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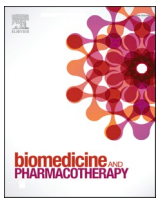

Gender differences and pharmacological regulation of angiogenesis induced by synovial fluids in inflammatory arthritis

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ABSTRACT

Several mediators including cytokines, growth factors and metalloproteinases (MMP) modulate pathological angiogenesis associated with inflammatory arthritis. The biological factors underlying sex disparities in the incidence and severity of rheumatic musculoskeletal diseases are only partially understood. We hypothesized that synovial fluids (SFs) from rheumatoid arthritis (RA) and psoriatic arthritis (PsA) patients would impact on endothelial biology in a sexually dimorphic fashion. Immune cell counts and levels of pro-angiogenic cytokines found in SFs from RA and PsA patients ($n = 17$) were higher than in osteoarthritis patients ($n = 6$). Synovial VEGF concentration was significantly higher in male than in female RA patients. Zymography revealed that SFs comprised solely MMP-9 and MMP-2, with significantly higher MMP-9 levels in male than female RA patients. Using *in vitro* approaches that mimic the major steps of the angiogenic process, SFs from RA and PsA patients induced endothelial migration and formation of capillary-like structures compared to control. Notably, endothelial cells from female donors displayed enhanced angiogenic response to SFs with respect to males. Treatment with the established anti-angiogenic agent digitoxin prevented activation of focal adhesion kinase and SF-induced *in vitro* angiogenesis. Thus, despite higher synovial VEGF and MMP-9 levels in male patients, the responsiveness of vascular endothelium to SF priming was higher in females, suggesting that gender differences in angiogenic responses were mainly related to the endothelial genotype. These findings may have implications for pathogenesis and targeted therapies of inflammatory arthritis.

1. Introduction

Inflammatory arthritis including rheumatoid arthritis (RA) and psoriatic arthritis (PsA) is a chronic and destructive disease characterized by synovial inflammation and hyperplasia of the lining cells [1,2]. The volume of synovial fluid (SF) increases, resulting in joint swelling and pain. Blood-derived cells infiltrate the sublining of the synovium and secrete a variety of effector molecules that promote inflammation and joint destruction. The synovium becomes locally invasive at the interface with cartilage and bone [3,4]. Notably, the SF proteome in RA comprises almost 1000 proteins [5].

Processes that occur in degenerative (primarily osteoarthritis, OA) and chronic inflammatory joint diseases may impact on endothelial function [6]. Cytokines such as interleukin (IL)-1β, IL-6 and IL-10, growth factors and metalloproteinases (MMP) such as MMP-9 released by synovial fibroblasts, immune cells and endothelial cells into the synovial microenvironment [7] may promote or inhibit the formation of new vessels. These mediators, in turn, can increase the synthesis of inflammatory cytokines and MMPs by synovial cells in a vicious circle. In many rheumatologic conditions, angiogenesis is observed since the earliest phases of disease and contributes substantially to synovial inflammation [2,8,9]. Newly formed blood vessels offer an entrance for inflammatory cells to the synovial membrane, eventually resulting in MMP-mediated cartilage and bone destruction. Increasing evidence for a major role of angiogenesis in RA and PsA as well as the success of anti-angiogenic treatments in oncology opened the perspective of directly targeting angiogenesis in arthritis [1,10–12]. For instance, vascular endothelial growth factor (VEGF)-inhibitors targeting neovascularization have been proven beneficial in oncology and may also hold therapeutic potential for inflammatory diseases [13,14]. Early

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studies in mice with collagen-induced arthritis showed that anti-VEGF agents significantly reduce disease severity and the number of blood vessels [15]. However, to date, no clinical studies have been performed in patients with RA/PsA or other arthritic diseases [6,12].

Sex-based differences in the incidence of many rheumatic musculoskeletal diseases are well documented, but the biological factors underlying these disparities are only partially understood [16]. Sex and gender differences are also evident in the clinical manifestations and treatment response, but the underlying mechanisms and drivers of these differences have not been fully identified [17,18]. We recently showed that endothelial cells derived from female donors are more responsive to chemotactic stimuli than those from male donors [19], at least in part due to differential tyrosine phosphorylation and activation of protein tyrosine kinase 2 (FAK). By interfering with the latter mechanism, digitoxin treatment at therapeutic concentrations abolishes the baseline sex difference in serum-induced endothelial cell migration [19,20]. On these grounds, we hypothesized that SFs from arthritic patients would impact on endothelial biology in a sexually dimorphic fashion. Hence, potential gender differences were investigated in the levels of inflammatory cells and soluble mediators affecting angiogenesis in the SF from RA and PsA patients, using SF from OA patients as non-inflammatory controls. We also tested the capacity of inflamed SFs to affect the gender-specific migration, formation of capillary-like structures and FAK phosphorylation status of human endothelial cells.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly delivered umbilical cords by collagenase digestions as described previously [21]. Umbilical cords were collected after delivery, from full-term normal pregnancies at the Obstetrics and Gynaecology Unit of Padua University Hospital. The donors gave their informed consent, and the collected cords were non-identifiable. The procedure was approved by the local Ethics Committee. The cell sex was assessed as described previously [19,22]. Cells were grown in medium M199 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 15 % FBS (GIBCO, Life Technologies), gentamicin (40 μg/mL, Invitrogen by Thermo Fisher, San Giuliano Milanese, Italy), endothelial cell growth factor (ECGS; 100 μg/mL) and heparin (100 IU/mL, Invitrogen), at 37 °C in a humidified 5 % CO_2 atmosphere. Cells were used for experiments from passages 2–5.

2.2. Synovial fluid collection

Synovial fluids (SFs) were collected by arthrocentesis from swollen knees of patients with RA, PsA and osteoarthritis (OA) attending the outpatients' clinic of the Rheumatology Unit at Padua University Hospital. The primary purpose for joint aspiration was therapeutic relief and/or diagnosis. Discarded samples were studied under protocols including written informed consent and approved by the local Institutional Review Board. SFs were obtained from 23 patients (8 RA, 9 PsA and 6 OA) with mild-to-moderate disease who presented after January 2019 due to acute pain. Patients already on treatment with glucocorticoids and/or anti-cytokine drugs were excluded from the study. Routine SF analysis consisted of total and differential white blood cell (WBC) count by light microscopy using a Bürker counting chamber and prestained slides for cell morphology (Testsimplets®), respectively. All SFs tested from RA and PsA donors were characterized by a high inflammatory index (WBC > 2000) and the absence of crystals and pathogens. SFs were centrifuged at 3000 *rpm* for 10 min and stored at −20 °C until further analysis.

2.3. ELISA assays

The following cytokines, chemokines, growth factors and MMP were measured in SFs after appropriate dilutions in PBS using enzyme-linked immunosorbent assay (ELISA) kits for interleukin (IL)-1β, IL-6, IL-8, IL-10, MCP-1 (Invitrogen), TGFβ (eBioscience by Thermo Fisher Scientific), VEGF (BioLegend, San Diego, CA) and MMP-9 (Biotechne, R&D Systems, Minneapolis, USA), according to the manufacturers' instructions.

2.4. Zymography

SF samples from RA, PsA and OA patients were diluted 1:10 in PBS and mixed with nonreducing Laemmli sample buffer (final concentration 1X). SF samples were loaded and electrophoresed on 10 % sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) containing copolymerized gelatin (2 mg/mL). After washing three times in 2.5 % Triton X-100 and then with bidistilled water, zymograms were incubated overnight at 37 °C. After that, zymograms were fixed with fixation buffer (50 % methanol and 4.6 % acetic acid) and stained with Brilliant Blue G (0.25 % p/v) in 45 % methanol and 9 % acetic acid. After destaining in 50 % methanol and 7.5 % acetic acid, MMP activity was identified as "cleared" (degraded) regions against a dark background. Densitometry analysis of bands was performed using the ImageJ version 1.47 software (National Institutes of Health).

