THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

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

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CONTENTS

1 ABBREVIATIONS

2-DG	2-Deoxyglucose
4-HNE	4-Hydroxy-2-nonenal
aCCP	Anti-cyclic citrullinated peptide
ACE	Angiotensin converting enzyme
ACPA	Anti-citrullinated protein antibody
ADAM	A Disintegrin and Metalloproteinase
ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin motifs
ADP	Adenosine diphosphate
AIA	Adjuvant-induced arthritis
Ang1	Angiopoetin-1
Ang2	Angiopoietin 2
Anti-β2GPI	Anti-phospholipid cofactor β2GPI
Anti-CarP	Anti-carbamylated protein
Anti-hsp60/65	Anti-heat shock protein 60/65
Anti-oxLDL	Antibodies to oxidized low density lipoprotein
AS	Ankylosing spondylitis
APS	Antiphospholipid syndrome
ATP	Adenosine triphosphate
ATP5B	Adenosine triphosphate synthase subunit β
BAFF	B cell activating factor
BASDAI	Bath Ankylosing Spondylitis Disease Activity Index
BV	Vascular layer
bFGF	Basic fibroblast growth factor
CAM	Cellular adhesion molecules
ccIMT	Common carotid intima-media thickness
CEP	Citrullinated enolase peptide
CIA	Collagen induced arthritis
CM	Conditioned media
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
CTGF	Connective tissue growth factor
CV	Cardiovascular
CVD	Cardiovascular disease
CZP	Certolizumab-pegol
DAS28-CRP	Disease activity score using swollen and tender joints from 28 joint count, patient global health assessment and C-reactive protein level
DAS28-ESR	Disease activity score using swollen and tender joints from 28 joint count, patient global health assessment and erythrocyte sedimentation rate
DAPI	4′,6-Diamidino-2-phenylindole
DCE-MRI	Dynamic contrast–enhanced magnetic resonance imaging
DKK-1	Dickkopf-1
DMARD	Disease-modifying antirheumatic drug
ECAR	Extracellular acidification rate
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ELR	Glutamyl-leucyl-arginyl
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial precursor cell
ERK	Extracellular signaling kinases
ESR	Erythrocyte sedimentation rate
ET-1	Endothelin-1
ETC	Electron transport chain
ETN	Etanercept

EULAR	European League Against Rheumatism
FAK	Focal adhesion kinase
FCCP	Trifluorocarbonylcyanide phenylhydrazone
FDA	Food and Drug Administration
FGF-1	Fibroblast growth factor-1
FGF-2	Fibroblast growth factor-2
FMD	Flow-mediated vasodilation
KGF	Keratinocyte growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage-colony stimulating factor
GLUT1	Glucose transporter 1
GWAS	Genome-wide association study
HB-EGF	Heparin-binding endothelial growth factor
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
hpf	High-power field
HRE	Hypoxia response element
hsCRP	High-sensitivity C-reactive protein
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intracellular adhesion molecule-1
ICAM-3	Intracellular adhesion molecule-3
ICOS	Inducible T-cell costimulator
IGF-1	Insulin-like growth factor-1
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible nitric-oxide synthase
JAK	Janus tyrosine kinase
JAM	Junctional adhesion molecule
JNK	c-Jun N-terminal kinase
LL	Lining layer
LTβ-R	Lymphotoxin- β receptor
mAB	Monoclonal Antibody
mtDNA	Mitochondrial DNA
mtROS	Mitochondrial reactive oxygen species
MAPK	Mitogen Activated Protein Kinase
MI	Myocardial infarction
MIF	Monocyte/macrophage migration inhibitory factor
MMP	Matrix metalloproteinase
M. oryzae	Methylobacterium oryzae
Mt	Mitochondrial
NF-κB	Nuclear Factor-κB
NIK	NF-κB inducing kinases
NO	Nitric-oxide
NSAID	Non-steroidal anti-inflammatory drug
OCR	Oxygen consumption rate
OD	Optical density
OPG	Osteoprotegerin
oxLDL	Oxidized low-density lipoprotein
OXPHOS	Oxidative phosphorylation
PAF	Platelet activating factor
PECAM-1	Platelet endothelial cell adhesion molecule
PI3K	Phosphoinositide 3-kinase (PI3K)
piHDL	Pro-inflammatory high-density lipoprotein
PlGF	Placenta growth factor
PDGF-B	Platelet-derived growth factor subunit B

PDGFR	Platelet-derived growth factor receptor
PFA	Paraformaldehyde
PKM2	Pyruvate kinase isozyme 2
PPARγ	Peroxisome-proliferator-activated receptor γ
PsA	Psoriatic arthritis
PWV	Pulse-wave velocity
RA	Rheumatoid arthritis
RANK	Receptor activator of NF-κB
RANTES	Regulated on Activation Normal T cell Expressed and Secreted
RASFC	Primary rheumatoid arthritis synovial fibroblast cell
RASFC-CM	Primary rheumatoid arthritis synovial fibroblast cell conditioned media
RF	Rheumatoid factor
RMC	Random mutation capture assay
ROS	Reactive oxygen species
RPMI 1640 medium	Roswell Park Memorial Institute 1640 medium
S1P	Sphingosine-1-phosphate
SAA	Serum amyloid-A
SC	Subcutaneous
sFasL	Soluble Fas Ligand
SFK	Src family kinases
SL	Sublining layer
SLE	Systemic lupus erythematosus
SOST	Sclerostin
Spk-1	Sphingosine kinase-1
ST	Synovial tissue
STAT	Signal transducer and activator of transcription
suPAR	Soluble urokinase plasminogen activator receptor
T0	Time point 0 or baseline
T3	Time point - 3 months after starting biological therapy
T6	Time point - 6 months after starting biological therapy
T12	Time point - 12 months after starting biological therapy
TCA	Tricarboxylic acid
TGF-β	Transforming GF-β
TH17	T-helper type 17 cells
Tie2	Tyrosine kinase receptor-2
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor α
TNFi	Tumour necrosis factor α inhibitor
TSP-1	Thrombospondin-1
uPA	Urokinase plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vWF:Ag	von Willebrand factor antigen

2 INTRODUCTION

Angiogenesis is an outgrowth from proliferating blood vessels and acts as an early event in the inflamed joint tissue. It is governed by a tightly controlled balance of pro- and anti-angiogenic stimuli, which promote or inhibit generation and proliferation of new endothelial cells, vascular morphogenesis and vessel remodelling. *De novo* capillary formation is crucial in maintaining the supply of various nutrients, as well as oxygen to the inflamed tissue. Local and systemic expression of angiogenic factors may indicate a constant remodeling of synovial vasculature. Redox signaling is closely related to angiogenesis and can alter angiogenic responses of synovial cells. In this review, we discuss key issues about endothelial pathology in inflammatory arthritis followed by review of angiogenic processes and main angiogenic mediators within the joint. We review broader aspects of angiogenesis, related systemic inflammation and autoimmune atherosclerosis. We discuss the hypoxia-vascular endothelial growth factor (VEGF)/Ang/Tie2 system and its related therapeutic implications in detail with further review of various mediator protein targets and intracellular regulatory pathway targets with their current and potential future role in preclinical or clinical setting whilst ameliorating inflammation.

Our experiments aim to reflect on the detrimental effects of hypoxia and related oxidative stress on aerobic respiration (with a bioenergetic switch towards anaerobic glycolysis in the inflamed synovial tissue and human endothelium) and mitochondrial mutagenesis with their coupled relationship with dysfunctional angiogenesis. In addition, some markers of angiogenesis may readily be measured in the patients' sera and levels of these markers may correlate with atherosclerosis, vascular pathophysiology and systemic inflammatory activity of arthritides as we will demonstrate below.

2.1. The endothelium and the angiogenic process in inflammation

2.1.1. 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) [1-3] and finally forms a primitive vascular network. Numerous physiological processes are featured by angiogenesis, such as reproduction, development and repair, but pathological implications also exist including cancer development, psoriasis, vasculitis, atheroscerosis and inflammatory arthritis [4, 5]. Inflammatory arthritis is one of the disease prototypes, 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 [3, 6]. 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 endothelium. Critical changes, such as vasodilation and increased permeability promote vascular injury and regeneration [7-9]. 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 [1, 10, 11]. The highly dysregulated synovial microvasculature leads to a local deficit in oxygen supply. This is paired with an increased energy demand from the activated infiltrating or resident cells resulting in a hypoxic microenvironment as well as mitochondrial dysfunction [12].

2.1.2 Local angiogenic mediators in the joint

Several inflammatory cytokines, chemokines, chemokine receptors, proteases, extracellular matrix (ECM) molecules, growth factors and cell adhesion molecules have been recognised in the neovascularisation of the inflamed tissue [2, 11, 13-15]. In this section we are going to discuss about those that are involved in the perpetuation and maintenance of the angiogenic process, the main mediators are listed in Table 1.

Table 1: Some angiogenic mediators implicated in inflammatory arthritis

Chemokines and their receptors	CXCL1, CXCL5, CXCL7, CXCL8, CXCL12, CXCL13, CXCL16, CCL2, CCL3, CCL5, CCL20, CCL21, CCL23, CCL28. CX3CL, CCR7, CXCR4, CXCR5, CCR10
Matrix molecules	Type I collagen, fibronectin, vitronectin, laminin, tenascin, proteoglycans, heparin, heparan sulphate, CD147, ADAM, ADAMTS
Cell adhesion molecules	β_1 and β_3 integrins, E-selectin, P-selectin, CD34, VCAM-1, endoglin, PECAM-1, VE-cadherin, Le^y/H, MUC18, JAM
Growth factors	VEGF, Ang2, Tie2, Ang1, bFGF, aFGF, PDGF, EGF, IGF-I, HIF-1, TGF-β, HGF, MIF, PlGF, FGF-1. FGF-2, HGF, HB-EGF, KGF, CTGF
Cytokines	TNF-α, IL-6, IL-11, IL-15, IL-18, IL-17A, IL-11
Proteases	MMPs, plasminogen activators
Others	Angiogenin, angiotropin, pleiotrophin, PAF, substance P, prolactin, SAA, ET-1, COX2, EPO, adenosin, histamin, thrombin, S1P, TLR, leptin, resistin

The process of vessel formation starts with abundant production of *growth factors (GF).* High levels of VEGF and angiopoetin-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 tyrosine kinase receptor-2 (Tie2) and by competing with angiopoetin-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 [16-20]. 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 (NO), endothelin-1 and growth factors (GF): monocyte/macrophage migration inhibitory factor (MIF), placenta GF (PlGF), fibroblast GF-1 and -2 (FGF-1 and FGF-2), epidermal GF (EGF), hepatocyte GF (HGF), heparin-binding endothelial GF (HB-EGF), keratinocyte GF (KGF), insulin-like GF-1 (IGF-1), connective tissue GF (CTGF), platelet-derived GF-β (PDGF-β), and transforming GF-β (TGF-β) [2, 3, 11, 13, 15, 21, 22]. Subsequently, as the vessel matures, at later stages Ang1 becomes important, recruiting pericytes into the newly formed basement membrane to facilitate the blood flow process [13, 15, 21, 23].

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 implicated in synovial angiogenesis [3, 24].

Chemokines are chemotactic cytokines that also modulate inflammatory angiogenesis and many has been implicated from the CXC, CC, C and CX3C families. The pro-angiogenic nature of most CXC chemokines has been associated with the glutamyl-leucyl-arginyl (ELR) amino acid motif within their structure, and many CXC chemokines induce angiogenesis in rheumatoid arthritis (RA) [3, 25-28]. ELR-containing CXC chemokines with angiogenic nature such as CXCL1, CXCL5, CXCL7, CXCL8, CXCL10, CXCL13 and CXCL16 have been frequently described in inflammatory synovial tissue [25, 27-33]. Those CXC chemokines without ELR motif appear to be angiostatic in nature with the exception of CXCL12, which exerts angiogenic effect when binding to its receptor CXCR4. The CXCL12/CXCR4 axis is fundamental in vasculogenesis as it attracts EPCs to line the newly formed blood vessels [2, 33, 34]. Chemokines may also act indirectly to promote angiogenesis through the attraction of immune cells to the site of inflammation. The CC family holds chemoattractants such as CCL2, CCL3 and CCL5 that are chemotactic for monocytes, lymphocytes and macrophages, which further induce angiogenesis. CCL21 promotes neovascularisation indirectly via other proinflammatory cytokine secretions in synovial tissue, but CCL20 and CCL28 are also implicated in angiogenesis with an effect on B-cell and endothelial cell migration [25, 28, 31, 32, 35, 36]. CX3C chemokines have 3 amino-acids between two cysteine residues. CX3CL1 (fractalkine) has a role in leukocyte recruitment and can also promote vessel formation in inflammatory angiogenesis and atherosclerosis [25, 37-39].

Among *extracellular matrix (ECM) components*, type I collagen, fibronectin, laminin, vitronectin, tenascin and proteoglycans are ECM components that mediate, while thrombospondin-1 (TSP-1) is a glycoprotein that regulates endothelial cell adhesion and neovascularization. As described above, some growth factors bind to proteoglycans during angiogenesis [2, 3, 10, 40].

With respect to *cellular adhesion molecules (CAMs),* most β_1 and β_3 integrins, E-selectin, the L-selectin ligand CD34, selectin-related glycoconjugates including Lewis^y/H and MUC-18, intracellular adhesion molecule-1 and -3 (ICAM-1, ICAM-3), vascular cell adhesion molecule-1 (VCAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1), endoglin and junctional adhesion molecules (JAMs) are expressed on the endothelial cell surface and promote neovascularisation. Elevated levels of ICAM-1, ICAM-3 and VCAM-1 were observed not only in RA synovial tissue, but in synovial fluid as well [41-45]. The $\alpha_V\beta_3$ integrin has significant importance as this CAM is involved in osteoclast activation leading to erosions, as well as synovial neovascularisation in RA [46, 47]. Focal adhesion kinases (FAK) modulate $\alpha_V\beta_3$ integrin signaling. FAKs is expressed in the RA synovium suggesting their role in synovial inflammatory angiogenesis [48, 49]. Other angiogenic factors, such as chemokines may act via integrin-dependent pathways [28, 32].

Matrix-degrading enzymes, such as matrix metalloproteinases (MMP), A Disintegrin and Metalloproteinase (ADAM) and A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) proteases digest the ECM, release growth factors and other angiogenic mediators and thus promote inflammatory angiogenesis [1, 50, 51].

Other angiogenic factors implicated in angiogenesis are serum amyloid-A (SAA), endothelin 1 (ET-1), members of the cyclooxygenase-2 (COX-2)-prostaglandin E_2 network, angiogenin, angiotropin, pleiotrophin, platelet-activating factor (PAF), substance P, erythropoietin, adenosine, histamine, prolactin, thrombin, sphingosine-1-phosphate (S1P), toll-like receptors (TLR) [2, 3, 14, 52].

2.1.3. 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 to 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 [53, 54]. 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 [55-58]. 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 [57, 59, 60]. 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 angiogenic process. HIF is composed of HIF-α and HIF-β subunits. Among the three isoforms of HIF-α subunits, HIF-1α and HIF-2α share structural and functional similarities and in hypoxic conditions they are able to translocate to the nucleus and form dimers with HIF-1β. 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-1α to stabilize, dimerize with HIF-1β thus initiating the transcription of genes containing HRE. Both HIF-1α and HIF-2α are strongly expressed in the RA synovium. However, hypoxia may also act via HIF-independent regulatory pathways including the peroxisome-proliferator-activated receptor γ (PPARγ). 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 [16, 55, 56, 61, 62].

2.1.4 Cellular crosstalks in response to inflammatory synovial angiogenesis

Inflammatory arthropathies are featured by perpetuated local joint inflammation followed by enhanced angiogenesis. The local angiogenic response to inflammation leads to

development of immature blood vessel structures and poor pericyte recruitment with destabilization of the vasculature. Inflammatory cells enter the synovial tissue and maintain the inflammatory state. The inflammatory cell infiltrate - brought by the increased vascular supply - results in a higher cellular energy demand in the synovium that consumes tissue oxygen [12]. It has been described that the inflammatory synovium is markedly hypoxic [55]. The shift in the redox equilibrium favours the accumulation of local reactive oxygen species (ROS) and these products trigger further oxidative stress with damage of biologically important proteins and lipids. The lipid peroxidation leads to formation of aldehydes, from which the cardinal one is 4-hydroxy-2-nonenal (4-HNE). It exhibits reactivity with proteins, DNA and phospholipids and is an inducer and mediator of oxidative stress [63]. Increased tissue expression of 4-HNE has been described in inflammatory arthritides when examined via immunhistochemistry and was paralleled by inverse reduction of synovial tissue oxygen levels. This suggests that hypoxia and lipid peroxidation are both early, overlapping events in the process of inflammation [63]. The synovial cells have an ability to adapt their metabolism in response to the inflammatory state and a shift in their metabolism occurs favouring glycolysis. This provides the energy supply for the rapid proliferation, however much less efficient than the trycarboxilic acid cycle. It has also been described that the above shift affects the respiratory complexes inducing dysfunction of the electron transport chain that serves the physiological function of mitochondria linked respiration. The accumulating ROS at the same time result in further oxidative stress and mitochondrial DNA damage [12].

