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**Investigating protein and gene biomarker expression,
specifically chemokine (C-X-C motif) ligand 16 (CXCL16),
associated with cardiovascular diseases using a combination
of *in vitro* and *in vivo* techniques**

Jillian Mary Gahan



A thesis submitted to the University of Dublin for the degree of Doctor of Philosophy

Department of Clinical Medicine

University of Dublin

Trinity College – January 2014

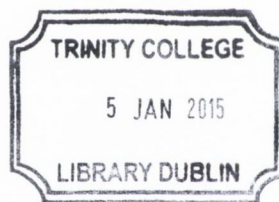
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Julian Gale

Summary

A patient based study was designed to analyze serum concentrations of 10 known inflammatory markers, GM-CSF, IFN- γ , IL-10, IL-12p70, IL-1 β , IL-2, IL-6, IL-8, TNF- α and CXCL16 in asymptomatic and symptomatic pre-operative atherosclerotic patients and healthy controls. This was carried out using the electrochemiluminescence and enzyme-linked immunosorbent assays. The inflammatory markers under investigation have all been associated with CVDs, either a pro-inflammatory, anti-inflammatory or both. Comparing first time pre-operational carotid atherosclerotic patients against healthy controls, five out of the ten cytokines/chemokines mentioned above had significantly different serum levels; IL-10, IL-8, IL-6, TNF- α and CXCL16. Patients were divided into sub-groups; age, statin therapy, risk factor presence, % stenosis and asymptomatic or symptomatics to investigate for significant association. Significant differences were found between asymptomatics and symptomatics, smokers, non-smokers and ex smokers, % stenosis and over and under 65 yo. It was then decided to further investigate the relationship between TNF- α and CXCL16 as both cytokines appeared to have an important impact in groups analysed.

As TNF- α and CXCL16 expression differed between patients and controls, both significantly (CXCL16 was higher in controls), it was deemed appropriate to investigate their association in protein expression studies in endothelial cells, the site of plaque development. Using a novel biochip, which was compatible with confocal laser microscopy, and a pulsatile pump, endothelial cells (EC) were subjected to a number of different conditions such as static v pulsatile, stimulated v unstimulated and time dependent in order to elucidate the expression patterns of the receptor form of CXCL16. As ICAM-1 is expressed in normal, disturbed, unstimulated and TNF- α stimulated environments, it was decided to use ICAM-1 as a positive control. The main findings from this set of experiments concluded that normal pulsatile flow of 10 dyne/cm², increases but does not appear to induce ICAM-1 expression

yet it initiates CXCL16 expression in ECs. TNF- α also increases ICAM-1 expression and mildly induces CXCL16 expression. Combining both stimulants showed ICAM-1 increase drastically in intensity and over 24 h expression was sustained. However, ECs stimulated with both TNF- α and pulsatile flow did not sustain CXCL16 over 24 h and its expression appeared to be exhausted. OxPAPC was also investigated as a stimulant for ECs. It had no effect on ICAM-1 or CXCL16. Normal laminar flow up-regulating ICAM-1 and inducing CXCL16 may pose a threat to normal endothelial function, because together they bind monocytes and oxLDL which infiltrate the endothelial barrier and build up in the *intima* depending on the amount of receptors expressed. If pro-inflammatory cytokines are a presence already, specifically in individuals who possess atherosclerotic risk factors, they too will heighten the inflammatory response and contribute to plaque development or rupture. A third aim was to investigate the CXCL16 gene for evidence of allelic expression imbalance. Unstable angina pectoris and post myocardial infarct patients and lymphoblastoid cell lines heterozygous for the gene were tested. This was also done for IL-18 as it has been identified as a CXCL16 inducer. Clinical samples revealed imbalance in both CXCL16 and IL-18, but inactivated cell lines only showed positive AEI for IL-18. Because TNF- α has shown significant findings in the previous two studies, it was used to stimulate cell lines to investigate its potential at causing allelic imbalance in CXCL16 gene. In cell lines stimulated with 100 ng/ μ l TNF- α 50% showed positive imbalance.

The work that was carried out in these studies involves many methods and technologies to incorporate *in vivo* and *in vitro* work on gene and protein expression to contribute to atherosclerotic research and to ultimately develop therapeutics and diagnostics for the prevention and treatment of this complex disease.

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Firstly I would like to thank my supervisors Dr Ross Murphy for providing me with the opportunity to undertake this research for the last three years and not doubting my ability in the beginning, and in particular Dr Anthony Ryan who never got annoyed with me, especially towards the end when I wasn't the easiest to communicate with. I am extremely grateful that not once throughout the three years neither of them made me feel not worthy of being there and no question was too silly. I would also like to thank the Royal City of Dublin Hospital Trust who have supported this project from the beginning.

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A special thanks to Karl, if I didn't have you to talk to almost every day it would have been tougher, I'm pretty sure you had a lot to put up with. You were great support and encouragement.

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grateful and appreciate everything, maybe I'll show you by moving out! My thesis is dedicated to you.

Statement of Work

This work was undertaken by the author with the Cardiology research group, Department of Clinical Medicine, Institute of Molecular Medicine, Trinity College Dublin and St James' Hospital.

LIST OF ABBREVIATIONS

ACS	Acute coronary syndrome
AEI	Allelic expression imbalance
ApoE	Apolipoprotein E
CAD	Coronary artery disease
cDNA	Complementary DNA
CRP	C-reactive protein
Ct	Threshold amplification cycle
CVD	Cardiovascular disease
ddNTP	Dideoxy nucleoside triphosphates
EC	Endothelial cell
ECL	Electrochemoluminescence
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ESS	Endothelial shear stress
GM-CSF	Granulocyte macrophage- colony stimulating factor
HDL	High density lipoprotein
HPAEC	Human pulmonary artery endothelial cells
HUVEC	Human umbilical vein endothelial cell
ICAM	Intracellular cell adhesion molecule
IFN	Interferon
IHF	Irish heart foundation
IL	Interleukin
LD	Linkage disequilibrium
LDL	Low-density lipoprotein
LXR	Liver X receptor
MCP-1	Monocyte Chemoattractant protein-1
MI	Myocardial Infarction
MM-LDL	Minimally-modified low density lipoprotein

MMP	Matrix metalloproteinase
MSD	Mesoscale discovery
NF-κβ	Nuclear factor - κβ
NK	Natural killer
OxLDL	Oxidised low density lipoprotein
OxPL	Oxidised phospholipids
PAPC	1-palmitoyl-2-arachidonoyl-sn-3-glycero-phosphorylcholine
PCR	Polymerase chain reaction
PECAM	Platelet endothelial cell adhesion molecule-1
PEIPC	palmitoyl-2-(5,6-epoxyisoprostane E2)-snglycero- 3-phsphocholine
PEIPC	palmitoyl-2-(5,6-epoxyisoprostane E2)-snglycero- 3-phsphocholine
PGPC	1-palmitoyl-2-glutaroyl-sn-glycerophosphatidylcholine
RA	Rheumatoid arthritis
RFLP	Restriction fragment length polymorphisms
ROS	Reactive oxygen species
SAP	Stable angina pectoris
SLE	Systemic lupus erythematosus
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
SREBP	Sterol regulatory endothelial binding protein
SS	Shear stress
TF	Transcription factor
TNF-α	Tumour necrosis factor
UAP	Unstable angina pectoris
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VNTR	Variable number tandem repeats
WHO	World Health organisation

TABLE OF CONTENTS

CHAPTER 1. GENERAL INTRODUCTION	20
1.1 CARDIOVASCULAR DISEASE	21
1.2 ATHEROSCLEROSIS	22
1.3 RISK FACTORS ASSOCIATED WITH ATHEROSCLEROSIS	23
1.4 STAGES OF ATHEROSCLEROSIS	24
1.4.1 The endothelium monolayer	24
1.4.2 Fatty streak and foam cell formation	26
1.4.2.1 Cellular composition of atherosclerotic plaques	27
1.4.2.2 EC and SMC communication	27
1.4.3 Plaque disruption and rupture	27
1.5 HEMODYNAMIC FORCES AND ARTERY ARCHITECTURE	29
1.6 SHEAR STRESS	30
1.6.1 Shear gradients	31
1.6.2 Types of shear stress and flow	31
1.6.2.1 Laminar flow	31
1.6.2.2 Disturbed flow	32
1.6.2.3 Oscillatory ESS	32
1.6.2.4 Pulsatile / Pulsatile ESS	32
1.6.2.5 Steady shear stress	33
1.6.2.6 Low SS	33
1.7 MECHANOTRANSDUCTION	35
1.8 INFLAMMATION AND ATHEROSCLEROSIS	37
1.9 BIOMARKERS AND TRIGGERS OF ATHEROSCLEROSIS	38
1.9.1 Anti-inflammatory cytokines	39
1.9.1.1 Interleukin 10 (IL-10)	39
1.9.2 Predominately Pro-inflammatory cytokines	40
1.9.2.1 Tumour Necrosis Factor alpha (TNF- α)	40
1.9.2.2 Interferon gamma (IFN- γ)	40
1.9.2.3 Interleukin 6 (IL-6)	41
1.9.2.4 Interleukin 2 (IL-2)	41
1.9.2.5 Granulocyte Macrophage Colony Stimulating Factor (GMCSF)	42
1.9.2.6 Interleukin 1 beta (IL-1 β)	42
1.9.2.7 Interleukin 12p70 (IL-12p70)	42
1.9.2.8 Interleukin 18 (IL-18)	43
1.9.3 The network of serum cytokines in this study	44
1.9.4 Inflammatory mediator targets	45
1.9.5 Anti-inflammatory therapeutics	45
1.9.6 Scavenger receptors in atherosclerosis	46
	10

1.9.7 Chemokines	46
1.9.7.1 Interleukin 8 (IL-8)	47
1.9.7.2 CXCL16	47
1.9.8 Adhesion molecules	50
1.9.8.1 ICAM-1	51
1.9.9 Lipid accumulation and oxidation	52
1.9.9.1 OxPAPC	53
1.10 STATINS- EXISTING TREATMENT FOR ATHEROSCLEROSIS	56
1.11 OTHER UP AND COMING THERAPEUTICS	58
1.11.1 Endothelial therapeutics	58
1.11.2 NAD(P)H oxidases	58
1.11.3 Cell based therapeutics	58
1.12 EVOLUTION AND CONTRIBUTION OF IN VITRO MODELS IN RESEARCH	59
1.12.1 EC-SMC co-culture models	62
1.12.2 Flow models and Drug delivery	63
1.13 GENOME-WIDE ASSOCIATION STUDIES (GWAS)	63
1.14 GENETIC VARIATION	64
1.15 SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS)	64
1.16 LINKAGE DISEQUILIBRIUM (LD)	65
1.17 HYPOTHESES AND OBJECTIVES	65
CHAPTER 2 HIGH SENSITIVITY CYTOKINE DETECTION IN SERUM OF CAROTID ATHEROSCLEROTIC PATIENTS	67
2.1 INTRODUCTION	68
2.1.1 Pro and anti-inflammatory markers analysed in this study	68
2.1.1.1 Stable angina pectoris (SAP) and Unstable angina pectoris (UAP)	68
2.1.1.2 Myocardial Infarction (MI)	69
2.1.1.3 Carotid endarterectomy	70
2.1.1.4 Mouse models	70
2.1.2 Cytokine detection	71
2.1.3 Hypotheses & Objectives	72
2.2 MATERIALS & METHODS	74
2.2.1 Patient recruitment	74
2.2.2 Blood sampling and PBL isolation	75
2.2.3 Enzyme-linked immunosorbent assay (ELISA)	75
2.2.4 Electrochemilluminescence	77
	11

2.3 RESULTS	81
2.3.1 Electrochemoluminescence	81
2.3.1.1 Carotid atherosclerotic patients' v healthy control comparison of serum cytokine levels	81
2.3.1.2 Comparing serum cytokine levels of carotid atherosclerotic patients' <65 and >65 years of age	84
2.3.1.3 Comparing serum cytokine levels of asymptomatic and symptomatic carotid atherosclerotic patients'	84
2.3.1.4 Comparing serum cytokine levels of carotid atherosclerotic patients' with one or two risk factors associated with atherosclerosis.	86
2.3.1.5 Comparing serum cytokine levels of carotid atherosclerotic patients' with and without hypertension.	87
2.3.1.6 Comparing serum cytokine levels of carotid atherosclerotic patients', who smoke, used to smoke and have never smoked.	87
2.3.1.7 Comparing serum cytokine levels of carotid atherosclerotic patients' who have more or less than 80% stenosis in one or both carotid arteries	88
2.3.2 Enzyme-linked immunosorbent assay	91
2.3.2.1 Carotid atherosclerotic patients' v healthy control comparison of serum CXCL16 levels	91
2.3.2.2 Carotid atherosclerotic patients' <65 and >65 years of age serum CXCL16 levels	92
2.3.2.3 Asymptomatic v symptomatic carotid atherosclerotic patients' serum CXCL16 levels	93
2.3.2.4 Serum CXCL16 levels in carotid atherosclerotic patients' with one and two or more risk factors associated with atherosclerosis.	94
2.3.2.4 Serum CXCL16 levels in carotid atherosclerotic patients' with above or below 80% stenosis.	95
2.4 DISCUSSION	96
2.4.1 ECL	97
2.4.2 ELISA	101
2.4.3 Concluding remarks	102
2.4.4 Future work	103
2.4.5 Limitations	103
CHAPTER 3 OPTIMIZATION OF A NOVEL MICROFLUIDIC BIOCHIP FOR PROTEIN EXPRESSION ANALYSIS IN ENDOTHELIAL CELLS UNDER PULSATILE SHEAR STRESS	104
3.1 INTRODUCTION	105
3.1.1 Primary triggers	105
3.1.2 Secondary triggers	107
3.1.3 Hypotheses & Objectives	109
3.2 MATERIALS AND METHODS	111
3.2.1 Thawing HUVECs for Cell culture	111
3.2.2 Subculturing cells	111
3.2.3 Cell counting	111
3.2.4 Optimization of Vena8 Fluoro+™ Biochip: For cell receptor studies under conditions mimicking physiological flow.	112
3.2.5 Optimization of iKima™ Pump with Apple Application for iPod Touch / iPhone	115
3.2.6 Stimulation of biochips with oxPAPC and TNF- α	118
	12

3.2.7 Experimental design	119
3.2.8 ELISA	120
3.2.9 Lysing cells for RT-PCR	120
3.2.10 RNA quantification using spectrophotometry	120
3.2.11 Fixing cells	120
3.2.12 Immunofluorescent staining	121
3.2.13 Confocal Laser Scanning Microscopy (CLSM)	121
3.2.14 Image processing analysis	121
3.2.15 Immunofluorescence quantification in endothelial cell cultures	123
3.2.16 Statistical analysis	124
 3.3 RESULTS	 125
3.3.1 Enzyme-linked immunosorbent assay (ELISA)	125
3.3.2 Real-time PCR (RT-PCR)	125
3.3.3 Optimization of Vena8 Fluoro+™ Biochip	125
3.3.3.1 The effect of pulsatile shear stress v static on EC receptor expression	125
3.3.3.2 The effect of TNF- α and oxPAPC stimulation on EC receptor expression	134
3.3.3.4 The effect of time on EC receptor expression sustainability	143
 3.4 DISCUSSION	 150
3.4.1 Primary triggers on EC receptor expression	150
3.4.2 Secondary triggers oxPAPC and TNF- α on EC receptor expression	151
3.4.3 Sustainability of EC receptor expression under primary and secondary triggers	153
3.4.4 Concluding remarks	154
3.4.2 Limitations	155
3.4.1 Future work	156
 CHAPTER 4 INVESTIGATING ALLELIC EXPRESSION IMBALANCE <i>IN VIVO</i> AND <i>IN VITRO</i>	 157
 4.1 INTRODUCTION	 158
4.1.1 Allelic expression imbalance (AEI)	158
4.1.3 SNPs in CXCL16 and IL-18	159
4.1.3.1 CXCL16 rs2277680	159
4.1.3.2 IL-18 rs360717	160
4.1.4 Epigenetics	161
4.1.5 Histone acetylation dependent allelic expression imbalance	161
4.1.6 Histone modifications and CVD	162
4.1.7 Hypotheses & Objectives	162
 4.2 MATERIALS AND METHODS	 163
4.2.1 Samples	163
4.2.1.1 Centre d'Etude du Polymorphisme Humain (CEPH)	163
4.2.1.2 Cell lines and Culture	163
4.2.1.3 Patient recruitment for clinical samples	163
4.2.1.4 Blood sampling and PBL isolation	164
4.2.2 TNF- α Treatment of cell lines	164
4.2.2.1 Treatment of cell lines with inhibitor L-Sulforaphane	164
4.2.3 RNA extraction	165

4.2.4 DNA extraction	165
4.2.5 DNA and RNA quantification by spectrophotometry	165
4.2.5 Cdna synthesis	165
4.2.6 Marker SNPs	166
4.2.7 Genotyping clinical samples for CXCL16 and IL-18 SNP and cell lines for IL-18 SNP	166
4.2.7.1 Analysing genotypes of clinical samples and cell lines	167
4.2.8 Allelic expression imbalance	168
4.2.8.1 Allelic real-time quantification	168
4.2.8.2 Analysis of AEI results	169
4.2.8.3 Calculating AEI using threshold amplification cycle data (Ct)	170
 4.3 RESULTS	 171
4.3.1 Genotyping	171
4.3.2 AEI at CXCL16 in ACS clinical samples	172
4.3.3 AEI at CXCL16 in cell lines	173
4.3.4 AEI at CXCL16 in 10 ng/μl TNF-α stimulated cell lines	174
4.3.5 AEI at CXCL16 in 100 ng/μl TNF-α stimulated cell lines	175
4.3.6 AEI at CXCL16 in cell line 07348 treated with L-Sulforaphane	176
4.3.7 AEI at CXCL16 in cell line 12234 treated with L-Sulforaphane	177
4.3.8 AEI at IL-18 in ACS clinical samples	178
4.3.9 AEI at IL-18 in cell lines	179
4.3.10 AEI at IL-18 in 10 ng/μl TNF-α stimulated cell lines	180
4.3.10 AEI at IL-18 in 100 ng/μl TNF-α stimulated cell lines	181
 4.4 DISCUSSION	 182
4.4.1 IL-18	182
4.4.2 CXCL16	184
4.4.3 Concluding remarks	185
4.4.4 Limitations	186
4.4.5 Future directions	186
5.1.1 Concluding remarks	194
 CHAPTER 5 GENERAL INTRODUCTION	 188
 CHAPTER 6 REFERENCES	 196

INDEX OF TABLES

Table 1.1 Types of CVD	21
Table 1.2 Risk Factors associated with atherosclerosis	23
Table 1.3 The area of vasculature and associated dyne/cm ² and intimal thickness	33
Table 1.4 Cone and plate flow apparatus v parallel-plate flow chamber	59
Table 2.1 Characteristics of atherosclerotic patients and healthy controls for ECL	74
Table 2.2 Characteristics of atherosclerotic patients and healthy controls for ELISA	76
Table 2.3 Eight point standard curve used in the enzyme-linked immunosorbent assay	76
Table 2.4 Eight point standard curve used in the multi-array sandwich immunoassay	78
Table 2.5 LLOD is a calculated concentration based on a signal 2.5 standard deviations above the blank (zero) calibrator	79
Table 2.6 Median, standard deviation (SD), p value and Mann-Whitney test of each cytokine based on the comparison of 58 patients and 10 controls involved	82
Table 2.7 Median, standard deviation (SD), p value and Mann-Whitney test of each cytokine based on the comparison of 58 patients divided into < and > 65 years of age	84
Table 2.8 Median, standard deviation (SD), p value and Mann-Whitney test of each cytokine based on the comparison of 58 patients divided into asymptomatics and symptomatics	85
Table 2.9 Median, standard deviation (SD), p value and Mann-Whitney test of each cytokine based on the comparison of 58 patients divided into patients with 1 and 2 or more risk factors	86
Table 2.10 Median, standard deviation (SD), p value and Mann-Whitney test of each cytokine based on the comparison of 58 patients divided into patients' with and without hypertension (HTN)	87
Table 2.11 Median, standard deviation (SD), p value (ANOVA) of each cytokine based on the comparison of 58 patients divided into patients', who smoke, used to smoke and have never smoked	88
Table 2.12 Median, standard deviation (SD), p value and Mann-Whitney test of each cytokine based on the comparison of 58 patients divided into patients' who have more or less than 80% stenosis in one or both carotid arteries	89
Table 2.13 Median, standard deviation (SD), p value and Mann-Whitney test of CXCL16 based on the comparison of 23 patients and 10 controls involved	91
Table 2.14 Median, standard deviation (SD), p value and Mann-Whitney test of CXCL16 based on the comparison of 23 patients divided into < and > 65 years of age	92
Table 2.15 Median, standard deviation (SD), p value and Mann-Whitney test of CXCL16 based on the comparison of 23 patients divided into asymptomatics and symptomatics	93
Table 2.16 Median, standard deviation (SD), p value and Mann-Whitney test of CXCL16 based on the comparison of 23 patients divided into patients with 1 and 2 or more risk factors	94
Table 2.17 Median, standard deviation (SD), p value and Mann-Whitney test of CXCL16 based on the comparison of 23 patients divided into patients with hypertension and without	94

Table 2.18 Median, standard deviation (SD), p value and Mann-Whitney test of CXCL16 based on the comparison of 23 patients divided into patients with < or > 80% stenosis in either internal carotid artery	95
Table 3.1 Technical and specifications of the Vena8 Fluoro+™ Biochip:	114
Table 3.2 Performance specifications of the Vena8 Fluoro+™ Biochip:	114
Table 3.3 Pump specifications of iKima™ Pump:	115
Table 3.4 Controller specifications of iKima™ Pump:	116
Table 3.5 The variables for each experiment are listed below	119
Table 4.1 Clinical characteristics of study groups	164
Table. 4.2 Steps and reagents involved in cDNA synthesis	166
Table 4.3 Location, Minor Allele Frequency and number of heterozygotes for AEI marker SNPs used	166
Table 4.4 Components, volumes and program for SNP genotyping assay for clinical and CEPH samples	167
Table 4.5 Components, volumes and program for AEI assay for clinical and CEPH samples	169

INDEX OF FIGURES

Figure 1.1 The normal endothelium monolayer, <i>intima media</i> and adventitia	25
Figure 1.2 Fatty streak and plaque formation	26
Figure 1.3 Rupture of plaque	28
Figure 1.4 Role of shear stress in atherosclerosis	34
Figure 1.5 The diagram illustrates the effect of shear stress on endothelial sensors	36
Figure 1.6 Mechanical stimuli signal similar to chemical stimulants	37
Figure 1.7 An interconnecting network of cells and cytokines potentially associated with atherosclerosis	39
Figure 1.8 Chromosome 11, location of <i>IL18</i>	43
Figure 1.9 Chromosome 17, location of <i>CXCL16</i>	47
Figure 1.10 Signalling pathways induced by oxPAPC and oxPOVPC. Studies are ongoing for TLR4 involvement	55
Figure 1.11 Parallel-plate flow channel	60
Figure 1.12 A Rectangular Flow Chamber and A Step Flow Chamber	61
Figure 2.1 The three steps involved in MSD technology	71
Figure 2.2 Layout of 96 well plate used in ECL and ELISA	77
Figure 2.3 Cytokine layout of Multiplex plate	78
Figure 2.4 Standard curve plot of MSD results	79
Figure 2.5 Serum concentrations IL-10, IL-6, IL-8 and TNF- α in pg/ml of atherosclerotic patients' v healthy volunteers	83
Figure 2.6 Serum concentrations IL-6, IL-8 in pg/ml of asymptomatic and symptomatic atherosclerotic patients	85
Figure 2.7 Serum concentrations of IL-8 and IL-6 in pg/ml of atherosclerotic patients' who are currently smokers, ex-smokers, never smoked (non-smokers)	88
Figure 2.8 Serum concentrations of GM-CSF, IL-12p70, IL-2, IL-6, IL-8 and TNF- α in pg/ml of atherosclerotic patients' who have stenosis of <80% and >80%	
Figure 2.9 Comparison of cytokine levels in vulnerable groups of carotid endarterectomy patients.	90
Figure 2.10 Serum concentrations of CXCL16 in pg/ml of atherosclerotic patients' v healthy volunteers	92
Figure 2.11 Serum concentrations of CXCL16 in pg/ml of atherosclerotic patients' <65 and >65 years of age	93
Figure 2.12 Serum concentrations of CXCL16 in pg/ml of asymptomatic and symptomatic atherosclerotic patients'	94
Figure 3.1 Counting grid on haemocytometer	112
	16

Figure 3.2 Diagram of Vena8 Fluoro+™ Biochip	113
Figure 3.3 iKima pump App	118
Figure 3.4 The Vena8 Fluoro+™ Biochip and iKima™ Pump	118
Figure 3.5 Flow chart of experimental design	122
Figure 3.6. Luminosity scale of Image and Surface plot of area stained by ICAM-1	123
Figure 3.7 A histogram measuring the area of cells stained using pixel count and pixel luminosity	124
Figure 3.8 ICAM-1 and CXCL16 expression in unstimulated ECs at 6 h under pulsatile and static conditions	127
Figure 3.9 ICAM-1 and CXCL16 expression in unstimulated ECs at 24 h under pulsatile and static conditions	128
Figure 3.10 ICAM-1 and CXCL16 expression in 10 ng/μl TNF-α stimulated ECs at 6 h under pulsatile and static conditions	130
Figure 3.11 ICAM-1 and CXCL16 expression in 10 ng/μl TNF-α stimulated ECs at 24 h under pulsatile and static conditions	131
Figure 3.11.1 Comparison of TNF-α, oxPAPC and unstimulated EC expression of ICAM-1 in a pulsatile and static environment	132
Figure 3.12 ICAM-1 and CXCL16 expression in 50μg/ml oxPAPC stimulated ECs at 24 h under pulsatile and static conditions	133
Figure 3.13 Comparison of ICAM-1 and CXCL16 expression in 10 ng/μl TNF-α stimulated and unstimulated ECs at 6 h under static conditions	135
Figure 3.14 Comparison of ICAM-1 and CXCL16 expression in 10 ng/μl TNF-α stimulated and unstimulated ECs at 24 h under static conditions	136
Figure 3.15 Comparison of ICAM-1 and CXCL16 expression in 10 ng/μl TNF-α stimulated and unstimulated ECs at 6 h under pulsatile conditions	138
Figure 3.16 Comparison of ICAM-1 and CXCL16 expression in 10 ng/μl TNF-α stimulated and unstimulated ECs at 24 h under pulsatile conditions	139
Figure 3.17 Comparison of ICAM-1 and CXCL16 expression in 50μg/ml oxPAPC stimulated and unstimulated ECs at 24 h under static conditions	141
Figure 3.18 Comparison of ICAM-1 and CXCL16 expression in 50μg/ml oxPAPC stimulated and unstimulated ECs at 24 h under pulsatile conditions	142
Figure 3.19 Comparison of ICAM-1 and CXCL16 expression in unstimulated ECs at 6 and 24 h under static conditions	144
Figure 3.20 Comparison of ICAM-1 and CXCL16 expression in 10 ng/μl TNF-α stimulated ECs at 6 and 24 h under static conditions	145
Figure 3.21 Comparison of ICAM-1 and CXCL16 expression in unstimulated ECs at 6 and 24 h under pulsatile conditions	147

Figure 3.22 Comparison of ICAM-1 and CXCL16 expression in 10 ng/μl TNF-α stimulated ECs at 6 and 24 h under pulsatile conditions	149
Figure 4.1 Example of a graph of allelic discrimination for genotyping samples	168
Figure 4.2 Scatterplot of the AEI assay results for the 22 ACS samples for rs2277680	172
Figure 4.3 Scatterplot of the AEI assay results for the 10 lymphoblast cell line samples for rs2277680	173
Figure 4.4 Scatterplot of the AEI assay results for rs2277680 in the 10 lymphoblast cell line samples stimulated by 10ng/μl TNF-α	174
Figure 4.5 Scatterplot of the AEI assay results for the 10 lymphoblast cell line samples for rs2277680 in all 10 cell lines stimulated with 100 ng/μl TNF-α	175
Figure 4.6 Scatterplot of the AEI assay results for rs2277680 in cell line 07348 treated with DMSO, inhibitor only, inhibitor plus 10 ng/μl TNF-α, 10 ng/μl TNF-α only, and a no treatment control	176
Figure 4.7 Scatterplot of the AEI assay results for rs2277680 in cell line 12234 treated with DMSO, inhibitor only, inhibitor plus 10 ng/μl of TNF-α, 10 ng/μl TNF-α only and no treatment control	177
Figure 4.8 Scatterplot of the AEI assay results for the 8 ACS samples for rs360717	178
Figure 4.9 Scatterplot of the AEI assay results for the 9 lymphoblast cell line samples for rs360717	179
Figure 4.10 Scatterplot of the AEI assay results for all 3 lymphoblast cell line samples for rs360717 in all 3 cell lines stimulated with 10 ng/μl of TNF-α	180
Figure 4.11 Scatterplot of the AEI assay results for all 3 lymphoblast cell line samples for rs360717 in all 3 cell lines stimulated with 100 ng/μl of TNF-α	181

CHAPTER 1

General Introduction

1.1 Cardiovascular diseases (CVDs)

Cardiovascular diseases (CVD) affect the cardiovascular system which includes the heart, arteries and veins (Maton 1993) (Table 1.1). Statistics released from the World Health Organisation (WHO) on the global atlas on CVD prevention and control revealed that in 2008 17.3 million people died from CVD (WHO). Their statistics also predict that more than 23 million people will die annually from the disease and that it will be the major global killer within the next fifteen years (Hansson and Libby, 2006, WHO). Classified as a disease of the western world, CVDs in fact, have highest mortality rates across the Middle East and central Africa (WHO). In Ireland, each year an average of 2,000 people die from stroke, more than a combined total of deaths from breast, prostate and bowel cancer (Irish Heart Foundation, IHF).

Table 1.1 Types of CVD (WHO)

Types of CVD	Definition
1. Coronary heart disease	Disease of the hearts blood vessels
2. Stroke	Obstruction of blood supply to the brain (ischemic/haemorrhagic stroke)
3. Congenital heart disease	Holes in the heart, abnormal heart chambers and valves caused by genetic/environmental factors
4. Rheumatic heart disease	Streptococcal bacteria damage the heart muscle & Valves
5. Peripheral arterial disease	Disease of leg and arm arteries
6. Aortic aneurysm	Rupture of aorta
7. Deep venous thrombosis & pulmonary Embolism	Blood clots in leg veins
8. Other CVD	Tumours of the heart, brain, heart muscle & valves
9. Other risk factors on the vascular system	Inflammation , drugs, alcohol, toxins

Apart from mortality rates CVDs also hugely impact global burden. In 2002 coronary artery disease (CAD) and stroke were the second and third highest diseases to impact on the lives of men behind HIV/AIDS while in women they were the third and fourth behind depressive disorders and HIV/AIDS (WHO). An estimated 30,000 people in Ireland are living with disabilities acquired by stroke (IHF).

CAD and stroke are the two most common sub-groups of CVD globally and both are caused by atheroma formation within major arteries. “Atherosclerosis remains the leading cause of morbidity and mortality in the Western world, the causes of atherosclerosis are multifactorial and knowing them could allow earlier prevention and detection of the disease (Shaaban and Duerinckx, 2000)”.

1.2 Atherosclerosis

Atherosclerosis is a chronic progressive disease which is mainly characterized by hyperlipidemia / hypercholesterolemia and fibrous elements in large and medium sized arteries and results in the thickening of the arterial wall (Lusis, 2000 and Libby, 2000). In fact, atherosclerosis is a systemic disease that can affect not only the heart, but also the brain, kidney, aorta and peripheral arteries (Muller et al., 2010). The build up of plaque in arteries begins in adolescence and evolves through the decades. However, not all individuals become symptomatic (Libby, 2006). Obstruction of blood flow through the coronary arteries results in a myocardial infarction, which may be fatal, and obstruction of blood flow to arteries supplying the brain results in stroke. Stroke sufferers experience irreversible loss of brain cells. Ischemic gangrene and abdominal aortic aneurysms are also results of impaired blood flow (Hansson and Libby, 2006 and Sherer and Shonfeld, 2006).

1.3 Risk Factors associated with atherosclerosis

Table 1.2 Risk Factors associated with atherosclerosis (Lusis, 2000)

Genetic & (Genetic variations)	Environmental	Mendelian disorders
• Blood pressure (Angiotensinogen, b2-adrenergic receptor, Alpha-adducin)	• High fat diet	• ApoAI deficiency (apoAI) Tangier disease (ABC1 transporter)
• Type II Diabetes	• Lack of exercise	• Familial hypercholesterolaemia (LDL receptor) • Familial defective apoB-100 (apoB)
• Systemic inflammation (RA)	• Infectious agents	• Haemostasis disorders
• Metabolic syndrome	• Smoking	• Homocystinuria (cystathionine b-synthetase)
• Elevated levels of lipoprotein(a) (Apolipoprotein(a))	• Low anti-oxidant levels	• MODY1 (hepatocyte nuclear factor 4a)5, MODY2 (glucokinase) MODY3 (hepatocyte nuclear factor 1a)
• Gender (male)		• Glucorticoid-remediable aldosteronism • Liddle's syndrome • Mineralocorticoid receptor
• Reduced HDL levels(Hepatic lipase, ApoAI-CIII-AIV cluster, Cholesteryl ester transfer protein, Lipoprotein lipase)		
• Elevated LDL/VLDL (ApoE)		
• Coagulation (Fibrinogen B, Plasminogen activator Promoter polymorphism inhibitor type 1, Factor VIII)		

1.4 Stages of Atherosclerosis

1.4.1 The endothelium monolayer

“The healthy endothelium is a monolayer of cells that is an active paracrine, endocrine and autocrine organ exerting many important homeostatic functions” (Reriani et al., 2010). It is located between blood and tissue (Figure 1.1) and involved in all stages of arterial lesion development. Its initial activation is crucial to atheroma initiation. The endothelium plays a principle role in the regulation and maintenance of vascular tone, cellular adhesion, anticoagulant properties, smooth muscle cell (SMC) proliferation, signal transduction, lipid homeostasis and vessel wall inflammation (Davies 1995, Davignon and Ganz, 2004). In 1977 Ross et al., put forward their response to injury hypothesis which suggested that injury to arterial ECs results in desquamation and lesion development. Sources of injury include; hyperlipidemia, homocystine, metabolites, immunologic injury, infectious agents and mechanical factors. These injuries lead to endothelial disruption and detachment which expose underlying tissue and give access to an excess amount of harmful molecules such as cholesterol and macrophages (Ross et al., 1977).

Activation of the endothelial cell (EC) induces the synthesis and secretion of adhesion molecules, selectins, chemokines and cytokines such as tumour necrosis factor alpha (TNF- α), vascular endothelial growth factor (VEGF), and interleukins (ILs) (Sima et al., 2009).

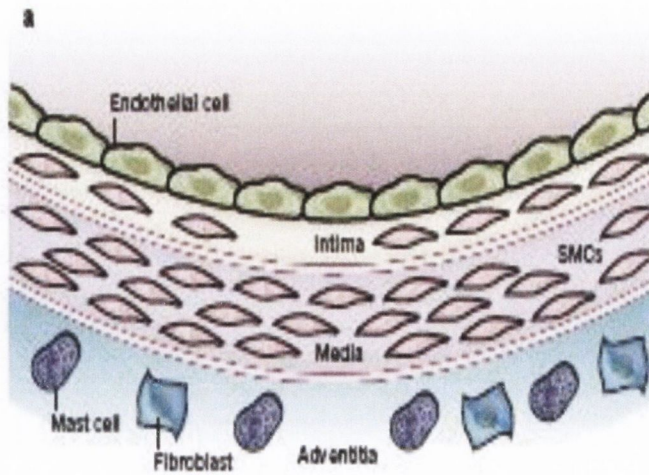


Figure 1.1 The normal endothelium monolayer, *intima media* and adventitia. Atheroma initiation involves all three of these cell layers. The *intima media* is mostly composed of smooth muscle cells and the adventitia contains a mixture of fibroblast and mast cells. (Libby et al., 2011)

Activation of EC dysfunction is firstly seen in increased barrier permeability allowing excess lipoproteins and monocytes to infiltrate the *intima* and inner layers more easily. Reduced efflux of the lipoproteins allows for their subsequent enzymatic modification, oxidation and glycation. There are many enzymatic sources of reactive oxygen species (ROS) in the mammalian cell, including mitochondrial respiration, lipoxygenase, cyclooxygenase, xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and nitric oxide (NO) synthase, the latter three have been studied extensively in CVD (Cai and Harrison, 2000).

Lipid oxidation triggers a number of events including endothelium release of chemotactic factors, mainly monocyte chemoattractant protein 1 (MCP-1), vascular cell adhesion molecule - 1 (VCAM-1) and intracellular adhesion molecule - 1 (ICAM-1), and E/P selectin expression on the vessel wall. This molecule combination encourages leukocyte rolling and infiltration into where monocytes and macrophages rapidly phagocytose the oxidised low density lipoprotein (oxLDL) using receptors such as CXCL16. Unmodified LDL does not contribute to foam cell formation, as it is undetectable to the scavenger receptors SR-A and CD36 of macrophages (Berliner and Watson, 2005).

Fatty streak and foam cell formation

The cholesterol rich macrophages are known as foam cells and over time these cells die and form the necrotic core of the fibrous lesion. This is a precursor to the advanced lesion which is usually formed by the addition of SMCs and SMC extracellular matrix (Figure 1.2) (Lusis, 2000). Cholesterol is a major component in the membranes of mammalian cells. As previously mentioned, an excessive amount leads to atherosclerosis progression. Accumulation of cholesterol can be due to an increase in cholesterol uptake, a non-functioning cholesterol trafficking system and reduced cholesterol efflux (Li et al., 2013).

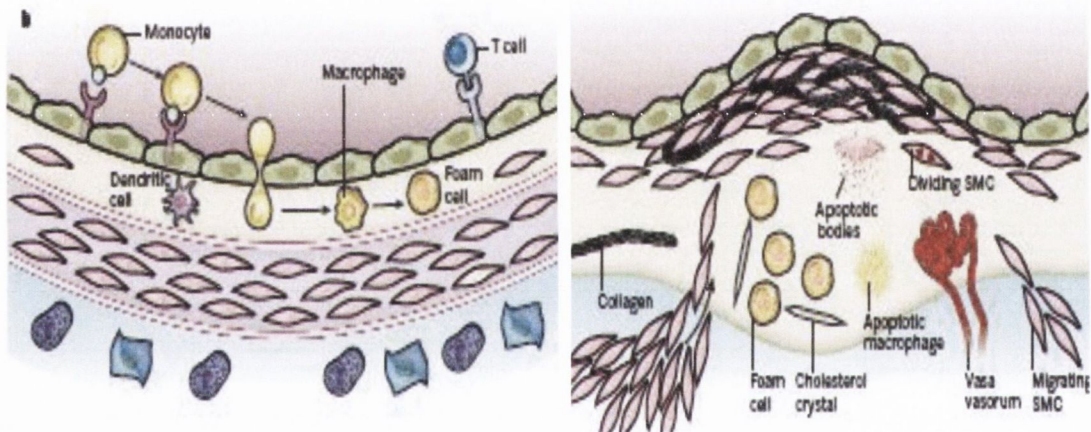


Figure 1.2 Fatty streak and plaque formation (Libby et al., 2011). The diagram to the left illustrates the binding of monocytes to the endothelium adhesion molecules and subsequent infiltration from the blood flow into the arterial wall and foam cell formation. The diagram to the right illustrates the beginning of the atheroma and the different cells involved.

Apolipoprotein E (ApoE) is essential for the sufficient metabolism of triglyceride rich lipoproteins. This apolipoprotein is secreted by macrophages and encourages cholesterol secretion to the High Density Lipoprotein (HDL). Its presence is crucial for the prevention of foam cell formation. Animal knock out studies ApoE ^{-/-} show that without the gene for this protein atherosclerotic plaque progresses more rapidly. In the late 90s studies showed the involvement of CD40 and its ligand CD40L in advanced lesion

development as it is expressed on SMCs, ECs and macrophages. This combination is the trigger for multiple events including cytokine production, adhesion

molecule expression and matrix metalloproteinases (MMP) production (Schönbeck et al., 2000).

1.4.1.1 Cellular composition of atherosclerotic plaques

The composition of atherosclerotic plaques is a crucial factor in determining rupture, thrombosis and clinical events (Randi et al., 2003). The atherosclerotic plaque is composed of a necrotic lipid core (>40%) of esterified cholesterol and cholesterol crystals and dead white blood cells with a monolayer of endothelial cells covering it. The fibrous cap which separates blood from the plaque components cap include matrix molecules such as collagen, elastin, and proteoglycans, derived from SMCs (Shah, 2003). Cap thinning is usually followed by plaque rupture.

1.4.1.2 EC and SMC communication

Myoendothelial bridges connect ECs and SMCs (Spagnoli et al., 1982; Little et al., 1995) and communicate through paracrine pathways (Cunningham and Gotlieb, 2005). Flow models have confirmed the bidirectional cross-talk between the two cell types as the EC monolayer is the only cell exposed to the SS. Depending on flow type, SMCs respond differently to disturbed shear stress by regulating cell proliferation, migration and differentiation contributing to lesion size. Activated, they also increase expression of growth factors and cytokines i.e. MCP-1 and VCAM-1. However, in laminar flow SMCs contribute to an atheroprotective environment (Chiu et al., 2004).

1.4.2 Plaque disruption and rupture

Plaque rupture and thrombus formation are specific events that takes place in coronary atherosclerosis which leads to a myocardial infarct, carotid atherosclerosis however, involves the development of plaque which leads to the thickening of the arterial wall only and prevents

blood flow causing stroke. Both types of atherosclerosis have common risk factors and initial development stages (Figure 1.1-1.2) such as ICAM-1 expression, and low shear stress association, however the extent of atherosclerosis differs depending on the vessel under investigation (femoral, abdominal, coronary or carotid) and also the specific region of the artery (i.e. carotid bifurcation) (Bots et al., 2007).

Studies have shown significant correlation between mean internal media thickness (IMT) and advancing coronary artery disease (CAD). Kablak-Ziembicka et al, concluded that IMT increases with advancing CAD (Kablak-Ziembicka et al., 2004) and Hodis et al found IMT to predict risk of coronary events in a more significant manner than coronary arterial measure of atherosclerosis and lipid measurements (Hodis et al., 1998). A review by Bots et al., discussed the relationship of carotid and coronary disease from the literature published and concluded that multiple lines of evidence support the measurement of IMT to predict cardiovascular events for future treatment options (Bots et al., 2007). This project investigated protein and gene expression of the same molecules in both types of arterial disease to investigate if the same markers were valid.

“Understanding the distribution of mechanical stress in an atherosclerotic plaque is an important prerequisite to understanding plaque rupture. The stresses within the plaque are determined by the complex mechanical properties and variable mixture of plaque components (Lee et al., 1991).”Plaques can be either high-risk vulnerable plaques or stable and low risk.

Events leading to a focal weakening in plaque structure and potential rupture include a necrotic lipid pool, macrophage infiltration, pulsatile and circumferential stresses and various plaque components such as inflammation mediators (Lutgens et al., 2003).

Features of vulnerable plaque include, outward remodelling, large lipid core (>40%), inflammatory cell infiltration of fibrous cap (macrophages > 25 cells per 0.3mm diameter), increased neovascularity and a thin fibrous cap (<65 μ m) of smooth muscle cells and collagen

and potential thrombosis due to contact between platelets and a highly thrombogenic necrotic core (Figure 1.3) (Shah, 2003, Virmani et al., 2006). “They are also characterised by increased circulating ICAM, VCAM, MMP, TNF- α , IL-6, IL-10 and IL-18” (Sima et al., 2009). Physical plaque disruption can be caused by a number of ways. Superficial erosion, micro-haemorrhaging of the microvascular channels due to neoangiogenesis and fracturing of the fibrous cap. Very few studies have focused on endothelial desquamation as an initiator of lesion disruption. Therefore little information is available on the mechanisms involved (Virmani et al., 2006, Hansson 2001, Hansson et al., 2002).

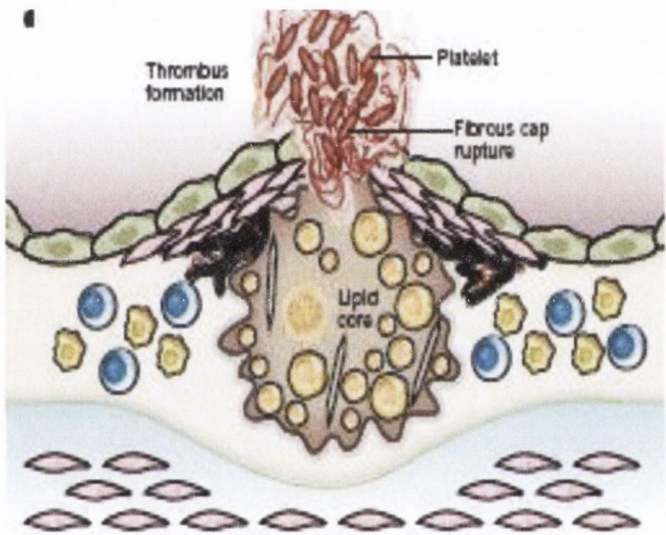


Figure 1.3 Rupture of coronary plaque. This event involves the release of platelets and the necrotic core into the arterial blood flow which results in a myocardial infarct (Libby et al., 2011).

Richardson et al 1989 demonstrated an important role of circumferential stress within a fibrous lesion. An increase in circumferential stress is caused by the fibrous cap decreasing in thickness. Richardson et al research involved investigating the relevance of these stresses to the stresses of atherosclerotic tissue failure. They found that plaques do not usually fracture at circumferential stresses lower than 300kPa. Richardson concluded that there were three factors in explaining the high circumferential stress and fracture association. Firstly an increase in blood pressure is directly proportional to increases in circumferential stress which

may lead to a myocardial infarct. Therefore a chronic rise in blood pressure is inevitably dangerous (Richardson et al., 1989).

As well as circumferential stress, pulsatile pressure causes dynamic stresses which weaken the plaques stable environment. In addition, evidence for stromelysin mRNA has been found in human atherosclerotic plaques; this protease may be one of the macrophage proteases causing plaque weakening (Henney et al., 1991). Dysregulated extracellular matrix (ECM) metabolism may contribute to vascular remodeling during the development and complication of human atherosclerotic lesions. Matrix metalloproteinases (MMPs) are a family of enzymes that degrade ECM components in human atherosclerotic plaques. There are three MMP classes (interstitial collagenase, MMP-1; gelatinases, MMP-2 and MMP-9; and stromelysin, MMP-3). Gelatinases specialized in the digestion of collagen fragments, basement membrane-type collagens, or elastin; interstitial collagenase (MMP-1) which digests fibrillar collagens; and stromelysin (MMP-3), which degrades proteoglycans, fibronectin, laminin, elastin, and can activate the zymogen forms of the other MMPs as well (Galis et al., 1994).

1.5 Hemodynamic forces and artery architecture

The endothelium monolayer being the dividing factor between the vascular wall and blood flow is subjected to fluid forces of greater magnitude than any other tissue (Davies, 1986). The magnitude can be estimated by Poiseuille's law, which states "that shear stress is proportional to blood flow viscosity and inversely proportional to the third power of the internal radius" (Malek et al., 1999). Pulsatile blood flow and pressure produce hemodynamic forces such as cyclic stretch, hydrostatic pressure and fluid shear stress on the EC (Tseng et al., 1995). Hemodynamic parameters such as disturbed and oscillatory flow and low shear stress have been implicated in the initiation and development of cardiovascular diseases (Anssari-Benam et al., 2013). Hemodynamic forces impact regions of curvature such as coronary arteries and bifurcations such as the carotid artery (Back et al., 2013).

1.6 Shear stress

“ Endothelial shear stress (ESS) is a biomechanical force on the endothelial surface that is determined by blood flow, vessel geometry and fluid viscosity that is computationally estimated using fluid dynamics models and is expressed in units of dynes/cm” (Cunningham and Gotlieb., 2005). “It is a product of shear rate and blood viscosity” (Chatzizisis et al., 2007). Shear rate is the change in blood flow from the arterial wall to the centre of the lumen. Blood viscosity is the ability of blood to flow; it measures the internal friction that causes blood to resist flow.

In an equation, shear stress is expressed as $\tau = 4\mu Q/\pi r^2$ where μ is the viscosity, Q is the flow rate and r is the vessel radius. The Reynolds number (Re) measures the stability of flow: $Re = 4\rho Q/\pi\mu D$ (g/cm²) where ρ is blood density, D is vessel diameter, μ is blood viscosity and Q is flow rate. “This represents a ratio of inertial fluid momentum to viscous frictional forces” (Cunningham and Gotlieb., 2005).

Shear stress of ≥ 15 dyne/cm² induces endothelial quiescence and a gene expression profile which is atheroprotective, shear stress of ≤ 4 dyne/cm², stimulates an atherogenic phenotype and is therefore present at sites of atherosclerosis (Malek et al., 1999). Shear stress differs between arteries and veins. In the venous system, shear stress ranges from 1-6 dyne/cm² while the arterial network experiences a greater and higher range 10-70 dyne/cm². However, this range is subject to arterial architecture as a shear stress as low as 0.5 dyne/cm² can occur in areas of curvature and bifurcation (Malek et al., 1999).

1.6.1 Shear gradients

Shear gradients are divided into two categories- Temporal gradients are the change in shear stress that occurs in the same location over a short period of time. A spatial gradient is the difference in shear stress between two points of a cell at the same time. White et al 2001

developed an *in vitro* model that separated these gradients which previously hadn't been achieved. The findings suggested temporal gradients acted as stimulants for EC proliferation and therefore considered significant in atherosclerotic-susceptible regions. Spatial gradients had no greater influence on EC proliferation than steady uniform shear stress (White et al., 2001). Significant stresses are created in the vasculature by the mechanical environment.

1.6.2 Types of shear stress and flow

The type of flow depends on the region of the vasculature; the carotid bulb varies in flow types. High shear occurs in the medial wall of the bulb whilst oscillatory stress occurs on the lateral wall.

1.6.2.1 Laminar flow

It has been suggested that undisturbed laminar flow is atheroprotective and stimulates cellular responses which are vital for endothelial cell function (Traub and Berk, 1998) and a healthy vascular system. Undisturbed laminar flow promotes an anti-inflammatory, antithrombotic, anticoagulative, profibrinolytic and antihypertrophic state (Cunningham and Gotlieb, 2004). This type of blood flow pattern is mainly seen in linear areas of the vasculature and has a high shear stress and rate of average 12 dyne/cm^2 (Yoshizumi et al., 2003).

1.6.2.2 Disturbed flow

Laminar flow transforms into disrupted flow, which is classified as a decrease in shear stress with forward and retrograde flow specifically at regions of curvature such as the carotid artery (Figure 1.4). Shear stress rate is typically $<4 \text{ dynes/cm}^2$. Areas of disturbed flow are associated with temporal and spatial shear gradients and experience recirculation, flow separation and reattachment (Estrada et al., 2011). This alters the homeostatic environment of vascular cells and down-regulates eNOS, endothelial repair and disturbs cellular alignment. Leukocyte adhesion, ROS and lipoprotein permeability (Figure 1.2) are all up-regulated, contributing to the formation of a site of inflammation which starts as a fatty streak,

progresses to an atheroma and finally a complex or vulnerable plaque which may rupture (Cunningham and Gotlieb, 2005).

1.6.2.3 Oscillatory ESS

Oscillatory stress is “periodic flow reversal with time-averaged shear stress approaching zero” that varies with the cardiac cycle and causes recirculation vortexes. Due to significantly low shear stress, cells subjected to oscillatory flow do not orientate perpendicular to direction of flow. This flow pattern occurs at the outer wall of the carotid sinus and has a shear stress range of approx -7 to +4 dynes/cm². Studies have shown correlation between fatty streak and lesion development and oscillatory flow resulting in a pro-atherogenic environment (Ku et al., 1985).

1.6.2.4 Pulsatile / Pulsatile ESS

Due to PSS the vessel wall undergoes a cyclic stress composed of radial and circumferential factors and the EC experiences a hemodynamic shear stress (Imberti et al., 2002). *In vivo* ECs are exposed to pulsatile pressure (normal: 120=80 mm Hg) and cyclic stretch (normal: 6–20%) at approx 80 bpm (Estrada et al., 2011). Depending on the area of vasculature shear stress varies (Table 1.3). Pulsatile flow creates a region of flow separation and reversal at the sinus’s outer wall.

Table 1.3 The area of vasculature and associated dyne/cm² and intimal thickness (Ku et al., 1985)

Area of vasculature	Pulsatile shear stress dyne/cm ² (Mean)	Intimal thickness
Common carotid	7	0.10 ± 0.02 mm to 0.15 ± 0.03 mm
Proximal internal carotid	26	0.14 ± 0.05 mm
Midpoint carotid sinus	17	0.10 ± 0.02 mm to 0.15 ± 0.03 mm
Distal carotid sinus	45	0.06 ± 0.01 mm to 0.09 ± 0.04 mm
External carotid	13.5	0.27 + 0.15 mm to 0.08 ±0.02 mm

1.6.2.5 Steady shear stress

Steady shear stress is a flow pattern which is temporally and spatially uniform and used in many experimental preparations. This usually induces many of the same EC responses as pulsatile shear stress but has qualitative and quantitative differences. Steady shear stress causes cells to reorientate their axis parallel to the direction of blood flow; this decreases the cells resistance, lowering the shear stress (Traub and Berk, 1998).

1.6.2.6 Low SS

Multiple roles of low ESS in atherosclerosis have been suggested. This is why low ESS may be a main causative factor in coronary artery disease. Low ESS promotes LDL permeability, uptake and synthesis, it promotes oxidative stress and the production of ROS which oxidise LDL, and it reduces NO bioavailability which is atheroprotective (Figure 1.4) (Li et al., 2003). Low ESS most importantly induces inflammatory mediator expression and is capable of activating transcription factors such as NF- κ B. When activated NF- κ B upregulates gene expression of adhesion molecules VCAM- and ICAM-1 and chemotactic factors such as MCP-1 and pro-inflammatory molecules TNF- α , IFN- γ and IL-1 (Chiu et al., 2004).