2.5. Chemotaxis assay

Chemotaxis experiments were performed in a 48-well modified Boyden chamber (Neuro Probe, Gaithersburg, MD) using 8 μm nucleopore polyvinyl pyrrolidine-free polycarbonate filters coated with 10 μg/mL collagen as described elsewhere [23]. As chemotactic stimuli, SFs from RA, PsA and OA patients were added to lower chambers after appropriate dilution (10 % in M199 supplemented with heparin). M199 plus 15 % FBS and heparin was used as a positive migration control, while M199 plus 1% FBS and heparin was used as a negative control. Upper chambers were filled with 50 μL HUVEC suspension (1.6×10^5 cells/mL in M199 with 1 % FBS and heparin) in the presence or absence of digitoxin as indicated. After 6-h incubation at 37 °C, non-migrating cells on the upper filter surface were removed by scraping. The cells that had migrated to the lower side of the filter were stained with hematoxylin/eosin, and densitometry was performed using ImageJ. Each experiment was performed in sextuplicate. Results are reported as optical density (OD) arbitrary units.

2.6. Tubulogenesis assay

HUVECs (7×10^3 cells/well) exposed to SFs from RA, PsA and OA patients were plated into a thin layer (50 μL) of basement membrane matrix (Matrigel™, Corning Corp., Corning, NY, USA) in 96-well plates and incubated at 37 °C for 4 h in the presence or absence of digitoxin as indicated. One image per well was captured at 4X under a bright field inverted microscope (Nikon Eclipse Ti, Shinagawa, Tokyo, Japan) equipped with a digital camera. Images were analysed using Angiogenesis Analyzer, a plugin developed for the ImageJ software. Data on topological parameters (number of junctions, master segments, meshes) of the capillary-like network were analysed in each well. Junctions were measured as pixels with 3 neighbours and master segments define segments delimited by two junctions.

2.7. Western blot

HUVECs (3×10^5cells) were seeded in 35-mm dishes in complete culture medium and lysed with 80 μL lysis buffer as described [19,20]. Proteins (40 μg) were separated on SDS-PAGE and transferred onto Amersham Hybond-P polyvinylidene difluoride membranes. Membranes were then blocked and probed using rabbit primary monoclonal antibodies to FAK, phospho-FAK Y576/577 (1:1000, Cell Signaling Technology) and GAPDH (1:10 000, Abcam). After three washing steps, membranes were incubated with rabbit secondary horseradish

peroxidase–conjugated antibodies (Vector Laboratories, Burlingame, CA) at 1:10 000 dilution. Bands were detected by chemiluminescence using the Westernbright™ Quantum (Advansta, Menlo Park, CA, USA). Images were acquired with Azure Imaging System, and densitometry was performed using ImageJ. Data are expressed as relative protein levels with respect to the loading control GAPDH.

2.8. Statistical analysis

All experiments were performed in at least 3 independent replicates. Data are reported as mean ± standard error (SEM). The Kolmogorov-Smirnov test was used to analyse the normal distribution of continuous variables. For normally distributed data, two-way or one-way ANOVA followed by Dunnett's or Bonferroni's *post-hoc* tests were used for multiple comparisons, and intra-group comparisons were evaluated by the paired-samples *t* test. For non-normal distributed data, Wilcoxon signed-rank test was performed to demonstrate significant differences within groups, and Mann-Whitney U test was chosen for comparison between independent groups. Pearson correlation analysis was used to determine associations. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). A P value of < 0.05 was considered statistically significant.

3. Results

3.1. Characterization of synovial fluids from arthritis patients

Sex differences in inflammatory arthritis are well described in the literature, but the underlying mechanisms are only partially understood [16]. We explored the cell composition in the SF from inflammatory arthritis (RA and PsA) patients with respect to OA patients as non-inflammatory controls. The latter SFs contained very few WBC and almost no PMN. No differences were found in the counts of synovial WBC and PMN between female and male RA and PsA patients (Table 1).

Angiogenesis is essential in the pathogenesis of joint inflammatory disorders, and in turn an array of inflammatory mediators promote or inhibit formation of new vessels [6]. We measured the levels of cytokines and growth factors released during the inflammatory phase into the SF from RA and PsA patients using OA patients as non-inflammatory controls (Table 1). Levels of the inflammatory and chemotactic cytokines IL-1β, IL-6 and IL-8 in RA were higher than in PsA patients, while those in OA patients were barely detectable, as previously shown [24]. Levels of IL-10, which plays a diverse role in angiogenesis, were also higher in SFs from RA patients than in PsA patients, again with barely detectable levels in OA patients. In contrast, synovial concentrations of the pro-angiogenic mediators MCP-1 and TGFβ did not differ among groups. VEGF, the main mediator of angiogenesis, is found in the SF and serum of patients with inflammatory arthritis, and its expression correlates with disease severity [25]. Although synovial VEGF levels did not differ among groups, levels in female RA patients were significantly lower than in males (Table 1). Concentrations of all other inflammatory mediators tested were comparable between males and females, consistent with the lack of gender differences in WBC and PMN counts.

3.2. Metalloproteinase activity in synovial fluids from patients with inflammatory arthritis

Angiogenesis involves migration of endothelial cells into surrounding stroma/tissues: in this context, MMPs produced by cells in the inflammatory microenvironment are critically important [26]. Moreover, elevated levels of MMP-2 and MMP-9 have been detected in the SF of RA and PsA patients, and could play a role in joint tissue damage and pathological angiogenesis [27–29].

Gelatine zymography revealed that all SF samples from RA and PsA patients comprised MMP-9 (triplet of 92, 130, and 225 kDa) and MMP-2 (72 kDa; Fig. 1A). Levels of MMP-9 in the SFs of RA patients were higher than in PsA patients (Fig. 1B–D). By contrast, levels of MMP-2 in the SFs from RA and PsA patients were similar to those in OA patients (Fig. 1E). The presence of the 72-kDa MMP-2 in all samples may reflect constitutive expression in the synovial compartment. MMP-2 was the only MMP present in SFs from OA patients (Fig. 1A), which contained very few PMNs. To further explore the relevance of MMP-9, the major isoform expressed by PMNs [26], synovial concentration was measured by ELISA. MMP-9 was significantly higher in SFs from RA than in PsA patients, with only barely detectable levels in SF from OA patients (Table 1). The widely ranging % PMN in SFs (15–80 %) was significantly associated with the amount of each MMP-9 forms as measured by zymography (Fig. 2A–C) as well as with the MMP-9 concentration as measured by ELISA (*data not shown*). Correlations between synovial MMP-9 and IL-1β (Fig. 2D), IL-6 (Fig. 2E) and IL-8 (Fig. 2F)

Table 1
Leukocyte counts and concentrations of cytokines, growth factors and MMP-9 in the synovial fluid from inflammatory arthritis and osteoarthritis patients including sex-disaggregated analysis for inflammatory arthritis.

