An investigation of epigenetic effects in Atherosclerosis

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

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2016

Declaration

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Trinity College – December 2016

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Summary

Epigenetic mechanisms may explain some of the missing heritability associated with CVD and how external factors such as diet, environment and lifestyle contribute to disease development and progression. This thesis aimed to investigate the role of epigenetics in atherosclerosis- from initiation to plaque development and its subsequent outcomes.

Injury to the endothelium and subsequent inflammation represent a critical starting point for atherosclerosis. A primary objective of this study was to evaluate the use of epigenetic targeting agents SFN and SAHA (Vorinostat) on the expression of CXCL16, IL8, IL18 and miR210 and miR145, in TNFα stimulated endothelial (EA.hy926) and macrophage (THP-1) cell lines. The findings of this thesis suggest that the expression of CXCL16, IL8, IL18 and the indicated miRNAs can be altered by histone deacetylase inhibitors SFN and SAHA. However, the amount of HDACi (SFN and SAHA) and duration of treatment may have disparate effects and depend on the pre-existing inflammatory background in this case cytokine levels. This could limit potential use in the clinic.

As CXCL16 plays a multifaceted role in all stages of atherosclerosis, genetic variation which influences gene expression is of particular interest. An AEI investigation of the CXCL16 SNP rs2277680 was undertaken to determine allelic expression imbalance in the presence and absence of epigenetic modifying agents. These experiments found that AEI can be induced following treatment with various ETAs, proposing an epigenetic mechanism for AEI. The question remains as to whether all cells produce mRNA from both alleles, albeit at different levels, or one allele is silenced completely (monoallelic expression) and the observations are the result of heterogeneous mixtures of cells with either one or the other allele silenced. However, monoallelic expression is unlikely as AEI can be observed following 6 h treatment, a

time point which is too short to be attributed to cell turnover. This study also investigated the stability of AEI over multiple passages during cell culture. These results suggest that AEI is predominately stable, with considerable noise, over cell culture but becomes less predictable with increasing passage number. This study also raises the question as to the effectiveness and reproducibility of using cells which have been frozen in DMSO due to its potential for altering allelic expression imbalance.

Finally, the expression of miRNAs 145 and 210 has previously been shown to be reduced in patients with plaques which are prone to rupture. However, an association in stroke recurrence prediction from an initial cardiac event has not been examined. This is the first study to demonstrate an inverse relationship between miR145 and CD68 macrophages in stroke recurrence in a clinical data set. This finding confirms the role of miR145 in CVD, and as a biomarker of decreased stroke recurrence, a potential therapeutic target and predictive marker. Data generated in this study suggest that decreased miR145 expression is associated with a greater risk of stroke recurrence. Levels of miR145 were significantly increased in non-recurrent patients when compared against those who had stroke recurrence after initial stroke/TIA, but before carotid endarterectomy (P=0.0360). Furthermore, miR145 expression was also shown to be significantly associated with histological features of plaque inflammation and instability, such as increased cap and plaque macrophage count, evidence of intraplaque haemorrhage, luminal thrombus with lipid nanocapsules and the degree of plaque stability (P=0.0207, 0.0258, 0.0447, respectively).

The work detailed in this thesis describes different methodologies that can be used to help elucidate and alter the epigenetic mechanisms affecting gene expression in atherosclerosis.

The results unequivocally show an influence of histone acetylation and RNA interference in the development and stability of atherosclerotic plaque.

Acknowledgements

Firstly, I would like to thank my supervisors; Dr Ross Murphy and Dr Anthony Ryan for giving me the opportunity to pursue this research and for their continuous encouragement, support and expertise over the last 3 years.

I would also like to thank the Royal City of Dublin Hospital Trust and The Institute of Cardiovascular Science, St. James Hospital, who have supported this project from the beginning.

This project wouldn't have been possible without the collaboration of Prof Peter Kelly, Dr Michael Marnane and Gillian Horgan of the Dublin Carotid Atherosclerosis Stroke study who kindly helped us with sample collection. To Dr Steven Gray thank you, for your expertise and guidance throughout.

To Ben a special thank you for all your help over the last 3 years. To everyone else in the reading room past and present (Ciara, Emer and Aoife) thank you for the making the whole experience bearable, making me laugh every day and answering my millions of questions both work and non-work related. I couldn't have done it without you.

Outside of work, I would like to say a huge thank you to all my friends especially Niamh, Michelle, Niamh G and Amelia – thank you for your encouragement, support and for putting up with me in general.

To Gene, thanks for all the positivity (even though sometimes it wasn't received too well). Thanks for always believing in me even when I didn't believe in myself. If I can be half as supportive to you finishing up I'll be doing well.

Finally and most importantly, I would like thank my family - my parents Paula and Trevor, brother Nathan and grandmother Shelia –who have supported me from day one and have given me everything I could ever need or want. Everything I have ever achieved would not have been possible without your constant love and support. This thesis is dedicated 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.

Publications arising from this thesis

Byrne, M.M., Murphy, R.T., Ryan, A.W., 2014. Epigenetic modulation in the treatment of atherosclerotic disease. Front. Genet. 5. doi:10.3389/fgene.2014.00364

Gahan, J.M., Byrne, M.M., Hill, M., Quinn, E.M., Murphy, R.T., Anney, R.J.L., Ryan,A.W.,2015. Detecting Allelic Expression Imbalance at Candidate Genes Using 5' Exonuclease Genotyping Technology. Methods Mol. Biol. Clifton NJ 1326, 93–103. doi:10.1007/978-1-4939-28392_10

List of Abbreviations

ACS Acute Coronary Syndrome

AEI Allelic expression imbalance

AIS Acute Ischemic Stroke

Ala Alanine

AMI Acute myocardial Infarction

ApoE Apolipoprotein E

ASM Allele specific methylation

BCP 1-Bromo-3-chloropropane

CAD Coronary artery disease

cDNA Complementary DNA

CEA Carotid endarterectomy

CEPH Centre de'Etude du Polymorphism Humain

CEU Northern Europeans from Utah

CHD Coronary Heart Disease

CNS Central Nervous System

CRP C-reactive protein

CSO Central Statistics office

Ct Threshold amplification cycle

CTCL Cutaneous T-Cell Lymphoma

CURC Curcumin

CVD Cardiovascular disease

DAC 5-Aza-2'-deoxycytidine

DAMP Danger associated molecular pattern

DMEM Dulbecco's modified Eagle Media

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

DNMT DNA methyl-transferase

DNMTi DNA methyl-transferase inhibitor

EBV Epstein-Barr Virus

EC Endothelial cell

ECM Extracellular matrix

ETA Epigenetic Targeting agent

EtOH Ethanol

EPC Endothelial Progenitor Cell

eQTL Expression quantitative trait loci

EWAS Epigenome-Wide association studies

FBS Fetal Bovine Serum

FDA Food and Drug administration

FDG ^{18}F-Flurodeoxyhlucose

gDNA Genomic DNA

GSM Grey scale medium

GST Glutathione S transferase

GWAS Genome-wide association studies

H Histone

HapMap Haplotype Map

HDAC Histone deacetylase

HDACI Histone deacetylase inhibitor

HDL High density lipoprotein

HIF Hypoxia Inducible factor

HO-1 Heme Oxygenase-1

ICAM Intracellular cell adhesion molecule

IFN Interferon

IFNγ Interferon gamma

IHF Irish heart foundation

IKB Inhibitor of kappaB protein

IL Interleukin

Jap Japanese

LD Linkage disequilibrium

LDL Low-density lipoprotein

LncRNA Long non-coding RNA

LSS Low shear stress

LTR Long Terminal Repeat

MCP-1 Monocyte Chemoattractant protein-1

MDS Myelodysplastic syndrome

Meth Methanol

MI Myocardial Infarction

miRNA micro-RNA

MMP Matrix metalloproteinase

MnSOD Manganese superoxide dismutase

ncRNA Non-coding RNA

NF-κB Nuclear factor - κβ

NO Nitric Oxide

NQO-1 NAD(P)H dehydrogenease [quinone] 1

Nt Nucleotide

NTC Non template control

OPS Oxford Plaque Study

OxLDL Oxidised low density lipoprotein

PA Protocatechuic aldehyde

PAMP Pattern associated molecular pattern

PCI Percutaneous Coronary Intervention

PCR Polymerase chain reaction

PMA Phorbol 12-myristate 13-acetate

Pre-miRNA Precursor miRNA

Pri-miRNA Primary miRNA

PTM Post-translational Modification

RA Rheumatoid arthritis

RISC RNA induced silencing complex

RNA Ribonucleic Acid

RNAi RNA interference

RNA-Seq RNA Sequencing

ROS Reactive oxygen species

RPMI Roswell Park Memorial Institute

RT Room temperature

PTM Post translational modification

SAHA Suberoylanilide hydroxamic acid

SFN Sulforaphane

siRNA Small interfering RNA

SLE Systemic lupus erythematosus

SMC Smooth muscle cell

SNP Single nucleotide polymorphism

SRF Serum Response Factor

SS Shear stress

STEMI ST-Elevation Myocardial Infarction

T1D Type-1 Diabetes

TF Transcription factor

TIA Transient ischemic attack

TLR Toll-like Receptor

TNF-α Tumour necrosis factor

TNFRSF1A TNF receptor 1

TNFRSF1B TNF receptor 2

TSA Trichostatin A

TSG Tumour Suppressor Gene

UGT uridine 5' – diphospho-glucuronosyltransferase

UTR Untranslated region

Val Valine

VCAM Vascular cell adhesion molecule

VEGF Vascular endothelial growth factor

VSMC Vascular Smooth muscle cell

WHO World Health organisation

WTCCC Wellcome Trust Case Control Consortium

Table of Contents

Index of Tables

Index of Figures

Chapter 1: General Introduction

1.1 Cardiovascular Disease

Cardiovascular diseases (CVD) affect the heart, arteries and veins (Maton 1993). The term encompasses coronary heart disease, which commonly manifests as heart attack, cerebrovascular disease, which results in stroke and a number of other conditions including raised blood pressure (hypertension), peripheral artery disease, congenital heart disease and heart failure. Major causes of CVD include smoking, physical inactivity, unhealthy diet, alcohol consumption and familial factors.

Data from the World Health Organisation (WHO) 2015 have identified CVD as the primary cause of death globally, resulting in more deaths annually than any other disease. In 2012, 31% of all global deaths could be attributed to CVD, thus posing a huge burden on health systems worldwide which is likely to increase as the population ages. Current pharmacological treatments available include aspirin, beta blockers, angiotensin converting enzyme inhibitors and statins. Surgical options include bypass, angioplasty, valve repair and replacement and transplant. The estimated cost for the treatment of CVD can be as high as 200 billion euro each year (Voelter-Mahlknecht, 2016). According to the CSO (Central Statistics Office, Ireland), circulatory diseases accounted for 30.6% of deaths in Ireland, the greatest of any aliment, accounting for 8,899 deaths annually of these, 4,329 were due to ischemic heart disease and 1,837 stroke (Figure 1.1). It has been estimated that by 2030, nearly 23.6 million people will die from CVD worldwide (Mathers and Loncar, 2006).

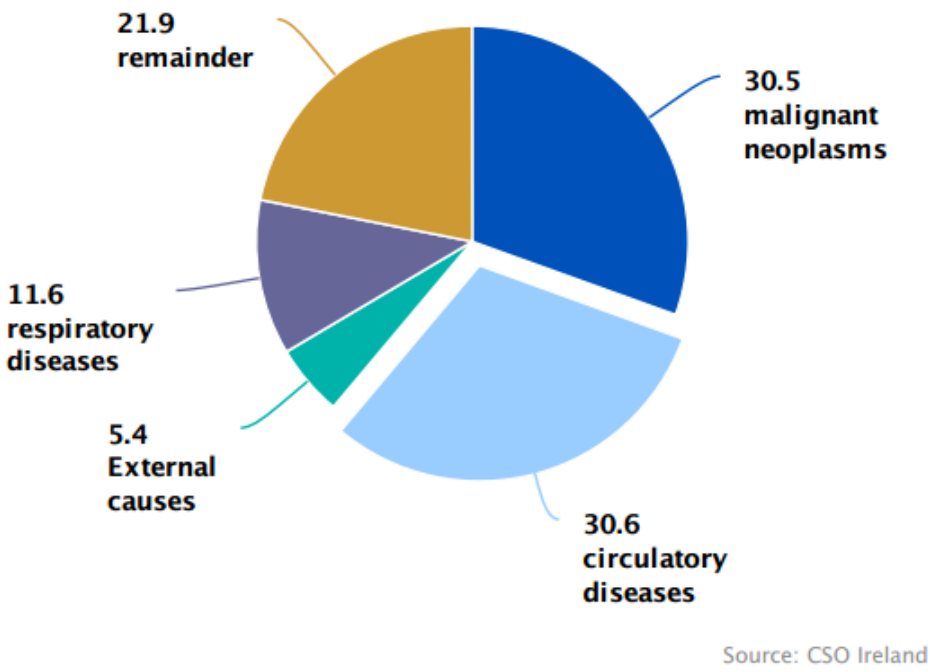

Figure 1.1: Principal causes of mortality in Ireland.

The principal causes of death in Ireland 2014 according to the CSO. Circulatory disease accounted for 30.6% of deaths in Ireland in 2014.

1.2 Atherosclerosis Disease Pathogenesis

Atherosclerosis gives rise to CAD through the formation of a slowly progressing lesion, leading to luminal narrowing of the arteries due to the development of a plaque. Following plaque rupture the most common forms of CVD manifest as acute coronary syndrome (ACS), myocardial infarction (MI) and stroke.

1.2.1 The Endothelium

"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).The vascular endothelium is responsible for coordinating and maintaining a multitude of activities essential for good vascular health, including preservation of vascular tone, inhibition of extracellular matrix (ECM) deposition, prevention of smooth muscle cell (SMC) proliferation, vasodilation and constriction of the arteries. It is regulated by various environmental cues and

mechanical forces such as shear stress, along with angiogenic and inflammatory stimuli (Abraham and Distler, 2007; Davignon and Ganz, 2004).

1.2.2 Endothelial Injury and Atherosclerosis Initiation.

In 1977, Ross *et al* put forward their response to injury hypothesis suggesting that injury to the endothelium is the first and initiating step in atherosclerosis development. Causes of such injury include but are not limited to hypercholesteremia, hypertension, smoking, diabetes, familial history of cardiac events and environmental factors resulting in endothelial activation (Ross et al., 1977). The activated endothelium induces the synthesis and secretion of various adhesion molecules, selectins, chemokines and cytokines such as TNFα, due to a build-up of reactive oxygen species (ROS) and nitric oxide (NO) as a result of oxidative stress (Deanfield et al., 2007; Goutzourelas et al., 2015; Kokura et al., 1999; Ross, 1993; Sima et al., 2009; Woywodt et al., 2002; Zou et al., 2006). This process is also accompanied by the loss of endothelial integrity and increased permeability, allowing excess lipoproteins and monocytes to infiltrate the *intima* media and inner layers of the endothelium.

Endothelial dysfunction has been recognised as an early marker for atherosclerosis, prior to ultrasonic evidence of plaque development (Lüscher and Barton, 1997). Statins are competitve inhibitors of HMG-CoA reductase, the first step in a pathway which cumulatives in cholesterol synthesis and are used mainly for their cholesterol lowering activity. Treatment with statins has been shown to improve endothelial dysfunction in patients with coronary risk factors and has reduced mortality rates by over one third (Cholesterol Treatment Trialists' (CTT) Collaborators et al., 2012) (Figure 1.2). Statins have not only been shown to reduce oxidative stress burden, but are capable of reducing the concentration of circulating markers of inflammation (Davignon and Ganz, 2004; Ridker et al., 2009).

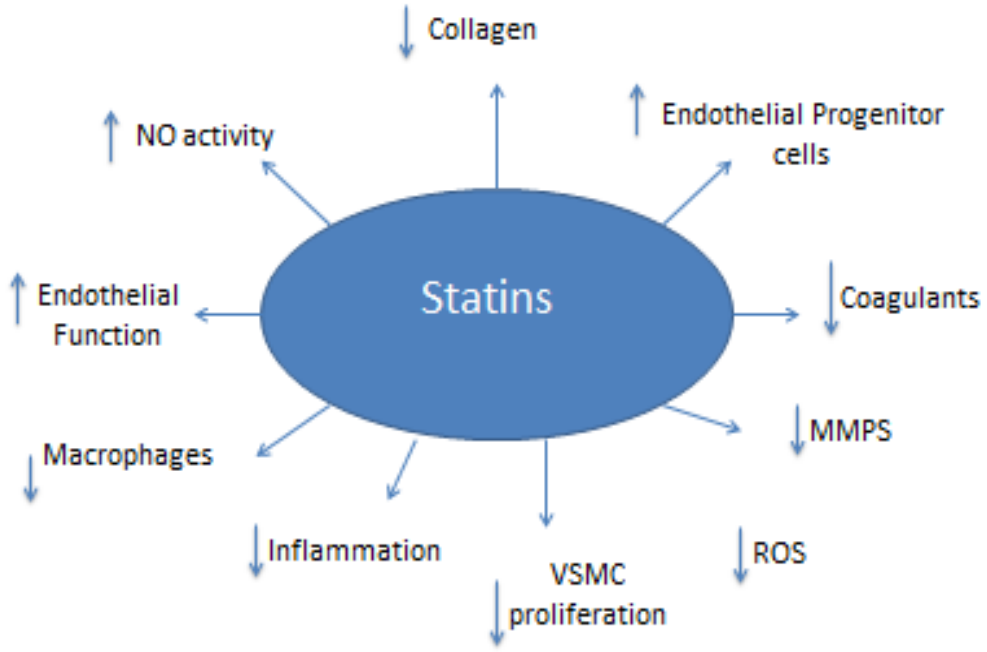

Figure1. 2: Pleotropic role of Statins.

Statins are the number one treatment for CVD worldwide and have been shown to have multiple beneficial pleiotropic effects. These effects can include but are not limited to maintenance of endothelial cell function, increased production of endothelial progenitor cells and Nitric Oxide (NO). Other beneficial effects include the decreased matrix metalloproteinase (MMP) expression, macrophage recruitment and reactive oxygen species production (ROS), ultimately acting to reduce inflammation. Modified from (Liao and Laufs, 2005).

1.3 Stages of Plaque Development

1.3.1 Plaque Initiation

As mentioned previously, atherosclerosis development begins primarily in response to injury of the endothelium with the formation of a fatty streak. The development of a fatty streak can be described as the first stage of plaque development, formed in response to the increased permeability of the endothelium and subsequent infiltration by lipids and monocytes (Hadi et al., 2005). Hypercholesteremia and hypertension are major risk factors as a result of increased LDL levels (Hansson et al., 2006). Upon entering the *intima* these lipids become modified via oxidation and release bioactive phospholipids which trigger endothelial activation. Activated

endothelial cells express several types of leukocyte adhesion molecules, which cause blood cells rolling on the vascular surface to adhere at the site of activation(Eriksson et al., 2001; Hansson et al., 2006). Such activation occurs at sites of low shear stress. Once adherent to the endothelium, leukocytes migrate into the underlying intima in response to chemoattractant stimuli including chemokines such as monocyte chemoattractant protein-1 (MCP-1) (Hansson et al., 2006).

1.3.2 Plaque Maturation

Mature plaques develop over time from the initial fatty streak and are known as atheromata. Atheromas contain a wider variety of cells types with a more complex structure consisting of a core containing foam cells and extracellular lipid droplets (Figure 1.3) (Jonasson, Holm et al. 1986).

The core region is surrounded by a cap which consists of SMCs and collagen rich material. Immune cells located at the edge of the cap actively express inflammatory cytokines such as IFN-γ and TNFα. These cytokines then activate vascular cell adhesion molecule (VCAM-1) in response to cholesterol accumulation in the *intima* (Cybulsky and Gimbrone 1991). VCAM-1 expression is largely responsible for the recruitment and retention of immune and inflammatory cells to the plaque, promoting disease progression and plaque enlargement (Hansson and Libby 2006). Plaque enlargement results in intra-plaque hypoxia, inflammation and local neovascularisation, which can be used to identify high risk plaques (Kwon et al., 1998). Death of lipid-laden foam cells leads to the formation of a necrotic core, consisting of other cellular debris and lipids such as esterified cholesterol, cholesterol crystals, dead white blood cells, which is then covered by a monolayer of endothelial cells which form the fibrous

cap. Dead foam cells account for >40% of the necrotic core with a lipid core of >40% identifying a highly vulnerable plaque (Shah, 2014; Virmani et al., 2006).

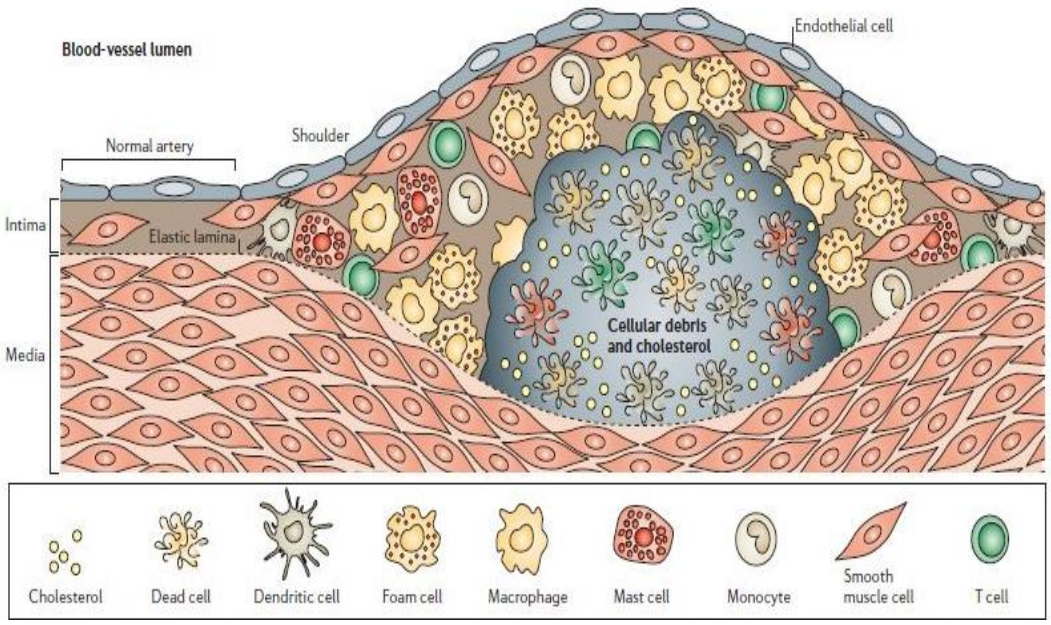

Figure 1.3: Cellular composition of a mature atherosclerotic plaque.

The plaque core consists of lipids and cellular debris from dead cells. Surrounding the core is cap composed of smooth muscle cells and collagen fibres which act to stabilise the plaque. Immune cells located within the plaque are often found in an activated state, which aids inflammation (Hansson and Libby 2006).

1.3.3 Plaque Disruption and Shear Stress

Sites of atherosclerosis and plaque development are located at regions where the arteries branch/curve, as blood flow is non-linear and exerts low shear stress (LSS) (Warboys et al., 20110. LSS upregulates inflammatory signalling in endothelial cells and modulates miRNAs expression to promote inflammation and monocyte recruitment (Loyer et al., 2013; Mondadori dos Santos et al., 2015; Zhou et al., 2014). LSS stimulates the production of ROS by endothelial cells which can oxidise LDL (Napoli et al., 2006). Oxidised Low-density lipoprotein (OxLDL) contributes to inflammation and in turn increases the expression of adhesion molecules. LSS also stimulates arterial remodelling, indicative of a highly vulnerable plaque.

Plaque vulnerability can be judged through a combination of fibrous cap thickness, necrotic core thickness and the degree of arterial remodelling (Poller, Tank et al. 2013).

Overproduction of matrix metalloproteinases (MMPs) is common in this inflammatory environment and is encouraged through proinflammatory cytokine expression. Due to their activity as collagenases, MMPs play an important role in the degradation of the fibrous cap, which ultimately results in plaque rupture and the severe clinical outcomes mentioned previously. MMP1, 8 and 13 have been shown to be over expressed in human atheroma and co-localise with plaque macrophages (Figure 1.4) (Ait-Oufella et al., 2011) .

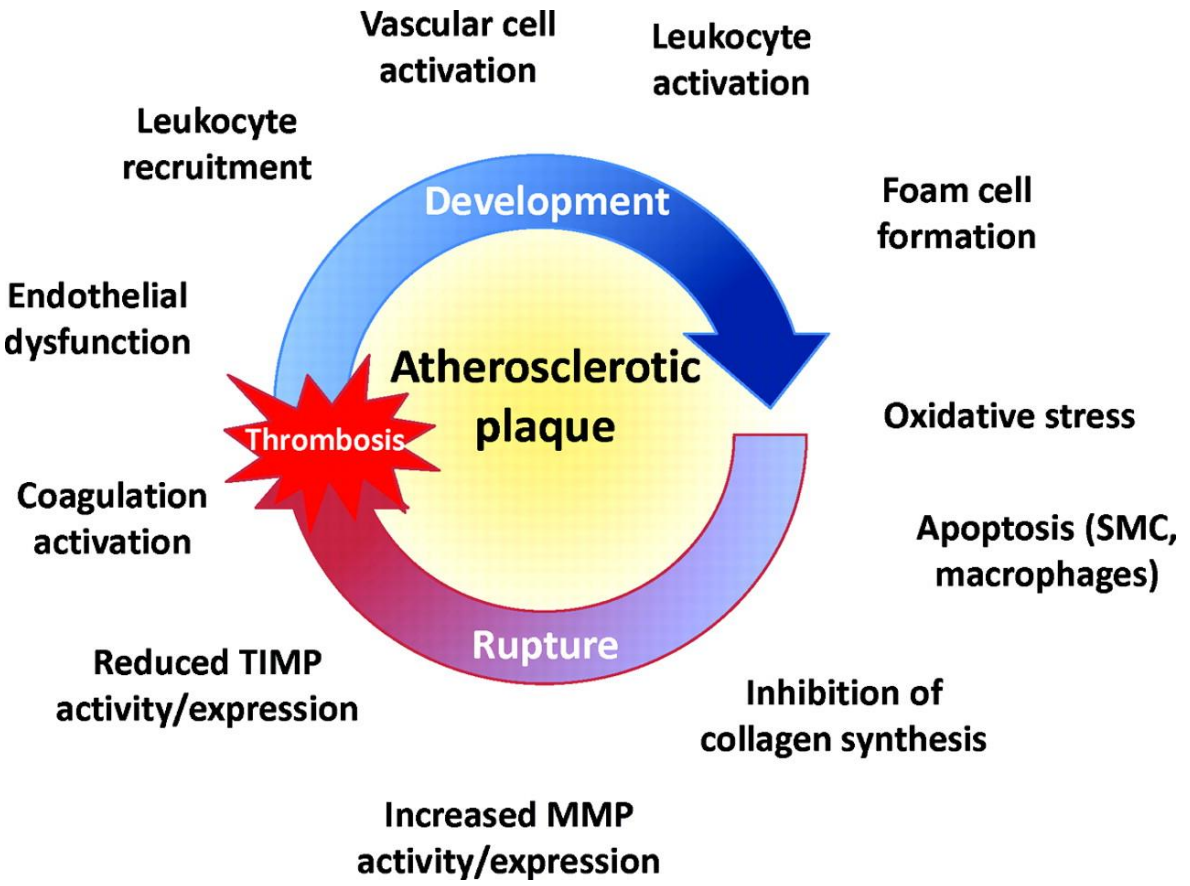

Figure 1.4: Stages and factors leading to plaque development and rupture.

Plaque formation initiates as a result of endothelial dysfunction arising from injury to the endothelium. This is followed by leukocyte recruitment and activation, macrophage uptake of oxLDL leading to foam cell formation,

oxidative stress and hypoxia leading to apoptosis and the formation of necrotic core. Increased MMP activity and shear stress degrade the fibrous cap of the plaque resulting in rupture (Ait-Oufella et al., 2011)

1.4 Inflammation and Atherosclerosis

Plaque development has been described as a disturbed balance of lipid accumulation, giving rise to chronic inflammation of the arterial wall (Moore and Tabas 2011, Weber and Noels 2011).

The presence of inflammatory mediators can be noted in all phases of atherogenesis from the initial fatty streak to maturation and finally the clinical manifestations commonly observed (Ross, 1999). The role which inflammation plays in plaque development was first noted in the mid-19th century by Virchow and in 1977 Ross *et al* put forward their response to injury hypothesis, identifying inflammation as a key driving force in atherosclerosis development and complication (Ross et al., 1977).

The role inflammation plays in atherosclerosis can be seen in the variety of immune cells found in plaques, such as lymphocytes, monocytes and macrophages. These immune cells act as conductors of a highly coordinated inflammatory response orchestrated by various cytokines and chemokines. This response can be pro- or anti-inflammatory, with many markers being used to detect early thrombotic atherogenic events such as CRP, IL18 and IL6 (Lamon and Hajjar, 2008). The accumulation of oxLDL cholesterol in the endothelium has been linked to activation of the inflammasome and the subsequent activation of a pro-inflammatory cascade. Modified lipids such as oxLDL are recognised as pathogen associated molecular patterns (PAMPS) which activate proinflammatory cytokines TNFα, IFN-γ, IL18, IL-6, IL-1b, MMPs, T-cells and leukocytes, with activated leukocytes acting in both the initiation and maintenance of inflammation throughout atherosclerosis (Ammirati et al., 2015; Hansson et al., 2015).

It is therefore evident that the immune response plays a crucial role in every phase of atherogenesis, through the accumulation of immune and inflammatory cells and subsequent activation and recruitment of proinflammatory chemokines and cytokines (Hansson 2006).

1.5 Biomarkers and Triggers of Atherosclerosis

The term “Biomarker” has been defined by the WHO in coordination with the United Nations and the International Labour Organization, as “Any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease” (Strimbu and Tavel, 2010). In compliance with this a biomarker can be any protein, DNA or mRNA which can be measured to assess biological and pathological processes in addition to pharmacological responses. This allows biomarkers to be used as both predictive and prognostic markers in disease treatment, and identifies them as critical components in complex diseases.

