

**SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
(PHD)**

**DOWNSTREAM EFFECTS OF TARGETING ANGIOGENESIS IN THE
INFLAMED JOINT AND SYSTEMIC VASCULATURE IN
INFLAMMATORY ARTHRITIS**

Emese Balogh, MD

Supervisor: Prof. Zoltán Szekanecz, MD, PhD, DSc



**UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF CLINICAL MEDICINE**

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Downstream effects of targeting angiogenesis in the inflamed joint and systemic vasculature in inflammatory arthritis

By Emese Balogh, MD

Supervisor: Prof. Dr. Zoltán Szekaneecz, MD, PhD, DSc

Doctoral School of Clinical Medicine, University of Debrecen

Head of the **Examination Committee**: Prof. Dr. Norbert Németh, MD, PhD, DSc

Members of the Examination Committee: Prof. Dr. Edit Bodolay, MD, PhD, DSc

Prof. Dr. György Nagy, MD, PhD, DSc

The Examination takes place at the Department of Rheumatology 1st floor Library (Faculty of Medicine and Division of Rheumatology) on Friday, 22nd of November 2019, at 11 am.

Head of the **Defense Committee**: Prof. Dr. Norbert Németh, MD, PhD, DSc

Reviewers: Dr. Miklós Fagyas, MD, PhD

Prof. Dr. András Komócsi, MD, PhD, DSc

Members of the Defense Committee: Prof. Dr. Edit Bodolay, MD, PhD, DSc

Prof. Dr. György Nagy, MD, PhD, DSc

The PhD Defense takes place at the Faculty Conference Centre (Kenézy Villa) on Friday, 22nd of November 2019, at 13:00 pm.

1 INTRODUCTION

1.1 The endothelium and the angiogenic process in inflammation

1.1.1 Endothelial pathology in the inflamed joint in inflammatory arthritis

Angiogenesis is the formation of new capillaries from pre-existing blood vessels and needs to be differentiated from another neovasculatory process termed vasculogenesis, where capillary sprouting originates from pre-existing precursors (endothelial precursor cells – EPCs) and finally forms a primitive vascular network. Inflammatory arthritis is one of the prototypic diseases where angiogenesis plays an important role in the morphological alterations of the vascular endothelial integrity. This is characterized by a cascade of multiple events where existing blood vessels grow new capillary sprouts in a dysregulated fashion. The imbalance between positive and negative angiogenic regulators drive the process with constant remodeling in a tightly controlled step by step manner. Angiogenic mediators activate endothelial cells through their receptors switching various signal transduction pathways on. Proteases are produced by activated cells and degrade the basement membrane of the endothelium and the interstitium resulting in a leaky endothel. Critical changes such as vasodilation and increased permeability promotes vascular injury and regeneration. Endothelial cells migrate to form new capillary sprouts. Some of them proliferate and undergo intensive mitosis in the midsection of the sprout, whereas others at the tip of the sprout only migrate. Lumen forms when two sprouts anastomose with each other into a capillary loop. Tube formation is finalized by new basement membrane synthesis and vessel stabilization through pericyte recruitment. Efficiency of oxygen supply to the synovium is poor due to the highly dysregulated synovial microvasculature. This along with the increased energy demands of activated infiltrating immune cells and inflamed resident cells leads to an hypoxic microenvironment as well as mitochondrial dysfunction.

1.1.2 Local angiogenic mediators in the joint

Several inflammatory cytokines, chemokines, chemokine receptors, proteases, matrix molecules, growth factors and cell adhesion molecules have been recognised in the neovascularisation of the inflamed tissue. In this section we are going to discuss about those that are involved in the perpetuation and maintenance of the angiogenic process.

The process of vessel formation starts with abundant production of *growth factors (GF)*, high levels of vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang2) that interact to control angiogenesis by inducing endothelial cell proliferation and sprouting. VEGF has outstanding importance from factors regulating new vessel formation. Ang2 acts as a partial agonist to the receptor tyrosine kinase-2 (Tie2) and by competing with angiopoietin-1 (Ang1) for Tie2 binding subsequently replaces the full agonist activity of Ang1 with a much lower activity. The relative balance of Ang2-Ang1 determines the activation state of Tie2 and Ang2 eventually acts towards the inhibition of vessel maturation and in the presence of VEGF promotes abnormal capillary sprouting. Several VEGF isoforms exist, VEGF-A being considered the major regulator in angiogenesis. Other regulators that act through either a VEGF dependent or independent path are interleukins (IL), such as IL-6, IL-17, IL-18, nitric-oxide, endothelin-1, monocyte/macrophage migration inhibitory factor (MIF), placenta GF (PIGF), fibroblast GF (FGF-1 and FGF-2), epidermal GF (EGF), hepatocyte GF (HGF), heparin-binding endothelial growth factor (HB-EGF), keratinocyte GF (KGF), insulin-like GF (IGF-1), connective tissue GF (CTGF), platelet-derived GF (PDGF), and transforming GF- β (TGF- β). Subsequently, as the vessel matures, at later stages angiopoietin-1 (Ang1) becomes important, recruiting pericytes into the newly formed basement membrane to facilitate the blood flow process.

The main *pro-inflammatory and pro-angiogenic cytokines* are Tumor Necrosis Factor- α (TNF- α), IL-1, IL-6, IL-8, IL-15, IL-17, IL-18, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage-colony stimulating factor (GM-CSF), and oncostatin M may also exert direct effect on the endothelium or act indirectly with via promoting excretion of pro-angiogenic molecules from inflammatory cell types.

Chemokines and chemokine receptors also have a well defined role in inflammatory angiogenesis and many has been implicated in inflammatory arthritis from the CXC, CC, C and CX3C families. Some of them are highly expressed on synovial tissue. Chemokines may also act indirectly to promote angiogenesis through the attraction of immune cells to the site of inflammation.

Extracellular matrix (ECM) components and cell adhesion molecules (CAMs) mediate and thrombospondin-1 (TSP-1) is a glycoprotein that regulates endothelial cell adhesion and neovascularization via various intracellular signaling pathways. Some growth factors bind to proteoglycans during angiogenesis.

Proteases digest the ECM, release growth factors and other angiogenic mediators and thus promote inflammatory angiogenesis.

Further important angiogenic factors are not described in this thesis due to little relevance to the author's research activity.

1.1.3 Regulatory networks in synovial angiogenesis

The *hypoxia-HIF-VEGF-Ang/Tie* system is of outstanding importance in inflammatory arthritis associated angiogenesis. Hypoxia is defined as cellular demand for molecular oxygen that exceeds the vascular supply, leading to a bioenergetic crisis. Explanatory mechanisms for hypoxia in the rheumatoid synovium were postulated by three hypotheses: On one hand capillary closure is mediated by synovial hyperplasia, synovial effusion and joint movements within a rigid capsule; secondly the metabolic demand increases due migration, proliferation and distance increment between proliferating cells and nearby blood vessels; then the expression of angiotensin converting enzyme (ACE) induces the formation of angiotensin II which is responsible for vasoconstriction and enhanced hypoxia. The angiogenic neovascular network is dysfunctional in arthritis and fails to restore tissue oxygen homeostasis leaving the inflamed synovial tissue and synovial fluid markedly hypoxic. This serves an angiogenic drive in the inflammatory tissue and has been associated with disease activity and increased expression of angiogenic VEGF. Furthermore, hypoxia enables abnormal cellular metabolism, and mitochondrial dysfunction with overproduction of reactive oxygen species (ROS) and perpetuation of inflammation, which ultimately leads to the outgrowth of immature, unstable microvasculature. Angiogenic factors such as VEGF is induced by hypoxia and hypoxia inducible factors (HIF-1 and HIF-2) in RA. HIF is a heterodimeric transcription factor and acts as a key regulator in the induction of the angiogenetic process. HIF is composed of HIF- α and HIF- β subunits. Among the three isoforms of HIF- α subunits, HIF-1 α and HIF-2 α share structural and functional similarities and in hypoxic conditions they are able to translocate to the nucleus and dimerize with HIF-1 β . This heterodimer binds to hypoxia-response elements (HRE) enabling the transcription of HIF-dependent genes. One of the most important target genes containing HREs is VEGF. Under normoxic conditions prolyl-hydroxylases hydroxylate HIF- α that later undergoes proteosomal degradation, while hypoxia is an inhibitor of HIF hydroxylation allowing HIF-1 α to stabilize, dimerize with HIF-1 β thus initiating the transcription of genes containing HRE. Both HIF-1 α and HIF-2 α are strongly expressed in the

rheumatoid arthritis (RA) synovium. However, hypoxia may also act via HIF-independent regulatory pathways including the peroxisome-proliferator-activated receptor- γ (PPAR γ). The Ang1/Tie2 complex interacts with VEGF during vessel stabilization in the neoangiogenesis. In contrast, Ang2, an antagonist of Ang1, inhibits vessel maturation. Both Ang1 and Tie2, as well as VEGF have been detected in the RA synovium even in very early phase of the disease. Hypoxia also stimulates the production of CXCL12, a major angiogenic chemokine described above, by RA synovial fibroblasts.