				Female		Male	
	RA	PsA	OA	RA	PsA	RA	PsA
Patients, *n*	8	9	3–6	5	4	3	5
WBC, n*10^3/mm^3 (range)	18 ± 3 (12–32)	14 ± 2 (7–27)	~ 0.1	19 (12–32)	13 (7–21)	16 (12–23)	15 (8–27)
PMN, % (range)	81 ± 3 (68–92)	59 ± 8 (15–86)	~ 0.1	78 (68–86)	57 (40–86)	85 (74–92)	60 (15–82)
IL-1β, pg/mL (range)	10.6 ± 2.7 (3.9–23.0) *,°°	1.9 ± 0.5 (0.0–5.3) *	N.D.	9.6 ± 3.6 (3.4–23)	2.0 ± 1.2 (0.0–5.3)	12.3 ± 4.9 (3.9–20.9)	1.9 ± 0.3 (1.0–2.7)
IL-6, ng/mL (range)	12.1 ± 2.9 (3.9–24.6) **,°	5.0 ± 1.7 (1.0–16.3) *	0.3 ± 0.1 (0.1–0.4)	13.6 ± 4.4 (4.6–24.6)	7.6 ± 3.5 (1.0–16.3)	9.5 ± 3.2 (3.9–15.0)	2.9 ± 0.8 (1.1–5.4)
IL-8, pg/mL (range)	619 ± 186 (300–1654) *,°	228 ± 48 (29–443) **	13 ± 3.0 (6–19)	548 ± 284 (61–1654)	196 ± 90 (29–443)	737 ± 209 (375–1098)	253 ± 57 (69–367)
IL-10, pg/mL (range)	18.5 ± 3.3 (1.6–27.2) **,°°	6.8 ± 1.5 (1.0–16.4) *	0.4 ± 0.3 (0.0–1.0)	14.3 ± 4.2 (1.6–26.8)	7.3 ± 0.9 (4.8–8.8)	25.6 ± 0.9 (24.1–27.2)	6.3 ± 2.8 (1.0–16.4)
MCP-1, pg/mL (range)	396.4 ± 157 (23–1171)	166 ± 63 (31–535)	152 ± 51 (16–279)	313 ± 217 (23–1171)	109 ± 64 (31–298)	536 ± 241 (117–952)	212 ± 104 (38–535)
TGF-β, ng/mL (range)	4.9 ± 1.0 (2.3–10.6)	3.7 ± 0.4 (2.3–5.9)	5.2 ± 2.6 (2.4–10.4)	4.9 ± 1.5 (2.3–11)	3.2 ± 0.4 (2.3–4.2)	4.9 ± 1.2 (2.7–6.9)	4.0 ± 0.7 (2.8–5.9)
VEGF, pg/mL (range)	848 ± 214 (303–2167)	486 ± 105 (238–1264)	505 ± 119 (185–753)	501 ± 71 (303–695)§	610 ± 223 (255–1264)	1425 ± 380 (914–2167)	387 ± 67 (271–547)
MMP-9, ng/mL (range)	1127 ± 278 (137–2674) **,°°	297 ± 96 (78–1074) *	0.1 ± 0.1 (0.02–0.17)	665 ± 158 (137–1090)§	363 ± 237 (109–1074)	1898 ± 405 (1310–2674)	254 ± 63 (78–496)

RA, rheumatoid arthritis; PsA, psoriatic arthritis; OA, osteoarthritis; IL, interleukin; WBC, white blood cells; PMN, polymorphonuclear leukocytes; MCP-1, monocyte chemoattractant protein 1; TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factor; MMP-9, metalloproteinase 9; N.D., not detectable
* $p < 0.05$ *vs* OA; ** $p < 0.01$ *vs* OA; °$p < 0.05$ *vs* PsA; °°$p < 0.01$ *vs* PsA; *t*-test; § $p < 0.05$ *vs* Male, *t*-test.

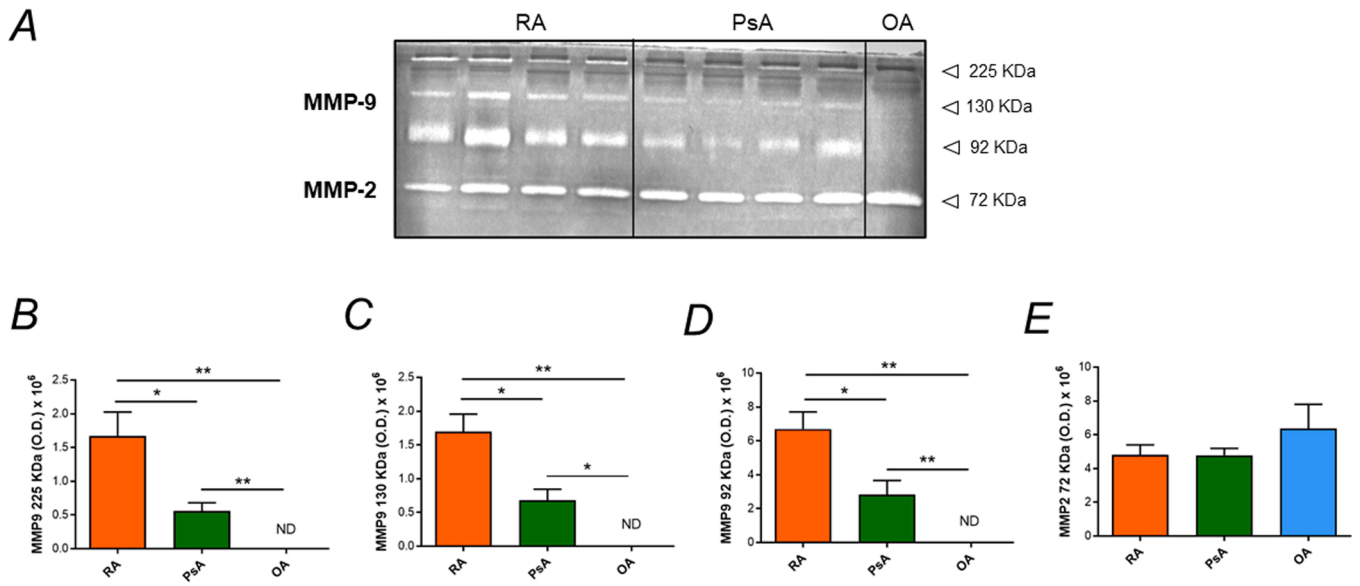

Fig. 1. Gelatine zymography of SF samples from inflammatory arthritis patients. MMP-9 and MMP-2 levels were determined as described in Materials and Methods. A. Representative gel. B-E. Levels of MMP-9 225 kDa (B), MMP-9 130 kDa (C), MMP-9 92 kDa (D), and MMP-2 72 kDa (E) in SFs from RA ($n = 4$), PsA ($n = 4$) and OA ($n = 3$) patients. Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *t*-test. RA, rheumatoid arthritis; PsA, psoriatic arthritis; OA, osteoarthritis; ND, non-detectable.