Atherosclerosis is a highly complex inflammatory disease involving the recruitment, activation and subsequent secretion of a variety of cytokines (Figure 1.6) and chemokines some of which are already in use in the clinic as tools for risk assessment in patients bearing atherosclerotic lesion. Notable examples include fibrinogen, serum amyloid A (SAA), IL6 and lipoprotein associated phospholipase a predictor of ischemic stroke (Ammirati et al., 2015; Nambi et al., 2009; Oei et al., 2005). The most widely used marker in the clinic remains C-reactive protein (CRP) which has been shown to reproducibly predict the risk of stroke in several epidemiologic studies (Ridker et al., 2000, 1997; Rost et al., 2001). However, conflicting data exist questioning the association, or strength thereof, CRP may have. It has been noted that although high serum (hs-CRP) predicts the presence of a plaque in the carotid artery, it does

not associate with the degree of stenosis (Puz et al., 2013). Moreover, Halvorsen and colleagues could not establish an association between CRP and the presence of carotid plaque in a cross-sectional study involving 5341 individuals (Chapman et al., 2004; Halvorsen et al., 2009). These conflicting data highlight the need for improved markers in both diagnosing and predicting cardiovascular events. In the instance of AMI, it is estimated that 15-20% of the patients have no traditional risk factors and would be classified as low risk using current prediction models, again highlighting the need for more specific informative cytokines and chemokines (Bye et al., 2016; Khot et al., 2003). These inflammatory mediators can however have varied and multiple effects on cellular homeostasis depending on cell type, timing and context and also possess the ability to act synergistically with parings including IL12 and IL18, TNFα and IFN-γ, MCP-1 and IL8 (Tedgui and Mallat, 2006). Therefore it is necessary to categorise markers and their association with the different stages of disease progression. MiRNAs have also been identified as promising disease biomarkers due to their association with a variety of diseases and because stable miRNA can be detected easily in circulation (Cortez and Calin, 2009).

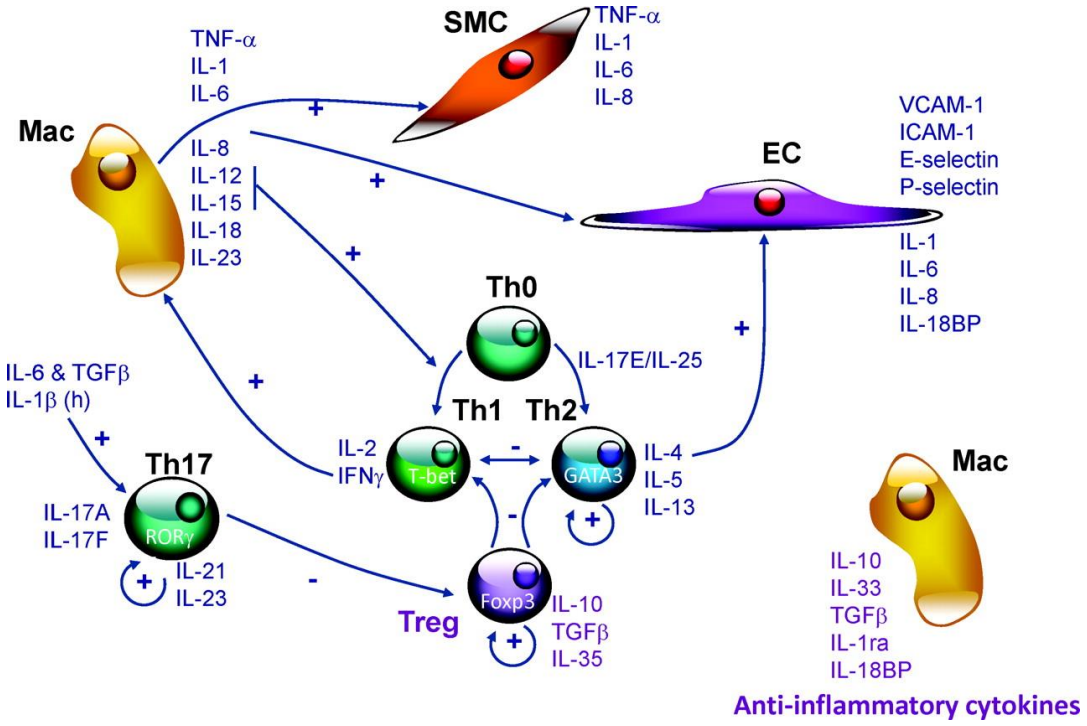

Figure1.5: Cytokines produced by inflammatory and vascular cells in Atherosclerosis.

This Image outlines the complex interconnected relationship of inflammatory cytokines, with known roles in atherosclerosis development. Macrophages are known to play a crucial role in CVD; this activity is achieved through various different cytokine produced. Macrophage derived cytokines activate SMC and ECs to produce additional proinflammatory mediators e.g. IL6, IL8 and TNFα. Furthermore, IL12 and IL18 known inducers of IFN-γ, act to induce the differentiation of naïve T-Cells into pro-atherogenic Th1 cells. Macrophages also produce anti-inflammatory cytokines such as IL-10 and TGFβ which promote anti-atherogenic Treg cell differentiation (Ait-Oufella et al., 2011).

1.6 Cytokines and Chemokines associated with CVD

1.6.1 TNFα

TNFα can be described as a pleotropic proinflammatory cytokine, produced by activated monocytes and T-lymphocytes. TNFα is known to play a major role in CVD progression through the induction of vascular inflammation, monocyte adhesion and vascular oxidative stress (Nakai et al., 1999).TNFα is secreted by Th1 effector cells, macrophages and NK cells (Gerthoffer, 2007; Hansson et al., 2006). Cells stimulated with TNFα have been shown to secrete a variety of inflammatory cytokines, with TNFα itself activating its own positive

feedback loop along with the upregulation of NF-κB (Turner et al., 2010). TNFα acts via 2 types of receptor, namely TNF receptor 1 (TNFRSF1A) and TNF receptor 2 (TNFRSF1B), which can exist in both soluble and membrane-bound forms, which likely explains how low doses of TNFα such as 8 pg/ml are enough to induce NF-κB activation (Khabar et al., 1995; Turner et al., 2010). TNFα activates NF-κB via the ubiquitination and degradation of its inhibitor, inhibitor of kappaB proteins (IKB) (Turner et al., 2010; Zandi et al., 1998). NF-κB has been described as a master regulator of inflammation, due to its proinflammatory and cytotoxic activity.

Epidemiological studies reveal that TNFα is upregulated in the plasma and arteries of patients with vascular complications (Ridker et al., 2000). Based on this and other observations, it is likely that TNFα is heavily involved in the pathogenesis of atherosclerosis, through the activation of pathways which upregulate pro-inflammatory cytokines, such as the activation of NF-κB in ECs and thus it could provide a novel target in preventing vascular endothelial dysfunction (Ait-Oufella et al., 2011; Bruunsgaard et al., 2000; Kleinbongard et al., 2010; McKellar et al., 2009). TNFα is inviolved in the regulation of thrombosis and coagulation through NF-κB , Sp1, JNK, P38 and STAT3 pathways (Gerthoffer, 2007). It is constitutively expressed by adipose tissue and therefore likely to be associated with the increased plasma levels of TNFα commonly observed in obese and insulin-resistant patients, many of which also suffer from cardiovascular complications (Dandona et al., 1998; Hotamisligil et al., 1993; Nakai et al., 1999). Furthermore serum levels of TNFα and L-Selectin were shown to be associated with larger plaque size as estimated by ultrasound imaging in a cohort of 1016 subjects (Andersson et al., 2009).

1.6.2 IL18

Intelerleukin-18 (IL18) is an 18kDa molecule, first described as an IFN-γ inducing factor in endotoxin-challenged mice (Puren et al., 1999). IL18 is located on chromosome 11 at 11q22.2-q22.3, close to the linkage region for systemic lupus erythematosus (SLE) in European populations, elevation of which is associated with genetic polymorphism and disease activity in lupus. The receptor for IL18, IL18R is a heterodimer consisting of an alpha chain for ligand binding and a beta chain responsible for signalling. Absence of this receptor leads to reduced NK-cell IFN-γ production and impaired Th1 cell signalling and differentiation (Hoshino et al., 1999; Torigoe et al., 1997).

IL18 functions in the regulation of the Th1 cell response, through the induction of IFN-γ. IFN-γ is a Th1 cytokine produced by T and NK cells following synergistic activation from pro-atherogenic IL-12 and IL18 (Tedgui and Mallat, 2006). In fact it has been suggested that the pro-atherogenic effect of IL18 is mediated by IFN-γ, as the promotion of IL18-mediated atherosclerosis was ablated in IFN-γ deficient $ApoE^{-/-}$ mice (Whitman et al., 2002).

IL18 has been described as a major independent inflammatory predictor of 30-day major adverse cardiac events and unfavourable outcomes after acute myocardial infarct (AMI) (Youssef et al., 2007). IL18 has also been suggested as a predictor of cardiocerebal vascular events in dialysis patients (Chang et al., 2015) and 60 day adverse clinical outcome in patients with ST-segment elevation acute myocardial infarction (STEMI) undergoing Percutaneous coronary intervention (PCI) (Gao et al., 2010). Furthermore, studies have shown that IL18 mediates atherosclerosis and CVD death in patients exhibiting CAD (Mallat et al., 2001; Whitman et al., 2002). In addition, atherosclerotic lesions express high levels of IL18 and IL18R

in macrophages, T-cells and SMCs, known players in the process of lesion development. This expression pattern was not observed in normal aorta (Gerdes et al., 2002; Mallat et al., 2001).

1.6.3 IL8

IL8/ CXCL8 is a chemokine known to participate in all stages of atherosclerosis from vascular inflammation to cardiac remodelling (Apostolakis et al., 2009). The biological effects of IL8 are mediated through 2 receptors ; CXCR1 and CXCR2 (Williams et al., 2000).

IL8 plays a crucial role in the shift from acute to chronic inflammation by mediating the release of MCP-1 and the interleukin 6 receptor (IL-6R/CD126) from neutrophils located within the inflammatory infiltrate (Velásquez et al., 2014).The association of IL8 serum and gene expression levels with the risk of coronary heart disease (CHD) has been investigated in a few small studies with contradictory findings (Romuk et al., 2002; Velásquez et al., 2014; Zhou et al., 2001). The EPIC-Norfolk prospective population study reported that baseline IL8 levels where higher in cases than controls, in addition to reporting that future risk of CAD increased with increasing IL8 in serum (Boekholdt et al., 2004).

In contradiction to its proinflammatory role, it has been suggested that in the long term IL8 expression exerts a protective effect on cardiac remodelling, via the mobilization and homing of circulating endothelial progenitor cells (EPCs) from the bone marrow to the injured myocardium, which could result in myocardial tissue repair (Apostolakis et al., 2009; Velásquez et al., 2014)

1.6.4 CXCL16

CXCL16 can be described as a triple threat, combining three functions into one molecule, acting as a chemokine, cytokine, scavenger receptor and adhesion molecule, all of which play key roles in the development of atherosclerosis.

CXCL16 and its receptor CXCR6 were identified in 2000 by three groups (Matloubian et al., 2000; Shimaoka et al., 2000; Wilbanks et al., 2001). The CXCL16/SR-PSOx gene is located on chromosome 17p13 and its protein product exists in both membrane and soluble forms. Membrane-bound CXCL16 is a 254-amino acid, 30 kDa type 1 transmembrane glycoprotein, consisting of CXC chemokine, mucin stalk, transmembrane and cytoplasmic domains. It uniquely does not share any structural homology with any other scavenger receptors in its class. It is expressed on dendritic cells and functions largely as an adhesion molecule for CXCR6 bearing T-cells (Shimaoka et al., 2004). The extracellular domain of CXCL16 is cleaved by MMP ADAM 10 to generate soluble CXCL16 (Abel et al., 2004; Sheikine and Hansson, 2006). CXLC16 is a known T-cell activator, its expression is increased by pro-inflammatory stimuli which enhance ox-LDL uptake and foam cell formation, suggesting it may play a role in early lesion development and maturation (Lehrke et al., 2007; Yamauchi et al., 2004).

CXCL16 acts as a chemoattractant for a variety of immune cells and is highly expressed in monocyte-derived macrophages, dendritic cells, B-cells and SMCs (Sheikine and Sirsjö, 2008). T-cells and endothelial cells also have CXCL16 expression detectable at mRNA level (Sheikine and Sirsjö, 2008). Studies have revealed that the binding of CXCL16 to its receptor CXCR6 facilitates the migration of immune cells to lymphoid organs and sites of inflammation such as atherosclerotic lesions, therefore identifying CXCL16 as a key target in atherosclerosis and lesion development (Sheikine and Sirsjö, 2008). The soluble form of CXCL16 also plays a role

in stimulating SMC proliferation and cell to cell adhesion in cell culture models (Chandrasekar et al., 2004).

CXCL16 was demonstrated to be involved in atherosclerotic lesion development by Minami *et al* in 2001. They noted the expression of CXCL16 mRNA and protein in coronary and carotid plaque lesions, but not in the intact aortic wall (Minami et al., 2001; Sheikine and Sirsjö, 2008). Further investigations revealed abundant expression of CXCL16 mRNA in human atherosclerotic lesions, while unaffected vessels exhibited low levels of the transcript, staining of carotids also revealed CXCL16 and CXCR6 protein in macrophage and T-cell rich regions of the plaque (Wågsäter et al., 2004; Wuttge et al., 2004). These results identify CXCL16 as an important component of atherosclerotic lesions and suggest its participation in the conversion of macrophages to foam cells and the migration of T-cells to the lesion (Sheikine and Sirsjö, 2008).

The cytokines above are just some of principal cytokines involved in atherosclerosis; other important cytokines involved in the process include IL2, IL6, IL7, IL10, sCD40L and M-CSF. These cytokines were not included in this study due to various limiting factors and therefore, it was necessary to perform our investigation on the indicated subset of cytokines based on the literature and their contribution to disease development.

It is clear nevertheless that cytokines play a critical role in inflammation and in all stages of atherosclerosis, suggesting that future therapies and patient profiling should target these cytokines to better treat atherosclerosis (Figure 1.7).

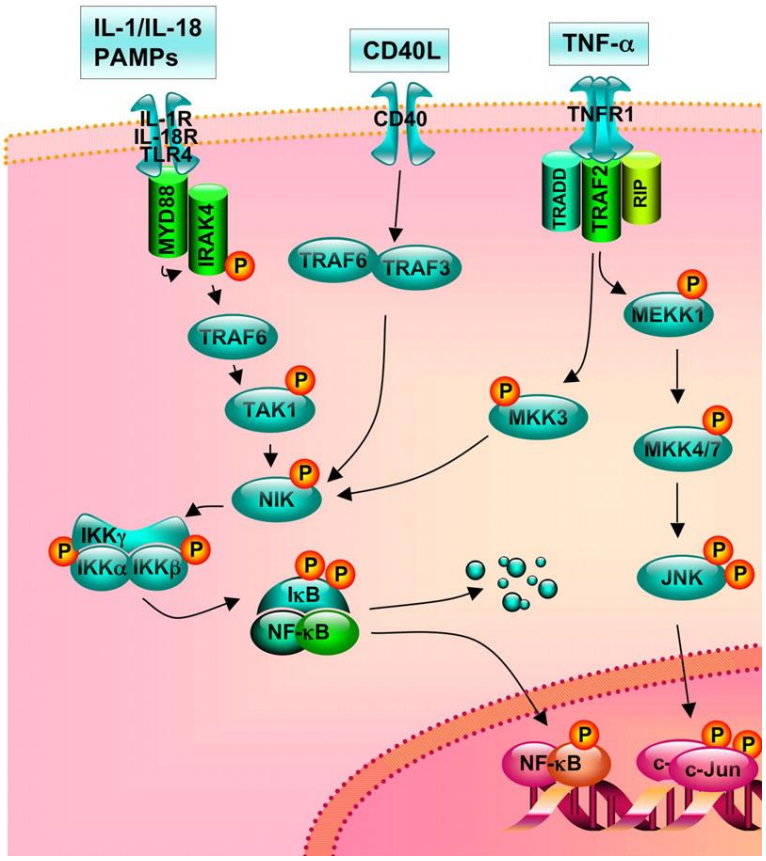

Figure 1.6: Inflammatory signalling pathways involved in atherogenesis.

Many of these signalling pathways are activated in response to cytokine expression e.g. IL18 and TNFα, and act to further drive the inflammatory response, making them attractive therapeutic targets. For instance both IL18 and TNFα activate NF-κB , known to be a master regulator of inflammation. TNFα also acts in the activation of c-Jun, known to play a role in cellular proliferation and differentiation and co-operate with NF-κB to protect from apoptosis (Tedgui and Mallat, 2006).

1.7 MiRNAs

1.7.1 Discovery

MiRNAs were first discovered in 1993 by Ambros *et al*, with further insights being provided by Wightman and colleagues in relation to their work on *lin-4* in *C.elegans* (Lee et al., 1993; Wightman et al., 1993). It was noted that lin-4 was able to control the timing of *C.elegans* larval development through the repression of lin-14. What was unique about this was that lin-4 produced a short ncRNA, approximately 22 nucleotides (nts) in length, which had complementarity to the 3' UTR of lin-14 and acted to inhibit its translation (Wightman et al.,

1993). This was followed by the identification of a second miRNA let-7. Let-7 was shown to be responsible for the regulation of genes with critical roles in developmental such as; lin-28, lin-41, lin-42 and daf, regulation of which was once more achieved via complementarity with the 3'UTR of the genes it regulated (Reinhart et al., 2000). These Let-7 ncRNAs where not only found in *C.elegans* but in various other animal taxa, including vertebrates, ascidians, hemichordates, molluscs, annelids and arthropods (Pasquinelli et al., 2000). This strong conservation across species has helped identify miRNAs as components of a highly conserved ancient regulatory system, responsible for key developmental processes (Grosshans and Slack, 2002; Kaikkonen et al., 2011). An estimated 5000 miRNA sequences have been identified to date in the human genome alone, with the identification of many more likely as a result of duplication and maturation of sequences (Londin et al., 2015; O'Driscoll, 2006).

1.7.2 miRNA Biogenesis

Mature miRNAs are approximately 18-25nts in length. However, primary miRNAs (pri-miRNA) are much longer, requiring processing to reach maturation. Pri-miRNAs are generated in the nucleus following transcription by RNA polymerase II and III, followed by generation of precursor miRNA by the micro-processor complex (Figure 1.8) (Borchert et al., 2006). Drosha, a double stranded RNA specific type II endonuclease, is an essential component of this microprocessor complex and necessary for the generation of a 60-70nt stem loop precursor miRNA (pre-miRNA) (Lee et al., 2003, 2002). Following cleavage by Drosha the pre-miRNA is then transported to the cytoplasm by exportin 5, a nuclear transport protein. Once in the cytoplasm the stem loop is further modified by a component of the RNA silencing complex (RISC) known as Dicer endonuclease, which acts to cleave to pre-miRNA into mature miRNA or siRNA, which are then incorporated into the RISC to induce either translational repression

or mRNA cleavage (Figure 1.8) Other key components of RISC include TRBP, a dsRNA binding protein and Argonaut protein (Ago2) an endonuclease which cleaves mRNA (Figure 1.8) (Lee, Nakahara et al. 2004).

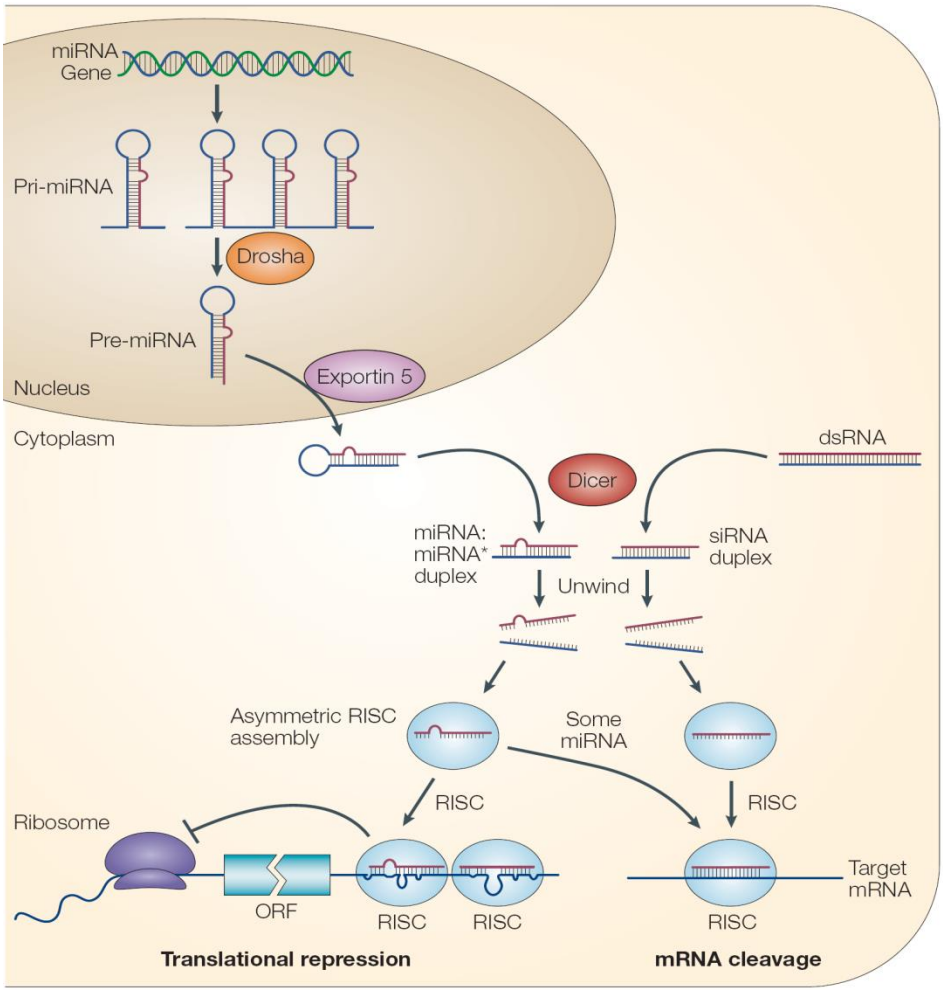

Figure 1.7: miRNA Biogenesis.

miRNA are transcribed in the nucleus by RNA polymerase II and III, to form a stem loop precursor known as primary miRNA (pri-miRNA). The double stranded hairpin is then cleaved by a double stranded RNA specific type II endonuclease known as Drosha and becomes known as precursor miRNA (pre-miRNA). Following this it is exported from the nucleus to the cytoplasm by Exportin 5, a nuclear transport protein. In the cytoplasm, an endonuclease known as Dicer cleaves and unwinds the pre-miRNA duplex resulting in the formation of miRNA and siRNA. These are then unwound and incorporated into the RNA silencing complex (RISC) to induce either translational repression or mRNA cleavage (He and Hannon, 2004).

MiRNAs act in the repression of translation at the initiation step, and less frequently inhibit translation through the degradation and deadenylation of mRNAs (Asirvatham et al., 2008; Hill et al., 2011; Krol et al., 2010). The miRNA-gene interaction facilitates the recruitment of chromatin modifying proteins to nuclear sites resulting in heterochromatic silencing (Grewal and Moazed, 2003). Regulation of gene expression via miRNAs has been shown to participate in embryonic development and are subject to environmental and nutritional factors (Asirvatham et al., 2008b; Bhattacharyya et al., 2006; Chiou, 2007; van Rooij et al., 2007).

1.7.3 MiRNAs as Disease Biomarkers

MiRNAs are promising disease biomarkers and therapeutic targets as they are believed to regulate more than 60% of human genes and are known to be implicated in a wide range of biological activities including; proliferation, differentiation and apoptosis, making them key players in disease diagnosis, prediction and treatment (Figure 1.1) (Corcoran et al., 2011; Li and Kowdley, 2012; Ng et al., 2012; Png et al., 2012; Rayner et al., 2011; Taganov et al., 2006; Zhang et al., 2012).

One of the most widely investigated and understood associations of miRNAs and disease is observed in various forms of cancer. MiRNAs are known to have expression patterns specific to cancer type and can provide information as to developmental lineage, stage wise progression and outcome (Iorio et al., 2005; Lu et al., 2005; Volinia et al., 2012). One of the first examples of an association between miRNAs and cancer was noted by the deletion of miR15 and miR16 in 65% of B-cell chronic lymphocytic leukaemia (CLL) patients (Calin et al., 2002). MiRNAs have also been shown to participate in multiple regulatory pathways in addition to common inflammatory pathways such as that of NF-κB (Jopling et al., 2005;

Junker et al., 2009; Karolina et al., 2011; Lanford et al., 2010; Li and Kowdley, 2012; Pogribny et al., 2010)

Disease		**miRNA**
Cancer	B-CLL	miR15, miR16
	Breast Cancer	miR125b,miR145, miR21, miR155, miR210
	Lung Cancer	miR155, let7a
	Gastric Cancer	miR145
	Liver Cancer	miR29b
Viral	HCV	miR122, miR155
	HIV-1	miR28, miR125b, miR150, miR223, miR382
	Influenza Virus	miR21, miR223
Immune-Related	Multiple Sclerosis	miR145, miR34a, miR155, miR326
	Systemic Lupus Erythematosus	miR146a
	Type II Diabetes	miR144, miR146a,1 miR50, miR182, miR103, miR107
	Non-alcoholic liver disease	miR200a, miR200b, miR429, miR122, miR451, miR27
	Non-alcoholic steatohepatitis	miR29c, miR34a, miR155, miR200b
Neurodegenerative	Parkinson's disease	miR 30b, miR 30c, miR 133b, miR 184,let7
	Alzheimer's disease	miR 29b-1, miR 29a,9

Table 1.1: miRNAs associated with common diseases. Table outlining the different miRNAs associated with disease. Many miRNAs are associated with multiple diseases, suggesting them as essential components of regulatory pathways, targeting of which is likely to result in multiple off target effects. Modified from (Li and Kowdley, 2012).

1.7.4 miRNAs and Cardiovascular Disease

Studies are currently investigating a potential link between miRNA expression profiles, plaque development, and rupture. miRNA expression profiles are likely to differ between

atherosclerotic plaques and healthy arteries, and may provide useful markers and targets in atherosclerosis (Figure 1.9) (Cipollone et al., 2011; Hao et al., 2014; Menghini et al., 2014; Raitoharju et al., 2011; Wierda et al., 2010). Many of the genes downregulated due to miRNAs include those involved in the regulation of signal transduction, transcription, and vesicular transport. Those which were upregulated are thought to be involved in the key processes of atherosclerosis (Raitoharju et al., 2011; Wierda et al., 2010).

Cipollone *et al*. (2011) investigated whether a unique miRNA signature was associated with plaque instability in humans. They identified five miRNAs, miR100, miR127, miR145, miR133a, and miR133b which had altered expression levels. Expression of these miRNAs was greater in symptomatic plaques from patients with ischemic stroke, suggesting a role for miRNAs in plaque instability and rupture, but also as predictive biomarkers. They confirmed their findings through endothelial cell transfection with miR145 and miR133a to identify their role in the modulation of stroke-related proteins. They noted that > 71% of patients affected by stroke had miRNA expression levels higher than asymptomatic patients and that many of the affected miRNA regulated genes are involved in modulation of inflammation. Raitoharju and colleagues in a similar study identified miR21, miR210, miR34a and miR146a/b as upregulated in human atherosclerotic plaques (Raitoharju, Lyytikainen et al. 2011). Further findings implicate miRNAs such as miR24 and miR21 in the regulation of MMPs, known to play a role in fibrous cap thinning and plaque rupture (Di Gregoli et al., 2014; Fan et al., 2014). miRNAs can regulate macrophage phenotype alterations from pro-inflammatory to anti-inflammatory, and can be altered by shear stress (Alexy et al., 2014; Boettger et al., 2009; De Paoli et al., 2014). It is likely that such endothelial miRNAs are selectively regulated by arterial flow conditions (shear

stress), particularly the combination of pro-atherogenic LSS and oxidized low-density lipoprotein (oxLDL), which induce the upregulation of miR92a (Loyer et al., 2014).

Circulating miRNAs have also attracted considerable attention as biomarkers for the risk stratification of patients (Deiuliis et al., 2014). Fichtlscherer *et al* identified four miRNAs which were reduced in patients with CAD compared to healthy controls, miR126, miR17, miR92a, and miR155. Muscle enriched miRNAs were also found to be more highly expressed in patients with CAD compared to volunteers (Fichtlscherer et al., 2010; Stellos and Dimmeler, 2014).