2.1.5 Targeting angiogenic mediators and angiostatic compounds

The *VEGF-dependent pathway* described above has been extensively targeted and VEGF or VEGF receptor (VEGFR) inhibitors have been tried mainly in cancer studies revolutionizing anti-angiogenesis therapies by now. Antibodies to VEGF or VEGFRs, as well as soluble VEGFR constructs have been tried in malignancies. Limited number of pre-clinical studies has also been conducted in arthritis [16, 17, 19, 64]. Probably the most well-known VEGF inhibitor, the anti-VEGF-A antibody bevacizumab has been approved in various forms of metastatic cancer including colorectal, lung, metastatic renal cell carcinoma, glioblastoma, ovarian and breast malignancies [65, 66]. The anti-VEGFR2 tyrosine kinase inhibitor ramucirumab showed potent anti-tumor activity in clinical trials then gained Food and Drug Administration (FDA) approval for the treatment of advanced gastric, gastroesophageal cancer and non-small cell lung cancer [67, 68]. Other novel antiangiogenic molecules such as

regorafenib (anti-VEGFR-2), and aflibercept (VEGF inhibitor) have been approved for metastatic colorectal cancers, gastrointestinal stromal tumors and hepatocellular carcinoma treatment following promising results with several phase III randomized trials [69, 70]. Pazopanib, a multi-tyrosine kinase inhibitor of VEGFR and PDGF receptor (PDGFR) α and β was also effective in the amelioration of angiogenesis *in vitro* and *in vivo* in phase II clinical trials in renal cell carcinoma and other solid tumours. Pazopanib has been approved by FDA for the treatment of advanced renal cell carcinoma and soft tissue sarcoma [71, 72]. With respect to arthritis, vatalanib (PTK787) and an anti-VEGFR-1 antibody exerted significant angiostatic and anti-arthritic effects in animal models of arthritis, whereas a soluble VEGFR-1 chimeric protein also inhibited synovial endothelial proliferation in arthritic models [16, 64, 73, 74]. Soluble Fas ligand (sFasL, CD178) is a functional inhibitor of the 165–amino acid form of VEGF (VEGF165), and it inhibited angiogenesis in arthritis [75]. A collagen induced arthritis (CIA) animal model revealed the significance of early VEGF inhibition in arthritis showing major reduction in disease severity with anti-VEGF sera treatment initiated before the onset of arthritis compared to treatment started after arthritis development [76] . Peroxisome proliferator-activated receptor γ (PPARγ) ligands rosiglitazone and pioglitazone inhibited VEGF-induced angiogenesis [77]. A soluble Tie2 receptor transcript delivered via an adenoviral vector to mice attenuated the incidence and severity of CIA [78]. A bispecific antibody Ang2 targeting peptide genetically fused to adalimumab enhanced anti-TNF-α efficacy [79]. HIF-1 may also be targeted in arthritis, as well as in cancer. No advanced human clinical trials have been conducted yet. A pilot trial of the oral HIF-1α inhibitor topotecan significantly decreased histological HIF-1α and VEGF expression along with a decrease in tumour blood flow and vessel permeability *in vivo* assessed by dynamic contrast–enhanced magnetic resonance imaging (DCE-MRI) [80]. The benzophenone analogue, BP-1, a HIF-1α inhibitor, ameliorated adjuvant-induced arthritis (AIA) in rats [81]. The proteasome inhibitor bortezomib in combination with bevacizumab was investigated in a Phase I clinical trial in advanced refractory malignancies and results suggested the inhibition of HIF-1 related angiogenic pathway [82]. YC-1, a superoxide-sensitive stimulator of soluble guanylyl cyclase and a HIF-1 inhibitor may potentially be used to suppress inflammatory angiogenesis [83].

Antirheumatic agents in current use such as rofecoxib, dexamethasone, chloroquine, sulphasalazine, methotrexate, azathioprine, cyclophosphamide, leflunomide, thalidomide, tacrolimus, minocycline, anti-TNF-α biologics and Janus kinase (JAK) inhibitors nonspecifically suppress angiogenesis [3, 19, 64, 84]. TNF-α blockade by infliximab was shown to reduce VEGF, Ang1 and Tie2 expression as well as vascularity within the RA synovium [85, 86].

Certolizumab pegol inhibits TNF-dependent angiogenesis [87]. The anti-IL-6 receptor antibody tocilizumab also decreased serum levels of VEGF and synovial neovascularization in RA [88]. The JAK inhibitor tofacitinib demonstrated inhibitory effects on migration, invasion and pro-angiogenic cytokine secretion in psoriatic arthritis in vitro [84]. IL-17 has been implicated in inflammatory angiogenesis, therefore, anti-IL-17 blockade may also be feasible in this respect [89].

Direct or indirect approaches may aim to target *chemokines* and their receptors. Some non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, traditional and biologic disease modifying antirheumatic drugs (DMARDs) may, in addition to their other anti-inflammatory effects, inhibit chemokines and chemokine receptors [25, 28, 32]. Briefly antioxidants, such as N-acetyl-L-cysteine and 2-oxothiazolidine-4-carboxylate, the bioflavonoid quercetin, the lipid-lowering simvastatin, green tea extracts inhibited the expression of various chemokines in vitro. PPARγ agonists, such as glitazones and oriental medicines, such as triptolide, lingzhi, curcumin, tongbiling, honokiol, cool-cool and others also exert anti-arthritic effects, which may, in part, be explained by chemokine and chemokine receptor inhibition. With respect to specific molecular targeting with neutralizing antibodies to various chemokine ligands blocked arthritis both therapeutically and preventatively in various RA animal models [25, 28, 32]. Due to the complexity of the several regulatory loops in the chemokine network, most of these approaches failed in human RA. There has only been a very limited number of human anti-chemokine trials and therefore we do not aim to discuss those in detail [90]. Without being exhaustive few examples are given from recent chemokine and chemokine receptor targeting research. An anti-CCL2 antibody, ABN912, was evaluated in a randomized, placebo-controlled study, but there was no detectable clinical benefit that would suggest that targeting of a single chemokine may not be effective in arthritis [91]. Some CXCR antagonists have been used in animal models and show antitumor activity in human cancer. From CCR antagonists the ones inhibiting CCR1 (CP-481,715 and MLN3897), CCR2 (MLN1202) and CCR5 (AZD5672, SCH351125, maraviroc) reached the stage of phase I and II clinical trials in RA with disappointing results [28, 32, 90].

Antibiotic derivatives, such as minocyclin, fumagillin analogues, deoxyspergualin, roxithromycin and clarithromycin also inhibit angiogenesis and VEGF release. Synthetic fumagillin derivatives, TNP-470 and PPI-2458 inhibit VEGF and VEGF-dependent angiogenesis. Minocycline, roxithromycin and clarithromycin exerted moderate, but significant clinical effects [2, 14, 19, 64].

Traditional *herbal remedies* may also influence angiogenesis. Scopolin, a coumarin derivative found in Erycibe obtusifolia Benth; celastrol, an active ingredient of Tripterygium wilfordii, also known as "Thunder God Vine"; or fisetin, the active ingredient of Rhus verniciflua Stokes may also have angiostatic effects in arthritis [2, 14, 19, 64]. Resveratrol, curcumin and tetramethylpyrazine in combination also appeared to inhibit the production of angiogenic cytokines in a mouse arthritis model [92].

2.1.6 Intracellular pathways and potential therapeutic targets

The relevant signaling pathways implicated in the pathogenesis of inflammatory angiogenesis are highlighted on Figure 1.

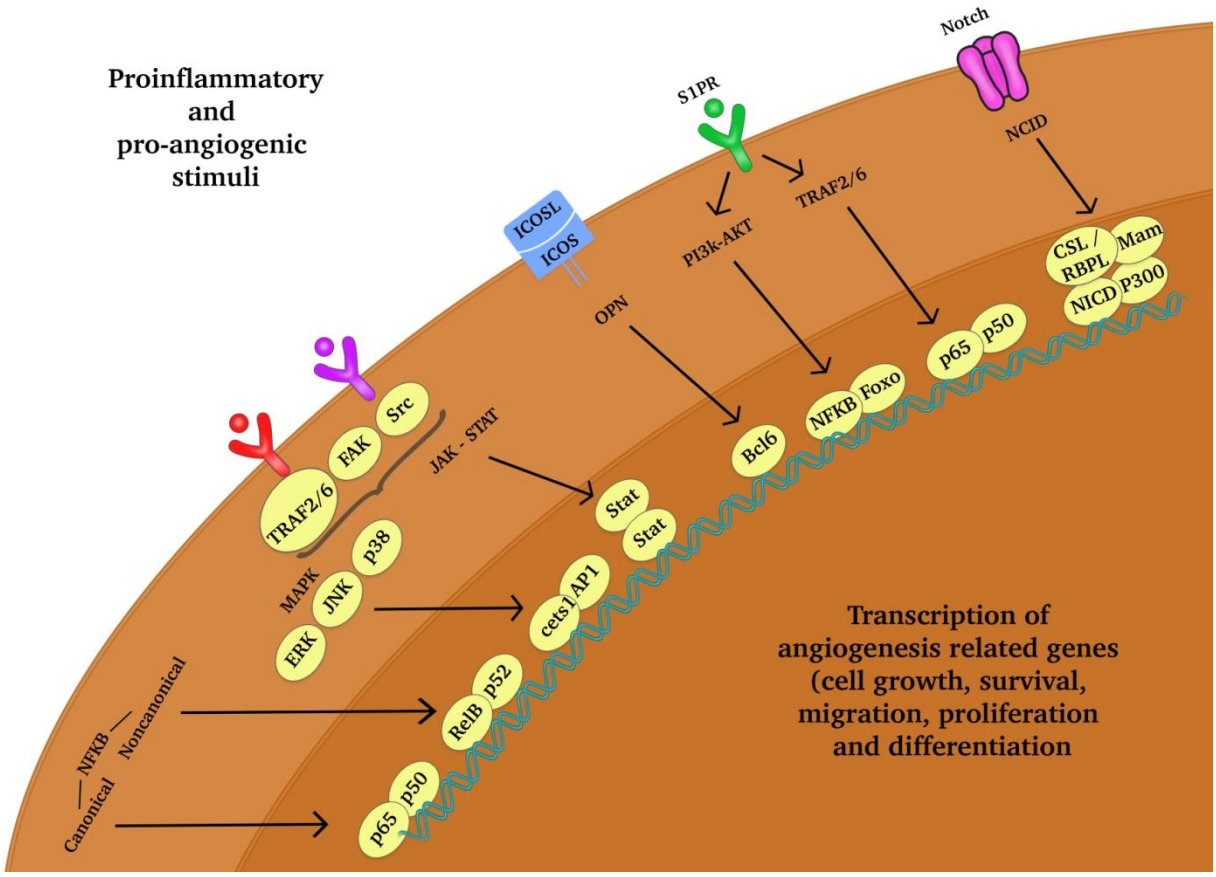

Figure 1. Sigaling pathways implicated in inflammatory angiogenesis

One of the most recognised signal transduction pathway modulating inflammation is the *Nuclear Factor-κB (NF-κB)* pathway. NF-κB signaling follows a canonical and a non-canonical route. Canonical NF-κB signalling is fast acting mainly via the actions of RelA/p50 DNA binding whereas the non-canonical pathway is slower and uses RelB/p52

heterodimerisation for gene transcription [93, 94]. Both pathways have potent pro-angiogenic potential in pathologies including cancer and autoimmune rheumatic diseases and they mediate the production of various pro-angiogenic molecules such as TNF-α, IL-1β, IL-6, CCL2 and IL-8 (CXCL8), COX-2, inducible nitric-oxide synthase (iNOS), MMPs and the adhesion molecules ICAM-1 and VCAM-1 [93-95]. Numerous compounds with reported inhibitory effects on NF-κB signalling have been developed, but larger clinical trials are lacking, may be due to concerns about toxicity associated with global NF-κB inhibition or off-target effects. NF-κB targeting selective for a specific cell type could offer a solution. As an example, a specific recombinant protein "sneaking ligand contruct" has been developed that binds to cytokine-activated endothelial cells and appears to be effective in inflammatory states *in vivo* [96]. The canonical and non-canonical pathways both can be triggered by activation of lymphotoxin- β receptor (LTβ-R), CD40, the B cell activating factor (BAFF) receptor and receptor activator of NF-κB (RANK) [97]. Direct inhibition of NF-κB inducing (NIK) kinases or IKKα homodimers of the non-canonical pathway may serve a potential therapeutic target [21, 98-100]. In RA synovial inflammation, NIK can be targeted using small molecule inhibitors [98]. BAY32-5915, a specific IKKα inhibitor has been reported in melanoma treatment [99]. NIK silencing may potentiate anti-VEGF therapy in cancers [100].

Mitogen Activated Protein Kinases (MAPK) include extracellular signaling kinases (ERK), the c-Jun N-terminal kinases (JNK) and the p38 mitogen activated kinases. All these kinases have been detected in RA synovial tissues with peak expression of ERK on synovial blood vessels [21, 101, 102]. One transcription factor known to be activated by the MAPK family of proteins is Ets-1. Ets-1 promotes angiogenesis through the p38 MAPK in endothelial cells with downstream effect of VEGF synthesis, it also regulates the production of proangiogenic molecules (TNF-α, CCL2) by lymphocytes [103, 104]. Targeting of all three kinases have been attempted. Small molecule p38 inhibitors appeared effective in animal studies, but showed lack of efficacy or failed due to toxicity in clinical trials may be due to the fact that p38 also exerts anti-inflammatory effects [21, 102]. Moreover, blocking one kinase may lead to the compensatory activation of others that regulating the same genes [105]. Clinical trials using semapimod in inflammatory bowel disease were promising [106, 107]. Blocking kinases that are located more upstream (such as MKK3/6) may serve a better therapeutic potential [102]. Among ERK inhibitors, FR180204 significantly amliorated arthritis in CIA mice [108]. Examining noncompetitive inhibitors of MEK1/2 (PD184352 and PD0325901) have also reached clinical trial stage as potential anticancer agents [109, 110]. However, no clinical trials in chronic inflammatory diseases have been performed so

far. Granzyme B gene silencing rat CIA model revealed suppressed activation of MAPK pathway by reduced phosphorylation of ERK and MEK [111]. A direct inhibitor of JNK activity, SP600125 decreased paw swelling in the rat AIA model with associated inhibition of radiographic damage [112]. Later this JNK inhibitor was replaced by more selective compounds. At present, several companies develop such JNK inhibitors, mostly in preclinical arthritis models [113, 114].

The *phosphoinositide 3-kinase (PI3K)* pathway has also been implicated in regulation of cellular processes such as cell growth, survival, migration, proliferation and differentiation. The PI3K family of kinases (known as protein kinase B) signals through Akt. Akt is essential for angiogenic processes both in physiological and pathological conditions and an activated pathway is able to initiate the transcription of VEGF, endothelial nitric-oxide synthase (eNOS), HIF-1α and HIF-2α and E-selectin modulating neovascularisation and chemotaxis [115, 116]. First generation broad spectrum PI3K inhibitors have entered preclinical studies mainly with major targets of cancer. Wortmannin and LY294002 have set safety concerns, but newer agents have been introduced into phase I clinical trials (BEZ235, BGT226, BKM120, XL765, XL147, GDC0941, GSK1059615, SF1126, PX-866, CAL-101) offering innovative targeted treatment for the future [117-119]. Lately idelalisib showed efficacy in treatment of haematologic malignancies [120].

The *Inducible T-cell costimulator (ICOS)* facilitates the differentiation and function of follicular helper T cells and inflammatory T cells. ICOS has been shown to be a key costimulatory pathway which controls induction and maintenance of murine CIA and elevated levels of $ICOS^+$ T cells has been found in the synovial fluid of patients with RA [121, 122].

Signalling by *Janus tyrosine kinase (JAK)* is a novel pathway that gained great interest in treatment of inflammatory arthritis. In the process of signalling throughout the activation of JAK subunits the crosslinked receptor recruits signal transducers and activators of transcription (STAT) proteins, which then become phosphorylated and are able to translocate to the nucleus to activate gene transcription. STATs are principle regulators of many pro-inflammatory genes including IL-17, ICAM-1 [123]. In RA, expression of activated STAT proteins has been visualized through immunohistochemistry. Several activators of the JAK/STAT pathway have been implicated in angiogenesis such as IL-6 activating STAT1 and STAT3, or GM-CSF activating JAK2/STAT3 leading to neovascularization in the chorioallantoic membrane assay [124-126]. Tofacitinib - blocking JAK1 and JAK3 -, then baricitinib - blocking JAK1 and JAK2 - demonstrated safety and efficacy and gained approval

for application in the clinical setting in treatment of RA (tofacitinib and baricitinib) and psoriatic arthritis (tofacitinib) [127-130]. Both filgotinib and upadacitinib showed promising results in patients with RA in phase IIb or III studies [131, 132]. The angiostatic effects of the JAK inhibitor AZD1480 have been demonstrated in malignancies [133].

The *Focal Adhesion Kinase (FAK)* pathway is essential in physiological signalling and contributes to angiogenesis in chronic inflammatory diseases and cancer [48, 49, 134]. Growth factor, integrin and cytokine receptors can active FAK and this pathway for migratory processes indespensible of angiogenesis. FAK acting through the integrin $\alpha_V\beta_3$ also plays a significant role in cell adhesion. FAK expression is known to be higher in patients both with rheumatoid arthritis, osteoarthritis and several cancer types compared to healthy subjects [48]. FAK deletion in mice injected with lung carcinoma cells leads to a significant decrease in angiogenic responses to VEGF and the endothelial cells isolated from these mice exhibit decreased proliferation and increased apoptosis [135]. Targeting FAK directly via FAK-silenced RNA or with combined PP2 inhibition with its downstream src pathway showed some anti-angiogenic effect. Phase I and II clinical trials using retroviruses expressing p53 indirectly into cancer cells (AD5CMV-p53) or direct FAK phosphorylation with small molecules (TAE226, PF-562,271, PF-04554878, GSK2256098) has been tried [21, 135].

