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**Investigating the effects of the disease-modifying anti-rheumatic
drug methotrexate on the vascular endothelium**

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Philosophy (PhD)

Statement of Originality

I, Marie Beatrice Lang, confirm that the work presented in this thesis is my own. I have indicated where work has been derived from other sources at the necessary places in this thesis.

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Für meine Großeltern,
Elisabeth und Ingomar Lang

Abstract

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease that associates with increased mortality from cardiovascular disease (CVD). Methotrexate (MTX), a folate analogue used as disease-modifying anti-rheumatic drug (DMARD), reduces CVD morbidity and mortality in RA patients, possibly by improving endothelial function. The aim of this study was to investigate the molecular effects of MTX in quiescent and activated vascular endothelial cells (EC).

In EC pre-treated with tumour necrosis factor (TNF)- α , MTX independently increased the activity of mitogen-activated protein kinase (MAPK) p38 and Akt and caused cell cycle arrest due to folate depletion. In contrast, MTX did not affect cell signalling, proliferation or gene expression in EC exposed to different types of shear stress. To tease out the underlying mechanism, folate or one carbon metabolism (OCM) was investigated in EC cultivated under static or shear stress conditions. Endothelial OCM was fundamentally altered by shear stress, with potentially important implications for MTX uptake and function. Using endothelial colony forming cells (ECFC) isolated from RA patients before and after treatment initiation with MTX and hydroxychloroquine, it was shown that systemic inflammation primes ECFC towards a pro-inflammatory state that may be reversible with DMARD treatment.

These data suggest that MTX acts on vascular EC in a folate-dependent manner. However, since endothelial OCM was largely downregulated by shear stress, this implies that MTX may have limited direct effects on EC *in vivo* and provides a potential explanation for the controversies around the vascular effects of folate and anti-folate therapy. Although findings in ECFC from patients with RA support anti-inflammatory effects of MTX containing therapy, the data in this thesis do not support a direct MTX-mediated anti-atherogenic effect on the endothelium but suggest that the drug may limit EC dysfunction in combination with other drugs indirectly by reducing systemic inflammation.

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Publications and Presentations

Publications

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Conference presentations

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Abbreviations

5-formylTHF, leucovorin or folinic acid
5-meTHF, 5-methyl THF
5,10-me+THF, 5,10-methenyl THF
5,10-meTHF, 5,10-methylene THF
7-OH MTX, 7-hydroxy MTX
A20, TNF Alpha Induced Protein 3
ABC, ATP-binding cassette transporter
Abl, Tyrosine-protein kinase ABL1
ACPA, anti-citrullinated peptide antibodies
ACS, acute coronary syndrome
Ad0, recombinant empty vector adenovirus
ADCK3, aarF domain containing kinase 3
AGE, advanced glycation end product
AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside
ALDH1L1, Aldehyde Dehydrogenase 1 Family Member L1
ALDH1L2, Aldehyde Dehydrogenase 1 Family Member L2
ALOX5, Arachidonate 5-Lipoxygenase
AMP, adenosine monophosphate
AMPK, AMP-activated protein kinase
AnV, Annexin V
ANXA5, Annexin A5
ApoE, apolipoprotein E
ARAF, Serine/threonine-protein kinase A-Raf
Arg, Tyrosine-protein kinase ABL2
ATIC, 5-Aminoimidazole-4-Carboxamide Ribonucleotide Formyltransferase + IMP
Cyclohydrolase
ATP, adenosine triphosphate
BAFF, B-cell activating factor of the TNF family
BH₂, dihydrobiopterin
BH₄, tetrahydrobiopterin
BMX, BMX non-receptor tyrosine kinase
BOEC, blood outgrowth endothelial cells
BRAF, Serine/threonine-protein kinase B-raf
BrdU, bromodeoxyuridine
BSA, bovine serum albumin
CAD, coronary artery disease
CCL2, C-C motif chemokine ligand 2 (MCP1)
CD39, ectonucleoside triphosphate dephosphorylase 1
CD73, ecto-5'-nucleotidase
cdk, cyclin dependent kinase
CDK13, cyclin dependent kinase 13 (CHED)
CDK2, cyclin-dependent kinase 2
CDK3, cyclin dependent kinase 3
CEC, circulating EC
CFR, coronary flow reserve
CIA, collagen-induced arthritis model
cIMT, carotid intima-media thickness
CLA, cutaneous lymphocyte-associated antigen
CO₂, carbon dioxide

COPD, chronic obstructive pulmonary disease
 CREB, cAMP response element-binding protein
 CRP, C-reactive protein
 CVD, cardiovascular disease
 DAMPA, 2,4-diamino-N10-methylpteroic acid
 DAS28-ESR, disease activity score in 28 joints
 DF, disturbed flow
 DHF, dihydrofolate
 DHFR, Dihydrofolate Reductase
 DHODH, dihydroorotate dehydrogenase
 DMARD, disease-modifying anti-rheumatic drug
 DMSO, dimethyl sulfoxide
 DN-AMPK α 1, dominant-negative AMPK α 1
 DNA, deoxyribonucleic acid
 dTMP, deoxythymidine monophosphate
 DTT, dithiothreitol
 dUMP, deoxyuridine monophosphate
 DUSP1, Dual Specificity Phosphatase 1
 E2F1, E2F Transcription Factor 1
 EC, endothelial cell
 ECFC, endothelial colony forming cells
 ECG, electrocardiogram
 ECM, extracellular matrix
 EDTA, ethylenediaminetetraacetic acid
 ENG, endoglin
 eNOS, endothelial nitric oxide synthase
 EPC, endothelial progenitor cells
 EphA2, Ephrin type-A receptor 2
 EphA4, Ephrin type-A receptor 4
 EphA5, Ephrin type-A receptor 5
 EphB2, Ephrin type-B receptor 2
 EphB3, Ephrin type-B receptor 3
 ERK, extracellular-signal regulated kinases
 ESR, erythrocyte sedimentation rate
 EULAR, European League against Rheumatism
 F-MTX, FITC-conjugated methotrexate
 FA, folinic acid
 FCS, foetal calf serum
 FLT1, vascular endothelial growth factor receptor 1 (VEGFR1)
 FMO, fluorescence minus one
 FOX, forkhead box
 FPGS, Folylpolyglutamate Synthase
 FS, forward scatter
 Fyn, Tyrosine-protein kinase Fyn
 GAPDH, Glyceraldehyde 3-phosphate dehydrogenase
 GART, Phosphoribosylglycinamide Formyltransferase, Phosphoribosylglycinamide Synthetase, Phosphoribosylaminoimidazole Synthetase
 GGH, Gamma-Glutamyl Hydrolase
 GM-CSF, granulocyte-macrophage colony-stimulating factor
 GMP, good manufacturing practice

GWAS, genome wide association study
 H-LSS, high laminar shear stress
 h, hour
 H₂O₂, hydrogen peroxide
 HAEC, human aortic endothelial cells
 HBSS, Hank's balanced salt solution
 HC, healthy control
 HCQ, hydroxychloroquine
 HIF1 α , hypoxia-inducible factor 1 α
 HO1, heme oxygenase 1
 HOX, homeobox
 HSC, haematopoietic stem cell
 HSP27, heat shock protein 27
 HUVEC, human umbilical vein endothelial cells
 Hz, Hertz
 IGF1, insulin-like growth factor 1
 IKK, I κ B kinase
 IFN, Interferon
 IL, Interleukin
 IQR, interquartile range
 IRR, insulin receptor-related receptor
 ITGAV, integrin alpha V
 I κ B α , NF κ B Inhibitor Alpha
 JAK1, Tyrosine-protein kinase JAK1
 JNK, c-Jun N-terminal kinase
 kDa, kilo Dalton
 KDR, kinase insert domain receptor (VEGFR2)
 KIT, KIT Proto-oncogene, Receptor Tyrosine kinase (c-kit)
 KLF2, Krüppel-like factor 2
 KLF4, Krüppel-like factor 4
 L-LSS, low laminar shear stress
 LCK, LCK Proto-Oncogene, Lck family tyrosine kinase
 LDL, low-density lipoprotein
 LPS, lipopolysaccharide
 LSS, laminar shear stress
 M-CSF, macrophage colony-stimulating factor
 MAC, myeloid angiogenic cell
 MAP4K4, Mitogen-Activated Protein Kinase Kinase Kinase Kinase 4
 MAPK, mitogen-activated protein kinase
 MAPKAPK2 or 3, Mitogen-activated protein kinase-activated protein kinase 2 or 3
 MAT, methionine S-adenosyltransferase
 MCP1, monocyte chemoattractant protein 1 (CCL2)
 MFT, mitochondrial folate transporter (SLC25A32)
 min, minutes
 MnSOD, manganese superoxide dismutase
 MRI, magnetic resonance imaging
 MSK1, mitogen- and stress-activated protein kinase 1
 MTFMT, Mitochondrial Methionyl-tRNA Formyltransferase
 MTHFD1, Methylenetetrahydrofolate Dehydrogenase, Cyclohydrolase +
 Formyltetrahydrofolate Synthetase 1

MTHFD1L, Methylenetetrahydrofolate Dehydrogenase (NADP+ Dependent) 1 Like.
MTHFD2, Methylenetetrahydrofolate Dehydrogenase (NADP+ Dependent) 2,
Methenyltetrahydrofolate Cyclohydrolase
MTHFD2L, Methylenetetrahydrofolate Dehydrogenase (NADP+ Dependent) 2 Like
MTHFR, Methylenetetrahydrofolate Reductase
mTOR, mammalian target of rapamycin
MTR, Methionine synthase
MTX, methotrexate
N/A, not available
NADH, nicotinamide adenine dinucleotide
NADPH, nicotinamide adenine dinucleotide phosphate
NFkB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NLRP3, NOD-, LRR- and pyrin domain-containing protein 3
NO, nitric oxide
NQO1, NAD(P)H Quinone Dehydrogenase 1
Nrf2, nuclear factor (erythroid-derived 2)-like 2
NRP1, neuropilin 1
ns, not significant
NSAID, non-steroidal anti-inflammatory drug
NSTEMI, non-ST-elevation myocardial infarction
OCM, one carbon metabolism
OSS, oscillatory shear stress
p70S6K, ribosomal protein S6 kinase beta 1
Pa, Pascal
pAcABG, para-acetamidobenzoylglutamate
PAH, pulmonary arterial hypertension
PAI-1, plasminogen activator inhibitor 1
PBMC, peripheral blood mononuclear cells
PBS, phosphate-buffered saline
PCI, percutaneous coronary intervention
PCR, polymerase chain reaction
PDGF, platelet-derived growth factor
PECAM1, platelet-endothelial cell adhesion molecule 1
PGF, placental growth factor
PHGDH, Phosphoglycerate Dehydrogenase
PI, propidium iodide
PI3K, phosphatidylinositol-3 kinase
Pim1, Pim-1 Proto-Oncogene, Serine/Threonine Kinase
PKA, Protein kinase A
PKC, protein kinase C
PKG, Protein kinase G
PKM2, pyruvate kinase M2
PRKX, protein kinase x-linked
PtdIns, phosphoinositides
PTGS2, cyclooxygenase 2 (COX2)
PTK, phospho-tyrosine kinase activity array
PTK2, protein tyrosine kinase 2
RA, rheumatoid arthritis
RAF1, RAF proto-oncogene serine/threonine-protein kinase
Rb, retinoblastoma protein

RF, rheumatoid factor
 RFC, reduced folate carrier (SLC19A1)
 RNA, ribonucleic acid
 ROS, reactive oxygen species
 RSK2, Ribosomal Protein S6 Kinase A3
 RSK3, Ribosomal Protein S6 Kinase A2
 RT, room temperature
 SAH, S-adenosylhomocysteine
 SAM, S-adenosylmethionine
 SCORE, Systematic Coronary Risk Evaluation criteria
 SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
 sec, seconds
 SELE, E-selectin
 SEM, standard error of the mean
 SERPINE1, PAI-1
 SHMT1, Serine Hydroxymethyltransferase 1
 SHMT2, Serine Hydroxymethyltransferase 2
 SIRT1, Sirtuin 1
 SLE, systemic lupus erythematosus
 SPHK1, sphingosine kinase 1
 SRC/HCK/BLK/LCK/FGR/YES1 Proto-Oncogene, Src family tyrosine kinase
 SREBP2, sterol regulatory element-binding protein 2
 SS, side scatter
 Stat3, signal transducer and activator of transcription 3
 STEMI, ST-elevation myocardial infarction
 STK, phospho-serine/threonine kinase activity array
 TAB1, TAK1-binding protein 1
 TBS-T, Tris-buffered saline with 0.1% Tween-20
 TBS, Tris-buffered saline
 TCA cycle, tricarboxylic acid cycle
 TEK, Angiopoietin-1 receptor (Tie2)
 TFPI, tissue factor pathway inhibitor
 TGF β , transforming growth factor beta
 THF, tetrahydrofolate
 TNFSF10, TNF superfamily member 10
 TNF α , tumor necrosis factor alpha
 TRKB, neurotrophic receptor tyrosine kinase 2
 TRKC, neurotrophic receptor tyrosine kinase 3
 TYMS, Thymidylate Synthetase
 Tyro3, Tyrosine-protein kinase receptor TYRO3
 UF, undisturbed flow
 ULN, upper limit of normal
 UT, untreated
 VE-Cadherin, vascular endothelial cadherin
 VEGF, vascular endothelial growth factor
 VEGFR2, VEGF receptor 2
 VSMC, vascular smooth muscle cells
 yrs, years

1 Chapter 1 - Introduction

1.1 Atherosclerosis

1.1.1 A brief introduction

Atherosclerosis is a common chronic inflammatory disease of arterial blood vessels, in which plaques develop progressively in the arterial wall due to lipid deposition and reactive inflammatory responses [1]. Plaque progression causes vessel remodelling, which ultimately leads to luminal narrowing, in the case of plaque rupture to thrombotic occlusion, and ischaemia. Depending on the primary vascular bed affected, atherosclerosis manifests as coronary artery disease (CAD), cerebrovascular disease or peripheral artery disease. Cardiovascular disease (CVD) is the most common cause of death in Europe and other parts of the world [2] and accounts for the increased mortality of patients with chronic systemic inflammatory diseases, including rheumatoid arthritis [3] (section 1.3). CVD risk factors include hypertension, diabetes, dyslipidaemia, obesity, smoking and family history of CVD; many of which are modifiable by primary prevention. This section gives an overview of the pathogenesis of atherosclerosis, highlighting major cell types and disease processes involved.

Early atherogenesis and plaque formation

The first event in atherogenesis is the activation of endothelial cells (EC) by cell stressors that represent traditional CVD risk factors (e.g. lipids or smoking) in haemodynamically susceptible arterial vessel segments (section 1.2.4) [1, 4]. Through the induction of oxidative stress and other mechanisms, these stimuli increase the expression of cell adhesion molecules and production of pro-inflammatory chemokines, promote vascular permeability, impair nitric oxide (NO) production and favour apoptosis in EC [4-7] (section 1.2.2). Following the loss of EC barrier integrity [4], circulating apoprotein B-containing lipoproteins, most prominently low-density lipoproteins (LDL), enter the arterial wall by endothelial transcytosis [8] and bind to extracellular matrix (ECM) molecules in the subintimal space [9] (Figure 1). Retained lipoproteins are modified on their lipid or protein moieties [10], most commonly by oxidation in an enzymatic or non-enzymatic manner to produce oxidised LDL [11]. These processes fuel the release of oxidised phospholipids, further EC activation and induction of innate and adaptive immunity [11]. Monocytes are trafficked into the vessel wall by binding to endothelial adhesion molecules and differentiate into macrophages, which ingest the modified lipoproteins by scavenger receptor-mediated uptake to become so-called foam cells [12]. The cholesterol crystals formed are consequently phagocytosed by macrophages [13], triggering activation of

the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome [14] and production of the pro-inflammatory cytokines interleukin (IL)-1 β and IL18 [15]. T-cells are also recruited into the early atherosclerotic lesion, where CD4⁺ Th1 helper cells promote the inflammatory response by the expression of the pro-inflammatory mediators interferon (IFN)- γ , IL2, IL3 and tumour necrosis factor (TNF)- α [16]. These processes fuel a self-perpetuating cycle of lipid modification, endothelial and inflammatory cell activation.

Plaque progression

Vascular smooth muscle cells (VSMC) clonally expand in the neointima and take up lipids to form foam cells that transdifferentiate into macrophage- and osteochondrogenic-like cells [17]. Lipid accumulation and overloading causes macrophage and VSMC cell death [12, 17] but lesional macrophages fail to remove debris and apoptotic cells (a process termed efferocytosis), which contributes to the formation of the necrotic core [12]. As a reactive response, VSMC produce ECM proteins such as collagen and elastin, which form the fibrous cap that overlays the growing necrotic core [17]. Osteochondrogenic-like VSMC induce calcification of the plaque [17]. Plaque progression induces vessel thickening, whereby initially the vessel remodels outward to keep the lumen diameter constant, but ultimately the growing atheroma expands into the lumen and can reduce vessel patency [1].

Plaque destabilisation and rupture

Plaque destabilisation is mediated by multiple mechanisms: VSMC produce less ECM molecules due to VSMC death, senescence and direct inhibition by pro-inflammatory mediators [12, 17]. Macrophages and VSMC express ECM-degrading molecules, which cause thinning of the fibrous cap [17]. Unstable plaques are characterised by a large lipid core, low VSMC number and thin fibrous cap. Rupture of the plaque exposes thrombogenic material to the blood stream, leading to thrombus formation, vessel occlusion and ischaemia.

Endothelial erosion

Thrombi can also develop on non-ruptured plaques, which often display little lipid content, few inflammatory cells and a thick fibrous cap [18, 19]. Under these circumstances, thrombus formation is thought to be mediated by loss of EC overlying a developing atheroma (termed endothelial erosion), which has been suggested to occur in response to disturbed shear stress, neutrophil accumulation and endothelial apoptosis [20].

Clinical outcomes

The predominant clinical manifestation of atherosclerosis is CAD, which presents as chest pain and shortness of breath on exertion or at rest, classified as stable angina pectoris or acute coronary syndrome (ACS) respectively. ACS describes unstable angina pectoris, non-ST-segment elevation myocardial infarction (NSTEMI) and STEMI, which are categorised based on changes in cardiac troponin T and the electrocardiogram (ECG). Complications after an acute coronary event may include heart failure and arrhythmia. Management is based on life-style modifications, risk factor modification, nitrate, anti-platelet and anti-coagulant therapy, beta blocker treatment and reperfusion by percutaneous coronary intervention (PCI) [21]. Lowering lipid levels with statin therapy has evolved as a central measure, with marked effects of LDL lowering on CVD events [1]. Despite great advances in understanding atherogenesis, therapy and secondary prevention, residual CVD risk remains, which needs to be targeted by primary prevention and additive, alternative therapeutic strategies, potentially including anti-inflammatory therapy [1] (section 1.3.4).

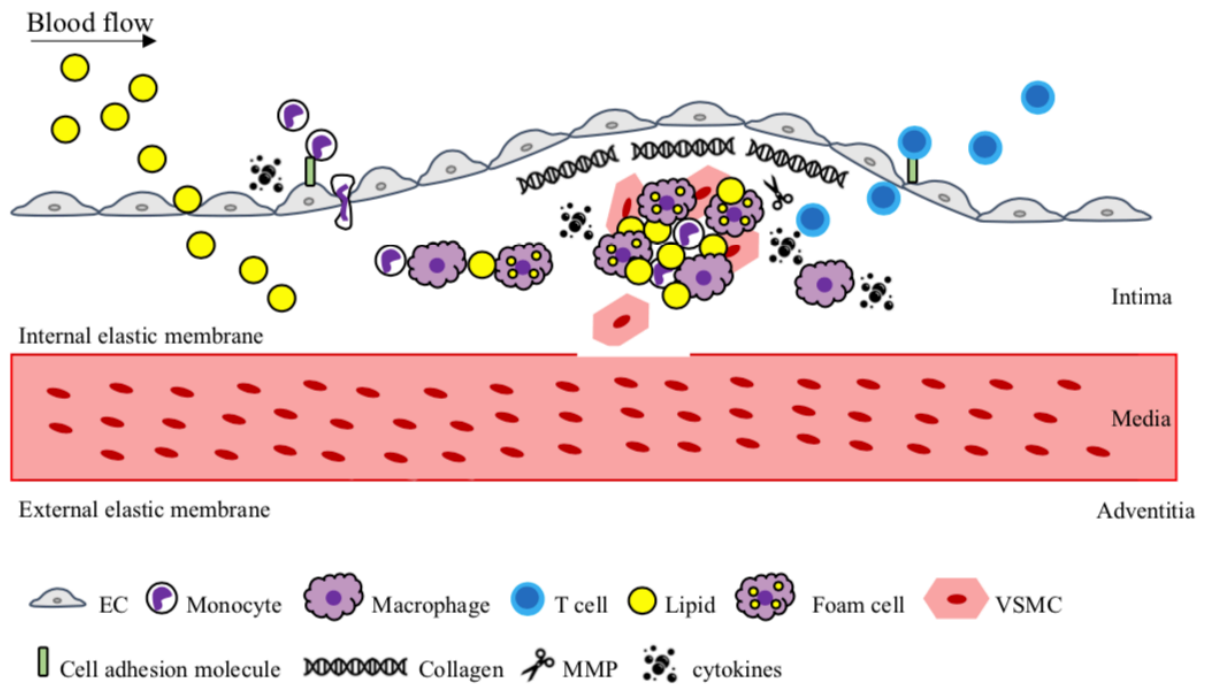


Figure 1. Hallmarks of atherogenesis. Schematic representation of cells and molecules involved in atherogenic plaque formation, progression and destabilisation. Depicted disease processes are simplified and are not exhaustive. Illustration adapted from Libby et al [1]. EC, endothelial cell. VSMC, vascular smooth muscle cell. MMP, matrix-metalloproteinase.

1.2 Endothelial cells in health and disease

This thesis focuses on the role of EC in atherogenesis in the context of rheumatoid arthritis (section 1.3). Before outlining the effects of rheumatoid arthritis on EC function, it is important to review the main concepts of EC biology, with a special focus on the role of fluid shear stress on vascular EC.

1.2.1 Endothelial cells in vascular homeostasis

The lumen of all blood vessels, that is arteries, arterioles, capillaries, venules and veins, is lined by a monolayer of EC. Being in contact with the circulating blood and surrounding tissue, EC represent an important node for systemic and local communication. The main functions of EC are to maintain vascular and tissue homeostasis by regulating the transport of nutrients, oxygen and other molecules to the surrounding tissues, vascular tone, vascular permeability, haemostasis, inflammation, angiogenesis and vascular repair [22]. It is increasingly recognised that EC are a plastic and heterogenous cell type that can adapt to changes in the microenvironment. Depending on their location in the vascular tree, the organ they support, vessel size, vessel type, haemodynamic forces and interaction with specific tissue-resident cell types, EC adopt specialised phenotypes and functions [23, 24] (exemplified below).

Vascular tone

EC produce several vasoactive substances, including prostacyclin, endothelin 1, prostanoids and NO, the latter being the most important [22]. EC constitutively express the enzyme endothelial nitric oxide synthase (eNOS) [25], which catalyses the conversion of arginine and oxygen to NO and citrulline in the presence of its cofactor tetrahydrobiopterin (BH₄) [26]. NO is a potent vasodilator that stimulates VSMC relaxation [27], while also conferring anti-inflammatory and anti-thrombotic actions by inhibiting platelet [28] and leukocyte [29] adhesion to the endothelium and platelet aggregation [30]. Shear stress and other stimuli such as acetylcholine increase eNOS expression and NO formation in EC [27, 31], mechanisms which are utilised to measure endothelial-dependent vasorelaxation *in vivo*. EC can also react to vasoactive cues from the circulation or the autonomic nervous system, including catecholamines, bradykinin and adenine nucleotides [22]. The systemic vascular tone is regulated by the arterioles, also termed resistance arteries.

EC barrier integrity

To facilitate blood-tissue exchange, capillaries cover a large surface area and exhibit very slow blood flow rates [24]. Transport of fluids and nutrients is mediated by passive diffusion between cells and transcytosis [23]. Depending on their location and functional requirement in a specific organ, capillaries display a continuous or discontinuous EC monolayer with or without transcellular pores known as fenestrations, which dictates basal permeability [23]. Tight junctions are multiprotein complexes that connect neighbouring cells at the apical region and form a barrier for paracellular transport.

Haemostasis

EC express pro- and anti-thrombotic molecules to coordinate and maintain vessel haemostasis [32]. In the basal state, EC maintain an anti-thrombotic phenotype by expressing thrombomodulin (TM), tissue factor pathway inhibitor, plasminogen activators (tissue or urokinase type, tPA or uPA), and by generating NO and prostacyclin. Following vascular injury, EC participate in clot formation by expressing the pro-coagulant molecules von Willebrand factor (vWF) and plasminogen activator inhibitor (PAI-1). These molecules are heterogeneously expressed in different vascular beds [23].

Angiogenesis and vascular repair

During development or following vascular injury, EC are able to form new blood vessels from mature EC, a process termed angiogenesis. This complex process involves programs that increase EC proliferation and migration following stimulation with angiogenic growth factors and hypoxia [22].

1.2.2 EC dysfunction

Persistent perturbation of any of the above homeostatic mechanisms may eventually lead to endothelial dysfunction [33]. The term describes a pro-inflammatory, pro-coagulant, more permeable and less vasoactive endothelial state. Because endothelial dysfunction can occur within every tissue, it is implicated in many different diseases, including atherosclerosis and rheumatoid arthritis (section 1.3.3). Different stimuli can impair EC function, including CVD risk factors and pro-inflammatory cytokines.

Oxidative stress and reduced NO bioavailability

Hyperglycaemia, advanced glycation end products (AGE), smoking and oxidised LDL trigger the formation of reactive oxygen species (ROS) in EC [34-36] by one or more of the following mechanisms: activation of NADPH oxidases [37-39], xanthine oxidases [40] and/or interference with the mitochondrial electron transport chain [38]. Increased ROS formation can result in the activation of the pro-inflammatory nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB) pathway [41, 42] (see below) and decreased NO bioavailability in EC. Increased oxidative stress leads to the formation of superoxide, which reacts with NO to form peroxynitrite [43]. BH₄ is oxidised by peroxynitrite, limiting its bioavailability [44]. In the absence of BH₄ and arginine, eNOS can only catalyse the production of superoxide uncoupled from further reactions to produce NO, in a process termed eNOS uncoupling [26]. This process further increases oxidative stress and decreases NO bioavailability, leading to an attenuated vasodilatory response and contributes to the loss of the anti-thrombotic and anti-inflammatory EC phenotype.

Inflammation, increased vascular permeability and thrombosis

Pro-inflammatory EC activation can occur via two distinct pathways, which differ in the rapidity of response and dependence on gene expression [45]. Participation in acute inflammatory reactions is a physiological and important endothelial function. However, persistent EC activation causes detrimental effects on the vasculature.

Type I activation is mediated by agonists such as histamine or thrombin, which induce G-protein coupled receptor signalling and Ca²⁺ release, increasing blood flow by promoting the production of prostacyclin and NO, vascular permeability by increasing intercellular gap formation and neutrophil recruitment via P-selectin and platelet activating factor (PAF) exposure on the endothelial surface. These reactions occur in post-capillary venules and are transient, lasting 10-20min [45].

Type II activation is promoted by pro-inflammatory cytokines, commonly TNFα and IL1, but also other mediators, which induce signalling events that converge on the activation of the canonical NFκB pathway (described in section 3.1) [46]. Following its nuclear translocation, NFκB mediates the transcriptional regulation of the endothelial cell adhesion molecules E-selectin [47], intercellular adhesion molecule (ICAM)-1 [48] and vascular cell adhesion

molecule (VCAM)-1 [49], promoting leukocyte recruitment and adhesion to the endothelium [50-52]. Leukocyte activation and arrest on the EC monolayer is facilitated by the endothelial chemokines monocyte chemoattractant protein (MCP)-1 and IL8 [53], both also regulated by NF κ B [54, 55]. TNF α increases endothelial permeability by inducing the formation of intercellular gaps, as demonstrated in respectively treated murine venous endothelium [56]. NF κ B activation also increases vascular susceptibility to thrombosis. TNF α reduces the levels of eNOS [57], TM [58], and tPA [59], while increasing PAI-1 expression in EC [59]. Supporting its role in endothelial haemostasis, NF κ B inhibition prevents lipopolysaccharide (LPS)-induced fibrin deposition in transgenic mice that overexpress a mutant of the inhibitor protein I κ B α in the endothelium [60]. Endothelial-specific genetic deletion of the NF κ B essential modulator (NEMO), a regulatory subunit of the I κ B kinase (IKK) required for NF κ B activation, reduces atherosclerotic lesion formation in apolipoprotein E (ApoE) knockout mice, highlighting the importance of endothelial NF κ B signalling in driving atherogenesis [61]. Type II activation takes longer to come into action (a couple of hours) and is only terminated after the removal of the inciting stimulus [45].

Angiogenesis in chronic inflammation

A sustained inflammatory response induces angiogenesis to maintain immune cell recruitment and survival of activated immune cells in the inflamed tissue [45]. Macrophages release angiogenic factors such as vascular endothelial growth factor (VEGF) [62] and TNF α [63] that promote neovascularisation, with other cell types and mediators also involved [45]. While angiogenesis is crucial for vascular repair after injury, pathological angiogenesis is a hallmark of atherosclerosis [64] and synovial pannus formation in rheumatoid arthritis [65].

1.2.3 Signalling pathways implicated in endothelial “cytoprotection”

As critical step in atherogenesis, EC dysfunction precedes the manifestation of overt CVD and predicts future CVD events in the general population [66]. Primary prevention aims at avoiding the induction of EC dysfunction, e.g. by life-style modification and risk factor reduction. There are currently no therapies available that are directed at reversing established EC dysfunction [33]. Several mechanisms have been described that decrease the endothelial susceptibility to cell stressors, such as laminar shear stress (section 1.2.4) and the expression of anti-oxidant, anti-apoptotic and anti-inflammatory molecules [67]. It has been suggested that activation of distinct endothelial signalling pathways, including the Akt, AMP-activated protein kinase

(AMPK), mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) signalling pathways, may confer protection from EC activation and hence represent potential treatment avenues [67].

Akt

Akt is a serine/threonine protein kinase (syn. protein kinase B) that is activated by growth factors such as VEGF and insulin-like growth factor (IGF)-1, insulin, angiopoietin 1, ROS, shear stress and statins [68, 69]. One of the main functions of Akt is to promote cell survival and inhibit apoptosis [70, 71], achieved by the phosphorylation and inactivation of the pro-apoptotic proteins caspase-9 [71] and Bad [72] in EC. Akt also drives EC migration, barrier integrity, proliferation [73] and cell cycle progression, the latter two by phosphorylating transcription factors of the forkhead box (FOX) family [74] and the cyclin-dependent kinase inhibitor p21^{cip1} [75]. Moreover, Akt phosphorylates eNOS [76] and regulates developmental angiogenesis in murine retinas [77]. Akt signalling is also important during atherogenesis: genetic deletion of Akt1 increases atherosclerotic lesion size in ApoE-knockout mice [78], while endothelial-specific expression of constitutively active Akt decreases neointimal formation after carotid artery ligation in mice compared to controls [79].

AMPK

AMPK is a serine/threonine protein kinase that is activated by cellular energy depletion (e.g. nutrient deprivation causing ATP depletion and a change in the AMP/ATP ratio), but also other forms of cell stress such as hypoxia and genotoxic stress, shear stress, vasoactive substances (e.g. VEGF, thrombin) and drugs, including statins and metformin [80-83]. The kinase induces cellular pathways to promote energy homeostasis and cell survival. In vascular EC, AMPK activation mediates eNOS phosphorylation, regulation of vascular tone, mitochondrial biogenesis, inhibition of apoptosis, protection from oxidative stress, inhibition of NFκB signalling and EC proliferation and regulation of angiogenesis (reviewed in [81]). AMPK activation, using 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) as an AMPK activator or EC-specific expression of constitutively active AMPK, confers atheroprotective effects by reducing atherosclerotic lesion size in ApoE-deficient mice [84] or protecting from diabetes mellitus-induced vascular dysfunction after carotid artery injury [85].

MAPK ERK1/2, JNK, p38, ERK5

The MAP kinase pathway consists of parallel sequential signalling cascades that activate one of the four effector serine/threonine kinases: extracellular-signal regulated kinases (ERK)1/2, c-Jun N-terminal kinase (JNK), p38 or ERK5 [86]. ERK1/2 is activated by growth factors [87] and pro-inflammatory cytokines [88], and promotes EC survival [87] and proliferation [89]. Stress stimuli such as pro-inflammatory cytokines [88], ROS [90] and disturbed flow induce JNK activation [91], priming EC at atherosusceptible sites for apoptosis [92]. p38 signalling is associated with both driving EC homeostasis and activation. Growth factors [89], pro-inflammatory cytokines [88], oxidative stress [93] and shear stress [94] can activate p38 signalling in EC. p38 inhibits EC proliferation and cell cycle progression by regulating the retinoblastoma (Rb) protein and cyclin-dependent kinase inhibitor p27^{Kip1} [89]. p38 induces EC apoptosis through the phosphorylation and downregulation of the anti-apoptotic molecule Bcl-xL [95]. VEGF-induced p38 activation increases EC migration and actin microfilament reorganisation by downstream phosphorylation of heat shock protein 27 (HSP27), which regulates actin polymerisation [96]. ROS-mediated activation of p38 causes stress fibre formation [97] and endothelial barrier dysfunction [93] by increasing cell contraction and paracellular endothelial permeability [98]. TNF α -triggered p38 activation mediates the induction of pro-inflammatory molecules in EC, including VCAM1 [99] and MCP1 [100]. Laminar shear stress activates ERK5 in EC, resulting in the increased expression of the transcription factors Krüppel-like factors (KLF)-2 and KLF4 and induction of an anti-inflammatory and anti-apoptotic EC phenotype (section 1.2.4) [101-103].

PKC

The protein kinase C (PKC) family has eleven members that have tissue-specific functions [104]. The novel isoform PKC ϵ has been implicated in having cardioprotective effects during ischaemic pre-conditioning in different animal models [105]. It is not clear how PKC ϵ is physiologically activated in EC, although growth factors and shear stress have been suggested [106]. PKC ϵ activates cAMP response element-binding protein (CREB) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signalling in EC, increases the expression of heme oxygenase (HO)-1 and suppresses TNF α -induced ICAM1 expression [107]. PKC ϵ also promotes NF κ B activation, which selectively drives the expression of anti-apoptotic and anti-inflammatory proteins A20 and Bcl2 but does not affect the levels of pro-inflammatory molecules [106]. PKC ϵ mediates the VEGF-induced activation of Akt and increase in eNOS

activity [108]. Activation of PKC ϵ using a specific peptide increases EC survival after serum deprivation [109] and induces EC proliferation and migration [110].

Importantly, it is clear that signalling pathways are not a binary molecular switch that will only promote one cellular function. Rather, they represent complex, interconnected cellular programs that achieve different effects in a context-dependent manner, that is for example dependent on other co-stimulatory signals, cellular state or duration of signal activation. It remains to be investigated if the activation of a single signalling cascade is sufficient to induce EC protection from injurious stimuli during atherogenesis.

1.2.4 Haemodynamics in the vascular wall: fluid shear stress

The heart pumps blood through the vascular system in the cardiac cycle, driven by the contraction (systole) and dilation (diastole) of the heart muscle. This generates a pulsatile blood flow and pressure pattern that shapes haemodynamic forces, such as fluid shear stress and cyclic strain, which act on EC in the vascular wall [111]. Fluid shear stress is a frictional force generated by the blood flow that acts in parallel on EC and is defined as force per unit area ($1 \text{ N/m}^2 = 1 \text{ Pascal (Pa)} = 10 \text{ dyn/cm}^2$) [112]. Cyclic strain is a tensile force that is produced by the pulsating blood flow and stretches the vessel circumferentially [113]. The haemodynamic forces in a given vessel segment are influenced by the local vessel geometry, vessel specification (arterial versus venous), tissue stiffness, blood pressure, blood composition and presence of pathology (e.g. stenosis) [111, 114]. Shear stress and cyclic strain are sensed by EC and transduced into biological cues, which regulate EC phenotype and function [111, 113]. This section focuses on the effects of fluid shear stress on EC biology.

Fluid shear stress is characterised by blood flow patterns that differ according to their direction and magnitude [112]. Laminar shear stress (LSS) is a unidirectional flow, where fluid travels in parallel layers, and occurs in straight vessel segments [112] (Figure 2). The cardiac cycle generates a pulsatile flow that changes the magnitude. In *in vitro* experiments, LSS is modelled as either pulsatile or continuous, steady flow. The latter is not physiological but is applied as simplified flow form in computational analyses and biological experiments using a time-averaged magnitude [112, 115]. Disturbed flow is defined by a form of flow reversal, including flow separation, flow reattachment, reciprocating flow and recirculation eddies [112]. Oscillatory shear stress (OSS) is a type of disturbed flow that changes direction in a reciprocating manner, resulting in a minimal net flow [112]. Turbulent flow is characterised by non-uniform, chaotic flow patterns, where the flow velocity changes in time and space [22]. Disturbed flow occurs at vessel bifurcations, curvatures, branch points, downstream of luminal obstructions (stenosis, atherosclerotic plaque or stents) and aneurysms [111] (Figure 2).

Shear stress activates mechanosensitive molecules on the EC surface, which translate the mechanical force into distinct biological programs. Endothelial mechanotransduction is facilitated by several different structures, including the glycocalyx, G-protein coupled receptors, ion channels, primary cilia and a junctional mechanosensory complex consisting of platelet-endothelial cell adhesion molecule (PECAM)-1, vascular endothelial (VE)-cadherin and VEGF receptor (VEGFR)-2 [116]. Shear stress induces cell signalling pathways that

regulate EC morphology and function via different mechanisms (see below). Depending on their location in the vascular tree, EC are exposed to different types of shear stress, which have specific biological effects and have so far been best characterised in the context of atherogenesis. It is well appreciated that atherogenesis primarily occurs in vessel areas that are subjected to disturbed flow (e.g. curvatures, bifurcations), whereas straight vessel segments exposed to high LSS are less susceptible [111]. Many *in vitro* and *in vivo* studies have demonstrated that LSS and OSS induce atheroprotective or atheroprone endothelial phenotypes respectively by modulating EC homeostasis and dysfunction [111] (Figure 2). Importantly, it is thought that OSS itself is not sufficient to drive atherogenesis but primes EC towards an atheroprone state, which is fully activated by the additional presence of other stressors.

Cell signalling

Shear stress modulates multiple cell signalling cascades in EC, which initiate specific transcriptional and biological pathways. Acute onset of LSS causes the transient activation of Akt [76], AMPK [117], ERK1/2 [88, 118], ERK5 [101] and protein kinase A (PKA) [119, 120] that drive the expression of KLF2 [101, 117], a master regulator of homeostatic shear stress responses [101], and mediate the phosphorylation or increased expression of eNOS [76, 118, 120]. PKC ϵ is required for the shear-stress induced activation of ERK1/2 [121]. LSS promotes phosphorylation of p38 [88], which regulates shear-induced cell alignment [94]. Following morphological and functional adaptation to LSS, it is thought that these cell signalling pathways are turned off again [122]. OSS leads to the priming or activation of the MAPK JNK [91, 123], p38 [91, 124], NF κ B [125, 126], Hippo [127], Wnt [128] and TGF β [129] signalling pathways in EC, which promote endothelial inflammation. Disturbed flow results in a sustained activation of cell signalling pathways as the local shear stress constantly changes magnitude and direction [122]. This is supported by the fact that enhanced kinase phosphorylation can be found in atheroprone regions *in vivo* [91, 123, 126].

Transcriptional regulation

LSS induces the expression of the transcription factor KLF2 [130] via activation of AMPK [117] and MEK5/ERK5/MEF2 [101] signalling pathways. KLF2 transcriptionally regulates the expression of many LSS-sensitive genes, including eNOS, TM and repression of IL8 [101]. Activated by LSS via the same pathway, KLF4 also regulates the expression of eNOS and inhibits TNF α -induced NF κ B activation and IL8 and MCP1 expression [102]. LSS promotes

the nuclear accumulation of Nrf2 that drives the expression of anti-oxidant genes such as NAD(P)H quinone dehydrogenase (NQO)-1 and HO1 [131]. These transcriptional programs contribute to the vasoactive, anti-inflammatory, anti-thrombotic and anti-apoptotic EC phenotype associated with LSS. In contrast, disturbed flow results in the loss of endothelial NO production, pro-inflammatory EC activation, increased permeability and thrombosis. NFκB is primed for activation in regions of disturbed flow *in vivo* [126], promoting endothelial inflammation as outlined above. Hypoxia-inducible factor 1α (HIF1α) is predicted to be among the most highly activated transcription factors in EC subjected to disturbed flow [132, 133]. It has been shown that HIF1α is activated by NFκB signalling and ROS and increases the expression of glycolytic genes in EC under disturbed flow, promoting endothelial glycolysis, pro-inflammatory activation and EC proliferation [133, 134]. A recent RNA sequencing analysis of shear-exposed HUVEC shows that the expression of the transcription factor E2F1 is upregulated under OSS, suggesting that it may facilitate cell cycle progression and proliferation under these conditions [132]. In addition, KLF2 expression is lower in vessel segments subjected to disturbed flow compared to LSS *in vivo* [135], indicating that its homeostatic gene expression pattern is reduced in these regions.

Proliferation, cell cycle and apoptosis

Endothelial proliferation and apoptosis are increased in EC located in areas prone to atherosclerotic plaque formation, as demonstrated in the aortas of guinea pigs [136], pigs [137] and human carotid arteries [138] respectively. Concomitantly, EC in atheroprone areas are more permeable [137] and have shorter telomeres [139], suggestive of high proliferative turnover. Loss of the endothelial cell layer is apparent over plaques in human coronary arteries with advanced atherosclerosis [140]. It has been suggested that low/disturbed flow causes EC injury and cell death in these areas, which is compensated by increasing endothelial turnover to support regeneration [136, 137]. Alternatively, increased EC proliferation may amplify cell turnover and replicative senescence, resulting in apoptosis at susceptible sites. These processes are believed to contribute to early atherogenesis but also later in the disease course by promoting endothelial denudation of plaques (endothelial erosions) [18, 138].

In vitro cell experiments confirm the role of shear stress in the regulation of EC proliferation and apoptosis. High LSS inhibits EC proliferation [141] by arresting the cells in the G0/G1 phase of the cell cycle, which is the result of increased JNK phosphorylation, increased

activation of p53, expression of p21, hypo-phosphorylation of Rb [142] and activation of Notch signalling [143]. In contrast, turbulent flow increases EC proliferation [144]. Mechanistically, it has been shown that OSS can induce Smad1/5 signalling, which causes the activation of mTOR and p70S6K pathways and differential expression of cyclin A and p21 [145, 146]. Furthermore, differential phosphorylation of AMPK and Akt by LSS and OSS can modulate cell cycle progression via mTOR signalling [147]. LSS protects EC from apoptosis [148], while OSS primes for apoptosis, partly via activating JNK signalling [92].

Inflammation

LSS prevents cytokine-mediated pro-inflammatory activation of EC [88]. NFκB signalling mediates the endothelial expression of cell adhesion molecules VCAM1, ICAM1 and E-selectin in response to disturbed flow (as outlined above), facilitating increased monocyte adhesion [149]. Disturbed flow induces the expression of sterol regulatory element-binding protein 2 (SREBP2), a transcription factor involved in cholesterol biosynthesis, resulting in the activation of the NLRP3 inflammasome and increased expression of MCP1, VCAM1 and E-selectin in EC [150].

It is beyond the scope of this section to comprehensively outline all shear stress-mediated effects on the endothelium. Further important aspects of EC biology that are modulated by shear stress include: EC morphology, EC migration, vascular permeability, ROS production, unfolded protein response [151], autophagy [152], vascular development, endothelial identity specification, vascular remodelling and epigenetics (reviewed in [111, 115, 153, 154]). The regulation of EC metabolism under static and shear stress conditions is reviewed in section 1.5.1.

Despite the acknowledgement that shear stress is an important regulator of EC function, endothelial *in vitro* experiments do not routinely apply shear stress due to the technical and experimental complexities and cost. Future work aims to integrate higher order models of EC culture, which include exposure to cyclic strain, shear stress and interacting cell types.

The next section introduces the chronic systemic inflammatory disease rheumatoid arthritis, its proposed effects on vascular function during atherogenesis and potential anti-inflammatory treatment avenues.

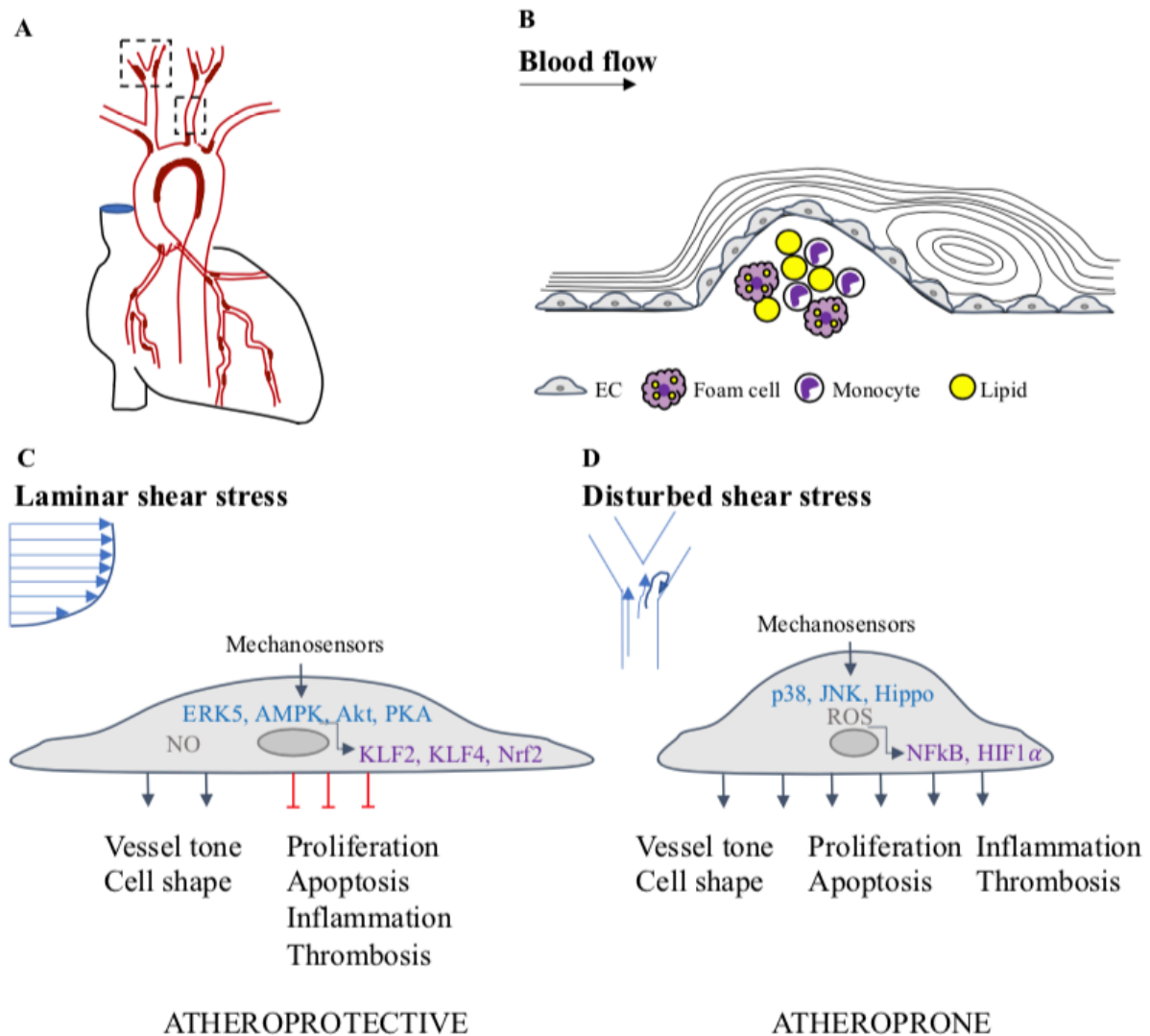


Figure 2. Shear stress modulates endothelial phenotype and function. (A) Schematic representation of the anatomy of the proximal aorta and its branches. Left and right coronary arteries supply the heart and arise from the sinus aortae. Areas prone to atherosclerotic plaque formation are indicated in dark red. Dashed boxes represent areas of LSS (right) or OSS (left). (B) Schematic representation of shear stress patterns upstream (elevated shear stress) and downstream (disturbed flow) of an atherosclerotic plaque region. (C) Laminar, unidirectional shear stress induces an atheroprotective endothelial phenotype by regulating cell signalling, transcription and functional pathways. (D) Disturbed shear stress promotes endothelial activation and dysfunction. List of mechanisms not exhaustive. Illustrations adapted from Noguchi et al [155], Yurdagul et al [156] and Gijssen et al [157].

1.3 Rheumatoid arthritis

1.3.1 Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease that leads to an abnormal immune response against the synovium, the membrane that lines the inner capsule of synovial joints [158]. This causes synovial inflammation, local matrix destruction and damage to the articular cartilage and bone. The prevalence of RA is 0.24% worldwide but is higher in Europe and the United States (0.44%) [159]. Women are affected twice as often as men [159] and are usually diagnosed at ages 40-60. The aetiology of RA remains unknown. Several genetic loci have been associated with an increased risk for developing RA, the most important being the class II human leukocyte antigen (HLA)-DRB1*04 and HLA-DRB1*01 alleles [160]. Specifically, an amino acid sequence shared by these and other HLA alleles (called the shared epitope) is thought to influence antigen binding and confer disease susceptibility. The current paradigm suggests that environmental factors (e.g. smoking) induce the generation of citrullinated antigens in genetically predisposed individuals, causing the formation of anti-citrullinated protein antibodies (ACPA) and antibodies against immunoglobulins (rheumatoid factor, RF) among others. It has been suggested that a “second hit” is then needed to promote autoantibody-mediated activation of synovial cells to initiate synovitis in the joint (reviewed in [161]).

Patients classically present with swollen and tender joints, most commonly in the hands, wrists and feet, with prolonged morning stiffness, which develops gradually and in a symmetrical pattern. Non-specific symptoms such as tiredness, fever and weight loss may occur. Although primarily affecting the joint, other organ systems may also be involved, including the skin (rheumatoid nodules), lungs (pleurisy and fibrosis), eyes (scleritis) and cardiovascular system (section 1.3.2) [162]. The disease course is variable and can be gradually progressive or more typically relapsing and remitting. If left untreated, joint damage and destruction are progressive and irreversible, leading to the impairment of daily activities and poor quality of life. Diagnosis of RA is based on the patient’s clinical history and examination, laboratory findings (serology and acute phase markers) and exclusion of other diagnoses. The 2010 ACR/EULAR classification criteria for RA [163] (Table 1) are not diagnostic but were established for patient stratification in clinical trials [158]. RA is commonly treated with the synthetic disease-modifying anti-rheumatic drug (DMARD) methotrexate (section 1.4), as monotherapy or in combination with other synthetic (e.g. hydroxychloroquine (HCQ), sulfasalazine, JAK inhibitors) or biological DMARD (e.g. TNF α or IL6 pathway inhibitors) [164]. Short-term

treatment with glucocorticoids facilitates the start or transition to a new DMARD until it reaches its maximum effect. Non-steroidal anti-inflammatory drugs (NSAID) are given to reduce symptoms. Treatment should be started as early as possible after diagnosis and aims at achieving disease remission, the near complete reduction of disease activity, or low disease activity within three to six months after treatment initiation to limit disease symptoms and progression [164]. Evaluation of disease activity is performed using different clinical scores, e.g. the Disease Activity Score in 28 joints (DAS28) score [158].

Table 1. 2010 ACR/EULAR classification criteria for RA

Number and type of affected joints	Serology	Acute phase	Symptom duration	Points
1 large joint	RF and ACPA negative	CRP and ESR normal	< 6 weeks	0
2-10 large joints		Abnormal CRP or abnormal ESR	≥ 6 weeks	1
1-3 small joints	RF or ACPA low positive			2
4-10 small joints	RF or ACPA high positive			3
>10 joints (with ≥ 1 small joint)				5
Score ≥ 6 points needed for classification as definite RA				

Affected joints represent any swollen or tender joint on examination. Imaging modalities (ultrasound, MRI) may be used to identify additional joints. Large joint (shoulder, elbow, hip, knee or ankle). Small joints (the metacarpophalangeal joint, the proximal interphalangeal joint, the second to fifth metatarsophalangeal joints, the interphalangeal joint of the thumb and the wrist). RF, rheumatoid factor. ACPA, anti-citrullinated protein antibody. Low positive, above the upper limit of normal (ULN). High positive, greater than three times above ULN. ESR, erythrocyte sedimentation rate. CRP, C-reactive protein [163].

1.3.2 Cardiovascular disease in RA

Patients with RA have a 40% increased risk of death compared to those without RA, as demonstrated in the Nurses' Health Study that followed women with RA over 36 years [165]. CVD is an important contributor to this increased mortality: patients had a 45% increased risk of death from CVD in the former study [165]. A meta-analysis similarly demonstrated that patients with RA have an 50% increased risk of CVD-related mortality compared to the general population [166]. Patients with RA have higher rates of myocardial infarction (MI), heart failure, cardiac arrest and peripheral artery disease compared to those without RA [167]. Myocarditis, pericarditis, valvular heart disease and conduction abnormalities may occur, but are either rare or not clinically relevant [168].

Both traditional CVD risk factors (hypertension, diabetes, obesity, dyslipidaemia and smoking) and RA-specific factors (RF, ACPA, ESR, CRP, disease activity) increase the risk for CVD events in patients, estimated as 49% of events driven by traditional and 30% by RA-specific risk factors [169]. Systemic inflammation is thought to cause vascular damage (discussed in the next section) but may also modify traditional CVD risk factors. For example, unlike CVD patients in the general population, patients with RA have lower levels of total cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) [170]. In addition to lowering lipid levels, inflammation may induce the modification of lipoprotein epitopes and render them dysfunctional, as suggested in a small study on HDL from active RA patients [171].

The clinical presentation and pathological features of CVD differ in RA compared to the general population. Patients with RA less frequently report a history of angina pectoris or acute chest pain, associated with an increased rate of undetected MI and recurrent ischaemic events compared to non-RA controls [172, 173]. One study suggested that RA patients have a more severe clinical presentation compared to the general population, as they are more likely to display ST-segment elevation MI, higher levels of troponin and have a worse outcome [174]. RA patients were demonstrated to have a higher burden of carotid [175] and coronary atherosclerotic plaque [176] compared to controls measured by ultrasonography and CT-angiography respectively. In contrast to the latter report, an autopsy study comparing coronary plaques in RA patients and matched controls with CVD showed that RA patients were less likely to have multiple vessel disease or high grade stenotic lesions, but more vulnerable plaques and arterial inflammation [177]. Supporting this, RA patients displayed a higher uptake of ¹⁸F-fluorodeoxyglucose in the aorta compared to controls measured by PET-CT, suggestive

of the presence of aortic inflammation [178]. It seems likely that both the greater prevalence and suggested inflammatory-mediated vulnerability of plaques contribute to CAD in RA [176].

Current CVD risk prediction scores do not accurately identify RA patients at higher CVD risk [168]. This was demonstrated in a study of 1050 RA patients, showing that the Framingham and Systematic Coronary Risk Evaluation (SCORE) scores, both established in the general population, underestimated future CVD events in these patients [179]. The QRISK2 algorithm, which includes RA as independent risk factor, overestimated CVD events in the same study. The Expanded Risk Score for CVD in RA (ERS-RA) score takes into account traditional CVD and RA-specific risk factors such as disease activity, but also underestimated CVD risk in 665 patients with RA [180]. The most recent EULAR recommendations for CVD management in RA support the use of national guidelines or the SCORE criteria [181] with application of a 1.5 multiplication factor to assess CVD risk once every five years [182]. Management includes life-style modifications, risk factor reduction and control of disease activity with DMARD therapy based on studies showing that the cumulative inflammatory burden increases CVD risk [183] and that reduction of disease activity is associated with fewer CVD events in RA patients [184].

1.3.3 Vascular/EC function in RA

Vascular morphology and function are compromised in patients with RA, affecting both the micro- and macrovasculature (reviewed in [185, 186]). Several small studies have suggested that microvascular function is impaired in RA patients compared to controls as demonstrated by reduced coronary flow reserve (CFR), the ratio between resting and maximal myocardial blood flow, in the absence of obstructive coronary artery disease [187-189]. A large number of reports show that macrovascular endothelial-dependent vasodilation, measured as reactive hyperaemia after vessel occlusion using flow-mediated dilatation (FMD), is decreased in RA patients compared to controls, in both treatment-experienced patients with long-standing disease [190-195] and treatment-naïve patients with early RA [196]. However, other studies have not found any changes in FMD in patients with RA compared to control groups [197-199]. Interestingly, one report suggested that micro- and macrovascular function do not correlate in RA patients [200], implying that they may represent different disease processes [186]. Indices of arterial stiffness and vascular morphology, such as the carotid intima/media thickness (cIMT), are increased in RA patients compared to controls [189, 191, 196, 201],

suggestive of ongoing arterial remodelling and atherogenesis. DMARD treatment improves FMD and cIMT measures in patients with inflammatory arthritis including RA [202, 203], emphasising the impact of inflammation on the vasculature.

Systemic inflammation may cause vascular damage and contribute to atherogenesis at all stages, from initiation of endothelial activation to plaque destabilisation [186, 204] (Figure 1). Atherosclerosis and RA share many inflammatory disease mechanisms, including cytokine profiles and inflammatory cell involvement, potentially indicating that these also drive atherogenesis in RA [204]. Pro-inflammatory cytokines such as TNF α and IL6, are produced by synovial cells [158] and enter the systemic circulation in RA [205]. These cytokines are thought to promote endothelial activation by inducing pro-inflammatory pathways in atheroprone regions (section 1.2.2). Macrophages and T-cells represent two major inflammatory cell types in the RA synovium [158]. During atherogenesis, monocytes and T-cells (CD4⁺ type 1 helper cells) migrate into the vessel wall and promote plaque formation, progression and destabilisation by foam cell formation and the expression of pro-inflammatory, pro-thrombotic and ECM-degrading molecules [204] (section 1.1.1). Although these mechanisms have not been formally demonstrated in the vasculature of RA patients due to the limited accessibility of tissue samples, they align with the observed effects of inflammation on synovial EC [65] and experimental models of endothelial cytokine exposure or atherosclerosis (reviewed in [1, 46, 204, 206]). Recent reports suggest that autoantibodies may also contribute to atherogenesis and show that ACPA can induce a pro-inflammatory and a pro-oxidant phenotype in healthy monocytes and neutrophils compared to synthetic IgG *in vitro* [207], and platelet activation in RA patients [208].

Despite the evidence for inflammatory-driven vascular dysfunction in RA, measures of vascular function have not been conclusively correlated with clinical parameters of inflammation (reviewed in [185, 186]). For example, of the seven studies showing reduced FMD in patients with RA mentioned above, four report no correlation of FMD with CRP, ESR or DAS28 [190, 193, 194, 196], while three demonstrate a relationship of FMD with CRP [191, 192] and/or ESR, DAS28 and disease duration [195]. It is important to note that these studies in general rely on small sample sizes ($n < 100$), which may limit their power to accurately detect these relationships. Further work is required to address these questions at a molecular level and

in larger clinical studies measuring vascular function and inflammation using different tests and parameters.

1.3.4 Anti-inflammatory therapy for cardiovascular disease

The unequivocal relationship between atherogenesis and inflammation has triggered the question whether anti-inflammatory therapy is capable of reducing CVD outcomes in the general population, as seen in patients with RA [184]. Studies evaluating different anti-inflammatory strategies in varying patient populations have so far achieved mixed results. Inclacumab, a monoclonal antibody against P-selectin, reduced myocardial damage compared to placebo in 544 patients undergoing PCI after non-ST-segment elevation MI in the SELECT-ACS trial [209, 210] but long-term endpoints were not assessed. The LATITUDE trial showed that losmapimod, a p38 MAPK inhibitor, did not reduce recurrent CVD events compared to placebo in 3503 patients after acute MI followed over 6 months [211]. The CIRT trial evaluated methotrexate for the secondary prevention of CVD events in patients with pre-existing CAD [212] (discussed in section 1.4.2). The CANTOS study was the first large-scale clinical trial to demonstrate the proof of principle for anti-inflammatory therapy in CVD. Treatment with canakinumab, a monoclonal antibody against IL1 β , was associated with a 15% reduced risk for recurrent CVD events compared to placebo in 10061 patients with previous MI and elevated CRP levels [213]. The LoDoCo [214] and COLCOT trials [215] evaluated the use of low-dose colchicine, which blocks the NLRP3-inflammasome, in patients with stable CAD or recent MI respectively, showing that the drug reduced recurrent CVD events in both patient populations. These studies imply that the NLRP3-inflammasome-IL1 β pathway may be a valuable target in CVD. Importantly, many open questions remain, relating to the identification of patients who are most likely to benefit from anti-inflammatory therapy (stable versus acute disease, inflammatory parameters), timepoint of anti-inflammatory intervention (primary versus secondary prevention) and which inflammatory pathways to target.

1.4 Methotrexate

Methotrexate (MTX) is a synthetic structural folate analogue that inhibits cellular folate metabolism. The drug is used in the treatment of multiple malignancies, chronic systemic inflammatory diseases and in the termination of ectopic pregnancies.

1.4.1 MTX in the treatment of RA

Following the first use of the anti-folate drug aminopterin in the treatment of childhood leukaemia in 1948 [216], MTX was developed as a compound that is easier to synthesise than aminopterin [217]. Although designed as anti-cancer drug, MTX was found to have anti-inflammatory effects in patients with RA and psoriatic arthritis in the 1960-70s [218, 219]. Randomised placebo-controlled trials followed and confirmed the efficacy of low-dose MTX in patients with RA [220-223]. MTX is a conventional synthetic DMARD that reduces symptoms and disease progression, shown to lower the number of tender and swollen joints, joint damage, radiographic progression, acute phase response and to improve physical function and quality of life in patients with RA in a meta-analysis [224]. MTX is still part of the first line treatment for RA, given either as monotherapy or in combination with other DMARD in a long-term, low-dose regimen [164]. Despite the advent of novel targeted molecular therapies including TNF α , IL6 and JAK pathway inhibitors, MTX remains an important cornerstone in the treatment of RA due to its low cost and the fact that combination therapy of MTX with these agents is generally superior to monotherapy [164], as shown for TNF α inhibitors [225, 226]. Patients taking MTX also receive folic acid (up to 1-5mg daily) or folinic acid (<7mg weekly) to reduce the drug's side effects and increase drug adherence [227]. The toxicity of MTX is usually well managed. The most common adverse effects of MTX are gastrointestinal symptoms (nausea, diarrhoea, oral ulcers), non-specific symptoms (headache, fatigue), transient elevations of liver enzymes, fever and haematological abnormalities including neutropenia. Severe adverse effects are rare but include hepatotoxicity, myelosuppression, pulmonary toxicity, risk of infection and nephrotoxicity [158]. Contraindications to MTX include pregnancy, alcohol consumption, immunodeficiency syndromes and acute renal failure. The response rate to MTX is about 40-60% in RA patients [228, 229], with current studies aiming to find biomarkers to predict response/non-response to the drug [230].

1.4.2 MTX and its role in atherosclerosis

MTX has gained considerable attention in the rheumatologic and cardiovascular community on account of its beneficial cardiovascular effects in patients with RA. In a prospective study of 1240 RA patients, MTX use was reported to correlate with a 70% reduced mortality from CVD [231]. The QUEST-RA study showed that MTX use was associated with a 15% decreased risk for overall CVD events in patients with RA, with a 18% reduced risk for MI and 11% for stroke [232]. In a meta-analysis of observational studies including patients with RA, psoriasis or polyarthritis, MTX was associated with a 21% decreased risk for CVD events [233]. Similar associations were found in a consecutive meta-analysis [234]. Triple therapy of MTX, sulfasalazine and HCQ was shown to reduce cIMT and improve FMD in patients with early RA [202]. In contrast, the CORRONA study did not find any effect of MTX on CVD outcomes in 10156 RA patients [235], while an early report suggested that MTX use was associated with increased CVD mortality in 623 patients with RA [236].

The proposed vasculoprotective effect of MTX is thought to be mediated by the reduction of the overall inflammatory burden and not by amelioration of traditional CVD risk factors [237]. However, this contention needs to be investigated further as there are conflicting studies on the matter. There are reports suggesting that MTX use (alone or in combination with other DMARD) does not affect blood pressure or lipid levels [238], but other studies conclude that MTX reduces blood pressure [239] and increases lipid levels [240] in patients with RA, either compared to those not receiving MTX (but prescribed other treatments) or at follow-up. A study in 387 RA patients showed that MTX use correlated with an approximately 50% reduced chance of having metabolic syndrome, associated with reduced fasting glucose levels, triglyceride levels and higher HDL levels but not with waist circumference, blood pressure or insulin resistance [241]. Yet, it is well known that inflammation can influence some parameters measured in metabolic syndrome and it may be difficult to disentangle a causal relationship. Although it was proposed that MTX may have detrimental CVD effects by increasing circulating homocysteine levels (section 1.5.2.4.4), this is not clinically relevant as supplementation with folic acid reverses this effect [242].

The concept that MTX may have anti-atherogenic actions is also evident in animal studies. Acute MTX treatment decreases infarct size after LAD occlusion in a canine MI model in an adenosine-dependent manner [243]. In a rabbit model of atherosclerosis, MTX decreases the size of the atherosclerotic lesions, intima-media ratio and macrophage migration into the intima

[244]. This may be supported by the inhibition of foam cell formation as MTX increases the expression of the cholesterol 27-hydroxylase and ABCA1 transporter in THP-1 monocytes, both involved in reverse cholesterol transport [245]. MTX treatment decreases intramyocardial vasculopathy and end-organ damage in a mouse model of SLE, suggesting that MTX may limit inflammation-mediated vascular injury [246].

These observations led to the hypothesis that anti-inflammatory therapy with MTX may also reduce CVD risk in the general population. The Cardiovascular Inflammation Reduction Trial (CIRT) tested if low-dose MTX (15-20mg/week) reduced recurrent CVD events in 4786 patients with previous MI or multivessel coronary disease and diabetes or metabolic syndrome compared to placebo [212]. MTX did not affect CVD outcomes or reduce inflammatory markers (IL6, IL1 β , CRP) in this study population, although these were also not elevated at baseline. This may suggest that CVD in the general population and in patients with RA is different with regards to e.g. inflammatory impact or relevant disease pathways. In addition, the time point of MTX treatment may be critical (primary versus secondary prevention). Taken together, while the literature supports the notion that MTX reduces CVD morbidity and mortality in patients with RA, it is not completely understood how MTX achieves its anti-inflammatory and ultimately atheroprotective effects at the molecular level.

1.4.3 Pharmacokinetics of MTX

MTX can be administered by oral, subcutaneous, intramuscular, intravenous or intrathecal routes, with the latter two being reserved for cancer therapy. Standard doses of MTX in the treatment of RA are 7.5-25mg per week, compared to the high doses given in cancer therapy over days (>500mg/m²). The onset of action of MTX is slow, becoming apparent approximately one month after treatment initiation in patients with RA [247]. Oral administration of MTX shows a large variation (50-80%) in bioavailability [248]. Due to the structural similarities of MTX and folic acid (Figure 4), the drug utilises the same absorption and excretion route as folates (described in section 1.5.2). Briefly, MTX is taken up in the intestine by active transport via the proton-coupled folate transporter (PCFT; SLC46A1) following oral administration and is consequently delivered to the liver and systemic tissues. MTX has a short plasma half-life (about 8h) and 30-70% of the drug is bound to serum albumin [249]. MTX is excreted in the urine, a small fraction as metabolites 7-hydroxyMTX (7-OH MTX) [250] or 2,4-diamino-N¹⁰-methylpteroic acid (DAMPA) [251]. Patients with renal

impairments must be monitored for MTX toxicity and should receive a lower dose. MTX is taken up into target cells predominantly via the reduced folate carrier (RFC; SLC19A1) [252]. Folate transporters (FR α , β and δ) can also facilitate MTX transport, their expression being cell-type specific [253]. Intracellularly, MTX is subjected to polyglutamation by the enzyme folylpolyglutamate synthetase (FPGS), increasing its affinity to folate-dependent enzymes and its cellular retention [252]. MTX is exported from the cell by the ATP-binding cassette (ABC) proteins ABCC1–ABCC5 and ABCG2 [254].