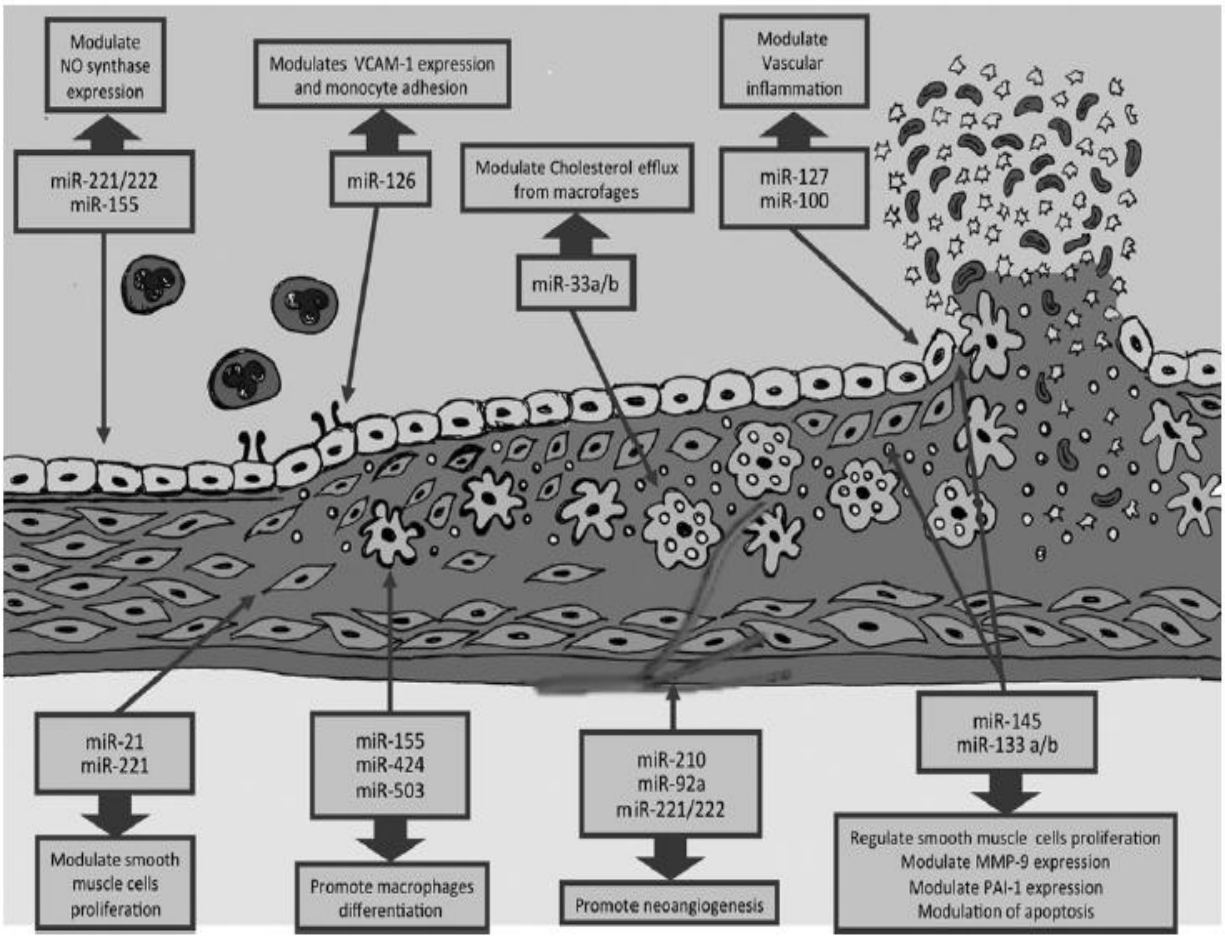

Figure 1.8: Main roles for miRNAs in atherosclerotic plaque development, progression and disruption.
miRNAs play a role in every stage of atherosclerosis from initiation (miR221,miR155, miR21,miR424,miR503,miR12) and maturation (miR33a/b,miR210,miR221,miR222,miR127,miR100) to rupture (miR145,miR133a/b). Identifying miRNAs to be just as important as cytokines in the identification, prevention and treatment of atherosclerosis related cardiac events (Santovito et al., 2012).

1.7.5 MiRNAs in the Clinic

The use of miRNAs in diagnostics and treatment is promising with numerous opportunities available, including; organ targeted RNAi using viral vectors or synthetic RNA, and therapeutic strategies based on the modulation of miRNA function (Poller et al., 2013).

One of the first successful applications of RNAi therapeutics in a clinical setting is that of RNAi drug ALN-PCS on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) (Abifadel et al., 2003; Zhao et al., 2006; Fitzgerald et al., 2014). Treatment resulted in a mean 70% reduction in circulating PCSK9 plasma protein and a mean 40% reduction in LDL cholesterol from baseline relative to placebo (Fitzgerald et al., 2014). In addition to this SPC3649a LNA-modified oligonucleotide has also potential in the treatment of hepatitis C infection via the targeting of miR122, which is necessary for viral replication along with fatty acid and cholesterol metabolism (Li and Kowdley, 2012). This anti-Mir was delivered intravenously and resulted in depletion of mature miR122 and a dose dependent lowering of cholesterol. Furthermore it demonstrated prolonged suppression, with apparently no viral resistance or side effects (Elmén et al., 2008; Lanford et al., 2010).

At present there are ~163 active clinical trials investigating the therapeutic potential of miRNA-mediated approaches, with approximately 94 assessing the role of miRNAs as disease biomarkers and a further 62 investigating the association of miRNAs with CVD (clinicaltrials.gov). Challenges remain with issues such as specificity. The 3' UTR of a single mRNA can be targeted by multiple miRNAs. miRNAs also exert many different actions dependent on cell type; hence miRNA modulation therapies require precise cellular targeting and suitable delivery methods. For example, miR144-3p has been shown to accelerate plaque

formation through the post-transcriptional regulation of ABCA1. ABCA1 has a critical role in cellular cholesterol efflux and the formation of HDL. Inhibition of ABCA1 through miR144-3p has been shown in THP-1 cells and in ApoE$^{-/-}$ mice to increase inflammatory cytokine secretion and accelerate plaque formation (Hu et al., 2014a). This highlights how essential it is for appropriate miRNA targeting in atherosclerosis as one miRNA can influence multiple pathways, a concept which is applicable to all other common diseases.

1.8 Epigenetics

Epigenetic processes are defined as heritable modifications in gene expression effected by chromatin-based modifications that do not alter the genetic sequence (Schuettengruber, Martinez et al. 2011). Epigenetic mechanisms respond to environmental changes and may contribute to the proportion of unexplained inheritance. For instance, individual variation in epigenetic modification of genes can explain a larger part of phenotypic variation in humans than differences in genotype alone (Turan, Katari et al. 2010).

Epigenetic mechanisms include DNA methylation, post-translational modification of histone proteins by acetylation and methylation and RNA mechanisms which act to alter chromatin structure, and in turn gene expression (Figure 1.10) (Bonasio, Tu et al. 2010). Chromatin in the nucleus is tightly coiled around histone proteins, known as nucleosomes. A nucleosome is associated with an octameric core of histone proteins, consisting of two H3-H4 histone dimers surrounded by two H2A-H2B dimers (Handy et al., 2011). The degree to which the chromatin is condensed or coiled reflects its transcriptional activity and can be divided into euchromatin and heterochromatin (Figure 1.10). Euchromatin is generally associated with an uncoiled chromatin state allowing binding and access of transcriptional machinery, whilst heterochromatin is synonymous with gene repression as the transcriptional machinery is

prevented from binding due to it tightly coiled state of the chromatin (Figure 1.10) (Schleithoff, Voelter-Mahlknecht et al. 2012). Both chromatin structure and gene accessibility are regulated by modification to both the DNA and the histone tails which protrude from the nucleosome at the N-terminal, which in turn determines transcription factor binding accessibility (Handy et al., 2011).

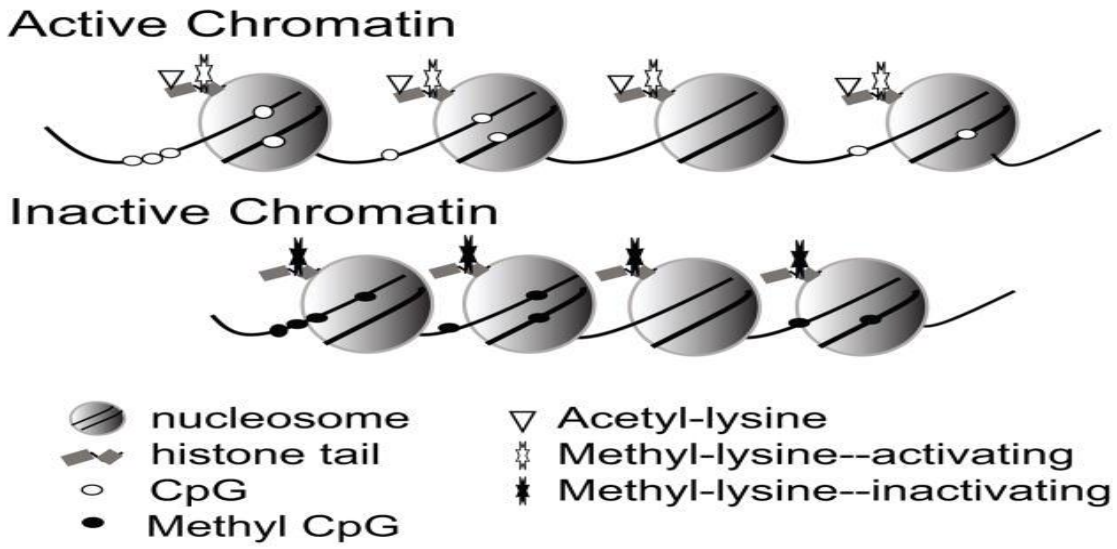

Figure 1.9: Chromatin States.

The types of modifications made to chromatin ultimately determine its transcriptional status. Modifications to chromatin can result in an active transcriptional state (uncoiled) or an inactive (tightly coiled) transcriptional state. Modifications to chromatin such as methylation result in gene repression and an inactive state, whereas acetylation results in an active chromatin state and ultimately gene expression (Handy et al., 2011)

1.8.1 Epigenetic Mechanisms

Due to the reversible nature of epigenetic mechanisms and their ability to regulate the transcriptional status of genes, they pose attractive therapeutic options. In relation to CVD targeting such mechanisms could prove beneficial in reducing inflammatory gene expression and disease progression. As mentioned previously, epigenetic mechanisms include DNA

methylation, post translational modifications (PTMs) of histone proteins and RNA interference (RNAi).

1.8.1.1 DNA methylation; DNA methylation represents one of the most commonly inherited and essential epigenetic marks in mammalian cells. Methylation occurs most frequently on the C5 position of cytosine residues in CpG dinucleotide sequences and is largely associated with transcriptional repression via the inhibition of transcription factor binding (Bird, 1986; Burdge and Lillycrop, 2010; de Nigris et al., 2002; Dupont et al., 2009; Schleithoff et al., 2012; Yan et al., 2010). DNA methylation is catalysed by DNMTs (DNA methyltransferases) and can be reversible through the action of DNMT inhibitors (DNMTi). DNMT1 acts as a 'maintenance' methyltransferase, responsible for maintaining CpG dinucleotide methylation states through replication cycles, maintaining the optimal cellular identity of cells throughout life. DNMT3a and DNMT3b are responsible for establishing DNA methylation patterns during embryonic development (Dupont et al. 2009, Burdge and Lillycrop 2010, Yan et al. 2010).

1.8.1.2 Post translational Modifications; Histone proteins can be post translationally modified in a number ways which affect chromatin structure. Histone acetylation, methylation, and phosphorylation are carried out by various families of enzymes which determine promoter accessibility (De Ruijter et al., 2003; Arrowsmith et al., 2012). Histone methylation is dynamically regulated by histone methyltransferases and histone demethylases and can result in transcriptional silencing, through their action on specific lysine residues (Schleithoff, Voelter-Mahlknecht et al. 2012). In general terms, histone acetylation results in an open chromatin structure permitting transcription factor binding and therefore is largely associated with transcriptional activation. However, this open structure may also facilitate access for

transcriptional repressors (Jaenisch and Bird, 2003). Acetylation of histone proteins is carried out by histone acetyltransferase (HAT) and, like methylation; its effects are reversible through the activity of histone deacetylases (HDAC), of which there are four different classes with specialised histone targets (Figure 1.11). PTMs to histones are often the result of the integration of environmental cues at a cellular level and therefore have important roles in diseases related to lifestyle, diet and early life exposure and thus are likely to be implicated in many common diseases such as cardiovascular disease and cancer.

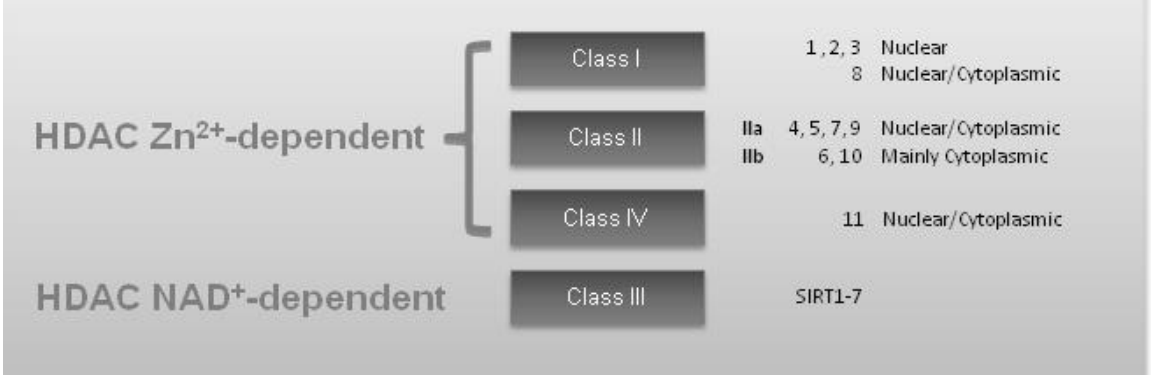

Figure 1.10: HDAC classes.

HDACs are divided into different classes based on function and DNA sequence similarity, whether they are zinc or NAD+ dependent. HDAC class I, II and IV are zinc dependent. HDAC1, 2, 3 and 8 are categorised as class I, with HDAC4,5,6,7,9,10 and 11 falling into class II and class IV respectively. Class III is NAD^+ and is composed of SIRT1-7 (Lemoine and Younes, 2010).

1.8.1.3 RNA mechanisms; Chromatin-based regulation of gene expression is largely influenced by non-coding RNA (ncRNA). NcRNA's are defined by their number of nucleotides; short ncRNA consist of ≤ 200 nucleotides and long ncRNA consist of > 200 nucleotides (LncRNA). ncRNAs have the ability to alter gene expression in a variety of ways and play regulatory roles during development, the response to stress and environmental stimuli. ncRNAs can be classified into three groups miRNAs, siRNAs, and LncRNAs (Mercer et al., 2009; Ponting et al., 2009;

Kaikkonen et al., 2011). The majority of non-protein coding transcripts belong to the group LncRNA (Ponting et al., 2009). LncRNAs are characterised by nuclear localisation, and may be classified as sense, anti-sense, bidirectional, intronic or intergenic (Ponting et al., 2009). LncRNA regulates transcription via the recruitment of chromatin remodelling complexes, which mediate epigenetic changes in genetic regulation. siRNAs act to silence the locus from which they are derived while miRNAs regulate gene expression from a different locus as discussed previously (see section 1.7). Computational and biological evidence suggest miRNA-mediated regulation represents a fundamental mechanism of post-transcriptional regulation with diverse effects (Yang et al., 2015). It is likely that miRNAs target gene promoters and direct transcriptional gene silencing through the recruitment of Argonaut proteins and the formation of epigenetic remodelling complexes that suppress gene expression by promoting histone deacetylation, methylation and DNA methylation (Hawkins et al., 2009; Kim et al., 2006, 2008).

1.8.2 Epigenetics and Atherosclerosis

Epigenetic mechanisms may explain some of the missing heritability associated with CVD and how external factors such as diet, environment and lifestyle contribute to disease development and progression. Epidemiological evidence and clinical data suggest that lifestyle modifications to nutrition and exercise can reduce disease risk, the underlying mechanism of which is likely to be epigenetic (Wang et al., 2013). However, the role such modifications play in atherosclerosis and plaque rupture is poorly understood in comparison to the established role they play in cancer.

Altered patterns in methylation correlate with CVD (Castro et al., 2006; Kim et al., 2007, 2010; Chen et al., 2010; McNeil et al., 2011; Soriano-Tárraga et al., 2014; van Kampen et al., 2014;

Yamada et al., 2014). Research on genomic DNA isolated from human atherosclerotic lesions has shown hypomethylation (Sharma et al., 2008). Hypermethylation has also been identified in the promoter regions of various genes associated with atherosclerosis, e.g. CXCL16, superoxide dismutase, estrogen receptor alpha, endothelial nitric oxide synthase (Castro et al., 2006; Jia et al., 2013; Libby et al., 2010; Zaina et al., 2014). In addition, global DNA hypermethylation has been noted in patients with increased inflammation as measured by CRP, and have identified it as one of the strongest independent risk factors for CVD mortality (Wierda et al., 2010; Stenvinkel et al., 2007).

Aside from DNA methylation, various histone modifications also contribute to plaque vulnerability and rupture with HDAC featuring prominently (Stein and Matter, 2011; Bleijerveld et al., 2013; Findeisen et al., 2013; Bentzon et al., 2014; Eom and Kook, 2014; Pucci et al., 2014). For instance, HDAC3 has been identified as the sole HDAC upregulated in ruptured lesions, inhibition of which shifts the phenotype of plaque macrophages to anti-inflammatory and reduces lipid accumulation, making it a key therapeutic target against plaque rupture (Hoeksema et al., 2014). Shear stress has long been known to induce alterations in the expression of various inflammatory markers and has also been shown to alter histone acetylation patterns in response to shear stress. Low shear stress decreases global histone acetylation and in turn increases acetylation at the c–fos promoter in SMCs (Hastings et al., 2007). HDAC is also responsible for the repression of type 1 collagen, which has been identified as a key step in atherogenesis, prevention of which may lead to plaque stabilisation (Kong et al., 2009; Weng et al., 2014).

miRNAs and LncRNAs have also been identified as key regulators in the development of atherosclerosis via epigenetic mechanisms (Motterle et al., 2012; Holdt et al., 2013; Chen et

al., 2014; Hu et al., 2014; Liu et al., 2014, Vausort et al., 2014). For example, the LncRNA ANRIL, a mediator of epigenetic regulation, located on chromosome 9p21 locus was identified as one of the most replicated markers of CAD, MI and ischemic stroke. High levels of this LncRNA have correlated with the severity of atherosclerosis and could be used in patient stratification to enable more specialised and tailored treatment prior to a cardiac event (Holdt et al., 2010).

The above brief examples highlight that a multitude of epigenetic mechanisms partake in the regulation of all aspects of CVD from development to rupture, and hold great potential in aiding both diagnosis and prognosis (Figure 1.12). Given the reversible nature of these modifications, future drug discovery investigations must take this into account while guarding against issues such as tissue specificity and off-target effects. It is also becoming apparent with time, that many of these epigenetic marks are likely to be induced as a result of environmental and nutritional changes and show a transgenerational mode of inheritance (Aldrich and Maggert, 2015; Kaati et al., 2002; Nadeau, 2009; Nilsson and Skinner, 2015; Pembrey et al., 2014; Skinner et al., 2012).

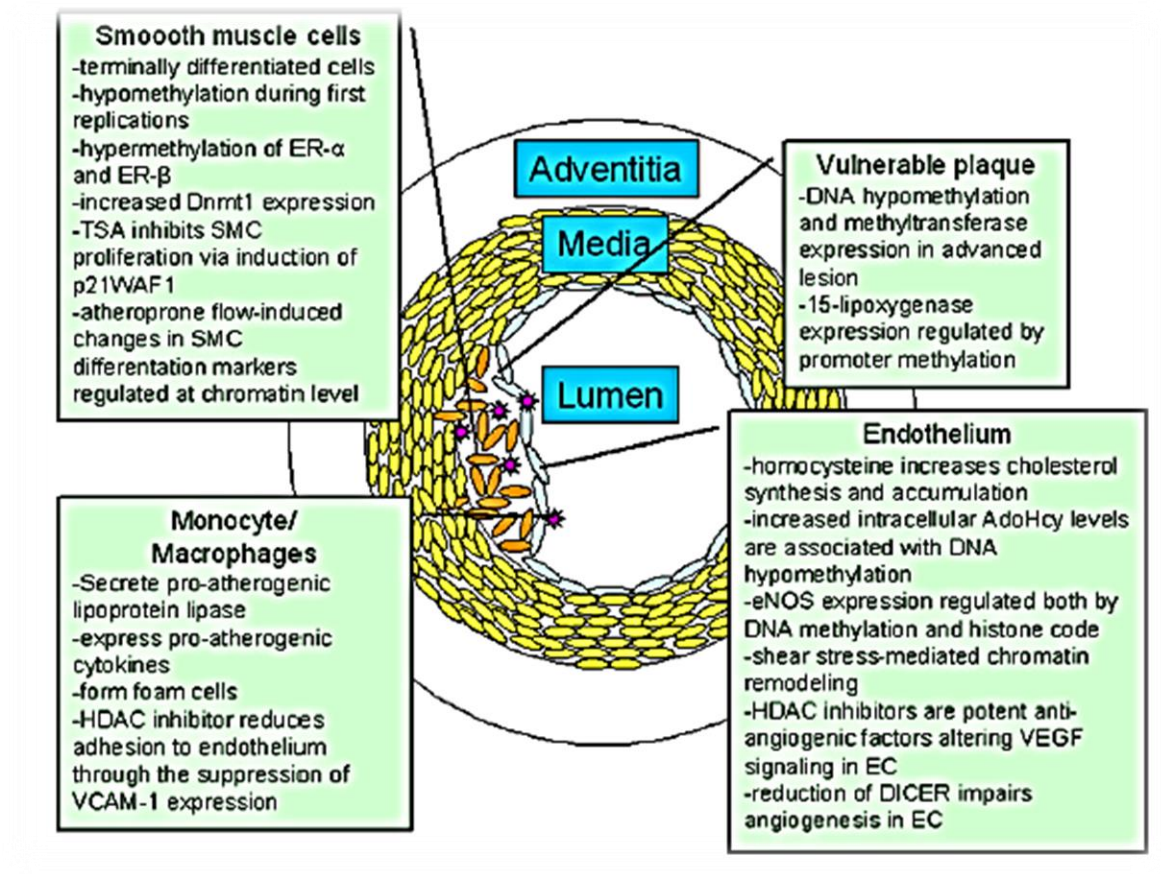

Figure 1.11: Summary of epigenetic effects in atherosclerosis and vascular wall.

Highlights the way in which histone modifications and inhibitors of such modifications can alter the endothelium, smooth muscle cells (SMCs) and contribute to plaque vulnerability(Turunen et al., 2009).

1.8.3 Transgenerational Epigenetic Inheritance

Transgenerational epigenetic inheritance refers to the transmission of epigenetic modifications from one generation to the next, affecting the traits of offspring without altering the primary structure of DNA (Daxinger and Whitelaw, 2010; Kilpinen and Dermitzakis, 2012; Skinner et al., 2012). One of the first observations of this was reported by Paul Krammer in his work with midwife toad in 1909, which later attracted controversy (Vargas, 2009).

Multiple epidemiological and population studies have identified transgenerational epigenetic effects left on populations due to environmental and nutritional changes. Studies include the

Overkalix study and the Dutch famine of 1944 (Lalande 1996,Painter et al., 2008). These studies noted that the children of women pregnant during the Dutch famine where more susceptible to diseases such as diabetes and obesity in late life (Painter et al., 2008). In addition to this, they were also smaller compared to children whose mothers were not pregnant during the famine, the reason for which is likely to be the inheritance of epigenetic marks which have been altered as a result of environmental change, in this case, the famine (Painter et al., 2008). Furthermore Kaati *et al* using data from 3 cohorts born in 1890, 1905 and 1920 in the Overkalix parish (Northern Sweden), followed until death or 1995 noted that overeating during a child's slow growth period before their pre-pubertal peak in growth velocity influenced their descendant's risk of death from CVD and diabetes (Kaati et al., 2002). These initial studies of Overkalix and the Dutch famine were among the first to identify a link between nutrition, epigenetics and disease risk.

Human and animal studies on transgenerational epigenetic events support the observations of Overkalix and the Dutch famine, whereby intrauterine environments can predispose offspring to disease later in life through the dysregulation of epigenetic modification such as DNA methylation. Transcriptomic profiling of mice overfed during preconception and gestation highlighted that maternal obesity induces extensive changes in gene expression, altering white adipose tissue transcriptomics associated with DNA methylation of CpG sites at key pro-adipogenic factors, and ultimately increasing adipogenesis in offspring prior to the development of obesity (Borengasser et al., 2013). Dysregulation of developmentally important miRNAs may explain epidemiological studies suggesting that the offspring of obese women are significantly more at risk for a range of congenital heart defects, myocardial hypertrophy, insulin resistance and cardiovascular disease, once more suggesting that

epigenetic mechanisms underlie the inheritance of many common diseases (Maloyan, Muralimanoharan et al. 2013). Barker *et al* in 1989 suggested that long term cellular memory can affect the development of CVD while investigating birth weight and death from ischemic stroke, this mechanism which was not yet known to be epigenetic, highlighting a role for epigenetic mechanisms in maintaining long term cellular memory (Barker et al., 1989).

These studies suggest how common diseases during adulthood may be strongly influenced by maternal and paternal environment, with the best-known examples being nutrition. However other environmental factors such as smoking and exercise are known to alter epigenetic pathways and ultimately gene expression and disease susceptibility(Horsburgh et al., 2015; Knopik et al., 2012). Transgenerational epigenetic inheritance may help explain some of the genetic diversity noted in many common diseases and disease susceptibility.

1.9 Epigenetic Targeting Agents

As mentioned previously, epigenetic processes are important regulators of gene expression and are therefore likely to a play a role in the regulation of genes which are essential to development and progression of a wide variety of common diseases. Several epigenetic targeting agents have been approved by the FDA; mainly for the treatment of blood related disorders (see Table 1.2).

Agent	Epigenetic mechanism	Application	Year of approval
Vorinostat	HDAC	CTCL	2006
Romidepsin	HDAC	CTCL	2009
5-Azacytidine	DNMT	MDS	2004
Decitabine	DNMT	MDS	2006
Ruxolitinib	JAK1/2	Myelofibrosis	2011

Table 1.2: List of FDA approved epigenetic drugs.
(Byrne et al., 2014)

The use of drugs which target the enzymes responsible for epigenetic modifications such as DNMTs and HDACs have considerable therapeutic potential in CVD and other inflammatory disorders, through the restoration/ removal of modifications which lead to aberrant gene expression (Table 1.3). Ideal therapeutic targets and a starting point for such investigations would include protein coding genes that have an already established relevance in CVD (Poller et al., 2013). Dual therapies are undergoing trials whereby a HDACi and DMNT are combined. Such treatment options are likely to be used in addition to chemotherapy and interferon treatment in cancer (Lustberg and Ramaswamy, 2011). On a cautionary note however, side effects such as toxicity and non-specific gene modulation have limited their use as cancer preventative agents and consequently, their use in other common diseases, thus highlighting a need for greater specificity, better drug design and a deeper understanding of the affected molecular pathways (Zhang et al., 2013).

Compound	Targets	Indications	Highest clinical status	Further information
Panobinostat	HDAC1, HDAC2, HDAC3 and HDAC6	Oncology	Phase III	ClinicalTrials.gov identifiers: NCT01034163 and NCT01023308
Belinostat	HDAC1, HDAC2, HDAC3 and HDAC6	Oncology	Phase II	ClinicalTrials.gov identifiers: NCT00873119, NCT00865969, NCT00431340 and others
Entinostat	HDAC1 and HDAC2	Oncology	Phase II	ClinicalTrials.gov identifiers: NCT00866333, NCT00828854, NCT01349959 and others
Mocetinostat	HDAC1 and HDAC2	Oncology	Phase II	–
Resminostat	HDAC1, HDAC3 and HDAC6	Oncology	Phase II	ClinicalTrials.gov identifier: NCT01037478
Givinostat	HDAC (class I and II)	Inflammation, oncology	Phase II	ClinicalTrials.gov identifiers: NCT00928707 and NCT01261624
SB939	Pan-HDAC	Myelofibrosis	Phase II	ClinicalTrials.gov identifiers: NCT01112384 and NCT01200498
CUDC-101	HDACs, EGFR and HER2	Oncology, solid tumours	Phase Ib	ClinicalTrials.gov identifier: NCT01171924
PCI-24781	HDAC (class I and II)	Oncology	Phase I/II	ClinicalTrials.gov identifier: NCT01027910
4SC-202	HDAC (class I)	Oncology	Phase I	ClinicalTrials.gov identifier: NCT01344707
AR-42	HDAC (class I and II)	Oncology	Phase I	ClinicalTrials.gov identifier: NCT01129193
CG200745	Pan-HDAC	Oncology	Phase I	ClinicalTrials.gov identifier: NCT01226407
ACY-1215	HDAC6	Oncology	Phase I/II	ClinicalTrials.gov identifier: NCT01323751
EVP-0334	HDAC (class I)	Alzheimer's disease	Phase I	See the EnVivo Pharmaceuticals website
RG2833	HDAC3	Friedreich's ataxia	Preclinical (investigational new drug)	See the RepliGen website
SEN196	SIRT1	Huntington's disease	Phase II	See the Siena Biotech website

EGFR, epidermal growth factor receptor; HDAC, histone deacetylase; SIRT1, sirtuin 1.

Table 1.3: Epigenetic Drugs undergoing clinical trials.
The table above provides insight into a subset of the epigenetic drugs undergoing clinical trials, the number of which is likely to have increased from 2012, noting that the vast majority of research is in the field of oncology (Arrowsmith et al., 2012).

Statins, one of the most successful and widely used drugs against atherosclerosis and CVD, have been suggested to have an epigenetic effect. Statins are known to induce a wide variety of different effects such as improved endothelial cell function and blood flow, including decreased LDL oxidation, increased plaque stability, reduced VSMC proliferation and vascular inflammation, the mechanism behind which is likely to be epigenetic (Schiano et al., 2015;

Voelter-Mahlknecht, 2016). However, statin therapy is not flawless, issues remain with 20% of patients becoming resistant and a further 10% suffering from myopathy, highlighting the need for new more targeted disease therapies (Harper and Jacobson, 2007; Hennessy and Moore, 2013).

1.9.1 SAHA (Vorinostat)

SAHA (suberoylanilide hydroxamic acid) is a HDACi also known as Vorinostat/Zolinza and is one of a few epigenetic drugs to be approved by the FDA for the treatment of T-cell lymphoma. SAHA is an orally active potent inhibitor of HDAC activity, inhibition of which is likely to be carried via the binding of SAHA to the zinc ion catalytic domain of the enzyme (Mann et al., 2007; Yoo and Jones, 2006). Currently, research and clinical trials are investigating the use of SAHA in the treatment of other cancers, and in combination with existing therapies (Arrowsmith et al., 2012; Krautkramer et al., 2015; Nakazawa et al., 2016; Yang et al., 2015).

