF = vascular endothelial growth factor.

To examine the relative expression of other angiogenic factors released by L-PRF, a human cytokine antibody array was performed on CM and exudate. Angiogenic factors, such as EGF, IL-8 and MCP-1 were identified in both CM and exudate (Figure 4A-B). The most abundant cytokines measured in the CM and exudate were quantified (Graph 4B). Four of the most abundant cytokines found in CM, namely ENA-78, GRO, IL-8 and neutrophil activating protein-2 (NAP-2), bind to the same receptor; the IL-8 receptor beta (IL8RB), which is also known as CXCR2. Statistical analysis of the difference in relative cytokine expressions between CM and exudate could not be performed due to the low sample size. However, the relative expressions of GRO, IL-8, ENA-78 and EGF seemed to be higher in the CM compared to the exudate, while leukemia inhibitory factor (LIF), leptin and TGF-β tend to be higher in the exudate (Figure 4B).

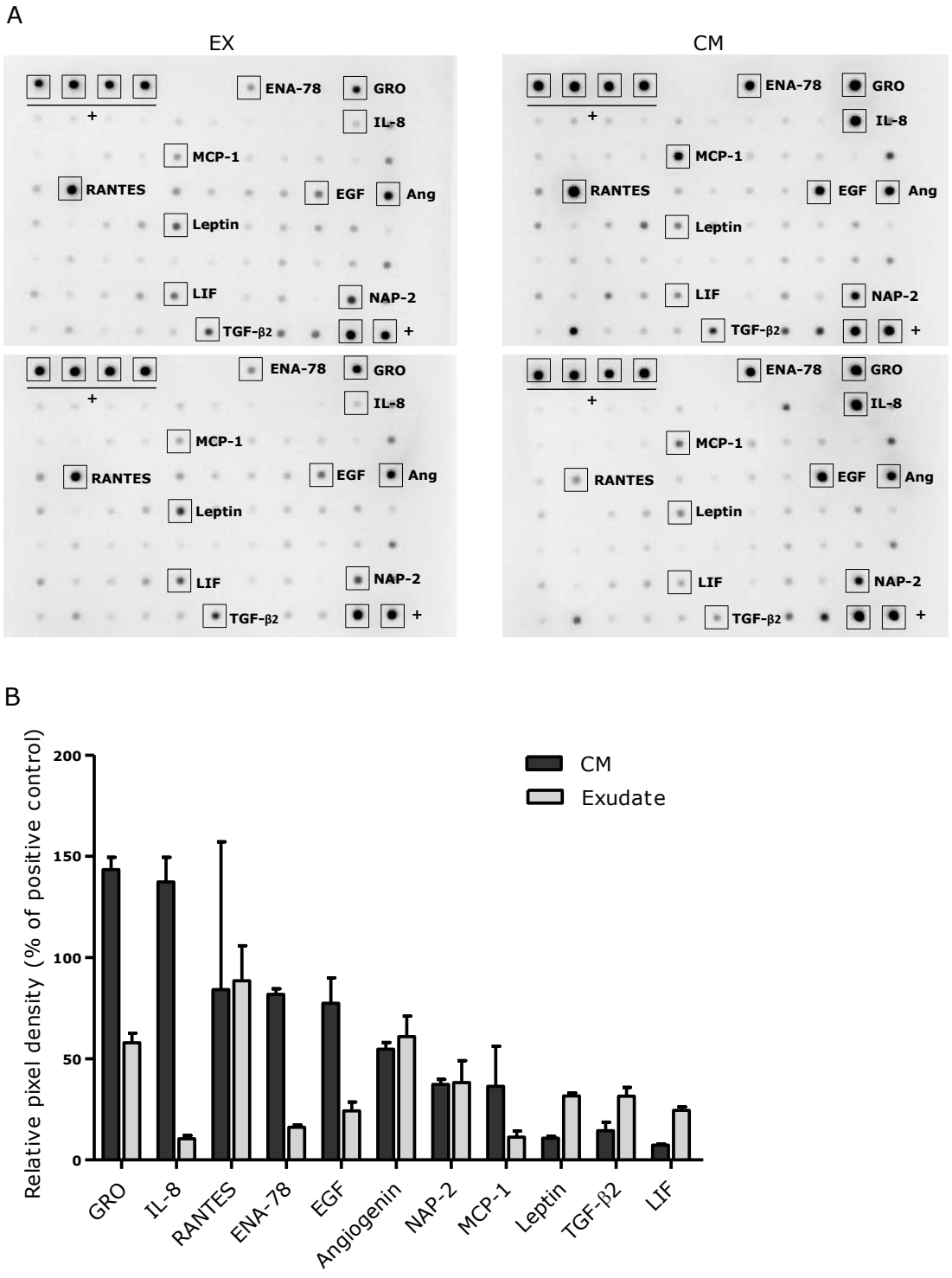

Figure 4: Cytokine profile of L-PRF CM and exudate. Angiogenic cytokines were detected in L-PRF exudate and CM by a Human Cytokine Antibody Array (A). The relative expression of the most abundant factors measured in the CM and exudate were quantified (B). Data (n=2) are represented as mean ± SEM. CM = conditioned medium; EGF = epidermal growth factor; GRO = growth-regulated oncogene; IL-8 = interleukin-8; LIF = leukemia inhibitory factor; L-PRF = leukocyte- and platelet-rich fibrin; MCP-1 = monocyte chemoattractant protein-1; NAP-2 = neutrophil activating protein-2; TGF-β2 = transforming growth factor-beta 2.

3.2 L-PRF shows angiogenic effects *in vitro*

After examination of secreted angiogenic proteins by L-PRF, the effect of L-PRF on HUVEC was investigated. In this part of the analysis of the angiogenic potential, it was explored whether L-PRF has an impact on the HUVEC viability, migration and tube formation. Furthermore, it was determined whether the effects were mediated by the CXCR2 pathway.

3.2.1 L-PRF increases endothelial cell viability

The influence of L-PRF CM and exudate on HUVEC viability was determined by an MTT assay. HUVEC were incubated for 48h with αMEM 0% FBS (negative control), αMEM 10% FBS (positive control), 100% CM, 50% CM, 1%, 3% or 10% exudate in αMEM 0% FBS. Addition of 100 nM SB225002 was performed to examine the involvement of the CXCR2 pathway in the effect of L-PRF on the endothelial viability. SB225002 is a selective inhibitor of CXCR2 with an IC_{50} of 22 nM. Incubation of HUVEC with L-PRF exudate, 1% 3% and 10%, resulted in a 5 to 6-fold increase in HUVEC viability, which was significantly different from the negative control, while CM showed no significant effect on HUVEC viability compared to the negative control condition (Figure 5A). Addition of the CXCR2 antagonist SB225002 did not cause a significant decrease in HUVEC viability compared to the same conditions without SB225002 (Figure 5B).