The *Src kinase* signalling is involved in crucial biological processes leading to angiogenesis, such as cell cycle control, cell adhesion, migration and proliferation. Src kinases are activated by various growth factors and cytokines. VEGF and basic fibroblast GF (bFGF) mediated activation is well recognised in endothelial cells, however, it seems to de dispensable for bFGF-mediated cell growth [136]. Due to the ability of crosslinking with various other pathways the Src family of kinases modulate several cellular processes. In combination with JNK activation Src plays a central role in IL-18-induced angiogenesis in RA [137]. It also regulates, in parallel with the PI3K pathway, soluble E-selectin-induced angiogenesis [138]. The proto-oncogen src is another one of the multiple therapeutic targets of angiogenic pathways [139]. Inhibition of src gene was attempted in many early phase clinical trials through targeting src protein, a non-receptor tyrosine kinase from the src family kinases (SFK). Its complex function partly remains unclear and limitations as a single agent make clinical applications challenging [139]. Inhibition of Src kinases targeted through their small-molecule inhibitors lately attracts attention, mainly in cancer treatments [139]. Dasatinib is the first FDA approved prototype as a SFK/ABL dual inhibitor for the treatment of chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia [139, 140]. Later bosutinib and ponatinib both gained approval for leukemias and

several other agents are in clinical trials [139]. Periploca forrestii Schltr., a classical traditional Chinese medicine also appeared to reduce paw swelling of rats in a CIA model with associated downregulation of Src activity [141].

Sphingosine-1-phosphate (S1P) and related pathological angiogenesis recently gained considerable interest. This bioactive lipid molecule has been shown to play a role in physiological processes such as cell growth, differentiation, migration, survival and angiogenesis [142, 143]. It is generated by the sphingosine kinase-1 (Spk-1), which has been implicated in the regulation of TNF-α-dependent release of IL-1β and IL-6 [143]. S1P promotes the persistence of activated CD4 T cells in inflamed sites hence perpetuating the inflammatory process [144]. Synovial fluid levels of S1P were found to be higher of patients with RA as compared to OA. Inhibiting the production of S1P leads to attenuated expression of TNF-α, IL-6, IL-1β, CCL2/MCP-1 and MMP-9 in RA mononuclear cells *in vitro* [145]. Safingol (l-threo-dihydrosphingosine), a putative inhibitor of Spk-1 entered phase-I clinical trials and showed downregulation of S1P [146]. An Spk-1 inhibitor, ginsenoside compound K has been studied in the amelioration of cell migration, proliferation and offers new therapeutic target in cancer treatment [147]. Data about an anti-S1P antibody also suggest that it has a role in the suppression of tumor angiogenesis and proliferation in mouse xenograft models *in vivo* [143].

Notch signalling is a highly conserved pathway that has been implicated in endothelial cell interactions, proliferation, differentiation and angiogenesis [148]. Notch canonical ligands (Jagged-1 and -2 [JAG1, 2] and Delta-like 1, 3, and 4) - expressed in mammalian cells - binding to their notch receptors (Notch 1-4) are able to induce γ-secretase mediated cleavage and release of the intracellular receptor domain. The nuclear translocation of notch activates target genes of Hes-1 and HESR1 promoting the transcription of angiogenic proteins [148]. High expression of Notch ligands and receptors were confirmed on the endothelium and actual tumor cells of different malignancies playing a role in vessel maturation signals [149]. Targeting the Notch pathway serves potential therapeutic target in inflammatory arthritis. However a previous phase II trial using RO4929097 compound investigated the side effects and safety in tumour angiogenesis inhibition in metastatic melanoma, probably due to inadequate therapeutic drug levels further investigations were not indicated [150]. Several other Notch inhibitors such as therapy (MK-0752, BMS-906024, PF-03084014, LY900009) attracted the attention and been clinically evaluated mainly in the field of cancer therapy [21, 151, 152]. Monoclonal antibodies (mAb) against Notch receptor and ligand competitors such as OMP-59R5 (humanized mAb), OMP-21M18 (humanized anti-Dll4 mAb), A5226A,(mAb

against the extracellular domain of nicastrin) are under development and EGF-like domain 7 (a soluble Notch ligand decoy) showed inhibitory effects on tumor neovascularisation in vitro [152]. The Notch receptor domain γ-secretase provides promising target for receptor inhibitors and several products (the dipeptide inhibitor z-Ile-Leu-CHO (GSI-I), GSI MRK-003, GSI PF-03084014, BMS-708163) have been tried in the preclinical setting with anti-angiogenic or anti-proliferative effects and currently are in clinical trials in malignancies [21, 152]. Nanoparticle delivery of Notch-1 siRNA significantly decreased paw swelling and arthritis scores in an animal study of rheumatoid arthritis highlighting that nanomedicine delivery of small molecules could be a promising future therapeutic approach [153].

2.2. Autoimmune-inflammatory atherosclerosis

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

RA and other inflammatory rheumatic conditions are associated with increased cardiovascular (CV) morbidity and mortality triggered by autoimmune atherosclerosis [39, 154, 155]. Various immune cells, pro-inflammatory cytokines and chemokines, GFs, proteases, CAMs, as well as other mediators play crucial role in the process of inflammatory atherosclerosis and angiogenesis [156-158]. Some of the above could serve as potential biomarkers of these vascular events [157]. 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 [155, 156, 158, 159]. 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 MMPs in the pathology of atherosclerotic rupture and thrombosis (via erosion of the fibrous caps) as well as joint destruction [155, 156, 158, 159]. 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 [155, 156, 158, 159].

2.2.2 Triggering factors in autoimmune atherosclerosis

Strong genetic background features either RA either cardiovascular disease (CVD) and atherosclerosis. Numerous susceptibility alleles such as *HLADRB1, PTPN22, TRAF1/C5, STAT4, PADI4, IRF5, FCGR, IL2RA, IL2RB, CD40, CCR6, CCL21* have been revealed in RA, genome-wide association studies (GWAS). More than 40 SNPs have been associated with RA [160, 161]. Looking at atherosclerosis, a large population based (>100,000 Europeans) GWAS study revealed association of >30 genes in coronary atherosclerosis including matrix molecule (e.g. *ADAMTS7, ANKS1A, COL4A1*), lipid (e.g. *LPA, LDLR*), chemokine (e.g. *CXCL12*) and other genetic loci [162, 163]. *HLADRB1* is associated with RA [164] and CVD as well [165] highlighting the possibility of having common genetic denominators for the two conditions. Gonzalez-Gay and their group performed studies primarily on single non-HLA alleles such as *MTHFR, SMAD3, CD40, IRF5, OPG* and others (reviewed in [166, 167]. As the association of RA and atherosclerosis with complex genetic signatures has not yet been elucidated, we have recently performed the analysis of genetic signatures that may determine RA-associated atherosclerosis [168]. Theoretically, single genetic alleles or complex signatures may reflect autoimmune atherosclerosis, however, the assessment of genes as biomarkers may have several limitations.

Environmental factors such as diet, smoking, alcohol, infections and silica are all risk factors amongst many others that have been implicated in the pathogenesis of RA and atherosclerosis [156, 169]. Among infectious agents, Porphyromonas gingivalis bacteria and periodontitis may provide link between RA and atherosclerosis [170].

Genetic and environmental factors may be associated with autoantibody production as known from several autoimmune diseases. The focus of research in RA targeted studies examining anti-citrullinated (ACPA) and anti-carbamylated proteins (anti-CarP) as well as rheumatoid factor (RF) [171]. Atherosclerotic plaques are known to express citrullinated proteins [172], which may drive ACPA-dependent atherogenesis of RA. Members of my research group [173] and others also demonstrated the association of RF and ACPA in the vascular pathology of RA [174, 175]. Both arthritis and atherosclerosis are linked to

periodontitis. Gingival bacteria produce α-enolase and anti-citrullinated enolase peptides (CEP) have been associated with the pathogenesis of RA [170, 176, 177]. Other autoantibodies, such as antibodies to oxidized low density lipoprotein (anti-oxLDL), the phospholipid cofactor β2GPI (anti-β2GPI), anti-phosphorylcholine and anti-heat shock protein 60/65 (anti-hsp60/65) also play role in the pathogenesis of autoimmune atherosclerosis [158, 178-182]. My colleagues also demonstrated association of antiphospholipid and anti-oxLDL antibodies with CVD [178, 179, 181].

2.2.3 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 [156-158]. Few authors examined the role of these mediators in autoimmune atherosclerosis and CVD, where the most meaningful marker appears to be the C-reactive protein (CRP), independently predicting CV risk [183]. My colleagues demonstrated marked expression of ICAM-1 adhesion molecule and increased TNF-α, IL-6, IL-8 and interferon γ (IFN-γ) production from the vasculature of inflammatory aortic aneurysms [184-186]. It was also found that Von Willebrand factor antigen (vWF:Ag) production is perpetuated in RA as sign of endothelial activation [187]. Higher expression of TNF-α and IL-18 were observed in the aortic adventitia of RA patients with CVD when compared to CVD patients only [188]. Ultrasound evaluation of patients with rheumatoid arthritis previously highlighted endothelial dysfunction by assessing flow mediated vasodilation (FMD), arterial stiffness via pulse-wave velocity (PWV) test and carotid atherosclerosis by measuring common carotid intima-media thickness (ccIMT) and assessing the presence of plaques [189]. Kerekes et al found correlations between impaired FMD and increased serum TNF-α, IL-6 and CRP levels, but also between increased ccIMT with longer disease duration, and increased TNF-α and CRP levels. Increased ccIMT however negatively correlated with lower IL-4 and IL-10 levels [173]. Increased neovacularisation, primarily stimulated by pro-angiogenic factors including 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 [2, 14, 157, 158][190]. CAMs, such as VCAM-1, ICAM-1 and E-selectin have been linked to endothelial dysfunction associated with atherosclerosis and angiogenesis in RA [41, 186,

191]. Among proteases, the urokinase plasminogen activator (uPA) system has a selected role in fibrinolysis, matrix degradation, inflammation and angiogenesis. The cell-bound, GPI-anchored protein uPA receptor (CD87) can be detected on synovial tissue, leucocytes and endothelial cells, but may be released from the cell membrane into a soluble form as well (suPAR). High levels of suPAR have been described with a number of autoimmune diseases [192, 193]. We demonstrated that suPAR levels correlated with RF and anti-cyclic citrullinated peptide (aCCP) levels in patients with RA [194]. NT-proBNP, a marker of myocardial function, also related to angiogenesis, has been found to be elevated in early inflammatory arthritis predicting increased all-cause CV mortality [195]. The bone marker osteoprotegerin (OPG) also associated with bone destruction in RA, has been linked to atherosclerosis and CVD. High OPG levels correlated not only with CVD morbidity but also with mortality in RA [196-198].

2.2.4 Cardiovascular and angiogenic biomarkers in monitoring therapeutic efficacy

The European League Against Rheumatism (EULAR) published recommendations for the cardiovascular risk management of patients with RA primarily focusing on prevention. Given that systemic inflammation drives the vascular pathology, the treatment of the underlying rheumatological condition is crucial [155]. Numerous studies on large patient cohorts suggested that conventional synthetic and biologic DMARDs may halt the development of secondary CVD [199-202]. In association with their clinical efficacy, targeted therapies may reduce the production of potential biomarkers of atherosclerosis and angiogenesis [157, 201]. It was found that adalimumab significantly decreased levels of soluble vWF:Ag in an early RA study [187]. Treatment with TNF inhibitors significantly decreased levels of VEGF in patients with ankylosing spondylitis (AS) and levels of PDGF-BB in RA patients [190]. Circulating oxLDL/β2GPI complex and anti-oxLDL/β2GPI antibody (AtherOx) levels were significantly reduced in sera of RA patients following TNFi therapy [181]. Adalimumab decreased NT-proBNP levels in a Dutch RA cohort [203]. My colleagues supported that TNF inhibition significantly suppressed NT-proBNP levels in RA patients. Moreover, seropositive patients had higher NT-proBNP levels compared to seronegatives [181]. Anti-TNF-α therapy was also able to decrease suPAR levels in RA, most markedly in those having a very high baseline suPAR measurement [194]. Tocilizumab therapy decreased markers of haemostasis such as fibrinogen and D-dimer in RA [204]. IL-6 blockade also reduced levels of serum amyloid A (SAA) and pro-inflammatory high-density

lipoprotein (piHDL) containing serum amyloid-A (SAA), all of which has pathogenic role in inflammation, atherogenesis and angiogenesis [204, 205]. Finally targeted therapies may differentially regulate the expression of those bone biomarkers (OPG, SOST and DKK-1), which have a role in vascular calcification and inflammatory joint destruction [198, 206]. It is yet to be elucidated whether a single or multi-biomarker approach should be preferable. Combination of laboratory biomarkers of inflammation, proteolysis, coagulation and imaging markers were tried to reflect vascular pathophysiology and effects of therapy [207]. Currently, the 13-component multi-biomarker assay, as well as other tests have been analyzed in this manner [208, 209].

3 AIMS

3.1 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 rheumatoid arthritis 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 TNFi treatment is able to reduce synovial tissue angiogenesis in patients with RA.

3.2. 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

- angiogenic activity indicated by angiogenic biomarkers in relation to atherosclerosis, vascular pathophysiology, oral health and some other clinical parameters.

4 PATIENTS AND METHODS

4.1. Study 1

4.1.1 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.

The PhD student recruited some patients and performed 60% of knee arthroscopies for sample collection. Douglas J Veale, Carl Orr and Chin-Teck Ng recruited other patients and completed the rest of the knee arthroscopies.

4.1.2 Rheumatoid arthritis synovial fibroblast cell culture

RA synovial tissue (collected via knee arthroscopy) was 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 % CO_2. Dissociated RASFC 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. First RASFC were seeded onto 96-well plates and into T25 flasks. RASFC cells were cultured in the presence of 4-hydroxy-2-nonenal (4-HNE,2.5 μM; Cayman Chemical, Ann Arbor, MI, USA) – which is a highly reactive compound from lipid peroxidation - or vehicle basal medium (0.1 % ethanol). The 4-HNE concentration was selected based on a cell viability assay used in previous

reports [210]. Following stimulation, the effect of amplified oxidative stress on mitochondrial function, cellular metabolism and angiogenic responses was assessed as described below.

The PhD student contributed to 60 % of passaging, seeding and culturing with 4-HNE and the remaining work was carried out by colleagues.

4.1.3 Human umbilical vein endothelial cell culture

We supplemented HUVECs (Lonza, Walkerville, MD, USA) were incubated in MCDB (Thermo Fisher Scientific) 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). Cell cultures were kept at 37 °C in humidified air with 5 % CO_2 and harvested with trypsin-ethylenediaminetetraacetic acid (Lonza). Cells from passages between 20 and 30 were used for further experiments.

The PhD student contributed to culturing and passaging in 60 % and the rest was completed by Monika Biniecka.

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

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), reflecting oxidative phosphorilation (OXPHOS) and glycolysis, respectively, were measured before and after treatment with modulators of OCR and ECAR, such as oligomycin (2 μg/ml), trifluorocarbonylcyanide 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). Oligomycin is an inhibitor of ATP synthase (complex V) favouring glycolysis, reducing OCR and increasing ECAR. FCCP disrupts the mitochondrial membrane potential, the electron flow becomes uninhibited through the electron transport chain and complex IV related oxygen consumption maximizes. Antimycin inhibits complex III and shuts down mitochondrial respiration, enabling the calculation of nonmitochondrial respiration. 2-DG is a glucose analog which binds glucose hexokinase and inhibits glycolysis, reduces ECAR. 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. We

rinsed with unbuffered DMEM plus 10 mM glucose, 1 mM sodium pyruvate and 2 mM L-glutamine, pH 7.4 assay medium. Thereafter incubation with assay medium for 30 minutes at 37 °C in a non-CO_2 incubator taken place. Ccells were stimulated then with 4-HNE (2.5 μM) and vehicle basal medium for a period of 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.

The PhD student contributed to all cell seeding and incubation with assay medium. Stimulation with 4-HNE and OCR/ECAR measurements were obtained by Monika Biniecka.

4.1.5 *In vitro* mitochondrial dysfunction and mitochondrial DNA mutagenesis

Reactive oxygen species (ROS) production was examined wit the DCFDA Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, Cambridge, UK). RASFC seeded onto 96-well plates (clear bottomed and dark sided) at a density of 2.5×10^4 cells/well were allowed to attach overnight. Cells were washed in 1× buffer and stained with 25 μM2 ′,7′-dichlorofluorescin diacetate in 1× buffer for 45 minutes at 37 °C and 5 % CO_2. After staining, cells were washed, treated with 4-HNE and incubated at 37 °C in 5 % CO_2. We assessed ROS fluorescence signal with SpectraMax Gemini system (Molecular Devices, Sunnyvale, CA, USA) with excitation and emission wavelengths of 485 nm and 538 nm, respectively. Mean fluorescence values were measured from 4 wells for each condition.

To characterise the frequencies of random mutations in RASFC exposed to 4-HNE for 24 hours, we used a mitochondrial random mutation capture assay. The assay consisted 4 steps of organelle separation, mitochondrial DNA (mtDNA) extraction, mtDNA digestion and qPCR-amplification using a previously reported protocol [211]. To minimize the nuclear DNA contamination fresh cell cultures were used with isolation of mitochondria prior to mtDNA extraction. MtDNA extraction was completed via lysis with a Proteinase K and SDS rich buffer and addition of RNase, then 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. The next step was the PCR amplification of the single DNA molecules, which 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 H_2O. Two primer pairs were used, one of which flanked the TaqαI restriction site (to quantify the DNA molecules with mutation), the other one didn't (to quantify every mtDNA molecule present). The annealing temperature was 60 °C. 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 37 °C incubation period is the only uncommon step compared to standard real-time PCR, which allows the uracil-DNA glycosylase to destroy contaminating products. 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′-CCTCAACAGTTAAATCAACAAAACTGC-3′ (forward) and 5′-GCGCTTACTTTGTAGCCTTCA-3′ (reverse).