In addition to its in use T-cell lymphoma it has been suggested to have anti-fibrotic and anti-inflammatory potential against pulmonary fibrosis, and it has been shown along with Trichostatin A (TSA) to interfere with the sprouting of capillaries from the aorta (Marumo et al., 2010; Wang et al., 2009; Yoon and Eom, 2016).

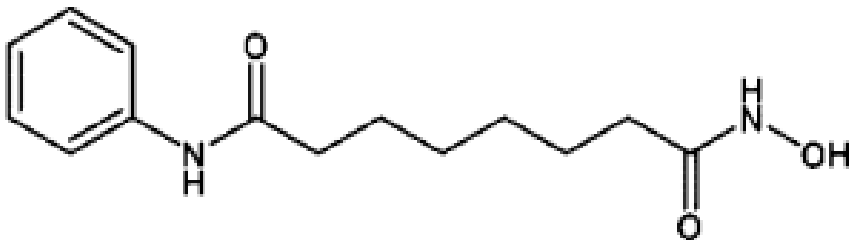

Figure 1.12: Chemical structure of SAHA

1.9.2 DAC

DAC (Decitabine) is a DNA methyltransferase inhibitor of 5-aza-2'-deoxycytidine. Decitabine has been studied in several phase II trials for solid tumours as well as in different types of leukaemia. The drug has been shown to have very limited efficacy against solid tumours but exhibits higher activity for the treatment of haematological malignancies (“Decitabine,” 2003).

DAC is a highly attractive epigenetic targeting agent as it holds the potential to induce the re-expression of aberrantly hypermethylated genes such as ERα, ERβ and collagen receptor COL15A1, with relatively low toxicity to SMC and ECs (Connelly et al., 2013; Kim et al., 2007). A combination of DAC and SAHA was used to inhibit growth of ovarian cancer in cell lines and xenografts and was shown to induce the expression of genes involved in apoptosis, G2/M arrest, autophagy and imprinted tumour suppressor genes (TSG) (Chen et al., 2011).

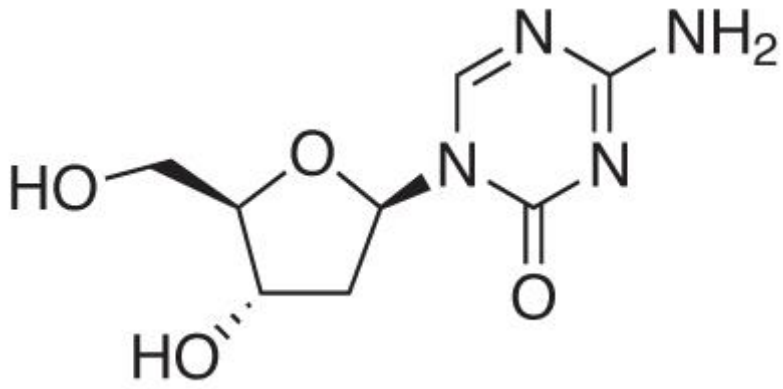

Figure 1.13: Chemical structure of DAC

Some bioactive food components have been shown to affect epigenetic signalling pathways such as DNA methylation, which has promoted research interest into the relationship between nutrition and epigenetics, as to the potential role dietary components may play in disease prevention. As many of these components are commonly present in the diet they provide a more accessible therapeutic option, particularly if they play a preventative role. This field of

research has become known as nutritional epigenetics (Jang and Serra, 2014; Mayne et al., 2016; Niculescu, 2012).

1.10 Nutraceuticals and Epigenetics

The treatment of a variety of multifactorial diseases with herbs and plant extracts has been noted in many ancient societies. Recently we have begun to look back on these treatments and their potential use in modern medicine. Identifying the underlying molecular mechanisms of these active food components still remains elusive. It is likely however, that many of these bioactive dietary components mediate epigenetic modifications, many of which may prove beneficial as they could potentially delay and/or prevent the progression of many chronic illnesses (Choi and Friso, 2010; Meeran et al., 2010).

Cruciferous vegetable such as kale, cabbage, brussel sprouts and broccoli contain bioactive chemical components such as sulforaphane, which have been shown to influence miRNA expression and inhibit HDACs and DNMTS (Royston and Tollefsbol, 2015). This amongst other observations and epidemiological studies has led to the suggestion of an “Epigenetic Diet”, whereby a diet rich in cruciferous vegetables decreases the risk of developing cancer and may have additional benefits in disease associated with lifestyle, such as diabetes and heart disease (Tang et al., 2008)

1.10.1 Sulforaphane

Sulforaphane[1-isothiocyanato-4-(methylsulfinyl)-butane] (SFN) derived from glucoraphanin is thought to be principally responsible for the health benefits associated with cruciferous vegetables due to its activity as a HDACi (Myzak et al., 2007; Elbarbry and Elrody, 2011). SFN

became the subject of considerable research interest due to a finding by Prochaska and colleagues in the 1980s. In this study they assessed a variety of fruit and vegetables for ability to induce anti-carcinogenic phase II enzymes, of which SFN induced the highest amount (Prochaska and Santamaria, 1988).

SFN is a competitive inhibitor of HDAC due to its affinity for the active pocket of HDAC enzymes by interaction with external amino acid residues located in the active site. SAHA also has a similar mechanism of action (Ho and Dashwood, 2011). This competitive inhibition can be seen in all isothiocynates and has been demonstrated both *in vitro* and *in vivo*. Targets of SFN are likely to include class I and class II HDACs and more recently DNMTs have been identified as targets (Meeran et al., 2012; Myzak et al., 2004; Su et al., 2014). Two non-exclusive mechanisms have been proposed to explain the beneficial role of SFN in health, these include its epigenetic role as HDACi and DNMTi and its ability to induce Nrf2 expression (Kaufman-Szymczyk et al., 2015). SFN has also been shown to regulate miRNA expression (Kala et al., 2013).

1.8.4.1.1 SFN and Inflammation

SFN acts as an indirect antioxidant to induce the expression of several enzymes via epigenetic modifications of the Nrf2 pathway (Bai et al., 2013; Zhang et al., 2013). Nrf2 is a transcription factor which plays an essential role in the regulation of cellular defence antioxidants and it can be activated by SFN, ROS and hypoxia. SFN is known to enhance the nuclear translocation of Nrf2 leading to increased mRNA and protein levels of Nrf2 target genes heme oxygenase-1 (HO-1), NAD(P)H dehydrogenase [quinone] 1 (NQO-1), uridine 5'-diphospho-glucuronosyltransferas (UGT), glutathione S transferases (GST), ferretin, Thioredoxin, Thioredoxin reductase 1, and manganese superoxide dismutase (MnSOD) (Zhang et al., 2013).

It is likely that SFN epigenetically regulates NRF2 via alterations to promoter methylation, leading to Nrf2 reactivation and ultimately the maintenance of cellular ROS balance and reduction in inflammatory cytokine release (Su et al., 2014; Yu et al., 2010; Zhang et al., 2013).

Numerous epidemiological and animal studies support sulforaphanes role as an anti-oxidant and epigenetic modifier, suggesting that the risk of atherosclerosis and CVD could potentially be reduced by its use (Evans, 2011; Fowke et al., 2006; Juurlink, 2012; Kim et al., 2012; Miao et al., 2012; Yoo et al., 2013; Zakkar et al., 2009), with further studies suggesting that consumption of broccoli leads to a reduced risk in CVD mortality (Cornelis et al., 2007; Genkinger et al., 2004; Hung et al., 2004; Yochum et al., 1999). Applications of SFN in CVD can include, but are not limited to, suppression of inflammatory mediators e.g. VCAM-1, NF-κB and its subsequent targets, and the induction of changes in vascular and inflammatory cell physiology (Evans, 2011; Hoffmann et al., 2002; Miao et al., 2012; Zakkar et al., 2009). SFN also has uses in the pharmacological activation of Nrf2, particularly under low shear stress conditions to suppress p38 activation, VCAM-1 expression and ROS production and further inflammatory cytokine release (Chen et al., 2009; Zakkar et al., 2009).

1.8.4.1.2 SFN and Cancer

SFN has a well-established chemo-preventative role for variety cancer types including breast cancer, colon cancer and prostate and has been widely investigated as therapeutic option (Alumkal et al., 2015; Atwell et al., 2015; Higdon et al., 2007; Li et al., 2011; Myzak et al., 2007; Rajendran et al., 2015; Royston and Tollefsbol, 2015; M. Yang et al., 2016).

Treatment with SFN in cancerous cells induces apoptosis, inhibits migration and invasion, induces cell cycle arrest and in some instance has anti-leukemic effects (Ferreira de Oliveira et al., 2014; Hussain et al., 2013; Pledgie-Tracy et al., 2007; Rudolf et al., 2014; Zhang and

Tang, 2007). The chemo-preventative effects of SFN are attributed to its ability to induce the reactivation of p21 transcription, via hyper acetylation of histone H3 and H4 on the p21 promoter and the SFN-dependent inhibition of anti-apoptotic NF-κB signalling pathways (Heiss et al., 2001; Lubecka-Pietruszewska et al., 2015; Moon et al., 2012; Mottet et al., 2009).

Sulforaphane is a natural dietary agent, which contains bioactive components and has demonstrated potential as a nutraceutical in cancer prevention through its effects as an epigenetic modifier (Meeran et al., 2010). Furthermore, it is rapidly absorbed following consumption, and unlike synthetic epigenetic drugs such as SAHA, has very low toxicity, requiring only a low concentration to induce a response in some instances (Myzak et al., 2007; Royston and Tollefsbol, 2015). Although possessing less toxicity, it is also less specific than synthetic drugs like SAHA and DAC and issues may arise from interindividual metabolic variation. Off-target effects are also likely to be noted, for instance increased long terminal repeat(LTR) acetylation, which may impair genome stability. This effect is only transient but may lead to lasting genomic damage (Baier et al., 2014). As the locus specificity of SFN is uncertain, there is a need to pursue gene specific expression through modulation of epigenetic marks in distinct genomic loci (Baier et al., 2014; de Groote et al., 2012). In theory, such gene-specific editing can be achieved by fusing enzymes or inhibitors to gene specific DNA binding domains (Baier et al., 2014).

1.10.2 Curcumin

Curcumin (diferuloymethane) a DNMTi and HDACi, is a polyphenol present in turmeric spice, with a diverse range of molecular targets, ranging from transcription factors and growth factors to cytokines and genes responsible for apoptosis and cell proliferation (Schiano et al., 2015; Vahid et al., 2015). Curcumin was demonstrated to reduce the extent of atherosclerotic

lesions and induce changes in the expression of genes involved in cell adhesion, transendothelial migration in ApoE$^{-/-}$ mice fed 0.2% curcumin over four months. Furthermore when packaged with SFN, Curcumin has shown potential use in the treatment of pancreatic cancer (Coban et al., 2012; Sutaria et al., 2012).

1.10.3 Protocatechuic Aldehyde (PA)

Protocatechuic aldehyde (PA) is a HDACi isolated from the aqueous extract of the root of the *Salvia miltiorrhiza* herb. Like SFN, PA has both epigenetic and antioxidant activity (Kong et al., 2014). There is evidence to suggest that PA can inhibit migration and proliferation of vascular smooth muscle cells, ultimately exerting a protective effect on endothelial cells (Kong et al., 2014; Moon et al., 2012; Wei et al., 2013; Zhou et al., 2005). It has been shown to reduce myocardial infarct size and the activities of cardiac troponin in serum (10 and 20 mg/kg) (Wei et al., 2013; Wei et al., 2012).

Cardiovascular disease	Compound[a]	Epigenetic alteration	Target and effect	Tissue/cell type	Study model
DNA methylation					
Atherosclerosis and CHD	DAC	Gene-specific DNA methylation	ERα and ERβ upregulation	Coronary plaque/SMCs; ECs	*In vitro*
	DAC	Gene-specific DNA methylation	COL15A1 upregulation	Aortic SMCs	*In vitro*
	Resveratrol	Gene-specific DNA methylation	SIRT1 upregulation	ECs	*In vitro*
	Acetylsalicylic acid	Gene-specific DNA methylation	ABCA1	Leukocytes	Human
	Cocoa	Global DNA methylation	DNMTs and MTHFR downregulation	Leukocytes	*In vitro*/human (NCT00511420)
	Folic acid	Global DNA methylation	Multiple gene expression regulation	Leukocytes	Human (NCT00266487)
Histone modification					
Atherosclerosis and CHD	TSA	HDAC activity	ERα and ERβ upregulation	Coronary plaque/SMCs; ECs	*In vitro*
	Simvastatin and Fluvastatin	HDAC activity	Inflammatory gene downregulation	ECs	*In vitro*
Cardiac hypertrophy	Curcumin	p300 HAT activity	GATA4 downregulation	Cardiomyocytes	*In vitro*/animal model
Myocardial infarction	TSA	HDAC activity	TNFα downregulation; Akt-1 phosphorylation	Cardiomyocytes	Animal model

[a]Abbreviations: DAC, 5-aza-2-deoxycytidine (demethylating agent); TSA, trichostatin A (histone deacetylase inhibitor).

Table 1.4: CVD- associated epigenetic alterations and compounds for therapy.
(Schiano et al., 2015)

These modifying agents present a new angle and possibilities for the treatment and prevention of CVD through the use of dietary supplements in the prevention of atherosclerosis, and may provide a starting point for the prevention and management of other chronic illnesses (Table 1.4).

1.11 Genome-wide association studies (GWAS)

The first successful GWAS application was published in 2005 for age related macular degeneration (Dewan et al., 2006; Klein et al., 2005) however a study published by the Wellcome Trust Case Control Consortium (WTCCC) in 2007 was generally accepted as the starting point for GWAS of complex disease (Burton et al., 2007). Since then, GWAS meta-analyses have identified over 2000 loci robustly associated with one or more complex traits (Evangelou and Ioannidis, 2013). GWAS provides an alternative and unbiased approach to candidate gene studies for identifying disease-associated variants, as prior knowledge of function is not required. GWAS identify common risk variants associated with heritable traits and disorders through the analysis of SNP frequencies in a defined experimental and control group (Visscher et al., 2012). Linkage disequilibrium (LD) describes the non-random association of alleles at different loci generated through evolutionary forces such as mutation, drift and selection (Visscher et al., 2012). Loci which are closer together exhibit stronger LD than loci further apart, with larger population sizes diluting the strength of LD for a given distance (Visscher et al., 2012). LD can occur in blocks that may exceed >100 kb around a given SNP (Reich et al., 2001).

SNPs are single nucleotide changes in the genome, a common form of genetic variation within the genome. SNP variation underlies disease susceptibility and has formed the basis of multiple association studies. A SNP defines a site of variation where the reference or alternate allele may be present at divergent frequencies within a given population. In some instances, the reference allele will be found at a substantially higher frequency and as such may provide a selective advantage over the alternate allele (Barreiro et al., 2008). SNPs are normally found within non-protein coding regions of the genome (regulatory SNP), but there are an estimated

60,000 located within coding regions of the genome (non-synonymous SNP) and untranslated regions (UTRs) (Sachidanandam et al., 2001). 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).

GWAS of CVD has identified a substantial genetic component; to date 48 genomic loci have been found, harbouring a number of SNPs known to have a significant association with CVD (Nikpay et al., 2015). A number of SNPs on chromosomal regions 9p21.3, 1q41, 3q22.3, 1p13.1, 2q36.3 amongst others have been suggested as risk loci for coronary disease and myocardial infarction (Erdmann et al., 2009; Esparragón et al., 2012; Haver et al., 2014; Bellenguez et al., 2012; Li et al., 2013; Nioi et al., 2016; Shen et al., 2008).

For instance, genetic variants on chromosome 13q34 at COL4A1/COL4A2 locus have been shown to affect vascular cell survival, atherosclerotic plaque stability and ultimately the risk of MI (Yang et al., 2016). However, issues remain with many of the regions identified by GWAS. Many studies have failed to replicate findings due to the heterogeneity of clinical phenotypes and populations studied, suggesting that although GWAS is a powerful tool in the identification of disease-associated variants greater validation is required before such markers can be used in risk assessment, prevention or as therapeutic targets (Ndiaye et al., 2011).

1.12 Experimental Design Summary/Aims and objectives

Epigenetic mechanisms are dysregulated in a variety of diseases. Altering these mechanisms through the use of epigenetic targeting agents (ETAs) such as SAHA (vorinostat) and DAC (Decitabine) has been shown to be effective in the treatment of cancer. Various dietary components have the ability to induce these variations which can significantly affect genome stability, mRNA and protein expression and metabolic changes. These compounds have the potential to regulate epigenetic modifications. Evidence suggests that cruciferous vegetables such as broccoli may help prevent and/or delay various inflammatory disorders. SFN is thought to be principally responsible for the health benefits associated with cruciferous vegetables due to its activity as a HDACI. Curcumin, another histone deacetylase inhibitor found in turmeric spice also offers potential as a dietary atheroprotective agent.

Epigenetic mechanisms such as DNA methylation (DNMT and DNMTi), histone acetylation (HAT and HDACi), and RNAi (miRNAs) are known to be altered in cardiovascular disease and thus may be suggested as potential targets in disease prevention and treatment.

1. The vascular endothelium is the initiating site of plaque development, and it is likely that epigenetic mechanisms participate in this process. In order to investigate this further, two cell lines; Ea.Hy926 and THP-1 (these cell lines are representative of cell types commonly found in atherosclerotic lesions) were analysed to assess the effect of alterations through HDACi (SFN) on key inflammatory genes *CXCL16*, *IL8* and *IL18* and miRNAs 145, 210 associated with plaque rupture. Ea.Hy926 cells are a human umbilical vein cell line. Ea.Hy926 was established by the fusion of primary human umbilical vein cells with a thioguanine-resistant clone of A549 by exposure to polyethylene glycol (PEG). Hybrid clones were selected in HAT medium and screened

for factor VIII-related antigen. These cells via Electron photomicrographs demonstrate characteristics of differentiated endothelial cell functions such as angiogenesis, homeostasis/thrombosis, blood pressure and inflammation. THP-1 cells are a human monocytic cell line from peripheral blood. Differentiation in macrophages can be induce by treatment with PMA.

These experiments will determine the potential of nutritional supplementation to decrease inflammation in the vascular endothelium and reduce the likelihood of plaque development and subsequent rupture.

2. Due to the multifaceted role CXCL16 plays in all stages of atherosclerosis, SNPs within this gene are of particular interest. This study focused on rs2277680 as it has a relatively high frequency across multiple populations and little or no information is known about it or the role it may have in atherosclerosis. This chapter will determine whether AEI can be induced and altered by ETAs, along with the effectiveness and reproducibility of using cells which have been frozen in DMSO and along with the potential of DMSO for altering epigenetic marks within samples.

3. The expression of miRNAs 145 and 210 has previously been shown to be upregulated in patients with plaques prone to rupture. However, an association with stroke recurrence prediction from an initial cardiac event has not been examined. I aimed to determine if miR145 and 210 could be used as potential biomarkers in predicting stroke recurrence in a clinically characterised subset of patients (Marnane et al., 2014).

Chapter 2: Role of HDACi on Vascular Endothelial Health

2.1 Introduction

Atherosclerosis initiates primarily as a result of injury to the endothelium, resulting in the activation and recruitment of a variety of cells which secrete cytokines, chemokines and other inflammatory mediators which contribute to atheroma formation, as such they are important disease biomarkers.

Inflammatory mediators may be considered as primary or secondary triggers in atherosclerosis, as they play roles in both the initiation and propagation of the disease. Underlying disorders, such as rheumatoid arthritis (RA), type 1 diabetes (T1D) and systemic lupus erythematosus (SLE) are known to be associated with exacerbated atherosclerosis development as a consequence of a pre-existing inflammatory condition, increased expression of pro-inflammatory cytokines, chemokines and endothelial activation prior to injury of the endothelium (Beckman et al., 2002; Frostegård et al., 2005; Pearson et al., 2003; Sakurada et al., 1996).

Endothelial activation upregulates pro-inflammatory chemokines such as IL8, MCP-1, VCAM-1, ICAM-1 and E-Selectin which enable the adhesion of monocytes to endothelial cells, further contributing to lesion development (Nallasamy et al., 2014). Different levels of circulating pro and anti-inflammatory cytokines have been reported between different CVD types and control models (Bucova et al., 2008).

2.1.1 TNFα Stimulation of Cell lines

TNFα has been described as a pleiotropic pro-inflammatory cytokine, known to play roles in the disruption of vascular function and the development of vascular disease, through mediating the interaction of invading monocytes with vascular ECs, thus triggering ECM deposition in aortic vessels (Gerthoffer, 2007). TNFα is a known activator of pathways which upregulate pro-inflammatory cytokines such as NF-κB in ECs. NF-κB is involved in the regulation of over 300 genes involved in processes such as inflammation, immunity and cell survival, in addition to being upregulated in the plasma and arteries of patients with vascular complications (Aggarwal, 2000; Hayden and Ghosh, 2008; Ridker et al., 2000; Turner et al., 2010; Zandi et al., 1998). This identifies TNFα as heavily involved in the pathogenesis of atherosclerosis and provides a rationale for the use of stimulation with TNFα to create an inflammatory response comparable to that observed in CVD patients.

2.1.2. Pro inflammatory markers analysed in this study

CXCL16

CXCL16 acts as a chemokine, scavenger receptor and adhesion molecule, all of which play a vital role in the development of atherosclerosis. CXCL16 expression is increased by pro-inflammatory stimuli, which enhance ox-LDL uptake and foam cell formation, key aspects in atherosclerotic lesion development (Lehrke et al., 2007). CXCL16 has been shown to participate in atherosclerotic lesion development (Minami et al., 2001; Sheikine and Sirsjö, 2008; Wågsäter et al., 2004; Wuttge et al., 2004). CXCL16 mediates T-cell adhesion to the endothelium , and as a chemokine drives migration, proliferation and inflammation in SMCs (Sheikine and Sirsjö, 2008). Stimulation of cells with TNFα and IFN-γ induces expression of

CXCL16 (Abel et al., 2004) and increases whole blood *CXCL16* mRNA and circulating CXCL16 levels, in a time and dose-dependent manner (Lehrke et al., 2007).

CXCL16 expression has been suggested as a biomarker for predicting patient outcomes. However, conflicting evidence exists as increased and decreased CXCL16 expression have been associated with CAD and plaque instability, respectively (Lehrke et al., 2007; Mitsuoka et al., 2009; Sheikine et al., 2006). Soluble CXCL16 levels did not statistically differ between statin and non-statin users, suggesting that CXCL16-mediated cardiovascular issues may not be resolved through conventional therapies. Furthermore, studies in ApoE$^{-/-}$ mice have noted that IFN-γ treatment rapidly increases *CXCL16* mRNA in lesions but not in unaffected areas. Mice injected with IL18 (a known inducer of IFN-γ) showed a similar response (Tenger et al., 2005; Wuttge et al., 2004).

IL18

IL18, as mentioned previously, has been described as a major independent inflammatory predictor of 30-day major adverse cardiac events and unfavourable outcomes following AMI (Youssef et al., 2007). Studies have shown that IL18 mediates atherosclerosis and is associated with a higher death rate in patients exhibiting CAD (Mallat et al., 2001a). A similar observation was noted by Whitman et al in mice (Whitman et al., 2002), in addition to being expressed at high levels in atherosclerotic lesion macrophages, T-cells and SMCs, an expression pattern which was not observed in normal aorta (Gerdes et al., 2002; Mallat et al., 2001). Serum levels of IL18 have also been used in predicting cardiovascular events in end stage renal patients, where IL18 levels positively correlate with CRP in 171 patients tested, identifying IL18 as a predictor of cardio-cerebral vascular events in dialysis patients (Chang et al., 2015).

IL18's function in atherosclerosis is mediated by interleukin 18 receptor (IL18R) and NaCl co-transporter (NCC), a 12-transmembrane-domain ion transporter protein preferentially expressed in the kidney (Wang et al., 2015). NCC is expressed in atherosclerotic lesions, where it co-localises with IL18r. In $ApoE^{-/-}$ mice, blockade or inhibition of IL18 reduces atherosclerosis and lesion formation (Elhage et al., 2003), whilst overexpression enhances lesion burden and inflammation (Tenger et al., 2005; Whitman et al., 2002). Furthermore, a combined deficiency in both IL18R and NCC has been shown to protect from atherosclerosis (Wang et al., 2015).

IL8

IL8 is a chemokine with pleiotropic effects on cardiovascular homeostasis. IL8 production is stimulated by danger associated molecular patterns (DAMPS) which are key mediators of the inflammatory response. For instance oxLDL stimulates secretion of IL6, IL8, monocyte MCP-1, and TNFα by signalling through CD36 and Toll-like receptor (TLR)-2, -4, and/or -6 pathways (Bekkering et al., 2015; Miller et al., 2011).

The association of IL8 serum and gene expression levels with the risk of coronary heart disease (CHD) has been investigated in a number of studies with discordant findings (Romuk et al., 2002; Velásquez et al., 2014; Zhou et al., 2001). In hospital based studies, circulating IL8 levels were higher in patients with AMI or unstable angina when compared against patient controls (free of previous clinically diagnosed MI) (Velásquez et al., 2014). Furthermore, in a cohort of 101 patients, serum levels of IL8 were found to be enhanced in patients with unstable plaques when compared to those with stable lesions (Pelisek et al., 2009). It is likely that the role of IL8 in atherosclerosis may change over time from proinflammatory to cardio protective, following an MI event IL8 promotes neutrophil homing to the site of injury (Riesenberg et al.,

1997) promoting new vessel generation, facilitating wound healing and myocardial tissue repair (Frangogiannis, 2004; Velásquez et al., 2014).

The above description aims to provide a reasoning and background basis in support of the proinflammatory markers used in this study, and how decreasing the expression of such epigenetically through the use of epigenetic modulators may provide a novel and effective method in disease prevention and progression.

2.1.3 Epigenetics and HDACi

The use of drugs which target the enzymes responsible for epigenetic modifications such as DNMTS and HDACs have considerable therapeutic potential in CVD and other inflammatory disorders. CVD therapeutic targets include protein coding genes that have an already established relevance in CVD (Egger et al., 2004; Poller et al., 2013; Zhang et al., 2013).

Sulforaphane, SAHA and Curcumin

SFN is thought to be principally responsible for the health benefits associated with cruciferous vegetables due to its activity as a HDACi (Elbarbry and Elrody, 2011; Myzak et al., 2007). Targets of SFN are likely to include class I and class II HDACs and more recently DNMTs (Meeran et al., 2012; Myzak et al., 2004; Su et al., 2014). Two non-exclusive mechanisms have been proposed to explain the beneficial role of SFN in health, these include its epigenetic role as a HDACi and DNMTi along with its ability to induce Nrf2 expression and its subsequent antioxidant activity (Kaufman-Szymczyk et al., 2015). SFN has also been shown to regulate miRNA expression (Kala et al., 2013).

A phase one study of biomarkers for metabolism and oxidative stress revealed decreased total and LDL cholesterol following ingestion of 100 g daily of broccoli over one week,

improving cholesterol metabolism and reducing oxidative stress (Murashima et al., 2004), with similar benefits being observed in additional studies (Aiso et al., 2014; Bahadoran et al., 2013; Murashima et al., 2004; Suido et al., 2002). Pharmokinetic studies suggest that mature broccoli and immature broccoli cress can generate a plasma SFN concentration of 1 μM to 60 nM (Evans, 2011). However, a deleterious effect of SFN on vascular health has been noted at concentrations which exceed 10 μm, with most *in vitro* studies opting for 10 μM or less (Liu et al., 2008).

Under experimental conditions, ECs cultured with SFN have been shown to suppress p38, VCAM-1 and ROS activation, key steps in atherogenesis (Chen et al., 2009; Zakkar et al., 2009). In a porcine model, pre-treatment with SFN resulted in a 90% reduction in p38 and 50% reduction of NF-ƙB, along with protection against renal injury following cardiopulmonary bypass (CPB) (Nguyen et al., 2014). This study also noted that TNFα and IL8 transcripts were enhanced in whole blood following CPB. In addition, treatment with SFN 2 h-post CPB significantly decreased their expression.

Due to the dual role of SFN as both a HDACi and antioxidant, it is vital that any additional antioxidant effects SFN may induce, and their contribution to CVD, be recognised. Deletion of essential antioxidant genes such as *HO-1*, which SFN indirectly regulates via NRf2 have resulted in enhanced atherosclerosis, while overexpression of such acted to reduce lesion formation (Chen et al., 2002; Kaneda et al., 2002; Orozco et al., 2007). Furthermore, Nrf2 knockout mice have a more severe atherosclerotic progression rate, while in contrast, overexpression of Nrf2 in mice reduced inflammation following injury (Levonen et al., 2007; Sussan et al., 2008).

In addition to SFN another HDACi Curcumin (diferuloymethane) a DNMTi and HDACi was also investigated. Curcumin (CURC) like SFN can also be sourced from the diet and is commonly found in turmeric spice. Curcumin was demonstrated to reduce the extent of atherosclerotic lesions and induce changes in the expression of genes involved in cell adhesion, transendothelial migration in ApoE$^{-/-}$ mice feed 0.2% curcumin over four months (Coban et al., 2012). As CURC like SFN has a diverse range of molecular targets (Schiano et al., 2015; Vahid et al., 2015), a more specific HDACi was used in the form of SAHA (Vorinostat). SAHA is an already FDA approved drug for use in the treatment of CTCL.

Thus we can see from the above examples that SFN has already been demonstrated to have a beneficial effect on cardiovascular health in both patient and animal studies. In this particular study it is difficult to separate out whether any effect observed is due to SFNs activity as a HDACi or antioxidant. Therefore, we also tested a similar but more specific HDACi SAHA (Vorinostat), which is FDA approved and already in use for the treatment of CTCL.

2.1.4 miRNAs and Epigenetic Targeting agents

Various types of miRNAs have been implicated in both the regulation and epigenetic regulation of cardiovascular disease, the activities of which can differ widely among cell types, exerting pro or anti-atherogenic functions (Alexy et al., 2014; De Paoli et al., 2014; Hu et al., 2014; Wierda et al., 2010).