1.1.4 Targeting angiogenic mediators and angiostatic compounds

The *VEGF-dependent pathway* has been extensively targeted and VEGF or VEGF receptor (VEGFR) inhibitors have been tried mainly in cancer studies. Limited number of pre-clinical studies has also been conducted in arthritis with promising results in collagen induced arthritis (CIA) murine models. A bispecific antibody Ang2 targeting peptide genetically fused to adalimumab also enhanced anti-TNF efficacy. IF-1 may also be targeted in arthritis, as well as in cancer.

Indirect targeting is feasible via *conservative antirheumatic therapy*. Antirheumatic agents nonspecifically suppress angiogenesis. TNF- α blockade by infliximab was shown to reduce VEGF, Ang1 and Tie2 expression as well as vascularity within the RA synovium. Certolizumab pegol inhibits TNF-dependent angiogenesis. The anti-IL-6 receptor antibody tocilizumab also decreased serum levels of VEGF in RA. The JAK inhibitor tofacitinib demonstrated inhibitory effects on migration, invasion and pro-angiogenic cytokine secretion in psoriatic arthritis in vitro. IL-17 has been implicated in inflammatory angiogenesis, therefore, anti-IL-17 blockade may also be feasible in this respect. The anti-IL-6 receptor antibody tocilizumab also decreased serum levels of VEGF in RA.

Specific molecular targeting with neutralizing antibodies to various *chemokine* ligands blocked arthritis both therapeutically and preventatively in various RA animal models. Due to the complexity of the several regulatory loops in the chemokine network, most of these approaches failed in human RA.

Antibiotic derivatives such as minocyclin, fumagillin analogues, deoxyspergualin, roxithromycin and clarithromycin also inhibit angiogenesis and VEGF release.

Traditional *herb compounds* have also been implicated in angiogenesis. Scopolin, celastrol or fisetin, resveratrol, curcumin and tetramethylpyrazine in combination also appeared to inhibit the production of angiogenic cytokines in a mouse arthritis model.

1.1.5 Intracellular pathways and potential therapeutic targets

Numerous signalling pathways (NFκB, MAPK, PI3K, ICOS, JAK/STAT, FAK, Src, Spk-1, Notch, neurological signaling) have been implicated in the pathogenesis of angiogenesis in inflammatory arthritis and targeting has been trialed mainly in cancer studies, but some data are available from arthritis research as well.

1.2. Autoimmune-inflammatory atherosclerosis

1.2.1 Autoimmune atherosclerosis as a systemic manifestation of angiogenesis in inflammatory arthritis

The RA and other inflammatory conditions are associated with increased cardiovascular morbidity and mortality triggered by autoimmune atherosclerosis. Various immune cells, proinflammatory cytokines and chemokines, growth factors, proteases, soluble and cell-expressed adhesion molecules as well as other mediators play crucial role in the process of inflammatory atherosclerosis and angiogenesis. Some of the above could serve as potential biomarkers of these vascular events. Traditional as well as inflammatory risk factors predict arthritis related atherosclerosis and it is postulated that in RA the vascular pathology is driven by the perpetuated systemic inflammation. Traditional risk factors and their role will not be discussed as inflammatory pathways are the most relevant with relation to biomarkers. Numerous shared pathogenic mechanisms exist between arthritis and inflammatory atherosclerosis. Both the inflammatory synovium and the vessel wall are infiltrated by inflammatory cells promoted by endothelial activation. Synovial inflammation and the atherosclerotic plaque formation, progression and rupture are mediated by inflammatory T-cells and macrophages. Synovial inflammation is facilitated by hypoxia related neovascularisation and the production of angiogenic VEGF. Angiogenic factors and new blood vessels also indicate an increased risk for rupture. The final event of tissue degradation is mediated by matrix metalloproteinases (MMPs) in the pathology of atherosclerotic rupture and thrombosis (via erosion of the fibrous caps) as well as joint destruction. The triangle of

genetic, environmental lifestyle related factors and autoimmunity all affect synovial inflammation and atherosclerosis via triggering systemic inflammation leading to endothelial dysfunction, arterial stiffness and atherosclerosis.

1.2.2 Systemic inflammatory and angiogenic mediators in autoimmune atherosclerosis

The crucial event is the local accumulation of inflammatory cells and mediators both in synovitis and in autoimmune atherosclerosis. Many inflammatory cells and factors have been implicated in this respect and majority of pro-inflammatory mediators also induce angiogenesis. Our group and other authors also examined the role of these mediators in autoimmune atherosclerosis and cardiovascular disease (CVD). Increased neovascularisation - primarily stimulated by pro-angiogenic factors such as VEGF, PDGF, FGF and HGF – observed in the vessel wall is associated with atherosclerosis. Various pro-inflammatory cytokines, chemokines, adhesion molecules, proteases and others have been described in the synovial as well as the systemic atherosclerotic angiogenic process. A previous study documented increased levels of Ang2 and PDGF that has positively correlated with longer RA disease duration. Elevated Ang1 and Ang2 levels also showed positive correlation with increased C-reactive protein (CRP). Higher serum PDGF concentrations correlated with increased common carotid intima-media thickness (ccIMT) as well.

2 AIMS

2.1 Study 1

In this study we examine the distinct interplay between a dysregulated angiogenesis, oxidative stress, altered cellular bioenergetics and mitochondrial dysfunction in inflammatory arthritis. For this purpose RA synovial fibroblast cell (RASFC) and human umbilical vein endothelial cell (HUVEC) cultures were examined to determine:

- the detrimental effects of oxidative stress on cellular energy metabolism, including alterations in aerobic and anaerobic respiration in RASFC and HUVEC

- the effects of altered bioenergetics on ROS production, mitochondrial DNA susceptibility for point mutations and activity change of mitochondrial complexes in RASFC
- the acceleration of pro-inflammatory and pro-angiogenic responses when RASFC exposed to oxidative stress
- that oxidative stress induced RASFC activation is able to enhance pro-angiogenic profile of HUVEC
- that there is an interface between regulatory mechanisms of angiogenesis, oxidative stress and altered energy metabolism when examined on human synovial tissue of patients with RA
- that Tumor Necrosis Factor- α inhibitory (TNFi) treatment is able to reduce synovial tissue angiogenesis in patients with RA.

2.1 Study 2

In this study we examined RA and AS patients to have insight into the complexity of angiogenesis regulation in case of inflammatory arthropathies. We aimed to examine:

- the effects of one-year TNFi therapy on the production of some serum biomarkers of angiogenesis
- the angiogenic activity indicated by angiogenic biomarkers in relation to atherosclerosis, vascular pathophysiology, oral health and some other clinical parameters.

Results of this have not been published or accepted for publication yet.

3 METHODS

3.1 Study 1

3.1.1 Patient recruitment, knee arthroscopies and sample collection

Fifteen patients were recruited having actively inflamed knee joint due to RA disease activity from the Rheumatology Department of St. Vincent's University Hospital, Dublin, Ireland. All of them failed conventional disease modifying drug therapy and awaited biological treatment. Disease activity was measured with 4 variable disease activity score using swollen and tender joints from 28 joint count, patient global health assessment and CRP level (DAS28-CRP) at all timepoints. Patients underwent knee arthroscopy prior to (T0) and 3 months after initiation of biologic treatment (T3). ST biopsies were collected and primary synovial fibroblasts were isolated for histological analyses.

3.1.2 Rheumatoid arthritis synovial fibroblast (RASFC) cell culture

RASFC biopsies obtained with arthroscopy were digested with 1 mg/ml collagenase type I (Worthington Biochemical, Lakewood, NJ, USA) in Gibco RPMI 1640 medium (Thermo Fisher Scientific, Paisley, UK) for 4 hours at 37 °C in humidified air with 5 % CO₂. Dissociated cells were plated in RPMI 1640 medium supplemented with 10 % Gibco FCS (Thermo Fisher Scientific), 20 mM 4-(2hydroxyethyl)-1-piperazineethanesulfonic acid (Thermo Fisher Scientific), penicillin (100 U/ml), streptomycin (100 U/ml) and amphotericin B (Fungizone 0.25 µg/ml; Invitrogen, Plymouth, MN, USA). Cells were grown to confluence and used between passages 4 and 7. RASFC were seeded onto 96-well plates and into T25 flasks and cultured in the presence of 4-hydroxi-nonenal (4-HNE, 2.5 µM; Cayman Chemical, Ann Arbor, MI, USA), a highly reactive end product of lipid peroxidation or vehicle basal medium (0.1 % ethanol). The concentration of 4-HNE used in the experiments was based on a cell viability assay and previously published studies. Following stimulation, the effect of amplified oxidative stress on mitochondrial function, cellular metabolism and angiogenic responses was assessed as described below.