The PhD student completed the reactive oxygen species assay to 80 % of each step and helped with extraction/digestion/amplification of DNA to 20 %. Rest of the processes were carried out by Monika Biniecka.

4.1.6 Examination of mitochondrial complex I–V activity

The direct effect of 4-HNE on all complexes of the mitochondrial respiratory chain (complexes I-V) was assessed with complexes I–V OXPHOS activity assay kits (Abcam). These assays use bovine heart mitochondria, a rich source of OXPHOS complexes. The activity of mitochondrial complexes I–V was measured as per the manufacturer's instructions. Briefly, OXPHOS complex I (NADH ubiquinone oxidoreductase) catalyses electron transfer from NADH to the electron carrier, ubiquinone, at the same time it pumps protons across the inner mitochondrial membrane. This oxidative reaction was monitored by spectrophotometry at optical density (OD) 340 nm. OXPHOS complex II (succinate-coenzyme Q reductase) is responsible for electron transfer from succinate to the electron carrier, ubiquinone. The product, ubiquinol, is used by complex III in the respiratory chain, and fumarate is necessary to maintain the tricarboxylic-acid (TCA) cycle. The production of ubiquinol in the presence of 4-HNE was monitored at OD 600 nm. To examine OXPHOS complex III activity, succinate

(electron donor of complex II) and oxidised cytochrome c (electron acceptor of complex III) were added to the mitochondria to start the electron transfer reaction that takes place during OXPHOS. The rate of coupled complex II+III reaction was measured by monitoring the conversion of oxidised cytochrome c into reduced form, observed as an increase in absorbance at OD 550 nm. OXPHOS complex IV (cytochrome c oxidase) transfers electrons from reduced cytochrome c to molecular oxygen and concomitantly pumps protons across the inner mitochondrial membrane. The post-oxidative reaction was recorded as a decrease in absorbance at OD 550 nm. OXPHOS complex V makes about 95% of a cell's adenosine-triphosphate (ATP) using energy generated by the proton-motive force and can also function in the reverse direction in the absence of a proton-motive force, hydrolysing ATP to generate adenosine diphosphate (ADP) and inorganic phosphate. The production of ADP by ATP synthase can be coupled to the oxidation of NADH to NAD+, and the progress of the coupled reaction in the presence of 4HNE was monitored as a decrease in absorbance at OD 340 nm. Results were calculated using SoftMax Pro 5.3 microplate analysis software (Molecular Devices). The activity of complexes I, II, IV and V is proportional to the decrease in absorbance, and the linear rate of reduction in absorbance over time was calculated. The activity of complex III is proportional to the increase in absorbance, and the linear rate of increase in absorbance over time was calculated. 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.

The PhD student performed measurement of optical densities and software analysis with the help of Monika Biniecka in this experiment.

4.1.7 Quantification of pro-angiogenic mediators in RASFC

We examined the effects of oxidative stress on secretion of angiogenic markers (VEGF, Ang2, PDGF-B), bFGF, IL-8, regulated on activation, normal T cell expressed and secreted (RANTES) and ICAM-1. For this RASFC were seeded into 96-well plates to confluency. RASFC then were serum-starved for 24 hours and 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).

The PhD student completed measurements for VEGF, Ang2 and PDGF-B secretion along with seeding and 4-HNE stimulation for the rest of the experiments. Monika Biniecka and Trudy McGarry completed measurements for bFGF, IL-8, RANTES and ICAM secretion.

4.1.8 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, RASFC were stimulated with 4-HNE (24 hours), then the conditioned media (CM) was harvested (4-HNE RASFC-CM). For basal medium, we used fibroblast-conditioned media from RASFC cultured in the absence of 4-HNE (basal RASFC-CM). HUVEC culture was stimulated with 10 % 4-HNE RASFC-CM or basal RASFC-CM. To make sure that the pro-angiogenic effects on HUVEC function were not due to the 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 μM), which is the same concentration as that in the 10 % 4-HNE RASFC-CM. We have exposed HUVEC to 4-HNE RASFC-CM or 4-HNE RPMI medium for a period of 24 hours, then assessed pro-angiogenic responses of endothelial cells described in the following sections. The PhD student contributed to completing cultures and stimulations and received help from Monika Biniecka for the rest.

HUVEC transwell invasion chambers were set to examine HUVEC invasion (BD BioCoat Matrigel invasion chambers, BD Biosciences, Wokingham, UK).. Cells were seeded at a density of 2.5×10^4 per well in the migration chamber on 8-μm 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. We removed non-migrating HUVEC from the upper surface by gentle scrubbing. Invading cells attached to the lower membrane were 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. The PhD student performed seeding, fixing and staining. Scoring under microscope was obtained by Monika Biniecka and Ursula Fearon.

HUVEC tube formation was examined using the following method: Matrigel (50 μl; 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 % CO_2. HUVEC

were removed from culture, trypsinised and resuspended at a concentration of 4×10^4 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 ×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. The PhD student performed the tube formation assay and microscopic scoring was carried out by Monika Biniecka and Ursula Fearon.

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. Cell stimulation was performed with 10 % 4-HNE RASFC-CM or 4-HNE RPMI for a period of 24 hours. 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 ×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. The PhD student performed seeding, wound scratch and stimulation. Monika Biniecka and Ursula Fearon completed the semi-quantitative analysis under microscope.

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 % CO_2. Stimulation was carried out with 10 % 4-HNE RASFC-CM or 4-HNE RPMI for 24 hours. Following incubation, cells were washed with PBS, fixation made with 4 % PFA and staining with 1 % crystal violet solution with subsequent washing and drying 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. The PhD student carried out PBS wash, fixing and staining of the stimulated HUVEC, other parts of the experiment were carried out by Monika Biniecka.

Finally we *quantified pro-angiogenic mediators from HUVEC.* HUVECs were seeded into 96-well plates and left overnight at 37 °C and 5 % CO_2. The day after we stimulated the cells with 10 % 4-HNE RASFC-CM or 4-HNE RPMI for 24 hours. Next, supernatants were

harvested, and angiogenic protein levels (Ang2 and PDGF-B) were quantified by using a specific ELISA (R&D Systems). This experiment was performed by the PhD student.

4.1.9 Immunofluorescence staining of RASFC and synovial tissue

Single-immunofluorescence staining was performed on RASFC following 24-hour cell stimulations with 4-HNE. To visualise immunoexpression of VEGF, we fixed cells in 4 % PFA and staining was performed with primary rabbit antibody against VEGF (Abcam). To demonstrate ST co-expression of markers of angiogenesis, oxidative stress and bioenergetics, dual-immunofluorescence staining was carried out onsynovial tissue cryostat sections. Steps of the procedure included fixing of ST sections with acetone (for 10 minutes) then co-incubation 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).

This experiment was completed by the PhD student in 60 % and Monika Biniecka and Ursula Fearon helped out in counterstaining, nuclear staining and microscopic evaluation.

4.1.10 Immunohistochemistry and scoring of synovial tissue

Immunohistochemistry (IHC) was performed using 7-μm cryostat synovial tissue (ST) sections and the DAKO ChemMate EnVision kit (Dako/Agilent Technologies, Glostrup, Denmark) with defrosting sections at room temperature for 20 minutes, fixing in acetone for 10 minutes and washing in PBS for 5 minutes. Non-specific binding was blocked with 1% casein in PBS for 20 minutes. Incubation 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) was performed. Immunoglobulin G control antibodies were used as negative controls. Following 1 hour 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 layer (BV) using a well-established and validated semi-quantitative scoring method [26], 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).

The PhD student completed the experiment, Monika Biniecka and Ursula Fearon evaluated with microscopic scoring.

4.1.11 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.

The author participated in collecting and interpreting the data (50%), but Monika Biniecka, Ursula Fearon and Douglas J Veale all contributed greatly in data analysis.

4.2. Study 2

4.2.1 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.

The PhD student did not contribute to patient recruitment, this was carried out by colleagues at the Rheumatology Department, namely Edit Végh, Szilvia Szamosi, Gabriella Szűcs and Sándor Szántó.

4.2.2 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.

The PhD student did not contribute to history taking, disease activity assessment, this was carried out by colleagues at the Rheumatology Department, namely Edit Végh, Szilvia Szamosi, Gabriella Szűcs and Sándor Szántó.

4.2.3 Laboratory measurements

Serum high sensitivity C reactive protein (hsCRP; normal: ≤ 5mg/l) and IgM rheumatoid factor (RF; normal: ≤ 50 IU/ml) 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; normal: ≤ 25 IU/ml). The assay was performed according to the manufacturer's instructions. Erythrocyte sedimentation rate (ESR) was determined by the traditional Westergren method (mm/h). The above measurements were performed by Gábor Nagy (UD Department of Laboratory Medicine) and Anita Pusztai.

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. Eighty percent of these measurements were performed by the PhD student with some help from Jennifer McCormick and Monika Biniecka when performing VEGF ELISAs.

Anti-CEP-1 IgG was measured in the serum samples using an in-house peptide ELISA, as previously described [212]. The measurement was performed by Karin Lundberg in Karolinska Institute, Stockholm. 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.

4.2.4 Assessment of vascular physiology by ultrasound

Brachial artery FMD was assessed as described before [173, 213, 214]. An ultrasound examination was performed on the right arm using 10 MHz linear array transducer (ultrasound system: HP Sonos 5500) by a single trained sonographer after 30 minutes resting in a temperature-controlled room (basal value for FMD). A B-mode longitudinal section was obtained of the brachial artery above the antecubital fossa. In order to assess FMD, 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. Flow velocities, the baseline

diameter, as well as FMD were ECG gated and detected offline. FMD values were expressed as % change from baseline (resting) value (FMD%). In our previous work, we divided RA patients into "high (normal) FMD" and "low (impaired) FMD" subsets by defining a cutoff value of 5% [173]. We used the same cut-off in the present studies. Assessment was performed by György Kerekes and Edit Végh.

The ccIMT measurements were carried out as described before [173, 214, 215]. Briefly, a duplex ultrasound system (HP Sonos 5500, 10 MHz linear array transducer) was used to assess the common carotid arteries by a single observer. Longitudinal high-resolution B-mode ultrasound scan were employed over both right and left common carotid arteries and were R-synchronized and recorded. The offline measurements were performed 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. In our previous work, we divided RA patients into "high (increased) IMT" and "low (normal) IMT" subsets by using a cut-off value of 0.65 mm [173]. We used the same cut-off in the present studies. Assessment was performed by György Kerekes and Edit Végh.

With respect to arterial stiffness, 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 as described before [214, 216]. If an artery is elastic, PWV is low. With decreased arterial elasticity, PWV rises. The arteriograph assesses this parameter from the oscillometric data obtained from the 35 mmHg suprasystolic pressure of the brachial artery. In order to obtain reproducible results, the patient had to rest in a supine position for at least 10 minutes before the assessment in a quiet room. PWV is expressed in m/s. Based on our previous experience [214] we used a cut-off value of 8 m/s, where PWV $\leq$ 8 m/s and PWV > 8 m/s indicated "low (normal)" and "high (increased)" PWV, respectively. Assessment was performed by György Kerekes and Edit Végh.

4.2.5 Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (IBM) software. Data are expressed as the mean ± 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.

The PhD student contributed to data analysis in 30 % and the rest of the calculations were completed by Katalin Hodosi and Zoltán Szekanecz.

5 RESULTS

5.1 Study 1

5.1.1 Oxidative stress alters cellular bioenergetics in RASFC and HUVEC *in vitro*

Previous studies by members of my research team demonstrated altered cellular bioenergetics in RASFC in the presence of hypoxia [60], and also demonstrated increased oxidative stress in the inflamed synovium [59]. First we further investigated whether oxidative stress in the inflamed joint is involved in metabolic reprogramming of RASFC and HUVEC. **Figure 2A** demonstrates representative OCR and ECAR profiles before and after injections of oligomycin, FCCP, antimycin A and 2-DG in basal and 4-HNE-stimulated RASFC. We show, 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 ($p<0.001$) and reserve capacity ($p< 0.01$), similarly ATP synthesis showed a non-significant tendency of reduction ($p=0.1$) (**Figure 2B**). This metabolic reprogramming was further accompanied by increased levels of basal glycolysis ($p<0.01$), glycolytic capacity ($p<0.01$), and unchanged glycolytic reserve ($p=0. 2$) in RASFC subjected to oxidative stress **(Figure 2B).**

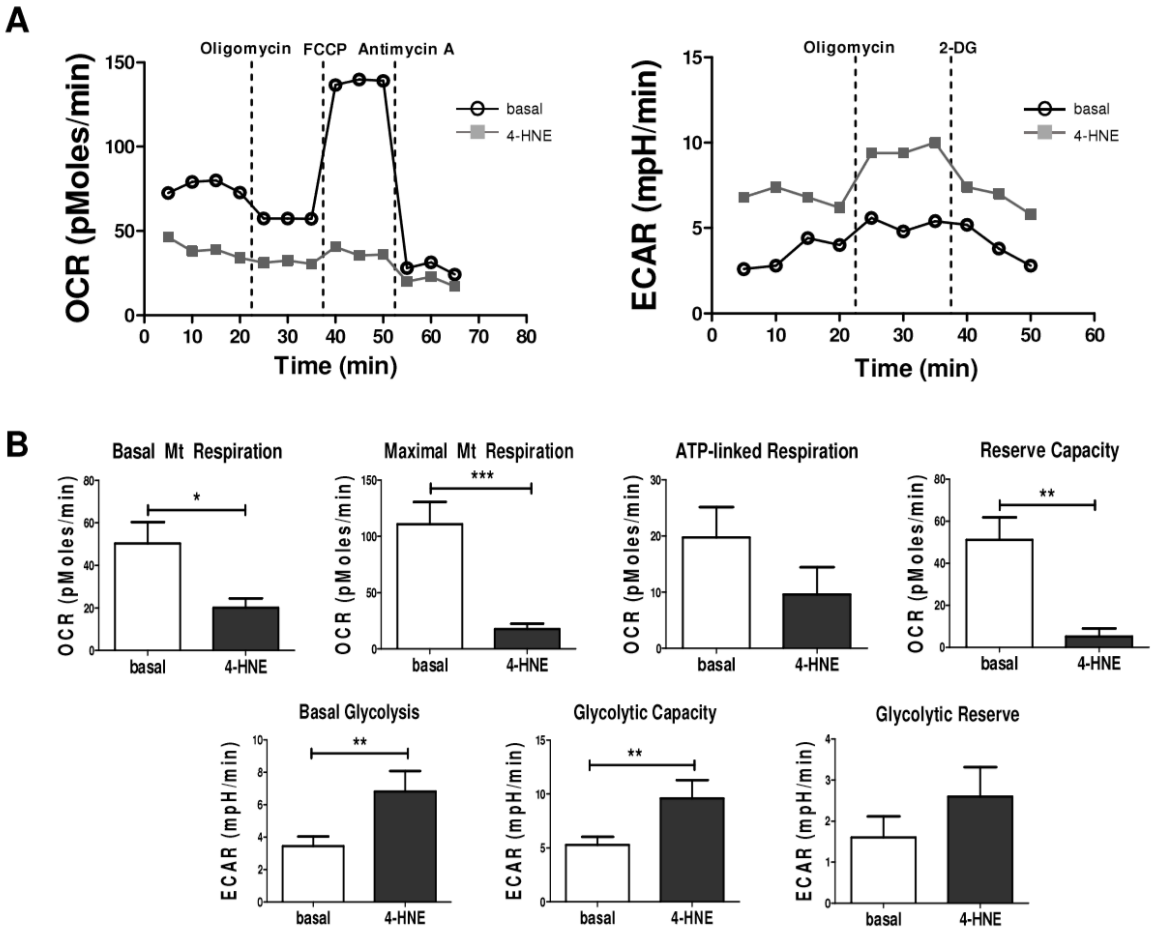

Figure 2. Bioenergetic metabolism in primary rheumatoid arthritis synovial fibroblast cells (RASFC) subjected to 4-hydroxy-2-nonenal (4-HNE)-induced oxidative stress. **A.** Representative oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) Seahorse analyser profiles before and after injections of oligomycin, trifluorocarbonylcyanide phenylhydrazone (FCCP), antimycin A and 2-deoxyglucose (2-DG) in RASFC in the presence and absence of 4-HNE. **B.** Bar graphs demonstrate quantification of basal mitochondrial (Mt) respiration, maximal Mt respiration, adenosine triphosphate (ATP) synthesis, reserve capacity, basal glycolysis, glycolytic capacity and glycolytic reserve in RASFC (n=5) subjected to oxidative stress. Data are presented as mean±SEM. *p<0.05, **p<0.01, and ***p<0.001, significant differences from basal level

Representative HUVEC OCR and ECAR profiles before and after injections of oligomycin, FCCP, antimycin A and 2-DG are shown on **Figure 3A**. Similarly to RASFC, 4-HNE inhibited basal mitochondrial respiration, maximal mitochondrial respiration, ATP synthesis and reserve capacity (all $p<0.01$) with concomitant elevation of basal glycolysis ($p<0.01$) and glycolytic reserve ($p<0.05$) in HUVEC exposed to oxidative stress **(Figure 3B).**