In this study we also aimed to assess the effect of HDACi SFN and SAHA on miRNA 145 and 210 expression in EA.hy 926 and THP-1 cells. These miRNAs were selected based on known associations with plaque instability, but little is known about their response to epigenetic modification (Chen and Juo, 2012; Cipollone et al., 2011; Raitoharju et al., 2013). SAHA in a previous study has been shown to epigenetically regulate the miR-17-92 cluster,

demonstrating the potential for miRNA expression to be altered following treatment with epigenetic targeting agents (ETAs) such as SAHA and its potential use in CVD treatment through the reduction of disease associated miRNAs (Yang et al., 2015).

2.2. Aims and Objectives

- Determine the effect of HDACi SFN and SAHA on the mRNA expression of defined inflammatory genes with known roles in atherosclerosis; *CXCL16*, *IL8* and *IL18* in EA.hy 926 and THP-1 cell lines.

- Determine the effect differential TNFα level has on the activity of SFN, SAHA and inflammatory markers described above CXCL16, IL8 and IL18 in EA.hy 926 and THP-1 cell lines.

- Assess the effect HDACi pre-treatment with SFN or SAHA, followed by stimulation with TNFα has on miR145 and miR210 expression in EA.hy 926 and THP-1 cell lines.

2.3 Materials and Methods

2.3.1 Cell lines and Culture

2.3.1.1 EA.hy 926

Human umbilical vein cell line EA.hy 926 was cultured with DMEM (Dulbecco's modified Eagle Media) medium supplemented with 10% foetal bovine serum (FBS) (v/v) and 5% Penstreptomycin (v/v) in 75 cm^2 flasks and incubated in a humidified incubator at 37 °C and 5% CO_2. Ea.hy 926 cells are a somatic endothelial cell hybrid, produced by the fusion of A549/8 (lung endothelial cells) with human umbilical vein endothelial cells (Lieber et al., 1976). This fusion cell line is one the most frequently used and best characterised human vasculae EC lines (Edgell et al., 1983). This cell line expresses von willebrand factor and upregulates ICAM-1, VCAM-1 and E-Selectin upon stimulation with TNFα (Thornhill et al., 1993; Edgell et al., 1983).

2.3.1.2 THP-1

THP-1 cells were cultured with RPMI 1640 (Roswell Park Memorial Institute) (Sigma-Aldrich, St. Louis, Missouri, USA) medium supplemented with 10% FBS (v/v) and 5% Penstreptomycin (v/v) (Sigma-Aldrich, St.Louis, Missouri,USA) in 75 cm^2 flasks and incubated in a humidified incubator at 37 °C and 5% CO_2. Penstreptomycin stock solution contains 10,000 units penicillin and 10 mg streptomycin/mL. Cells were differentiated into macrophages by incubation with RPMI 1640 supplemented with 10% FBS (v/v) and 5% Penstreptomycin (v/v), containing 25 ng/ml Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, Missouri, USA) at 37 °C, 5% CO_2 for 72 h prior to treatment.

2.3.2 Treatment of cell lines with epigenetic targeting agents

Cells were seeded in 24 well plates at a density of 2 x 10^5 cells per ml in triplicate. Cells were treated with 10 µM concentration of either SFN (Cayman, Ann-Arbour, Michigan, USA) or SAHA (Sigma-Aldrich, St. Louis, Missouri, USA) for 3 h. Both SFN and SAHA were reconstituted and stocks formed in DMSO. SFN preparation was as follows; 10 mg was dissolved in 1ml DMSO, giving a stock solution of 56.4 mM. From this stock to prepare a 3 ml solution of 10 µM, 0.81 µl of SFN was added to 2.999.1 µl of medium. For SAHA, a 18.91 mM stock was prepared using DMSO. 1 µl of this stock solution was then added to 999 µl of DMSO to create a substock of 18,910 µM. To prepare a 3 ml solution of 10 µM SAHA, 1.58 µl of the substock was added to 2, 998 µl of medium.

2.3.3 Treatment with TNFα

Following pre-treatment with SFN or SAHA, cells were treated with either 5 ng/µl, 10 ng/µl and 15 ng/µl of TNFα for 4, 16 and 24 h. Medium was removed and the samples lysed in 1 ml of Tri-Reagent and stored at -80 °C until further use.

2.3.4 RNA Extraction

RNA extraction was performed using Tri-Reagent (Sigma-Aldrich, St. Louis, Missouri, USA) as per manufacturer's instructions. The RNA pellet was resuspended in 30 µl nuclease-free water. RNA was stored at -80 °C.

2.3.5 RNA quantification using spectrophotometry

RNA was quantified using the Nanodrop 8 sample spectrophotometer ND-8000 (Nanodrop Tech, DE, USA).

2.3.6 cDNA Synthesis

300 ng/µl of RNA from each treatment was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Massachusetts, USA). The cDNA-synthesis reaction was performed at 42 °C for 90 min. The reaction was stopped by denaturing at 70 °C for 10 min. See table 2.1.

Reagents	1 Reaction	Program
5X reaction Buffer	2 µl	42 °C 90 min
Ribolock	0.025 µl	70 °C 10 min
dNTP (10mM)	1 µl	
Revertaid	1 µl	
Random Hexamer	0.5 µl	
Total	4.5 µl	

Table 2.1: Components, volumes and program for cDNA synthesis

2.3.7 miRNA Reverse Transcription

First strand cDNA synthesis for miRNA using miRCURY LNA Universal RT microRNA PCR (Exiqon, Denmark) as per manufacturer's instructions

2.3.8 Real Time PCR

SYBR-Green (Bioline, UK) RT-qPCR analysis was performed on an Eco Real-Time PCR System (Illumina, San Diego, USA) as per manufacturer's instructions. cDNA was added to an Eco 48 well plate, compatible with the system. Analysis was performed for IL8, CXCL16, IL18 and TNFα using *GAPDH* as a reference gene. All real-time PCR calculations, reagents and conditions are shown in Table 2.2 below. Each reaction was performed in triplicate.

Reagents	**1 Reaction**	**Program**
SensiFast SYBR	5 µl	95 °C 15 min
Forward Primer (10µm)	0.4 µl	40 cycles of 1 min @
Reverse Primer (10µm)	0.4 µl	94 °C, 58 °C, 72 °C
cDNA	4.6 µl	
Total	10.4 µl	

Table 2.2: Components, volumes and program for qPCR analysis

2.3.9 microRNA PCR

MicroRNA PCR was carried out using miRCURY LNA Universal RT microRNA PCR (Exiqon) as per manufacturer's instructions on an Eco Real-Time PCR System (Illumina)

2.3.10 Statistical Analysis

Statistical analysis for all data was performed using Prism GraphPad version 5 and analysed using an unpaired t-test with Welch's correction (N = 3). Comparisons considered significant if $p < 0.05$.

2.4 Results

2.4.1 TNFα expression following pre- treatment for 3 h with epigenetic targeting agents

The effect epigenetic modifiers SFN (10 µM), SAHA (10 µM) and CURC (10 µM) have on TNFα expression was assessed prior to stimulation with TNFα (5 ng/µl, 10 ng/µl and 15 ng/µl) in THP-1 and EA.hy926 cell lines (Figure 2.1). The contribution of EtOH and DMSO was also analysed as a control, as these solvents were used to reconstitute SFN, SAHA and CURC. The resulting data suggest that treatment with epigenetic modifiers such as SFN, SAHA, CURC and common reconstitution agents DMSO and EtOH act to the decrease the expression of inflammatory marker TNFα, in EA.hy 926 cells lines following 3 h incubation (Figure 2.1). In THP-1 cells pre-treatment with SFN, SAHA, DMSO and CURC for 3 h does not increase TNFα expression, treatment with EtOH increased TNFα expression although not significantly (Figure 2.1, 3) (P = 0.3429), thus confirming the need to stimulate with TNFα to induce an inflammatory environment as is commonly observed in atherosclerosis.

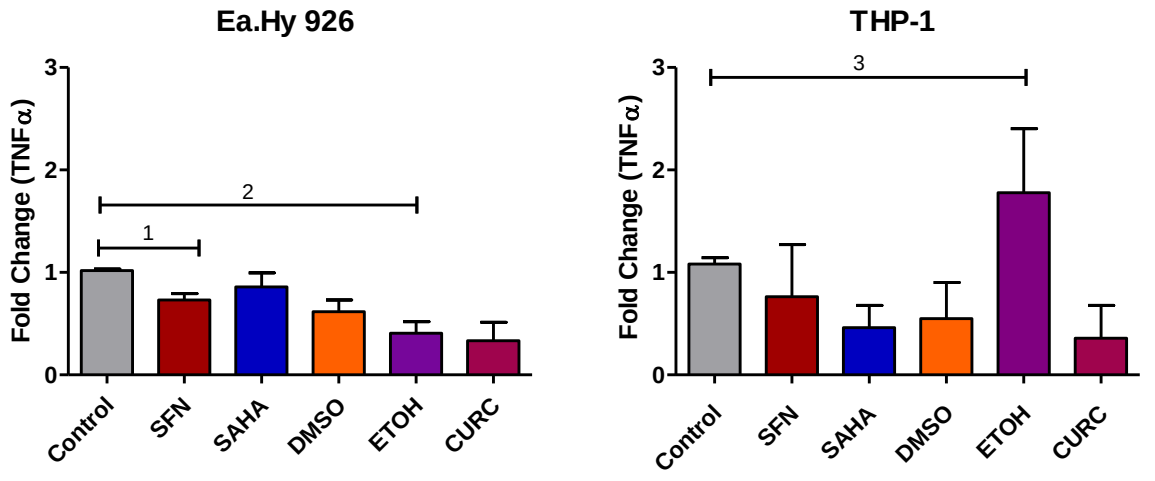

Figure 2.1: TNFα Induction following treatment (3 h) with SFN, SAHA, DMSO, ETOH, CURC (10 µM).

None of the treatments acted to significantly increase TNFα expression when compared to untreated control samples in either EA.hy 926 or THP-1 cells. In EA.hy 926 treatment with SFN (1) (P= 0.0472) and EtOH (2) (P=

0.0319) acted to significantly decrease TNFα expression. THP-1 cells demonstrated reduced TNFα expression following treatment with SFN, SAHA, DMSO and CURC. Treatment with EtOH increased TNFα expression although not significantly (3) (P = 0.3429).

2.4.2 *CXCL16, IL18 and IL8* expression following treatment (3 h) with epigenetic targeting agents.

The impact of pre-treatment for 3 h with SFN (10 µM), SAHA (10 µM), CURC (10 µM), DMSO and EtOH on the expression of *CXCL16*, *IL18* and *IL8* was investigated (Figure 2.2), prior to stimulation experiments with indicated amounts of TNFα. In EA.hy 926 cells the expression of *IL18* and *IL8* was upregulated following treatment with ETAs (SFN, SAHA, CURC, DMSO and ETOH) and also with the carrier solvents DMSO and EtOH (Figure 2.2). *CXCL16* expression was decreased following all treatments but showed a significant reduction following treatment with EtOH (Figure 2.2, 2) (P=0.0174). THP-1 cells demonstrated reduced expression of *CXCL16* and *IL8* following treatments, with SFN significantly reducing the expression of both genes (Figure 2.2, 5 and 6 respectively) (P=0.0127, P=0.0089). *IL8* expression was also significantly decreased following treatments SAHA (P=0.0003), CURC (P=0.0012), DMSO (P=0.0002), EtOH (P=0.0239). In contrast, an increase in *IL18* expression was recorded in response to treatment with ETAs and vechicle controls but these increases were not significant (Figure 2.2, 7).

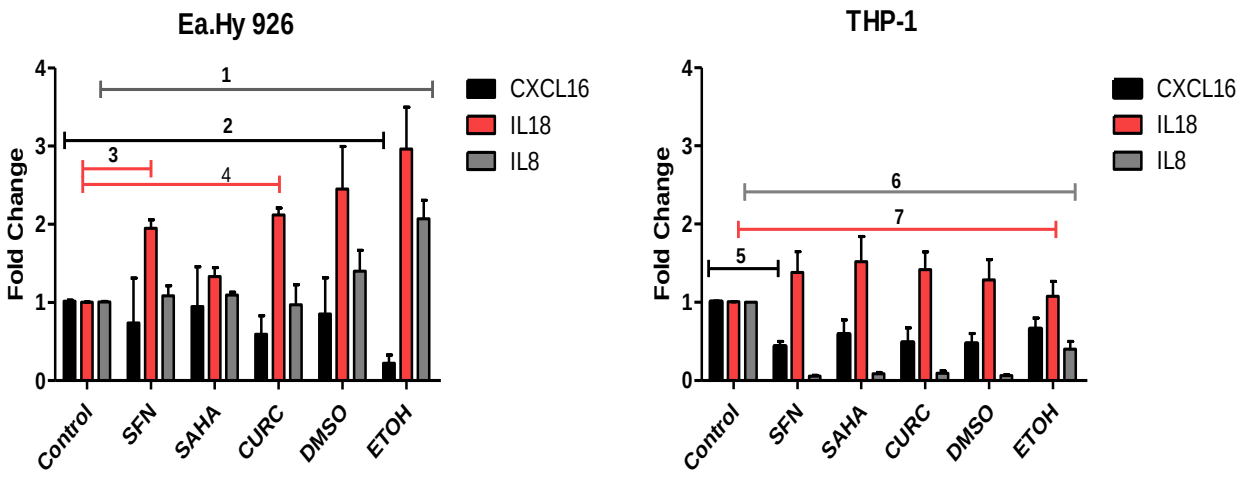

Figure 2.2: Inflammatory gene expression (*CXCL16, IL18, IL8*) following treatment with epigenetic modifiers (SFN, SAHA, CURC, DMSO, EtOH (10 µM)).

In EA.hy 926 cells treatment with EtOH significantly decreased *CXCL16* (2) (P=0.0174) and increased *IL8* (1) (P=0.0460) expression. Treatment with SFN (3) (P=0.0174) and CURC (4) (P=0.0058) acted to significantly increase *IL18* expression. THP-1 differentiated cells showed a significant decrease in *CXCL16* expression upon treatment with SFN (5) (P=0.0127). *IL8* expression was significantly decreased after all treatments (6) SFN (P=0.0001), SAHA (P=0.0003), CURC (P=0.0012), DMSO (P=0.0002), EtOH (P=0.0239). *IL18* expression was increased following all such treatments (7) but not significantly.

2.4.3 Investigation into the expression of *CXCL16, IL8* and *IL18* following pre-treatment with SFN or SAHA and stimulation with TNFα over 4, 16 and 24 h in THP-1 cell line

To determine the potential of epigenetic targeting agents in atherosclerosis and the role they may play in reducing inflammatory gene expression, the effect of SFN, was assessed by pre-treating the cells with SFN (10 µM) for 3 h, prior to stimulation with 5 ng/µl, 10 ng/µl, and 15 ng/µl of TNFα, a known inducer of inflammatory gene expression. This activity was also investigated with the more specific FDA approved HDACi SAHA (10 µM) under identical conditions (Figure 2.3).

Pre-treatment with SAHA followed by 4 h stimulation with TNFα prevented an increase in *CXCL16* expression, compared to levels induced by TNFα alone at 5, 10 and 15 ng/µl. SFN acts in a similar manner but is not as effective (Figure 2.3, A). Pre-treatment with SAHA and SFN followed by 16 h stimulation with 5 and 15 ng/µl of TNFα reduced *CXCL16* when compared to levels induced by TNFα alone (Figure 2.3, B). Pre-treatment with SFN and stimulation with 10 ng/µl of TNFα for 16 h increased *CXCL16* when compared to unstimulated cells (P=0.0108) (Figure 2.3, B, 1). TNFα stimulation following pre-treatment of SAHA in EA.hy 926 cells for 24 h, lead to a significant increase in *CXCL16* expression when subsequently stimulated with TNFα 5ng/µl (P=0.0154), TNFα 10ng/µl (P=0.0055) (Figure 2.3, C, 2+3). SFN pre-treatment also significantly increased *CXCL16* expression following stimulation with 15ng/µl of TNFα (P=0.0889) (Figure 2.3, C, 4).

IL8 expression (Figure 2.4) following pre-treatment with SAHA in the presence or absence of TNFα increased across all time points measured when compared with *IL8* expression induced by TNFα alone over the same time course and TNFα amounts. This induction was significant at 24 h, SAHA+ TNFα 5 ng/µl (P=0.0025) and SAHA+TNFα 10 ng/µl (P=0.0054) (Figure 2.4, C, 2+3). The response induced by SFN varied from reducing *IL8* at 4 h (Figure 2.4, A), to increasing it at 16 h (Figure 2.4, B). At 24 h, SFN pre-treatment only reduced *IL8* expression upon stimulation with TNFα 5 ng/µl (Figure 2.4, C).

IL18 expression (Figure 2.5) after 4 h was not greatly altered by treatment with SFN or SAHA or by TNFα alone, with the exception of pre-treatment with SFN followed by stimulation with 5 ng/µl of TNFα (P=0.0292) (Figure 2.5, A, 1) which increased expression. SAHA treatment reduced *IL18* expression at 16 h, with the exception of SAHA+TNFα 15 ng/µl (Figure 2.5, B, 2). The effects of SFN varied at both 16 and 24 h from reducing to increasing expression.

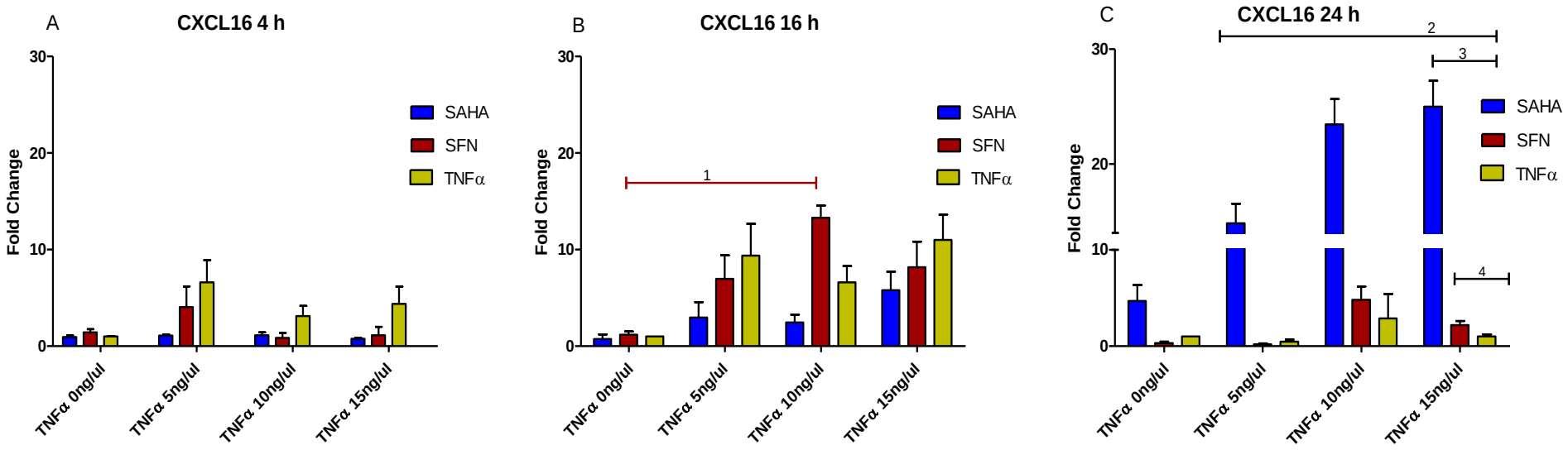

Figure 2.3: Time and dose-dependent effect of TNFα on CXCL16 expression in EA.hy cells pre-treated with ETAs

CXCL16 expression following pre-treatment (3 h) with either SAHA or SFN followed by stimulation with 5 ng/µl, 10 ng/µl, 15ng/µl TNFα over 4, 16 and 24 h. (A) At 4 h the effect of SFN and SAHA was to decrease CXCL16 expression following stimulation with all amounts TNFα. (B) At 16 h all treatments accompanied by TNFα stimulation increased *CXCL16* expression above levels observed in untreated and SAHA/SFN alone cells (No TNFα stimulation). Pre-treatment with SFN and stimulation with 10 ng/µl of TNFα for 16 h increased *CXCL16* when compared to untreated cells (P=0.0108) (1). (C) Pre-treatment with SAHA increased *CXCL16* expression in dose-dependent manner when compared to the effects of treatment with TNFα alone, a significant increase in expression was noted at 5 ng/µl, 10 ng/µl and 15 ng/µl (P=0.0154,0.0055,0.0104 respectively) (2+3). SFN pre-treatment followed by stimulation with TNF 15 ng/µl for 24 h also induced greater *CXCL16* levels that was observed by treatment with 15 ng/ul of TNFα alone (P=0.0007) (4).

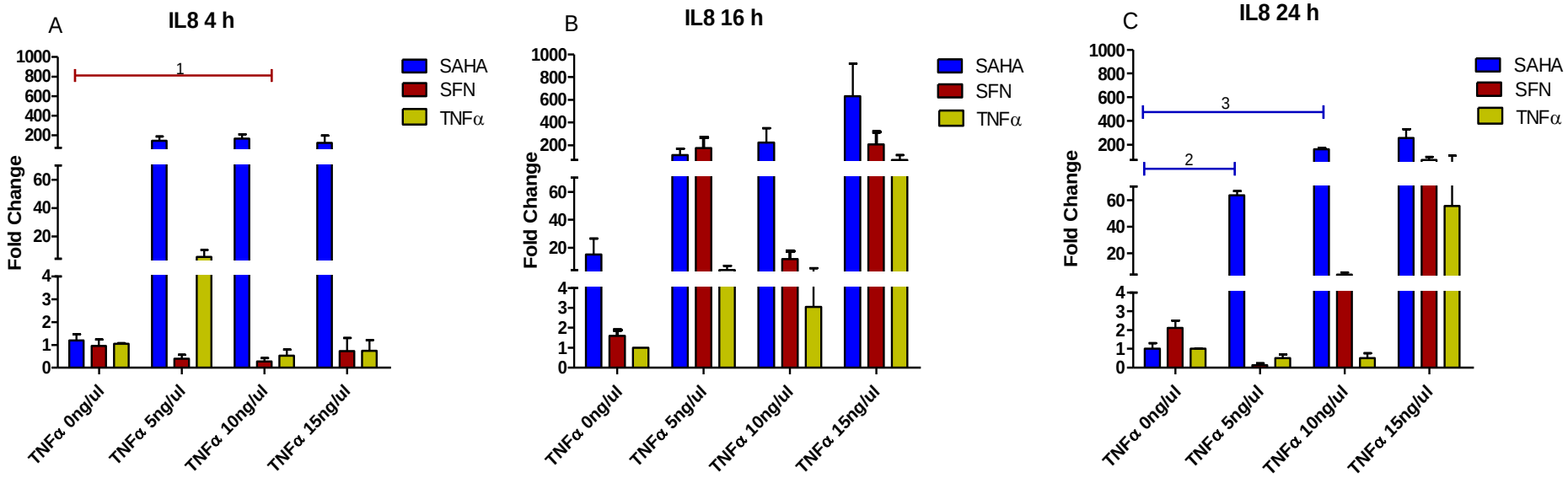

Figure 2.4: Time and dose-dependent effect of TNFα on IL8 expression in EA.hy cells pre-treated with ETAs

IL8 expression following pre-treatment (3 h) with either SAHA or SFN followed by stimulation with 5, 10 15 ng/µl TNFα over 4, 16 and 24 h. (A) SFN pre-treatment followed by 4 h TNFα stimulation reduced the inflammatory response induced by TNFα, significantly reducing *IL8* expression at TNFα 10 ng/µl when compared to untreated cells (P=0.0430) (1). (B) SFN and SAHA pre-treatment followed by 16 h TNFα stimulation increased *IL8* expression. (C) 24 h stimulation with TNFα saw SAHA increase *IL8* expression across all amounts of TNFα, dependent on the amount of TNFα. This increase is significant following pre-treatment and stimulation with 5 ng/µl and 10 ng/µl (P=0.0025, 0.0054) (2+3). Pre-treatment with SFN increases *IL8* across all stimulations with the exception of TNFα 5 ng/µl, an increase in *IL8* expression is observed in response to increasing amounts of TNFα and is noted from 5 ng/µl to 15 ng/µl.

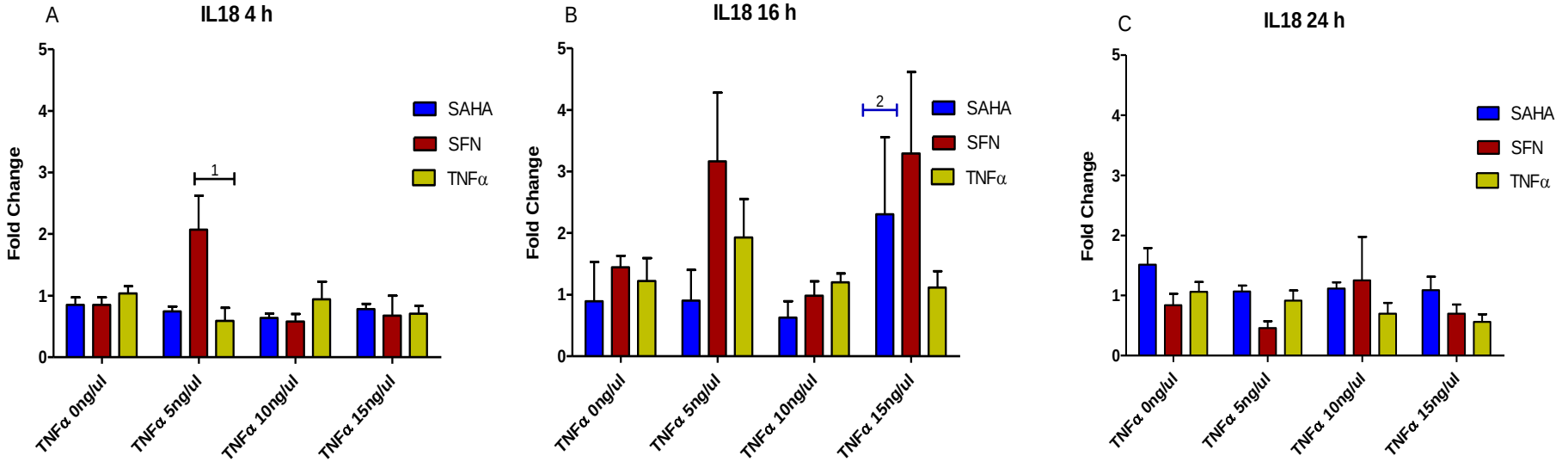

Figure 2.5: Time and dose-dependent effect of TNFα on IL18 expression in EA.hy cells pre-treated with ETAs

IL18 expression following pre-treatment (3 h) with either SAHA or SFN followed by stimulation with 5, 10 15 ng/µl TNFα over 4, 16 and 24 h. (A) SAHA and SFN pre-treatment does not greatly alter *IL18* expression upon stimulation with TNFα, excluding SFN and TNFα 5 ng/µl whereby SFN significantly increases *IL18* expression above levels induced by 5 ng/µl of TNFα alone (P=0.0292) (1). (B) Pre-treatment with SAHA reduces *IL18* expression at TNFα 0, 5 and 10 ng/µl. increased expression as a result of SAHA treatment is noted at TNFα 15 ng/µl. The effect of SFN varies depending on the amount of TNFα used, increasing *IL18* at TNF 0, 5 and 15 ng/µl and reducing *IL18* at 10 ng/µl. (C) At 24 h SAHA acts to increase *IL18* expression across all stimulations of TNFα. The effect of SFN varies depending on the amount of TNFα, with greater amounts 10 ng/µl and 15 ng/µl increasing *IL18* expression above levels induced by TNFα alone.

2.4.4 Investigation into the expression of *CXCL16, IL8* and *IL18* following pre-treatment with SFN or SAHA and stimulation with TNFα over 4, 16 and 24 h in THP-1 cell line

An analysis of *CXCL16*, *IL8* and *IL18* under identical conditions previously outlined (Section 2.4.3) was undertaken on differentiated THP-1 cells. Treatment with SFN lowered TNFα induced expression of *CXCL16* following 4h treatment with 10 ng/µl and 15 ng/µl when compared to expression levels induced by treatment with TNF 10 ng/ul and 15 ng/ul alone (Figure 2.6, A, 1,2,3). Pre-treatment with SAHA alone (3 h) acted to increase *CXCL16* expression to levels greater than those observed in untreated cells and SFN pre-treatment (3 h) (Figure 2.6, A, 4). 16 h stimulation with TNFα saw *CXCL16* expression increase in response to increasing amounts of TNFα. HDACis SFN and SAHA acted to match or lower *CXCL16* expression induced by TNFα, with the exception of SFN+TNF 10 ng/µl whereby SFN increased expression (Figure 2.6, B). Once more at 24 h an increase in *CXCL16* expression was noted in response to increasing amounts of TNFα, with SFN and SAHA acting to reduce this effect when combined with greater amounts of TNFα e.g. SFN+TNF 15 ng/µl (P=0.0833), SAHA+TNF 10 ng/µl (P=0.0375), SAHA+TNF 15 ng/µl (P=0.0399) (Figure 2.6, C, 5,6,7).

In relation to *IL8*, TNFα acted once more in an dose-dependent manner to increase *IL8* expression, following 4, 16 and 24 h stimulation with varying amounts of TNFα (5,10 and 15 ng/µl)(Figure 2.7, A,B,C). SAHA pre-treatment effectively reduced the effect induced by TNFα, restoring *IL8* levels (Figure 2.7, A, B, C). The effect of SFN on *IL8* expression varied, with lower amounts of TNFα inducing greater *IL8* expression when combined with TNFα, significantly at 5 ng/µl TNFα (P=0.0457) (Figure 2.7, A, 1). The strength of such induction decreased with increasing amounts of TNFα, SFN+TNFα 15 ng/µl (P=0.4408) (Figure 2.7, A, 2).The effects of stimulation with TNFα for 16 h are decreased when pre-treated with SFN although not as

effectively as SAHA (Figure 2.7, B). 24 h stimulation with TNFα when combined with SFN pre-treatment increases *IL8* expression, the opposite of which can be noted following pre-treatment with SAHA (Figure 2.7, C).