3.1.3 Human umbilical vein endothelial cell (HUVEC) culture

HUVECs (Lonza, Walkerville, MD, USA) were incubated in MCDB (Thermo Fisher Scientific) supplemented with L-glutamine (Thermo Fisher Scientific), 0.5 ml epidermal growth factor (Thermo Fisher Scientific), 50 ml FCS (Thermo Fisher Scientific), 0.5 ml of hydrocortisone, penicillin (100 U/ml; Bioscience), streptomycin (100 U/ml; Bioscience) and

Fungizone (0.25 µg/ml; Bioscience). Cells were cultured at 37 °C in humidified air with 5 % CO₂ and harvested with trypsin-ethylenediaminetetraacetic acid (Lonza). Cells were used between passages 20 and 30.

3.1.4 Oxygen consumption rate and extracellular acidification rate measured using Seahorse technology

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), reflecting OXPHOS and glycolysis, respectively, were measured before and after treatment with oligomycin (2 µg/ml), trifluorocarbonyl cyanide phenylhydrazone (FCCP; 5 µM), antimycin A (2 µM) and 2-deoxyglucose (2-DG; 25 mM) using the Seahorse XF24 analyser (Agilent Technologies, Santa Clara, CA, USA). RASFC and HUVEC were seeded at 30,000 cells per well in a Seahorse XF96 cell culture microplate (Agilent Technologies) and allowed to adhere for 24 hours. Cells were rinsed with assay medium (unbuffered DMEM supplemented with 10 mM glucose, 1 mM sodium pyruvate and 2 mM L-glutamine, pH 7.4) before incubation with assay medium for 30 minutes at 37 °C in a non- CO₂ incubator. Following incubation, cells were stimulated with 4-HNE (2.5 µM) and vehicle basal medium for 2 hours. Four baseline OCR and ECAR measurements were obtained over 28 minutes before injection of specific metabolic inhibitors. Moreover, to challenge the metabolic capacity of the RASFC and HUVEC, three OCR and ECAR measurements were obtained over 15 minutes following injection with oligomycin, FCCP, antimycin A and 2-DG.

3.1.5 In vitro mitochondrial dysfunction and mitochondrial DNA mutagenesis

ROS production was assessed using the DCFDA Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, Cambridge, UK). RASFC were seeded into clear-bottomed, dark-sided 96-well plates at a density of 2.5×10⁴ cells/well and allowed to attach overnight. Cells were washed in 1× buffer and stained with 25 µM 2',7'-dichlorofluorescein diacetate in 1× buffer for 45 minutes at 37 °C and 5 % CO₂. After staining, cells were washed, treated with 4-HNE and incubated at 37 °C in 5 % CO₂. ROS fluorescence signal was measured using the SpectraMax Gemini system (Molecular Devices, Sunnyvale, CA, USA) with excitation and emission wavelengths of 485 nm and 538 nm, respectively. Mean fluorescence values from four wells for each condition were obtained. To characterize the frequencies of

random mutations in RASFC exposed to 4-HNE for 24 hours, we used a mitochondrial random mutation capture assay. Mitochondrial DNA (mtDNA) was extracted using a previously reported protocol. Following extraction, 10 µg of mtDNA was digested with 100 U of TaqαI restriction enzyme (New England Biolabs, Ipswich, MA, USA), 1× bovine serum albumin, and a TaqαI-specific digestion buffer (10 mM Tris HCl, 10 mM MgCl₂, 100 mM NaCl, pH 8.4) for 10 hours, with 100 U of TaqαI added to the reaction mixture every hour. PCR amplification was performed in 25-µl reaction mixtures containing 12.5 µl of 2× SYBR Green Brilliant Master Mix (Stratagene, La Jolla, CA, USA), 0.1 µl of uracil DNA glycosylase (New England Biolabs), 0.7 µl of forward and reverse primers (10 pM/µl; Integrated DNA Technologies, Skokie, IL, USA), and 6.7 µl of H₂O. The samples were amplified using a Roche LightCycler 480 Instrument (Roche Diagnostics, Indianapolis, IN, USA), according to the following protocol; 37 °C for 10 minutes, 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Samples were kept at 72 °C for 7 minutes and following melting-curve analysis were immediately stored at -80 °C. The primer sequences used were as follows: for mtDNA copy number, 5'-ACAGTTTATG TAGCTTACCTCC-3' (forward) and 5'-TTGCTGCG TGCTTGATGCTTGT-3' (reverse); for random mutations, 5'-CCTCAACAGTTAAATCAACAAAACACTGC-3' (forward) and 5'-GCGCTTACTTTGTAGCCTTCA-3' (reverse).

3.1.6 Examination of mitochondrial complexes I–V activity

Mitochondrial complexes I–V OXPHOS activity assay kits (Abcam) were used to screen the direct effect of 4-HNE on all complexes of the mitochondrial respiratory chain. These assays are performed using whole bovine heart mitochondria, a rich source of OXPHOS complexes. The activity of mitochondrial complexes I–V was measured as per the manufacturer's instructions via assessing the decrease of absorbance of intermediates at 340-600 nm optical density. For each complex, results are graphically demonstrated as the percentage of enzymatic activity in the presence of 4-HNE relative to the percentage of basal activity.

3.1.7 Quantification of pro-angiogenic mediators in RASFC

To assess the effects of oxidative stress on secretion of VEGF, Ang2, PDGF-B, basic fibroblast growth factor (bFGF), interleukin (IL)-8, regulated on activation, normal T cell expressed and secreted (RANTES) and intercellular adhesion molecule (ICAM), RASFC were seeded into 96-well plates. Confluent RASFC were serum-starved for 24 hours and then cultured with 4-HNE for 24 hours. Supernatants were harvested, and protein secretion levels were quantified using MSD assays (Meso Scale Discovery, Rockville, MD, USA) or specific enzyme-linked immunosorbent assays (ELISAs) (R&D Systems, Minneapolis, MN, USA).

3.1.8 Induction of pro-angiogenic mechanisms of HUVEC in response to oxidative stress-activated RASFC

To examine if oxidatively activated RASFC could further affect pro-angiogenic mechanisms of HUVEC, RA fibroblast cells were stimulated with 4-HNE for 24 hours, and conditioned media (CM) were harvested. (4-HNE RASFC-CM). As a basal medium, we used fibroblast-conditioned media from RASFC cultured in the absence of 4-HNE (basal RASFC-CM). Next, the culture of HUVEC was supplemented with 10 % 4-HNE RASFC-CM or basal RASFC-CM. To ensure that the effects on HUVEC function were not due to residual 4-HNE in the 10 % 4-HNE RASFC-CM, HUVEC were also cultured with RPMI 1640 medium containing 4-HNE (4-HNE RPMI) at the same concentration (0.25 μ M), which is the same concentration as that in the 10 % 4-HNE RASFC-CM. Following 24-hour exposure of HUVEC to 4-HNE RASFC-CM or the abovementioned 4-HNE RPMI medium, pro-angiogenic responses of endothelial cells were assessed as described in the subsections that will follow.

HUVEC transwell invasion chambers were set. BD BioCoat Matrigel invasion chambers (BD Biosciences, Wokingham, UK) were used to examine HUVEC invasion. Cells were seeded at a density of 2.5×10^4 per well in the migration chamber on 8- μ m membranes pre-coated with Matrigel. HUVEC media containing 10 % 4-HNE RASFC-CM or 4-HNE RPMI were separately placed in the chamber, and cells were allowed to migrate for 48 hours. Non-migrating HUVEC were removed from the upper surface by gentle scrubbing. Cells that had invaded were attached to the lower membrane and fixed with 4 % paraformaldehyde (PFA) and stained with 0.1 % crystal violet. To assess the average number of invading HUVEC, cells were counted in five random high-power fields.

HUVEC tube formation was examined using the following method: Matrigel (50 μ l; BD Biosciences, San Jose, CA, USA) was plated in 96-well culture plates after thawing on ice and allowed to polymerise for 30 minutes at 37 °C in humidified air with 5 % CO₂. HUVEC were removed from culture, trypsinised and resuspended at a concentration of 4 \times 10⁴ cells/ml in endothelial cell growth medium. Five hundred microliters of cell suspension was added to each chamber in the presence of 10 % 4-HNE RASFC-CM or 4-HNE RPMI and cultured for 8 hours. The tube analysis was determined from five sequential fields (magnification \times 10) with a focus on the surface of the Matrigel by two blinded observers and a connecting branch between two discrete endothelial cells was counted as 1 tube.