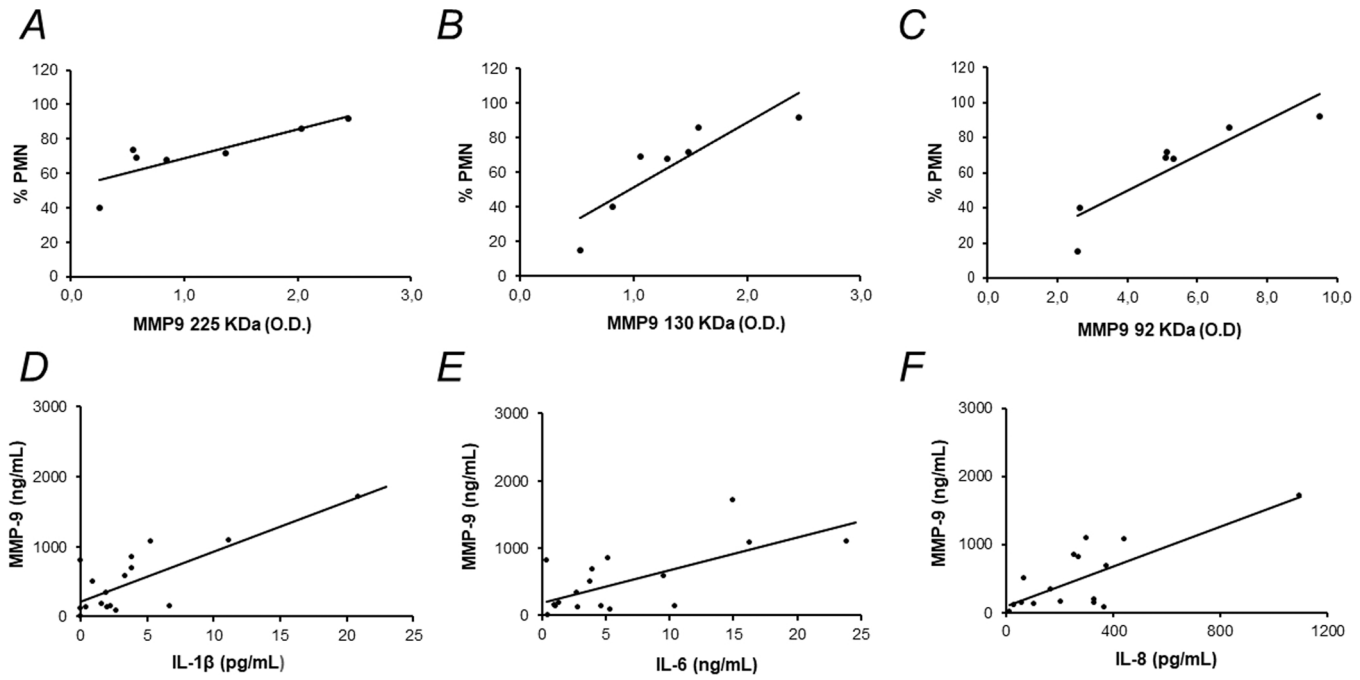

Fig. 2. Correlation between MMP-9 abundance and percentage of PMN or concentration of proangiogenic cytokines in SFs from chronic arthritis patients. PMN levels in the SFs of RA and PsA patients correlated with 225-KDa MMP-9 (A, $n = 7$; Pearson correlation coefficient = 0.83, $p = 0.02$), 130-KDa MMP-9 (B, $n = 7$; Pearson correlation coefficient = 0.9, $p = 0.008$) and 92-KDa MMP-9 (C, $n = 7$; Pearson correlation coefficient = 0.9, $p = 0.007$) as measured by zymography. Synovial concentration of MMP-9 as measured by ELISA correlated with synovial concentrations of IL-1β (D, $n = 17$; Pearson correlation coefficient = 0.8, $p = 0.001$), IL-6 (E, $n = 17$; Pearson correlation coefficient = 0.7, $p = 0.003$) and IL-8 (F, $n = 16$; Pearson correlation coefficient = 0.8, $p = 0.001$).

concentrations were also significant.

Sex-disaggregated analysis of synovial MMP data in RA patients ($n = 5$ females and $n = 3$ males) revealed for the first time, to the best of our knowledge, that levels of all three MMP-9 forms in males were higher than in females (Fig. 3). Accordingly, MMP-9 concentration in the SF from RA patients was about 2.5-fold higher in males than in females ($p < 0.05$, Table 1), suggesting a possible role for this enzyme as a sex-specific biomarker of disease severity. Conversely, MMP-9 levels did not differ between male and female PsA patients (Fig. 3).

3.3. Synovial fluids from inflammatory arthritis patients induced in vitro angiogenesis

The angiogenic potential of HUVECs exposed to well-characterized SFs from inflammatory arthritis patients has not been assessed so far. We explored potential functional alterations induced by SFs using a

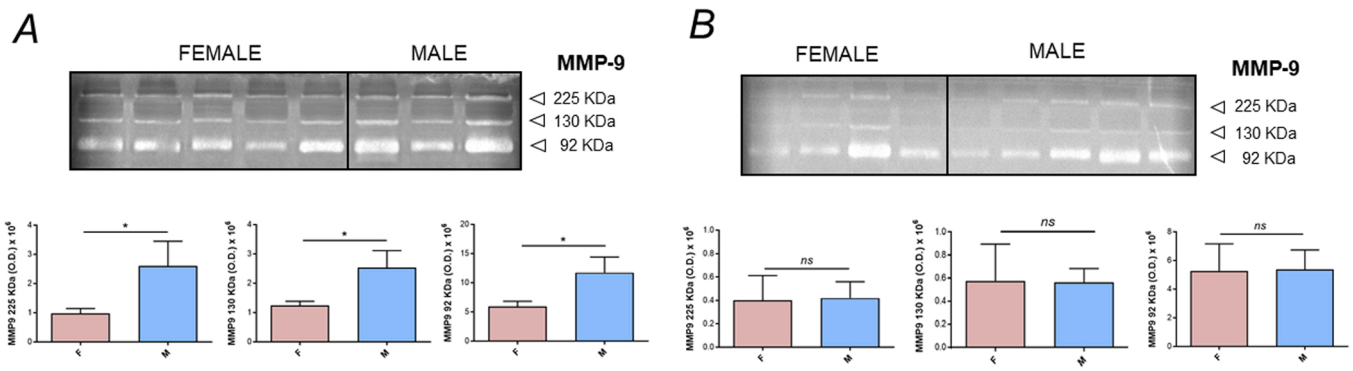

Fig. 3. Sex-disaggregated analysis of zymography of SF samples. Levels of 225-KDa, 130-KDa and 92-KDa MMP-9 were assessed in the SFs of RA (A) and PsA patients (B). The assay was conducted as described in the legend to Fig. 1. Data are expressed as mean ± SEM of 3 male and 5 female RA patients (A), and 5 male and 4 female PsA patients (B). * $p < 0.05$, *t*-test. F, female; M, male.

number of *in vitro* experimental approaches that mimic the major steps of the angiogenic process including tube formation and migration of SF-primed HUVECs [30].

Exposure to SFs from RA, PsA and OA patients for 24 h did not affect HUVEC viability using an MTT assay (Supplementary Fig. 1). Chemotaxis assays are used to measure HUVEC migration in response to an attractant gradient, an essential step in pathological synovial angiogenesis. Therefore, we investigated the effect of SFs derived from RA and PsA patients in a Boyden chamber using SFs from OA patients as controls. As shown in Fig. 4, the migration of HUVECs induced by inflamed SF from RA and PsA patients was greater than that induced by the SF from OA patients, likely due to the different composition of cytokines and chemokines. Of note, the migration induced by SF from OA patients was greater than that induced by the negative control (Fig. 4).

Tubularization recapitulates several steps of the *in vivo* angiogenic process, and can be investigated using Matrigel assays. All SFs from RA, PsA and OA patients induced the formation of capillary-like structures compared to negative control. As shown in Fig. 5, most topological parameters (junctions, master segments, meshes and total mesh area) of the capillary-like network were comparable among groups, as observed in earlier studies using SFs from RA and OA patients [31].

In view of gender differences in synovial VEGF and MMP-9 levels as described in Section 3.2, we compared the angiogenic response of fHUVECs and mHUVECs when exposed to the same SF from inflammatory arthritis patients (Fig. 6). fHUVECs migrated more than mHUVECs (both $n = 5$) in response to SF from RA (Fig. 6A) and PsA patients (Fig. 6B). Sexual dimorphism was also detectable in the Matrigel assay. In fact, the formation of capillary like-structures was significantly enhanced in fHUVECs *vs*. mHUVECs (both $n = 5$) exposed to SFs from RA (Fig. 7A, B) and PsA patients (Fig. 7A, C). After testing the differences in arthritic angiogenesis due to the sex of the HUVECs, we tested the role of the sex of arthritic SF donors. Selected experiments showed that: mHUVEC migrated equally to male RA SF as female RA SF (Supplemental Fig. 2); fHUVEC had similar tube formation in response to male and female RA SF (Supplemental Fig. 3); mHUVEC migrated to male RA SF and fHUVEC migrated to female RA SF, and fHUVECs still out-migrated mHUVECs (Supplemental Fig. 4). Thus, despite higher synovial levels of proangiogenic factors in male RA patients, the response of vascular endothelium to the SF microenvironment was more intense in cells from female donors. Such a sexual dimorphism may be relevant in inflammatory arthritis pathogenesis and/or progression.