SAHA effectively reduces the expression of *IL18* both when used alone and as a pretreatment (Figure 2.8, A, B, C). SFN is not as consistent, having both a beneficial and detrimental effect on *IL18* expression depending on the amount of TNFα used (Figure 2.8, A, B, C).

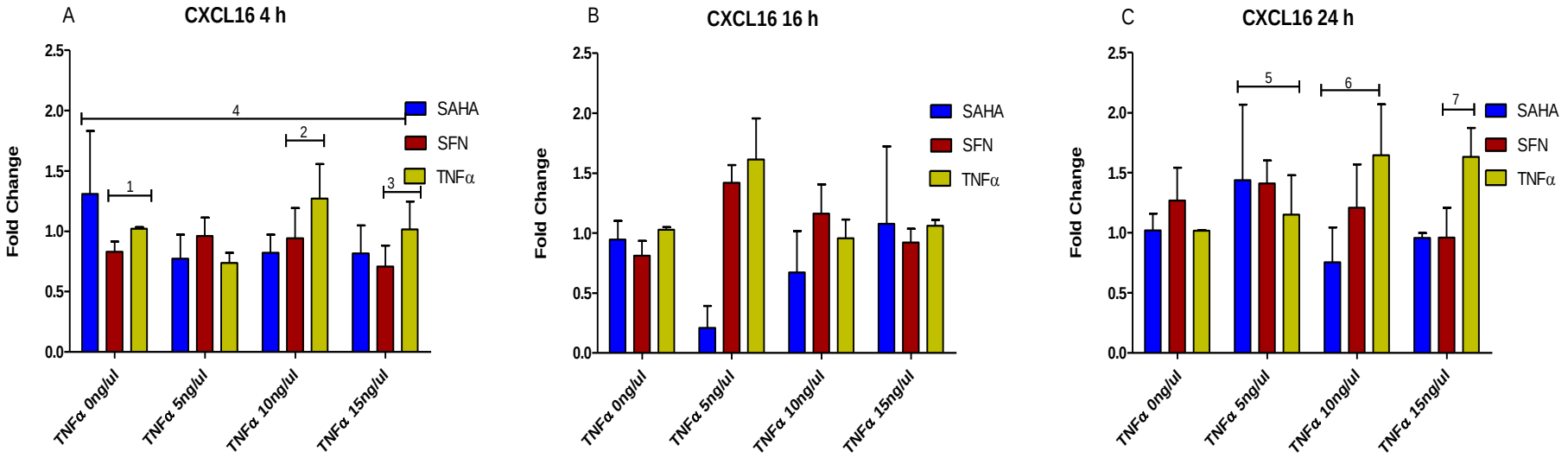

Figure 2.6: Time and dose-dependent effect of TNFα on CXCL16 expression in THP-1 cells pre-treated with ETAs

CXCL16 expression following pre-treatment (3 h) with either SAHA or SFN followed by stimulation with 5, 10 1 5ng/µl TNFα over 4, 16 and 24 h. (A)Treatment with SFN acts the decrease the inflammation induced by TNFα in all treatments except 5 ng/ul of TNFα. SAHA except when used alone matches and/or reduces *CXCL16* expression induced by TNF (B) Following 16 h stimulation a mixed response is observed depending the amount of TNFα used for both SAHA and SFN. (C) The effect of pre-treatment with TNFα varies depending on the amount used, SAHA pre-treatment prior to stimulation with 10 ng/µl and 15 ng/µl of TNFα reduced *CXCL16* expression when compared to effect of 5 ng/µl and 10 ng/µl of TNFα alone (P=0.1338,0.0399). SFN pre-treatment prior to stimulation with 15 ng/µl of TNFα reduced *CXCL16* expression when compared to the effect 15 ng /µl of TNFα alone (P=0.0833).

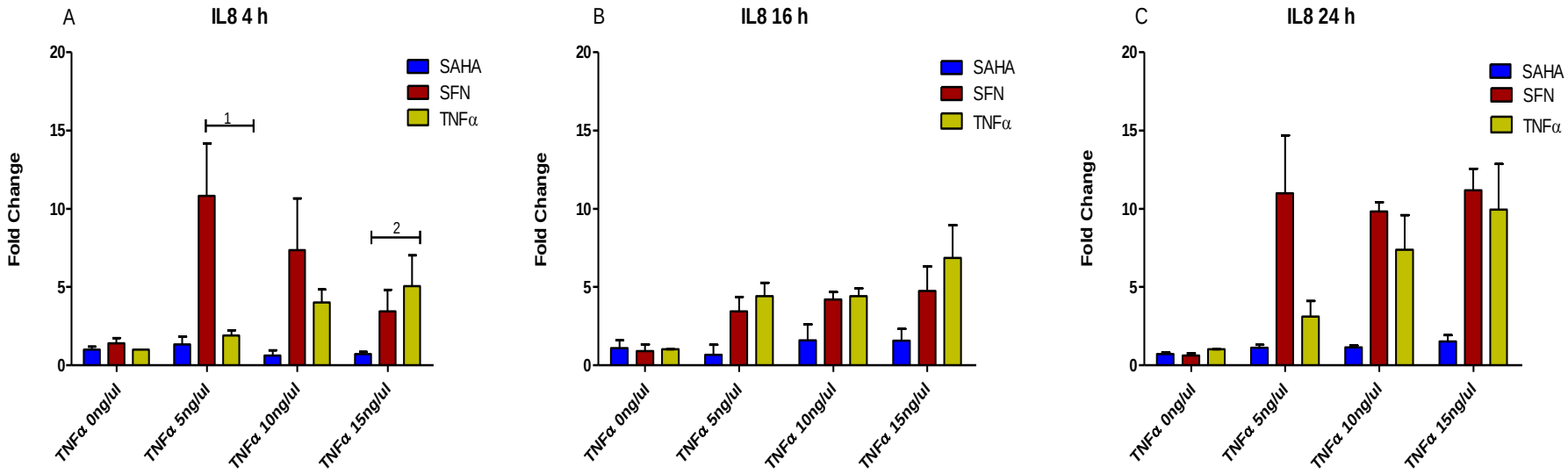

Figure 2.7: Time and dose-dependent effect of TNFα on IL8 expression in THP-1 cells pre-treated with ETAs

IL8 expression following pre-treatment (3 h) with either SAHA or SFN followed by stimulation with 5, 10 15 ng/µl TNFα over 4, 16 and 24 h.(A) Pre-treatment with SAHA effectively lowered IL8 expression when compared to levels induced by TNFα across all stimulations. The effect of SFN on *IL8* expression varied, with less TNFα inducing greater IL8 expression when combined with TNFα, significantly at 5 ng/µl TNFα (P=0.0457) the strength of such induction decreased with increasing amounts of TNFα, SFN+TNFα 15 ng/µl (P=0.4408) (Figure 2.7, A, 2). (B) SFN and TNFα appear to increase *IL8* in a manner dependent on the amount of TNFα used. SFN reduced *IL8* induction at 15 ng/ul when compared to levels induced by 15 ng/µl of TNFα alone. SAHA reduces *IL8* expression across all stimulations of TNFα. (C) The combination of SFN and TNFα greatly increase *IL8* expression, SAHA decreases expression in response to induction with TNFα significantly at 15 ng/µl when compared to levels induced by TNFα 15 ng/µl alone.

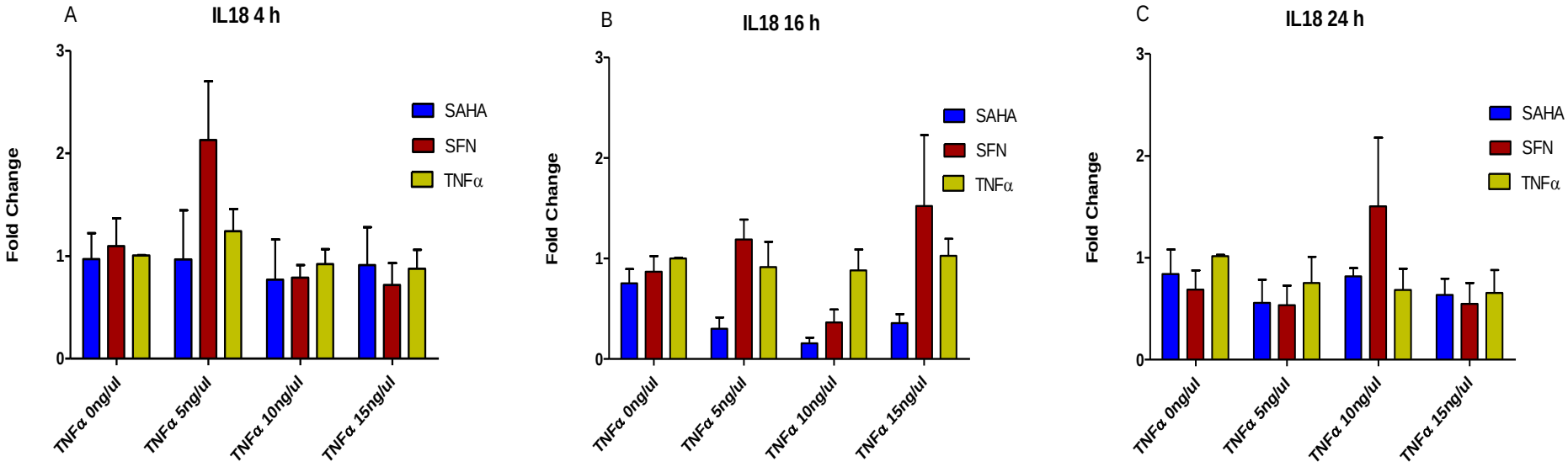

Figure 2.8: Time and dose-dependent effect of TNFα on IL18 expression in THP-1 cells pre-treated with ETAs

IL18 expression following pre-treatment (3 h) with either SAHA or SFN followed by stimulation with 5, 10 15 ng/µl TNFα over 4, 16 and 24 h. (A) The effect of pre-treatment with SFN or SAHA varies across all amounts of TNα, pre-treatment with SAHA and stimulation with TNFα don't affect levels greatly when compared to levels observed in untreated cells. (B) SAHA reduces *IL8* expression across all stimulations of TNFα, the effect of SFN varies across stimulation from reducing to increasing expression. (C) At 24 h the effect of pre-treatment with SAHA or SFN does not greatly alter IL18 expression when compared to untreated cells, with the exception on SFN and TNF 10 ng/ul.

2.4.5 miRNA expression following pre- treatment for 3 h with epigenetic targeting agents

The expression levels of the miRNAs, miR210 and miR145, following pre-treatment with SFN (10 µM), SAHA (10 µM) and DMSO were measured under the indicated amounts of TNFα stimulation in the THP-1 and EA.hy926 cell lines. THP-1 cells did not produce any detectable levels of miR145 but did express the 5s ribosomal RNA therefore, results could only be obtained for miR210. Levels of miR210 are not significantly altered following treatment with SFN or SAHA when compared to levels expressed in untreated control cells. Treatment with DMSO alone increased miR210 expression levels although not significantly (P=0.075) (Figure 2.9, A, 1). Levels of miR210 and miR145 could be detected in EA.hy 926 cells. DMSO treatment increased miR210 expression in EA.hy 926 (P=0.0069) (Figure 2.9, B, 2), whilst decreasing the amount of miR145 expression below that of untreated cells following 3 h incubation (P=0.0024) (Figure 2.9, B, 3). SFN acted to increase levels of miR210 and miR145, while SAHA reduced expression however, this was not significant.

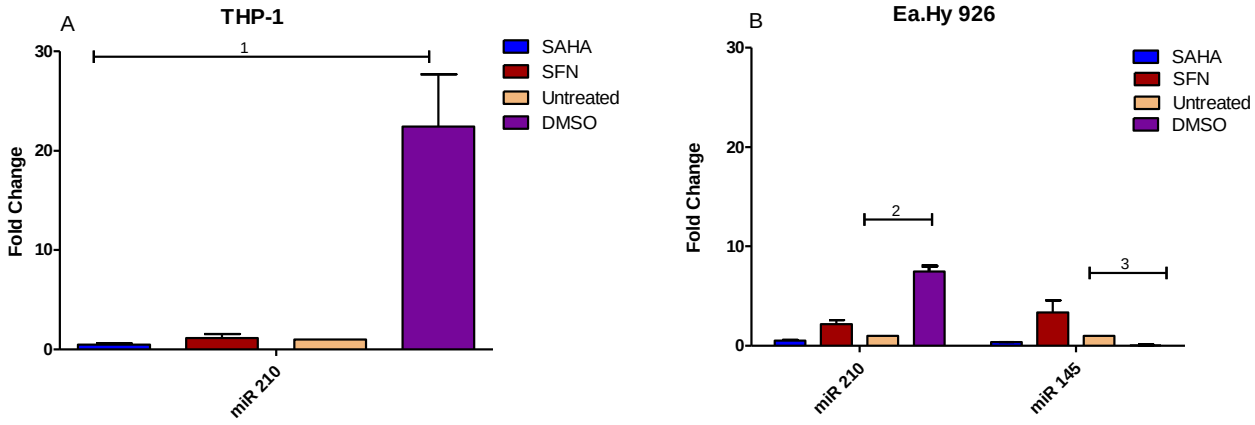

Figure 2.9: miR210 and 145 Induction following treatment (3 h) with SFN, SAHA and DMSO.

The expression of miR210 appears to be highly susceptible to treatment with DMSO in both cell THP-1 and EA.hy926 cell lines. Treatment with DMSO (10µM) alone increased miR210 expression levels although not significantly (P=0.075) (A, 1). DMSO treatment increased miR210 expression in EA.hy926 (P=0.0069) (B, 2), whilst

decreasing the amount of miR145 expression below that of untreated cells following 3 h incubation (P=0.0024) (B, 3). SFN acted to increase slightly the expression of miR210 and 145 in both cell lines although not significantly. SAHA reduced miRNA expression in both cell lines but was not significant.

2.4.6 Is the expression of miR145 and miR210 altered by HDACi pre-treatment with SFN or SAHA and stimulation with TNFα over 4, 16 and 24 h in the EA.hy 926 cell line?

4 and 16 h stimulation with TNFα (5 ng/µl, 10 ng/µl, and 15 ng/µl) increased levels of miR145 in a manner dependent on the amount of TNFα and in most instances stimulation with TNFα induced the lowest levels of miR145 and miR210 when compared against levels induced following pre-treatment with SFN or SAHA (Figure 2.10, Figure 2.11). The activity of HDACi seems to be more apparent when used in combination with greater amounts of TNFα (15 ng/µl), decreasing levels of miR145 (Figure 2.10, TNF 15 ng/µl).

The lowest expression levels of miR210 were noted following pre-treatment with SFN and SAHA alone, followed by 4 and 16 h incubation (Figure 2.11, A, B). SAHA acts in a TNFα dependent manner at 4 h to increase miR210 levels (Figure 2.11, A). SFN and TNFα have an inverse relationship at 4 h, with TNFα stimulation alone decreasing miR210 expression at 10 ng/µl of TNFα. When 10 ng/µl of TNFα is used in combination with SFN miR210 expression is increased although not significantly (Figure 2.11, A).

SAHA when used alone followed by 16 h incubation in medium only reduced miR210 expression. This is also observed when followed by TNFα stimulation, inducing the lowest expression levels of all treatment combinations (Figure 2.11, B). 24 h stimulation with TNFα sees SAHA pre-treatment increase the expression of miR210 up to 10 ng/ul of TNFα then reduces the expression following stimulation with 15 ng/ul of TNFα for 24 h (Figure 2.11, C).

Thus, confirming that the activity of HDACi's SFN and SAHA can vary between cell lines, amounts of TNFα and the duration of exposure to TNFα.

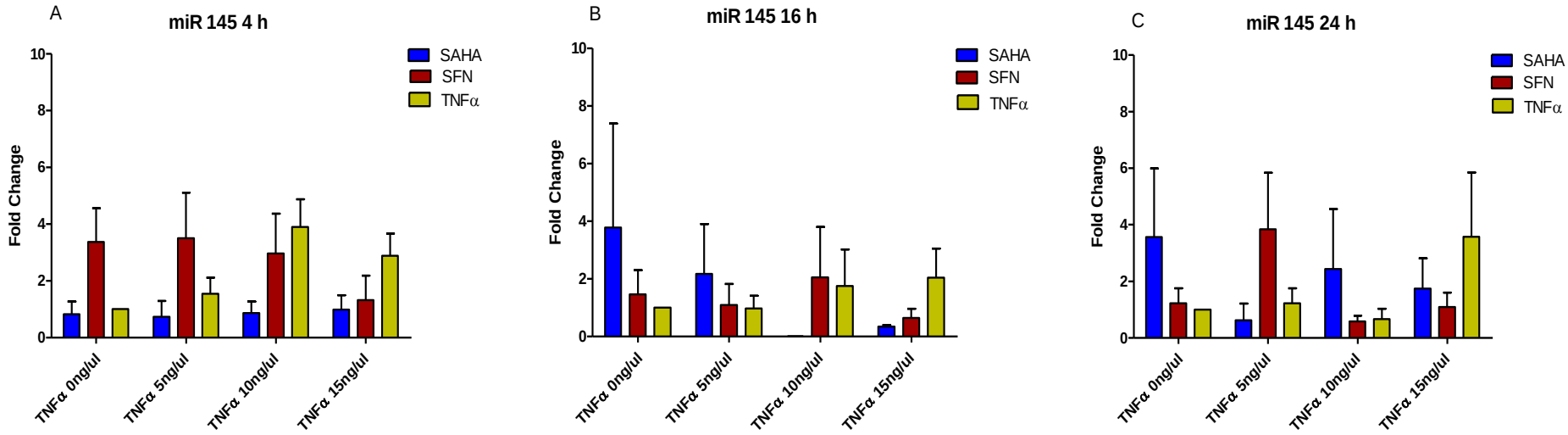

Figure 2.10: EA.hy 926 expression of miR145 following pre-treatment with SFN/SAHA followed by stimulation with TNFα 5, 10 and 15 ng/µl.

(A) An inverse relationship between SFN and TNFα can be noted, as the amount of TNFα increases so does miR145 expression, in contrast to this as the amount of TNFα decreases samples pre-treated with SFN express greater miR145. The effect of SAHA of miR145 remains constant across all amount of TNFα. (B) At 16 h TNFα treatment acts in a similar manner to 4 h. SAHA acts in the opposite manner to TNFα, when used alone the greatest induction of miR145 can be noted. When used as a pretreatment before TNFα stimulation SAHA acts to decrease miR145 expression, in a manner dependent on the amount of TNFα used. (C) The effect on TNFα on miR145 expression acts once more in a manner dependent on the amount of TNFα. The effect of miR145 due to SFN and SAHA varies depending on the amount of TNFα used.

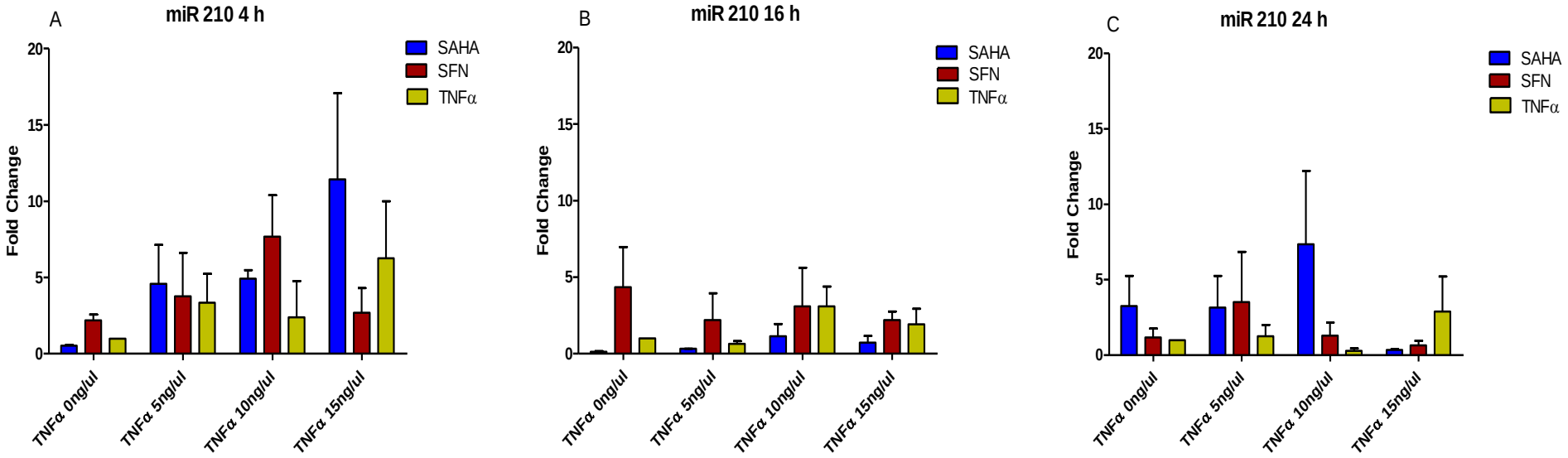

Figure 2.11: EA.hy 926 expression of miR210 following pre-treatment with SFN/SAHA followed by stimulation with TNFα 5, 10 and 15 ng/µl.

(A) A gradual increase in the expression of miR210 can be noted with increased amounts of TNFα and when pre-treated with either SFN or SAHA. Expression of miR210 as a result of SAHA pretreatment increases in response to the amount of TNFα used. (B) At 16 h an overall decrease in miR210 expression is noted, with SFN acting as the most potent inducer of expression at this time point. (C) At 24 h the activity of SFN, SAHA and TNFα varies in response to the amount of TNFα used, with pretreatment acting to increase levels of miR210 when compared to levels induced by TNFα alone with the exception of 15 ng/µl.

2.4.7 Is the expression of miR210 altered by HDACi pre-treatment with SFN or SAHA and stimulation with TNFα over 4, 16 and 24 h in THP-1 cell lines

The treatment of activated THP-1 cells with SFN did not greatly alter the expression of miR210 following 4 h stimulation with TNFα 5,10 and 15 ng/µl, exceptions were noted by increased miR210 expression following treatment with TNFα 10 ng/µl and when pre-treated with SAHA followed by stimulation with 15 ng/µl of TNFα (Figure 2.12, A). Following 16 h stimulation with TNFα the expression of miR210 increased in response to increasing TNFα amounts, a similar response was noted in SFN pre-treated cells prior to TNFα stimulation although induction levels where lower (Figure 2.12, B). The effect of SAHA followed by 16 h TNFα stimulation varied from decreasing to increasing miR210 expression levels across all stimulations with TNFα (Figure 2.12, B). 24 h stimulation with TNFα appears to lower miR 210 expression depending the amount of TNFα used (Figure 2.12, C). SAHA acts to lower miR210 levels below those observed in untreated cells, an effect which is maintained throughout TNFα stimulation. Untreated cells at 24 h exhibited the highest expression of miR210 (Figure 2.12, C). As none of the data described above was found to be significant, caution should be exercised when interpreting them.

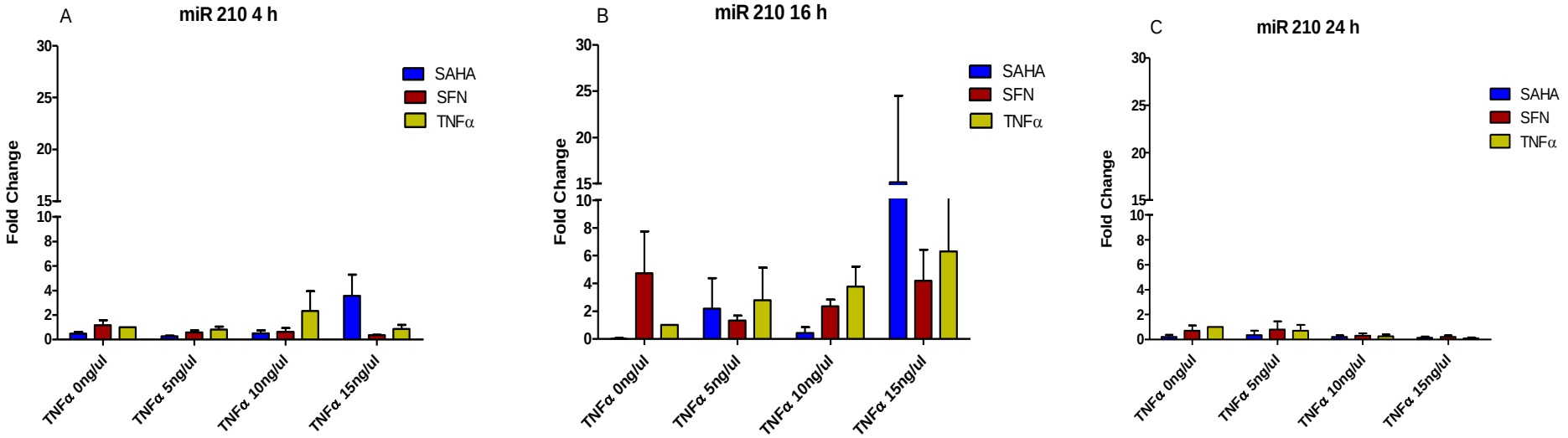

Figure 2.12: THP-1 expression of miR210 following pre-treatment with SFN/SAHA followed by stimulation with TNFα 5, 10 and 15 ng/µl.

The expression of miR210 in THP-1 cells following pretreatment with SFN/SAHA and stimulation indicated amounts of TNFα shows a varied response both within time points and across time points. (A) The expression of miR210 is greatly altered to levels above or below those observed in untreated cells. Non-significant increases can be noted when treated with 10 ng/µl and when pre-treated with SAHA followed by stimulation with 15 ng/µl of TNFα. (B) The strongest induction of miR210 can be noted at this time point. miR210 expression increases in response to increasing amounts of TNFα. With an inverse relationship being noted between SFN and SAHA, pretreatment with SFN increases expression whilst pretreatment with SAHA reduces miR145 expression. (C) At 24 h very little if any miR210 is detectable with induction levels remaining roughly the same across all amounts of TNFα used.

2.5 Discussion

In this chapter we aimed to investigate the effect of HDACi SFN and SAHA on vascular endothelial health, as the endothelium is the initial starting point from which atherosclerosis develops and therefore is an important target in disease prevention. To that end, the HDACi activity of SFN and SAHA was assessed in two cell lines; EA.hy 926 (Endothelial) and PMA differentiated THP-1 cells (Macrophage), in order to contribute to our understanding with regard to the use of SFN and SAHA as anti-inflammatory agents in the diet and potentially in the clinic.

To create an *in vitro* inflammatory environment, similar to that observed in atherosclerosis, cells were stimulated using an optimised range of amounts of TNFα over a defined time course. This state of inflammation, in turn induces the upregulated expression of key inflammatory genes associated with atherosclerosis; *CXCL16*, *IL8* and *IL18* along with associated miRNAs, miR210 and miR145.

In EA.hy 926 cells, SFN consistently lowered the expression *of CXCL16, IL8* and *IL18* following 4 h of stimulation with 10 ng/µl of TNFα and 24 h with 5 ng/µl of TNFα. However, SFN also increased the expression of these genes following 24 h stimulation with 10 and 15 ng/µl of TNFα. It is also of note that 3 h of SFN pre-treatment and 16 h of incubation with TNFα increased the expression of *IL8, IL18* and *CXCL16* in comparison to untreated control cells. The opposite of which can be noted in THP-1 cell line, at the 16 h time point cells pre-treated with SFN only, showed a down regulation of the above genes. Stimulation with 15 ng/µl of TNFα for 4 h following pre-treatment with SFN decreased *CXCL16, IL8* and *IL18* expression.

HDACi SAHA was used in addition to SFN as it is approved by the FDA and already used in the clinic for the treatment of CTCL. Furthermore, issues remain to be resolved as to the specificity

SFN and determining whether the anti-inflammatory effects observed are due to its activity as a HDACi or anti-oxidant. Overall, when compared to the effects of SFN in this study SAHA performs better as a HDACi, which is likely to due to its greater specificity. In EA.hy926 cells, SAHA pre-treatment lowers *CXCL16* expression following 4 and 16 h stimulation with TNFα regardless of the amount used. At 24 h however, SAHA pretreatment acts to increase *CXCL16* expression levels above levels induced by TNFα. As the amount of TNFα used increases so to does *CXCL16* expression. IL8 expression appears to be further induced by pre-treatment with SAHA, once more depending on the amount of TNFα. When compared against SFN for activity against *IL8* SAHA is not as effective at decreasing IL8 expression, with SFN decreasing IL8 expression effectively at 4 h. IL18 expression in EA.hy 926 cells does not appear to be greatly reduced by pre-treatment with SFN/SAHA or by subsequent stimulation with TNFα. THP-1 cells react more consistently in relation to the dampening activity of SAHA on inflammatory gene expression, with pretreatment followed by stimulation with TNFα decreasing the expression of *CXCL16, IL8* and *IL18* across all variables. In addition to inflammatory gene expression, the activities of SFN and SAHA pre-treatment followed by TNFα stimulation on the expression of miR210 and miR145 was also assessed in the same samples, the results of which varied across time points and the amount of TNFα used.

This analysis reveals a complex relationship between the HDACis SFN, SAHA and TNFα, where different permutations of time and concentration yield variable expression levels of key inflammatory markers of atherosclerosis. It must be noted that although SFN/SAHA significantly lowered the expression of *CXCL16, IL8* and *IL18* in some instances, it also significantly increased them in others. This observation is supported by a study carried out by Halil *et al*, in which the pro/ anti- inflammatory effects of broad spectrum HDACis such as TSA

and SAHA were identified to be separable over a concentration range, with differential effects being observed on macrophage inflammatory responses to LPS (Halili et al., 2010). This study by Halil suggests a dual role for HDACis TSA and SAHA in cellular proliferation and the negative regulation of inflammatory gene expression, once more suggesting the effect of HDACi is dependent on the pre-existing inflammatory environment and the gene of interest. Pre-treatment with 10 μM SAHA significantly reduced TNFα expression and prevented the nuclear translocation of Nf-kb in LPS stimulated mouse macrophages, with similar results being noted after 3 h pre-treatment with TSA or SAHA (Bode et al., 2007; Chong et al., 2012).