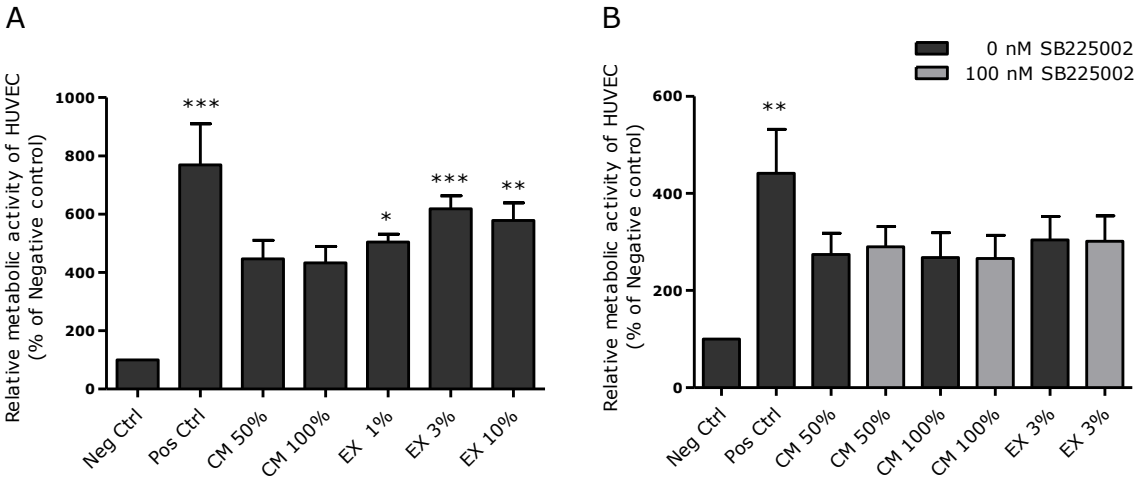

Figure 5: L-PRF exudate increases HUVEC viability. (A) The effect of L-PRF (n=10) CM and exudate on HUVEC viability *in vitro* was examined. αMEM 0% FBS and αMEM 10% FBS were used as a negative and positive control respectively. (B) To examine the involvement of the CXCR2 pathway, HUVEC were incubated with L-PRF CM or exudate 3% with (100 nM) or without (0 nM) addition of SB225002 (n=6). All values are represented as mean ± SEM. *** = p-value < 0.001, **= p-value < 0.01 and * = p-value <0.05. CM = conditioned medium; EX = exudate; FBS = fetal bovine serum; HUVEC = human umbilical vein endothelial cells; L-PRF = leukocyte- and platelet-rich fibrin.

3.2.2 L-PRF is able to stimulate endothelial migration

Another important step in the angiogenic process is EC migration. A transwell migration assay was performed to examine whether L-PRF can induce HUVEC migration. CM and exudate were able to

significantly increase the migration of HUVEC 15 to 19 times and 10 to 25 times respectively compared to the negative control (Figure 6A). For the CM conditions, the effect of adding SB225002 was tested. Treatment of HUVEC with SB225002 did not significantly attenuate the increase in HUVEC migration caused by L-PRF CM, although a trend towards decreased migration was observed (Figure 6B).

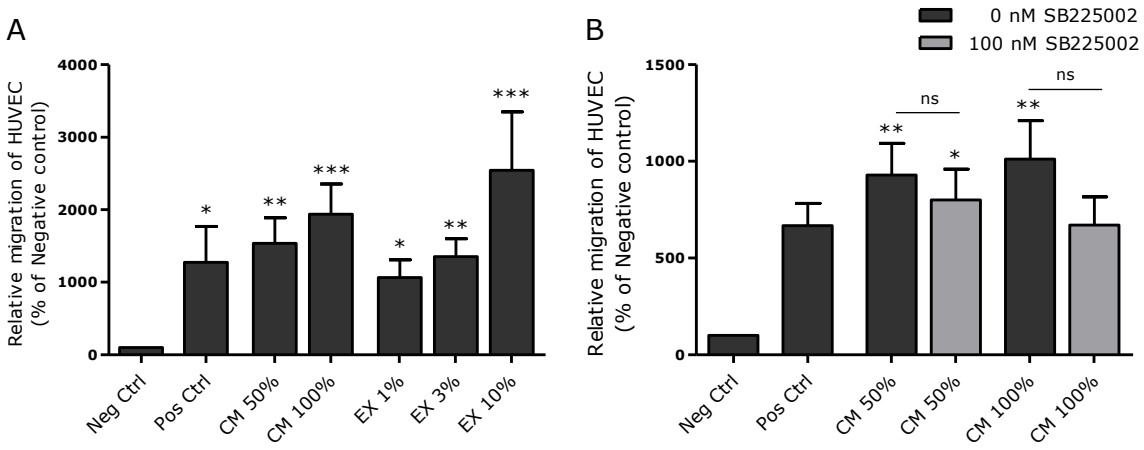

Figure 6: L-PRF increases endothelial cell migration. (A) The influence of L-PRF (n=10) CM and exudate on the endothelial cell migration was determined. αMEM 0% FBS and αMEM 10% FBS were used as a negative and positive control respectively. (B) The effect of the CXCR2 antagonist SB225002 on CM-induced HUVEC migration was assessed (n=8). Data are shown as mean ± SEM. *** = p-value < 0.001, ** < 0.01 and * < 0.05. CM = conditioned medium; EX = exudate; FBS = fetal bovine serum; HUVEC = human umbilical vein endothelial cells; L-PRF = leukocyte- and platelet-rich fibrin.

3.2.3 L-PRF induces endothelial cell tube formation

The effect of L-PRF on the ability of HUVEC to form capillary-like structures was examined in a tube formation assay. The total branching length and number of nodes, two parameters to assess the amount of tube formation, were determined. CM 100% and exudate 1% were able to significantly increase both tube formation parameters (Figure 7A-B). A four-fold increase in number of nodes and a 2 to 3-fold increase in total branching length was observed. No significant effect was observed when HUVEC were incubated with 50% CM in αMEM 0% FBS. However a trend towards increased tube formation was observed, since CM 50% caused a two-fold increase in total branching length and number of nodes (Figure 7A).

In a second experiment, HUVEC were pre-incubated with the CXCR2 antagonist SB225002 before seeding in CM, to examine whether the *in vitro* effect of L-PRF on the tube-forming activity of HUVEC was mediated by CXCR2. There was no significant decrease in tube formation when the L-PRF conditions with (100 nM) and without (0 nM) SB225002 were compared (Figure 8). CM 50% in combination with 100 nM SB225002 even caused a significant increase in tube formation compared to CM 50% without SB225002 and compared to the negative control.