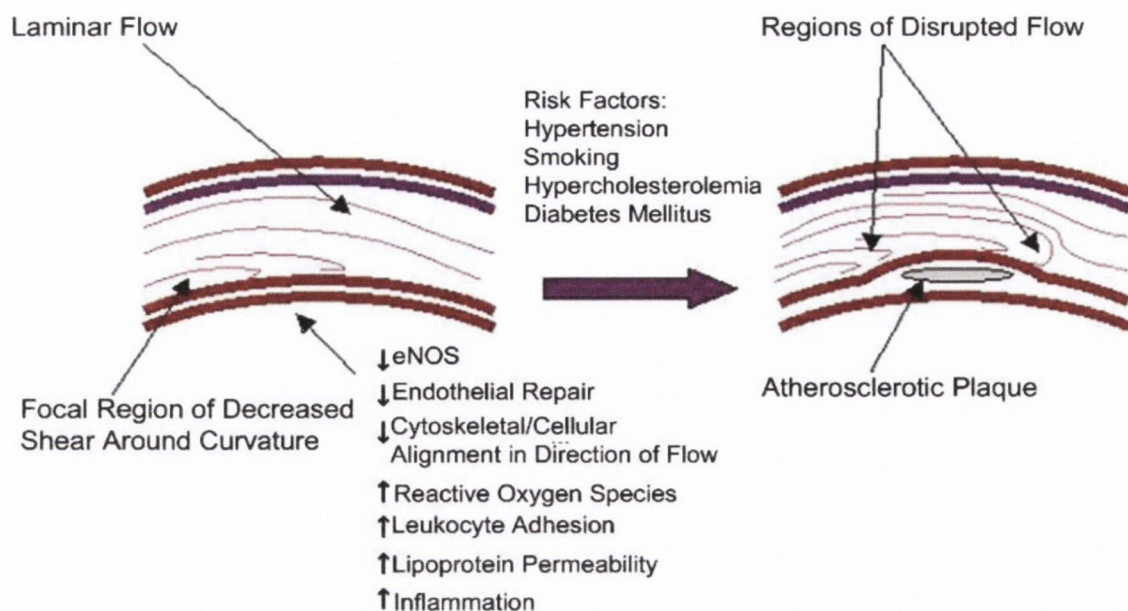


Figure 1.4 Risk factors in combination with an increase in ROS, leukocyte adhesion, lipoprotein permeability, inflammation and a decrease in eNOS, endothelial repair and cytoskeletal/cellular alignment lead to a disruption in laminar flow. Role of shear stress in atherosclerosis (Cunningham and Gotlieb, 2005)

Low ESS promotes ECM degradation in the vascular wall and fibrous cap which are both made of collagen and elastin fibres. Low ESS upregulates MMP gene expression, in particular MMP2/9, these matrix degrading proteins are released from macrophages, VSMCs and endothelial cells upon activation of transcription factors (TFs) which upregulate pro-inflammatory cytokines $\text{TNF-}\alpha$, IL-1, $\text{INF-}\gamma$. Low ESS may play a potential role in plaque calcification and neovascularisation as VEGF is shown to be upregulated (Catzizisis et al., 2007). Sakamoto et al 2006 reported migration of SMCs was decreased by shear stress of 1.5 Pa, however 0.1 Pa shear stress did not suppress migration (Sakamoto et al., 2006).

The most cited factors toward initiation of atherosclerotic plaque in the current literature are abnormalities in shear stress and flow patterns. However, clinical findings suggest that plaque still forms at arterial sites that are associated with steady and high shear stress rates (Anssari-Benam and Korakianitis, 2013). A review of 27 studies on low and oscillatory shear stress revealed in fact that the evidence supporting its part in atherosclerosis was less

robust than assumed (Peiffer et al., 2013).

1.7 Mechanotransduction

Cells sense their physical environment by translating mechanical forces into biochemical signals which results in a mechanosensitive feedback loop known as mechanotransduction. Mechanotransduction regulates cellular functions including differentiation, proliferation, migration, apoptosis and homeostasis. Disturbing this feedback process alters cell signalling events, such as gene and protein expression, and leads to pathophysiological changes, in this case atherosclerosis (Wheeler-Jones 2005).

There are many forms of endothelial responses to fluid shear stress. Mediators of mechanotransduction can be divided into two groups: In cell associated and surface/extra-cellular associated mediators. In cell associated mediators consist of cytoskeleton (Microfilaments, Microtubules, Intermediate filaments) and nuclei (Ion channels, Nuclear lamina, Chromatin, Gene expression). Surface/extra-cellular mechanosensors include membranes (Ion channels, Caveolae, Surface receptors, Lipid bilayer), surface processes (Primary cilium, Stereocilia), cell-cell adhesions (Cadherins, Gap junctions), ECM (Fibronectin, Collagen, Proteoglycans, Basement membrane) and cell-ECM adhesions (Focal adhesions, Integrins) (Figure 1.5) (Davies, 1995 and Takahashi et al., 1997).

Mediators of mechanotransduction activate signalling molecules (Figure 1.6), nitric oxide, cGMP, G-proteins and G-protein coupled receptors, ROS, inositol triphosphate, protein kinases, calcium influx and transcription factors, early growth factor-1, NF- κ B and activator protein-1 within minutes of surface response. After several hours changes can then be identified through cytoskeleton organisation in the direction of flow and cell elongation (Orr et al., 2006). Signalling also results in growth arrest/proliferation, inflammatory/anti-inflammatory gene activation via the shear stress response elements located on the promoter

regions (Chien 2007).

According to experimental results, laminar flow is capable of mechanoreceptor activation and transduction however cells adapt to the environment and begin to down regulate pathways. Disturbed flow was able to sustain activation of NF- κ B and tissue factor promoting inflammation and thrombosis (Orr et al., 2006).

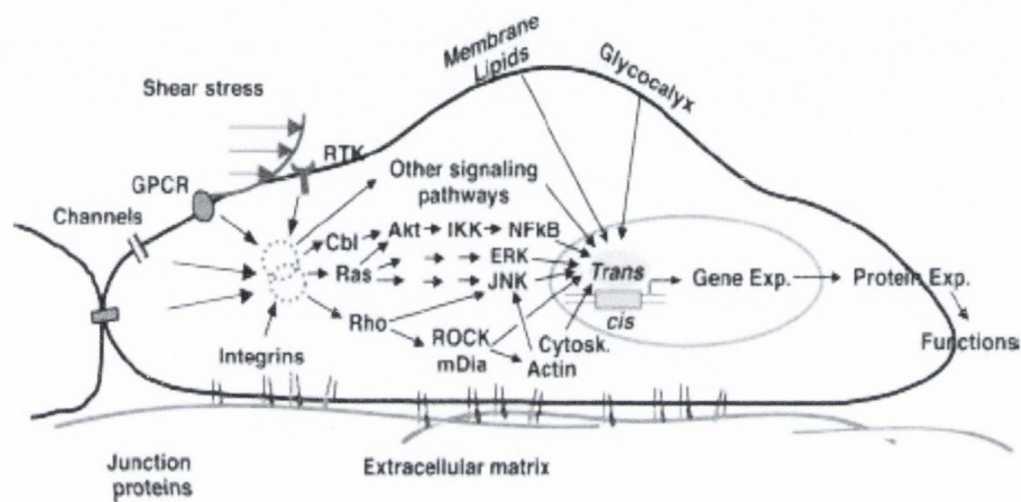


Figure 1.5 The diagram illustrates the effect of shear stress on endothelial sensors (Chien 2007). A number of surface and subendothelial activation pathways activate excessive protein expression which encourages atheroma formation.

Mechanotransduction in ECs has been studied with both *in vivo* and *in vitro* approaches. Many *in vitro* models have been designed to study this process as they have the advantage of controlling experimental variables. However, *in vivo* studies are important as follow up studies to verify and determine applicability of *in vitro* results.

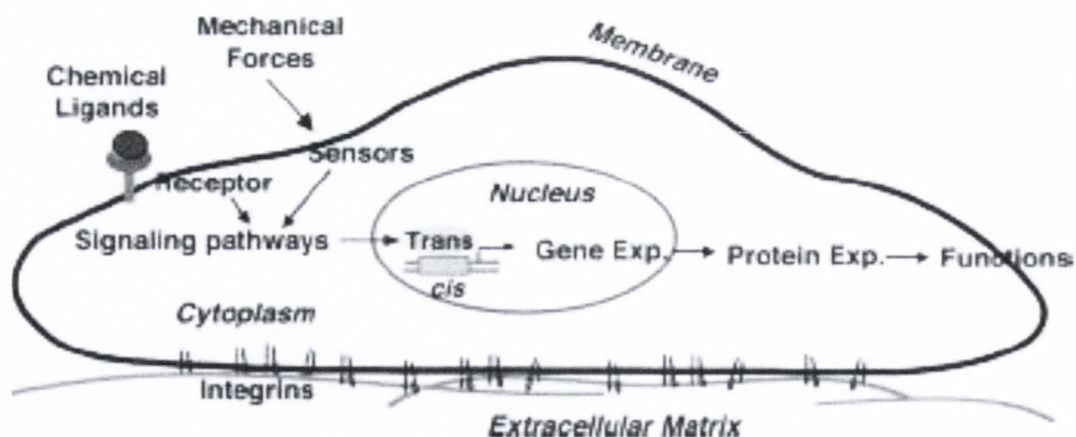


Figure 1.6 Mechanical stimuli signal similar to chemical stimulants (Chien 2007) and both types of stimulants, in a disturbed environment, produce a greater potential environment for atheroma development.

1.8 Inflammation and atherosclerosis

In the mid 19th century Virchow established the importance of inflammation in atherogenesis and in 1977 Ross et al, put forward their response to injury hypothesis. Inflammation is regulated by the presence of immune cells; vascular cells, lymphocytes, monocytes, macrophages, and mast cells, which may be generally classified as pro or anti-inflammatory and secrete over 50 types of cytokines. These inflammatory mediators co-ordinate the innate and adaptive immune response in atherosclerosis (McLaren and Ramji, 2009).

They can be split into two categories, class I and II, based on the structural homology of their receptors. Interleukins, colony stimulating factors and Interferons signal via the Janus kinase. However, cytokine families IL-1 (IL-1 α , IL-1 β , IL-1 α R, and IL-18) and TNF- α activate nuclear factor- κ B (NF- κ B) and mitogen-activated protein (MAP) kinase (Tedgui and Mallat, 2006). Cytokines are not the primary trigger in atherosclerosis; they are released by cells as an injury response at sites of inflammation. So far, inflammatory mediators that have been used as a useful tool to detect early to thrombotic atherogenic events include CRP, IL-18, IL-6 (Lamon and Hajjar, 2008).

1.9 Biomarkers and triggers of atherosclerosis

As atherosclerosis is a complex inflammatory disease, there are many influential biomarkers which contribute to the disease's progression as well as biomarkers from atherosclerosis related diseases such as periodontal disease and autoimmune diseases i.e. Diabetes, RA, SLE. Biomarkers can be proteins, DNA and mRNA which are measured to assess biological, pathological processes and pharmacological responses. Biomarkers can be classified as early, predictive and prognostic biomarkers depending on disease stage (Huang et al., 2010).

Identifying biomarkers for disease and finding therapeutic targets is crucial to research and treatment and overall a populations mortality and morbidity rate (Uno and Nicholls, 2010). A good approach in investigating biomarkers in a progressive disease such as atherosclerosis that may take decades to show symptoms would be to identify separate markers associated with the different stages of the disease. However ongoing research has particularly focused on some of the following pro and anti-inflammatory mediators. Atherosclerotic associated cells as discussed earlier: ECs, SMCs, and macrophages are all producers of important biomarkers (Figure 1.7).

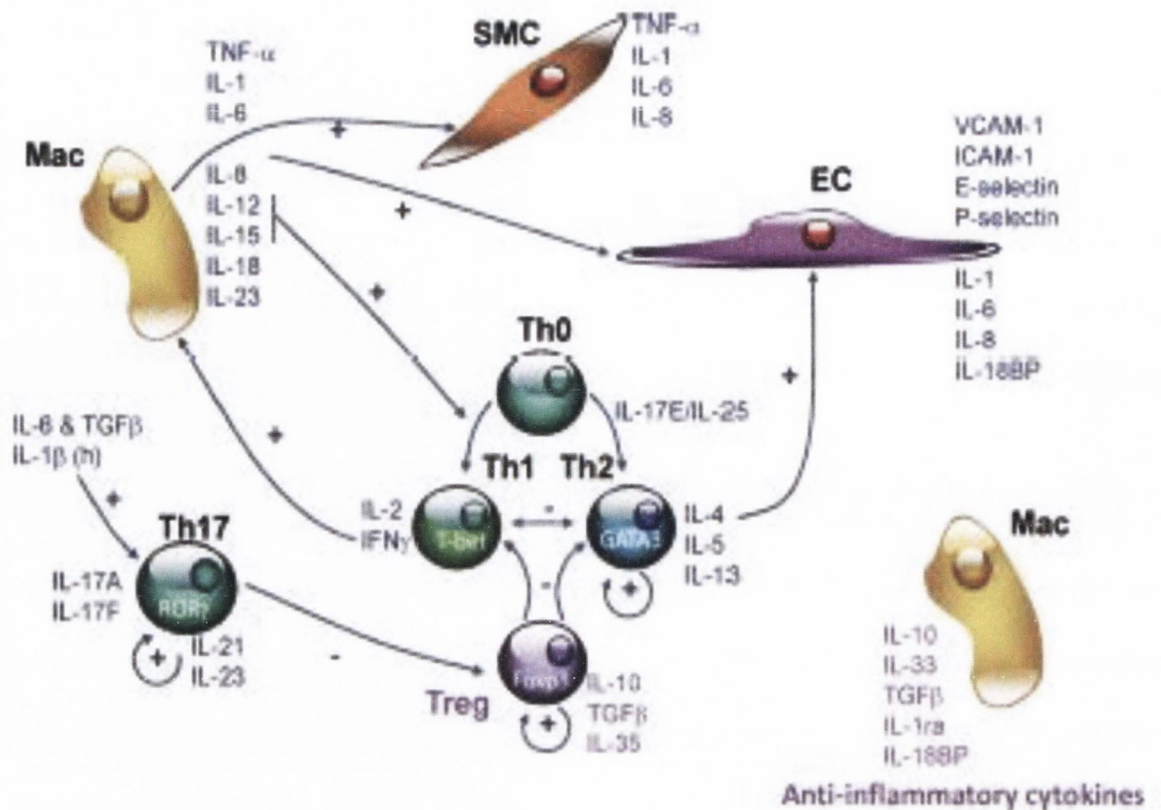


Figure 1.7 An interconnecting network of cells produce cytokines potentially associated with atherosclerosis (Ait-Oufella et al., 2011). The diagram above illustrates the various cytokines associated with the different types of cells involved such as macrophages, smooth muscle cells, endothelial cells and T cells. Each cell produces a specific set of cytokines.

1.9.1 Anti-inflammatory cytokines

1.9.1.1 Interleukin 10 (IL-10)

IL-10 is classified as a pleiotropic anti-inflammatory lymphokine that suppresses expression of IFN- γ , TNF- α , GM-CSF and proliferation of T cells. For the immune system to be fully functional there must be a balance of pro and anti-inflammatory molecules in systemic circulation. IL-10 deficiency has been reported in numerous disease associated with inflammation including RA and SLE (Pyo et al., 2003) and UAS, patients demonstrate an imbalance of TNF- α and IL-10 levels in serum (Waehe et al., 2002). IL-10 deficiency in atherosclerosis leads to an increase in fatty streak formation and proteolytic and coagulant activity (Caligiuri et al., 2003) as it inhibits

Th1 cytokine production (Fiorentino et al., 1991), reduces CC chemokine production that would result in leukocyte homing and downregulates ICAM-1. It also contributes to prevention of plaque destabilization by reducing MMP production. However, like other cytokines involved in atherosclerosis, serum levels of IL-10 in patients vary between studies and disease type (Stenvinkel et al., 2005). IL-10 plays a cross-regulatory role with IL-12, and inhibits its oxLDL induced release (Uyemura et al., 1996).

1.9.2 Predominately Pro-inflammatory cytokines

1.9.2.1 Tumour Necrosis Factor alpha (TNF- α)

TNF- α is a pleiotrophic pro-inflammatory cytokine and it is mainly exported by monocytes and macrophages. TNF signals through the TNF receptor (TNFR) (Fragoso et al., 2013), TNFR is found on endothelial cells and ultimately activates NF- κ B which is responsible for cell survival, proliferation, inflammation and immune regulation. Many inflammatory marker genes consist of an NF- κ B promoter region, therefore if protein expression is regulated by TNF- α it may be considered as a pro-inflammatory marker. It is involved of all stages of atheroma formation: initiation, development, susceptibility, severity and response to treatment (Fragoso et al., 2013). TNF- α is involved in the production of chemokines (IL-6, CRP) and cytokines, expression of adhesion molecules (ICAM-1, VCAM-1), recruitment of leukocytes, induction of smooth muscle cell proliferation and lipid metabolism (decreases HDL activity) (Bruunsgaard et al., 2000). TNF- α concentration is 200 times higher in intimal thickenings of atherosclerotic aortas than in corresponding serum samples (Rus and Vlaicu, 1991). A study carried out on patients suffering from RA and AS treated with anti-TNF- α for 1 year had a significant reduction in aortic stiffness (Angel et al., 2012).

1.9.2.2 Interferon gamma (IFN- γ)

IFN- γ is the only type II interferon, type I interferons include α , β and δ . Cells which produce

IFN- γ include activated T lymphocytes (CD4⁺ Th1 cells), natural killer cells, monocytes/macrophages, dendritic cells and B cells. Cytokines IL-12 and IL-18 released by antigen presenting cells activate IFN- γ secretion from these cells. IFN- γ has multiple pro-atherogenic roles. IFN- γ signalling activates immune cells such as T-cells, macrophages and NK cells, class I and II major histocompatibility complex (MHC) molecules, cytokine production and increased expression of adhesion molecules and chemokines at site of lesion. (Harvey and Ramji, 2005, Tenger et al., 2005). IFN- γ is known as a pro-inflammatory cytokine but is likely to play both pro and anti-inflammatory role in atherosclerosis (Muhl and Pfeilschifter, 2003). IFN- γ is involved in multiple stages of atherosclerosis as a pro-inflammatory cytokine: foam cell formation, immune response and plaque development (McLaren et al., 2009). Therapeutics focusing only on this cytokine have vast potential in prevention of atheroma development, a study demonstrating IFN- γ neutralisation by a plasmid encoding soluble IFN- γ R reduced lesion advances in mouse models (Gotsman and Lictman, 2007).

1.9.2.3 Interleukin 6 (IL-6)

IL-6 is a glycoprotein produced by monocytes/macrophages, adipose tissue and endothelial cells. IL-6 stimulates SMC proliferation, MCP-1 secretion from macrophages and ICAM-1 expression on ECs. Elevated IL-6 plasma concentrations have been associated with morbidity and mortality rates in unstable angina (Koenig and Khuseyinova, 2007). In carotid artery and coronary artery patients, IL-6 had significantly higher expression levels than controls and correlated with the lumen diameter of the common carotid and was considered a useful biomarker (Larsson et al., 2005). IL-6 and IL-8 have shown significantly higher levels in fibrous plaque than the normal arterial wall (Rus et al., 1996).

1.9.2.4 Interleukin 2 (IL-2)

IL-2 is a known angiogenic factor which has multiple inducers, including IL-4, IL-6, TNF- α

and itself. It has been shown to be increased in coronary artery disease (CAD) and stable angina (SA) patients but not acute coronary syndrome (ACS) (Ozeren et al., 2003). In a study on carotid endarterectomies by Frostegard et al., the imbalance of pro and anti-inflammatory cytokines were significantly different, IL-2 and IFN- γ were present in 30-50% of plaques (Frostegard et al., 1999).

1.9.2.5 Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)

GM-CSF is a cytokine responsible for the differentiation of progenitor cells into mature granulocytes and macrophages and the proliferation of DCs (Alberts-Grill et al., 2013). It is a key mediator in the response to injury phase of inflammation initiation. Protein and mRNA studies have confirmed the presence of GM-CSF in non-diseased arteries and its up-regulation in atherosclerotic human coronary arteries (Plenz et al., 1997). The expression of GM-CSF in diseased arteries can be due to the excessive amount of oxLDL, which induces cytokine release by endothelial cells. The ability of GM-CSF to regulate granulocytes and macrophages contributes to plaque progression (Shaposhnik et al., 2007).

1.9.2.6 Interleukin 1 beta (IL-1 β)

Of the IL-1 family IL-1 β is the most predominant isoform circulating in humans. Vascular cells can both produce and be targets of IL-1 β signalling. IL-1 β signals through the MAPK pathway by binding to IL-1RI (receptor) and activates TFs and NF- κ B which results in pro-inflammatory gene expression (Chamberlain et al., 2006). In atherosclerosis, IL-1 β is considered a significant contributor in all stages of the disease; it increases adhesion molecule expression, vascular permeability and SMC proliferation (Apostolakis et al., 2008).

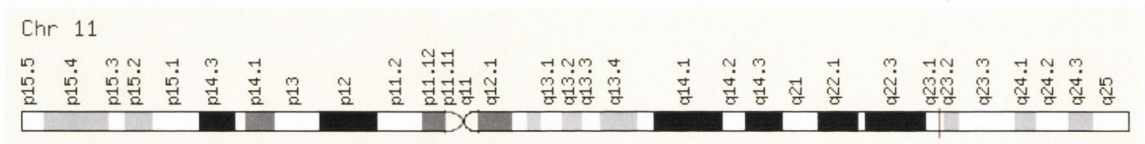
1.9.2.7 Interleukin 12p70 (IL-12p70)

IL-12 is an important regulator of Th1 and Th2 cell responses. It is produced by activated monocytes and is a T cell growth factor. IL-12 expression may be initiated by monocyte

activation by oxLDL but not minimally modified (MM)-LDL. IL-12 p40 mRNA and IL-12 p70 protein have been found in abundance in atheroma (Uyemura et al., 1996).

1.9.2.8 Interleukin 18 (IL-18)

IL-18 is a member of the IL-1 family alongside IL-1 β . A wide range of cells express this pro-inflammatory IFN- γ inducing cytokine such as monocytes/macrophages, T and B cells, dendritic cells and epithelial cells (Apostolakis et al., 2008). *IL-18* gene is found on chromosome 11: position 11q22.2-q22.3 (Figure 1.8) (Okamura et al., 1995).



(<http://www.genecards.org/cgi-bin/carddisp.pl?gene=IL18&search=IL-18>)

Figure 1.8 Chromosome 11, location of *IL18*.

IL-18 is associated with inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus and skin diseases, psoriasis and atopic dermatitis (Sims and Smith, 2010). Unlike most cytokines, IL-18 is constitutively expressed without a stimulant. In atherosclerosis, IL-18 has many roles; it induces cytokines GM-CSF, TNF- α , IL-1 β , IL-6 and chemokine IL-8, which significantly contribute to chronic inflammation at the site of lesion development. The most studied influence IL-18 has on immune cells is its ability to stimulate a potent amount IFN- γ from Th1 cells. A combination of IL-12 and IL-18 also generates IFN- γ on a wider range of cells, CD8 $^{+}$ T cells, B cells and NK cells (Arend et al., 2008).

IL-18 studies have shown the presence of IL-18 co-localized with macrophages in atherosclerotic lesions while absent in healthy arterial regions. In mouse models, IL-18 enhances atherosclerosis through IFN- γ release (Whitman et al., 2002). Also, elevated levels of IL-18 in carotid and aorta atheromas correlated with the increasing potential of plaque

destabilization. However, studies show conflicting results describing the role of IL-18 as pro- or anti-atherosclerotic, depending on the experimental model (Arend et al., 2008). IL-18 has been shown to impact on elevating CXCL16 expression in lesions along with IFN- γ (Tenger et al., 2004). In aortic smooth muscle cells CXCL16 dependent aortic smooth muscle cell (ASMC) proliferation is mediated by IL-18 (Chandrasekar, et al., 2005). Apart from immune cells IL-18 also has an effect on endothelial cell expression, IL-18 upregulates adhesion molecules ICAM-1 and VCAM-1 (Apostolakis et al., 2008).

1.9.3 The network of serum cytokines in this study

There are numerous molecules that form a network in atherosclerosis and activate each other and self activate using monocytes/macrophages, smooth muscle cells and endothelial cells. Numerous inter-relationships have been confirmed and suggested in association with atherosclerosis (Figure 1.7).

TNF- α levels are associated with increased IL-6 levels in vivo and in vitro (Mendell et al., 1997, Bruunsgaard et al., 2000). TNF- α and IFN- γ up-regulate the expression of CXCL16 from ECs and SMCs (Harvey and Ramji, 2005, Tenger et al., 2005). As IFN- γ down-regulates scavenger receptors, this could explain its role in regulating CXCL16 as an anti-atherogenic factor too. IFN- γ is primarily a pro-inflammatory regulator but induces anti-inflammatory protein IL-8, and IL-12 controls the production of IFN- γ in an inflamed environment (Harvey and Ramji, 2005). These cytokines are multi-factorial in atherosclerosis and regulate each other, IL-1 β and TNF- α induce IL-6, while IL-6 inversely regulates TNF- α expression (Akira et al., 1990).

IL-1 β induces IL-6, IL-8 and CXCL16 (Smith et al., 2008, Chamberlin et al., 2006). IL-10 also down-regulates expression of IFN- γ , TNF- α and GM-CSF (Pyo et al., 2003). IL-10 also

inhibits IL-12 (Frostegard et al., 1999). More research is required to better understand the complex network of cells and their cytokines in inflammatory diseases.

1.9.4 Inflammatory mediator targets

Members of each cytokine family have been identified as key players in the pathogenesis of atherosclerosis and all cell types found in atherosclerotic plaque produce or respond to the cytokines involved. In fighting disease, the balance between pro and anti-inflammatory mediators is crucial and therefore controlling expression of cytokines through the use of therapeutics is potentially promising. The limiting step in interleukin therapeutics is identifying which cytokines are compensatory, contributory or associated with atherosclerosis (Von Der Thusen et al., 2003). Different approaches to regulating interleukins involve inhibiting them and their receptors (anti-sense therapy), inhibiting interleukin processing (natural/synthetic ICE inhibitors), neutralization of proinflammatory interleukins (anti-cytokine antibodies, soluble cytokine receptors and cytokine-binding proteins), IL receptor antagonists (IL-1ra), up-regulation of anti-inflammatory interleukins (IL-9, IL-10, IL-11, IL-4, IL-3), inhibition of IL-induced gene expression (NF- κ B, AP-1, STAT) and finally inhibition of interleukin signalling (tyrosine kinase inhibitors e.g. tyrphostins) (Von Der Thusen et al., 2003).

If these interleukin based therapeutics cannot stand alone, the introduction of combination therapy including more than one of the above interleukin targeted therapies should be considered.

1.9.5 Anti-inflammatory therapeutics

Two recent placebo controlled clinical trials on anti-inflammatory based agents have been carried out. Both agents are used to treat inflammatory driven diseases but could prove beneficial for CVD. Methotrexate given at a low dose significantly reduced the number of vascular events and cardiovascular related deaths in RA patients and is a potential cost

effective method for risk prevention in patients with an inflammatory disorder (Maini et al., 1998). Another anti-inflammatory agent which has undergone clinical trials is canakinumab. This antibody neutralizes IL-1 β and reduces CRP, and would be particularly useful for the secondary prevention of cardiovascular disease (Libby et al., 2011).

1.9.6 Scavenger receptors in atherosclerosis

Scavenger receptors are pattern-recognition receptors (PRR) expressed on macrophages, SMCs, monocytes, platelets, endothelial and epithelial cells. They are classified into eight groups (A-H) of membrane and soluble proteins. Their function is to bind to pathogens and macromolecular complexes in order to regulate intracellular signalling (Stephen et al., 2010). In atherosclerosis they mediate internalization of modified lipoproteins, lipoproteins which have undergone oxidation or enzymatic modification, a precursor of foam cell development (Hansson & Libby, 2006).

1.9.7 Chemokines

Chemoattractants are functional messengers classified into inflammatory and homeostatic chemokines. Inflammatory chemokines are secreted by vascular cells such as endothelial and smooth muscle cells and recruit activated macrophages and T cells to sites of inflammation, in this case atheroma formation (Hansson & Libby 2006). Chemokines also play a role in lymphocyte differentiation and angiogenesis (Yamauchi et al., 2004).

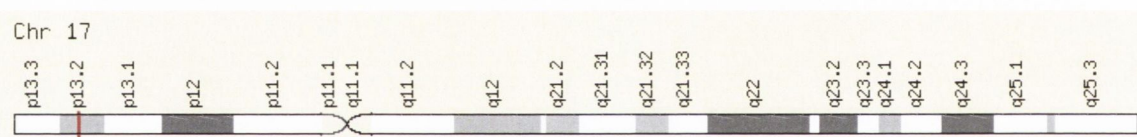
Chemotactic cytokines are generally divided into four main groups, two major CC and CXC, and two minor CX3C and XC (Kraaijeveld et al., 2007), depending on the NH₂-terminal Cysteine motif (Zernecke and Weber, 2010, Yamauchi et al., 2004). Approximately fifty chemokines have been reported on so far (Kraaijeveld et al., 2007) and many play a role in the development of lesion formation.

1.9.7.1 Interleukin 8 (IL-8)

IL-1 and TNF- α stimulate fibroblasts, ECs, keratinocytes and mononuclear cells to synthesize IL-8 chemokine production. IL-8 is a regulator of neutrophil and vascular endothelial cell migration and considered an angiogenic factor. Unstable angina pectoris (UAP) patients have significantly higher levels of IL-8 in their circulation compared to healthy controls (Ozeren et al., 2003). Statins significantly reduce the level of IL-8 and IL-6 in hypercholesterolemic patients (Rezaie-Majd et al., 2002).

1.9.7.2 CXCL16

CXCL16/SR-POX gene has five exons, spans 6283 bp and is found on chromosome 17, position 17p13.2 (Figure 1.9) (Huang et al., 2010).



(<http://www.genecards.org/cgi-bin/carddisp.pl?gene=CXCL16&search=CXCL16>)

Figure 1.9 Chromosome 17, location of CXCL16

CXCL16, also known as SR-PSOX, is a multi-functional molecule composed of four domains, a chemokine domain attached to a mucin stack fused to a transmembrane and cytoplasmic domain (Lehrke et al., 2007, Chandrasekar et al., 2003). It can be described as a scavenger receptor, adhesion molecule and soluble chemokine thus making it a triple threat in disease. Expression of CXCL16 has been reported on T-cells, dendritic cells, macrophages and cytokine stimulated ECs and SMCs (Zernecke et al., 2010) and B cells (Lougaris et al., 2012). CXCL16 is a known regulatory protein of tissue injury, fibrosis and inflammation. The role of CXCL16 has been controversial in clinical samples of many diseases therefore it is of critical importance to elucidate the consequences of its expression.

Soluble CXCL16

The disintegrin and metalloproteinase ADAM10 has the ability to cleave CXCL16 resulting in a soluble chemoattractant for CXCR6 associated cells. CXCR6 has been detected on a variety of cell types, interstitial lymphocytes, NK T cells, monocytes, dendritic cells, a subset of CD4⁺ or CD8⁺ T cells and CD4⁺ effector memory T cells, NK T cells, and a subset of Foxp3⁺ regulatory T cells in humans (Zernecke & Weber., 2009).

CXCL16 as a chemokine signals via heterotrimeric G proteins/PI3K/PDK-1/Akt/IKK/I κ B activating NF- κ B. In Human aortic smooth muscle cells (HASMC), CXCL16 expression increased NF- κ B binding activity resulting in up regulation of TNF- α , I κ B phosphorylation and degradation, cell to cell adhesion and ASMC proliferation (Chandrasekar et al., 2003). Soluble CXCL16 has been reported to have a more definite role as a novel prognostic marker in predicting recurrence of liver metastases in colorectal cancer patients (Matsushita et al., 2011) and as a protector against experimental glomerular injury (Izquierdo et al., 2014) compared to cardiovascular disease. Therefore, it is important to clarify its role in atherosclerosis as it does not act as a negative or positive influence on disease.

Receptor CXCL16

CXCL16 is also a class G scavenger receptor which binds phosphatidyl-serine, oxidised lipoproteins, apoptotic cells and bacteria (Murphy et al., 2005, Lehrke et al., 2007). Pro-inflammatory cytokines associated with increased CXCL16 expression include IFN- γ , IL-18, LPS and to a lesser extent TNF- α , IL-12 and IL-15 (Wagsater et al., 2004).

CXCL16 & atherosclerosis

Studies have shown CXCL16 to play a role in various steps of atheroma initiation and formation, including vascular cell proliferation (Lehrke et al., 2007), endocytosis of oxLDL, but not naïve LDL, leading to foam cell formation (Minami et al., 2001) and CD8⁺ t cell recruitment and IFN- γ production (Yamauchi et al., 2004). CXCL16 was also found to have

an effect on IL-8 and MCP-1 release (Smith et al., 2008).

The location of CXCL16 expression has been confirmed at sites pre-disposed to atheroma formation and has not been detected on the endothelium of healthy arteries. Increased expression has been reported in lesion prone areas with no visible plaque and in macrophage rich regions of advanced lesions (Hofnagel et al., 2011).

Looking at the individual roles of the different forms of CXCL16 it appears to have a pro-atherogenic role. However, the role of CXCL16 in CAD has been controversial.

At present, more research needs to be invested into classifying this molecule as a biomarker, causative factor or therapeutic target. As there is no current reliable biomarker for ischemic stroke this chemokine could be a potential possibility (Sheikine and Sirsjo, 2008). Chapter 2 investigates further the serum levels and plaque presence of CXCL16 in humans and attempts to distinguish the pro or anti-atherosclerotic role.

Atherosclerosis is but one disease that CXCL16 and its receptor CXCR6 assist in inflammatory and therefore disease development. Other auto-immune and inflammatory related diseases have identified the chemokine/receptor duo as important biomarkers.

CXCL16 in inflammatory diseases

Sarcoidosis is an immunomediated disorder of unknown causes. It affects many organs including lungs, liver and heart. CXCR6 and CXCL16 play a pro-inflammatory role in the pathogenesis of T-cell Alveolitis in sarcoidosis. Sarcoid CD4+ T cells in the bronchoalveolar express CXCR6 which then binds to CXCL16 on the epithelial/epithelioid cells and pulmonary macrophages (Agostini et al., 2005).

CXCL16 is also associated with Irritable Bowel Disease (IBD). The soluble form of the chemokine, which we know as an attractant for CXCR6- expressing T cells, significantly increases in patients with Ulcerative Colitis and Crohn's Disease compared to healthy controls (Lehrke et al., 2007).

Gout, an inflammatory arthritis, is also shows a correlation between increased CXCL16 expression and severity of the disease progression. It was also found to be correlated with renal function in the same cohort (Gong et al., 2012).

Precancerous lesions of proliferative inflammatory atrophy have been proposed to form prostatic intraepithelial neoplasia. A study revealed out of 37 chemokines associated with prostate cancer cell lines, CXCL16 had a significant positive correlation with the stage and grade of prostate cancer (Darash-Yahana et al., 2009). This chemokine enhances leukocyte recruitment enabling malignancy and CXCL16 and CXCR6 were also found on tumour-associated lymphocytes, proposing it as a tumour promoter. There is also a strong link between TNF- α and IL-1 β levels and the expression level of CXCL16 in prostate epithelial cells (Lu et al., 2008).

However, CXCL16 does not seem to be associated with a bad prognosis for all cancers, the increase in CXCL16 in colorectal cancerous tissue is an indicator of a better prognosis for colon rectal cancer (CRC). To date it is unclear to why CRC tumour cells upregulate CXCL16 (Hojo et al., 2007).

This multi-functional protein has proved itself to be a marker of importance in many diseases like TNF- α and IFN- γ , so investing research and time to uncover all its possible roles in disease will no doubt be beneficial to not one but multiple diseases.

Therapeutics opportunities

Inhibition of chemokines is a potential approach to treating atherosclerosis. In current literature so far, receptor antagonists for RANTES, CCR5 and CXCR3 have reduced lesion development.

However, limitations exist using cytokine and chemokine blockers. Targeting the lesion site specifically while not affecting the rest of the hosts defence system is a major challenge (Braunersreuther et al., 2007).

1.9.8 Adhesion molecules

Direct cell-cell interactions are maintained and regulated by cell adhesion molecules. The expression of adhesion molecules directs inflammatory responses (Walpole et al., 1994). Early adhesion molecules include P and E selectins, which slow down leukocyte movements along the endothelium wall so adhesion molecules such as VCAM-1 and ICAM-1 can attach to the ligand on leukocytes (Leeuwenberg et al., 1992, Libby, 2006). Soluble forms of these adhesion molecules are also found in the supernatant of cytokine activated cultured endothelial cells (Pigott et al., 1992).

In atherosclerosis, adhesion molecules are upregulated by cytokines and shear stress to facilitate the attachment of leukocytes to the activated endothelium and permit their transmigration into the *intima* where they accumulate and form foam cells at sites predisposed to atherosclerosis (Frank and Lisanti, 2008).

1.9.8.1 ICAM-1

Intercellular adhesion molecule 1 (ICAM-1) is a member of the immunoglobulin superfamily along with ICAM-2/3, vascular cell adhesion molecule 1 (VCAM-1) and platelet endothelial cell adhesion molecule 1 (PECAM-1). They are glycoprotein receptors found on the endothelial membrane and each possess a different number of extracellular Ig domains (Blankenberg et al., 2003).

ICAM-1 and atherosclerosis

ICAM-1 expression is distributed around the cell under normal shear stress with an abundance in the junctional regions. In normal healthy coronary arteries ICAM-1 is expressed. However, it has also been identified in all plaque types in both ECs and macrophages (Davies et al., 2005). In inflamed regions ICAM-1 expression on ECs is controlled by cytokines such as VEGF and TNF- α and synthesized via RhoA activation and the NF- κ B pathway (Thompson et al., 2002, Kim et al., 2001). In murine model studies,

ICAM-1 deficiency alone did not prevent lesion formation and Cybulsky et al., concluded VCAM-1 as a more significant role in atheroma initiation (Cybulsky et al., 2001). Walpola reported that an increase in SS upregulates ICAM-1 expression in rabbit carotid arteries while a reduction in SS is proportionate to ICAM-1 expression (Walpola et al., 1994).

Circulating ICAM-1 in CAD

In human studies, circulating levels of ICAM-1 have been measured in coronary and carotid artery disease patients. Both produce higher levels compared to healthy controls but levels do not vary between these two groups (Hwang et al., 1997). An upregulation of ICAM-1 in circulation has also been linked to myocardial infarctions and future coronary events (Luc et al., 2003). This may suggest a promising molecular marker for atherosclerosis. However, systemic reduction of monocyte adhesion could be potentially dangerous as a therapeutic strategy against atherosclerosis. Nonetheless, mice models with significant adhesion molecule reductions look promising (Nagel et al., 1997).

1.9.9 Lipid accumulation and oxidation

An increasing amount of research has supported that low-density lipoprotein (LDL) particles trapped in the subendothelial space play a vital role in the initiation of fatty streaks and progression of fibrotic plaque in atherosclerosis (Furnkranz et al., 2005). All lipoproteins contain cholesterol which is surrounded by a water soluble and fat soluble surface which allow its transportation throughout the blood. However LDL, specifically harmful in CVD, and HDL, reported to help protect against plaque formation, have structural differences. The most significant being HDL has a higher amount of protein and lower amount of cholesterol which cause it to function differently. As LDL has a lower density it remains in the bloodstream for longer and is more likely to accumulate at the site of injury (Zhang et al., 2011). Various inflammatory diseases such as cancer, diabetes, rheumatoid arthritis and in this case atherosclerosis, feature lipid oxidation an important pathological event (Leitinger.,

2005). A build up of lipoproteins in the intima initiate inflammation and oxidation occurs along with inflammatory activation of cell types such as the epithelium, fibroblasts, neutrophils, monocytes and in the case of atherosclerosis, the endothelium (Birukov et al., 2013).

Formation of oxPLs

Oxidised phospholipids (OxPL) form from (poly) unsaturated diacyl- and alk(en)ylacyl glycerophospholipids by radical induced mechanisms either the oxidation of LDLs (enzymatic) or from the membranes of apoptotic cells (non-enzymatic). Enzymes such as myeloperoxidase, 12/15 lipoxygenase, phospholipase A2, sphingomyelinase and NADPH oxidase are significant enzymes involved oxidative modification (Birukov et al., 2013). The oxidation of arachidonic acid containing membrane phospholipids (LDL) results primarily in MM-LDL. OxPLs have been identified in atherosclerotic plaque and circulation (Ashraf et al., 2008).

Sterol regulatory element-binding proteins are key transcription factors involved in the modulation of genes implicated in cholesterol biosynthesis and LDL uptake. SREBP1, one of the three family members, can be activated by shear stress and TNF- α (Pastorino and Shulga, 2008) in the presence of sterols via integrins and activation is prolonged by disturbed flow. The activation of SREBP1 is a result of transcriptional activation of genes which are regulated by sterol response elements (Li et al., 2002). OxPAPC has been identified as a regulator of SREBPs (Gargalovic et al., 2006). The subsequent activation of these transcription factors results in the over-induction of lipogenic enzymes and subsequent cholesterol biosynthesis which the cell is not capable of controlling.

1.9.9.1 OxPAPC

MM-LDL differs from highly oxidised LDL undergoing less linoleic acid oxidation and little to no protein modification. MM-LDL, unlike oxLDL is involved in monocyte recruitment

and macrophage transformation (Watson et al., 1997). The formation of minimally modified LDL assists in monocyte adhesion and stimulates chemokine MCP-1 and cytokines for plaque progression. The biological activity of MM-LDL is caused by the oxidation of 1-palmitoyl-2-arachidonoyl-sn-3-glycero-phosphorylcholine (PAPC) which then yields fragmented 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-phosphatidylcholine (POVPC), 1-palmitoyl-2-glutaroyl-sn-glycerophosphatidylcholine (PGPC), lysophosphatidyl choline (lyso-PC), and full length products palmitoyl-2-(5,6-epoxyisoprostane E2)-sn-glycero-3-phosphocholine (PEIPC), all of which have been shown to accumulate in atherosclerotic lesions (Birukov et al., 2013).

OxPAPC signalling

Oxidized LDLs (oxLDLs) mediate the inflammatory response by activating inflammatory and oxidative stress gene expression via different signalling pathways, receptors, protein kinases and transcription factors. A negative feedback loop forms as oxidative stress induces inflammatory cytokine and chemokine production which then induce free radical production (Leonarduzzi et al., 2011). Figure 1.10 shows the pathway and potential pathway which oxPAPC signals through to result in the induction of monocyte adhesion (Leitinger, 2005).

Biological function of OxPLs

The roles of OxPLs are quite diverse but typically relate to pro-inflammatory events such as cytokine stimulation, chemokine production, cell adhesion, coagulation and platelet activation (Ashraf et al., 2008). Specifically in atherosclerosis oxPLs form foam cells and initiate fatty streaks (Greig et al., 2012) and stimulate endothelial cells to secrete chemokines MCP-1 and IL-8 and monocyte recruitment to sites of vascular injury (Birukov et al., 2013). However studies on the role of oxPAPC in monolayer permeability seem to be contradictory, reporting on both a protective and pathological effect.

It is known that disruption of the endothelial barrier is a key step in atheroma formation. High concentrations of oxPAPC (50-100 µg/ml) increase endothelial disruption and permeability but low levels (5-20 µg/ml) increase barrier function, protect against LPS-induced injury and induce anti-inflammatory genes (Birukova et al., 2013). Once high concentrations of oxPAPC disrupt the endothelium, signalling mechanisms are activated (Starosta et al., 2011).

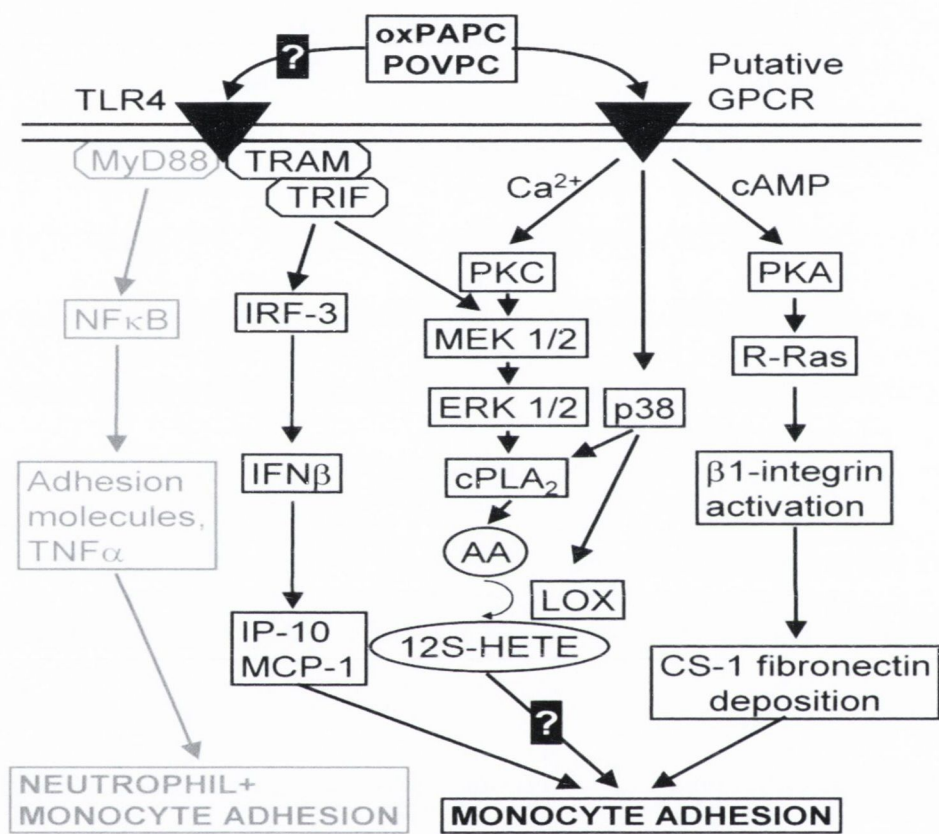


Figure 1.10 Signalling pathways induced by oxPAPC and oxPOVPC. Studies are ongoing for the interaction of TLR4 and oxPAPC/POVPC. oxPAPC binds to GPCRs and signals through the various pathways above to increase monocyte adhesion (Birukova et al., 2013).

However it not only depends on the oxPAPC concentration but the components also. In human pulmonary artery endothelial cells (HPAECs) PEIPC enhances barrier protection whereas increased levels of fragmented products of POPC, POVPC, PGPC and lyso-PC trigger signalling mechanisms and barrier disruptive effects (Birukova et al., 2013).

Apart from lower oxPAPC concentrations and products being anti-inflammatory it also has

an anti-inflammatory role inhibiting the binding of LPS to LPS-binding protein therefore blocking LPS presentation to TLR-4 thus inhibiting the NF- κ B pathway (Mackman et al., 2003) (Figure 1.10).

1.10 Statins- existing treatment for atherosclerosis

Statins or 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors are lipid lowering drugs and used in the treatment of hypercholesterolemia, which is a main contributor to cardiovascular disease and thus morbidity and mortality rates. Many clinical trials such as the Scandinavian Simvastatin Survival Study (4S) and Cholesterol and Recurrent Events (CARE) have demonstrated the positive effect of statin therapy (Liao and Laufs, 2005). Statins are used as both primary and secondary preventatives of arterial disease and their pleiotrophic effects on cellular functions and signalling in inflammatory associated sites (Iwata et al., 2012). As excess cholesterol impairs endothelial production and increases nitric oxide degradation, the administration of statins also improves endothelial function.

Statins function by inhibiting the enzyme HMG-CoA reductase which is responsible for catalyzing the conversion of HMG-CoA to mevalonic acid in cholesterol biosynthesis. A reduction of circulating cholesterol triggers an increase in LDL receptor expression which facilitates clearance of LDL and LDL precursors. Statins also play a minor role in elevating HDL levels (Maron et al., 2000, LaRosa et al., 1999). Statins stimulate and upregulate the enzyme endothelial NO synthase (eNOS) which increases NO production. In hypoxic and hyperlipidemia conditions statins also restore eNOS activity and prevents further endothelial dysfunction (Liao and Laufs, 2005).

Statins activate the PI3K/Akt pathway which induces endothelial progenitor cell (EPC) differentiation. These discoveries lead to a therapeutic goal of rescuing tissue from critical ischemia by stimulation of neovascularization (Dimmeler et al. 2001).

Other indirect beneficial roles that statins are involved in include decreasing SMC production *in vitro* and studies have shown simvastatin and pravastatin specifically reduce macrophage proliferation. Macrophages degrade collagen which is the structural backbone of the fibrous cap (Liao and Laufs, 2005). In general and unrelated to cholesterol metabolism, statins protect against plaque progression and rupture by inhibiting lymphocyte and other blood mononuclear cell growth (Maron et al., 2000).

Statins also affect plasminogen activator inhibitor -1 and fibrinogen levels and pravastatin lowers C-reactive protein levels. Studies have suggested statins as anti-inflammatory drugs, the mechanisms involved in reducing the number of inflammatory cells have yet to be elucidated but they possess the ability to inhibit adhesion molecule expression (ICAM-1) (Liao and Laufs, 2005).

Lipid related targeting

New and old trials remain constant in emphasizing the importance of lowering and overall clearance of LDL in high risk patients. Emerging treatments such as PCSK9 inhibitors key target is LDL. The role of HDL is less clear, the emerging risk factor collaboration have provided robust data on HDL as an undisputable risk factor for CVD (Chong and Bachenheimer, 2000). To date there are still no clinical trials supporting the theory that targeting HDL will reduce CV outcome. Using novel biomarkers and pharmacogenomics approaches are ongoing to help improve therapeutics for dyslipidemia management (EAS report 2013).

Nuclear receptors, liver X receptors (LXRs) are activated by oxysterols and play an important role in controlling lipid homeostasis, specifically in regulating cholesterol and fatty acid metabolism in macrophages. As dyslipidemia is a main risk factor in atherosclerosis these receptors play an influential role in disease progression. Studies have shown correlation

between LXR activity and the pathogenesis of atherosclerosis which suggests it could be an attractive target for therapeutics (Joesph and Tontonoz, 2003). However there has only been one phase 1 study so far in humans. The non-specific LXR agonist increased efflux pathways ABCG1 and ABCA1 (Laurencikiene and Ryden, 2012).

1.11 er up and coming therapeutics

1.11.1 Endothelial therapeutics

Endothelial damage evolution and severity in advanced atheroma remains elusive, partly due to a lack of in vivo quantifiable methods. Recently endothelial damage was quantified in an ApoE deficient mouse model using a multifunctional perfluorocarbon nanoparticle (PFC NP). It concluded that endothelium damage correlated with the length of the animal-fat diet consumption. The model also investigated PFC NP as a drug deliverer, loading the particle with a nf-kb inhibitor reduced inflammation (Pant et al., 2013).

1.11.2 NAD(P)H oxidases

Activation of NOX proteins, a new NADPH oxidase subfamily, signal through MAPK, tyrosine kinases and transcription factors to stimulate ROS production. ROS plays a pathophysiological role in inflammation, endothelial dysfunction, hypertrophy and angiogenesis (Cai et al., 2003). Therapeutics against NADPH oxidases have the ability to lower ROS levels and potentially protect against vascular injury (Paravicini and Touyz, 2008).

1.11.3 Cell based therapeutics

Foam cells are an essential requirement in all stages of the pathogenesis of atherosclerosis. Studies elucidating cellular processes associated with macrophages have suggested numerous therapeutic targets such as, monocyte recruitment and cholesterol uptake and evacuation (Choudhury et al., 2005).

1.12 Evolution and contribution of in vitro models in research

Various *in vitro* microfluidic models have been constructed to replicate the *in vivo* environment as studying cellular responses *in vivo* is too complex. These models examine the physiological response of endothelial and smooth muscle cells to hemodynamic forces and allow a controlled and accessible environment. The two types of shear stress in vitro models most commonly used at the beginning of the microfluidic revolution were the cone and parallel plate flow chambers and elucidated the morphological and biological responses of the endothelial monolayer to shear stress (Table 1.4) (Leong et al., 2013).

Table. 1.4 Cone and plate flow apparatus v parallel-plate flow chamber

	Cone and plate flow apparatus	Parallel-plate flow chamber
Components :	rotating cone, stationary plate	Two parallel plates
Flow:	produced by axisymmetric rotation of the cone above the plate	Flow is created by a prescribed pressure gradient
How it works:	Gap width and azimuthal cone surface velocity increase as distance increases from axis of rotation	Pressure head, syringe pump, peristaltic pump or pulsatile pump creates a pressure gradient
Advantages:	shear stress can be changed by varying cone angle and rotation speed	
Variants:	Parallel disk device	Tapered parallel plate flow chamber

Dewey et al, 1981 used a modified cone-plate viscometer that produced a uniform fluid shear stress. This study focused on morphological responses such as shape, orientation and size. On exposure to 1-5 dynes/cm², Dewey concluded that endothelial cell proliferation was comparable to that in a static environment and when exposed to a laminar flow of 5-10 dynes/cm², cell shape adjusted. In a time-dependent manner cells became oriented with flow and transformed from a polygonal shape to an ellipsoidal shape. This study was the first to suggest that endothelial functions such as surface protein expression

and cytoskeleton assembly were sensitive to mechanical forces such as shear stress, and that normal vessel wall physiology was essential for preventing atherosclerosis (Dewey et al., 1981).

To date the parallel plate flow chambers and its derivatives are more widely used as they facilitate a peristaltic pump or pulsatile pump. They also accommodate more intrinsic research such as biological responses: gene and protein expression of ECs. Hsiai et al, 2001 designed a model that assessed the affect of shear stress rate and pulsatile versus oscillatory flow on monocyte binding to oxidised lipid treated endothelial cells (Figure 1.11). This system allowed for real-time observation, pressure, flow and temperature monitoring and a range of shear stress from 0-200 dyne/cm². The main advantage of this model is the ability to extract RNA post experiment.

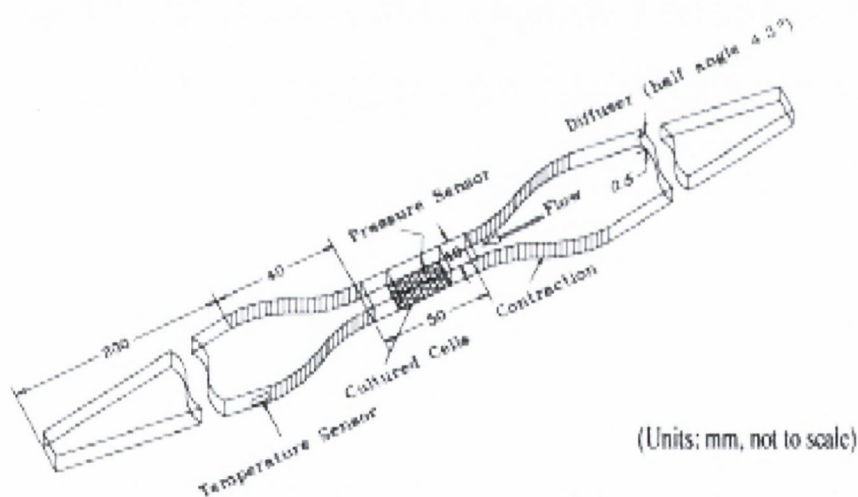


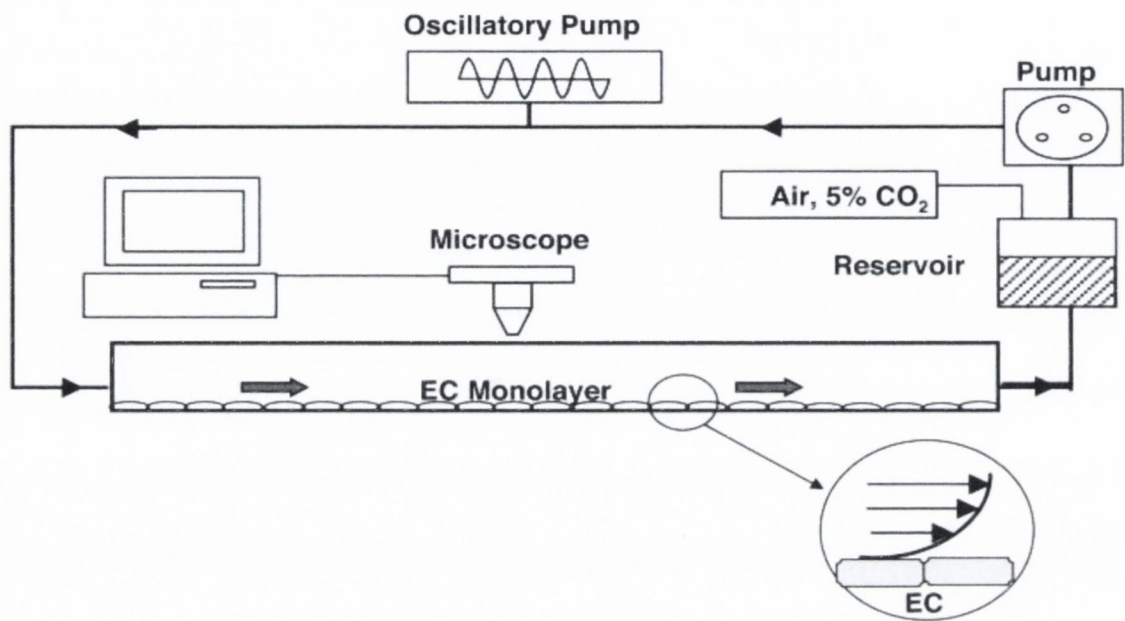
Figure 1.11 Parallel-plate flow channel. Symmetrical contractions and diffusers were connected to the inlet and outlet of the parallel channel to provide uniform flow fields across the width of the channel (Hsiai et al., 2001).