3.4. Pharmacological control of synovial fluid-induced angiogenesis

We previously reported that the cardiac glycoside digitoxin inhibits angiogenesis *in vitro* and *in vivo* in a sex-specific fashion [19] *via* inhibition of protein tyrosine kinase 2 (FAK), a key player in cell migration control [20]. Therefore, we investigated the effect of digitoxin at a concentration within the therapeutic range on HUVEC migration, using SFs from inflammatory arthritis patients as chemoattractant stimuli. As shown in Fig. 8A–B, treatment with 25 nM digitoxin reduced HUVEC migration induced by inflamed SFs from RA and PsA patients. Additional experiments were performed to test whether digitoxin would equally impact the effects of male or female derived RA SFs on cells from the same donor. As shown in Fig. 8C, the effect of digitoxin was comparable when HUVECs were exposed to male or female derived RA synovial fluids. Digitoxin treatment also inhibited tubularization of HUVECs seeded in Matrigel and primed by SF samples from RA (Fig. 9A,B) and

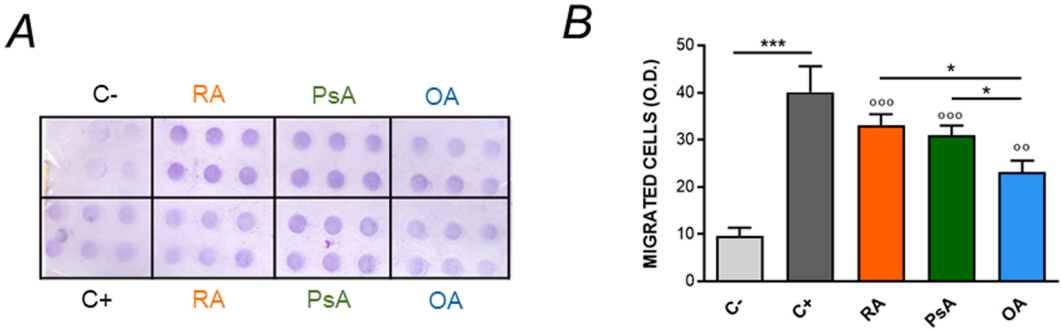

Fig. 4. Migration of pooled HUVECs exposed to SFs from patients with inflammatory arthritis. A. Representative images of migrated HUVECs on the bottom of a filter membrane of a modified 48-well Boyden chamber after 6 h incubation at 37 °C as detailed in Materials and Methods. M199 supplemented with 15 % FBS, ECGF (100 μg/mL) and heparin was used as a positive control (C+), while M199 supplemented with 1 % FBS and heparin was used as a negative control (C-). B. Migration of HUVECs induced by SF of RA ($n = 7$), PsA ($n = 9$) and OA ($n = 3$) patients, the latter being used as non-inflammatory controls. Each SF sample was tested in sextuplicate. Cell migration is shown as optical density (O.D) values. Data are expressed as mean ± SEM of 5 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *t*-test; °°$p < 0.01$, °°°$p < 0.001$ *vs* C-, *t*-test. RA, rheumatoid arthritis; PsA, psoriatic arthritis; OA, osteoarthritis.

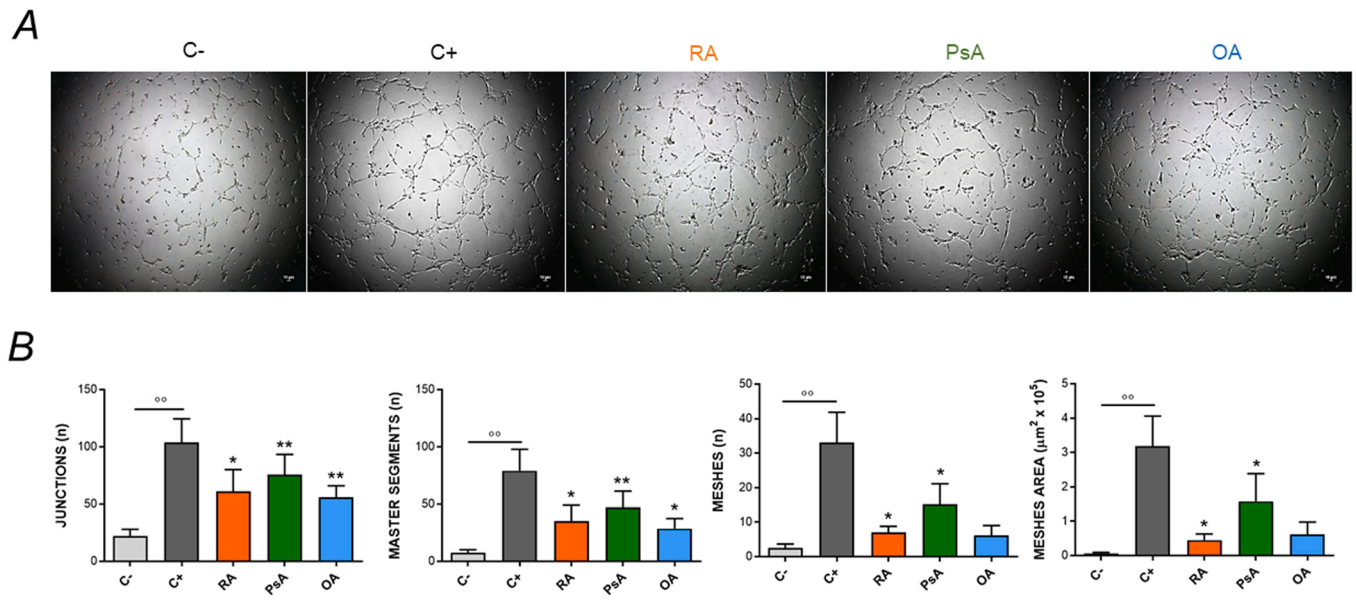

Fig. 5. Capillary tube formation in pooled HUVECs exposed to SFs from patients with inflammatory arthritis. A. Representative images of Matrigel assay using SF samples at 15 % dilution. M199 supplemented with 15 % FBS and heparin was used as a positive control (C+), while M199 supplemented with heparin was used as a negative control (C-). One micrograph image per well was taken after 4-h incubation; scale bar: 10 μm. B. Specific parameters of capillary tube formation (junction, master segment, meshes and meshes area) following exposure to SF from RA, PsA and OA (all $n = 6$) patients as measured using Angiogenesis Analyser (ImageJ). Data are expressed as mean ± SEM of 6 independent experiments. * $p < 0.05$, ** $p < 0.01$ *vs* C-, *t*-test; °$p < 0.05$, °°$p < 0.01$, *t*-test. RA, rheumatoid arthritis; PsA, psoriatic arthritis; OA, osteoarthritis.

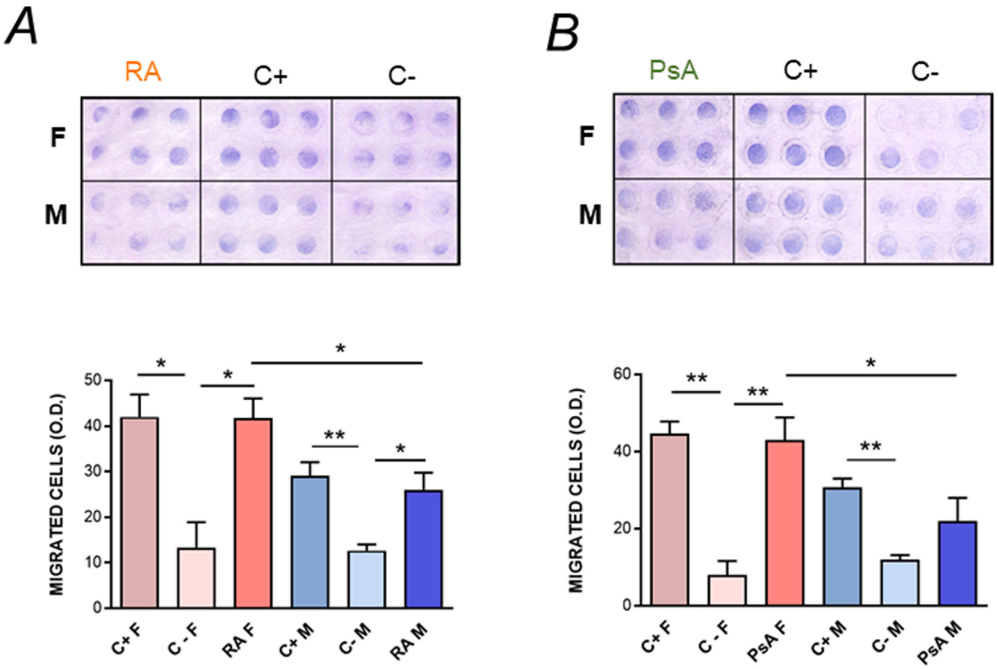

Fig. 6. Effect of HUVEC donor's sex on migration in a chemotaxis assay upon exposure to SF from patients with RA (A) and PsA (B) as a chemoattractant agent. HUVECs from male and female donors (both $n = 5$) were exposed to the SF from the same RA or PsA (both $n = 3$) patients in each independent experiment. Experiments were performed as described in the legend to Fig. 4. *Upper panels:* Representative images of migrated cells. *Lower panels:* Migration of HUVECs in response to controls and SFs shown as optical density (O.D) values. Each SF sample was tested in sextuplicate. Data are expressed as mean ± SEM of 5 independent experiments. * $p < 0.05$, ** $p < 0.01$; *t*-test. RA, rheumatoid arthritis; PsA, psoriatic arthritis; F, female; M, male.