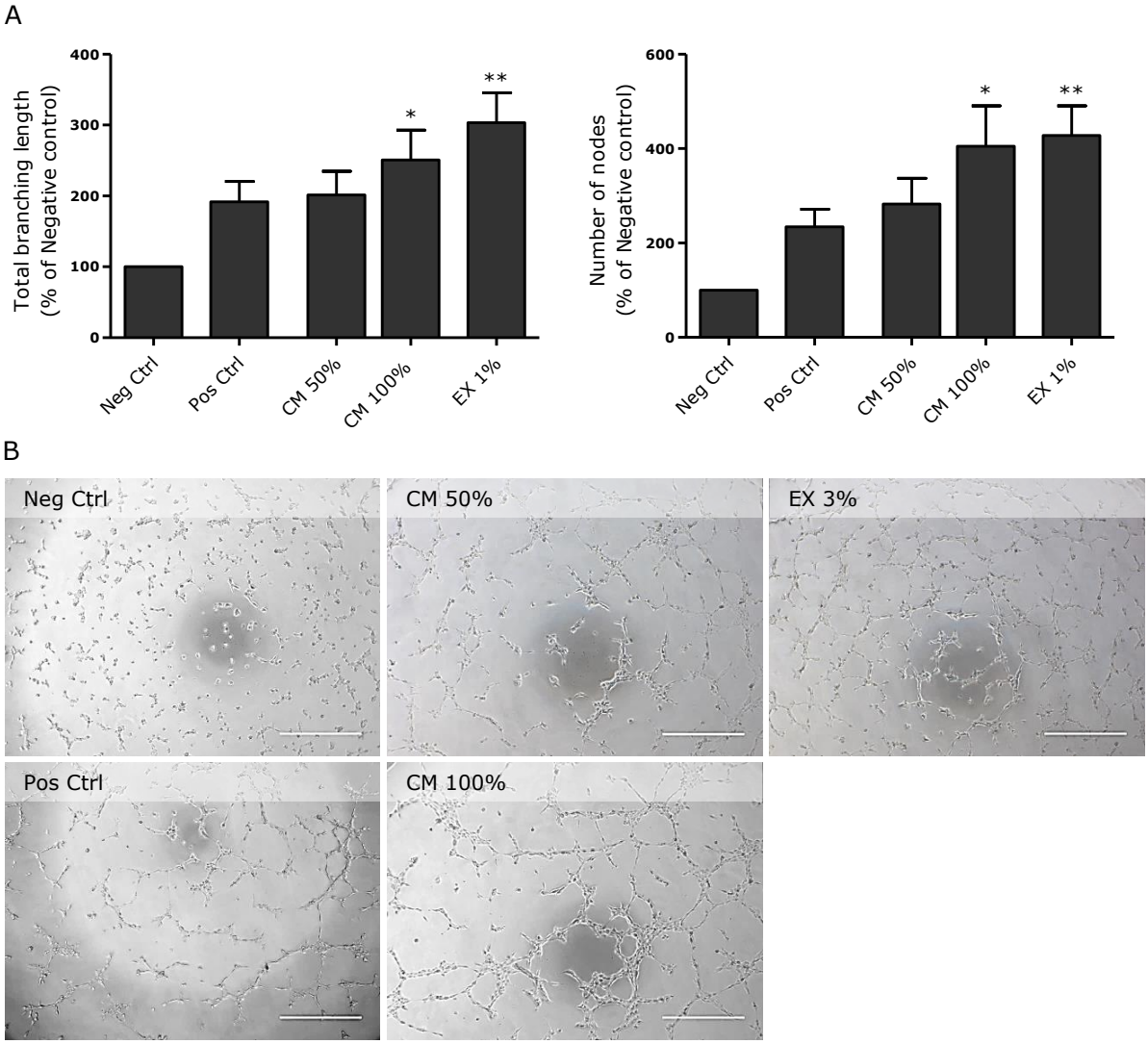

Figure 7: L-PRF induces endothelial cell tube formation. Impact of L-PRF (n=10) CM and exudate on HUVEC tube formation. αMEM 0% FBS and αMEM 10% FBS served as a negative and positive control respectively. Data are shown as mean ± SEM. **= p-value < 0.01, * < 0.05. Scale bar = 100 µm. CM = conditioned medium; EX = exudate; FBS = fetal bovine serum; HUVEC = human umbilical vein endothelial cells. L-PRF = leukocyte- and platelet-rich fibrin.