Chachisvilis et al, 2006 used a 2mm parallel flow channel with 30 dynes/cm² to analyse the effect of shear stress on GPCR conformational change (Chachisvilis et al., 2006).

A review by Chien in 2006 discussed *in vitro* flow chamber studies on mechanotransduction in response to shear stress. Two variations of a parallel plate flow chamber, rectangular and

step, investigated different types of shear stresses on the endothelial monolayer mechanotransduction and homeostasis (Figure 1.12) (Chien, 2006).

A



B

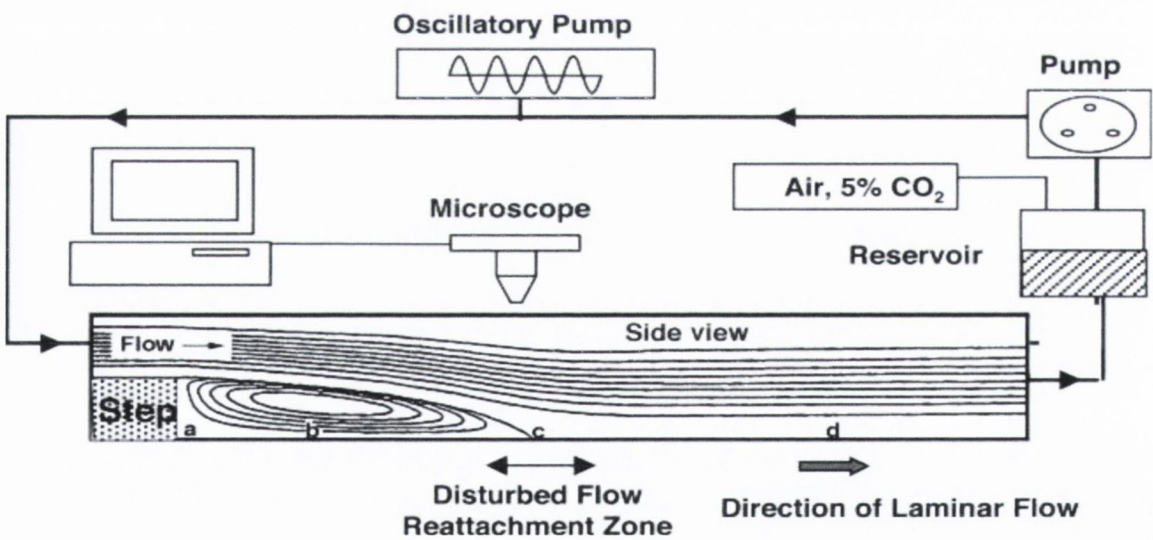


Figure 1.12 A) A Rectangular Flow Chamber allows for multiple shear stress conditions, including pulsatile (12 ± 4 dyne/cm²), reciprocating (0.5 ± 4 dyne/cm²) and steady (12 dyne/cm²) shear stress and a static (0.5 dyne/cm²) environment. B) A Step Flow Chamber was used for experiments examining the effect of disturbed shear.

Pulsatile dynamics on the EC have also been studied *in vivo* like *in vitro* experiments. Ku et al., designed a glass carotid bifurcation model which had a pulse wave form and capable of producing systolic and diastolic intervals. Flow velocity of the carotid bifurcation was measured using a Plexiglas model and laser Doppler velocimetry.

1.12.1 EC-SMC co-culture models

The EC- SMC interactions are critical to blood vessel function and failure to communicate properly results in barrier and signalling dysfunction which leads to vascular disease. *In vitro* experiments have not only investigated the effect of shear stress on the EC monolayer but on both the EC and SMC together interacting. These coculture models represent a significant advance towards replicating a more *in vivo* environment *in vitro*.

In the mid 90's a co-culture, *in vitro* pulsatile model was developed (Redmond et al., 1995). Redmond examined the effect that flow induced changes in ECs had on the SMCs. A novel transcapillary EC-SMC co-culture model was developed that allowed chronic exposure of both cells to a controlled mechanical stress. The model replicated an artery in appearance, ECs grew on the inner surface and SMCs grew on the outer surface of a perfused elastic capillary. EC and SMC populations were maintained as separate populations so biochemical and molecular analysis could be analysed for an individual population.

Chiu et al, 2003 introduced a parallel plate coculture flow system that had similar investigative intentions to Redmond et al. this adaptation of already available *in vitro* models had the advantage of real time microscopic visualization of both cell types under flow and morphological and biochemical analysis. The aim of this study focused on EC and SMC interacting to regulate adhesion protein expression and found that SMCs play a critical role in mediating ICAM-1, V-CAM-1 and E-selectin on ECs under flow and static conditions (Chiu et al., 2003).

The microfluidic models mentioned are designed to give in depth observations at the direct effect of shear stress on endothelial and smooth muscle cell protein expression and pathway regulation in a controlled environment, and thus can expand our knowledge on the impact of flow *in vivo*.

1.12.2 Flow models and Drug delivery

In the last few years vascular targeted drug delivery applications have been an important area of interest for the future of vascular disease treatment. Studies have focused on the binding efficiency of these vascular targeted drug carriers in varying conditions of particle size, shear stress, flow type i.e. laminar and blood composition. Charoenphol et al., used in vitro plate flow chambers to examine particle size and discovered that micron sized particles and not nanoparticles were optimal for drug delivery to medium-large sized arteries (Charoenphol et al., 2011).

In chapter 3 the Cellix *Vena8 Fluoro+TM Biochip* and iKima pump was used to investigate protein expression using Immunofluorescence. This model is specialized for cell receptor-ligand studies under conditions mimicking physiological flow.

1.13 genome-wide association studies (GWAS)

Genome wide association studies are a hypothesis free agnostic approach to genotyping and studying single nucleotide polymorphisms (SNPs) simultaneously. In the past couple of years GWAS meta-analyses have identified numerous loci associated with diseases and provided knowledge of allele architecture of complex traits (Evangelou and Ioannidis, 2013).

1.14 Genetic variation

Gene expression variation is likely heritable and significant in disease susceptibility (Epstein et al., 2009). Variations in the DNA sequence can be divided into structural (short and large insertion/ deletions and translocations) and sequence variations (SNPs). Gene cloning made

DNA variants easily detectable, the first variants tested were restriction fragment length polymorphisms (RFLPs), and development of polymerase chain reaction (PCR) meant analysis of SNPs, copy number variants (insertion/deletion polymorphisms) and variable number tandem repeats (VNTRs) could be investigated further (Hirschhorn et al. 2002).

1.15 Single nucleotide polymorphisms (SNPs)

Single nucleotide polymorphisms (SNP) are single nucleotide changes in the genome and are not homogenously distributed. Two alleles represent the SNP and natural selection fixates the allele of the SNP which is most favourable for adaptation (Barreiro et al., 2008).

Within the human genome there are over 11 million SNPs with a frequency greater than 1%. (Wang and Elbein, 2007). The SNP Consortium and the International Human Genome Sequencing Consortium uncovered more than 95% 1.42 million SNPs that were distributed on average every 1.9 kilobase (kb). SNPs usually occur in non-coding regions (regulatory SNP) of the genome; an estimated 60,000 can be located within coding (non-synonymous SNP) and untranslated regions known as exons (Sachidanandam et al., 2001). There are a number of reasons why frequency and distribution of SNPs need to be understood. Marker SNPs can be used to map polygenic disease loci (1-2% of point mutations are in non-coding regions, Epstein et al., 2009), history, migrations and population size can be studied and distribution of variation can shed light on evolutionary forces such as selection, mutation and genetic drift (Nachman, 2001).

The chromosome region 9p21.3 has been identified as major risk locus for coronary disease and myocardial infarction. Gschwendtner et al, 2009 investigated the locus among stroke patients and genotyped individuals for 15 SNPs which were found in the critical interval of 9p21. Six SNPs were found to be associated with atherosclerotic stroke and independent to other arterial diseases (Gschwendtner et al., 2009).

SNPs have a low recurrent mutation rate making them stable markers (Sachidanandam et al., 2001) and have great potential for preventing, predicting and treating diseases (Hirschhorn et al., 2002). The ability of mapping SNPs offers the possibility of using linkage disequilibrium (LD) also map disease related genes (Reich et al., 2001).

1.16 Linkage disequilibrium (LD)

LD is the frequency at which some combinations of alleles occur due to what's expected from a random formation of haplotypes of neighbouring alleles (Reich et al., 2001). The amount of LD is dependent on the difference between observed and expected allele frequencies based on a homogenous population and influenced by multiple factors such as the rate of mutation and recombination, genetic linkage, genetic drift and non-random mating (Cheung et al., 2009). LD occurs in blocks that can extend >100kb around a SNP (Reich et al., 2001).

1.17 Hypotheses and objectives

Despite the advances in atherosclerotic research there are still many questions left unanswered and uncertainties in the pathological processes and relationships in stages of atheroma formation such as lipoprotein oxidation and inflammation. Further investigation also continues into genetic and environmental risk factors associated with atherosclerosis. "Understanding how to combine experimental and clinical science will provide further insight into atherosclerosis and could lead to new clinical applications" (Libby et al., 2012). This thesis as a whole aimed to combine *in vivo* and *in vitro*, protein and genetic studies to contribute to atherosclerotic research. This was carried out by assessing known circulatory biomarkers in vascular patients and performing correlation analysis, optimizing and validating an *in vitro* pulsatile model for protein expression studies and investigating the occurrence of allelic imbalance in clinical samples and TNF- α stimulated and unstimulated

cell lines.

Objectives

1. To investigate known and potential anti and pro inflammatory cytokines that are involved in carotid plaque development by measuring their levels in a highly sensitive and higher dynamic range platform in order to generate a cytokine network profile that will help in the diagnosis and prognosis of cardiovascular disease patients.
2. Optimize and validate a pulsatile pump and immunofluorescent biochip which facilitates a laminar pulsatile flow which allows protein expression to be quantified from qualitative data.
3. To examine if allelic expression imbalance is present or absent in SNPs that could be common in cardiovascular disease using cell lines and patient samples to allow for further association analysis, and to test if allelic imbalance is affected by the introduction of pro-inflammatory cytokine TNF- α as it is a known adhesion molecule inducer in current literature and throughout this project.

Overall the aim was to possibly identify new ways to prevent first time or re- occurring thrombosis.

CHAPTER 2

High Sensitivity Cytokine Detection in serum
of carotid atherosclerotic patients

2.1 Introduction

2.1.1 *Pro and anti-inflammatory markers analysed in this study*

Mediators in innate and acquired immunity are considered as contributors to atheroma formation, as explained previously in the stages of plaque initiation and progression, and therefore important in the search for significant biomarkers (Hansson et al., 2002).

Inflammation and its mediators may be considered as primary or secondary triggers in atherosclerosis. Underlying diseases associated with atherosclerosis which promote an existing pro-inflammatory environment include, rheumatoid arthritis (RA) (Sakurada et al., 1996), diabetes (Beckman et al., 2002) and systemic lupus erythrocyte (SLE) (Frostegard et al., 2005). These diseases may assist atheroma formation by producing excessive amounts of pro-inflammatory cytokines and chemokines, which activate the EC and result in receptor expression and leukocyte binding. In patients without these diseases, artery architecture and altered hemodynamics may be considered primary triggers of atheroma formation, as it leads to response to injury.

Studies on different CVDs report different levels of circulating pro and anti-inflammatory cytokine between disease types and between disease and control models. Main immune mechanisms are involved in CVDs: Non-specific innate immunity includes cytokines TNF- α , IL-1, IL-6, chemokines (MCP-1/CCL2, CXCL16) MMP-9, complement, acute phase proteins, histamin, chymase, tryptase, endogenous vasoconstrictors and specific adaptive immunity cytokines IFN- γ , IL-12, IL-2, IL-4, IL-10, IL-17, IL-33, TGF- β (Bucova et al., 2008a).

2.1.1.1 *Stable angina pectoris (SAP) and Unstable angina pectoris (UAP)*

IL-10 is the only inflammatory cytokine of this study which is solely an anti-inflammatory molecule. Studies show that in unstable angina patients circulatory levels of IL-10 are

significantly lower compared to stable angina patients (Smith et al., 2001) and controls (Ranjit et al., 2004). Much debate exists about the role of CXCL16 in atherosclerosis. Sheikine et al, 2005 unstable and stable angina patients expressed lower levels of CXCL16 than controls and CXCL16 levels did not correlate with CAD severity. Therefore these authors concluded CXCL16 to be atheroprotective (Sheikine et al., 2005). In stable and unstable angina patients CXCL16 levels are upregulated compared to controls. These findings conflicted with Sheikine et al and concluded that CXCL16 was a potential inflammatory mediator and marker (Smith et al., 2007).

A significantly higher level of IL-6 and TNF- α is found in UAP patients compared to SAP patients (Smith et al., 2001, Ridker et al., 2000 and Mizia-Stec et al., 2003). Comparing UAP patients to healthy controls, levels of IL-12 (Yamashita et al., 2003), TNF- α IL-6, IL-1 β (Ranjit et al., 2004), IFN- γ (Mazzone et al., 2001) and IL-8 (Ozeren et al., 2003) were found to be significantly elevated. Changes in IL-2 levels have been found to be significantly higher in stable angina patients compared to controls (Mazzone et al., 1999) however no significant change occurs between unstable angina patients and healthy controls (Ozeren et al., 2003).

2.1.1.2 Myocardial Infarction (MI)

In MI serum samples many of the same results apply. Levels of circulating TNF- α , IL-1 β and IL-6 are used as markers for myocardial ischemia (Roth-Isigkeit et al., 1999). In many tissues IL-10 modulates TNF- α expression, and a decrease in the IL-10 to TNF- α ratio correlates with depressed cardiac function (Kaur et al., 2006). Potential markers which also show elevated levels in MI patients compared to controls include IL-2 (Mazzone et al., 1999) and IFN- γ (Patel et al., 2009). Chronic heart failure and disease severity can also be characterized by elevated CXCL16 plasma levels (Dahl et al., 2009). Shintani et al, 2001 revealed that no

significant change occurred in levels of GM-CSF and IL-8 between acute myocardial patients and controls (Shintani et al., 2001).

2.1.1.3 Carotid endarterectomy

All of the mediators assessed in this study have been identified in human atherosclerotic plaque (Ait-Oufella et al., 2011). A study done by Profumo et al, 2008 looked at cytokine expression in the peripheral blood of patients with less than 70% stenosis undergoing a carotid endarterectomy. TNF- α , IFN- γ , IL-1 β , IL-6, IL-8 and IL-10 had significantly higher levels in patients than controls (Profumo et al., 2008). IL-6 specifically seems to be an independent and useful biomarker (Larsson et al., 2005). GM-CSF is found in every atheroma-associated cell, ECs macrophages, T lymphocytes, SMCs (Sugiyama et al., 2001). Unlike the controversial role of CXCL16 in UAP and SAP patients, CXCL16 serum levels show a significant increase associated with carotid atherosclerosis (Wang et al., 2010).

2.1.1.4 Mouse models

Mouse models have also been studied for circulating cytokine levels and a review by Kleemann et al., in 2008 summarized the contribution and relevance of some of the cytokines involved in this study in atherosclerotic mouse models. The concluding remarks stated that only a few cytokines, IL-1, IL-12, IFN- γ and TNF- α , had sufficient data consistent with pro-atherosclerotic classification and. Mouse studies revealed TNF- α gene disruption reduced plaque development (Ohta et al., 2005). IL-10 was the only cytokine which had sufficient data that supported a consistent anti-atherosclerotic role while IL-6 and GM-CSF exerted both pro and anti-atherogenic effects (Kleemann et al., 2008).

The improved knowledge of serum biomarkers is essential for the development of atherosclerotic research and developing tests which could predict plaque build up based upon a cytokine serum measurement.

2.1.2 Cytokine detection

Cytokines and chemokines were measured from serum samples of patients and healthy volunteers using Electrochemoluminescence (ECL) and Enzyme-linked immunosorbent assay (ELISA). Since the 1970s ELISAs have been considered the standard cytokine measurement method because of their accurate and sensitive detection of the cytokine of interest which produce quantitative and reproducible results (Leng et al., 2008). However, a number of limitations exist with the ELISA format. This study focuses on profiling multiple cytokine levels in the serum of vascular patients and controls and an ELISA is specific for measuring one cytokine. Both assays measure in pg/ μ l, however, with an ELISA, detectable levels are not as low as ECL and do not measure accurately below 15 pg/ μ l. ECL includes three elements, light, chemical and electrochemical (Figure 2.1). When non-radioactive labels attached to the electrode are electrochemically stimulated light is emitted at ~ 620 nm. It enables minimal background signal, only labels bound near the electrode surface are detectable and allows for signal amplification which improves sensitivity.

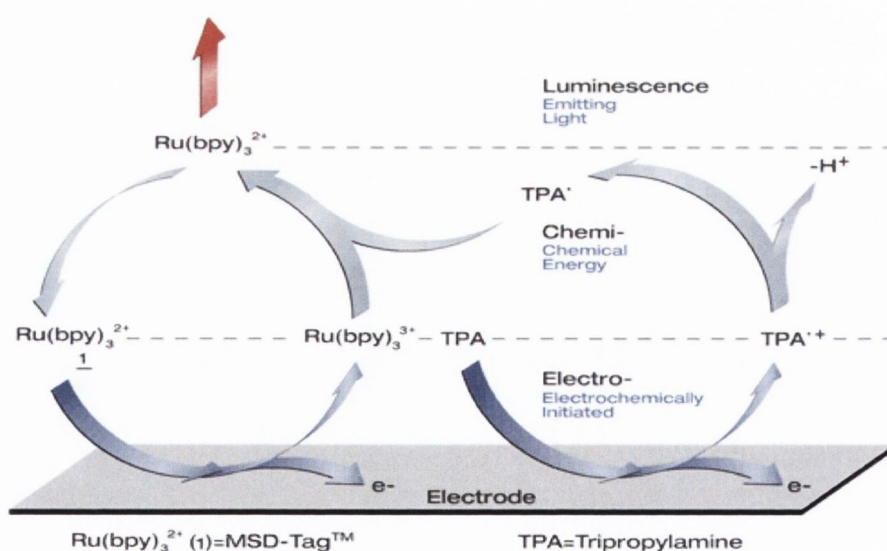


Figure 2.1. The three steps involved in a mesoscale discovery plate: electrochemical, chemical and light. This combination provides better sensitivity and a higher dynamic range than the commonly used ELISA test.

The multi-spot plate used in this study is designed for high throughput multiplexing of biomarkers. An ELISA was used first for CXCL16 before using a more sensitive ECL test, as the ELISA measured detectable levels of CXCL16 the ECL assay was not needed to measure serum CXCL16 levels as basal levels were >15pg/ml.

A previous multiplex study measured the same cytokines GM-CSF, IFN- γ , IL-1 β , IL-2, IL-10, IL-12p70, TNF α , IL-6, and IL-8 as this study but in the serum of post myocardial infarction and unstable angina patients. IL-10 and IFN- γ showed higher expression levels in the MI group (Patel et al., 2009). This method is high throughput and promising for identifying numerous novel biomarkers in many, not just vascular diseases. As cytokines are part of a network and influence each other, it is advantageous to do a multiplex array to investigate any correlation that may not be considered likely (Huang et al., 2012). The MSD platform opens up results to a vast array of analyses.

2.1.3 Hypotheses & Objectives

The aims of this chapter were to further investigate inflammatory markers associated with atherosclerosis and to contribute to the current knowledge supporting the anti or pro atherosclerotic roles. Firstly, the aim was to assess the key cytokines in association with vascular disease in comparison to healthy volunteers by measuring serum concentrations of nine cytokines and chemokines in vascular disease patients using the highly sensitive technique ECL and chemokine CXCL16 using an ELISA. Using this information it was then fundamental to determine if there was a significant association between patient serum cytokine levels and risk factors (smoking/hypertension), degree of stenosis or between asymptomatic and symptomatic patients.

- It was hypothesized that patients with more than one risk factor associated with atherosclerosis may have higher levels of pro-inflammatory cytokines. The cytokine

profile measured was chosen based on the current literature regarding important biomarkers in vascular disease.

- It was also interesting to see if any cytokines displayed the same expression pattern as many can induce or change each other's expression. As CXCL16 has shown conflicting results in the current literature, we wanted to investigate its levels in carotid artery disease. The purpose of cytokine profile screening gives us the ability to determine the risk of heart attack or stroke as clinical laboratory testing is currently limited (Shah et al., 2010).
- This chapter examined known inflammatory cytokines which play a part in atherosclerosis and evaluated whether serum levels could help predict potential rupture, specifically with increasing stenosis percentage.
- Finally, it was also of interest to see if serum samples had more or less pro-inflammatory than anti-inflammatory cytokines in both patients and controls, as a balance is necessary for healthy arteries (Frostegard et al., 1999)

2.2 Materials & Methods

2.2.1 Patient recruitment

Asymptomatic and symptomatic atherosclerotic patients due to undergo carotid endarterectomies were recruited in St James’ Hospital, Dublin, Ireland via the Department of Cardiology. Written consent was gained from each patient after the patient was informed of the study’s details. Patients who suffered from RA, SLE and Renal failure/transplant were excluded from the study. Risk factors, smoking, hypertension and diabetes were noted and a Doppler scan provided plaque type and % stenosis. Statin therapy was also recorded. Patient details were obtained and approval was given by the St. James’s Hospital / AMNCH Research Ethics Committee. The patient cohort consists of 58 subjects and 10 healthy controls (Table 2.1) for ECL and 23 subjects and 10 healthy controls for ELISA (Table 2.2).

Table 2.1 Characteristics of atherosclerotic patients and healthy controls for ECL

	Atherosclerotic patients n= 58	Controls n=10
Age, mean S.E.M	72±8.8	54±3.5
Sex (M/F)	47/11	6/4
BMI (M/F)	26±8.9/25±9.6	25 ± 2.58
Asymptomatic	27	-
Symptomatic	31	-
Smoker	14	-
Ex -smoker	30	-
Non -smoker	8	10
Diabetes Type II	14	-
Hypertension	14	-
Unknown risk	2	
No risk factor	4	
1 risk factor	26	
2/more risk factors	22	
% Stenosis internal carotid (Right and left)		
< 80%	33	-
>80%	25	-
Statin on Admission		
Yes	47	
Aspirin	20	-
β-blockers	10	-
None	11	

Table 2.2 Characteristics of atherosclerotic patients and healthy controls for ELISA

	Atherosclerotic patients n= 23	Controls n=10
Age, mean S.E.M	72±9.6	54±3.5
Sex (M/F)	18/5	6/4
BMI (M/F)	25±6.2/21±1.2	25± 2.58
Asymptomatic	7	-
Symptomatic	16	-
Hypertension	15	-
1 risk factor	11	
2 risk factors	10	
Unknown risk factors	2	

2.2.2 Blood sampling and PBL isolation

The patient's blood sample was taken within two hours of arrival to St James' Hospital after signing a consent form agreeing to participation in the study. The blood samples were collected and allowed to rest for 30 minutes prior to serum isolation to ensure adequate clotting. Healthy volunteers were recruited independently for controls to determine baseline levels.

Serum was then isolated from the blood samples by centrifugation at 4000 rpm for 10 minutes. Using a sterile pasteur pipette (Sigma Aldrich, Germany) serum was removed from the sample and stored in 1ml eppendorf microcentrifuge tubes (Thermo Fisher Scientific, USA) at -80°C until further analysis.

2.2.3 Enzyme-linked immunosorbent assay (ELISA)

The concentration of CXCL16 was determined from the serum samples of the 23 patients and 7 healthy controls mentioned previously by enzyme-linked immunosorbent assay (ELISA). Human CXCL16 DuoSet (R&D systems, USA) was used to measure the quantity of CXCL16. Kit content included Capture Antibody, Detection Antibody, Recombinant Standard and Streptavidin conjugated to horseradish-peroxidase (Streptavidin-HRP). All contents were diluted with Reagent Diluent (1% PBS/BSA) according to manufacturer's

guidelines.

High binding 96-well microtiter plates (Greiner Bio-one) were coated with 100µl per well of anti-human CXCL16 capture antibody, the plate was sealed and incubated overnight at room temperature. The capture antibody solution was aspirated and wells were washed three times with 400µl of wash buffer (R&D Systems, USA). Non-specific binding sites were blocked with 300µl of Reagent Diluent at room temperature for a minimum of 1 hour. The aspiration was repeated and three washes were performed. Sample and standards were added (Figure 2.2). Standards provided by the manufacturer were diluted in Reagent Diluent and used to produce a standard curve (Table 2.3).

Table 2.3 Eight point standard curve used in the enzyme-linked immunosorbent assay

Standard	Human CXCL16 (pg/ml)	Dilution factor
100x stock	1000000	
Dilution. Stock calibrator	10000	1000
Standard (STD)-01	1000	2
(STD)-02	500	2
(STD)-03	250	2
(STD)-04	125	2
(STD)-05	62.5	2
(STD)-06	31.25	2
(STD)-07	15.6	2
(STD)-08	0	n/a

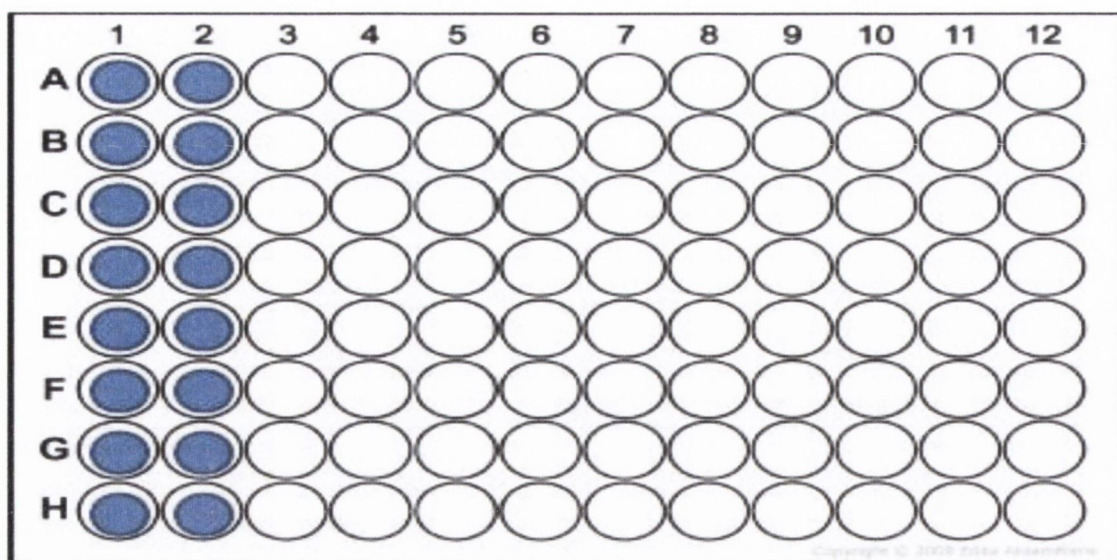


Figure 2.2 Layout of 96 well plate used in the ELISA for CXCL16 expression in serum samples. Standards were added in duplicate according to calculations in Table 2.2.2 in rows A-H and Columns 1-2. Samples were then added in duplicate in rows A-H columns 3-12.

100µl of sample and standards were added per well, plates were sealed with an adhesive strip (R&D Systems, USA) and incubated at room temperature for 2 h. Aspiration and washing was repeated. 100µl of detection antibody was added to each well and incubated for 2 hours covered with an adhesive strip. Aspiration and washing was repeated. 100µl of Streptavidin-HRP working solution was then added to each well. The plate was covered with an adhesive strip and incubated at room temperature for 20 min avoiding direct light. Aspiration and washing was repeated. 100µl of Substrate solution (R&D Systems, USA) was added per well and incubated for 20 min at room temperature avoiding direct light. 50µl of stop solution (R&D Systems, USA) was added directly to the substrate solution. The plate was lightly tapped for a thorough mix. The optical density was determined using a microplate reader (Mason Technology, Ireland) at 450nm.

2.2.4 Electrochemilluminescence

A multi-array sandwich immunoassay kit (Meso Scale Discovery MSD®, Maryland, USA) was used to quantify the cytokine serum concentrations of the patients. The human

Proinflammatory 9-plex multispot 96 well plates (MSD # N05007A-1) (Figure 2.3) was used. Standards provided by the manufacturer were diluted in Diluent 2 and used to produce a standard curve (Table 2.4).

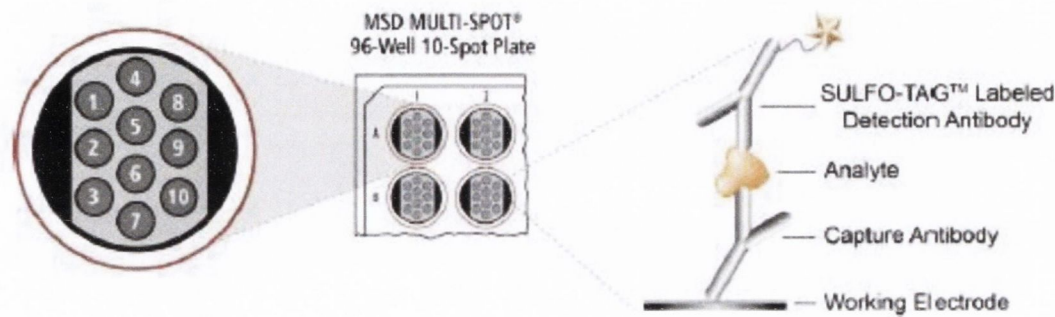


Figure 2.3 The 10 spot per well layout of an MSD plate. The 1=IL-2, 2=IL-8, 3=IL12p70, 4=IL-1 β , 5=GM-CSF, 6=BSA blocked, 7=IFN- γ , 8=IL-6, 9=IL-10, 10=TNF- α . The analyte is captured in a similar way to the ELISA using a capture and detection antibody.

Table 2.4 Eight point standard curve used in the multi-array sandwich immunoassay

Standard	Human Proinflammatory 9-plex Calibrator blend (pg/ml)	Dilution factor
100x stock	1000000	
Dilution. Stock calibrator	10000	100
Standard (STD)-01	2500	4
(STD)-02	625	4
(STD)-03	156	4
(STD)-04	39	4
(STD)-05	9.8	4
(STD)-06	2.4	4
(STD)-07	0.61	4
(STD)-08	0	n/a

The layout of 96 well plate used in MSD Electrochemilluminescence was that of Figure 2.2. Each well contained an electrode and capture antibody for each of the cytokines in Figure

2.3. Standards calculated in Table 2.4 were added to rows A-H and Columns 1-2 in duplicate. Samples were then added in duplicate to rows A-H and columns 3-12.

The calculations of cytokine serum levels were generated by the MSD DISCOVERY WORKBENCH software which was incorporated into the mesoscale plate reader (Figure 2.4). The lower limits of detection (LLOD) were as follows (Table 2.5).

Table 2.5 LLOD is a calculated concentration based on a signal 2.5 standard deviations above the blank (zero) calibrator.

	IL-2	IL-8	IL-12p70	IL-1β	GM-CSF	IFN-γ	IL-6	IL-10	TNF-α
LLOD (PG/ML)	0.35	0.090	1.4	0.36	0.20	0.53	0.27	0.21	0.50

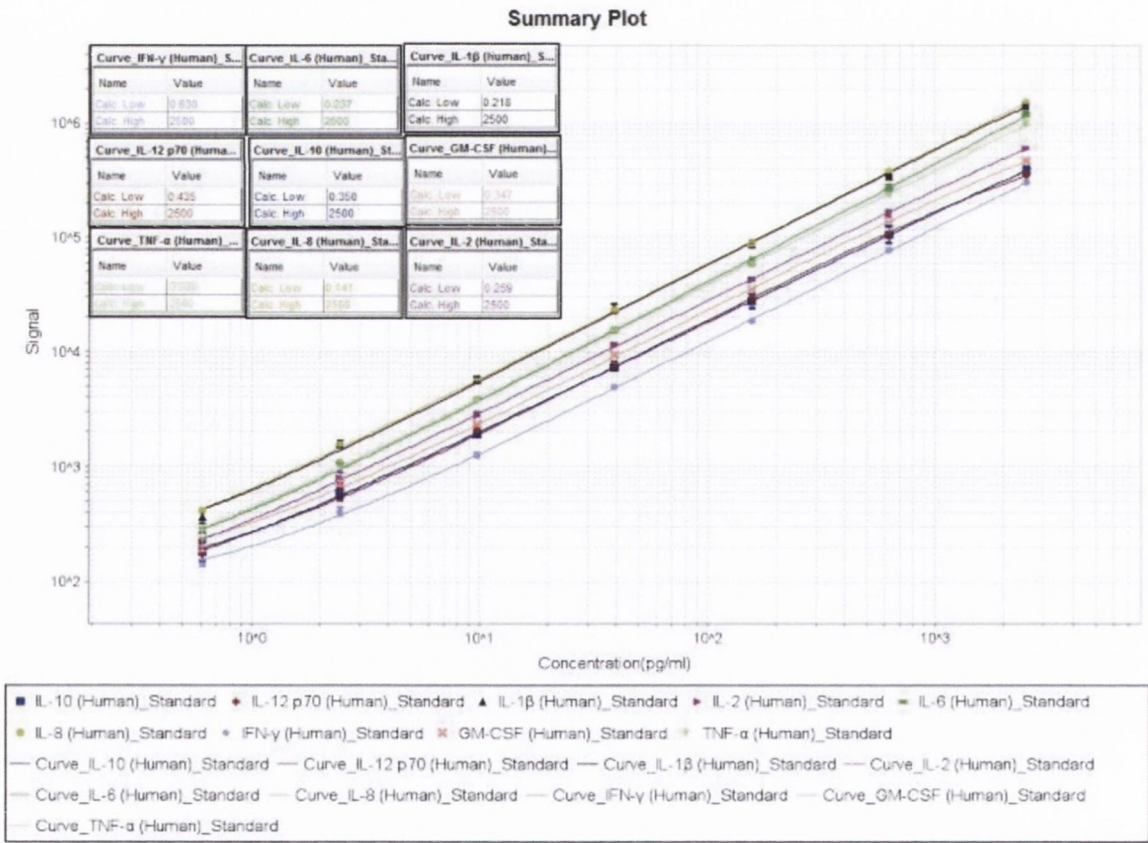


Figure 2.4 Standard curve plot of MSD. Results allow the determination of cytokine serum levels and the minimum sensitivity level (LLOD) for each cytokine.

2.2.5 Statistical analysis

Data generated was transferred to Microsoft excel 2007 (Microsoft) and GraphPad prism 5 (Graphpad Software Inc, California, USA) for statistical analysis. A p-value <0.05 was considered significant. Mann-Whitney non-parametric tests were used to compare two groups and an ANOVA was used for comparing more than three groups.

2.3 Results

The ECL and ELISA results were analysed separately. 58 patients were assessed in the ECL while only 23 patients were assessed for CXCL16 serum levels using an ELISA. Male patients had an average BMI termed overweight and females were in normal range.

2.3.1 *Electrochemoluminescence*

The nine cytokines analysed using ECL were divided into groups: carotid atherosclerosis patients v healthy controls, age, level of statin therapy, asymptomatic and symptomatic patients, risk factor and percentage of stenosis. Analysis of results led to the discovery that values of the following cytokines, GM-CSF (28/58), IFN- γ (10/58), IL-12p70 (43/58), IL-1 β (25/58) and IL-2 (25/58) were below the lower limits of detection. High values and outliers evident in the results were also associated with several patients.

2.3.1.1 *Carotid atherosclerotic patients' v healthy control comparison of serum cytokine levels*

Four out of the nine cytokines showed a significance difference when comparing patients and controls (Figure 2.5). IL-10 ($p=0.0190$), IL-8 (0.0003), IL-6 (0.0096) and TNF- α (< 0.0001) (Table 2.6). Cytokines GM-CSF, IFN- γ , IL-12p70, IL-1 β and IL-2 had insignificant differences. These five cytokines had values recorded below the LLOD. Outliers were present in all groups except TNF- α and IFN- γ . IL-10 was the only cytokine to show a significantly higher level in serum in the control group which reflects on its predominant anti-inflammatory potential. Although TNF- α showed the strongest significance between the two groups, IL-8 had overall the highest levels in patients serum. Due to the potential bias that age and statin therapy may influence patients' serum cytokine levels, significance testing was carried out comparing patients' age.

Table 2.6 Median, standard deviation (SD), p value and Mann-Whitney test of each cytokine based on the comparison of 58 patients and 10 controls involved.

Cytokine	Patients n=58	SD	Controls n=10	SD	P value	Mann-Whitney U
GM-CSF	0.120096	6.576059	0.327154	2.941852	0.6958	267
IFN- γ	1.187724	2.3668	2.058665	2.522874	0.0759	187
IL-10	1.816925	24.91485	7.495081	3.58058	0.019	154
IL-12 P70	0.36866	41.57718	1.43296	4.68497	0.7619	272
IL-1 β	0.164455	0.787272	0.492052	0.336889	0.1209	200
IL-2	0.378181	12.89967	0.926288	0.631628	0.4618	247
IL-6	2.017493	54.48914	1.115554	7.089154	0.0096	140
IL-8	20.02073	301.1386	9.997641	5.393339	0.0003	136
TNF- α	5.527738	3.272491	1.101132	1.271686	< 0.0001	18

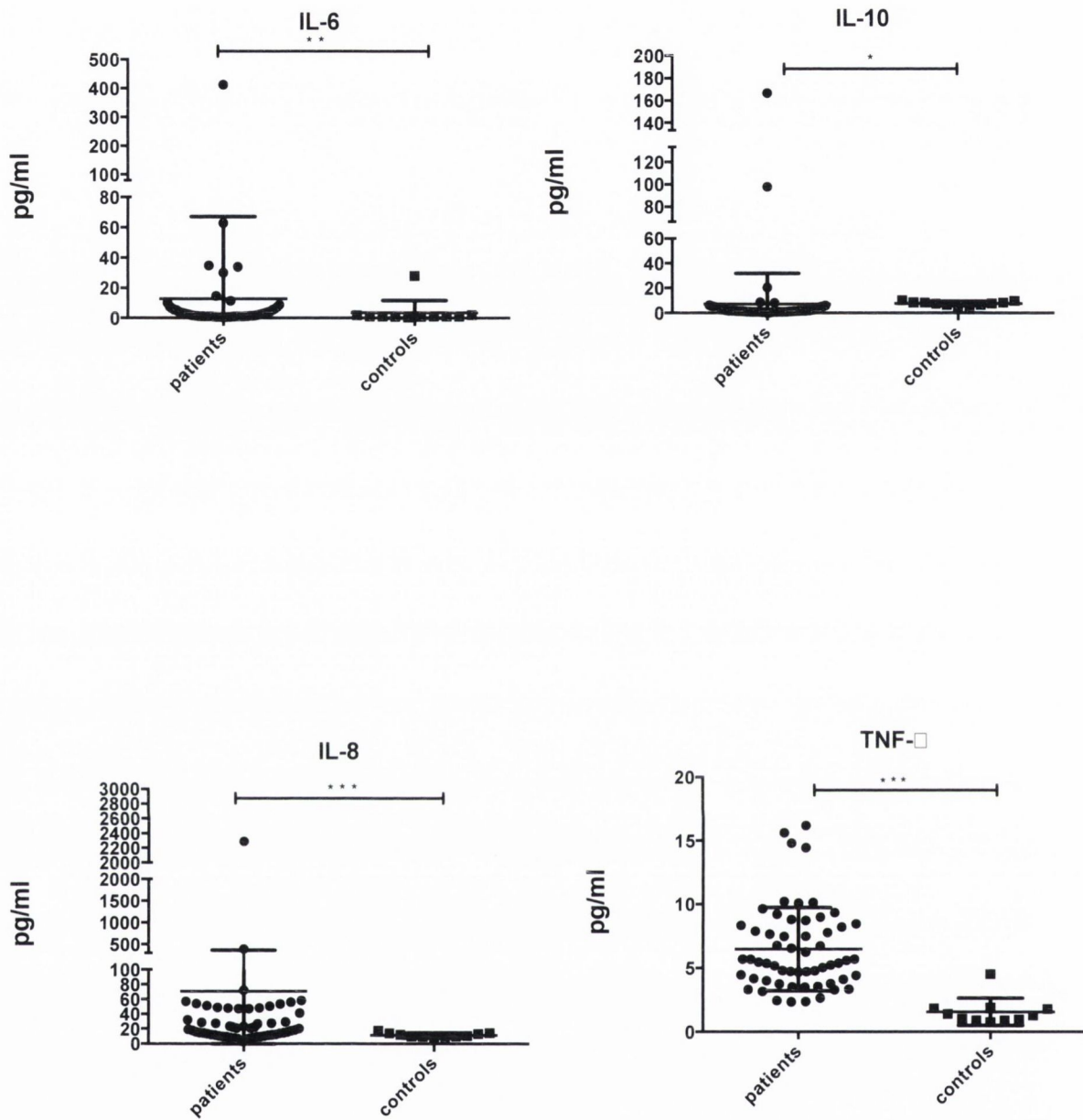


Figure 2.5 Serum concentrations of IL-10, IL-6, IL-8 and TNF- α in pg/ml of atherosclerotic patients' (n=58) v healthy volunteers (n=10). Each sample was done in triplicate and median and standard deviation are shown. Significance was tested using a Mann-Whitney test $* < 0.05$, $** < 0.01$ and $*** < 0.001$.

2.3.1.2 Comparing serum cytokine levels of carotid atherosclerotic patients' <65 and >65 years of age

No significant difference was found between any of the nine cytokines when comparing serum levels in patients below 65 years and over (Table 2.7). However, all of the outliers are seen in patients 65 and older.

Table 2.7 Median, standard deviation (SD), p value and Mann-Whitney test of each cytokine based on the comparison of 58 patients divided into < and > 65 years of age.

Cytokine	<65 n=12	SD	>65 n=46	SD	P value	Mann-Whitney U
GM-CSF	0.344678	0.690813	0.098001	7.366406	0.4472	236
IFN- γ	1.204138	0.792014	1.134477	2.630057	0.6245	250
IL-10	1.723502	2.09869	1.816925	27.89364	0.7808	261
IL-12 P70	0.217397	1.093047	0.276204	46.69532	0.4521	236.5
IL-1 β	0.153218	0.447335	0.094262	0.874851	0.5866	221
IL-2	0.711665	1.101755	0.347531	14.47498	0.2781	219
IL-6	1.645724	9.595307	2.605646	60.95757	0.342	226
IL-8	21.01026	16.57677	20.02073	337.8222	0.8855	268
TNF- α	5.972363	3.421225	5.418332	3.26205	0.5202	242

2.3.1.3 Comparing serum cytokine levels of asymptomatic and symptomatic carotid atherosclerotic patients'

Asymptomatic patients are classified as low risk under the subject of percentage stenosis by the National Cholesterol Education Program (NCEP). Two out of the nine cytokines showed a significance difference when comparing serum levels between asymptomatics and symptomatics (Figure 2.6). Both IL-6 (p=0.0046) and IL-8 (p=0.0016) levels were found to be higher in asymptomatic patients when outliers were not considered. No other significant differences were detected (Table 2.8).

Table 2.8 Median, standard deviation (SD), p value and Mann-Whitney test of each cytokine based on the comparison of 58 patients divided into asymptomatics and symptomatics.

Cytokine	Asymptomatics n=27	SD	Symptomatics n=31	SD	P value	Mann-Whitney U
GM-CSF	0.066967	0.051644	0.004093	0.157201	0.3172	354
IFN- γ	1.189212	1.890827	1.180932	0.774845	0.4131	365.5
IL-10	1.96129	1.494933	1.083835	5.336507	0.1852	333
IL-12 P70	0.277685	0.235788	0.221912	0.530068	0.1366	322.5
IL-1 β	0.197862	0.488711	0.134705	0.679869	0.374	361
IL-2	0.29969	0.236065	0.167644	0.148523	0.8516	406
IL-6	5.684883	10.68438	2.792447	105.8115	0.0046	236
IL-8	47.82313	18.05203	19.54847	10.53517	0.0016	215
TNF- α	5.197248	2.115263	3.781804	1.63672	0.5747	382

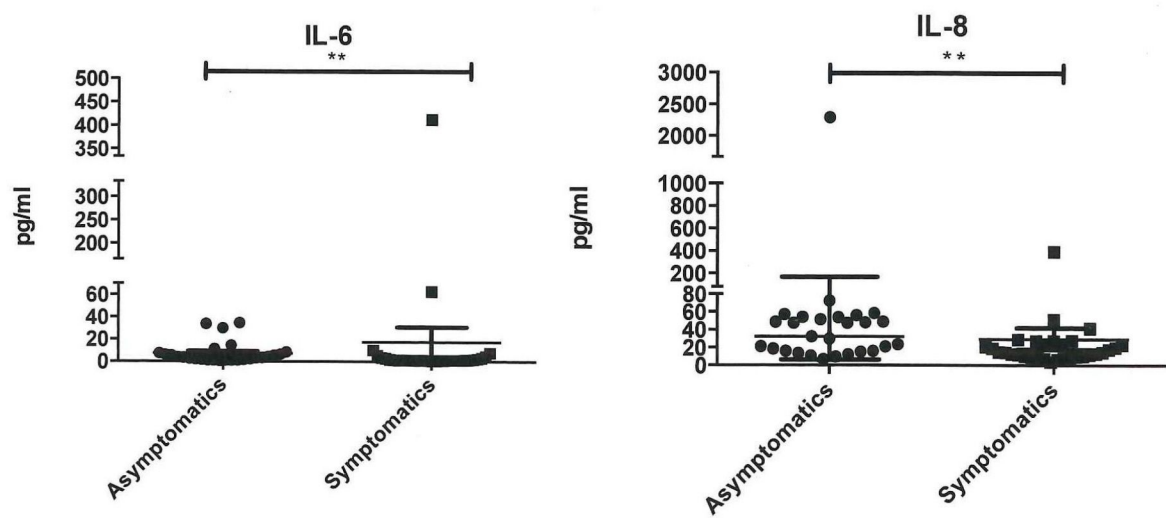


Figure 2.6 Serum concentrations IL-6, IL-8 in pg/ml of asymptomatic (n=27)and symptomatic (n=31) atherosclerotic patients. Each sample was done in triplicate and median and standard deviation are shown. Significance was tested using a Mann-Whitney test *<0.05, **<0.01 and ***<0.001.

2.3.1.4 Comparing serum cytokine levels of carotid atherosclerotic patients' with one or two risk factors associated with atherosclerosis.

Patient risk factor data can be seen in Table 2.9. In this study risk factors; smoking and hypertension were analysed for correlation with cytokine serum data. Patients with diabetes were included also. Patients who were ex-smokers were not included as a risk factor as it had been a year or more since individuals had given up and it could not interfere with cytokine readings. No trend or significance was observed between patients who had one or two or more risk factors (Table 2.9).

Table 2.9 Median, standard deviation (SD), p value and Mann-Whitney test of each cytokine based on the comparison of 58 patients divided into patients with 1 and 2 or more risk factors

Cytokine	1 Risk factor n=26	1 SD	2 Risk factors n=22	2 SD	P value	Mann-Whitney U
GM-CSF	0.117621	0.77172	0.10742	8.628709	0.9834	284.5
IFN- γ	1.422871	2.098342	1.125894	1.634118	0.828	275
IL-10	1.88365	18.99521	1.554651	1.81769	0.4257	247
IL-12 P70	0.277685	1.118003	0.342945	1.014866	0.3789	243
IL-1 β	0.088909	0.654545	0.139212	0.385537	0.131	195
IL-2	0.490495	1.014834	0.352749	3.922797	0.893	279
IL-6	2.242196	80.3559	1.738953	15.27766	0.7019	267
IL-8	16.8061	16.95155	26.23797	79.46068	0.1564	217
TNF- α	4.78595	3.780693	5.697574	1.944512	0.4882	252

2.3.1.5 Comparing serum cytokine levels of carotid atherosclerotic patients' with and without hypertension.

No significant differences were detected comparing serum levels of patients with and without hypertension (Table 2.10). Outliers were evident in both groups.

Table 2.10 Median, standard deviation (SD), p value and Mann-Whitney test of each cytokine based on the comparison of 58 patients divided into patients' with and without hypertension (HTN)

Cytokine	HTN n=42	SD	NO HTN n=16	SD	P value	Mann-Whitney U
GM-CSF	0.10742	6.254372	0.109699	8.050939	0.6217	267.5
IFN- γ	1.165837	1.903827	1.278972	3.440206	0.813	281
IL-10	1.8708	15.05093	1.183845	2.430279	0.2936	238
IL-12 P70	0.331467	1.018378	0.46177	5.730218	0.6028	266
IL-1 β	0.179588	0.529667	0.143962	1.312459	0.8425	283
IL-2	0.381467	2.888432	0.33969	25.85267	0.8573	284
IL-6	2.412613	63.83238	1.885485	2.723132	0.8277	282
IL-8	20.02073	59.33943	21.12608	606.792	0.9623	291
TNF- α	5.697574	2.808388	5.290689	4.045486	0.9774	292

2.3.1.6 Comparing serum cytokine levels of carotid atherosclerotic patients', who smoke, used to smoke and have never smoked.

Using an ANOVA all four groups were assessed for significant differences of each cytokine. Results concluded only IL-8 had significant findings when comparing the difference of all four groups (Figure 2.7). However, when a Mann-Whitney was performed between two groups, both IL-8 and IL-6 showed important differences (Table 2.11). Mann-Whitney tests were performed between smokers and non-smokers, smokers and ex-smokers and ex-smokers and non-smokers. IL-6 (p=0.0185) had significantly higher levels in patients who were ex-smokers compared to smokers and IL-8 (p=0.0070) had significantly higher levels in non-smokers compared to smokers (Figure 2.7).

Table 2.11 Median, standard deviation (SD), p value (ANOVA) of each cytokine based on the comparison of 58 patients divided into patients', who smoke, used to smoke and have never smoked

Cytokine	Smokers	SD	Ex-smokers	SD	Non-smokers	SD	No risk	SD	p value
GM-CSF	0.479091	0.54726	0.078418	9.077837	0.162904	0.931471	0.043826	0.448425	0.7122
IFN-Y	1.125894	1.265731	1.143113	2.779662	1.608662	2.107193	1.416601	3.065425	0.8106
IL-10	1.507499	1.049553	1.8708	3.904755	2.622566	33.6129	1.3497	2.275994	0.1031
IL-12 P70	0.970655	0.928079	0.273672	4.017371	0.324273	0.665431	0.301241	0.244571	0.7769
IL-1B	0.139212	0.371027	0.167294	0.99066	0.08224	0.688753	0.454566	0.570778	0.7837
IL-2	0.50031	1.040817	0.281371	17.89889	0.420746	0.988978	0.33969	0.510076	0.8161
IL-6	1.287037	8.446688	2.787228	75.17781	4.310065	9.636144	5.517269	2.268133	0.7848
IL-8	15.92164	10.90911	18.17449	17.04608	44.46927	19.74175	26.15172	19.90138	0.0194
TNF-a	6.122527	3.262741	5.130122	3.465268	6.227429	2.100564	5.527738	1.414111	0.9139

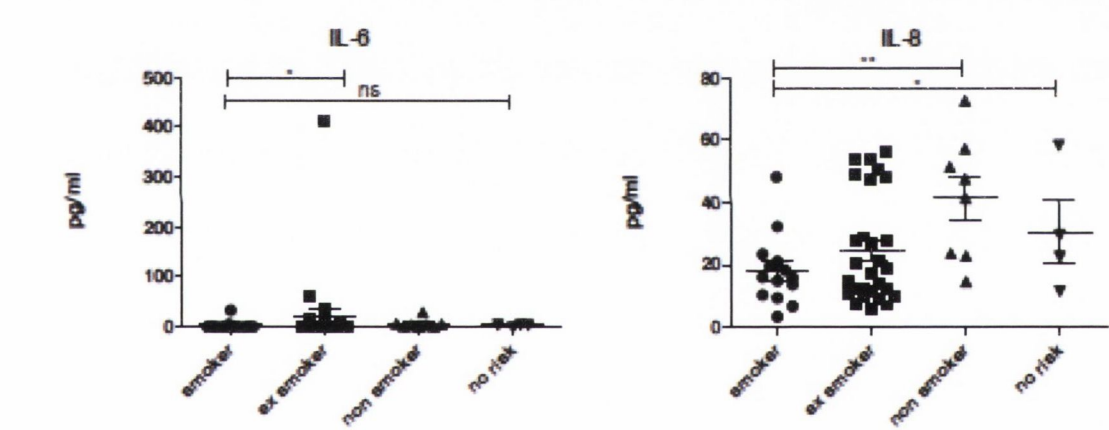


Figure 2.7 Serum concentrations of IL-8 and IL-6 in pg/ml of atherosclerotic patients' who are currently smokers (n=13), ex-smokers (n=39), never smoked (non-smokers n=8) and those who are not associated with any risk factor (n=4). Significance was tested using a Mann-Whitney test between 2 groups and an ANOVA comparing all four groups, *<0.05, **<0.01 and ***<0.001. Only IL-6 and IL-8 showed significance comparing individual groups and IL-8 showed a significant difference between all four groups.

2.3.1.7 Comparing serum cytokine levels of carotid atherosclerotic patients' who have more or less than 80% stenosis in one or both carotid arteries

Patients with less than 80% in stenosis in one or both arteries had over 20% stenosis in one or both arteries. Six out of nine cytokines had significantly different values when comparing the two levels of stenosis (Table 2.12). The following p values were found GM-CSF (p=0.0057), IL-12p70 (p=0.0193), IL-2 (p=0.0083), IL-6 (p=0.0193), IL-8 (p=0.0143) and TNF-α (p=0.0461). Excluding outliers, higher levels of TNF-α, GM-CSF, IL-12p70 and IL-2 and lower levels of IL-6 and IL-8 were found in patients with >80% stenosis (Figure 2.8). Outliers were found in both groups.