1.4.4 Mechanism of action of MTX

The mechanism of action of MTX in the treatment of RA is still incompletely understood. Over the past decades, multiple drug effects have been described (Figure 3).

Inhibition of folate metabolism

Based on its action in cancer cells, inhibition of folate or one carbon metabolism (OCM, introduced in section 1.5) has been considered as mechanism underlying the anti-inflammatory effects of MTX. Polyglutamated MTX inhibits the folate-dependent enzymes dihydrofolate reductase (DHFR) [255] and thymidylate synthetase (TYMS) [256], which are required for nucleotide synthesis, DNA replication and cellular proliferation (Figure 3). Therefore, MTX may exert anti-inflammatory actions by inhibiting the proliferation of immune cells. This is supported by studies showing that MTX reduces the cellular proliferation of T-cells, B-cells [257] and monocytes [258] and induces apoptosis in activated T-cells by DHFR inhibition [259]. MTX inhibits the proliferation of activated, but not resting, T-cells by limiting purine synthesis, which can be rescued with hypoxanthine [260]. However, the anti-inflammatory effects of MTX are not entirely explained by folate-dependent mechanisms because folic acid supplementation does not reduce the efficacy of the drug despite ameliorating its toxic effects in patients [230], suggesting that other mechanisms are in place.

Adenosine release

One of the best-established anti-inflammatory mechanisms of MTX relates to its ability to promote adenosine release from different cell types. MTX inhibits the enzyme 5-Aminoimidazole 4-Carboxamide Ribonucleotide Formyltransferase/IMP Cyclohydrolase (ATIC) [261] that is part of the *de novo* purine synthesis pathway [262] (Figure 3). Inhibition of ATIC leads to increased levels of its substrate AICAR. This metabolite inhibits the enzymes

adenosine deaminase (ADA) and AMP deaminase (AMPDA) [261], increasing the levels of adenine nucleotides which are in turn converted by the ectonucleoside triphosphate dephosphorylase 1 (CD39) and ecto-5'-nucleotidase (CD73) to adenosine. Adenosine is a molecule with pleiotropic functions that plays an important role in inflammation and its resolution [263]. MTX has been shown to increase adenosine release *in vitro* and *in vivo*, resulting in reduced neutrophil adherence to EC or fibroblasts and reduced leukocyte accumulation in a murine air pouch model of inflammation [264, 265]. This effect is dependent on adenosine as MTX did not reduce inflammation in mice deficient in the adenosine receptor ADORA2A or CD73 [266, 267]. Additionally, MTX limits the formation of neutralising anti-drug antibodies against TNF α inhibitors in both an adenosine- and B-cell activating factor of the TNF family (BAFF)-dependent manner in mice [268]. Further supporting this concept, MTX increases the excretion of AICA, a metabolite of AICAR, in the urine of patients with psoriasis, indicative ofATIC inhibition [269]. Importantly, MTX-induced adenosine release may also confer other effects based on receptor subtype binding and afflicted cell type. There are four different adenosine receptors, ADORA1, ADORA2A, ADORA2B and ADORA3, which show differential expression patterns and mediate different effects of adenosine in various cell types [263]. It has been suggested that adenosine may also mediate some of the adverse effects of MTX, for example hepatic fibrosis and tiredness [230].

Interference with methylation reactions

Inhibition of DHFR by MTX may affect cellular methyl reactions by interfering with the recycling of homocysteine to methionine and reducing the production of the central methyl-donor S-adenosylmethionine (SAM) (Figure 5). MTX also reduces the expression and activity of the enzyme methionine S-adenosyltransferase (MAT) that catalyses the formation of SAM [270]. SAM is required for Wnt-signalling induced endocytosis of extracellular proteins and lysosome activity, which is inhibited by MTX in cancer cells [271]. Furthermore, MTX treatment reduces the methylation of the G-protein Ras and inhibits downstream signalling pathways affecting cancer cell proliferation *in vitro* [272]. However, contrary to this, it has been shown that patients with treatment-naïve RA display a global DNA hypomethylation pattern in T-cells and monocytes, which was reversed with MTX treatment [273]. This mechanism and its relevance need to be investigated further.

Pro-inflammatory cytokine and adhesion molecule expression

Low-dose MTX treatment can interfere with pro-inflammatory cytokine production and adhesion molecule expression (see section 1.4.5), supporting the concept that MTX inhibits inflammatory cell influx into inflamed sites by reducing immune cell activation and trafficking [274]. For example, MTX reduces the expression of IL1 β , TNF α , VCAM1 and E-selectin and inflammatory cell infiltration in synovial samples from RA patients [275]. Although the mechanism underlying this anti-inflammatory effect is not clear, MTX has been shown to inhibit NF κ B signalling in different cell types including T-cells and macrophages [276-278].

ROS production

MTX induces the generation of ROS in monocytes [258] and T-cells [279], which activates JNK signalling and primes T-cells for apoptosis. The latter report showed that this effect was rescued by BH₄ supplementation. This suggests that MTX inhibits the DHFR-dependent BH₄ salvage pathway (Figure 3) and induces nitric oxide synthase uncoupling, generating ROS.

Other mechanisms

Recently, MTX has been shown to inhibit JAK/STAT signalling in *Drosophila* cells and cancer cell lines [280]. MTX can bind to the high-mobility group box 1 (HMGB1) protein and inhibit its interaction with the receptor for advanced glycation end products (RAGE), limiting HMGB1-mediated TNF α release in RAW 264.7 cells [281]. The relevance of these mechanisms remains to be established in patients with RA.

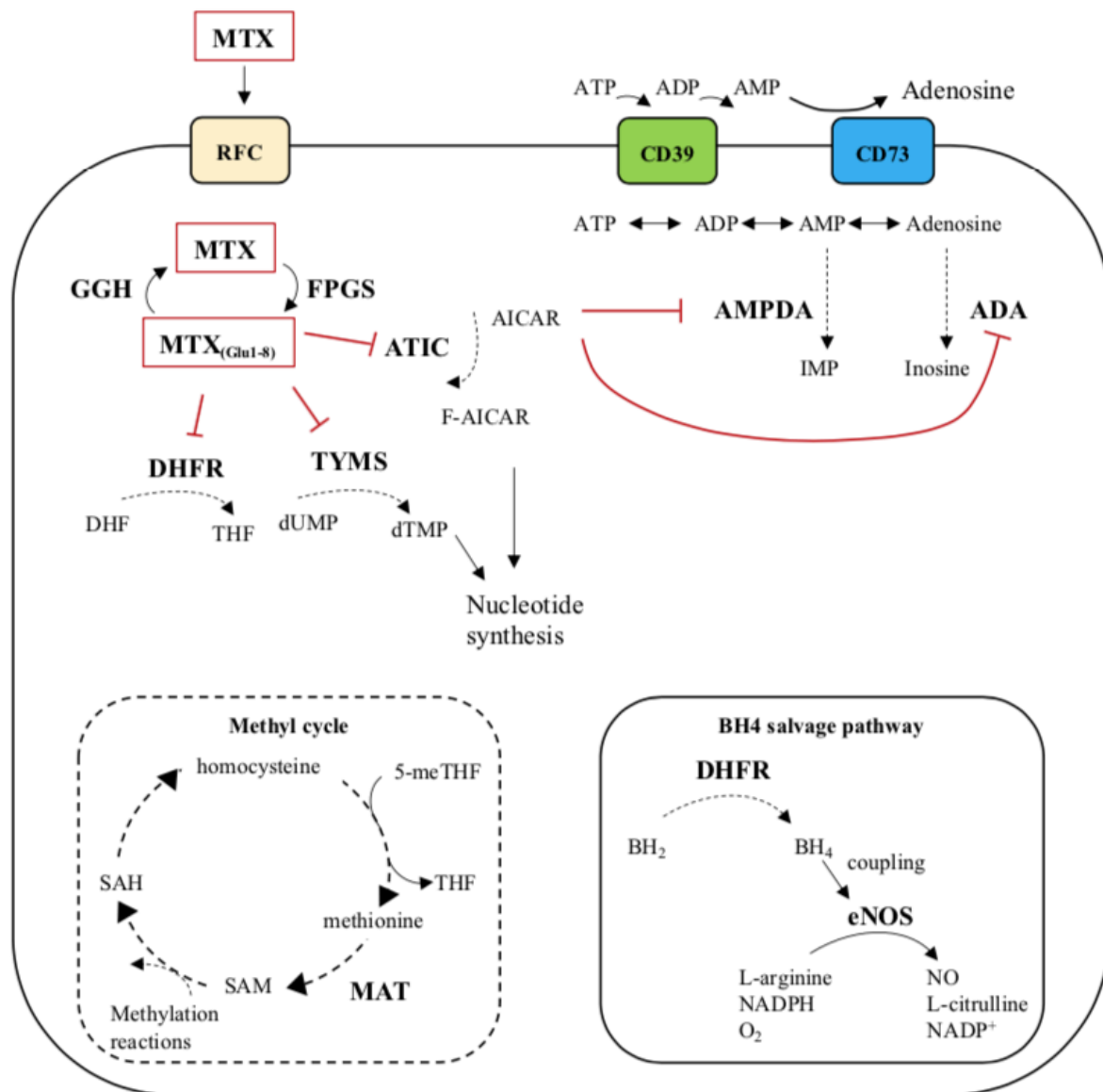


Figure 3. Mechanism of action of MTX. Illustration adapted from Cronstein et al [230]. ADA, adenosine deaminase. ADP, adenosine diphosphate. AICAR, 5-Aminoimidazole-4-Carboxamide Ribonucleotide. AMP, adenosine monophosphate. AMPDA, AMP deaminase. ATIC, 5-Aminoimidazole-4-Carboxamide Ribonucleotide Formyltransferase + IMP Cyclohydrolase. ATP, adenosine triphosphate. BH₂, dihydrobiopterin. BH₄, tetrahydrobiopterin. CD39, ectonucleoside triphosphate dephosphorylase 1. CD73, ecto-5'-nucleotidase. DHF, dihydrofolate. DHFR, Dihydrofolate Reductase. dTMP, deoxythymidine monophosphate. dUMP, deoxyuridine monophosphate. F-AICAR, formyl-AICAR. FPGS, Folylpolylglutamate Synthase. GGH, Gamma-Glutamyl Hydrolase. IMP, inosine monophosphate. MAT, methionine adenosyltransferase. NO, nitric oxide. RFC, reduced folate carrier (SLC19A1). SAH, S-adenosyl-homocysteine. SAM, S-adenosyl-methionine. THF, tetrahydrofolate. TYMS, Thymidylate Synthetase. 5-meTHF, 5-methyl THF.

1.4.5 Effects of MTX on cell types involved in atherosclerosis

MTX most likely achieves its immunomodulatory effects via interference with multiple cellular processes in a variety of cell types and this may also apply to its proposed anti-atherogenic effects. The reported molecular effects of MTX on cell types involved in atherogenesis, including EC, VSMC, macrophages and T-cells (section 1.1.1), are outlined below.

T-cells

MTX has anti-proliferative effects in activated T-cells and inhibits T-cell activation, as outlined above. MTX inhibits the production of the cytokines IL4, IL13, IFN γ and TNF α after T-cell activation in whole blood cultures [282] and decreases the expression of the adhesion molecules cutaneous lymphocyte-associated antigen (CLA) and ICAM1 in activated T-cells *in vitro* [283]. Supporting this, MTX treatment decreases the percentage of TNF α -positive CD4⁺ T-cells and increases IL10-positive T-cells in patients with early RA [284]. MTX induces apoptotic priming of T-cells by increasing ROS, activating JNK and increasing the expression of p53 and p21 via BH₄-depletion [279, 285]. MTX inhibits NF κ B activation in T-cells in a BH₄- and folate-dependent manner [277] and by inducing the expression of the long non-coding RNA lincRNA-p21 [286].

Monocyte/Macrophages

The reports on the molecular effects of MTX on monocytes/macrophages have described multiple, sometimes contradicting, mechanisms. MTX causes growth inhibition, ROS release and the production of the pro-inflammatory cytokines IL1 and IL6 in U937 monocytes [258, 287]. Furthermore, MTX was shown to induce pro-inflammatory gene expression in GM-CSF-conditioned human macrophages via TYMS inhibition [288], which was argued to induce tolerance to further pro-inflammatory activation by inhibiting cytokine-induced cytokine production and NF κ B signalling, and by inducing A20 expression [278]. M-CSF-conditioned human macrophages that express high levels of the folate receptor (FR)- β inhibit T-cell activation via adenosine signalling upon pro-inflammatory stimulation [289]; a mechanism which can be promoted by MTX. Interestingly, MTX inhibits cellular proliferation in undifferentiated monocytes but not synovial macrophages [290]. These observations suggest that MTX may differentially affect monocytes and macrophages based on their polarisation status [291]. As mentioned above, MTX may inhibit foam cell formation and macrophage migration during atherosclerosis.

VSMC

There is only one report on the effects of MTX on VSMC, which shows that MTX does not inhibit VSMC proliferation *in vitro* but that MTX-coated stents reduce neointimal hyperplasia in porcine coronary arteries [292].

Vascular EC

It has been proposed that MTX has protective effects on EC because the drug improves endothelial function (measured as FMD or reactive hyperaemic index) in patients with inflammatory arthritis, either alone [203] or in combination with other DMARD [202]. Yet, there is limited and often contradicting literature on the molecular actions of MTX in the vasculature. MTX has been reported to inhibit cell proliferation [293-295] and induce apoptosis in EC [296]. The drug inhibited angiogenesis in a rabbit model of neovascularisation [295] but had no effect on angiogenesis in a murine collagen-induced arthritis (CIA) model using Matrigel depots or in a placenta angiogenesis assay [297]. MTX activates the AMPK and CREB signalling pathways to mediate the induction of anti-oxidant, anti-inflammatory and anti-apoptotic molecules in EC *in vitro* [246]. MTX increases adenosine release in EC *in vitro* [264], reduces the TNF α -induced expression of VCAM1 and ICAM1 on HUVEC [293] and attenuates inflammatory cell recruitment to the vessel in different *in vivo* models of vascular injury [244, 246]. However, MTX induces the expression of the pro-inflammatory mediators complement component C3 and IL8 in an endothelial epithelial hybrid cell line (EAhy 926) [298]. In line with inhibiting the DHFR-driven recycling of BH₄, MTX has been shown to decrease NO production and also prevent folic acid-induced increase in NO production in bovine aortic EC *in vitro* [299]. Furthermore, MTX decreases the intracellular levels of BH₄ and causes eNOS uncoupling in a murine EC line [300]. These observations suggest that MTX may induce EC dysfunction by decreasing NO bioavailability. In a rat arthritis model, MTX did not show any effect on endothelial function as measured by acetylcholine-induced relaxation of aortic rings [301]. These studies clearly emphasise the need to better understand the effects of MTX on vascular EC in the context of atherogenesis.

Due to the importance of folate metabolism for MTX pharmacokinetics and speculatively function, the next chapter outlines the basic principles of OCM in health and disease, with a special focus on endothelial OCM.

1.5 One carbon metabolism

1.5.1 Introduction to EC metabolism

EC metabolism has been at the centre of interest in the vascular biology field in the past years as it has been demonstrated that EC actively regulate metabolic pathways according to their functional state [302]. Dysregulation of metabolic pathways has been described in various vascular pathologies including atherosclerosis [303]. This section features a brief introduction into the key concepts of EC metabolism.

EC generate most of their energy by glycolysis, in contrast to other cell types that rely predominantly on mitochondrial oxidative phosphorylation for ATP production [304]. This may facilitate efficient oxygen supply to the surrounding tissues, enable angiogenesis in hypoxic conditions and decrease oxidative stress in EC [304]. Glycolysis is required for EC proliferation and angiogenesis [304]. EC have fewer mitochondria than other cell types (percent of cytosolic volume in rat EC 2-5% [305] versus cardiomyocytes 32% [306]) and only show a small rate of glucose, fatty acid or glutamine oxidation [304]. Based on these observations, it is thought that mitochondria are not required for endothelial energy production but rather for cell signalling, redox homeostasis, biomass synthesis and detoxification of waste products [302]. In line with this idea, it was recently shown that the mitochondrial respiratory chain complex III promotes EC proliferation by maintaining NAD levels and macromolecule synthesis [307]. Likewise, fatty acid oxidation (FAO) does not affect ATP production but is utilised for nucleotide synthesis in proliferating EC [308]. Fatty acid metabolism is also involved in the regulation of endothelial permeability, migration, differentiation, lipid membrane composition and lipid transfer to other cell types [302]. The role of amino acid metabolism in EC has been to date best explored for glutamine, arginine and serine. Glutamine is required for macromolecule synthesis, replenishment of the tricarboxylic acid (TCA) cycle and redox homeostasis, supporting EC proliferation and angiogenesis [309]. Arginine is a critical substrate for endothelial NO synthesis [310]. Serine is used for nucleotide and heme synthesis in EC, needed for redox homeostasis, cell proliferation and angiogenesis [311].

Most studies have investigated the regulation of endothelial metabolism in cells grown in static culture, which models a relatively proliferative state when compared to EC *in vivo*. Recent reports have demonstrated that endothelial quiescence is accompanied by metabolic adaptations: Quiescent EC (induced either by constitutive activation of the transcription factor forkhead box O (FOXO)-1 [312] or contact inhibition [313]) decrease mitochondrial

respiration and/or glycolysis compared to proliferating cells [312, 313]. Moreover, quiescent EC do not merely repress metabolic processes but can redirect the utilisation of distinct pathways, as shown for FAO which promotes nucleotide synthesis during proliferation [308] but maintains redox homeostasis in quiescent EC [313]. LSS is a potent inducer of EC quiescence and reduces glycolysis and mitochondrial content in EC [314]. In contrast, disturbed flow increases endothelial glycolysis but reduces mitochondrial respiration by activating HIF1 α [124, 133]. Of note, hypoxia similarly drives glycolysis [315] and inhibits the expression of key mitochondrial genes in EC [316]. Further studies are needed to understand the role of metabolic pathways in EC in different functional states and vascular beds and determine whether these can be clinically targeted for therapeutic benefit in vascular diseases.

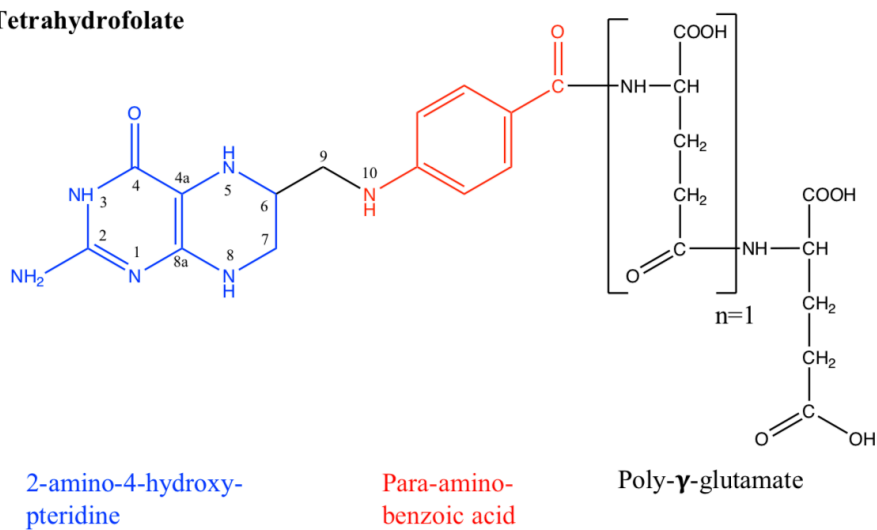
1.5.2 One carbon metabolism

OCM has received little attention in the vascular community in recent years and is poorly characterised in the vasculature. OCM describes the reactions that transfer and interconvert one carbon (1C) units on the backbone of the bioactive folate derivative tetrahydrofolate (THF) and take place in the cytosol, mitochondria and nucleus [317]. These folate intermediates supply different biosynthetic pathways, including nucleotide synthesis, amino acid metabolism, redox state, methylation reactions and mitochondrial protein synthesis. This section covers the principles of folate biology, biochemistry and its role in health and disease.

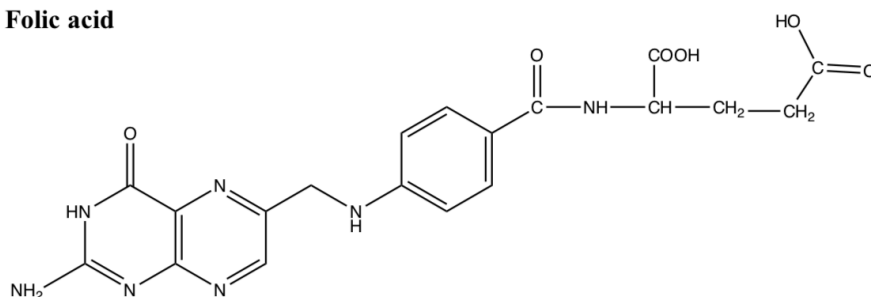
1.5.2.1 Folate nomenclature and dietary sources

Folates are water soluble B vitamins that share a common structure consisting of a 2-amino-4-hydroxy-pteridine ring, a para-aminobenzoic acid (PABA) moiety and variable number of glutamate residues (Figure 4) [318, 319]. Folates occur naturally in foods including green leafy vegetables, legumes, nuts, dairy products and meat. Vitamin supplements or fortified foods often contain synthetic folate compounds, mainly in the form of folic acid [319]. THF is a reduced form of folate that can accept 1C units in different oxidation states. The synthetic folic acid has a fully oxidised pteridine ring, only one glutamate residue [319] (Figure 4) and needs to be converted to THF prior to entering OCM. Folinic acid (leucovorin; 5-formylTHF) is a natural by-product of OCM that does not serve a biosynthetic function but is believed to represent a more stable folate intermediate used for cellular storage which can be reintroduced into OCM by methenyl-THF synthetase (MTHFS) (Figure 5A) [317, 318].

Tetrahydrofolate



Folic acid



Methotrexate

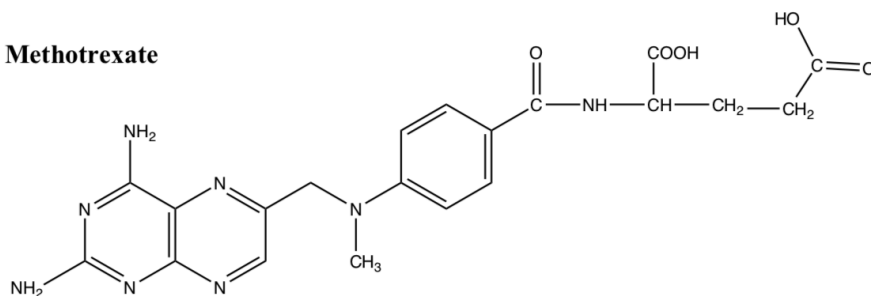


Figure 4. Chemical structures of tetrahydrofolate, folic acid and methotrexate. Illustration adapted from Zheng et al [318] and Visentin et al [252].

1.5.2.2 Folate uptake, transport, retention and excretion

The majority of natural folates are present in the diet in a reduced methylated form with polyglutamate chains of variable lengths [320]. Upon ingestion, the folate polyglutamate tail needs to be cleaved to a monoglutamate residue by the enzyme gamma glutamyl hydrolase (GGH) [321] in the proximal small intestine to facilitate epithelial uptake via the proton-coupled folate transporter (PCFT) [253]. PCFT actively transports reduced folates and folic acid in symport with protons into intestinal cells, optimally at an acidic pH [253]. Following intestinal absorption, folates enter the portal system and are transported to the liver. Conversion of folic acid to THF is mediated by a two-step reaction by the enzyme DHFR (folic acid-DHF-THF) (Figure 5A). It is not clear if this step occurs in the intestinal mucosa or in the liver [322]. Folates can be processed in the liver and released into the systemic circulation or bile, where they enter enterohepatic recirculation [322]. Folates are mostly reabsorbed in the proximal tubules of the kidney [253] and cleaved folate products in the form of para-acetamidobenzoylglutamate (pAcABG) and pterins can be excreted in the urine [318, 323].

5-methylTHF (5-meTHF) is the most abundant folate in plasma [322] and is taken up into tissues predominantly by the ubiquitously expressed reduced folate carrier (RFC) [253]. RFC transports reduced folates (with lower affinity for folic acid) in exchange with organic anions at neutral pH [253]. Folates can also be taken up peripherally by receptor-mediated endocytosis by the folate receptors FR α , β and δ , which show differential expression patterns in various cell types [253]. Of note, FR α and β are absent in EC [324, 325]. Inside the cell, the enzyme folylpolyglutamate synthase (FPGS) adds glutamate residues to folates in a sequential manner (n=1-8) [326]. Polyglutamated folates have higher affinities for folate-dependent enzymes [327], are believed to be poor substrates for folate transporters [328] and maintain intracellular folate levels [329]. FPGS depicts slower binding rates for 5-meTHF and folic acid compared to other reduced folates [326], indicating that 5-meTHF needs to be converted to THF by the enzyme methionine synthase (MTR) prior to its polyglutamation [318, 330]. Folate transport into the mitochondria is conferred by the mitochondrial folate transporter (MFT; SLC25A32) [331]. Folate polyglutamation occurs separately in the cytosol and mitochondria [332], mediated by mitochondrial and cytosolic splice variants of the FPGS gene [333]. The enzyme GGH removes glutamate residues from polyglutamated folates, facilitating folate export out of the cell via ABC transporters ABCC1-5 and ABCG2 [253].

1.5.2.3 Cytosolic and mitochondrial OCM

OCM mainly takes place in the cytosol and mitochondria in parallel, interconnected biochemical pathways (Figure 5A), facilitated by separately encoded cytosolic and mitochondrial enzymes that catalyse (almost) identical biochemical reactions and produce most of the folate intermediates in both compartments. These enzymes attach 1C units to THF at the 5-position nitrogen atom of the pteridine ring and 10-position nitrogen of the PABA moiety (Figure 4) and interconvert them between methyl-, methylene-, methenyl- or formyl-groups [317]. Mitochondrial OCM is the main route of 1C production [334], which is channelled into the cytosol as formate to fuel biosynthetic reactions [335]. The direction of the metabolic flux is determined by the NAD(P)H gradient in the different compartments [317].

1.5.2.3.1 Mitochondrial OCM

Mitochondrial OCM is required for shuttling 1C units into the cytosol, mitochondrial protein translation, glycine synthesis and maintenance of redox homeostasis.

Serine and glycine metabolism

THF is shuttled into the mitochondria, where it is converted to 5,10-methyleneTHF (5,10-meTHF) by the enzyme serine hydroxymethyltransferase (SHMT)-2 using serine as 1C donor in a reversible reaction that also generates glycine [336, 337] (Figure 5A). Glycine is a substrate for the synthesis of proteins (collagen), heme, glutathione, creatine and functions as a neurotransmitter [338]. The glycine cleavage system (GCS) is a multi-protein unit that catalyses the conversion of glycine, THF and NAD into CO₂, NH₃, NADH and 5,10-meTHF [339].

Mitochondrial protein synthesis

5,10-meTHF is utilised for the production of deoxythymidine monophosphate (dTMP) by thymidylate synthase (TYMS) or as a methyl donor for the mitochondrial translation of respiratory chain enzymes [340]. In addition, 5,10-meTHF can be converted to 10-formylTHF in a two-step reaction catalysed by the bifunctional enzymes methylenetetrahydrofolate dehydrogenase (NADP⁺-dependent) 2, methenyltetrahydrofolate cyclohydrolase (MTHFD2) [341] or methylenetetrahydrofolate dehydrogenase (NADP⁺-dependent) 2 like (MTHFD2L) [342] via their dehydrogenase and cyclohydrolase domains (Figure 5A). The enzyme mitochondrial methionyl-tRNA formyltransferase (MTFMT) utilises 10-formylTHF for the N-

formylation of methionine tRNA^{Met} required for initiation of mitochondrial protein synthesis [343].

The existence of the two isozymes MTHFD2 and MTHFD2L may be explained by their differential expression patterns [344]. MTHFD2 has been shown to be expressed in embryos and transformed cancer cell lines but not adult tissues [345, 346], with a proposed special role in haematopoietic cells [317, 346]. In contrast, MTHFD2L expression gradually increases with embryonic development and is maintained in adult murine tissues [344]. However, a recent report found that MTHFD2 is widely expressed in human adult tissues using RNA sequencing datasets and at a higher level than MTHFD2L, with prominent expression in arterial samples [347]. Further work is required to understand the relative importance of these two isozymes.

Mitochondrial 1C shuttling into the cytosol and redox homeostasis

10-formylTHF can be converted to NADPH and CO₂ by the enzyme aldehyde dehydrogenase 1 family member L2 (ALDH1L2). For transport into the cytosol, 10-formylTHF needs to be converted to formate by the enzyme methylenetetrahydrofolate dehydrogenase (NADP⁺-dependent) 1 like (MTHFD1L) [348, 349] (Figure 5A).

1.5.2.3.2 Cytosolic OCM

The cytosolic compartment of OCM facilitates *de novo* purine synthesis, thymidylate synthesis, methionine metabolism and maintenance of methylation reactions.

Purine synthesis

Formate is shuttled from the mitochondria into the cytosol, where it is converted by the trifunctional cytosolic enzyme methylenetetrahydrofolate dehydrogenase, cyclohydrolase + formyltetrahydrofolate synthetase 1 (MTHFD1) [350]: first to 10-formylTHF by its formyltetrahydrofolate synthetase activity, then to 5,10-methenylTHF (5,10-me⁺THF) via its cyclohydrolase and finally to 5,10-meTHF via its dehydrogenase catalytic domains. Cytosolic 10-formylTHF is utilised for *de novo* purine synthesis, mediated by the enzymes ATIC and phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase (GART) [262]. Alternatively, this substrate can be removed by aldehyde dehydrogenase 1 family member L1 (ALDH1L1) to produce CO₂ and NADPH.

Thymidylate synthesis

5,10-meTHF can be used as a co-factor for the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) (and DHF) by TYMS [262]. DHF is then converted back to THF by DHFR in a NADPH-consuming reaction.

Serine and glycine metabolism

5,10-meTHF can be utilised to produce serine and THF by the cytosolic SHMT1 enzyme in a reversible reaction that consumes glycine [351].

Methylation reactions

5,10-meTHF can be irreversibly converted to 5-meTHF by the NADPH-dependent enzyme methylene tetrahydrofolate reductase (MTHFR) [352]. Methionine synthase (MTR) catalyses the vitamin B12-dependent reaction of homocysteine to methionine using 5-meTHF as a co-factor [353], now entering the methyl cycle as methionine (Figure 5B). Methionine and ATP are used to produce S-adenosylmethionine (SAM) by the enzyme methionine S-adenosyltransferase (MAT). SAM is one of the most important cellular methyl donors, required for methylation of DNA, proteins and lipids, for the transsulfuration pathway and polyamine synthesis [354]. S-adenosylhomocysteine (SAH) is formed from SAM after donation of its methyl group. SAH is converted to homocysteine and adenosine by the enzyme SAH hydrolase [354].

1.5.2.3.3 Nuclear OCM

Several OCM enzymes can enter the nucleus (Figure 5B). This has been shown for TYMS, DHFR, and SHMT1 following SUMOylation [355, 356] and SHMT2 α , a splice variant of SHMT2 [357], MTHFD1 [358] and MTHFD2 [359]. The exact role of the nuclear folate pathway remains to be determined.

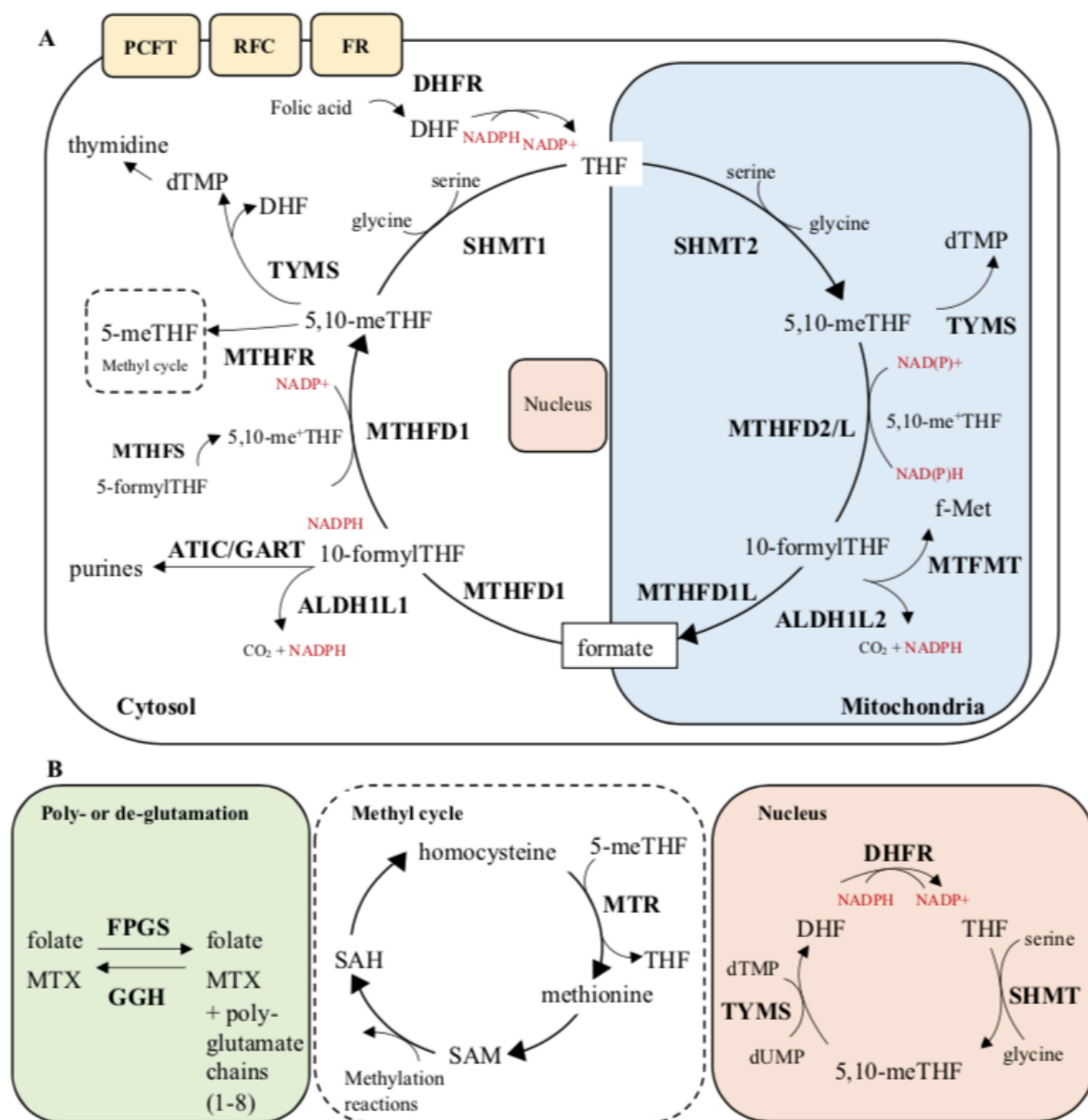


Figure 5. Schematic representation of mammalian OCM. (A) Illustration of mammalian cytosolic and mitochondrial OCM pathways. (B) Graphical representation of the biochemical processes involved in folate poly-(de)-glutamation, the methyl cycle and nuclear OCM. Illustrations adapted from Ducker [317], Tibbetts [335] and Leung et al. [360]. ALDH1L1, Aldehyde Dehydrogenase 1 Family Member L1. ALDH1L2, Aldehyde Dehydrogenase 1 Family Member L2. ATIC, 5-Aminoimidazole-4-Carboxamide Ribonucleotide Formyltransferase + IMP Cyclohydrolase. DHF, dihydrofolate. DHFR, Dihydrofolate Reductase. dTMP, deoxythymidine monophosphate. FPGS, Folylpolyglutamate Synthase. FR, folate receptor. GART, Phosphoribosylglycinamide Formyltransferase, Phosphoribosylglycinamide Synthetase, Phosphoribosylaminoimidazole Synthetase. GGH, Gamma-Glutamyl Hydrolase. MTFMT, Mitochondrial Methionyl-tRNA Formyltransferase. MTHFD1, Methylenetetrahydrofolate Dehydrogenase, Cyclohydrolase, Formyltetrahydrofolate Synthetase 1. MTHFD1L, Methylenetetrahydrofolate Dehydrogenase (NADP⁺ Dependent) 1 Like. MTHFD2, Methylenetetrahydrofolate Dehydrogenase (NADP⁺ Dependent) 2,

Methenyltetrahydrofolate Cyclohydrolase. MTHFD2L, Methylenetetrahydrofolate Dehydrogenase (NADP+ Dependent) 2 Like. MTHFR, Methylenetetrahydrofolate Reductase. MTHFS, Methenyltetrahydrofolate Synthetase. MTR, methionine synthase. PCFT, Proton-Coupled Folate Transporter (SLC46A1). RFC, reduced folate carrier (SLC19A1). SAH, S-adenosyl-homocysteine. SAM, S-adenosyl-methionine. SHMT1, Serine Hydroxymethyltransferase 1. SHMT2, Serine Hydroxymethyltransferase 2. THF, tetrahydrofolate. TYMS, Thymidylate Synthetase. 5-meTHF, 5-methylTHF. 5,10-meTHF, 5,10-methylene-THF. 5,10-me+THF, 5,10-methenyl-THF. 5-formyl-THF, folinic acid.

1.5.2.4 OCM in health and disease

The usage of OCM pathways and relative contribution to biosynthetic processes is different in various cell types and tissues and is thought to correlate with cellular metabolic demand [317], suggesting high tissue specificity of this metabolic pathway. In highly proliferating tissues, e.g. during embryonic development, OCM-mediated supply of nucleotides is critical for cellular replication. The role of OCM in non-proliferative adult tissues is less well understood. It has been suggested that OCM in the liver and kidney mainly facilitates amino acid metabolism, synthesis of choline and creatine and methylation reactions [317]. The importance of OCM in health and disease is illustrated by the presence of genetic variants in the OCM pathway, folate deficiency or biological states requiring increased OCM.

1.5.2.4.1 Genetic variants

Genetic variants or mutations in human OCM genes have been linked to haematological, vascular, neurological and developmental disorders. DHFR deficiency due to homozygous mutations in the corresponding gene is associated with megaloblastic anaemia and neurological abnormalities [361]. Individuals carrying the variant C677T in the MTHFR gene have higher circulating levels of homocysteine, which is variably associated with vascular diseases including CAD, stroke and hypertension, neurological diseases and cancer [362]. A variant in the MTHFD1 gene was associated with neural tube defects [363]. Variants in the MTHFD1L gene have been associated with CAD, neural tube defects and Alzheimer disease [364-367].

1.5.2.4.2 Folate deficiency

The clinical presentation of folate deficiency features neural tube defects during foetal development and macrocytic anaemia in the adult due to defective haematopoiesis [368]. Folate deficiency can be caused by decreased intake or absorption (alcoholism, eating disorders, malabsorption syndromes after bariatric surgery or inflammatory bowel diseases),

unmet increased demand (pregnancy, haemodialysis), medication (anti-folate drugs, anticonvulsants) or rare genetic disorders and is treated with folate supplementation [368].

1.5.2.4.3 Role of OCM in immunity and cancer

OCM has a long-established role in immunity and cancer as anti-folate drugs such as MTX have been used as anti-inflammatory and anti-proliferative agents for decades [317]. However, only recently have studies begun to understand the molecular mechanisms of OCM regulation and function in immune and cancer cells. Recent evidence shows that mitochondrial OCM is upregulated in activated T-cells [369] and LPS-induced macrophages [370]. OCM enzyme expression and flux are increased in various cancer types and cell lines. Overexpression of DHFR, TYMS, ATIC, MTHFD2, SHMT2 and MTHFD1L has been demonstrated in multiple human tumours [371, 372]. The transcription factors MYC [373], mTOR complex 1 (mTORC1)-induced ATF4 [374] and Nrf2 [372] regulate the expression of mitochondrial OCM enzymes in cancer cells. MTHFD2, SHMT2 and MTHFD1L facilitate cancer cell growth [371-373, 375], presumably due to their role in mitochondrial-driven nucleotide production for proliferation and/or protection against oxidative stress in cancer cells [372, 376]. Targeting mitochondrial OCM for cancer therapy is currently sought after but may be complicated by the fact that there is pathway compensation [334], multiple OCM isozymes and a dependency of growth restriction on the availability of metabolic factors such as for example serine in the extracellular milieu [317].

1.5.2.4.4 Role of OCM in CVD

The role of OCM in CVD is incompletely understood but multiple lines of evidence suggest a link between the two. Genetic variants of MTHFR can lead to increased blood levels of homocysteine and associate with vascular disease [362]. Increased levels of homocysteine have been associated with CVD. Homocysteine is an established independent risk factor for CVD including CAD and stroke in the general population [377, 378] and multiple studies have shown that homocysteine has detrimental effects on the vasculature, causing increased oxidative stress, inflammatory activation, thrombosis and eNOS dysfunction (reviewed in [379]). These observations suggested that reducing homocysteine with vitamin B supplementation would mitigate CVD risk and was addressed in three large clinical studies. The Vitamin Intervention for Stroke Prevention (VISP) trial investigated the effects of a high versus low dose regimen of folic acid (2.5mg vs 20µg), vitamin B6 (25mg vs 200µg) and B12

(0.4mg vs 6µg) on the risk of recurrent stroke, myocardial infarction (MI) or death in 3680 patients with previous ischaemic stroke over a follow-up period of two years [380]. The Norwegian Vitamin trial (NORVIT) examined the effect of vitamin B intervention (given as three different regimens: 1) folic acid 0.8mg, vitamin B12 0.4mg and vitamin B6 40mg; 2) folic acid 0.8mg and vitamin B12 0.4mg; or 3) vitamin B6 40mg) or placebo on the recurrence of MI, stroke or death in 3749 patients with acute MI, with a median follow-up period of 3.3 years [381]. The Heart Outcomes Prevention Evaluation (HOPE)-2 trial studied the composite endpoint of MI, stroke or death in 5522 patients with vascular disease or diabetes receiving either a combination of folic acid (2.5mg), vitamin B6 (50mg) and vitamin B12 (1mg) or placebo over five years [382]. None of these studies found any cardiovascular benefit of folate supplementation despite lowering homocysteine levels, questioning the causality of increased homocysteine levels in CVD [383]. This lack of effect may have been caused by 1) the timing of treatment, arguing that folate may have a beneficial effect in primary rather than secondary prevention, 2) the folate dose, speculating that folate food fortification programs may have already achieved maximum benefit or even 3) detrimental effects of folate due to the potential promotion of the methyl cycle and consequent epigenetic changes or folate-dependent increases in proliferation [379, 383]. Despite the abundant *in vitro* and patient data, the role of homocysteine and folate in CVD remain unsolved.

Beyond homocysteine, other variants and epigenetic modifications in OCM genes have been associated with CVD (summarised in Table 2). A recent paper showed that variants in genes compromising a network related to MTHFD2 function in EC (e.g. SHMT2, MARS, DDIT3, SORT1, CEBPB) were associated with CAD risk [384]. The same paper argued that there may be a link between glycine levels and MTHFD2 in atherosclerosis, as it has been previously shown that plasma glycine levels inversely associate with the risk of acute MI in patients undergoing coronary angiography [385]. Promoter hypermethylation in the MTHFD1 or SHMT1 genes have been detected in patients with essential hypertension [386, 387]. Although these reports are suggestive of the importance of OCM in the vasculature, more work is required to understand the role of OCM in atherogenesis.

Table 2. Association of OCM modifications with CVD

Gene	Variation	Function	Association	Reference
MTHFR	C677T	5-meTHF production Methyl cycle	Hypertension Stroke CAD	[362]
MTHFD1	Promoter hypermethylation	5,10-meTHF 10-formylTHF production	Hypertension	[386]
MTHFD1L	Variant	Formate production	CAD	[364, 365]
MTHFD2 Network	Variants in functional network	Amino acid metabolism	CAD	[384]
SHMT1	Promoter hypermethylation	Serine and glycine synthesis 5,10-meTHF + THF production	Hypertension	[387]

1.5.2.4.5 Endothelial OCM

The regulation and role of endothelial OCM is not well understood. Insights are provided from studies that treated EC or vessels with folic acid and homocysteine or depleted EC from folate or DHFR. OCM is thought to maintain BH₄ levels, eNOS coupling and NO bioavailability in EC. Folic acid promotes BH₄ and NO production by increasing DHFR expression and activity in bovine aortic EC [299]. Folic acid modulates eNOS activity by altering its phosphorylation [388] and can abrogate homocysteine-induced apoptosis in EC *in vitro* [389]. High dose folic acid (5mg daily) improves FMD in patients with CAD independent of homocysteine lowering [390, 391]. 5-meTHF also promotes NO production in cultured EC [392] and increases endothelial-dependent vasorelaxation in vessels from patients with CAD, proposedly by inhibiting peroxynitrite-mediated BH₄ oxidation [393].

In contrast, EA.hy926 cells grown in media containing low folate levels were shown to accumulate in the S-phase of the cell cycle and display increased permeability and a pro-inflammatory phenotype compared to those subjected to high folate levels, suggesting that folate deficiency causes EC dysfunction [394, 395]. DHFR is critical to the BH₄ salvage pathway in EC as DHFR depletion reduces BH₄ and NO levels due to eNOS uncoupling [300, 396]. While homozygous deletion of DHFR is embryonic lethal, heterozygous DHFR knockout

mice show no overt abnormalities but challenge of these mice with angiotensin II induces hypertension and abdominal aortic aneurysms due to increased eNOS uncoupling and mitochondrial oxidative stress [397]. However, the importance of DHFR in the endothelial BH₄ salvage pathway has been challenged by studies showing that DHFR activity is low in human EC [398] and its expression is reduced by LSS [399]. While one may argue that the relevance of OCM in endothelial homeostasis may come into play under pathologically relevant conditions such as atherosclerosis, the clinical studies outlined above have not supported a protective role of folate supplementation in CVD.

There are very few studies that have investigated the function of other OCM enzymes in EC. A recent paper showed that MTHFD2 and related proteins (MTHFD2 network) can be induced in EC by oxidised phospholipids [384]. Loss of endothelial MTHFD2 inhibits EC proliferation, migration and angiogenesis, which is rescued with glycine. MTHFD2 expression is also increased in human atherosclerotic carotid plaques and in EC from partially ligated murine carotid arteries. The authors argued that oxidised phospholipids induce MTHFD2 to maintain endothelial ATP production during atherosclerosis [384]. In a second study, *de novo* serine synthesis (modelled by the depletion of its key enzyme, phosphoglycerate dehydrogenase (PHGDH)) is essential for angiogenesis and EC proliferation [311]. Mechanistically, the authors showed that serine is required for heme synthesis, necessary for maintaining endothelial mitochondrial respiration and redox homeostasis. Interestingly, PHGDH was also identified as a member of the MTHFD2 EC network in the first study described [384]. These observations suggest that these pathways are closely linked in EC and regulate angiogenesis, amino acid metabolism and mitochondrial homeostasis.

Depletion of the pyruvate kinase M2 (PKM2), an ATP producing enzyme at the last step of glycolysis, results in mitochondrial dysfunction, downregulation of OCM protein levels, decrease in SAM levels and hypomethylation of endogenous retroviral elements in EC [400]. This paper indirectly suggests that OCM-driven SAM synthesis is linked to glycolysis and may be relevant for innate immunity. Further studies are required to determine OCM flux, metabolic output and function in EC in different (patho)-physiological states. Understanding OCM in different vascular cell types will provide more insight into its proposed role in CVD.

1.6 Endothelial colony forming cells

Endothelial colony forming cells (ECFC) are putative endothelial progenitor cells that can be isolated from the mononuclear cell fraction of blood, express markers of mature endothelial cells and have the ability to clonally expand and participate in angiogenesis *in vitro* and *in vivo* [401]. This chapter reviews their history, characterisation, origin and potential as disease model, diagnostic and therapeutic tool.

1.6.1 Endothelial progenitor cells

New blood vessels can be formed by two processes, classically categorised as either vasculogenesis or angiogenesis. Vasculogenesis is the *de novo* formation of blood vessels from progenitor cells which occurs exclusively during embryonic development, while angiogenesis is defined as blood vessel formation from pre-existing mature EC that takes place throughout development and adulthood [402]. During vasculogenesis, blood vessels are generated by the aggregation of mesodermal cells that differentiate into haemangioblasts, forming so-called blood islands. Haemangioblasts are precursor cells for both haematopoietic stem cells (HSC) and angioblasts. The latter are endothelial progenitor cells (EPC) and differentiate into EC, which form the primary capillary plexus that will consequently develop into the vasculature via angiogenesis [402]. According to this model, EPC exist only during embryogenesis and have no role in vascular homeostasis later in life.

1.6.1.1 Myeloid angiogenic cells (formerly early EPC)

This paradigm was challenged by a seminal paper by Asahara et al. in 1997, who for the first time described the presence of putative EPC in human peripheral blood that could differentiate into EC-like cells *in vitro* and participate in what is now termed postnatal vasculogenesis *in vivo*. To identify circulating adult EPC, the authors used the markers CD34 and Flk1 (KDR; VEGFR2) because these are expressed on both angioblasts and HSC [403]. Of note, CD34 and Flk1 are also expressed by other cell types (Table 3). Putative EPC were isolated from the mononuclear cell fraction from peripheral blood by CD34⁺ or Flk1⁺ selection, cultivated *in vitro* and consequently shown to express endothelial markers CD31 and eNOS, to produce NO and locate to capillaries of ischaemic vessels in animal models of hindlimb ischaemia [403]. Based on the vast potential of this ground breaking observation, multiple studies followed which established the concept that EPC are mobilised from the bone marrow after vascular insult, migrate to the site of injury and facilitate vessel repair (reviewed in [404]). These reports

used different protocols to isolate and characterise EPC, focusing on their *in vitro* adhesion, growth, morphology, acetylated LDL uptake and cell surface marker expression (reviewed in [404, 405]). Of particular importance in the EPC field is a paper by Hill et al. [406], who established a colony formation assay to quantify EPC numbers (defined as colony forming units (CFU) Hill assay) that became commercially available. For this assay, PBMC were pre-plated on fibronectin coated dishes for 48h, after which the non-adherent cells were re-plated (to avoid contamination with other cell types) and evaluated for colony formation after seven days, which reportedly expressed CD31 and VEGFR2 [406]. The CFU assay was used to correlate EPC numbers with disease outcomes, e.g. showing that the levels of circulating EPC predicted future CVD events in patients with CAD [407]. However, it was consequently demonstrated that EPC generated using the CFU-Hill assay or similar protocols were not true EPC because they cannot differentiate into mature EC [408-410] or form blood vessels themselves *in vitro* [410] or *in vivo* and lack proliferative capacity [408]. Rather, they are of haematopoietic origin, retain the expression of monocyte/macrophage markers despite also featuring endothelial attributes and maintaining phagocytic activity and LDL uptake which macrophages are also capable of [408, 411, 412]. The cells analysed by these initial studies have now been named myeloid angiogenic cells (MAC) (Figure 6) [413]. Nevertheless, this body of work showed that MAC, despite not representing bona fide EPC, contribute to angiogenesis by paracrine signalling [409].

Table 3. Markers used for EPC characterisation

Marker	Function	Expression	Ref.
CD34	Transmembrane phosphoglycoprotein that can bind to L-selectin (CD62L); function not clear	Haematopoietic stem and progenitor cells, mesenchymal stromal cells, muscle satellite cells, keratocytes, interstitial cells, epithelial progenitor cells	[414]
CD133 or Prominin-1	Transmembrane glycoprotein Self-renewal, tumourigenesis	Haematopoietic stem cell, foetal brain stem cells, embryonic epithelium, prostatic epithelial stem cells, myogenic cells, cancer stem cells	[415, 416]
KDR, Flk1, VEGFR2	Vascular Endothelial Growth Factor Receptor 2; tyrosine protein kinase involved in VEGF signalling	EC, haematopoietic stem and progenitor cells, megakaryocytes, retinal progenitor cells	[417-420]
CD146	Cell adhesion molecule angiogenesis, vascular permeability, leukocyte transmigration	EC, smooth muscle cells, pericytes, T-cells, B-cells, NK cells	[421]
CD144	VE-cadherin Intercellular junction Vascular integrity	EC	[422]
CD31 or PECAM	Platelet/endothelial cell adhesion molecule 1; Intercellular junction leukocyte trafficking, mechanotransduction, angiogenesis	EC, granulocytes, monocytes and platelets	[423]
CD36	Pattern recognition receptor inflammation, thrombosis, atherosclerosis	Monocytes, macrophages, microvascular EC, adipocytes, platelets, cardiomyocytes	[424]
CD14	Pattern recognition receptor innate immune response	Monocytes, macrophages; non-myeloid cells including EC, epithelium, smooth muscle, pancreatic islet cells, fibroblasts and spermatozoa	[425]
CD45	Receptor protein tyrosine phosphatase immune cell activation	Leukocytes	[426]

1.6.1.2 ECFC

In 2000, Lin et al. described a second cell type with endothelial characteristics that could be similarly isolated from human peripheral blood by plating PBMC on collagen-coated plates. These cells appeared later in culture (after 2-4 weeks) and showed a more typical cobblestone morphology compared to MAC (round cells surrounded by spindle shaped cells) [427]. These cells were termed blood outgrowth EC (BOEC) and shown to express CD34, the endothelial markers CD146, VEGFR2, VE-cadherin, CD31, CD36 but not the monocyte marker CD14. Interestingly, the authors contrasted these cells with circulating mature EC termed circulating EC (CEC) that can be directly isolated from blood by CD146⁺ selection and thought to shed from mature vessels, concluding that these represent two independent cell types. The precursor cell giving rise to the blood outgrowth cells remained unidentified.

1.6.1.2.1 ECFC characterisation

As characterised by this initial study, BOEC or ECFC are defined by their late appearance in culture when cultivating the mononuclear cell fraction in endothelial growth medium, cobblestone morphology and expression of endothelial but importantly not haematopoietic surface markers [408] (Figure 6). The above-mentioned studies on MAC emphasised that there is no single surface marker signature that is specific to EPC and that the functional and molecular characterisation of these cells is very important [428]. ECFC have a high proliferative and colony forming potential in culture [429], a characteristic that supports their putative progenitor status. Underlining this notion further, ECFC were reported to undergo similar [430] or more [431] population doublings compared to HUVEC. In line with their endothelial lineage, ECFC can form tubes *in vitro* [410, 432] and participate in angiogenesis *in vivo* [408, 410]. In contrast, differentiated EC did not have angiogenic capacities in mouse models of hindlimb ischaemia [410, 433]. Hinting at the unique angiogenic potential of ECFC, a series of studies have reported their ability to contribute to vascular repair by aiding neovascularisation and improving tissue perfusion and function *in vivo* in animal models of hind limb ischaemia [434, 435], MI [436], stroke [437], traumatic brain injury [438] and ischaemic retinopathy [439]. Mechanistically, this is thought to be achieved by the direct incorporation of ECFC into the vessel wall, paracrine effects and direct interaction with mesenchymal stem cells [434-436, 440, 441].

ECFC have not been well characterised at a molecular level. Recent global gene expression analyses using RNA sequencing comparing adult ECFC to HUVEC and human coronary artery

EC showed that ECFC in general share a very similar endothelial transcriptional signature but express a mixture of markers classically associated with either arterial (HEY2), lymphatic (VEGFR3, LYVE1) and venous (NRP2) differentiation, suggesting that ECFC are not yet fully committed to a specific endothelial phenotype [442]. In line with this, ECFC cultures isolated from adult peripheral blood comprised a mixed pool of cells with characteristics of either blood or lymphatic EC based on the presence or absence of lymphatic markers VEGFR3, LYVE1 and prox-1 [443]. Other studies proposed that ECFC display microvascular characteristics based on their expression of CD36 [427] (Table 3) and specific homeobox (HOX) genes [444]. Due to the proposed heterogeneity within a given ECFC population to produce colonies with different proliferative potential, several reports investigated the phenotype and molecular signature of CD34⁺ and CD34⁻ ECFC [445-447]. However, these studies produced contradictory results on the proliferative, vasculogenic capacity and maturity of either cell population. A different study reported that low versus high aldehyde dehydrogenase (ALDH) activity in ECFC was associated with a higher proliferative capacity and reduced tissue necrosis in a murine ischaemia model [448]. Other reports demonstrated ECFC-specific expression of the endothelial protein Eglf7 [449], pro-angiogenic factor cytokine-like 1 [450] and CD133 (intracellularly) [451] and their involvement in the angiogenic capacity of these cells *in vitro* or *in vivo*.

1.6.1.2.2 ECFC origin

The origin and the precursor of ECFC have not been identified yet, partly due to the fact that there are no specific markers to detect these *in vivo*. However, it is now largely accepted that ECFC are derived from precursor cells that express CD34 but not CD45 or CD133 [452]; a rare population in cord blood [453]. Lin et al. argued that ECFC were bone-marrow derived, showing that ECFC mostly carried the donor genotype in patients receiving sex-mismatched bone-marrow transplants [427]. However, this was challenged by a consequent study reporting that ECFC could not be isolated from bone-marrow derived mononuclear cells [430]. Since the initial study by Lin et al, ECFC have been isolated from human cord blood [429], adipose tissue [454], lung [455] and placenta [456] (although two of these studies [454, 455] only used CD31 as selection marker), suggesting that ECFC may be derived from tissue-resident precursor cells. Ingram et al put forward the concept that vascular EC in culture display a mixture of cells with differential proliferative potential, similar to ECFC [457]. Based on this and other findings, it was suggested that ECFC may represent highly proliferating resident cells in the

vessel wall [458], although definite proof is lacking. Yet, there is also the possibility that ECFC do not exist *in vivo* but are cell populations only generated under *in vitro* culture conditions that favour endothelial outgrowth from PBMC. Although this question remains to be addressed, ECFC may represent powerful tools in vascular biology and regenerative medicine.

1.6.1.2.3 ECFC in vascular disease

On the basis that ECFC may represent bona fide EC that make up the vascular endothelium, patient-derived ECFC have been characterised in a variety of diseases featuring vascular impairment, including von Willebrand disease, pulmonary arterial hypertension (PAH), diabetes, sickle cell disease and chronic obstructive pulmonary disease (COPD) (reviewed in [401]). These studies have enabled valuable insight into disease-specific effects on endothelial function on a phenotypic and molecular level. This section reviews work undertaken with ECFC from patients with chronic systemic inflammatory diseases and CVD.

Chronic systemic inflammatory diseases

There are only three studies that evaluated ECFC numbers or phenotype in patients with chronic systemic inflammatory diseases. To date, two studies have been performed in treatment-experienced RA patients, showing that ECFC colony frequency was higher in patients [459] and that patient-derived ECFC were more proliferative, angiogenic and pro-inflammatory primed compared to controls [460]. This phenotype was dependent on sirtuin (SIRT)-1, a NAD-dependent deacetylase, and aligned with the features of synovial EC (section 6.5). In patients with systemic sclerosis, one study reported unchanged ECFC numbers compared to controls [461].

Cardiovascular disease (CVD)

In patients with acute MI, the frequency of ECFC colonies was increased compared to controls [462] and ECFC frequency at time of MI positively correlated with a reduction in infarct size at follow-up after 6 months [463]. In patients with established and significant CAD (defined as one or more coronary arteries with stenosis >70% evaluated by coronary angiography), ECFC frequency was higher when compared to those without significant stenosis [464]. As yet, ECFC phenotype has not been investigated in patients with acute MI. However, other studies have shown no major differences in ECFC colony frequency or phenotype in patients with stable ischaemic cardiomyopathy [465], stable CAD [466] or premature CAD [467, 468]

compared to controls. Of note, ECFC were not affected by premature CAD, although there were functional impairments in local vascular wall EC isolated from the same patients [467], challenging the concept that ECFC can represent the local vasculature in every disease setting. However, the local vascular EC may have been injured during sampling using a wire-loop in that study. It is interesting to speculate that ECFC may be mobilised in acute or more unstable CAD where vessel injury prevails when compared to stable CAD, and if so, that may offer a potential explanation for these observations.

1.6.1.2.4 Therapeutic avenues using ECFC

Beyond their use as an *ex vivo* endothelial disease model, ECFC have been explored for their utility and feasibility for transplantation into ischaemic tissues to improve vascular repair (as outlined above). In addition, ECFC are an attractive tool for tissue engineering and are being investigated for their ability to vascularise engineered scaffolds that can be used for example in wound healing. ECFC are also currently being explored for gene therapy in haemophilia A, von Willebrand disease and cancer (all reviewed in [401]). Based on their potential role in vascular repair and increased presence in states of acute vascular injury, ECFC may serve as a marker of vascular damage. An attractive concept would be to use ECFC frequency as a diagnostic or predictive tool in different disease setting, as exemplified by [463]. However, further studies are required to evaluate the robustness and feasibility of such an approach.

1.6.1.2.5 Current challenges in the ECFC field

Despite efforts to streamline the definition and reporting of ECFC [413, 469], it is still difficult to interpret and compare ECFC studies since different isolation protocols, surface markers and nomenclatures are still being used. It will be important to identify the origin and innate function of ECFC *in vivo* in order to understand the biology of EPC and their role in endothelial regeneration. Furthermore, this will also allow a better understanding of the implication of measuring ECFC frequency and function in patients. Key challenges currently limiting the introduction of ECFC into clinical studies include both biological and practical parameters, specifically the fact that ECFC cannot be isolated from some individuals while others yield limited cell numbers, a lack of markers to specifically detect ECFC or their precursor cells and their laborious isolation and culture protocols for which there is non-compliance with current good manufacturing practice (GMP) regulations for cell therapy [401]. Importantly however, several studies have aimed at improving ECFC detection, isolation efficiencies and their

proliferative/angiogenic capacities (reviewed in [470]). Following the confusion and setbacks of the initial MAC studies, it is important to standardise ECFC research to advance insight into EPC biology and their implementation into the clinic.

1.6.1.3 Resident vascular progenitor/stem cells

In parallel to work in the ECFC field, several reports have investigated whether resident endothelial progenitor or stem cells exist in the vascular wall using different approaches, including surface marker expression, wound healing assays, transcriptional profiling and lineage tracing. In line with the initial hypothesis of Ingram et al [457], Patel and colleagues described the existence of a hierarchy of progenitor to differentiated EC within the vessel wall, distinguishing three different cell types according to their cell surface expression of CD34, VE-Cadherin, CD31, VEGFR2, vasculogenic capacity and transcriptional signature [471]. The authors proposed that endovascular progenitors are quiescent cells that reside in the endothelium and can give rise to transient amplifying and differentiated EC during murine wound healing in a SOX-18 dependent manner. Similarly, another report showed that ischaemia-induced vascular injury was repaired in part by the clonal expansion of VE-Cadherin positive cells in different animal models [472]. Fang et al defined putative resident vascular stem cells using the markers CD31, endoglin, Sca1, c-Kit, showing that this cell population had a high proliferative potential and was capable of forming blood vessels in a Matrigel assay *in vivo* from a single cell [473]. Supporting this, Wakabayashi et al demonstrated the presence of CD157 expressing vascular stem cells resident in the vasculature of multiple organs, which were shown to clonally expand and contribute to vessel formation during homeostasis and after vascular injury in the murine liver [474]. Other putative vascular stem cell populations have been described (reviewed in [475]). Importantly, the presence of these EPC needs to be confirmed in humans. It is not clear if these studies refer to the same progenitor cell population due to the use of different surface markers. In some studies, it is difficult to differentiate between true EPC or simply activated, more proliferative mature EC. It would be interesting to investigate whether these resident EPC could potentially represent ECFC precursors. Further studies are needed to better characterise these cells, understand their innate biological function and utility in regenerative medicine.

Endothelial progenitor cells		
MAC	ECFC	Resident vascular progenitor cell
CD31+, CD14+, CD45+ CD146-, CD34-  • Myeloid phenotype • Phagocytosis • Contributes to angiogenesis in a paracrine manner • Hematopoietic origin	CD31+, CD146+ CD14-, CD45-  • EC phenotype • Clonal expansion • Tube formation <i>in vitro</i> • Vasculogenesis <i>in vivo</i> • Vascular repair • Origin in vessel wall?	No consensus markers  • Isolation from vessel EC • Clonal expansion • Vasculogenesis <i>in vivo</i> • Endothelial regeneration • Vessel wall

Figure 6. Cell types characterised in the search for endothelial progenitor cells. Illustration adapted from Medina et al. [413]. EC, endothelial cell; ECFC, endothelial colony forming cell; MAC, myeloid angiogenic cell.

1.7 Hypothesis and Aims

While there is currently no targeted treatment for CVD in patients with RA, the clinical demonstration that the anti-folate drug MTX reduces CVD events and mortality in RA suggests that the drug may confer anti-atherogenic actions. However, the mechanisms underlying this effect are poorly characterised. It has been proposed that the drug's beneficial vascular effects are mediated by improving endothelial function, but it is not clear if MTX has direct anti-inflammatory effects on the vascular endothelium.

The objective of this thesis was to investigate the effects of MTX on vascular EC in the context of atherogenesis, modelled by TNF α - and shear stress-induced EC activation *in vitro*. A particular focus of this thesis was to determine whether the drug activates protective cell signalling pathways in EC that may shield the cell from inflammatory and shear-stress induced insults. The drug's mechanism of action in EC was addressed by studying endothelial folate metabolism under static and shear stress conditions. Finally, the impact of inflammation and dual DMARD therapy upon EC was evaluated in ECFC isolated from patients with active RA before and after treatment initiation.

Insights into the direct and indirect endothelial effects of MTX will enable us to understand how the drug may achieve its beneficial vascular outcomes in patients with RA.

1.7.1 Hypothesis

MTX prevents or reduces EC dysfunction induced by pro-inflammatory stimuli or disturbed flow.

1.7.2 Aims of the study

- 1) To understand the effects of MTX on vascular EC grown under static conditions
- 2) To validate the effects of MTX on vascular EC subjected to shear stress
- 3) To characterise folate metabolism in EC
- 4) To determine the effects of systemic inflammation and DMARD therapy on the frequency and function of ECFC derived from patients with RA

2 Chapter 2 - Materials and Methods

2.1 Cell culture

2.1.1 Isolation of human umbilical vein endothelial cells (HUVEC)

Human umbilical vein endothelial cells (HUVEC) were isolated according to a previous protocol [476]. In brief, human umbilical veins were washed with Hank's balanced salt solution (HBSS; Sigma). HUVEC were harvested after incubation with 0.5mg/mL collagenase-A (Roche) for 15min at 37°C and 5% CO₂, followed by washing with HBSS. The cells were centrifuged at 300g for 8min at room temperature (RT) and resuspended in M199 growth medium (Table 4). The cells were seeded on 25cm² flasks (Sarstedt) pre-coated with 1% gelatin (Sigma) and grown at 37°C and 5% CO₂.

2.1.2 Cell propagation

HUVEC (either isolated in house or commercially sourced through Promocell) and human aortic endothelial cells (HAEC; Promocell) were cultured in 75cm² flasks (Sarstedt) pre-coated with 1% gelatin in M199 growth medium (Table 4) or Endothelial Growth Medium (EGM)-2 (Promocell) supplemented with 10% foetal calf serum (FCS; Labtech), EGM-2 SupplementPack (Promocell) and penicillin/streptomycin (100µg/mL; Sigma). The cells were passaged by washing with HBSS (without Ca²⁺ and Mg²⁺; Sigma) and incubating with 0.25% trypsin/1mM EDTA (Sigma) for 3min at 37°C. The cells were centrifuged at 300g for 5min at RT, resuspended and counted using a Cellometer Auto T4 Bright Field cell counter (Nexcelom Bioscience). Cells were seeded at 10 000 cells/cm² in 75cm² flasks. HUVEC and HAEC were used at passages 3-5 either as single donors or pools from multiple donors.

2.1.3 Cell freezing and thawing

For cryopreservation, cells were washed, harvested by trypsinisation and centrifuged at 300g for 5min. Cell pellets (0.75x10⁶ cells) were resuspended in FCS supplemented with 10% DMSO (Sigma) and stored in a freezing container allowing a cooling rate of 1°C/min (Mr. Frosty, Thermo Fisher) at -80°C for 48h. Cell stocks were then transferred to liquid nitrogen for long-term storage. Cell stocks were quickly thawed at 37°C and seeded into 1% gelatin-coated flasks in EGM-2 (10%FCS).