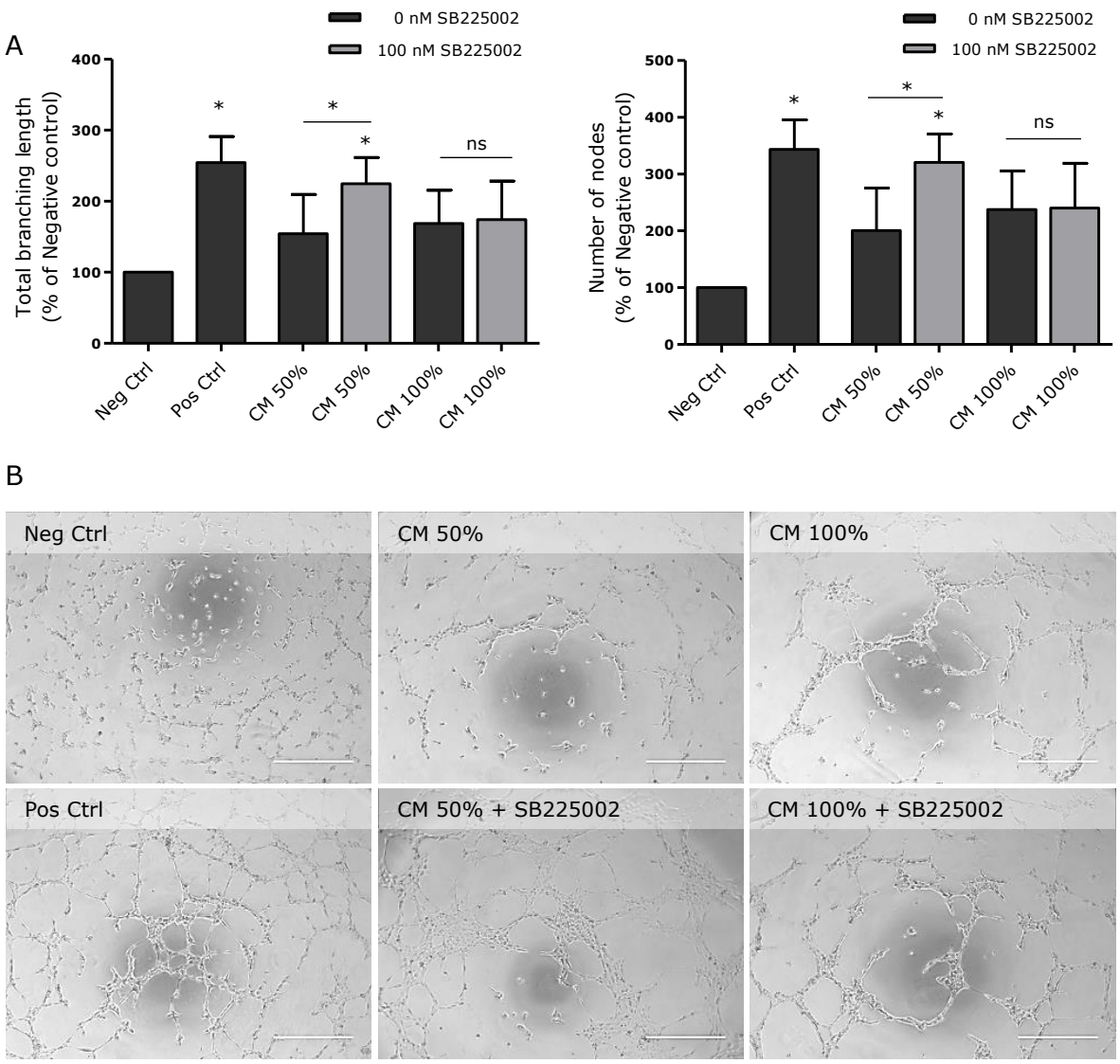

Figure 8: Effect of L-PRF on endothelial cell tube formation is not mediated by CXCR2. Effect of CXCR2 antagonist SB225002 on L-PRF CM induced HUVEC tube formation. αMEM 0% FBS and αMEM 10% FBS were used as a negative and positive control respectively. Data are shown as mean ± SEM and correspond to n=7. *** = p-value < 0.001, **= p-value < 0.01 and * = p-value < 0.05. CM = conditioned medium; FBS = fetal bovine serum; HUVEC = human umbilical vein endothelial cells; L-PRF = leukocyte- and platelet-rich fibrin.

3.2.4 IL-8 does not affect endothelial cell viability, migration or tube formation

Since the CXCR2 antagonist SB225002 was not able to attenuate the effects of L-PRF on HUVEC behavior, its functionality was examined using IL-8. According to literature, IL-8 is able to increase EC viability, migration and tube formation and binds to CXCR2 with a high affinity. The effect of different concentrations of IL-8, namely 1, 10 and 100 ng/ml, on the EC behavior was examined in an MTT assay, transwell migration assay and tube formation assay. These effects were compared to the IL-8 conditions in which HUVEC were pre-incubated with 100 nM SB225002. IL-8 seemed not to affect the HUVEC viability (Figure 9A), migration (Figure 9B) or tube formation (Figure 9C-D). When adding SB225002, a trends towards decreased viability and increased tube formation

were noticed. These observations could not be tested for statistical significance since the experiments were only performed twice.

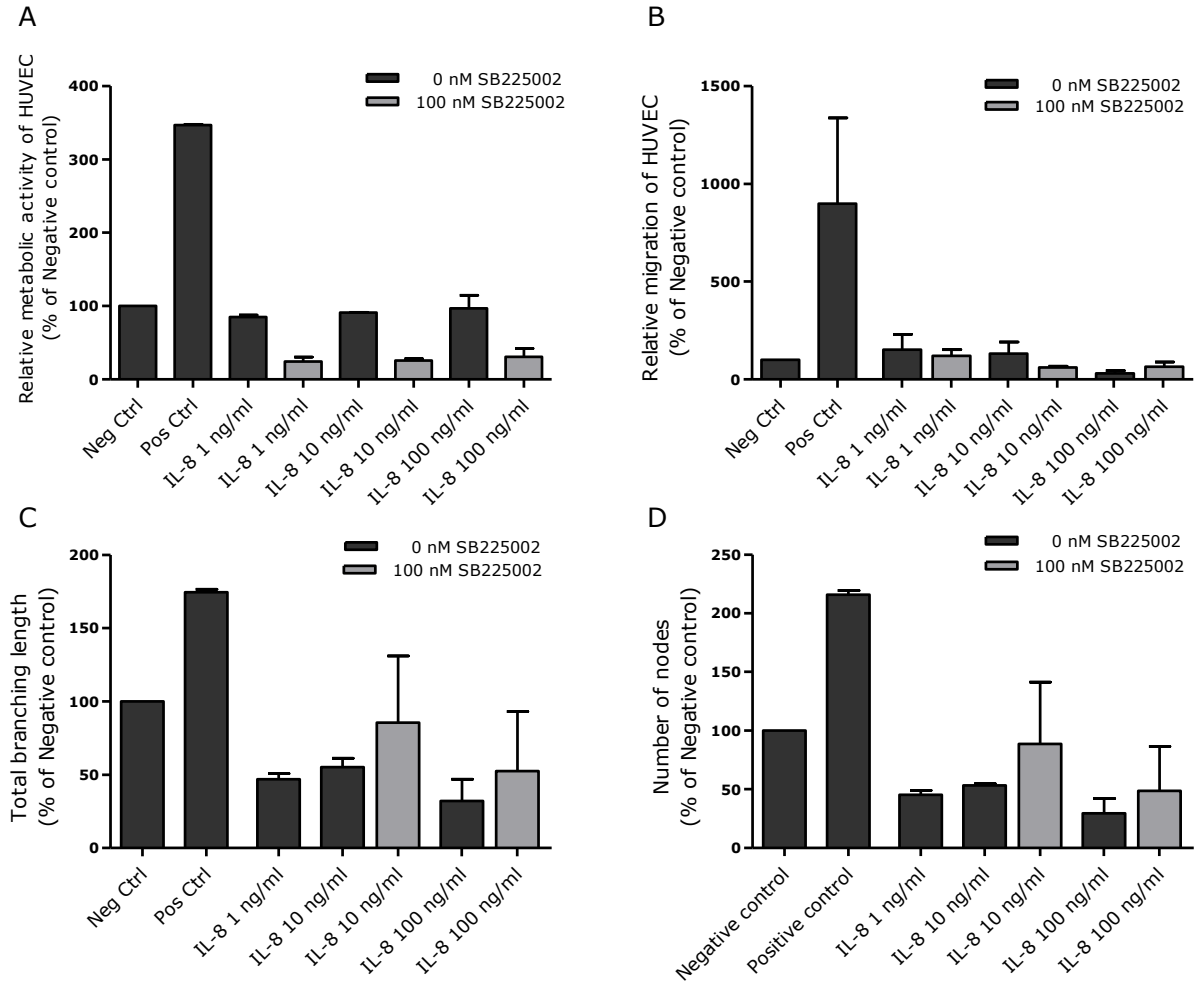

Figure 9: IL-8 does not affect endothelial cell viability, migration and tube formation. The effect of IL-8, whether or not in combination with 100 nM SB225002, on HUVEC viability (A), HUVEC migration (B) and HUVEC tube formation (C,D) was examined. αMEM 0% FBS and αMEM 10% FBS served as a negative and positive control respectively. For each experiment, data are shown as mean ± SEM and correspond to n = 2 assays. No significance tests were performed since the number of repeats was less than three. IL-8 = interleukin-8; HUVEC = human umbilical vein endothelial cells.

3.3 CXCR2 surface expression on HUVEC

Since IL-8 did not increase HUVEC viability, migration and tube formation and SB225002 was not able to attenuate the effects of L-PRF on HUVEC behavior, CXCR2 expression on HUVEC was verified by flow cytometry. A cell surface expression of CXCR2 was observed in 6.5 % ± 1,945 (mean ± SEM) of the analyzed HUVEC (Figure 10).