Table 2.12 Median, standard deviation (SD), p value and Mann-Whitney test of each cytokine based on the comparison of 58 patients divided into patients' who have more or less than 80% stenosis in one or both carotid arteries

Cytokine	>80% n=25	SD	<80% n=33	SD	P value	Mann-Whitney U
GM-CSF	0.924464	5.990478	0.08069	7.069246	0.0043	211.5
IFN-γ	1.194515	3.048172	1.180932	1.681938	0.9	404
IL-10	1.902262	32.9041	1.752342	16.97553	0.5096	370
IL-12 P70	1.010558	63.15707	0.313242	0.88785	0.0322	263
IL-1β	0.191984	1.04207	0.134705	0.521682	0.5987	378.5
IL-2	1.296712	19.26945	0.313666	3.229331	0.0143	244
IL-6	1.682519	6.951921	3.54029	71.68061	0.0282	263
IL-8	14.19172	454.7239	27.04291	65.43403	0.0079	223
TNF-α	7.652849	4.212686	5.164349	1.78093	0.0461	285

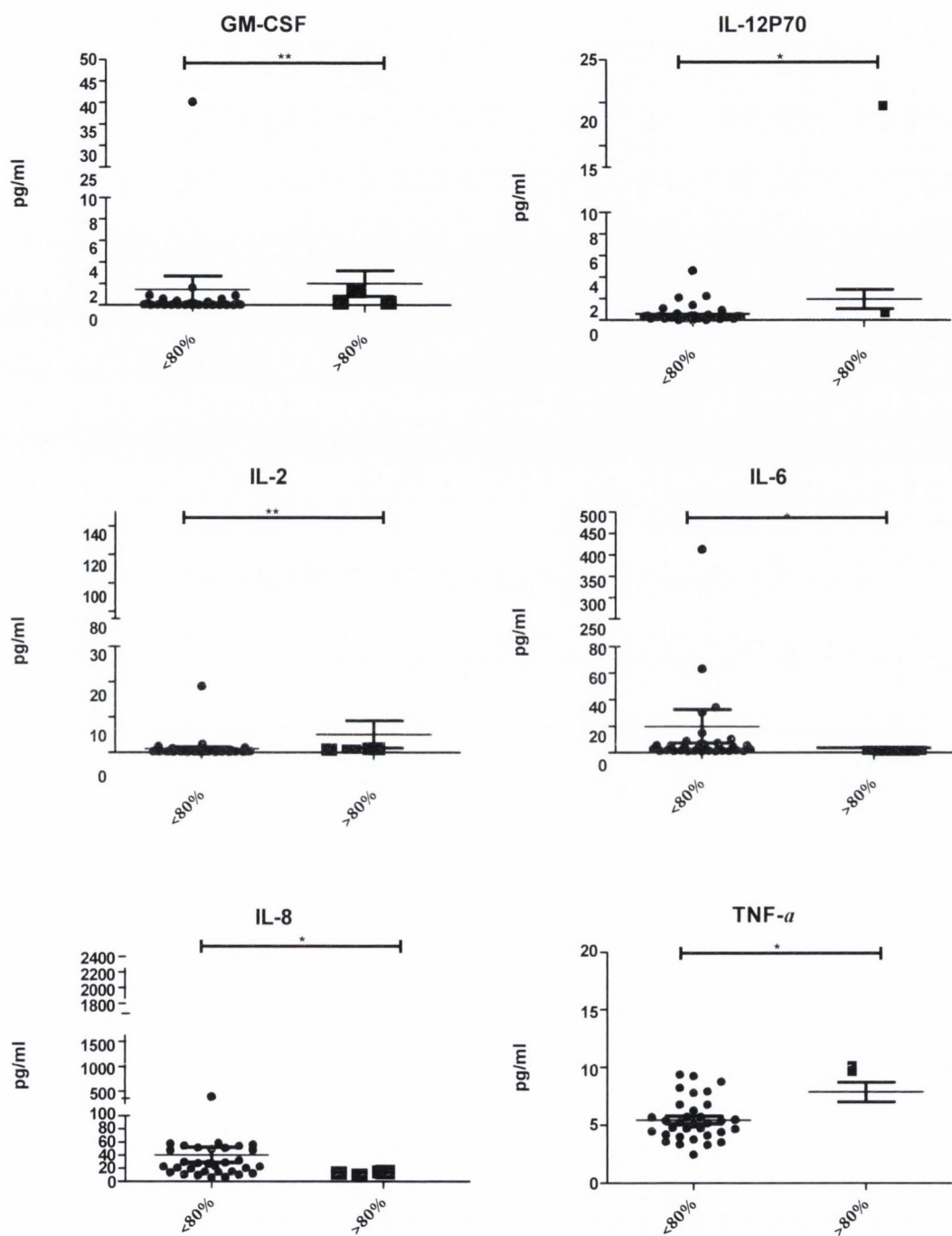


Figure 2.8 Serum concentrations of GM-CSF, IL-12p70, IL-2, IL-6, IL-8 and TNF- α in pg/ml of atherosclerotic patients' who have stenosis of <80% (n=33) and >80% (n=25). Each sample was done in triplicate and median and standard deviation are shown. Patients were grouped according to %stenosis in either right or left internal carotid artery. Significance was tested using a Mann-Whitney test *<0.05, **<0.01 and ***<0.001

- 5... 2.3.1.8 Investigating levels of each individual cytokine analysed between five vulnerable groups of
6... atherosclerotic patients to identify if there is a significant change.

Using a one way ANOVA there was a significant difference in expression of TNF- α between carotid patients who were hypertensive, symptomatic, ex-smokers and had more than 80% stenosis. The median level pg/ml was higher in the patients with more than 80% stenosis, the patients with least amount of TNF- α were hypertensive. Symptomatic patients and ex-smokers had similar pattern of expression. The remaining eight cytokines showed no difference in expression among the groups analysed. Therefore it may be suggested that TNF- α is a distinguishable cytokine expressed at a higher level when stenosis increases.

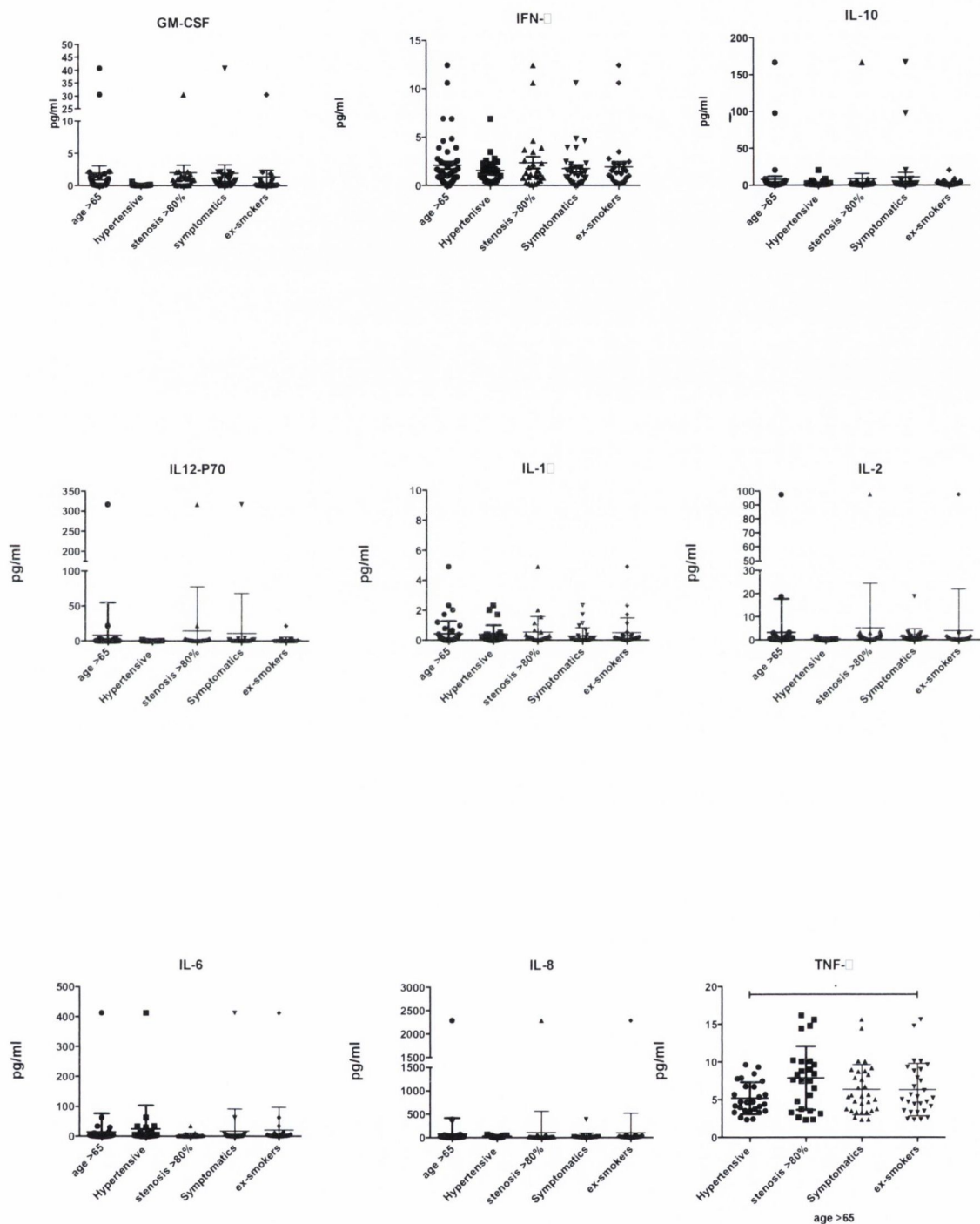


Figure 2.9 Comparison of cytokine levels in vulnerable groups of carotid endarterectomy patients. Groups were analysed using a one way ANOVA to identify if there was a significant difference among patients over 65 years (n=46), hypertensive (n=27), more than 80% stenosis (n=25), symptomatic (n=31) and ex-smokers (n=39). TNF- α was the only cytokine to show a significant difference when the group levels were analysed p= 0.0457.

2.3.2 Enzyme-linked immunosorbent assay

CXCL16 serum levels were measured and association analysis was carried out for the following groups;; Carotid atherosclerotic patients' v healthy controls, patients <65 v >65 years, asymptomatic v symptomatic and risk factors. Interestingly, no patient had an unusually high or low amount of CXCL16.

2.3.2.1 Carotid atherosclerotic patients' v healthy control comparison of serum CXCL16 levels

As shown in Figure 2.9 CXCL16 levels were significantly higher in healthy controls compare to carotid artery patients (p=0.0277) (Table 2.13). Serum levels of CXCL16 in controls had a larger range in values than patient values. No unusual outliers were present in either group. As previously discussed patient and controls were not ideally age matched therefore it was interesting to analyse if significance existed between patients who were classified as old age pensioners and younger.

Table 2.13 Median, standard deviation (SD), p value and Mann-Whitney test of CXCL16 based on the comparison of 23 patients and 10 controls involved

Cytokine	Patients n=23	SD	Controls n=10	SD	P value	Mann-Whitney U
CXCL16	50.77778	22.80291	0.327154	2.941852	0.0277	50.5

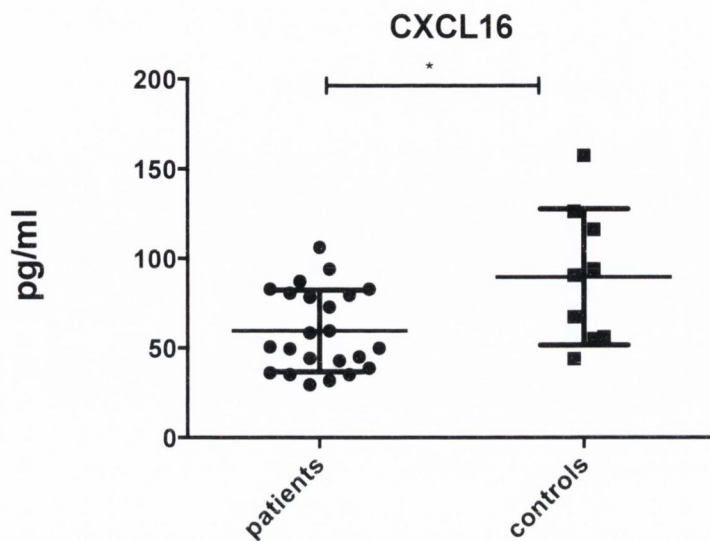


Figure 2.10 Serum concentrations of CXCL16 in pg/ml of atherosclerotic patients' (n=23) v healthy volunteers (n=10). Each sample was done in triplicate and median value and standard deviation are shown. Significance was tested using a Mann-Whitney test $p < 0.05$

2.3.2.2 Carotid atherosclerotic patients' <65 and >65 years of age serum CXCL16 levels

Evidently there were a larger number of patients who were over the age of 65, however measuring the difference of the median values; patients under 65 had a significantly higher level of serum CXCL16 ($p=0.0208$) (Figure 2.10).

Table 2.14 Median, standard deviation (SD), p value and Mann-Whitney test of CXCL16 based on the comparison of 23 patients divided into < and > 65 years of age

Cytokine	<65 n=6	SD	>65 n=17	SD	P value	Mann-Whitney U
CXCL16	80.77778	18.48505	45.22222	20.14883	0.0208	17.5

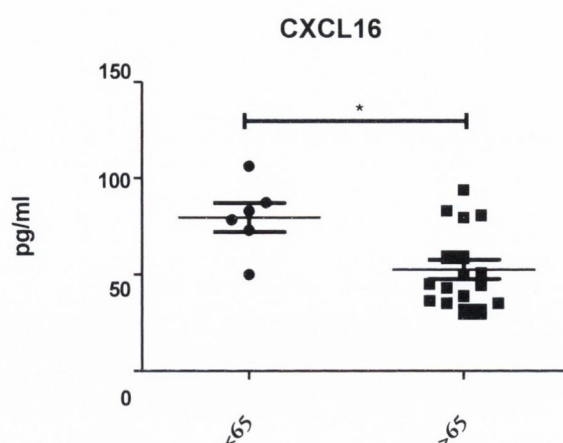


Figure 2.11 Serum concentrations of CXCL16 in pg/ml of atherosclerotic patients' <65 (n=6) and >65 (n=17) years of age. Each sample was done in triplicate and median value and standard deviation are shown. Significance was tested using a Mann-Whitney test *<0.05

2.3.2.3 Asymptomatic v symptomatic carotid atherosclerotic patients' serum CXCL16 levels

There were fewer asymptomatic patients than symptomatic patients when comparing serum CXCL16 levels. The range of symptomatics varied. However, comparing median values asymptomatics had significantly higher CXCL16 (p=0.0450) (Table 2.15) levels than symptomatics (Figure 2.11).

Table 2.15 Median, standard deviation (SD), p value and Mann-Whitney test of CXCL16 based on the comparison of 23 patients divided into asymptomatics and symptomatics.

Cytokine	Asymptomatics n=7	SD	Symptomatics n=17	SD	P value	Mann-Whitney U
CXCL16	78.55556	17.58488	44.83333	22.55823	0.045	25.5

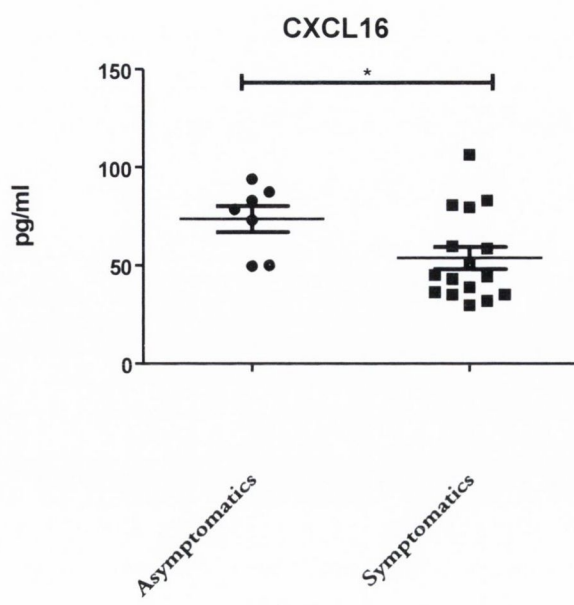


Figure 2.12 Serum concentrations of CXCL16 in pg/ml of asymptomatic (n=7) and symptomatic (n=17) atherosclerotic patients'. Each sample was done in triplicate and median value and standard deviation are shown. Significance was tested using a Mann-Whitney test *<0.05

2.3.2.4 Serum CXCL16 levels in carotid atherosclerotic patients' with one and two or more risk factors associated with atherosclerosis.

Both groups had a varied range of values and no significant difference was evident between patients who had either one risk factor or two or more (Table 2.16). The same result was also seen when comparing patients with and without hypertension (Table 2.17).

Table 2.16 Median, standard deviation (SD), p value and Mann-Whitney test of CXCL16 based on the comparison of 23 patients divided into patients with 1 and 2 or more risk factors.

Cytokine	1 Risk factor n=10	1 SD	2 Risk factors n=7	2 SD	P value	Mann- Whitney U
CXCL16	55.22223	20.28413	58.55556	22.97117	0.8071	32

Table 2.17 Median, standard deviation (SD), p value and Mann-Whitney test of CXCL16 based on the comparison of 23 patients divided into patients with hypertension (HTN) and without.

Cytokine	HTN n=15	SD	non-HTN n=6	SD	P value	Mann- Whitney U
CXCL16	50	24.23312	79.66667	14.43019	0.6282	52

2.3.2.4 Serum CXCL16 levels in carotid atherosclerotic patients' with above or below 80% stenosis.

As seen in Table 2.18 there were no significant differences in serum CXCL16 levels between < and >80% stenosis. A larger number of patients had > 80% also.

Table 2.18 Median, standard deviation (SD), p value and Mann-Whitney test of CXCL16 based on the comparison of 23 patients divided into patients with < or > 80% stenosis in either internal carotid artery

Cytokine	>80% n=16	SD	<80% n=7	SD	P value	Mann-Whitney U
CXCL16	58.55556	14.47079	50.22223	25.75515	0.8937	53.5

2.4 Discussion

Discovering new biomarkers and new relationships between biomarkers or risk factors in at risk populations could reduce the amount of reoccurring heart attack, stroke and deaths. A more detailed knowledge of circulating triggers could facilitate tailored therapy and new treatments (Shah et al., 2010). The study of blood biomarkers by ECL in combination with other high throughput technologies such as blood RNA profiling, which has correlated CAD severity with expression of 160 genes, would contribute greatly to predicting the risk of cardiovascular events (Shah et al., 2010).

Aukrust et al, 2008 reviewed potential biomarkers of cardiovascular disease and published main criteria needed for a biomarker to be considered important in diagnosis and prognosis. One of the main requirements was the ability to measure these biomarkers using inexpensive and standardized commercial assays which both have been provided in the technologies used in this chapter. The most valued biomarker in CVDs is CRP so this chapter aimed to discover other potential markers such as CXCL16 and IL-8. One of the challenges mentioned in this review was the fact chemokines are found at low levels in serum/plasma and often under the LLOD, this was not the case for this study as no value in any patient for either chemokine was below the LLOD. This study also covered the criteria needed to move inflammatory marker measurements into the clinic, “Studies that investigate the ability of combined measurements of several chemokines to give prognostic information” and “Studies that examine the predictive value of new chemokines, such as CXCL16” (Aukurst et al., 2008).

Both ECL and ELISA results were grouped based on age, risk factors and symptoms, ECL data was further analysed for association with % stenosis. Patient numbers were unsatisfactory to proceed with all groups for CXCL16 analysis.

and dose dependent manner stimulates IL-8 and MCP-1 release (Ni et al., 2009). In relation to hypertension pulmonary arterial hypertension (PAH) patients have shown significantly higher levels of IL-1B, IL-2, IL-6, IL-8, IL-10 and IL-12p70 (Soon et al., 2010). Currently IL-17 has been recognized as a potential therapeutic target for preventing angiotensin II-induced hypertension. IL-17 is a synergistic cytokine and TNF- α is one of many cytokines it forms an alliance with to modulate an inflammatory response (Madhur et al., 2010). However, this study reported no such findings and no difference in cytokine levels were detected in patients with and without hypertension.

Patients with diabetes were also included but due to a low number of diabetic patients its association with cytokine levels was not assessed. It is however known that IL-6 and TNF- α are up-regulated in diabetic patients compared to controls, as IL-8 is stimulated by TNF- α it is also known to increase in levels (Verma et al., 2013). Diabetes was included as a risk factor when comparing one or two or more risk factors. According to these results the amount of risk factors a patient had did not make a significant difference in cytokine levels. It was then decided to investigate if one particular risk factor was associated with increasing or decreasing cytokine levels. Current literature suggests cytokines/chemokines IL-1 β , IL-6, IL-8 and MCP-1 are expressed at a higher level in smokers compared to non-smokers (Kuschner et al., 1996). IL-8 was the only cytokine to show significantly different levels when all four groups were analysed using an ANOVA. However, when separate Mann-Whitney tests were performed IL-8 and IL-6 showed significant changes, both with different groups. IL-6 appeared to be expressed more in ex smokers compared to smokers and IL-8 showed more expression in non-smokers, values in non-smokers varied and deviated from the median more so than smokers. As most patients had more than one risk factor it is difficult to single out a risk factor as the cause of the change in cytokine levels.

Stenosis - TNF- α , IFN- γ , IL-1 β , IL-6, IL-8 and IL-10 have shown significantly higher levels in patients with less than 70% stenosis undergoing a carotid endarterectomy than controls (Profumo et al., 2008). This study looked at above and less than 80% stenosis and found GM-CSF, IL-2, IL-12p70, IL-6, IL-8, and TNF- α to have different levels between these two groups. The only two cytokines which showed a higher rate in patients with less than 80% were IL-6 and IL-8. It was noted that GM-CSF, IL-2, IL-12p70 had <LLOD considered. This data correlates with data seen in asymptomatic v symptomatic cytokine levels. Asymptomatics who typically have lower stenosis showed the same, lower IL-6 and IL-8 levels. Plaques which are classified as non-flow limiting stenosis are accountable for more cases of rupture and thrombosis (Shah, 2003). Therefore the association of IL-6 and IL-8 should be of further interest.

Statins- The majority of patients included in this study were currently being treated with a statin, and statins have been reported to affect the levels of serum cytokines. As this study was completed on a relatively small cohort and statin therapy and concentrations varied among patients it was not taken into consideration. Ten of the patients included in the study were on no form of statin when this study was carried out and a third were being treated with a low dose of 10-20mg of simvastatin and atorvastatin. Lyngdoh et al 2011 reported no association between statins and IL-1 β , IL-6 or TNF- α but found association with a decrease in CRP levels (Lyngdoh et al., 2011). However, the opposite findings exist in a study by Iwata et al which found IL-6 and IL-8 mRNA levels inhibited significantly by statins. Therefore, the evidence of statins affecting circulating cytokine levels is still questionable.

All p values were presented without correction for multiple testing, such as a Bonferroni correction. Although in this case we have tested multiple hypotheses, a strict Bonferroni correction is not appropriate, because the levels of the individual cytokines tested are not independent of each other, a prerequisite for the Bonferroni correction.

Establishing a trend amongst the cytokines in each association study was not achieved. Indeed in some cytokine level analysis all nine cytokines showed no significance when compared. The most noticeable relationship was that of IL-6 and IL-8, one did not show a significant difference in levels without the other.

2.4.2 ELISA

It has been confirmed that CXCL16 plasma levels are extremely stable under basal conditions and studies have shown that CXCL16 may have favourable biomarker characteristics for clinical epidemiologic and epidemiologic application (Lehkre et al., 2007 and Aukurst et al., 2008). As previously discussed conflicting data on CXCL16s role exists. Smith et al., 2008 found no correlation between CXCL16 and CAD severity based on coronary angiography in post MI patients (Smith et al., 2008) and neither did Sun et al., in SAP patients (Sun et al., 2008). Yi et al., discovered that CXCL16 expression levels do not affect the plaque size but the plaque stability and could potentially be a marker of destabilization (Yi et al., 2011). These two studies proposed the opposite to the Sheikine study in 2006, claiming that decreased CXCL16 levels supported the atheroprotective role of CXCL16. These data contribute to the already existing controversial findings of the role of CXCL16 in CAD and found that healthy controls and carotid atherosclerosis patients do have a significant difference;; CXCL16 levels were lower in patients' serum. The only cytokine to show this pattern of expression between patients and controls was IL-10, therefore CXCL16 could be atheroprotective. As risk factors and age are known to impact cytokine profiles in CVD

patients, it was interesting to see if CXCL16 was influenced by such factors. Using the over 65 and under age bracket, CXCL16 levels were significantly higher in patients less than 65 years of age. However, a fraction of over 65s had similar pg/ml CXCL16 to those under 65.

As previously mentioned combining expression profiling with arrays for personalised therapy for at risk patients is something to research further. Gargalovic et al., used individual EC cultures from 12 heart transplant donors to confirm common genetic variations have a significant impact on cytokine expression, specifically IL-8, in response to oxPAPC.

Many of the cytokines analysed in this study effect one another's expression levels. Soluble CXCL16 increases IL-8 release in VSMCs from CAD patients and IL-1 β mediates CXCL16 release from ECs and PBMCs (Smith et al., 2008). The only group that found both CXCL16 and IL-8 significantly different were asymptomatics and symptomatics. CXCL16 and IFN- γ take part in a feedback loop system in which both are inducers of the other, Lehrke suggested that this helps regulate atherogenic effects however the two did not display similar effects in this study (Lehrke et al., 2007)

2.4.3 Concluding remarks

As expected, IL-6 showed relevance in a number of patient groups analysed in this study as it is already regarded as a useful tool for clinicians along with CRP levels. Regarding patients' v healthy control data the cytokines/chemokines of further interest included IL-8, IL-6, TNF- α , IL-10 and CXCL16. The cytokines/chemokines that showed significant differences most frequently were IL-6 and IL-8, which were associated with patients and controls, asymptomatics v symptomatics, stenosis and smoking. Other known markers that normally show association with CVDs such as IL-1 β and IFN- γ did not produce any significant results.

On top of statin treatment for lipid control in atherosclerotic patients it would be beneficial to control inflammation as well as immune cells and cytokines play such a central role in plaque

development and rupture. Using these significant *in vivo* findings it was decided to further investigate the relationship of TNF- α on EC expression, in particular CXCL16.

To date this multi-array method has not been published regarding the use of carotid patients, however analytes such as cTroponin I and Myosin binding protein-C have been analysed in myocardial infarct patients. As discussed in the introduction, carotid and coronary atherosclerosis have both similarities and differences; this type of platform could be a potential way of investigating them in relation to inflammatory mediator expression. So far, comparing the results of carotid patients in this project and of coronary patients in Patel et al., 2009, IL-10 was significant in both and IFN- γ in coronary and TNF- α , IL-8 and IL-6 in carotid. This MSD platform allows for an increasing dynamic and sensitivity scale for low levels of inflammatory mediators. The detection of small changes amongst an array of cytokines can potentially be preventative in the further progression of disease in atheroma patients and could help establish a difference of expression in asymptomatic and symptomatic patients. This type of multiple cytokine measuring could be an essential part to the diagnosis and prognosis of at risk patients.

2.4.4 Future work

A larger cohort could potentially shed more light on the association of CXCL16 levels and the groups which were only analysed for ECL, such as smoking and statin therapy. The number of patients was insufficient to group patients into more than two groups. Also because there was a significant difference in CXCL16 patients over and under 65 years, data should be age matched when analysing this chemokine. For future carotid and coronary comparisons it would also be useful to screen both carotid and coronary patients serum samples using MSD and a combination of lipid, protein, anti/pro-inflammatory mediators to highlight the similarities and differences which will be of importance in the ongoing debate of IMT and CAD relations.

2.4.5 Limitations

The majority of patients that took part in this study were already on a type of statin before cytokine serum values were measured. Statins have known to lower CXCL16 and other cytokine expression levels, both simvastatin and atorvastatin lowered CXCL16 serum levels over a 6 month period (Smith et al., 2008). Simvastatin also down-regulated circulating levels of IL-6, IL-8 after 6 weeks and all statins lowered levels in cultured models in a time-dose dependent manner (Rezaie-Majd et al., 2002). However, this study measured the difference between each cytokine in the serum of patients receiving treatment and not. No significance was found no matter what dose they received. Also five out of the nine cytokines analysed by ECL included levels below the LLOD, these remained included in the analysis as they were still above 0.00. However, between patient and control data not one of the five showed significant difference in serum levels. The only analysis that they showed significance in was % stenosis. Comparing clinical findings is always controversial as patients have different backgrounds genetically and environmentally.

CHAPTER 3

Optimization of a novel microfluidic biochip for protein
expression analysis in endothelial cells under pulsatile shear
stress

3.1 Introduction

The atherosclerotic environment is complex and consists of many factors that contribute to plaque initiation, progression and rupture. In chapter 2 two circulating proteins identified with different expression levels between patients and controls were CXCL16 and TNF- α , therefore were ideal contributing factors of atherosclerosis to be further assessed in a controlled environment. For a positive control it was decided to use ICAM-1 as a marker as literature has reported on TNF- α and ICAM-1s relationship (Chiu et al., 2004, Chen et al., 2004, Krunkosky et al., 2000). In the majority of existing studies ECs response to shear stress and stimulants are analysed by protein expression using western blots and gene expression by mRNA assays. This study uses a completely new format to analyse the presence and amount of protein expression. The Cellix Biochip model was specific for the requirements necessary in this study, an EC only, laminar flow pulsatile environment.

3.1.1 Primary triggers

In the current literature, one of the main and initial triggers in atheroma formation is irregular shear stress patterns in arteries. Altered hemodynamics occurring in areas of curvature or bifurcations and hypercholesterolemia all have the potential as primary triggers in atherosclerosis by activating the endothelium monolayer. Pro-atherogenic conditions include, low, reversing and oscillatory shear stress and temporal variations of shear stress (Anssari-Benam and Korakianitis, 2013). However, clinical findings have found plaque development in sites of steady and high shear stress implying that additional factors play an equally large role in plaque formation. Other hemodynamic factors like circumferential stress waves combined with the effect of wall shear stress can cause endothelial cell mechanotransduction (Anssari-Benam and Korakianitis, 2013). The expression of two EC receptors; ICAM-1 and CXCL16 were focused on. Previous data published regarding their relationship with shear stress is sparse and are based on alternative methodologies.

ICAM-1 expression is controlled by hemodynamic stress, due to the shear stress response element activation in its promoter region (Nagel et al., 1999). Shear stress response elements (SSREs), within promoters of endothelial genes interact with transcription factors such as NF- κ B, EGR-1, c-Jun and c-Fos and have been reported as responsive to different types of flow. All four SSREs show increasing levels from static to uniform to disturbed flow.

In a mouse model of carotid artery ligation Ni et al., identified CXCL16 as one of many mechanosensitive genes which responds to disturbed flow. A genome wide microarray study was carried out on endothelial RNAs isolated from atherosclerotic plaque in mice. After 12 hr a number of genes involved in cell proliferation and morphology including CXCL16 were regulated by disturbed flow (Ni et al., 2010).

ICAM-1 is expressed in normal ECs of the healthy artery (Davies et al., 1993). Studies have shown ICAM-1 up-regulation in HUVECs is time and shear stress dependent (Nagel et al., 1999) and normal to high shear stress (over 20 dyne/cm² is atheroprotective) is associated with an increase in ICAM-1, specifically in junctional regions (Walpole et al., 1995). However, in atheroma-susceptible regions of the arteries ICAM-1 expression is also up-regulated (Frank and Lisanti, 2008) and circulating levels of ICAM-1 have been associated with myocardial infarctions and coronary death (Luc et al., 2003) suggesting its significance as a biomarker. Low SS which occurs at plaque sites (Stone et al., 2007) increases ICAM-1 regulation in ECs (Chatzizisis et al., 2007). Although data on ICAM-1 expression levels and role in atherosclerosis initiation are not conclusive (Cybulsky et al., 2001) Estrada et al., reported that ECs do not have a significantly different ICAM-1 expression pattern between disturbed and normal flow conditions (Estrada et al., 2011). In

conclusion based on the current literature, ICAM-1 is present in all conditions, healthy or diseased, high or low shear stress, and whether or not it is a marker of disease remains undecided.

As discussed in the introduction, low SS regulates EC gene expression via NF- κ B activation, therefore a median range of SS, between low and high shear stress as classified in the literature (0.5-20 dyne/cm²), and a healthy (unstimulated) and disease-like (stimulated) environment were chosen to investigate and clarify the induction and regulation of CXCL16 and ICAM-1.

3.1.2 Secondary triggers

The secondary triggers which facilitate plaque development are immune cells, cytokines and chemokines, but can also be primary triggers from an existing inflammatory disease i.e. RA, SLE (Sakurada et al., 1996, Sherer and Shoenfeld, 2006). In this chapter two triggers were investigated, a cytokine TNF- α and an oxidised lipid oxPAPC.

TNF- α can alter EC gene expression that promotes an inflammatory environment. *In vitro* models and mouse models have been used to investigate cytokine stimulation on EC expression. Under laminar flow in a parallel plate flow chamber, TNF- α stimulated ECs show an increase in mRNA ICAM-1 expression in ECs and when static conditions returned, ICAM-1 expression did not persist. TNF- α activates ICAM-1 expression through TNFR1 binding and NF- κ B pathway (Zhou et al., 2007). On the promoter region of ICAM-1 gene there is a NF- κ B specific binding site, which suggests its pro-inflammatory connection (Chiu et al., 2004). Chiu et al found TNF- α (100u/ml) stimulated ICAM-1 mRNA expression at 4 hours. However, introducing a shear stress of 20 dyne/cm² significantly increased ICAM-1 mRNA expression. In ischemic mouse studies, ICAM-1 expression was lower in mice that

received anti-TNF- α mAb than that of control subjects, confirming that ICAM-1 expression was attenuated by blocking TNF- α (Yang et al., 1998).

IFN- γ and TNF- α are both known inducers of endogenous CXCL16 in ECs (Abel et al., 2004) and conflicting data remain for SMCs as Wagsater et al., only found 10 ng/ μ l IFN- γ to be a significant inducer of CXCL16 (Wagsater et al., 2006). Other cells and cytokines associated with inflammatory disease have had an impact on CXCL16 expression. In RA patients TNF- α enhances CXCL16 expression in macrophages and contributes to the inflammatory cascade (van der Voort et al., 2005). Little research has been carried out on TNF- α and CXCL16 relationship, specifically under pulsatile flow conditions. A specific concentration of TNF- α was used, due to already established findings on ICAM-1 and CXCL16 expression and it was also seen in the carotid patient serum levels in chapter 2 (Abel et al., 2004).

The literature on the role oxPAPC plays in atherogenesis is divided. Some studies suggest the concentration to be of vital importance, Starosta et al, 2011 found oxPAPC to have a dose-dependent effect on EC permeability; low doses (5-20 μ g/ml) were considered atheroprotective and high doses (100 μ g/ml) pro-atherogenic. Other studies are divided when its role as a stimulant is questioned, Amberger et al, 1996 showed chronic exposure by moderately-highly oxidised LDL induces VCAM-1, ELAM-1 and ICAM-1 (Amberger et al., 1996). However, Birukov 2006 reported that EC activation can be triggered by incubation with oxidised lipids such as oxLDL and oxPAPC, but they ruled out ICAM-1 related expression (Birukov, 2006). So far a number of studies which have investigated the effect of oxPAPC have not established it as a trigger for CXCL16. Gargalovic et al, uncovered 1000 genes, 600 upregulated and 400 down-regulated by 50 μ g/ml oxPAPC in ECs, none of which were CXCL16 or ICAM-1 (Gargalovic et al., 2006). OxPAPC is however known as a

molecule which has the ability to increase chemokine IL-8 and MCP-1 production from ECs (Birukov, 2006).

oxPAPC also has an effect on SREBP increasing activity in some cell lines (Gargalovic et al., 2006). SREBPs are transcription factors which regulate genes involved in cholesterol synthesis and receptor-mediated LDL uptake. Using a parallel plate flow channel and a laminar SS of 12 dyne/cm², studies showed an increase in LDLR expression, and a sustained increase using a disturbed flow. SS also regulates SREBPs through EC integrins (Liu et al., 2002).

3.1.3 Hypotheses & Objectives

For this chapter, the objectives were to build an *in vitro* microfluidic model that investigates the effect of shear stress with different stimuli on the expression of different proteins involved in atherogenesis, to optimize and validate the *in vitro* microfluidic model for expression related studies. Using this model, the aim was to use quantitative & qualitative techniques to signify the difference in EC receptor expression under a series of different conditions and triggers associated with atherosclerosis. Overall, to investigate if initiating factors (cytokines, lipids or shear stress) by the effect they have on important EC receptor expression.

The effect of primary and secondary triggers on ECs was of particular interest. In this chapter the primary trigger; shear stress and secondary triggers; cytokine TNF- α and minimally oxidised lipid oxPAPC on ICAM-1 and CXCL16 receptor expression from ECs was studied. A time scale of 6 and 24 h was also incorporated into the study to investigate continuous expression.

- As CXCL16 was identified as a mechanosensitive gene to disturbed flow, this study hypothesized it could be present under laminar flow also. Because TNF- α has shown

effect on CXCL16 expression, it was expected to see a bigger increase combining the two effects compared to just shear stress regulation.

- Because ICAM-1 has a SSRE in its promoter region and has a NF- κ B binding site, this study aimed to investigate if TNF- α ECs under normal flow increased regulation of ICAM-1.
- We also aimed to investigate a component of highly oxidised LDL and gain insight on the potential activating power of oxPAPC and its effectiveness at promoting atherogenic environment or protecting against an atherogenic environment. As both oxPAPC and SS have an effect on TFs that regulate receptor-mediated LDL uptake, and CXCL16 is a receptor for oxLDL we thought it would be interesting to quantify the effect on expression. It was also interesting to distinguish if oxPAPC could potentially activate ICAM-1 expression.
- ICAM-1 expression is present in normal arteries and in lesion sites to facilitate the binding of monocytes through LFA-1 for transmigration and subsequent foam cell formation. As CXCL16 is also expressed on the EC, has a controversial role and binds CXCR6 on leukocytes to facilitate transmigration also, the scale of CXCL16 expression compared to that of a known pro-atherogenic factor such as ICAM-1 was of particular interest.

3.2 Materials and Methods

3.2.1 Thawing HUVECs for Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained frozen in liquid nitrogen in cryovials from PromoCell, Germany. The cryovials were thawed in a 37°C waterbath for 90 seconds and transferred to a Nunc 25cm³ flask (Thermoscientific, MA, USA) containing 10ml of pre-heated endothelial cell growth medium (PromoCell, Germany) using a sterile transfer pipette (Sarstedt, Germany) in a sterile environment. Cells were then incubated at 37°C and 5% CO².

3.2.2 Subculturing cells

When cells reached ~90% confluency they were transferred from the incubator to a sterile environment. All media were removed with a 10ml transfer pipette. Cells were then washed with 5ml PBS (Gibco, Invitrogen, CA, USA) and 5 ml pre-warmed accutase (PromoCell, Germany) was used, the flask was then incubated for 2-3 minutes at 37°C. The detached cells were transferred to a 15ml falcon (Sarstedt, Germany) and placed in a centrifuge (Eppendorf, Germany) for 5mins at 400xg at 21°C. Cell passages 5-7 were used.

3.2.3 Cell counting

Before starting experiment, cells were quantified using a haemocytometer. The haemocytometer was wiped with 70% ethanol. 10µl of thoroughly mixed cell suspension was pipetted directly onto the top and bottom grid. A fresh coverslip was carefully placed onto the haemocytometer avoiding air bubble formation that would displace cells and skewer cell count. The haemocytometer was placed on the light microscope stage and using the 10x objective lens the cells in each of the four corner squares were counted. Only the inner 16 smaller squares of each of the four larger squares were used for cell count. The total is divided by four to give a mean number of cells per 0.1µl. To determine how much of the

stock cell suspension was used the mean value was multiplied by 10,000 to give concentration of cells/ml. HUVECs were seeded into each of the eight channels on the Vena8 Fluoro+TM Biochip (Cellix, Ireland) (Figure 3.1) at a concentration of 100,000/20 μ l.

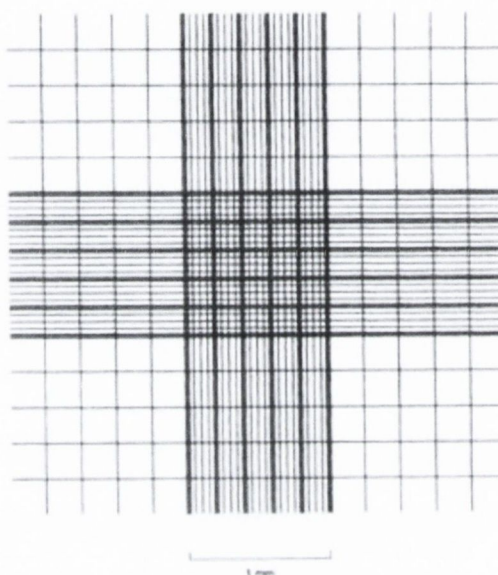


Figure 3.1 Counting grid on haemocytometer. 4 corner squares made up of 16 smaller squares.

3.2.4 Optimization of Vena8 Fluoro+TM Biochip: For cell receptor studies under conditions mimicking physiological flow.

Features of the Vena8 Fluoro+TM Biochip include; 8 parallel enclosed microcapillaries, suited for confocal microscopy observation combined with flow based experiments (Figure 3.2), 20x, 40x, 60x, 100x short working distance magnification microscopy; 60x, 100x oil immersion microscopy, brightfield / phase contrast / fluorescent microscopy, low level fluorescence imaging, low fluorescent background, compatible with confocal microscopy, suitable for a wide range of cell suspensions and whole blood, easy to coat microcapillaries with a range of different adhesion molecules, biochip plastic is optically clear permitting

detailed microscopy studies, 0.05 – 450 dyne/cm² shear stresses and real time imaging under flow conditions.

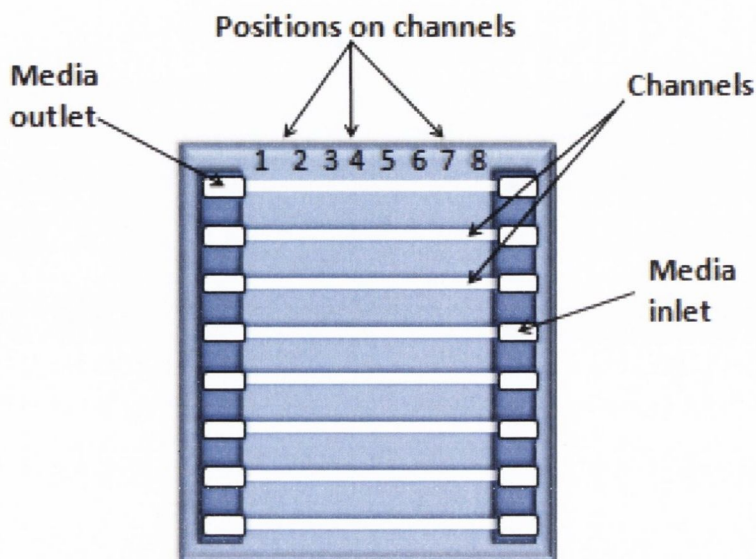


Figure 3.2 Diagram of Vena8 Fluoro+TM Biochip. Media flows in through media inlet and out through media outlet where it flows back to a 50ml pyrex flask embedded in the iKima pump. The positions are for image and analysis purposes.

Sterile biochips were opened in a sterile environment and 20ul of fibronectin (R&D Systems, USA) was pipetted into all eight channels. The chip was then placed in a sterile petri dish (Sarstedt, Germany) overnight at 4°C for coating. Cells were harvested and counted and 20µl of 1.5×10^6 per 100µl were pipetted into each channel. The chip was incubated for 15-20 min at 37°C 5% CO². Cells were observed under a light microscope at 10x to examine for even spreading throughout. The reservoirs at either side of each channel were filled with 30µl of

media and the chip was returned to a sterile petri dish and incubated at 37°C for a further 1.5-2 hours for sufficient attachment to the fibronectin monolayer.

Table 3.1 Technical and specifications of the Vena8 Fluoro+™ Biochip:

Material:	Acrylic, Topas
Number of channels per biochip:	8
Volume of each channel:	0.8 µL
Dimensions of each channel:	400 µm (W) x 100 µm (D) x 28 mm (L)
Dead volume at input port:	0.1 µL
Thickness of bottom substrate:	0.17 mm

Table 3.2 Performance specifications of the Vena8 Fluoro+™ Biochip:

Range of proteins for coating Biochip	VCAM, ICAM, MAdCAM, Fibronectin, vWF, Fibrinogen, Collagen etc.
Minimum Sample Volume:	~10 µL
Cell types	• T-cells: primary & cell lines e.g. HUT 78 • Monocytes: primary and cell lines; e.g. THP-1 • Eosinophils • Neutrophils • Platelets • PBMCs, whole blood, etc.
Minimum Sample Volume:	~10 µL
Maximum Sample Volume:	100 µL (Vena8 Fluoro+ microwells)
Shear Stress Precision:	<0.5% CV
Shear Stress Range for cell suspension:	0.05 - 10 dyne/cm ² ; steps of 0.05 dyne/cm ² (100 µL syringe)
Shear Stress Range for whole blood:	2.25 – 450 dyne/cm ² (1 mL syringe)
Volumetric Flow Rates:	100 nL/minute - 20 µL/minute (100 µL syringe);; 5 µL/minute – 1 mL/minute (5 mL syringe)
Sample Volume Aspiration Accuracy:	±1%
Shear Stress Accuracy:	±0.5%
Sample Volume Aspiration Precision:	<1% CV

3.2.5 Optimization of iKima™ Pump with Apple Application for iPod Touch / iPhone

The Microfluidic pump aids cell culture under physiological conditions and is controlled by the iKima™ Application for use with the iPod Touch and iPhone. It connects easily to the biochip via fully autoclaveable tubing and delivers pulses of fresh media to cells seeded inside the microchannels of the biochip. The iKima™ App is available for download from Apple’s iTunes Store.

Table 3.3 Pump specifications of iKima™ Pump:

Volumetric Flow Rates	Perfusion mode: 15 - 35 mL/hr (*) +/- 4%
Shear Stress Range	Perfusion mode: ~2.5 to ~6.5 dyne/cm ²
Dead Volume	< 300 µL
Working Pressure	30 psi – 2 bars maximum
Flow Direction	• Dispense only • Two modes: wash and perfusion (controlled only by Kima controller via iPod Touch / iPhone)
Additional features	• Plug and Play with Kima controller and iKima Application (available for iPod Touch / iPhone) • Featured with a standard 100mL bottle holder
Dimensions	190mm x 85mm x 90mm (L x W x H)
Weight	< 800g (bottle and tubing not included)
Conditions of use	• Temperature: 10°C to 60°C • Humidity: < 98% RH • Altitude: <2000m

Table 3.4 Controller specifications of iKima™ Pump:

General specifications	Controller with: • 2 modes of pumping: wash and perfusion • 4 sockets which can control 4 iKima pumps Independently • Universal dock station (power + communication) to use with iKima application (available for download on iTunes) • Wi-Fi communication • USB communication • Tactile power switch • LEDs for power / status information
Power Requirements	Input: 100 – 240V AC / 47 - 63Hz
	Output: 12V DC – 3.33A max
Dimensions	128mm x 81mm x 104mm (W x D x H)
Weight	< 550g (including power supply)
Conditions of use	• Temperature: 10°C - 40°C • Humidity: < 90% RH • Altitude: < 2000m

The iKima App was downloaded from the iPhone App store. All tubing and bottles were autoclaved prior to starting the experiment and the pump set up and biochip attachment all took place within a biosafety sterile hood. The iKima pump (Cellix, Ireland) was washed with 70% ethanol and then sterile distilled H₂O after using a 5ml sterile syringe (BD bioscience, NJ, USA). 30ml of sterile endothelial cell growth media (PromoCell, Germany) was placed in the 100ml pyrex bottle of the iKima apparatus. Tubing was connected to the pyrex bottle via the inlet port of the pump. Separate tubing with 8 way cable pins was also connected from the outlet port of the pump and rested in a sterile petri dish before biochip connection. The tubing was then washed with media for 2-3 min until there was a steady flow with no air bubbles evident. Perfusion was started using the iKima app (Figure 3.3), a cycle of 2 min perfusion and 20 min pause was applied at a shear stress rate of 10dyne/cm². The 8-way cable with pins was then inserted into the reservoirs of the biochip and the outlet pins were connected to the

same media bottle for recirculation of the same media. The iKima pump and biochip were then transferred into a 37°C incubator according to time specific requirements (Figure 3.4). Once time lapsed the apparatus was taken out of the incubator and put into a sterile biosafety hood where the cells within the channels were fixed.



Figure 3.3 iKima pump App 1. Set-up -Select iKima App on iPod Touch or iPhone. Connect iPod Touch to iKima controller: Press "Connect" button. 2. Wash out- Select "wash out" button 3. Flow assay- The user can select two flow rates for two different time periods. These two flow rates are then continuously looped or cycled until the user stops the device



Figure 3.4 The Vena8 Fluoro+™ Biochip and iKima™ Pump with Apple Application functioning together after the set up of both separately. This apparatus provided a pulsatile laminar flow for EC analysis.

3.2.6 Stimulation of biochips with oxPAPC and TNF- α

HUVECs were stimulated by both an oxidised lipoprotein oxPAPC (Cambridge Bioscience, England) and a cytokine TNF- α (R&D systems, USA) dependent on time and concentration (Table 3.5). The stimulants were heated to 37C and added to the endothelial growth cell

media before the pump was attached to the biochip and the stimulated media circulated the system for the experiments duration.

3.2.7 Experimental design

Table 3.5 The variables for each experiment are listed below.

Stimulant	Time (hours)	Concentration (µg/ml)	Flow rate (dyne/cm ³)	Receptor
TNF- α	6	.01	10	ICAM-1 CXCL16
TNF- α	24	.01	10	ICAM-1 CXCL16
TNF- α	6	.01	Static	ICAM-1 CXCL16
TNF- α	24	.01	Static	ICAM-1 CXCL16
OXAPC	6	50	10	ICAM-1 CXCL16
OXAPC	24	50	10	ICAM-1 CXCL16
OXAPC	24	50	Static	ICAM-1 CXCL16

3.2.8 ELISA

The 20 ml of media used was collected and an ELISA was carried out to test for the presence of soluble CXCL16. High binding 96-well microtiter plates (Greiner Bio-one) were coated with 100µl per well of anti-human CXCL16 capture antibody, the plate was sealed and incubated overnight at room temperature. The capture antibody solution was aspirated and wells were washed with 400µl of wash buffer (R&D Systems, USA) x3. Non-specific binding sites were blocked with 300µl of Reagent Diluent at room temperature for a minimum of 1 hour. The aspiration was repeated and three washes were performed. Sample and standards were added. Standards provided by the manufacturer were diluted in Reagent Diluent and used to produce a standard curve. The full 20ml of media was used as sample.

3.2.9 Lysing cells for RT-PCR

Excess media were removed from biochip wells. 20ul of lysis buffer (Qiagen) was pipette into each channel and incubated at room temperature for 2-3 mins. An additional 20ul of lysis buffer was added and a pipette was used to remove the buffer from well and channel. RNA extraction was carried out using the An RNeasy micro extraction kit (Qiagen) was used for RNA extraction from $\sim 10^5$ cells per treatment per cell line according to the manufacturer's protocol.

3.2.10 RNA quantification using spectrophotometry

Concentrations of RNA and DNA were measured using the Nanodrop 8 sample spectrophotometer ND-8000 (Nanodrop Tech, DE, USA).

3.2.11 Fixing cells

20µl of 4% PFA (Sigma-Aldrich, Germany) was added to each well of the biochip once experiment completed and incubated for 15 min at 37°C. 20µl of PBS x2 was then added to make sure all of the PFA was washed out. Excess liquid was removed from the reservoirs and

topped up with 30µl of PBS each side. Biochips were stored at 4°C until ready for immunostaining.

3.2.12 Immunofluorescent staining

All antibody solutions were made in 3% PBS/BSA (Sigma-Aldrich, Germany). Cells were blocked by pipetting 20µl of 3% PBS/BSA into each channel and incubated at room temperature for 30min. 20µl of primary antibodies rabbit anti-human CXCL16 (dilution 1:200) (Peprotech, USA) and mouse anti-human ICAM-1 (dilution 1:10) (R&D Systems, USA) were added and incubated for 2 hours at room temperature. Two channels were used for a control with no primary antibody only 3% PBS/BSA solution. Channels were washed with 20µl x3 with PBS-0.01% Tween 20 (Sigma-Aldrich, Germany) for 2-3 min each. Hoechst 33258 (Invitrogen, CA, USA), Alexa Fluor 488 anti-mouse (Invitrogen, CA, USA) and Alexa Fluor 568 goat anti-rabbit (Invitrogen, CA, USA) were added at a 1/1000 dilution. Wash step was repeated and channels were left in 20µl of PBS. Cells were then ready for fluorescent microscopy.

3.2.13 Confocal Laser Scanning Microscopy (CLSM)

A Zeiss LSM 510 laser module was used for imaging biochips and gathering qualitative data. Eight images were taken per channel per biochip. Images were taken across the channel from left to right and by a 63x objective lens. All images were taken with the same microscope and laser settings.

3.2.14 Image processing analysis

ImageJ was used for all image analysis, quantitative and qualitative. Figure 3.5 shows a flow diagram of how the experimental procedure was performed.

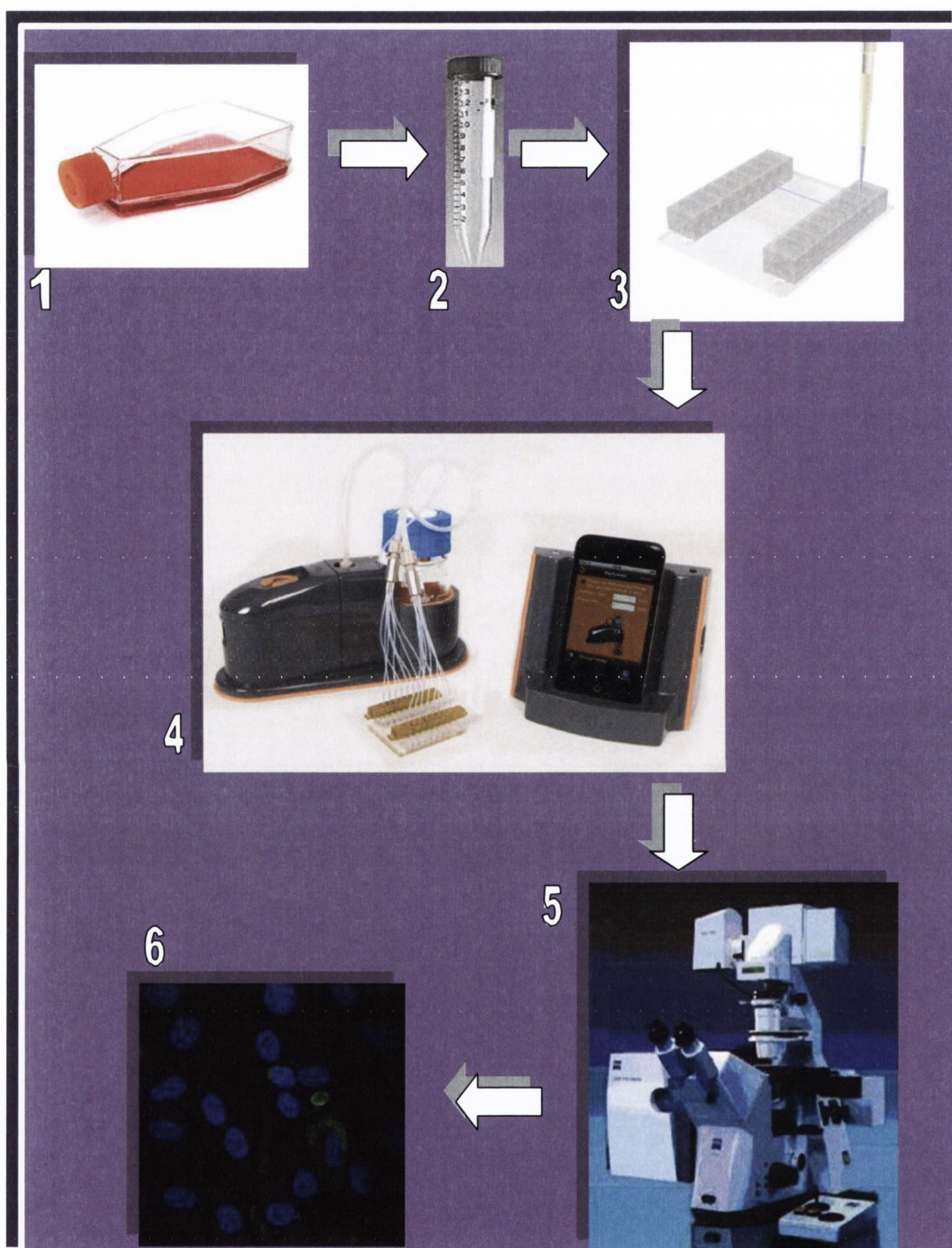


Figure 3.5 Flow chart of experimental design. 1)Cell culture and growth 2)Cell harvesting 3)Cell seeding into biochip 4)Pump connection and flow initiation 5) Confocal imaging 6) In cell analysing of intensity pixels, quantitative and qualitative results

3.2.15 Immunofluorescence quantification in endothelial cell cultures

Histograms

The darkness or brightness in an image may be determined by examining its image histogram. The histogram of a image provides a distribution of the pixels in an image as a function of luminosity, in this case on a scale of 0–255. A peak in the shadow regions of the histogram corresponds to the black background in the image.