Table 4. M199 media formulations

Reagents and company	Growth Medium	Assay Medium
M199 medium (Sigma)	M199	M199
Foetal calf serum (FCS; Labtech)	20%	10%
L-glutamine (Sigma)	2mM	2mM
Penicillin/Streptomycin (Sigma)	100µg/mL	100µg/mL
Endothelial cell growth supplement (ECGS; Merck)	30µg/mL	15µg/mL
Heparin 1000 I.U./ml (Wockhardt UK)	10units/mL	10units/mL

2.1.4 Cell treatments

HUVEC and HAEC were seeded at a density of 1×10^5 cells per well in a 6-well plate (Nunc) in M199 assay medium (Table 4) and cultured overnight. Cells were treated with the pharmacologic agent or control in assay medium as indicated in Table 5.

Table 5. Pharmacologic agents

Reagents and company	Diluent	Concentration
Folinic acid (Sigma)	H ₂ O (HyClone, Endotoxin-free; GE Life Sciences)	500nM
Hydrogen peroxide (H ₂ O ₂ ; Sigma)	-	200µM
Methotrexate (MTX; Hospira UK)	Supplied in solution containing sodium chloride, sodium hydroxide, hydrochloric acid and water; Media as control	100nM
Tumour necrosis factor α (TNF α ; R&D Systems)	0.2%BSA/PBS (Sigma); Media as control	0.01–1ng/mL

2.1.5 Folate depletion experiments

To deplete folic acid, cells were grown in either RPMI (“high folate”; Gibco) or folic acid-free RPMI (“low folate”; Gibco) supplemented with 10%FCS, 100µg/mL Penicillin/Streptomycin and 15µg/mL ECGS. Folic acid levels in FCS and ECGS were determined using the ARCHITECT i2000SR analyser (Abbott) (kindly supported by John Morris, Department of Pathology, Hammersmith Hospital) and are depicted in Table 6. The final concentration of folic acid was 1.91nM and 2.3µM in the low and high folate RPMI respectively.

Table 6. Folic acid levels in FCS and endothelial growth factors

Folic Acid Levels		
	ng/ml	nM
FCS (Labtech)	8.4	19.0
ECGS (Merck)	0.8	1.8

2.1.6 siRNA transfection

HUVEC were seeded at a density of 2.5×10^5 cells per well in a 6-well plate in EGM-2 and cultured overnight. The cells were washed and fed with pre-warmed OptiMEM (Gibco). HUVEC were transfected with non-targeting control siRNA (sictrl) (siGENOME non-targeting siRNA, Dharmacon) or specific siRNA (Table 7) using the transfection reagent geneFECTOR (Venn Nova) according to the manufacturer's instructions. The siRNA and geneFECTOR were diluted in OptiMEM, incubated for 5min at RT and added to the cells for 6h at 37°C and 5% CO₂. The cells were recovered in EGM-2 supplemented with 2%FCS, EGM-2 Supplement Pack and penicillin/streptomycin (100µg/mL) media and harvested at the indicated timepoints for further experimental analyses.

Dr. Claire Peghaire (Vascular Sciences, Imperial College London) kindly provided cDNA of HUVEC transfected with control siRNA (Allstars Negative control siRNA; Qiagen) or siRNA against KLF2 (Table 7), which she prepared using the following protocol: HUVEC (2×10^5 cells per well) were seeded onto 1% gelatin coated 6-well plates in EGM-2 (Lonza) and incubated over night at 37°C and 5% CO₂. The next day, the cells were transfected with either control siRNA or siRNA directed against KLF2 using AtuFect01 lipid (final concentration, 1µg/mL; Silence Therapeutics) according to the manufacturer's instructions. AtuFect and siRNA were diluted in pre-warmed OptiMEM and incubated for 30min at 37°C. The cells were fed with EGM-2 and the transfection mix was added. The cells were harvested after 48h.

Table 7. siRNA sequences

Target Gene	siRNA	Target sequence	Conc.	Company
	siGENOME non-targeting siRNA pool #1	UAGCGACUAAACACAUCAA UAAGGCUAUGAAGAGAUAC AUGUAUUGGCCUGUAUUAG AUGAACGUGAAUUGCUCAA	20nM	Dharmacon
	AllStars negative control siRNA	Proprietary sequence	20nM	Qiagen
DHFR	Hs_DHFR_5 FlexiTube siRNA	AACCTCCACAAGGAGCTCATT	20nM	Qiagen
MTHFD1	Hs_MTHFD1_7 FlexiTube siRNA	CAGATCACCATCCATCTTCAA	20nM	Qiagen
SHMT1	Hs_SHMT1_7 FlexiTube siRNA	CTGACGGAGCTGGGCTACAA A	20nM	Qiagen
MTHFD2	Hs_MTHFD2_7 FlexiTube siRNA	TAGGATTATTCCTTGCTATTA	20nM	Qiagen
SHMT2	Hs_SHMT2_7 FlexiTube siRNA	CCGGGAGATCCCTTACACATT	20nM	Qiagen
KLF2	Hs_KLF2_7 FlexiTube siRNA	CACCTGGCGCTGCACATGAAA	20nM	Qiagen
NRP1	SMARTpool siRNA	CGAUAAAUGUGGCGAUACU GGACAGAGACUGCAAGUAU GUAUACGGUUGCAAGAUAA AAGACUGGAUCACCAUAAA	40nM	Dharmacon

2.1.7 Adenoviral transfection

Cells were seeded at a density of 1×10^5 cells per 6-well and left to reach 70% confluence. On the day of transfection, medium was changed to M199 base media and cells were infected with the virus at the desired multiplicity of infection (MOI = the ratio of infectious agent (number of infected particles) over infection targets (number of cells)). Calculation of MOI was performed as illustrated below:

- Total infectious units needed (ifu) = total cell number / well x desired MOI
- Amount of viral stock to get desired MOI (ml) = total ifu / ifu/ml (viral titre)

The cells were incubated at 37°C and 5% CO₂ for 2h, after which the medium was changed to assay medium. Eight hours post transfection, the cells were treated with media or MTX (100nM) for 48h, after which they were harvested for downstream analyses. The adenoviral (Ad) vector dominant-negative (DN)-AMPK α 1 (MOI 200) was a kind gift from Prof. David Carling (Imperial College London) [477]. The Ad0 vector was used as control (MOI 200) [478].

2.1.8 Plasmid transfection (Dr. Claire Peghaire)

HUVEC were seeded at 2×10^5 cells per well onto 6-well plates in EGM-2 (Lonza). The next day, the cells were transfected with control pcDNA3.1 (Invitrogen) or KLF2 plasmid PpyCAG-KLF2-IB (Addgene plasmid #60441) using the transfection reagent GeneJuice (Merck Chemicals) according to the manufacturer's protocol. Briefly, 3 μ l of GeneJuice reagent per 1 μ g of plasmid were added to 300 μ l OptiMEM. After 5min incubation at RT, 1 μ g plasmid DNA was added to the transfection reagent and the mix was incubated for another 15min at RT. Cells were incubated with the lipid/DNA mix in EGM-2. Cells were harvested 24h after transfection.

2.1.9 NF κ B reporter luciferase assay (Dr. Jose Lopez Ayala, MSc student)

HUVEC were plated in 6-well plates at a density of 1×10^5 cells per well and grown to reach 70% confluence. The cells were transfected with NF κ B Luciferase adenovirus (MOI 200; Vector Biolabs) diluted in M199 base medium for 2h at 37°C. The medium was removed and the cells were treated with MTX (100nM) for 48h, after which TNF α (0.1 ng/ml) was added to the cells for 4h. The luciferase activity was measured using the Luciferase Assay System (Promega) according to the manufacturer's instructions. In brief, the cells were washed with PBS and lysed with 1x cell culture lysis reagent (Promega) for 5min on a shaker. The cell lysate was centrifuged for 1min at 17900g to remove debris. The cell lysate was mixed with Luciferase Assay Reagent (Promega) and luminescence was measured on a plate reader.

2.1.10 Proliferation assay

Cell proliferation was measured by incorporation of bromodeoxyuridine (BrdU) into DNA using a 96 well-based BrdU Cell Proliferation assay ELISA kit (Cell Signaling). The assay was performed according to the manufacturer's instructions.

- For investigating the role of OCM in EC proliferation, EC were transfected with siRNA as described above for 24h. Cells were then seeded at 2500 cells per well of a 96 well plate in triplicate in EGM-2 and grown for 48h. The BrdU assay was performed as stated below and cell proliferation was expressed relative to sictrl (Chapter 5).
- For evaluating the proliferative capacity of patient- and HC-derived ECFC, cells were seeded at 2500 cells per well of a 96 well plate in triplicate in EGM-2. The next day, the cells were serum starved (0% FCS) or maintained in 2% FCS for 24h. The medium was then restored to either 0.5 or 2% FCS, or kept in 2%, for 24h. The next day, the BrdU assay was performed. Cell proliferation was expressed as ratio of BrdU incorporation (measured at 450nm) in 2% FCS versus 0.5% recovered cells (Chapter 6). Optimisation assays performed in HUVEC to determine serum concentrations and media conditions are shown in Figure 7.

BrdU was added into the culture medium for 4h at 37°C. The cells were then fixed and denatured for 30min at RT and stained with an anti-BrdU antibody for 1h at RT. After washing, the cells were incubated with an HRP-conjugated secondary antibody for 30min. The cells were washed and TMB substrate was added for 15min. The reaction was stopped by adding STOP solution and quantified on a plate reader measuring absorbance at 450nm (Bio-TEK).

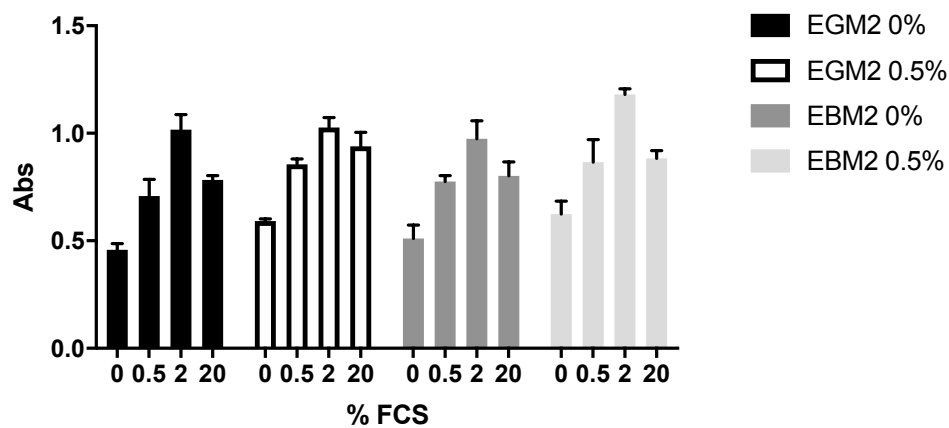


Figure 7. Optimisation of the BrdU proliferation assay in HUVEC. HUVEC were seeded on a 96well plate at 2.5K cells per well. The next day, the cells were serum starved with either 0 or 0.5% FCS in EGM-2 or EBM-2 media for 24h. The cells were then either kept in starvation media or restored with 0.5, 2 or 20% FCS respectively for 24h. At the end of the experiment, cells were subjected to the BrdU assay. Values show mean $\pm$ SEM (n=1; three technical replicates). Abs, absorbance.

2.1.11 Cell cycle analysis

Cells were seeded at a density of 1×10^5 cells per well in a 6-well plate and cultured overnight.

Cell treatments varied depending on the experimental question.

- To evaluate whether MTX alters cell cycle progression in pro-inflammatory activated EC in a folate-dependent manner, cells were treated with media or $\text{TNF}\alpha$ (0.1 ng/ml) for 8h, after which MTX (100nM) was added for 48h. After the first 24h of MTX treatment, folinic acid (500nM) was added for 24h (Chapter 3).
- To determine if MTX alters cell cycle progression in EC under shear stress, EC were subjected to shear stress for 48h, followed by addition of media or MTX (100nM) for 48h under shear stress. For one experiment, EC were exposed to shear stress for 24h, after which media or $\text{TNF}\alpha$ (0.1 ng/ml) were added. After 24h, MTX (100nM) was added for 48h under shear stress (Chapter 4).

Cells were harvested by trypsinisation, washed twice in 1xPBS and fixed in ice-cold 70% ethanol overnight at 4°C. Fixed cells were washed twice in 1xPBS and stained with a permeabilisation buffer containing PBS, 0.1% Triton X-100 (Sigma), 0.05M EDTA (Sigma), propidium iodide (50µg/ml; Sigma) and RNase A (400µg/ml; Sigma) for 30min at 37°C. Cells were filtered through a 50µm cell strainer (Sysmex) and analysed by flow cytometry on a CyAN ADP flow cytometer (Beckman Coulter), counting at least 10 000 events per sample. For cell cycle analyses of EC exposed to shear stress, 5000 events were counted per sample. Data was analysed using the FlowJo software. Figure 8 depicts the gating strategy used.

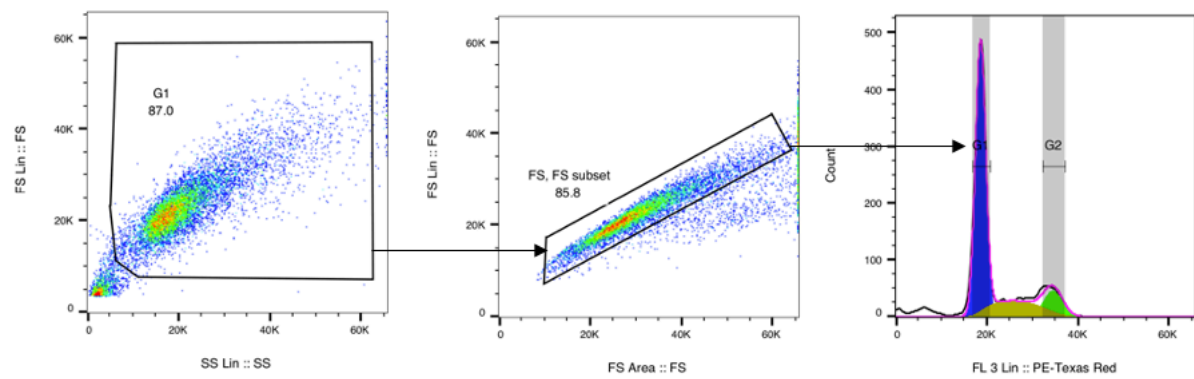


Figure 8. Gating strategy for cell cycle analyses by flow cytometry. The endothelial cell population was selected in gate G1 using the forward and side scatter. From this selection, doublet cells were gated out by plotting forward scatter area versus the forward scatter. These cells were then analysed for the distribution in the cell cycle using PI and the FlowJo software. Representative images shown. Phases of cell cycle coloured as following: G1, blue; S, yellow, G2/M, green. At least 10 000 events were counted.

2.1.12 Apoptosis assay

Cells were seeded at a density of 1×10^5 cells per well in a 6-well plate and cultured overnight. The next day, the cells were treated with M199 assay media (control), MTX (100nM) for 48h or hydrogen peroxide (H_2O_2 ; 200 μM) for 3h. Serum starvation was performed for 48h in M199 with 0%FCS. Cells were harvested by trypsinisation, washed twice in 1xPBS and stained with Annexin V and propidium iodide (PI) in Annexin V binding buffer for 15min at RT in the dark using the Pacific Blue Annexin V Apoptosis detection kit with PI (BioLegend). Annexin V binding buffer was added to each tube and the cells were filtered through a 50 μm cell strainer. The cells were analysed by flow cytometry counting at least 10 000 events per sample.

2.1.13 Shear stress experiments

2.1.13.1 Parallel plate model

EC were subjected to shear stress using a commercially available parallel flow system (Quad fluidic unit; Ibidi). In principal, cells were seeded on microslides and exposed to shear stress at constant flow rates using an air pump controlled by computer software (Ibidi) (Figure 9). The experiments were performed according to the manufacturer's instructions. In brief, the perfusion sets (red, 15cm; Ibidi) and media were pre-incubated at 37°C and 5% CO_2 overnight to degas. The slides (0.4 Luer μ -slide, Ibidi) were coated with 1% gelatin for 1h at 37°C and dried overnight at 37°C. The next day, cells were seeded at a density of 2.5×10^5 cells per slide, incubated at 37°C and 5% CO_2 for 6-8h to achieve a monolayer and then attached to the pre-equilibrated perfusion sets mounted on the fluidic unit system. Using this set up, the system allowed the application of either continuous high laminar shear stress (H-LSS; 20dyn/cm²), low laminar shear stress (L-LSS; 5dyn/cm²) or oscillatory shear stress (OSS; ± 5 dyn/cm², 2Hz) at a given time. Shear stress was applied to EC for a duration of 48-96h in a humidified incubator at 37°C and 5% CO_2 . Cells were pre-conditioned with shear stress for 48h because morphological changes become apparent at this time point. L-LSS was used as internal control for OSS experiments (same shear rate but unidirectional flow pattern). Static controls were grown on 6-well plates in parallel to the shear stress experiments because cells cannot be cultivated on the μ -slides for longer than 24h under static conditions due to the small medium reservoir. The application of shear stress to EC was validated by qPCR, immunoblotting and cell cycle analyses (Chapter 4 and 5). Cells were treated with pharmacological agents as indicated and harvested for downstream applications. Appropriate loading controls for shear stress experiments were determined by immunoblot analysis (Figure 10).

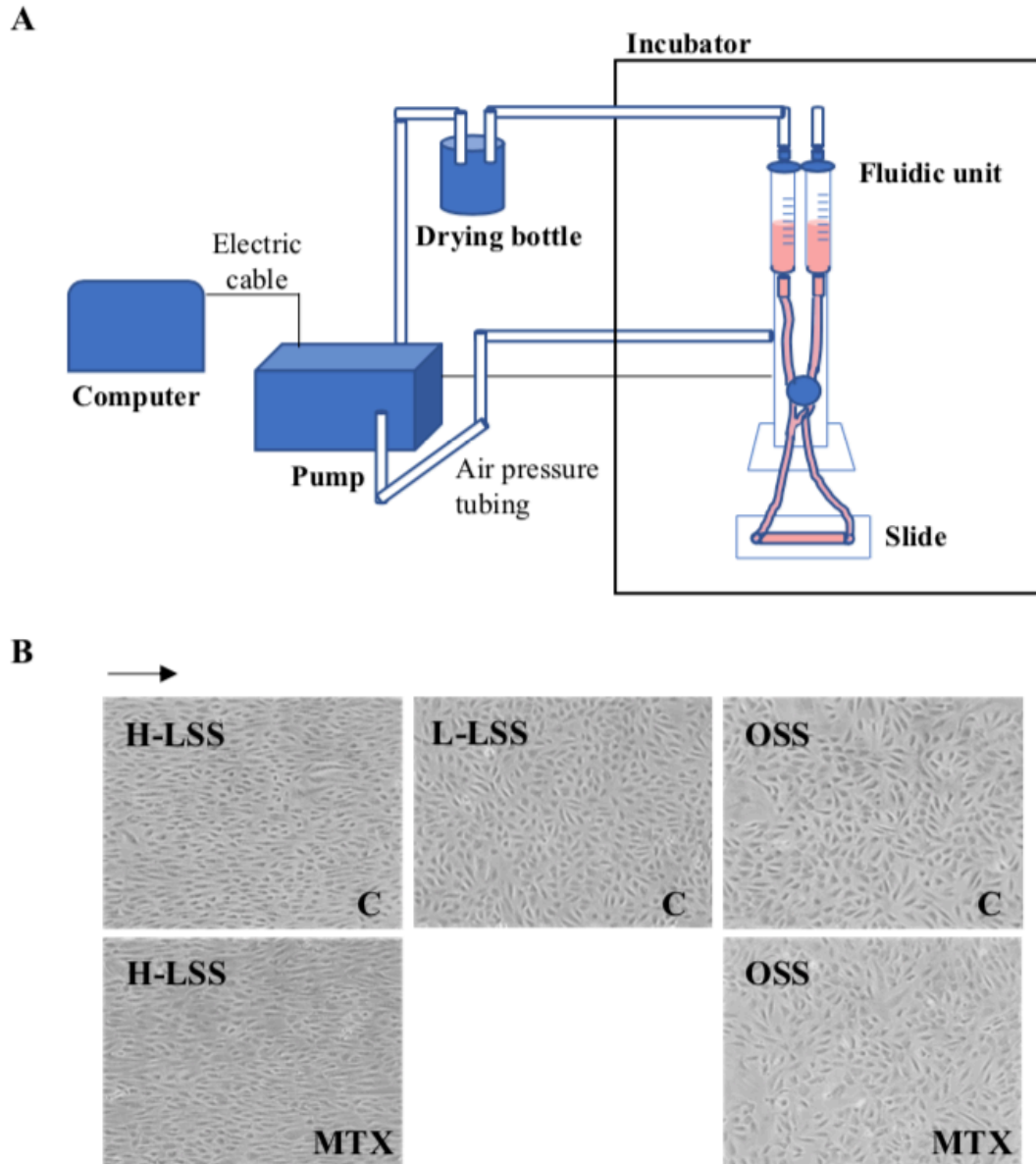


Figure 9. Characterisation of parallel plate model. (A) Schematic representation of experimental setup of parallel plate system (Ibidi). Illustration adapted from Ibidi Pump System Instruction manual. (B) Representative images of HAEC pre-conditioned with high laminar (H-LSS; 20dyn/cm^2), low laminar (L-LSS; 5dyn/cm^2) or oscillatory (OSS; $\pm 5\text{dyn/cm}^2$, 2Hz) shear stress for 48h before addition of media (control, C) or MTX (100nM) for 48h ($n>3$). Total exposure to shear stress 96h. Arrow indicates direction of flow. Magnification 20x.

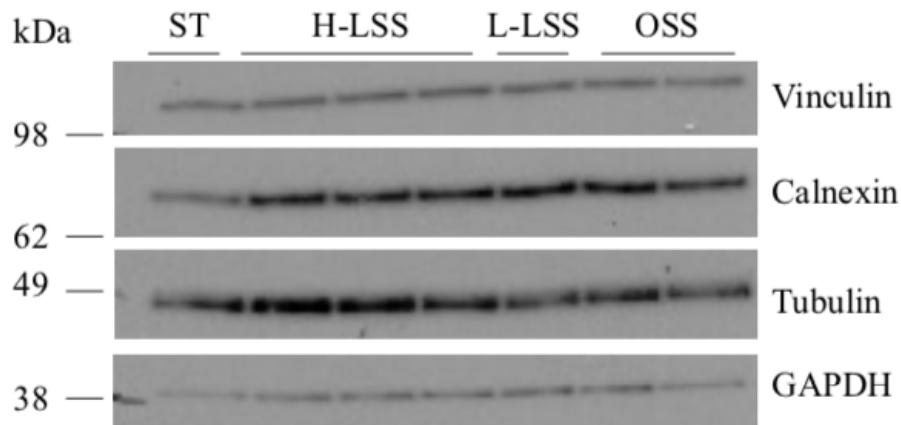


Figure 10. Validation of loading controls for shear stress experiments. Immunoblot analysis of HAEC subjected to static culture (ST), high LSS (H-LSS; 20dyn/cm²; three technical replicates), low LSS (L-LSS; 5dyn/cm²; one technical replicate) or OSS ($\pm$ 5dyn/cm², 2Hz; two technical replicates) for 96h (n=1). Whole cell protein lysates were separated by SDS-PAGE. Proteins were detected with antibodies against Vinculin (124kDa), Calnexin (67kDa), α -tubulin (50kDa) and GAPDH (39kDa).

2.1.13.2 Orbital shaker model

To validate the findings generated with the parallel flow system, selected experiments were performed using the orbital shaker model in collaboration with Dr. Christina Warboys (Imperial College London) as described previously [479-481]. Shear stress is induced by the movement of the medium over the cells resulting in different flow patterns in the periphery (undisturbed flow; UF) or centre of the well (disturbed flow; DF) (Figure 11). UF is characterised by a uniaxial flow of high shear stress with fluctuating magnitude, while DF produces a low multidirectional flow [482]. Cells were seeded at confluence on 6-well plates pre-coated with 1% gelatin, incubated for 6-8h and put on an orbital shaker platform (Grant Instruments) located in the tissue culture incubator at 37°C and 5% CO₂. The radius of orbit of the orbital shaker was 10mm and the rotation rate was set to 150rpm. Cells were pre-conditioned with shear stress for 48h, after which media or MTX were added for 48h under shear stress. For a single experiment, cells were subjected to shear stress for 72h. Cells were harvested from different areas of the well (as indicated by the cartoon) for downstream applications.

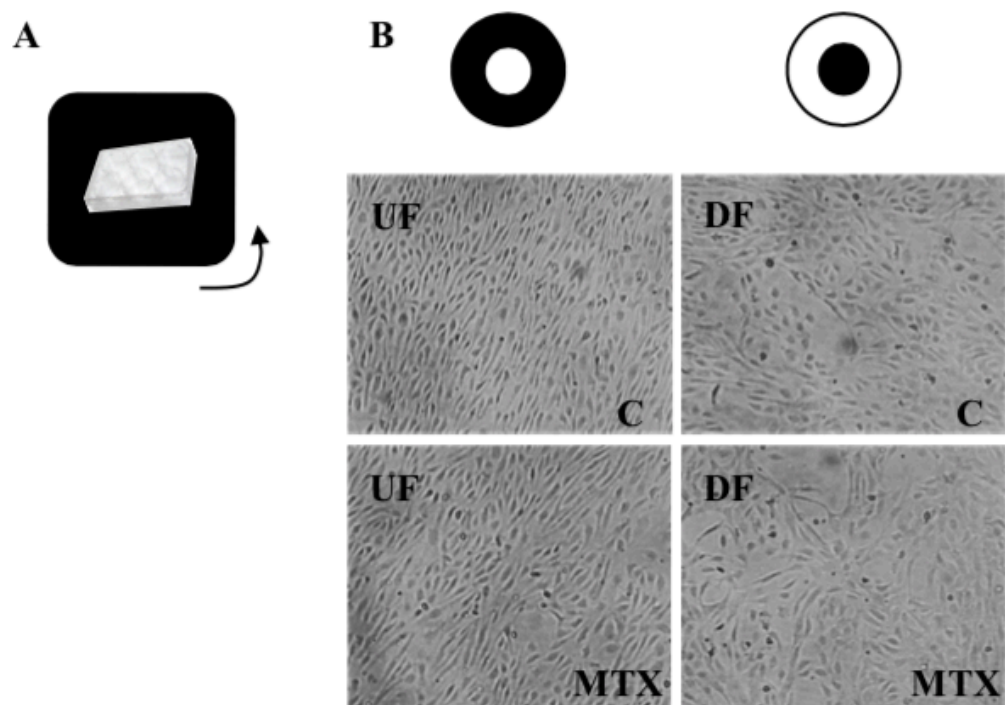


Figure 11. Characterisation of orbital shaker model. (A) Schematic representation of experimental setup. (B) Representative images of HAEC pre-conditioned with shear stress for 48h before addition of media (control, C) or MTX (100nM) for 48h using the orbital shaker model (n=3). Images taken from different areas of the well which experience differing shear rates: undisturbed flow (UF) is achieved in the periphery of the well, whereas disturbed flow (DF) occurs in the centre of the well (as indicated in the cartoon). Magnification 20x.

2.1.14 Endothelial colony forming cells (ECFC)

Subject recruitment, peripheral blood mononuclear cell (PBMC) isolation, ECFC propagation and characterisation were performed in collaboration with Dr. Robert Maughan and Dr. Charis Pericleous. Study recruitment was kindly supported by Antonia Greeves and Mary “Jess” Borbon (Hammersmith Hospital).

2.1.14.1 Study subjects

All patients were recruited in the Early Inflammatory Arthritis clinic at Hammersmith Hospital (Imperial College London). Patient enrolment was guided by the following inclusion criteria: clinical diagnosis of RA, active disease as judged by DAS28-ESR score [483] and no prior treatment with DMARD. Exclusion criteria included active malignancy, current infection or pregnancy as these represent contraindications for therapy, and other systemic inflammatory autoimmune diseases. Treatment-naïve patients with active RA were recruited prior to initiating dual DMARD therapy comprising methotrexate (MTX; 10-25mg/week) and hydroxychloroquine (HCQ; 200-400mg/day) according to the local standard of care. RA patient management was guided by the 2016 EULAR guidelines on RA patient management with DMARD therapy [484]. Patients with treatment-naïve active RA were started on treatment as soon as possible after diagnosis with the aim to reach disease remission or low disease activity. Some of the patients received bridging therapy with short-term glucocorticoid treatment. RA patients were recruited for follow-up four to six months after DMARD treatment initiation. This timepoint is within the clinically implemented time frame for the assessment of treatment response [484]. Treatment response was evaluated using the EULAR response criteria and DAS28-ESR activity score [485, 486]. Healthy controls (HC) were also recruited. Patient blood samples were collected for plasma, PBMC and ECFC isolation. The clinical parameters DAS28-ESR disease activity score, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were recorded in patients at baseline and follow-up. Written informed consent was obtained for all subjects prior to their inclusion. Ethical approval for this research has been granted by the Wales Multicenter Research Ethics Committee (17/WA/0161) via Imperial College Tissue Bank.

2.1.14.2 PBMC isolation

ECFC were isolated from peripheral blood and cultured using an established protocol [487]. Whole blood was drawn into sodium heparin containing tubes (Greiner) and centrifuged at 400g for 10min. The plasma fraction (upper layer) was transferred into a new tube without disturbing the buffy coat (leukocytes and platelets) and further depleted of platelet contamination by centrifugation at 3000g for 10min. Plasma samples were aliquoted and stored at -80°C. For PBMC isolation, a modified version of the StemCell SepMate method was used. The remaining fractions were diluted first 1:1 with 1xPBS (HyClone; GE Life Sciences), then 1:1 with PBS supplemented with 2% FCS (HyClone) and carefully layered onto a SepMate tube (StemCell) containing Ficoll paque (GE Life Sciences). PBMC were isolated by density centrifugation at 1200g for 10min (brake on). The PBMC containing fraction was poured into a fresh tube, washed with PBS 2%FCS and centrifuged at 350g for 15min (brake on half). The cells were washed and centrifuged at 350g for 10min (brake on half) two more times. PBMC were counted using a haemocytometer, resuspended in EGM-2 supplemented with 20% FCS (HyClone), EGM-2 SupplementPack (Promocell) and penicillin/streptomycin (100µg/mL) and plated on flasks pre-coated with rat-tail collagen 1 (Corning) for 1h at 37°C and 5% CO₂. The medium was changed after 48h and every two to three days thereafter. Cultures were assessed every other day for the appearance of colonies between 7-35 days post-seeding.

2.1.14.3 ECFC colony generation capacity

ECFC frequency was evaluated using their colony generation capacity *in vitro*, quantified by counting the number of emerging colonies every other day after PBMC seeding from day 7 until the first passage. Following ISTH guidelines [488], ECFC colonies were identified as “well-circumscribed colonies with a cobblestone appearance and with more than 50 adherent cells” of endothelial morphology. Colony generation is presented as final colony number before first split per 10⁷ PBMC seeded. The number of days to first split were also recorded for each ECFC culture.

2.1.14.4 ECFC propagation

Once established, ECFC colonies were washed with PBS, sub-cultured by trypsinisation and expanded at a cell seeding density of 10 000 cells/cm². In general, ECFC were expanded to passage 3, after which cells were cryopreserved. Functional experiments were conducted using cryopreserved ECFC at passage 5 on a sub-set of the study cohort.

2.1.14.5 Evaluation of endothelial purity of ECFC samples (Dr. Robert Maughan)

ECFC were assessed for endothelial purity measuring the following endothelial CD31, CD144, CD146, myeloid CD14 and leukocyte CD45 markers by flow cytometry. Briefly, cryopreserved ECFC were thawed in pre-warmed HBSS supplemented with 1% FCS (HBSS 1%FCS), spun at 350g for 5min and resuspended in HBSS 1%FCS. 1×10^5 cells were added per tube and stained with Zombie NIR (in PBS) for 20min in the dark at RT to determine cell viability. The cells were washed in PBS and centrifuged at 350g for 5min. The cells were resuspended in Cell Staining buffer (BioLegend) and stained with conjugated anti-CD31, CD144, CD146, CD14, CD45 antibodies (Table 8) for 20min at RT. The cells were washed, resuspended in Cell staining buffer and filtered through a 50 μ m cell strainer. FACS analyses were performed using a CyAN ADP flow cytometer (Beckman Coulter), counting at least 10 000 cells. Gating strategies were configured using fluorescence minus one (FMO) controls in stained HUVEC and are depicted in Figure 12. Viability gates were set using HUVEC that were heat shocked for 65°C for 2min, followed by incubation on ice for 2min and staining with Zombie NIR.

Table 8. Antibodies used for flow cytometry

Antibody	Clone	Fluor	Species	Company (Reference #)	Concentration
CD31	WM59	FITC	mouse	BioLegend (#303104)	5 μ l
CD144	16B1	PE	mouse	EBioscience (#12-1449-82)	10 μ l
CD146	P1H12	PE	mouse	BioLegend (#361004)	5 μ l
CD14	M5E2	APC	mouse	BioLegend (#301808)	5 μ l
CD45	2D1	BV510	mouse	BioLegend (#368526)	5 μ l
Zombie NIR			-	BioLegend (#423106)	1:3750

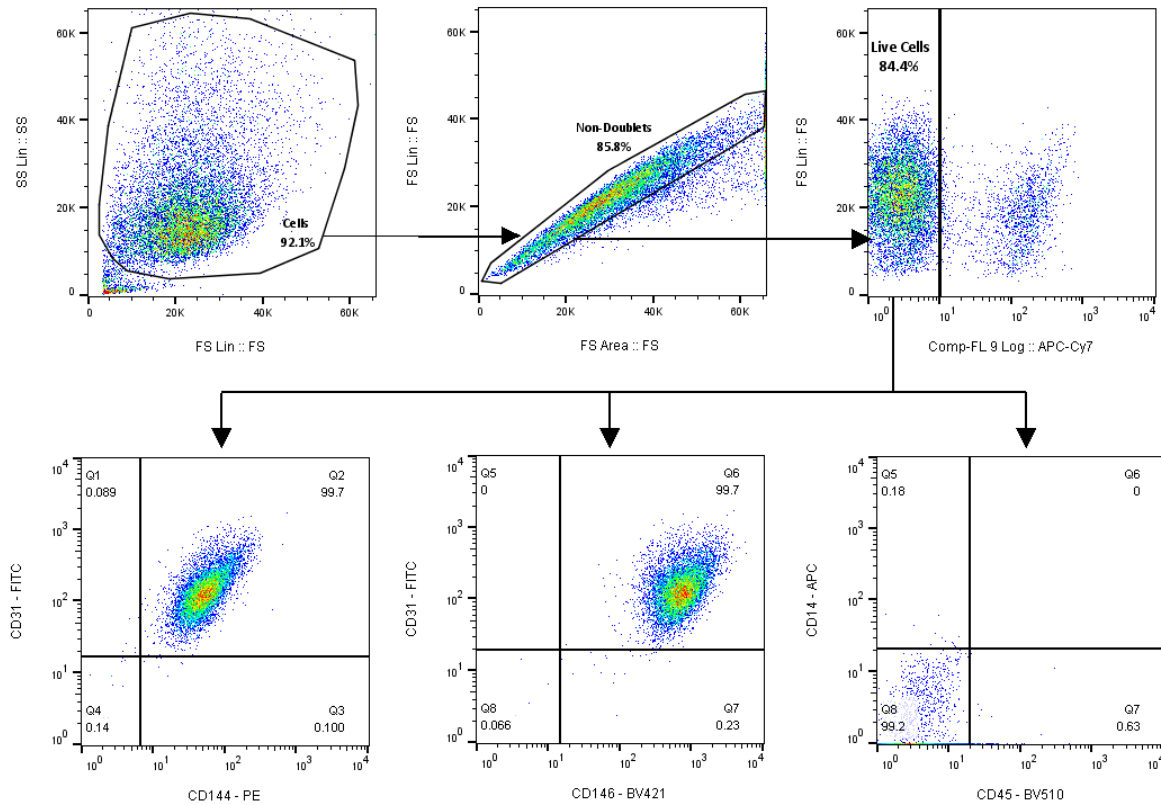


Figure 12. Evaluation of the endothelial purity of ECFC samples from the ECFC-RA study cohort by flow cytometry. Gating strategy for flow cytometric analyses of ECFC for endothelial purity as measured by the CD31⁺/CD144⁺/CD146⁺/CD14⁻/CD45⁻ cell population. Representative images shown. FS, forward scatter. SS, side scatter. This experiment was performed by Dr. Robert Maughan.

2.1.14.6 Inflammatory activation of ECFC derived from HC and RA patients

ECFC were seeded at a density of 1×10^5 cells per well on a 12-well plate in EGM-2. The cells were exposed to a concentration series of TNF α (0.01-1ng/mL) in EGM-2 for 4h. The cells were harvested for RNA extraction. The induction of inflammatory cell adhesion molecules was assessed by measuring mRNA expression levels by qPCR.

2.2 RNA

2.2.1 RNA isolation

Cells were washed twice with PBS and lysed in 350 μ L RNeasy Lysis Buffer (RLT; Qiagen) supplemented with β -mercaptoethanol (10 μ L/mL). After harvesting the lysate, RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA concentrations were measured by UV spectrophotometry using a NanoDrop (Thermo Scientific).

2.2.2 First strand cDNA synthesis

RNA (500ng-1 μ g) was mixed with 4 μ L of Quanta qScript cDNA mix (Quanta Biosciences) and water in a total volume of 20 μ L. The reaction was run for 5min at 22°C, 30min at 42°C and 5min at 85°C.

2.2.3 Quantitative real-time polymerase chain reaction (qPCR)

A master mix of 12.5 μ L IQ SYBR Green Supermix (Bio-Rad), 1 μ L of primer mix containing the forward and reverse primers (5 μ M each) and water was prepared. cDNA (5 μ L of a 1:30 dilution) was added to 20 μ L of master mix. The PCR protocol included a denaturation step of 95°C for 3min and 40 cycles of 95°C for 10sec and 58°C for 45sec. Real-time PCR reactions were performed using a Bio-Rad CFX96 thermocycler. Target gene expression was normalised to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results were analysed using the Bio-Rad CFX Manager Software version 2.0 (Bio-Rad) and comparative cycle threshold method ($\Delta\Delta C_t$). Oligonucleotide sequences of the primers used are listed in Table 9.

Table 9. Human oligonucleotide sequences used for qPCR analyses

Primer	Oligonucleotide sequence 5' to 3'
ACTB_fw	GAG CTA CGA GCT GCC TGA CG
ACTB_rev	GTA GTT TCG TGG ATG CCA CAG GAC T
ALOX5_fw	TGGCGCGGTGGATTCATA
ALOX5_rev	ACTTCAGGTACCAGTCGTCA
ATIC_fw	CGTGATAAGCCCGGAAACAG
ATIC_rev	AGAGACACTAAATAAGGCGAGC
CCL2_fw	AGTCTCTGCCGCCCTTC
CCL2_rev	CTTGCTGCTGGTGATTCTTC
DHFR_fw	CACAACCTCTTCAGTAGAAGGTAA
DHFR_rev	CTGCCACCAACTATCCAGAC
DUSP1_fw	GGC CAT TGA CTT CAT AGA CTC C
DUSP1_rev	ACA AAC TCA AAG GCC TCG TC
FPGS_fw	ATGGAGTACCAGGATGCCGT
FPGS_rev	GGCTTCCAACGTGTGTCTGAG
GAPDH_fw	CAA CAG CCT CAA GAT CAT
GAPDH_rev	GAG TCC TTC CAC GAT ACC
GART_fw	ACCCGGTGTTCGGTTTCATT
GART_rev	CAGCGTATGTTCCCTTCCTC
GGH_fw	GCATCCAGAGAAAGCACCTT
GGH_rev	TGGTTGTTTTTCCGAGCTTCA
ICAM1_fw	ATGCCCAGACATCTGTGTCC
ICAM1_rev	GGGGTCTCTATGCCCAACAA
KIT_fw	ACGCACCTGCTGAAATGTAT
KIT_rev	CACAGAATGGTCTACCACGG
KLF2_fw	GCA AGA CCT ACA CCA AGA GTT CG
KLF2_rev	CAT GTG CCG TTT CAT GTG C
MRT_fw	CAACCCGAAGGTCTGAAGAA
MRT_rev	CCTCGGAAGTGTTCTTCGTT
MTHFD1_fw	GCTGAAGTCTACACGAAGCA
MTHFD1_rev	CAGGCATTGTGCTCATCGTT
MTHFD1L_fw	GGGACTCCATCGTCAGAGAA
MTHFD1L_rev	TCACCTGCCTGGATAATTGC
MTHFD2_fw	CTGAGTGTGATCCTGGTTGG
MTHFD2_rev	GTCTCACTGTTGATTCCCACA
MTHFD2L_fw	GGTGTGAGGACCAGCAGAGA
MTHFD2L_rev	CCATGATTCCACACCTCGCT
NRP1_fw	GAAAAATCGAATGCTGAT
NRP1_rev	AATCCGGGGGACTTTATCAC
PTGS2_fw	GGCCATGGGGTGGACTTAAA
PTGS2_rev	ACCGTAGATGCTCAGGGACT
SELE_fw	TACCTACCTGTGAAGCTCCC
SELE_rev	GCTTTCCGTAAGCATTTCCG
SHMT1_fw	AAGTTCGGGGTTTGGGGTT
SHMT1_rev	GAAGCTGCCTAGCGACG

SHMT2_fw	CTTCTCTTTGTTTTGGGCGG
SHMT2_rev	GCAGAAGTTCTCTGAGGCAA
SLC19A1_fw	TGCTACCTTTGCTTCTACGG
SLC19A1_rev	TGATCTCGTTCGTGACCTGC
THBD_fw	ACGACTGCTTCGCGCTCTACCC
THBD_rev	CACCGAGGAGCGCACTGTCATTA
TNFAIP3_fw	GGCGTTCAGGACACAGAC
TNFAIP3_rev	TCCCATTCATCATTCAGTTC
TNFSF10_fw	GAGGTCCTCAGAGAGTAGCA
TNFSF10_rev	TTCTTGGAGTTTGGAGAAGACAA
TYMS_fw	TGGTTTATCAAGGGATCCACAA
TYMS_rev	CAGTTGGTCAACTCCCTGTC
VCAM1_fw	CTACGCTGACAATGAATC
VCAM1_rev	GCAACTGAACACTTGAC

2.2.4 qPCR arrays

Samples were run on pre-designed qPCR arrays (384 well format; 4 samples per plate) with primers against 84 targets important in endothelial biology, 5 reference genes, 6 positive controls and 1 control for genomic DNA contamination (Qiagen; for complete gene list see the Appendix Table 12). RNA isolation was carried out as stated above. cDNA synthesis was performed using the RT² First Strand Kit (Qiagen). 400ng total RNA was added to Buffer GE and water to a total volume of 10µl (Genomic DNA elimination mix), incubated for 1min at 42°C and 5min at 4°C. This mix was added to Buffer BC3, Control P2, RE3 Reverse Transcriptase mix and water to a total volume of 20µl. Reverse transcription was performed for 15min at 42°C and was stopped by incubating the mix for 5min at 95°C. Each reaction was diluted with 91µl RNase free water and stored at -20°C. Prior to analysis on the qPCR array, all samples were tested for genomic DNA contamination and equal loading. A mix of cDNA (2.8ng/reaction), 2x RT² SYBR Green Mastermix and water were added to the qPCR plates (4 samples per plate; 3 biological replicates). The PCR program was 1 cycle of 50°C for 2min and 95°C for 10min, followed by 40 cycles of 95°C for 15sec and 60°C for 1min. The reaction was run on a QuantStudio Flex 6 Thermocycler (Applied Biosciences). The arrays contained primers for five house-keeping controls (ACTB, B2M, GAPDH, HPRT1, RPLP0), which were stable across all samples. Target gene expression was normalised to GAPDH. The results were analysed using the RT² Profiler PCR data analysis excel spreadsheet supplied by the manufacturer by unpaired T-tests. Genes were excluded if they did not pass the Ct threshold of >35 or melt curve criteria. Chosen target genes were validated by qPCR using different primers.

2.2.5 Primer validation

Oligonucleotide primers for quantitative real-time reactions were designed using the Primer-BLAST website (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) [489]. Each primer was validated on a dilution series of EC cDNA samples to evaluate the melt curves and efficiencies of the respective primers. Representative images are shown in Figure 13.

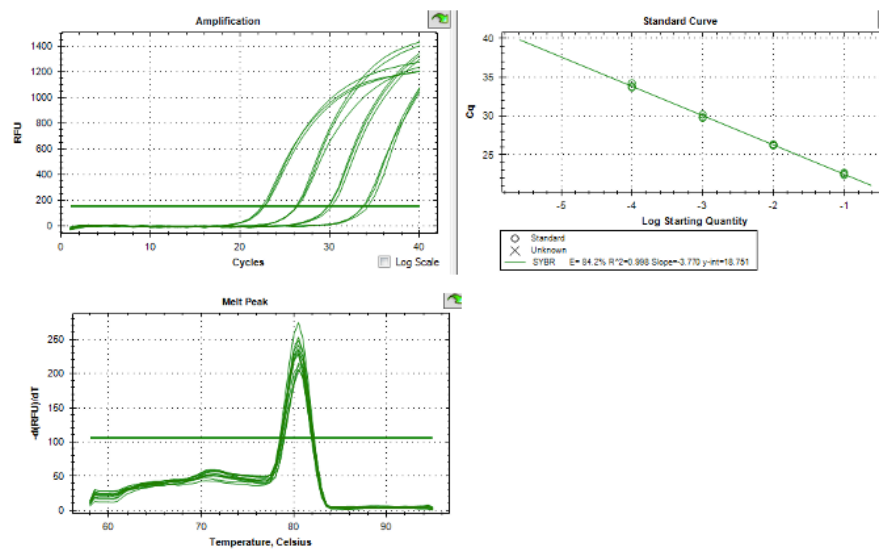


Figure 13. Primer validation for quantitative real-time PCR. HUVEC cDNA was serially diluted at 1:10, 1:100, 1:1000 and 1:10000. Primers were evaluated for melt curves and efficiencies. Representative graphs shown.

2.3 Protein

2.3.1 Determination of protein concentration

Cells were washed twice in ice-cold PBS and lysed in RIPA buffer (Thermo Scientific; 80µl per 6-well) supplemented with complete protease and phosphatase inhibitors (Roche). Cell lysates were spun for 15min at 17900g and 4°C, and the supernatants were harvested. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher) or DC protein assay (BioRad) as per manufacturer's protocol on a microplate reader (Bio-TEK).

2.3.2 Immunoblot analysis using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were prepared by mixing 2–10µg of protein lysate, NuPAGE LDS Sample Buffer (Invitrogen), 1M dithiothreitol (DTT) (Sigma) and water, followed by heating at 100°C for 5–10min. The proteins were separated by SDS-PAGE using a NuPAGE Novex 4–12% gradient Bis-Tris Protein Gels (Thermo Fisher) and NuPage MOPS SDS running buffer (Thermo Fisher) for 1.5h at 150V. Proteins were blotted using the iBlot 2 dry blotting system and the iBlot 2 PVDF Regular Stacks (Invitrogen) for 10.5min at 20V. Membranes were blocked with 5% BSA/TBS-T (Tris-buffered saline (TBS) with 5% w/v BSA; 0.1% Tween-20) for 1h at RT, followed by incubation with the primary antibodies (Table 10) diluted in 5% BSA/TBS-T at 4°C overnight. The membranes were washed three times with TBS-T (TBS with 0.1% Tween-20) for 5min and incubated with the HRP-coupled secondary antibodies in 5% BSA/TBS-T for 1h at RT. After washing three times with TBS-T for 10min, the membranes were incubated with ECL chemiluminescence (GE Healthcare) for 1min and proteins were detected with Carestream Kodak BioMax light films (Sigma).

Alternatively, the blots were probed with the secondary antibody goat anti-mouse IgG Dylight 800 in 5% BSA/TBS-T for 1h at RT. The washing steps were performed using TBS-T, followed by a final wash in TBS. The proteins were detected with the Odyssey CLx imaging system (LI-COR Biosciences).

Table 10. Antibodies used for immunoblot detection

Antibody	Species	Company (Reference #)	Concentration
p-Akt S473	Rabbit	Cell Signaling (#4060)	1:2000
Akt	Rabbit	Cell Signaling (#4685)	1:2000
p-AMPK T172	Rabbit	Cell Signaling (#2535)	1:2000
AMPK α	Rabbit	Cell Signaling (#2532)	1:2000
Calnexin	Rabbit	Cell Signaling (#2679)	1:1000
p-ERK1/2 T202/Y204	Rabbit	Cell Signaling (#9101)	1:1000
Anti-mouse IgG Dylight 800	Goat	Invitrogen (#SA535521)	1:10000-20000
GAPDH	Rabbit	Cell Signaling (#2118)	1:10000
GAPDH	Mouse	MAB374, EMB Millipore	1:10000
HRP-tagged anti-rabbit IgG	Swine	DAKO (#P0399)	1:5000–1:40000
HRP-tagged anti-mouse IgG	Goat	DAKO (#P0447)	1:5000–1:40000
ICAM-1	Mouse	SN from Nancy Hogg's lab (clone 15.2)	1:4
I κ B α	Mouse	Cell signaling (#L35A5)	1:3000
p-Jak1 Y1034/1035	Rabbit	Cell Signaling (#74129)	1:1000
MTHFD2	Rabbit	Abcam (#ab151447)	1:1000
p-p38 T180/Y182	Rabbit	Cell Signaling (#4511)	1:5000
p38	Rabbit	Cell Signaling (#8690)	1:5000
p-p65 S536	Rabbit	Cell Signaling (#3033)	1:1000
p65	Rabbit	Assay Designs (#KAS-TF110)	1:1000
p-PKC ϵ S729	Rabbit	Millipore (#06-821-1)	1:1000
PKC ϵ	Rabbit	Santa Cruz (#sc214)	1:1000
SHMT2	Rabbit	Cell Signaling (#12762)	1:1000
Thrombomodulin	Mouse	Santa Cruz (#sc13164)	1:500
α -Tubulin	Mouse	Cell Signaling (#3873)	1:20000
VCAM-1	Rabbit	Cell Signaling (#12367)	1:1000
Vinculin	Rabbit	Cell Signaling (#13901)	1:10000

2.3.3 Phospho-kinase activity arrays

Phospho-serine/threonine (STK) or tyrosine (PTK) kinase activity arrays (PamChip) were used to measure kinase activity in a given sample according to the manufacturer's instructions (Pamgene). The principle of the assay is briefly outlined: The PamChip arrays feature porous ceramic membranes, which are coated with 144 or 196 peptides (STK or PTK respectively) with known phosphorylation motifs. Incubation of the arrays with cell lysate allows kinases, which are active in the cell lysate, to phosphorylate peptides with their respective substrate motif. Phosphorylation events are captured by a phosphorylation specific primary antibody, followed by detection with a fluorescently labelled secondary antibody and signal detection (PTK arrays use a directly fluorescently labelled primary antibody for signal detection). The first output of the arrays is the log₂ fold change and significance (-log₁₀ p-value) of

differentially phosphorylated peptides in a treatment compared to control condition, which are analysed using a paired t-test (for two groups) or one-way ANOVA (for multiple groups) and depicted using volcano plots. Secondly, the upstream kinase analyses provide predictions on which kinases are responsible for the changes in peptide phosphorylation between two groups. Importantly, this scoring method relies on the collective change of peptide phosphorylation within a given group, not on statistical significance or fold change of individual peptides. For this, the pattern of differentially phosphorylated peptides is bioinformatically converted into a ranking of putative active kinases using functional class scoring and permutation analyses according to predicted and empirically validated kinase/substrate interactions (scoring method developed by Pamgene).

The kinase ranking is performed according to four different parameters: normalised kinase statistic (or effect size), significance, specificity and final score. The normalised kinase statistic represents the degree of differential phosphorylation of peptides in the treatment versus control group contributing to the ranking of the kinase (e.g. the higher the value, the higher the kinase activity in the treatment group). The significance score reflects the significant contribution of the experimental group (treatment or control) to the kinase statistic in the permutation analyses (permutation of samples between experimental groups). The specificity score describes the contribution of a specific peptide to the score (permutation of peptides in peptide sets). The final score is a combined measure of the significance and specificity scores. For presentation purposes, the top 15% of putative kinase hits (ranked by their final score and normalised kinase statistic) are displayed.

Cells were washed with ice-cold PBS and lysed on ice using the M-PER Mammalian Extraction Buffer (Thermo Fisher) containing Halt Phosphatase Inhibitor Cocktail and Halt Protease Inhibitor Cocktail, EDTA free (Thermo Fisher). Cell lysates were spun for 15min at 17900g and 4°C, and the supernatants were harvested. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher). Cell lysates were aliquoted and stored at -80°C until further use. STK or PTK PamChip arrays were placed into the PamStation12 machine (Pamgene) and were blocked with 2%BSA for 30min at RT, followed by washing with PK wash buffer. Sample mix consisting of 1µg or 5µg cell lysate (STK or PTK respectively), 4mM ATP, BSA, water, (1M DTT PTK only), proprietary buffers and antibodies were incubated on the arrays. Arrays were then washed with PK buffer (PTK) or 1x PBS/0.01% Tween (STK). For STK arrays, a secondary FITC-labelled antibody was added for

signal detection. After washing, peptide spot intensity was captured using a CCD camera. Image and data analyses were performed using the BioNavigator software (Pamgene). The phospho-kinase activity arrays were validated by immunoblot analyses for specific targets.

2.3.4 Dihydrofolate Reductase (DHFR) activity assay

DHFR activity was measured in cell lysates using a DHFR activity assay kit (Sigma). The kinetic assay measures DHFR activity as the decrease of its co-factor NADPH (used for the conversion of dihydrofolic into tetrahydrofolic acid) over time detected by a decrease in absorbance. HUVEC were washed twice with ice-cold PBS and lysed in CellLytic M mammalian cell lysis/extraction reagent (Sigma) supplemented with complete protease and phosphatase inhibitors (Roche). The lysates were spun at 17900g for 15min and concentrated using the Amicon Ultra 0.5mL Centrifugal Filters (Ultracel 3K; Millipore). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher). 60µM NADPH, 50µM dihydrofolic acid (DHF), recombinant DHFR enzyme or varying concentrations of cell lysate were diluted in assay buffer and mixed in a 96-well format. Reactions were run sequentially. DHFR activity was instantly recorded by reading the absorbance every 15 seconds for 2.5min at 340nm and RT on a plate reader. Inhibition of the reaction was achieved by pre-incubation of the assay mix with 1µM MTX. Reactions without substrate or co-factor were used as negative controls.

2.3.5 Tetramethylrhodamine methyl ester (TMRM) and Mitotracker live-staining

Cells were incubated with TMRM (100nM; Thermo Fisher) and Mitotracker Deep Red (300nM; Thermo Fisher) in EGM-2 for 30min at 37°C and 5% CO₂. Cells were washed three times with HBSS containing Ca²⁺ and Mg²⁺ (Gibco) and nuclei were counterstained with Hoechst 33342 (5µg/ml; Sigma) in HBSS. Cells were live imaged on a LSM780 Confocal microscope (Zeiss) using a 40x objective on a heated stage (37°C). Z-stack images of six different fields were taken in collaboration with Dr. Claudio Raimondi (QML). Image quantification was performed in ImageJ (v2.0).

2.3.6 Quantification of mitochondrial morphology

Quantification of mitochondrial morphology was performed by visual scoring of the cells into two groups, either “punctate” or “intact”, based on their Mitotracker staining pattern (method adapted from [490]). In a given image, the number of cells in each group were counted and expressed as percentage of the total cell count. 400-650 cells in total were counted per condition from three individual experiments (n=3). Representative images are shown in Figure 14.

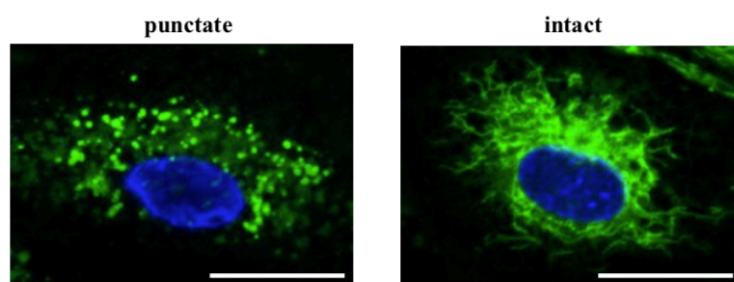


Figure 14. Categorisation of punctate or intact mitochondrial morphology by Mitotracker staining. HUVEC were transfected with sictrl for 24h and subjected to static culture for an additional 48h. The cells were stained with Mitotracker (green) and Hoechst (blue) for the visualisation of mitochondria and nuclei respectively. Cells were imaged on a Zeiss LSM Confocal 780. Representative images shown. Scale bar 20 μ m.

2.3.7 Quantification of mitochondrial network

Quantification of the mitochondrial network was performed using the Mitochondrial Network analysis (MiNA) macro tool in ImageJ published by Valente et al [491]. Briefly, images of single cells were selected and pre-processed using the unsharp mask filter, local contrast enhancement (CLAHE) and median filter. Using the ridge detection tool, the fluorescence intensity of the processed image was simplified into a skeleton overlay. The parameters analysed were the mitochondrial footprint (area of the mitochondrial signal over background), mean number of branches per network and mean branch length. Per condition, a total of 30 cells were analysed on a single cell basis from three individual experiments (n=3). Representative images of quantified cells are shown in Figure 15.

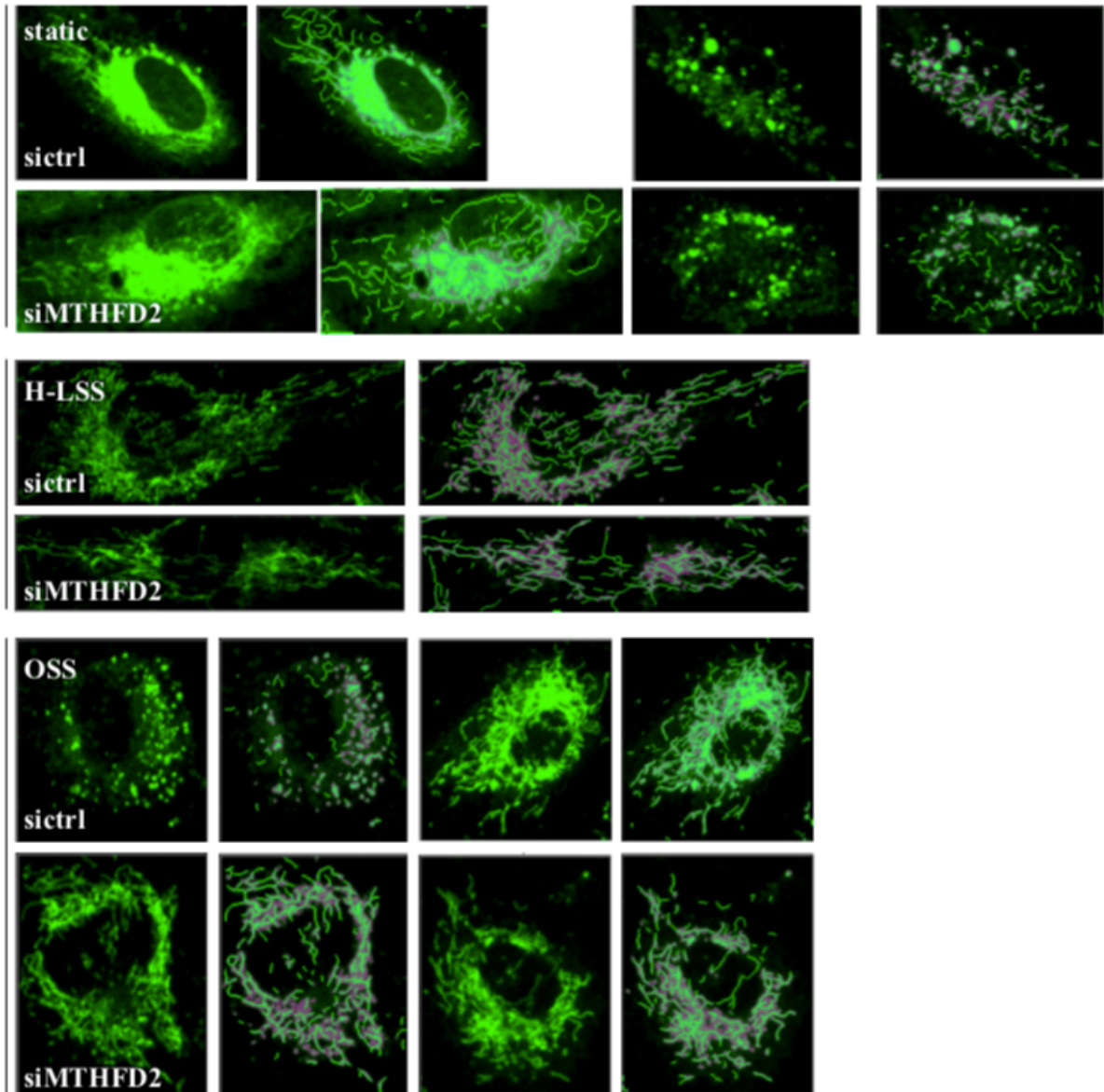


Figure 15. Quantification of the mitochondrial network in MTHFD2-depleted EC. HUVEC were transfected with sictrl or siRNA against MTHFD2 for 24h and subjected to either static culture, H-LSS or OSS for an additional 48h. The cells were stained with Mitotracker and imaged on a Zeiss LSM Confocal 780. Quantification of the mitochondrial network was performed using the Mitochondrial Network analysis (MiNA) macro tool using ImageJ published by Valente et al. [491]. Images were pre-processed as stated. Skeleton maps were applied to the processed images using the ridge detection method. The parameters analysed are the area of the mitochondrial signal (mitochondrial footprint), mean number of branches per network and mean branch length. Representative images shown for each condition of an unprocessed image (left) versus its skeleton map (right)). Image brightness was adjusted for presentation.

2.4 Ultra-performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS) (Dr. Kit-Yi Leung)

Folate metabolites were measured in cell lysates by Dr. Kit-Yi Leung (UCL) using an established UPLC-MS/MS protocol described in [492]. HUVEC were subjected to static conditions, H-LSS or OSS for 48h in EGM-2 (10%FCS) using the parallel plate model, harvested by trypsinisation, counted and aliquoted at a given cell number. Cell pellets were washed with PBS and centrifuged at 300g for 5min. After removal of PBS, the cell pellets were snap frozen on dry ice and stored at -80°C. Five individual experiments were pooled to achieve 1.8×10^6 cells per condition for one LCMS run.

Sample preparation, UPLC-MS/MS handling and data analyses were performed by Dr. Kit-Yi Leung. Cell pellets were resuspended in a buffer of 20mM ammonia acetate (Sigma), 0.1% ascorbic acid (Sigma), 0.1% citric acid (Sigma) and 100mM DTT (Sigma) at pH 7. After sonication, proteins were precipitated by mixing with acetonitrile (2x sample volume; Thermo Fisher) and centrifugation for 15min at 12000g and 4°C. Supernatants were lyophilised and stored at -80°C. For analysis, lyophilised samples were diluted in ultrapure water, centrifuged for 5min at 12000g and 4°C and analysed by reversed-phase UPLC (Acquity UPLC BEH C18 column; Waters). The solvents used for UPLC analyses were: Buffer A: 5% methanol (Thermo Fisher), 95% water and 5mM dimethylhexylamine (Fluka) at pH 8.0; Buffer B: 100% methanol, 5mM dimethylhexylamine. After pre-equilibration of the column with 95% Buffer A: 5% Buffer B, the UPLC was run on 95% Buffer A: 5% Buffer B for 1min, followed by a gradient of 5-60% Buffer B over 9min and then 100% Buffer B for 6min before re-equilibration for 4min. The flow rate was 500nl/min. The UPLC was coupled to a XEVO-TQS mass spectrometer (Waters) operating in negative-ion mode using the following settings: capillary 2.5kV, source temperature 150°C, desolvation temperature 600°C, cone gas flow rate 150 L/h and desolvation gas flow rate 1200 L/h. Folate metabolites were measured by multiple reaction monitoring.

The peak areas of individual folates were analysed using MassLynx software (Waters) [493]. The total peak area of each folate (sum of mono- and polyglutamated forms) was expressed as a percentage of the total folate in each sample.

2.5 Data analysis and statistics

Data presented in this thesis are representative of at least three independent experiments and shown as the mean $\pm$ standard error of the mean (SEM) unless indicated otherwise. Data analysis was carried out using the GraphPad Prism 8.0 Software (Graph Pad Software, CA, USA). Data were analysed by unpaired T-tests or one-way ANOVA and Sidak test for multiple comparisons as appropriate, unless indicated otherwise. For ANOVA analyses, the omnibus ANOVA p-value is given in the text. Patient data were shown as median (and interquartile range, IQR) and analysed using non-parametric Mann Whitney U tests or Wilcoxon matched pairs signed rank tests for paired data. Quantification of immunoblots was performed using the ImageJ software (v2.0). Flow cytometry data was analysed using the FlowJo software (BD Life Sciences). P-values <0.05 were considered significant.

3 Chapter 3 - The effects of MTX on static EC

3.1 Introduction

Although it is long recognised that patients with RA have an increased risk of premature death from cardiovascular disease [168], there is still no targeted treatment strategy to improve vascular function and reduce CVD events in this patient population. The current management of CVD in RA focusses on lifestyle recommendations, the reduction of traditional CVD risk factors and control of disease activity [182]. The latter recommendation originates from studies showing that the inflammatory burden in RA correlates with increased CVD risk [183] and that DMARD treatment such as MTX lowers CVD events in RA patients [182, 233]. The molecular mechanisms contributing to this beneficial effect on vascular function, however, are poorly understood.

As reviewed in section 1.3.3, patients with RA display impaired vascular function, likely induced by the potent systemic pro-inflammatory environment present in these patients [204]. $\text{TNF}\alpha$, a key pathogenic pro-inflammatory cytokine in RA that circulates in the peripheral blood, is thought to contribute to the activation of the canonical $\text{NF}\kappa\text{B}$ signalling pathway in vascular EC [204]. Briefly, $\text{TNF}\alpha$ -induced $\text{NF}\kappa\text{B}$ signalling is mediated by the activation of TNF receptors, which regulate the intracellular assembly of ubiquitin ligases and adaptor proteins that build a platform for the binding and activation of the $\text{I}\kappa\text{B}$ kinase (IKK) complex. Activated IKK phosphorylates $\text{I}\kappa\text{B}$, leading to its proteasomal degradation and release of the bound $\text{NF}\kappa\text{B}$ complex $\text{p}50/\text{p}65$ that translocates into the nucleus to induce target gene expression [46, 494]. IKK can also phosphorylate $\text{p}65$, which increases its transactivation ability [495]. The duration and extent of $\text{NF}\kappa\text{B}$ signalling is regulated by negative feedback loops, one of which is controlled by the ubiquitin modifying enzyme A20 [496]. $\text{NF}\kappa\text{B}$ activation results in the increased expression of endothelial cell adhesion molecules and inflammatory cell recruitment [46]; initiating events in atherogenesis (section 1.1.1).

One experimental treatment avenue aims to prevent or promote the reversal of inflammatory-driven EC dysfunction and early atherogenesis. Protecting EC from inflammatory insults may be achievable by inducing specific “cyto-protective” signalling pathways and the expression of anti-oxidant and anti-apoptotic molecules in EC [67]; concepts which have been explored in EC in studies on organ transplantation and ischaemic preconditioning during ischaemia/reperfusion injury [497]. Endothelial “cyto-protective” signalling pathways include

AMP-activated protein kinase (AMPK), Akt, mitogen-activated protein kinases (MAPK) and protein kinase C (PKC), which are implicated in EC survival, proliferation and apoptosis [67] as presented in section 1.2.3.

Supporting this idea, a previous study from our lab demonstrated that MTX activates AMPK and CREB signalling pathways in EC, mediating the expression of the anti-oxidant and anti-inflammatory molecules HO1 and manganese superoxide dismutase (MnSOD) [246]. Our group further showed that MTX reduces intramyocardial vasculopathy and end-organ damage in a mouse model of SLE. AMPK is a master kinase of endothelial homeostasis, regulating eNOS phosphorylation, vascular tone, apoptosis, redox status, NF κ B signalling and angiogenesis in vascular EC (reviewed in [81] and introduced in section 1.2.2). Based on the finding that MTX can activate AMPK in EC [246], it was hypothesised that MTX may have additional AMPK-dependent, but also independent effects in EC, which may mediate protection of EC from inflammatory activation and hence atherogenesis. The goal of this chapter was to explore the molecular effects of MTX in EC in the context of pro-inflammatory activation with TNF α and folate supplementation to model the patient vasculature. Specifically, the aim was to comprehensively assess (a) the effects of MTX on cell signalling and functional pathways in TNF α -treated EC, and (b) whether MTX inhibits TNF α -induced pro-inflammatory cell signalling and NF κ B activation in EC. The identification of novel effects of MTX in EC may explain and contribute to its anti-atherogenic effects in patients with RA.