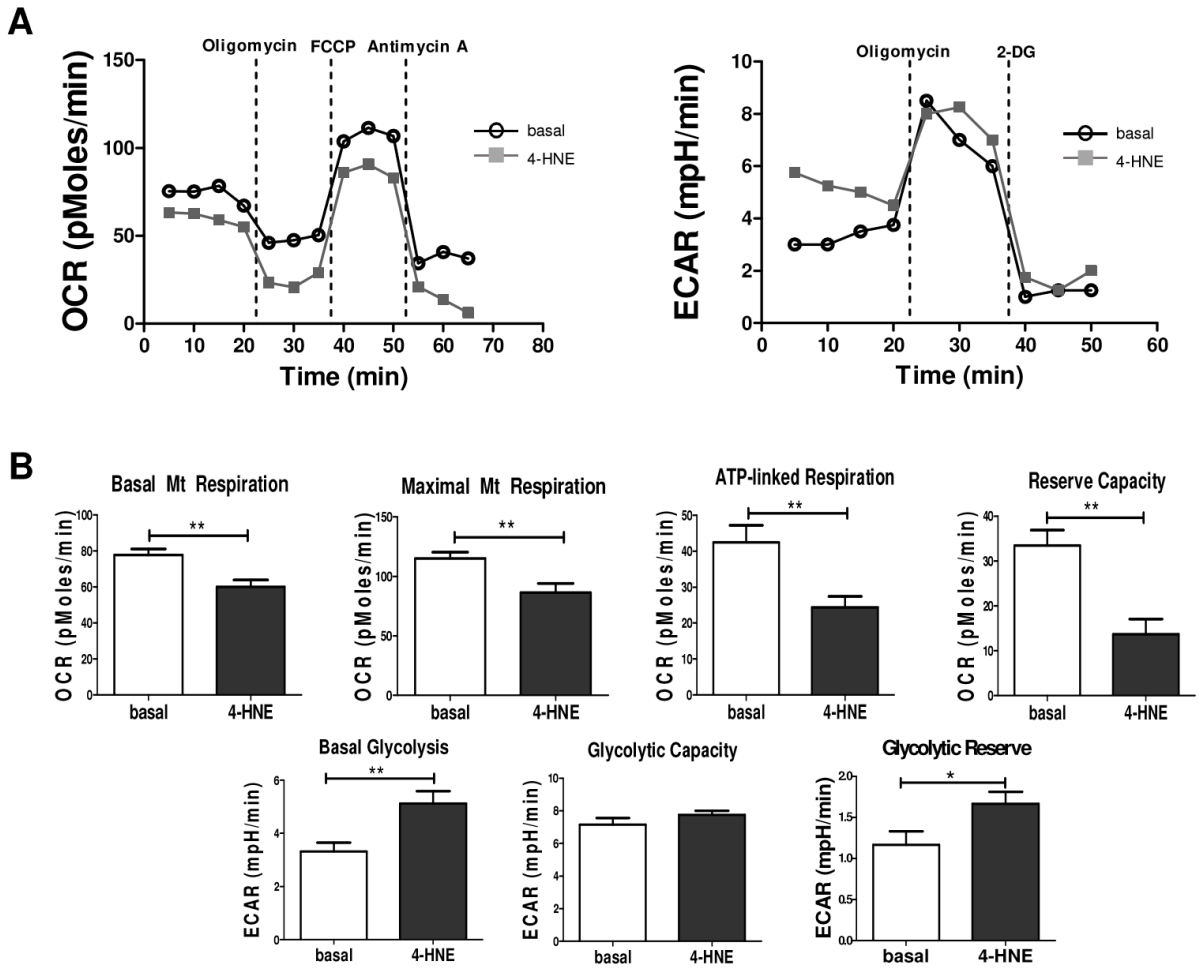

Figure 3. Bioenergetic metabolism in human umbilical vein endothelial cells (HUVEC) subjected to 4-hydroxy-2-nonenal (4-HNE)-induced oxidative stress. **A.** Representative oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) Seahorse analyser profiles before and after injections of oligomycin, trifluorocarbonylcyanide phenylhydrazone (FCCP), antimycin A and 2-deoxyglucose (2-DG) in HUVEC in the presence and absence of 4-HNE. **B.** Bar graphs demonstrate quantification of basal mitochondrial (Mt) respiration, maximal Mt respiration, adenosine triphosphate (ATP) synthesis, reserve capacity, basal glycolysis, glycolytic capacity and glycolytic reserve in HUVEC (n=3) subjected to oxidative stress. Data are presented as mean±SEM. *p<0.05 and **p<0.01, significant differences from basal level

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

My colleagues have previously shown that increased mtDNA mutation frequency and mitochondrial dysfunction in the RA joint were strongly associated with synovial inflammation and hypoxia [217, 218]. We have also reported, at a functional level, induction of pro-angiogenic responses of endothelial cells in the presence of oxidative stress [219]. 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 a non-significant trend of increment in mtDNA point mutations in RASFC in the presence of 4-

HNE compared with basal cells ($p<0.001$ and $p=0.06$, respectively) **(Figure 4A).** 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 $p<0.01$). Non-significant enzymatic activity decline following 4-HNE stimulation was also detected for complex I by 9 %, complex II by 22 % and complex V by 12 % (all $p=0.2$)**(Figure 4B).**

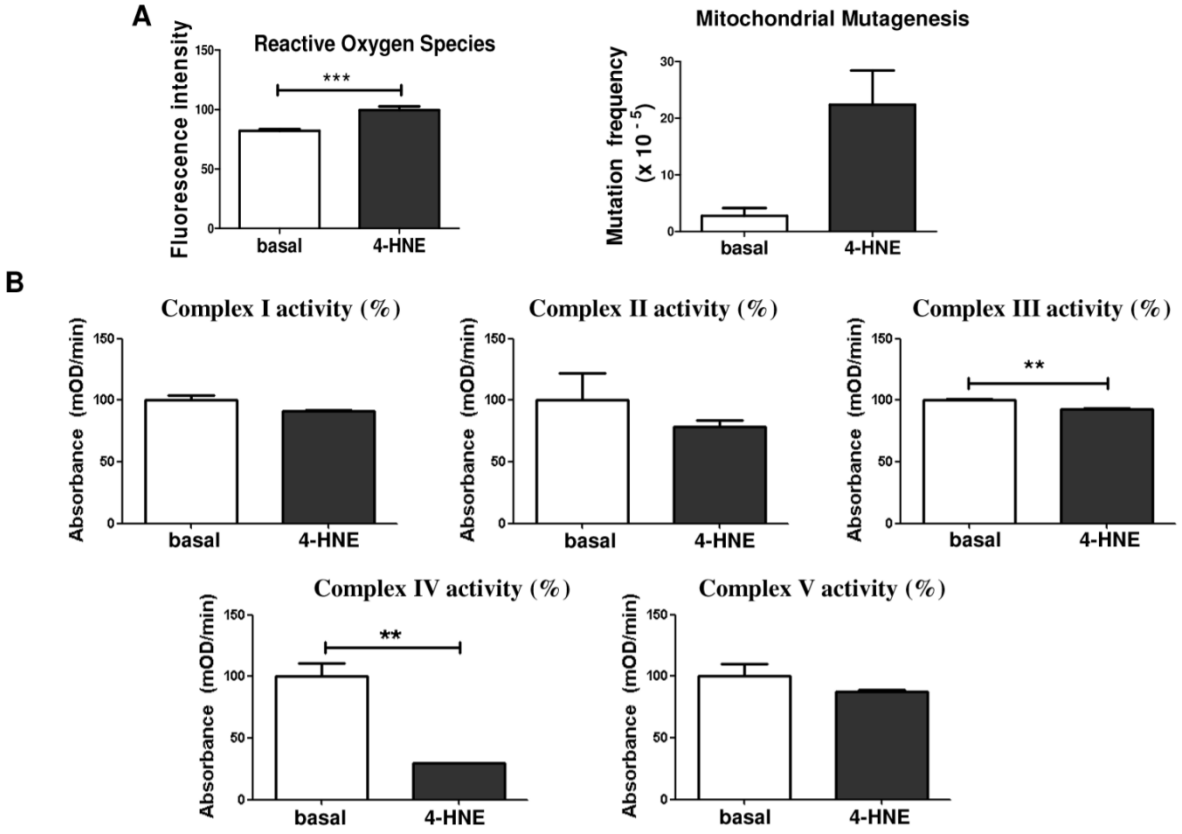

Figure 4. Mitochondrial mutagenesis and activity of enzymes of mitochondrial oxidative phosphorylation (OXPHOS) complexes under 4-hydroxy-2-nonenal (4-HNE)-induced oxidative stress. **A.** Bar graphs demonstrate increased production of reactive oxygen species (n=7), paralleled by non-significant greater frequency of mitochondrial DNA mutation (n=5) in primary rheumatoid arthritis synovial fibroblast cells (RASFC) in response to 4-HNE. **B.** Activity of mitochondrial OXPHOS complexes I–V in the presence of 4-HNE. 4-HNE reduces the activity of complex I by 9%, complex II by 22%, complex III by 8%, complex IV by 70% and complex V by 12% (all complexes measured in triplicate). 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. Data is represented as Mean ± SEM, **$p<0.01$; ***$p<0.001$ significantly different to basal

5.1.3 *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. **Figure 5** demonstrates increased VEGF immunofluorescence staining 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 $p<0.01$]).

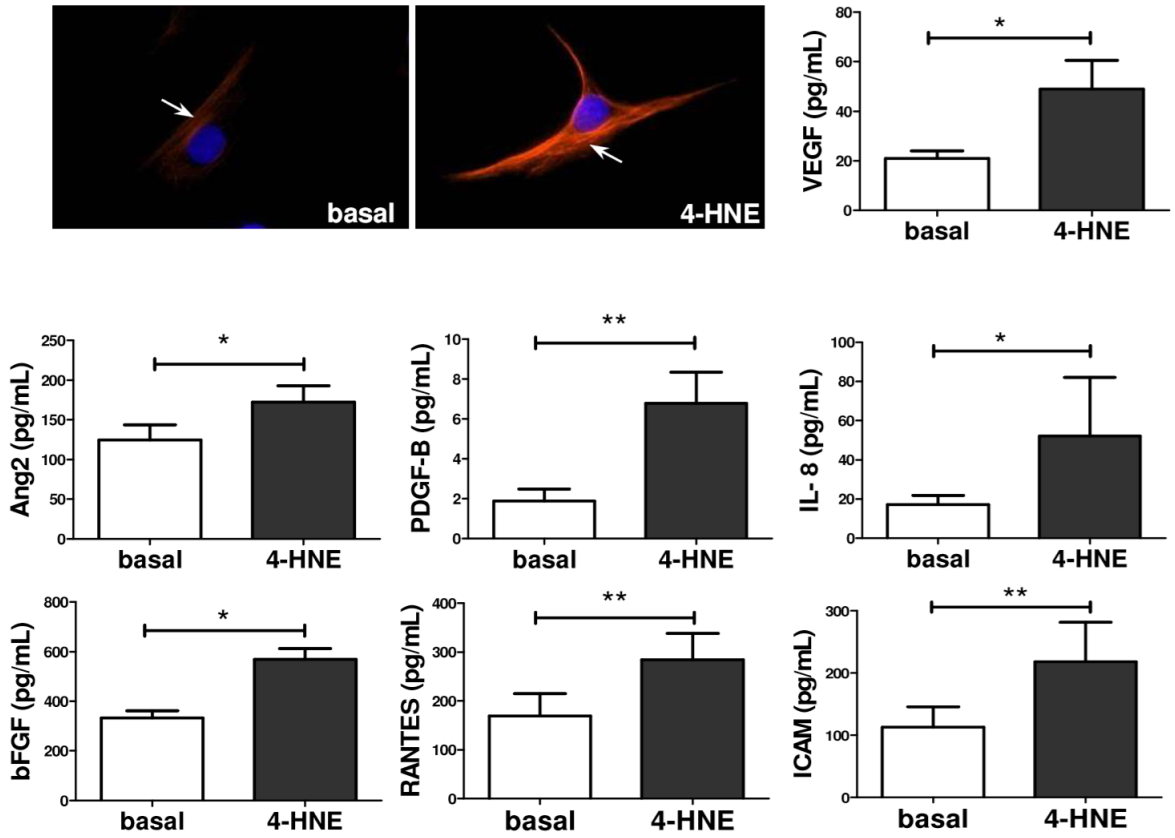

Figure 5. 4-Hydroxy-2-nonenal (4-HNE) induces pro-angiogenic and pro-inflammatory mechanisms in primary rheumatoid arthritis synovial fibroblast cells (RASFC). Increased vascular endothelial growth factor (VEGF) immunofluorescence in RASFC subjected to 4-HNE compared to the basal cells and quantification of VEGF, angiopoietin 2 (Ang2), basic fibroblast growth factor (bFGF), interleukin (IL)-8, platelet-derived growth factor subunit B (PDGF-B), regulated on activation, normal T cell expressed and secreted (RANTES), intercellular adhesion molecule (ICAM) in RASFC supernatants (n=7) following cell culture with 4-HNE. Data are presented as mean ±SEM. *$p<0.05$ and **$p<0.01$, significant differences from basal level. Red =VEGF; blue=4′,6-diamidino-2-phenylindole–; magnification of photomicrographs ×40

These findings, along with our previously published in vitro study showing TNF-α-induced mitochondrial dysfunction [218], 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). **Figure 6A** shows changes of macroscopic vascularity and ST expression of VEGF, Ang2 and Tie2 from T0 to T3. **Figure 6B** graphically illustrates decreases in ST VEGF ($p=0.1$), Ang2 ($p<0.005$) and Tie2 ($p<0.005$) after TNFi therapy.

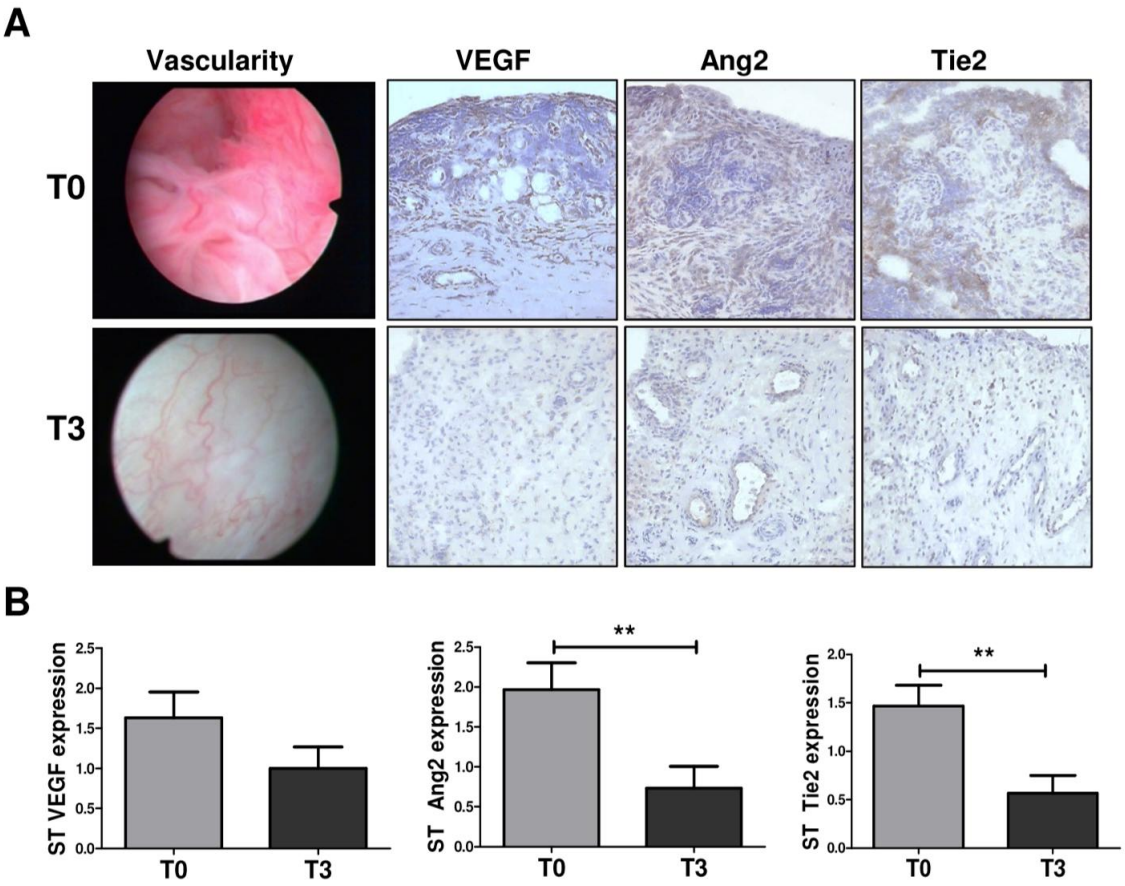

Figure 6. Synovial tissue expression of angiogenic markers in patients with RA. **A.** Representative images demonstrating macroscopic vascularity and ST VEGF, Ang2 and Tie2 immunostaining at baseline (T0) and 3 months after the commencement of biologic treatment (T3). Magnification of photomicrographs ×20. **B.** Baseline and 3 months post-TNFi quantification of ST VEGF, ST Ang2 and ST Tie2 in patients with RA (n=15). Data are presented as mean ± SEM. *$p<0.05$; **$p<0.01$. (TIF 5466 kb)

5.1.4 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 [220]. 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). **Figure 7A** demonstrates the effect of basal or 4-HNE RASFC-CM on invasion, the formation of tube-like structures and migration of HUVEC. **Figure 7B** graphically illustrates 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 basal or 4-HNE RASFC-CM.