Calculating Background

The background was calculated by measuring pixel luminosity of images stained for secondary antibody only. The darker green/black area in Figure 3.6 (A) represents the background. The background in all images for one channel per experiment (biochip) was averaged. Backgrounds for ICAM-1 and CXCL16 were averaged separately. An average cut off of 30 ± 5.9 (ICAM-1) and 50 ± 7.1 (CXCL16) on the luminosity scale, corresponding to the point where fluorescence in the image starts to become visible was used.

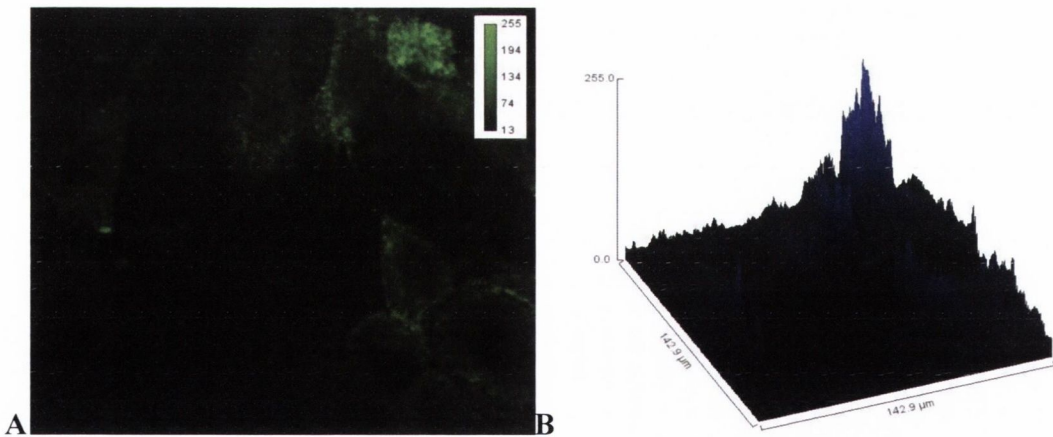


Figure 3.6.Both image A and B are the same, ECs treated for 6 h pulsatile shear stress at 10 dyne/cm² and stained for ICAM-1. Image A is an example of how the 0-255 luminosity scale is measured. Image B is a 3-D surface plot of what was measured. The image and surface plot are showing what the histogram measures.

Calculating area of cells stained

Values from 30 or 50 to 255 were integrated to obtain an Area Under the Curve Value corresponding to the fluorescence that results when the primary antibody binds to the antigen (ICAM-1/CXCL16) and the secondary antibody (with the covalently attached fluorescent probe) interacts with the primary antibody. Figure 3.6 (B) is a 3-D representation of what is measured in the following equation.

Area of cells stained: = SUMPRODUCT (A30:A255, (B30:B255)/2). A = pixel luminosity count 30/50-255 and B = the number of pixels at each count. This equation uses ICAM-1s range as an example. Every image had a total pixel count combining the full scale of the pixel luminosity after the cut off point.

These values were then normalized by the number of cells in each image field of view, cells were counted manually. This method was taken from McMullen et al with modifications (McMullen et al., 2010).

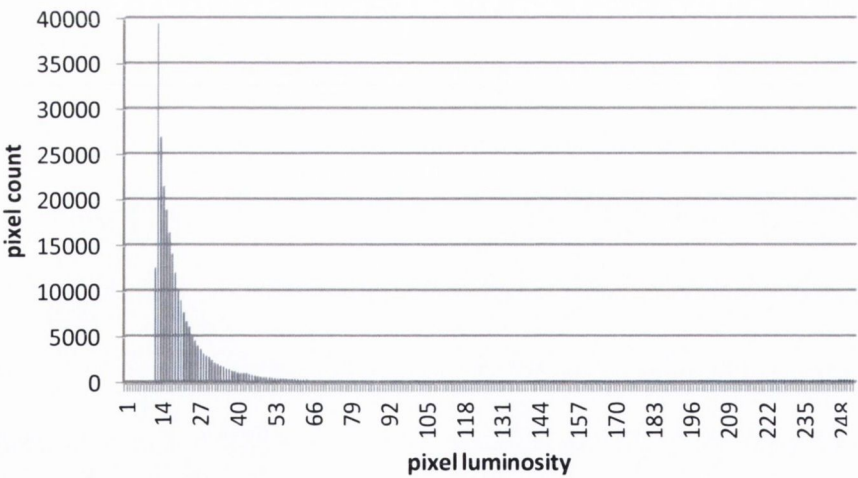


Figure 3.7 A histogram measuring the area of cells stained using pixel count and pixel luminosity. This total area was divided by the number of cells per image and used as the amount of fluorescence generated by the antibody labeled protein.

3.2.16 Statistical analysis

Values from 8 images from each duplicate channel from both duplicate biochips were

averaged and input into GraphPad prism 5 for analysis. Unpaired t tests were carried out for significance testing using a p value of <0.05 .

3.3 Results

3.3.1 Enzyme-linked immunosorbent assay (ELISA)

There was no soluble CXCL16 detected in the media of the biochip experiments using the ELISA kit described in chapter 2. A standard 8 fold dilution was used in duplicate and a negative control of media only was used. Both the negative control and biochip media produced the same optical density as the blank in the standard.

3.3.2 Real-time PCR (RT-PCR)

After RNA extraction and quantification using a spectrophotometer, the required amount of RNA (20ng/ml) to proceed with RT-PCR was not achieved. As a control, the same number of cells were seeded in duplicate using wells in a 96 well plate which produced enough RNA (>20ng/ml) for cDNA conversion. This was a novel attempt at retrieving cells from the biochip platform which still needs further optimization to succeed. For future experiments using two biochips (16 separate channels) may produce the necessary RNA quantity.

3.3.3 Optimization of Vena8 Fluoro+TM Biochip

Expression of CXCL16 and ICAM-1 was investigated quantitatively and qualitatively. Through CSLM imaging at 63x we visualised the presence/absence or increase/decrease of protein expression and we were then able to quantify expression as previously explained using ImageJ. Multiple variables were examined and results were divided into the following sections for comparisons: Pulsatile shear stress v Static, stimulated v unstimulated and time points 6 h v 24 h.

Using histograms we calculated the area under the curve to give us a value for the amount of staining per image and normalised it to cell count which would interpret as the amount of protein expression (CXCL16 or ICAM-1) per cell. Using CSLM images we evaluated

morphological features and trends in EC expression. One image per treatment is shown.

3.3.3.1 The effect of pulsatile shear stress v static on EC receptor expression

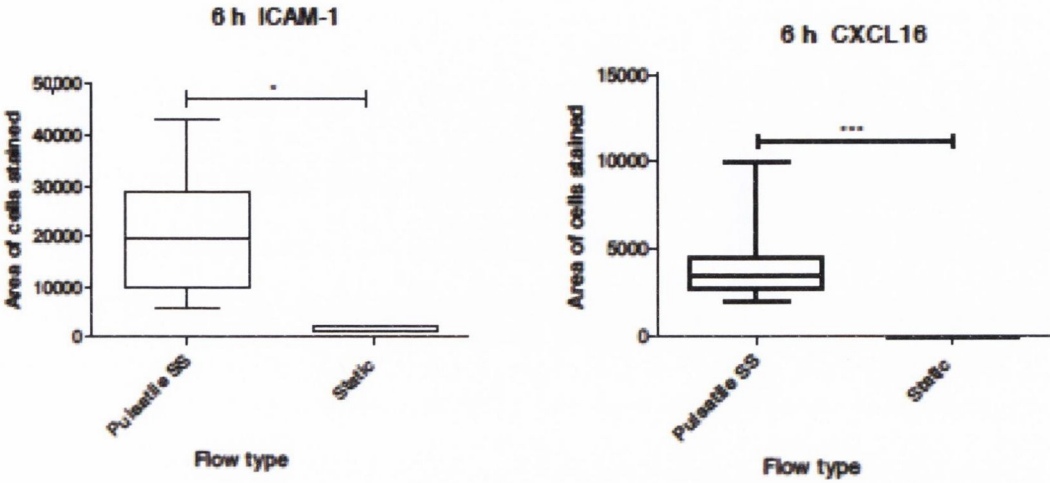
Qualitatively, ECs unstimulated at 6h under pulsatile shear stress show ICAM-1 expression while ECs under the same conditions without pulsatile SS show ICAM-1 expression in a few cells but throughout the cytoplasm (Figure 3.8 (ii) A and B). Under pulsatile conditions, ICAM-1 expression is also not present in every cell. Each cell positive for staining shows a distribution of protein expression throughout the cells cytoplasm. Some cells show intense

staining surrounding the nuclei. The quantitative values correspond to the images, there is a significant difference of $p = 0.0144$ between pulsatile and static conditions (Figure 3.8 (i)). The large range in area stained per cell in pulsatile conditions replicates the images that show both cells which are positive for staining and negative.

CXCL16 expression does not appear in unstimulated ECs in static conditions at 6 h. This result is also replicated in the quantitative values. In unstimulated ECs under pulsatile flow at 6 h CXCL16 is induced and its expression is localised around the nuclei in most cells imaged. Unstimulated ECs under pulsatile SS for 24 h show an induction of CXCL16 expression compared to ECs in a static environment. Both qualitative and quantitative results show no evidence of CXCL16 expression in a static environment. The majority of cells show CXCL16 around the nuclei and some cells show intensity spreading throughout the cytoplasm. CXCL16 appears intensified along the cells' edge (Figure 3.9), this resembles ICAM-1 expression pattern under pulsatile flow at 24 h unstimulated.

At 24 h unstimulated, static conditions showed no influence on ICAM-1 expression as there was little staining in resting ECs. However pulsatile SS induced ICAM-1 expression in 99% of cells imaged. Some cells positive for ICAM-1 staining showed an unusual pattern in staining. A halo like shape of intense staining formed in the cell's cytoplasm towards the edges. These ECs *in vivo* would be side by side in a monolayer, it appears the intense staining in these images occurs at the cell to cell junction and the surface which directly contacts blood flow. The images correspond to the values acquired. A p value of < 0.0001 shows significant difference in ICAM-1 expression between pulsatile and static conditions.

i)



ii)

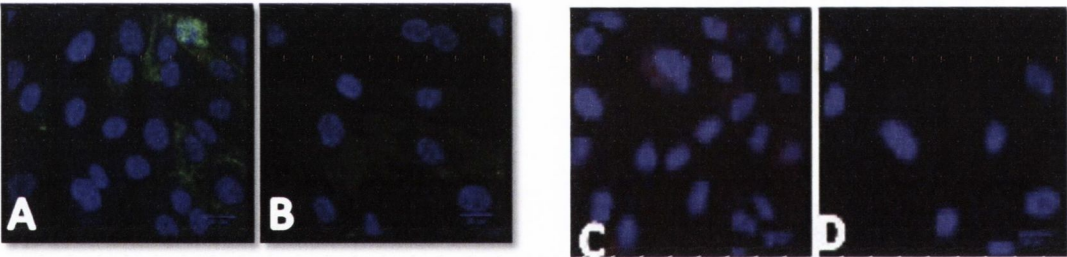
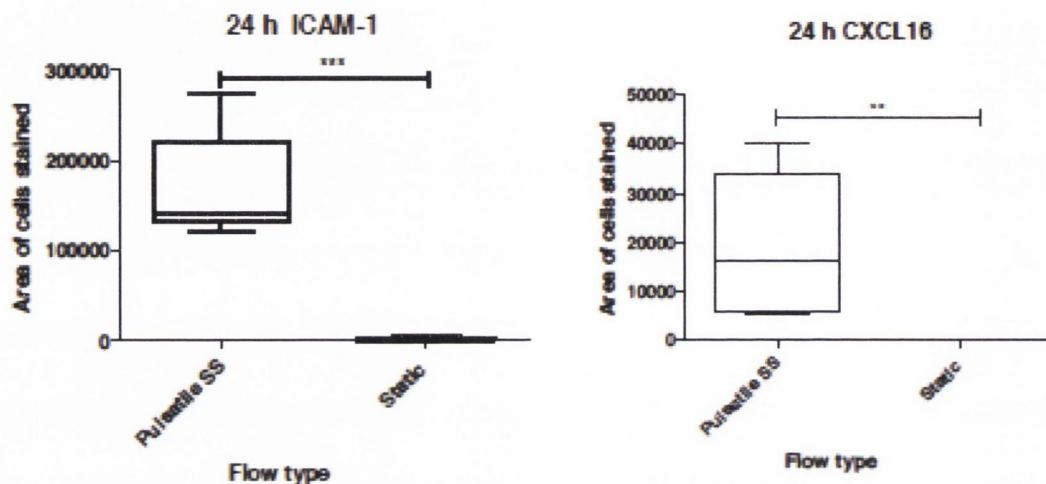


Figure 3.8 i) The two graphs represent the area stained by ICAM-1 (left) and CXCL16 (right) per cell. Results compared EC expression of ICAM-1 and CXCL16 for 6 h between pulsatile shear stress of 10 dyne/cm² and without pulsatile SS in static conditions, each parameter was tested in duplicate. There is a significantly higher expression level of both ICAM-1 and CXCL16 per cell under pulsatile SS using an unpaired t-test, ICAM-1 $p = 0.0144$ and CXCL16 $p = 0.0003$ ii) A-D are images taken of ECs by CSLM at 63x. Nuclei are stained blue, ICAM-1 is green and CXCL16 is red. A) EC ICAM-1 expression under Pulsatile SS of 10 dyne/cm² for 6 h B) EC ICAM-1 expression under static conditions for 6 h C) EC CXCL16 expression under Pulsatile SS of 10 dyne/cm² for 6 h D) EC CXCL16 expression under static conditions for 6 h

i)



ii)

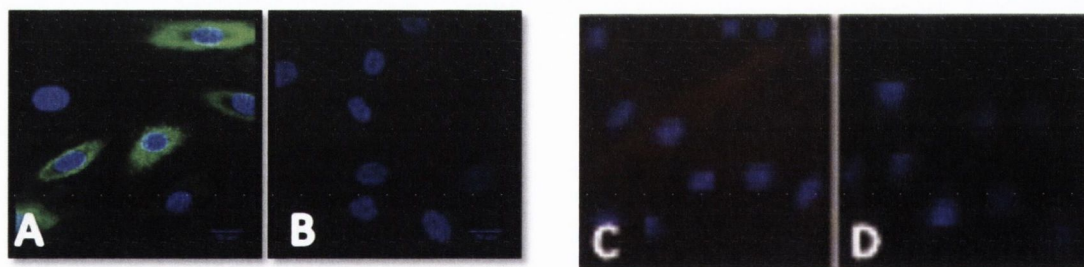


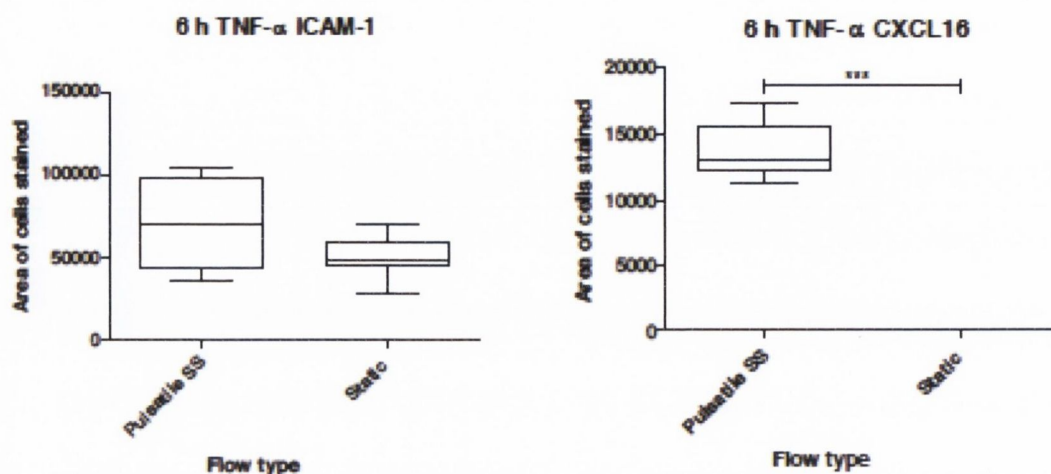
Figure 3.9 i) The two graphs represent the area stained by ICAM-1 (left) and CXCL16 (right) per cell. Results compared EC expression of ICAM-1 and CXCL16 for 24 h between pulsatile shear stress of 10 dyne/cm² and without pulsatile SS in static conditions, each parameter was tested in duplicate. There is a significantly higher expression level of both ICAM-1 and CXCL16 per cell under pulsatile SS using an unpaired t test, ICAM-1 $p = < 0.0001$ and CXCL16 $p = 0.0022$ ii) A-D are images taken of ECs by CSLM at 63x. Nuclei are stained blue, ICAM-1 is green and CXCL16 is red. A) EC ICAM-1 expression under Pulsatile SS of 10 dyne/cm² for 24 h B) EC ICAM-1 expression under static conditions for 24 h C) EC CXCL16 expression under Pulsatile SS of 10 dyne/cm² for 24 h D) EC CXCL16 expression under static conditions for 24 h

ECs stimulated with 10ng/ μ l of TNF- α at 6 h in a static environment show ICAM-1 expression is induced in all cells visible (Figure 3.10 (ii) B). When a pulsatile SS is introduced under the same conditions, images show a slight increase in expression intensity; again a halo type pattern appears in some cells (Figure 3.10 (ii) A). When the images were analysed quantitatively looking at area stained, no significant difference was evident between the two flow conditions on EC ICAM-1 expression when stimulated with 10ng/ μ l TNF- α at 6 h, which corresponds to the qualitative data (Figure 3.10 (i)).

Static ECs in the presence of TNF- α for 6 h show no CXCL16 expression (Figure 3.10 (ii) D), which is seen qualitatively and quantitatively. However, under pulsatile flow all cells imaged showed positive red staining for CXCL16 (Figure 3.10 (ii) C). Again CXCL16 expression was most intense around the nuclei. Quantitative results show a narrow range of values, all cells analysed had similar amount of area stained for CXCL16. Comparing pulsatile SS and static EC CXCL16 expression with TNF- α stimulation at 6 h, there was a significant induction and increases of CXCL16 under pulsatile flow, $p < 0.0001$.

ECs stimulated with 10ng/ μ l at 24 h in a static environment show ICAM-1 expression in the majority of cells (Figure 3.11 (ii) B). However, the introduction of pulsatile shear stress increases ICAM-1 expression and intensity in all cells (Figure 3.11 (ii) A). Images of ECs at 24 h TNF- α under pulsatile SS show little to no areas of unstained cells. This change is represented in the corresponding values. A p value of < 0.0001 signifies the difference between the two treatments (Figure 3.11 (i)). At 24 h same conditions, expression of CXCL16 was lower in static ECs than pulsatile but not significantly, this was evident in both images and values (Figure 3.11 (i) and (ii) C and D).

i)



ii)

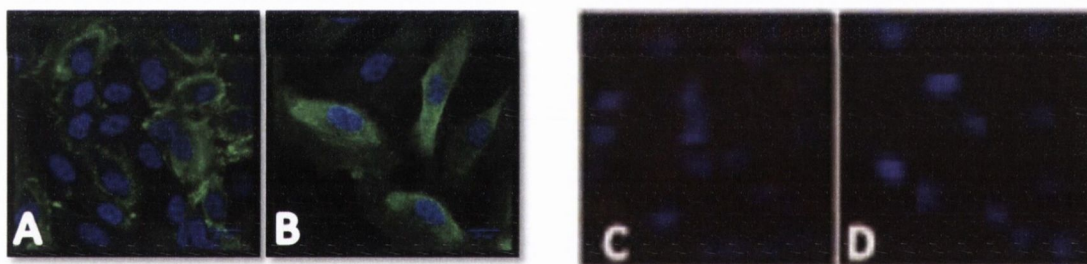
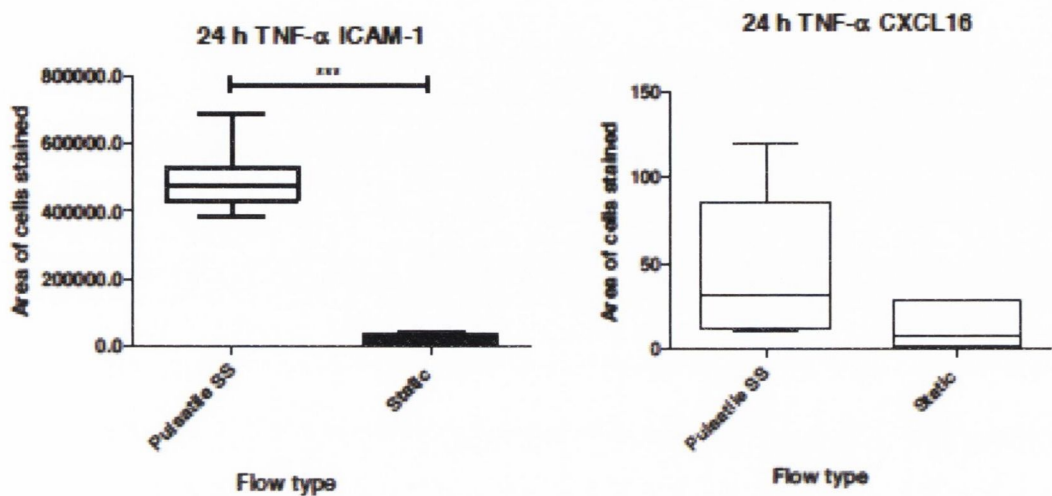


Figure 3.10 i) The two graphs represent the area stained by ICAM-1 (left) and CXCL16 (right) per cell. Results compared expression of ICAM-1 and CXCL16 by ECs stimulated with 10ng/ μ l TNF- α for 6 h between pulsatile shear stress of 10 dyne/cm² and without pulsatile SS in static conditions, each parameter was tested in duplicate. There is a significantly higher expression level of both ICAM-1 and CXCL16 per cell under pulsatile SS using an unpaired t test, ICAM-1 $p = 0.0867$ and CXCL16 $p < 0.0001$ ii) A-D are images taken of TNF- α stimulated ECs by CSLM at 63x. Nuclei are stained blue, ICAM-1 is green and CXCL16 is red. A) EC ICAM-1 expression under Pulsatile SS of 10 dyne/cm² for 6 h B) EC ICAM-1 expression under static conditions for 6 h C) EC CXCL16 expression under Pulsatile SS of 10 dyne/cm² for 6 h D) EC CXCL16 expression under static conditions for 6 h

i)



ii)

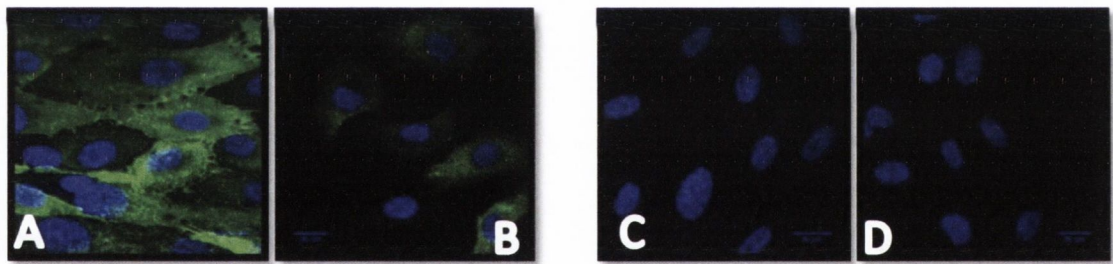


Figure 3.11 i) The two graphs represent the area stained by ICAM-1 and CXCL16 per cell. Results compared expression of ICAM-1 and CXCL16 by ECs stimulated with 10ng/μl TNF-α for 24 h between pulsatile shear stress of 10 dyne/cm² and without pulsatile SS in static conditions, each parameter was tested in duplicate. There is a significantly higher expression level of ICAM-1 per cell under pulsatile SS using an unpaired t test $p = < 0.0001$, there is no significant difference in CXCL16 expression ii) A-D are images taken of TNF-α stimulated ECs by CSLM at 63x. Nuclei are stained blue, ICAM-1 is green and CXCL16 is red. A) EC ICAM-1 expression under Pulsatile SS of 10 dyne/cm² for 24 h B) EC ICAM-1 expression under static conditions for 24 h C) EC CXCL16 expression under Pulsatile SS of 10 dyne/cm² for 24 h D) EC CXCL16 expression under static conditions for 24 h

The combination of oxPAPC and pulsatile SS has a significant increasing effect on ICAM-1 expression in ECs compared to oxPAPC stimulated ECs in a static environment. This is evident in both histogram values and confocal images (Figure 3.12 (i) and (ii) A and B). Comparing stimulants, oxPAPC and TNF- α , in both normal pulsatile and static conditions on EC ICAM-1 expression it is evident the TNF- α /pulsatile conditions have the strongest effect at promoting ICAM-1 expression (Figure 3.11.1). Qualitatively, no CXCL16 expression is present at 24 h pulsatile SS or static in oxPAPC stimulated ECs, so no values were available to graph above the cut off value 50.

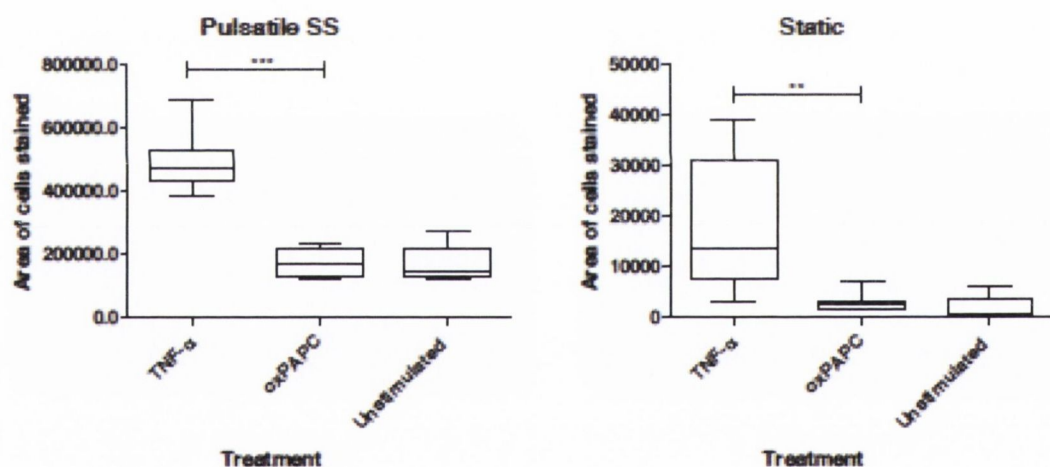
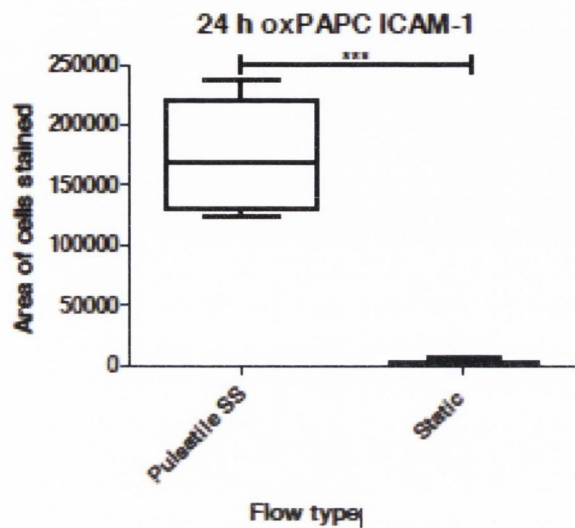


Figure 3.11.1 The two graphs represent the area stained by ICAM-1 per cell under three separate treatments in pulsatile SS and static conditions. Under pulsatile SS conditions TNF- α has a significant increase on ICAM-1 expression compared to oxPAPC $p < 0.0001$ and under static conditions $p=0.0083$, using an unpaired t-test

i)



ii)

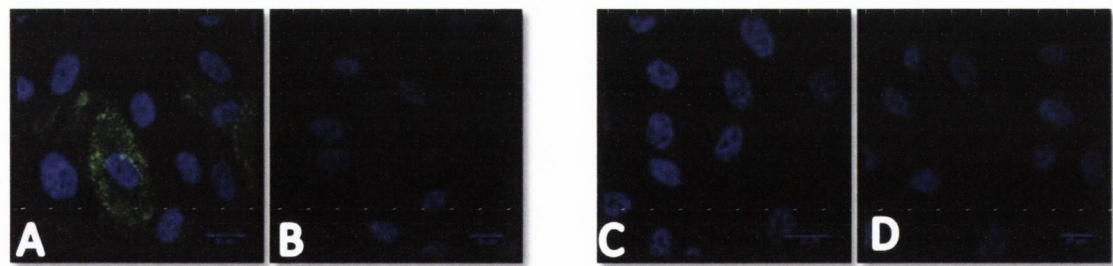


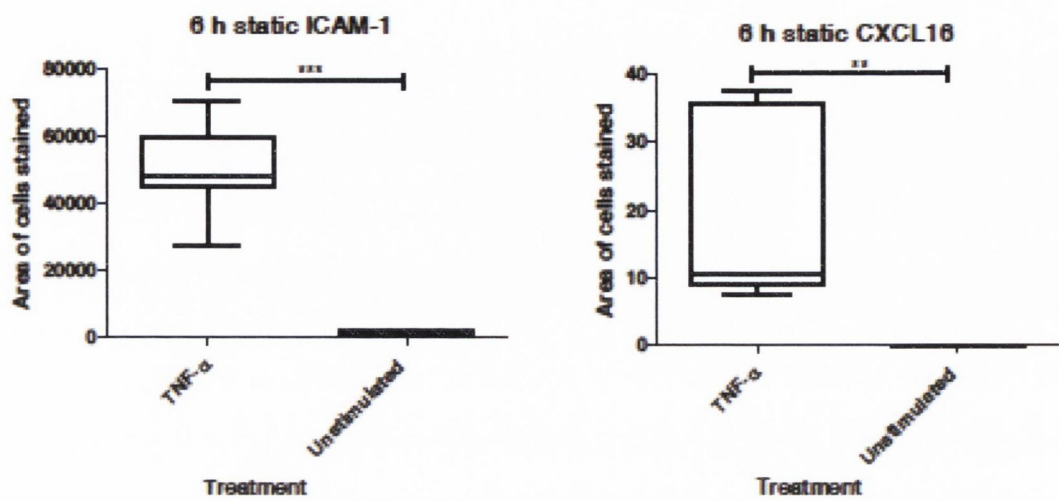
Figure 3.12 i) The graph represents the area stained by ICAM- per cell. There is no graph for CXCL16 area stained as the values did not exceed background staining. Results compared expression of ICAM-1 by ECs stimulated with 50µg/ml oxPAPC for 24 h between pulsatile shear stress of 10 dyne/cm² and without pulsatile SS in static conditions, each parameter was tested in duplicate. There is a significantly higher expression level of ICAM-1 per cell under pulsatile SS using an unpaired t test, $p = < 0.0001$, there is no significant difference in CXCL16 expression ii) A-D are images taken of oxPAPC stimulated ECs by CSLM at 63x. Nuclei are stained blue, ICAM-1 is green and CXCL16 is red. A) EC ICAM-1 expression under Pulsatile SS of 10 dyne/cm² for 24 h B) EC ICAM-1 expression under static conditions for 24 h C) EC CXCL16 expression under Pulsatile SS of 10 dyne/cm² for 24 h D) EC CXCL16 expression under static conditions for 24 h

3.3.3.2 *The effect of TNF- α and oxPAPC stimulation on EC receptor expression*

In a static environment at 6 h, ICAM-1 expression was weak in most images and pixel luminosity values were extremely low, however with the addition of TNF- α ICAM-1 expression was induced in the majority of cells, a p value of < 0.0001 shows this significant increase and correlation with qualitative data (Figure 3.13 (i) and (ii) A and B). 24 h ICAM-1 expression is similar to 6 h results. Values and images show little ICAM-1 in an unstimulated static environment but with TNF- α stimulation there is a significant increase $p = 0.0265$ (Figure 3.14 (i) and (ii) A and B).

The luminosity values which interpret area stained suggest a minimal difference in CXCL16 expression between unstimulated ECs and TNF- α stimulated ECs at 6 h static (Figure 3.13 (i)). This is reflected in the images; qualitatively no CXCL16 is visible with both treatments (Figure 3.13 (ii) C and D). These results are also seen when comparing CXCL16 expression at 24 h between unstimulated and TNF- α stimulated ECs (Figure 3.14 (i) and (ii) C and D).

i)



ii)

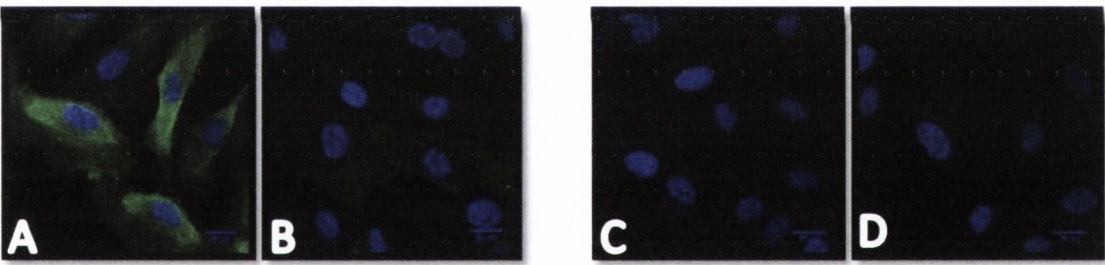
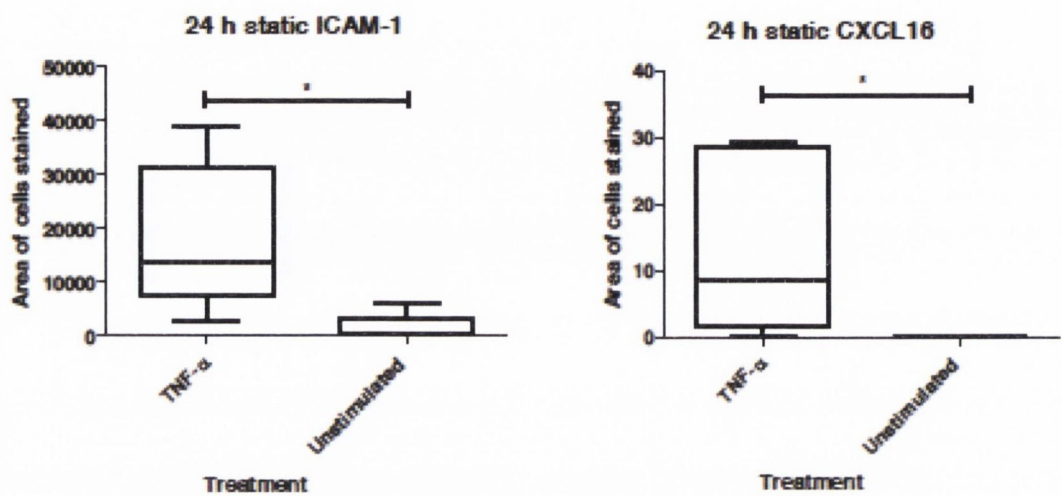


Figure 3.13 i) The two graphs represent the area stained by ICAM-1 and CXCL16 per cell. Results compared ICAM-1 and CXCL16 expression by TNF- α (10 ng/ μ l) stimulated and unstimulated EC's under static conditions for 6 h, each parameter was tested in duplicate. There is a significantly higher expression level of both ICAM-1 and CXCL16 per cell when 10 ng/ μ l TNF- α is present, ICAM-1 $p = < 0.0001$ and CXCL16 $p = 0.0014$ using an unpaired t test ii) A-D are images taken of ECs by CSLM at 63x. Nuclei are stained blue, ICAM-1 is green and CXCL16 is red. A) TNF- α stimulated EC ICAM-1 expression under static conditions for 6 h B) EC ICAM-1 expression under static conditions for 6 h C) TNF- α stimulated EC CXCL16 expression under static conditions for 6 h D) EC CXCL16 expression under static conditions for 6 h

i)



ii)

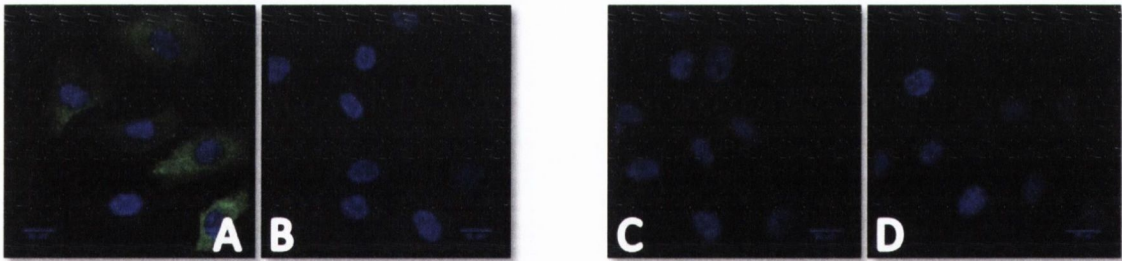
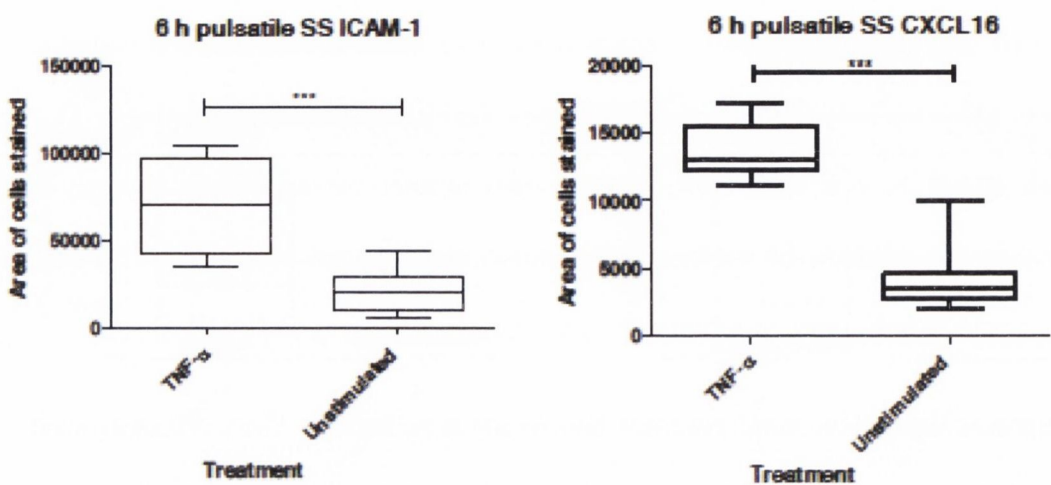


Figure 3.14 i) The two graphs represent the area stained by ICAM-1 and CXCL16 per cell. Results compared ICAM-1 and CXCL16 expression by TNF- α (10 ng/ μ l) stimulated and unstimulated ECs under static conditions for 24 h, each parameter was tested in duplicate. There is a significantly higher expression level of both ICAM-1 and CXCL16 per cell with 10 ng/ μ l TNF- α , ICAM-1 $p = 0.0265$ and CXCL16 $p = 0.0224$ using an unpaired t test ii) A-D are images taken of ECs by CSLM at 63x. Nuclei are stained blue, ICAM-1 is green and CXCL16 is red. A) TNF- α stimulated EC ICAM-1 expression under static conditions for 6 h B) EC ICAM-1 expression under static conditions for 6 h C) TNF- α stimulated EC CXCL16 expression under static conditions for 6 h D) EC CXCL16 expression under static conditions for 6 h

Comparing ICAM-1 expression between TNF- α stimulated and unstimulated ECs under pulsatile flow at 6 h shows evidence of ICAM-1 up-regulation when combined with a primary trigger (pulsatile SS) and secondary trigger (TNF- α) of atherosclerosis. Quantitative and qualitative results show a significant increase, ICAM-1 $p = 0.0003$, CXCL16 $p < 0.0001$, (Figure 3.15 (i) and (ii) A and B) when both pulsatile shear stress and cytokine stimulation are combined. ECs with pulsatile flow and TNF- α have double the amount of staining as ECs unstimulated. At 24 h, a p value of < 0.0001 demonstrates the significant increase in ICAM-1 due to the addition of another pro-atherosclerotic trigger being TNF- α . These results can be seen in both types of analyses (Figure 3.16 (i) and (ii) A and B).

CXCL16 expression under the same treatment follows the same pattern. There is a significant increase in CXCL16 area stained in TNF- α stimulated ECs under pulsatile flow compared to unstimulated ECs under pulsatile flow at 6 h (Figure 3.15 (i)). In unstimulated ECs CXCL16 expression is localised around the nucleus but its expression spreads with the addition of TNF- α (Figure 3.15 (ii) C and D). At 24 h comparing the same conditions gave very different results both quantitatively and qualitatively. CXCL16 was not present visually or in values in ECs under 24 h TNF- α stimulation and under pulsatile shear stress (Figure 3.16 (i) and (ii) C). However unstimulated ECs (Figure 3.16 (ii) D) show CXCL16 expression in the cell majority and cover a significant area (Figure 3.16 (i)).

i)



ii)

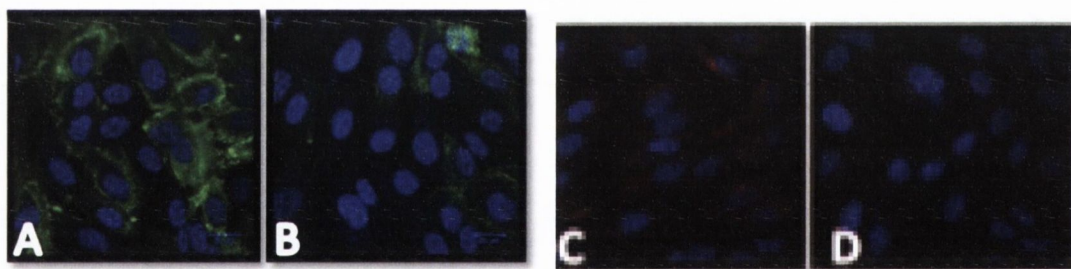
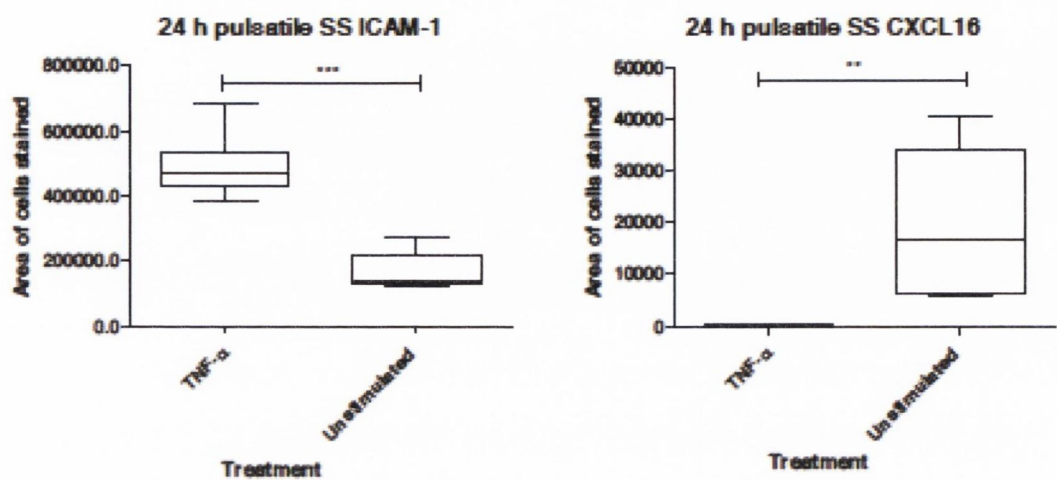


Figure 3.15 i) The two graphs represent the area stained by ICAM-1 and CXCL16 per cell. Results compared ICAM-1 and CXCL16 expression by TNF- α (10 ng/ μ l) stimulated and unstimulated ECs under pulsatile SS at 10 dyne/cm² for 6 h, each parameter was tested in duplicate. There is a significantly higher expression level of both ICAM-1 and CXCL16 per cell under pulsatile SS and 10 ng/ μ l TNF- α , ICAM-1 $p = 0.0003$ and CXCL16 $p < 0.0001$ using an unpaired t test ii) A-D are images taken of ECs by CSLM at 63x. Nuclei are stained blue, ICAM-1 is green and CXCL16 is red. A) TNF- α stimulated EC ICAM-1 expression under pulsatile SS at 10 dyne/cm² for 6 h B) EC ICAM-1 expression under pulsatile SS at 10 dyne/cm² for 6 h C) TNF- α stimulated EC CXCL16 expression under pulsatile SS at 10 dyne/cm² for 6 h D) EC CXCL16 expression under pulsatile SS at 10 dyne/cm² for 6 h

i)



ii)

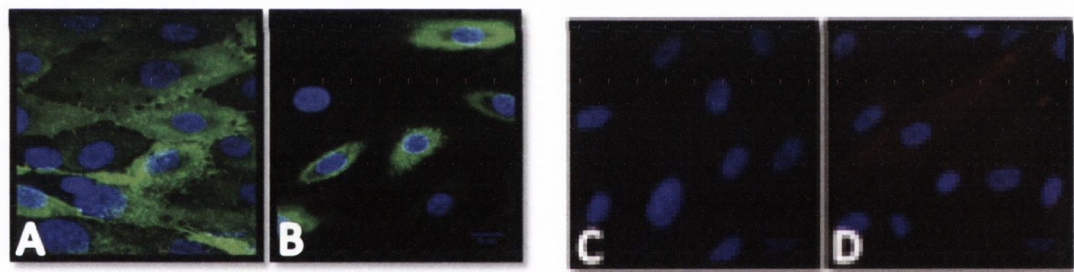
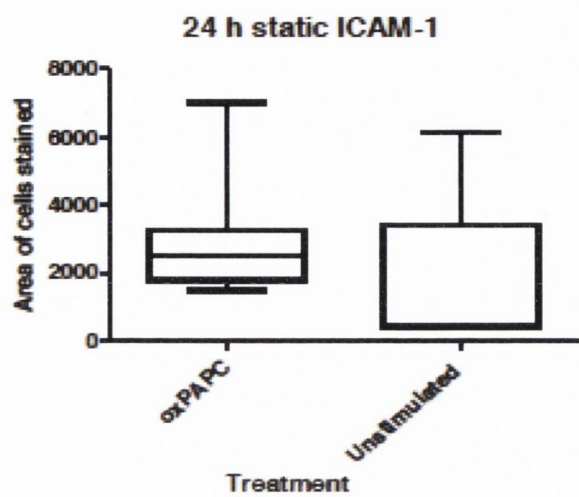


Figure 3.16 i) The two graphs represent the area stained by ICAM-1 and CXCL16 per cell. Results compared ICAM-1 and CXCL16 expression by TNF- α (10 ng/ μ l) stimulated and unstimulated ECs under pulsatile SS at 10 dyne/cm² for 24 h, each parameter was tested in duplicate. There is a significantly higher expression level of ICAM-1 per cell under pulsatile SS and 10 ng/ μ l TNF- α , ICAM-1 $p = < 0.0001$ using an unpaired t test. CXCL16 expression by TNF- α stimulated ECs is significantly lower under 24 h pulsatile SS $p = 0.0023$ ii) A-D are images taken of ECs by CSLM at 63x. Nuclei are stained blue, ICAM-1 is green and CXCL16 is red. A) TNF- α stimulated EC ICAM-1 expression under pulsatile SS at 10 dyne/cm² for 24 h B) EC ICAM-1 expression under pulsatile SS at 10 dyne/cm² for 24 h C) TNF- α stimulated EC CXCL16 expression under pulsatile SS at 10 dyne/cm² for 24 h D) EC CXCL16 expression under pulsatile SS at 10 dyne/cm² for 24 h

Under static conditions at 24 h, there is no significant difference in ICAM-1 expression between oxPAPC stimulated and unstimulated ECs. For both treatments there is a wide range of values varying in difference of about 6000 (Figure 3.17 (i)). This skews significance testing. Images show a random ICAM-1 staining pattern, and it is evident ICAM-1 is expressed at higher levels in oxPAPC treated cells (Figure 3.17 (ii) A and B). No graph is available for CXCL16 expression under the same conditions, the images confirm the absence of red staining (CXCL16 expression) and that values were not present above the cut off 50 to incorporate into a quantitative reading.

Again the difference in ICAM-1 expression in oxPAPC stimulated and unstimulated ECs is not significantly different under a pulsatile SS environment (Figure 3.18 (i)). However, under the same conditions CXCL16 expression is significantly different. The ECs stimulated with oxPAPC appear to have no red staining; it would suggest oxPAPC extinguishes CXCL16 expression (Figure 3.18 (ii) C and D).

i)



ii)

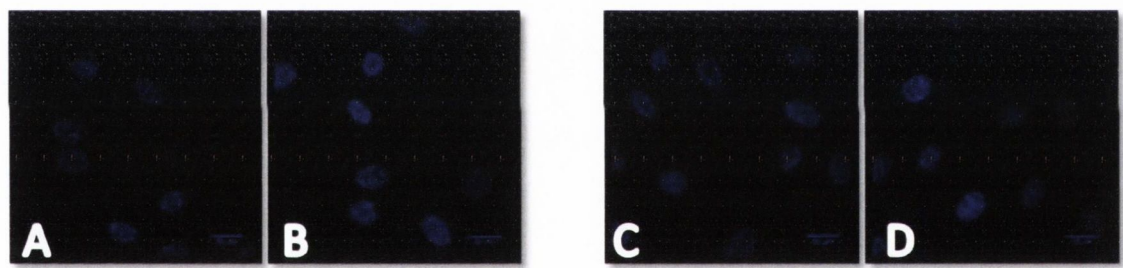
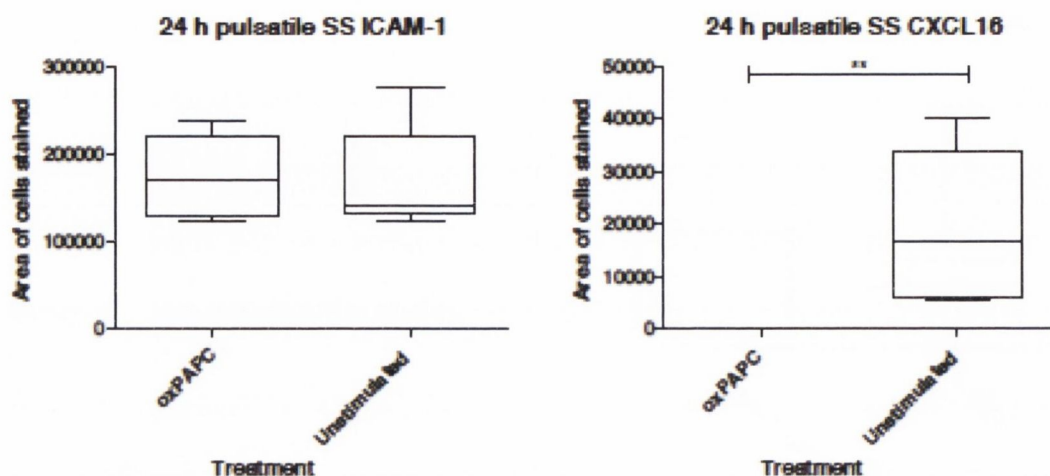


Figure 3.17 i) The graph represent the area stained by ICAM-1 per cell. There is no graph for CXCL16 area stained as the values did not exceed background staining. Results compared ICAM-1 and CXCL16 expression by oxPAPC (50µg/ml) stimulated and unstimulated ECs under static conditions for 24 h, each parameter was tested in duplicate. There is no significant difference expression level of either ICAM-1 or CXCL16 per cell with 50 µg/ml oxPAPC using an unpaired t test ii) A-D are images taken of ECs by CSLM at 63x. Nuclei are stained blue, ICAM-1 is green and CXCL16 is red. A) oxPAPC stimulated EC ICAM-1 expression under static conditions for 6 h B) EC ICAM-1 expression under static conditions for 6 h C) oxPAPC stimulated EC CXCL16 expression under static conditions for 6 h D) EC CXCL16 expression under static conditions for 6 h

i)



ii)

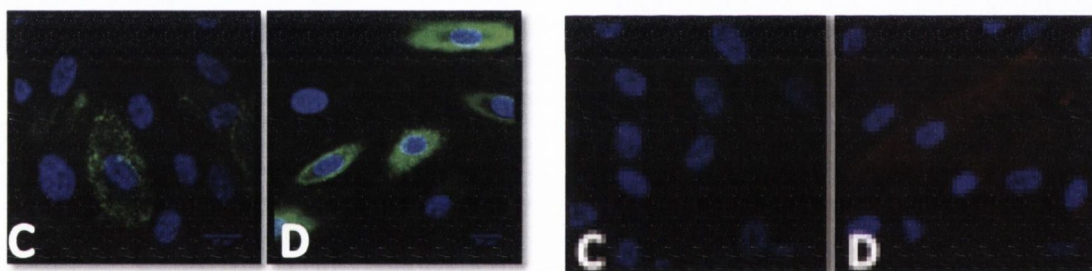


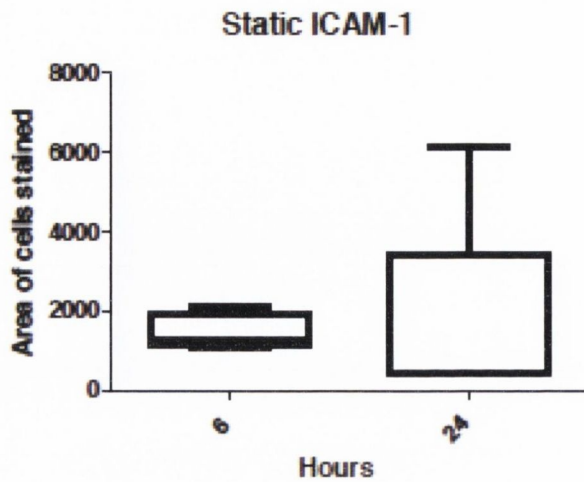
Figure 3.18 i) The two graphs represent the area stained by ICAM-1 and CXCL16 per cell. Results compared ICAM-1 and CXCL16 expression by oxPAPC (50 μ g/ml) stimulated and unstimulated ECs under pulsatile SS at 10 dyne/cm² for 24 h, each parameter was tested in duplicate. There is no significant difference expression level of ICAM-1 per cell under pulsatile SS and 50 μ g/ml oxPAPC. CXCL16 expression by oxPAPC stimulated ECs is significantly lower under 24 h pulsatile SS $p = 0.0023$ using an unpaired t test ii) A-D are images taken of ECs by CSLM at 63x. Nuclei are stained blue, ICAM-1 is green and CXCL16 is red. A) oxPAPC stimulated EC ICAM-1 expression under pulsatile SS at 10 dyne/cm² for 24 h B) EC ICAM-1 expression under pulsatile SS at 10 dyne/cm² for 24 h C) oxPAPC stimulated EC CXCL16 expression under pulsatile SS at 10 dyne/cm² for 24 h D) EC CXCL16 expression under pulsatile SS at 10 dyne/cm² for 24 h

3.3.3.4 The effect of time on EC receptor expression sustainability

ECs in a static environment show a decrease in ICAM-1 expression from 6 to 24 h. This decrease does not however interrupt as a significant decrease (Figure 3.19 (i) and (ii) A and B). No quantitative results for CXCL16 are presented as values above the cut off 50 did not exist.