3.2 Hypothesis of the chapter

MTX causes the activation of protective signalling pathways in EC, which prevent or reduce inflammatory-driven EC dysfunction.

3.3 Aims of the chapter

- 1) Determine the effect of MTX on cell signalling pathways in EC
- 2) Evaluate whether MTX inhibits TNF α -induced NF κ B signalling in EC
- 3) Investigate whether MTX affects cell cycle progression in EC
- 4) Determine if the effects of MTX are folate-dependent in EC

3.4 Results

3.4.1 MTX does not induce apoptosis in HAEC

To demonstrate that MTX does not reduce cell viability in EC, HAEC were treated with MTX (100nM) for 48h and stained with Annexin V (AnV) and propidium iodide (PI) to evaluate apoptosis and necrosis by flow cytometry (Figure 16). This timepoint and MTX concentration, which is achievable in the plasma of RA patients receiving low-dose MTX [249], had been previously used in the group [246] and in other reports [264, 277, 279, 295]. Serum starvation (SS) or hydrogen peroxide (H₂O₂) were used as positive controls for the induction of apoptosis and necrosis respectively, and markedly increased the proportion of AnV/PI double positive cells as expected (one-way ANOVA; $p=0.0009$) (Figure 16A, B and C). MTX did not significantly increase the percentage of AnV, PI or double positive cells, suggesting that the drug did not induce apoptosis or necrosis in EC at that timepoint (Figure 16C). Furthermore, MTX did not significantly affect the cell number of similarly treated HUVEC after 48h (Figure 16D).

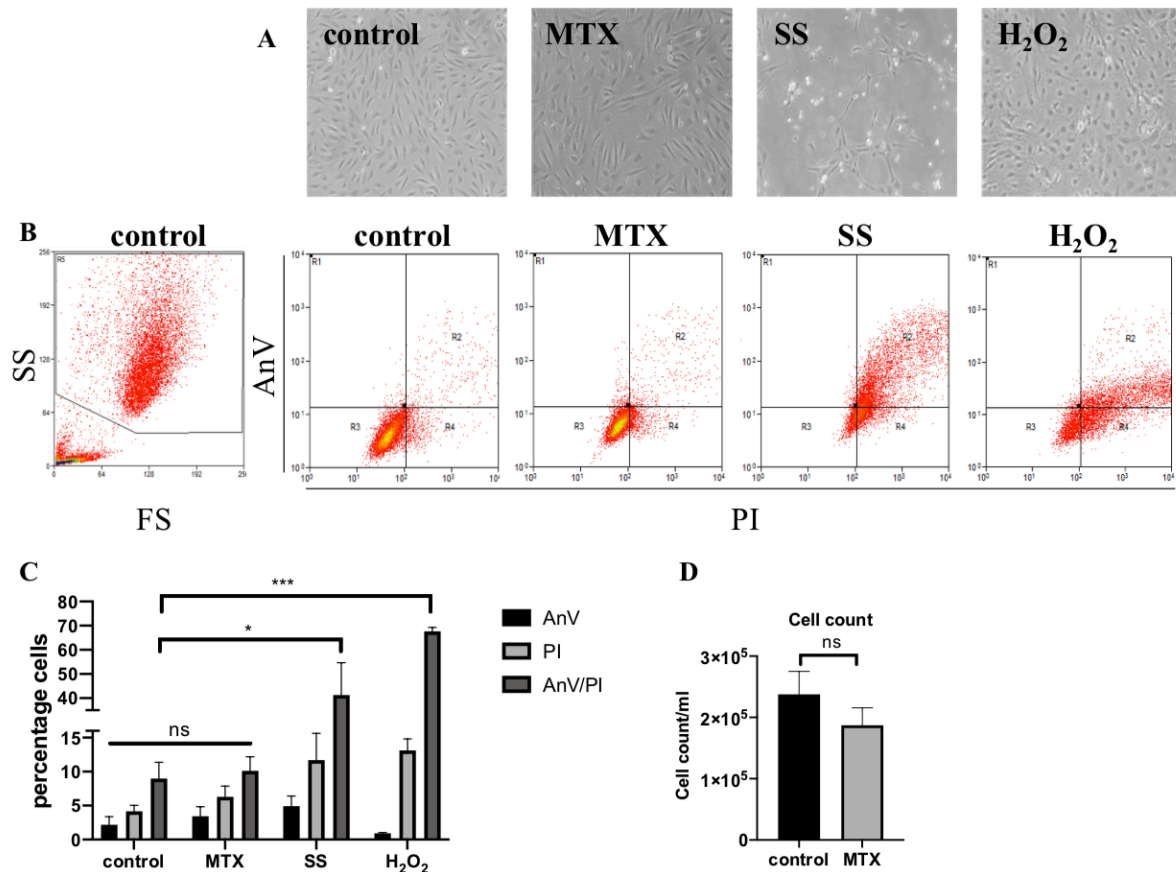


Figure 16. MTX does not induce apoptosis in EC. (A-C) HAEC were treated with either media containing 10% FCS (control), MTX (100nM; 48h), H₂O₂ (200μM; 3h) or were serum starved in 0% FCS (SS; 48h). (A) Representative bright field images (magnification 20x) of respectively treated cells. (B) Flow cytometry profiles of cells treated as described above. Cells were stained with annexin V (AnV) and propidium iodide (PI) and analysed by flow cytometry (n=3). (C) Quantification of AnV, PI or double positive HAEC treated as stated above. Data shown as percentages of cells positive for AnV, PI or both (n=3). Data analysed using a one-way ANOVA and Sidak test for multiple comparisons. (D) Cell count of HUVEC grown in the absence (control) or presence of MTX (100nM) for 48h (n=5). Unpaired t-test. Values represent means +/- SEM. ns, not significant. * p<0.05. ** p<0.01. *** p<0.001.

3.4.2 MTX induces multiple cell signalling pathways in HAEC

To study the effects of MTX on cell signalling in inflammatory-activated EC as a model of the patient vasculature, HAEC were treated with TNF α (0.1ng/mL) for 8h before adding MTX for 48h to give the following conditions: control (media), MTX, TNF α and TNF α +MTX. The cells were then harvested for analyses on serine-threonine (STK) and tyrosine (PTK) phospho-kinase activity arrays (Figure 17 and Supplementary Figure 1; for assay principal see section 2.3.3). All conditions were compared to control.

Figure 17A depicts volcano plots of changes in peptide phosphorylation for each treatment versus control from the STK arrays. MTX alone induced the differential phosphorylation of 51 peptides compared to control ($\log_2$ FC range -0.69 to 1.21; $p < 0.05$) in the STK arrays (Figure 17A). When bioinformatically grouped together, differential phosphorylation of these peptides translated into an increased activity of kinases in the PKG, PKA, PKC, Akt and MAPK (MAPKAPK2 and 3) families among others (Figure 17C). TNF α treatment altered the phosphorylation of 55 peptides ($\log_2$ FC range -0.19 to 1.41; $p < 0.05$) and increased the activity of kinases in the MAPK pathway (ERK5, p38, JNK, MSK1, MAPKAPK2 and 3, RSK2) as expected. Co-treatment of TNF α and MTX altered the phosphorylation pattern of 73 peptides ($\log_2$ FC range -0.09 to 2.34; $p < 0.05$), resulting in the increased activities of PKA, PKG, PKC, Akt and kinases in the MAPK pathway. Comparing the predicted kinase profiles from each condition demonstrated that MTX and co-treatment of TNF α and MTX displayed the highest number of shared targets (73% of kinases), while TNF α alone showed equal overlaps with the other conditions (27% of kinase hits each) in the STK arrays.

On the PTK arrays, MTX treatment significantly affected 55 peptides ($\log_2$ FC range -0.8 to 1.1; $p < 0.05$), which was predicted to be the result of the increased activity of members of the Src tyrosine kinase family (Src, Lck, Lyn, Blk, Fgr, Fyn, Frk) and Abl kinases (Abl and Arg) (Supplementary Figure 1). TNF α promoted the regulation of 16 peptides ($\log_2$ FC range -3.45 to 0.85; $p < 0.05$), causing a putative increase in kinase activity in the Eph/Ephrin pathway (EphA8, EphA4), neurotrophic receptor tyrosine kinases (TRKB and TRKC), and Src kinases (Src, Blk). Combined TNF α and MTX treatment altered the phosphorylation of 84 peptides ($\log_2$ FC range 0.29 to 3.49; $p < 0.05$). This resulted in the increased activities of Src kinases (Src, Lck, Lyn) and the insulin receptor-related receptor (IRR) kinase among others. In the PTK arrays, all conditions showed the same number of shared kinase hits. These findings

demonstrated that MTX had independent effects on cell signalling in EC. Although there were some commonalities (e.g. Akt, MAPAPAK2/3, Src), the majority of MTX- and TNF α -activated kinases were different. Interestingly, co-treatment of TNF α and MTX showed a dominance for MTX-induced pathways.

The results of the phospho-kinase arrays were independently validated by immunoblotting for Akt, p38 (as MAPK target) and PKC ϵ (as PKC target) (Figure 17D and E) as these pathways have been previously associated with roles in EC homeostasis [67]. TNF α significantly increased the expression of its known target protein VCAM1 (292.4-fold compared to control; $p < 0.0001$), which was used as positive control. Of note, MTX showed a trend to also induce VCAM1 protein expression, albeit at far lower levels compared to TNF α , and did not further affect VCAM1 levels in combination with TNF α . MTX induced the phosphorylation of Akt (3.1-fold; $p = 0.0127$) and p38 (13.9-fold; $p = 0.0071$) in EC compared to control (Figure 17E), but interestingly did not activate p38 at an earlier timepoint in HAEC (Supplementary Figure 2). Moreover, despite not reaching statistical significance, MTX seemed to also promote PKC ϵ phosphorylation in EC (2-fold; $p = 0.4079$). In contrast, TNF α alone showed a trend to increase the phosphorylation of p38 (3.2-fold, ns) but not Akt or PKC ϵ . Pre-treatment with TNF α did not affect the MTX-mediated induction of Akt or p38 phosphorylation (Figure 17E).

Taken together, these results suggested that MTX did not affect TNF α -activated cell signalling in these experiments. MTX independently increased the activity of multiple cell signalling pathways in EC independent of prior pro-inflammatory activation with TNF α , including Akt and p38 pathways.

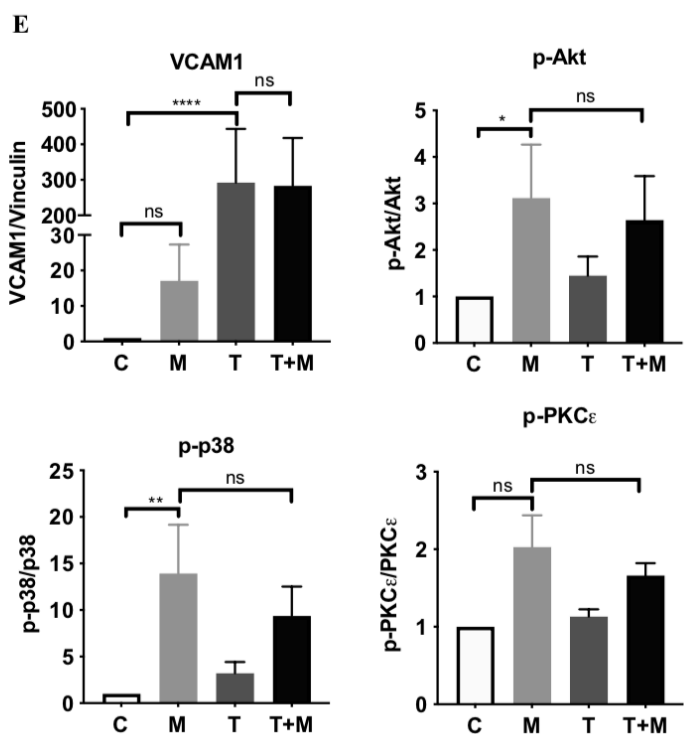
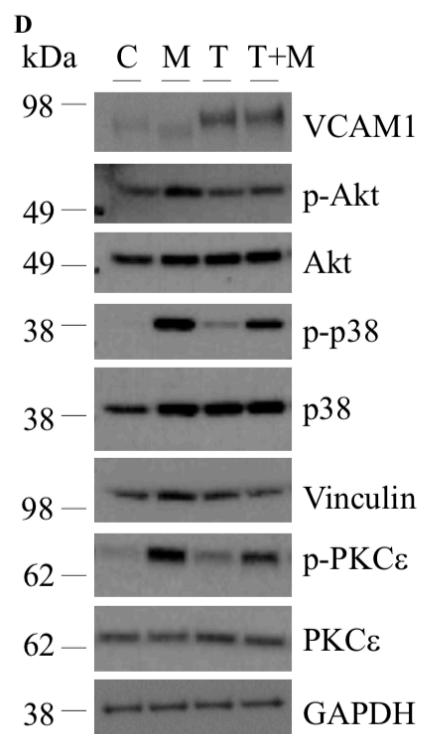
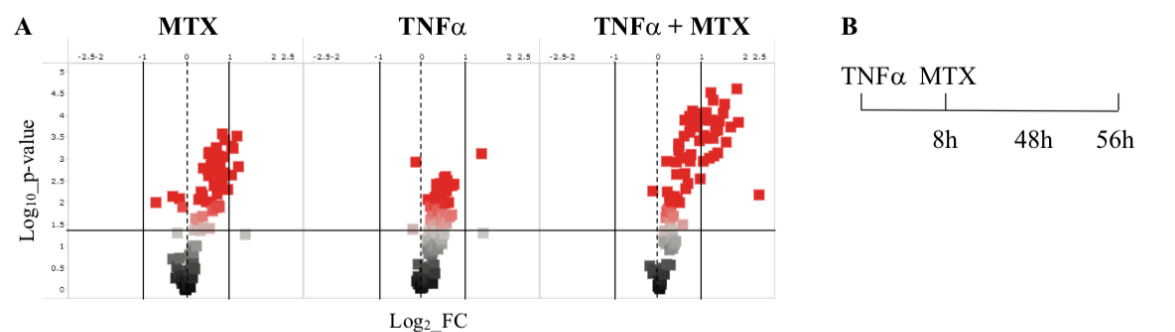


Figure 17. MTX induces multiple cell signalling pathways in EC independent of prior pro-inflammatory activation with TNF α . (A-E) HAEC were pre-treated with media (control) or TNF α (0.1ng/ml) for 8h. Media or MTX (100nM) were added to the cells for another 48h (TNF α treatment for total of 56h). Experimental outline shown in (B). (A) Volcano plots of differentially phosphorylated peptides in a given condition over control from STK arrays (n=3). (C) Top 15% of kinase hits from STK arrays performed on respectively treated cells. Final Score indicates combined measure of significance and specificity of permutation analyses. The kinase statistic is computed by the overall change of the peptide set that represent a kinase (value >0 means that kinase is more active in test over control sample). (D) Validation of phospho-kinase arrays by immunoblotting of cell lysates from HAEC treated as described above (C, control; M, MTX; T, TNF α ; T+M, TNF α + MTX). Whole protein lysates were separated by SDS-PAGE. Proteins were detected with antibodies against VCAM1 (95kDa), p-p38 T180/Y182 (38kDa), p38 (38kDa), p-Akt S473 (60kDa), Akt (60kDa), p-PKC ϵ S729 (82kDa), PKC ϵ (82kDa), Vinculin (124kDa) and GAPDH (39kDa). Representative blots shown (n=4-5). (E) Quantification of VCAM1 levels and p38, Akt and PKC ϵ phosphorylation in HAEC treated as described above (n=4-5). Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. Values represent means $\pm$ SEM. ns, not significant. * p<0.05. ** p<0.01. *** p<0.001. **** p<0.0001.

3.4.3 MTX also induces p38 and Akt phosphorylation in HUVEC

Because HUVEC are more readily available and more cost-effective to work with than HAEC, it was examined whether MTX also induced Akt and p38 phosphorylation in HUVEC. To this end, time course experiments were performed in which HUVEC were treated with media (control) or MTX at time points ranging from 2h to 48h (Figure 18A and B). Our group had previously demonstrated that MTX induces the phosphorylation of AMPK in EC, which was used as a positive control. MTX caused an approximately 2-fold increase in AMPK phosphorylation at 48h (p=0.013), in line with previous results [246]. Compared to control, Akt phosphorylation was increased 3.7-fold (p=0.006) by MTX only at the 48h timepoint, while a double peak was seen for p38 phosphorylation: a 2.5-fold induction at 2h (p=0.003), which returned to baseline at 4h and rose again at 48h (10.6-fold induction; p=0.0047). These experiments suggested that MTX similarly induced Akt and p38 phosphorylation in HUVEC and HAEC. The 48h time point gave the most prominent activation of p38 and Akt and was therefore chosen for further experiments.

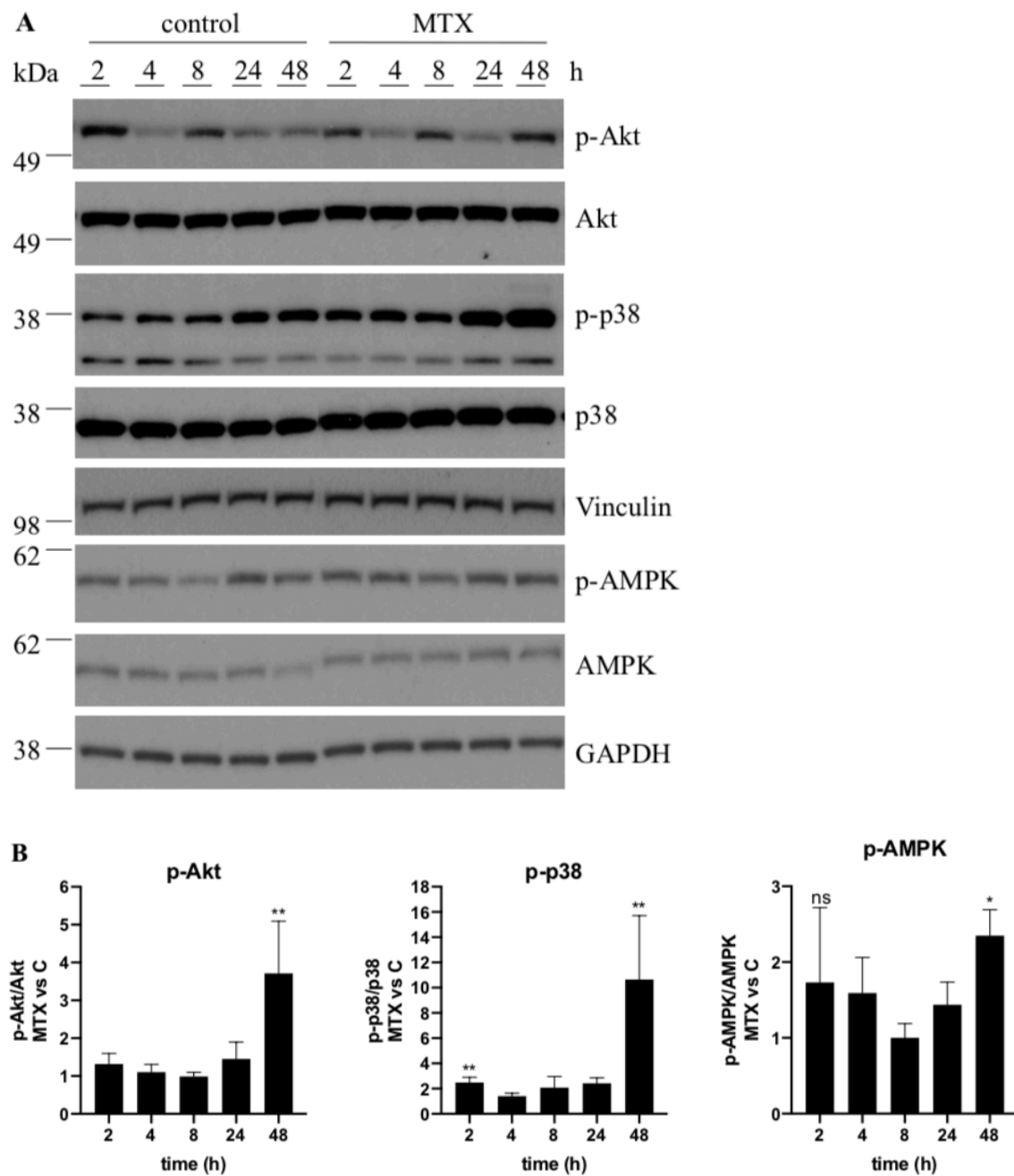


Figure 18. MTX induces p38 and Akt phosphorylation in HUVEC. (A) Immunoblot analyses of HUVEC treated with media (C, control) or MTX (100nM) for indicated time points. Whole protein lysates of respectively treated cells were separated by SDS-PAGE. Proteins were detected with antibodies against p-AMPK α T172 (62kDa), AMPK α (62kDa), p-p38 T180/Y182 (38kDa), p38 (38kDa), p-Akt S473 (60kDa), Akt (60kDa), GAPDH (39kDa) and Vinculin (124kDa). Representative blots shown (n=4-5). For presentation purposes, the order of the lanes was re-arranged. (B) Quantification of Akt, p38 and AMPK α phosphorylation levels in HUVEC treated as described above (n=4-5). Values represent means $\pm$ SEM. Data shown as fold change over the respective control. Unpaired T-Test. ns, not significant. * $p < 0.05$. ** $p < 0.01$.

3.4.4 MTX-induced phosphorylation of p38, but not Akt, is AMPK-dependent

To determine if there is cross talk between MTX-induced AMPK, p38 and Akt signalling, HUVEC were left untreated (UT) or transfected with adenoviruses either expressing an empty vector (Ad0) as a control or dominant negative (DN)-AMPK α 1, followed by treatment with MTX for 48h (Figure 19A and B). DN-AMPK α 1 was well expressed in the respectively treated cells and reduced AMPK phosphorylation at baseline. MTX induced the phosphorylation of AMPK (2.5-fold; $p=0.002$), p38 (2.5-fold; $p=0.0093$) and, although not significantly, Akt (11.5-fold; $p=0.0541$) in Ad0-transfected cells as expected. This response was completely abrogated for p38, but not Akt, in the presence of DN-AMPK α 1, demonstrating that phosphorylation of p38 by MTX is AMPK-dependent, while Akt is activated by MTX in a different manner.

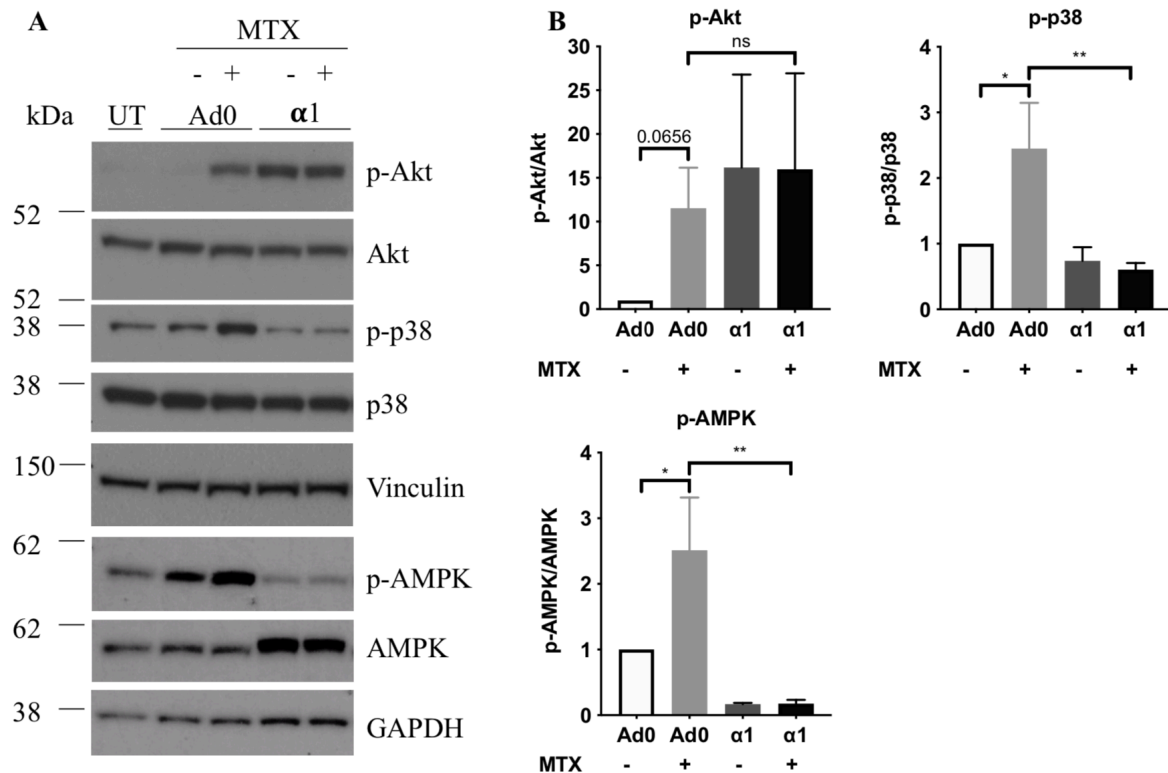


Figure 19. MTX-induced phosphorylation of p38 is AMPK-dependent. (A) Immunoblot analyses of HUVEC, which were either untreated (UT), transfected with a recombinant empty vector adenovirus (Ad0) or adenovirus expressing dominant negative (DN)-AMPK $\alpha 1$ D157A ($\alpha 1$). Media or MTX (100nM) were added to the cells 8h post adenoviral transfection for a further 48h. Whole protein lysates of respectively treated cells were separated by SDS-PAGE. Proteins were detected with antibodies against p-AMPK α T172 (62kDa), AMPK α (62kDa), p-p38 T180/Y182 (38kDa), p38 (38kDa), p-Akt S473 (60kDa), Akt (60kDa), GAPDH (39kDa) and Vinculin (124kDa). Representative blots shown (n=3). (B) Quantification of p38, AMPK α and Akt phosphorylation levels in HUVEC treated as described above (n=3). Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. ns, not significant. * $p < 0.05$. ** $p < 0.01$. Values represent means $\pm$ SEM. The DN-AMPK $\alpha 1$ adenovirus was kindly provided by Prof. David Carling (Imperial College London).

3.4.5 MTX does not inhibit TNF α -induced NF κ B activation in EC

Previous reports showed that AICAR, a known AMPK activator, inhibited NF κ B signalling in EC [498], while MTX abrogated NF κ B activation in other cell types [277, 288]. To understand if MTX inhibits pro-inflammatory NF κ B signalling in EC, HUVEC were pre-treated with MTX and then incubated with TNF α for 15min (cell signalling) or 4h (transcriptional activity by luciferase assay and gene expression) (Figure 20B), giving the following conditions: control (media), MTX, TNF α and MTX+TNF α .

TNF α promoted the transcriptional activity of NF κ B as expected (54.8-fold compared to control; $p=0.0067$). MTX pre-treatment did not affect this outcome (TNF α 54.8-fold vs MTX+TNF α 75.4-fold; ns) (Figure 20A). In line with activation of the NF κ B signalling pathway, TNF α increased the phosphorylation of p65 (95-fold; $p=0.0059$) and caused the degradation of I κ B α (60% reduction; $p=0.0123$) in EC compared to control (Figure 20C and D). However, MTX did not prevent TNF α -induced phosphorylation of p65 or degradation of I κ B α . Similarly, TNF α induced the mRNA expression of known NF κ B target genes such as VCAM1 (963.9-fold; $p=0.0002$) and E-selectin (294.7-fold; $p<0.0001$) compared to control, while ICAM1 (144.8-fold; ns) and A20 (16-fold; ns) did not reach statistical significance. MTX did not inhibit the mRNA expression of these target genes, and co-treatment of MTX+TNF α even increased E-selectin or A20 expression compared to TNF α alone (Figure 20E). Surprisingly, although not reaching statistical significance, MTX seemed to independently induce the expression levels of these NF κ B target genes 5- to 15-fold compared to control. Taken together, MTX did not inhibit TNF α -induced activation of NF κ B signalling in EC.

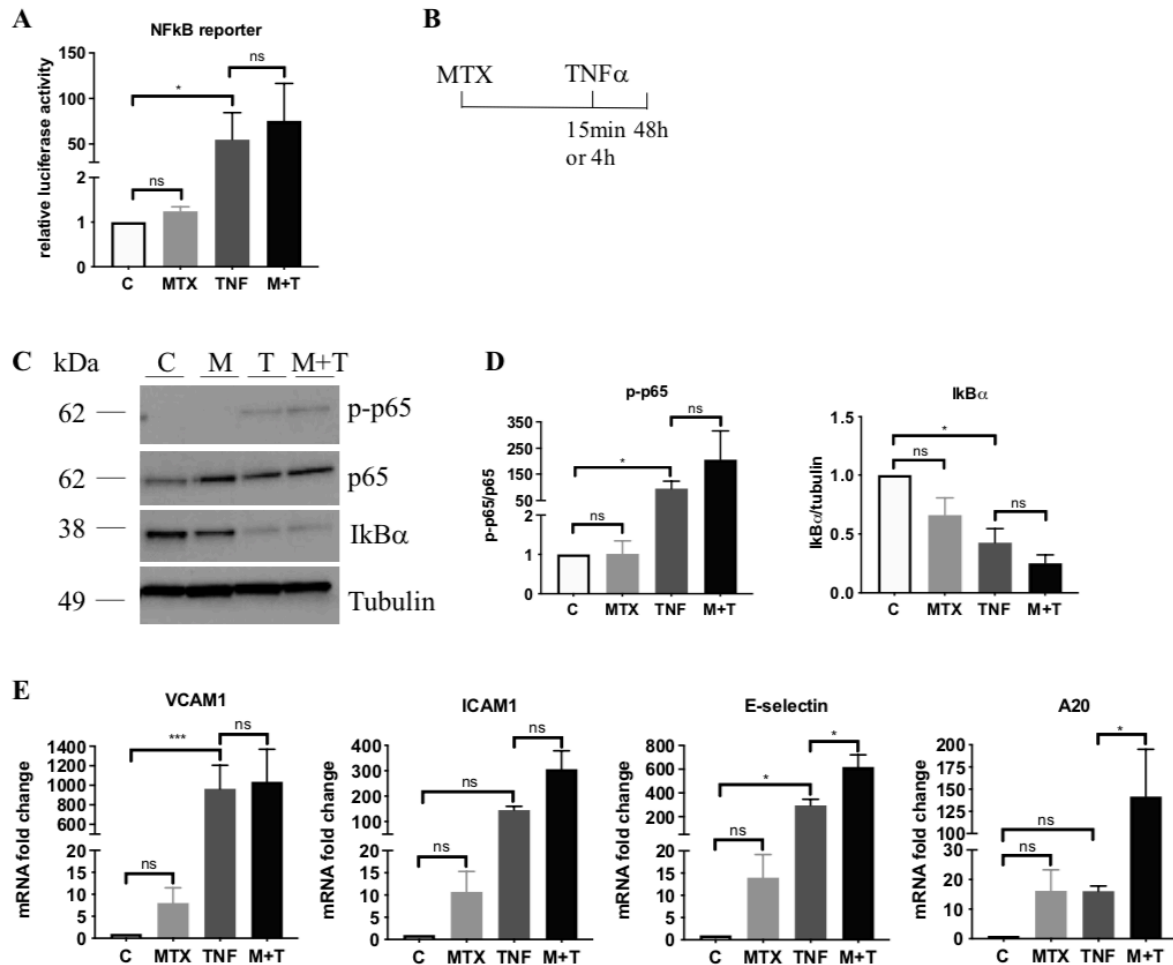


Figure 20. MTX does not inhibit TNFα-induced NFκB activation in EC. (A) NFκB luciferase reporter assay. HUVEC were transfected with a recombinant adenovirus expressing a NFκB luciferase reporter. Two hours post transfection, cells were treated with media (C, control) or MTX (100nM) for 48h. Media or TNFα (0.1ng/ml) were added for 4h alone or in combination with MTX (M+T). NFκB activity was measured using a Luciferase Reporter Assay System. Data shown as fold change of relative luciferase activity normalised to the control (n=4). (B-E) HUVEC were treated with media (C, control) or MTX (M; 100nM) for 48h. Media or TNFα (T; 0.1ng/ml) were added alone or in combination with MTX (M+T) for 15min (cell signalling) or 4h (gene expression) as outlined in (B). (C) Immunoblot analyses of respectively treated cells. Whole protein lysates were separated by SDS-PAGE. Proteins were detected with antibodies against p-p65 S536 (65kDa), p65 (65kDa), IκBα (39kDa) and α-tubulin (50kDa) (n=3-4). (D) Quantification of p-p65 and IκBα protein levels in HUVEC treated as described in (B) (n=3-4). (E) Gene expression analyses of HUVEC treated as described above by qPCR. Gene expression normalised to GAPDH (n=4). Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. Values represent means +/- SEM. ns, not significant. * p<0.05. ** p<0.01. *** p<0.001. Data in part produced by Jose Lopez Ayala (MSc student) under the supervision of Marie Lang.

3.4.6 MTX causes S-phase cell cycle arrest in EC in a folate-dependent manner

Both Akt and p38 signalling are involved in regulating cell cycle progression (section 1.2.3). Therefore, it was hypothesised that Akt and p38 activation by MTX may affect this process in EC. Because MTX is a folate analogue known to inhibit folate-dependent enzymes in different cell types [230], it was investigated whether any changes in cell cycle progression induced by MTX could be reversed by folate supplementation. To determine if MTX affected the cell cycle in EC via folate depletion in the context of pro-inflammatory activation, HUVEC were treated with TNF α for 8h before adding MTX for 48h. After the first 24h of MTX treatment, folinic acid (FA) was added for another 24h (Figure 21A), giving the following conditions: control (media and vehicle), MTX, TNF α , TNF α +MTX, FA, MTX+FA, TNF α +FA and TNF α +MTX+FA. The cells were then harvested for cell cycle analyses by PI staining and flow cytometry (Figure 21B and C).

MTX increased the percentage of cells in the S-phase of the cell cycle (MTX 30.95% vs control 17.6%; ANOVA $p=0.0001$), while reducing the number of cells in the G2/M phase compared to control (MTX 6.35% vs control 11.1%; ANOVA $p<0.0001$) (Figure 21B, upper panel, and 21C), suggesting that the drug induced S-phase arrest in EC. TNF α alone had no effects on cell cycle progression and did not affect MTX-induced cell cycle arrest (S-phase: MTX 30.95% vs TNF α +MTX 29.2%, ns; G2/M phase: MTX 6.35% vs TNF α +MTX 5.2%, ns). FA alone had no effects on the distribution of cells in the different phases of cell cycle but rescued MTX-treated cells from undergoing S-phase arrest (MTX 30.95% vs MTX+FA 20.2%) (Figure 21B, lower panel, and 21C). FA was also able to reverse MTX-induced changes in cell cycle in the presence of TNF α (S-phase: TNF α +MTX 29.2% vs TNF α +MTX+FA 10.9%; G2/M phase TNF α +MTX 5.2% vs TNF α +MTX+FA 16.1%).

These results demonstrated that MTX induced cell cycle arrest in S-phase in EC independent of prior pro-inflammatory activation with TNF α . This effect was rescued by supplementation with FA, suggesting that MTX prevented cell cycle progression by inhibiting folate metabolism in EC.

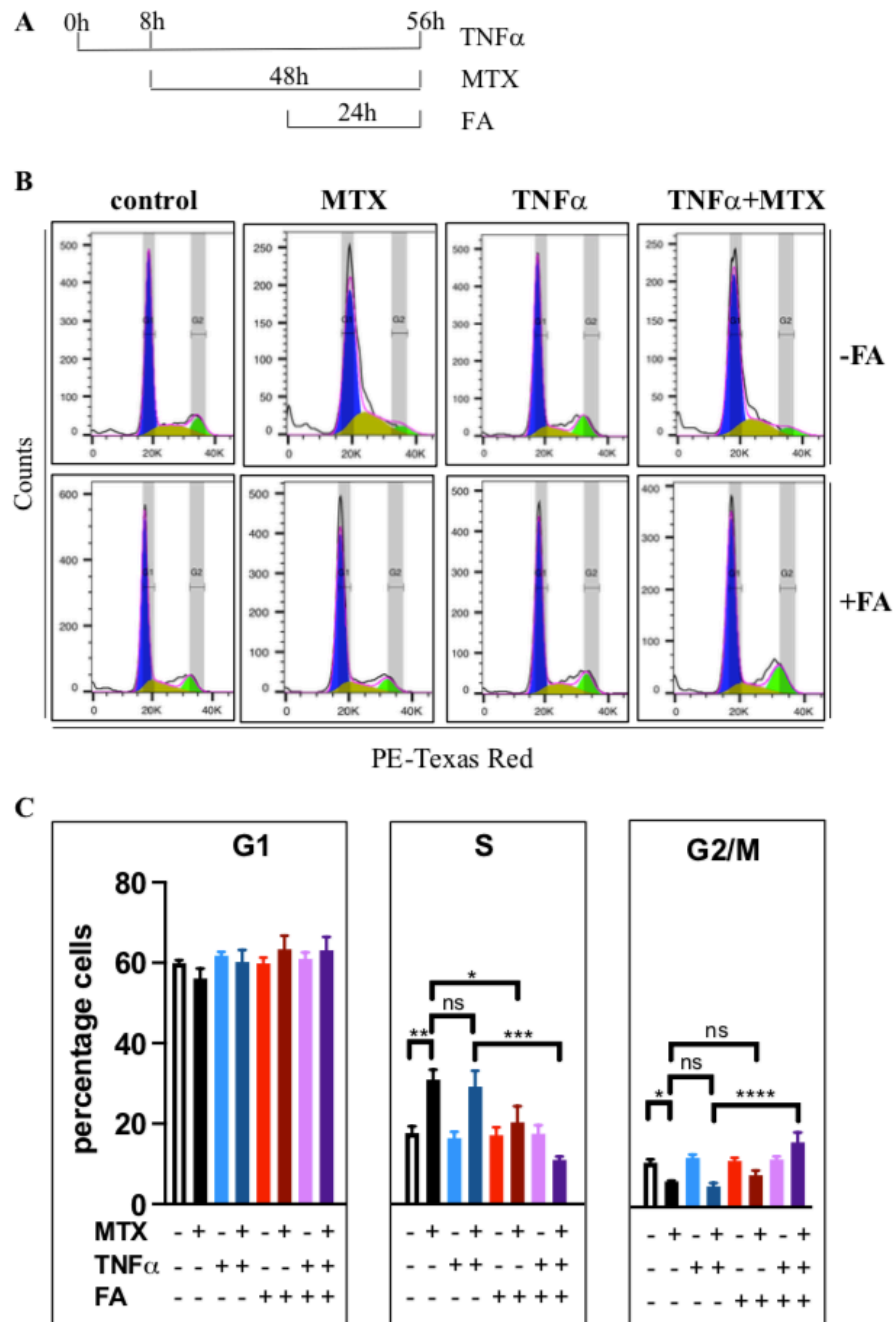


Figure 21. MTX induces S-phase cell cycle arrest in EC in a folate-dependent manner. (A-C) HUVEC were treated with media (control) or TNF α (0.1ng/ml) for 8h. Media or MTX (100nM) were then added for 48h. After the first 24h of MTX treatment, vehicle or folinic acid (FA; 500nM) were added for 24h as outlined in (A). (B) Representative flow cytometry profiles of respectively treated cells stained with propidium iodide (PI) and analysed by flow cytometry (n=4). Phases of cell cycle coloured as following: G1, blue; S, yellow, G2/M, green. (C) Quantification of cell cycle experiments performed as stated above (n=4). Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. Values represent means $\pm$ SEM. ns, not significant. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$.

3.4.7 MTX-induced changes in cell signalling and gene expression are folate-dependent

To understand if the effects of MTX on Akt and p38 signalling and gene expression were also dependent on folate inhibition in EC, HUVEC were pre-treated with MTX for 24h, after which FA was added for another 24h (total MTX exposure 48h) (Figure 22A), resulting in the following experimental conditions: control (media and vehicle), MTX, FA and MTX+FA co-treatment. The cells were then harvested for immunoblot (Figure 22B and C) or gene expression analyses (Figure 22D and E).

FA abrogated the MTX-induced phosphorylation of p38 (13.9- vs 1.9-fold induction; $p=0.0229$) and Akt (2.73- vs 0.52-fold; $p=0.0297$), but had no effects on the baseline phosphorylation levels of these kinases compared to control as evaluated by immunoblot analyses (Figure 22B and C). Of note, FA was also able to revert MTX-induced p38 phosphorylation in the presence of $\text{TNF}\alpha$ (Supplementary Figure 3). At the gene expression level, MTX robustly induced the mRNA expression of the anti-inflammatory molecules A20 (39.8-fold; $p=0.0129$) and dual specificity phosphatase 1 (DUSP1) (6-fold; $p=0.0008$) (Figure 22D), while also increasing the levels of the pro-inflammatory cell adhesion molecule VCAM1 (7.3-fold; $p=0.0341$) compared to control (Figure 22E). A similar trend was observed for ICAM1 (16.6-fold; $p=0.0509$) and E-selectin (SELE) (30.6-fold; $p=0.1433$). FA reversed the MTX-induced expression of A20, DUSP1 and VCAM1 back to baseline levels, showing a similar, yet non-significant, effect for ICAM1 and E-selectin.

These findings implied that the MTX-induced activation of Akt and p38 signalling and increase in mRNA expression of A20, DUSP1 and VCAM1 were caused by folate depletion in EC.

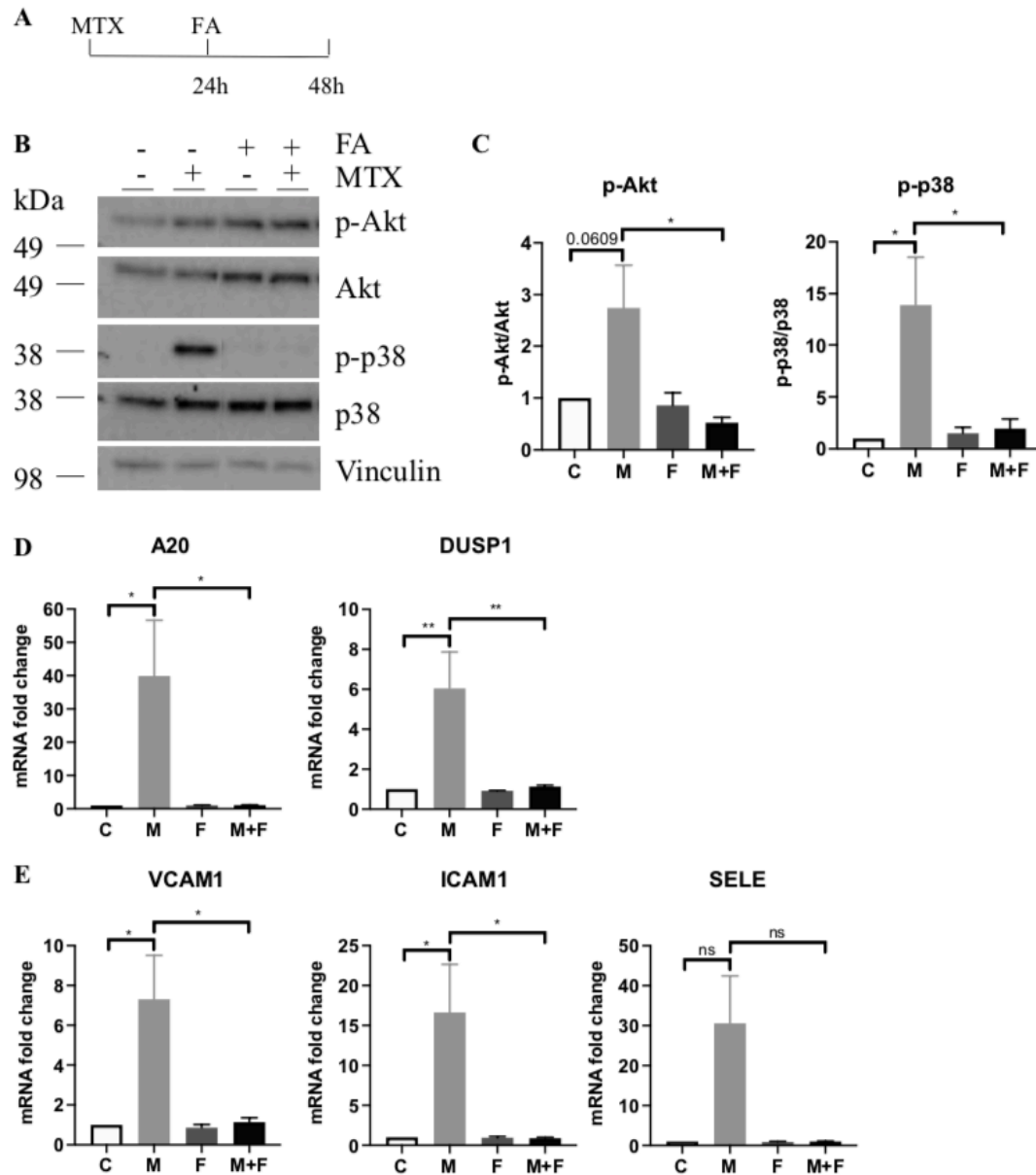


Figure 22. MTX-induced changes in cell signalling and gene expression are folate-dependent in EC. (A-E) HUVEC were treated with media (control) or MTX (100nM) for 48h. After the first 24h of MTX treatment, folinic acid (FA; 500nM) or vehicle control were added for 24h as outlined in (A). (B) Immunoblot analyses of HUVEC treated as above. Whole protein lysates of respectively treated cells were separated by SDS-PAGE. Proteins were detected with antibodies against p-p38 T180/Y182 (38kDa), p38 (38kDa), p-Akt S473 (60kDa), Akt (60kDa) and Vinculin (124kDa) (n=4). For presentation purposes, the order of the lanes was re-arranged. (C) Quantification of p38 and Akt phosphorylation in HUVEC treated as described above (n=4). (D+E) Gene expression analyses of anti-inflammatory (D) and pro-inflammatory molecules (E) in respectively treated cells by qPCR. Data were normalised to GAPDH and shown as fold change over control (n=5). C, control; M, MTX; F, FA; M+F, MTX+FA. Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. Values represent means $\pm$ SEM. ns, not significant. * $p < 0.05$. ** $p < 0.01$.

3.5 Discussion

The goal of this chapter was to identify novel effects of MTX in vascular EC in the context of a pro-inflammatory environment, which may explain the drug's anti-atherogenic actions in patients with RA. In this chapter, MTX independently increased the activity of multiple cell signalling pathways, induced the phosphorylation of p38 and Akt and cell cycle arrest in TNF α -activated EC. These effects were caused by MTX-induced folate depletion. MTX did not inhibit TNF α -induced NF κ B activation or target gene expression in EC. These results were unexpected in light of previous reports demonstrating that MTX reduced CVD risk and improved endothelial function in patients with inflammatory arthritis including RA [203, 233] and question the validity of using static *in vitro* experiments to model this response.

The results from the phospho-kinase activity arrays indicated that MTX induced the activity of multiple kinases in EC, most of which were part of the AGC kinase family (protein kinase A, G and C) and MAPK pathway. Although not shown in Figure 17, AMPK was among the top 26 ranked putative active kinases in MTX-treated EC in line with previous findings [246]. It is not clear how MTX achieves the activation of these pathways in EC but it is interesting to note that the MTX-induced kinases are known to be activated by cyclic AMP/GMP (PKA [499] and PKG [500]), lipids (PKC [501]), nucleotide depletion (AMPK) [81] and growth factors (Akt) [502]; possibly hinting at a metabolic mode of activation by MTX. These kinase families form a central cell signalling nexus and induce pleiotropic context-dependent effects in EC, including cell survival, proliferation, apoptosis, angiogenesis, eNOS phosphorylation and inflammatory cell adhesion [81, 104, 500, 502, 503]. This implies that MTX treatment could affect fundamental cellular functions in EC. Reports on the effects of MTX on cell signalling in EC are sparse. However, there is evidence that MTX can regulate MAPK and Akt signalling pathways in other cell types as the drug has been shown to modulate the activation of JNK in Jurkat T-cells and human PBMC [279], p38 in lung epithelial cell lines [504], Ras in mouse embryonic fibroblasts [272] and Akt in cancer cells [505], which conferred effects on cell proliferation, apoptosis or inflammation.

MTX did not inhibit TNF α -activated cell signalling in EC following long- or short-term pro-inflammatory activation. In the kinase arrays, low-dose, long-term TNF α treatment promoted the activation of multiple kinases in the MAPK pathway, which are well known to be TNF α -inducible [506]. MTX did not prevent the TNF α -induced activation of MAPK signalling.

Rather, co-treatment of MTX and TNF α seemed to achieve additive effects on cell signalling, mirroring the kinase profile induced by MTX alone. Although it can be argued that the time point chosen for the TNF α treatment was too long to see reversal of cell signalling, MTX also did not inhibit TNF α -induced p38 phosphorylation (data not shown) and NF κ B signalling in EC when TNF α was added acutely to MTX-treated EC (15min to 4h). This was demonstrated at the level of NF κ B transcriptional activation, cell signalling and target gene expression. Parallel to the lack of inhibition, MTX even induced the expression of pro-inflammatory cell adhesion molecules and the anti-inflammatory molecules A20 and DUSP1, which constitute a negative feedback loop for NF κ B and MAPK activation respectively [507], in a folate-dependent manner. MTX alone did not activate canonical NF κ B signalling in this study but could potentially affect non-canonical pathways. These findings were unexpected and in contrast to earlier studies in other cell types and EC, in which MTX inhibited TNF α -induced NF κ B activation [276, 277] and cell adhesion molecule expression [293]. These divergent responses may be explained by several factors. The MTX concentration and timepoint used in this study were identical to the conditions used on T-cells and fibroblast like synoviocytes by Spurlock et al. [277], suggesting that MTX may have cell type-specific effects on NF κ B activation. In comparison to the studies performed in HUVEC [276, 293], the most noticeable shared difference is the shorter MTX treatment time points used: 2-8h versus the 48h timepoint in this study. It remains to be tested if shorter MTX pre-treatment would result in the inhibition of TNF α -induced NF κ B activation in EC in this system. No other effects of the drug were noticeable at shorter timepoints in this study. These results demonstrated that MTX activated cell signalling in EC independent of TNF α .

Due to their importance in EC homeostasis, Akt, the MAPK p38 and PKC ϵ were chosen as targets for further validation by immunoblotting, while AMPK was a known target for MTX as previously reported by our group. In EC, Akt activation is associated with cell survival and inhibiting apoptosis, cell cycle progression and eNOS phosphorylation [508], while p38 signalling regulates cell proliferation, inflammation and apoptosis [86]. PKC ϵ has been reported to promote cell survival, eNOS activation, anti-inflammatory and anti-apoptotic pathways in EC [67]. Indeed, MTX increased the phosphorylation of Akt and p38, but not PKC ϵ , in HAEC and also in HUVEC at 48h (and not at earlier time points). Interestingly, AMPK activation was necessary for MTX-induced p38, but not for Akt, phosphorylation, suggesting cross talk between AMPK and p38 signalling upon MTX treatment in EC. In T-

cells, AMPK was shown to induce p38 autophosphorylation by recruitment to the TAB1 (TAK1-Binding Protein 1) complex, which inhibited T-cell proliferation and induced senescence [509]. In cancer cells, metabolic stress activated AMPK-p38 signalling to promote the maintenance of cellular energy status and cell survival by increasing mitochondrial oxidative phosphorylation [510]. In contrast, AMPK and Akt have antagonistic effects on cell metabolism and inversely regulate each other in different cell types [511-513], matching the observation that EC expressing DN-AMPK α 1 showed higher baseline Akt phosphorylation compared to control (Figure 19). However, other studies show that AMPK can activate Akt signalling to induce eNOS phosphorylation in EC [514, 515]. It is difficult to extrapolate the functional outcomes of cell signalling pathways from different studies due to their context-dependent functions. Due to the implication of these pathways in the regulation cell proliferation, cell cycle and survival, further experiments were performed to study the role of MTX in endothelial cell cycle regulation.

In this study, MTX induced cell cycle arrest in S-phase in EC, which was completely rescued by supplementation with folinic acid. This effect was again independent of prior pro-inflammatory activation with TNF α . Furthermore, folinic acid also reversed the MTX-mediated phosphorylation of p38 and Akt, implying that the changes in cell signalling were linked to the cell cycle arrest. Folate is required for cellular nucleotide synthesis and therefore for cell cycle progression [317]. MTX has been shown to induce cell cycle arrest in cancer cells [516]. EA.hy 926 cells grown under low folate conditions also accumulate in S-phase [394] and activate p38 signalling [395]. Interestingly, MTX caused a sustained activation of Akt in cancer cells, which impaired cell cycle progression and induced apoptosis via regulation of CDK2 [505]. These results suggest that low-dose MTX leads to a metabolic block by inhibiting the folate pathway in EC, which causes the activation of p38 and Akt concurrent with cell cycle arrest. Activation of these cell signalling pathways by MTX may attempt to replenish cellular energy levels and preserve cell survival.

MTX promoted both the activation/expression of molecules or pathways that are considered “protective” (AMPK, Akt, A20) or “deleterious” (pro-inflammatory cell adhesion molecules) in EC. In this regard, it is noteworthy to point out the findings of a recent study, which investigated the molecular effects of MTX on macrophages. The paper reported that MTX inhibited LPS-induced NF κ B activation and induced A20 expression in macrophages, which

was proposed as a novel biomarker of MTX response in RA patients [278]. Importantly, however, the authors argued that MTX induced an overall pro-inflammatory response in macrophages [288], which limited further pro-inflammatory activation [278]. Whether the inhibition of secondary pro-inflammatory activation and upregulation of A20 in the context of a generalised pro-inflammatory response is sufficient to claim anti-inflammatory effects of the drug is debatable. This point emphasises the critical question whether the induction of a stress response by an anti-metabolite drug can confer a protective outcome, which is inherently difficult to prove in an *in vitro* context. Although MTX did not induce apoptosis or necrosis in the current experimental setup, it is possible that the drug may increase apoptosis at a later time point as a consequence of cell cycle arrest; an effect that has been demonstrated in cancer cells. Given that MTX did not inhibit TNF α -mediated pro-inflammatory activation and induced cell cycle arrest, the results from this chapter suggest that the beneficial effects of MTX on the vasculature are achieved in a different manner.

The data showed that MTX inhibited folate metabolism in EC. Multiple reports demonstrate folate-dependent effects of MTX in different cell types (e.g. [276, 277]). MTX has been previously shown to inhibit the folate pathway, reduce cell proliferation and elicit a pro-inflammatory phenotype in an endothelial-epithelial hybrid cell line [298], similar to the effects seen in the current study. Moreover, MTX inhibited the folic acid mediated induction of NO production by inhibiting DHFR in bovine aortic EC [299], suggesting that folate inhibition may have deleterious effects in EC. However, the biological relevance of these findings is unclear as it is thought that the effects of MTX are not mediated via folate depletion *in vivo* due to the fact that folate supplementation does not abrogate the drug's therapeutic efficacy in patients.

There are some limitations to the study. First, it is difficult to accurately model the exposure of vascular EC to MTX in terms of concentration and duration of drug treatment due to the lack of pharmacokinetic data of MTX uptake and metabolism in the vasculature *in vivo*. In clinical practice, MTX is administered at a dose of 10-25mg per week [164] and has a relatively short half-life in plasma (about 8h), where it achieves a concentration of about 100-500nM in RA patients [249]. However, MTX plasma concentrations do not correlate with its clinical efficacy in patients with RA [517]. Rather, MTX is taken up by cells, where it is retained as a polyglutamated form for weeks (as shown in erythrocytes in RA patients [518]). The

conversion rate or concentrations of polyglutamated MTX are not known for most cell types including vascular EC. Secondly, the concentration of folinic acid (500nM) used in this study was higher than that achieved in patient blood after routine folic or folinic acid supplementation (10-50nM) [242, 519], but was used to demonstrate a proof of principle of the drug's mechanism of action. Lastly, the phosphokinase activity arrays used in this study are not able to capture all affected cellular signalling events in EC because the peptides on the arrays do not equally represent substrates for all kinase families. Therefore, MTX may affect other endothelial cell signalling pathways which were not measured using this system. In contrast, some of the proposed kinases are not functionally relevant or expressed in EC, such as for example Src kinases Lyn, Lck, Blk [520], and were therefore not followed up.

The results of this chapter are controversial to the current school of thought of MTX actions on the vasculature. It is very clear that MTX has beneficial cardiovascular effects in patients with RA [233] and in animal models of atherosclerosis [244] and chronic systemic inflammation [246]. Importantly, there is no clinical evidence that MTX causes adverse effects on the vasculature in patients treated with a long-term, low-dose regimen [212, 227, 521]. This highlights the apparent inconsistency of the clinical effects of the drug and the findings of this chapter, which may be explained by two different hypotheses. The first possibility is that MTX may achieve its anti-inflammatory effects on the vasculature in the clinical setting through the inhibition of immune cell activation and not via direct actions on EC. In general, it is difficult to dissect the individual contribution of different cell types to a systemic *in vivo* response, which often only allows speculation about causality. This is especially difficult in a scenario where a drug affects multiple cell types such as MTX. As described in section 1.4.5, MTX is well known to inhibit the proliferation and function of immune cells (reviewed in [230]), resulting in the decreased release of pro-inflammatory mediators, which consequently may lead to the reduced activation of vascular EC, and decreased adhesion to EC. In other words, it is plausible that control of disease activity with MTX may prevent endothelial activation and later vascular dysfunction. Supporting this argument, MTX did not affect EC function in a rat arthritis model [301]. However, if the endothelium is inert to the drug, this does not explain why MTX induced multiple cellular events in EC in this chapter.

The second hypothesis argues that MTX does have anti-inflammatory effects on vascular EC, which are, however, context-dependent and, therefore, not sufficiently modelled by performing static *in vitro* experiments. It is clear that *in vitro* experiments cannot recapitulate the complex

biological *in vivo* environment. EC phenotypes are thought to be plastic and heterogenous *in vivo*, depending on their location in the vascular tree, local tissue requirements [23] and shear stress forces [111]. Numerous studies have shown that EC under shear stress acquire different phenotypes compared to static EC [111]. Dictated by the local haemodynamic forces, EC form a highly quiescent and non-proliferative monolayer in the adult vasculature that becomes activated and proliferative during atherogenesis or states of vascular injury requiring angiogenesis or wound healing. Culture of EC grown under static conditions does not reflect quiescent endothelium and is at best able to represent certain aspects of proliferative endothelial phenotypes but lacks further biological context. Shear stress is also an important determinant of EC susceptibility to atherogenesis, which can be used to study atheroprotective and atheroprone endothelial phenotypes [111]. Therefore, I hypothesised that MTX may have different effects in shear-exposed quiescent and activated EC, which may provide evidence for the drug's anti-atherogenic actions in patients. Taken together, I speculated that the results obtained so far were not entirely representative of the drug's actions on the vasculature *in vivo*. To better model the (patho)-physiological environment of EC, I aimed to study the effects of MTX in EC subjected to shear stress in the next chapter.

4 Chapter 4 – The effects of MTX on EC subjected to shear stress

4.1 Introduction

In light of reports showing that MTX can improve vascular function and reduce CVD risk in patients with inflammatory arthritis [203, 233], I hypothesised that MTX may have different effects in EC exposed to shear stress compared to static conditions. Shear stress is a mechanical force that is generated by the flowing blood, and to which EC are physiologically subjected to in the vasculature. As outlined in section 1.2.4, shear stress can fundamentally influence endothelial phenotype and function by inducing distinct signalling, transcriptional and functional programs in EC, which can protect or prime EC to atherogenesis [115].

Two main types of shear stress have been used in the literature to model EC phenotypes in regions of low or high probability of atherosclerotic plaque formation, termed laminar or disturbed flow respectively (section 1.2.4). Using a commercially available parallel plate system (section 2.1.13.1), laminar shear stress (LSS) was modelled by applying continuous unidirectional shear stress at two different shear rates: high (H-LSS; 20dyn/cm²) and low (L-LSS; 5dyn/cm²). Disturbed flow was mimicked by oscillatory shear stress (OSS) ($\pm$ 5dyn/cm²; 2Hz), defined by a low shear rate and directional reversibility. These conditions, which reflect wall shear stress values and patterns achieved in human coronary [522] and carotid arteries [523, 524], are often used in this field [157]. Moreover, they induce EC phenotypes similar to those seen *in vivo* [111]. Selected results were validated in a different model of shear stress using the orbital shaker system, as introduced in section 2.1.13.2.

As discussed in section 1.2.4, H-LSS results in an atheroprotected, quiescent and homeostatic endothelial phenotype characterised by NO production, expression of anti-inflammatory and anti-thrombotic molecules and reduced proliferation. In contrast, OSS causes endothelial activation defined by the expression of pro-inflammatory and pro-thrombotic proteins, increased proliferation, cell turnover, apoptosis and vascular permeability, resulting in EC priming towards an atheroprone state (reviewed in [111]). In this thesis, three parameters contributing to these endothelial profiles were investigated in more detail: cell signalling, gene expression signatures and cell cycle progression (outlined in section 1.2.4). These were selected both for comparison to the experiments from the previous chapter on static EC, and to investigate the effects of MTX on shear stress-induced endothelial phenotypes.

It has been shown that patients with RA develop signs of endothelial dysfunction early in the disease course [196]. As part of the first-line treatment for patients with RA [164], MTX has the opportunity to prevent or reduce these initial pathological changes. A previous report showed that MTX reduced intimal macrophage infiltration and atherosclerotic lesion size in cholesterol-fed rabbits [244], suggesting that the drug may prevent inflammatory cell recruitment into the vessel wall. Modelling OSS-induced endothelial dysfunction allows to address how MTX may affect this process by for example studying the drug's effect on endothelial adhesion molecule expression. Likewise, MTX may shield EC from injury in the quiescent regions subjected to LSS by inducing protective signalling pathways. No other study had previously examined the drug's effect on EC under shear stress. Treating EC with MTX under shear stress provided a model to investigate the drug's influence on endothelial quiescence and early atherogenesis; two important endothelial states in health and disease, which are only sub-optimally recapitulated with static culture.

4.2 Hypothesis of the chapter

MTX causes the activation of protective signalling pathways in EC under H-LSS and/or reduces OSS-driven EC activation.

4.3 Aims of the chapter

- 1) Determine the effects of MTX on cell signalling in EC subjected to shear stress
- 2) Validate findings in EC subjected to the orbital shaker model
- 3) Determine the effects of MTX on cell cycle progression in EC subjected to shear stress
- 4) Investigate the effects of MTX on OSS-induced gene expression in EC

4.4 Results

4.4.1 MTX differentially affects cell signalling in EC subjected to shear stress using a parallel plate model

To examine the effects of MTX on cell signalling in EC under shear stress, HAEC were pre-conditioned with high laminar (H-LSS; 20dyn/cm²), low LSS (L-LSS; 5dyn/cm²) or oscillatory shear stress (OSS; $\pm$ 5dyn/cm², 2Hz) for 48h. Media (control) or MTX were added for a further 48h under H-LSS or OSS using a parallel plate model (Figure 23A), giving the following conditions: H-LSS control, H-LSS MTX, L-LSS control, OSS control and OSS MTX. The cells were harvested and analysed by phospho-kinase activity arrays and immunoblotting (Figure 23+24 and Supplementary Figure 4). Throughout this chapter, the effect of individual shear stress conditions was compared between the control samples (e.g. H-LSS control vs OSS control), while MTX was always compared to its control in the respective shear condition (e.g. OSS control vs OSS MTX).

Characterisation of EC morphology and phenotype under shear stress

Application of shear stress changed EC morphology in line with the literature [111]: H-LSS induced the alignment and elongation of EC, whereas OSS caused a polygonal, cobblestone-like phenotype (Figure 9). All cell lysates were tested by immunoblotting for the protein expression levels of TM and VCAM1, which were used as positive controls for the application of H-LSS and OSS respectively (reviewed in [111]). EC exposed to OSS expressed lower protein levels of TM (85% reduced; $p < 0.0052$) and higher levels of VCAM1 (625-fold induction; $p < 0.0002$) compared to H-LSS as expected (Figure 23B and C). L-LSS induced VCAM1 (32-fold) and reduced TM protein expression (60% reduction) compared to H-LSS, despite not reaching statistical significance. MTX did not affect the morphology or the protein levels of TM or VCAM1 compared to control-treated EC subjected to either H-LSS or OSS ((Figure 9; Figure 23B and C). Additional characterisation at the cell cycle level is presented in a later section of this chapter (Figure 27). These results supported the validity of using this model to induce shear stress responses in EC.

Phospho-kinase activity arrays

OSS induced the differential phosphorylation of 6 peptides in the STK ($\log_2$ FC range -1.39 to -0.59; $p < 0.05$) and 22 peptides in the PTK arrays ($\log_2$ FC range -0.21 to 0.63; $p < 0.05$) in EC compared to H-LSS (Supplementary Figure 4). Interestingly, OSS reduced the activity of PKA, PKG and Akt signalling, while increasing the activity of tyrosine kinases in the Eph/Ephrin (EphA4, EphA5) and Src families (Lck, Lyn, Fyn, Frk) in EC compared to H-LSS.

MTX treatment did not lead to significant changes in peptide phosphorylation in EC subjected to H-LSS compared to its control when analysed on STK arrays (Figure 23D). The upstream kinase analyses reported an increased activity of kinases involved in MAPK (ARAF, BRAF, RSK3), Akt and PKC signalling in MTX-treated EC under H-LSS (Figure 23E). In EC subjected to OSS, MTX treatment resulted in the decreased phosphorylation of 24 peptides in the STK arrays ($\log_2$ FC range -0.11 to -0.51; $p < 0.05$) (Figure 23D). These changes resulted in a decreased activity of similar kinases in the MAPK pathway (RSK3, ARAF, BRAF, p38, Pim1 and 3) (Figure 23E). AMPK was not among the putative kinase hits in MTX-treated cells under H-LSS or OSS, in contrast to the static experiments. In the PTK arrays MTX induced the phosphorylation of 9 peptides in EC under H-LSS compared to control ($\log_2$ FC range 0.15 to 0.44; $p < 0.05$), resulting in the increased activity of kinases in the Src family (Lck, Lyn, Frk and Fgr) and Abl kinases (Abl, Arg) among others (Figure 24B and C). In EC subjected to OSS, MTX led to the differential phosphorylation of three peptides ($\log_2$ FC range -0.49 to 0.27; $p < 0.05$). The top kinase hits included kinases involved in Eph/Ephrin signalling (EphA2, EphB3 and EphB2) and Jak-Stat signalling (Jak1), showing a higher or lower kinase activity respectively in MTX versus control treated EC under OSS.

These results showed that MTX had more modest effects on cell signalling in EC subjected to shear stress compared to static culture. In contrast to Chapter 3, MTX caused the differential phosphorylation of a small number of peptides in EC under shear stress, suggesting that the overall effect sizes of these changes in kinase activity were small. Although there was an overlap in the kinase families regulated by MTX under static and shear stress conditions (including MAPK, Akt, PKC and Abl), MTX differentially regulated the activity of these kinases in EC under different conditions. These results highlighted that MTX may achieve specific, context-dependent effects on cell signalling in EC under static, H-LSS and OSS conditions.