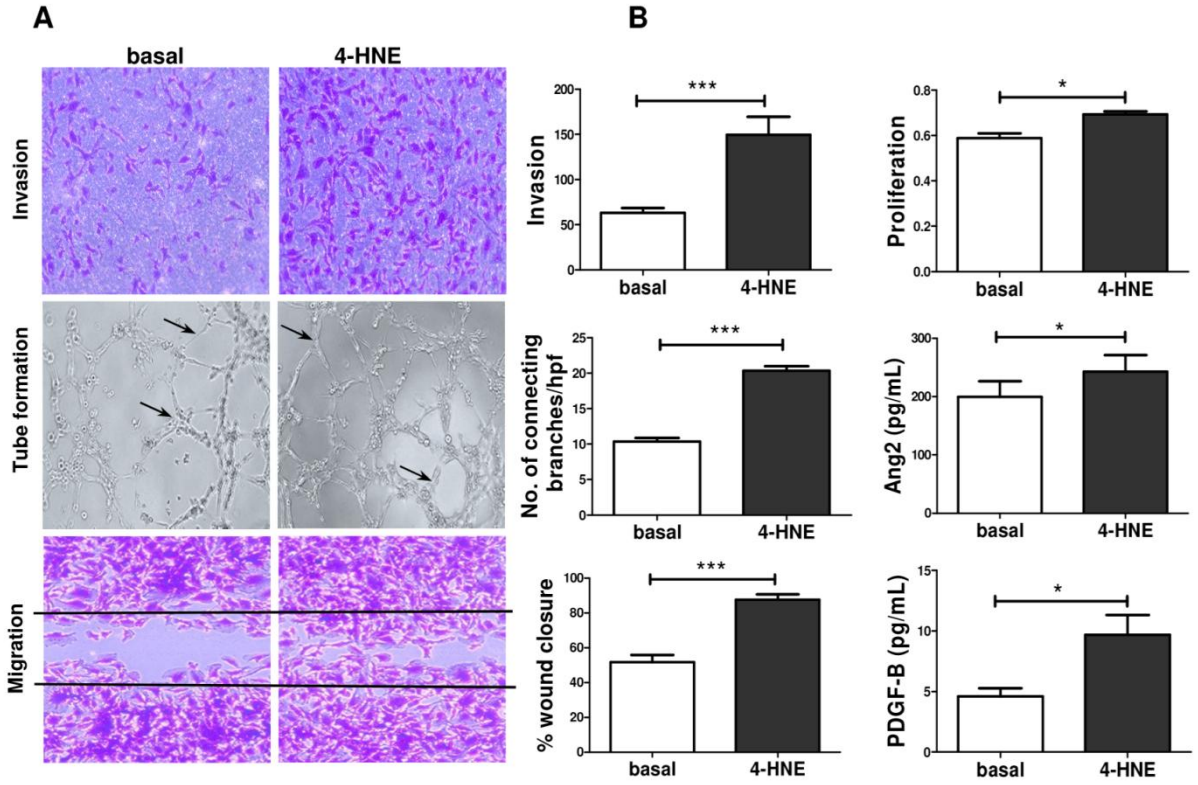

Figure 7. Effects of primary rheumatoid arthritis synovial fibroblast cell (RASFC)-conditioned media on angiogenic responses of human umbilical vein endothelial cells (HUVEC). **A**. Representative images demonstrating invasion, the formation of tube-like structures and migration of HUVEC cultured in the presence of basal or 4-hydroxy-2-nonenal (4-HNE)-supplemented conditioned media. Magnification ×10 of photomicrographs demonstrating invasion, tube formation (arrows indicate connecting branches) and cell migration. **B.** Bar graphs demonstrate an increase in the number of invading, proliferating and migrating HUVEC, a higher number of connecting branches formed between HUVEC, and greater angiopoietin 2 (Ang2) and plateletderived growth factor subunit B (PDGF-B) release from HUVEC exposed to 4-HNE-supplemented conditioned media (n=6). Data are presented as mean±SEM. *p<0.05and***p<0.001, significant differences from basal level. hpf High-power field

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 μM; 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 with 4-HNE RPMI 1640 control media further supports the direct effect of 4-HNE on RASFC-induced angiogenesis in the inflamed joint **(Figure 8).**

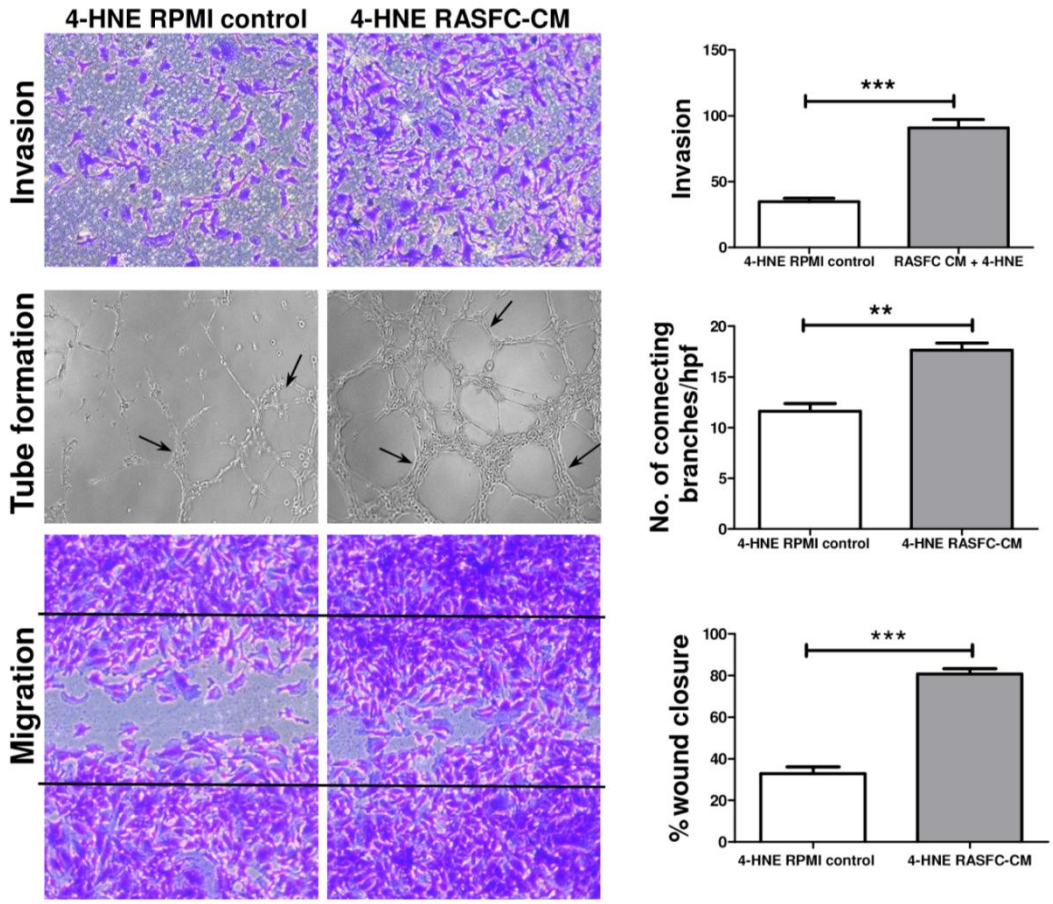

Figure 8. Effects of oxidatively activated RASFC on angiogenic responses of HUVEC. To confirm that the increase in proangiogenic responses of HUVEC is due to oxidatively activated RASFC and not residual 4-HNE present in the conditioned media, HUVEC were cultured in the presence of RPMI 1640 media supplemented with 4-HNE (0.25 μM; 4-HNE RPMI 1640 control), which was at the same concentration of 4-HNE in the 10% RASFC conditioned media. Representative images and bar graphs demonstrate higher invasion, greater number of formed tube-like structures and greater cell migration across the wound in HUVEC in response to 4-HNE RASFC-conditioned media (4-HNE RASFC-CM; n=6) than in response to 4-HNE RPMI 1640 control. Data are presented as mean±SEM. **p<0.01and ***p<0.001, representing significant differences from control. Magnification of photomicrographs demonstrating invasion, tube formation (arrows show connecting branches) and cell migration ×10. (TIF 8263 kb)

5.1.5 Association between synovial 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 [60]. ST 4-HNE expression was associated with increased expression of VEGF (r=0.63; p=0.015) and Tie2 (r=0.56; p=0.029), GAPDH (r=0.60; p=0.03) and with reduced levels of ATP5B (p=−0.52, p=0.017). Furthermore, representative 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, is demonstrated in **Figure 9.**

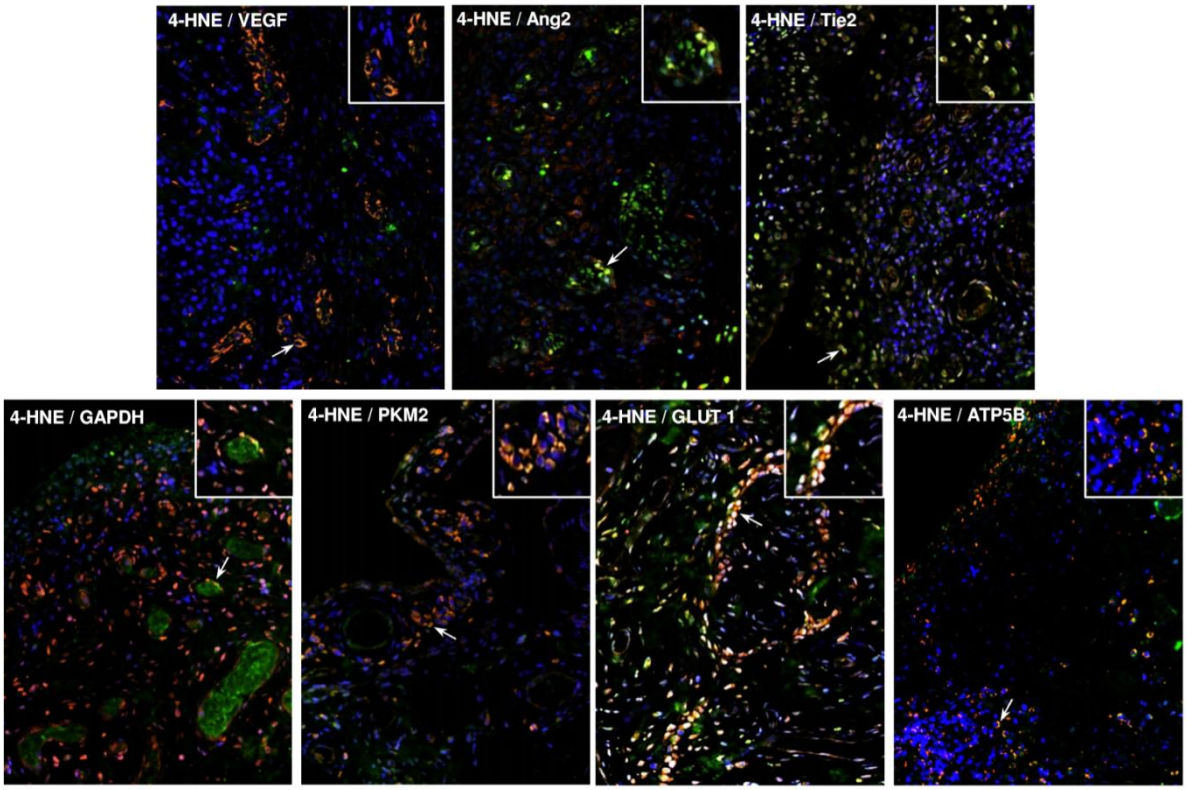

Figure 9. Synovial tissue (ST) angiogenesis, oxidative stress and cellular bioenergetics. To support the concept that oxidative stress, angiogenesis and energy metabolism are interconnected processes that co-exist during the inflammation milieu, double-immunofluorescence staining was performed. ST slides were co-incubated with primary mouse antibody against human 4-hydroxy-2-nonenal (4-HNE) and with primary rabbit antibodies against angiogenic factors (vascular endothelial growth factor [VEGF], angiopoietin 2 [Ang2], tyrosine kinase receptor [Tie2]), glycolytic proteins (glyceraldehyde 3-phosphate dehydrogenase [GAPDH], pyruvate kinase isozyme 2 [PKM2], glucose transporter 1 [GLUT1]) and a mitochondrial marker (adenosine triphosphate synthase subunit β [ATP5B]). Representative merged immunofluorescence images demonstrate examples of co-localisation (yellow) of 4-HNE with VEGF, Ang2, Tie2, GAPDH, PKM2, GLUT1 and ATP5B. Cells stained green are positive for 4-HNE only; cells stained red are positive only for VEGF, Ang2, Tie2, GAPDH, PKM2, GLUT1 and ATP5B. Arrows indicate examples of co-localisation. Magnification of photomicrographs ×20, insets show high-power magnification of co-localisation. Representative images show single immunofluorescence of 4-HNE, VEGF, Ang2, Tie2, GAPDH, PKM2, GLUT1 and ATP5B along with their controls. Isotype-matched antibodies are shown in Additional file 3: Figure S3 and Additional file 4: Figure S4

Figure 10 - 11 show single images of VEGF, Ang2, Tie2, GAPDH, PKM2, GLUT1 and ATP5B (all in red), single images of 4-HNE immunofluorescence (in green), as well as single images of DAPI (in blue), along with their controls with isotype-matched antibodies.

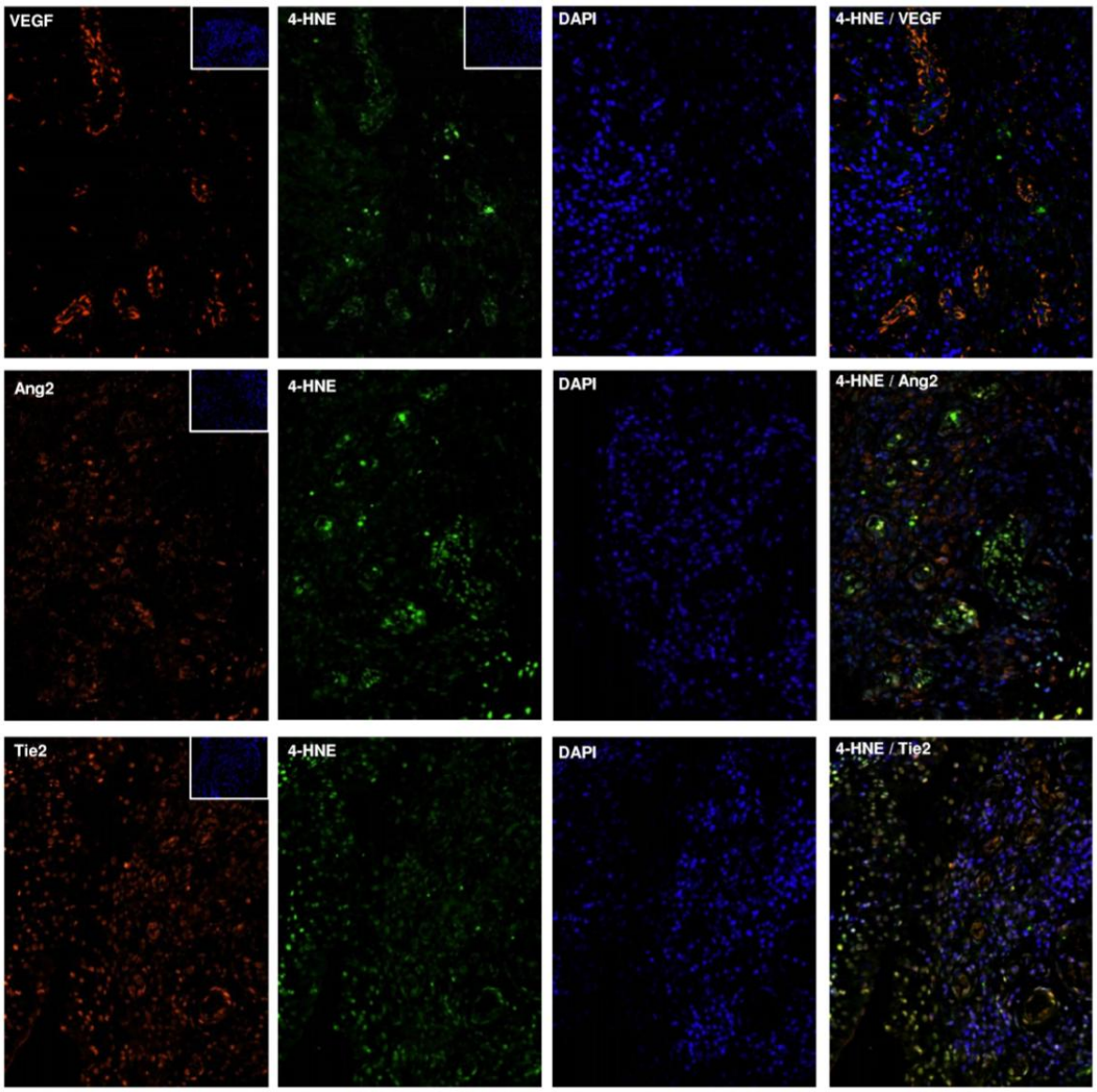

Figure 10. ST angiogenesis and oxidative stress. Representative images of immunofluorescent staining between markers of angiogenesis and 4-HNE in inflamed synovial tissue of patients with RA: VEGF, Ang2 and Tie2 (red); 4-HNE (green); DAPI (blue); and merged images (yellow). Insets show negative control staining with isotype-matched antibodies. Magnification of photomicrographs ×20.