In a static environment ICAM-1 expression levels decrease from 6 to 24 h in TNF- α stimulated cells (Figure 3.20 (i) and (ii) A and B). In the same conditions, there is no difference in CXCL16 expression (Figure 3.20 (i) and (ii) C and D).

i)



ii)

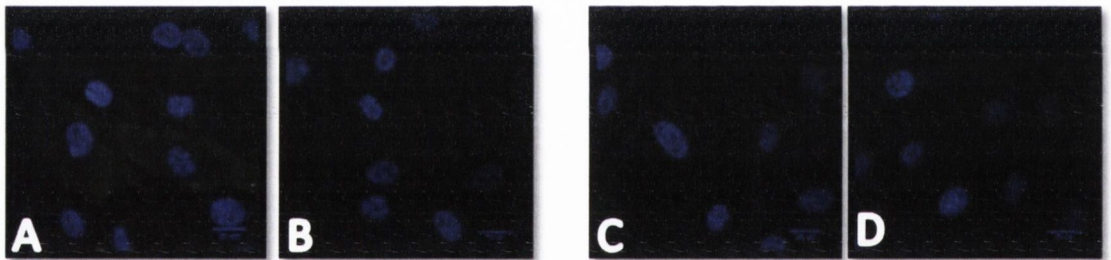
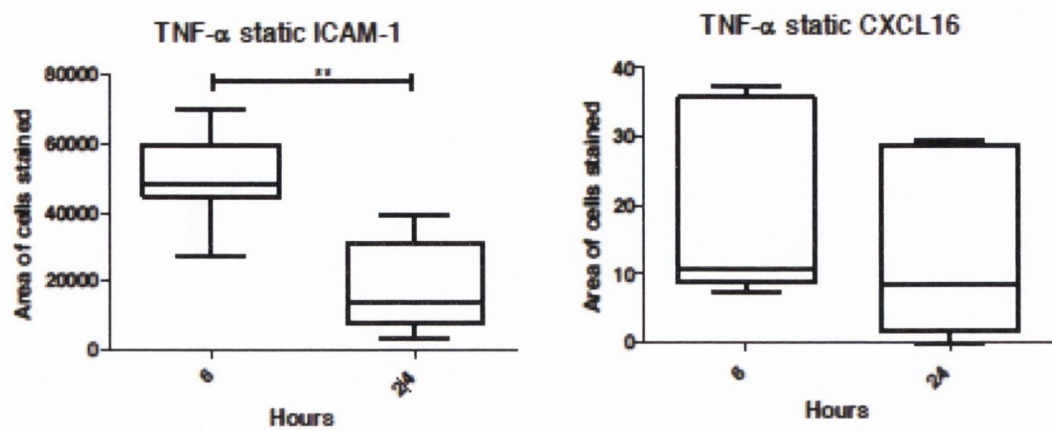


Figure 3.19 i) The two graphs represent the area stained by ICAM-1 and CXCL16 per cell. Results compared EC expression of ICAM-1 and CXCL16 at time points 6 and 24 h under static conditions, each parameter was tested in duplicate. There is no significant difference between ICAM-1 or CXCL16 at any time points using an unpaired t test ii) A-D are images taken of ECs by CSLM at 63x. Nuclei are stained blue, ICAM-1 is green and CXCL16 is red. A) EC ICAM-1 expression under static conditions for 6 h B) EC ICAM-1 expression under static conditions for 24 h C) EC CXCL16 expression under static conditions for 6 h D) EC CXCL16 expression under static conditions for 24 h

i)



ii)

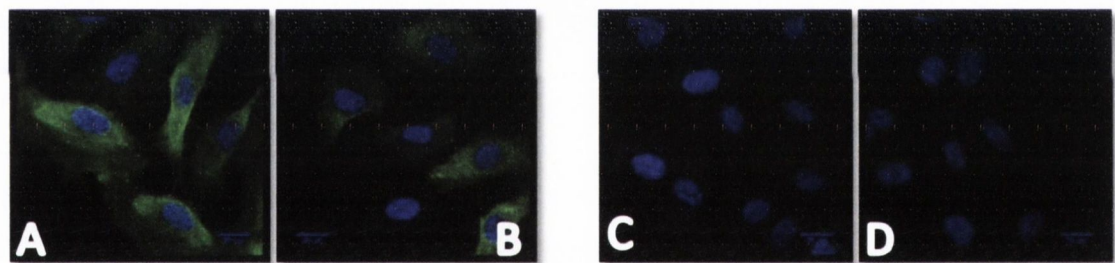
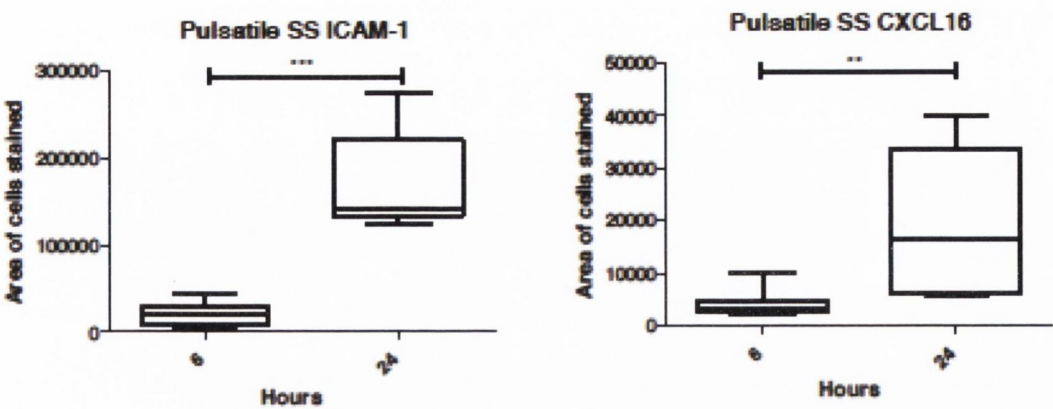


Figure 3.20 i) The two graphs represent the area stained by ICAM-1 and CXCL16 per cell. Results compare TNF-α (10 ng/μl) stimulated EC expression of ICAM-1 and CXCL16 at time points 6 and 24 h under static conditions, each parameter was tested in duplicate. There is a significant decrease of ICAM-1 at 24 h $p = 0.0011$. CXCL16 expression shows no significant change between the two time points using an unpaired t test ii) A-D are images taken of ECs by CSLM at 63x. Nuclei are stained blue, ICAM-1 is green and CXCL16 is red. A) TNF-α stimulated EC ICAM-1 expression under static conditions for 6 h B) TNF-α stimulated EC ICAM-1 expression under static conditions for 24 h C) TNF-α stimulated EC CXCL16 expression under static conditions for 6 h D) TNF-α stimulated EC CXCL16 expression under static conditions for 24 h

Both images and values show a significant increase in ICAM-1 expression when comparing 6 h and 24 h unstimulated ECs under pulsatile flow. A p value of < 0.0001 demonstrates the significant increase over 6-24 h time point (Figure 3.21 (i)). Visually ICAM-1 is intensified around the cytoplasm edges at 24 h while at 6 h ICAM-1 appears to be more intense closer to the nuclei. In both conditions, EC ICAM-1 expression varies between cells within the same fields (Figure 3.21 (i) A and B). Like ICAM-1 regulation, CXCL16 was present at 6 h but at 24 h expression was up-regulated significantly ($p=0.0070$). In a couple of cells imaged CXCL16 staining could be seen spreading through the cytoplasm to the cells edge at 24 h. Again like in the 24 h images of ICAM-1, there was a high intensity of CXCL16 around the edges in a few ECs. Qualitative and quantitative data showed that time had an increasing effect on ICAM-1 and CXCL16 expression in unstimulated ECs under pulsatile flow (Figure 3.21 (i) and (ii) C and D).

i)



ii)

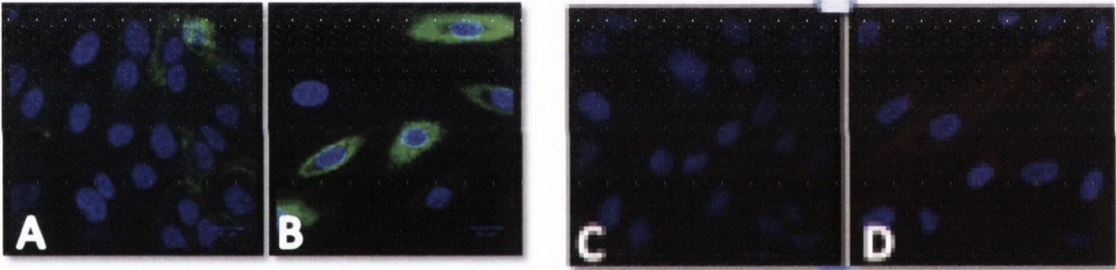
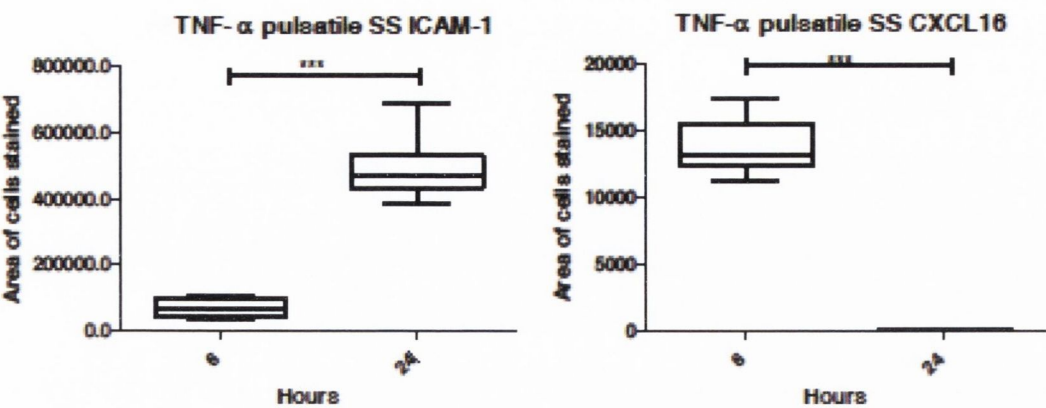


Figure 3.21 i) The two graphs represent the area stained by ICAM-1 and CXCL16 per cell. Results compare EC expression of ICAM-1 and CXCL16 at time points 6 and 24 h under pulsatile SS of 10 dyne/cm², each parameter was tested in duplicate. Between 6 and 24 h there is a significant increase in ICAM-1 and CXCL16 expression $p < 0.0001$ and $p = 0.0070$ using an unpaired t test ii) A-D are images taken of ECs by CSLM at 63x. Nuclei are stained blue, ICAM-1 is green and CXCL16 is red. A) EC ICAM-1 expression under pulsatile SS for 6 h B) EC ICAM-1 expression under pulsatile SS for 24 h C) EC CXCL16 expression under pulsatile SS for 6 h D) EC CXCL16 expression under pulsatile SS for 24 h

Qualitatively, ICAM-1 staining intensified from 6 to 24 h and every cell, from nuclei to cell-cell junction was positive for ICAM-1 expression (Figure 3.22 (ii) A and B). The combination of TNF- α , pulsatile flow and 24 h had the largest staining area per cell out of all the treatments that were experimented in this study. Time made a significant impact on ICAM-1 regulation $p < 0.0001$ (Figure 3.22 (i)). The same three parameters had the opposite effect on CXCL16 expression, from 6 h to 24 h there was a significant down regulation of CXCL16 $p < 0.0001$ in ECs. CXCL16 staining was not visible in images however values showed slight expression (Figure 3.22 (i) and (ii) C and D).

i)



ii)

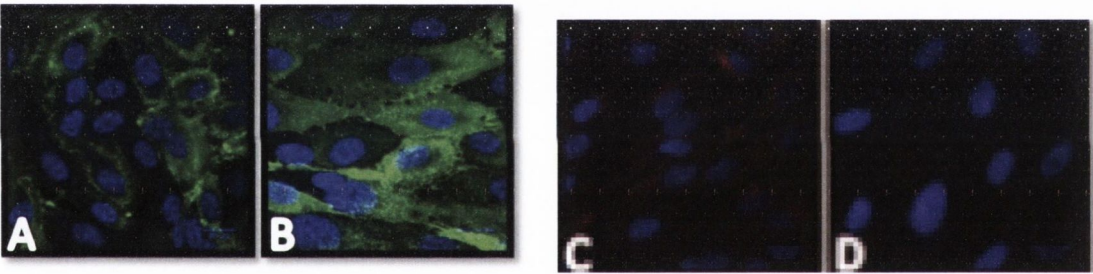


Figure 3.22 i) The two graphs represent the area stained by ICAM-1 and CXCL16 per cell. Results compare TNF- α (10 ng/ μ l) TNF- α stimulated EC expression of ICAM-1 and CXCL16 at time points 6 and 24 h under pulsatile SS of 10 dyne/cm², each parameter was tested in duplicate. Between 6 and 24 h there is a significant increase in ICAM-1 expression in TNF- α stimulated ECs under pulsatile SS $p < 0.0001$, and a significant decrease from 6 to 24 h in CXCL16 expression under the same conditions using an unpaired t test ii) A-D are images taken of ECs by CSLM at 63x. Nuclei are stained blue, ICAM-1 is green and CXCL16 is red. A) TNF- α stimulated EC ICAM-1 expression under pulsatile SS for 6 h B) TNF- α stimulated EC ICAM-1 expression under pulsatile SS for 24 h C) TNF- α stimulated EC CXCL16 expression under pulsatile SS for 6 h D) TNF- α stimulated EC CXCL16 expression under pulsatile SS for 24 h

3.4 Discussion

A number of novel approaches to investigate triggers and receptors involved in plaque development were attempted in this chapter. This chapter provides qualitative and quantitative evidence of the effects of pulsatile shear stress and stimulation using oxidised lipoproteins and cytokines on HUVEC receptor expression. A combination of pulsatile shear stress, stimulation and incubation period, produce different expression levels of ICAM-1 and CXCL16 as opposed to each individual parameter effect alone.

3.4.1 Primary triggers on EC receptor expression

SS on CXCL16

The expression of CXCL16 was of particular interest as it is a triple threat with adhesion, receptor and chemotactic roles and expressed on multiple cell types, macrophages, SMCs and ECs. Its role in atherogenesis is also debated on whether or not it is pro-atherogenic or athero-protective. In the past CXCL16 has been observed as a gene responsive to disturbed flow in mouse endothelium (Ni et al.,2010), in this study pulsatile shear stress was capable of inducing CXCL16 expression as no CXCL16 was evident in ECs in a static environment. This clarifies that CXCL16 is responsive to normal pulsatile shear flow.

SS on ICAM-1

There are many studies investigating the ICAM-1 response to various shear stresses and flow types. ICAM-1 appears to be a highly sensitive response element in these types of conditions. By comparing area of cell stained for ICAM-1 this study supports the theory that laminar pulsatile flow increases ICAM-1 but does not induce it, as ICAM-1 is present in a static environment. However the levels of ICAM-1 in static ECs are far less. These findings support the presence of ICAM-1 in healthy arteries in undisturbed areas of the vasculature. The morphology of ECs observed at 24 h pulsatile flow for both ICAM-1 and CXCL16, showed a

More intense halo-type shape around the cells edge. Increased expression of junction adhesion molecules have been documented in HUVECs (Hisano et al., 1999).

Overall this study showed that pulsatile laminar flow increased ICAM-1 expression and induced CXCL16 expression in HUVECs in a controlled *in vitro* model. There were significant increases in unstimulated ECs expression of receptors subjected to pulsatile flow compared to a static environment. ICAM-1 was also more sensitive to regulation by pulsatile shear stress than CXCL16. ICAM-1 could participate in excess monocyte adhesion and potentially be a huge factor in early plaque development under normal conditions.

3.4.2 Secondary triggers oxPAPC and TNF- α on EC receptor expression

The role of ICAM-1 expression due to stimulation and shear stress has been studied more extensively than CXCL16 and its role is clear in assisting in plaque initiation and build up. Part of this study investigated two potential endothelial cell activators oxPAPC and TNF- α on regulating receptors involved in plaque initiation.

OxPAPC on ICAM-1

As oxLDL has been extensively studied regarding its role in atherogenesis, it was of interest to define the roles of its components. POVPC and PGPC are abundant products in oxidized LDL and have been identified as major players in “the activation of endothelial cells and induction of leukocyte binding” (Ashraf et al., 2008). So far current literature reports that oxPAPC facilitates adhesion of monocytes through B1-integrin activation (Leonarduzzi et al., 2012) and the majority of studies have not confirmed it as a stimulant for up-regulation of ICAM, VCAM, or E-selectin, but rather to induce MCP-1, connecting segment-1, and P-selectin (Fruhwirth et al., 2007). In a static and pulsatile environment unstimulated ECs and oxPAPC stimulated ECs showed no significant difference in ICAM-1 expression at 24 h. Therefore oxPAPC had no significant effect combining these factors supporting previous

findings that oxPAPC does not influence the induction or regulation of ICAM-1 expression in this specific environment.

OxPAPC on CXCL16

Both qualitative and quantitative results support the lack of effect oxPAPC had on CXCL16 expression at 24 h. oxPAPC neither induced or increased CXCL16 expression, therefore it can be presumed this component of highly oxidised LDL is not responsible for CXCL16 expression in ECs.

Supporting Gargalovic et al., this study found no significant difference in protein CXCL16 and ICAM-1 expression on ECs, as oxPAPC has not been confirmed as a NF- κ B activating molecule it is most likely not involved in significantly promoting a pro-inflammatory environment.

TNF- α on ICAM-1

TNF- α on the other hand had a more significant effect on both ICAM-1 and CXCL16 expression in ECs. TNF- α , along with IL-1 β and LPS are known inducers of ICAM-1 in ECs (Myers et al., 1992). This study shows that ICAM-1 did not require TNF- α stimulation or shear stress for ICAM-1 expression and in fact TNF- α only up-regulated its expression, albeit significantly. ICAM-1 appears to be expressed without any stimulant necessary and stimulants only modulate its expression. Therefore monocytes are capable of binding to the endothelial cell in any circumstance.

TNF- α on CXCL16

Studies suggest IFN- γ is a major initiator of CXCL16 expression. According to Wagsater et al, IFN- γ had the biggest effect on elevated CXCL16 expression from aortic smooth muscle cells (AoSMC) (Wagsater et al.). IFN- γ CXCL16 regulation was also confirmed by a study

done by Wuttge (Wuttge et al., 2004) in macrophages *in vitro* and in lesions *in vivo*. Studies on IFN- γ appear to agree on its effect on CXCL16, TNF- α however, like many parameters investigated in this study is the subject of some controversy. TNF- α stimulation of CXCL16 has been reported as insignificant (Wagsater et al., 2004) and significant (Abel et al., 2004). In cancer research, TNF- α induces CXCL16 primary prostate epithelial cells (Darash-Yahana et al., 2009). Similar to Abel et al., this study concluded that TNF- α did induce CXCL16 in ECs in a static environment, but only a minor amount of CXCL16 was expressed. However the induction of this receptor will increase oxLDL binding if it is present, with the up-regulation of ICAM-1 in the same area this will produce all the criteria necessary for fatty streak formation.

Looking at the effect of only 6 h TNF- α on ICAM-1 and CXCL16 expression in ECs in a static environment, results showed that TNF- α had a small inducing effect on CXCL16 and increased ICAM-1 significantly. In a pulsatile environment TNF- α only had an effect on increasing expression of both ICAM-1 and CXCL16.

3.4.3 Sustainability of EC receptor expression under primary and secondary triggers

6 h and 24 h time points were used to investigate the sustainability of stimulants and shear stress to regulate surface receptors. Both ICAM-1 (Panettieri et al., 1995) and CXCL16 (Wagsater et al., 2004) are detectable within 6 h of stimulation; 24 h was therefore used as a sustainability measure. With no stimulant or shear stress CXCL16 did not increase from 6 to 24 h and ICAM-1 lowered expression levels from 6 to 24 h. In a static environment TNF- α stimulated cells decreased expression of ICAM-1 from 6 to 24 h and CXCL16 showed no significant difference between time-points, both time points showed minimal expression levels. ECs under pulsatile shear stress however expressed a significantly higher level of ICAM-1 and CXCL16. As expected from previous results ICAM-1 increased from 6 to 24 h

under TNF- α stimulation however pulsatile flow decreased CXCL16 at 24 h. This study reveals that CXCL16 is not chronically exposed with both a primary and secondary trigger of inflammation. In contrast ICAM-1 is. A study by Leeuwenberg et al., reported that EC ICAM-1 expression reaches a plateau at 24 h and is constant for 96 h when activated (Leeuwenberg et al., 1992).

3.4.4 Concluding remarks

Overall findings suggest that a combination of TNF- α and pulsatile flow has the most significant effect on increasing ICAM-1 levels and extending time-points increases that value again, however it is not the same for CXCL16 expression. The regulation of CXCL16 expression appears to be controlled by pulsatile flow and TNF- α at 6 h. CXCL16 expression is maintained and sustained at 24 h unstimulated pulsatile ECs. However, the combination of stimulant and pulsatile flow appear to exhaust the expression of surface CXCL16 in ECs. Disturbed flow has shown to sustain activation of receptors and transcription factors. This study now shows laminar pulsatile flow is also responsible for ICAM-1 sustainability and CXCL16 without cytokine stimulation as well.

Chiu et al found a combination of TNF- α stimulation and high shear stress increased EC mRNA ICAM-1. This study found applying a lower pulsatile shear stress of 10 dyne/cm² to unstimulated HUVECs induced ICAM-1 and CXCL16 expression at 6h and 24h. With the addition of 10ng/ μ l TNF- α stimulation for 6h to cells under pulsatile shear stress ICAM-1 expression area increased and CXCL16 expression intensified. Other publications have shown that TNF- α stimulated ECs only express ICAM-1 in an environment with flow and not static (Zhou et al., 2007).

The activation of CXCL16 and its abundance is critical, as more CXCL16 surface receptor produced will result in oxLDL uptake, if cholesterol synthesis is compromised and the

endothelial cell wall disrupted fatty streak formation is inevitable. Also if ADAM10 is present in the surrounding environment, CXCL16 could potentially be cleaved and made soluble. Soluble CXCL16 then recruits monocytes to the site of inflammation. The co-expression of CXCL16 and ICAM-1 at 6 h under pulsatile flow and TNF- α stimulation is potentially a pro-atherogenic environment, binding both monocytes and oxLDL.

3.4.2 Limitations

A few limitations existed within the experiment, specifically regarding the biochip and the pulsatile pump. Originally a biochip with a Y shaped channel was designed to investigate the ECs expression at the bifurcation. However, the cells would not seed evenly along the diverging channels and cells accumulated causing blockages in the channels. As this issue could not be resolved a straight channel biochip was used. The iKima pump had a set range of dyne/cm² therefore there were not many options of varying shear stress rates. In this study we used a pulsatile flow consisting of pulse for 2 min and rest for 20. This was decided on throughout the optimization of the biochip. The endothelial cells would detach from the fibronectin lining the channels when a more frequent pulse, more than 2 min, was used. The combination of 10 dyne/cm² and a pulse for 2 min every 20 min was the result of a highly optimised technique. Other obvious limitations noted are the absence of other native artery cells such as smooth muscle cells. However, this is the desired environment we required for specifically looking at EC involvement in plaque development.

However, not all attempts were successful. ELISA results did not detect any soluble CXCL16 being expressed. This may have been due the vast volume (20ml) of media compared to the cell density (10⁵ per channel). A significant amount of soluble CXCL16 has been reported to be released by 10 ng/ μ l TNF- α stimulated ECs (Abel et al.,2004). The RT-PCR encountered set backs also also, lysing cells in the channel and regaining them proved unsuccessful. A main part of

this study was optimizing and validating an *in vitro* pulsatile flow model that produced a controlled and stable environment for investigating EC surface receptor expression.

3.4.1 Future work

For this specific biochip, future studies could focus on analysing and imaging drug delivery using nano or micron particles, as these chips are capable of live and fixed imaging. The biochip could be a potential platform to investigate and research emerging therapeutics which target adhesion molecules or endothelial cells. As this study has shown there is great potential for quantification of expression using imaging. Using the method discussed other stimulators and receptors could be analysed. The environment sustained in this study was very controlled and specific. Future work could also include using siRNA cells, knocking down specific players in the shear stress response of ECs.

Chapter 4

Investigating allelic expression imbalance *in vivo* and *in vitro*

4.1 Introduction

The previous two chapters have discussed the presence and abundance of cytokines and receptors, and in particular CXCL16. In this chapter, CXCL16 gene expression in individuals was investigated to further investigate the importance of CXCL16 expression and its role in atherosclerosis. IL-18 has been identified as an inducer of many potent cytokines in atherosclerosis, including CXCL16.

Gene expression is affected by cis and trans-acting genetic and epigenetic variation. Expression quantitative trait loci (eQTL) mapping characterizes the influence variation has on expression. However, for cis-acting regulatory variation, allele specific approaches are required (Pastinen, 2010). Cis-regulatory mutations affect morphological, physiological and neurological phenotypes and cause, among others, B-thalassemia, haemophilia and atherosclerosis (Epstein et al., 2009).

4.1.1 Allelic expression imbalance (AEI)

Allele imbalance or allele specific expression contributes to human phenotypic variation (Schadt et al., 2003). Allelic expression imbalance is when one haplotype is expressed more than another in a heterozygous individual due to a marker SNP resulting in excess mRNA and overall variation in a population and can be quantified by real-time PCR. Detecting AEI depends on the strength of allelic imbalance (evidence of widespread departure from 1:1 allelic ratio) at the marker heterozygous SNP (Heap et al., 2009). Advantages of using allelic discrimination on cis effects include eliminating environmental and trans-acting influences and detecting differences in allele expression of heterozygotes (Pastinen, 2010).

So far, we have attempted to analyse potential regulators of *CXCL16* in carotid patients and CXCL16 receptor expression in an *in vitro* model. In this chapter cis-acting regulatory elements were investigated in CXCL16 and IL-18 for their effect on expression in clinical

samples of stable angina pectoris patients and heterozygous cell lines from the Centre d'Etude du Polymorphisme Humain (CEPH).

4.1.3 SNPs in CXCL16 and IL-18

Both CXCL16 and IL-18 are involved in multiple stages of atherosclerosis and influence each other's expression. Therefore, monitoring their gene expression in normal and diseased populations is vital, so that patterns of expression can be assessed and associated accordingly.

SNPs are selected on the basis of:

- 1) They must be on the mature RNA-exonic 5'UTR or 3'UTR
- 2) They must be far enough from the splice junction to make sure the primers don't overlap with intrinsic sequence
- 3) Heterozygosity must be high enough to make sure you get enough heterozygote individuals or cell lines

Candidate SNPs were identified on the Applied Biosystems website (Assays on demand). The SNP assayed is only a proxy- it does not need to be functionally relevant in the disease process- it is just a marker for imbalance; which could be due to other cis-acting SNPs.

4.1.3.1 CXCL16 rs2277680

The SNP rs2277680 is a missense mutation, has an allele change of C-T and a residue change from A [Ala] to V [Val] at position 181. The HapMap project has revealed that the SNP is relatively common in many populations by frequency of the minor allele G; 0.44 in Japanese, 0.58 in CEU, 0.75 in Nigeria and 0.45 in India making it an accessible polymorphism in a population for research. However, studies carried out looking at this particular allele change have not supported its association with CVD. Lundberg et al, did not find a significant difference in allele frequency between patients and controls, there was an associated

difference in expression of the V-allele between stenosis groups. The V-allele showed a direct correlation with increasing stenosis (Lundberg et al., 2005). Petit et al came to the same conclusion but in post-myocardial infarct patients. Using a large cohort no association was found between the V181 allele and the protection or pre-disposition to CHD (Petit et al., 2011). A study by Hang et al., focused on four particular SNPs, rs2250333, rs2304973, rs2277680 and rs1051009 of the Chinese population and reported that only rs3744700 showed significance between controls and cases and there was no evident link with severity of stenosis and SNP rs2277680 (Huang et al., 2010). We decided to further investigate rs2277680 for allelic expression imbalance in cell lines activated by TNF- α .

4.1.3.2 *IL-18* rs360717

A number of studies have several polymorphisms in functional regions of the *IL-18* gene. The potential genetic variability of IL-18 is related to cardiovascular phenotypes such as serum levels and mortality (Tiret et al., 2005). The SNP mutation rs360717 is a 5'UTR mutation, has an allele change of C-T at position 105. Using the AtheroGene cohort Barbaux et al, found haplotypes that combine rs360717 and rs5744292 in complete linkage disequilibrium have been associated with lower circulating levels of IL-18 (Barbaux et al., 2007). However, Koch et al., recently reported that individuals with rs360717 and four other SNPs of *IL-18* gene were associated with an increased risk of acute myocardial infarction (AMI) (Koch et al., 2011).

Currently there are few studies investigating these two SNPs, pubmed cites 8 papers which include SNP rs360717 and 2 papers citing SNP rs2277680.

4.1.4 Epigenetics

Epigenetics is the study of changes in gene expression caused by post-translational modifications to histones and nucleic acids without changing the nucleotide sequence (Spector, 2012). Histones are highly conserved small basic proteins found mainly in the nucleus which help pack 2m of DNA into a chromatin structure. Chromatin regulates the access of factors involved in transcription, regulation and repair (Xhemalce et al., 2011). Histones may be post-translationally modified to alter the chromatin structure and this may have significant roles in biological processes that are involved in the expression of DNA. Such modifications include, histone acetylation, phosphorylation and methylation. Their function is mediated through specific enzymatic activity of recruited proteins and complexes (Bannister and Kouzarides, 2011). Inhibition of these enzymes has been targeted as a new strategy in cancer treatment as they are potent inducers of growth arrest and are responsible for the apoptosis of tumour cells (Monneret, 2005). Histone deacetylases (HDAC) inhibitors specifically show a promising future in the fight against cancer and in this chapter in particular, the effects of L-Sulforaphane are investigated.

L-Sulforaphane is a naturally occurring isothiocyanate found in green and yellow vegetables and induces the detoxification of enzymes that participate in xenobiotic metabolism (Zhang et al., 1992).

4.1.5 Histone acetylation dependent allelic expression imbalance

The importance of AEI in disease is becoming more evident as Fischer et al., found that AEI of the *BAPX1* gene predisposes individuals to oculo-auriculo-vertebral spectrum (OAVS) as AEI was only evident in patients and relatives and could occur *de novo*. AEI most likely affects the total BAPX1 mRNA, owing to variation between individuals. Allelic expression also appeared to be heavily skewed and this was due to the involvement of histone deacetylation (Fischer et al., 2006).

4.1.6 Histone modifications and CVD

Methylation changes have been reported in pathological genes of atherosclerosis such as, superoxide dismutase, endothelial nitric oxide synthase and 15-lipoxygenase. No significant data about histone modifications in atheromas are available. However, it could explain some of the environmental and dietary effects on atherogenesis (Turunen et al., 2009).

4.1.7 Hypotheses & Objectives

The aims of this chapter were to genotype ACS patients for CXCL16 and IL-18 SNPs and to test for an imbalance in allele expression that could be due to genetic variations. This study also genotyped 10 cell lines for the same SNPs and tested for allelic imbalance in these. Keeping with the investigation into the effect TNF- α has on CXCL16 expression, one of the hypotheses was

- that stimulation of these heterozygous cell lines with 10 and 100 ng/ μ l TNF- α could potentially alter allele specific expression. As the previous chapters have discussed TNF- α stimulation on protein expression and levels in atherosclerosis patients, it was decided to further study its stimulatory effect on gene expression using the concentration from previous chapters in this experiment, as well as a much higher concentration (100ng/ μ l) (as seen in atheromas) but at the same time point.
- Finally, this study wanted to test the hypotheses that the inhibitor L-Sulforaphane, a HDAC inhibitor, could potentially effect and regulate allele expression.

Overall this study attempted to gain further insight into CXCL16 and IL-18 genetic expression associated with an atherosclerotic environment using molecular techniques and to investigate if histone modification plays a part in AEI.

4.2 Materials and Methods

4.2.1 Samples

4.2.1.1 Centre d'Etude du Polymorphisme Humain (CEPH)

Cell lines involved originated from the CEPH European (CEU) (<http://www.cephb.fr/>). These EBV-transformed lymphoblastoid cell lines have been genotyped by the HapMap consortium in an attempt to locate specific regions which share genetic variations within and between populations (<http://www.hapmap.org>). Both the genotype data and gene expression (<http://www.sanger.ac.uk>) data are publicly available.

4.2.1.2 Cell lines and Culture

All EBV-transformed lymphoblastoid cell lines GM10856, 10861, 12239, 12234, 12003, 07348, 10846, 11881, 11995, 12717, 11839, 12864, 12872, 10863, 12155 and 12006 used have been genotyped as HapMap CEU samples and were obtained from the Coriell Institute. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 20% foetal bovine serum (FBS) in 75cm³ flasks at 37°C and 5% CO².

4.2.1.3 Patient recruitment for clinical samples

A total of 48 patient samples and 8 healthy controls from a previous study (Patel, 2011) were used. These patients were ACS diagnosed and due to undergo a coronary angiography (Table 4.1). Patients were recruited through the department of cardiology in St James' Hospital and all gave informed consent in accordance with the criteria set out by the Ethics Board Committee. Patients who had a history of auto-immune disease, renal failure, recent infection, immunosuppressive therapy and malignancy were excluded. The sample population was of Irish descent.

Table 4.1 Clinical characteristics of study groups. Mean values are presented with the S.E.M (standard error mean)

	Unstable Angina n = 12	Myocardial Infarction n = 36
Age	62.6 (±3.17)	67 (±2.33)
Sex (M/F)	10/4	36/12
Days since diagnosis	6.9 (±1.06)	6.08 (±0.69)

4.2.1.4 Blood sampling and PBL isolation

Blood sampling and serum isolation was carried out as in chapter 2.

4.5.2.2 TNF- α Treatment of cell lines

CXCL16 heterozygous cell lines GM10856, 10861, 12239, 12234, 12003, 07348, 10846, 11881, 11995, 12717 and *IL-18* heterozygous cell lines GM10861, 10856 and 11995 were activated with TNF- α . Cells were seeded in 6 well plates (Sarstedt) at $\sim 10^5$ and treated with 10 and 100 ng/ μ l of TNF- α (R&D systems, USA) concentration and at time point 6 h. Media were removed and centrifuged for 5 min at 1800 rpm at room temperature. The supernatant was removed and 350 μ l of lysate buffer was added (Qiagen). Lysates were stored at -80°C until further use.

4.2.2.1 Treatment of cell lines with inhibitor L-Sulforaphane

Cells were seeded in 6 well plates at $\sim 10^5$ and treated with L-Sulforaphane (Sigma-Aldrich, USA). A dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) only control and TNF- α (10 ng/ μ l) only control were used. After 6 h, cells were lysed for RNA extraction as previously described.

4.2.3 RNA extraction

An RNeasy micro extraction kit (Qiagen) was used for RNA extraction from $\sim 10^5$ cells per treatment per cell line according to the manufacturer's protocol. Clinical RNA samples had previously been extracted from a previous study in our laboratory (Patel, 2011).

4.2.4 DNA extraction

DNA extraction of both clinical samples and $\sim 10^5$ cultured cells from each cell line was performed at the BioBank in the Institute of Molecular Medicine, Trinity College.

4.2.5 DNA and RNA quantification by spectrophotometry

Concentrations of RNA and DNA were measured using the Nanodrop 8 sample spectrophotometer ND-8000 (Nanodrop Tech, DE, USA).

4.2.5 cDNA synthesis

1 μ g of RNA from each of the HapMap CEPH lymphoblast cell lines and from each of the clinical samples was reverse transcribed into cDNA using a RETROscript Kit (Ambion, Applied Biosystems, USA) according to the manufacturer's instructions. cDNA synthesis using the RETROscript Kit (Ambion, Applied Biosystems, USA) is a two step process (Table 4.2). **Step 1.** 2 μ l of oligo (dt) primers was added into an eppendorf tube and 10 μ l of 1 μ g RNA. The mixture was run for 4 minutes at 72°C on a thermal cycler (PTC-200). **Step 2.** The following concentrations and volumes made up the master mix which was added to the mixtures after step 1. The complete mixture (20 μ l) was then run at 42°C for 2 hours and held at 4°C.

Table. 4.2 Steps and reagents involved in cDNA synthesis

Step 1		Step 2	
Reagents	μl	Reagents	μl
Template RNA	10	RT Buffer	2
Oligo (dt) primers	2	dNTP mix	4
Total	12	Reverse Transcriptase	0.5
		RNase inhibitor	0.5
		RNase free water	1
		Total	8

4.2.6 Marker SNPs

The following marker SNPs were chosen in order to quantify allelic discrimination in heterozygote individuals (Table 4.3). Using a SNP with a high minor allele frequency indicates a larger population of heterozygotes, which is desirable as it increases the number of individuals available for the study.

Table 4.3 Location, Minor Allele Frequency and number of heterozygotes for AEI marker SNPs used (Petit 2011)

Gene	Marker	Location in Gene	Minor Allele Frequency	HapMap CEPH Heterozygotes
CXCL16	rs2277680	Exon 4	0.58	41
IL-18	rs360717	Exon 1	0.46	19

4.2.7 Genotyping clinical samples for CXCL16 and IL-18 SNP and cell lines for IL-18 SNP

All 10 cell lines used were heterozygous for SNP rs2277680 (CXCL16). Genotyping was carried out on all cell lines for rs360717 (IL-18) and clinical samples for rs2277680 (CXCL16) and rs360717 (IL-18). Allelic discrimination was performed using TaqMan SNP

genotyping assay (Applied Biosystems) for rs2277680 and rs360717 on the ABI 7900HT Fast Real Time PCR system (Applied Biosystems). All real-time PCR calculations, reagents and conditions are shown (Table 4.4) and each reaction was replicated in triplicate. A positive homozygote control and a negative water control were used. Threshold amplification cycle (Ct) was used to determine relative allele frequency (RAF), where $RAF = 1 / (2^{\Delta Ct} + 1)$ and $\Delta Ct' = [(Ct \text{ allele1 (cDNA)} - Ct \text{ allele2(cDNA)}) - (Ct \Delta gDNA)]$. cDNA threshold values were normalised against genomic DNA for assay-specific variability (Quinn, 2011).

Table 4.4 Components, volumes and program for SNP genotyping assay for clinical and CEPH samples

Component	Vol. Required for 1 reaction (µl)	Program
Taqman 2x universal mx	5	50°C 2 min
40x stock of SNP genotyping assay	0.125	95°C 10 min
DNase free water	3.875	95°C 15 sec x40 60°C 1 min
Total volume	9	

4.2.7.1 *Analysing genotypes of clinical samples and cell lines*

When the run was completed a post read was performed to determine which clinical samples were heterozygotes. The allelic discrimination option was selected. The area of the plate in use was highlighted and markers VIC-NFQ and FAM-NFQ selected. Information about which markers to be used can be found on the CD which accompanies the assay. Following the commands “Connect”, “post read” and “analyse” the Results tab was generated and showed an image (Figure 4.1) of the samples in clusters. These clusters were manually labelled and once the heterozygotes are identified the results tab showed what wells on the plate these had heterozygote samples for the specific SNP.

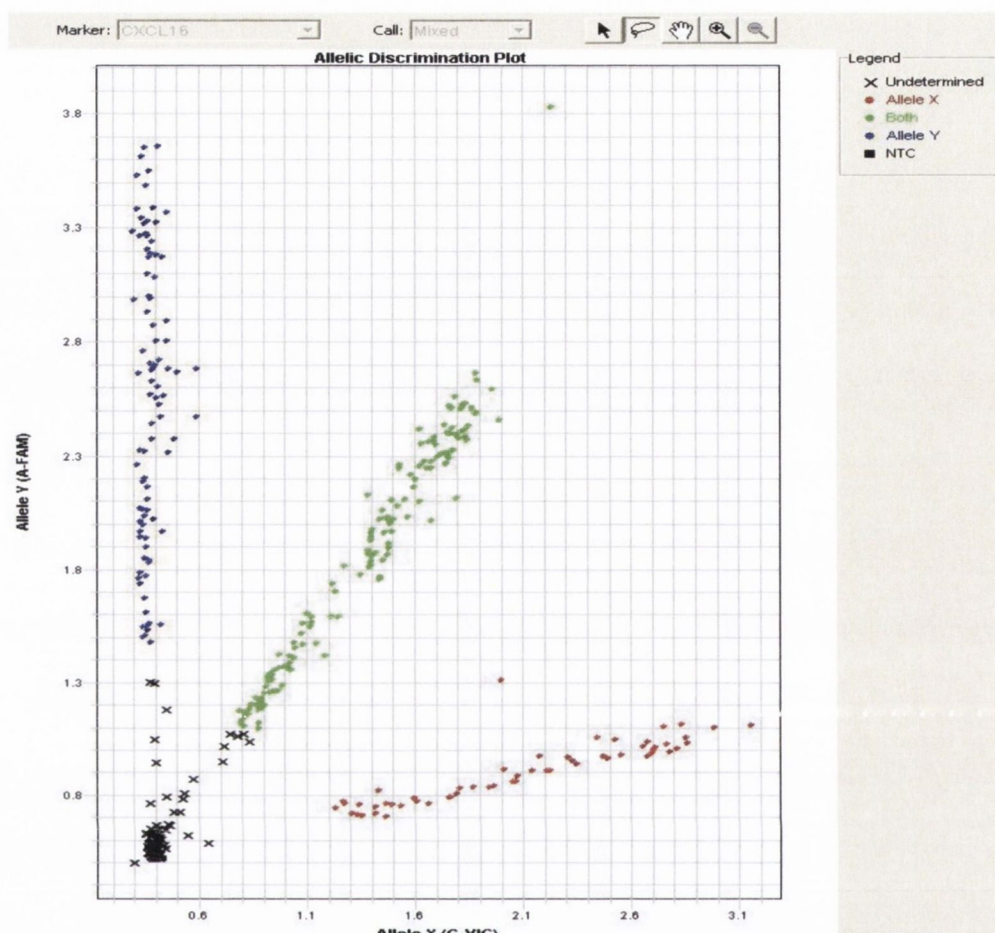


Figure 4.1 Example of a graph of allelic discrimination for genotyping samples. The blue and red clusters of dots are homozygotes and the green are heterozygotes

4.2.8 Allelic expression imbalance

4.2.8.1 Allelic real-time quantification

Allelic expression imbalance was detected and quantified using Taqman allelic discrimination assays (Applied Biosystems). The assay determines the amount of cDNA and DNA at the specific SNP being studied. The assay works by labelling each allele with a probe that has both a fluorescent dye and quencher. The quencher prevents fluorescence until Taq polymerase, which contains 5'-3' exonuclease activity, cleaves the quencher off the probe,

resulting in the release of the fluorescent dye. The level of dye released is measurable in real-time and determines the amount of cDNA/DNA template that is present. Allelic quantification for both rs360717 (IL-18) and rs2277680 (CXCL16) was performed using the same assay for genotyping and on the ABI 7900HT Fast Real Time PCR system (Applied Biosystems). All real-time PCR calculations, reagents and conditions are shown in Table 4.5 and each reaction was replicated in triplicate. A positive homozygote control and a negative water control were also used.

Table 4.5 Components, volumes and program for AEI assay for clinical and CEPH samples

Component	Vol. Required for 1 reaction (µl)	Program
Taqman 2x universal mx	5	50°C 2 min 95°C 10 min 95°C 15 sec x40 60°C 1 min
40x stock of SNP genotyping assay	0.25	
DNase free water	2.75	
cDNA/DNA	20 ng	
Total volume	9	

4.2.8.2 Analysis of AEI results

Differential allelic expression of the *IL-18* and *CXCL16* gene was investigated by the allelic imbalance method. This method compares the ratio of genomic DNAs of each allele to that of cDNA (Figure 4.2) (constitutively equal to 1:1) in individual who are heterozygotes for a given transcribed polymorphism. Both alleles have an influential effect in heterozygous individuals once the genetic background and experimental/environmental conditions are the same, therefore the confounding effects are limited (Barbaux et al., 2007).

4.2.8.3 Calculating AEI using threshold amplification cycle data (Ct)

The threshold amplification cycle data (Ct) is used to determine relative allele frequency (rF). To correct for inequalities in allelic ratios caused by the assay the cDNA, Ct values were normalised against the gDNA Ct values. The equation used to calculate rF is:

$$rF = [1 / (2^{\Delta Ct'^*} + 1)]$$

$$* \Delta Ct' = [(Ct \text{ allele-1 (cDNA)} - Ct \text{ allele-2 (cDNA)}) - (\Delta Ct \text{ gDNA})]$$

The Ct value for cDNA is normalized to the Ct value of gDNA which represents a 1:1 ratio of both alleles. The rF values range between 0 and 1. 0.0 is the total expression of allele-1, 1.0 is the total expression of allele-2 and 0.5 represents equal expression of both alleles. A positive AEI (+) result is evident by ratios greater than 20% (i.e. $0.4 > AEI > 0.6$).

4.3 Results

4.3.1 Genotyping

22 patients in total were heterozygous for CXCL16 rs2277680, 9 were unstable angina (UA) patients and 13 were post myocardial infarcts (MI), no controls were heterozygous. 8 patients of the same 22 in total were heterozygous for IL-18 rs360717, 5 were MI and 3 were UA. Out of the 16 cell lines, 10 were heterozygous for CXCL16 rs2277680 GM10856, 10861, 12239, 12234, 12003, 07348, 10846, 11881, 11995, 12717 and 9 were heterozygous for IL-18 rs360717 GM10856, 10861, 11995, 11839, 12864, 12872, 10863, 12155 and 12006.

4.3.2 AEI at CXCL16 in ACS clinical samples

Using the marker SNP rs2277680, 5 out of 22 heterozygous individuals were found to be positive for AEI (Figure 4.2). This result indicates that approximately a quarter of the ACS heterozygous population display allelic imbalance at CXCL16. All individuals who displayed allelic imbalance for CXCL16 were above 0.6. Three of the five individuals had a high level of imbalance over 0.9. Two were UA patients and three were MI.

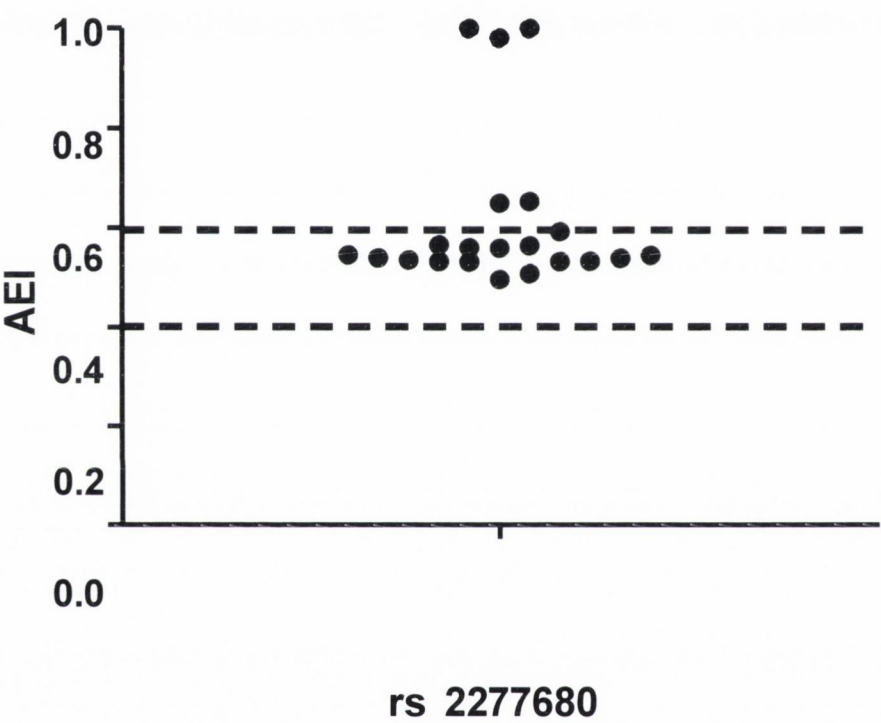


Figure 4.2 Scatterplot of the AEI assay results for the 22 ACS samples for rs2277680. Positive AEI (AEI values >0.6 (n=5) or <0.4 (n=0)) was observed in 5 individuals

4.3.3 AEI at CXCL16 in cell lines

All 10 heterozygous cell lines using marker SNP rs2277680 at CXCL16 showed no AEI (Figure 4.3). Expression of both alleles was between 0.4 and 0.6.

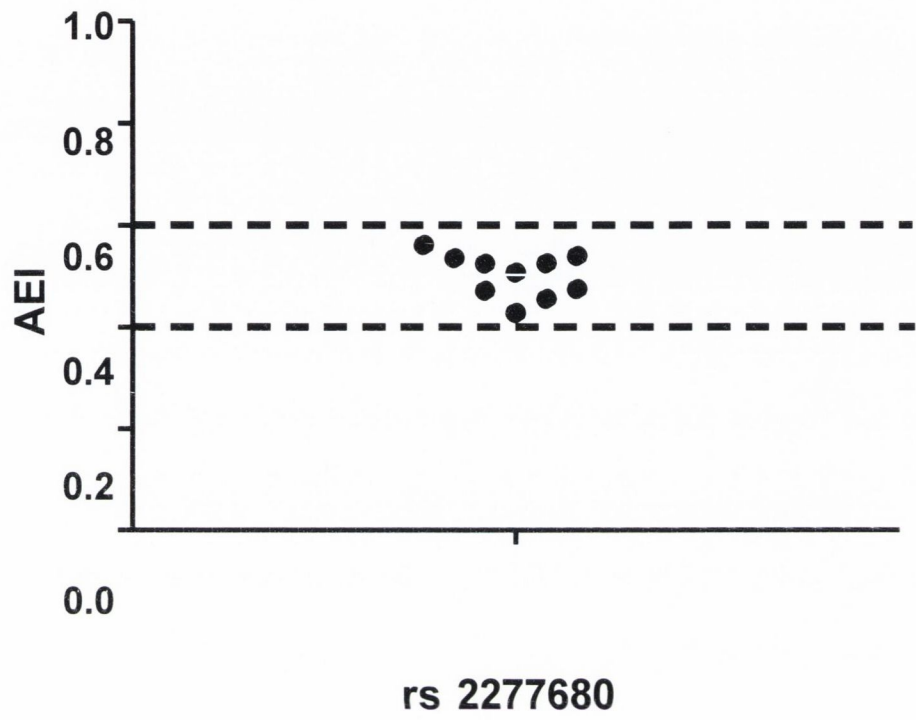


Figure 4.3 Scatterplot of the AEI assay results for the 10 lymphoblast cell line samples for rs2277680. There were no cell lines that showed positive AEI (AEI values >0.6 or <0.4)

4.3.4 AEI at CXCL16 in 10 ng/μl TNF-α stimulated cell lines

All 10 heterozygous cell lines using marker SNP rs2277680 at CXCL16 showed no AEI (Figure 4.4). Expression of both alleles was between 0.4 and 0.6. An unpaired t-test was carried out comparing the AEI values of unstimulated v stimulated cell lines (Figure 4.3 and Figure 4.4), no significant difference was detected. GM10861 (0.42 to 0.58) and GM12003 (0.45 to 0.58) had the biggest increases in AEI out of the 10.

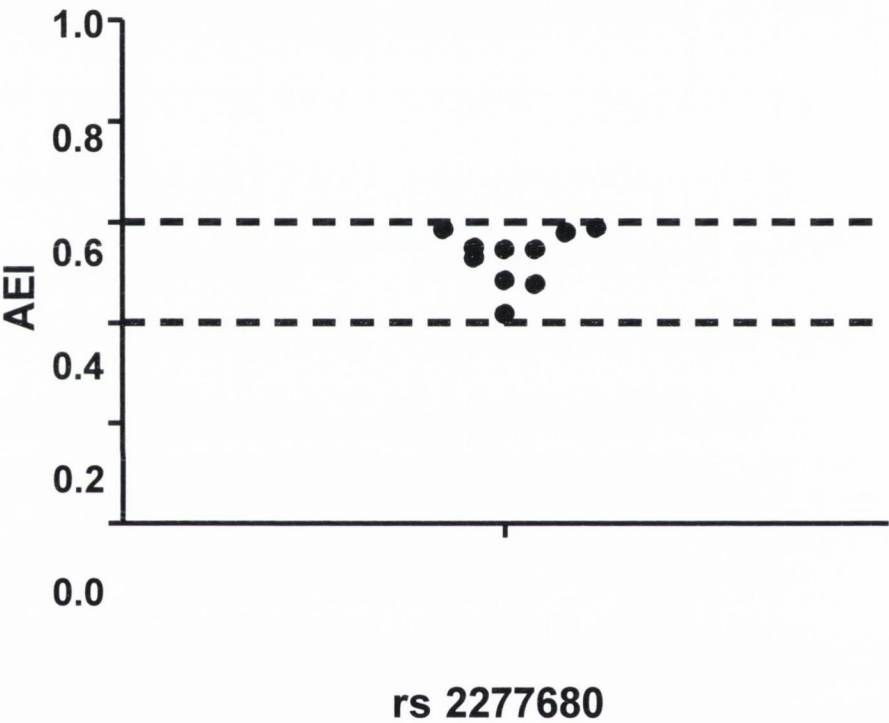


Figure 4.4 Scatterplot of the AEI assay results for rs2277680 in the 10 lymphoblast cell line samples stimulated by 10ng/μl TNF-α. There were no cell lines that showed positive AEI (AEI values >0.6 or <0.4)

4.3.5 AEI at CXCL16 in 100 ng/μl TNF-α stimulated cell lines

AEI carried out on all 10 heterozygous cell lines treated with 100 ng/μl revealed that 50% showed imbalance at rs2277680 (Figure 4.5). All five displaying allelic imbalance, GM11995, GM12717, GM10856, GM10846 and GM11881, were above 0.6 and between 0.8 and 1.0 indicating a strong differential expression.