HUVEC wound repair assay was performed with seeding HUVEC onto 24-well plates and growing to confluence. A single scratch wound was induced through the middle of each well with a sterile pipette tip. Cells were subsequently stimulated for 24 hours with 10 % 4-HNE RASFC-CM or 4-HNE RPMI. HUVEC migration across the wound margins from 8 hours was assessed and photographed using a phase-contrast microscope. Semi-quantitative analysis of cell repopulation of the wound was assessed. Briefly, images of the scratch wound assays were taken at \times 10 magnification. The mean closure of the wound was manually calculated from the average of three individual measurements from each wound. This process was repeated for all technical replicates. Measurement of scratches at timepoint 0 were designated as 100 % open. From this, the percentage of closure for all scratches was calculated.

HUVEC proliferation was assessed with a crystal violet cell proliferation assay in the presence of RASFC conditioned media. HUVEC were seeded into 96-well culture plates at a density of 5000 cells/well and left overnight at 37 °C and 5 % CO₂. Next, cells were stimulated with 10 % 4-HNE RASFC-CM or 4-HNE RPMI for 24 hours. Following cell culture, cells were washed with PBS, fixed in 4 % PFA and stained with 1 % crystal violet solution. Plates were washed with tap water and then dried overnight. Cells were resuspended in 1 % Triton X-100 solution (Sigma-Aldrich, St. Louis, MO, USA), and cell number was measured with a microplate reader at a wavelength of 550 nm.

Finally we *quantified pro-angiogenic mediators from HUVEC*. HUVECs were seeded into 96-well plates and left overnight at 37 °C and 5 % CO₂. The following day, cells were stimulated with 10 % 4-HNE RASFC-CM or 4-HNE RPMI for 24 hours. Next, supernatants were harvested, and protein secretion levels of Ang2 and PDGF-B were quantified by using a specific ELISA (R&D Systems).

3.1.9 Immunofluorescence staining of RASFC and synovial tissue (ST)

Single-immunofluorescence staining was performed on RASFC following 24-hour cell stimulations with 4-HNE. To visualise immunoexpression of VEGF, cells were fixed in 4 % PFA and stained with primary rabbit antibody against VEGF (Abcam). To demonstrate ST co-expression of markers of angiogenesis, oxidative stress and bioenergetics, dual-immunofluorescence staining was performed on cryostat synovial sections. ST sections were fixed with acetone for 10 minutes and co-incubated with primary mouse antibody against human 4-HNE (GENTAUR, Kampenhout, Belgium) and with primary rabbit antibodies against VEGF, Ang2, Tie2, ATP5B and glucose transporter 1 (GLUT1) (all from Abcam), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Trevigen, Gaithersburg, MD, USA) and pyruvate kinase isozyme 2 (PKM2) (Abgent, San Diego, CA, USA). Following overnight incubation in a humidified chamber, RASFC and ST samples were incubated with Invitrogen Alexa Fluor 488-conjugated goat Invitrogen Superclonal™ anti-mouse secondary antibody (Thermo Fisher Scientific) and Cy™3-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 60 minutes and counterstained with 4',6-diamidino-2-phenylindole (DAPI) nuclear stain (Sigma-Aldrich) for 10 minutes. Samples were mounted with Molecular Probes antifade mounting medium (Thermo Fisher Scientific) and assessed by immunofluorescence microscopy (Olympus BX51; Olympus, Hamburg, Germany).

3.1.10 Immunohistochemistry (IHC) and scoring of synovial tissue

IHC was performed using 7-µm cryostat ST sections and the DAKO ChemMate EnVision kit (Dako/Agilent Technologies, Glostrup, Denmark). Sections were defrosted at room temperature for 20 minutes, fixed in acetone for 10 minutes and washed in PBS for 5 minutes. Non-specific binding was blocked using 1 % casein in PBS for 20 minutes. The sections were incubated with rabbit monoclonal primary antibodies against human VEGF, Ang2, Tie2, ATP5B (all from Abcam), GAPDH (Trevigen) and mouse monoclonal antibodies against human 4-HNE (GENTAUR). Immunoglobulin G control antibodies were used as negative controls. Following 1hour incubation with primary antibody, endogenous peroxidase activity was blocked using 0.3 % hydrogen peroxide for 5 minutes. Slides were incubated for 30 minutes with secondary antibody/horseradish peroxidase (Dako/ Agilent Technologies).

3,3'-Diaminobenzidine (1:50) was used to visualise staining, and Mayer's haematoxylin (BDH Laboratories, Poole, UK) was incubated for 30 seconds as a counterstain prior to mounting in DPX mounting media. Slides were scored separately for lining layer (LL), sublining layer (SL) and vascular region (BV) using a well-established and validated semi-quantitative scoring method, where the percentage of cells that were positive for a specific marker was compared with the percentage of cells that were negative. Percentage positivity was graded using a 0–4 scale, where 0 = no stained cells, 1= 1–25%, 2=25–50%, 3=50–75 % and 4=75–100% stained cells. Images were captured using an Olympus DP50 light microscope and AnalySIS software (Olympus Soft Imaging Solutions, Lakewood, CO, USA).

3.1.11 Statistical analysis

IBM SPSS Statistics version 20 for Windows software (IBM, Armonk, NY, USA) was used for statistical analysis. Wilcoxon's signed-rank test, Spearman's rank correlation coefficient and the Mann-Whitney U test were used for analysis of non-parametric data. Parametric data were analysed using one-way analysis of variance. All p values were two-sided, and p values less than 0.05 were considered statistically significant.

3.2 Study 2

3.2.1 Patient recruitment

Fifty-three patients, out of which 36 with RA and 17 with AS were enrolled in the study with active disease (indicated by DAS28-ESR>5.2 or Bath Ankylosing Spondylitis Disease Activity Index (BASDAI)>4 despite maximized conservative therapy set by national protocols). All of them were about to start TNFi therapy, which was continued for the next 12 months. Among the 36 RA patients, 20 received etanercept (ETN) 50 mg weekly subcutaneously (SC) and 16 received certolizumab pegol (CZP) (400 mg at 0, 2 and 4 weeks followed by 200 mg twice weekly SC dose). All 17 AS patients received ETN 50 mg weekly SC. Measurements were carried out at baseline and 12 months after TNFi treatment initiation. We recruited 8 age-matched healthy controls for single serum sample collection as well. The study was approved by the Hungarian Scientific Research Council Ethical Committee (approval No. 14804-2/2011/EKU). Written informed consent was obtained from each patient and assessments were carried out according to the Declaration of Helsinki.

3.2.2 Clinical assessments

Detailed medical history was taken. We enquired about current smoking status, history of CVD during the past year in setting of a questionnaire. All patients' dental condition was recorded by a dentist. Number of missing teeth was counted. Based on periodontal charting according to current classification the presence of periodontitis was decided. Clinical examination was performed at baseline (T0), then at the 3rd (T3), 6th (T6) and 12th months (T12) of therapy.

3.2.3 Laboratory measurements

Serum high sensitivity CRP (hsCRP) and IgM rheumatoid factor (RF) were measured by quantitative nephelometry (Cobas Mira Plus-Roche), using CRP and RF reagents (both Dialab) and aCCPautoantibodies were detected in serum samples using a second generation Immunoscan-RA CCP2 ELISA test (Euro Diagnostica). Erythrocyte sedimentation rate (ESR) was determined by the traditional Westergren method (mm/h).

Among serum biomarkers of angiogenesis, VEGF (V-Plex, Meso Scale Diagnostics; pg/ml), PDGF-BB (DuoSet ELISA, R&D Systems; pg/ml), Ang1 (DuoSet ELISA, R&D Systems; pg/ml), Ang2 (QuantiKine ELISA, R&D Systems; pg/ml) and TSP-1 (DuoSet ELISA, R&D Systems; ng/ml) levels were determined by ELISA at baseline, as well as after 6 and 12 months of TNFi therapy.

Anti- Citrullinated enolase peptide-1 (anti-CEP1) IgG was measured in the serum samples using an in-house peptide ELISA, as previously described. Anti-CEP-1 IgG levels are presented as AU/ml, based on a standard curve. The cut-off for „positivity” and „negativity” was 3.7 AU/ml.

3.2.4 Assessment of vascular physiology by ultrasound

Brachial artery *flow mediated dilation (FMD)* was assessed (right arm, 10 MHz, HP Sonos 5500). Reactive hyperaemia was induced by release of a pneumatic cuff around the forearm inflated to suprasystolic pressure for 4.5 minutes. After deflation the maximal flow velocity and the arterial diameter was 90 seconds long continuously recorded. FMD values were expressed as % change from baseline (resting) value (FMD%).