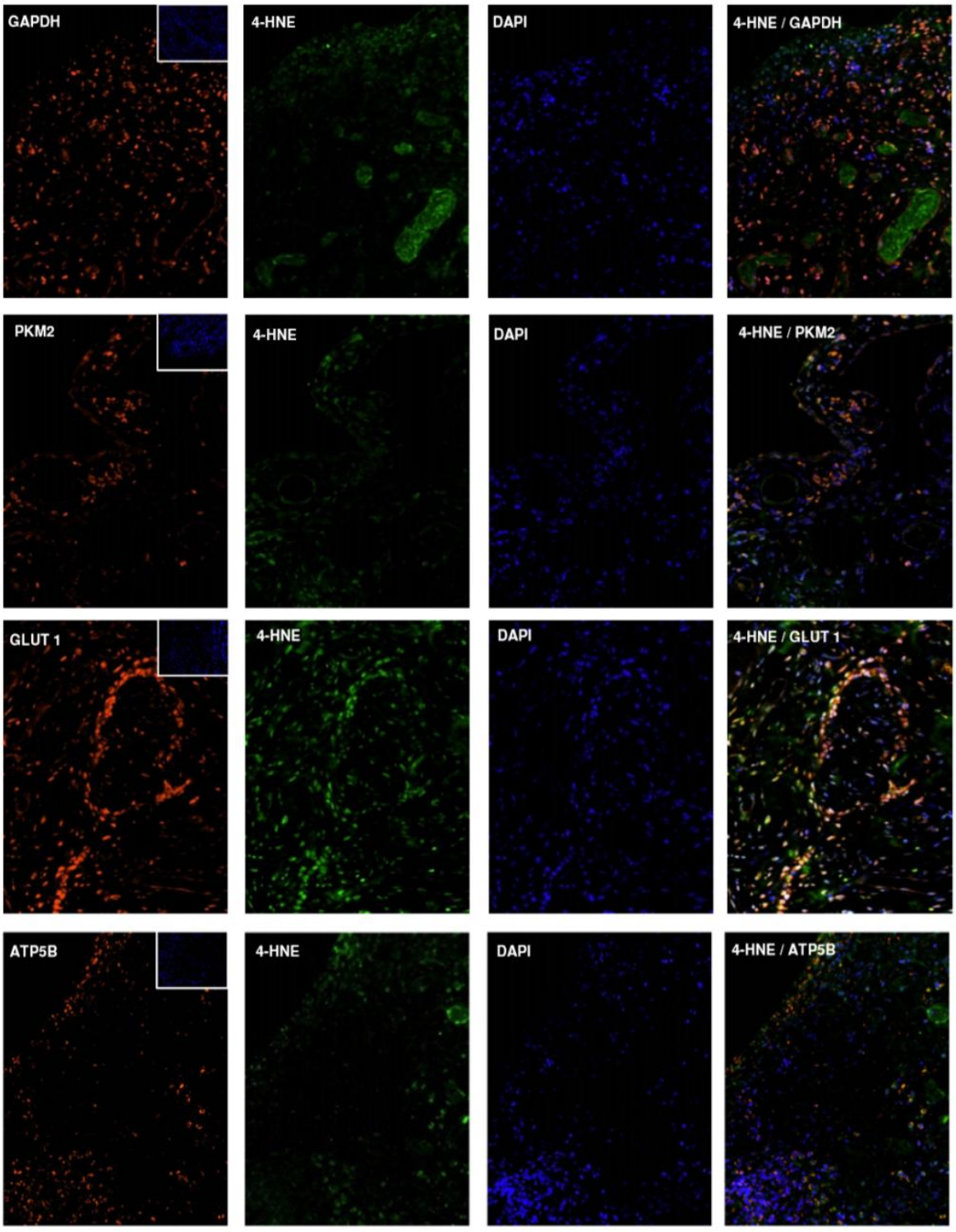

Figure 11. ST cellular bioenergetics and oxidative stress. Representative immunofluorescence images show co-localisation of the oxidative stress marker 4-HNE with glycolytic proteins (GAPDH, PKM2, GLUT1) and a mitochondrial marker (ATP5B) in inflamed ST of patients with RA: GAPDH, PKM2, GLUT1, and ATP5B (red); 4-HNE (green); DAPI (blue); merged images (yellow). Insets show negative control staining with isotype-matched antibodies. Magnification of photomicrographs × 20. (TIF 9536 kb)

5.2 Study 2

5.2.1 Baseline demographics

Patient characteristics are seen in **Table 2**. The combined arthritis cohort included 34 women and 19 men with mean age of 52.0±12.1 (range: 24-83) years. The mean±SEM age of the control group was 49.75±3.6 years with 6:3 female:male ratio, where all controls were non-smokers. Mean disease duration was 8.5±7.9 (range: 1-44) years for the combined arthritis cohort. 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 (**Table 2**).

Table 2. Baseline characteristics of a mixed cohort of patients

	RA	**AS**	**Total**
n	36	17	53
female:male	31:5	3:14	34:19
age (mean±SEM)(range), years	55.9±9.8 (35-83)	43.6±12.4 (24-72)	52.0±12.1 (24-83)
disease duration (mean±SEM) (range), years	9.1±8.3 (1-44)	7.2±7.0 (1-26)	8.5±7.9 (1-44)
smoking (current)	7	7	14
positive CV history	8	1	9
periodontitis (current)	10	5	15
tooth loss (current)	14	7	21
RF positivity, n (%)	26 (72)	-	-
aCCP positivity, n (%)	21 (58)	-	-
DAS28-ESR (baseline) (mean±SEM)	5.00±0.86	-	-
BASDAI (baseline) (mean±SEM)	-	5.79±1.19	-
Treatment (ETN, CZP)	20 ETN, 16 CZP	17 ETN	37 ETN, 16 CZP

Abbreviations: RA – rheumatoid arthritis, AS – ankylosing spondylitis, CV – Cardiovascular, RF – rheumatoid factor, aCCP – anti-cyclic citrullinated peptide, Disease activity score using swollen and tender joints from 28 joint count, patient global health assessment and erythrocyte sedimentation rate, BASDAI – Bath Ankylosing Spondyitis Disease Activity Index, ETN – etanercept, CTZ – certolizumab pegol

5.2.2 Clinical response to TNFi therapy in inflammatory arthritides

Although we used a mixed cohort of RA and AS patients in our present study, as RA and AS disease activity are assessed by different methods, we first determined the efficacy of biologic therapy in the two diseases. In RA (n=36), TNFi treatment resulted in significant decrease in DAS28-ESR at 6 and 12 months of treatment (3.11±0.84 and 3.02±0.96) compared to baseline (5.00±0.86; $p<0.001$). In AS (n=17), BASDAI significantly decreased from baseline 5.79±1.19 to 2.00±1.03 after 6 months, then to 1.86±1.04 after 12 months of therapy ($p<0.001$)..

5.2.3 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 inflammatory arthropathies (n=53) compared to age matched healthy controls (n=8): 267.8±133.3 vs 140.7±63.5 pg/ml **(Figure 12A).** In the cohort of our 53 patients with RA and AS, the 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. (**Figure 12B**). 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) (**Figure 12C**). 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) (**Figure 12D**). Serum Ang1 and TSP-1 levels did not change overtime during anti-TNF therapy (data not shown).

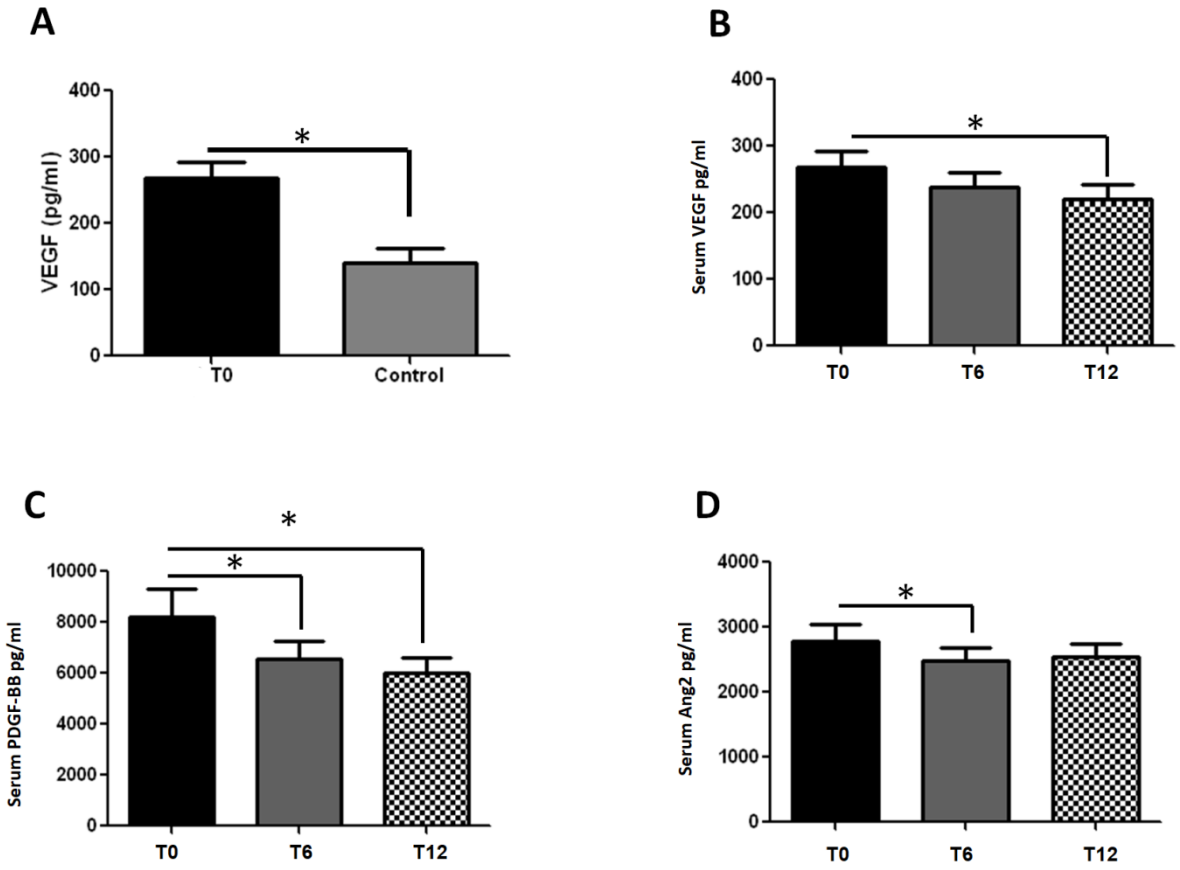

Figure 12. A: VEGF levels in the sera of a mixed cohort of patients with rheumatoid arthritis (RA) and ankylosing spondylitis (AS, n=53) at baseline compared to age matched healthy controls (n=8) **B/C/D:** Angiogenic marker levels in the mixed cohort of AS and RA at baseline (T0), 6 months (T6) and 12 months (T12) after initiation of TNFα inhibitory treatment with etanercept or certolizumab. **B.** Serum VEGF (pg/ml), **C.** Serum PDGF-BB (pg/ml), **D.** Serum Ang2 (pg/ml). *p<0.05, significant differences from baseline.

5.2.4 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 ($p<0.05$) (**Table 3**). 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) (data not shown). Current clinical periodontitis or tooth loss was not associated with elevated angiogenic marker levels (data not shown).

Table 3. Significant positive correlations between angiogenic marker levels and other parameters

	Disease duration	**CRP-0**	**CRP-12**	**CCP-0**	**CEP-0**	**ccIMT-0**
PDGF-BB-0						R=0.459 p=0.009
PDGF-BB-12	R=0.436 p=0.014					
Ang1-0		R=0.370 p=0.041				
Ang2-0	R=0.555 p=0.001	R=0.419 p=0.019	R=0.416 p=0.020			
Ang2-12	R=0.452 p=0.011	R=0.406 p=0.023	R=0.411 p=0.022			
TSP1-0				R=0.548 p=0.018	R=0.625 p=0.007	R=0.406 p=0.024

Abbreviations: CRP-0: C-reactive protein at baseline, CRP-12: C-reactive protein at 12 months of treatment, CEP-0: Citrullinated enolase peptide at baseline, ccIMT-0: Common carotid intima-media thickness at baseline, PDGF-BB-0: Platelet-derived growth factor subunit B levels at baseline, PDGF-BB-12: Platelet-derived growth factor subunit B levels at 12 months, Ang1-0: Angiopoetin 1 at baseline, Ang2-0: Angiopoetin 2 at baseline, Ang2-12: Angiopoetin 2 at 12 months of treatment, TSP1-0: Thrombospondin 1 at baseline.

Results of the univariate and multivariate analyses are indicated in **Table 4**. 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 ($p<0.05$) (**Table 4**).

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 ($p=0.002$) (**Table 3).**

Table 4. Univariate and multivariate analysis of determinants of angiogenic markers

Dependent variable	**Independent variable**	**Univariate analysis**				**Multivariate analysis**			
		B	**CI 95%**	**β**	**p**	**B**	**CI 95%**	**β**	**p**
PDGF-BB-0	**ccIMT-0**	31595.1	8346.4-54843.8	0.459	0.009				
PDGF-BB-12	**disease duration**	4612.8	3067.9-6157.7	0.436	0.014				
	smoking (current)	3866.5	1179.8-6553.3	0.480	0.006	3866.5	1179.8-6553.3	0.480	0.006
Ang1-0	**CRP-0**	1.423	0.064-2.781	0.370	0.041				
Ang2-0	**disease duration**	85.5	36.9-134.2	0.555	0.001	87.1	43.9-130.3	0.569	<0.001
	CV disease (history)	1121.3	102.9-2139.7	0.386	0.032				
	CRP-0	47.7	8.5-85.8	0.419	0.019	49.2	17.2-81.2	0.435	0.004
Ang2-12	**disease duration**	58.3	14.6-102.0	0.452	0.011	59.9	20.9-98.9	0.471	0.004
	CRP-0	38.6	5.6-71.6	0.406	0.023				
	CRP-12	68.9	10.8-127.0	0.411	0.022				
TSP1-0	**disease duration**	4.629	-0.05-9.309	0.688	0.002	4.538	0.571-8.506	0.433	0.028
	CEP-0	4.341	1.358-7.323	0.625	0.007	4.776	2.150-7.401	0.688	0.002
	ccIMT-0	525.0	75.8-974.3	0.406	0.024				

See text for abbreviations and explanations. *

6 DISCUSSION

6.1 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 proangiogenic effects, hence further triggering the vicious cycle [1, 2, 14, 15, 52]. The systemic inflammation secondary to overproduction of these mediators is linked to an increased risk of cardiovascular morbidity and quiescent or manifest autoimmune atherosclerosis [39, 155, 156, 201]. 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 proangiogenic 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 gylcolytic 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 via fission, fusion and mitophagy [57, 217, 218, 221]. Previous studies 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 [57, 217, 218, 221]. 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 [222]. 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 [63, 223]. 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

[224, 225]. 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 [226]. 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 [227]. 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 [60, 228]. Additionally, glucose-6-phosphate isomerase (glycolytic enzyme) activation by hypoxia up-regulated VEGF production, proliferation and invasion when examined RASFC and HUVEC [229]. Electrophilic lipids are able to adduct numerous glycolytic proteins as described by others, and PKM2, GAPDH, fructose bisphosphate aldolase A (aldolase A) as well as phosphoglycerate kinase 1 are all examples demonstrated from previous research [230-232]. 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 [227], as well as raised 18F-fludeoxyglucose uptake and glycolytic metabolism by oxLDL via upregulated GLUT1 expression and hexokinase activity [233]. 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 PI3K 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 [220]. Hypoxia and oxidative stress are crucial mediators of impaired angiogenesis that we demonstrated by experiments where RASFC originated pro-angiogenic and proinflammatory 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-1β, IL-18 and NF-κB and activation of the NLRP3 inflammasome [234, 235]. Upregulated angiogenic responses due to redox changes has similarly been described by others in HUVEC, keratinocytes, lung epithelial cells and retinal cells [219, 236].

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 vasularity 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 [85, 237, 238]. 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 [237, 238]. 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 [239].

6.2 Study 2

Synovial angiogenesis has outstanding importance in orchestrating local inflammatory response in inflammatory arthritis. This angiogenic cascade is associated with the overproduction of pro-inflammatory and/or pro-angiogenic cytokines, resulting in a vicious circle of events [3, 14, 15, 157]. The excessive production of the above molecules generate a systemic inflammatory process that is associated with an increased risk of atherosclerosis, CV morbidity and mortality [155, 156, 173, 240-242].

TNF-α has pro-inflammatory, but pro-angiogenic role as well regulating capillary sprouting via VEGF, Ang1 and Ang2 related pathways. [243]. TNF-α and numerous other pro-inflammatory mediators have potential pathogenetic role in inflammatory atherosclerosis [14, 244]. Earlier data suggests that TNFi therapy may mitigate angiogenesis [19, 85, 86, 245] and atherosclerosis [201, 202, 241] 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.

On our patient cohort it was 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 [85, 86]. In one of these studies [85], synovial expression of VEGF dropped following infliximab therapy and this was linked to an increased Ang2 expression. These seemingly controversial observations in relation to Ang2 expression may have been present due to the methodology of experiments completed via IHC, and not via direct measurements in serum levels. 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 [246, 247]. Inhibition of PDGFR pathway has also been shown to be linked to synovial fibroblast related ECM degradation in RA [247], 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 [248, 249]. PDGF related signaling has been implicated in tumor angiogenesis [250], but we have not found any reports on the possible effects of TNFi therapy on PDGF-BB in arthritis and TNFi therapy did not change serum PDGF-BB levels significantly throughout 12 months observation of our inflammatory arthropathy cohort. 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 [251-253]. 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.

Clear positive correlation was demonstrated between high VEGF and Ang2 levels, while Ang1 seems to be in part VEGF-independent [20, 254]. In addition, Ang2 may have outstanding effects in the initiation of the neoangiogenesis [20]. Excessive Ang2 expression was observed in early PsA [254]. 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 [20, 254]. 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 [1, 157]. 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 [255, 256]. Hashimoto et al [256] found correlations between arthritic active disease and high titers of angiogenic factors. Kurosaka et al [257] 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 [258] found no link between VEGF titer and aCCP status. Periodontitis and anti-CEP have been implicated in arthritides [170, 212], 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 enhaced by Porphyromonas gingivalis that we know acts as a contributor to the pathogenesis of periodontitis and RA [259]. 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 [157, 260, 261]. PDGF-BB and TSP-1 have been implicated in vascular smooth muscle cell proliferation and migration, as well as several other mechanisms underlying atherosclerosis [262-264]. 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.[265-267]. 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 [268].