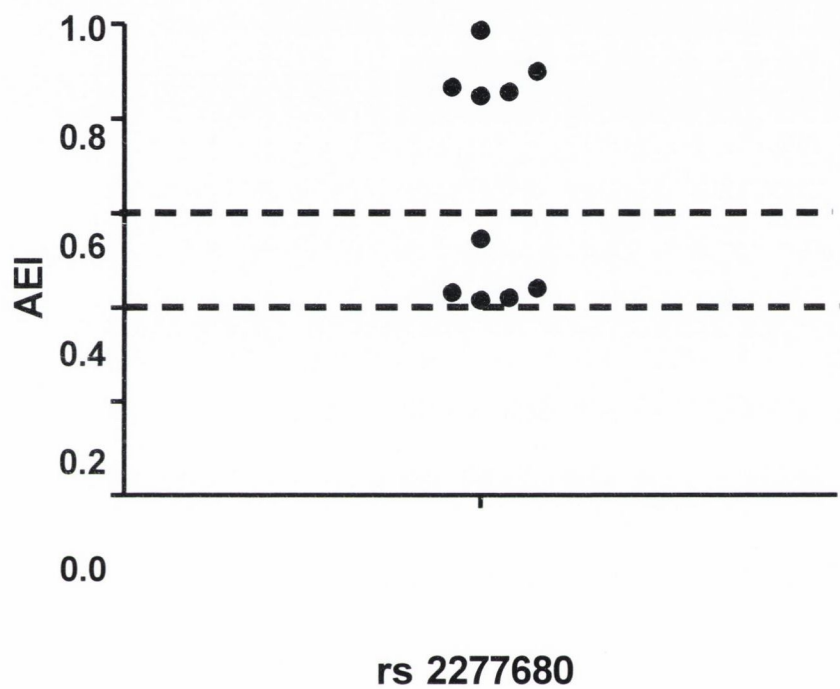


Figure 4.5 Scatterplot of the AEI assay results for the 10 lymphoblast cell line samples for rs2277680 in all 10 cell lines stimulated with 100 ng/μl TNF-α. Positive AEI (AEI values >0.6 (n=5) or <0.4 (n=0)) was observed in 5 cell lines, GM11995, GM12717, GM10856, GM10846 and GM11881

4.3.6 AEI at CXCL16 in cell line 07348 treated with L-Sulforaphane

AEI of CXCL16 at SNP rs2277680 in GM07348 under five different treatments was carried out to investigate the effect the inhibitor had on allelic expression in healthy individuals (Figure 4.6). As previously discussed this cell line showed no imbalance without TNF- α and with 10ng/ μ l TNF- α stimulation. However, 07348 treated with DMSO, inhibitor only and a combination of inhibitor and TNF- α displayed allelic imbalance at CXCL16. All positive AEI were above 6.0 and between 0.8 and 1.0 indicating a strong imbalance.

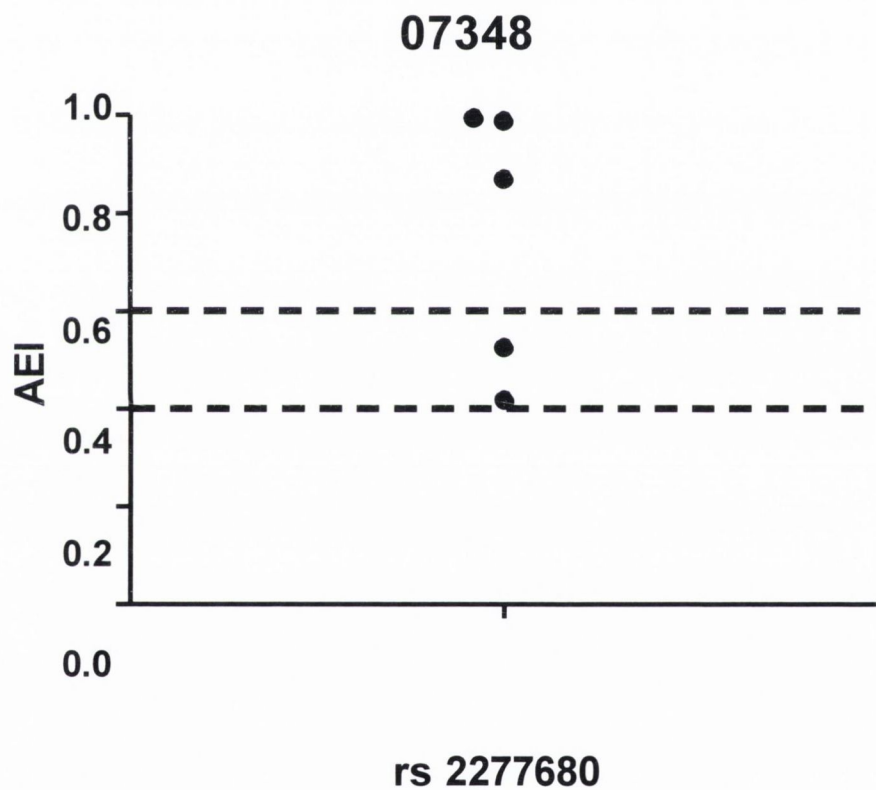


Figure 4.6 Scatterplot of the AEI assay results for rs2277680 in cell line 07348 treated with DMSO, inhibitor only, inhibitor plus 10 ng/ μ l TNF- α , 10 ng/ μ l TNF- α only, and a no treatment control. Positive AEI (AEI values >0.6 (n=3) <0.4 (n=0)) was observed in 07348 treated with DMSO, inhibitor only and inhibitor plus TNF- α . AEI values were DMSO=0.994, L-Sulphoraphane=0.870 and L-Sulphoraphane+TNF- α =0.989

4.3.7 AEI at CXCL16 in cell line 12234 treated with L-Sulforaphane

AEI of CXCL16 at SNP rs2277680 in GM12234 under five different treatments was carried out to investigate the effect the inhibitor had on allelic expression in healthy individuals (Figure 4.7). As previously discussed this cell line showed no imbalance without TNF- α and with 10ng/ μ l TNF- α stimulation. However, 12234 treated with DMSO, inhibitor only and a combination of inhibitor and TNF- α displayed allelic imbalance at CXCL16. All positive AEI were above 0.6 and between 0.8 and 1.0 indicating a strong imbalance.

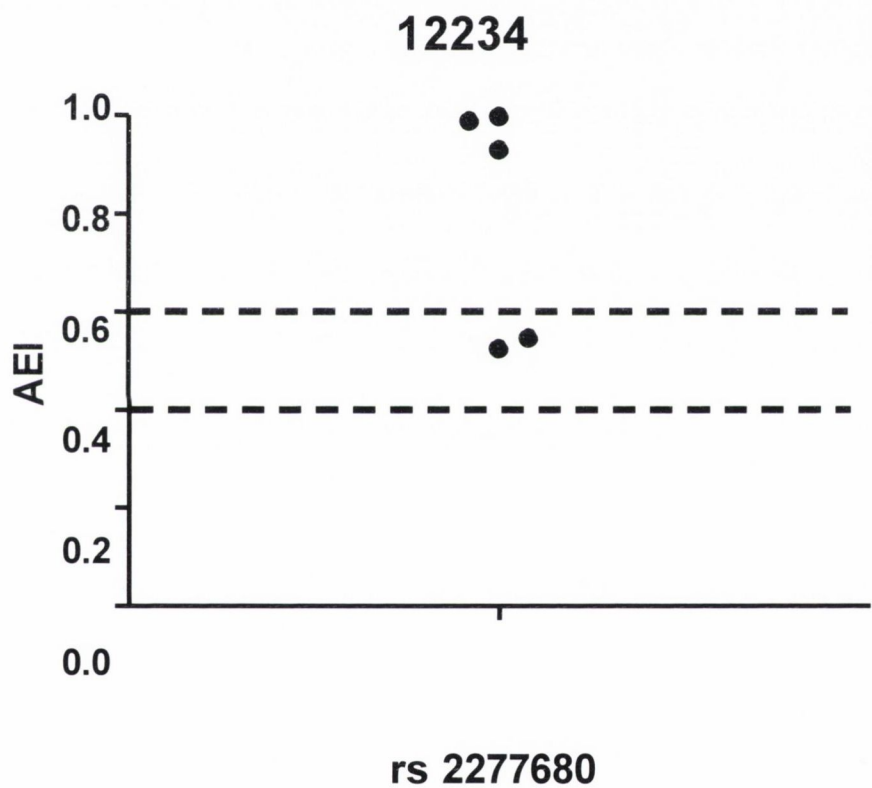


Figure 4.7 Scatterplot of the AEI assay results for rs2277680 in cell line 12234 treated with DMSO, inhibitor only, inhibitor plus 10 ng/ μ l of TNF- α , 10 ng/ μ l TNF- α only and no treatment control. Positive AEI (AEI values >0.6 (n=3) <0.4 (n=0)) was observed in 12234 treated with DMSO, inhibitor only and inhibitor plus TNF- α . DMSO=0.997, L-Sulphoraphane=0.930 and L-Sulphoraphane+TNF- α =0.988

4.3.8 AEI at IL-18 in ACS clinical samples

Using the marker SNP rs360717, all 8 heterozygous individuals were found to be positive for AEI (Figure 4.8). This result shows 100% of ACS heterozygous population display allelic imbalance at *IL-18*. All individuals who displayed allelic imbalance for *IL-18* were above 0.6. Three of the five individuals had a high level of imbalance over 0.9. 3 individuals were UA patients and 5 were MI patients.

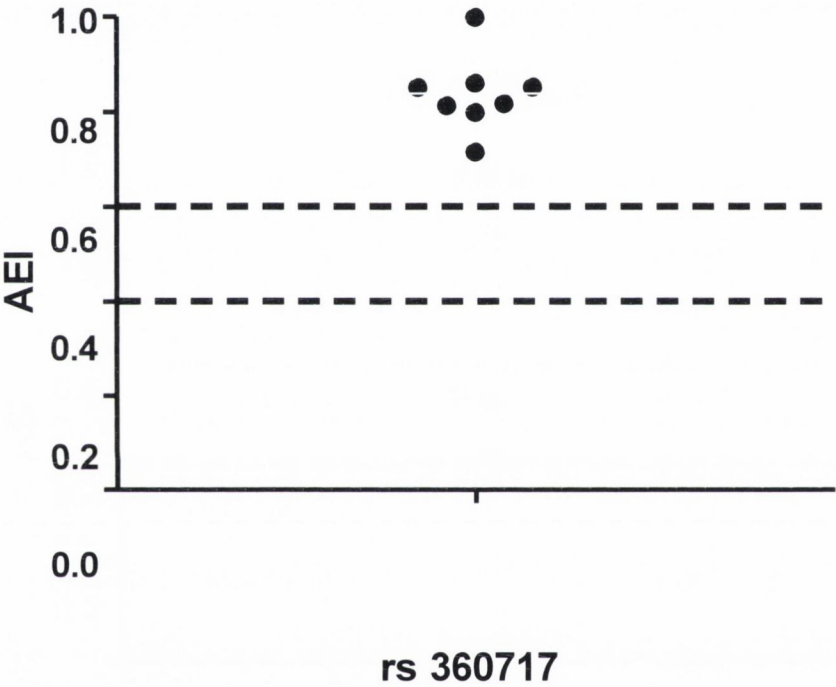


Figure 4.8 Scatterplot of the AEI assay results for the 8 ACS samples for rs360717. Positive AEI (AEI values >0.6 (n=8) or <0.4 (n=0)) was observed in all 8 individuals. UA AEI values were 0.999, 0.799 and 0.822 and MI AEI values were 0.853, 0.813, 0.861, 0.817, 0.716 and 0.851

4.3.9 AEI at IL-18 in cell lines

Using marker SNP rs360717, 8 heterozygous cell lines showed imbalance at *IL-18* (Figure 4.9). GM10863 was the only cell line that did not show AEI imbalance. Six AEI values were above 0.6 and two were below 0.4 (GM12155 and 12006). Four cell lines showed a high level of AEI imbalance between 0.8 and 1.0 (GM10861, 10856, 11995 and 12872), which gives an indication that a strong imbalance is present at *IL-18*.

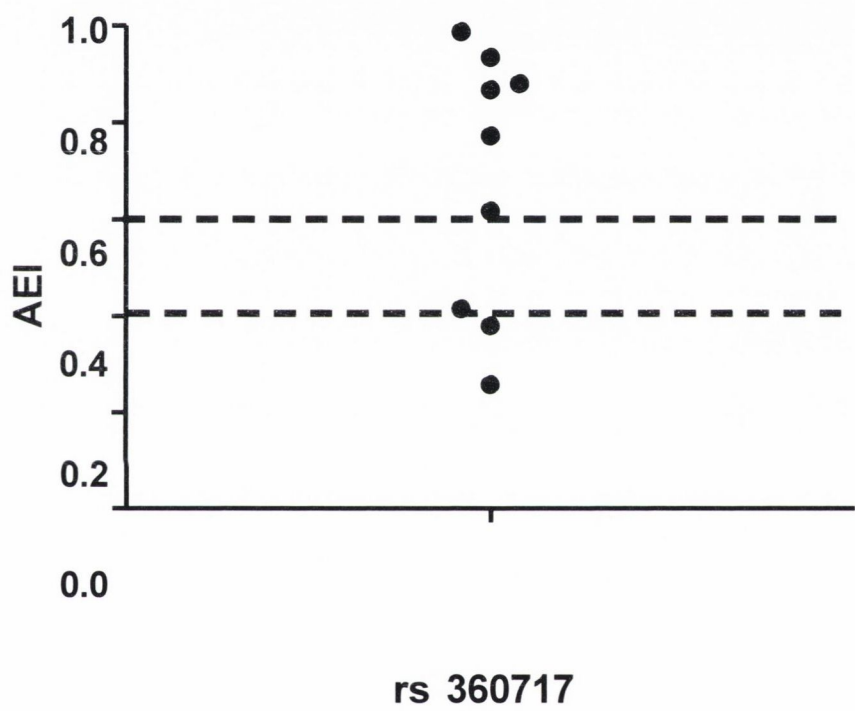


Figure 4.9 Scatterplot of the AEI assay results for the 9 lymphoblast cell line samples for rs360717. Positive AEI (AEI values >0.6 (n=3) or <0.4 (n=0)) was seen in 8 cell lines

4.3.10 AEI at IL-18 in 10 ng/ μ l TNF- α stimulated cell lines

Cell lines GM10861, 10856 and 11995 showed a decrease in AEI values with TNF- α stimulation, which suggests that the positive AEI imbalance was not as powerful as those seen in untreated IL-18 cell lines (Figure 4.10).

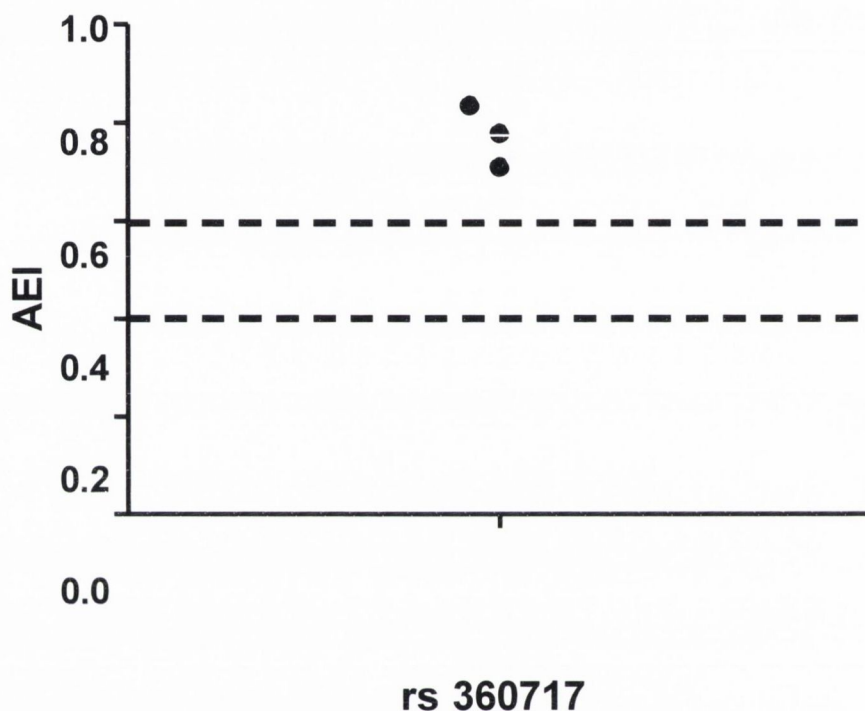


Figure 4.10 Scatterplot of the AEI assay results for all 3 lymphoblast cell line samples for rs360717 in all 3 cell lines stimulated with 10 ng/ μ l of TNF- α . Positive AEI (AEI values >0.6 (n=5) or <0.4 (n=0)) was observed in all 3 cell lines, GM11995, GM10856, GM10861.

4.3.10 AEI at IL-18 in 100 ng/μl TNF-α stimulated cell lines

All three IL-18 heterozygous cell lines show positive AEI when treated with a higher concentration of TNF-α. Cell lines GM10856 and GM10861 have similar values of AEI strength and allelic imbalance to those seen in the previous graph (Figure 4.10). Both values are around 0.7. However, with 100ng/μl TNF-α GM11995 has increased its AEI value showing stronger allelic imbalance. This value is similar to that seen in untreated cell lines for *IL-18* (Figure 4.11).

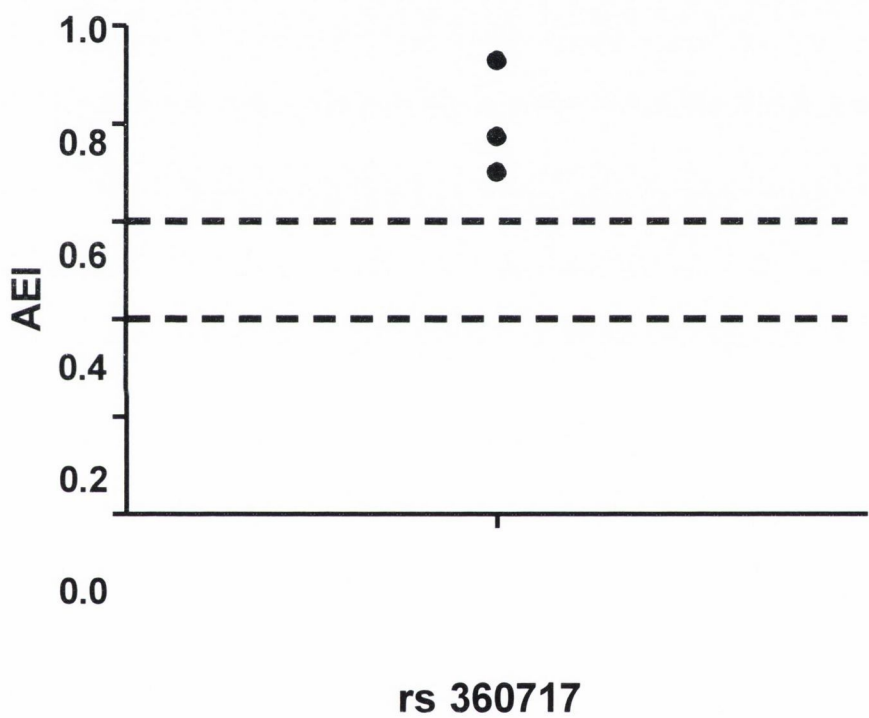


Figure 4.11 Scatterplot of the AEI assay results for all 3 lymphoblast cell line samples for rs360717 in all 3 cell lines stimulated with 100 ng/μl of TNF-α. Positive AEI (AEI values >0.6 (n=5) or <0.4 (n=0)) was observed in all 3 cell lines, GM11995, GM10856, GM10861.

4.4 Discussion

“An important question and a great challenge in human molecular genetics today is to decipher the mechanisms underlying complex and heterogeneous diseases that defy simple Mendelian Inheritance (Fischer et al., 2006)”. To associate common variants with disease it is essential to understand the frequency at which DNA variants compromise normal gene function. The purpose of this study was to detect evidence of AEI in two genes associated with atherosclerosis which would indicate the presence of cis-acting variants at the specific loci. As discussed in chapter 1 IL-18 is capable of increasing the expression of CXCL16 in lesions so it was interesting to investigate if their gene expression was similar. Also discussed previously was the impact of TNF- α , TNF- α can affect EC receptor expression, which may lead to an inflamed environment through multiple signalling pathways. Current literature has investigated the effect of inhibitor trichostatin A (TSA) on TNF- α treated EC expression, specifically adhesion molecules. Results suggested that L-Sulphoraphane is capable of reducing VCAM-1 expression thus attenuating the inflammatory response (Inoue et al., 2006). This study also aimed to elucidate the role inhibitors play on TNF- α treated cells and CXCL16 gene expression.

4.4.1 IL-18

A number of *IL18* single nucleotide polymorphisms have been associated with different diseases. Allelic variation in *IL18* can be related to the pathogenesis of cervical cancer (Yang et al., 2007) and some of *IL18* promoter polymorphisms, including rs360717, are associated with lowering IL-18 levels, which is an initiating factor in recurrent spontaneous miscarriage (Khateeb et al., 2011). However, lowering IL18 levels is associated with lowering cardiovascular risk (Barbeux et al., 2007) but opposite findings have also been reported in MI patients (Koch et al. 2001). In this study, fewer of the population examined were heterozygous for IL-18 than CXCL16. However, all individuals were heterozygous for IL-18

displayed AEI at *IL-18* indicating a very positive correlation between this SNP rs360717 and ACS patients.

Eight out of 9 lymphoblast cell lines heterozygous for *IL-18*, which originated from healthy individuals in the CEPH project, showed positive AEI. This further demonstrated the importance in investigating this SNP as a marker as both healthy and ACS individuals can have this allelic imbalance. The minor allele frequency (MAF) is relatively high in both populations, therefore further research is required.

All clinical samples heterozygous for *IL-18* displayed allelic imbalance in one direction, above 0.6, and cell lines showed AEI in both directions. The clinical data suggest the same allele expression is altered. CEPH samples display a strong imbalance evidently as four are positive AEI over 0.8. Clinical samples consist of a more varied imbalance over 0.6, but the majority are over 0.8. However, the AEI values for all three cell lines used in the TNF- α treated study decreased toward 0.6 when stimulated with 10 ng/ μ l TNF- α . Therefore, allelic expression began to become balanced. As discussed, this SNP has been associated with lowering *IL-18* levels and lowering atherogenesis potential. Therefore, if alleles are expressed equally, do *IL-18* levels increase, which would make sense when cell lines are influenced by a pro-inflammatory molecule. Because of this result it was interesting to investigate if a higher concentration of TNF- α restored balance of allele expression. GM11995 was the only cell line out of the three to significantly change AEI values, after treatment with 100ng/ μ l it increased in value towards 1.0, becoming more imbalanced, similar to that seen in untreated cell lines. Cell lines GM10861 and GM10856 values did not deviate significantly when TNF- α concentrated increased, which suggests that 10 and 100ng/ μ l of TNF- α had the same effect on allelic expression of *IL-18*.

4.4.2 CXCL16

As there is little information about rs2277680 this study provides additional data into AEI at this marker SNP. The SNP in *CXCL16* may have been more common in clinical samples than the *IL-18* SNP, however, the *CXCL16* SNP did not display positive AEI in all samples that it was present in. This suggests a weaker association with allelic imbalance at this marker SNP rs2277680 and ACS patients. The imbalanced alleles, like *IL-18*, all showed positive AEI over 0.6, three specifically showed a stronger allele expression around 1.0.

None of the CEPH individuals heterozygous for *CXCL16* rs2277680 displayed imbalance. This indicates that the heterozygote alleles are balanced in gene expression in these heterozygous cell lines. Therefore, the association of imbalance could be a potential risk factor as it is seen in ACS patients. A larger study needs to be carried out on CEPH cell lines using this marker SNP for *CXCL16* expression analysis. As all cell lines examined did not show imbalance for *CXCL16*, it was decided to stimulate the cells with a common cytokine associated with atherosclerosis and analyse the effects on gene expression of *CXCL16*. Even though *IL-18* showed imbalance in unstimulated CEPH cell lines it too was examined under the effect of $\text{TNF-}\alpha$. Two concentrations of $\text{TNF-}\alpha$ were used to stimulate the cell lines, 10 ng/ μl as it was previously used on *CXCL16* protein expression and 100ng/ μl as higher levels of $\text{TNF-}\alpha$ exist in plaque *in vivo* (Rus et al., 1991). The hypotheses that $\text{TNF-}\alpha$ could influence gene expression of *CXCL16* was confirmed when stimulated with 100ng/ μl . 5 out of the 10 cell lines had positive AEI above 0.8, GM1995, GM12717, GM10856, GM10846 and GM11881. The lower concentration 10 ng/ μl , which was used throughout the project, did not appear to affect allelic expression in *CXCL16* at marker SNP rs2277680.

In clinical samples both UA and MI patients had *IL-18* and *CXCL16* AEI at marker SNPs. Two patients had an imbalance of both *CXCL16* and *IL-18* gene expression, one UA and one

MI. Both patients also showed a strong imbalance over 0.8 for *CXCL16* and *IL-18*. *CXCL16* showed imbalance in 5 CEPH cell lines activated with a higher concentration of TNF- α , two of which showed imbalance for *IL18* in inactivated CEPH cell lines, GM10856 and GM11995.

TNF- α 10 ng/ μ l stimulated cell lines displayed no AEI, so without increasing the concentration, the effect of L-Sulphoraphane was investigated on gene expression. All three treatments; DMSO only, inhibitor only and TNF- α plus inhibitor changed the gene expression of cell lines 07348 and 12234 at one allele using the marker SNP rs2277680. As positive AEI was seen in the DMSO only treated cells, it remains unclear to whether or not the imbalance was caused directly by the inhibitor.

4.4.3 Concluding remarks

One of the most interesting findings in this study is the imbalance that occurred in *CXCL16* in clinical and CEPH was in one direction, above 0.6, indicating that one allele is responsible for the altered gene expression. Positive AEI imbalance in one direction was seen in patients for both IL-18 and CXCL16 in the some patients, so the effect of IL-18 on CXCL16 was evident.

In conclusion this study has shown that increasing TNF- α concentration has a positive AEI effect on CXCL16 using marker SNP rs2277680 in 50% of CEPH sample cell lines and either DMSO or inhibitor has the same effect when treating cell lines 07348 and 12234. These findings encourage further studies into the stability of allelic imbalance when a pro-atherosclerotic environment is present, both are novel findings in this field. As DMSO is a commonly used compound in freezing and storing cells it is vitally important to establish the individual source behind the positive AEI present. Knowledge of what alleles in a pro inflammatory environment are expressed could potentially reveal what to

examine in a patients genetics when treating inflammatory diseases.

Like the MSD platform discussed in chapter 2, a multiple cytokine profile on AEI could be highly important as an environment which contains many inflammatory mediators could escalate and increase a certain allele and thus protein expression. AEI can be assessed *in vitro* cell line samples under the influence of a number of different cytokines and chemokines and provide a more extensive knowledge on the effect of allele behavior. Chapter 2 revealed CXCL16 to have significantly higher levels in carotid patients compared to controls, and this chapter highlighted the AEI in a CXCL16 SNP in coronary patients. Future experiments should look at both MSD and AEI on the opposite patient sets for CXCL16 expression analysis. It would also be interesting to activate the cell lines used in this chapter under IFN- γ stimulation as it is an important mediator in CAD.

4.4.4 Limitations

Limitations involved in this study included the solution of DMSO which the inhibitor was dissolved in, which meant that the effect of the DMSO and the effect of the inhibitor could not be differentiated. This was a small study of ACS patients and CEPH individuals, as not all patients were heterozygous for either CXCL16 or IL-18 it would be interesting to carry out this study, for example, in the AtheroGene cohort.

Different populations have different mutations in their genetic make-up so the sample population should be from the same ancestry pool and the outcome of the study could vary depending on what population is studied. The AtheroGene cohort includes 1288 individuals with coronary artery disease which had >30% stenosis, participants were of German nationality and lived in the Rhein-Mainz area. Patients had either acute myocardial infarctions or unstable angina. The cohort has elucidated the impact of gene expression involved in CAD, such as von Willebrand factor (vWF), fibrinogen, thrombin-antithrombin (TAT) complexes, D-dimers, plasmin-antiplasmin (PAP)

complexes (Morange et al.,2006), and HDL (Silbernagel et al., 2013). It would be interesting to carry out the same experiments on participants of the AtheroGene cohort and further investigate if there are significant CXCL16 allelic expression differences.

4.4.5 Future directions

Further studies related to this chapter could include AEI analysis of all heterozygous cell lines for CXCL16 and IL-18 and due to existing results should investigate the properties of DMSO and L-Sulphoraphane as factors affecting AEI in CXCL16 and IL-18 expression. It would also be interesting to see what effect the inhibitor has on cell lines that showed AEI imbalance and investigate potential reversal, specifically on CXCL16 which showed AEI after 100ng/ μ l TNF- α treatment.

Chapter 5

General Discussion

Cardiovascular diseases remain the leading causes of morbidity and mortality in the developed world, despite the advances in prevention, diagnosis and treatment. The majority of these cases are due to plaque rupture, erosion and thrombosis (Phinikaridou et al., 2013). Atherosclerosis involves a mixture of cellular components such as immune cells, soluble molecules and circulating lipids at the gene and protein level that makes it a complex disease (Garrido-Urbani et al., 2013). In this project a number of components were investigated, such as circulating proteins, endothelial cell activation by mechanotransduction and inflammatory mediators and the ability of $\text{TNF-}\alpha$ to alter CXCL16 allelic expression.

Inflammation is the predecessor of many diseases i.e. cancers, autoimmune diseases and complex diseases such as atherosclerosis. Therefore, understanding its initiation and the components involved is important. Chemokines and cytokines produced from immune cells or cells that have been disrupted i.e. endothelial dysfunction are key targets of anti-atherosclerotic therapy. A number of new therapeutics were discussed in the Introduction. However, statins still remain the number one choice of treatment along with blood thinners such as aspirin. This thesis investigates cytokines as biomarkers which could be used for diagnostics if an appropriate association is discovered, IL-6 and CRP levels have already been established as key factors in CVDs. It also uses novel flow models to further investigate the potential biomarkers.

This research project used the combination of clinical and molecular techniques, protein and gene expression analysis to further elucidate the roles of potential and existing biomarkers in atherosclerosis. A number of inflammatory markers such as ICAM, CXCL16, oxPAPC have unclear roles in inflammation and their contribution to plaque development is undefined so it was interesting to see their expression levels in a number of different environments. On top of new insights into these controversial molecules, this thesis included new techniques such as

the MSD platform, the Cellix immunofluorescent Biochip and the method of quantification which was adapted from an existing method.

In chapter 2 patients serum cytokine/chemokine expression levels were analysed in multiple groups including, asymptomatic v symptomatic, risk factor association, degree of stenosis, plaque type and statin therapy. Interestingly GM-CSF, IL-2, IL-12p70, IFN- γ and IL-1 β did not have many values that were detectable and GM-CSF, IL-2, IL-12p70 only showed significant difference comparing stenosis %. Not many studies have focused on the levels of GM-CSF and IL-12p70 in serum samples of CVD patients. A previous study completed in the IMM showed no significance with these two cytokines in MI and UAPs (Patel et al., 2009). IL-2 however was present in unusually low levels, as it has many inducers including two, TNF- α and IL-6 which were found to have significant differences when comparing patients and controls in this study. IFN- γ and IL-1 β have both been identified in multiple stages of atherosclerosis (McLaren et al., 2005). However, they did not produce any significant differences in the groups that were compared. After MIs these two cytokines appear to rapidly increase (Roth-Isigkeit et al., 1999 and Patel et al., 2009). The levels of IFN- γ and IL-1 β may reflect the fact that no infarct had yet been experienced in these patients and this surge in levels may be a post-traumatic event only. Higher levels may occur at more localised sites of inflammation and may not be systemic, which may suggest none of the cytokines mentioned above have the potential to identify at risk atherosclerosis patients pre-stroke. IL-10, the only anti-inflammatory of the study, showed higher levels in controls which suggests that there is an unbalanced level of pro and anti-inflammatory mediators in these patients and IL-10 is down-regulated. As IL-10 suppresses the expression of TNF- α and UAP patients display an imbalance between these two cytokines (Waehre et al., 2002) it is not a surprise that carotid patients have shown an increase in TNF- α and a decrease in IL-10.

Without the balance of IL-10 levels stable plaques are threatened as MMP production may increase.

TNF- α , IL-6 and IL-8 also showed significant differences in patient v control data, as TNF- α is involved in IL-6 production this observation was expected (Bruunsgaard et al., 2000). Excluding outliers patients expressed more circulating TNF- α which may have affected increasing IL-6 production in carotid patients. As IL-6 plasma concentrations have been associated with morbidity and mortality rates in unstable angina patients it may also be considered as a marker for high risk atherosclerosis patients. IL-6 and IL-8 show ongoing correlation in this study in the patient groups therefore could both be useful to support the diagnosis of potential rupture patients. Overall the patient group which showed the most significant differences in cytokine levels was comparing % stenosis. As previously mentioned, patients with lesser stenosis are potentially more at risk of thrombolytic events so markers significantly different in the below 80% stenosis group could help monitor disease outcome. CXCL16 results proved significant also when patient groups and patient v control levels were compared. As well as IL-8, CXCL16 showed significance with IL-6 when comparing asymptomatics and symptomatics. As controversial findings exist for serum levels exist for CXCL16 it would appear to be beneficial to establish its role in atherosclerosis as it is significant in multiple analysis in this chapter. In conclusion, this chapter suggests that analysing a combination of serum levels of CXCL16, IL-6, IL-8, IL-10 and TNF- α could be useful when considering the potential risk of atherosclerotic events occurring in patients <65 years, with <80% stenosis and asymptomatic or symptomatic.

Using the information gathered in chapter 2 and the need for clarification for inflammatory biomarkers in plaque development, the relationship between stimulants present in atherosclerotic disease and the endothelial monolayer was looked at in more detail in the

Cellix Biochip with pulsatile pump. *In vitro* models have contributed hugely to cell expression and monocyte adhesion studies. Many studies have used the parallel plate or cone apparatus but have modified them in a way where they are novel each time. These models have become more complex over the years and facilitate many conditions which make them an ideal platform for analysis. They encompass endothelial cell culture, co-culture which consists of SMCs and ECs, and allow cells to be detached and analysed by a variety of methods including mRNA expression, flow cytometry, protein expression by western blotting and mainly Immunofluorescence. The *in vitro* models can be used to replicate what is seen *in vivo* but in a controlled environment so specific causes can be attributed to a certain condition. It is a successful way of investigating environments such as those seen in immunohistochemistry of carotid endarterectomies without background noise. Chapter 3 selected specific conditions based on the current literature on ICAM-1 and CXCL16 expression. ICAM-1 was used a type of positive control for the EC expression biochip model, it was known to be expressed in laminar flow and TNF- α stimulation (Chiu et al., 2004, Krunkosky et al., 2000). As lysing or detaching ECs from the fibronectin based channels was unachievable another form of analysis had to be accomplished. Looking at the area of cells stained was a suitable way of interrupting the effect a stimulant had on EC receptor expression. Three stimulants used were pulsatile flow with a shear stress of 10 dyne/cm², TNF- α and oxPAPC. Out of the three, pulsatile flow had the largest effect on inducing or increasing ICAM-1 and CXCL16 expression levels, followed by TNF- α and oxPAPC had little to no effect. CXCL16 expression was increased most significantly at 6 h under pulsatile flow in unstimulated ECs. The combination of TNF- α and pulsatile flow showed ICAM-1 covering 100% of EC surface in immunofluorescent images at 24 h. As ICAM-1 was at its maximum expression levels it can be suggested that it arterial areas of laminar flow which are subjected to cytokine stimulation can be potential sites of excess monocyte adhesion, EC

dysfunction and fatty streak formation. Also EC genes which are shear responsive induce cytokine and other pro-inflammatory creating the harmful environment itself. One of the main findings in this chapter was the response of CXCL16 to laminar flow as the gene was found responsive to disturbed SS (Ni et al., 2010). Both qualitative and quantitative results show receptor CXCL16 expression induced by laminar flow. The current literature suggests CXCL16 has an NF- κ B binding site (Chandrasekar et al., 2004) and is responsive to cytokine stimulation such as IFN- γ and TNF- α therefore pro-inflammatory potential (Abel et al., 2004). This study found CXCL16 to be induced by TNF- α however small the values appeared to be. However, the combination of TNF- α and pulsatile flow at 24 h did not sustain CXCL16 suggesting it may not be a long term threat at an inflammatory site as expression appeared to have fatigued. Considering the results from chapter 2 and 3, patients have shown higher levels of circulating CXCL16 than TNF- α , as CXCL16 may be considered more systemic as it is a chemokine whereas TNF- α would be more localised to the site of inflammation. Lower levels of CXCL16 in patients compared to controls may have been influenced by statin therapy as this has been reported (Smith et al., 2008). After 6 h TNF- α activated ECs in a laminar pulsatile flow of 10 dyne/cm² environment, all the potential factors for macrophage and oxLDL accumulation are present. Therefore disturbed flow may only increase the chances of plaque formation and not be an inducing factor.

Qualitatively, two main findings were noticed. At 24 h with no stimulation and pulsatile flow, extremely intensified halo shaped distribution of ICAM-1 were observed along the ECs edge at cell-to cell junctions and at 6 h, unstimulated ECs under pulsatile flow showed CXCL16 remained localised around the nucleus and spread throughout the cytoplasm by 24 h.

After investigating the relationship of TNF- α at a protein level, it was decided to return to a molecular level and investigate if the same concentration affected allelic expression of CXCL16 in CVD patients and CEPH individuals. Allelic imbalance was seen in CVD patients, CEPH cell lines showed no imbalance so they were activated with 10 ng/ μ l for 6 h, again, no imbalance was discovered. When TNF- α concentration were increased to 100 ng/ μ l an imbalance was achieved. Like the ACS patients, one allele appeared responsible for the imbalance occurring, as positive AEI was above 0.6 in all samples. As the higher concentration of TNF- α had an impact on genetic expression it would be interesting to use the same concentration on EC activation in the *in vitro* model and monitor CXCL16 expression. To our knowledge, this study is one of the first to investigate TNF- α stimulation on AEI effect in CEPH individuals. In the past few years, inhibitors have been identified as potential candidates for controlling the growth and spread of cancer cells and research is ongoing to investigate their properties as anti-cancer treatment. Now, inhibitors have been targeted for preventing the formation of fatty streaks. Cell lines in this study appeared to show positive AEI in the presence of L-Sulforaphane. However, as this was diluted in DMSO results are inconclusive as to the direct impact of the inhibitor or DMSO on allelic imbalance.

5.1.1 Concluding remarks

This project encompassed clinical data and molecular data, protein and genetic data that attempted to further elucidate markers that have undiscovered potential in the diagnosis or prognosis of future plaque rupture in individuals who are at risk or suffer from atherosclerosis.

In chapter 2 levels of circulating cytokines were measured below the ELISA level of sensitivity, an understudied subset of patients were investigated and correlations between cytokines and other risk factors were investigated. The *in vitro* model in chapter 3 provides a

relevant platform and research tool for further expression studies in a monitored environment and has the ability to incorporate multiple factors for investigation. It is also a suitable model for the evaluation of new molecules/drugs under pulsatile shear stress conditions. Static and pulsatile shear stress conditions on EC expression of ICAM-1 and CXCL16 were evaluated. Finally, chapter 4 showed AEI in clinical and CEPH cell lines, evaluated the effect TNF- α stimulation and L-Sulporaphane had on AEI in CEPH cell lines and showed that the measurement of cis-effects needs to take stimulation and chromatin modification into consideration.

Chapter 6

REFERENCES

- Abel, S., Hundhausen, C., Mentlein, R., Schulte, A., Berkhout, T.A., Broadway, N., Hartmann, D., Sedlacek, R., Dietrich, S., Muetze, B., Schuster, B., Kallen, K.-J., Saftig, P., Rose-John, S., Ludwig, A., 2004. The Transmembrane CXC-Chemokine Ligand 16 Is Induced by IFN- γ and TNF- α and Shed by the Activity of the Disintegrin-Like Metalloproteinase ADAM10. *J Immunol* 172, 6362–6372.
- Agostini, C., Cabrelle, A., Calabrese, F., Bortoli, M., Scquizzato, E., Carraro, S., Miorin, M., Beghè, B., Trentin, L., Zambello, R., 2005. Role for CXCR6 and its ligand CXCL16 in the pathogenesis of T-cell alveolitis in sarcoidosis. *American journal of respiratory and critical care medicine* 172, 1290–1298.
- Ait-Oufella, H., Salomon, B.L., Potteaux, S., Robertson, A.-K.L., Gourdy, P., Zoll, J., Merval, R., Esposito, B., Cohen, J.L., Fisson, S., 2006. Natural regulatory T cells control the development of atherosclerosis in mice. *Nature medicine* 12, 178–180.
- Ait-Oufella, H., Taleb, S., Mallat, Z., Tedgui, A., 2011. Recent Advances on the Role of Cytokines in Atherosclerosis. *Arterioscler Thromb Vasc Biol* 31, 969–979.
- Akira, S., Hirano, T., Taga, T., Kishimoto, T., 1990. Biology of multifunctional cytokines: IL 6 and related molecules (IL 1 and TNF). *FASEB J* 4, 2860–2867.
- Al-Khateeb, G.M., Sater, M.S., Finan, R.R., Mustafa, F.E., Al-Busaidi, A.S., Al-Sulaiti, M.A., Almawi, W.Y., 2011. Analysis of interleukin-18 promoter polymorphisms and changes in interleukin-18 serum levels underscores the involvement of interleukin-18 in recurrent spontaneous miscarriage. *Fertil. Steril.* 96, 921–926.
- Alberts-Grill, N., Denning, T.L., Rezvan, A., Jo, H., 2013. The role of the vascular dendritic cell network in atherosclerosis. *American Journal of Physiology - Cell Physiology*.
- Amberger, A., Maczek, C., Jürgens, G., Michaelis, D., Schett, G., Trieb, K., Eberl, T., Jindal, S., Xu, Q., Wick, G., 1997. Co-expression of ICAM-1, VCAM-1, ELAM-1 and Hsp60 in human arterial and venous endothelial cells in response to cytokines and oxidized low-density lipoproteins. *Cell stress & chaperones* 2, 94.
- Ambrose, J.A., Barua, R.S., 2004. The pathophysiology of cigarette smoking and cardiovascular disease An update. *J Am Coll Cardiol* 43, 1731–1737.
- Angel, K., Provan, S.A., Fagerhol, M.K., Mowinkel, P., Kvien, T.K., Atar, D., 2012. Effect of 1-year anti-TNF- α therapy on aortic stiffness, carotid atherosclerosis, and calprotectin in inflammatory arthropathies: a controlled study. *Am. J. Hypertens.* 25, 644–650.
- Anney, R., n.d. Allelic expression imbalance of the schizophrenia susceptibility gene CHI3L1, evidence of cis-acting variation and tissue specific regulation. [WWW Document]. URL http://www.academia.edu/915982/Allelic_expression_imbalance_of_the_schizophrenia_susceptibility_gene_CHI3L1_evidence_of_cis-acting_variation_and_tissue_specific_regulation (accessed 9.19.13).
- Anssari-Benam, A., Korakianitis, T., 2013a. Atherosclerotic plaques: Is endothelial shear stress the only factor? *Med. Hypotheses*.
- Apostolakis, S., Vogiati, K., Krambovitis, E., Spandidos, D.A., 2008. IL-1 cytokines in cardiovascular disease: diagnostic, prognostic and therapeutic implications. *Cardiovasc Hematol Agents Med Chem* 6, 150–158.
- Arend, W.P., Palmer, G., Gabay, C., 2008b. IL-1, IL-18, and IL-33 families of cytokines. *Immunological reviews* 223, 20–38.
- Ashraf, M.Z., Kar, N.S., Podrez, E.A., 2009. Oxidized phospholipids: Biomarker for cardiovascular diseases. *The International Journal of Biochemistry & Cell Biology* 41, 1241–1244.
- Aukrust, P., Halvorsen, B., Yndestad, A., Ueland, T., Øie, E., Otterdal, K., Gullestad, L., Damås, J.K., 2008a. Chemokines and Cardiovascular Risk. *Arterioscler Thromb Vasc*

Biol 28, 1909–1919.

- Bäck, M., Gasser, T.C., Michel, J.-B., Caligiuri, G., 2013. Biomechanical factors in the biology of aortic wall and aortic valve diseases. *Cardiovasc. Res.*
- Bannister, A.J., Kouzarides, T., 2011. Regulation of chromatin by histone modifications. *Cell Res* 21, 381–395.
- Barboux, S., Poirier, O., Godefroy, T., Kleinert, H., Blankenberg, S., Cambien, F., Tired, L., 2007. Differential haplotypic expression of the interleukin-18 gene. *European Journal of Human Genetics* 15, 856–863.
- Barreiro, L.B., Laval, G., Quach, H., Patin, E., Quintana-Murci, L., 2008. Natural selection has driven population differentiation in modern humans. *Nat Genet* 40, 340–345.
- Beckman JA, Creager MA, Libby P, 2002. Diabetes and atherosclerosis: Epidemiology, pathophysiology, and management. *JAMA* 287, 2570–2581.
- Berliner, J.A., Gharavi, N.M., 2008. Endothelial cell regulation by phospholipid oxidation products. *Free Radical Biology and Medicine* 45, 119–123.
- Berliner, J.A., Watson, A.D., 2005. A Role for Oxidized Phospholipids in Atherosclerosis. *New England Journal of Medicine* 353, 9–11.
- Birukova, A.A., Starosta, V., Tian, X., Higginbotham, K., Koroniak, L., Berliner, J.A., Birukov, K.G., 2013a. Fragmented oxidation products define barrier disruptive endothelial cell response to OxPAPC. *Translational Research* 161, 495–504.
- Birukova, A.A., Starosta, V., Tian, X., Higginbotham, K., Koroniak, L., Berliner, J.A., Birukov, K.G., 2013b. Fragmented oxidation products define barrier disruptive endothelial cell response to OxPAPC. *Translational Research* 161, 495–504.
- Blackman, B.R., Garcia-Cardena, G., Gimbrone, M.A., 2002. A new in vitro model to evaluate differential responses of endothelial cells to simulated arterial shear stress waveforms. *TRANSACTIONS-AMERICAN SOCIETY OF MECHANICAL ENGINEERS JOURNAL OF BIOMECHANICAL ENGINEERING* 124, 397–407.
- Blankenberg, S., Barboux, S., Tired, L., 2003. Adhesion molecules and atherosclerosis. *Atherosclerosis* 170, 191–203.
- Bochkov, V.N., Oskolkova, O.V., Birukov, K.G., Levonen, A.-L., Binder, C.J., Stöckl, J., 2010. Generation and Biological Activities of Oxidized Phospholipids. *Antioxidants & Redox Signaling* 12, 1009–1059.
- Bots ML., D. Baldassarre, A. Simon, E de Groot, D.H. O’Leary, W. Riley, J. J. Kastelein, and D. E. Grobbee 2007 Carotid intima-media thickness and coronary atherosclerosis: weak or strong relations? *European Heart Journal*, 28: 398–406
- Bruunsgaard, H., Skinhøj, P., Pedersen, A.N., Schroll, M., Pedersen, B.K., 2000. Ageing, tumour necrosis factor-alpha (TNF-alpha) and atherosclerosis. *Clin. Exp. Immunol.* 121, 255–260.
- Bucova, M., Bernadic, M., Buckingham, T., 2008. C-reactive protein, cytokines and inflammation in cardiovascular diseases. *Bratisl Lek Listy* 109, 333–340.
- Cai, H., Griendling, K.K., Harrison, D.G., 2003. The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. *Trends Pharmacol. Sci.* 24, 471–478.
- Cai, H., Harrison, D.G., 2000. Endothelial Dysfunction in Cardiovascular Diseases: The Role of Oxidant Stress. *Circulation Research* 87, 840–844.
- Caligiuri, G., Rudling, M., Ollivier, V., Jacob, M.-P., Michel, J.-B., Hansson, G.K., Nicoletti, A., 2003. Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice. *Molecular Medicine* 9, 10.
- Chachisvilis, M., Zhang, Y.-L., Frangos, J.A., 2006. G protein-coupled receptors sense fluid shear stress in endothelial cells. *PNAS* 103, 15463–15468.
- Chamberlain, J., Evans, D., King, A., Dewberry, R., Dower, S., Crossman, D., Francis, S., 2006. Interleukin-1beta and signaling of interleukin-1 in vascular wall and circulating cells modulates the extent of neointima formation in mice. *Am. J. Pathol.* 168, 1396–1403.
- Chandrasekar, B., Bysani, S., Mummidi, S., 2004. CXCL16 signals via Gi, phosphatidylinositol 3-kinase, Akt, I κ B kinase, and nuclear factor- κ B

and induces cell-cell adhesion and aortic smooth muscle cell proliferation. *Journal of Biological Chemistry* 279, 3188–3196.

- Chandrasekar, B., Mummidi, S., Valente, A.J., Patel, D.N., Bailey, S.R., Freeman, G.L., Hatano, M., Tokuhisa, T., Jensen, L.E., 2005. The pro-atherogenic cytokine interleukin-18 induces CXCL16 expression in rat aortic smooth muscle cells via MyD88, interleukin-1 receptor-associated kinase, tumor necrosis factor receptor-associated factor 6, c-Src, phosphatidylinositol 3-kinase, Akt, c-Jun N-terminal kinase, and activator protein-1 signaling. *Journal of Biological Chemistry* 280, 26263–26277.
- Chapman, C.M., McQuillan, B.M., Beilby, J.P., Thompson, P.L., Hung, J., 2006. Interleukin-18 levels are not associated with subclinical carotid atherosclerosis in a community population. The Perth Carotid Ultrasound Disease Assessment Study (CUDAS). *Atherosclerosis* 189, 414.
- Charoenphol, P., Mocherla, S., Bouis, D., Namdee, K., Pinsky, D.J., Eniola-Adefeso, O., 2011. Targeting therapeutics to the vascular wall in atherosclerosis—Carrier size matters. *Atherosclerosis* 217, 364–370.
- Chatzizisis, Y.S., Coskun, A.U., Jonas, M., Edelman, E.R., Feldman, C.L., Stone, P.H., 2007a. Role of Endothelial Shear Stress in the Natural History of Coronary Atherosclerosis and Vascular Remodeling. *Molecular, Cellular, and Vascular Behavior. J Am Coll Cardiol* 49, 2379–2393.
- Chatzizisis, Y.S., Coskun, A.U., Jonas, M., Edelman, E.R., Stone, P.H., Feldman, C.L., 2007b. Risk stratification of individual coronary lesions using local endothelial shear stress: a new paradigm for managing coronary artery disease. *Current opinion in cardiology* 22, 552–564.
- Chatzizisis, Y.S., Giannoglou, G.D., 2006. Pulsatile flow: a critical modulator of the natural history of atherosclerosis. *Medical hypotheses* 67, 338–340.
- Chatzizisis, Y.S., Giannoglou, G.D., Parcharidis, G.E., Louridas, G.E., 2007c. Is left coronary system more susceptible to atherosclerosis than right?: A pathophysiological insight. *International journal of cardiology* 116, 7–13.
- Chen, C., Chou, C., Sun, Y., Huang, W., 2001. Tumor necrosis factor alpha-induced activation of downstream NF-kappaB site of the promoter mediates epithelial ICAM-1 expression and monocyte adhesion. Involvement of PKCalpha, tyrosine kinase, and IKK2, but not MAPKs, pathway. *Cellular signalling* 13, 543.
- Cheung, V.G., Spielman, R.S., 2009. Genetics of human gene expression: mapping DNA variants that influence gene expression. *Nat Rev Genet* 10, 595–604.
- Chien, S., 2007. Mechanotransduction and endothelial cell homeostasis: the wisdom of the cell. *Am J Physiol Heart Circ Physiol* 292, H1209–H1224.
- Chiu, J.-J., Chen, L.-J., Chen, C.-N., Lee, P.-L., Lee, C.-I., 2004a. A model for studying the effect of shear stress on interactions between vascular endothelial cells and smooth muscle cells. *Journal of Biomechanics* 37, 531–539.
- Chiu, J.-J., Lee, P.-L., Chen, C.-N., Lee, C.-I., Chang, S.-F., Chen, L.-J., Lien, S.-C., Ko, Y.-C., Usami, S., Chien, S., 2004b. Shear Stress Increases ICAM-1 and Decreases VCAM-1 and E-selectin Expressions Induced by Tumor Necrosis Factor- α in Endothelial Cells. *Arterioscler Thromb Vasc Biol* 24, 73–79.
- Chong, P.H., Bachenheimer, B.S., 2000. Current, new and future treatments in dyslipidaemia and atherosclerosis. *Drugs* 60, 55–93.
- Choudhury, R.P., Fisher, E.A., 2009. Molecular Imaging in Atherosclerosis, Thrombosis, and Vascular Inflammation. *Arterioscler Thromb Vasc Biol* 29, 983–991.
- Collins, A.R., Lyon, C.J., Xia, X., Liu, J.Z., Tangirala, R.K., Yin, F., Boyadjian, R.,

- Bikineyeva, A., Praticò, D., Harrison, D.G., Hsueh, W.A., 2009. Age-Accelerated Atherosclerosis Correlates With Failure to Upregulate Antioxidant Genes. *Circulation Research* 104, e42–e54.
- Cunningham, K.S., Gotlieb, A.I., 2005. The role of shear stress in the pathogenesis of atherosclerosis. *Laboratory Investigation* 85, 9–23.
- Cybulsky, M.I., Iiyama, K., Li, H., Zhu, S., Chen, M., Iiyama, M., Davis, V., Gutierrez-Ramos, J.-C., Connelly, P.W., Milstone, D.S., 2001. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *Journal of Clinical Investigation* 107, 1255–1262.
- Darash-Yahana, M., Gillespie, J.W., Hewitt, S.M., Chen, Y.-Y.K., Maeda, S., Stein, I., Singh, S.P., Bedolla, R.B., Peled, A., Troyer, D.A., Pikarsky, E., Karin, M., Farber, J.M., 2009. The Chemokine CXCL16 and Its Receptor, CXCR6, as Markers and Promoters of Inflammation-Associated Cancers. *PLoS ONE* 4, e6695.
- Davies, P.F., 1995. Flow-Mediated Endothelial Mechanotransduction. *Physiol Rev* 75, 519–560.
- Davies, P.F., Remuzzi, A., Gordon, E.J., Dewey, C.F., Gimbrone, M.A., 1986. Turbulent fluid shear stress induces vascular endothelial cell turnover in vitro. *PNAS* 83, 2114–2117.
- Davignon, J., Ganz, P., 2004. Role of Endothelial Dysfunction in Atherosclerosis. *Circulation* 109, III–27–III–32.
- Davis, J.M., 3rd, Knutson, K.L., Strausbauch, M.A., Crowson, C.S., Therneau, T.M., Wettstein, P.J., Matteson, E.L., Gabriel, S.E., 2010. Analysis of complex biomarkers for human immune-mediated disorders based on cytokine responsiveness of peripheral blood cells. *J. Immunol.* 184, 7297–7304.
- Dewey, C.F., Jr, Bussolari, S.R., Gimbrone, M.A., Jr, Davies, P.F., 1981. The dynamic response of vascular endothelial cells to fluid shear stress. *J Biomech Eng* 103, 177–185.
- Diez, D., Wheelock, Å.M., Goto, S., Haeggström, J.Z., Paulsson-Berne, G., Hansson, G.K., Hedin, U., Gabrielsen, A., Wheelock, C.E., 2010. The use of network analyses for elucidating mechanisms in cardiovascular disease. *Molecular BioSystems* 6, 289.
- Dimmeler, S., Aicher, A., Vasa, M., Mildner-Rihm, C., Adler, K., Tiemann, M., Rütten, H., Fichtlscherer, S., Martin, H., Zeiher, A.M., 2001. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *Journal of Clinical Investigation* 108, 391–397.
- Elneihoum, A., Falke, P., Hedblad, B., Lindgärde, F., Ohlsson, K., 1997. Leukocyte activation in atherosclerosis: correlation with risk factors. *Atherosclerosis* 131, 79–84.
- Epstein, D.J., 2009. Cis-regulatory mutations in human disease. *Brief Funct Genomic Proteomic* 8, 310–316.
- Estrada, R., Giridharan, G.A., Nguyen, M.-D., Prabhu, S.D., Sethu, P., 2011. Microfluidic endothelial cell culture model to replicate disturbed flow conditions seen in atherosclerosis susceptible regions. *Biomicrofluidics* 5, 32006–3200611.
- Evangelou, E., Ioannidis, J.P.A., 2013. Meta-analysis methods for genome-wide association studies and beyond. *Nature Reviews Genetics* 14, 379–389.
- Fiorentino, D.F., Zlotnik, A., Mosmann, T.R., Howard, M., O'garra, A., 1991a. IL-10 inhibits cytokine production by activated macrophages. *The Journal of Immunology* 147, 3815–3822.
- Fiorentino, D.F., Zlotnik, A., Vieira, P., Mosmann, T.R., Howard, M., Moore, K.W., O'Garra, A., 1991b. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *The Journal of Immunology* 146, 3444–3451.
- Fisher, A.B., Chien, S., Barakat, A.I., Nerem, R.M., 2001. Endothelial cellular response to

- altered shear stress. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 281, L529–L533.
- Fragoso Lona, J.M., Sierra Martínez, M., Vargas Alarcón, G., Barrios Rodas, A., Ramírez Bello, J., 2013. [Tumor necrosis factor alfa in cardiovascular diseases: molecular biology and genetics.]. *Gac Med Mex* 149, 521–530.
- Frank, P.G., Lisanti, M.P., 2008. ICAM-1: role in inflammation and in the regulation of vascular permeability. *Am J Physiol Heart Circ Physiol* 295, H926–H927.
- Fröhlich, M., Sund, M., Löwel, H., Imhof, A., Hoffmeister, A., Koenig, W., 2003. Independent association of various smoking characteristics with markers of systemic inflammation in men. Results from a representative sample of the general population (MONICA Augsburg Survey 1994/95). *Eur. Heart J.* 24, 1365–1372.
- Frostegård, J., Ulfgren, A.K., Nyberg, P., Hedin, U., Swedenborg, J., Andersson, U., Hansson, G.K., 1999. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis* 145, 33–43.
- Fruhworth, G.O., Loidl, A., Hermetter, A., 2007. Oxidized phospholipids: from molecular properties to disease. *Biochim. Biophys. Acta* 1772, 718–736.
- Furnkranz, A., Schober, A., Bochkov, V.N., Bashtrykov, P., Kronke, G., Kadl, A., Binder, B.R., Weber, C., Leitinger, N., 2005. Oxidized phospholipids trigger atherogenic inflammation in murine arteries. *Arterioscler. Thromb. Vasc. Biol.* 25, 633–638.
- Galis, Z.S., Sukhova, G.K., Lark, M.W., Libby, P., 1994. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J. Clin. Invest.* 94, 2493–2503.
- Gargalovic, P.S., Gharavi, N.M., Clark, M.J., Pagnon, J., Yang, W.-P., He, A., Truong, A., Baruch-Oren, T., Berliner, J.A., Kirchgessner, T.G., Lusis, A.J., 2006a. The Unfolded Protein Response Is an Important Regulator of Inflammatory Genes in Endothelial Cells. *Arterioscler Thromb Vasc Biol* 26, 2490–2496.
- Gargalovic, P.S., Imura, M., Zhang, B., Gharavi, N.M., Clark, M.J., Pagnon, J., Yang, W.-P., He, A., Truong, A., Patel, S., Nelson, S.F., Horvath, S., Berliner, J.A., Kirchgessner, T.G., Lusis, A.J., 2006b. Identification of inflammatory gene modules based on variations of human endothelial cell responses to oxidized lipids. *PNAS* 103, 12741–12746.
- Garrido-Urbani, S., Meguenani, M., Montecucco, F., Imhof, B.A., 2013. Immunological aspects of atherosclerosis. *Semin Immunopathol.*
- Glass, C.K., Witztum, J.L., 2001. Atherosclerosis: The Road Ahead Review. *Cell* 104, 503–516.
- Gong, Q., Wu, F., Pan, X., Yu, J., Li, Y., Lu, T., Li, X., Lin, Z., 2012. Soluble C-X-C chemokine ligand 16 levels are increased in gout patients. *Clin. Biochem.* 45, 1368–1373.
- Gotsman, I., Lichtman, A.H., 2007. Targeting Interferon- γ to Treat Atherosclerosis. *Circulation Research* 101, 333–334.
- Greig, F.H., Kennedy, S., Spickett, C.M., 2012. Physiological effects of oxidized phospholipids and their cellular signaling mechanisms in inflammation. *Free Radical Biology and Medicine* 52, 266–280.
- Gschwendtner, A., Bevan, S., Cole, J.W., Plourde, A., Matarin, M., Ross-Adams, H., Meitinger, T., Wichmann, E., Mitchell, B.D., Furie, K., Slowik, A., Rich, S.S., Syme, P.D., MacLeod, M.J., Meschia, J.F., Rosand, J., Kittner, S.J., Markus, H.S., Müller-Myhsok, B., Dichgans, M., International Stroke Genetics Consortium, 2009. Sequence variants on chromosome 9p21.3 confer risk for atherosclerotic stroke. *Ann. Neurol.* 65, 531–539.