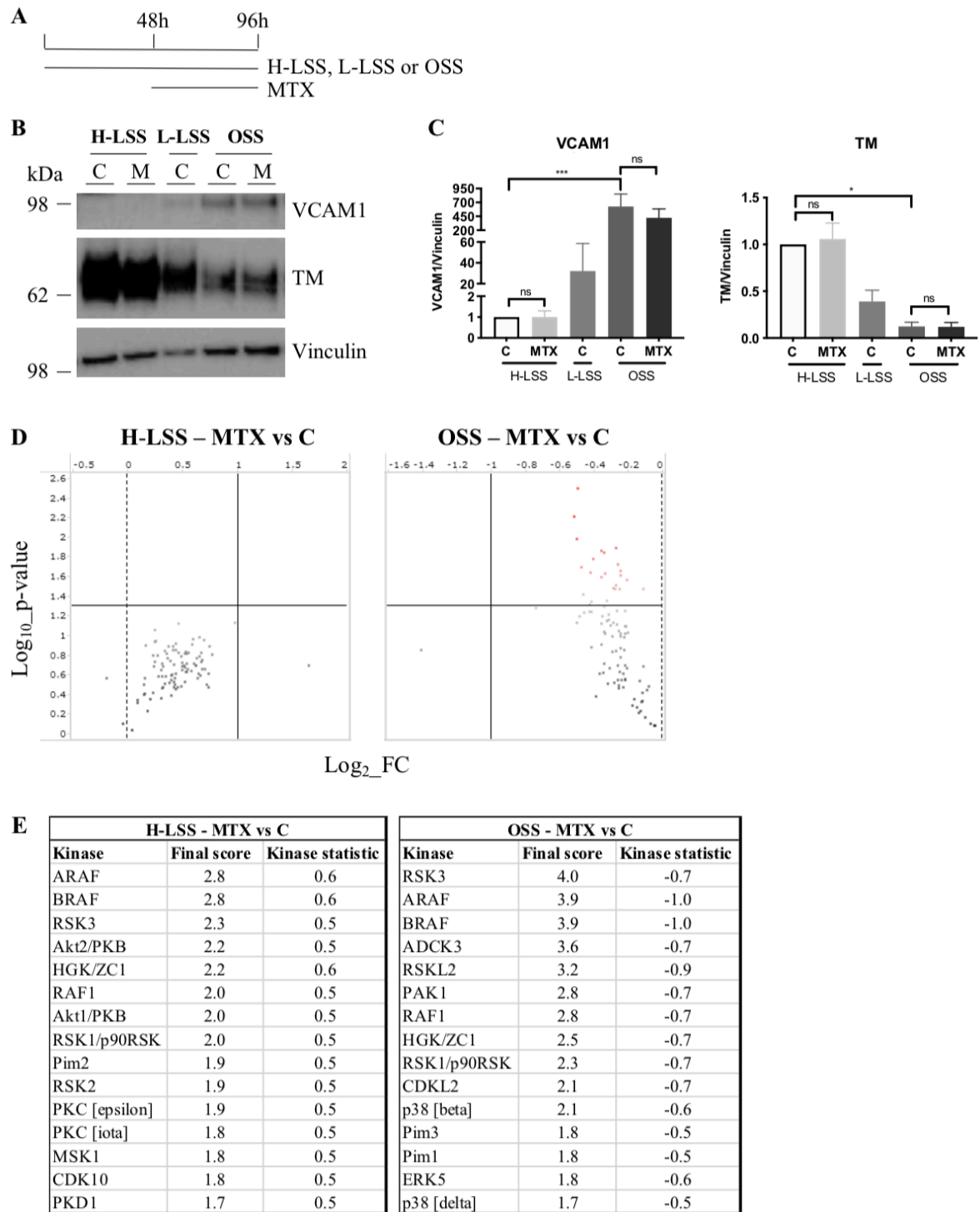


Figure 23. MTX differentially affects serine-threonine kinase activity in EC subjected to shear stress. (A-E) HAEC were pre-conditioned with high LSS (H-LSS; 20dyn/cm²), low LSS (L-LSS; 5dyn/cm²) or OSS ($\pm$ 5dyn/cm², 2Hz) for 48h. Media (control, C) or MTX (M; 100nM) were added to the cells for a further 48h under H-LSS or OSS (n=4-5). L-LSS served as internal control. Experimental outline shown in (A). (B) Immunoblot analyses of respectively treated cells. Whole protein lysates were separated by SDS-PAGE. Proteins were detected with antibodies against thrombomodulin (TM; 74kDa), VCAM1 (95kDa) and Vinculin (124kDa) (n=4-5). (C) Quantification of VCAM1 and TM protein expression levels in respectively treated HAEC. Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. Values represent means $\pm$ SEM. ns, not significant. * p<0.05. *** p<0.001. (D) Volcano plots of phospho-serine-threonine (STK) arrays performed on HAEC treated with MTX under H-LSS or OSS as stated above (n=4-5). Dots represent differentially phosphorylated peptides in a given condition comparing MTX versus control (C) samples subjected to either H-LSS or OSS. (E) Top 15% of kinase hits from STK arrays performed on respectively treated cells. Final Score indicates combined measure of significance and specificity of permutation analyses. The kinase statistic is computed by the overall change of the peptide set that represent a kinase (value >0 means that kinase is more active in test over control sample).

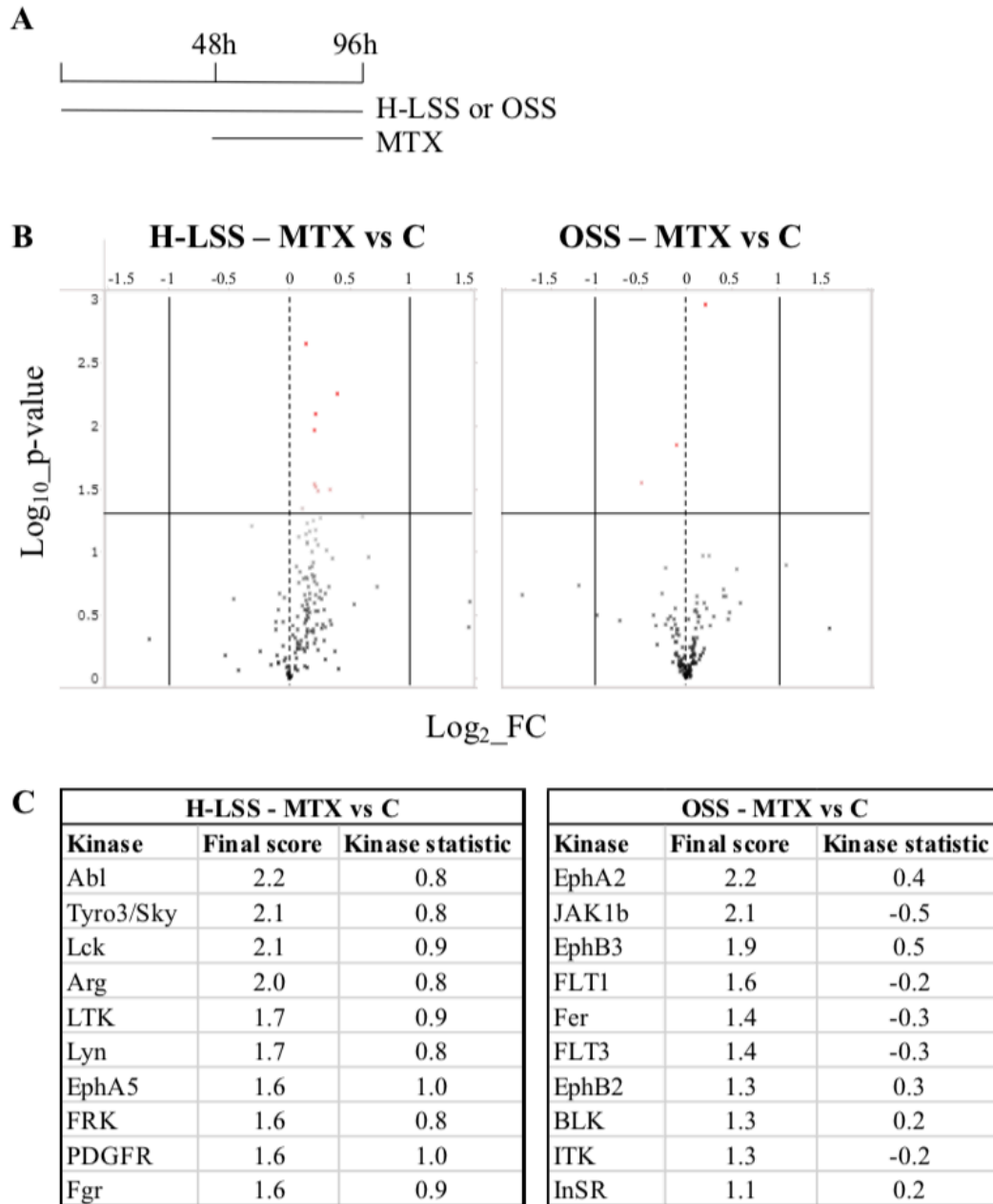


Figure 24. MTX differentially affects tyrosine kinase activity in EC subjected to shear stress. (A-C) HAEC were pre-conditioned with high LSS (H-LSS; 20dyn/cm²) or OSS ($\pm$ 5dyn/cm², 2Hz) for 48h. Media (control, C) or MTX (100nM) were added to the cells for a further 48h under the respective shear stress condition (n=3). Experimental outline shown in (A). (B) Volcano plots of phospho-tyrosine (PTK) arrays performed on HAEC treated respectively (n=3). Dots represent differentially phosphorylated peptides in a given condition comparing MTX versus control samples subjected to either H-LSS or OSS. (C) Top 15% of kinase hits from PTK arrays performed on respectively treated cells. Final Score indicates combined measure of significance and specificity of permutation analyses. The kinase statistic is computed by the overall change of the peptide set that represent a kinase (value >0 means that kinase is more active in test over control sample).

Validation of phospho-kinase activity arrays by immunoblotting

Akt, MAPK kinases p38, ERK1/2 (as downstream target of Raf kinases) and tyrosine kinase Jak1 (due to its importance in inflammatory signalling [525]) were chosen as targets for validation by immunoblotting (Figure 25).

In contrast to its effects in static EC, MTX did not affect p38 phosphorylation and demonstrated a trend to increase ERK1/2 phosphorylation ($p=0.15$) compared to its control in EC subjected to H-LSS (Figure 25A and C). In EC exposed to OSS, MTX showed a trend to decrease p38 phosphorylation ($p=0.13$), while ERK1/2 phosphorylation was not affected. Similar observations were made in preliminary experiments in HUVEC, where MTX induced p38 phosphorylation in EC under static conditions, but not under H-LSS or OSS (Supplementary Figure 5). MTX tended to increase Jak1 phosphorylation levels under H-LSS and OSS, although there was considerable variation in the two experiments examining Jak1 phosphorylation. It was not possible to conclusively report the effects of MTX on Akt signalling because its phosphorylation levels showed great variability in all examined conditions (data not shown). Of note, OSS did not induce p38 phosphorylation, tended to increase ERK1/2 and reduce Jak1 phosphorylation compared to H-LSS. L-LSS did not affect p38 or Jak1 and showed a trend to increase ERK1/2 phosphorylation levels compared to H-LSS.

Overall, MTX treatment was associated with small but non-significant effects on p38 and ERK1/2 phosphorylation in EC under H-LSS or OSS. Despite showing similar trends to the phospho-kinase activity arrays, these MTX-induced effects could not be conclusively validated by immunoblotting, probably owing to the small effect sizes, the n number and variability of these observed changes. Importantly, these results confirmed that MTX did not increase p38 phosphorylation in EC exposed to shear stress.

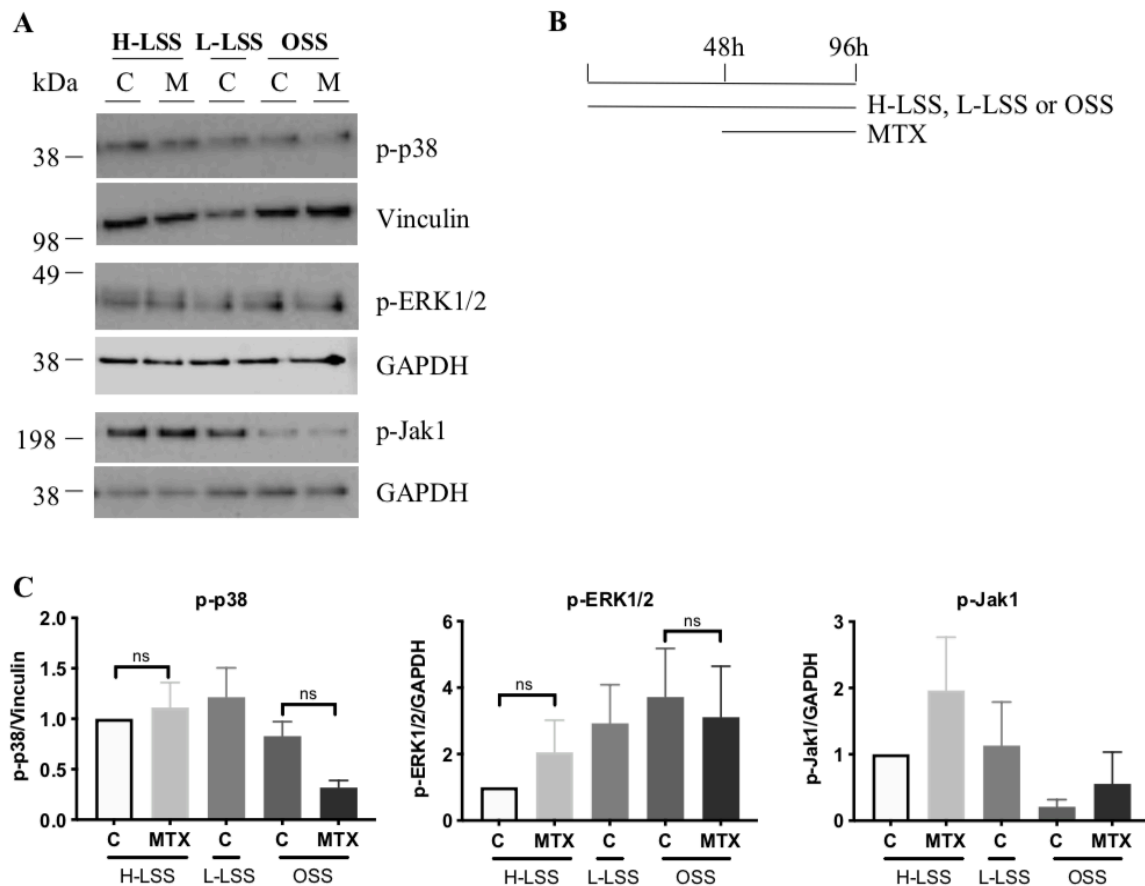


Figure 25. Validation of phospho-kinase activity arrays in MTX-treated EC subjected to shear stress. (A-C) HAEC were pre-conditioned with high LSS (H-LSS; 20dyn/cm²), low LSS (L-LSS; 5dyn/cm²) or OSS ($\pm$ 5dyn/cm², 2Hz) for 48h. Media (control, C) or MTX (100nM) were added to the cells for a further 48h under H-LSS or OSS (n=4). L-LSS served as internal control. Experimental outline shown in (B). (A) Immunoblot analyses of respectively treated cells. Whole protein lysates of respectively treated cells were separated by SDS-PAGE. Proteins were detected with antibodies against p-p38 T180/Y182 (38kDa), p-ERK1/2 T202/Y204 (42/44kDa), p-Jak1 Y1034/1035 (130kDa), GAPDH (39kDa) and Vinculin (124kDa) (p-p38 n=3; p-ERK1/2 n=4; p-Jak1 n=2). (C) Quantification of p38, ERK1/2 and Jak1 phosphorylation levels in respectively treated HAEC. Data shown as fold change compared to H-LSS control condition. Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. Values represent means $\pm$ SEM. ns, not significant.

4.4.2 MTX does not induce Akt or p38 phosphorylation in EC subjected to shear stress using the orbital shaker model

To examine the effects of MTX on Akt and p38 signalling in another *in vitro* model of shear stress, HAEC were exposed to undisturbed (UF) or disturbed flow (DF) using the orbital shaker model. The cells were pre-conditioned with shear stress for 48h, followed by addition of media (control) or MTX for a further 48h under shear stress (Figure 26A), giving the following conditions: UF control, UF MTX, DF control and DF MTX. The cells were analysed by qPCR and immunoblotting (Figure 26B-D). Shear stress conditions were compared using the control samples (UF control vs DF control), while MTX was always compared to its control under UF or DF respectively (e.g. UF control vs UF MTX).

Characterisation of EC morphology and phenotype under shear stress

Similar to the parallel plate model, EC acquired distinct morphologies according to the applied shear stress: EC in the periphery of the well (UF) were more aligned and elongated compared to those in the centre (DF), where the cells displayed a polygonal shape (Figure 11). Unlike the parallel plate model, DF did not induce VCAM1 ($p=0.48$) or reduce TM ($p=0.83$) protein expression compared to UF (Figure 26C and D). Therefore, additional validation was carried out by analysing the gene expression of KLF2, E-selectin (SELE), VCAM1 and TM. As expected, DF reduced the mRNA expression of KLF2 by 46% ($p<0.0001$) and tended to increase E-selectin 2-fold but did not change the expression of VCAM1 ($p=0.44$) or TM ($p=0.82$) compared to UF (Figure 26B). These results demonstrated that the orbital shaker method sufficiently induced shear stress-dependent changes in EC morphology and phenotype, which were however not as pronounced as in the parallel plate model.

Characterisation of MTX-treated EC under UF and DF

MTX did not affect the morphology of EC in either shear condition (Figure 11). Furthermore, MTX also did not alter the mRNA or protein expression of VCAM1, TM or KLF2 in EC under UF or DF (Figure 26B, C and D), in line with the findings on VCAM1 and TM protein expression in the parallel plate model.

MTX showed a trend to reduce Akt but did not affect p38 phosphorylation levels compared to control in EC subjected to UF (Figure 26C and D). Despite not reaching statistical significance, MTX seemed to cause a reduction of both Akt (by approximately 70%) and p38 (by 45%) phosphorylation under DF. Of note, p38 and Akt phosphorylation were not significantly affected by DF compared to UF.

In contrast to Chapter 3, MTX also did not induce Akt or p38 phosphorylation in EC exposed to shear stress using the orbital shaker model. Consistent with the parallel plate model, MTX treatment showed a trend to decrease p38 phosphorylation in EC exposed to disturbed flow, suggesting that this may be a reproducible effect of MTX on this pathway, despite remaining non-significant. It was not possible to compare the effects of MTX on Akt signalling in the two shear stress systems due to the highly variable phosphorylation levels in the parallel plate model. Taken together, these results demonstrate that MTX did not promote Akt or p38 signalling in EC subjected to shear stress using two independent shear stress models, providing further evidence that MTX may have different effects on cell signalling in EC cultured under static conditions and those exposed to shear stress. All further shear stress experiments were performed using the parallel plate model because the orbital shaker model did not induce the expression of all examined well-established shear-inducible targets in EC.

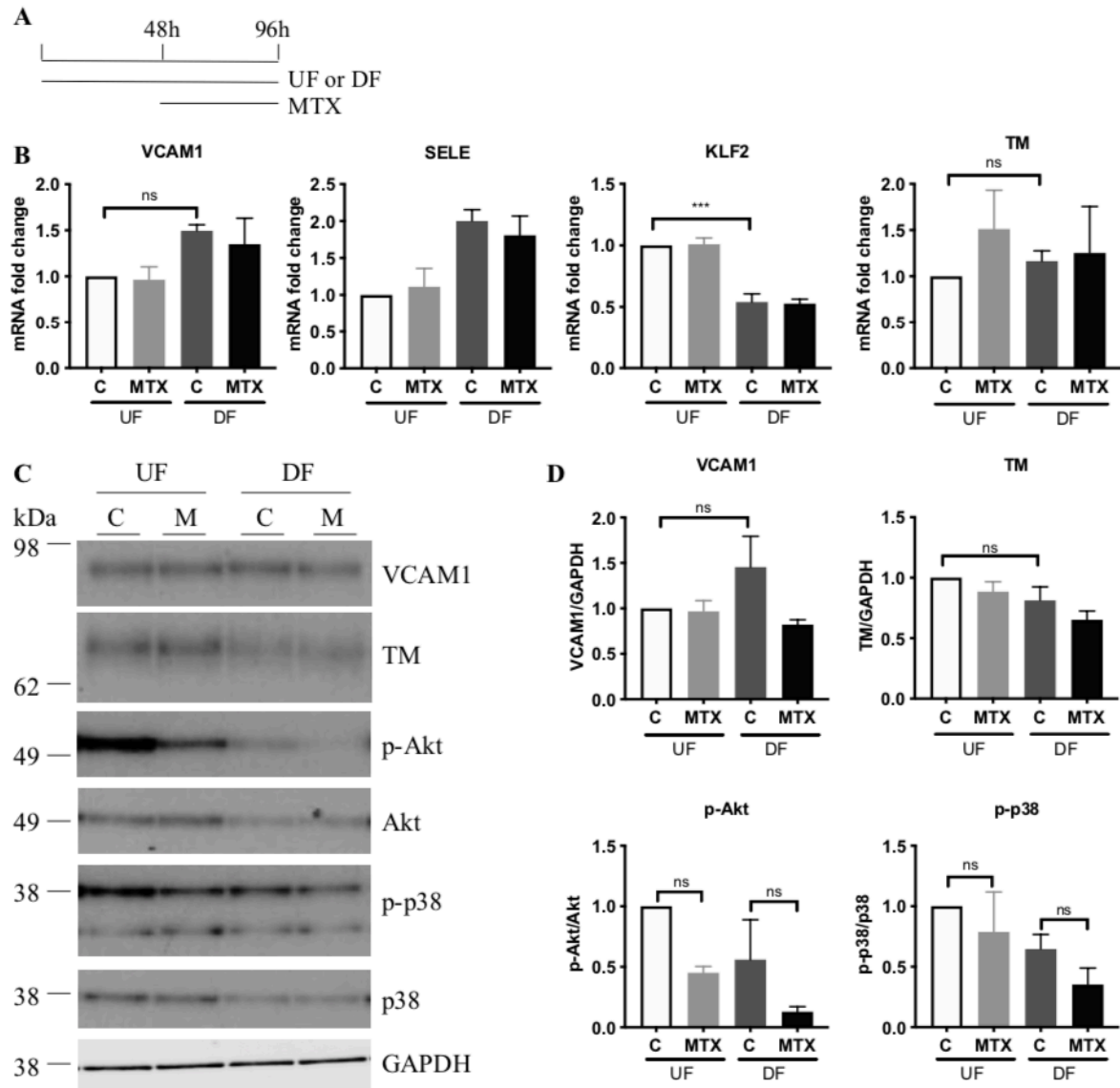


Figure 26. MTX does not induce Akt or p38 phosphorylation in EC subjected to shear stress using the orbital shaker model. (A-D) HAEC were pre-conditioned with undisturbed (UF) or disturbed flow (DF) using the orbital shaker model for 48h. Media (control, C) or MTX (100nM) were added for a further 48h under shear stress (n=3), as shown in (A). (B) Gene expression analyses of respectively treated HAEC. Gene expression was analysed by qPCR and normalised to GAPDH (n=3; except SELE n=2). (C) Immunoblot analyses of HAEC treated as described above. Whole cell protein lysates were separated by SDS-PAGE. Proteins were detected with antibodies against p-p38 T180/Y182 (38kDa), p38 (38kDa), p-Akt S473 (60kDa), Akt (60kDa), thrombomodulin (TM; 74kDa), VCAM1 (95kDa) and GAPDH (39kDa) (n=3). (D) Quantification of VCAM1, TM, p-p38 and p-Akt levels in HAEC treated as described above (n=3). Data shown as fold change compared to UF control. One-way ANOVA and Sidak test for multiple comparisons. Values represent means $\pm$ SEM. ns, not significant. *** $p < 0.001$.

4.4.3 MTX does not inhibit the cell cycle of EC subjected to shear stress

To understand the effects of MTX on cell cycle progression in EC under shear stress, HUVEC were pre-treated with H-LSS or OSS for 48h before addition of media (control) or MTX for a further 48h under shear stress using the parallel plate model. Cell cycle progression was evaluated by staining the cells with PI and analysis by flow cytometry (Figure 27).

As expected, EC subjected to OSS were more proliferative than those exposed to H-LSS, demonstrated by a decreased percentage of cells in G1 (OSS 75.3% vs H-LSS 85.7%; $p=0.0003$) and increased proportion of cells in the G2/M phase (OSS 11.7% vs H-LSS 5.4% $p=0.0028$) of the cell cycle (Figure 27B and C). MTX treatment did not alter the proportion of cells in the G1, S- or G2/M phases of the cell cycle compared to control in EC under H-LSS or OSS; a finding that was in contrast to its ability to reliably induce S-phase arrest in EC under static conditions (Chapter 3, Figure 21).

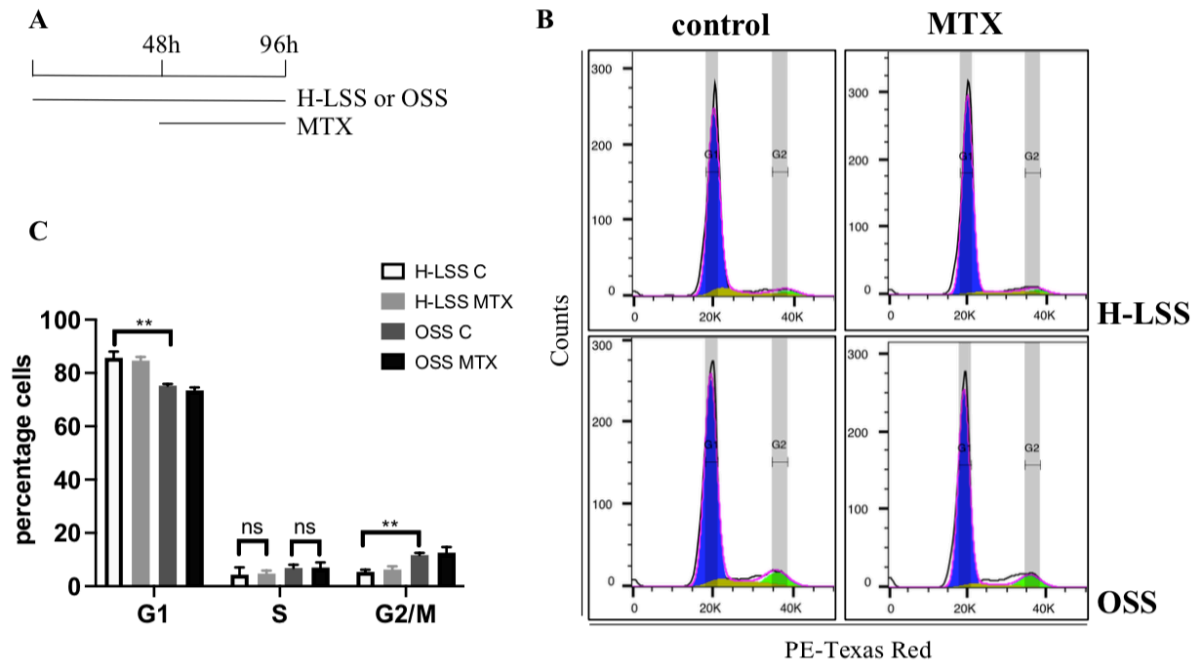


Figure 27. MTX does not induce cell cycle arrest in EC under shear stress. (A-C) HUVEC were pre-conditioned with H-LSS or OSS for 48h, after which cells were treated with media (control, C) or MTX (100nM) for a further 48h under shear stress. Experimental outline shown in (A). (B) Representative flow cytometry profiles of respectively treated cells stained with propidium iodide (PI) and analysed by flow cytometry (n=3-4). Phases of cell cycle coloured as following: G1, blue; S, yellow, G2/M, green. (C) Quantification of cell cycle experiments performed as stated (n=3-4). Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. Values represent means $\pm$ SEM. ns, not significant. * $p < 0.05$. ** $p < 0.01$.

It is thought that pro-inflammatory stimulation induces EC proliferation in patients with RA [65]. To test whether TNF α would alter the ability of MTX to affect cell cycle progression in EC grown under H-LSS or OSS, HUVEC were pre-conditioned with H-LSS or OSS and treated with media (control), TNF α and MTX as outlined in Figure 28A. The cells were stained with PI and analysed by flow cytometry (Figure 28). This experiment was only performed once.

TNF α did not seem to affect cell cycle progression in EC subjected to H-LSS compared to control (Figure 28B and C). TNF α showed a trend to reduce cell proliferation in EC under OSS, as it increased the number of cells in G1 phase and decreased the proportion of cells in the S- and G2/M phase compared to control. This was not affected by MTX co-treatment (Figure 28B and D). In summary, MTX did not induce cell cycle arrest in EC subjected to shear stress.

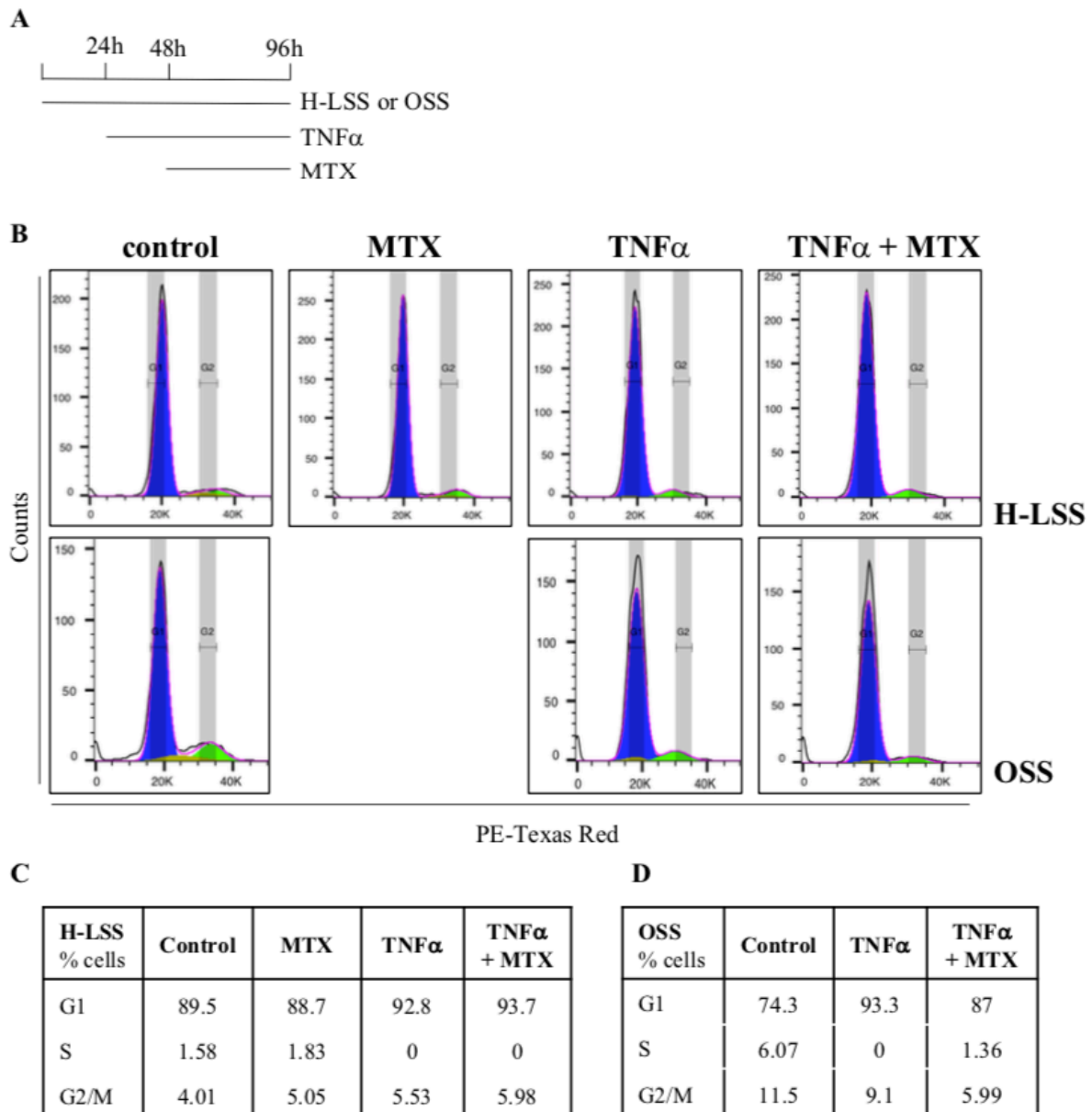


Figure 28. MTX may not induce cell cycle arrest in TNF α -activated EC under shear stress. (A+B) HUVEC were pre-conditioned with H-LSS or OSS for 24h, after which cells were treated with media (control) or TNF α (0.1ng/ml) for 24h. After this period, media or MTX (100nM) were added to the cells for a further 48h under shear stress. Experimental outline shown in (A). (B) Flow cytometry profiles of respectively treated cells stained with propidium iodide (PI) and analysed by flow cytometry (n=1). Phases of cell cycle coloured as following: G1, blue; S, yellow, G2/M, green. (C+D) Percentages of cells in G1, S and G2/M phases treated under H-LSS (C) or OSS (D) as described above (n=1).

4.4.4 MTX does not alter OSS-induced gene expression in EC

The results from this chapter so far did not support a role of MTX in significantly altering cell signalling or proliferation in EC subjected to shear stress. Therefore, I aimed to investigate the effects of MTX on other functions pertinent to EC. It is well established that OSS induces a pro-inflammatory, pro-thrombotic and pro-apoptotic endothelial phenotype by regulating the expression of numerous genes in EC (as outlined in section 1.2.4). To examine if MTX modulated OSS-induced endothelial gene signatures, HAEC were pre-treated with OSS for 48h and treated with media (control) or MTX for a further 48h under shear stress using the parallel plate model. In parallel, cells were subjected to low LSS (L-LSS) as control for OSS-induced gene expression, giving the following conditions: L-LSS control, OSS control and OSS MTX. The cells were analysed using commercially available endothelial-focused qPCR arrays (Qiagen) examining the expression of genes relevant in angiogenesis, inflammation, coagulation, platelet activation, apoptosis and vascular tone (for full gene list see Appendix, Table 12).

Figure 29 shows the results from the final grouped analysis focusing on EC subjected to OSS compared to L-LSS under control conditions. OSS significantly affected the expression of 18 genes (Figure 29A and B), of which 8 showed a $\log_2FC$ of greater than ± 0.9 (Figure 29C): OSS induced the mRNA expression of VCAM1, E-selectin and sphingosine kinase 1 (SPHK1) and reduced VEGFR2 (KDR) and HO1 (HMOX1) expression levels compared to L-LSS (Figure 29C), in line with previous reports [126, 149, 526-528]. Interestingly, OSS also increased the mRNA levels of endoglin (ENG), c-Kit (KIT) and integrin α_v (ITGAV).

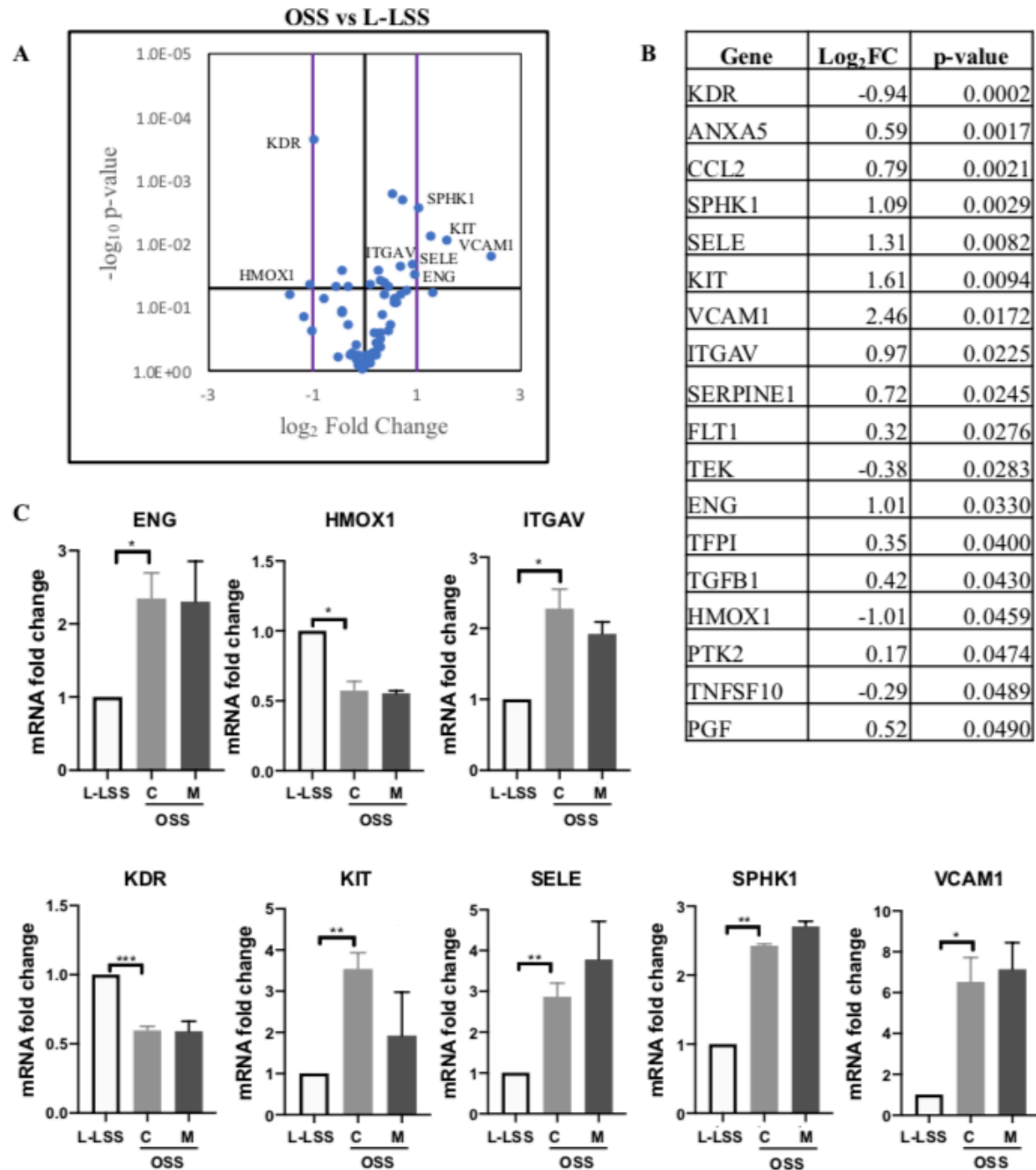


Figure 29. OSS alters gene expression in EC compared to low LSS. (A-C) HAEC were subjected to OSS ($\pm 5 \text{ dyn/cm}^2$, 2Hz) for 48h. Media (control, C) or MTX (M; 100nM) were added to the cells for another 48h under OSS. As control, HAEC were exposed to low LSS (L-LSS; 5 dyn/cm^2) for 96h. Gene expression analyses were performed using qPCR arrays ($n=3$). (A) Volcano plot of differentially expressed genes in HAEC subjected to OSS over L-LSS. Purple bars indicate fold change >2 ; horizontal black bar indicates a p-value <0.05 . (B) List of significant differentially expressed genes in EC subjected to OSS compared to L-LSS. (C) Representative differentially regulated genes in OSS-subjected EC compared to L-LSS. mRNA expression levels normalised to GAPDH and shown relative to L-LSS. Data were analysed using unpaired T-tests. Values represent means $\pm$ SEM. ns, not significant. * $p<0.05$. ** $p<0.01$. *** $p<0.001$.

Based on the grouped analysis of triplicate experiments, MTX did not significantly alter the expression of any of the genes measured (Figure 29C and 30). Interestingly, however, it became evident by examining this data more closely that five target genes ALOX5, CCL2, KIT, PTGS2 (COX2) and TNFSF10 were regulated by MTX in two of the three biological replicates. Figure 30A depicts a heatmap of log₂ fold changes of all genes measured in the three individual experiments. The heatmap was generated by comparing EC subjected to OSS versus L-LSS as the control, and shows the result for each gene in control and MTX-treated EC. The genes are grouped according to function - of note, some genes are implicated in multiple functional categories. The five target genes identified to be regulated by MTX (ALOX5, CCL2, KIT, PTGS2 and TNFSF10) are highlighted in red. The expression levels of these five genes were examined in a larger number of replicates by qPCR. However, MTX-mediated regulation of the chosen target genes in EC subjected to OSS could not be validated, despite showing a trend for c-Kit (Figure 30C). ALOX5 mRNA expression was not detectable in EC using in-house designed primers (data not shown). The complete data sets for all target genes are shown in Supplementary Figures 6-8. Overall, the data suggested that MTX did not modulate the expression of key endothelial genes in EC subjected to OSS.

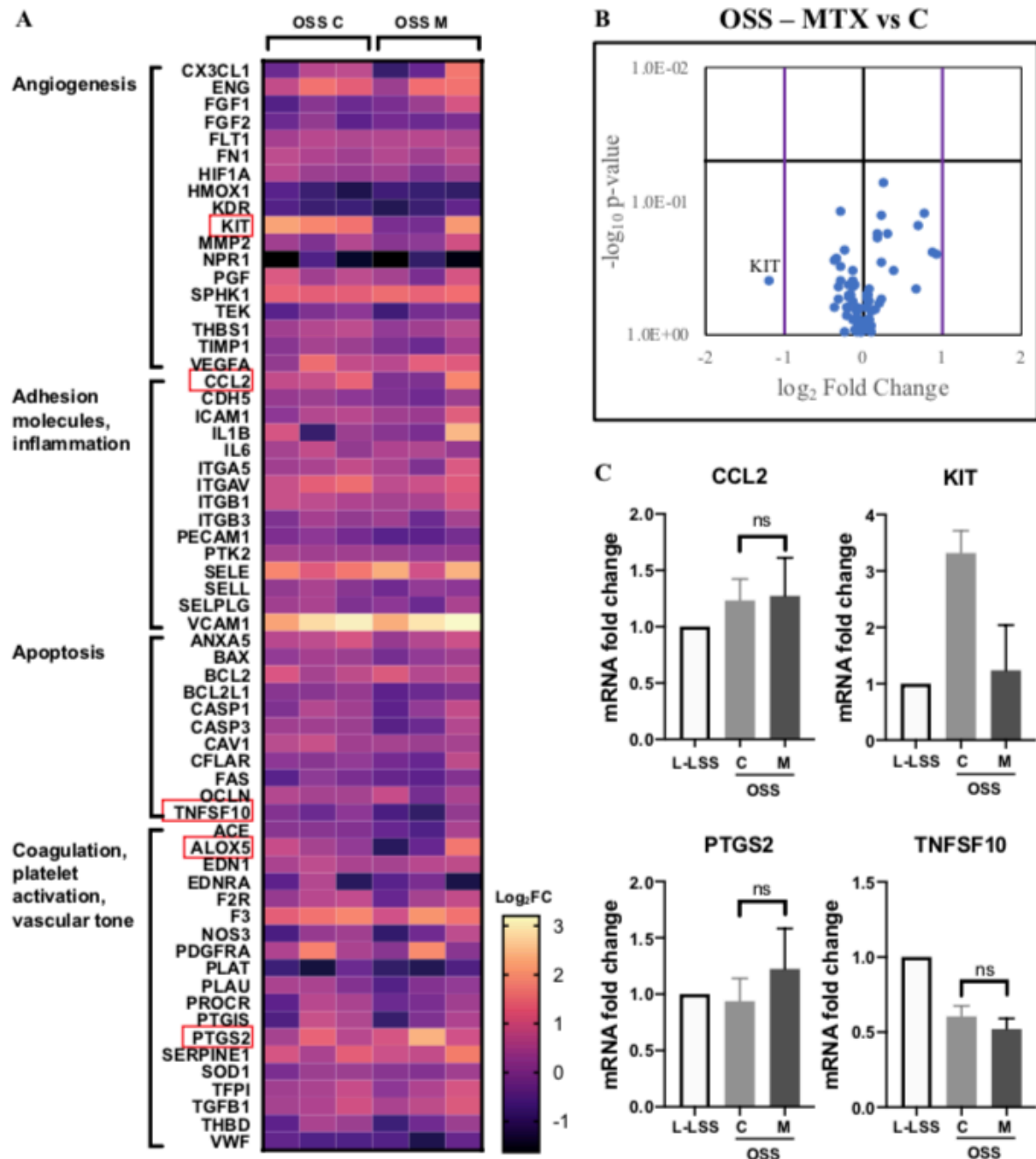


Figure 30. MTX does not affect gene expression in EC subjected to OSS. (A-C) HAEC were subjected to OSS ($\pm 5 \text{ dyn/cm}^2$, 2Hz) for 48h. Media (control, C) or MTX (M; 100nM) were added to the cells for another 48h under OSS. As control, HAEC were exposed to low LSS (L-LSS; 5 dyn/cm^2) for 96h. Gene expression analyses were performed using qPCR arrays (n=3). (A) Heat map of log₂FC of genes in EC subjected to OSS with and without MTX compared to L-LSS. Putative MTX target genes are highlighted with a red box. (B) Volcano plot of endothelial genes in HAEC subjected to OSS with and without MTX. Purple bars indicate fold change >2; horizontal black bar indicates a p-value <0.05. (C) Validation of putative MTX target genes by qPCR. mRNA expression levels normalised to GAPDH and shown relative to L-LSS (n=4-5; KIT n=2). Data were analysed using unpaired T-tests. Values represent means $\pm$ SEM. ns, not significant.

4.5 Discussion

This chapter addressed two questions: 1) Do EC exposed to shear stress respond differently to MTX compared to static conditions? 2) What are the effects of MTX treatment on shear induced EC phenotypes? The most striking finding of this chapter was that MTX indeed had different effects in EC subjected to shear stress conditions. In contrast to Chapter 3, MTX did not significantly modulate central cell signalling pathways, cell cycle progression or the expression of target genes implicated in fundamental endothelial functions in EC subjected to shear stress. These data support the position that the described MTX-induced effects in static EC most likely do not reflect the drug's actions on the vasculature *in vivo*. However, the results of this chapter did not identify alternative novel targets of the drug in EC under shear stress.

MTX did not have clear effects in EC subjected to shear stress as assessed in phospho-kinase activity arrays, cell cycle analyses and gene expression arrays. In the kinase arrays, MTX altered the phosphorylation of a small number of peptides under both H-LSS and OSS, resulting in very modest changes in kinase activity. The top kinase hits included Akt, PKC and MAPK; targets that were also identified under static conditions. Importantly, however, MTX caused different effects on the activity of these kinases under different conditions: the activity of Akt was increased under H-LSS but not OSS, while kinases in the MAPK pathway were regulated in an opposite manner by MTX under H-LSS and OSS. These results supported the possibility that the drug could have distinct effects in different areas of the vascular tree. However, none of the kinase targets selected for validation, including Akt, Jak1 and the MAPK p38 and ERK1/2, could be confirmed by immunoblotting. Akt phosphorylation levels were very variable across all conditions in the parallel plate model, making it difficult to draw any conclusions on its regulation by MTX. In line with the predictions from the kinase arrays, MTX treatment showed trends towards an increase in ERK1/2 phosphorylation under H-LSS and a decrease of p38 phosphorylation levels under OSS, but these effects did not reach statistical significance. Interestingly, a similar but also non-significant effect on p38 phosphorylation was observed in MTX-treated EC subjected to disturbed flow using the orbital shaker model, suggesting that this result is not a mere artefact. Because p38 signalling is activated by disturbed flow and promotes pro-inflammatory events in EC [124], it was intriguing to speculate that MTX may be able to inhibit p38 activation under OSS, despite requiring more experimental repeats to conclusively demonstrate this. However, the biological relevance of this proposed effect was challenged by two observations in this study: first, p38 was not phosphorylated by disturbed flow in either shear stress model, suggesting that MTX was not

reversing an OSS-mediated activation of this kinase. Secondly, OSS-induced adhesion molecule expression was not altered by MTX as measured at either the mRNA or protein level, highlighting also that MTX did not affect canonical downstream targets of the p38 pathway. Based on these findings and the limited feasibility of performing a high number of shear stress experiments, this pathway was not further investigated. Jak1 was chosen as a target for validation as the Jak/Stat signalling pathway is one of the main pro-inflammatory signalling hubs in inflammatory diseases including RA [525] and MTX had been previously shown to inhibit Jak/Stat signalling in drosophila and cancer cell lines [280]. Due to the small amount of cell lysate generated by these experiments, it was only possible to investigate the effects of MTX on Jak1 in two experiments, which demonstrated considerable variation in the levels of Jak1 phosphorylation in MTX-treated EC. To date, only one report investigated the actions of MTX in EC under shear stress, which was published during the writing of this thesis. The authors found that MTX inhibited Hippo signalling in EC under disturbed flow in an AMPK-dependent manner, resulting in decreased VCAM1 expression and monocyte adhesion to EC [529]. The Hippo pathway was not examined in this chapter because it is not represented on the kinase arrays, but MTX did not affect AMPK activity or VCAM1 expression in EC under OSS. These discrepancies may be explained by the different experimental setup used: the authors pre-treated HUVEC with MTX (100nM) for 48h in static culture before applying shear stress using a similar model and shear conditions. In this thesis, EC were subjected to shear stress before adding MTX, speculating that this reflected the clinical situation, where the vasculature is constantly exposed to shear stress before drug treatment. Given the results from Chapter 3 showing that MTX-treated EC are metabolically compromised, the biological validity and relevance of these findings can be questioned. Further work is required to prove that MTX induces AMPK activation in EC *in vivo* and whether this leads to the inhibition of Hippo signalling and ultimately atherogenesis in an EC-dependent manner. Taken together, MTX did not have significant effects on the examined cell signalling pathways in EC subjected to shear stress in this chapter. The predicted kinase hits were most likely the result of variable and/or small drug effects of unclear biological relevance (as demonstrated by p38). Importantly, MTX did not induce Akt or p38 signalling under shear stress using two independent models, suggesting that shear stress altered the endothelial response to MTX as hypothesised in Chapter 3.

Supporting this argument, MTX did not affect cell cycle progression in EC subjected to H-LSS or OSS. While it may seem plausible that MTX was not able to arrest cell cycle progression in EC with a low proliferative index under H-LSS, it is very interesting to note that the drug also did not inhibit proliferation in EC under OSS. This highlights that the proliferative phenotype of EC under static, H-LSS or OSS conditions is biologically different. The lack of MTX effect on cell proliferation in EC under shear stress aligns with the fact that the drug did not induce p38 or Akt signalling, which were causally linked in Chapter 3.

Furthermore, MTX did not alter the expression of selected endothelial genes in important pathways covering angiogenesis, vascular tone and inflammation under OSS. The only noteworthy finding was that MTX showed a trend to prevent OSS-mediated induction of c-Kit expression. c-Kit is a receptor tyrosine kinase, which can regulate Akt, MAPK and Stat signalling in different cellular contexts [530]. Although none of these effects reached significance, it is interesting to speculate that there is a relationship between c-Kit expression and MAPK signalling in EC, which seemed to be slightly inhibited by MTX under OSS. The lack of MTX effect may be explained by the comparably small number of genes examined using the qPCR arrays and further experiments are required to answer the question of whether MTX alters endothelial gene expression under shear stress (e.g. using RNA sequencing). The data from this chapter show that the endothelial response to MTX is fundamentally affected by shear stress. In line with the fact that MTX does not induce adverse effects on the vasculature in patients (as discussed in Chapter 3), MTX did not promote Akt or p38 signalling and consequent cell cycle arrest in EC under shear stress. However, no other MTX-dependent effects could be identified in EC under shear stress using kinase or gene expression arrays, highlighting the critical question whether MTX has direct anti-atherogenic effects on the vasculature or whether these are achieved indirectly by its actions on immune cells *in vivo*. More work is required to conclusively address this problem, but the results of this chapter currently do not support a direct protective role of MTX on the vasculature.

This study did not find pronounced MTX-mediated effects in EC under shear stress but did identify potentially novel targets of shear stress in EC in the kinase and gene expression arrays. Shear stress-induced EC signalling has been mostly investigated in short-term models (<24h), which mimic the acute response to the onset of flow, seen during vascular development or in pathological states such as ischaemia-reperfusion injury. The healthy adult vasculature does not often experience such acute and dramatic changes in shear stress. As there are very few

studies investigating the long-term effects of shear stress on EC, it is interesting to discuss the results of this study, comparing the effects of OSS to high LSS in EC after 4 days of flow. To my knowledge, this is the first report showing that disturbed flow regulates the Eph/Ephrin signalling pathway (EphA4 and EphA5) in EC. Eph/Ephrin signalling is implicated in driving vascular development, angiogenesis, arterial versus venous identity specification, vascular inflammation, permeability and atherosclerosis [531, 532]. EphA4 and EphA2 show a higher expression in the aortic arch compared to the thoracic aorta in ApoE knockout mice fed a Western diet [533]. EphA2 promotes endothelial inflammation [533] and atherosclerotic plaque formation in a mouse model [534]. In light of these reports, it seems plausible that OSS induces Eph/Ephrin signalling to sustain a pro-inflammatory endothelial phenotype and drive atherogenesis. Interestingly, Eph/Ephrin signalling can activate members of the Src family in other contexts [535, 536], which were also predicted to be more active in OSS-exposed EC compared to H-LSS. Of note, OSS seemed to reduce the phosphorylation of Jak1 compared to H-LSS in preliminary experiments (Figure 25). The Jak/Stat pathway is implicated in atherosclerosis as it has been shown that endothelial knockout of Stat3 reduced atherosclerotic lesion size in a mouse model [537]. Although there are no studies on the effects of OSS on this signalling pathway, LSS inhibited IL6-mediated activation of Jak/Stat signalling in EC [538]. Given these reports, it is currently unclear why OSS would reduce Jak1 phosphorylation. Taken together, these preliminary findings are novel but require validation by other methods to conclusively demonstrate the regulation of these signalling pathways by OSS.

OSS differentially regulated the expression of eight genes implicated in angiogenesis and inflammation compared to low LSS, of which three may be novel targets: endoglin, integrin α_v and c-Kit. Endoglin is a co-receptor for proteins of the TGF β pathway and is implicated in the regulation of vascular development, angiogenesis and vascular tone [539]. Endoglin expression is relatively low in quiescent EC but can be upregulated in states of inflammation, vascular injury and angiogenesis. High LSS reduced endoglin expression in human coronary artery EC [540], while low LSS increased endoglin mRNA expression in mouse embryonic EC [541]. Endoglin is expressed in human atherosclerotic lesions [542] but has contradictory roles in atherosclerosis [543]. Integrins are extracellular matrix proteins that form heterodimers consisting of an α and β subunit. Integrins are activated by shear stress as part of the mechanosensory complex in EC [122]. Integrin $\alpha_v\beta_3$ is expressed in EC in atherosclerotic plaques [544] and mediates OSS-induced endothelial inflammation and atherosclerotic plaque

formation in mice [545]. c-Kit has been shown to modulate migration, survival and tube formation in HUVEC [546] and neovascularisation in murine models [547]. Recently, it has been shown that global c-Kit deletion increases the number of atherosclerotic plaques in mice fed a high fat diet [548]. These data support the potential regulation of endoglin and integrin α_v by OSS in EC but are less clear for c-Kit. These target genes need to be validated by other methods in EC exposed to OSS but may provide novel insight into OSS-mediated gene regulation.

It is unclear why MTX did not have the same effects in EC subjected to shear stress and static culture. To rule out the possibility that the drug preparation did not work, the efficacy of MTX was always tested in EC under static conditions alongside the shear stress experiments (shown in Supplementary Figure 5). It is important to highlight that the experiments performed with MTX-treated EC under static and shear stress conditions are not directly comparable, which may account for the differences seen. Although the same concentration and timepoint of the drug were used in both conditions, it was not possible to exactly replicate the growth conditions for the cells under static and shear conditions using the parallel plate model. This was due to the different growth chambers used (flow slides), length of experiment (48h of pre-conditioning with shear stress) and perfusion system with excess media. The flow slides have a very small medium reservoir and are not designed for long-term culture (>24h) under static conditions. This issue was partly addressed by using the orbital shaker model where the cells were kept in the same chamber format and amount of medium, which encouragingly produced similar results to the parallel plate model.

Another possible explanation for the lack of drug effect under shear stress is that MTX may be differentially taken up or metabolised by EC under static and shear stress conditions. To visualise MTX uptake into the cells, EC were treated with increasing concentrations of a commercially available FITC-conjugated form of MTX (F-MTX) and analysed by flow cytometry (data not shown). In my hands, F-MTX was only detectable at high concentrations (10 μ M) in static EC. Further review of the literature revealed that F-MTX was not suitable to study MTX uptake because it was able to enter the cell by passive diffusion across the lipid membrane and did not require active transport mechanisms (unlike MTX) [549]. MTX levels and polyglutamation state can be measured using LCMS but this was technically not feasible in EC under shear stress due to the high cell number required. Older methods rely on the radioactive detection of an MTX isotope, which was also ruled to be not feasible with the

current parallel plate model. Intracellular localisation of MTX can be measured by making use of its high binding affinity to DHFR and its consequent inhibition. I tried to optimise a DHFR activity assay to assess MTX uptake into EC, but the assay was not sensitive enough for the amounts of cell lysate generated with the flow system (Supplementary Figure 9). Due to these technical difficulties, these questions remain to be addressed.

There are several limitations to the work presented in this chapter, many of which are explained by the intrinsic features of the shear stress model used. The parallel plate model is low throughput, technically challenging, expensive, lacks good comparability to static culture in long-term experiments and critically only generates small amounts of cellular material for downstream analyses. For these reasons, it is very difficult, and on occasion technically impossible, to address certain experimental questions or achieve sufficient repeats for a given experiment. Given that maximally only three or four experimental conditions could be run on this system at the same time under OSS or LSS respectively, it was not possible to optimise the timepoint and concentration of MTX or to routinely include other pharmacological agents in the system. In comparison, the orbital shaker model addressed some of these limitations by using the same plate format and facilitating more high-throughput experimental setups at a lower cost. However, it was clear that this system produced less discriminatory EC phenotypes in regions of undisturbed and disturbed shear stress, which may not accurately model endothelial profiles *in vivo*. In this chapter, only *in vitro* systems were used to study the effects of MTX on vascular EC under shear stress. The kinase and gene expression arrays used investigated only a selected number of kinases or genes.

Based on the observation that treatment with MTX, a folate antagonist, resulted in the activation of cell signalling and cell cycle arrest by folate depletion in EC under static conditions (Chapter 3) but did not induce the same effects under shear stress, I speculated that folate metabolism may be differentially regulated in EC under shear stress and static culture. To date, there is no report on the role and regulation of folate metabolism in EC exposed to shear stress. I hypothesised that folate metabolism may provide the key to better understanding the effects of MTX on the vasculature. Folate metabolism may serve different functional roles in EC under shear stress compared to static conditions and the identification of these mechanisms may provide insights into how MTX may affect these. For example, folate metabolism is an important cellular source of nucleotides, which are required for proliferation [317]. It seems plausible that cells exposed to shear stress may alter their metabolic

requirements for folate as proliferation is strongly modulated under these conditions, providing potential evidence for why MTX did not affect cell cycle progression in these cells. This chapter had not identified any novel MTX targets, which may be explained by the fact that this biological baseline is not well defined, limiting the ability to ask the correct experimental questions. Although it is well established that MTX is a folate inhibitor, it can be argued that studying folate metabolism does not contribute to understanding of the biology of the drug because folate antagonism is not considered to be the main mechanism of action of MTX in patients with rheumatic diseases [230]. However, there is evidence to suggest that folate metabolism may be more important than previously acknowledged. Two independent reports showed that folinic acid supplementation at high doses can reverse the anti-inflammatory effects of MTX in patients with RA [550, 551]. Furthermore, while many reports have shown that MTX inhibits folate-dependent enzymes in multiple cell types relevant to RA [230], nobody has formally linked its actions on folate metabolism and its anti-inflammatory effects. Although it is not possible to exclude that the inhibition of folate metabolism by low-dose MTX is an *in vitro* phenomenon, this did not seem very likely given the extensive literature on its folate-dependent effects. Beyond MTX, it was of great interest to see if folate metabolism was differentially regulated in EC under shear stress as this has not been addressed previously. Therefore, these considerations led to the decision to study folate metabolism in EC under static and shear stress conditions in the next chapter.

5 Chapter 5 – One carbon metabolism in EC

5.1 Introduction

MTX is a structural folate analogue known to disrupt folate metabolism in cancer cells by inhibiting the folate-dependent enzymes DHFR, TYMS and ATIC [274]. Although the role of this pathway in the anti-inflammatory effects of MTX is contended, the experiments from Chapters 3 and 4 indicate the importance of folate metabolism in the drug's effects on EC.

OCM encompasses biochemical reactions that transfer one carbon units between different folate intermediates, which serve as substrates for cellular nucleotide synthesis, amino acid metabolism, methylation reactions and contribute to redox homeostasis (section 1.5.2). OCM is compartmentalised in the cell, where a distinct set of enzymes perform similar chemical reactions in parallel in the cytosol and mitochondria (and to some extent in the nucleus). The proper function of OCM is critical at all stages of life due to its implication in embryogenesis, haematopoiesis, immune cell activation, CVD and cancer.

The role of OCM is not well understood in primary cells including EC [317]. As outlined in section 1.5.2.4.5, it is thought that OCM contributes to EC homeostasis by maintaining eNOS-dependent NO production through increasing the bioavailability of its co-factor BH₄ [552]. DHFR is part of the BH₄ salvage pathway and contributes to NO bioavailability in EC [396]. Moreover, folic acid or its derivative 5-methyl-tetrahydrofolate (5-meTHF) increase BH₄ levels in EC [299] and endothelial-dependent vasorelaxation in vessels from patients with CVD [393]. However, folate supplementation has failed to reduce cardiovascular events in clinical trials [380-382], implying that OCM is still incompletely understood in vascular cells.

In the previous chapters, it was demonstrated that MTX exerted folate-dependent effects on cell cycle progression and signalling in static EC which could not be replicated under shear stress conditions. This raised the possibility that OCM may be differentially regulated in these two settings and hence provide a basis for the drug's differential actions. Shear stress is well known to alter endothelial metabolism (section 1.5.1), supporting this hypothesis. As OCM is generally poorly characterised in EC, the aim of this chapter was to delineate its role and regulation in EC under static and shear stress conditions, proposing that insights into this pathway may provide a molecular foundation for better understanding the effects of MTX in the vasculature. The role of OCM was assessed in EC by analysing gene expression, metabolite

levels, cell proliferation and mitochondrial morphology and function. Cell proliferation was studied because OCM-dependent nucleotide synthesis is essential for proliferation in many cell types [317]. OCM may also play a role in EC proliferation as mild folate deficiency caused cell cycle alterations in an EC-epithelial hybrid cell line [394]. Mitochondria were selected as an experimental readout because mitochondrial OCM facilitates mitochondrial protein synthesis [340, 343] and is thought to be the main route for generating formate [334], which is used for biosynthetic processes in the cytosol. Inhibition of the mitochondrial respiratory chain is reported to cause an upregulation of OCM genes in EC [307] and inhibit serine-driven formate production in cancer cells [553], providing evidence for a close functional relationship between OCM and mitochondria. Importantly, normal mitochondrial function is important for EC homeostasis and dysfunction is evident in vascular diseases such as diabetes and atherosclerosis [554].

In this chapter, the regulation and function of endothelial OCM was explored under static and shear stress conditions. Investigating its role in EC may shed light on the implications of folate and anti-folate therapy in vascular health and disease.

5.2 Hypothesis of the chapter

OCM is differentially regulated in EC under static and shear stress conditions.

5.3 Aims of the chapter

- 1) Determine the expression levels of genes involved in OCM in EC subjected to static and shear stress conditions
- 2) Explore the transcriptional regulation of OCM in EC
- 3) Measure folate metabolite levels in EC subjected to static conditions and shear stress
- 4) Define the role of OCM in EC proliferation
- 5) Explore the role of OCM in mitochondrial morphology and membrane potential in EC

5.4 Results

5.4.1 OCM is differentially regulated in EC by shear stress

5.4.1.1 OCM gene expression in shear-exposed EC in a published RNA sequencing dataset

A schematic overview of genes involved in OCM is given in Figure 31A. To evaluate if the expression of these genes is altered in EC by shear stress, transcript levels of selected genes were first screened in a previously published RNA sequencing dataset of HUVEC subjected to either pulsatile (PS) or oscillatory shear stress (OSS) over a time period of 24h using a parallel plate model [132].

From the publicly available data, transcript levels of genes implicated in folate transport and de-glutamation (SLC19A1, GGH) showed a trend towards being reduced by both PS and OSS over the 24h time course, while the levels of the enzyme required for folate polyglutamation (FPGS) were not affected (Figure 31B). Interestingly, the transcript levels of many enzymes in the cytosolic OCM compartment (DHFR, TYMS, MTHFD1 and SHMT1) were downregulated in EC under both shear stress conditions (Figure 31C). For example, the expression of DHFR, one of the main MTX targets, was decreased down to 35% by PS and to 50% by OSS respectively at 24h, compared to the 1h timepoint. The levels ofATIC and MTHFR, enzymes required for purine metabolism and maintenance of the methylation cycle respectively, were not altered under these conditions. In contrast to their cytosolic counterparts, mitochondrial MTHFD2, SHMT2 and MTHFD1L transcript levels were unaffected by shear stress (Figure 31D). These findings suggested that OCM genes may be differentially regulated in EC by shear stress.

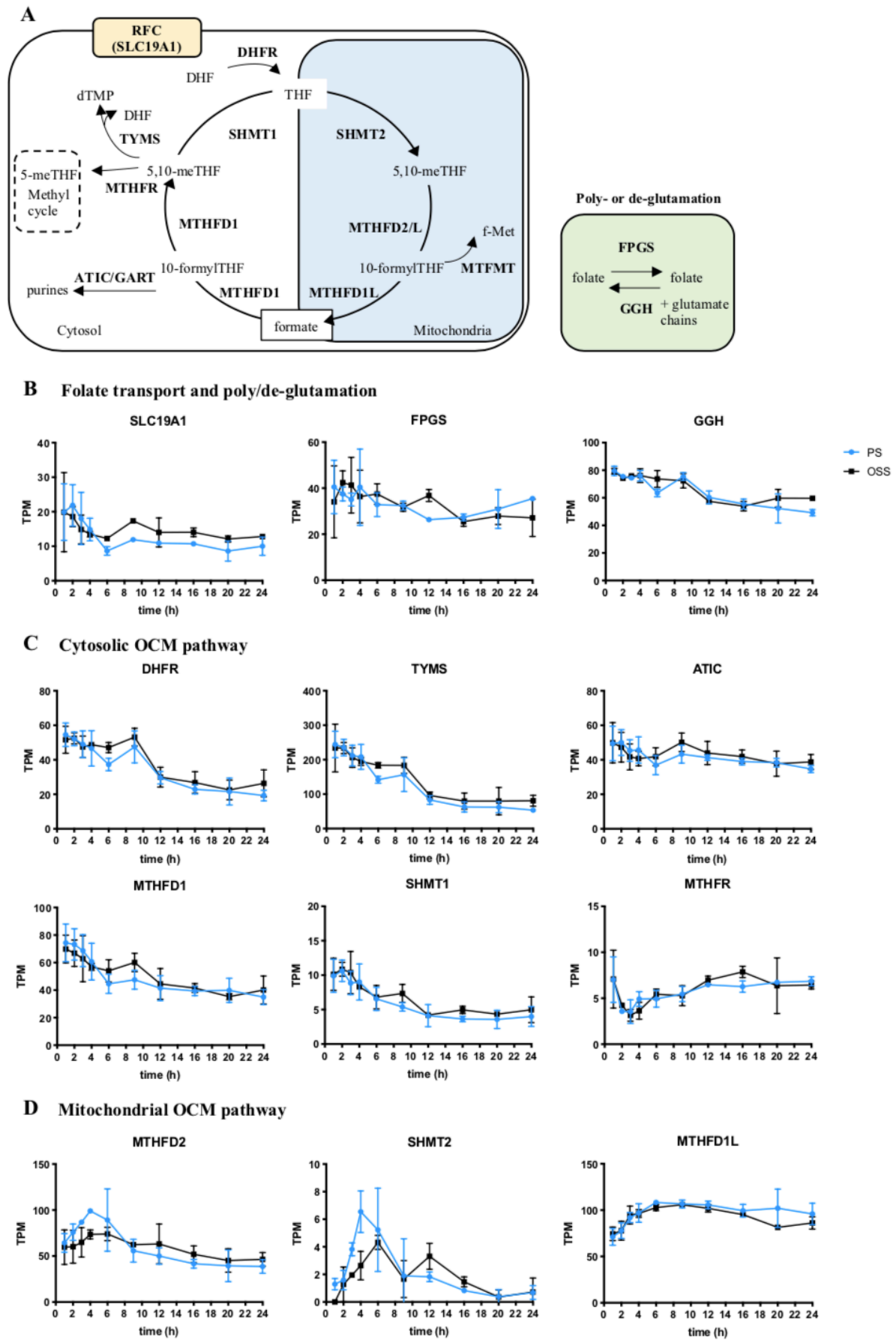


Figure 31. Transcript levels of transporters and enzymes related to OCM in HUVEC exposed to shear stress in a published RNA sequencing dataset. (A) Schematic representation of mammalian OCM. (B-D) Data obtained from publicly available RNA sequencing dataset (Gene Expression Omnibus (GEO) GSE103672) published by Ajami et al. [132]. Transcripts per million (TPM) of selected transporters and enzymes related to OCM in HUVEC subjected to pulsatile (PS, $12 \pm 5 \text{ dyn/cm}^2$) or oscillatory shear stress (OSS, $0.5 \pm 5 \text{ dyn/cm}^2$) using a parallel plate model over a period of 24h. (B) Proteins involved in cellular folate transport and (de-)polyglutamation. (C) Enzymes in cytosolic OCM compartment. (D) Enzymes in mitochondrial OCM compartment. Data extraction from RNA sequencing dataset kindly supported by Dr. Robert Maughan.