However, SFN still is an attractive HDACi option as it can be easily obtained from the diet and has little or no toxicity, proving an easily accessible preventative option in cardiovascular treatment. THP-1 cells pre-treated with SFN (1-5 ug/ml) for 2 h, inhibited TNFα dose-dependent induced adhesion of THP-1 monocytic cells and protein expression of VCAM1 (Kim et al., 2012). In EA.hy 926 cells, protection was induced by SFN upon lysophosphatidiylcholine (LPC) induced injury however, whether this was due to its activity as a HDACi or antioxidant remains to be determined (Li et al., 2015). Other studies have also shown the ability of SFN to reduce vascular inflammation (Chen et al., 2009; Kim et al., 2012; Kwon et al., 2012; Rubanyi, 1988; Xue et al., 2008; Zhu et al., 2008). It must be noted that although many studies suggest a protective role for SFN in CVD, the physiological relevant dose at which to induce an optimum response remains elusive. It has been suggested that achievable plasma SFN levels in rodents and humans is < 2 μm following consumption, which may not be sufficient to induce a response, requiring alternative delivery methods to reach efficacy (Hanlon et al., 2009; Li et al., 2010; Riedl et al., 2009; Ye et al., 2002a). It is likely that peak plasma levels of SFN are

obtained within 1.5 h, with levels reaching around 50 % 3 h following consumption (Hanlon et al., 2009; Ye et al., 2002b).

Furthermore, inter-individual variation is also likely to play a role in response to treatment. For instance, patients with CVD may have a variable range of TNFα in serum which could influence the concentration of SFN necessary to induce a protective response based on the described above cell line work. Numerous studies suggest that the physiological levels of TNFα in plasma, mouse tissue and in serum are in reality much lower than those used to induce an experimental response, typically ~10 ng/ml but have been shown to reach greater levels in CAD patients (Damas et al., 1989; Matalka et al., 2005; Nakai et al., 1999; Patel et al., 2009; Turner et al., 2010). A further consideration is that the ability of human gut microflora to hydrolyse glucopahrin and SFN for absorption in the large intestine varies among individuals (Glade and Meguid, 2015). This, along with TNFα levels, should be taken into prior consideration when trialling such a therapy.

The inflammatory markers investigated in this study have a known association with atherosclerosis. Translating epigenetic-altering treatments from the lab to the bedside would encounter a number of obstacles not accounted for in cell line studies. The effects of these treatment in patients may vary throughout the various stages of disease development; and the timing of patient sampling may determine the candidate set of markers used. Genetic variants and gender have also been shown to influence the expression of inflammatory genes in CVD patients (Aslanian and Charo, 2006; Lundberg et al., 2005; Velásquez et al., 2014; Vogiatzi et al., 2008; Zhang et al., 2011).

2.6 Limitations and Future work

This study was carried out in cell lines, which are not an ideal model due to alterations in gene expression patterns and genomic instability observed over time (Landry et al., 2013). This study also does not take into account the effect such treatments may have on other cell types commonly affected by atherosclerosis such as T cells, dendritic cells, mast cells, and SMCs. Furthermore, passage number and the effect of DMSO on gene expression levels must also be considered. However, precautions have been taken in form of vehicle controls (DMSO and EtOH only) and assessment of AEI throughout cell culture passages (P1-P30) (See chapter 3).

Future avenues would include an investigation into the transcriptional activity of NF-kB following treatment with SFN/SAHA, to gain further insight into the pathway affecting the alterations in gene expression described above. As Nrf2, an antioxidant transcription factor induced by SFN has been shown to attenuate NF-kB signalling (Li et al., 2008; Wardyn et al., 2015). In addition, Chromatin immunoprecipitation (ChIP) of CXCL16, would help determine the association of CXCL16 with transcription factors on promoter regions (such as Nrf2), or other DNA binding sites associated with regulation of gene expression and could provide additional insight into the epigenetic mechanisms involved. Finally tailoring the action of SFN through combined treatments and improving targeting to desired cell types by delivery using solid lipid nanoparticles would prove beneficial in eliminating off target effects (Sutaria et al., 2012). Drug delivery using nanoparticles has been shown to increase bioavailability, allow for sustained and controlled drug release, in addition to increasing stability and solubility. The nanometer size range allows for increased uptake by cells through enhanced permeation and retention effect. The ultimate goal of such an investigation would

be towards dietary intervention in both healthy and sick individuals to determine the true value of HDACi in the diet.

It should also be noted that DMSO provided the pharmacological starting point for the development of SAHA, an FDA approved HDACi. DMSO has been shown to induce growth arrest and terminal differentiation in transformed cells, and is also likely to epigenetically alter miRNA expression (Marks and Breslow, 2007). The ability of DMSO to induce unexpected changes in cell fates which are controlled epigenetically by DNA methylation and histone modifications suggest an epigenetic activity for DMSO (Czysz et al., 2015; Iwatani et al., 2006).

The cell lines used in this study EA.hy 926 and THP-1 may also have been expanded to include primary endothelial cells such as HUVECs, as Ea.hy 926 cells are a somatic endothelial cell hybrid, produced by the fusion of A549/8 (lung endothelial cells) with human umbilical vein endothelial cells (Lieber et al., 1976). Based on this EA.hy 926 cells may not provide the most definitive description of endothelial cell activity. THP-1 cells were also used in this study and are a human monocytic cell line from the peripheral blood which can be differentiated into macrophages, a key player in atherosclerosis.

2.7 Concluding remarks

This investigation suggests that the concentration of HDACi (SFN and SAHA) and duration of treatment may have contradictory functions and depend on the pre-existing inflammatory background which could limit potential use in the clinic. SFN, although an attractive and cost-effective option, is a broad-spectrum inhibitor and how many genes it may affect remains unknown. This knowledge would be essential in order to prevent any undesired off-target effects. This could be as little as 2 %, as is the case for TSA (Choi et al., 2005). Therefore, highlighting the need for specific analysis into the individualised role of HDACs in CVD, and

identifying the network of genes modulated by a given HDAC. With regard to disease markers such as *CXCL16* and miRNAs, a similar approach is required and further elucidations necessary as to the specific roles each marker plays in the stages of disease development. The action of a specific miRNA is dependent on cell type; hence miRNA modulation therapies would require more precise cellular targeting and suitable delivery methods.

Chapter 3: An analysis of the epigenetic contribution to allelic expression imbalance

3.1 Allelic Expression Imbalance

Allelic Expression Imbalance (AEI) , whereby one allele is expressed significantly more or less than the other, can be observed in 5-20 % of autosomal genes, in some cases resulting in monoallelic expression (Nussbaum et al., 2016).AEI is likely to act in a tissue-specific manner and has been shown to differ in regions of the adult human brain and in F1 progeny mice (Buonocore et al., 2010; Campbell et al., 2008).

AEI is known to play roles in human development, many congenital diseases and has been suggested as the cause of increased susceptibility to various adult onset diseases including; CVD, type 2 diabetes, psychiatric disorders and cancers, along with contributing to non-pathological variability in drug response (Butler, 2009; Hill et al., 2011; Hirota et al., 2004; Johnson et al., 2008; Locke et al., 2015; Nussbaum et al., 2016; Quinn et al., 2010)

Analysis of AEI is extremely useful alongside GWAS, with GWAS identifying candidate genetic polymorphisms implicated in disease development that can then be investigated for AEI (Johnson et al., 2008). As the majority of susceptibility loci identified by GWAS are in non-protein coding regions, it suggests that these polymorphic sites impact the level of gene expression through the recruitment of transcription factors or ncRNAs, which may contribute to AEI and ultimately affect disease susceptibility (Locke et al., 2015).

3.1.2 AEI and Epigenetics

Gene expression can be altered by *cis* and *trans* regulatory elements. *Trans*-acting regulatory elements can regulate genes on a different chromosome or distal site from the gene from which they were transcribed (Wittkopp and Kalay, 2012). *Cis*-acting regulatory elements are located close to the gene which they regulate (Wittkopp and Kalay, 2012).

Trans-acting regulatory elements include transcription factors and miRNAs, trans acting elements operate equally on both chromosomes so no imbalance of allelic expression is seen (Jones and Swallow, 2011). AEI therefore arises as a result of variation in *cis* acting factors, which disrupts transcription factor binding and alters gene expression (Wittkopp and Kalay, 2012). These *cis* factors can be enhancers, repressors or promoters, with locations both up and downstream of the transcriptional start site (Buonocore et al., 2010). *Cis*-acting variation can occur as a result of epigenetic modifications such as DNA methylation and DNA polymorphisms (Buonocore et al., 2010; Pastinen and Hudson, 2004; Schilling et al., 2009) or as a result of SNPs in the promoter. *Cis*-acting modifications may explain approximately 25-35 % of interindividual differences in gene expression, and are likely to be responsible for a considerable proportion of the observed phenotypic diversity and variation in susceptibility to complex diseases (Bray et al., 2003; Buonocore et al., 2010; Lo et al., 2003; Pastinen and Hudson, 2004; Yan et al., 2002).Further complexity arises from the presence of multiple alternative gene promoters, exons and variations in chromosome structure (Kleinjan and van Heyningen, 2005).

It is likely that AEI can have either a genetic or epigenetic basis, the genetic component being explained by heterozygosity at DNA sequence variants affecting *cis* regulatory elements, while the epigenetic influence may be due to allele specific modifications of the DNA or chromatin (Buonocore et al., 2010). Epigenetic modifications may explain some of the AEI throughout the genome, due to their ability to skew allele specific DNA methylation (ASM) (Schalkwyk et al., 2010). Data suggests that ~90 % of ASM is *cis* in nature and in many instances tissue specific (Schalkwyk et al., 2010). Schalkwyk *et al* identified allele specific DNA methylation at >35,000 sites across the genome and hypothesised that a spectrum of ASM is

likely with heterogeneity between individuals and across tissues. This heterogeneity may be the cause for the small effect sizes and lack of replication often seen in genetic association studies.

Epigenetic modifications to gene expression are often misinterpreted due to regulatory polymorphisms. For instance, classically imprinted autosomal loci have preferential expression of an allele resulting in mono-allelic expression that is independent of sequence variation via epigenetic mechanisms (Pastinen and Hudson, 2004). It is has been proposed that many assayed genes are subject to an imprinting mechanism occurring in only a portion of cells and that a significant proportion of human genes are subject to random epigenetic allele silencing which can persist in clonal cell lines (Buonocore et al., 2010; Gimelbrant et al., 2007; Schalkwyk et al., 2010; Schilling et al., 2009). Non-imprinted AEI is common among human genes but has been linked to disease in only one instance (Fischer et al., 2006). Fischer *et al* suggested that prolonged cell culture and treatment with HDACi trichostatin A (TSA) can lead to the reactivation of the downregulated allele in *BAPX1* in patients with oculo-auriculo vertebral spectrum. The study of immortalised lymphoblastoid cells from the CEPH families has also contributed to our knowledge of AEI and the mechanisms of its action, whereby evidence for the mendelian transmission of AEI, imprinted transmission of AEI, and discordant allelic expression in individuals carrying haplotypes identical by descent has been identified (Fischer et al., 2006).

In an effort to identify how common AEI is the human genome, Lo *et al*, examined allele specific expression of 1063 SNPs. Of this subset, 602 genes showed heterozygosity in kidney and liver tissues of seven individuals, of which 326 (54 %) had preferential expression of one allele, with 170 showing a greater than fourfold difference between the two alleles, many of

which are not found in known imprinting domains but distributed throughout the genome (Lo et al., 2003). Such modifications have demonstrated Mendelian inheritance, with estimates suggesting that AEI may affect approximately 20-50% of human genes.

In conclusion, AEI is a significant contributor to genetic variability and is commonly observed throughout the human genome. As AEI can be altered by *cis* modifications which include epigenetic mechanisms such as DNA methylation and acetylation, there is potential to alter such patterns through HDACi and DNMTi and ultimately direct ASM patterns to the most favourable allele.

3.1.2.3 Establishment and Maintenance of Allelic Status

As mentioned previously, the establishment and maintenance of allelic expression plays a critical role in developmental processes ranging from lineage commitment to X chromosome inactivation, and has been implicated in various disease phenotypes. In order to maintain such expression patterns throughout multiple cell division cycles, epigenetic mechanisms play a role. It was initially thought that DNA methylation acted in the maintenance of monoallelic expression. However, treatment of cells with DNMTi 5-Azacytidine failed to restore biallelic expression, highlighting the role of additional mechanisms such as histone modification in the maintenance of allelic expression (Eckersley-Maslin and Spector, 2014; Jeffries et al., 2016). In the case of cells of a neural lineage which have monoallelic expression, overall depletion of the active histone marks H3K4me3, H3lysine9, H3k36me3, but increased expression of the repressive mark H3K27me3 have been shown (Eckersley-Maslin and Spector, 2014). In B lymphocytes, the presence of silent and activating histone marks within a gene body were sufficient to distinguish between monoallelic and biallelic expression (Nag et al., 2013).

However, questions remain concerning the stability of such allelic expression patterns across multiple cell culture passages. Recent evidence suggests that a significant proportion of human genes are subject to random epigenetic allele silencing which can persist in clonal cell lines (Buonocore et al., 2010; Gimelbrant et al., 2007; Schalkwyk et al., 2010; Schilling et al., 2009).

A defining characteristic of random monoallelic expressed genes is unequal transcription at each allele and the inheritance of this across mitosis and successive cell divisions up to at least 15 passages in culture (Eckersley-Maslin and Spector, 2014; Gendrel et al., 2014). However, many groups continue to culture cells up to 30 passages before thawing out new stocks. Cell lines with passage numbers of greater than 30 are more likely to acquire genetic abnormalities compared to lower passage cells (Esquenet et al., 1997; Lin et al., 2003; O'Driscoll et al., 2006). Stable inheritance is not always observed, and many monoallelically expressed genes are subject to fluctuations in allele expression, often as a result of transcriptional bursting which can lead to temporary AEI between alleles, lasting minutes or hours and could reflect random stochastic activation of the two alleles (Eckersley-Maslin and Spector, 2014). Fischer *et al* also suggested that prolonged cell culture and treatment with HDACi TSA led to the reactivation of the downregulated allele at *BAPX1* in primary cells from patients with oculo-auriculo vertebral spectrum(Fischer et al., 2006), demonstrating that AEI is not always stable in cultured cells and can be influenced by treatment with ETAs such as TSA.

3.1.3 Methods for detecting AEI

To detect AEI within a population, it is first necessary to genotype the loci of interest followed by expression quantification of the various alleles of the gene, and finally association of the levels of allelic expression with genotype (Albert and Kruglyak, 2015). Methods for detecting

AEI include allelic expression profiling, eQTL, transcriptome sequencing and haplotype specific chromatin immunoprecipitation (haploCHIP).

The AEI assay identifies the presence of regulatory variation i.e. changes in transcript levels due to *cis* acting sequence differences without directly identifying or requiring knowledge of specific regulatory variants and aids in the identification of gene variants for association studies in human disease. In this study AEI was detected using relative allelic expression, whereby the levels of mRNA transcribed from the two alleles are compared in individual samples. This method makes use of SNPs within the mRNA sequence, allowing the RNA transcribed from each allele to be distinguished and quantified in heterozygous subjects, a departure from genomic 1:1 ratio of the two alleles in cDNA reflect heterozygosity and can be used to detect the effects of *cis*-regulatory variation in individual samples (Buonocore et al., 2010).

Expression quantitative trait loci (eQTLs) are regions of the genome containing DNA sequence variants which influence the expression level of associated genes (Albert and Kruglyak, 2015). The identification of eQTL permits the association of disease-associated SNPs with candidate genes (Albert and Kruglyak, 2015). As mentioned previously, this is a two-stage process involving genotyping of individuals, subsequent expression quantification and association of expression levels with genotype to identify the presence of any eQTLs. This method can also be used to detect global genomic AEI.

Transcriptome sequencing has become feasible due to the development of high throughput next generation sequencing. This offers an alternative method to array based global genomic eQTL variant mapping (Heap et al., 2010). Transcriptome sequencing can detect somatic mutations in addition to a more accurate quantification of allele specific gene expression

(Tuch et al., 2010). Transcriptomic sequencing involves the resequencing of allelic haplotypes separately and can therefore be used to identify AEI accurately as long as the polymorphism present for which the individual is heterozygous may be used as a marker (Heap et al., 2010).

The final method for detecting AEI is haplotype specific chromatin immunoprecipitation (haploCHIP). This method does not rely on polymorphisms in the primary transcript but instead involves the use of CHIP and mass spectrometry to identify different protein-DNA interactions which reflect different levels of allelic expression (Knight et al., 2003). This method operates on the basis that the level of phosphorylated RNA polymerase II associated with the secretion of chromatin correlates with the transcriptional activity of genes at corresponding loci. Although less widely used in comparison to eQTL and relative expression profiling, it has had some experimental success. HaploCHIP was used to quantify the expression of different alleles of the PAI-1 gene, which may have a promoter polymorphism associated with increased MI and decreased stroke risk (Hultman et al., 2010).

These methods are not flawless and ultimately highlight the need for multidimensional studies which link DNA sequence variation and epigenetic modifications. Such studies will be important for the detailed characterisation of human genetic variation function (Kilpinen and Dermitzakis, 2012). This genome blueprint will enable functional and phenotypic interpretation of personalised genomes in the future.

3.1.4 AEI and Disease Susceptibility

AEI studies offer approaches for connecting genotype to disease susceptibility based on changes in gene expression, as opposed to changes in the structure of the encoded protein (Yan et al., 2002).

3.1.4.1 AEI in Development and Congenital Disorders

Genomic AEI has a significant role in normal human development, including X-Chromosome inactivation and imprinting (Nussbaum et al., 2016). AEI is responsible for the monoallelic expression of one X chromosome in XX females and compensating expression levels in XY males (Kiefer, 2007).

Imprinting of specific genes plays a role in the development of the placenta, foetal growth and organogenesis. This activity is achieved by the monoallelic expression of selected genes in a manner specific to the parental origin of the chromosome on which the allele resides (Kiefer, 2007; Szabó and Mann, 1995). Approximately 1 % of all autosomal genes are imprinted (Jirtle and Skinner, 2007). Imprinting is likely to be the result of inherited epigenetic modifications to alleles at specific loci which ultimately influence the expression of these alleles in development, highlighting epigenetic modifications as the underlying mechanism responsible for allele silencing in imprinting (Peters, 2014; Spies et al., 2015).

As a result of the role AEI plays in developmental pathways, it is not surprising that AEI can have profound effects leading to various congenital disorders (Fischer et al., 2006), such as Prader-Wili syndromes, Angelman syndrome, Beckwith Wiedemann syndrome and Wilms' Tumours (Bittel and Butler, 2005; Butler, 2009; Cassidy and Schwartz, 1998; Peters, 2014). An example of note linking both AEI and epigenetics is that of Oculo-Auriculo-Vertebral Spectrum (OAVS). This disorder is a highly heterogeneous congenital disorder, characterised by malformations of structures which are derived from the first and second pharyngeal arches (Beleza-Meireles et al., 2014). This disorder is likely to be attributed to a combination of environmental, epigenetic and genetic susceptibility factors (Beleza-Meireles et al., 2014). AEI of the *BAPX1* gene was found to be significantly associated with expression on the OAVS

phenotype in some patients, furthermore this AEI was shown to be associated with histone acetylation and subsequently increased expression of *BAPX1* gene (Fischer et al., 2006).

3.1.4.2 AEI in Common Complex Diseases

AEI has also been shown to underlie a number of adult onset complex diseases including CVD, type 2 diabetes (Locke et al., 2015) , psychiatric disorders (Hill et al., 2011; Quinn et al., 2010). Furthermore, somatic acquisition of AEI can lead to loss of imprinting, a key step in the development of many different cancers (Jelinic and Shaw, 2007; Peters, 2014). AEI can also alter drug response in patients (Johnson et al., 2008).

AEI in CVD

AEI has been proposed as a possible contributing factor to explain a vast amount of complexity associated with CVD and the degree of patient variability observed. In an effort to identify genes associated with altered drug response in CVD and CNS diseases through the use AEI methodology, nine out of 18 candidate CVD genes analysed where shown to exhibit AEI (Johnson et al., 2008). These were genes with known roles in drug response, coagulation, inflammation, lipid metabolism and contractile function (Johnson et al., 2008).

GWAS has also helped identify multiple polymorphisms associated with increased CVD susceptibility, yet the mechanism by which this occurs remains largely unknown, as a large amount of these polymorphisms are located in non-protein coding regions of associated genes. However, the presence of eQTLs has been identified in > 45 % of genes identified by GWAS to be influential in CVD contained at least *cis*-eSNP, suggesting that *cis*-acting variation may contribute towards increased susceptibility to CVD conferred by polymorphic variation in these genes (Zhang et al., 2014). Consequently, SNPs within HATs and HDACs may also

influence disease susceptibility, for example, HDAC9 has been found to play a role in mouse macrophage polarisation, cholesterol efflux and atherosclerosis (Cao et al., 2014). SNPs in the intron of HDAC9 have been shown to have an association with stroke in two independent studies (Smith, 2014). Whether the effect of HDAC9 is due to a primary histone modification (SNP) or deacetylation of effector proteins remains to be shown. It does however indicate a need for specification in the future but also the potential to alter genetic and epigenetic events to induce a more beneficial response.

3.1.5 CXCL16 - rs2277680

Due to the multifaceted role CXCL16 plays in all stages of atherosclerosis, SNPs within this gene have proven to be of particular interest. One particular SNP located on CXCL16 is rs2277680, a missense mutation located in exon four. This missense mutation results in an allele change from C – T, changing alanine [Ala] to valine [Val] at position 181. This SNP has a relatively high frequency across multiple populations (frequencies of the minor allele G according to HapMap 0.44 Japanese, 0.58 CEU, 0.75 Nigeria, 0.45 India), making it an ideal polymorphism in population research and CVD. Although an ideal candidate for studying the association between SNPs in CXCL16 and CAD, contradictory evidence exists as to the association, if any, which it may have with CVD. An initial promising study by Lundberg *et al* in 2005 reported that the severity of coronary artery stenosis can be associated with the V181 polymorphism, yet a significant difference in allele frequencies between both groups studied was not detected (Lundberg et al., 2005). Patients with this polymorphism experienced more severe coronary artery stenosis in addition to having a smaller luminal diameter when compared to healthy controls. In 2010 and 2011 further investigations reached similar conclusions, Huang *et al* also looked at the association between four SNPs in *CXCL16* and

stenosis, and found that only rs3744700 showed a significant difference in allele frequency between patients and controls. Petit *et al* detected no significance when looking for an association between SNPs in CXCL16 and post myocardial infarct patients (Huang et al., 2010; Petit et al., 2011).

We decided to further investigate rs2277680 as nothing is known about AEI at this SNP in CEPH cell lines. The effect stimulation with TNFα had on AEI at the SNP was also assessed, as it is commonly elevated in CVD patients. Furthermore, an investigation into the capabilities histone modifying agents may have, on allelic expression at rs2277680 was carried out.

3.1.6 Complex diseases: An outcome of Genetic and Epigenetic interaction

Many of the common complex diseases which burden society are the result of underlying genetic variation and epigenetic interactions. Disease-associated SNPs have the potential to create CpG sites, of which 70-80 % may become methylated and potentially alter disease susceptibility (Jabbari and Bernardi, 2004). Such interactions have been shown to play a role in age-dependent susceptibility to insulin resistance whereby, a polymorphism in the *NDUFB6* gene creates a promoter CpG site which becomes methylated acting to confer greater disease risk (Handy et al., 2011; Ling et al., 2007).

Allele specific methylation (ASM) is also prevalent and contributed to by CpG SNPs in the human genome (Shoemaker et al., 2010). ASM is often cell type specific with approximately 38-88 % of regions being dependent on the presence of heterozygous SNPs in CpG dinucleotides which influence methylation potential (Shoemaker et al., 2010).

CpG SNPs are likely to be an important class of *cis*-regulatory polymorphisms which connect genetic variation to individual variability of the epigenome, furthermore, as CpG dinucleotides are highly mutable, there are likely to be many CpG rare variants in individual genomes (Li et al., 2009; Shoemaker et al., 2010). CpG-SNPs have been identified in six inflammatory related genes and have shown an association with CHD, highlighting the important roles such modifications play in disease development and progression (Chen et al., 2016).

3.1.7 ETA and AEI

One of the primary aims of this study was to assess the potential effect of ETAs SFN, DAC, CURC and PA had on AEI. This study stemmed from an original observation made by our group (Jillian Gahan PhD Thesis) which noted the ability of AEI to be induced in two heterozygous cell lines GM12234 and GM07348 following treatment with 10 µM SFN, DMSO and SFN+TNFα (100 ng/µl) (Figure 3.1). We attempted to replicate these findings and further expand on this observation through the use of additional epigenetic modifiers over a longer time course.

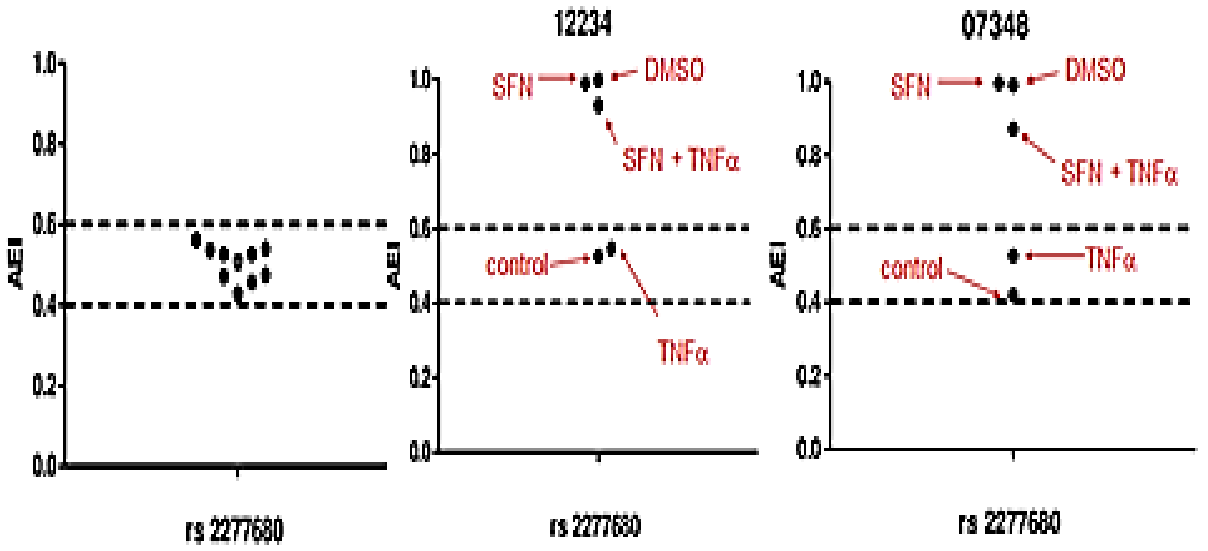

Figure 3.1: Primary AEI observation.

Initial data obtained from (Gahan, 2014). Noted the ability of 2 out of 10 heterozygous CEPH cell lines to be induced into AEI upon treatment with SFN (10 µM), DMSO and SFN+TNFα (100 ng/µl). AEI is rF > 0.6 or rF < 0.4.

Based on the above observation (Gahan, 2014), the study was expanded to include additional ETAs. SAHA (Vorinostat)is an FDA approved HDACi with greater specificity than SFN (Yang et al., 2015). Curcumin(CURC) is a dietary HDACi found in turmeric spice known to reduce the extent of atherosclerotic lesions and inhibit HDAC1,HDAC2 and HDAC8 (Coban et al., 2012; Vahid et al., 2015). Protocatechuic aldehyde (PA) is an established HDACi, isolated from the aqueous extract of the root of the *Salvia miltiorrhiza* herb, which may inhibit migration and proliferation of VSMCs, in addition to reducing the expression of adhesion molecules (Moon et al., 2012; Zhou et al., 2005). DAC (5-aza-2'-deoxycytidine) is a DNA methyltransferase inhibitor similar to azacitidine, used as a treatment option for MDS. In addition to the ETAs described above, their reconstitution vehicles were also assessed as controls, these included DMSO, EtOH and Methanol.

3.2 Aims and Objectives

- Evaluate the hypothesised epigenetic influence on AEI of the SNP rs2277680 in GM12234 and GM07348 CEPH cells following treatment with the epigenetic modifying agents; SFN, Curcumin, SAHA, DAC and PA, and how this may influence inflammatory gene expression.

- Assess the epigenetic stability of CEPH cell lines under conditions of long term culture by evaluating changes towards AEI and the extent of which AEI is induced at rs2277680 in GM12234 and GM07348.

- Examine the potential of ETAs (for example SFN) to restore or induce allelic expression imbalance.

3.3 Materials and Methods

3.3.1 Centre d'Etude du Polymorphisme Humain (CEPH)

CEPH cell lines obtained from Corriell Cell Repositories (Camden, NJ) were used throughout the study. These EBV-transformed lymphoblastoid cell lines from multigenerational normal, healthy human volunteers have been genotyped by the HapMap consortium as part of the effort to locate specific regions which share genetic variations within and between populations (http://www.hapmap.org). Both the genotype data and gene expression data are publicly available (ftp://ftp.ncbi.nlm.nih.gov/hapmap/).

3.3.2 Cell lines and Culture

CEPH cell lines GM12234 and GM07348 have been genotyped as HapMap CEU samples and are known to be heterozygous at CXCL16 rs2277680. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 20 % FBS non-heat inactivated (v/v) in 75 cm^2 flasks and incubated in a humidified incubator at 37 °C and 5% CO_2. Cells where seeded at 6 x 10^6 in 20 ml and split every 2-3 days, 1 ml of cell suspension was taken every passage and gDNA and RNA extraction performed. The procedure is summarised in the schematic below (Figure 3.2).