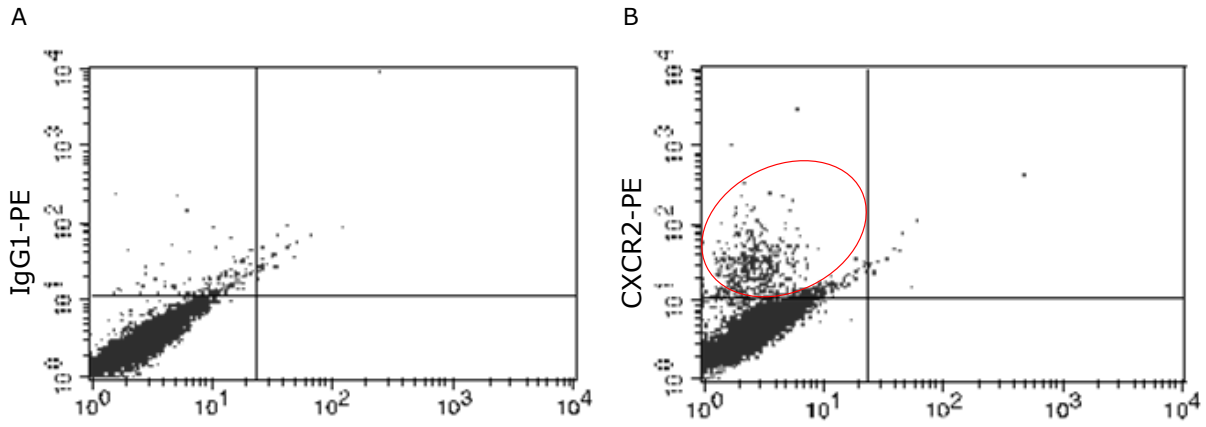

Figure 10: Low levels of CXCR2 surface expression on HUVEC. HUVEC were stained for CXCR2 and analyzed by means flow cytometry. A small CXCR2-positive HUVEC population was observed (upper left, B). CXCR2 expression was determined on three independent HUVEC populations at different time points and compared to an isotype control (A). HUVEC = human umbilical vein endothelial cells.

3.4 L-PRF induces blood vessel formation *in ovo*

The effect of L-PRF on the distinct steps of the angiogenic process, namely cell viability, migration and tube formation was examined *in vitro*. However, these steps are interdependent processes of the angiogenic response and *in vivo* assays permit a more accurate investigation of the effect on the complete angiogenic process.

A CAM assay was performed to investigate whether L-PRF demonstrates angiogenic effects in an *in ovo* setting. Fertilized eggs were incubated for three days with matrigel droplets containing 50% CM or 50% exudate. The CXCR2 antagonist SB225002 was added to the CM condition to evaluate whether the angiogenic effect of L-PRF is mediated by the CXCR2 pathway. Matrigel droplets containing VEGF, FGF-2, IL-8 or αMEM 10% FBS were used as positive controls, while αMEM 0% FBS served as a negative control. Pictures were taken and blood vessels intersecting two concentric circles placed around the matrigel droplets were counted. CM and exudate caused a significant 1.4-fold increase in number of intersecting blood vessels for both circles compared to the negative control (Figure 11). SB225002 was not able to attenuate this effect. Unlike the *in* vitro test, IL-8 significantly induced blood vessel formation. As regards to the tested positive controls, FGF-2 and IL-8 were able to cause a significant increase in number of blood vessels. VEGF, however, showed a trend to more angiogenesis but this was not statistically different.