PsA patients (Fig. 9A,C). These findings suggest that the effect of digitoxin is independent from the pro-angiogenic trigger.

To confirm the mechanism of anti-angiogenic effect, HUVECs were exposed to SFs from RA and PsA patients in the presence of digitoxin to assess tyrosine phosphorylation as an index of FAK activation. Treatment with the drug for 6 h prevented the accumulation of phospho-FAK (Y576/577) induced by SFs from patients with RA (Fig. 10A) and PsA (Fig. 10B); there was also a trend to a decrease at the earlier time point in RA.

4. Discussion

Inflammatory arthritis forms such as RA and PsA are chronic systemic disorders characterized by synovitis, joint erosion and pathological angiogenesis. The SF from RA and PsA patients contains a variety of pro-inflammatory factors and promotes angiogenesis [32,33]. Because the prevalence, severity and response to treatment of these conditions differ between males and females [34], we set out to investigate potential gender differences in the impact of SF from inflammatory arthritis patients on the angiogenic process. The experimental approach

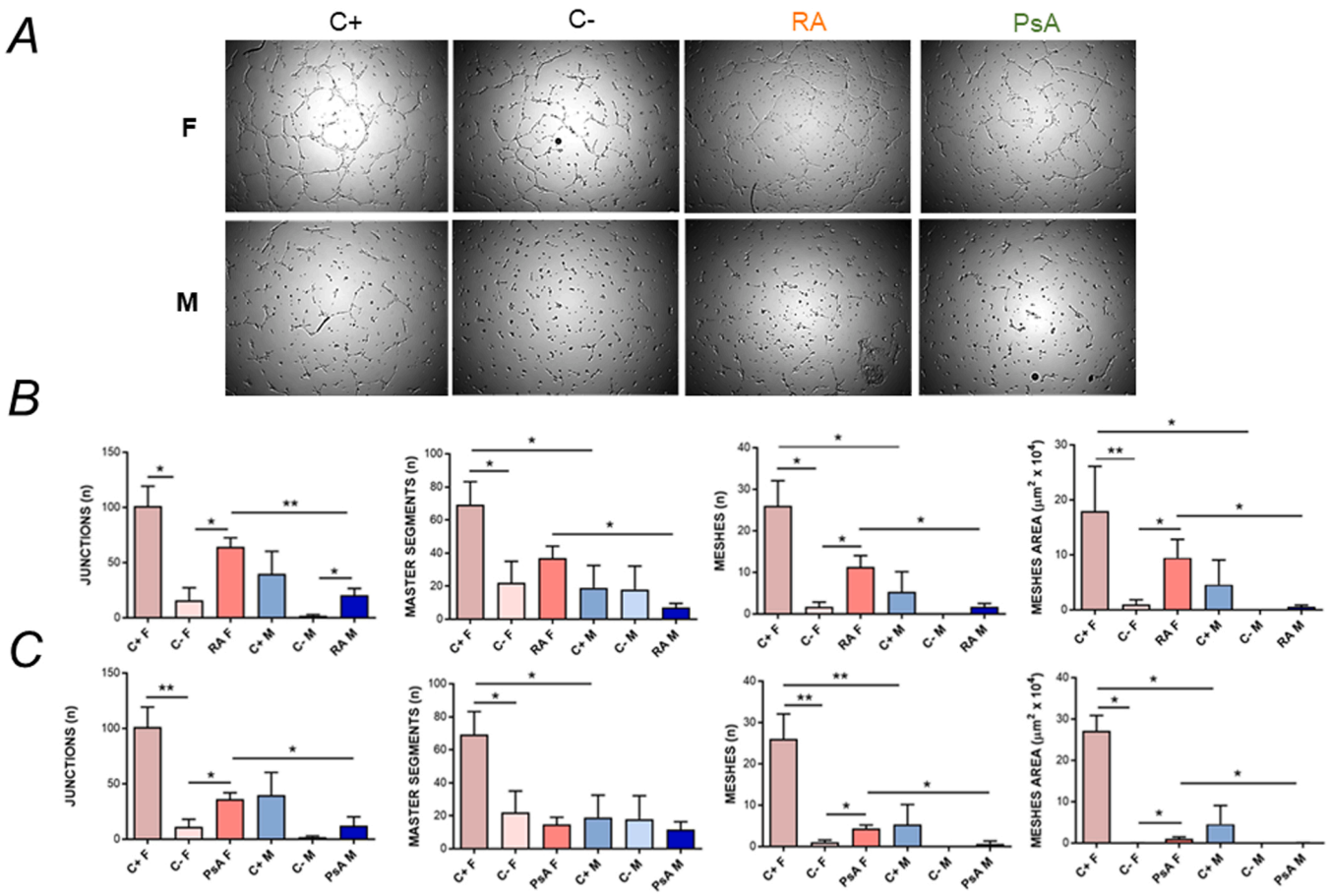

Fig. 7. Effect of HUVEC donor's sex on capillary tube-like formation upon exposure to SF from patients with inflammatory arthritis. HUVECs from male and female donors (both $n = 3$) were exposed to the SF from the same patients with RA and PsA (both $n = 5$) in each independent experiment. The assay was carried out as described in the legend to Fig. 5. A. Representative images of Matrigel assays. B, C. Specific parameters of capillary tube formation (junction, master segment, meshes and meshes area) in response to SF from patients with RA (B) and PsA (C). Data are expressed as mean ± SEM of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *t*-test. RA, rheumatoid arthritis; PsA, psoriatic arthritis; F, female; M, male.

was two-fold: in a first aspect, we characterized the composition and biological effects of the SF from male and female patients on pooled HUVECs; in a second aspect, we tested the biological effects of the same SF samples on HUVECs from male *vs*. female donors.

No gender differences in WBC and PMN counts were observed in inflamed SFs from of RA and PsA patients. Similarly, the synovial concentration of cytokines and growth factors was comparable except for VEGF, which was almost three times as abundant in SFs from male as in female RA patients. To the best of our knowledge, this had not been reported before. The dual activities of VEGF as an endothelial cell mitogen and a modulator of changes in vascular permeability are relevant to the pathogenesis of RA and PsA [6,13]. In addition, there is evidence of sex-specific outcomes in cancer patients treated with the anti-VEGF antibody bevacizumab [reviewed in 14]. In a real-world study at an ophthalmological hospital, women with diabetic macular oedema have been found to display less visual improvement with anti-VEGF medications than men [35], suggesting a sexually dimorphic pathophysiological role of VEGF in different settings.