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.

7 CONCLUSIONS

O 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 angiopoetin/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.

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9 SUMMARY

9.1 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.

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 mutatagenesis 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.

9.2 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.

10 ÖSSZEFOGLALÁS (SUMMARY IN HUNGARIAN)

10.1 Első vizsgálat

Az inflammatórikus artritiszek helyi és szisztémás gyulladással jellemezhetők, s olyan események körkörös kaszkádját vonják maguk után, mely egy bioenergetikus krízis alapjául szolgál. Az endotélium megváltozott biológiája és a különféle proinflammatórikus mediátorok egy defektív angiogenezishez vezetnek, amely tovább súlyosbítja a szinoviális gyulladást, proliferációt és destruktív pannusz képződését. A gyulladt ízület egy hypoxiás mikrokörnyezetet teremt és az oxidatív stressz dominálása a légzési láncban változásokat idéz elő, mégpedig az anaerob glikolízis előnyben részesítésével. A további reaktív oxigénvegyületek felhalmozódása és a lipid peroxidáció kedvez a mitokondriális mutációk létrejöttének. A hypoxia, oxidatív stressz, mitokondriális alulműködés, bioenergetikai állapotváltozás, illetve a proangiogén és proinflammatórikus mediátorok felhalmozódása egymással szerves kölcsönhatásban zajlik többféle sejt szignáltranszdukciós folyamatot is involválva.

Munkánkban az oxidatív stressz szerepét értékeltük a sejt bioenergetikájára, illetve a proangiogén és proinflammatórikus válaszokra olyan primer rheumatoid artritisz szinoviális fibroblaszt sejtek (RASFC) és humán köldökvéna endoteliális sejtek (HUVEC) vizsgálatával, amelyeket oxidative stressz markerrel, 4-Hydroxy-2-nonenállal (4-HNE) inkubáltunk. Az extracelluláris savasodási sebesség (ECR), az oxigénfogyasztás sebessége (OCR), a mitokondriális DNS stabilitás és a proangiogén, valamint a proinflammatórikus folyamatok kerültek értékelésre Seahorse készülék használatával, I-V enzimkomplex aktivitási vizsgálatokkal, random mutációs mitokondriális jelölővizsgálatokkal, enzimhez csatolt immunoszorbent (ELISA) módszerrel és funkcionális vizsgálatokkal az érképződés, migráció és invázió vizsgálatára. Az angiogén markerek expressziója a szinóviális szövetben (ST) immunhisztokémiával (IHC) került elemzésre olyan rheumatoid artritiszes betegek esetében, akik térdízületi artroszkópián estek át tumor nekrózis faktor-α inhibitórikus (TNFi) kezelés előtt, illetve 3 hónappal azt követően.

A 4-HNE általi oxidatív stressz az energiametabolizmus megváltozásához vezetett a bazális, maximális és adenozin-trifoszfát kapcsolt mitokondriális légzési folyamat és

rezervkapacitás gátlásával RASFC és HUVEC sejtekben, amely az III. és V. oxidatív foszforilációs enzimkomplexek csökkent működését vonta maga után. Ellenkezőleg, a 4-HNE stimulatív hatással volt a bazális glikolízisre, glikolízis kapacitásra és rezervre, mely a mitokondriális mutációk számának növekedésével és reaktív oxigénvegyületek felhalmozódásával járt. A 4-HNE a RASFC proangiogén aktivitását is stimulálta, amely a HUVEC további inváziós, migrációs és érképződési képességét növelte, illetve belőle proangiogén mediátorok felszabadulását segítette elő. Az angiogenesis markerei, mint a vaszkuláris endoteliális növekedési factor (VEGF), tirozin-kináz receptor 2 (Tie2), és ligandja az angiopoetin 2 (Ang2) együttes expresziót mutattak az oxidatív stressz és az oxigén metabolizmus markereivel a gyulladt szinóviális szövetben vizsgálva. A TNFi terápia szignifikánsan csökkentette a ST makroszkópikus vaszkularitásfokozódását és az Ang2/Tie2 mikroszkópos expresszióját RA-s betegek esetében.

Az oxidatív stressz hatása megváltoztatja a sejtválaszt egy bioenergetikai átállással az anaerob légzés irányába, amely a gyulladás és a defektív angiogenezis felgyorsulásához vezethet RA-ban.

10.2 Második vizsgálat

A gyulladás és a defektív angiogenezis nemcsak helyileg, hanem szisztémásan is megmutatkozik autoimmun vaszkuláris plakk-képződés és atheroszklerózis formájában. Több terápiás target is bemutatásra került, melyek ígéretes jövőbeni stratégiák lehetnek az inflammatórikus artritiszek kezelésében. A kifejezett szisztémás gyulladás az atheroszklerózis, CV morbiditás és mortalitás rizikójának emelkedésével járhat. Vizsgálatunkban azt vettük szemügyre, hogy a TNFi kezelés megváltoztatja-e az angiogén mediátorok szintjét 6 és 12 hónappal a terápia kezdetét követően, valamint azt, hogy az angiogén mediátorok megemelkedett szintje kapcsolódik-e az FMD/ccIMT/PWV által bemutatott megváltozott vaszkuláris fiziológiához. Hasonlóan meg kívántuk ítélni, hogy az angiogén válasz változása egyrészt korrelál-e az orális egészségmegváltozással, másrészt hogy a dohányzás ténye azt befolyásolja-e.

A TNFi terápia klinikailag hatékonynak bizonyult mind RA, mind pedig AS kezelésében, amit a DAS28-ESR és BASDAI indexek szignifikáns csökkenése tükrözött. A TNFi-vel való egy éves kezelés a szérum VEGF, PDGF-BB és Ang2 értékek szignifikáns

csökkenését eredményezte. Úgy gondoljuk, hogy a TSP-1 komplex, együttesen anti- és pro-angiogén hatásmechanizmusa vezethet azon megfigyelésünkhöz, mely szerint a szérum TSP-1 szintje a TNFi terápia 6. és 12. hónapjára szignifikánsan nem változott.

Egyértelmű pozitív korrelációt írtunk le a magas VEGF és Ang2 szintek között, amíg az Ang1 hatása valamelyest VEGF-től függetlennek tűnt. Mindezen túl az Ang2 szintje korrelált a CV megbetegedések jelenlétével alátámasztva az angiogenezis és inflammatórikus atheroszklerózis közötti összefüggést. Az Ang2 és a PDGF-BB szintje korrelált a betegségi időtartammal, illetve az Ang1 és Ang2 összefüggést mutatott a CRP-vel, így igazolva az intenzív angiogenezis, hosszú betegségi időtartam és szisztémás gyulladás együttes átfedését. Ezen felül megfigyeltük, hogy a magas kezdeti Ang2 szintek jól korrelálnak a terápiát követő perzisztensen emelkedett CRP szintekkel, hasonlóan eredményt látva amikor a 12. havi Ang2 értékeket hasonlítjuk a kezdeti CRP értékekhez.

A kezdeti aCCP és anti-CEP autoantitestek jelenléte szintén korrelált a TSP-1 értékekkel az autoimmunitás és neoangiogenezis átfedését tükrözve.

A kezdeti PDGF-BB és TSP-1 összefüggést mutatott a ccIMT-vel azt sugallva, hogy ezen angiogén mediátorok szereppel bírnak az autoimmun atheroszklerózis folyamatában.

A többváltozós regressziószámítás alapján a PDGF-BB szintje dohányosok esetében szignifikánsan magasabb volt a nem dohányosokhoz viszonyítva a TNFi terápia 12. hónapjában, amely azt sugallja, hogy a dohányzás ténye a gyulladás folyamatát, valamint a neovaszkularizációt felerősíti és a terápiás választ csökkenti.

Összefoglalásként elmondhatjuk, hogy az angiogenesis kiemelt szereppel bír az inflammatórikus arthropathiák pathogenezisében, valamint a arthritiszekkel társuló felgyorsult inflammatórikus atheroszklerózisban is. Néhány angiogén mediátor biomarkerként funkcionálhat az angiogenezis, gyulladás és atheroszklerózis együttes megjelenésének igazolásában arthritiszek esetében.

11 PUBLICATIONS

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Candidate: Emese Balogh
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Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. **Balogh, E.**, Biniecka, M., Fearon, U., Veale, D. J., Szekanecz, Z.: Angiogenesis in inflammatory arthritis.
   Isr. Med. Assoc. J. 21, 345-352, 2019.
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Total IF of journals (all publications): 46,988
Total IF of journals (publications related to the dissertation): 8,524

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22 July, 2019

DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár

11.3 Conference abstracts

- 12th April 2006: Scientific Student Meeting (TDK), Debrecen:
  Evaluation of the effectivity of ultrasound therapy for patients with peripheral arterial disease using laser doppler scanner flowmetry and radioisotopic techniques
  E Balogh
  Oral presentation, abstract book p. 142.

- 19th February 2007: Scientific Student Meeting (TDK), Debrecen:
  Hand microcirculation functional imaging (thermography and laser doppler scanner) post - radial artery harvesting in case of coronary bypass surgery - postoperative study
  E Balogh
  Oral presentation, Abstract book: p. 135.

- 8th September 2007: XIV. Congress of Hungarian Society of Surgery of the Hand, Hajdúszoboszló:
  New non-invasive diagnostic technique -laser doppler scanner- in preoperative routine examinations of radial artery harvesting for coronary bypass surgeries
  E Balogh, Z Galajda, N Szabó, I Horváth, I Garai, Z Csiki
  Oral presentation, Abstract book: p.19 A-0018

- 11th October 2007: Angiology Days in Nyíregyháza, Nyíregyháza:
  Systemic and local effects of a dietary product with high dose of flavonoids on patients with Raynaud's syndrome
  Z Csiki, **E Balogh**, Z Galajda, J Szentmiklósi, N Szabó, I Horváth, Cs Kerékgyártó, I Garai, Gy Major, P Molnár, M Zeher
  Oral presentation, Journal of Angiologic diseases: 2007/suppl. 2., p.22-23.

- 12th October 2007: Angiology Days in Nyíregyháza, Nyíregyháza:
  Hand microcirculation functional imaging (thermography and laser doppler scanner) post-radial artery harvesting in case of coronary bypass surgery - late postoperative study
  Z Csiki, **E Balogh**, N Szabó, I Horváth, I Garai, Zs Kromplák, A Szász, Cs Kerékgyártó, Gy Major, P Molnár, Z Galajda
  Poster presentation, Journal of Angiologic diseases: 2007/suppl. 2., p.34.

- 8th November 2007: XIV. Congress of Hungarian Society of Cardiac Surgery, Eger:
  Routine examinations of the hand radial artery with laser doppler scanner before coronary bypass surgeries
  E Balogh, Zs Kromplák, M Pakucs, Cs Kerékgyártó, Cs Papp, Z Galajda, Z Csiki
  Oral presentation**,** Journal of Cardiologica Hungarica: suppl. D: 37:D1

- 8th December 2007: XV. Congress of Hungarian Society of Hypertonia, Budapest:
  Efficacy of metoprolol alone and metoprolol-felodipin combination on the digital microcirculation of patients with primary Raynaud's syndrome
  Z Csiki, **E Balogh**, E Vaszily, Cs Kerékgyártó, P Molnár, I Horváth, N Szabó, I Garai, M Zeher
  Poster presentation, Hypertony and Nephrology 11 (S3): p. 70.

- 3rd April 2008.: Scientific Student Meeting (TDK), Debrecen:

Routine examinations of the hand with laser doppler scanner before coronary bypass surgeries with radial artery
E Balogh
Oral presentation, Abstract book: p. 43.

- 4th-5th December 2009.: Hungarian Rheumatologyst Association – Meeting of Young Rheumatologysts. Cserkeszőlő:
  Biological therapy and hepatitis-C positive patients
  E Balogh
  Oral presentation

- 3th-5th December 2010.: Hungarian Rheumatologyst Association – Meeting of Young Rheumatologysts. Kóspallag-Nagyírtáspuszta:
  A novel TNF-Ang2/Tie-2 mediated effect on leukocyte cell influx and DNA damage in inflammatory arthritis
  E Balogh, M Biniecka, CT Ng, D J Veale, U Fearon
  Oral presentation

- 25-28th May 2011.: EULAR (European League against Rheumatism annual congress 2011 – London, UK:
  A novel TNF-Ang2/Tie-2 mediated effect on leukocyte cellinflux and DNA damage in inflammatory arthritis.
  E Balogh, M Biniecka, C T Ng, D J Veale, U Fearon
  Poster presentation

- 6th-9th June 2012.: EULAR (European League Against Rheumatism) annual congress 2012 – Berlin, Germany
  A comparative analysis of PsA spondylitis patients with PsA and AS 12 months after initiation of TNFi therapy
  J M Dias, **E Balogh**, L Harty, E O'Flynn, A Grier, M Molloy, P Minnock, E Molloy, O FitzGerald, D J Veale
  Poster presentation

- 6th-9th June 2012.: EULAR (European League Against Rheumatism) annual congress 2012 – Berlin, Germany
  Nicotinamide Adenine Dinucleotide Phosphate Oxidase Mediated Angiogenesis And Inflammation In The Arthritic Joint.
  M Biniecka, W Gao, C T Ng, **E Balogh**, D J Veale, U Fearon
  Poster presentation

- 20th-21st September 2012.: ISR (Irish Society for Rheumatology) – Belfast, UK
  High Acute Phase Reactants predict Cardiovascular Events and Rapid Radiological Progression in Inflammatory Arthritis
  E Balogh, C T Ng, T Saber, J M Dias, R Mullan, U Fearon, D J Veale
  Oral presentation

- 20th-21st September 2012.: ISR (Irish Society for Rheumatology) – Belfast, UK
  Comparison of Remission Criteria in a Biologic-treated Rheumatoid Arthritis Patient Cohort
  E Balogh, J M Dias, R Mullan, L Harty, P Gallagher, M Molloy, E O'Flynn, A

O'Kelly, M O'Neill, L Moore, M Murray, O FitzGerald, U Fearon, D J Veale
Poster presentation

- 20th-21st September 2012.: ISR (Irish Society for Rheumatology) – Belfast, UK
  The EULAR Scleroderma Trials and Research Group (EUSTAR): Results from one participating centre in Ireland.
  M Murray, **E Balogh**, D J Veale
  Poster presentation

- 9th-14th November 2012.: ACR (American College of Rheumatology) – Washington D.C. – USA
  A Novel Angiopoetin2/TEK Tyrosine Kinase Receptor Mediated Effect On Leukocyte Cell Influx and Oxidative Damage in Inflammatory Arthritis
  E Balogh, C T Ng, D J Veale, U Fearon, M Biniecka
  Poster presentation

- 4-6th April 2013.: Controversies in Rheumatology and Autoimmunity – Budapest, Hungary
  Altered angiogenesis, oxidative damage and synovial hypoxia in the inflammatory joint.
  E Balogh, CT Ng, D J Veale, U Fearon, M Biniecka
  Oral presentation

- 4-6th April 2013.: Controversies in Rheumatology and Autoimmunity – Budapest, Hungary
  High acute phase reactants predict cardiovascular events and rapid radiological progression in inflammatory arthritis.
  E Balogh, CT Ng, T Saber, R Mullan, U Fearon, D J Veale
  Oral presentation

- 19-20th September 2013.: Irish Society for Rheumatology, Autumn Meeting - Knightsbrook Hotel, Trim
  Interleukin-34 regulates angiogenesis in Inflammatory Arthritis
  E Balogh, M Connolly, M Biniecka, J McCormick, D J Veale, U Fearon
  Oral Presentation

- 5th-7th March 2015.: European Workshop for Rheumatology Research , Budapest, Hungary:
  Effects of anti-TNF therapy on markers of angiogenesis and vascular pathology in arthritis: A comparative approach
  E Balogh, E Végh, Gy Kerekes, A Váncsa, P Csomor, L Pogácsás, F Balázs, J McCormick, M Biniecka, S Szántó, G Szűcs, U Fearon, DJ Veale, Z Szekanecz
  Poster Presentation

- 24-25th of September 2015: Irish Society for Rheumatology, Naas, Ireland:
  Adult onset Still's Disease as a rare manifestation of ASIA syndrome
  E Balogh, D Duke, N Walsh, C Power, G Harbourne, J O'Dowd, C Barry, P O'Connell
  Poster presentation

- 26th of April 2017: British Society for Rheumatology Annual Meeting –

Birmingham:
Real-life data on comparison between second tumour necrosis factor inhibitor verus biologics with different mode of action after secondary failure of initial tumour necrosis factor inhibitor in rheumatoid arthritis
M Shipa, M Di Cicco, **E Balogh**, E Roussou
Poster presentation

- 3-8th of November 2017: ACR/ARHP Annual Meeting – San Diego, USA:
  Tailoring Second-Line Biologic Therapy in Rheumatoid Arthritis: New Findings on the Usefulness of Antibody Status to Optimise Drug Selection
  M Shipa, M Di Cicco, **E Balogh**, A Mian, D Mukerjee, E Roussou
  Poster presentation

12 KEYWORDS/TÁRGYSZAVAK:

12.1 Keywords

Angiogenesis

Inflammatory arthritis

Rheumatoid arthritis

Ankylosing spondylitis

Oxidative stress

Hypoxia

Atherosclerosis

Angiogenic mediators

Intracellular pathways

TNFα inhibition

Therapy

Targets

Bioenergetic metabolism

12.2 Tárgyszavak (Keywords in Hungarian)

Angiogenezis

Inflammatórikus arthritisz

Rheumatoid arthritisz

Spondylitisz ankylopoetika

Oxidatív stressz

Hypoxia

Atheroszklerózis

Angiogén mediátorok

Intracelluláris útvonalak

TNF gátlás

Terápia

Célpontok

Bioenergetikai metabolizmus

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