5.4.1.2 OCM gene expression in shear-exposed EC using the parallel plate model

To validate these findings in EC exposed to the static and shear stress conditions used in Chapters 3 and 4, HUVEC were subjected to either static culture, H-LSS (20 dyn/cm^2), L-LSS (5 dyn/cm^2) or OSS ($\pm 5 \text{ dyn/cm}^2$, 2Hz) for 48h using the parallel plate model. The expression of selected OCM genes was analysed by qPCR and is shown relative to the static condition (Figure 32 and Supplementary Figure 10).

EC exposed to shear stress showed a typical expression pattern of shear-inducible target genes as expected (Figure 32B and Supplementary Figure 10): H-LSS induced the mRNA expression of KLF2 (17.8-fold; $p < 0.0001$) and TM (29-fold; $p = 0.0061$), while OSS increased VCAM1 expression (56.4-fold; $p = 0.0127$) compared to the static condition, showing a similar trend for E-selectin. L-LSS induced the expression of all control genes in EC albeit to a lesser extent, producing an intermediate phenotype.

The mRNA expression of the reduced folate carrier (RFC; gene name SLC19A1) was reduced by H-LSS (by about 50%; $p = 0.0156$) but was not affected by OSS in EC compared to static conditions, showing a differential regulation between H-LSS and OSS (Figure 32B (heatmap) and 32C (individual gene plot)). The mitochondrial isoform of FPGS, an enzyme required for folate polyglutamation, was not affected by shear stress, while the expression of its functional counterpart (GGH) was reduced by H-LSS (by 65%; $p = 0.0041$) compared to static condition. In line with the published RNA sequencing data, the mRNA expression levels of the cytosolic enzymes DHFR ($p = 0.0059$), TYMS ($p = 0.0027$) and MTHFD1 ($p < 0.0001$) were reduced by both H-LSS and OSS by 50-70% compared to static conditions (Figure 32D). Again, ATIC expression was not altered by shear stress. GART, an enzyme required for purine biosynthesis, was downregulated by H-LSS (by 40%) and OSS (by 20%; $p < 0.0001$) compared to static EC.

Enzymes localised in the mitochondrial compartment all showed a similar pattern of regulation (Figure 32E): mRNA levels of MTHFD1L ($p=0.0003$), SHMT2 ($p=0.0025$) and MTHFD2 ($p=0.025$) were increased 1.6 to 1.9-fold under OSS compared to H-LSS. In line with its shear rate, L-LSS showed an intermediate response between H-LSS and OSS for most genes.

The protein expression levels of MTHFD2 and SHMT2 were evaluated in HUVEC as treated above in a single experiment. In line with the gene expression data, MTHFD2 expression was increased by OSS compared to H-LSS (Supplementary Figure 11). SHMT2 protein levels were lower in all shear stress conditions compared to static EC, requiring further validation.

These results suggested that several key genes in the OCM pathway are differentially regulated in HUVEC under static and shear stress conditions, showing similar gene expression patterns to those seen in the RNA sequencing dataset. While genes in the cytosolic pathway were generally either not affected or downregulated by all shear conditions compared to static culture, members of the mitochondrial pathway were differentially expressed between H-LSS and OSS, potentially pointing towards different metabolic requirements for folate under these conditions.

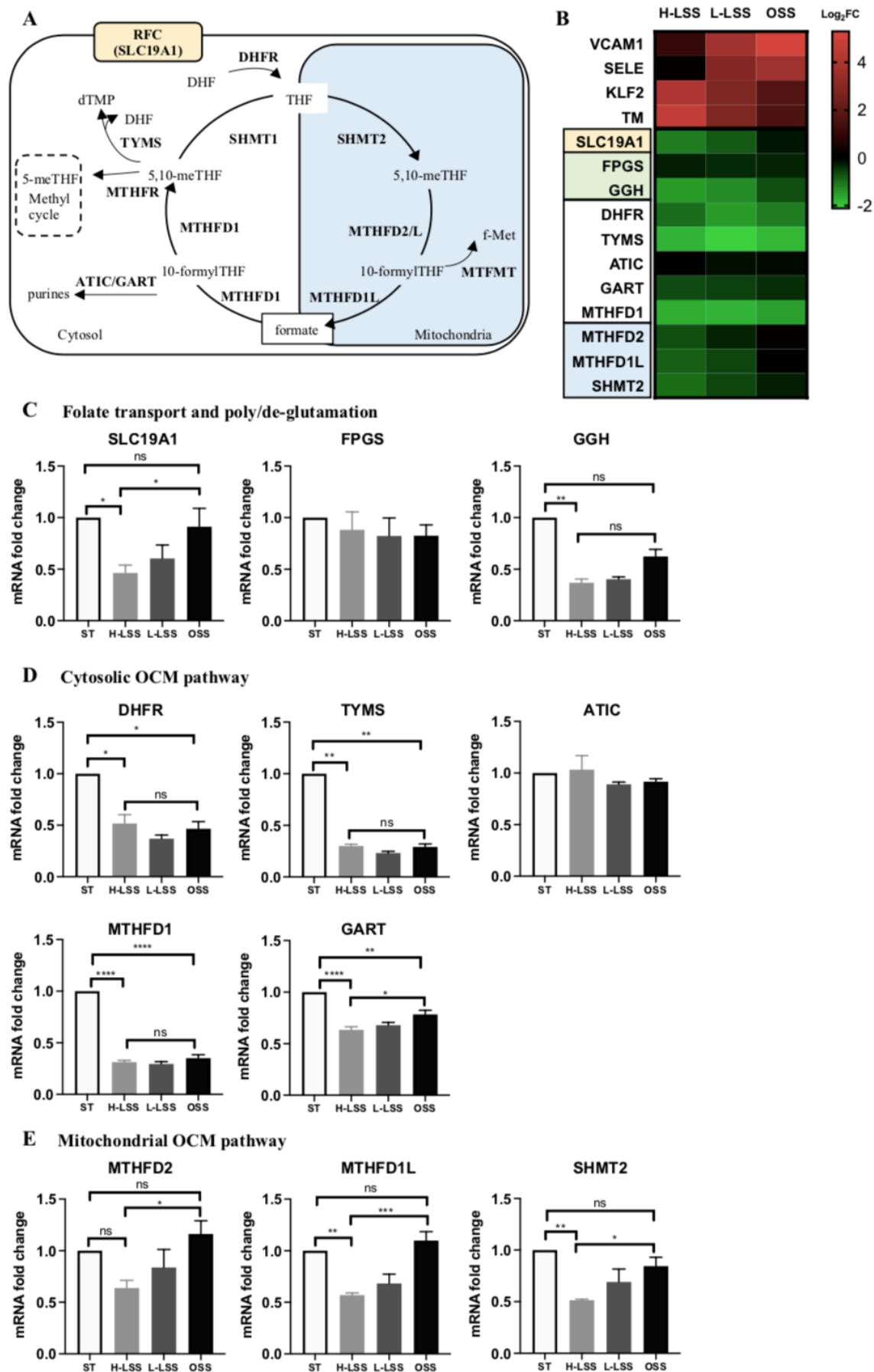


Figure 32. OCM gene expression is differentially regulated in HUVEC exposed to shear stress and static culture. (A) Schematic representation of mammalian OCM. (B-E) Gene expression analyses of HUVEC subjected to static culture (ST), high LSS (H-LSS; 20dyn/cm²), low LSS (L-LSS; 5dyn/cm²) or OSS ($\pm$ 5dyn/cm², 2Hz) for 48h by qPCR (n=3). (B) Heat map of log₂fold changes of the expression of selected genes related to OCM and shear stress-related controls (VCAM1, SELE, KLF2 and TM) in respectively treated HUVEC. Different functional categories are highlighted by coloured boxes. Proteins involved in cellular folate transport (yellow) and (de-)polyglutamation (green). Enzymes in cytosolic compartment (white). Enzymes in mitochondrial compartment (blue). (C-E) mRNA expression levels of genes in the OCM pathway in HUVEC treated as described above. mRNA expression levels normalised to GAPDH and shown relative to static conditions. Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. Values represent means $\pm$ SEM. ns, not significant. * p<0.05. ** p<0.01. *** p<0.001. **** p<0.0001.

To confirm that these findings were not specific to HUVEC, the expression of the same OCM genes, including the additional targets SHMT1 and MTR, was also evaluated in HAEC using the same experimental setup (Figure 33A-D and Supplementary Figure 10). Similar to HUVEC, H-LSS induced the mRNA levels of KLF2 (16.9-fold; p=0.0004) and TM (11.8-fold; p=0.01), while OSS increased VCAM1 (20.2-fold; p=0.0413) compared to static EC (Figure 33A and Supplementary Figure 10). The mRNA levels of RFC (SLC19A1) were decreased by H-LSS (by 70%, p=0.0211) but again not changed by OSS compared to static EC (Figure 33B). Mitochondrial FPGS expression was not altered in EC by shear stress, while GGH mRNA levels were reduced by both H-LSS and OSS to a similar extent (p=0.0017). In line with the results generated in HUVEC, the expression levels of the cytosolic enzymes DHFR (p=0.0077), TYMS (p=0.001) and MTHFD1 (p<0.0001) were reduced by 65-80% by all shear stress conditions compared to static culture, showing a similar trend for SHMT1 (Figure 33C). The mRNA levels of ATIC and MTR were not changed but GART expression was reduced by H-LSS (p=0.0176) compared to the static condition. The mitochondrial enzymes (MTHFD2, MTHFD1L, SHMT2) again tended to be more highly expressed in EC under OSS compared to H-LSS, although this only reached statistical significance for MTHFD1L (p=0.0003) (Figure 33D).

To validate these results in a different shear stress model, the expression of a select number of OCM genes was assessed in HAEC subjected to the orbital shaker for 72h. This experiment was only performed once (Figure 33E (heatmap) and Supplementary Figure 10 (individual gene plots)). The expression of RFC (SLC19A1) was reduced under both shear conditions, but FPGS and GGH levels were not affected. Supporting the previous observations, DHFR and TYMS mRNA levels were decreased by shear stress compared to static culture, while ATIC expression was not changed.

In general, all genes showed a similar expression pattern in HUVEC and HAEC, supportive of a universal shear stress-induced regulation of OCM in EC. This is not likely to be an effect specific to the parallel plate system as similar results were generated with the orbital shaker model (although this was only examined in a single experiment). Taken together, the expression levels of OCM genes were found to be differentially regulated in EC under shear stress as evaluated bioinformatically in a publicly available RNA sequencing dataset and experimentally in two different EC types.

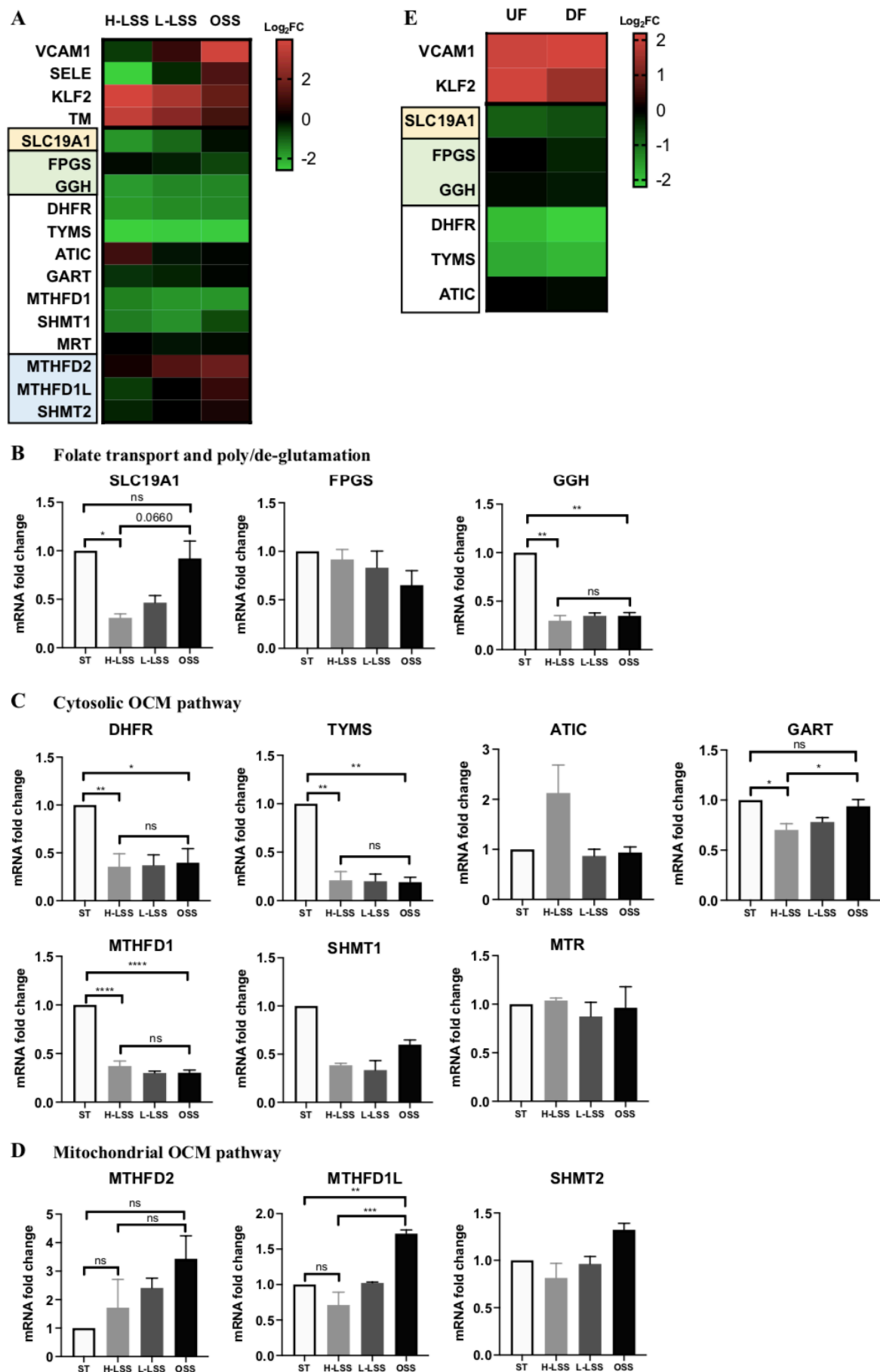


Figure 33. OCM gene expression is differentially regulated in HAEC exposed to shear stress and static culture. (A-D) Gene expression analyses of HAEC subjected to static culture (ST), high LSS (H-LSS; 20dyn/cm²), low LSS (L-LSS; 5dyn/cm²) or OSS ($\pm$ 5dyn/cm², 2Hz) for 48h by qPCR (n=3). (A) Heat map of log₂fold changes of the expression of selected genes related to OCM and shear stress-related controls (VCAM1, SELE, KLF2 and TM) in respectively treated HAEC. (B-D) mRNA expression levels of genes in the OCM pathway in HAEC treated as described above using the parallel plate model. mRNA expression levels normalised to GAPDH and shown relative to static conditions (n=3; SHMT1 n=2). (E) Gene expression analyses of HAEC subjected to static culture, undisturbed (UF) or disturbed flow (DF) for 72h using the orbital shaker model (n=1). Heat map of log₂fold changes of the expression of selected genes. Different functional categories are highlighted by coloured boxes. Proteins involved in cellular folate transport (yellow) and (de-) polyglutamation (green). Enzymes in cytosolic compartment (white). Enzymes in mitochondrial compartment (blue). Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. Values represent means $\pm$ SEM. ns, not significant. * p<0.05. ** p<0.01. *** p<0.001. **** p<0.0001.

5.4.1.3 KLF2 does not regulate the transcription of selected OCM genes in static EC

As shear stress extensively modulated the expression of endothelial OCM genes, it raised the question whether this is achieved by direct transcriptional regulation. Shear stress induces distinct transcriptional programs to fine-tune EC phenotypes, with the transcription factor KLF2 being one of the key regulators of shear-sensitive genes in EC [101, 132] (section 1.2.4). As KLF2 overexpression recapitulates many aspects of shear-induced phenotypes and has been used as a model of LSS in previous reports [314, 555], it was explored whether KLF2 coordinated the transcriptional regulation of OCM genes in static EC.

To understand if KLF2 can bind to regulatory regions of OCM genes, the promoter regions of DHFR, MTHFD1 and MTHFD2 were screened for either established or putative KLF2 binding sites using the UCSC Genome Browser, JASPAR database for transcription factor binding motifs and previous reports. The results of the transcription factor binding analyses are shown as an example on the human DHFR promoter (Supplementary Figure 12). Four putative KLF2 binding sites were identified within 400bp upstream of the transcription start site in the DHFR promoter region, two of which overlapped with established Sp1 binding sites [556]. Moreover, three putative KLF2 binding sites were found in the MTHFD1 promoter within 550bp upstream of the translation start site. In the MTHFD2 promoter region, one putative KLF2 binding site was found within 400bp downstream of the translation start site (data not shown).

To examine if KLF2 regulates the expression of these genes in EC, KLF2 expression was either increased using an overexpression plasmid or reduced by siRNA depletion in HUVEC grown under static conditions (Figure 34). HUVEC were transfected with sictrl for 48h (as part of a different experiment), followed by transfection with control pcDNA or KLF2 overexpression plasmids for 24h and gene expression analyses by qPCR (Figure 34A). KLF2 overexpression was confirmed by measuring KLF2 mRNA levels (10.2-fold induction compared to control; $p=0.0085$). However, DHFR, MTHFD1 and MTHFD2 mRNA levels were not different in EC overexpressing KLF2 compared to control. For KLF2 depletion, HUVEC were transfected with a non-targeting control siRNA (sictrl) or siRNA against KLF2 for 48h and analysed by qPCR (Figure 34B). siRNA depletion of KLF2 caused a 60% reduction ($p<0.0001$) of KLF2 expression compared to control but did not affect MTHFD1 mRNA expression levels (other genes not measured). These results demonstrated that KLF2 did not regulate the gene expression of DHFR, MTHFD1 or MTHFD2 in static EC.

Evidently, these experiments were exploratory and do not conclusively address whether KLF2 regulates OCM gene expression in EC under shear stress. However, KLF2 overexpression strategies in static EC have been used in other reports to model shear stress-specific responses and are at the very least indicative of showing a (non)-response. These results suggested that shear-induced regulation of OCM may be mediated by a more complex transcriptional program that either involves KLF2 and co-operating factors or other transcription factors.

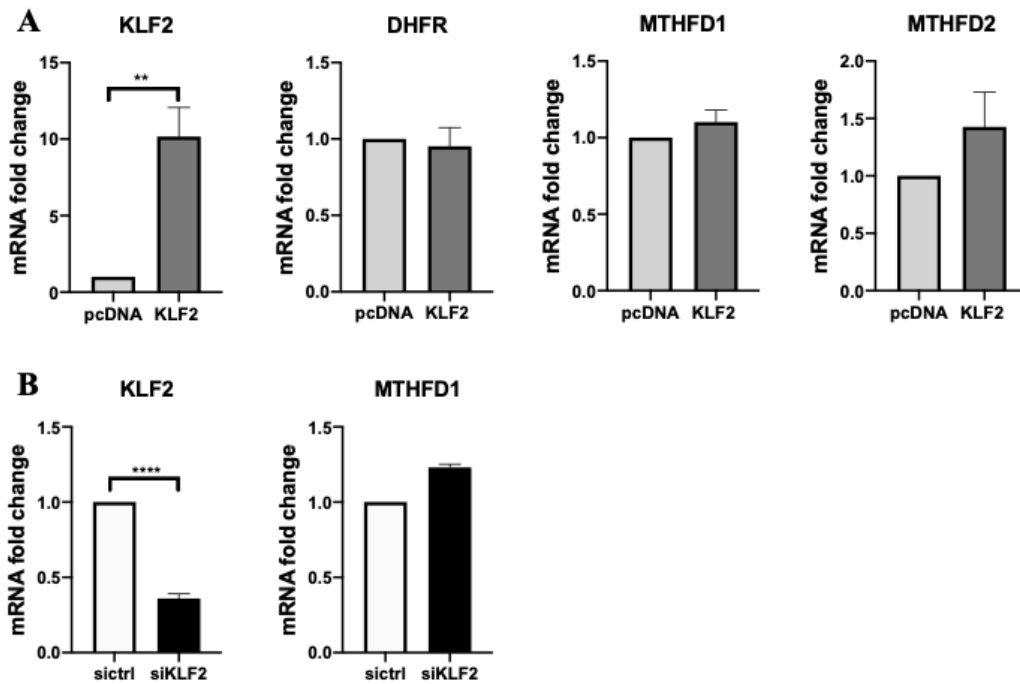


Figure 34. KLF2 does not regulate the expression of selected genes in the folate pathway. (A) Gene expression analyses of HUVEC transfected with non-targeting siRNA (siCtrl) for 48h, followed by transfection with control pcDNA or KLF2 plasmids for 24h, by qPCR (n=3). (B) Gene expression analyses of HUVEC transfected with a non-targeting siRNA (siCtrl) or siRNA against KLF2 (siKLF2) for 48h by qPCR (n=3). mRNA expression levels normalised to GAPDH and shown relative to control. Values represent means $\pm$ SEM. Unpaired Student's t-test. ns, not significant. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$. All samples prepared and kindly provided by Dr. Claire Peghaire (Imperial College London).

5.4.2 Folate metabolites in EC subjected to shear stress measured by LCMS

To understand if the differential expression of OCM genes causes metabolic alterations in EC under static and shear stress conditions, folate metabolite levels were measured in HUVEC subjected to static culture, H-LSS or OSS in EGM-2 (10%FCS) for 48h by LCMS (Figure 35). Five individual experiments were pooled for one LCMS run.

EC exposed to shear stress displayed approximately 75% lower levels of folate metabolites compared to those grown under static culture (Figure 35B). Interestingly, total folate metabolite levels were not different between EC under H-LSS or OSS. Figure 35C shows the levels of individual folate metabolites expressed as percentage of total folate levels in each condition. The levels of 5-meTHF were not altered in EC exposed to shear stress compared to static culture. THF, 5,10-me⁺THF and 5,10-meTHF levels were lower in EC under shear stress compared to static EC, while DHF was not detectable in any condition. Remarkably, the levels of 10-formylTHF, required for *de novo* purine synthesis and mitochondrial protein synthesis, were higher in EC exposed to H-LSS and OSS compared to static EC. Of note, the pattern of folate metabolites in static EC was similar to those measured in a previous report in human fibroblasts but not lymphocytes [493].

Although these findings need to be validated in further experiments, they provide initial evidence to support the hypothesis that OCM is differentially regulated on a metabolic level in EC under static compared to shear stress conditions, in line with the observed gene expression patterns in these cells. However, so far, no differences in folate metabolite levels in EC subjected to H-LSS compared to OSS have been found.

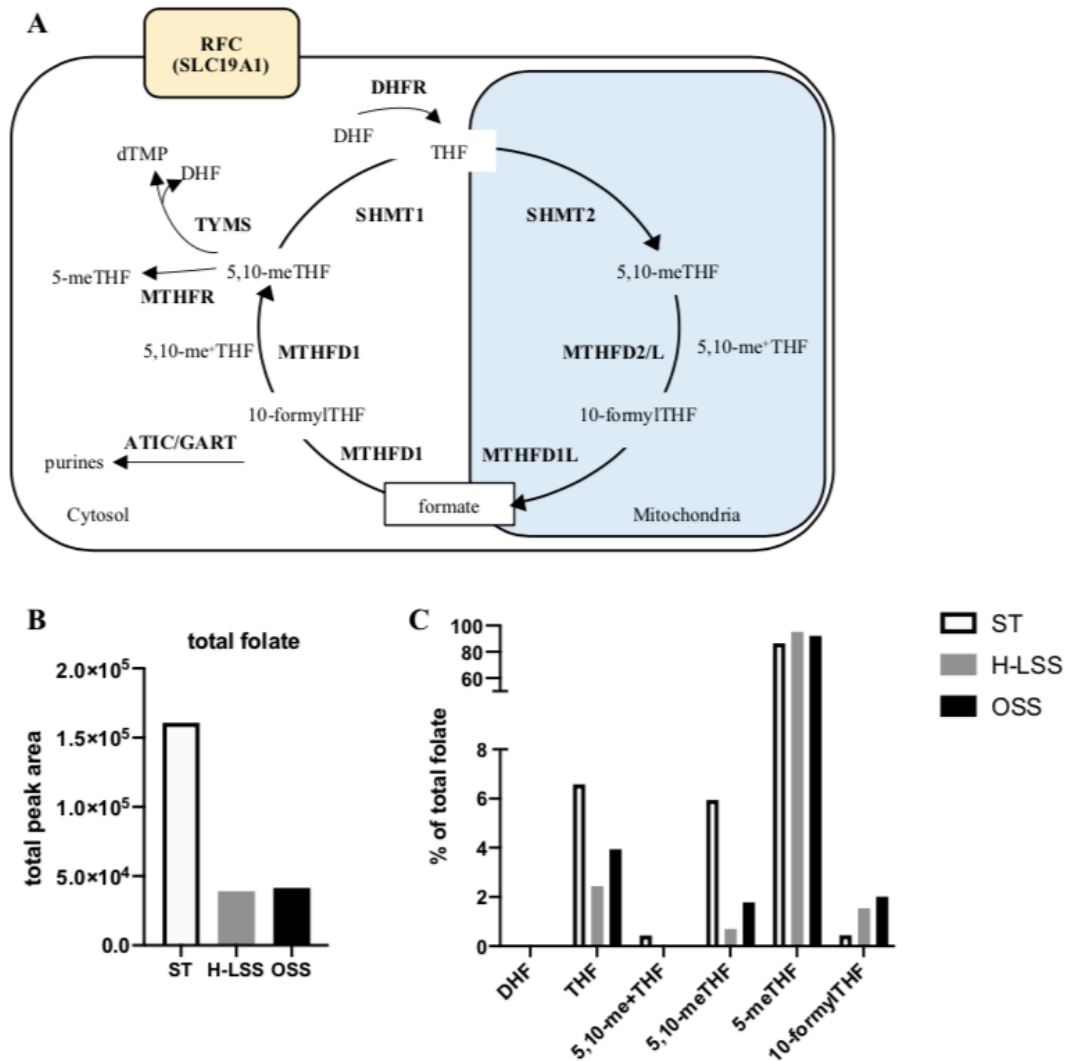


Figure 35. Folate metabolite levels in EC subjected to shear stress. (A) Schematic representation of mammalian OCM. (B+C) Folate metabolite profiles of HUVEC, kept under static conditions (ST) or subjected to H-LSS (20dyn/cm²) or OSS ($\pm$ 5dyn/cm², 2Hz) for 48h analysed by LCMS (in collaboration with Dr. Kit-Yi Leung, UCL). Five individual shear stress experiments were pooled for one LCMS run (n=1). These experiments were performed in EGM-2 (10%FCS). (B) Sum of the total peak area of each individual folate metabolite to give the total folate levels in each condition. (C) Folate metabolite levels shown as percentage of total folate in each condition. DHF, dihydrofolate. THF, tetrahydrofolate. 5,10-meTHF, 5,10-methylene-THF. 5,10-me+THF, 5,10-methenyl-THF. 10-formylTHF.

5.4.3 Interference with folate metabolism affects proliferation in static EC

There is no data on the function of OCM in EC subjected to shear stress and only few reports in EC grown under static conditions (section 1.5.2.4.5). OCM is required for cell proliferation in other cell types based on its contribution to nucleotide synthesis [317]. To understand if OCM promotes cell cycle progression in EC, HUVEC were either grown in folate-depleted medium or transfected with siRNA against cytosolic and mitochondrial OCM enzymes. Because these data were generated late in the project, the impact of OCM on cell proliferation was only evaluated in EC grown under static conditions.

To induce folate depletion, HUVEC were grown in RPMI medium containing low or high levels of folic acid for fourteen days, after which the cells were stained with PI and analysed by flow cytometry (Figure 36). Cells grown in media containing low levels of folic acid showed a higher percentage of cells in G1 (low folate 59.9% vs high folate 42.8%; $p=0.0128$) and a lower percentage of cells in G2/M (low folate 16.2% vs high folate 30.5%; $p=0.0026$) phases of the cell cycle compared to those grown in high folate levels (Figure 36A and B). The cell count was not significantly different between cells grown under low or high folate conditions, although a trend for lower cell numbers was seen in the low folate condition (Figure 36C).

These results showed that folate depletion caused an accumulation of cells in the G1 cell cycle phase in EC, suggesting that folic acid is required for cell proliferation in EC grown under static conditions, similar to other cell types.

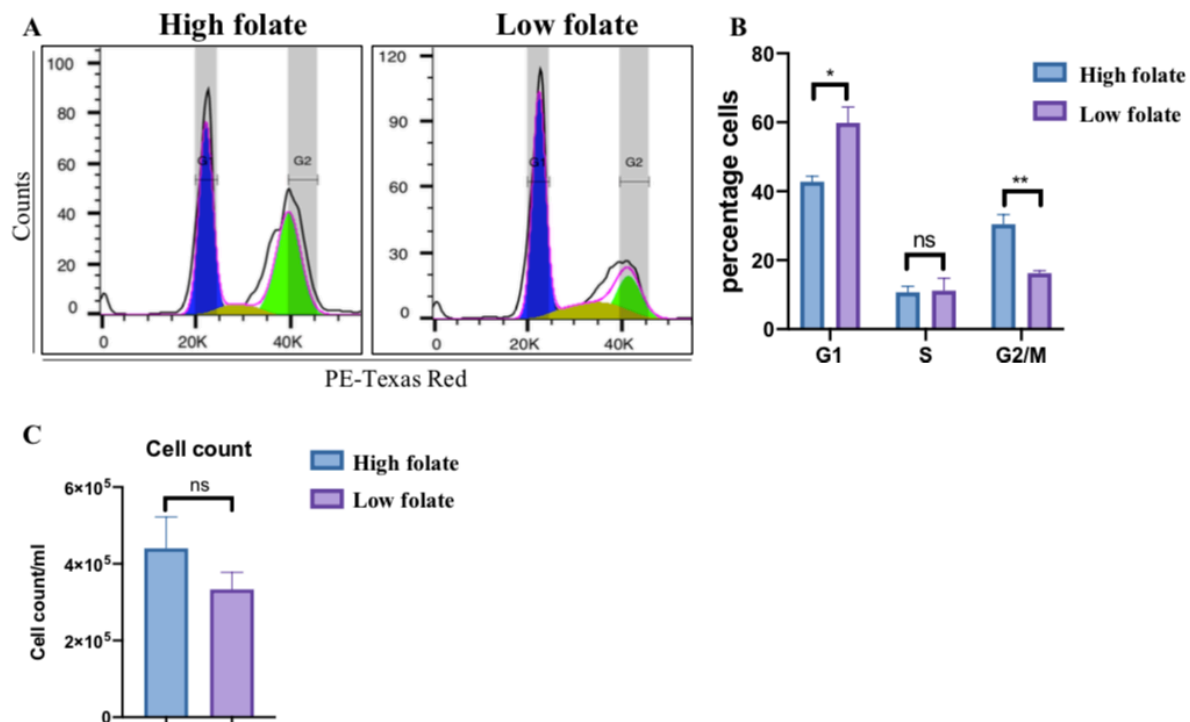


Figure 36. Depletion of folic acid leads to G1 cell cycle accumulation in EC under static conditions. (A) Representative flow cytometry profiles of HUVEC grown in RPMI medium containing low or high levels of folic acid for 14 days. Low folate media (1.91nM folate) = folic acid free RPMI supplemented with 10% FCS, P/S and endothelial growth factors. High folate media (2.3 μ M folate) = complete RPMI supplemented with 10% FCS, P/S and endothelial growth factors. Cells were stained with PI and analysed by flow cytometry (n=4). Phases of cell cycle coloured as following: G1, blue; S, yellow, G2/M, green. (B) Quantification of percentages of cells within the different cell cycle phases of HUVEC grown under low folate or high folate conditions (n=4). (C) Cell counts of HUVEC grown under the respective conditions for 14 days (n=4). Data were analysed using an unpaired t-test. Values represent means $\pm$ SEM. ns, not significant. * p<0.05. ** p<0.01.

To better understand the individual contribution of OCM enzymes to EC proliferation, HUVEC were transfected with non-targeting control siRNA (sictrl) or siRNA against the cytosolic enzymes DHFR, MTHFD1 and SHMT1 for 48h in EGM-2 (2%FCS), stained with PI and analysed by flow cytometry. Cell counts were performed 72h after transfection (Figure 37).

SiRNA depletion of DHFR, MTHFD1 and SHMT1 was confirmed at the mRNA level by qPCR (Figure 37A). DHFR mRNA expression levels were reduced by over 90% ($p=0.0008$) in siDHFR treated cells compared to sictrl; a response that was not seen with the other specific siRNA. Similarly, MTHFD1 ($p<0.0001$) and SHMT1 ($p=0.0032$) expression were reduced by over 90% by siMTHFD1 or siSHMT1 respectively compared to sictrl. Although MTHFD1 mRNA levels were significantly induced by siSHMT1 treatment, this effect was very small (1.3-fold induction) and was not considered to be a strong compensatory mechanism.

Cells transfected with siRNA against DHFR tended to display more cells in G1 compared to sictrl but otherwise showed a similar cellular distribution in the other cell cycle phases (Figure 37B and C). Although depletion of MTHFD1 did not significantly affect cell cycle progression, a trend towards an increase in the percentage of cells in G1 and a decrease in cells in the S-phase was observed compared to sictrl. siRNA depletion of SHMT1 increased the percentage of cells in the G1 phase (siSHMT1 66.4% vs sictrl 56.9%; $p=0.0241$) and showed a trend towards decreased cells in the S-phase compared to sictrl. After 72h of transfection, siRNA depletion of MTHFD1 significantly reduced the cell number compared to sictrl ($p=0.0092$). A similar non-significant trend was seen for DHFR- but not for SHMT1-depleted cells (Figure 37D).

In contrast to other cell types [557], these results demonstrated that siRNA depletion of DHFR did not alter cell proliferation in EC. Depletion of MTHFD1 decreased the cell count and showed a trend to inhibit cell cycle progression, indicating that MTHFD1 may promote EC proliferation in line with its central role in OCM. SHMT1 knockdown induced cell accumulation in G1 but this did not translate into lower cell counts at a later timepoint, suggesting that SHMT1 may not be required for proliferation; in agreement with the more important role of mitochondrial OCM in 1C production.

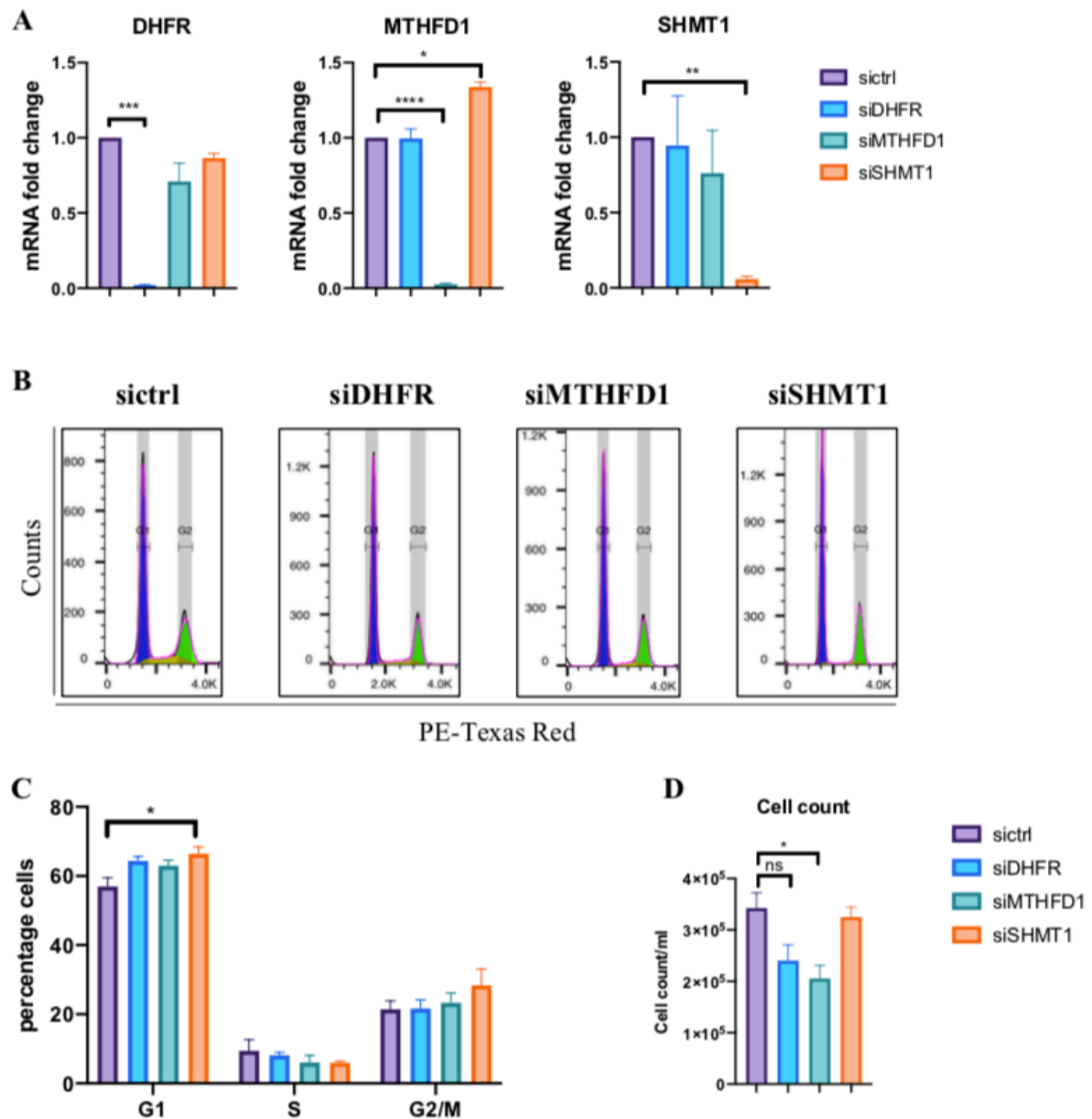


Figure 37. Effects of DHFR, MTHFD1 and SHMT1 depletion on cell cycle progression in static EC. HUVEC were transfected with siCtrl or siRNA against DHFR, MTHFD1 or SHMT1 for 48h. (A) Gene expression analyses of respectively treated HUVEC by qPCR (n=3). (B) Representative flow cytometry profiles of respectively treated HUVEC. Cells were stained with propidium iodide (PI) and analysed by flow cytometry (n=4). Phases of cell cycle coloured as following: G1, blue; S, yellow, G2/M, green. (C) Quantification of percentages of cells within the different cell cycle phases of respectively treated EC (n=4). (D) Cell counts of HUVEC treated respectively 72h after transfection (n=4). Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. Values represent means $\pm$ SEM. ns, not significant. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$.

To address the question whether mitochondrial OCM enzymes have a role in EC proliferation, HUVEC were transfected with sictrl or siRNA against the mitochondrial enzymes MTHFD2 and SHMT2 for 48h, stained with PI and analysed by flow cytometry. Cell counts were performed 72h after transfection (Figure 38).

SiRNA depletion of MTHFD2 and SHMT2 was confirmed by qPCR (Figure 38A) and at the protein level by immunoblotting (Figure 38B and C). MTHFD2 mRNA levels were significantly reduced by 90% with siMTHFD2 ($p<0.0001$) when compared to sictrl. A similar observation was made for SHMT2 ($p<0.0001$) in respectively transfected cells. There was no compensatory regulation of MTHFD2, SHMT2 or their functional counterparts MTHFD1, MTHFD1L and SHMT1 in these EC (Figure 38A and Supplementary Figure 13).

Immunoblotting of cells transfected with sictrl demonstrated that the anti-MTHFD2 antibody detected a double band for MTHFD2 (Figure 38B). Interestingly, the same observation was made for SHMT2. Despite not reaching statistical significance, MTHFD2 protein expression was decreased in respectively transfected EC compared to control (Figure 38B and C). SHMT2 protein expression was reduced by about 70% ($p=0.0025$) in cells transfected with siRNA against SHMT2 compared to sictrl, mainly by affecting the upper band of SHMT2.

MTHFD2 or SHMT2 depleted cells did not show differences in cell cycle progression in EC (Figure 38D and E) and also did not affect cell proliferation measured by cell counting compared to sictrl (Figure 38F). These results suggested that siRNA-mediated depletion of the mitochondrial OCM enzymes MTHFD2 and SHMT2 did not affect cell proliferation in EC, unlike their role in cancer cells.

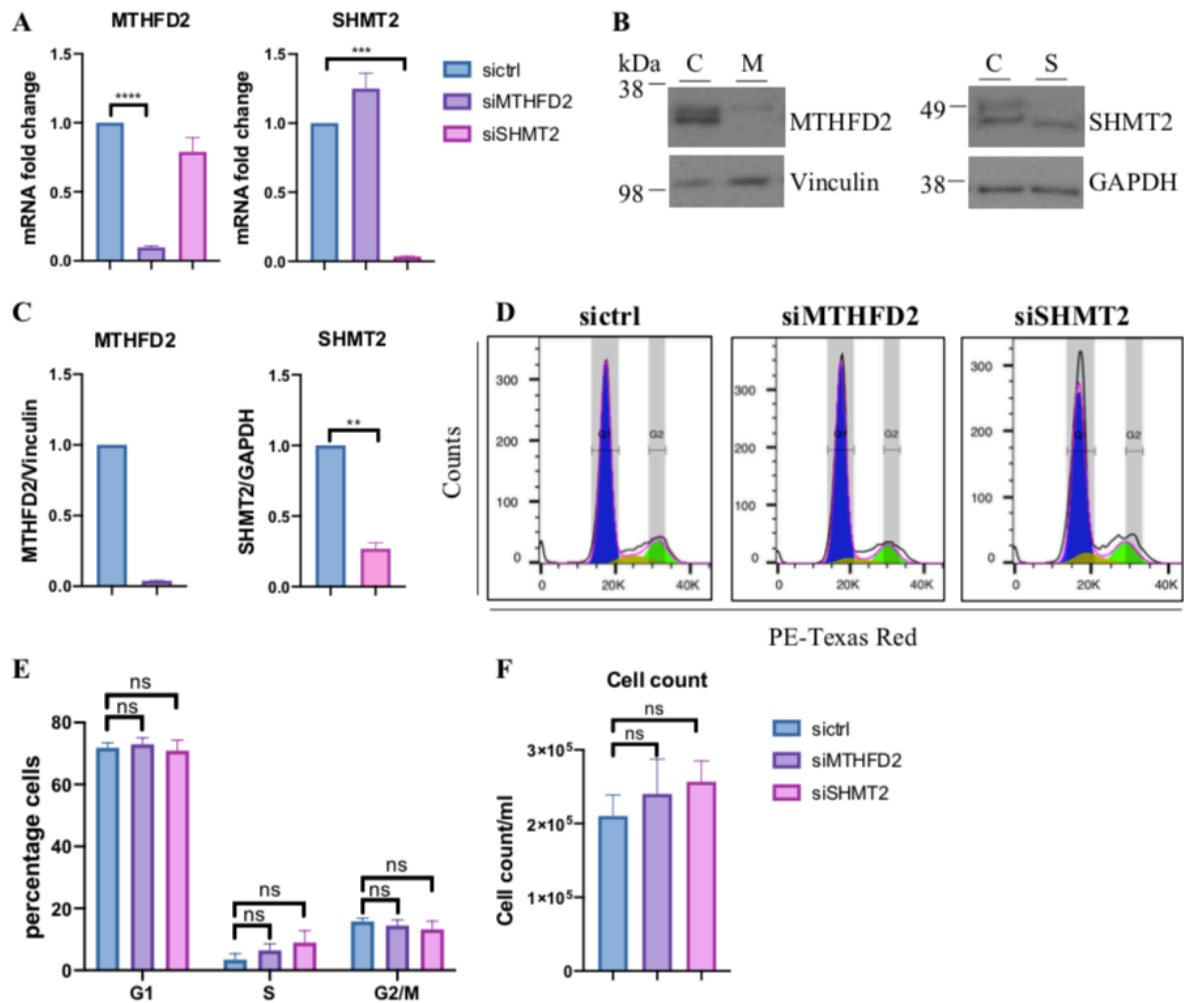


Figure 38. siRNA depletion of MTHFD2 or SHMT2 does not affect cell cycle progression in static EC. (A-F) HUVEC were transfected with sictrl (C) or siRNA against MTHFD2 (M) or SHMT2 (S) for 48h. (A) Gene expression analyses of respectively treated HUVEC by qPCR (n=4). (B) Immunoblot analyses of respectively treated HUVEC. Proteins were detected with antibodies against MTHFD2 (34kDa), SHMT2 (52kDa), GAPDH (39kDa) and Vinculin (124kDa) (n=3). For presentation purposes, the order of the lanes was re-arranged. (C) Quantification of MTHFD2 and SHMT2 protein levels in HUVEC treated as above (n=3). Unpaired T-test. (D) Representative flow cytometry profiles of respectively treated HUVEC. Cells were stained with propidium iodide (PI) and analysed by flow cytometry (n=3). Phases of cell cycle coloured as following: G1, blue; S, yellow, G2/M, green. (E) Quantification of percentages of cells within the different cell cycle phases of EC treated as described above (n=3). (F) Cell counts of HUVEC treated respectively at 72h after transfection (n=3). Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons unless stated otherwise. Values represent means $\pm$ SEM. ns, not significant. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$.

To directly compare the effects of the cytosolic and mitochondrial OCM enzymes on EC proliferation in a colorimetric assay, HUVEC were transfected with sictrl or siRNA against DHFR, MTHFD1, SHMT1, MTHFD2 or SHMT2 for 24h, re-seeded on 96 well plates and grown for an additional 48h. Cell proliferation was measured by BrdU incorporation (Figure 39). SiRNA depletion of MTHFD1 and SHMT1 decreased cell proliferation by 40-50% ($p < 0.0001$) compared to sictrl in EC (Figure 39). Knockdown of DHFR, MTHFD2 and SHMT2 had no effect on EC proliferation.

These results showed that depletion of MTHFD1, but not its mitochondrial counterpart MTHFD2, decreased EC proliferation in agreement with the previous data. Again, DHFR and SHMT2 knockdown did not alter proliferation. SHMT1 depletion led to a reduction in cell proliferation, in contrast to the results described above. This discrepancy remains unclear but may be related to the different experimental setups used. Taken together, OCM is required for cell cycle progression in static EC but the roles of the individual enzymes in cell proliferation differ from those described in cancer cells (with the exception of MTHFD1). Further experiments are required to evaluate the role of OCM in EC proliferation under shear stress.

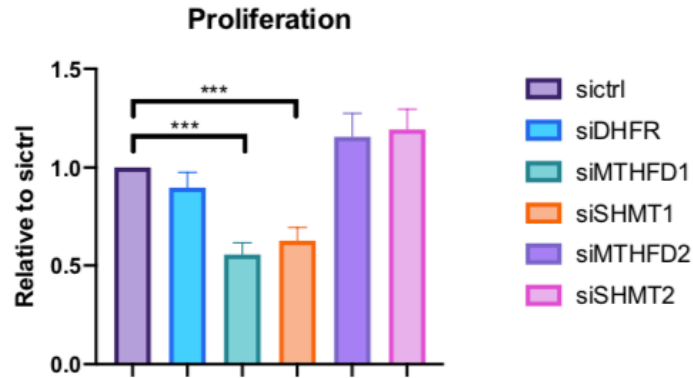


Figure 39. siRNA-mediated depletion of MTHFD1 and SHMT1, but not MTHFD2 or SHMT2, reduces EC proliferation. Proliferation assay of HUVEC transfected with sictrl or siRNA against DHFR, MTHFD1, SHMT1, MTHFD2 or SHMT2. After 24h, cells were re-seeded and grown for an additional 48h (n=3). Proliferation measured by BrdU incorporation. Data shown relative to sictrl. Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. Values represent means $\pm$ SEM. ns, not significant. *** $p < 0.001$.

5.4.4 MTHFD2 depletion alters mitochondrial morphology in EC under shear stress

Based on the observation that mitochondrial OCM genes were differentially regulated in EC under static and shear stress conditions and the proposed intricate role of OCM in mitochondria (outlined above), it was explored whether endothelial OCM affects mitochondrial form and/or function under different culture conditions. To investigate this relationship, the mitochondrial enzyme MTHFD2 was chosen as a target due to its higher expression under OSS compared to H-LSS in HUVEC in this chapter and its implication in pro-atherogenic responses in EC treated with oxidised phospholipids [384]. Mitochondrial function was evaluated in EC exposed to static and shear stress conditions by measuring mitochondrial morphology and membrane potential, which provide information about mitochondrial network dynamics and their functional integrity [554].

To evaluate if MTHFD2 regulates mitochondrial membrane potential and morphology, HUVEC were transfected with sictrl or siMTHFD2 for 24h. The cells were re-seeded on microslides and subjected to static culture, H-LSS or OSS for 48h using the parallel plate model. As a positive control, cells were transfected with siRNA against neuropilin 1 (NRP1), known to reduce mitochondrial membrane potential in EC [558]. The cells were live stained with the fluorescent dyes TMRM (as a marker of mitochondrial membrane potential), Mitotracker (to label mitochondria) and Hoechst (nuclear counterstain) and imaged on a Confocal microscope (Figure 40 and 41; Supplementary Figure 14).

siRNA depletion of MTHFD2 did not alter mitochondrial membrane potential as measured by the integrated signal density (measure of signal area and intensity) of TMRM/Mitotracker normalised to the cell count in EC under static or shear stress conditions compared to sictrl (Figure 40A and B). Depletion of NRP1 decreased the mitochondrial membrane potential in EC as expected, despite not reaching statistical significance (Supplementary Figure 14).

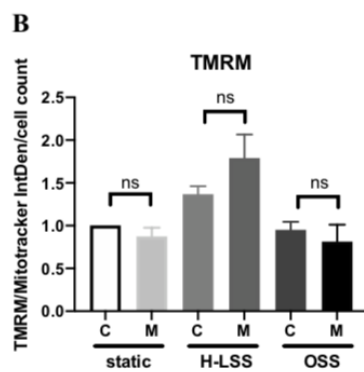
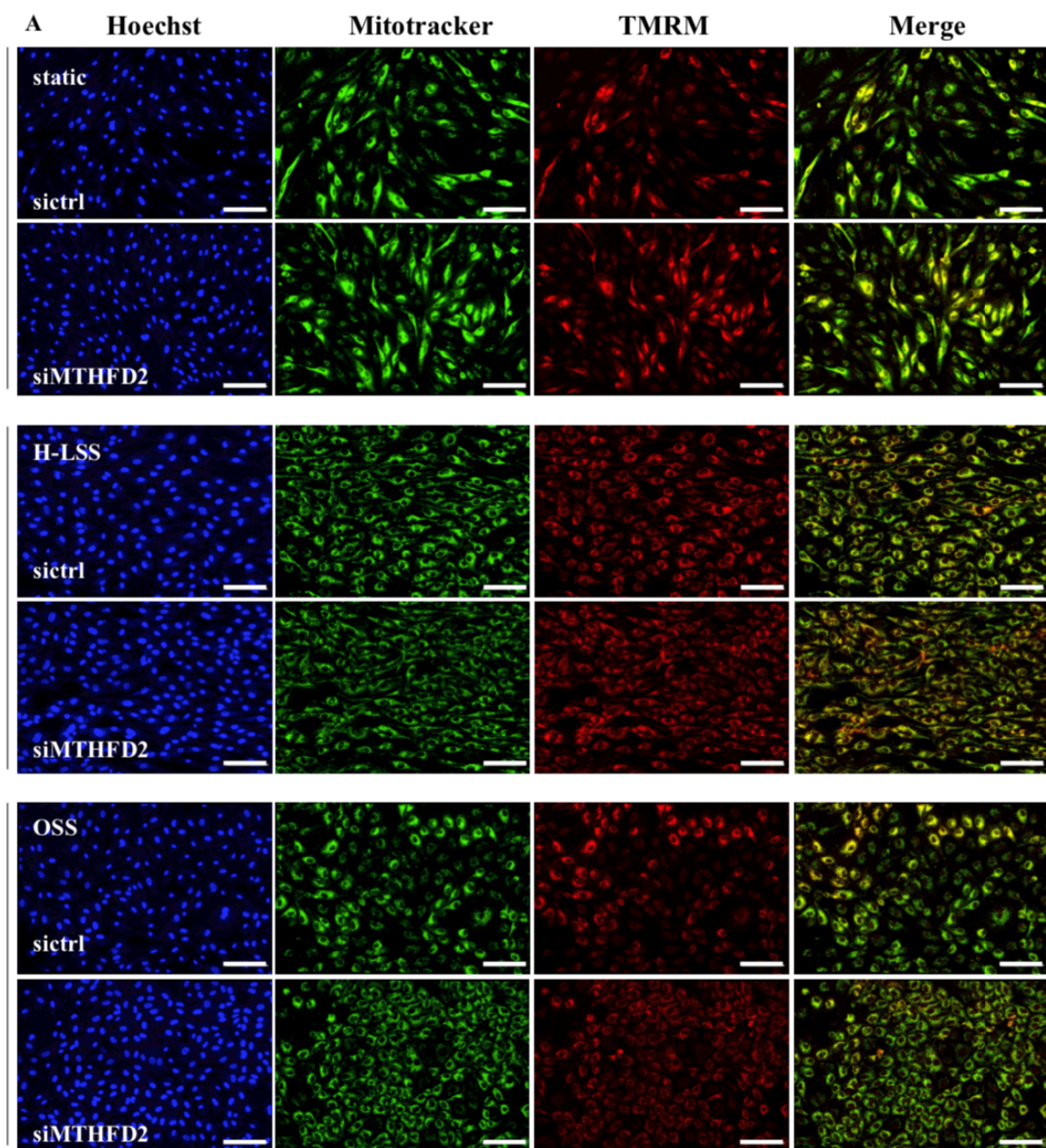


Figure 40. siRNA depletion of MTHFD2 does not alter mitochondrial membrane potential in EC under shear stress. (A+B) HUVEC were transfected with sictrl (C) or siRNA against MTHFD2 (siMTHFD2, M) for 24h, after which the cells were subjected to either static culture (upper panel), H-LSS (middle panel) or OSS (lower panel) for an additional 48h. The cells were live stained with TMRM and Mitotracker and imaged on a Zeiss LSM Confocal 780 using a 40x objective. Nuclei were counterstained with Hoechst. (A) Representative confocal images shown (n=3). Image brightness was adjusted for presentation. Scale bar 100µm. (B) Quantification of mitochondrial membrane potential as integrated signal density of TMRM/Mitotracker stain divided by the cell count. Data shown as fold change over static sictrl. Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. Values represent means +/- SEM. ns, not significant.

Qualitative assessment of the images suggested that the mitochondrial morphology was different in EC grown under static and shear stress conditions and influenced by MTHFD2 depletion (Figure 40 and 41). However, quantification of the mitochondrial phenotype using the integrated signal density of the Mitotracker stain normalised to the cell count did not show any differences in any condition (Figure 41B), suggesting that the integrated measure of signal area and intensity was not sufficient to quantify these observations.

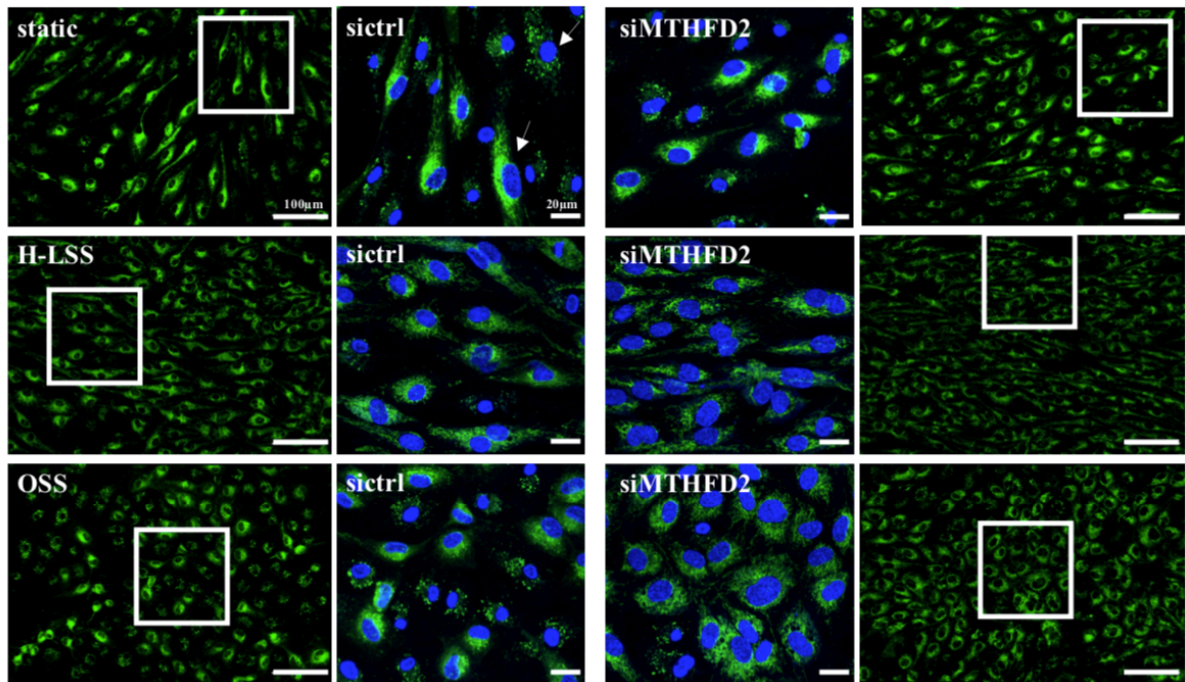
Mitochondrial network dynamics are balanced by two physiological processes, fission and fusion, by which mitochondria divide or fuse, resulting in either more punctate/fragmented or connected mitochondrial networks respectively. These mechanisms are required for e.g. cell division, removal of damaged mitochondria and distribution of metabolites, but fission can also be induced by cell stressors in EC, for example by high glucose exposure [554]. Categorisation of the cells as displaying either punctate or intact mitochondrial structures based on the Mitotracker signal (indicated by white arrows in Figure 41A; for representative images see Figure 14) showed that the majority of sictrl-transfected static EC displayed punctate staining (Figure 41A and C). In contrast, sictrl-treated EC subjected to H-LSS demonstrated more intact mitochondrial networks compared to static EC (H-LSS 86.7% vs static 33% cells with intact mitochondrial staining; two-way ANOVA $p < 0.0001$) (Figure 41C), suggesting that H-LSS promotes mitochondrial fusion. OSS induced the formation of both punctate and intact mitochondrial structures in EC to a similar degree, in line with the notion of increased cell stress under this condition. Depletion of MTHFD2 increased the percentage of cells with intact mitochondrial networks compared to sictrl-treated EC under OSS, but not under H-LSS or static culture (Figure 41C).

To assess the mitochondrial networks of these cells in more detail, the images were analysed on a single cell basis using a published macro tool for mitochondrial network analysis (MiNa) in ImageJ [491]. This tool provides the following quantitative outputs: mitochondrial footprint (Mitotracker signal area/background), number of branches per mitochondrial network and mean branch length. A network is defined by the presence of at least one node connecting three branches [491]. Sictrl-treated static EC depicted two different cell populations that differed in the number of branches per network and branch length (Figure 41A, left panel, and 41D), in line with the above quantification. In comparison, sictrl-treated EC under H-LSS displayed a higher mitochondrial coverage per cell as measured by the mitochondrial footprint (one-way ANOVA; $p < 0.0001$) and longer branches (one-way ANOVA; $p < 0.0001$) (Figure 41A and D), consistent with uniformly showing intact mitochondrial structures. Sictrl-treated EC under OSS produced a similar mitochondrial phenotype compared to the static condition.

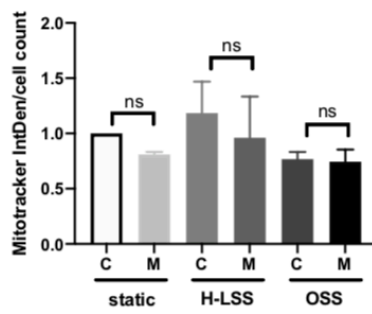
MTHFD2 depletion did not affect the mitochondrial morphology of static EC compared to sictrl (Figure 41A, right panel, and 41D). Interestingly, under H-LSS, siMTHFD2-treated cells displayed a reduced mitochondrial coverage (Figure 41D), reflected by an intact but seemingly less dense mitochondrial network compared to sictrl. In contrast, under OSS, MTHFD2-depleted EC exhibited higher mitochondrial coverage with a larger number of branches per network (one-way ANOVA; $p < 0.0001$) and longer branches (one-way ANOVA; $p < 0.0001$) compared to sictrl, consistent with the above quantification.

These data suggested that shear stress differentially affects mitochondrial morphology but not membrane potential in EC. Furthermore, these results implied that MTHFD2 may have different roles in mitochondria in EC under shear stress. Under H-LSS, MTHFD2 seemed necessary for the maintenance of an intact mitochondrial network, potentially promoting mitochondrial fusion. In contrast, under OSS, loss of MTHFD2 increased mitochondrial fusion, mirroring the mitochondrial phenotype of control cells under H-LSS. This may suggest that MTHFD2 mediates OSS-induced changes in mitochondrial morphology. Although the functional implications of these findings remain to be investigated, these data highlight that interference with OCM affects mitochondrial morphology in EC.

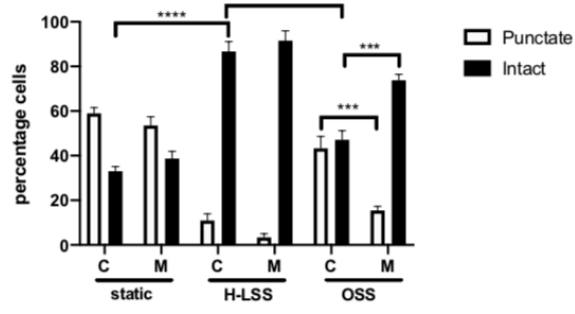
A



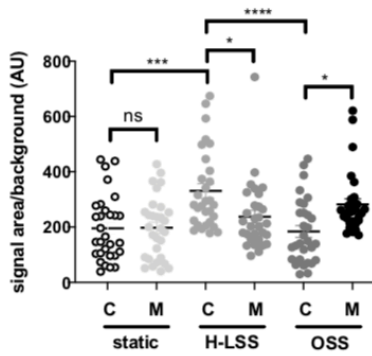
B Mitotracker



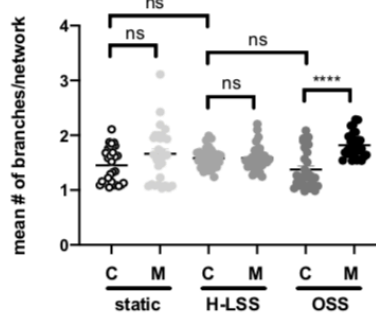
C Mitochondrial Morphology



D Mitochondrial footprint



Number of branches per network



Branch length

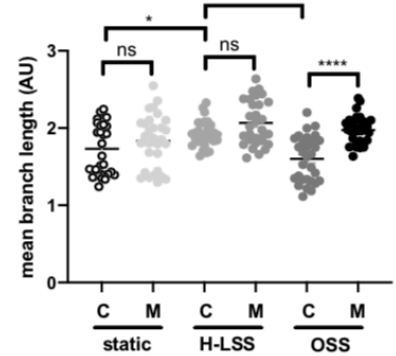


Figure 41. siRNA depletion of MTHFD2 alters mitochondrial morphology in EC under shear stress. (A-D) HUVEC were transfected with sictrl (C) or siRNA against MTHFD2 (siMTHFD2, M) for 24h, after which the cells were subjected to either static culture, H-LSS or OSS for an additional 48h. The cells were live stained with Mitotracker (green) and Hoechst (blue) and imaged on a Zeiss LSM Confocal 780 using a 40x objective. Same experiment as shown in Figure 40. (A) Representative confocal images shown (n=3). Image brightness adjusted for presentation. Scale bar 100µm for outer panels; 20µm for inner panels. (B) Quantification of mitochondrial coverage as integrated signal density of Mitotracker stain divided by the cell count. Data shown as fold change over static sictrl. (C) Quantification of percentage of cells showing punctate or intact mitochondrial structures (indicated by white arrows) as visually scored by Mitotracker staining in each condition. A total of 400-650 cells were counted from three individual experiments (n=3). Two-way ANOVA and Sidak test for multiple comparison. (D) Quantification of the mitochondrial network was performed using the Mitochondrial Network analysis (MiNA) macro tool using ImageJ published by Valente et al. [491]. The parameters analysed are the area of the mitochondrial signal (mitochondrial footprint), mean number of branches per network and mean branch length. Per condition, a total of 30 cells were analysed on a single cell basis from three individual experiments (n=3). Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. Values represent means +/- SEM. ns, not significant. * $p<0.05$. ** $p<0.01$. *** $p<0.001$. **** $p<0.0001$.

5.5 Discussion

The role of OCM is currently poorly understood in EC but may be instrumental in understanding the effects of MTX on the vasculature. This chapter investigated the regulation and function of OCM in EC subjected to static and shear stress conditions. Endothelial OCM was predominantly downregulated by shear stress at a transcriptional and metabolic level, suggesting that EC may have an altered demand for OCM *in vivo*. Interestingly, mitochondrial, but not cytosolic, OCM enzymes were differentially expressed in EC under OSS compared to H-LSS, potentially pointing towards different requirements for mitochondrial OCM under OSS. Supporting this idea, MTHFD2 depletion differentially affected mitochondrial morphology in EC exposed to H-LSS and OSS. These findings provide novel insights into the role of OCM in EC and may explain why folate and anti-folate therapy achieved contrasting effects in EC in *in vitro* and clinical studies.

Endothelial OCM was differentially regulated by shear stress at the gene expression level using independent bioinformatic and experimental approaches. These analyses revealed that the majority of genes related to OCM were downregulated in EC by H-LSS compared to static EC. In line with these results, the expression of OCM enzymes (DHFR, TYMS, MTHFD1, MTHFD2, SHMT1 and SHMT2) was reduced in contact-inhibited, quiescent EC compared to proliferating cells in a previous report [313]. EC subjected to LSS expressed lower levels of DHFR protein [399] and TYMS mRNA [559] compared to those grown under static conditions. These data agree well with the concept that major endothelial metabolic pathways, including glycolysis, are repressed in quiescent EC [313]. Supporting this, a recent report showed that H-LSS decreased glucose uptake and mitochondrial content in EC, the former by reducing the expression of glycolytic enzymes in a KLF2-dependent manner [314]. Of note, KLF2 did not regulate the expression of selected OCM genes in static EC. Although further experiments are needed to confirm these results under shear stress, these observations imply that the transcriptional programs coordinating the expression of OCM genes are more complex. Overall, these findings may reflect a reduced requirement for OCM-mediated biosynthetic processes during endothelial quiescence.

OSS induces a pro-inflammatory endothelial state by a HIF1 α -dependent increase in glycolysis and reduction of mitochondrial oxidative phosphorylation [133, 134]. Most of the examined cytosolic OCM genes were also repressed by OSS compared to static EC. In contrast, the mitochondrial enzymes MTHFD2, MTHFD1L and SHMT2 were increased under OSS

compared to H-LSS. Indeed, a recent paper showed that MTHFD2 and SHMT2 expression were increased in human atherosclerotic plaques and in murine EC from partially ligated carotid arteries [384], providing evidence for a potential OSS-mediated regulation of these proteins *in vivo*. There is no data on the regulation of MTHFD1L in EC but a genome wide association study showed an association between CAD and an intronic variant in this gene [560]. This pattern of regulation may be suggestive of an increased need for mitochondrial OCM in activated or more proliferative EC. Supporting this, LPS-induced macrophages showed a similar differential regulation of cytosolic and mitochondrial OCM enzymes compared to controls [370], suggesting that this may be an effect common to pro-inflammatory activation.