synovial pro-angiogenic cytokines, VEGF and MMPs modulate angiogenesis, which involves migration/invasion of endothelial cells into surrounding stroma/tissues. In this context, MMP-9 and 2 are critically important: a pivotal role of MMP-9 has been reported in the development of inflammatory joint disease [36]. Previous studies showed that total MMP-9 levels are significantly associated with VEGF levels in RA, but not in OA SFs [37]. Because VEGF stimulates the synovial membrane to secrete MMP-9, the proangiogenic properties of these factors have been suggested to be interdependent [38]. SF samples from RA and PsA patients in the present study comprised solely MMP-9 (triplet of 92, 130, and 225 kDa) and latent fibroblast-derived MMP-2 (72 kDa). The three MMP-9 bands correspond to MMP-9 trimer, neutrophil gelatinase-associated lipocalin (NGAL)-MMP-9 complex and pro MMP-9, respectively [39]. Of note, the proteolytic activity of each MMP-9 form was associated specifically with PMN infiltration and levels of the proangiogenic cytokines IL-1β, IL-6 and IL-8. In contrast, MMP-2 was the only MMP detected in SFs from OA patients. As OA SFs contained very few PMNs, MMP-2 was most likely released by resident fibroblasts and endothelial cells [40]. Our findings are also in line with the study by Makowski and colleagues [41], who reported the presence of MMP-2 and MMP-9 in SFs in proportion to PMN infiltration. However, the relative contribution of endothelium-derived MMP functional expression in SFs [42] could not be assessed in our study. In addition, other MMPs are involved in RA or PsA pathogenesis [43,44]. We also found higher synovial MMP-9 levels and activity in male as compared with female RA patients. This is consistent with reports that erosive disease and nodules in RA are more prevalent and develop earlier in men than in women [45,46]. Although the sample size of our study is limited, these observations highlight MMP-9 as a sex-specific disease biomarker in RA to be validated in larger studies with clinical endpoints. Eder et al. reported that men with PsA are more likely to develop axial involvement and radiographic joint damage [47]; our results, however, apparently rule out gender differences in synovial cytokine, growth factor or MMP levels as a mechanism underlying such clinical observations.

Inflammatory arthritic diseases have been associated with altered angiogenesis that promote the development of the hyperplasic

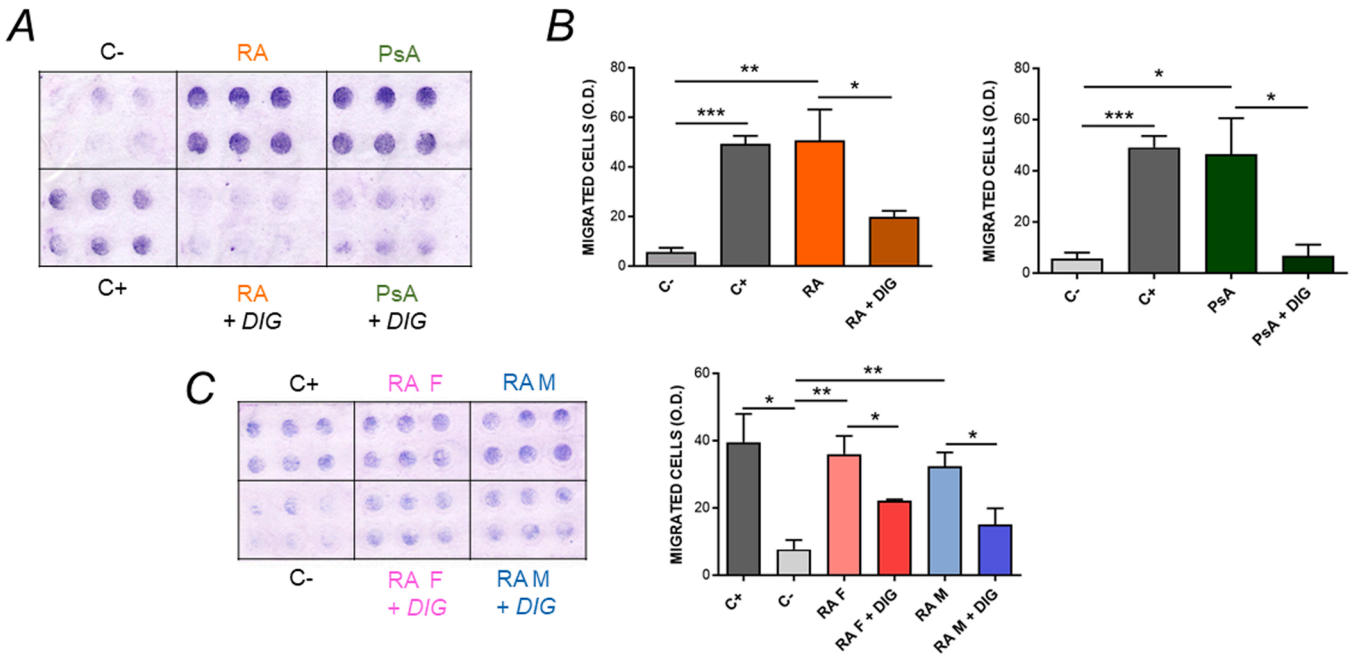

Fig. 8. Migration of HUVECs exposed to SFs from patients with inflammatory arthritis in the presence or absence of 25 nM digitoxin for 6 h. The assay was carried out as described in the legend to Fig. 4. A. Representative assay. B. Effect of digitoxin on pooled HUVEC migration induced by inflamed SFs of RA (*left panel*) and PsA (*right panel*) patients. Each SF sample was tested in sextuplicate. C. Representative assay (*left panel*) and bar graph (*right panel*) showing the effect of digitoxin on female HUVEC migration induced by male ($n = 3$) or female ($n = 4$) derived RA SFs. Migration is shown as optical density values (O.D). Data are expressed as mean ± SEM of 3–4 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *t-test*; °°$p < 0.01$, °°°$p < 0.001$ *vs.* C-, *t*-test. RA, rheumatoid arthritis; PsA, psoriatic arthritis; DIG, digitoxin.