Common carotid intima-media thickness (ccIMT) measurements were carried out as well (HP Sonos 5500, 10 MHz) over both right and left common carotid arteries 1 cm proximal to the carotid bulb in the far wall. IMT was defined as the distance between the first and second echogenic lines from the lumen taking the average of 10 measurements on both sides. IMT values were expressed in mm.

With respect to arterial stiffness, *pulse wave velocity (PWV)* was calculated automatically by a TensioClinic arteriograph system (Tensiomed Ltd, Budapest, Hungary) as the quotient of the distance between the jugular fossa and symphysis. If an artery is elastic, PWV is low. With decreased arterial elasticity, PWV rises.

3.2.5 Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (IBM) software. Data are expressed as the mean \pm SD for continuous variables and percentages for categorical variables. Continuous variables were evaluated by paired two-tailed t-test and Wilcoxon test. Nominal variables were compared between groups using the chi-squared or Fisher's exact test, as appropriate. Correlations were determined by Spearman's analysis. Univariate and multiple regression analysis using the stepwise method was applied to investigate independent associations between angiogenic biomarkers (dependent variables) and other clinical, laboratory and imaging parameters (independent variables). The β standardized linear coefficients showing linear correlations between two parameters were determined. The B (+95% CI) regression coefficient indicated independent associations between dependent and independent variables during changes. P values < 0.05 were considered significant.

4 RESULTS

4.1 Study 1

4.1.1 Oxidative stress alters cellular bioenergetics in RASFC and HUVEC in vitro

Previous studies by our group demonstrated altered cellular bioenergetics in RASFC in the presence of hypoxia, and also demonstrated increased oxidative stress in the inflamed synovium. First we further investigated whether oxidative stress in the inflamed joint is involved in metabolic reprogramming of RASFC and HUVEC. We demonstrated OCR and

ECAR profiles before and after injections of oligomycin, FCCP, antimycin A and 2-DG in basal and 4-HNE-stimulated RASFC. We showed, for the first time to our knowledge, that inhibition of OCR following 4-HNE-induced oxidative stress was associated with a shift in RASFC metabolism towards glycolysis. 4HNE reduced basal mitochondrial respiration ($p<0.05$), paralleled by a reduction in maximal mitochondrial respiration ($p<0.001$), ATP synthesis ($p=0.1$) and reserve capacity ($p<0.01$). This metabolic reprogramming was further accompanied by increased levels of basal glycolysis ($p<0.01$), glycolytic capacity ($p<0.01$) and glycolytic reserve ($p=0.2$) in RASFC subjected to oxidative stress.

Seahorse HUVEC OCR and ECAR profiles before and after injections of oligomycin, FCCP, antimycin A and 2-DG were analyzed as well. Similarly to RASFC, 4-HNE inhibited basal mitochondrial respiration, maximal mitochondrial respiration, ATP synthesis and reserve capacity (all $p<0.01$) with concomitant increase in basal glycolysis ($p<0.01$) and glycolytic reserve ($p<0.05$) in HUVEC exposed to oxidative stress.

4.1.2 Examination of mitochondrial mutagenesis and activity of enzymes of mitochondrial OXPHOS complexes under 4HNE-induced oxidative stress in RASFC

Our group previously shown that increased mtDNA mutation frequency and mitochondrial dysfunction in the RA joint were strongly associated with synovial inflammation and hypoxia. We have also reported, at a functional level, induction of pro-angiogenic responses of endothelial cells in the presence of oxidative stress. In the present study, we assessed the frequency of mtDNA mutations and mitochondrial dysfunction in RASFC subjected to 4-HNE. We observed increases in ROS production and mtDNA point mutations in RASFC in the presence of 4-HNE compared with basal cells ($p<0.001$ and $p=0.06$, respectively). 4-HNE protein adduction may alter protein activity; therefore, we next examined the activity of the individual proteins of mitochondrial OXPHOS complexes I–V. 4-HNE significantly reduced the activity of complex III by 8 % and complex IV by 70 % compared with basal values (both $p<0.01$). Lower enzymatic activity following 4-HNE stimulation was also detected for complex I by 9 %, complex II by 22 % and complex V by 12 % (all $p=0.2$).

4.1.3 In vitro secretion of pro-angiogenic and pro-inflammatory mediators under oxidative stress conditions in RASFC

As we found a close association of redox state with energy metabolism in RASFC, we next examined the effect of oxidative stress on angiogenic and inflammatory mediators from RASFC. Increased VEGF immunofluorescence was observed in RASFC cultured in the presence of 4-HNE compared with the basal cells. In addition, 4-HNE significantly increased secretion of key pro-inflammatory and pro-angiogenic mediators compared with basal RASFC (VEGF, Ang2, bFGF, IL-8 [all $p < 0.05$], PDGF-B, RANTES, ICAM [all $p < 0.01$]).

These findings, along with our group's previously published in vitro study showing TNF- α -induced mitochondrial dysfunction, further support the concept of the complex interplay between oxidative damage, oxygen metabolism and angiogenesis in RA. Therefore, we next determined angiogenic in vivo responses following TNFi in 15 patients with RA at baseline (T0) and 3 months after the commencement of biologic treatment (T3). Reduction of macroscopic vascularity and decreases in ST VEGF expression ($p = 0.1$), Ang2 ($p < 0.005$) and Tie2 ($p < 0.005$) was observed after TNFi therapy initiation when T0-T3 timepoints compared.

4.1.4 Oxidative stress-activated RASFC promote pro-angiogenic mechanisms in HUVEC

RASFC are known to be strongly involved in regulating pathological angiogenesis in the inflamed joint. Therefore, we next examined if the observed alterations in cellular bioenergetics and pro-inflammatory processes in RASFC in response to oxidative stress could subsequently influence pro-angiogenic mechanisms in HUVEC. We stimulated RASFC in the presence or absence of 4-HNE and harvested the supernatants, termed conditioned media (CM). The effect of basal or 4-HNE RASFC-CM on invasion, the formation of tube-like structures and migration of HUVEC was demonstrated on microscopic images. Markedly induced invasion ($p < 0.001$), proliferation ($p < 0.05$), number of formed tube-like structures ($p < 0.001$), cell migration across the wound ($p < 0.001$) and secretion of Ang2 and PDGF-B (both p values < 0.05) in HUVEC in response to 4-HNE RASFC-CM was seen when compared to basal RASFC-CM.

To confirm that the increase in pro-angiogenic responses of HUVEC was due to oxidatively activated RASFC and not to residual 4-HNE present in the CM, additional experiments were performed, consisting of RPMI 1640 media supplemented with 4-HNE (0.25 μ M; 4-HNE RPMI 1640 control), which would be at the same concentration of 4-HNE in the 10 % RASFC-CM. A significant increase in invasion ($p < 0.001$), number of formed tube-like structures ($p < 0.01$) and cell migration across the wound ($p < 0.001$) in HUVEC in

response to 4-HNE RASFC-CM compared to 4-HNE RPMI control media further supports the direct effect of 4-HNE on RASFC-induced angiogenesis in the inflamed joint.

4.1.5 Association between ST angiogenesis, oxidative stress and bioenergetics

Finally, the correlation of angiogenic factors with previously assessed markers of oxidative stress and metabolism in this patient cohort was examined. 4-HNE oxidative stress marker ST expression was associated with increased expression of angiogenic markers such as VEGF ($r=0.63$; $p=0.015$) and Tie2 ($r=0.56$; $p=0.029$), glycolytic protein of GAPDH ($r=0.60$; $p=0.03$) and with reduced levels of mitochondrial marker of ATP5B ($r=-0.52$, $p=0.017$). Furthermore, immunofluorescence images demonstrating co-localisation of 4-HNE with angiogenic factors (VEGF, Ang2, Tie2), as well as with mitochondrial (ATP5B) and glycolytic (GAPDH, PKM2, GLUT1) proteins were demonstrated.

4.2 Study 2

4.2.1 Baseline demographics

The cohort included 34 women and 19 men with mean age of 52.0 ± 12.1 (range: 24-83) years. Mean disease duration was 8.5 ± 7.9 (range: 1-44) years. At baseline RA patients had a mean DAS28-ESR of 5.00 ± 0.86 , while AS patients exerted mean BASDAI of 5.79 ± 1.19 .

4.2.2 Clinical response to TNFi therapy in inflammatory arthritides

In RA ($n=36$), TNFi treatment resulted in significant decrease in DAS28-ESR at 12 months of treatment (3.02 ± 0.96) compared to baseline (5.00 ± 0.86 ; $p<0.001$). In AS ($n=17$), BASDAI significantly decreased from 5.79 ± 1.19 at baseline to 1.86 ± 1.04 after 12 months of therapy ($p<0.001$).