It was not clear if the differential regulation of OCM genes would result in altered metabolite levels in EC. To date, there is no study that has investigated OCM metabolites in EC. While this experiment reflected only one technical run on the LCMS (performed with pooled samples from five independent experiments) and requires further validation, it will be discussed considering its technically challenging nature. The total folate levels were decreased in EC by both H-LSS and OSS compared to the static condition, possibly reflecting a decreased need for OCM under shear stress. However, three metabolites showed a distinct pattern of regulation that may give insight into specific functions of endothelial OCM. 5-meTHF was the predominant folate metabolite in static EC and was not altered by shear stress. EC may maintain this metabolite at high levels to produce SAM [317] (Figure 5). Supporting this, the levels of the enzyme MTR, which uses 5-meTHF to regenerate methionine from homocysteine, were not changed in EC under shear stress, indicating that this metabolite may be efficiently used for the methyl cycle. Moreover, EC under shear stress showed a lower relative abundance of 5,10-meTHF but increased levels of 10-formylTHF compared to static EC. 5,10-meTHF can be used as a substrate to produce 5-meTHF (MTHFR; methyl cycle), dTMP (TYMS; nucleotide synthesis), THF (SHMT; along with serine synthesis) or 10-formyl-THF (MTHFD) (Figure 5). Given that THF levels were reduced, this may point towards an increased use of 5,10-meTHF for the methyl cycle and/or for 10-formylTHF production, in line with the metabolite changes seen. 10-formylTHF can be utilised for purine (ATIC/GART), mitochondrial protein (MTFMT) or NADPH synthesis (ALDH1L1/2). The gene expression levels of MTFMT and ALDH1L1/2 were not evaluated in this chapter, but ATIC levels were not altered in EC under shear stress. This may indicate that 10-formylTHF fuels nucleotide synthesis in these conditions. Future experiments should address how 10-formylTHF is used

in EC under shear stress, by evaluating nucleotide levels, NADPH/NADP ratio and mitochondrial protein synthesis. Importantly, however, the levels of 5,10-meTHF and 10-formylTHF were low and at the detection limit of the assay, emphasising that these data need to be interpreted with caution.

There was no difference in the total folate levels or distribution of the metabolites in EC under H-LSS and OSS. This was unexpected due to the presumed differential metabolic requirements for EC in quiescence or activation. This finding may be explained by the following points: It is possible that OCM is not required to maintain OSS-induced EC activation. The increased expression of mitochondrial genes may not affect metabolite levels but impact on EC function in a non-enzymatic manner. Most of the folate metabolites can be produced in the cytosol and mitochondria with shuttling of THF and formate between compartments. This may have masked any changes in the OSS-induced mitochondrial metabolite profile. This experiment may not have captured increased mitochondrial metabolites under OSS because these could have already been utilised for reactions that were not measured (e.g. nucleotide synthesis). Because this experiment is a static snapshot of the metabolite profile, the increased abundance of a metabolite can reflect its increased production or decreased consumption [561]. Performing dynamic metabolite labelling experiments using isotope tracers would allow understanding of the direction and contribution of 1C units to different metabolites. These results indicated that shear stress reduces folate content in EC, in line with the gene expression results and a presumed overall decreased need for biosynthetic reactions. Based on the differential relative abundance of distinct OCM metabolites, EC may differentially use these metabolites for specific, yet still undefined, processes. It remains to be conclusively addressed if OCM has diverging roles in quiescent and activated EC.

To address the functional implications of OCM in EC, cell proliferation was analysed in static EC grown in folate-depleted media or transfected with siRNA against selected OCM genes. Folate-depletion experiments showed that folate was required for cell cycle progression, supported by findings from a previous report in EA.hy926 cells [394] and MTX-treated EC (Chapter 3). However, incubation with folate-depleted media caused EC accumulation in the G1 phase in this study, whereas it led to a prolonged S-phase in EA.hy926 cells [394] and in MTX-treated EC. It is unclear why this happened but may be related to the different media supplied.

siRNA-targeted depletion of OCM enzymes suggested that MTHFD1 is important for cell proliferation, as shown by the BrdU proliferation assay and cell counts (and a trend in the cell cycle data). Supporting this, CRISPR-targeted deletion of MTHFD1 did not yield viable cell clones in HEK293T cells, most likely due to the indispensable role of cytosolic 10-formylTHF production for purine synthesis [334]. DHFR, MTHFD2 and SHMT2 depletion showed no effect on cell proliferation, with contradicting results for SHMT1. These results were in contrast to previous reports in cancer cells: DHFR was essential for cancer cell growth and DHFR-deficient cells required thymidine, a purine and glycine for proliferation [557]. CRISPR-mediated deletion of SHMT2, but not SHMT1, caused a mild growth defect, which was more pronounced upon serine or glycine withdrawal [334]. siRNA-mediated depletion of MTHFD2 inhibited cancer cell proliferation [371] and decreased EC proliferation [384], although the latter to a minimal extent. This may be explained by the hypothesis that OCM has different roles in cancer cells compared to primary EC. As hinted at above, the presence of amino acids or nucleotide precursors can impact on the effects of enzyme deletion on proliferation, allowing the cells to bypass specific metabolic blocks. EC are kept in very rich media after siRNA transfection, which may supply high levels of these substrates and obscure a proliferative phenotype. Compensation of cytosolic and mitochondrial enzyme expression may occur after depletion of a specific enzyme. This was only partially tested in these experiments and needs to be conclusively addressed. Isoforms may also compensate for loss of a specific enzyme. It was noted that antibodies capturing MTHFD2 and SHMT2 proteins detected a double band for each enzyme respectively, which were incompletely removed by siRNA knockdown. There are two described murine MTHFD2 transcript variants [341] but currently no reports on functional protein isoforms. Multiple MTHFD2 protein bands were shown in EC in a recent report [384], which could represent endothelial transcripts, post translational modifications, degradation products or non-specific antibody signal. SHMT2 has two splice variants, translated into the mitochondrial SHMT2 protein (50kDa) and the cytosolic/nuclear isoform SHMT2 α (53kDa) [357]. siRNA depletion of SHMT2 reduced the upper but not lower band (Figure 38), suggestive of targeting of SHMT2 α but not SHMT2. However, the qPCR primers only targeted SHMT2, confirming loss of the mitochondrial isoform after siRNA depletion. The identity of the second band remains unclear. Although these experiments demonstrated the importance of OCM in proliferation, more work needs to be done to understand the individual contribution of OCM enzymes to this phenotype in static and shear-exposed EC, including optimisation of growth media and complete evaluation of compensatory mechanisms.

OCM was shown to regulate mitochondrial morphology in EC under shear stress in this chapter. Interestingly, shear stress per se differentially affected mitochondrial morphology but not membrane potential. EC under H-LSS revealed more intact mitochondrial networks compared to those under OSS, suggesting increased mitochondrial fusion. In contrast, previous reports showed that LSS increased mitochondrial fission compared to static EC [562] and mitochondria from intact arteries showed a more fragmented staining pattern compared to cultured EC [563]. A recent study, however, did not find any differences in Mitotracker staining in EC subjected to LSS or OSS [133]. These differences may be explained by the siRNA transfection procedure (sictrl), length of shear stress exposure, mitochondrial stain and imaging conditions. It is incompletely understood how shear stress affects mitochondrial function in EC. LSS has been shown to increase mitochondrial biogenesis [564, 565] as well as the production of mitochondrial reactive nitrite species which inhibit the electron transport chain [566], with conflicting reports on its impact on mitochondrial membrane potential [562, 564, 567]. OSS increases mitochondrial superoxide production [568]; a mechanism proposed to promote oxidative stress. It is interesting that both H-LSS [314] and OSS [133] downregulated mitochondrial content or oxidative phosphorylation in EC, suggesting that these opposing states similarly dampen mitochondrial metabolism. More work is required to understand the function of mitochondria in EC under shear stress.

This is the first report to demonstrate that MTHFD2 regulates mitochondrial dynamics in EC under shear stress. MTHFD2 depletion led to a thinning of the mitochondrial network in EC subjected to H-LSS, but strikingly had the opposite effect in EC under OSS, where loss of this enzyme increased mitochondrial coverage and networking. These experiments suggest that MTHFD2 may serve different roles in mitochondria under shear stress, in line with the observed differential expression of this gene under these conditions. Supporting this finding, depletion of PHGDH, an enzyme required for serine synthesis that is closely interlinked with OCM, caused structural mitochondrial defects in EC [311]. It is currently not clear how MTHFD2 affects mitochondrial morphology in this shear stress-dependent manner and what functional consequences this may have. MTHFD2 may regulate mitochondrial fission or fusion through enzymatic or non-enzymatic functions. MTHFD2 catalyses the two-step conversion of 5,10-meTHF to 10-formylTHF [317], promoting the upstream SHMT2-driven production of glycine. MTHFD2 knockdown has been shown to decrease levels of glycine and TCA cycle intermediates [373, 384], suggesting that this enzyme may affect mitochondrial dynamics on a metabolic level. Glycine is required for heme and glutathione synthesis and it has been shown

that decreased glycine levels cause mitochondrial respiratory defects in static EC [311]. MTHFD2 itself regulates cellular NADPH levels [569], implying that oxidative stress may be aggravated in MTHFD2-depleted EC. Moreover, MTHFD2 may affect cell function in a non-enzymatic manner, as it can support cell proliferation independent of one of its catalytic domains [359] and may have a role in RNA binding [570]. While OSS-induced endothelial inflammation was reliant on the activation of glycolysis and the repression of oxidative phosphorylation in EC [133], it remains to be determined if MTHFD2 is required to maintain an OSS-induced EC phenotype or if its upregulation serves as a compensatory mechanism to counteract cellular stress [384]. MTHFD2 may be important for inflammatory processes as its depletion abrogated the LPS-induced expression of IL1 β in macrophages [370]. In that regard, it is interesting to speculate that MTHFD2 may support nucleotide production in EC under OSS (as seen in static EC upon lipid exposure [384]) to fuel the OSS-driven pro-inflammatory response [571]. Further work should aim to understand if MTHFD2 contributes to nucleotide metabolism (ATP generation), redox homeostasis and endothelial activation under OSS.

The study has the following limitations. As previously explained, static cells were not cultured in the same growth chamber as those subjected to shear stress, which can undermine the direct comparison of static and shear stress conditions. To address this issue, the gene expression analyses were validated in a publicly available RNA sequencing dataset and preliminarily in the orbital shaker model. Nevertheless, it cannot be excluded that this may have impacted the metabolite analyses and requires further workup. The metabolite levels were only measured in one technical LCMS run (with pooled samples from five independent experiments due to the low cell yield in the parallel plate model) and also requires further validation. This work did not exhaustively measure all genes related to OCM, including the enzymes MTHFR, MTFMT and ALDH1L, proteins involved in cellular folate export and serine synthesis. For FPGS, only the mitochondrial but not the cytosolic isoform was measured. The gene expression analyses require confirmation at the protein level. Importantly, the experiments in this chapter were performed using different media, which may hamper the comparability of the gene expression (M199), metabolite (EGM-2), folate depletion (RPMI) and siRNA depletion experiments (EGM-2) due to the presence of different co-factors. The quantification of the mitochondrial network was not performed in a blinded manner and may have been limited by the siRNA transfection, comparably low magnification of the images and clumping of mitochondrial structures [491]. This study lacks confirmation of the proposed effects in an *in vivo* model.

In conclusion, this is the first report to characterise endothelial OCM under static and shear stress conditions (Figure 42). Endothelial OCM was significantly altered on a transcriptional and metabolic level by shear stress and may contribute to mitochondrial function in a shear stress-dependent manner. Further work is required to delineate the function of endothelial OCM in states of endothelial quiescence and activation and to validate these findings *in vivo*. These results may have implications for the vascular effects of folate supplementation. Previous clinical studies have not been successful in showing beneficial effects of folic acid in CVD patients. The data from this chapter suggest that folic acid and BH₂ may not be efficiently metabolised by DHFR in EC under shear stress due to its low expression pattern. This complements two previous studies that argue that DHFR-mediated BH₄ recycling will be low in EC *in vivo* [398, 399], potentially explaining the lack of clinical effect. Therefore, OCM may have a limited role in NO bioavailability *in vivo*.

These results may also explain why MTX had differential effects in EC in Chapters 3 and 4 (Figure 42). The data suggest that MTX uptake may be limited in EC under H-LSS due to the decreased expression of RFC, its main cellular transporter [252]. RFC expression was maintained in EC under OSS, suggesting that MTX may predominantly target EC at atheroprone sites. The expression of DHFR and TYMS, two main targets of MTX, were decreased under shear stress, potentially explaining why MTX did not affect cell proliferation in shear-exposed EC.ATIC expression was not altered by shear stress, implying that MTX may increase adenosine release in EC under OSS via ATIC inhibition. This point requires validation by measuring adenosine release from MTX-treated EC under shear stress. Endothelial adenosine production is likely maintained *in vivo* as the expression of most enzymes related to this process were not altered by shear stress in a published RNA sequencing dataset [132] (Supplementary Figure 15). However, the expression of ADORA2A, the receptor shown to mediate the anti-inflammatory effects of adenosine [265], was drastically decreased in EC by shear stress. This may suggest that adenosine does not directly exert anti-inflammatory effects on EC but may have paracrine effects on other cell types. These findings support that MTX may have local anti-inflammatory effects in atheroprone areas. Further work is required to understand the role of OCM and MTX in vascular EC, with a proposed focus on EC under OSS. Beyond MTX, it will be paramount to understand endothelial OCM as MTHFD2 specific anti-folates are currently sought after as anti-cancer drugs [373]. Taken together, these results demonstrate a novel aspect of EC metabolic regulation under shear stress which may have implications for the effects of folates and anti-folates on the vasculature.

6 Chapter 6 - The effects of systemic inflammation and DMARD therapy on ECFC derived from patients with RA

6.1 Introduction

CVD management in RA is complicated by the limited understanding of its pathogenesis, lack of accurate RA-specific CVD risk prediction scores and targeted treatments [168] (section 1.3.2). Early identification of patients at higher risk would benefit the timely management of CVD in RA. However, detecting vascular dysfunction in patients is impeded by the lack of reliable biomarkers and technical difficulties associated with existing tests of vascular function such as FMD, which currently prevent their inclusion into routine clinical practice [572]. Furthermore, vascular disease in RA patients exhibits important clinical differences from CVD in the general population (section 1.3.2) and remains poorly characterised due to limited access of patient tissue. Although systemic inflammation has been widely proposed to impair endothelial function and drive atherogenesis in RA [186] (section 1.3.3), this relationship is inconclusively demonstrated in studies correlating vascular function and clinical inflammatory markers [185, 186]. The molecular effects of systemic inflammation and its resolution on the vasculature remain incompletely understood and while effort has been made to address these questions in *in vitro* models (including this thesis), these are not able to fully reflect the complex systemic inflammatory environment, patient genotype, effect of interacting cell types and drug effects on preconditioned cells (time, dosing and metabolism of the drug) among other factors.

ECFC may represent tools to address some of these issues. These cells can be derived from circulating progenitor cells in the peripheral blood and are defined by the expression of endothelial markers, clonal expansion and high proliferative and vasculogenic capacity *in vitro* and *in vivo* [401] (section 1.6). Although the precise origin and function of these progenitor cells and their progeny remain unclear, ECFC have gained attention as potential markers and models of vascular damage in patients with various diseases. ECFC frequency, defined by the number of endothelial colonies generated *in vitro* from a given individual, has been suggested to be a marker of ongoing vascular injury and repair. This is based on studies showing that ECFC frequency is increased in patients with venous thromboembolic disease (VTD) [573], clinically significant CAD [464] and acute MI [462], where the presence of ECFC correlated with reduced infarct size [463]. Only one study so far has characterised ECFC in RA [459], showing an increased ECFC frequency in treatment-experienced RA patients compared to healthy controls. Furthermore, ECFC have been utilised as *ex vivo* models of vascular disease

[401], enabling the delineation of disease-specific effects on endothelial cells on a phenotypic and molecular level in a given patient population (section 1.6.1.2.3). Therefore, ECFC frequency and phenotype may offer different modalities of detecting and studying vascular dysfunction in RA.

Importantly, the work in the previous chapters of this thesis was directed towards understanding the effects of MTX on vascular EC in the context of atherosclerosis but had been limited by several aspects of *in vitro* culture (Chapter 3 and 4). As ECFC can be directly isolated from patients, these cells allow studying the impact of DMARD therapy (including MTX) on cells exposed to the patient-specific inflammatory environment and relevant drug levels *in vivo*. This may overcome some of the limitations faced in the previous chapters.

The ECFC-RA study is an ongoing study aiming to (i) evaluate ECFC frequency and function in active, treatment-naïve RA patients compared to healthy controls (HC) and (ii) understand the effects of DMARD treatment. Therefore, this study will determine whether ECFC can be used as tools to detect inflammation-driven vascular dysfunction in patients with RA, with the ultimate goal of defining a novel biomarker for compromised vascular function to identify RA patients at increased CVD risk. Crucially, the ECFC-RA study design makes it possible to prospectively address the impact of ongoing systemic inflammation and its reversal with therapy on ECFC from the same patient. The first set of results are presented in this chapter.

6.2 Hypothesis of the chapter

ECFC frequency is increased in patients with active RA compared to HC. ECFC from patients with active RA are more proliferative and express higher levels of pro-inflammatory markers compared to those isolated from HC. DMARD initiation will reverse these changes by reducing disease activity.

6.3 Aims of the chapter

- 1) Determine ECFC frequency in RA patients pre- and post-DMARD initiation, and HC
- 2) Correlate ECFC frequency in RA patients with clinical parameters of inflammation
- 3) Confirm endothelial purity of ECFC samples
- 4) Determine ECFC phenotype in terms of cell proliferation and gene expression signature in RA patients pre- and post-DMARD initiation compared to HC

6.4 Results

6.4.1 Characterisation of the study population

The outline of the ECFC-RA study is depicted in Figure 43. The study will prospectively evaluate ECFC frequency and function in 35 patients with active, treatment-naïve RA at baseline and four to six months after treatment initiation with MTX and HCQ according to the local standard of care, compared to 35 healthy controls (HC). This timepoint is within the clinically implemented time frame for the assessment of treatment response [164]. Study recruitment commenced in November 2018 and was still ongoing during the writing of this thesis. All data from the ECFC-RA study generated up to December 2019 are presented in this thesis.

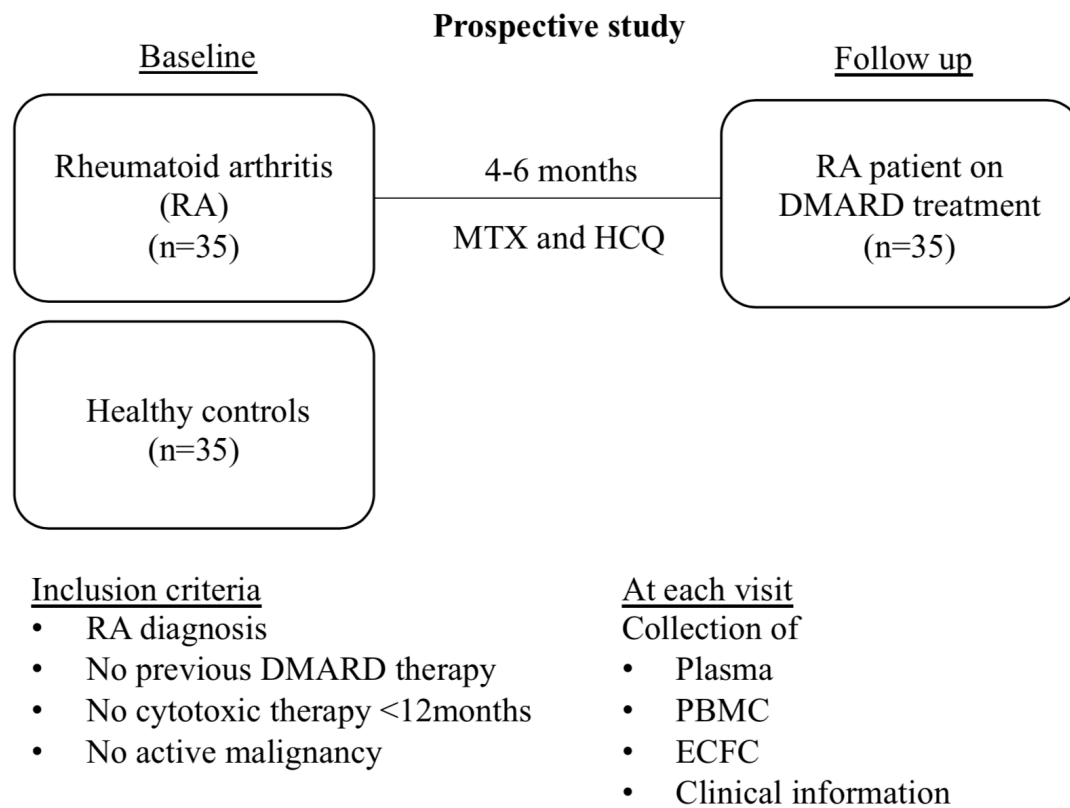


Figure 43. Design of the ECFC-RA study. Study recruitment was performed at the Early Arthritis Clinic at Hammersmith Hospital. DMARD, disease-modifying anti-rheumatic drug. ECFC, endothelial colony forming cell. MTX, methotrexate. HCQ, hydroxychloroquine. PBMC, peripheral blood mononuclear cells.

Demographic and clinical characteristics of the ECFC-RA study cohorts are outlined in Table 11. Nineteen patients with active, treatment-naïve RA (RA pre) were recruited. The median age of the treatment-naïve patients was 58 years (IQR 49.5-62) and 84.2% were female. 84.2% (16/19) of RA patients displayed moderate to high disease activity as demonstrated by their DAS28-ESR score (>3.2). One patient had a history of CVD and three treatment-naïve RA patients received statin medication. Fourteen healthy individuals were recruited for the healthy control group (HC). Although age (Mann Whitney Test; $p=0.0692$), sex (Fisher's exact test; $p=0.6781$) or ethnicity (Fisher's exact test; $p=0.1258$) were not statistically different in the HC and treatment-naïve RA patients, the HC group was slightly younger and was not as ethnically diverse. After DMARD initiation, ten patients were prospectively recruited at follow-up (RA post). The median follow-up time was 4.3 months (IQR 4.1-5.1). Four patients (21.1%) were lost at follow-up due to declined consent (1 patients) or refusal to initiate or continue DMARD therapy (3 patients). For the remaining five patients (26.3%), the follow-up timepoint had not been reached at the time of data collection for this thesis (December 2019). Of the ten patients followed up, two stopped HCQ treatment due to adverse effects. Seven RA patients (70%) responded to DMARD treatment at follow-up as evaluated by the EULAR response criteria, defined as no response (no change in DAS28-ESR or high disease activity level), moderate response (DAS28-ESR change by >1.2 without reaching low disease activity or by a decline of 0.6 to 1.2 plus reaching at least moderate disease activity (e.g. DAS28-ESR <5.1)) or good response to treatment (decline in score >1.2 and low disease activity (e.g. DAS28-ESR <3.2)) [486].

Table 11. Clinical characteristics of the ECFC-RA study cohort.

	Healthy controls	RA patients		p-value
	HC n=14	Pre n=19	Post n=10	
Age (yrs)	52 (37.5-57.5)	58 (49.5-62)	58 (49-59.5)	0.0692
Sex (female; %)	78.6	84.2	90	0.6781
Ethnicity				0.1258
Caucasian	11 (78.6)	10 (52.6)	4 (40)	
Black	-	5 (26.3)	5 (50)	
Asian	3 (21.4)	2 (10.5)	-	
Not specified	-	2 (10.5)	1 (10)	
Clinical parameters				
Disease duration	N/A			
- <6 months		10 (52.6)	5 (50)	
- <12 months		2 (10.5)	1 (10)	
- >12 months		7 (36.8)	4 (40)	
DAS28	N/A			
- <2.6		1 (5.3)	4 (40)	
- ≥ 2.6 and ≤ 3.2		2 (10.5)	0	
- >3.2 and ≤ 5.1		5 (26.3)	4 (40)	
- >5.1		11 (57.9)	2 (20)	
RF (% positive)	N/A	68.4	80	
ACPA (% positive)	N/A	63.2	70	
CRP (mg/L)	N/A	6.1 (2.4-14.9)	3.1 (1.5-13)	
ESR (mm/h)	N/A	30 (25.5-40.5)	34 (25.8-54.8)	
Co-morbidity				
Cardiovascular	N/A	1 (5.3)	1 (10)	
Medication				
Steroids (short-term)	N/A	11 (57.9)	1 (10)	
MTX	N/A	0	2 (20)	
MTX + HCQ	N/A	0	8 (80)	

Healthy controls (HC), treatment-naïve (RA pre) and treated (RA post) RA patients. Data shown as median with interquartile range (IQR) or counts (%). Mann Whitney Test for comparison of age between HC and RA pre groups. Fisher Chi Square Test to compare sex and ethnicity between HC and RA pre groups. Reported disease duration at time of diagnosis. Cardiovascular co-morbidities include history of myocardial infarction and stroke. DAS28-ESR, disease activity score in 28 joints. RF, rheumatoid factor. ACPA, anti-citrullinated peptide antibodies. CRP, C-reactive protein. ESR, erythrocyte sedimentation rate. MTX, methotrexate. HCQ, hydroxychloroquine. yrs, years. N/A, not available.

6.4.2 ECFC frequency is not different between RA patients and HC

A representative image of an early ECFC colony is shown in Figure 46. ECFC colony formation was observed in 88.9% (16/18) of RA patients before treatment initiation, in 100% (10/10) of patients on treatment and 92.9% (13/14) of HC (Figure 44A). Colony formation could not be recorded in one RA pre patient sample due to an infected culture. There was no statistical difference in ECFC frequency between HC and treatment-naïve RA patients (median ECFC number/ 10^7 PBMC, HC 0.88 vs RA pre 0.62; ns) (Figure 44A). The initiation of DMARD treatment did not significantly affect ECFC frequency in RA patients (RA pre 0.48 vs RA post 0.6; ns) (Figure 44B), even after stratification for treatment response (data not shown).

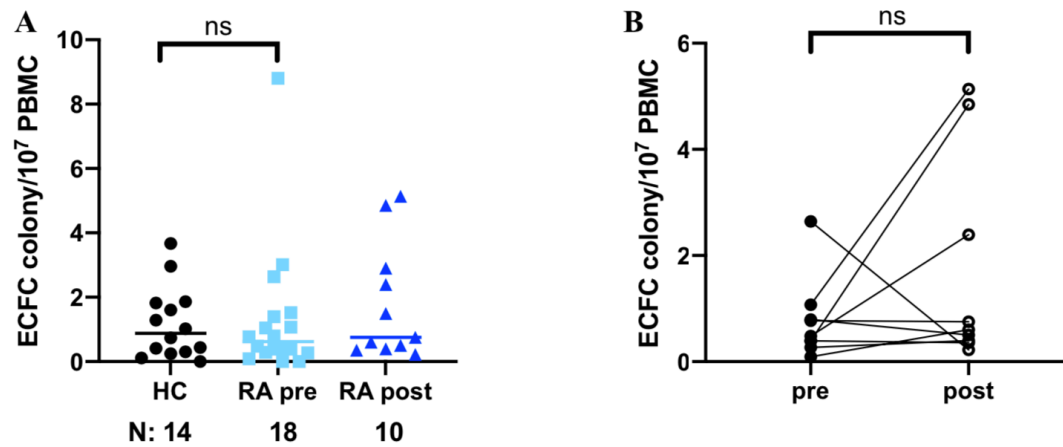


Figure 44. ECFC frequency in RA patients pre- and post-treatment initiation is comparable to HC. (A) ECFC frequency in HC (n=14), treatment-naïve (RA pre; n=18) and treated RA patients (RA post; n=10) shown as colony count per 10^7 PBMC seeded. Mann Whitney Test. (B) Longitudinal ECFC frequency (colony count per 10^7 PBMC) in the same RA patients pre- and post-treatment initiation (n=9). Wilcoxon matched-pairs signed rank test. Data shown as median ECFC frequency. Ns, not significant. PBMC, peripheral blood mononuclear cells.

6.4.3 Correlation of ECFC frequency with clinical parameters in RA patients

To investigate the relationship between systemic inflammation and ECFC frequency in RA patients, clinical markers of disease activity (DAS28-ESR score) and inflammation (ESR, CRP) were correlated with ECFC frequency (normalised colony number) and other related parameters, namely PBMC numbers seeded, absolute colony number, time of colony appearance and time of first colony split (in days) (Figure 45).

As expected, there was a positive correlation between absolute and relative colony frequencies ($\rho=0.89$; $p<0.0001$) (Figure 45A). ECFC frequency inversely correlated with the time of first colony appearance ($\rho=-0.5$; $p=0.049$), reflecting the observation that individuals with a large number of colonies display the first colony earlier than those donors with less colonies. This is further represented by the fact that the parameters time of colony appearance and time to first split associated positively ($\rho=0.75$; $p=0.0013$), indicating a higher proliferative potential of those colonies. DAS28-ESR activity score correlated with both ESR ($\rho=0.55$; $p=0.01$) and CRP ($\rho=0.59$; $p=0.0096$) as expected. Interestingly, there was a negative correlation of ECFC frequency with ESR levels ($\rho=-0.57$; $p=0.0145$), with a similar trend for CRP and DAS28-ESR score. Of note, ESR levels also associated with the number of seeded PBMC ($\rho=0.48$; $p=0.044$).

Following these observations, ECFC frequency was determined in RA patients stratified to normal or high levels of ESR or CRP. In line with the above correlations, ECFC frequency was lower in treatment-naïve RA patients with high compared to normal levels of ESR (normal 2.82 vs high 0.39; $p=0.0007$) (Figure 45B), with a similar trend noted for CRP (Figure 45C). This effect was not evident in RA patients on DMARD treatment. These results suggest that systemic inflammation may negatively affect ECFC frequency in treatment-naïve RA patients.

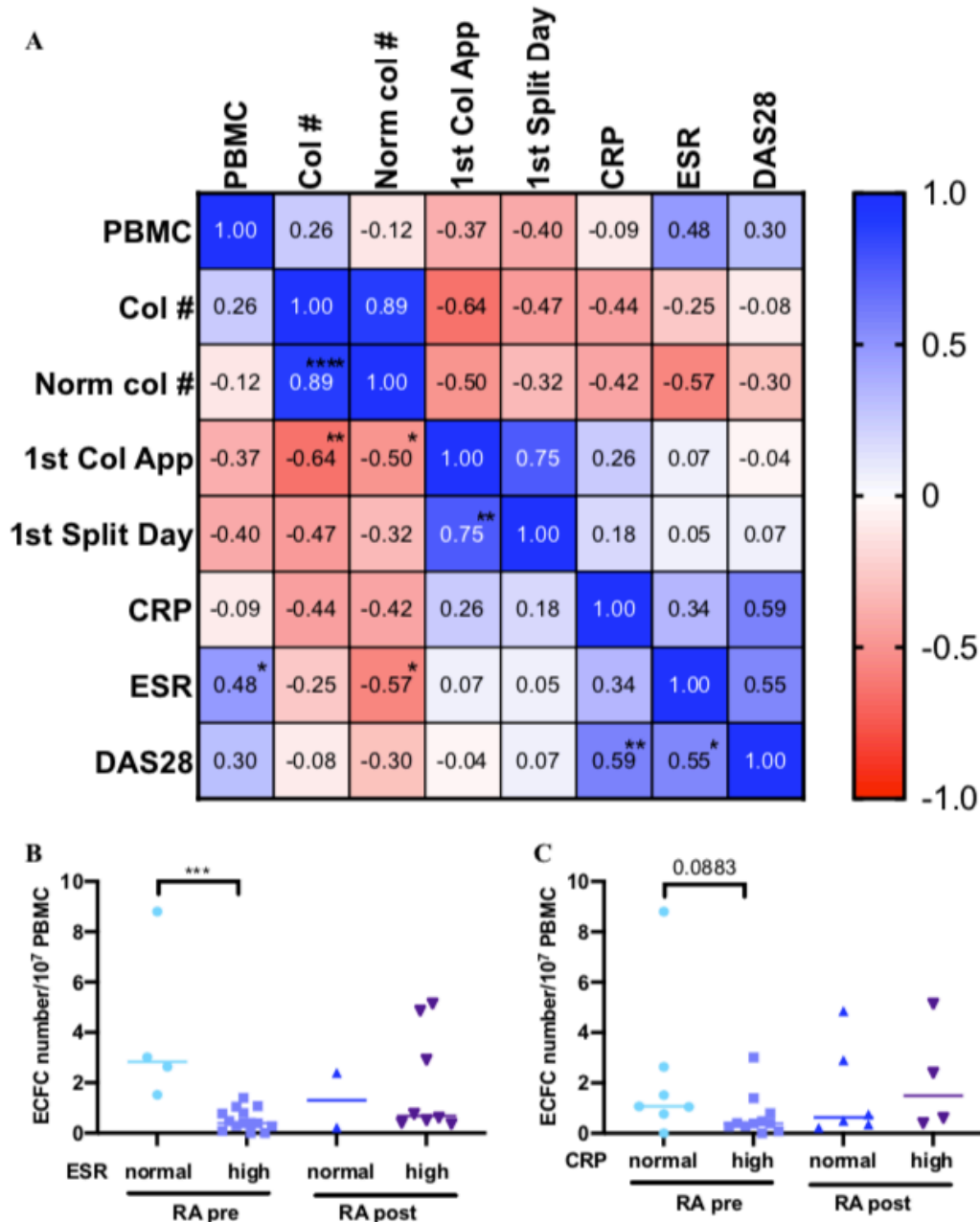


Figure 45. Correlation of ECFC frequency with clinical parameters of inflammation in RA patients. (A) Correlation matrix of ECFC frequency parameters PBMC number seeded (PBMC), absolute colony number (col #), colony number per 10^7 PBMC seeded (norm col #), first day of colony appearance (1st col App), first day of split of colony (1st split day) with clinical inflammatory measures DAS28 disease activity score, ESR and CRP levels in RA patients before treatment initiation (n=18). Spearman correlation (rho shown in correlation matrix). Significance indicated by asterisks. (B) ECFC frequency in RA patients after stratification for ESR levels (normal ESR<23mm/h; high ESR>23mm/h). RA pre “normal” n=4; RA pre “high” n=14; RA post “normal” n=2; RA post “high” n=8. (C) ECFC frequency in RA patients after stratification for CRP levels (normal CRP<5mg/L; high CRP>5mg/L). RA pre “normal” n=7; RA pre “high” n=11; RA post “normal” n=6; RA post “high” n=4. Data shown as median. Mann Whitney test. Ns, not significant. * p<0.05. ** p<0.01. *** p<0.001. **** p<0.0001.

6.4.4 Patient- and HC-derived ECFC display a bona-fide endothelial morphology

ECFC cultures from a subset of the study cohort were expanded to passage 5 and subjected to more detailed morphological and phenotypic characterisation. To verify the endothelial purity of the ECFC samples generated, seven RA pre, five RA post and six HC ECFC cultures were analysed by flow cytometry for their respective positivity for the endothelial markers PECAM1 (CD31), VE-cadherin (CD144) and MCAM (CD146) and negativity for the leukocyte and monocyte markers CD45 and CD14 respectively [413] (Figure 46A). A representative flow cytometry panel can be found in section 2.1.14.5.

All samples demonstrated >90% negativity for CD45 and CD14 expression and the majority of ECFC showed >90% positivity for CD31/CD144 and CD146 surface expression. There were two notable exceptions: one HC ECFC culture was only 66.6% positive for CD31/CD144 but 92.1% positive for CD146. One RA post sample demonstrated 81.7% and 81.6% positivity for both CD31/CD144 and CD146 respectively but was 95.4% negative for CD45/CD14 expression. Endothelial morphology of ECFC cultures was also verified by light microscopy, showing a typical endothelial monolayer with cobble-stone appearance (Figure 46B). These data indicated that the ECFC cultures generated in this study showed high endothelial purity and very low contamination with other cell types at passage 5.

A

		Percentage positivity		
Sample		CD31+/CD144+	CD31+/CD146+	CD45-/CD14-
HC	1	66.6	92.1	96.8
	2	96.7	97.3	93.4
	3	99.3	99.6	98.8
	4	98.6	99.6	99.1
	5	98	98.9	99.6
	6	98.1	99.3	99.6
RA pre	1	98.4	99.2	99.9
	2	99.7	99.7	99.2
	3	97.2	91.8	94.1
	4	95.1	96	97.7
	5	98.4	98.9	99.7
	6	99.7	99.6	99.7
	7	98.8	99.2	98
RA post	1	91.8	99.4	99.7
	2	99.6	99.6	99.4
	3	92.7	96.5	99.7
	4	81.7	81.6	95.4
	5	99.4	99.7	100

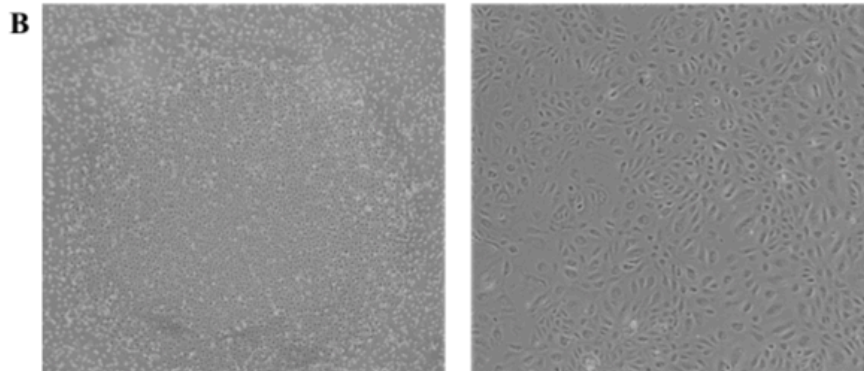


Figure 46. ECFC derived from HC and RA patients pre- and post-treatment initiation display a bona-fide endothelial morphology. (A) Quantification of endothelial purity of ECFC samples determined by flow cytometry based on the following marker profile: CD31⁺/CD144⁺/CD146⁺/CD14⁻/CD45⁻ (HC n=6; RA pre n=7; RA post n=5). Percentage positivity is reported. (B) Light microscopy images of a representative ECFC colony (left) and ECFC at passage 5 (right). Magnification 4x (left image) and 20x (right image). Endothelial purity of ECFC was determined by flow cytometry by Dr. Robert Maughan.

6.4.5 ECFC from treatment-naïve RA patients may be less proliferative compared to HC

To understand the effect of systemic inflammation and DMARD treatment on ECFC phenotype, cell proliferation was analysed in ECFC derived from HC, RA patients pre- and post-treatment initiation using a BrdU proliferation assay (Figure 47). For this experiment, a total of seven RA pre, five RA post and seven HC samples were used. Four RA patient samples were paired before and after treatment initiation.

Initial experiments were performed to optimise the culture parameters for the BrdU assay. For the final protocol, ECFC were serum starved in 0% FCS for 24h followed by recovery in 0.5% or 2% FCS for a further 24h (as outlined in section 2.1.10). Results are reported as the ratio of BrdU incorporation (measured at 450nm) in 2% FCS versus 0.5% recovered cells.

ECFC proliferation did not significantly differ in cells derived from RA patients compared to HC, although there was a trend for RA pre ECFC to be less proliferative (2% vs 0.5% ratio, RA pre 1.7 vs HC 2.4; $p=0.1282$) (Figure 47A). Initiation of DMARD treatment did not affect cell proliferation in RA patients ($p=0.25$) (Figure 47B). Although these experiments require further validation, the findings imply that ECFC from treatment-naïve RA patients may be less proliferative compared to HC.

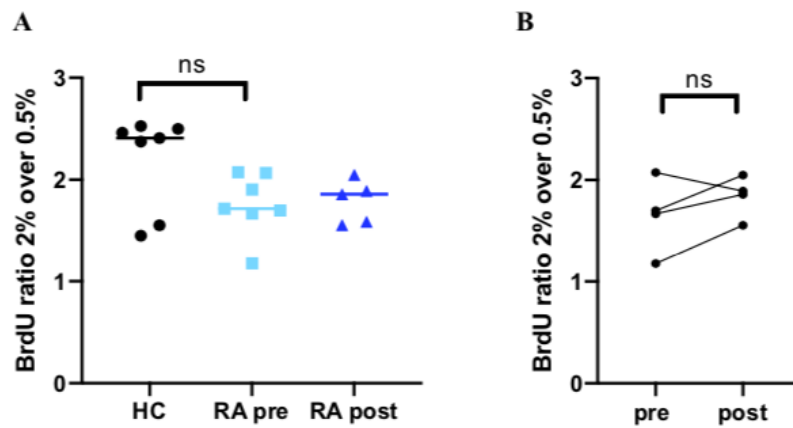


Figure 47. ECFC from treatment-naïve RA patients may be less proliferative compared to HC. Proliferation assay of ECFC from HC (n=7), treatment-naïve (n=7) and treated RA patients (n=5) as measured by BrdU incorporation. ECFC were serum starved in EGM-2 containing no serum for 24h, recovered in either 0.5% or 2% FCS EGM-2 for 24h and subjected to the BrdU assay. Proliferation expressed as serum induction from cells recovered in 2% over 0.5% FCS EGM-2. Data shown as median. **(A)** Mann Whitney Test for comparison of ECFC proliferation in HC (n=7) versus RA pre (n=7) groups or **(B)** Wilcoxon matched-pairs signed rank test for comparing RA patients pre- and post-treatment initiation (n=4). Ns, not significant

6.4.6 ECFC from treatment-naïve RA patients express higher levels of pro-inflammatory molecules compared to HC

Next, gene expression signatures of ECFC from HC and treatment-naïve RA patients were investigated using commercially available endothelial-focused qPCR arrays (for full gene list see Appendix, Table 12) (Figure 48).

Compared to HC, ECFC from treatment-naïve RA patients differentially expressed seven genes (Figure 48B), of which five (CCL2, ICAM1, IL1 β , VCAM1 and SELE) showed > 2-fold induction. Interestingly, these five genes are among the best-studied pro-inflammatory genes in EC (section 1.2.2), including the cell adhesion molecules ICAM1, VCAM1 and E-selectin and pro-inflammatory mediators CCL2 and IL1 β . No other functional pathway was differentially affected in ECFC from RA patients compared to control in this experiment. These data show that ECFC from treatment-naïve RA patients express higher levels of pro-inflammatory genes compared to HC, suggesting that these cells carry a pro-inflammatory signature.

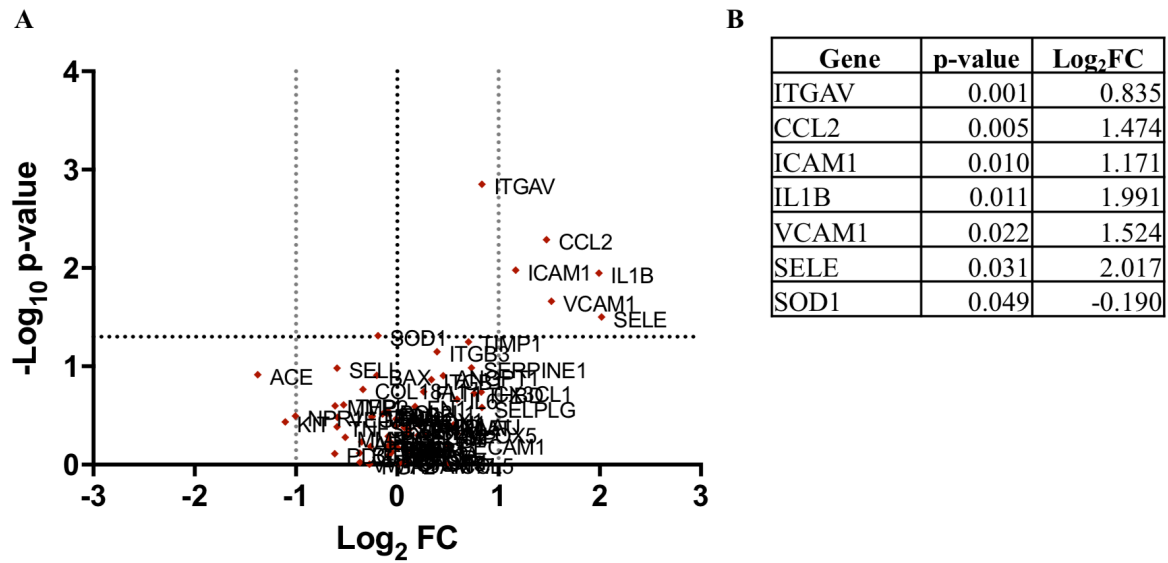


Figure 48. ECFC from treatment-naïve RA patients express higher levels of pro-inflammatory molecules compared to HC. (A+B) ECFC from HC (n=6) or treatment-naïve RA patients (n=3) were analysed using qPCR arrays at passage 5. **(A)** Volcano plot of differentially expressed genes in ECFC from RA patients over HC. Bars indicate fold change >2 (x-axis) or p-value <0.05 (y-axis). **(B)** List of significant differentially expressed genes in ECFC from RA pre samples compared to HC, expressed as log₂ fold change. Data were analysed using unpaired T-tests. qPCR array analyses were performed by Dr. Robert Maughan.

6.4.7 DMARD may reverse the pro-inflammatory phenotype of ECFC in RA patients

To validate the qPCR array findings and investigate the inflammatory state of patient-derived ECFC in more detail, ECFC from HC (n=7) and RA patients pre- and post-DMARD initiation (n=7 and n=5, respectively) were treated with increasing concentrations of TNF α for 4h. The expression levels of cell adhesion molecules (CAM) VCAM1, ICAM1 and E-selectin were measured by qPCR (Figure 49).

At baseline, the expression of all CAM tended to be higher and more heterogeneously regulated in ECFC from treatment-naïve RA patients compared to HC, despite not reaching statistical significance (Figure 49A). When stimulated with TNF α at a concentration of 0.01ng/mL, ECFC derived from treatment-naïve RA patients demonstrated significantly higher mRNA expression levels of VCAM1 (12.9-fold; p=0.0012), ICAM1 (3.7-fold; p=0.0006) and E-selectin (12.9-fold; p=0.0041) compared to HC. This effect was lost at higher TNF α concentrations, possibly due to saturation of CAM levels. Strikingly, initiation of DMARD treatment tended to reduce CAM expression levels on ECFC to comparable levels of the HC, although this did not reach statistical significance. Figure 49B shows the mRNA expression levels of E-selectin in TNF α -treated ECFC from four paired RA patient samples from before and after treatment initiation, emphasising this trend.

To validate these findings at the protein level, the expression of VCAM1 and ICAM1 were analysed in ECFC from all groups by Western blot (Figure 49C and D). These cells had been treated with media (control) or MTX for 48h for a different experiment (data not shown). In line with the mRNA data, VCAM1 and ICAM1 protein expression levels showed a non-significant elevated trend at baseline in ECFC from treatment-naïve RA patients compared to HC. A trend for lower VCAM1 protein expression in ECFC from treated compared to treatment-naïve RA patients was also observed. Taken together, these data demonstrated that ECFC derived from treatment-naïve RA patients displayed a primed pro-inflammatory state compared to HC, which may be reversible with DMARD treatment.

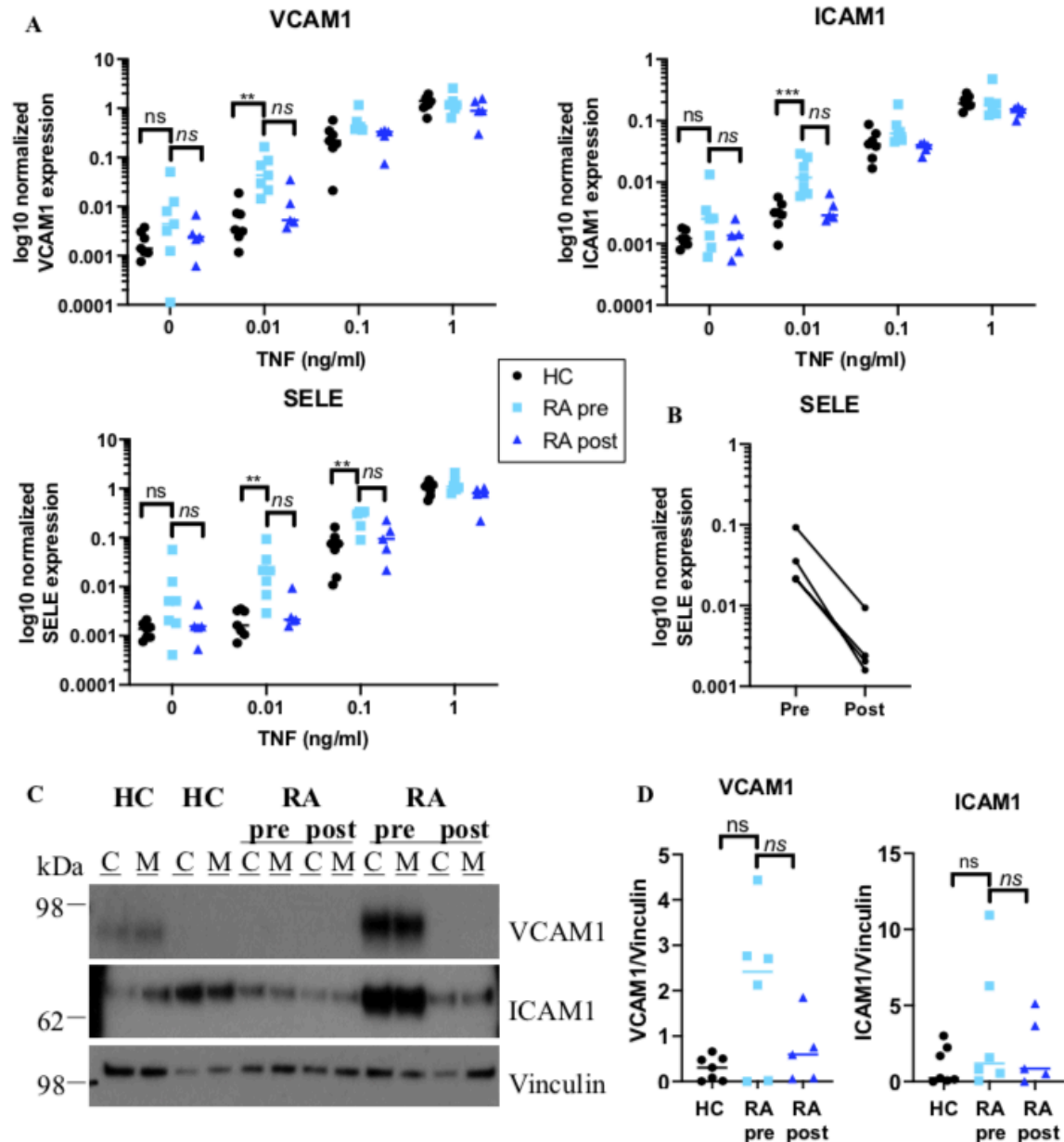


Figure 49. ECFC from treatment-naïve RA patients express higher levels of TNF α -induced cell adhesion molecules compared to HC and treated RA patients. (A) mRNA expression levels of VCAM1, ICAM1 and E-selectin (SELE) in baseline and TNF α treated (4h at indicated dose) ECFC from HC (n=7), treatment-naïve (n=6-7) or treated RA patients (n=5) measured by qPCR and normalised to GAPDH. (B) SELE mRNA expression levels in TNF α -treated (0.01ng/mL) ECFC from four RA patients pre- and post-treatment. (C) Immunoblot analyses of HC and patient ECFC treated *in vitro* with media (control; C) or MTX (M; 100nM, 48h). Whole cell protein lysates were separated by SDS-PAGE. Proteins were detected with antibodies against VCAM1 (95kDa), ICAM1 (90kDa) and Vinculin (124kDa) (HC n=7; RA pre n=6; RA post n=5). Representative blots from two HC and two RA patients are shown. (D) Quantification of VCAM1 and ICAM1 protein expression in control-treated ECFC from respective groups. Data shown as median. Mann Whitney Test (HC versus RA pre) and Wilcoxon matched-pairs signed rank test (RA pre versus post, indicated in *italic*). Ns, not significant. * p<0.05, ** p<0.01.

6.5 Discussion

The ECFC-RA study was established to determine if ECFC represent useful tools to identify and study inflammation-driven vascular dysfunction in patients with RA. In addition, the study aims to address whether DMARD therapy affects the vasculature in RA, one of the central questions of this thesis. The data presented in this chapter point towards important findings that the ECFC-RA study will build upon. Firstly, although ECFC frequency was not changed in RA patients compared to HC, there was an inverse correlation of this parameter with ESR levels, suggesting that systemic inflammation may lower the number of circulating ECFC in treatment-naïve RA patients. Second, ECFC derived from active, treatment-naïve RA patients displayed a primed pro-inflammatory state, which may be reversible with DMARD treatment. These data suggest that systemic inflammation may affect ECFC frequency and function and that DMARD confer anti-inflammatory effects on these cells. Despite the early study stage at the time of data collection for this thesis, the results are supportive of further exploring ECFC as marker and model of vascular inflammation in RA.

The ECFC-RA study is the first prospective study reporting ECFC frequency and function in treatment-naïve patients with active RA who initiate DMARD therapy. ECFC frequency overall was not altered in RA patients, neither cross-sectionally compared to HC nor prospectively after treatment initiation. However, correlation analyses of ECFC frequency and clinical inflammatory markers showed a negative association of ECFC frequency and ESR levels in treatment-naïve RA patients, with similar trends for CRP and DAS28-ESR. Supporting this, ECFC frequency was lower in active RA patients with higher ESR levels compared to those with normal levels. Although these findings require validation in a larger study cohort, they may imply that systemic inflammation reduces ECFC frequency in RA. The decreased circulating ECFC frequency may reflect inflammatory-induced vascular damage, compromising the local vascular progenitor pool [474] and ECFC release or increased homing of ECFC to inflamed sites [461, 574].

The reduced ECFC frequency seen in active RA patients is contradictory to findings from a previous cross-sectional study, which reported elevated ECFC colony numbers in RA patients compared to controls [459]. However, the patient cohort presented in that study comprised a heterogeneous group of RA patients in terms of treatment regimen, disease duration and disease activities: all patients received treatment with conventional or biologic DMARD (treatment duration not specified). Patients had the disease for a long time (mean disease

duration sixteen years) and a mean DAS28 score of 4.36, reflected by more patients having a score <2.6 (19% vs 5.3% in the ECFC-RA study) [459]. In contrast, our RA pre patient group was DMARD-naïve, 63.1% reported a disease duration of less than one year and had a mean DAS28 score of 5.34. Interestingly, the authors reported no correlation of ECFC frequency with clinical markers or treatment regimen in RA patients [459], likely due to the heterogeneity of their cohort parameters. To date, there are no other reports on ECFC frequency in RA patients nor any other chronic systemic inflammatory disease, apart from one study reporting unchanged ECFC colony counts in patients with systemic sclerosis compared to controls [461]. It remains to be determined whether systemic inflammation has general effects on ECFC frequency or if there are RA-specific mechanisms. These findings encourage further analysis of ECFC frequency in RA to understand whether this parameter could be used as a future biomarker of vascular injury. This will require careful patient stratification into active and inactive disease in order to tease out the impact of inflammation on ECFC frequency. Importantly, implementation of ECFC frequency into the clinical practice is currently limited by the long culture protocol. Although one study suggests the possibility to directly detect circulating ECFC from human cord blood [453], this method needs to be validated before its application in clinical routine. Moreover, large cohort studies are needed to first evaluate the correlation of ECFC frequency with different measures of vascular function, and subsequently with the occurrence of cardiovascular events to establish its use in prediction scores in RA.

ECFC derived from patients with active RA exhibited a pro-inflammatory and potentially less proliferative phenotype compared to HC. This pro-inflammatory phenotype was characterised by an increased expression of CAM at baseline and upon TNF α -induction. Of note, a non-significant trend for increased baseline phosphorylation levels of p38 in treatment-naïve RA ECFC samples compared to HC further supports this notion (data not shown). There is currently only one other report on the phenotype of ECFC derived from RA patients and HC [460], which was published during the writing of this thesis. The authors showed that ECFC from treatment-experienced RA patients with long-standing disease had a higher proliferation rate, greater angiogenic capacity and were primed to pro-inflammatory activation compared to HC. The latter was demonstrated by showing increased ICAM1 protein levels in RA ECFC compared to HC after TNF α stimulation (50ng/mL). SIRT1, a NAD-dependent deacetylase, was identified as driving force of this phenotype. SIRT1 expression was decreased in RA ECFC and synovial samples from RA patients, and EC-specific SIRT1 deletion prolonged the

persistence of arthritis in a methyl-bovine serum albumin-induced arthritis model. Comparison of this work with the ECFC-RA study is again complicated by the different patient populations analysed [460], making it difficult to assess the effect of active systemic inflammation or treatment on the ECFC phenotype. However, it is interesting to note that the treatment-experienced RA patients in the published study displayed on average moderate disease activity, with their ECFC being similarly activated in a pro-inflammatory manner, as found in this chapter. The differences in proliferation may be further explained by the different assays used (cell impedance versus BrdU assay). These findings warrant further functional characterisation of the ECFC in our study cohorts.

It is plausible to speculate that this pro-inflammatory phenotype is driven by systemic inflammation in the active RA patient as it is not present in healthy controls or paired treated patients. Notably, similar changes in EC morphology and function are also observed in TNF α -treated EC *in vitro* [46] and in the rheumatoid synovium, where EC in the venules develop a cuboidal and activated phenotype with increased expression of CAM and pro-angiogenic properties following prolonged exposure to the pro-inflammatory milieu [65], further supporting this concept. Interestingly, pro-inflammatory phenotypes were also described in ECFC derived from patients with vaso-occlusive diseases. ECFC from children with sickle cell anaemia showed differential pro-inflammatory activation in individuals at high or low risk of ischaemic stroke [575]. Higher levels of pro-inflammatory cytokines were found in the supernatants of ECFC derived from patients with VTD compared to healthy controls [573], highlighting the intricate role of inflammation in vascular dysfunction. Although the functional significance of the reported phenotypic changes remains to be validated further and established in the context of atherosclerosis, ECFC may prove powerful tools to characterise vascular disease in RA patients on a molecular level. In future studies, it will be interesting to analyse patient-derived ECFC in assays of migration, inflammatory cell recruitment and adhesion, angiogenesis and thrombosis; all thought to be dysregulated processes in RA.

It is remarkable that patient-derived ECFC maintain distinct, disease-specific, phenotypic signatures after passaging *in vitro*. This strongly suggests that epigenetic alterations are present in these cells. Supporting this argument, epigenetic changes are also found in other cell types isolated from RA patients. Global DNA hypomethylation has been reported in T-cells and monocytes from treatment-naïve RA patients, which was reversed with MTX treatment [273].

Interestingly, hypomethylation was specifically demonstrated in the IL6 promoter region in PBMC isolated from RA patients, emphasising a potential disease-driving mechanism [576]. Studies in fibroblast-like synoviocytes from RA patients have found differential methylation patterns in genes with known RA association [577]. Of note, it is unlikely that the observed pro-inflammatory phenotype was caused by contamination with mononuclear cells as the endothelial purity of the ECFC cultures was thoroughly validated. Additionally, it has been shown that EC can be epigenetically primed by different stimuli. Transient hyperglycaemia induces the pro-longed expression of p65 and inflammatory genes in aortic EC, changes that persisted following return to normoglycaemia [578]. Shear stress causes alterations in DNA methylation and acetylation in EC *in vitro* and *in vivo* [154], demonstrated for example by the fact that NFκB signalling is primed in EC located in regions of disturbed flow [126]. Pro-inflammatory stimulation of EC with various agents *in vitro* alters the consequent endothelial response to further activation, causing either tolerance or enhanced production or storage of pro-inflammatory molecules [579, 580]. These observations support the hypothesis that the pro-inflammatory state of ECFC from active RA patients may be explained by prior cytokine-induced epigenetic modifications that occurred *in vivo* and are conserved *ex vivo*. Similarly, data in ECFC from COPD patients provide evidence for epigenetic priming in other diseases, showing increased DNA damage and senescence in patient cells compared to controls [581]. It remains unclear whether epigenetic modulations are cause or effect of chronic inflammation in RA patients [582] and whether these also occur and account for the phenotype in our patient derived ECFC. Epigenetic markers are currently sought for the prediction of treatment response in RA patients [582]; an avenue potentially facilitated by patient-derived ECFC in the future. Further studies will aim to better understand if epigenetic modifications of inflammatory genes are present and sufficient to explain the pro-inflammatory phenotype of RA ECFC, by for example studying DNA methylation globally or at local promoters of genes of interest.

A central question in this thesis was how DMARD therapy affects the vascular endothelium in patients with RA. Thus, it was compelling that DMARD therapy with MTX and HCQ showed a strong trend to reverse the pro-inflammatory ECFC phenotype in the RA patient cohort, supporting the notion that DMARD therapy may prevent inflammatory activation of vascular EC. Due to the local standard of care of our patients with dual DMARD therapy, it was, however, not possible to address the individual contribution of these drugs to ECFC function. HCQ is an anti-malarial drug that is used in the treatment of SLE but also functions as a

DMARD in RA. It is usually prescribed in combination with MTX or other DMARD due to its lower efficacy in halting disease progression in RA patients [164]. It seems likely that MTX and HCQ acted synergistically to achieve this effect as both drugs have been associated with improvement of EC function [202] and a lower risk for CVD events in RA patients [233, 583]. Among other actions, HCQ has been shown to achieve direct anti-inflammatory effects on EC as it decreased TNF α -induced NF κ B activation, pro-inflammatory signalling and leukocyte adhesion [584] and normalised endothelial-dependent relaxation and thrombin generation in a mouse model of anti-phospholipid syndrome [585]. The data from this thesis suggest that MTX confers anti-inflammatory effects on the vasculature by achieving overall control of disease activity through its actions on immune cells and not, or only limited, through direct effects on the endothelium. These results highlight the importance of early aggressive treatment of RA patients with DMARD therapy beyond the effects on joint function.

Completion of the ECFC-RA study, which is currently ongoing, is required to validate these findings in more patients. The small portion of the study presented here is limited by the small sample sizes of the study groups, especially for the follow-up group at this stage. Although not statistically significant, the age and ethnic distribution of the subjects in the study cohorts were not sufficiently well balanced. Three patients in the treatment-naïve RA cohort received statins, which have been reported to increase ECFC frequency in patients with premature CAD [468].

In conclusion, these findings suggest that systemic inflammation affects the frequency of circulating ECFC and causes a pro-inflammatory ECFC phenotype in patients with RA that may be reversible with DMARD treatment. Future studies will need to address the applicability of ECFC as a biomarker and EC surrogate of vascular inflammation. Patient ECFC have been used as surrogates of the vascular endothelium in a variety of diseases but there are still several unanswered questions regarding their origin and function *in vivo* [401]. It is currently not clear if ECFC reflect arterial, venous, microvascular or macrovascular vessels (section 1.6.1.2.1). Therefore, it is difficult to conceptualise the pathogenetic implication of ECFC phenotypes in a specific vascular bed and context, leaving the question unanswered which aspect of the endothelial RA phenotype can be addressed with ECFC (e.g. synovial versus vascular EC). These infrequently discussed points are pertinent to not only this work but to the ECFC field as a whole and remain to be addressed.

7 Concluding remarks

7.1 Discussion and Future Work

7.1.1 MTX is an anti-folate drug

MTX has gained considerable interest as an anti-inflammatory drug for reducing CVD events in patients with RA [231, 233]. Despite its long history and attention from multiple disciplines, the drug's anti-inflammatory and potential anti-atherogenic mechanism of action remains a topic of debate. The goal of this thesis was to study the molecular actions of MTX on vascular EC and understand if these contribute to its anti-atherogenic effects in RA patients.

MTX activated cell signalling and induced cell cycle arrest in EC, which was reversed by folate supplementation (Chapter 3). Because MTX does not cause adverse vascular events in patients [521], it was speculated that these effects may have been a product of artificial culture conditions. To address this point, EC were treated with MTX under shear stress. Strikingly, MTX did not affect cell signalling or cell cycle progression in EC under LSS or OSS (Chapter 4). Following these observations, it was hypothesised that shear stress regulates endothelial folate metabolism, which was consequently demonstrated in Chapter 5. Thus, despite lacking a definite answer to the question of what effects MTX has in the vascular endothelium, these results strongly suggest an important role for endothelial folate metabolism in the drug's mechanism of action.

The observation that MTX interferes with endothelial folate metabolism is not surprising given that the drug is an anti-folate compound. Inhibition of the folate pathway is accepted as the main mechanism of action in cancer therapy. In rheumatology, however, the often-repeated dogma has been, and still is, that the anti-inflammatory effects of low-dose MTX therapy are folate-independent because the drug's immunomodulatory efficacy is not altered by folate supplementation in RA patients in the clinical practice [230]. However, I challenge this paradigm and speculate that folate metabolism is central to MTX's mechanism of action in the anti-inflammatory setting by providing the following arguments.

- 1) Folic or folinic acid are routinely given to RA patients receiving low-dose MTX therapy to reduce adverse effects [227]. Supplementation with high dose folinic acid (15mg or more) reduced the side effects but importantly also the clinical efficacy of MTX in RA patients [550, 551], providing proof of principle that the anti-inflammatory effects of MTX are

folate-dependent [550]. Therefore, clinical practice relies on the correct timing and dosage of folate to reduce the adverse effects of MTX without compromising its immunomodulatory function [227]. Studying the molecular actions of MTX and its dependency on folate *in vitro* has been complicated by the fact that the dynamics of cellular folate or MTX uptake and metabolism *in vivo* are not completely understood (outlined in section 3.5), making it difficult to model accurate concentrations of either agent experimentally. The selection of folate derivative is also crucial to examine competition with MTX in *in vitro* experiments. Unlike folic acid, the cellular utilisation and conversion of folinic acid into other folate derivatives is not dependent on the function of DHFR, one of the main targets inhibited by MTX, highlighting that folinic acid is more potent in rescuing MTX effects. Many studies have used folic acid to examine the folate dependency of MTX effects *in vitro*, which may have led to the underestimation of the folate pathway in the drug's mechanism of action. Another key point not considered in most publications is the impact of the selected growth medium on the MTX response *in vitro* by supplying different baseline levels of folate. I speculate that the routinely prescribed folate regimen (dose and timing) does not interfere with the drug's anti-inflammatory efficacy because of intricate cellular differences in uptake, metabolism, use and elimination of MTX and folates at certain concentrations in different cell types (high proliferating vs non-proliferating) *in vivo*, and most likely not due to another mechanism of action (but I acknowledge that I cannot exclude this possibility).

- 2) The literature describes several effects of low-dose MTX treatment on different cell types (section 1.4.5). Although rarely acknowledged or linked, I speculate that many if not most of the so far reported MTX functions to date could be explained by the direct or indirect inhibition of the folate pathway (section 1.5.2): interference with nucleotide metabolism and apoptosis (DHFR, TYMS, ATIC), increased adenosine release (ATIC and indirectly ADA and AMPDA) [264], AMPK activation (ATIC) [246, 586], ROS generation [258], interference with methylation pathways [273] (through dysregulation of folate metabolism and MAT inhibition [270]), BH₄ metabolism (DHFR) [300] and cell signalling [271, 272] (methylation pathways). In addition, some of these effects (e.g. inhibition of nucleotide synthesis and AMPK activation [587]) have also been described in cancer cells treated with high-dose MTX, supporting the argument that these observations rely on folate-dependent mechanisms regardless of dose.

- 3) The function of OCM is not well understood in non-proliferating/non-cancer cells (section 1.5.2.4). Many reports now show that immune cell activation is accompanied by a change in the cellular metabolic state (reviewed in [588]). It was recently reported that T-cell activation and survival relies on the upregulation of mitochondrial OCM [369]. In addition, BH₄ synthesis, which is metabolically linked to OCM, is required for T-cell proliferation and immune responses [589]. Similarly, OCM has been recently implicated in driving the production of IL1 β in LPS-activated pro-inflammatory macrophages [370, 590], suggesting that inhibition of OCM with MTX could reduce immune cell activation.
- 4) Genetic variants in ATIC, SLC19A1, GGH and DHFR, genes relating to the OCM pathway, have been implicated in predicting the response to MTX (efficacy or toxicity) in RA patients [591]. Genome wide association studies (GWAS) have associated loci in the DHFR, TYMS and FPGS genes with MTX (non-)response in a cohort of Indian RA patients [592]. Moreover, regulatory T-cells (Tregs) from RA patients unresponsive to MTX have been shown to express lower levels of CD39, a molecule involved in adenosine production, compared to those who respond [593]. This evidence further emphasises that the MTX response relies on a functioning folate pathway in RA.
- 5) Lastly, it is interesting to note that another agent used in the treatment of RA achieves anti-inflammatory effects by inhibiting *de novo* pyrimidine nucleotide synthesis. Leflunomide is a DMARD that is indicated in the first line treatment of RA when MTX is contraindicated [164]. The drug inhibits the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH), which converts dihydroorotate to orotate used for the synthesis of uridine monophosphate and ultimately pyrimidine nucleotides [594]. This supports the notion that MTX-mediated inhibition of the folate pathway and hence nucleotide synthesis may also contribute to, or even be sufficient for, its anti-inflammatory effects in RA.