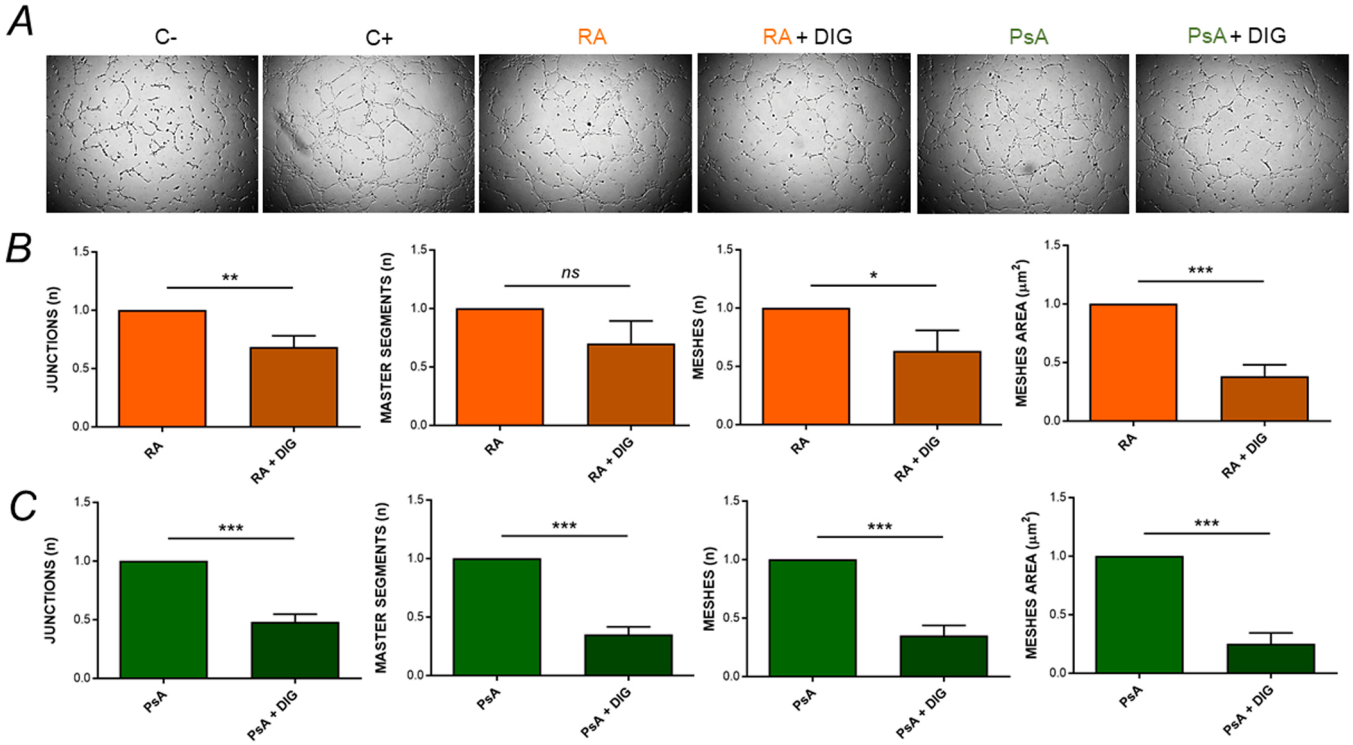

Fig. 9. Capillary tube formation in pooled HUVECs exposed to SFs from patients with inflammatory arthritis in the presence or absence of 25 nM digitoxin for 4 h. The assay was carried out as described in the legend to Fig. 5. A. Representative images of Matrigel assays. B. Formation of capillary like-structures induced by SF of RA patients ($n = 6$). Specific parameters of capillary tube formation (junction, master segment, meshes and meshes area) were measured using Angiogenesis Analyser (ImageJ). Values in the RA group were set as 1. Data are expressed as mean ± SEM of 6 independent experiments. * $p < 0.05$, *** $p < 0.001$, *t*-test. C. Formation of capillary-like structures induced by SFs of PsA patients ($n = 6$). Values in the PsA group were set as 1. Data are expressed as mean ± SEM of 6 independent experiments. ** $p < 0.01$, *** $p < 0.001$, *ns*, non-significant; *t*-test. RA, rheumatoid arthritis; PsA, psoriatic arthritis; DIG, digitoxin.

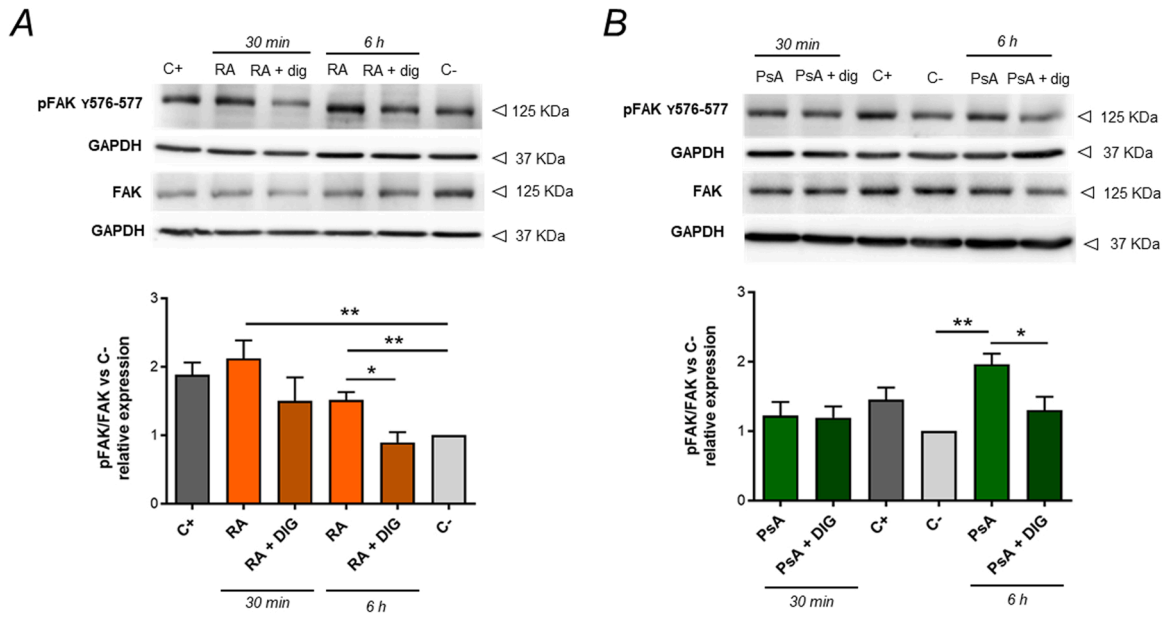

Fig. 10. Effect of SF from patients with RA (A) and PsA (B) on FAK activation at different time points in the presence and absence of 25 nM digitoxin. HUVECs from female donors were seeded in 35-mm dishes in M199 complete medium and stimulated with 10 % SF in serum-free M199 (no growth factors added). p-FAK levels were measured after 30 min and 6 h. *Upper panels*: Representative Western blot showing the abundance of FAK and p-FAK (Y576/577); GAPDH was used as loading control. *Lower panels*: Densitometry analysis of bands normalized to GAPDH levels. Values in the C- group were set as 1 (relative expression). Data are shown as the mean ± SEM of 3–4 independent experiments using SFs from RA and PsA patients (both $n = 3$). * $p < 0.05$, ** $p < 0.01$, *t*-test. RA, rheumatoid arthritis; PsA, psoriatic arthritis; DIG, digitoxin.

proliferative pathologic synovium [9]. We found that inflamed SFs derived from RA and PsA patients induced HUVEC migration and capillary network formation. Despite barely detectable levels of inflammatory and pro-angiogenic mediators, the SFs from OA patients also had the capacity to induce migration and some parameters of network formation. In fact, synovial levels of MCP-1, TGF-β and VEGF were comparable in the three groups. These findings also point out that the responsiveness of vascular endothelium to the complex biological matrix of SF from an arthritic joint may well differ from the responsiveness to individual agents when tested separately. It is worth noting that early morphological changes in endothelial cell cultures exposed to SFs are associated with a significantly longer duration of disease in patients with RA [31].

Of note, HUVECs from female donors migrated more than those from male donors in response to the SF from the same patients. A similar finding was reported in the context of VEGF-induced angiogenesis [48]. Topological parameters of the capillary-like network were also markedly enhanced in inflamed SF-exposed HUVECs from female compared to male donors. This agrees with previous reports from our group and others, pointing to sex as a key determinant of endothelial function [19,49]. The angiogenic potential of HUVECs from male donors was attenuated with respect to HUVECs from female donors when exposed to the same inflamed SFs despite higher synovial VEGF and MMP-9 levels in male RA patients (Table 1). This sexually dimorphic angiogenic capacity was also observed in response to PsA SFs, whose composition did not differ between male and female patients, and was maintained in the subgroup analysis of sex-disaggregated SFs (*data not shown*). This suggests that the endothelial genotype plays a more relevant role in gender differences in pathological angiogenesis of inflammatory arthritis with respect to the synovial microenvironment.

Finally, we explored the effect of an established anti-angiogenic pharmacological agent on SF-induced angiogenesis. Digitoxin treatment at therapeutic concentrations attenuated HUVEC migration and tubule network formation induced by inflamed SFs. The SF from RA and PsA patients induced tyrosine phosphorylation of FAK, which was prevented by digitoxin treatment at different time points. This is consistent with earlier studies using different activating stimuli [19,20], and suggests that the effect of digitoxin is independent from angiogenic trigger (s). It is conceivable that agents targeting shared upstream steps of signaling pathways relevant in SF-induced angiogenesis are endowed with improved efficacy.

The main limitation of this study is that it was performed in a small number of patients. In addition, access to patients' demographic and clinical data was incomplete. Therefore, conclusions made about sex differences do not rule out differences in other factors. HUVECs are derived from normal umbilical cords and have no relevance to the arthritic joint, whereas the RA and PsA SFs are from diseased patients. However, while confirmation in mature endothelial cell models is required, the findings of our study suggest that endothelial biology is more relevant than the sex of SF donors in shaping the angiogenic response in diseased patients. The present findings may also lay the ground for further studies with larger sample size and clinical endpoints.

In conclusion, gender differences in the composition of SFs from patients with different forms of chronic arthritis were limited, except for higher VEGF and MMP-9 levels in male *vs.* female RA patients. Inflamed SFs triggered *in vitro* angiogenesis as observed in migration and network formation assays, which was prevented by digitoxin treatment. HUVECs from female donors were significantly more responsive to inflamed SF exposure with respect to those from male donors, suggesting that gender differences in endothelial function are relevant to inflammatory arthritis pathogenesis and/or progression. These findings may have implications for pathogenesis and targeted therapy of chronic arthropathies.

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Conflict of interest statement

None to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113181.

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