4.2.3 Effects of TNF inhibition on the levels of angiogenic biomarkers

First, increased levels of serum VEGF, the master regulator of angiogenesis were confirmed and appeared to be significantly higher in patients with (RA+AS) arthropathies

(n=53) compared to age matched healthy controls (n=8): 267.8 ± 133.3 vs 140.7 ± 63.5 pg/ml. These high VEGF levels showed a tendency of non-significant reduction from a baseline of 267.9 ± 123.3 pg/ml to 239.8 ± 116.3 pg/ml ($p=0.082$) at 6 months followed by significant decrease to 220.9 ± 120.7 pg/ml at 12 months ($p=0.007$) of treatment in (RA+AS). Serum PDGF-BB levels significantly dropped after 6 months (6579.4 ± 3819.2 pg/ml; $p=0.015$) with even further reduction at 12 months (6020.4 ± 3216.1 pg/ml; $p=0.005$) compared to baseline (8187.4 ± 6282.0 pg/ml). Serum Ang2 concentrations significantly decreased after 6 months (2473.7 ± 1136.1 pg/ml; $p=0.024$) and maintained a tendency of reduction at 12 months (2537.2 ± 1156.1 pg/ml; $p=0.081$) vs baseline (2790.0 ± 1381.1 pg/ml). Serum Ang1 and TSP-1 levels did not change overtime during anti-TNF therapy

4.2.4 Correlations of angiogenic biomarkers with other parameters

In the simple Spearman's correlation analysis, PDGF-BB levels at 12 months of treatment, Ang2 at baseline and at 12 months correlated with disease duration ($p<0.05$). Baseline CRP showed correlation with baseline Ang1, as well as baseline and post-treatment Ang2 ($p<0.05$) levels. Post-treatment CRP correlated with pre- and post-treatment Ang2 ($p<0.05$). Baseline aCCP and anti-CEP levels, correlated with baseline TSP-1 ($p<0.05$). Baseline ccIMT exerted correlations with baseline PDGF-BB and baseline TSP-1 ($p<0.05$). When current smokers (n=14) and non-smokers (n=39) were compared, PDGF-BB levels at 12 months of treatment were significantly higher in smokers (8903.2 ± 5084.1 pg/ml) compared to non-smokers (4866.2 ± 1895.8 pg/ml; $p=0.01$). Current clinical periodontitis or tooth loss was not associated with elevated angiogenic marker levels.

According to the univariate analysis, PDGF-BB levels at baseline correlated with baseline ccIMT, while PDGF-BB after 12 months of treatment correlated with disease duration and current smoking status ($p<0.05$). Ang1 at baseline correlated with baseline CRP ($p<0.05$). Ang2 at baseline showed correlation with disease duration, the positive history of CV disease, as well as baseline CRP, while post-treatment Ang2 correlated with disease duration, as well as baseline and 12-month CRP ($p<0.05$). Finally, TSP-1 levels at baseline correlated with disease duration, as well as baseline ccIMT and CEP levels ($p<0.05$).

As suggested by the multivariate analysis, smoking was an independent predictor of post-treatment PDGF-BB levels ($p=0.006$). Disease duration determined baseline Ang2 ($p<0.001$), 12-month Ang2 ($p=0.004$) and baseline TSP-1 ($p=0.028$). Baseline CRP was an

independent predictor of baseline Ang2 ($p=0.004$). Finally, baseline CEP determined baseline TSP-1 levels ($p=0.002$).

5 DISCUSSION

5.1 Study 1

Synovial angiogenesis is a well recognized primary event in perpetuation of local inflammation in inflammatory arthritis. This defective neovasculatory process is associated with the abundant production of pro-inflammatory molecules, most of which has pro-angiogenic effects, hence triggering the vicious circle further. The systemic inflammation secondary to overproduction of these mediators is linked to an increased risk of cardiovascular morbidity and quiescent or manifest autoimmune atherosclerosis. In this work we reviewed the pathophysiology of inflammatory arthritis related angiogenesis affecting the joint as well as peripheral vasculature leading to vascular plaque formation. Learning from previous research we highlighted the most crucial pro-inflammatory mediators with dominant pro-angiogenic characteristics and those regulatory networks serving a potential therapeutic target of angiogenesis in the setting of inflammatory arthritis.

Oxidative stress is detrimental in inflammatory arthritis by reprogramming cellular bioenergetics via downregulation of OXPHOS and promotion of glycolysis that to our knowledge we demonstrated first using human RASFC and HUVEC cultures. The maximal and ATP-linked respiration and reserve capacity decreased reflecting on this change. However in presence of 4-HNE glycolytic capacity and reserve increased. The altered cellular bioenergetics were associated with high ROS production and mutations of the mtDNA with reduced activity of mitochondrial complexes III and IV. We demonstrated that oxidative stress promotes pro-inflammatory and pro-angiogenic marker production by RASFC and enhances the pro-angiogenic profile of HUVEC when stimulated by CM from 4-HNE stimulated RASFC. This has been featured by intense invasion, cell proliferation and migration, tube formation and secretion of pro-angiogenic mediators. Furthermore, colocalisation of angiogenic markers, oxidative stress markers and markers of altered energy metabolism were demonstrated in ST demonstrating an interface between these regulatory

mechanisms. In addition, TNFi treatment significantly reduced ST angiogenesis in patients with RA.

Hypoxia is a leading metabolic event in the inflamed ST of RA with overproduction of ROS and increased lipid peroxidation. At cellular level the mitochondrial function and integrity is also impaired by 4-HNE induced covalent modifications of mitochondrial DNA, lipids and other proteins. The respiratory chain metabolic properties, protein transport and mitochondrial dynamics and quality control have all been reported to suffer. From previous studies it is demonstrated that mtDNA mutation frequency and subsequent mitochondrial dysfunction correlated with increased macroscopic synovial vascularity, high levels of hypoxia, markers of oxidative stress and pro-inflammatory cytokines. The mitochondrial DNA is highly susceptible to oxidative damage that we further highlighted with in vitro studies on RASFC with a mitochondrial random mutation capture assay. The random mitochondrial point mutation frequency was quantified following stimulation by 4-HNE. This methodology relies on single molecule amplification to screen a large number of mtDNA molecules for the presence of unexpanded mutations that may appear following oxidative stress. 4-HNE has mutagenic potentials that was supported by elevated number of mutations of the mitochondria in RASFC exposed to oxidative stress. 4-HNE-guanine adducts have been detected in the p53 tumour suppressor gene in a human lymphoblastoid cell line, causing gene mutation and affecting cell cycle arrest, apoptosis, DNA repair and differentiation. Mitochondrial genome alteration is primarily lead by elevated mtROS levels. Our study describes increased production of ROS by RASFC exposed to 4-HNE, which indicates that 4-HNE has the potential of exacerbating ROS production and perpetuating the oxidative stress driven mitochondrial mutagenesis.

Oxidative stress in inflammatory arthritis may mediate angiogenesis through VEGF dependent or independent pathways involving ROS induced lipid peroxidation. Therefore we examined the mitochondrial DNA instability when affected by lipid peroxidation with its implications on respiratory metabolism. RASF and HUVEC cell lines were assessed in presence of 4-HNE and the two main energy metabolism pathways examined in presence of 4-HNE, where a switch of their bioenergetic profile from OXPHOS to anaerobic glycolysis was observed in response to the increased energy demand. This was associated with low maximal and ATP-linked respiration and reserve capacity, in contrast to increased glycolytic capacity and glycolytic reserve. The mitochondrial complex enzymatic activities decreased by oxidative stress mechanisms. The compensatory mechanisms of anaerobic glycolysis provide