- Hansson, G.K., 2001. Immune Mechanisms in Atherosclerosis. *Arterioscler Thromb Vasc Biol* 21, 1876–1890.
- Hansson, G.K., Libby, P., 2006. The immune response in atherosclerosis: a double-edged sword. *Nature Reviews Immunology* 6, 508–519.
- Hansson, G.K., Libby, P., Schönbeck, U., Yan, Z.-Q., 2002. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circulation research* 91, 281–291.
- Harvey, E.J., Ramji, D.P., 2005. Interferon-gamma and atherosclerosis: pro- or anti-atherogenic? *Cardiovasc. Res.* 67, 11–20.
- Heap, G.A., Yang, J.H.M., Downes, K., Healy, B.C., Hunt, K.A., Bockett, N., Franke, L., Dubois, P.C., Mein, C.A., Dobson, R.J., Albert, T.J., Rodesch, M.J., Clayton, D.G., Todd, J.A., van Heel, D.A., Plagnol, V., 2010. Genome-wide analysis of allelic expression imbalance in human primary cells by high-throughput transcriptome resequencing. *Hum. Mol. Genet.* 19, 122–134.
- Henney, A.M., Wakeley, P.R., Davies, M.J., Foster, K., Hembry, R., Murphy, G., Humphries, S., 1991. Localization of stromelysin gene expression in atherosclerotic plaques by in situ hybridization. *Proc. Natl. Acad. Sci. U.S.A.* 88, 8154–8158.
- Hirschhorn, J.N., Lohmueller, K., Byrne, E., Hirschhorn, K., 2002. A comprehensive review of genetic association studies. *Genet Med* 4, 45–61.
- Hisano, N., Yatomi, Y., Satoh, K., Akimoto, S., Mitsumata, M., Fujino, M.A., Ozaki, Y., 1999. Induction and suppression of endothelial cell apoptosis by sphingolipids: a possible in vitro model for cell-cell interactions between platelets and endothelial cells. *Blood* 93, 4293–4299.
- Hodis H.N., Wendy J. Mack, Laurie LaBree, Robert H. Selzer, Chao-ran Liu, Ci-hua Liu, and Stanley P. Azen. 1998 The Role of Carotid Arterial Intima-Media Thickness in Predicting Clinical Coronary Events. *Annals of Internal Medicine*, Volume 128 (4) : 262-269
- Hofnagel, O., Luechtenborg, B., Plenz, G., Robenek, H., 2002. Expression of the Novel Scavenger Receptor SR-PSOX in Cultured Aortic Smooth Muscle Cells and Umbilical Endothelial Cells. *Arterioscler Thromb Vasc Biol* 22, 710–711.
- Hojo, S., Koizumi, K., Tsuneyama, K., Arita, Y., Cui, Z., Shinohara, K., Minami, T., Hashimoto, I., Nakayama, T., Sakurai, H., 2007. High-level expression of chemokine CXCL16 by tumor cells correlates with a good prognosis and increased tumor-infiltrating lymphocytes in colorectal cancer. *Cancer research* 67, 4725–4731.
- Hsiai, T., Berliner, J.A., 2007. Oxidative Stress as a Regulator of Murine Atherosclerosis. *Current Drug Targets* 8, 1222–1229.
- Hsiai, T.K., 2008. Mechanosignal transduction coupling between endothelial and smooth muscle cells: role of hemodynamic forces. *AJP: Cell Physiology* 294, C659–C661.
- Huang, M., Han, Y., Zhang, X., Pei, F., Deng, J., Kang, J., Yan, C., 2010. An intron polymorphism in the CXCL16 gene is associated with increased risk of coronary artery disease in Chinese Han population: a large angiography-based study. *Atherosclerosis* 210, 160–165.
- Huang, Y., Zhu, X.Y., Du, M.R., Wu, X., Wang, M.Y., Li, D.J., 2006. Chemokine CXCL16, a scavenger receptor, induces proliferation and invasion of first-trimester human trophoblast cells in an autocrine manner. *Human reproduction* 21, 1083–1091.
- Hwang, S.-J., Ballantyne, C.M., Sharrett, A.R., Smith, L.C., Davis, C.E., Gotto, A.M., Boerwinkle, E., 1997. Circulating Adhesion Molecules VCAM-1, ICAM-1, and E-selectin in Carotid Atherosclerosis and Incident Coronary Heart Disease Cases The Atherosclerosis Risk In Communities (ARIC) Study. *Circulation* 96, 4219–4225.
- Imberti, B., Seliktar, D., Nerem, R.M., Remuzzi, A., 2002. The response of endothelial cells to fluid shear stress using a co-culture model of the arterial wall. *Endothelium* 9, 11–23.
- Inoue, K., Kobayashi, M., Yano, K., Miura, M., Izumi, A., Mataka, C., Doi, T., Hamakubo, T., Reid, P.C., Hume, D.A., Yoshida, M., Aird, W.C., Kodama, T., Minami, T., 2006. Histone deacetylase inhibitor reduces monocyte adhesion to endothelium through the

suppression of vascular cell adhesion molecule-1 expression. *Arterioscler. Thromb. Vasc. Biol.* 26, 2652–2659.

Iwata, A., Shirai, R., Ishii, H., Kushima, H., Otani, S., Hashinaga, K., Umeki, K., Kishi, K., Tokimatsu, I., Hiramatsu, K., Kadota, J., 2012. Inhibitory effect of statins on inflammatory cytokine production from human bronchial epithelial cells. *Clin. Exp. Immunol.* 168, 234–240.

Izquierdo MC, Martin-Cleary C, Fernandex-Fernandez B, Elewa U, Sanchez-Nino MD, Carrero JJ, Ortiz A., 2014. CXCL1^Δ in kidney and cardiovascular injury. *Cytokine Growth Factor Rev.*

Joseph, S.B., Tontonoz, P., 2003. LXRs: new therapeutic targets in atherosclerosis? *Current Opinion in Pharmacology* 3, 192–197.

Kablak-Ziemicka A, W Tracz, T Przewlocki, Pieniazek, A Sokolowski, and M Konieczynska., 2004 Association of increased carotid intima-media thickness with the extent of coronary artery disease *Heart.* Nov 2004; 90(11): 1286–1290.

Kaur, K., Sharma, A.K., Singal, P.K., 2006. Significance of changes in TNF- α and IL-10 levels in the progression of heart failure subsequent to myocardial infarction. *American Journal of Physiology - Heart and Circulatory Physiology* 291, H106–H113.

Kim, I., Moon, S.O., Kim, S.H., Kim, H.J., Koh, Y.S., Koh, G.Y., 2001. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J. Biol. Chem.* 276, 7614–7620.

Kim, K.J., Choi, S.I., Lee, M.S., Kim, J.A., Chun, E.J., Jeon, C.H., 2013. The prevalence and characteristics of coronary atherosclerosis in asymptomatic subjects classified as low risk based on traditional risk stratification algorithm: assessment with coronary CT angiography. *Heart* 99, 1113–1117.

Kleemann, R., Zadelaar, S., Kooistra, T., 2008. Cytokines and atherosclerosis: a comprehensive review of studies in mice. *Cardiovasc Res* 79, 360–376.

Koch, W., Kastrati, A., Böttiger, C., Mehilli, J., von Beckerath, N., Schömig, A., 2001. Interleukin-10 and tumor necrosis factor gene polymorphisms and risk of coronary artery disease and myocardial infarction. *Atherosclerosis* 159, 137.

Koenig, W., Khuseynova, N., 2007. Biomarkers of atherosclerotic plaque instability and rupture. *Arterioscler. Thromb. Vasc. Biol.* 27, 15–26.

Kraaijeveld, A.O., de Jager, S.C.A., van Berkel, T.J.C., Biessen, E.A.L., Jukema, J.W., 2007. Chemokines and atherosclerotic plaque progression: towards therapeutic targeting? *Curr. Pharm. Des.* 13, 1039–1052.

Krunkosky, T.M., Fischer, B.M., Martin, L.D., Jones, N., Akley, N.J., Adler, K.B., 2000. Effects of TNF-alpha on expression of ICAM-1 in human airway epithelial cells in vitro. Signaling pathways controlling surface and gene expression. *American journal of respiratory cell and molecular biology* 22, 685.

Ku, D.N., Giddens, D.P., Zarins, C.K., Glagov, S., 1985. Pulsatile flow and atherosclerosis in the human carotid bifurcation. Positive correlation between plaque location and low oscillating shear stress. *Arterioscler Thromb Vasc Biol* 5, 293–302.

Kuschner, W.G., D'Alessandro, A., Wong, H., Blanc, P.D., 1996. Dose-dependent cigarette smoking-related inflammatory responses in healthy adults. *Eur Respir J* 9, 1989–1994.

Lamon, B.D., Hajjar, D.P., 2008. Inflammation at the Molecular Interface of Atherogenesis. *Am J Pathol* 173, 1253–1264.

LaRosa JC, He J, Vupputuri S, 1999. Effect of statins on risk of coronary disease: A meta-analysis of randomized controlled trials. *JAMA* 282, 2340–2346.

Larsson, P.T., Hallerstam, S., Rosfors, S., Wallén, N.H., 2005. Circulating markers of inflammation are related to carotid artery atherosclerosis. *Int Angiol* 24, 43–51.

Laurencikienė, J., Rydén, M., 2012. Liver X receptors and fat cell metabolism. *Int J Obes*

(Lond) 36, 1494–1502.

Lee, R.T., Grodzinsky, A.J., Frank, E.H., Kamm, R.D., Schoen, F.J., 1991. Structure-dependent dynamic mechanical behavior of fibrous caps from human atherosclerotic

- plaques. *Circulation* 83, 1764–1770.
- Lee, Y.W., Kühn, H., Hennig, B., Neish, A.S., Toborek, M., 2001. IL-4-induced oxidative stress upregulates VCAM-1 gene expression in human endothelial cells. *Journal of molecular and cellular cardiology* 33, 83–94.
- Leeuwenberg, J.F., Smeets, E.F., Neefjes, J.J., Shaffer, M.A., Cinek, T., Jeunhomme, T.M., Ahern, T.J., Buurman, W.A., 1992. E-selectin and intercellular adhesion molecule-1 are released by activated human endothelial cells in vitro. *Immunology* 77, 543–549.
- Lehrke, M., Millington, S.C., Lefterova, M., Cumaranatunge, R.G., Szapary, P., Wilensky, R., Rader, D.J., Lazar, M.A., Reilly, M.P., 2007. CXCL16 is a marker of inflammation, atherosclerosis, and acute coronary syndromes in humans. *Journal of the American College of Cardiology* 49, 442–449.
- Leitinger, N., 2005. Oxidized phospholipids as triggers of inflammation in atherosclerosis. *Molecular Nutrition & Food Research* 49, 1063–1071.
- Leng, S.X., McElhaney, J.E., Walston, J.D., Xie, D., Fedarko, N.S., Kuchel, G.A., 2008. ELISA and Multiplex Technologies for Cytokine Measurement in Inflammation and Aging Research. *J Gerontol A Biol Sci Med Sci* 63, 879–884.
- Leonarduzzi, G., Gamba, P., Gargiulo, S., Biasi, F., Poli, G., 2012. Inflammation-related gene expression by lipid oxidation-derived products in the progression of atherosclerosis. *Free Radical Biology and Medicine* 52, 19–34.
- Leong, C.M., Voorhees, A., Nackman, G.B., Wei, T., 2013. Flow bioreactor design for quantitative measurements over endothelial cells using micro-particle image velocimetry. *Review of Scientific Instruments* 84, 045109.
- Li, G., Gu, H.-M., Zhang, D.-W., 2013. ATP-binding cassette transporters and cholesterol translocation. *IUBMB Life* 65, 505–512.
- Liao, J.K., Laufs, U., 2005. PLEIOTROPIC EFFECTS OF STATINS. *Annu Rev Pharmacol Toxicol* 45, 89–118.
- Libby, P., 2000. Changing concepts of atherogenesis. *Journal of Internal Medicine* 247, 349–358.
- Libby, P., 2006. Inflammation and cardiovascular disease mechanisms. *Am J Clin Nutr* 83, 456S–460S.
- Libby, P., Ridker, P.M., Hansson, G.K., 2011. Progress and challenges in translating the biology of atherosclerosis. *Nature* 473, 317–325.
- Libby, P., Ridker, P.M., Maseri, A., 2002a. Inflammation and atherosclerosis. *Circulation* 105, 1135–1143.
- Libby, P., Ridker, P.M., Maseri, A., 2002b. Inflammation and Atherosclerosis. *Circulation* 105, 1135–1143.
- Little, T.L., Xia, J., Duling, B.R., 1995. Dye tracers define differential endothelial and smooth muscle coupling patterns within the arteriolar wall. *Circ. Res.* 76, 498–504.
- Liu, Y., Chen, B.P.-C., Lu, M., Zhu, Y., Stemerman, M.B., Chien, S., Shyy, J.Y.-J., 2002. Shear stress activation of SREBP1 in endothelial cells is mediated by integrins. *Arterioscler. Thromb. Vasc. Biol.* 22, 76–81.
- Lougaris, V., Baronio, M., Vitali, M., Tampella, G., Soresina, A., Badolato, R., Plebani, A., 2012a. B cell responses to CpG correlate with CXCL16 expression levels in common variable immunodeficiency. *ScientificWorldJournal* 2012, 960219.
- Lougaris, V., Baronio, M., Vitali, M., Tampella, G., Soresina, A., Badolato, R., Plebani, A., 2012b. B cell responses to CpG correlate with CXCL16 expression levels in common variable immunodeficiency. *ScientificWorldJournal* 2012, 960219.
- Lu, Y., Wang, J., Xu, Y., Koch, A.E., Cai, Z., Chen, X., Galson, D.L., Taichman, R.S., Zhang, J., 2008. CXCL16 functions as a novel chemotactic factor for prostate cancer cells in vitro. *Molecular Cancer Research* 6, 546–554.

- Luc, G., Arveiler, D., Evans, A., Amouyel, P., Ferrieres, J., Bard, J.-M., Elkhailil, L., Fruchart, J.-C., Ducimetiere, P., 2003. Circulating soluble adhesion molecules ICAM-1 and VCAM-1 and incident coronary heart disease: The PRIME Study. *Atherosclerosis* 170, 169–176.
- Ludwig, A., Schulte, A., Schnack, C., Hundhausen, C., Reiss, K., Brodway, N., Held-Feindt, J., Mentlein, R., 2005. Enhanced expression and shedding of the transmembrane chemokine CXCL16 by reactive astrocytes and glioma cells. *Journal of neurochemistry* 93, 1293–1303.
- Lundberg, G.A., Kellin, A., Samneglaard, A., Lundman, P., Tornvall, P., Dimmeler, S., Zeiher, A.M., Hamsten, A., Hansson, G.K., Eriksson, P., 2005. Severity of coronary artery stenosis is associated with a polymorphism in the CXCL16/SR-PSOX gene. *Journal of internal medicine* 257, 415–422.
- Lusis, A.J., 2000. Atherosclerosis. *Nature* 407, 233–241.
- Lusis, A.J., Weiss, J.N., 2010. Cardiovascular Networks Systems-Based Approaches to Cardiovascular Disease. *Circulation* 121, 157–170.
- Lutgens, E., van Suylen, R.-J., Faber, B.C., Gijbels, M.J., Eurlings, P.M., Bijmens, A.-P., Cleutjens, K.B., Heeneman, S., Daemen, M.J.A.P., 2003. Atherosclerotic plaque rupture: local or systemic process? *Arterioscler. Thromb. Vasc. Biol.* 23, 2123–2130.
- Lyngdoh, T., Vollenweider, P., Waeber, G., Marques-Vidal, P., 2011. Association of statins with inflammatory cytokines: a population-based Colaus study. *Atherosclerosis* 219, 253–258.
- Mackman, N., 2003. How Do Oxidized Phospholipids Inhibit LPS Signaling? *Arterioscler Thromb Vasc Biol* 23, 1133–1136.
- Madhur, M.S., Lob, H.E., McCann, L.A., Iwakura, Y., Blinder, Y., Guzik, T.J., Harrison, D.G., 2010. Interleukin 17 Promotes Angiotensin II-Induced Hypertension and Vascular Dysfunction. *Hypertension* 55, 500–507.
- Maini, R.N., Breedveld, F.C., Kalden, J.R., Smolen, J.S., Davis, D., Macfarlane, J.D., Antoni, C., Leeb, B., Elliott, M.J., Woody, J.N., Schaible, T.F., Feldmann, M., 1998. Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis. *Arthritis Rheum.* 41, 1552–1563.
- Malek, A.M., Alper, S.L., Izumo, S., 1999. Hemodynamic shear stress and its role in atherosclerosis. *JAMA* 282, 2035–2042.
- Mallat, Z., Besnard, S., Duriez, M., Deleuze, V., Emmanuel, F., Bureau, M.F., Soubrier, F., Esposito, B., Duez, H., Fievet, C., Staels, B., Duverger, N., Scherman, D., Tedgui, A., 1999. Protective role of interleukin-10 in atherosclerosis. *Circ. Res.* 85, e17–24.
- Mallat, Z., Corbaz, A., Scoazec, A., Besnard, S., Lesèche, G., Chvatchko, Y., Tedgui, A., 2001. Expression of Interleukin-18 in Human Atherosclerotic Plaques and Relation to Plaque Instability. *Circulation* 104, 1598–1603.
- Marin, T., Gongol, B., Chen, Z., Woo, B., Subramaniam, S., Chien, S., Shyy, J.Y.-J., 2013. Mechanosensitive microRNAs - Role in Endothelial Responses to Shear Stress and Redox State. *Free Radic. Biol. Med.*
- Maron, D.J., Fazio, S., Linton, M.F., 2000. Current Perspectives on Statins. *Circulation* 101, 207–213.
- Maton, Anthea; Jean Hopkins, Charles William McLaughlin, Susan Johnson, Maryanna Quon Warner, David LaHart, Jill D. Wright (1993). *Human Biology and Health*. Englewood Cliffs, New Jersey: Prentice Hall. ISBN 0-13-981176-1
- Matsushita K, Toiyama Y, Tanaka K, Saigusa S, Hiro J, Uchida K, Inoue Y, Kusunoki M. Soluble CXCL16 in preoperative serum is a novel prognostic marker and predicts recurrence of liver metastases in colorectal cancer patients., 2012. *Ann*

Surg Oncol. Suppl 3:S518-27

Mazzone, A., Servi, S.D., Mazzucchelli, I., Bossi, I., Ottini, E., Vezzoli, M., Meloni, F., Lotzinker, M., Mariani, G., 2001. Increased concentrations of inflammatory mediators in unstable angina: correlation with serum troponin T. Heart 85, 571–575.

- McLaren, J.E., Ramji, D.P., 2009. Interferon gamma: A master regulator of atherosclerosis. *Cytokine & Growth Factor Reviews* 20, 125–135.
- Mendall, M.A., Patel, P., Asante, M., Ballam, L., Morris, J., Strachan, D.P., Camm, A.J., Northfield, T.C., 1997. Relation of serum cytokine concentrations to cardiovascular risk factors and coronary heart disease. *Heart* 78, 273–277.
- Minami, M., Kume, N., Shimaoka, T., Kataoka, H., Hayashida, K., Akiyama, Y., Nagata, I., Ando, K., Nobuyoshi, M., Hanyuu, M., Komeda, M., Yonehara, S., Kita, T., 2001. Expression of SR-PSOX, a Novel Cell-Surface Scavenger Receptor for Phosphatidylserine and Oxidized LDL in Human Atherosclerotic Lesions. *Arterioscler Thromb Vasc Biol* 21, 1796–1800.
- Mizia-Stec, K., Gasior, Z., Zahorska-Markiewicz, B., Janowska, J., Szulc, A., Jastrzebska-Maj, E., Kobielski-Gembala, I., 2003. Serum tumour necrosis factor- α , interleukin-2 and interleukin-10 activation in stable angina and acute coronary syndromes. *Coron. Artery Dis.* 14, 431–438.
- Monneret, C., 2005. Histone deacetylase inhibitors. *Eur J Med Chem* 40, 1–13.
- Morange, P.E., Bickel, C., Nicaud, V., Schnabel, R., Rupprecht, H.J., Peetz, D., Lackner, K.J., Cambien, F., Blankenberg, S., Tiret, L., AtheroGene Investigators, 2006. Haemostatic factors and the risk of cardiovascular death in patients with coronary artery disease: the AtheroGene study. *Arterioscler. Thromb. Vasc. Biol.* 26, 2793–2799.
- Mühl, H., Pfeilschifter, J., 2003. Anti-inflammatory properties of pro-inflammatory interferon- γ . *International Immunopharmacology* 3, 1247–1255.
- Müller, K., Aichele, S., Herkommer, M., Bigalke, B., Stellos, K., Htun, P., Fateh-Moghadam, S., May, A.E., Flather, M., Gawaz, M., Geisler, T., 2010. Impact of inflammatory markers on platelet inhibition and cardiovascular outcome including stent thrombosis in patients with symptomatic coronary artery disease. *Atherosclerosis* 213, 256–262.
- Murphy, J.E., Tedbury, P.R., Homer-Vanniasinkam, S., Walker, J.H., Ponnambalam, S., 2005. Biochemistry and cell biology of mammalian scavenger receptors. *Atherosclerosis* 182, 1–15.
- Myers, C.L., Wertheimer, S.J., Schembri-King, J., Parks, T., Wallace, R.W., 1992. Induction of ICAM-1 by TNF- α , IL-1 β , and LPS in human endothelial cells after downregulation of PKC. *Am. J. Physiol.* 263, C767–772.
- Nachman, M.W., 2001. Single nucleotide polymorphisms and recombination rate in humans. *Trends Genet.* 17, 481–485.
- Nageh, M.F., Sandberg, E.T., Marotti, K.R., Lin, A.H., Melchior, E.P., Bullard, D.C., Beaudet, A.L., 1997. Deficiency of Inflammatory Cell Adhesion Molecules Protects Against Atherosclerosis in Mice. *Arterioscler Thromb Vasc Biol* 17, 1517–1520.
- Nagel, T., Resnick, N., Dewey, C.F., Gimbrone, M.A., 1999. Vascular endothelial cells respond to spatial gradients in fluid shear stress by enhanced activation of transcription factors. *Arteriosclerosis, thrombosis, and vascular biology* 19, 1825–1834.
- Ni, C.-W., Qiu, H., Rezvan, A., Kwon, K., Nam, D., Son, D.J., Visvader, J.E., Jo, H., 2010. Discovery of novel mechanosensitive genes in vivo using mouse carotid artery endothelium exposed to disturbed flow. *Blood* 116, e66–e73.
- Ohta, T., Yamashita, N., Maruyama, M., Sugiyama, E., Kobayashi, M., 1998. Cigarette smoking decreases interleukin-8 secretion by human alveolar macrophages. *Respiratory Medicine* 92, 922–927.
- Okamura, H., Tsutsi, H., Komatsu, T., Yutsudo, M., Hakura, A., Tanimoto, T., Torigoe, K.,

- Okura, T., Nukada, Y., Hattori, K., 1995. Cloning of a new cytokine that induces IFN- γ production by T cells. *Nature* 378, 88–91.
- Orr, A.W., Helmke, B.P., Blackman, B.R., Schwartz, M.A., 2006. Mechanisms of Mechanotransduction. *Developmental Cell* 10, 11–20.
- Ozeren, A., Aydin, M., Tokac, M., Demircan, N., Unalacak, M., Gurel, A., Yazici, M., 2003. Levels of serum IL-1 β , IL-2, IL-8 and tumor necrosis factor- α in patients with unstable angina pectoris. *Mediators of inflammation* 12, 361–365.
- Panettieri Jr, R.A., Lazaar, A.L., Pure, E., Albelda, S.M., 1995. Activation of cAMP-dependent pathways in human airway smooth muscle cells inhibits TNF- α -induced ICAM-1 and VCAM-1 expression and T lymphocyte adhesion. *The Journal of Immunology* 154, 2358–2365.
- Pant, S., Deshmukh, A., GuruMurthy, G.S., Pothineni, N.V., Watts, T.E., Romeo, F., Mehta, J.L., 2013. Inflammation and Atherosclerosis—Revisited. *J CARDIOVASC PHARMACOL THER* 1074248413504994.
- Paracchini, S., Monaco, A.P., Knight, J.C., 2010. An allele-specific gene expression assay to test the functional basis of genetic associations. *J Vis Exp*.
- Paravicini, T.M., Touyz, R.M., 2008. NADPH Oxidases, Reactive Oxygen Species, and Hypertension: Clinical implications and therapeutic possibilities. *Diabetes Care* 31, S170–S180.
- Pastinen, T., 2010. Genome-wide allele-specific analysis: insights into regulatory variation. *Nat Rev Genet* 11, 533–538.
- Pastori, D., Carnevale, R., Pignatelli, P., 2013. Is there a clinical role for oxidative stress biomarkers in atherosclerotic diseases? *Intern Emerg Med*.
- Pastorino, J.G., Shulga, N., 2008. Tumor necrosis factor- α can provoke cleavage and activation of sterol regulatory element-binding protein in ethanol-exposed cells via a caspase-dependent pathway that is cholesterol insensitive. *J. Biol. Chem.* 283, 25638–25649.
- Patel, J.A., Kunimoto, M., Sim, T.C., Garofalo, R., Elliott, T., Baron, S., Ruuskanen, O., Chonmaitree, T., Ogra, P.L., Schmalstieg, F., 1995. Interleukin-1 α mediates the enhanced expression of intercellular adhesion molecule-1 in pulmonary epithelial cells infected with respiratory syncytial virus. *American journal of respiratory cell and molecular biology* 13, 602.
- Patel, K.D., Duggan, S.P., Currid, C.A., Gallagher, W.M., McManus, R., Kelleher, D., Murphy, R.T., Ryan, A.W., 2009. High sensitivity cytokine detection in acute coronary syndrome reveals up-regulation of Interferon Gamma and Interleukin-10 post Myocardial Infarction. *Clinical Immunology* 133, 251–256.
- Peiffer, V., Sherwin, S.J., Weinberg, P.D., 2013. Does low and oscillatory wall shear stress correlate spatially with early atherosclerosis? A systematic review. *Cardiovasc. Res.*
- Petit, S.J., Wise, E.L., Chambers, J.C., Sehmi, J., Chayen, N.E., Kooner, J.S., Pease, J.E., 2011. The cxcl16 a181v mutation selectively inhibits monocyte adhesion to cxcr6 but is not associated with human coronary heart disease. *Arteriosclerosis, thrombosis, and vascular biology* 31, 914–920.
- Phinikaridou, A., Andia, M.E., Lacerda, S., Lorrio, S., Makowski, M.R., Botnar, R.M., 2013. Molecular MRI of atherosclerosis. *Molecules* 18, 14042–14069.
- Pigott, R., Dillon, L.P., Hemingway, I.H., Gearing, A.J.H., 1992. Soluble forms of E-selectin, ICAM-1 and VCAM-1 are present in the supernatants of cytokine activated cultured endothelial cells. *Biochemical and Biophysical Research Communications* 187, 584–

- Plenz, G., Koenig, C., Severs, N.J., Robenek, H., 1997. Smooth Muscle Cells Express Granulocyte-Macrophage Colony-Stimulating Factor in the Undiseased and Atherosclerotic Human Coronary Artery. *Arterioscler Thromb Vasc Biol* 17, 2489–2499.
- Profumo, E., Buttari, B., Tosti, M.E., Siracusano, A., Ortona, E., Margutti, P., Capoano, R., Salvati, B., Riganò, R., 2008. Association of intracellular pro- and anti-inflammatory cytokines in peripheral blood with the clinical or ultrasound indications for carotid endarterectomy in patients with carotid atherosclerosis. *Clin. Exp. Immunol.* 152, 120–126.
- Pyo, C.-W., Hur, S.-S., Kim, Y.-K., Choi, H.-B., Hong, Y.-S., Kim, D.-W., Kim, C.-C., Kim, H.-K., Kim, T.-G., 2003. Polymorphisms of IL-1B, IL-1RN, IL-2, IL-4, IL-6, IL-10, and IFN- γ genes in the Korean population. *Human immunology* 64, 979–989.
- Raines, E.W., Ross, R., 1993. Smooth muscle cells and the pathogenesis of the lesions of atherosclerosis. *British heart journal* 69, S30.
- Randi, A.M., Biguzzi, E., Falciani, F., Merlini, P., Blakemore, S., Bramucci, E., Lucreziotti, S., Lennon, M., Faioni, E.M., Ardissino, D., Mannucci, P.M., 2003. Identification of differentially expressed genes in coronary atherosclerotic plaques from patients with stable or unstable angina by cDNA array analysis. *J. Thromb. Haemost.* 1, 829–835.
- Ranjit, S., Dazhu, L., Qiutang, Z., Yibo, F., Yushu, L., Xiang, W., Shen, C.L., Yuan, T., 2004. Differentiation of dendritic cells in monocyte cultures isolated from patients with unstable angina. *International Journal of Cardiology* 97, 551–555.
- Redmond, E.M., Cahill, P.A., Sitzmann, J.V., 1995. Perfused transcapillary smooth muscle and endothelial cell co-culture--a novel in vitro model. *In Vitro Cell. Dev. Biol. Anim.* 31, 601–609.
- Reich, D.E., Cargill, M., Bolk, S., Ireland, J., Sabeti, P.C., Richter, D.J., Lavery, T., Kouyoumjian, R., Farhadian, S.F., Ward, R., Lander, E.S., 2001. Linkage disequilibrium in the human genome. *Nature* 411, 199–204.
- Reriani, M.K., Lerman, L.O., Lerman, A., 2010. Endothelial function as a functional expression of cardiovascular risk factors. *Biomark Med* 4, 351–360.
- Rezaie-Majd, A., Maca, T., Bucek, R.A., Valent, P., Müller, M.R., Husslein, P., Kashanipour, A., Minar, E., Baghestanian, M., 2002. Simvastatin reduces expression of cytokines interleukin-6, interleukin-8, and monocyte chemoattractant protein-1 in circulating monocytes from hypercholesterolemic patients. *Arterioscler. Thromb. Vasc. Biol.* 22, 1194–1199.
- Richardson, P.D., Davies, M.J., Born, G.V., 1989. Influence of plaque configuration and stress distribution on fissuring of coronary atherosclerotic plaques. *Lancet* 2, 941–944.
- Ridker, P.M., Rifai, N., Stampfer, M.J., Hennekens, C.H., 2000. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation* 101, 1767–1772.
- Ridker PM, S.M., 2001. Novel risk factors for systemic atherosclerosis: A comparison of c-reactive protein, fibrinogen, homocysteine, lipoprotein(a), and standard cholesterol screening as predictors of peripheral arterial disease. *JAMA* 285, 2481–2485.
- Ross, R., Glomset, J., Harker, L., 1977. Response to injury and atherogenesis. *Am. J. Pathol.* 86, 675–684.
- Ross, R., Harker, L., 1976. Hyperlipidemia and atherosclerosis. *Science* 193, 1094–1100.
- Roth-Isigkeit, Borstel, Seyfarth, Schmucker, 1999. Perioperative serum levels of tumour-necrosis-factor alpha (TNF- α), IL-1 β , IL-6, IL-10 and soluble IL-2 receptor in patients undergoing cardiac surgery with cardiopulmonary bypass without and with

- correction for haemodilution. *Clinical & Experimental Immunology* 118, 242–246.
- Rus, F.G., Niculescu, F., Vlaicu, R., 1991. Tumor necrosis factor-alpha in human arterial wall with atherosclerosis. *Atherosclerosis* 89, 247–254.
- Rus, H.G., Vlaicu, R., Niculescu, F., 1996. Interleukin-6 and interleukin-8 protein and gene expression in human arterial atherosclerotic wall. *Atherosclerosis* 127, 263–271.
- Ruth, J.H., Haas, C.S., Park, C.C., Amin, M.A., Martinez, R.J., Haines, G.K., Shahrara, S., Campbell, P.L., Koch, A.E., 2006. CXCL16-mediated cell recruitment to rheumatoid arthritis synovial tissue and murine lymph nodes is dependent upon the MAPK pathway. *Arthritis & Rheumatism* 54, 765–778.
- Sachidanandam, R., Weissman, D., Schmidt, S.C., Kakol, J.M., Stein, L.D., Marth, G., Sherry, S., Mullikin, J.C., Mortimore, B.J., Willey, D.L., Hunt, S.E., Cole, C.G., Coggill, P.C., Rice, C.M., Ning, Z., Rogers, J., Bentley, D.R., Kwok, P.Y., Mardis, E.R., Yeh, R.T., Schultz, B., Cook, L., Davenport, R., Dante, M., Fulton, L., Hillier, L., Waterston, R.H., McPherson, J.D., Gilman, B., Schaffner, S., Van Etten, W.J., Reich, D., Higgins, J., Daly, M.J., Blumenstiel, B., Baldwin, J., Stange-Thomann, N., Zody, M.C., Linton, L., Lander, E.S., Altshuler, D., International SNP Map Working Group, 2001. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature* 409, 928–933.
- Sakamoto, N., Ohashi, T., Sato, M., 2006. Effect of Fluid Shear Stress on Migration of Vascular Smooth Muscle Cells in Cocultured Model. *Ann Biomed Eng* 34, 408–415.
- Sakurada, S., Kato, T., Okamoto, T., 1996. Induction of cytokines and ICAM-1 by proinflammatory cytokines in primary rheumatoid synovial fibroblasts and inhibition by N-acetyl-L-cysteine and aspirin. *Int. Immunol.* 8, 1483–1493.
- Saxer, T., Zumbuehl, A., Müller, B., 2013. The use of shear stress for targeted drug delivery. *Cardiovasc. Res.*
- Schächinger, V., Zeiher, A.M., 2000. Atherosclerosis-associated endothelial dysfunction. *Z Kardiol* 89 Suppl 9, IX/70–74.
- Schadt, E.E., 2009. Molecular networks as sensors and drivers of common human diseases. *Nature* 461, 218–223.
- Schönbeck, U., Sukhova, G.K., Shimizu, K., Mach, F., Libby, P., 2000. Inhibition of CD40 signaling limits evolution of established atherosclerosis in mice. *Proc. Natl. Acad. Sci. U.S.A.* 97, 7458–7463.
- Shaaban, A.M., Duerinckx, A.J., 2000. Wall shear stress and early atherosclerosis: a review. *AJR Am J Roentgenol* 174, 1657–1665.
- Shah, P.K., 2003. Mechanisms of plaque vulnerability and rupture. *J Am Coll Cardiol* 41, S15–S22.
- Shah, S.H., Granger, C.B., Hauser, E.R., Kraus, W.E., Sun, J.-L., Pieper, K., Nelson, C.L., Delong, E.R., Califf, R.M., Newby, L.K., 2010. Reclassification of cardiovascular risk using integrated clinical and molecular biosignatures: Design of and rationale for the Measurement to Understand the Reclassification of Disease of Cabarrus and Kannapolis (MURDOCK) Horizon 1 Cardiovascular Disease Study. *American Heart Journal* 160, 371–379.e2.
- Shaposhnik, Z., Wang, X., Weinstein, M., Bennett, B.J., Lusis, A.J., 2007. Granulocyte Macrophage Colony-Stimulating Factor Regulates Dendritic Cell Content of Atherosclerotic Lesions. *Arterioscler Thromb Vasc Biol* 27, 621–627.
- Sheikine, Y., Bang, C.S., Nilsson, L., Samnegård, A., Hamsten, A., Jonasson, L., Eriksson, P., Sirsjö, A., 2006. Decreased plasma CXCL16/SR-PSOX concentration is associated with coronary artery disease. *Atherosclerosis* 188, 462–466.
- Sheikine, Y., Sirsjö, A., 2008. CXCL16/SR-PSOX--a friend or a foe in atherosclerosis? *Atherosclerosis* 197, 487–495.

- Sherer, Y., Shoenfeld, Y., 2006. Mechanisms of Disease: atherosclerosis in autoimmune diseases. *Nature Clinical Practice Rheumatology* 2, 99–106.
- Shintani, S., Murohara, T., Ikeda, H., Ueno, T., Honma, T., Katoh, A., Sasaki, K., Shimada, T., Oike, Y., Imaizumi, T., 2001. Mobilization of Endothelial Progenitor Cells in Patients With Acute Myocardial Infarction. *Circulation* 103, 2776–2779.
- Shrestha, S., Irvin, M.R., Grunfeld, C., Arnett, D.K., 2013. HIV, Inflammation, and Calcium in Atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.*
- Silbernagel, G., Schöttker, B., Appelbaum, S., Scharnagl, H., Kleber, M.E., Grammer, T.B., Ritsch, A., Mons, U., Holleczer, B., Goliasch, G., Niessner, A., Boehm, B.O., Schnabel, R.B., Brenner, H., Blankenberg, S., Landmesser, U., März, W., 2013. High-density lipoprotein cholesterol, coronary artery disease, and cardiovascular mortality. *Eur Heart J* eht343.
- Sima, A.V., Stancu, C.S., Simionescu, M., 2009. Vascular endothelium in atherosclerosis. *Cell Tissue Res* 335, 191–203.
- Sims, J.E., Smith, D.E., 2010. The IL-1 family: regulators of immunity. *Nat. Rev. Immunol.* 10, 89–102.
- Smith, C., Halvorsen, B., Otterdal, K., Wæhre, T., Yndestad, A., Fevang, B., Sandberg, W.J., Breland, U.M., Frøland, S.S., Øie, E., 2008. High levels and inflammatory effects of soluble CXC ligand 16 (CXCL16) in coronary artery disease: down-regulatory effects of statins. *Cardiovascular research* 79, 195–203.
- Smith, D.A., Irving, S.D., Sheldon, J., Cole, D., Kaski, J.C., 2001. Serum levels of the antiinflammatory cytokine interleukin-10 are decreased in patients with unstable angina. *Circulation* 104, 746–749.
- Soon, E., Holmes, A.M., Treacy, C.M., Doughty, N.J., Southgate, L., Machado, R.D., Trembath, R.C., Jennings, S., Barker, L., Nicklin, P., Walker, C., Budd, D.C., Pepke-Zaba, J., Morrell, N.W., 2010. Elevated Levels of Inflammatory Cytokines Predict Survival in Idiopathic and Familial Pulmonary Arterial Hypertension. *Circulation* 122, 920–927.
- Spagnoli, L.G., Pietra, G.G., Villaschi, S., Johns, L.W., 1982. Morphometric analysis of gap junctions in regenerating arterial endothelium. *Lab. Invest.* 46, 139–148.
- Spector, Tim (2012). *Identically Different: Why You Can Change Your Genes*. London: Weidenfeld & Nicolson. p. 8
- Starosta, V., Wu, T., Zimman, A., Pham, D., Tian, X., Oskolkova, O., Bochkov, V., Berliner, J.A., Birukova, A.A., Birukov, K.G., 2012. Differential Regulation of Endothelial Cell Permeability by High and Low Doses of Oxidized 1-Palmitoyl-2-Arachidonyl-sn-Glycero-3-Phosphocholine. *Am J Respir Cell Mol Biol* 46, 331–341.
- Stenvinkel, P., Ketteler, M., Johnson, R.J., Lindholm, B., Pecoits-Filho, R., Riella, M., HEIMBÜRGER, O., Cederholm, T., Girndt, M., 2005. IL-10, IL-6, and TNF- α : central factors in the altered cytokine network of uremia—the good, the bad, and the ugly. *Kidney international* 67, 1216–1233.
- Stephen, S.L., Freestone, K., Dunn, S., Twigg, M.W., Homer-Vanniasinkam, S., Walker, J.H., Wheatcroft, S.B., Ponnambalam, S., 2010. Scavenger receptors and their potential as therapeutic targets in the treatment of cardiovascular disease. *Int J Hypertens* 2010, 646929.
- Sugiyama, S., Okada, Y., Sukhova, G.K., Virmani, R., Heinecke, J.W., Libby, P., 2001. Macrophage Myeloperoxidase Regulation by Granulocyte Macrophage Colony-Stimulating Factor in Human Atherosclerosis and Implications in Acute Coronary Syndromes. *The American Journal of Pathology* 158, 879–891.
- Sun, Y., Chang, Z., Zhang, S., 2008. Increased Serum CXCL16 Level Is a Marker for Acute

- Coronary Syndromes. *Archives of Medical Research* 39, 332–337.
- effector T cell recruitment. *Nature immunology* 4, 982–990.
- Takahashi, M., Ishida, T., Traub, O., Corson, M.A., Berk, B.C., 1997. Mechanotransduction in endothelial cells: temporal signaling events in response to shear stress. *J. Vasc. Res.* 34, 212–219.
- Tedgui, A., Mallat, Z., 2006. Cytokines in Atherosclerosis: Pathogenic and Regulatory Pathways. *Physiol Rev* 86, 515–581.
- Tenbaum, S., Arqués, O., Chicote, I., Tenbaum, S., Puig, I., G. Palmer, H., 2012. Standardized Relative Quantification of Immunofluorescence Tissue Staining. Protocol Exchange.
- Tenger, C., Sundborger, A., Jawien, J., Zhou, X., 2005. IL-18 accelerates atherosclerosis accompanied by elevation of IFN- γ and CXCL16 expression independently of T cells. *Arteriosclerosis, thrombosis, and vascular biology* 25, 791–796.
- Thompson, P.W., Randi, A.M., Ridley, A.J., 2002. Intercellular adhesion molecule (ICAM)-1, but not ICAM-2, activates RhoA and stimulates c-fos and rhoA transcription in endothelial cells. *J. Immunol.* 169, 1007–1013.
- Tiret, L., Godefroy, T., Lubos, E., Nicaud, V., Tregouet, D.-A., Barbaux, S., Schnabel, R., Bickel, C., Espinola-Klein, C., Poirier, O., Perret, C., Münzel, T., Rupprecht, H.-J., Lackner, K., Cambien, F., Blankenberg, S., AtheroGene Investigators, 2005. Genetic analysis of the interleukin-18 system highlights the role of the interleukin-18 gene in cardiovascular disease. *Circulation* 112, 643–650.
- Traub, O., Berk, B.C., 1998. Laminar Shear Stress Mechanisms by Which Endothelial Cells Transduce an Atheroprotective Force. *Arterioscler Thromb Vasc Biol* 18, 677–685.
- Tseng, H., Peterson, T.E., Berk, B.C., 1995. Fluid shear stress stimulates mitogen-activated protein kinase in endothelial cells. *Circ. Res.* 77, 869–878.
- Turunen, M.P., Aavik, E., Ylä-Herttuala, S., 2009. Epigenetics and atherosclerosis. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1790, 886–891.
- Tzima, E., 2006. Role of Small GTPases in Endothelial Cytoskeletal Dynamics and the Shear Stress Response. *Circulation Research* 98, 176–185.
- Uno, K., Nicholls, S.J., 2010. Biomarkers of inflammation and oxidative stress in atherosclerosis. *Biomark Med* 4, 361–373.
- Uyemura, K., Demer, L.L., Castle, S.C., Jullien, D., Berliner, J.A., Gately, M.K., Warrier, R.R., Pham, N., Fogelman, A.M., Modlin, R.L., 1996. Cross-regulatory roles of interleukin (IL)-12 and IL-10 in atherosclerosis. *Journal of Clinical Investigation* 97, 2130.
- Vaddi, K., Nicolini, F.A., Mehta, P., Mehta, J.L., 1994. Increased secretion of tumor necrosis factor-alpha and interferon-gamma by mononuclear leukocytes in patients with ischemic heart disease. Relevance in superoxide anion generation. *Circulation* 90, 694–699.
- Van Der Voort, R., Van Lieshout, A.W.T., Toonen, L.W.J., Slöetjes, A.W., Van Den Berg, W.B., Figdor, C.G., Radstake, T.R.D.J., Adema, G.J., 2005. Elevated CXCL16 expression by synovial macrophages recruits memory T cells into rheumatoid joints. *Arthritis & Rheumatism* 52, 1381–1391.
- Vasto, S., Candore, G., Balistreri, C.R., Caruso, M., Colonna-Romano, G., Grimaldi, M.P., Listi, F., Nuzzo, D., Lio, D., Caruso, C., 2007. Inflammatory networks in ageing, age-related diseases and longevity. *Mechanisms of Ageing and Development* 128, 83–91.
- Virmani, R., Burke, A.P., Farb, A., Kolodgie, F.D., 2006. Pathology of the vulnerable plaque. *J. Am. Coll. Cardiol.* 47, C13–18.

- VON DER THÜSEN, J.H., Kuiper, J., Fekkes, M.L., de Vos, P., van Berkel, T.J., AL BIESSEN, E., 2001. Attenuation of atherogenesis by systemic and local adenovirus-mediated gene transfer of interleukin-10 in LDLr^{-/-} mice. *The FASEB Journal* 15, 2730–2732.
- Waaagsäter, D., Hugander, A., Dimberg, J., 2004a. Expression of CXCL16 in human rectal cancer. *International journal of molecular medicine* 14, 65.
- Waaagsäter, D., Olofsson, P.S., Norgren, L., Stenberg, B., Sirsjö, A., 2004b. The chemokine and scavenger receptor CXCL16/SR-PSOX is expressed in human vascular smooth muscle cells and is induced by interferon γ . *Biochemical and biophysical research communications* 325, 1187–1193.
- Waehre, T., Halvorsen, B., Dam\aaas, J.K., Yndestad, A., Brosstad, F., Gullestad, L., Kjekshus, J., Frøland, S.S., Aukrust, P., 2002. Inflammatory imbalance between IL-10 and TNF α in unstable angina potential plaque stabilizing effects of IL-10. *European journal of clinical investigation* 32, 803–810.
- Walpol, P.L., Gotlieb, A.I., Cybulsky, M.I., Langille, B.L., 1995. Expression of ICAM-1 and VCAM-1 and Monocyte Adherence in Arteries Exposed to Altered Shear Stress. *Arterioscler Thromb Vasc Biol* 15, 2–10.
- Wang, H., Elbein, S.C., 2007. Detection of allelic imbalance in gene expression using pyrosequencing. *Methods Mol. Biol.* 373, 157–176.
- Wang, K., Liu, Z., Wang, R., Wang, Y., Zhang, G., Su, J., Kang, X., 2010. Chemokine CXC Ligand 16 serum concentration but not A181V genotype is associated with atherosclerotic stroke. *Clinica Chimica Acta* 411, 1447–1451..
- Watanabe, T., Fan, J., 1998. Atherosclerosis and inflammation: Mononuclear cell recruitment and adhesion molecules with reference to the implication of ICAM-1/LFA-1 pathway in atherogenesis. *International Journal of Cardiology* 66, Supplement 1, S45–S53.
- Watson, A.D., Leitinger, N., Navab, M., Faull, K.F., Hörkkö, S., Witztum, J.L., Palinski, W., Schwenke, D., Salomon, R.G., Sha, W., Subbanagounder, G., Fogelman, A.M., Berliner, J.A., 1997. Structural Identification by Mass Spectrometry of Oxidized Phospholipids in Minimally Oxidized Low Density Lipoprotein That Induce Monocyte/Endothelial Interactions and Evidence for Their Presence in Vivo. *J. Biol. Chem.* 272, 13597–13607.
- Weber, C., Noels, H., 2011. Atherosclerosis: current pathogenesis and therapeutic options. *Nat Med* 17, 1410–1422.
- White, C.R., Haidekker, M., Bao, X., Frangos, J.A., 2001. Temporal gradients in shear, but not spatial gradients, stimulate endothelial cell proliferation. *Circulation* 103, 2508–2513.
- Whitman, S.C., Ravisankar, P., Daugherty, A., 2002. Interleukin-18 enhances atherosclerosis in apolipoprotein E^{-/-} mice through release of interferon- γ . *Circulation research* 90, e34–e38.
- Wong, K.H.K., Chan, J.M., Kamm, R.D., Tien, J., 2012. Microfluidic models of vascular functions. *Annu Rev Biomed Eng* 14, 205–230.
- Wu, R., Shen, G., Morris, R., Patnaik, M., Peter, J.B., 2005. Elevated autoantibodies against oxidized palmitoyl arachidonoyl phosphocholine in patients with hypertension and myocardial infarction. *Journal of autoimmunity* 24, 353–360.
- Wuttge, D.M., Zhou, X., Sheikine, Y., Wåagsäter, D., Stemme, V., Hedin, U., Stemme, S., Hansson, G.K., Sirsjö, A., 2004. CXCL16/SR-PSOX is an interferon-gamma-regulated chemokine and scavenger receptor expressed in atherosclerotic lesions. *Arterioscler. Thromb. Vasc. Biol.* 24, 750–755.
- Yamauchi, R., Tanaka, M., Kume, N., Minami, M., Kawamoto, T., Togi, K., Shimaoka, T., Takahashi, S., Yamaguchi, J., Nishina, T., 2004. Upregulation of SR-PSOX/CXCL16

- and recruitment of CD8+ T cells in cardiac valves during inflammatory valvular heart disease. *Arteriosclerosis, thrombosis, and vascular biology* 24, 282–287.
- Yang, G.-Y., Gong, C., Qin, Z., Ye, W., Mao, Y., Bertz, A.L., 1998. Inhibition of TNF [alpha] attenuates infarct volume and ICAM-1 expression in ischemic mouse brain. *Neuroreport* 9, 2131–2134.
- Yi, G.-W., Zeng, Q.-T., 2008. Circulating CXCL16 is related to the severity of coronary artery stenosis. *Arch. Med. Res.* 39, 531–535.
- Mechanism. *Arteriosclerosis, thrombosis, and vascular biology* 21,
- Yoshizumi, M., Abe, J.-I., Tsuchiya, K., Berk, B.C., Tamaki, T., 2003. Stress and vascular responses: atheroprotective effect of laminar fluid shear stress in endothelial cells: possible role of mitogen-activated protein kinases. *J. Pharmacol. Sci.* 91, 172–176.
- Young, H.A., 2009. Cytokine multiplex analysis. *Methods Mol. Biol.* 511, 85–105.
- Zernecke, A., Liehn, E.A., Gao, J.-L., Kuziel, W.A., Murphy, P.M., Weber, C., 2006. Deficiency in CCR5 but not CCR1 protects against neointima formation in atherosclerosis-prone mice: involvement of IL-10. *Blood* 107, 4240–4243.
- Zernecke, A., Weber, C., 2010. Chemokines in the vascular inflammatory response of atherosclerosis. *Cardiovasc Res* 86, 192–201.
- Zhang, J.-X., Wang, Z.-M., Zuo, G.-F., Li, B., Zhang, J.-J., Tian, N.-L., Chen, S.-L., 2013. [Low shear stress induces human vascular endothelial cell apoptosis by activating Akt signal and increasing reactive oxygen species]. *Nan Fang Yi Ke Da Xue Xue Bao* 33, 313–317.
- Zhou, Z., Connell, M.C., MacEwan, D.J., 2007. TNFR1-induced NF- κ B, but not ERK, p38MAPK or JNK activation, mediates TNF-induced ICAM-1 and VCAM-1 expression on endothelial cells. *Cellular Signalling* 19, 1238–1248.
- Zhu, S.-N., Chen, M., Jongstra-Bilen, J., Cybulsky, M.I., 2009. GM-CSF regulates intimal cell proliferation in nascent atherosclerotic lesions. *J Exp Med* 206, 2141–2149.