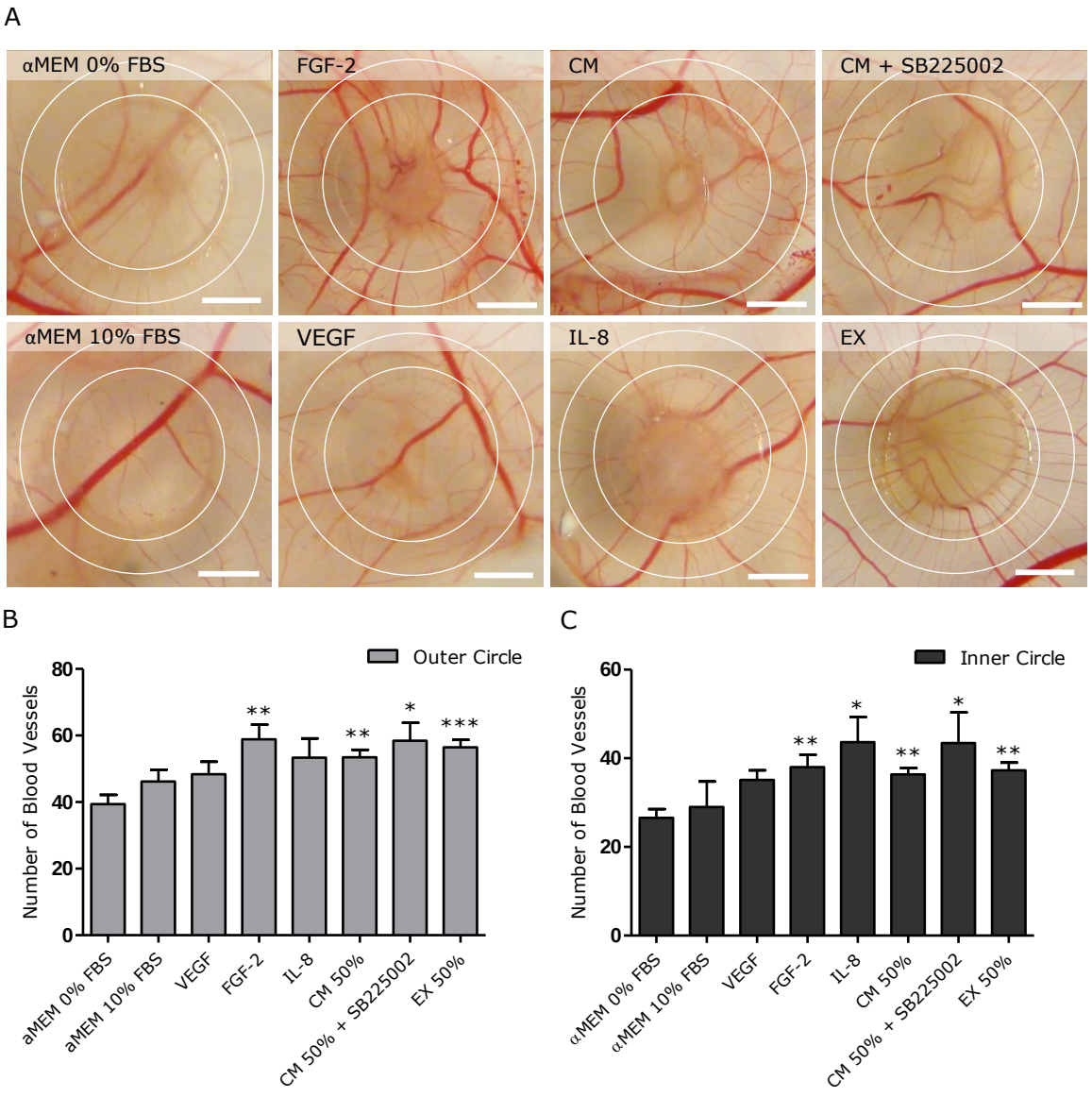

Figure 11: L-PRF induces blood vessel formation *in ovo*. All vessels intersecting two concentric circles (with a radius of 0.6 and 0.9 mm), digitally positioned around the matrigel droplets, were counted. αMEM 0% was used as a negative control, while αMEM 10% FBS, VEGF, FGF-2 and IL-8 were used as positive controls. CXCR2 antagonist SB225002 was not able to attenuate the effect of L-PRF. The assay was performed four independent times. Values are represented as mean ± S.E.M. and correspond to n=10 donors. *** = p-value < 0.001, **= < 0.01 and * = < 0.05. Scale bar = 2 mm. CAM = chorioallantoic membrane; CM = conditioned medium; FBS = fetal bovine serum; FGF-2 = fibroblast growth factor-2; IL-8 = interleukin-8; L-PRF = leukocyte- and platelet-rich fibrin; VEGF = vascular endothelial growth factor.

4 Discussion

Angiogenesis is a multistep process which is strictly regulated by counteracting actions of many pro- and anti-angiogenic molecules (1). Pathologies such as myocardial infarctions, stroke, neurodegeneration and chronic wounds are often associated with insufficient angiogenesis, which impedes recovery, and still there is no FDA approved therapeutic angiogenesis treatment available (2, 7). Autologous platelet concentrates are recently considered as a potential strategy to obtain effective therapeutic angiogenesis. Although L-PRF is nowadays applied in clinical settings such as oral and maxillofacial surgery and the treatment of chronic ulcers (23, 24), little is known about the mechanism of action of L-PRF. Therefore the angiogenic potential of L-PRF and the angiogenic steps and proteins involved were examined in the current study. First, the release of angiogenesis-related molecules by L-PRF was determined. Next, it was examined whether L-PRF has an effect on different types of angiogenesis related EC behaviors *in vitro*. Eventually, we investigated whether L-PRF can induce blood vessel formation *in ovo*.

The secretion of angiogenesis-related factors by L-PRF was determined by ELISA and a cytokine antibody array. Data obtained from the VEGF ELISA showed that L-PRF CM 96h contains 45 times more VEGF compared to the exudate. The significant difference in VEGF concentration can be explained by the organization of the fibrin network in L-PRF. Due to the absence of any additives, such as bovine thrombin and calcium chloride, L-PRF polymerizes slowly during the centrifugation process. This leads to the formation of equilateral junctions, which create a flexible and fine fibrin network (41). This kind of fibrin organization supports cytokine enmeshment and, consequently, cytokines are slowly released over time (42, 43). Since L-PRF CM is harvested after 96h of incubation with L-PRF, while the exudate is obtained by immediate compression of the L-PRF clot, the sustained release is likely to be a reason for the significant difference in VEGF concentration between CM and exudate (44). Another possible explanation for the difference in VEGF release is that activated leukocytes and platelets, present in the L-PRF matrix, continue to produce VEGF (21, 44). Although CM collection after 96h of incubation resulted in a 2-fold increase in VEGF concentration compared to CM 48h, this difference was not significant. However, the trend towards an increase between 48h and 96h could be related, as well, to the sustained release of cytokines over time. The VEGF concentration did not change significantly between the CM of 96h and 144h. This is not in line with the studies of Schär *et al.* and Ehrenfest *et al.* reporting a sustained VEGF release up to seven days (21, 44). However, Ehrenfest *et al.* examined the VEGF secretion in CM of L-PRF membranes, while we used uncompressed L-PRF clots. The compression of the fibrin matrix can affect its architecture and, therefore, the cytokine enmeshment (38). Besides, comparing the experimental set-up, the L-PRF clots in our study were kept in the same medium for 144h, while, in those studies, the medium is repeatedly changed and the total release was calculated afterwards. This repeated medium change creates every time a new cytokine-poor environment, stimulating cytokine release by the cells present in the L-PRF clot (44).

In our study, we performed all following experiments with CM 96h, because of the trend towards an increased VEGF-expression. Finally, it should be kept in mind that the VEGF secretion by L-PRF can differ from the obtained results when L-PRF is applied in a physiological situation in the body. Since αMEM without any supplementations is an extra stimulus for cytokine secretion by the present cells, a more biological environment could affect the L-PRF secretion behavior (44).

To examine the release of other angiogenic molecules, a cytokine antibody array was performed. Pro-angiogenic factors such as IL-8, ENA-78, EGF and MCP-1, were detected both in CM and exudate. The relative expressions of GRO, IL-8, ENA-78 and EGF showed a trend towards higher expressions in CM compared to exudate. The higher cytokine expressions in CM can again be addressed to the fibrin matrix architecture favoring cytokine enmeshment. However, certain cytokines, such as LIF, TGF-β2 and leptin, show a trend towards a higher presence in the exudate. Since the fibrin binding affinity of growth factors influences their incorporation in the matrix (45, 46), a possible explanation for the high expressions in exudate is that these cytokines bind to fibrin with a low affinity. Except for TGF-β2, no literature was found reporting on fibrin binding affinity of these cytokines. Martino *et al.* reported a modest binding of TGF-β2 to fibrin, but the release of TGF-β2 from the fibrin matrix was not verified (47). However, if the low binding affinity would be the only reason, the expression of these cytokines should be high in CM as well. The fact that they are not can indicate that cells present in L-PRF do not secrete these cytokines and, regarding the short half-life of cytokines (48), they will be degraded after 96h when extra production is absent. This supposition could be verified in future experiments by examining the leukocyte content of L-PRF and relating it to their cytokine expression. Furthermore, ENA-78 (CXCL5), GRO (CXCL1), IL-8 (CXCL8) and NAP-2 (CXCL7), four cytokines belonging to the cytokines with the highest relative expressions measured in CM, are found to bind with a high affinity to CXCR2, or IL-8 receptor beta (IL8RB) (49-51). These cytokines belong to the glutamic acid-leucine-arginine positive (ELR^+) subfamily of CXC chemokines. This ELR motif is responsible for the high binding affinity of these cytokines for CXCR2 (51). It was already proven by Strieter *et al.* that these ELR^+ cytokines have a pro-angiogenic effect (52). Furthermore, Addison *et al.* showed that the induction of angiogenesis by these ELR^+ chemokines is mediated by CXCR2 (53). These findings are supported by the results of Heidemann *et al.* showing that IL-8 exerts pro-angiogenic effects on EC via CXCR2 (54). It should be noted that in these studies, ECs different from HUVEC were used, namely intestinal microvascular ECs (54), dermal microvascular ECs (53) and bovine adrenal gland capillary ECs (52). Since the angiogenic effect on HUVEC was examined in this study, we verified whether HUVEC expressed CXCR2 as well and whether the effect of L-PRF on HUVEC was mediated by this receptor.