short term energy supply only and prolonged dependence may lead to a bioenergetic crisis with severe energy deficiency, that most probably perpetuates dysfunctional angiogenesis, cellular migration, invasion and pannus formation. We demonstrated consistent results with earlier studies showing 4-HNE-induced mitochondrial respiration deficiency when examined epithelial cells of the cardiac and pulmonary tissue. Inhibition of mitochondrial respiration following 4HNE stimulation could be due to reduced functionality from 4-HNE protein-adducts of proteins associated with the ETC and ATP synthase, or it could be due to a diminished ability of RASFC to detoxify 4-HNE because this process requires energy. A group using proteomic approach identified various 4-HNE-modified mitochondrial proteins in mice cardiac mitochondria after treatment with doxorubicin used in chemotherapy. These proteins were involved in the mitochondrial energy metabolism including ETC subunits such as NDUFS2 (complex I), SDHA (complex II) and ATP5B (complex V), as well as dihydrolipoamide dehydrogenase, a component of the TCA cycle. Finally, 4-HNE adduction lead to reduction in the activity of the mitochondrial proteins, declined OCR and increased ECAR profiles. Other studies found that 4HNE modified proteins are involved in metabolism, cellular adhesion, cytoskeletal reorganisation and anti-oxidation in human platelets. Using glycolytic inhibitors to block glycolytic processes generates weakened pro-inflammatory responses of RASFC and HUVEC as well as the severity of arthritis in K/BxN mice. Additionally, glucose-6-phosphate isomerase (glycolytic enzyme) activation by hypoxia up-regulated VEGF production, proliferation and invasion when examined RASFC and HUVEC. Electrophilic lipids are able to adduct numerous glycolytic proteins as described by others. PKM2, GAPDH, fructose bisphosphate aldolase A (aldolase A) and phosphoglycerate kinase 1 are all examples demonstrated from previous research. These covalent modifications can impair glucose metabolism and lead to the accumulation of glycolytic intermediates. This is in agreement with other results showing significant rise in lactic acid levels and ECAR by human platelets cultured in presence of 4-HNE, as well as raised ¹⁸F-fludeoxyglucose uptake and glycolytic metabolism by oxidized low-density lipoprotein (oxLDL) via upregulated GLUT1 expression and hexokinase activity. This response was mediated by HIF-1- α and reliant on ROS production. In turn, this metabolic effect of oxLDL was completely abrogated by Src (PP2) and phosphatidylinositol-3 kinase inhibitors, supporting the regulatory role of this pathway in glucose metabolism and immune cell activation.

RASFC are known to be strongly involved in regulating pathological angiogenesis in the inflamed joint. Hypoxia and oxidative stress are crucial mediators of impaired

angiogenesis that we demonstrated by experiments where RASFC originated pro-angiogenic and pro-inflammatory cytokine levels were induced by oxidative stress, 4-HNE. Stimulated migration, tube formation and pro-angiogenic mediator secretion was observed in HUVEC cells that were treated with CM from 4-HNE activated RASFC. Markers of angiogenesis and oxidative damage showed co-expression on ST when examined microscopically and TNFi therapy has shown to reduce ST angiogenesis in RA patients. Our data provide evidence that there is both direct and indirect pro-angiogenic stimulus in response to 4-HNE within the inflamed joint. Our findings are in agreement with other studies showing 4-HNE-induced expression of COX-2, IL-1 β , IL-18 and NF- κ B and activation of the NLRP3 inflammasome. Upregulated angiogenic responses due to redox changes has similarly been described by others in HUVEC, keratinocytes, lung epithelial cells and retinal cells.

The co-expression of angiogenic factors with oxidative stress markers, mitochondrial bioenergetics and glycolysis was examined in our study and 4-HNE expression was associated with increased expression of angiogenic markers, glycolysis markers and with reduced expression of markers of mitochondrial respiration. The co-existence of the above processes was confirmed by colocalisation of different markers with immunofluorescent method and it highlights the interplay between oxidative stress, altered bioenergetic profile and dysfunctional angiogenesis.

TNF- α is known to have pro-angiogenic role and regulates capillary sprouting through VEGF, Ang1 and Ang2 and their signaling. In our study, we assessed whether TNFi therapy would alter levels of angiogenic mediators when examined 3 months after initiation of treatment. TNFi therapy reduced ST expression of VEGF, Tie2 receptor and its Ang2 ligand, which further supports the strong link between angiogenesis and TNF- α . Reduction of macroscopic vascularity well correlated with reduced IHC expression of angiogenic markers such as VEGF, Tie2 receptor and its Ang2 ligand on ST, which highlights the coupled relationship between angiogenesis and TNF- α . Our data is in line with other studies showing reduced angiogenic marker expression and endothelial cell activation following TNFi therapy. Furthermore, other TNFi biologic agents such as etanercept and infliximab have been found to prove positive effect on oxidative damage in RA with significant reduction of serum and urinary levels of oxidative DNA damage markers and lipid peroxidation markers with concomitant improvement of DAS28 measured disease activity score. Inhibiting other pathways than TNF- α also have suppressant effect on levels of oxidative stress markers and in

a study IL-6 receptor blockade with tocilizumab those significantly reduced when compared to TNFi.

5.2 Study 2

Synovial angiogenesis has outstanding importance in orchestrating local inflammatory response in inflammatory arthritis. The excessive production of pro-inflammatory and pro-angiogenic cytokines generate a systemic inflammatory process that is associated with an increased risk of atherosclerosis, CV morbidity and mortality.

Earlier data suggests that TNFi therapy may mitigate angiogenesis and atherosclerosis linked to RA and AS. Our study examined whether TNFi treatment alters levels of angiogenic mediators at 6 and 12 months post-initiation of therapy. The unique novelty of this research comes from its complexity, as we examined the therapeutic effects of biologics on angiogenic biomarker levels in conjunction with distinct markers of vessel pathophysiology (FMD, ccIMT, PWV), oral health and further clinical data.

We demonstrated that TNFi treatment was clinically effective both in RA and AS which was indicated by significant reduction of DAS28-ESR and BASDAI indices, respectively.

Serum VEGF levels of the inflammatory arthritides cohort was significantly above the VEGF levels of an age-matched healthy control population that reflected the dominant angiogenesis in inflammatory arthritis. One-year treatment with TNF inhibition led to significant drop in serum VEGF, PDGF-BB and Ang2 levels. Few studies suggested suppression of VEGF excretion when TNFi biological therapy applied in RA and psoriatic arthritis (PsA) setting. It has been described that downstream mechanisms of PDGF-B has a cross-link with HIF-1 led pathways promoting tumor angiogenesis and metastasis through chemotactic and proliferative properties making it an attractive target for investigating tumor therapy. Inhibition of PDGFR pathway has also been shown to be linked to synovial fibroblast related ECM degradation in RA, and to our knowledge we are the first ones confirming that TNFi therapy reduces PDGF-B levels in sera of patients with inflammatory arthropathy, hence interfering with the inflammatory and cross-linking angiogenic processes. Inhibition of this signaling by imatinib RTK inhibitor has proven its benefits in ameliorating joint destruction in RA. There is controversial thoughts about the relevance of TSP-1 in

angiogenesis as its effect are mediated through anti-angiogenic as well as angiogenic properties and mostly investigated in tumor research. Possibly its complex mechanism of action contributed to our findings when no significant change was observed in serum TSP-1 levels 12 months after initiation of TNFi therapy.

We demonstrated clear positive correlation between high VEGF and Ang2 levels, while Ang1 seems to be in part VEGF-independent. In addition, Ang2 may have outstanding effects in the initiation of the neoangiogenesis. Excessive Ang2 expression was observed in early PsA [245]. Ang1/Ang2 ratio may vary during the pathogenesis of arthritis and angiogenesis: high levels of VEGF-dependent Ang2 may accompany early inflammation and vessel proliferation, while increased VEGF-independent Ang1 levels may be linked to the later vessel maturation stage. Indeed, we included patients with inflammatory arthropathy with high disease activity, which may well reflect on the observed early active synovial angiogenesis with predominance of Ang2 instead of Ang1. TNFi treatment was effective to suppress both VEGF and Ang2 in parallel.

In addition to demonstrating the effects of TNFi on angiogenic markers, we correlated their levels with markers of vascular pathophysiology, atherosclerosis, oral health and some other parameters. Ang2 levels correlated with the history of CV disease underlining the importance of angiogenesis in inflammatory atherosclerosis. Ang2 and PDGF-BB correlated with disease duration and Ang1 and Ang2 showed positive correlation with CRP underpinning that long disease duration and high degree of inflammation are both accompanied by active angiogenesis. Hashimoto et al found correlations between arthritic active disease and high titers of angiogenic factors. Kurosaka et al found that serum VEGF level was marker of RA activity, as well as a predictor of joint destruction; Ang1 level may be useful as an index of sustained arthritis, while Ang2 level may reflect a state of marked angiogenesis. Moreover, our findings indicating that baseline Ang2 correlated with post-treatment CRP and, *vica versa*, 12-month Ang2 also correlated with baseline CRP demonstrates the continuous interplay between systemic inflammation and angiogenesis throughout the observation period.

Baseline aCCP and anti-CEP antibodies also positively correlated with TSP-1 confirming an interplay between autoimmunity and neoangiogenesis. The association between angiogenesis and ACPA in RA is not well described due to lack of studies looking into this. An earlier study found no link between VEGF titer and aCCP status. Periodontitis and anti-CEP have been implicated in arthritides, but we found no data on direct links between

angiogenesis and periodontitis or anti-CEP in arthritis. It has however been observed that TSP-1 is enhanced by *Porphyromonas gingivalis* that we know acts as a contributor to the pathogenesis of periodontitis and RA. While anti-CEP correlated with TSP-1, none of the studied angiogenic factors showed association with current clinical periodontitis or tooth loss.