It is clearly beyond the scope of this thesis to prove this hypothesis, but the presented data suggest that shifting the focus back to investigating the role of folate metabolism in vascular EC and other cell types, will facilitate understanding the molecular actions of MTX in atherosclerosis. This thesis provides preliminary evidence that endothelial OCM is differentially regulated by shear stress, with potentially important implications for MTX uptake and action *in vivo* (Chapter 5). First, cytosolic OCM was dampened by shear stress, suggesting

that many previously described MTX effects that are dependent on DHFR or TYMS inhibition (e.g. eNOS uncoupling due to reduction of BH₄ recycling [300]) will most likely not occur in EC *in vivo*. Based on the expression profile of ATIC and RFC in EC exposed to OSS, I speculate that MTX may be preferentially taken up in atheroprone regions *in vivo*, where it may enhance local adenosine release by inhibition of ATIC. It is important to emphasise that this work did not identify any effects of MTX on cell signalling, cell cycle progression or gene expression in EC subjected to OSS (Chapter 4), implying that the proposed adenosine release may not affect EC directly but may promote a local anti-inflammatory environment.

It remains to be addressed if this proposed mechanism is valid and if it contributes to the drug's anti-atherogenic actions. Future work should determine intracellular MTX concentrations, polyglutamation, retention and adenosine release in/from MTX-treated EC exposed to H-LSS versus OSS *in vitro*, followed by validation in an animal model. If adenosine release is indeed increased in MTX-treated EC in atheroprone regions, it would be interesting to evaluate whether MTX-induced adenosine release mediates anti-atherogenic effects by for example studying inflammatory cell adhesion to EC under shear stress *in vitro* using adenosine receptor blockers. To globally assess if MTX has direct effects on EC under OSS, it would be informative to perform RNA sequencing, proteomics and metabolomics analyses under baseline conditions and pro-inflammatory challenge. These studies should be complemented by a more detailed characterisation of endothelial OCM (see below).

Elucidating the molecular effects of MTX has proven very difficult *in vitro* and *in vivo*, with many studies reporting contradictory results (section 1.4.5). This study has started to address two important layers of complexity modulating MTX response in EC: shear stress and its regulation of OCM. The data provide a basis for explaining the divergent effects of the drug in different biological settings. Taken together, I propose that the effects of MTX on a given cell type will be dependent on the regulation of OCM and expression pattern of adenosine receptors brought about by cellular specificity and its biomechanical microenvironment. The systematic work up of OCM regulation and function in different cell types and tissues *in vivo*, at baseline and upon challenge with different stimuli, will be essential for understanding the effects of MTX. Folate antagonism and consequent increase in adenosine production are so far the best demonstrated mechanisms of action of MTX. Although this does not formally rule out non-folate dependent effects of the drug, the lack thereof is very evident in the literature on a drug, which has been used in the clinical setting for over 70 years.

7.1.2 Endothelial OCM is differentially regulated by shear stress

It is the current school of thought that endothelial OCM facilitates the maintenance of NO bioavailability via BH₄ recycling (section 1.5.2.4.5). In line with this hypothesis, folate supplementation has been investigated as measure for the secondary prevention of CVD in the general population but has yielded disappointing results (section 1.5.2.4.4). This is the first report to holistically investigate OCM in EC under static and shear stress conditions.

The findings from this thesis show that endothelial OCM is tightly controlled by shear stress on a transcriptional and potentially also metabolic level. Both H-LSS and OSS systematically downregulated the expression of enzymes in the cytosolic OCM pathway, while showing differential expression profiles for enzymes in the mitochondrial pathway. This supports the concept that endothelial metabolism is generally repressed by atheroprotective shear but may be selectively activated by disturbed flow (section 1.5.1). The observed transcriptional profiles suggest that OCM has other roles in EC than the previously proposed regulation of BH₄ levels via DHFR, in line with other reports [398, 399]. While the abundance of folate metabolites was generally reduced by shear stress, three metabolites, 10-formylTHF, 5-meTHF and 5,10-meTHF, may show a unique pattern of regulation, potentially indicating a specific requirement for these metabolites under shear stress.

Mitochondria do not have a classic energy producing role in EC (section 1.5.1) and their functions under shear stress are incompletely understood (section 5.5). The OSS-induced expression of mitochondrial OCM enzymes suggests a functional role for this pathway in EC. Indeed, MTHFD2, a central enzyme in mitochondrial OCM, was shown to regulate mitochondrial morphology in EC under shear stress. The underlying mechanism and relevance of this finding remain to be addressed. MTHFD2 has been implicated to have a central role in EC in atherogenesis in a recent report [384], making it very tempting to speculate that mitochondrial OCM may drive EC activation and inflammation in atheroprone regions.

Future experiments should address whether endothelial OCM is dispensable or necessary for EC function *in vivo*. First, it will be necessary to confirm the proposed shear-mediated regulation of OCM at the protein and metabolic level. For this, OCM protein levels could be determined in EC located in atheroprotected and atheroprone regions in murine aortas by immunostaining. To understand the metabolic flux through OCM, it would be interesting to perform metabolite tracer experiments in EC under shear stress. The functional role of OCM

in EC could be further investigated by systematic targeted deletion of OCM enzymes in EC exposed to shear stress *in vitro* or using EC-specific OCM knockout mice and evaluation of the resulting effects on EC inflammation, proliferation, ROS production and metabolism.

There are many open questions regarding the regulation and role of OCM in EC but also other cell types relevant in atherogenesis. OCM has been closely linked to CVD but its role remains incompletely understood. By providing insight into the basic mechanisms of endothelial OCM, this work ultimately aims to understand its role in EC, and whether it contributes to atherosclerosis and represents a therapeutic target in patients.

7.1.3 ECFC may serve as a tool to detect and investigate vascular damage in RA patients

It is not clear how systemic inflammation and its reversal with DMARD therapy affects EC in RA patients, partly due to the limited accessibility of patient EC, limited availability of reliable tools to non-invasively assess vascular function in patients and difficulty associated with accurately modelling disease/drug responses *in vitro*. To address this, the ECFC-RA study was established to prospectively measure ECFC frequency and function in RA patients before and after DMARD initiation with MTX and HCQ, and in healthy controls.

The current data suggest that the frequency of ECFC from treatment-naïve RA patients may be reduced with increasing levels of inflammation, as shown by the inverse correlation of ECFC frequency and ESR levels. This may support the hypothesis that inflammation-mediated vascular damage reduces the number of circulating ECFC progenitor cells, perhaps due to direct damage to resident precursor pools or increased homing to the site of injury. ECFC from treatment-naïve patients displayed a primed pro-inflammatory signature compared to HC, suggesting that chronic inflammation causes systemic EC activation. Interestingly, inflammatory priming was lost in paired ECFC from treated patients, suggesting that DMARD may reverse this phenotype through direct or indirect actions on EC. This finding could provide a mechanism to explain how DMARD therapy reduces CVD events in patients.

These initial findings from the ECFC-RA study are exciting and will be further expanded in the full study, which is currently ongoing. There are still open questions regarding ECFC and their use in translational medicine. A recent paper on ECFC derived from a treatment-experienced RA cohort [460] demonstrated the utility of ECFC to molecularly dissect disease-

relevant pathways in RA, which were validated in synovial EC. The goal of this thesis was to characterise the effect of RA-related inflammation on EC in the context of CVD using ECFC, therefore trying to model the macrovasculature. Because the origin and molecular phenotype of ECFC is ill defined (section 1.6.1.2), it is difficult to pinpoint their pathophysiological significance to a particular organ; especially in diseases in which multiple vascular beds are affected. I speculate that systemic inflammation may produce a continuum of EC activation in different locations, causing vascular damage in the joint but also in the systemic circulation. It remains to be investigated which vascular bed is best represented by ECFC and if the molecular phenotype of synovial and systemic EC is different in RA. Although ECFC represent promising tools to study vascular function *ex vivo*, further work is required to define their origin, phenotype and function to avoid misinterpretation of their significance.

The data from this thesis support the evaluation of ECFC as a potential tool to detect and investigate vascular dysfunction in RA patients. Current efforts are directed at characterising these patient-derived ECFC in more detail, for example by RNA sequencing, along with ECFC from patients with other chronic systemic inflammatory diseases, which may help identify disease-specific and general inflammatory signatures. Moreover, these cells will be further investigated for their behaviour in angiogenesis, migration and thrombosis assays. More studies are needed to optimise ECFC detection and culture protocols and correlate ECFC parameters with other markers of vascular function in patients. In the future, ECFC may be used as a biomarker of vascular damage and to understand inflammatory disease mechanisms in the endothelium.

7.2 Conclusion

This thesis evaluated the question whether MTX protects EC from pro-inflammatory or OSS-induced dysfunction and whether this mechanism could contribute to the anti-atherogenic effects seen in RA patients. The data show that folate metabolism dictates the effects of MTX in EC, suggesting that MTX may induce endothelial adenosine release in atheroprone regions which may act on cells in the local surrounding. Co-treatment of MTX and HCQ may reduce the pro-inflammatory activation of EC in RA patients as demonstrated using patient-derived ECFC, implying that DMARD therapy may effectively prevent EC dysfunction. The data from this thesis suggest that MTX may achieve this via indirect effects on the endothelium by limiting systemic inflammation.

8 References

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9 Appendix

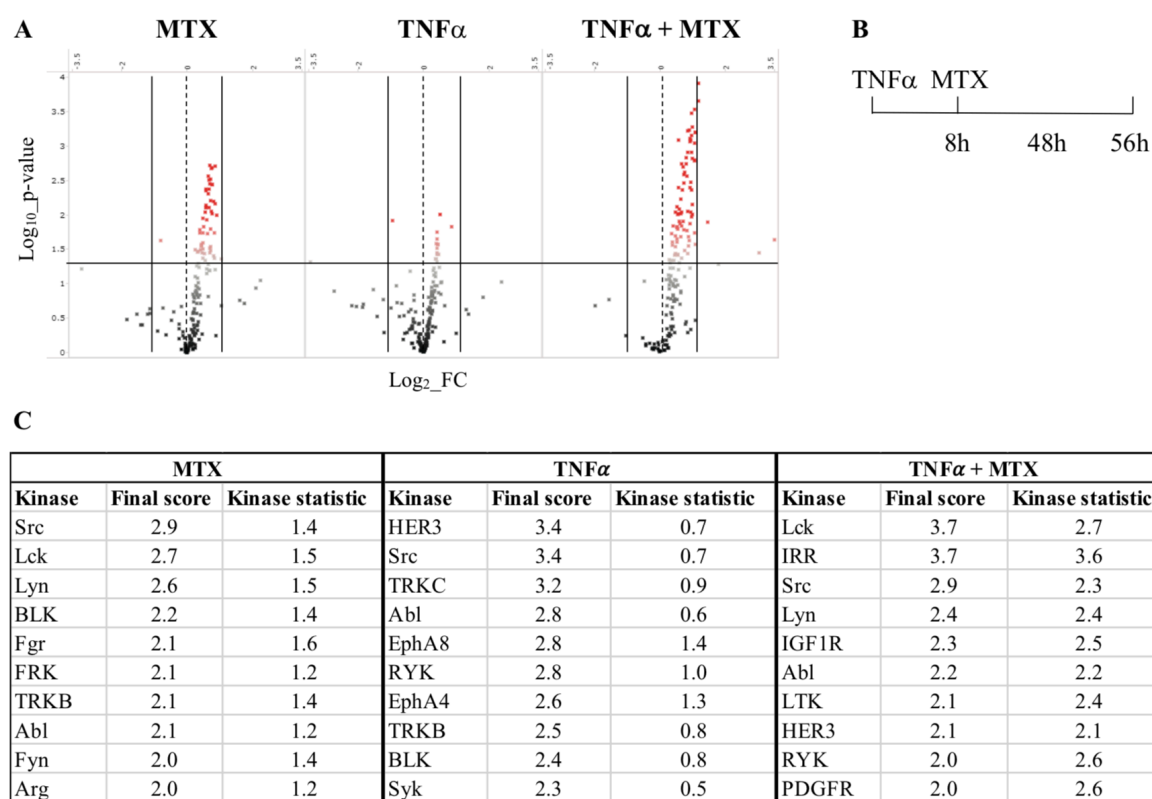
9.1 EC biology qPCR array targets

Table 12. Gene list of EC biology qPCR array (Qiagen)

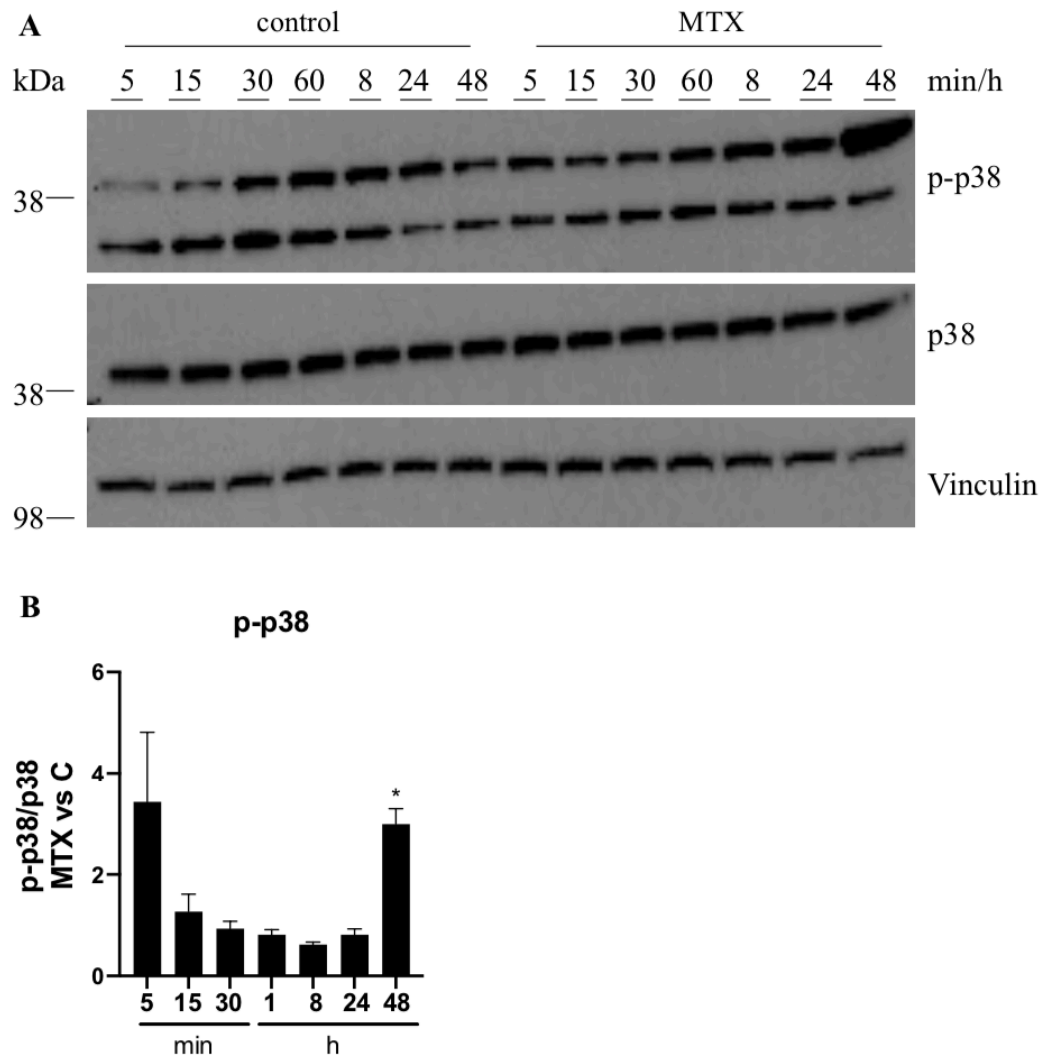
Gene symbol	Full gene name
ACE	Angiotensin I converting enzyme 1
ADAM17	ADAM metallopeptidase domain 17
AGT	Angiotensinogen
AGTR1	Angiotensin II receptor, type 1
ALOX5	Arachidonate 5-lipoxygenase
ANGPT1	Angiopoietin 1
ANXA5	Annexin A5
APOE	Apolipoprotein E
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
BCL2L1	BCL2-like 1
CALCA	Calcitonin-related polypeptide alpha
CASP1	Caspase 1, apoptosis-related cysteine peptidase
CASP3	Caspase 3, apoptosis-related cysteine peptidase
CAV1	Caveolin 1, caveolae protein, 22kDa
CCL2	Chemokine (C-C motif) ligand 2
CCL5	Chemokine (C-C motif) ligand 5
CDH5	Cadherin 5, type 2 (vascular endothelium)
CFLAR	CASP8 and FADD-like apoptosis regulator
COL18A1	Collagen, type XVIII, alpha 1
CX3CL1	Chemokine (C-X3-C motif) ligand 1
EDN1	Endothelin 1
EDN2	Endothelin 2
EDNRA	Endothelin receptor type A
ENG	Endoglin
F2R	Coagulation factor II (thrombin) receptor
F3	Coagulation factor III (thromboplastin, tissue factor)
FAS	Fas (TNF receptor superfamily, member 6)
FASLG	Fas ligand (TNF superfamily, member 6)
FGF1	Fibroblast growth factor 1 (acidic)
FGF2	Fibroblast growth factor 2 (basic)
FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor receptor)
FN1	Fibronectin 1
HIF1A	Hypoxia inducible factor 1, alpha subunit
HMOX1	Heme oxygenase (decycling) 1
ICAM1	Intercellular adhesion molecule 1
IL11	Interleukin 11
IL1B	Interleukin 1, beta
IL3	Interleukin 3 (colony-stimulating factor, multiple)
IL6	Interleukin 6 (interferon, beta 2)
IL7	Interleukin 7
ITGA5	Integrin, alpha 5 (fibronectin receptor)

ITGAV	Integrin, alpha V (vitronectin receptor, antigen CD51)
ITGB1	Integrin, beta 1 (fibronectin receptor, antigen CD29)
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
KDR	Kinase insert domain receptor (VEGFR2)
KIT	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KLK3	Kallikrein-related peptidase 3
MMP1	Matrix metalloproteinase 1
MMP2	Matrix metalloproteinase 2
MMP9	Matrix metalloproteinase 9
NOS3	Nitric oxide synthase 3
NPPB	Natriuretic peptide B
NPR1	Natriuretic peptide receptor A/guanylate cyclase A
OCLN	Occludin
PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide
PECAM1	Platelet/endothelial cell adhesion molecule
PF4	Platelet factor 4
PGF	Placental growth factor
PLAT	Plasminogen activator, tissue
PLAU	Plasminogen activator, urokinase
PLG	Plasminogen
PROCR	Protein C receptor, endothelial
PTGIS	Prostaglandin I ₂ (prostacyclin) synthase
PTGS2	Prostaglandin-endoperoxide synthase 2 (COX2)
PTK2	PTK2 protein tyrosine kinase 2
SELE	Selectin E
SELL	Selectin L
SELPLG	Selectin P ligand
SERPINE1	plasminogen activator inhibitor type (PAI) 1)
SOD1	Superoxide dismutase 1, soluble
SPHK1	Sphingosine kinase 1
TEK	TEK tyrosine kinase, endothelial
TFPI	Tissue factor pathway inhibitor
TGFB1	Transforming growth factor, beta 1
THBD	Thrombomodulin
THBS1	Thrombospondin 1
TIMP1	TIMP metalloproteinase inhibitor 1
TNF	Tumor necrosis factor
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
TYMP	Thymidine phosphorylase
VCAM1	Vascular cell adhesion molecule 1
VEGFA	Vascular endothelial growth factor A
VWF	Von Willebrand factor
ACTB	Actin, beta
B2M	Beta-2-microglobulin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPRT1	Hypoxanthine phosphoribosyltransferase 1
RPLP0	Ribosomal protein, large, P0

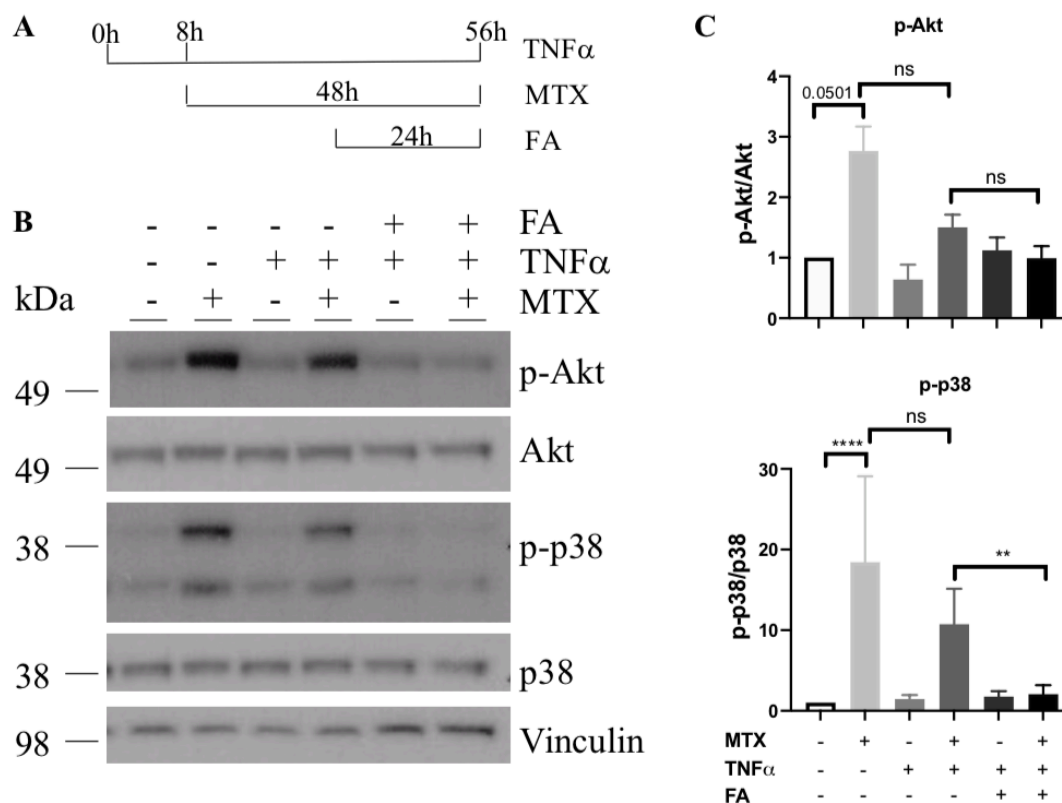
9.2 Supplementary Figures



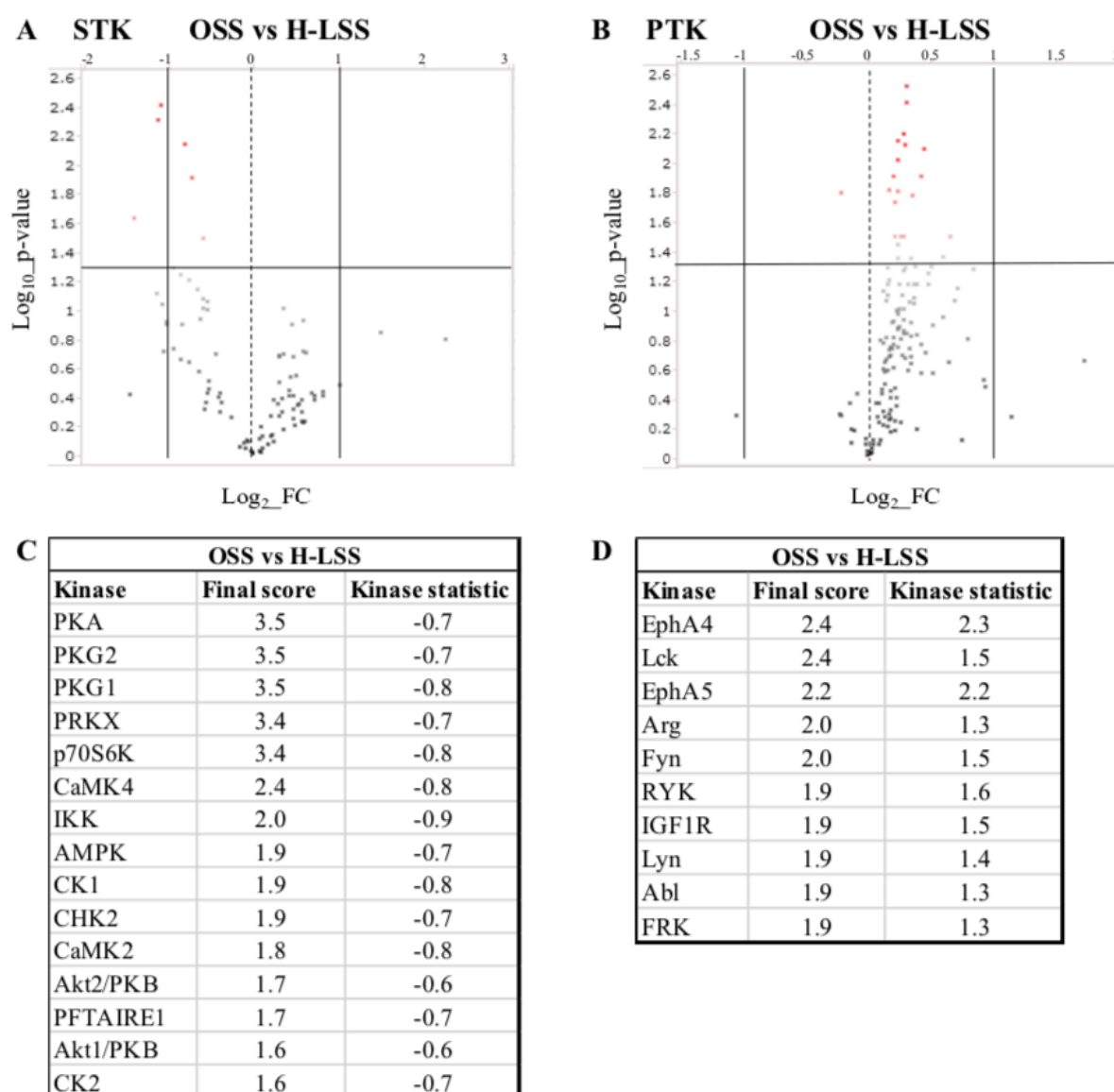
Supplementary Figure 1. MTX induces multiple cell signalling pathways in EC independent of prior pro-inflammatory activation with TNF α . (A-E) HAEC were pre-treated with media (control) or TNF α (0.1ng/ml) for 8h. MTX (100nM) was added to the cells for another 48h (TNF α treatment for total of 56h). Experimental outline shown in (B). (A) Volcano plots of differentially phosphorylated peptides in a given condition over control from PTK arrays (n=3). (C) Top 15% of kinase hits from PTK arrays performed on respectively treated cells. Final Score indicates combined measure of significance and specificity of permutation analyses. The kinase statistic is computed by the overall change of the peptide set that represent a kinase (value >0 means that kinase is more active in test over control sample).



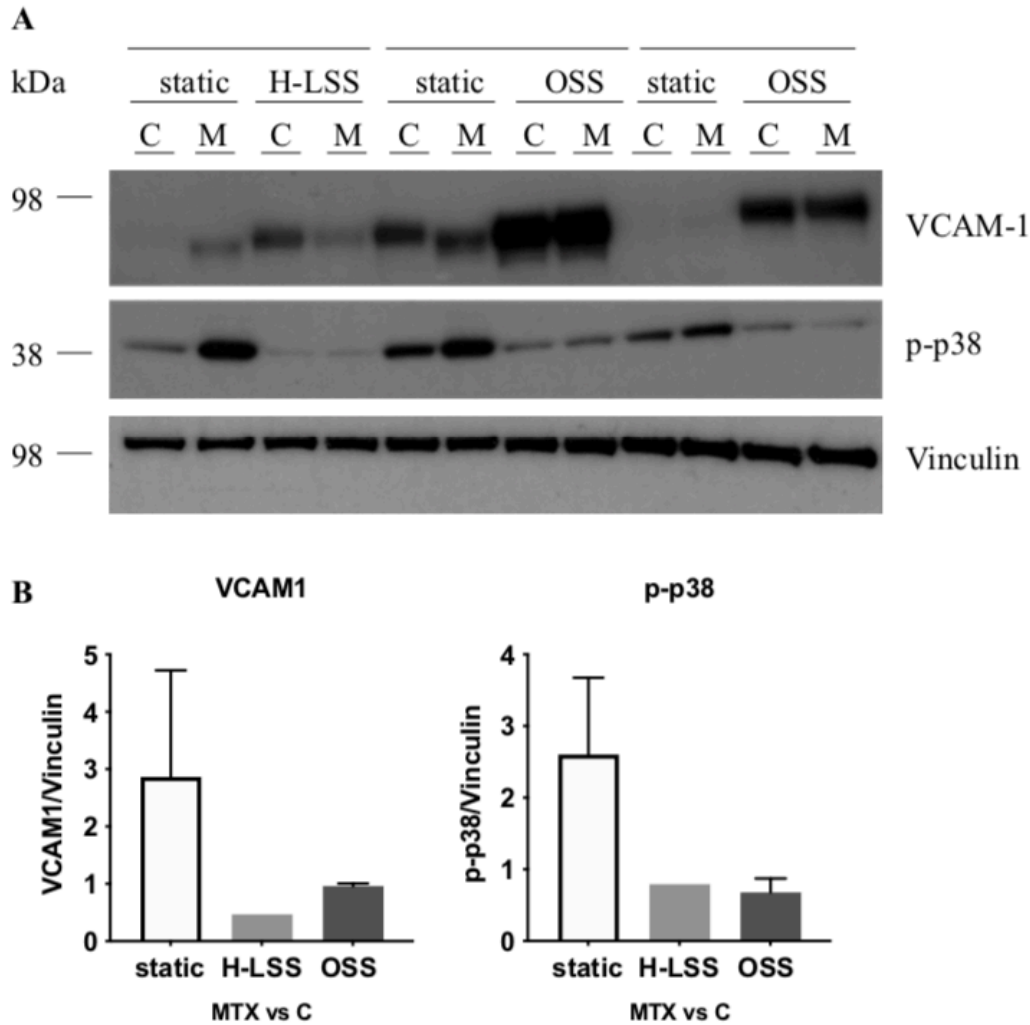
Supplementary Figure 2. Time course of MTX-induced p38 phosphorylation in HAEC. (A) Immunoblot analyses of HAEC treated with media (control, C) or MTX (100nM) for indicated time points. Whole protein lysates of respectively treated cells were separated by SDS-PAGE. Proteins were detected with antibodies against p-p38 T180/Y182 (38kDa), p38 (38kDa) and Vinculin (124kDa). Representative blots shown (n=3). (B) Quantification of p38 phosphorylation levels in HAEC treated as described above (n=3). Data shown as fold change over respective control. All values represent means $\pm$ SEM. Unpaired T-Test. ns, not significant. * $p < 0.05$.



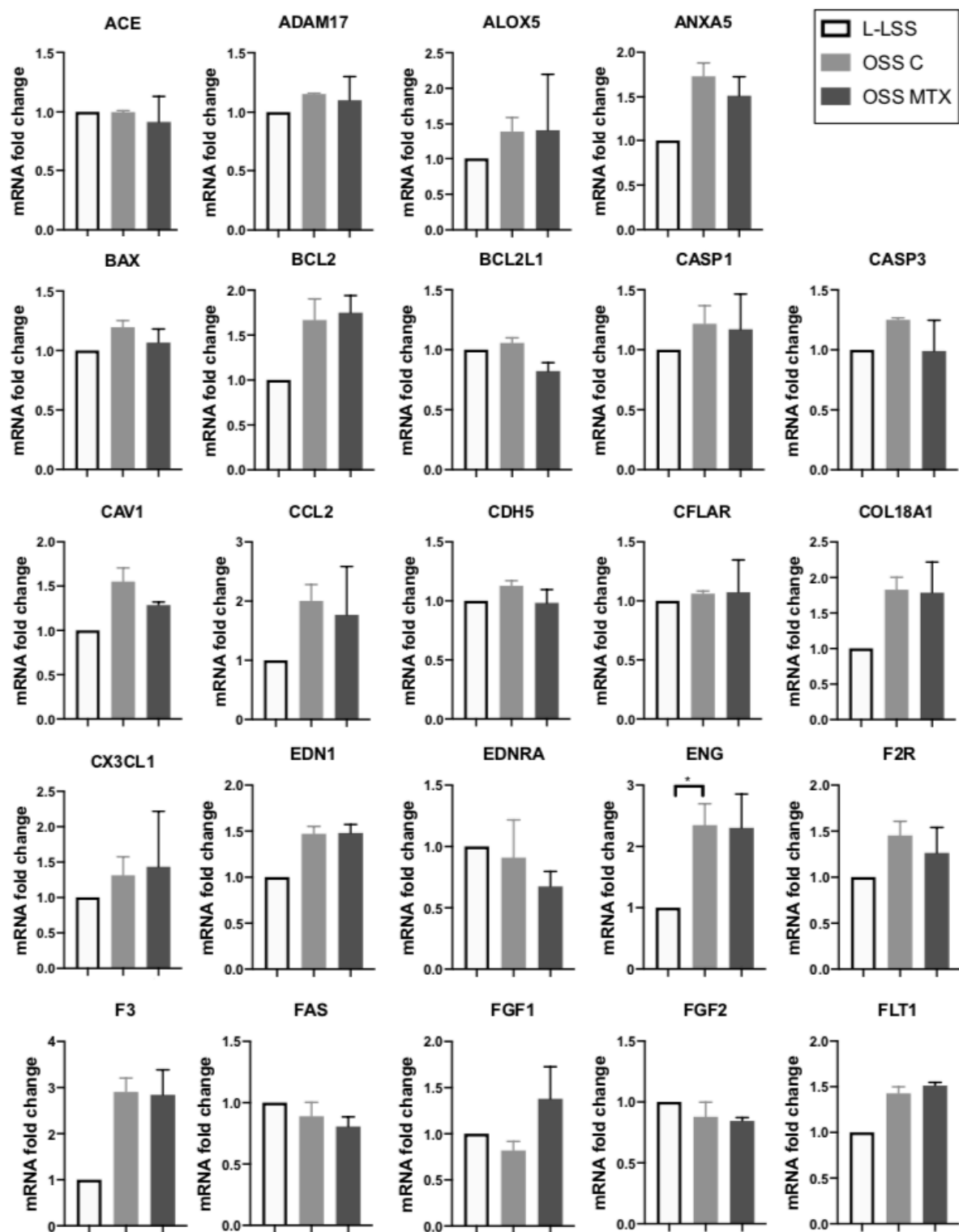
Supplementary Figure 3. MTX-induced p38 phosphorylation is rescued by FA in the presence of TNF α in EC. (A-C) HUVEC were pre-treated with media (control) or TNF α (0.1ng/ml) for 8h. Media or MTX (100nM) were then added for 48h. After the first 24h of MTX treatment, folinic acid (FA; 500nM) or vehicle control were added for 24h as outlined in (A). (B) Immunoblot analyses of HUVEC treated as above. Whole protein lysates of respectively treated cells were separated by SDS-PAGE. Proteins were detected with antibodies against p-p38 T180/Y182 (38kDa), p38 (38kDa), p-Akt S473 (60kDa), Akt (60kDa) and Vinculin (124kDa) (n=3). (C) Quantification of p38 and Akt phosphorylation in HUVEC treated as described above (n=3). Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. Values represent means $\pm$ SEM. ns, not significant. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$.



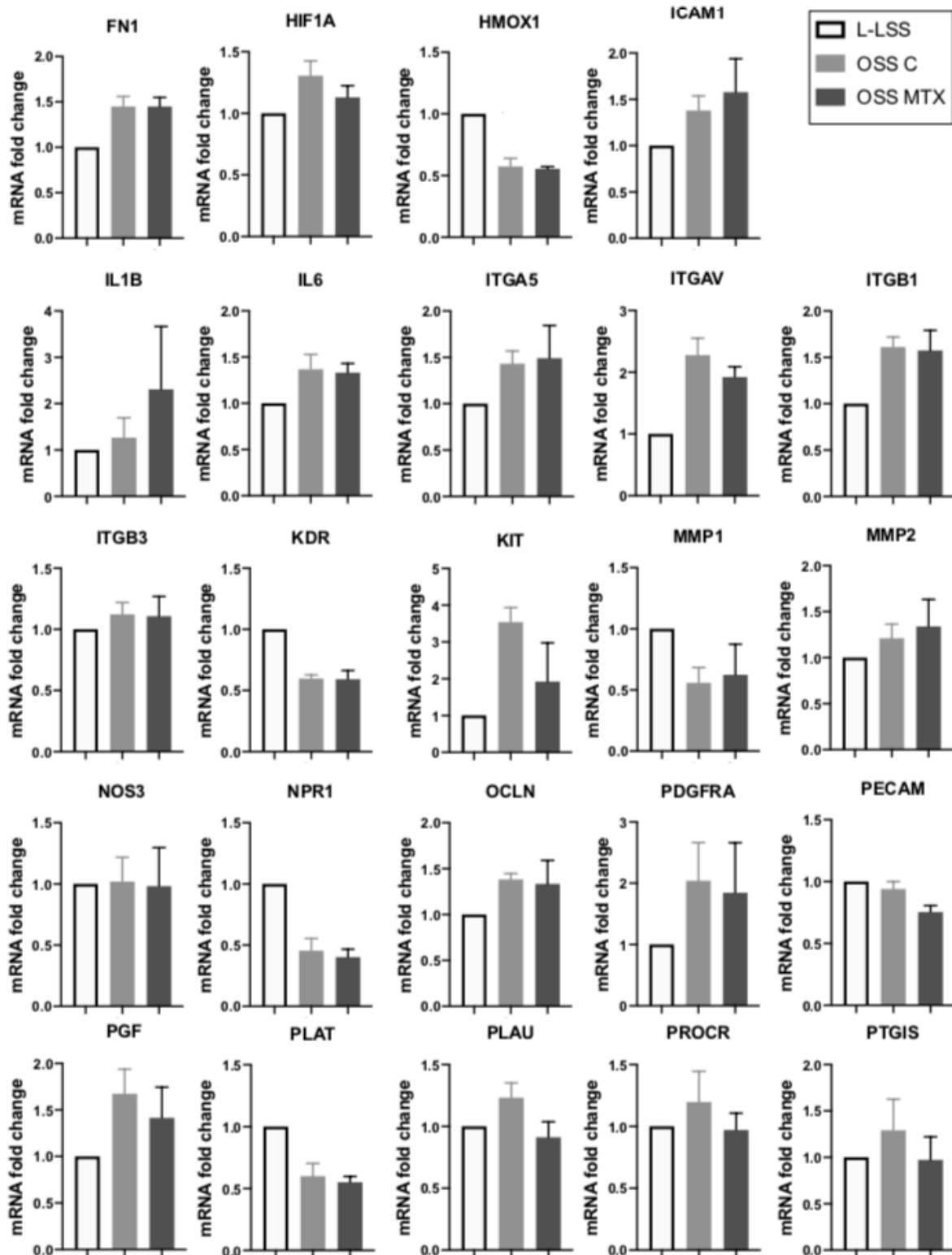
Supplementary Figure 4. Phospho-kinase activity arrays in EC subjected to H-LSS and OSS. HAEC were subjected to H-LSS (20dyn/cm²) or OSS (± 5 dyn/cm², 2Hz) for 96h. (A+B) Volcano plots of phospho-serine-threonine (STK) (A) or tyrosine kinase (PTK) (B) arrays performed on HAEC treated respectively (STK n=4-5; PTK n=3). Dots represent differentially phosphorylated peptides in a given array comparing OSS and H-LSS conditions (shown as increase or decrease in OSS over H-LSS). (C+D) Top 15% of kinase hits in STK (C) or PTK (D) arrays in EC comparing OSS and H-LSS conditions. Final Score indicates combined measure of significance and specificity of permutation analyses. The kinase statistic is computed by the overall change of the peptide set that represent a kinase (value >0 means that kinase is more active in test over control sample).



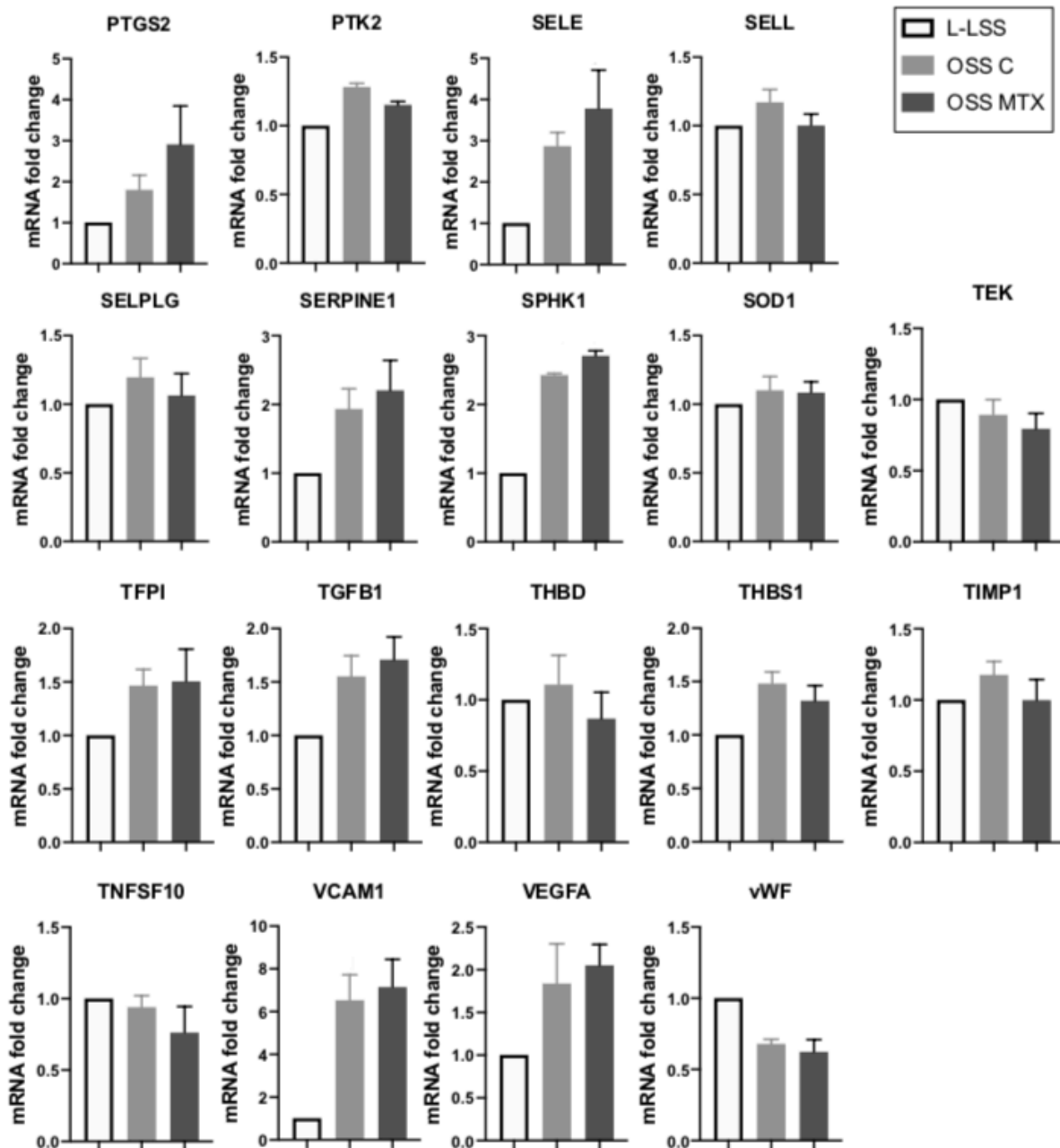
Supplementary Figure 5. MTX differentially affects cell signalling in EC subjected to shear stress or static conditions. (A+B) HUVEC were pre-conditioned with H-LSS (20dyn/cm²) or OSS ($\pm$ 5dyn/cm², 2Hz) for 48h. Media (control, C) or MTX (100nM) were added to the cells for a further 48h under the respective shear stress condition (n=1-2). Alternatively, HUVEC were grown under static conditions in the absence (C) or presence of MTX (100nM, 48h) (n=3). (A) Immunoblot analyses of respectively treated cells. Whole protein lysates were separated by SDS-PAGE. Proteins were detected with antibodies against VCAM1 (95kDa), p-p38 T180/Y182 (38kDa), p38 (38kDa) and Vinculin (124kDa) (n=1-2). (B) Quantification of VCAM1 protein and p38 phosphorylation levels in respectively treated HUVEC. Data shown as fold change of MTX versus control treatment in respective condition.



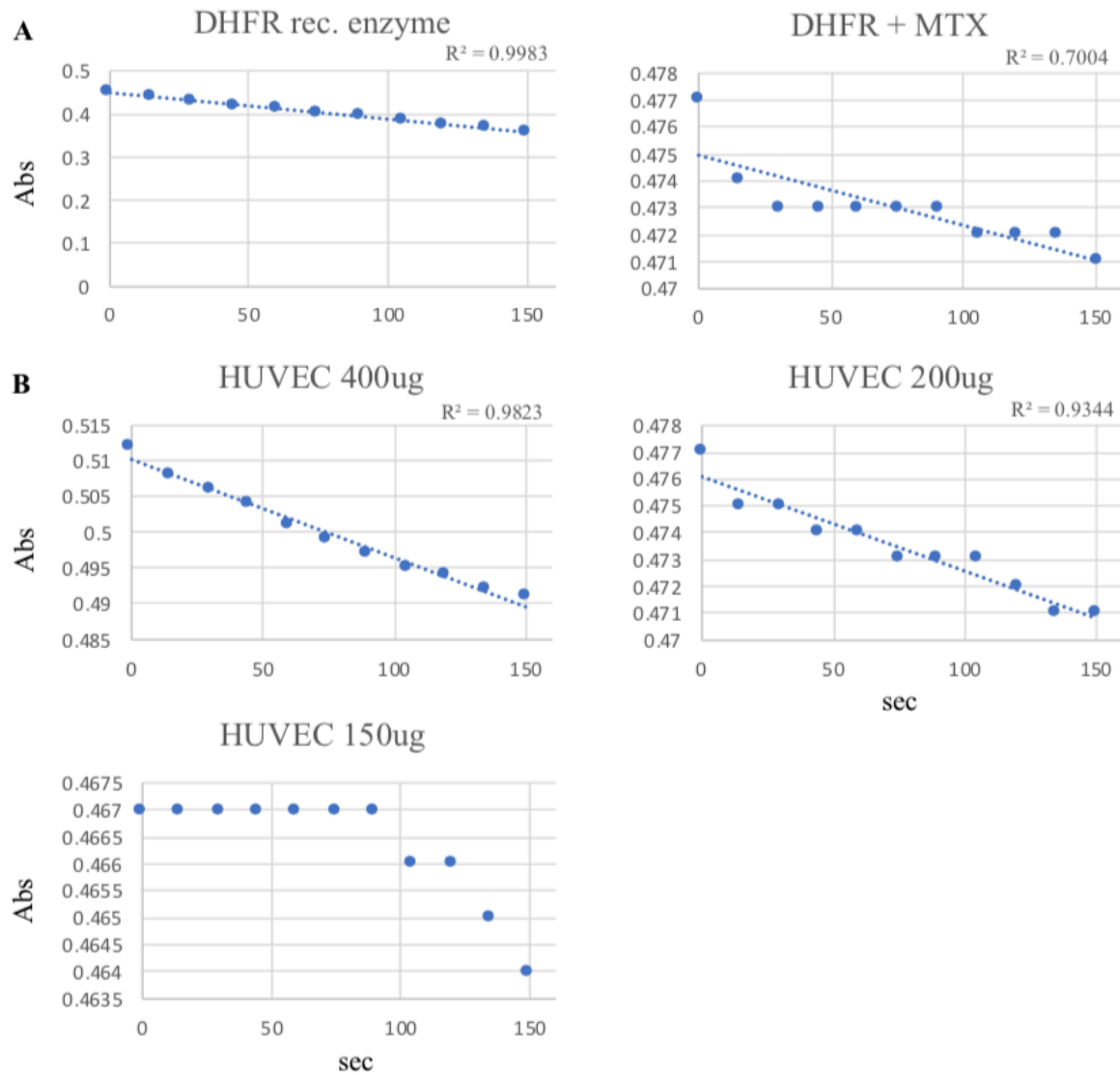
Supplementary Figure 6. Gene expression analyses of EC treated with MTX under OSS using qPCR arrays. HAEC were subjected to OSS ($\pm 5 \text{ dyn/cm}^2$, 2Hz). After 48h, media (control) or MTX (100nM) were added to the cells for another 48h under OSS. As control, EC were exposed to L-LSS (5 dyn/cm^2) for 96h. Gene expression analyses were performed using qPCR arrays. mRNA expression levels normalised to GAPDH and shown relative to low laminar shear condition ($n=3$). In alphabetical order.



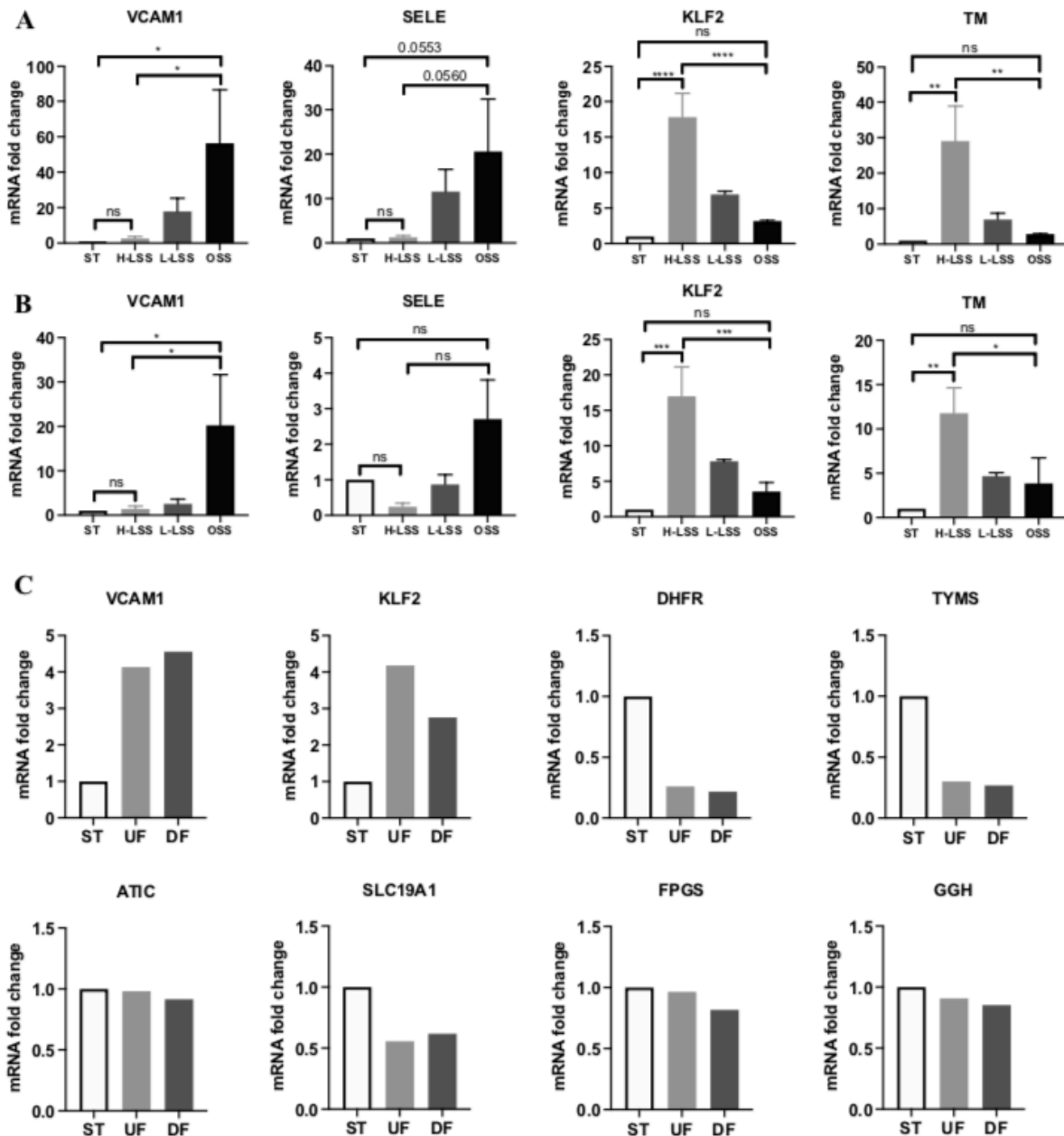
Supplementary Figure 7. Gene expression analyses of EC treated with MTX under OSS using qPCR arrays. HAEC were subjected to OSS ($\pm 5 \text{ dyn/cm}^2$, 2Hz). After 48h, media (control) or MTX (100nM) were added to the cells for another 48h under OSS. As control, EC were exposed to L-LSS (5 dyn/cm^2) for 96h. Gene expression analyses were performed using qPCR arrays. mRNA expression levels normalised to GAPDH and shown relative to low laminar shear condition ($n=3$). In alphabetical order.



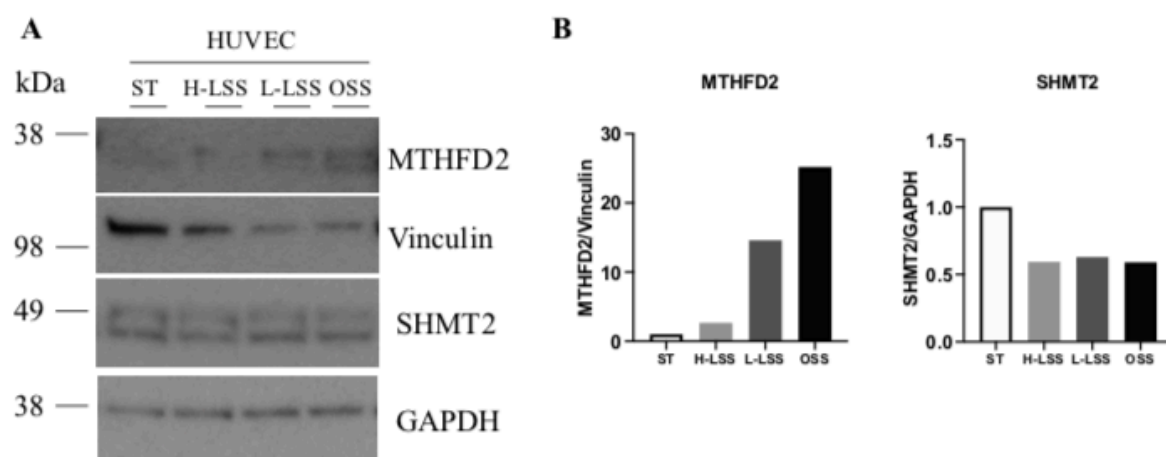
Supplementary Figure 8. Gene expression analyses of EC treated with MTX under OSS using qPCR arrays. HAEC were subjected to OSS ($\pm 5 \text{ dyn/cm}^2$, 2Hz). After 48h, media (control) or MTX (100nM) were added to the cells for another 48h under OSS. As control, EC were exposed to L-LSS (5 dyn/cm^2) for 96h. Gene expression analyses were performed using qPCR arrays. mRNA expression levels normalised to GAPDH and shown relative to low laminar shear condition ($n=3$). In alphabetical order.



Supplementary Figure 9. DHFR activity assay in HUVEC grown under static conditions. (A) Cell-free DHFR activity assay performed with recombinant DHFR enzyme in the absence (left) or presence of MTX (1 μ M) (right). (B) DHFR activity assay performed on HUVEC lysates of varying concentrations (n=1). Kinetic DHFR activity assay (DHFR assay Kit; Sigma) measures DHFR activity as decrease of its co-factor (NADPH) over time (sec), which is detected by absorbance on a plate reader. DHFR is either supplied as recombinant enzyme or in the cell lysate.



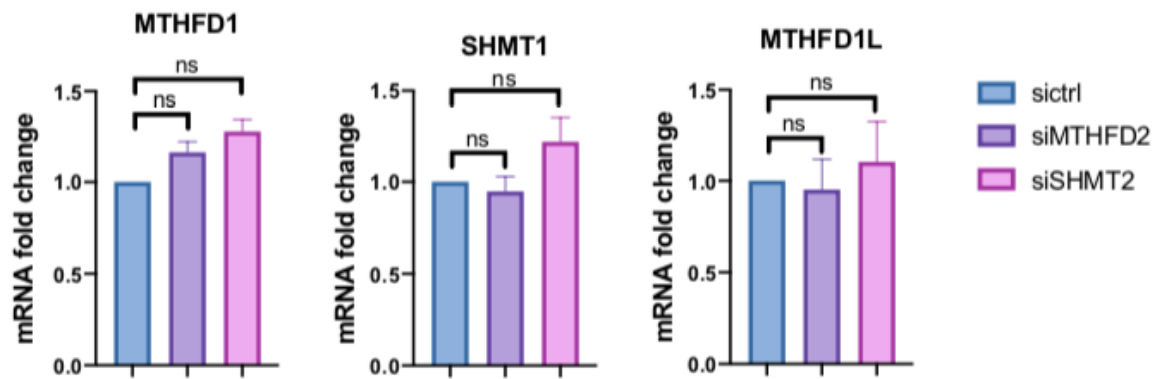
Supplementary Figure 10. Gene expression analyses of EC exposed to shear stress relating to Chapter 5 (Figures 32 and 33). (A+B) HUVEC (A) or HAEC (B) were subjected to static culture (ST) or H-LSS (20dyn/cm²), L-LSS (5dyn/cm²) or OSS ($\pm$ 5dyn/cm², 2Hz) for 48h using the parallel plate model (each n=3). (C) HAEC were subjected to static culture (ST), undisturbed (UF) or disturbed flow (DF) for 72h using the orbital shaker model (n=1). Gene expression analyses were performed by qPCR. mRNA expression levels normalised to GAPDH and shown relative to static conditions. Values represent means $\pm$ SEM. (A+B) Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. ns, not significant. * p<0.05. ** p<0.01. *** p<0.001. **** p<0.0001.



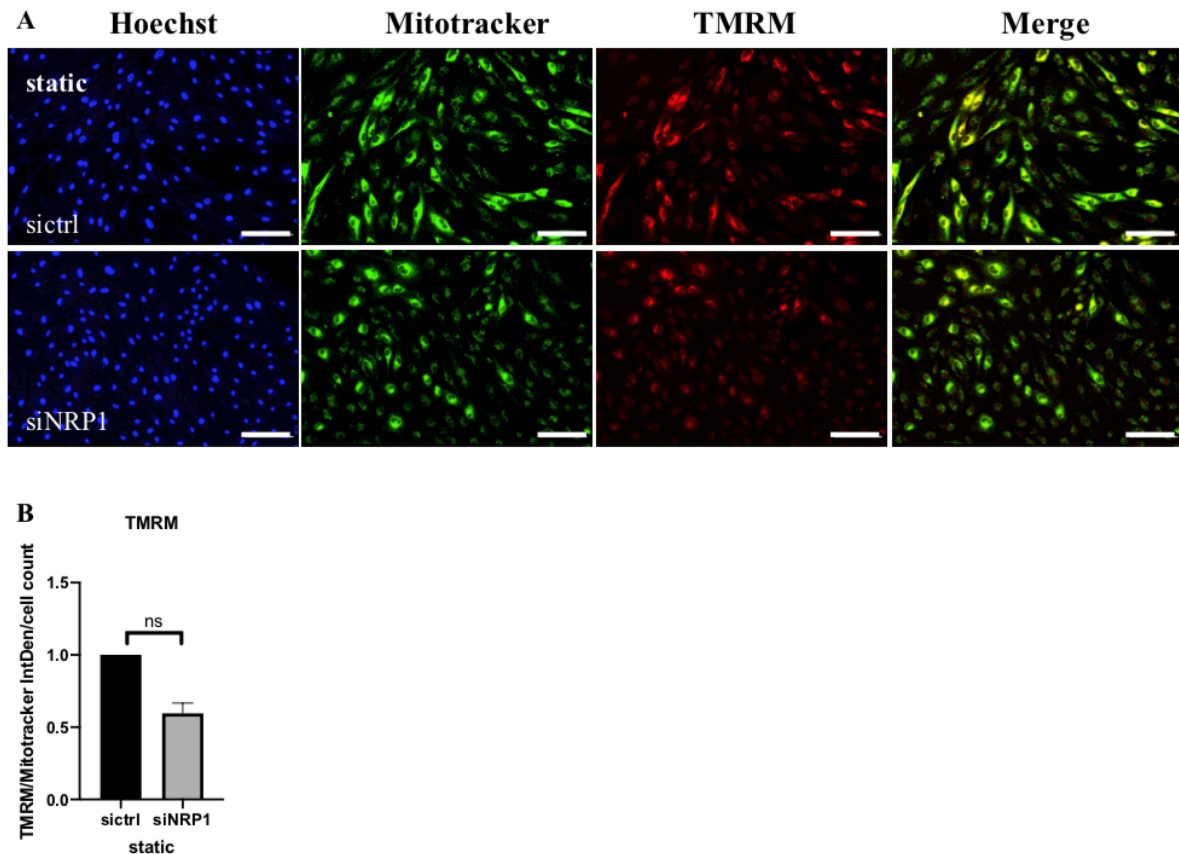
Supplementary Figure 11. MTHFD2 and SHMT2 protein expression in EC subjected to shear stress. (A) Immunoblot analyses of HUVEC, which were either kept under static conditions (ST) or subjected to H-LSS (20dyn/cm²), L-LSS (5dyn/cm²) or OSS ($\pm$ 5dyn/cm², 2Hz) shear stress conditions for 48h in EGM2 (2% FCS). Whole protein lysates of respectively conditioned cells were separated by SDS-PAGE. Proteins were detected with antibodies against MTHFD2 (34kDa), SHMT2 (52kDa), GAPDH (39kDa) and Vinculin (124kDa) (n=1). (B) Quantification of MTHFD2 and SHMT2 protein levels in respectively treated HUVEC (n=1).



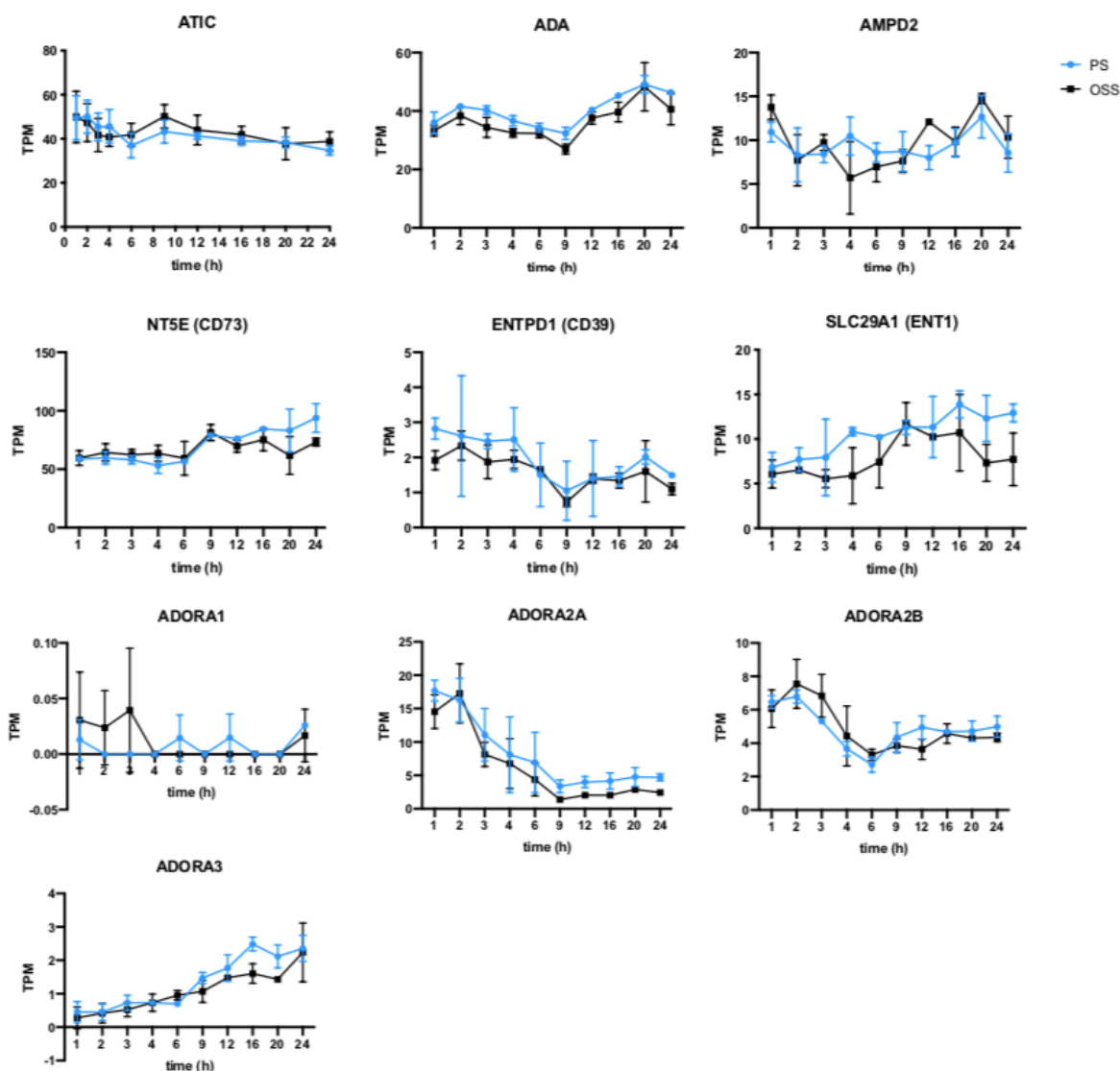
Supplementary Figure 12. Transcription factor binding motif analyses on the human DHFR promoter. (A) Human DHFR gene locus presented using the UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly (<http://genome.ucsc.edu/>). ENCODE data for H3K27Ac mark, SwitchGear Genomics transcription start sites and Chromatin State Segmentation by HMM from ENCODE/Broad for HUVEC indicate active promoter region. (B) Transcription factor binding motifs for KLF2 (MA1515.1), Sp1 (MA0079.1) and E2F1 (MA0024.1) were obtained from the JASPAR database (2019). (C) Genomic sequence of the DHFR gene (chr5:79,950,253-79,950,802) taken from the NCBI database (NG_023304.1). TSS (italic, grey background), initiation codon (bold) (both taken from Chen et al. [595]) and validated transcription factor binding sites for Sp1 (red) and E2F1 (blue) indicated in sequence [556, 596, 597]. Putative KLF2 binding sites are underlined (overlapping with Sp1 sites). TSS, transcription start site.



Supplementary Figure 13. siRNA depletion of MTHFD2 and SHMT2 is not compensated by regulation of cytosolic or mitochondrial counterparts in EC. Gene expression analyses of HUVEC transfected with sictrl or siRNA against MTHDF2 or SHMT2 for 48h by qPCR (n=3-4). Gene expression normalised to GAPDH and shown relative to sictrl. Data were analysed using a one-way ANOVA and Sidak test for multiple comparisons. Values represent means $\pm$ SEM. ns, not significant.



Supplementary Figure 14. siRNA depletion of NRP1 in EC under static conditions. (A+B) HUVEC were transfected with siNRP1 or siRNA against NRP1 for 72h under static conditions. The cells were live stained with TMRM and Mitotracker and imaged on a Zeiss LSM Confocal 780 using a 40x objective. (A) Representative confocal images of respectively treated cells. Image brightness adjusted for presentation. Scale bar 100µm. (n=3). (B) Quantification of mitochondrial membrane potential as integrated signal density of TMRM/Mitotracker stains divided by the cell count. Data shown as fold change over siNRP1. Data were analysed using ImageJ. Values represent means $\pm$ SEM. Unpaired T-test. ns, not significant.



Supplementary Figure 15. Transcript levels of molecules relevant in adenosine metabolism in HUVEC exposed to shear stress using a published RNA sequencing dataset. Data obtained from publicly available RNA sequencing dataset (Gene Expression Omnibus (GEO) GSE103672) published by Ajami et al. [132]. Transcripts per million (TPM) of selected transporters and enzymes related to one carbon metabolism in HUVEC subjected to pulsatile (PS, $12 \pm 5 \text{ dyn/cm}^2$) or oscillatory shear stress (OSS, $0.5 \pm 5 \text{ dyn/cm}^2$) using a parallel plate model over a period of 24h. Data extraction from RNA sequencing dataset kindly supported by Dr. Robert Maughan.