Evidence concerning the presence of CXCR2 on HUVEC has been conflicting. Petzelbauer *et al.* did not detect CXCR2 expression at mRNA level (55), while Murdoch *et al.* reported that HUVEC do express CXCR2 mRNA, while no CXCR2 expression could be detected at protein level (56). Li *et al.* detected CXCR2 mRNA expression in HUVEC as well and were able to confirm this CXCR2 expression on protein level, with 12,1% of the tested HUVEC being CXCR2-positive (57). Salcedo *et al.* reported that only a small portion of HUVEC expresses low levels of CXCR2 compared to

human microvascular dermal endothelial cells (HMEC), stating that the CXCR2 levels differ between EC types (58). Our flow cytometric analysis showed that only a small percentage of the examined HUVEC (6.5%) showed CXCR2 cell surface expression. In the future, it might be interesting to perform a flow cytometric analysis on permeabilized HUVEC to determine possible intracellular pools of CXCR2. Furthermore, CXCR2 protein levels could be analyzed after incubation of HUVEC with CM or exudate to determine whether paracrine stimulation will upregulate CXCR2 surface expression.

Besides examining the secretion of angiogenesis-related molecules, the effect of L-PRF on HUVEC behavior was assessed as well. Regarding the multiple stages of the angiogenic process, it was examined whether L-PRF affects the EC viability, migration and tube formation. Little has been published on the effect of this platelet concentrate on EC behavior. As regards to EC viability and proliferation, only the impact of other platelet concentrates on EC proliferation was reported (59) or the effect of L-PRF on the viability and proliferation of other cells, such as osteoblasts or stem cells (22, 60). Therefore, the impact of L-PRF on HUVEC viability was determined in the current study. The results indicate that L-PRF exudate was able to significantly increase HUVEC viability, while CM only showed a trend towards an increased HUVEC viability. This can be due to the fact that present growth factors are more concentrated in the small volume of exudate obtained after compressing compared to CM. The HUVEC viability was not affected in a concentration-dependent manner by exudate, since exudate 3% caused the highest significant increase. Saturation levels may have been reached and, consequently, exudate 10% did not lead to a more increased effect on HUVEC viability.

Another important step in the angiogenic process is the EC migration towards angiogenic factors. A transwell assay was performed to determine whether L-PRF induces HUVEC migration. The results of this assay indicated that CM as well as exudate caused a significant increase in HUVEC migration. Both CM and exudate influenced the HUVEC migration in a concentration-dependent manner. These findings are in line with the results reported by Schär *et al.*, indicating that L-PRF CM is able to induce HUVEC migration (21). After EC migration towards angiogenic stimuli, EC tube formation is another key step in the angiogenic process. Therefore, we investigated whether L-PRF influences the ability of HUVEC to aggregate into capillary-like structures by seeding HUVEC in L-PRF CM or exudate on growth factor-reduced Matrigel. CM 100% and exudate 1% were able to significantly increase both the number of nodes and total branching length, two parameters to quantify the amount of tube formation. CM 50% was not able to significantly increase EC tube formation, which is probably an indication that growth factors are too diluted at this concentration to evoke tube formation. However, a trend towards increased tube formation was still observed. Effects of L-PRF on EC tube formation have not been described before. However, Yin *et al.* reported that two other platelet concentrates, pure platelet-rich plasma (P-PRP) and leukocyte- and platelet-rich plasma (L-PRP), were also able to induce HUVEC tube formation (61).

Due to the observation that the ELR^+ cytokines, ENA-78, GRO, IL-8 and NAP-2 were abundantly present in the L-PRF CM and the knowledge that these cytokines can induce pro-angiogenic effects

mediated by CXCR2, we examined whether the effect of L-PRF CM on angiogenesis was (partially) mediated by CXCR2. Therefore, the viability assay, transwell migration assay and tube formation assay, as described earlier, were performed with SB225002, a selective CXCR2 antagonist. The results of the transwell migration assay showed no significant effect of pre-incubation of HUVEC with the SB225002. However, a trend towards decreased HUVEC migration was observed for the CM conditions with SB225002 compared to the CM conditions without CXCR2 antagonist. The observed trend is consistent with the data obtained from the array, namely the high expression of ELR^+ cytokines in CM. Future repetition of the experiment, however, will have to give more insight in the involvement of CXCR2 in the effect of L-PRF CM and exudate on HUVEC migration. Regarding the influence on HUVEC viability, the effect of L-PRF CM and exudate was not (mainly) mediated by CXCR2, as addition of SB225002 did not attenuate the effect. SB225002 neither decreased the effect of L-PRF on HUVEC tube formation. In fact, even a significant increased tube formation was observed when SB225002 was added to the CM 50% condition. It may be that SB225002 slows down the tube formation instead of blocking it or that, when CXCR2 is blocked, compensation pathways will cause tube formation, but over a longer period of time. The experiment should be repeated to further investigate this statement. This can be verified by evaluating tube formation on different time points. However, SB225002 did not cause an increase in tube formation in the condition of CM 100%.

Four possible explanations can be formulated for why inhibition of CXCR2 did not decrease the effect of L-PRF on HUVEC viability and tube formation. (i) First of all, it should be kept in mind that L-PRF is an autologous cocktail containing numerous growth factors, from which only known cytokines can be measured and evaluated. Other cytokines present in CM can substitute or cover the effect of the ELR^+ cytokines on angiogenesis. Therefore, it is difficult to relate the current results with studies focusing on the use of single recombinant growth factors, such as IL-8 (53, 54). (ii) Other explanations could be that the CXCR2 antagonist did not function optimally or (iii) that HUVEC are not responsive to ELR^+ cytokines. In order to verify these assumptions, the viability assay, transwell migration assay and tube formation assay were repeated using IL-8, instead of L-PRF, and SB225002. IL-8 was not able to increase HUVEC viability compared to the negative control condition. It was observed that SB225002 tends to decrease the values obtained with IL-8. However, no conclusion about the functionality of SB225002 can be made based on these results, since HUVEC did not proliferate more compared to the negative control. The obtained results suggest that IL-8 does not affect HUVEC migration or tube formation. However, these experiments should be repeated in the future since they were run too few times to calculate statistical significance. In literature, contradictory results regarding the effect of IL-8 on HUVEC were found. Koch *et al.* stated that IL-8 is able to induce HUVEC proliferation and chemotaxis, which is in line with the findings of Li *et al.*, reporting a direct effect of IL-8 on HUVEC proliferation, migration and tube formation (57, 62). However, Petzelbauer *et al.* found that IL-8 does not directly increase HUVEC proliferation or migration (55). They reported that IL-8 receptor expression and responsiveness of HUVEC, as demonstrated in other articles, may be regulated by culture conditions. Li *et al.* indeed used endothelial cell medium (EGM-2) for experimental