Baseline PDGF-BB and TSP-1 correlated with ccIMT suggesting that these angiogenic mediators play role in autoimmune atherosclerosis. Indeed, previous research highlighted that angiogenesis contributes to the pathogenesis of atherosclerosis. PDGF-BB and TSP-1 have been implicated in vascular smooth muscle cell proliferation and migration, as well as several other mechanisms underlying atherosclerosis. Yet, we have not found any reports with respect to the direct involvement of these angiogenic factors in arthritis-associated atherogenesis.

Smoking, atherosclerosis and CV disease has all been described in the pathogenesis of arthritides. In our multivariate analysis, PDGF-BB levels were significantly higher in smokers compared to non-smokers at 12 months of TNFi treatment. Interestingly, the direct association of smoking with inflammatory angiogenesis has not yet been evaluated. However, PDGF signalling has been implicated in cigarette smoke-induced pulmonary hypertension.

In summary, angiogenesis has outstanding role in pathogenesis of inflammatory arthropathies, as well as in inflammatory (accelerated) atherosclerosis associated with arthritides. The titers of some angiogenic mediators correlate with disease duration, CRP, RA-associated autoantibodies and carotid atherosclerosis. Twelve months TNFi therapy attenuated the production of some angiogenic mediators in both RA and AS. Thus, some angiogenic markers may be used as surrogate biomarkers that reflect the interplay of angiogenesis, inflammation and atherosclerosis in arthritides.

6 CONCLUSIONS

In this study we reviewed the crucial pathomechanisms of inflammatory synovial angiogenesis and autoimmune atherosclerosis. We described the most dominant pro-inflammatory molecules in the process of impaired angiogenesis and pinpointed possible future therapeutic targets in controlling the dysfunctional neovasculatory process. We examined the interplay of synovial cellular bioenergetics, oxidative stress and angiogenesis in RA. It has been shown that oxidative stress switched bioenergetics from OXPHOS to

anaerobic glycolysis responding to the high energy demand of the inflammatory joint. This creates a bioenergetic crisis that may contribute to a defective angiogenesis promoting further inflammation in RA. In addition, ST expression of the angiopoietin/Tie2 system can be reduced following TNFi therapy. Our results indicate that the levels of some angiogenic cytokines may correlate with disease duration, the level of inflammation, ACPA antibody reflected autoimmunity and systemic atherosclerosis in inflammatory arthritides (such as RA and AS). We demonstrated that TNFi therapy attenuates the production of certain angiogenic cytokines (VEGF, PDGF-BB and Ang2) in the same cohort. Thus, some pro-angiogenic markers may be useful as surrogate biomarkers to reflect the interplay between angiogenesis, inflammation and autoimmune inflammatory atherosclerosis in arthritides.

7 SUMMARY

7.1 Study 1

Inflammatory arthritis is characterised by local and systemic inflammation that is driven by a cascade of circular events that serve the platform for a bioenergetic crisis. Altered endothelial biology, activation of various pro-inflammatory mediators drive a dysfunctional angiogenesis, which further fuels synovial inflammation, proliferation and destructive pannus formation. The inflamed joint creates a hypoxic microenvironment and the domination of oxidative stress triggers alterations in the cellular respiratory chain responses shifting towards anaerobic glycolysis. The subsequent accumulation of reactive oxygen species and lipid peroxidation favours to mitochondrial mutagenesis. The hypoxia, oxidative stress, mitochondrial dysfunction, bioenergetic switch, accumulation of pro-angiogenic and pro-inflammatory mediators arise in a distinct interplay involving multiple cellular pathways leading to inflammation. Inflammation and dysregulated angiogenesis is not only localised at synovial tissue level, but also gains systemic involvement resulting in autoimmune vascular plaque formation and atherosclerosis. Numerous therapeutic targets have been identified that may offer promising future therapeutic strategies in treatment of inflammatory arthritis.

In our study we assessed the effect of oxidative stress on cellular bioenergetics and pro-angiogenic, pro-inflammatory responses using primary rheumatoid arthritis synovial fibroblast cells (RASFC) and Human umbilical vein endothelial cells (HUVEC) when

cultured with 4-Hydroxy-2-nonenal (4-HNE), a marker of oxidative stress. The extracellular acidification rate (ECR) and oxygen consumption rate (OCR), mitochondrial DNA stability and pro-angiogenic as well as pro-inflammatory mechanisms were examined with a Seahorse analyser, complex I–V activity assays, random mutation mitochondrial capture assays, enzyme-linked immunosorbent (ELISA) assays and functional assays, including angiogenic tube formation, migration and invasion. Synovial tissue (ST) angiogenic marker expression was examined by immunohistochemistry (IHC) in patients with rheumatoid arthritis (RA), who underwent knee arthroscopy prior to and 3 months after initiation of tumour necrosis factor- α inhibitor (TNFi) therapy.

In RASFC and HUVEC, oxidative stress by 4-HNE altered energy metabolism by inhibiting basal, maximal and adenosine triphosphate-linked mitochondrial respiration and reserve capacity, which was linked to the reduced activity of oxidative phosphorylation enzyme complexes III and IV. Inversely, 4-HNE stimulated basal glycolysis, glycolytic capacity and glycolytic reserve, coupled with an increase in mitochondrial mutagenesis and reactive oxygen species. 4-HNE also triggered pro-angiogenic activity of RASFC, which stimulated HUVEC invasion and cell migration, formation of angiogenic tube-like structures, and the release of pro-angiogenic mediators. Angiogenic markers of vascular endothelial growth factor (VEGF), tyrosine kinase receptor (Tie2) and its ligand angiopoietin 2 (Ang2) were co-expressed in the inflammatory synovium with markers of oxidative stress and oxygen metabolism. TNFi treatment significantly reduced ST macroscopic vascularity microscopic expression of Ang2/Tie2 in patients with RA.

Oxidative stress alters cellular responses with a bioenergetic switch towards anaerobic respiration, which may contribute to acceleration of inflammation and dysregulated angiogenesis in RA.

7.2 Study 2

Inflammation and dysregulated angiogenesis is not only localised at synovial tissue level, but also gains systemic involvement resulting in autoimmune vascular plaque formation and atherosclerosis in inflammatory arthritis. Numerous therapeutic targets have been identified that may offer promising future strategies in treatment of inflammatory arthritis. The enhanced systemic inflammation may lead to increased risk of atherosclerosis, CV morbidity and mortality. Our study examined whether TNFi treatment alters levels of angiogenic mediators at 6 and 12 months

post-initiation of therapy and whether increased levels of angiogenic mediators are associated with altered vascular physiology assessed by FMD, ccIMT, PWV. We also examined whether the angiogenic response is linked to changes in oral health or whether it is altered by smoking status.

TNFi treatment was clinically effective both in RA and AS which was indicated by significant reduction of DAS28-ESR and BASDAI indices, respectively. One-year treatment with TNF inhibition led to significant drop in serum VEGF, PDGF-BB and Ang2 levels, respectively. We hypothesize that the complex anti- and pro-angiogen mechanism of action of TSP-1 may have contributed to our findings when no significant change was observed in serum TSP-1 levels 6 or 12 months after initiation of TNFi therapy.

We demonstrated clear positive correlation between high VEGF and Ang2 levels, while Ang1 seems to be in part VEGF-independent. Moreover, Ang2 levels correlated with the history of CV disease underlining the importance of angiogenesis in inflammatory atherosclerosis. Ang2 and PDGF-BB correlated with disease duration and Ang1 and Ang2 showed positive correlation with CRP linking perpetuated angiogenesis to long disease duration and a systemic inflammatory state. Moreover, our findings indicate that high baseline Ang2 correlated with high post-treatment CRP and, *vica versa* when comparing 12-month Ang2 to baseline CRP.

Baseline aCCP and anti-CEP antibodies also positively correlated with TSP-1 confirming an interplay between autoimmunity and neoangiogenesis.

Baseline PDGF-BB and TSP-1 correlated with ccIMT suggesting that these angiogenic mediators play role in autoimmune atherosclerosis.

In our multivariate analysis, PDGF-BB levels were significantly higher in smokers compared to non-smokers at 12 months of TNFi treatment and this suggests that smoking may result in intensified inflammation, neovascularisation and poorer response to treatment as well.

In summary, angiogenesis has an outstanding role in pathogenesis of inflammatory arthropathies, as well as in inflammatory (accelerated) atherosclerosis associated with arthritides and some angiogenic markers may be used as surrogate biomarkers that reflect the interplay of angiogenesis, inflammation and atherosclerosis in arthritides.

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List of publications related to the dissertation

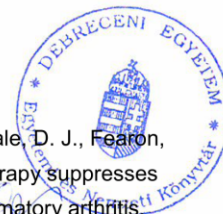
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