conditions, while we used αMEM 0% FBS in the current study. Besides, Petzelbauer *et al.* stated that some commercially-obtained HUVEC cultures can contain subpopulations of IL-8 responsive cells, which could be a reason for the discrepancy in results. Our results are in line with the finding of Salcedo *et al.*, as mentioned earlier, that HUVEC exhibit low responsiveness to IL-8 (58). (iv) A fourth explanation might be that not CXCR2, but CXCR1 is important for the induced EC behavior effects, since IL-8 can also bind to CXCR1. As for CXCR2, there is still controversy about CXCR1 expression on HUVEC. Salcedo *et al.* reported that only a small portion of HUVEC expresses low levels of CXCR1, as they described as well for CXCR2 (58). However, Li *et al.* showed that HUVEC express CXCR1 on mRNA and protein level and reported that 43% of the tested HUVEC were CXCR1 positive (57). This was in line with the findings of Murdoch *et al*. who reported CXCR1 mRNA and cell surface expression on HUVEC (56). Since findings remain controversial, future experiments have to point out whether HUVEC express CXCR1 and whether CXCR1 is involved in the angiogenic effects of L-PRF.

Since the different *in vitro* assays indicated a pro-angiogenic effect of L-PRF, the angiogenic potential of L-PRF was assessed *in ovo*. A CAM assay was performed, in which the multiple steps of angiogenesis are combined. During chick embryonic development, three extraembryonic membranes are formed, namely the yolk sac membrane, the amnion, and the chorioallantoic membrane (CAM). Since the CAM has a rich vascular system, it is often used to study (anti-) angiogenic effects. Human L-PRF CM and exudate could be applied in this assay, since the chick embryo lacks a mature immune system until 18 days into its development (63). Both L-PRF CM and exudate were able to induce blood vessel formation *in ovo*. This effect could not be attenuated by addition of SB225002. This observation is in line with the findings from the *in vitro* experiments and indicates that CXCR2 does not play a major role in the mediation of the angiogenic of L-PRF.

For future experiments, it is interesting to examine the angiogenic potential of L-PRF in an *in vivo* setting which mimics more closely the clinical applications of L-PRF. A wound healing model would be suitable to mirror the biological environment in which L-PRF is applied in clinical settings. Large animal models, such as a pig model, are often used in wound healing studies. However the use of these models impose certain requirements, including housing (59). A mouse model is a more practical and economical animal model, but murine wounds have a different wound healing process compared to human. While in humans, wounds are mainly closed by tissue regeneration, contraction accounts for a large part of wound closure in mice. To overcome the differences between wound healing in mice and human, a murine excisional wound splinting model, which prevents healing by contraction, is preferred. The splinting ring ensures that the wound will heal by granulation and re-epithelialization (64). For application of L-PRF on the wound, CM or exudate can be added to a biodegradable material such as Matrigel, as used for the CAM assay, or gelatin hydrogel. The effect of L-PRF on wound healing can be assessed by observing the closure of the wound over time. After isolation of the wound tissue, the effect of L-PRF on angiogenesis can be determined by evaluating the newly formed blood vessels using immunohistological staining against von Willebrand factor or CD146 (65). Roy *et al.* already reported that another platelet concentrate, namely platelet-rich fibrin matrix (PRFM), can improve impaired angiogenesis in a

porcine ischemic wound model (59). Furthermore, Notodihardjo *et al.* recently found that platelet-rich plasma releasate is able to induce wound healing and angiogenesis in the described murine wound splinting model (65).

The angiogenic effects of L-PRF support its use as a new strategy to promote angiogenesis. Previous studies reported recombinant growth factors as a treatment for insufficient angiogenesis (66, 67). However, the use of L-PRF offers several advantages over recombinant proteins, further supporting its use as a promising treatment strategy. First of all, L-PRF represents a greater similarity to the natural angiogenic process, since it is composed of multiple growth factors. Furthermore, its autologous origin will avoid rejection reactions. Since L-PRF can be produced as needed at the point of care from the patient's blood, storage time does not need to be considered as it does for recombinant proteins. The simple and fast procedure protocol make the use and production of L-PRF easy and cost-effective (17).

Taken together, the results demonstrate that L-PRF shows a profound pro-angiogenic effect *in vitro* and *in ovo*, while CXCR2 does not play a major role in this angiogenic potential of L-PRF. Further research is recommended to get more insight in the molecular mechanisms behind the angiogenic effects of L-PRF.

Conclusion

L-PRF is a second generation platelet concentrate which can be obtained by a simple and fast centrifugation protocol. Due to the absence of any biochemical handling, it is a complete autologous and natural product. L-PRF is currently used in clinical applications, such as oral surgery and the treatment of chronic wounds, for its capacity to enhance tissue healing. Despite its clinical applications, little is known about the mechanisms of action and biological features of this platelet concentrate. It is known that angiogenesis is a key process in tissue healing and, moreover, leukocytes, growth factors derived from platelets and a supportive fibrin matrix, as present in L-PRF, are important participants in the angiogenic process. Therefore, this study aimed to investigate whether L-PRF exerts angiogenic effects. A better understanding of the molecular mechanisms is required to better understand its clinical effects and interpatient variability and broaden its applications.

First, the angiogenic secretion profile of L-PRF was examined by an ELISA and human cytokine antibody array. L-PRF showed expression of angiogenic factors (VEGF, EGF, IL-8, MCP-1). Besides, it was found that four ELR^+ cytokines (ENA-78, GRO, IL-8 and NAP-2), which are known to be involved in angiogenesis and all bind to the same receptor (CXCR2), were abundantly present in CM. Therefore, not only the effect of L-PRF on HUVEC behavior in the different steps of the angiogenic process was examined, but also the involvement of CXCR2. By performing different *in vitro* angiogenic assays, it was found that L-PRF is able to enhance HUVEC viability, migration and tube formation. Next, the involvement of CXCR2 in these angiogenic effects was studied. Although the applied CXCR2 antagonist caused a trend towards decreasing the effect of L-PRF on HUVEC migration, no significant contribution of CXCR2 in any of the effects of L-PRF on HUVEC could be detected. This observation can be explained by the autologous variety of growth factors present in L-PRF, which makes CXCR2 only one of the many contributors to the observed angiogenic effect. Besides the angiogenic effects observed *in vitro*, also the results from the *in ovo* model showed a pro-angiogenic influence of L-PRF. This effect seemed again not to be mediated by CXCR2. In the future, it would be interesting to examine whether L-PRF exerts pro-angiogenic effects in a more biological environment, in which the effect of L-PRF can be stimulated or slowed down by other biological processes, such as in an *in vivo* wound healing model. Taken together, the current study provides new evidence on the angiogenic effects and the involved mechanisms of L-PRF *in vitro* and *in ovo*, which further supports the rationale to use L-PRF as a new strategy for therapeutic angiogenesis.

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