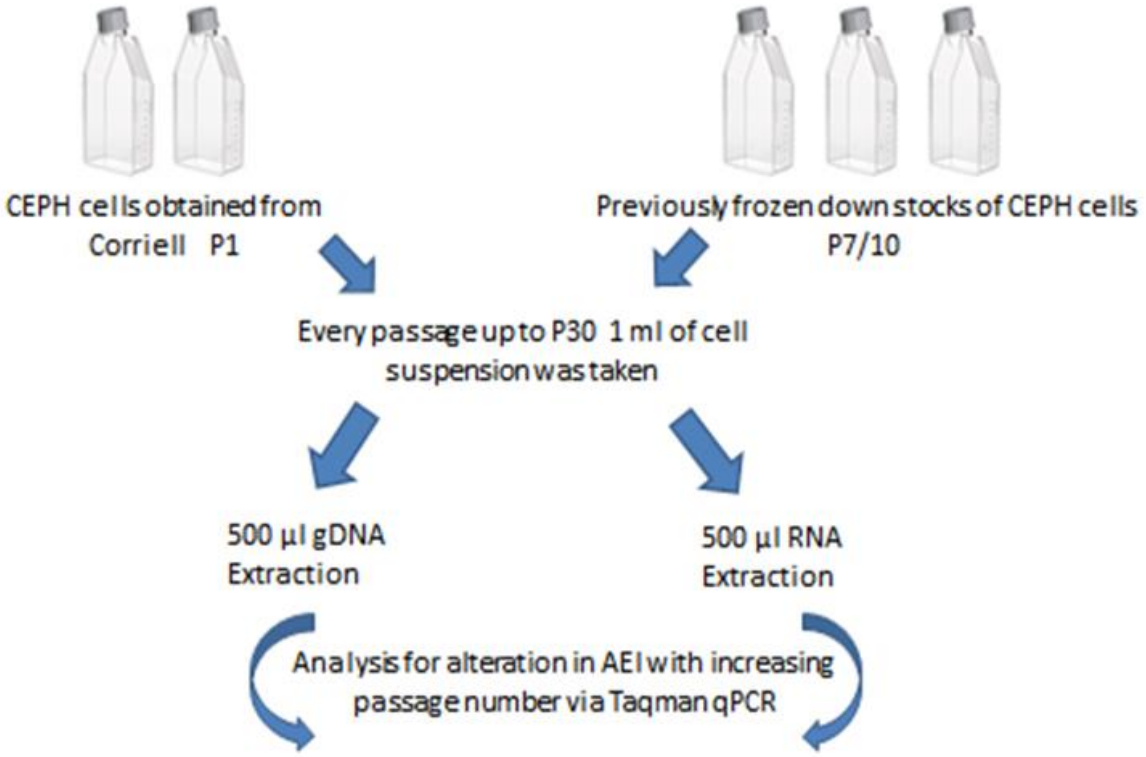

Figure 3.2: Schematic representation of cell culture procedure for analysis of alterations to AEI with passage number

3.3.3 TNFα Treatment of cell lines

CXCL16 heterozygous cell lines GM12234 and GM07348 were activated with TNFα. Cells were seeded in 6 well plates (Sarstedt) at ~10^6 per ml and treated with either 10 ng/µl or 100 ng/µl of TNFα for 6 and 24 h. Cells were collected by centrifugation for 5 min at 540 x g, medium removed and lysed in 1 ml of Tri-Reagent for RNA extraction.

3.3.4 Treatment of cell lines with ETA Sulforaphane

Cells were seeded in 6 well plates at ~10^6 and treated with 10 µM SFN (Sigma-Aldrich, USA) for 6h and 24 h (prepared as described in section 2.3.2). A dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) control was also used. For a 3 ml solution of 0.81 µl of DMSO was added to 2.999.1 µl of medium. Following the above incubation periods, cells were lysed in 1 ml of Tri-

Reagent. The same experimental set up was applied to all other inhibitors used, listed in Table 3.1 below. All treatments N=4. DAC was prepared as follows; 1mg/ml at 4.38 mM dissolved in methanol. For 100 nm 1 µl of this stock was added to 999 µl of methanol. In order to prepare a 3 ml solution of DAC (100 nm) 6.8 µl was added to 2993.3 µl of medium. PA was prepared as follows; 0.65 g of PA (MW 138.12) was dissolved in 5 ml of ethanol to give a 1 M stock solution. This stock was further diluted (1 µl to 999 µl of of ethanol) to give a 1000 µM solution. To prepare a 3 ml solution of PA (10 µM) for treatment 20 µl (1000 µM) was added to 2,980 µl medium.

Inhibitor name	***Concentration used***	***Incubation time***
Curcumin	10 µM	6+24 h
DAC	500 nM	72 h *Fresh media and DAC added every 24 h.
PA	10 µM/5 µM	24 h
SAHA	10 µM/5 µM	24 h
TNFα 100ng/ul +SFN	10 µM	6 h
SFN+CURC	10 µM	6/24 h
Vehicle Controls used		
ETOH	1 % ETOH - 10 µM 0.5 % ETOH – 5 µM *For a 3 ml solution	6 h
DMSO	0.09 % DMSO – 10 µM 0.045 % DMSO - 5 µM *For a 3 ml solution	6/24 h
Methanol	500 nM	72 h

Table 3.1: Table of inhibitors used.

This table highlights the concentration of ETA used and incubation time. For all treatments cells were seeded at ~10^6, and treated with ETA at concentrations and durations described above. Vehicle controls were also used to

determine whether any alterations in AEI could be the result of the vehicle and rather than the ETA, as data by (Gahan, 2014) indicate the ability of DMSO to induce AEI. All treatments N=4.

3.3.5 Genomic DNA extraction

Genomic DNA extraction was performed using GenElute DNA miniPrep kit (Sigma-Aldrich, USA) as per manufacturer's instructions.

3.3.6 RNA Extraction

Cells were thawed at room temperature (RT) for 5 min. 200 µl of BCP (1-bromo-3-chloro-propane) was added per 1 ml of Tri-Reagent. Samples were vortexed for 15 s and incubated at RT for 3 min, followed by centrifugation at 12,000 rpm for 15 min at 4 °C. The upper aqueous phase was transferred to a new labelled microcentrifuge tube, followed by the addition of 500 µl of Isopropanol. After vortexing for 30 s and incubation at RT for 10 min, samples were centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was aspirated and nucleic acids precipitated using 1 ml of 75% EtOH, vortexing and centrifugation at 7,500 rpm for 5 min at 4 °C. The resulting pellets were dissolved in 30 µl RNase free H_20, vortexing, and incubation at RT for 5 min to fully dissolve the pellet. RNA preparations were stored at -80 °C

3.3.7 cDNA Synthesis

1 µg of RNA from each treatment was reverse transcribed into cDNA using SensiFast cDNA Synthesis Kit (Bioline, UK) as per manufacturer's instructions. The cDNA-synthesis reaction was performed at 25 °C for 10 min, 42 °C for 15 min, and 85 °C for 5 min for deactivation. Reaction conditions are given in Table 3.2.

Reagents	**1 Reaction**	**Program**
RNA	1 µg	25 °C 10 min
5x TransAmp Buffer	4 µl	42 °C 15 min
Reverse Transcriptase	1 µl	85 °C 5 min

H_20	X	4 °C Hold
Total	10µl	

Table 3.2: Reagents used for cDNA synthesis

3.3.8 RT-qPCR Analysis Allelic expression imbalance

Allelic expression imbalance was detected and quantified using Taqman Allelic Discrimination Assays (Applied Biosystems, California, USA). The assay determines the amount of DNA (or cDNA) at the specific SNP (rs2277680) 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 DNA or cDNA template that is present. Allelic quantification for rs2277680 (CXCL16) was performed using the ABI 7900HT Fast Real Time PCR system (Applied Biosystems). All real-time PCR reagents and conditions are shown in Table 3.3. Each reaction was performed in triplicate, and a negative non-template control (NTC) control was also used.

Reagents	**1 Reaction**	**Program**
TaqMan 2x Universal Mix	5 µl	50 °C 2 min
40x Stock of SNP genotyping Assay	0.125 µl	95 °C 10 min
DNase Free H_20	1.875 µl	95 °C 15 s X 40
gDNA	2 µl	60 °C 1 min
Total	9 µl	

Table 3.3: Components, volumes and program for qPCR Analysis

3.3.9 Calculating AEI using threshold amplification cycle data (Ct)

The threshold amplification cycle data (Ct) is used to determine relative allele frequency (rF) (Gahan et al., 2015). 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:

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

*** ΔCt' = [(Ct allele-1 (cDNA) – Ct allele-2 (cDNA)) – (ΔCt 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. An rF value of 0.0 indicates the total expression of allele-1, while an rF of 1.0 indicates the total expression of allele-2. An rF of 0.5 represents equal expression of both alleles. A positive AEI (+) result is evident by ratios which deviate by greater than 20% (i.e. 0.4>rF>0.6).

3.3.8 RT-qPCR

SYBR-Green (Bioline, UK) RT-PCR analysis was performed on an Eco Real-Time PCR System (Illumina, California, USA) as per manufacturer's instructions. cDNA was added to an Eco 48 well plate, compatible with the system. Analysis was performed on samples which showed AEI at rs2277680, using *GAPDH* as a reference gene. All real-time PCR calculations, reagents and conditions are shown in Table 3.4 below. Each reaction was performed in duplicate.

Reagents	**1 Reaction**	**Program**
SensiFast SYBR	5 µl	95 °C 15min 40 cycles of 1 min @ 94 °C, 58 °C, 72 °C
Forward Primer (10 µm)	0.4 µl	
Reverse Primer (10 µm)	0.4 µl	
cDNA	4.6 µl	
Total	10.4 µl	

Table 3.4: Components, volumes and program for RT-qPCR analysis

3.3.9 Statistical Analysis

Statistical analysis for all data was performed using Prism GraphPad version 5 and analysed using a paired T-test (N = 4). Comparisons considered significant if $p < 0.05$.

3.4 Results

3.4.1 AEI at rs2277680 following treatment with SFN, DMSO, TNFα 100 ng/µl and SFN+TNFα 100 ng/µl.

AEI analysis was carried out in two CEPH cell lines; GM12234 and GM07348, based on a previous observation (Gahan, 2014) where treatment with ETAs SFN (10 µM) and DMSO induced AEI in balanced cell lines (Figure 3.1). The results below show the ability of DMSO to induce AEI in both GM12234 and GM07348, the direction of which varies depending on cell line. In GM07348 cells, treatment with SFN (10 µM) for 6 and 24 h acts to induce AEI (Figure 3.3, B). The same treatment in GM12234 did not however induce AEI in all samples (Figure 3.3, A). Treatment with TNFα (100 ng/µl) both alone and in combination with SFN (10 µM) did not affect allelic expression in GM12234 cells (Figure 3.3, A). SFN (10 µM) when combined with TNFα (100 ng/µl) in GM07348 acted to induce imbalance, which was not observed following treatment with TNFα (100 ng/µl) alone (Figure 3.3, B).

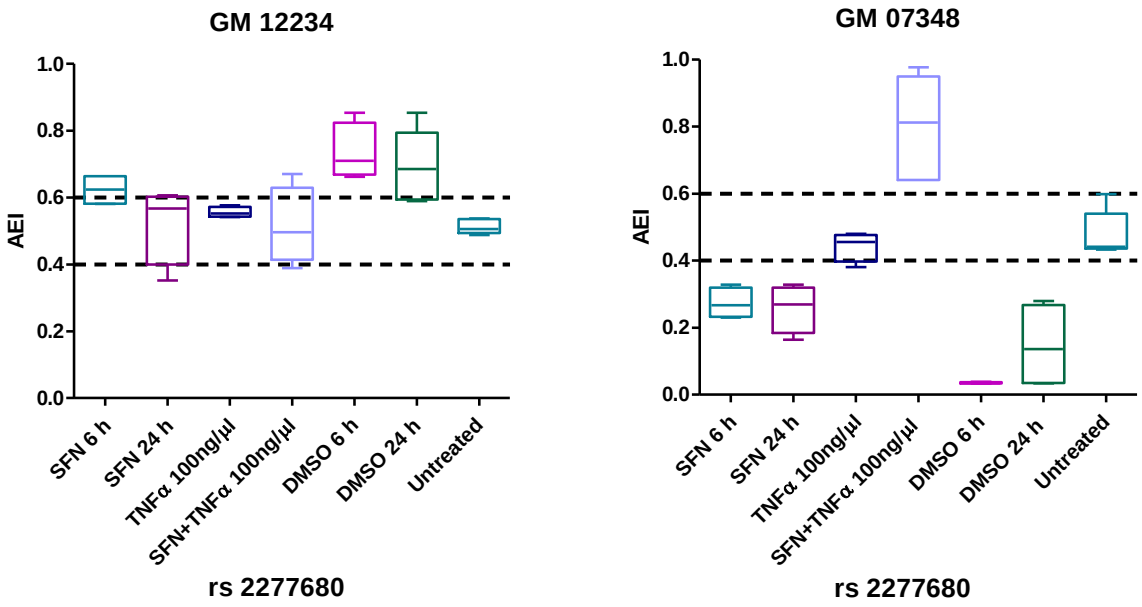

Figure 3.3: Box and whisker plot of AEI in GM12234 and GM07348.

The data was collected in an effort to replicate and expand the results observed in Jillian Gahan PhD Thesis, whereby the ability of SFN to induce AEI was noted in cell lines GM12234 and GM07348. In addition to the initial observation of AEI after 6 h of treatment with SFN (10 µM) a 24 h SFN (10 µM) time point was added. The above figure confirms the ability of DMSO to induce AEI, in both cell types following a 6 and 24 h incubation period. SFN (10µM) treatment for 6 and 24 h induces AEI in GM07348 but not GM12234. The combination of SFN (10 µM) and TNFα (100 ng/µl) only induced imbalance in GM07348, when used alone TNFα (100 ng/µl) did not alter allelic states. Data above representative of N=4. AEI = 0.4>rF>0.6

3.4.2 AEI at rs2277680 following treatment with SFN, CURC and SFN+CURC, 6 and 24 h

In addition to SFN, Curcumin (CURC) was also assessed. Curcumin has a similar activity to SFN as it a HDACi with dietary sources e.g. Turmeric spice. The ability of SFN (10 µM), CURC (10 µM) and a combination of SFN+CURC (10 µM each) to induce AEI was determined following 6 and 24 h treatment. The effect of incubation with SFN for 6 and 24 h varies between cell lines (Figure 3.4). AEI following treatment with SFN (6+24 h) can be noted in GM07348 (Figure 3.4, B) and in some replicates for SFN 6 h in GM12234 (Figure 3.4, A). Curcumin does

appear to be as effective at inducing imbalance in both cell lines however, a combination of SFN and Curcumin acts to induce imbalance in GM12234, which when both used alone did not induce imbalance (Figure 3.4, A). The combination of SFN and Curcumin (6+24 h) also altered the direction of imbalance (AEI<0.4) when compared to the direction altered the SFN 6 and 24 h (Figure 3.4, A).

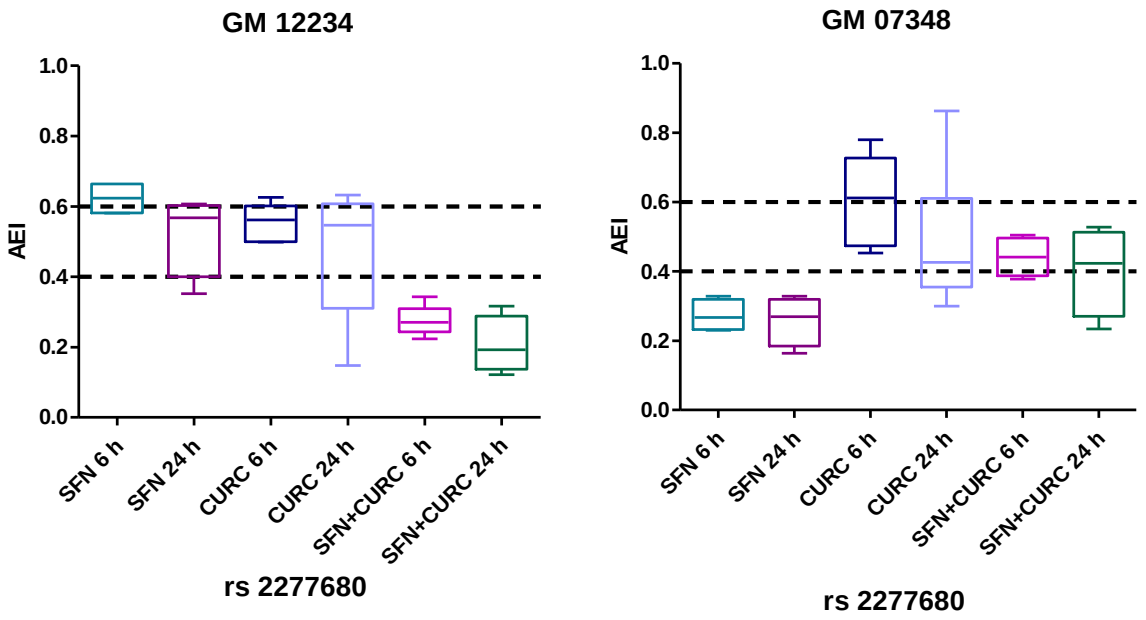

Figure 3.4: Box and whisker plot of AEI at rs2277680 following treatment with SFN, Curcumin, and SFN+Curcumin.

Allelic expression is not greatly altered by treatment with CURC (10 µM) for 6 and 24 h in both cell lines. When used in combination with SFN (10 µM) for 6 and 24 h GM12234 can be induced into imbalance (A), the effect of which is towards the opposite allele when compared to SFN alone 6 and 24 h. Allelic expression at GM07348 (B)does not appear to be greatly altered by the addition of curcumin or combination treatment. AEI = 0.4>rF>0.6.

3.4.3 AEI at rs2277680 following treatment with ETAs

The ability to induce AEI was also investigated for ETAs Protocatechuic Aldehyde (PA), suberoylanilide hydroxamic acid/Vorinostat (SAHA) and Decitabine (DAC). The ETAs, PA and

SAHA were investigated at 5 µM and 10 µM concentrations, over a 24 h incubation period. The effect of DAC was investigated at a concentration of 500 nM over 72 h, at 24 h intervals, the medium was replaced with fresh medium supplemented with DAC. GM12234 cell treatments with lower concentrations (5 µM) of PA, SAHA, DMSO and ETOH acted to induce imbalance in the same direction AEI<0.4 (Figure 3.5, A). Concentrations of 10 µM however, acted to change the direction of imbalance towards AEI>0.6 (Figure 3.5, A). The effect of the above ETAs was not as notable in GM07348. All replicates, N=4, where induced into AEI following treatment with SAHA (10 µM), DMSO (5 µM and 10 µM), this imbalance was all in the same direction AEI<0.4 (Figure 3.5, B). AEI was not observed following incubation with SAHA (5 µM) for 24 h (Figure 3.5, B). All other treatments PA (5 µM and 10 µM), DAC (500 nM), ETOH (5 µM and 10 µM) and Methanol (500 nM) did not act induce AEI in all replicates N=4.

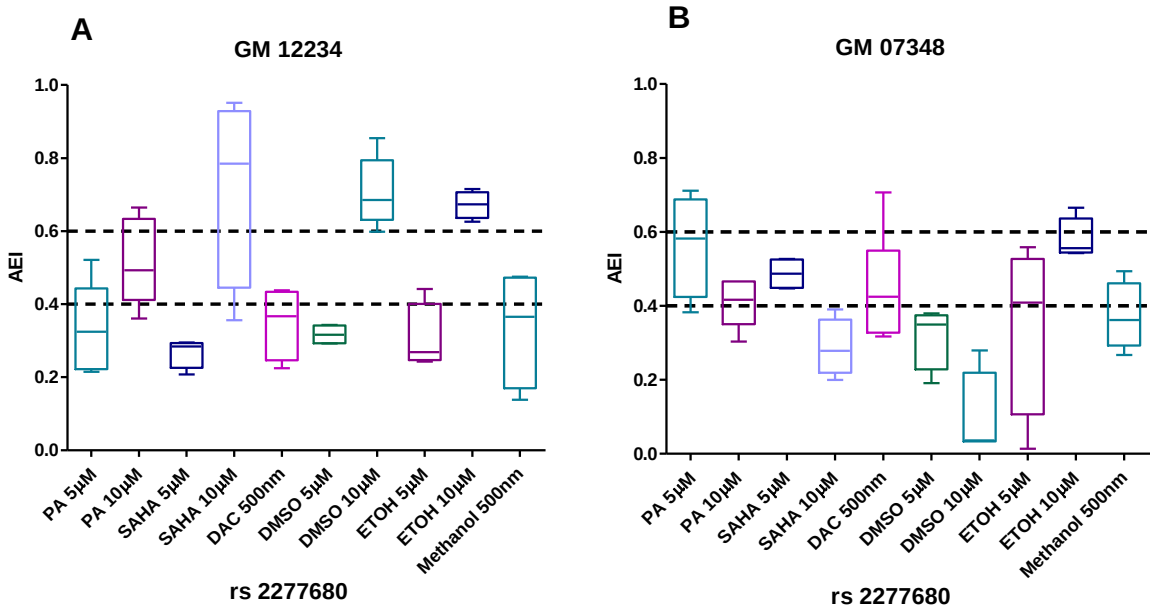

Figure 3.5: Box and whisker plot of AEI at rs2277680 following treatment with ETAs.

The effect of Protocatechuic Aldehyde (PA), suberoylanilide hydroxamic acid/Vorinostat (SAHA) following 24 h of treatment with concentrations of (5 µM) and (10 µM) was assessed along with the effect of Decitabine (DAC) 500 nM for 72 h. GM12234 can be induced into imbalance following treatment with SAHA (5 µM), DMSO (5 µM),

DMSO (10 µM), ETOH (5 µM) and ETOH (10 µM) (A). All other treatments with the exception of PA 10 µM acted to induce imbalance in most replicates (A). GM07348 showed complete AEI in 3 treatments SAHA (10 µM), DMSO (5 µM) and (10 µM). No AEI was noted in SAHA (5 µM), the remaining treatments showed a variety of different responses within replicates. AEI = 0.4>rF>0.6. All treatments shown above are representative of N=4.

3.4.4 CXCL16 expression analysis in imbalanced samples

Levels of absolute mRNA were determined in cases were AEI was noted in order to determine whether the affected allele is up- or down-regulated. GM12234 cells which showed imbalance appear to have increased expression of the affected allele when compared against untreated balanced control samples, with the exception SFN (10 µM) 6 h and DMSO (10 µM) 24 h which reduced CXCL16 expression levels although not significantly (P=0.1475, P=0.4420) (Figure 3.6, A, 1, 2). In GM12234 a (5 µM) concentration of DMSO for 24 h acts to increase CXCL16 expression (P=0.0399) (Figure 3.6, A, 3). This induction is not as strong, however, using the higher concentration of 10 µM (Figure 3.6, A). Furthermore, incubation with SFN and CURC appears to act in a time dependent manner, with expression increasing from 6 to 24 h (P=0.0425, P=0.0257) (Figure 3.6, A, 4). For GM07348 the opposite is true, with imbalanced samples showing significantly reduced levels of CXCL16 when compared to balanced control samples ($p<0.05$), with the exception of TNFα 100 ng/µl+SFN and EtOH (5 µM), which were not significant (Figure 3.6, B, 5, 6). Treatment with SFN (10 µM) 24 h significantly reduced levels of CXCL16 (P=0.0045) as did all of the samples analysed with the exception of EtOH (5 µM) (P=0.5597) and TNFα 100 ng/µl+SFN (P=0.1375) (Figure 3.6, B, 5, 6).

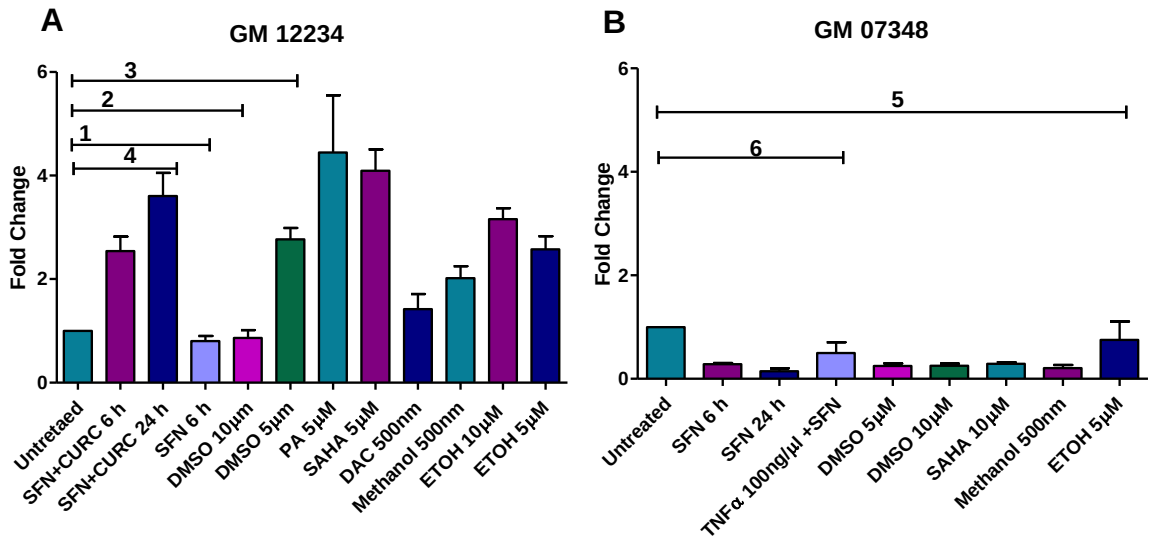

Figure 3.6: CXCL16 expression analysis imbalanced samples.

Levels of absolute mRNA for CXCL16 were determined in samples which showed imbalance. For GM12234, all treatments which induced imbalance acted to increase CXCL16 expression levels with the exception of SFN (10 µM) 6 h and DMSO (10 µM) 6 h which reduced CXCL16 although not significantly. Treatment with DAC (500 nM) and PA (5 µM) did not alter expression levels significantly. All other treatments acted to significantly increase CXCL16 expression ($P<0.05$). The opposite was noted for GM07348, where all treatments which induced AEI acted to decrease CXCL16 expression levels significantly ($P<0.05$) with the exception of TNFα 100 ng/µl+SFN and ETOH (5 µM) 24 h which did not significantly affect the expression of CXCL16.

3.4.5 Alterations to AEI at rs2277680 arising from long term cell culture of GM12234

As Gahan, 2014 noted AEI in the opposite direction in both cell lines, the epigenetic stability of GM12234 and GM07348 cell lines was determined following long-term cell culture up to passage 30 (P30). AEI was investigated in new stocks (P1) and also in older frozen stocks P 8/10. New stocks of cells that were not subjected to freezing in DMSO (shown in red in Figure 3.7 below) appeared to be relatively stable over time in a cell culture environment, with imbalance being noted in later passage numbers. By passage 30 two thirds of replicates showed imbalance in both directions (Figure 3.7, Red). Cells which have been previously

frozen in DMSO (shown in blue Figure 3.7 below) appear to be imbalanced when first thawed. With increasing passage number however, balance had been reached by P30.

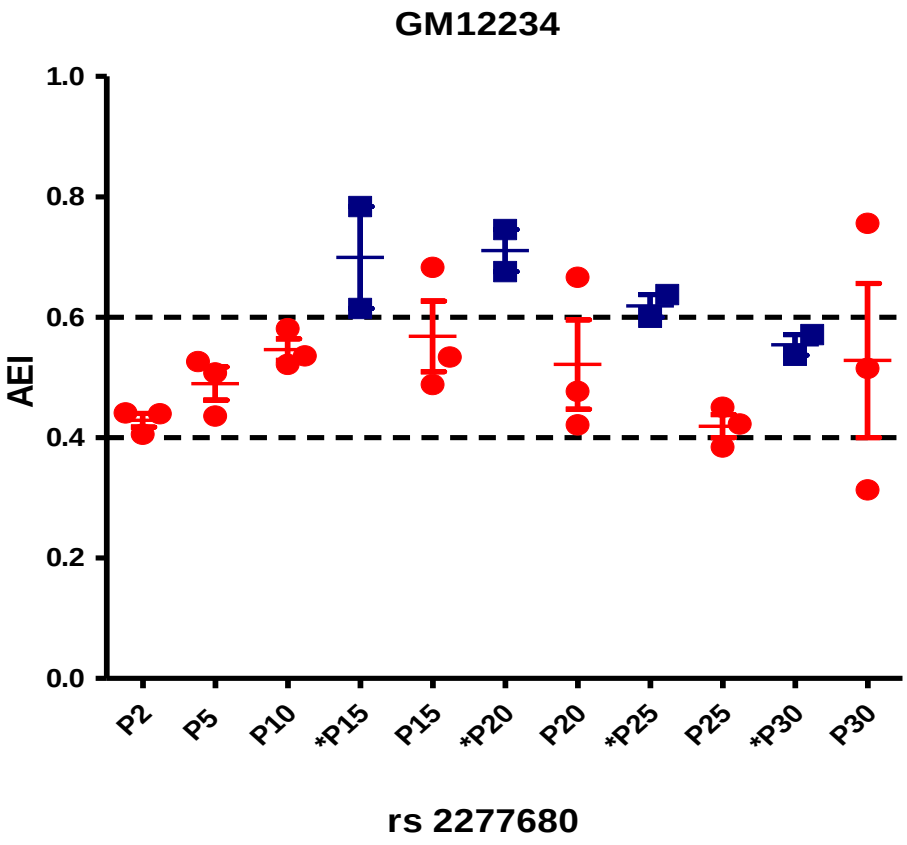

Figure 3.7: The effect of long-term cell culture on AEI at rs2277680 in GM12234 .

The impact of long-term cell culture on AEI was examined in a new stock of cells (P1) (Red) and older cells which had been previously frozen and were thawed for this analysis (Blue). The new cell line stock was investigated from P2 to P30. Previously frozen stocks were thawed at P8/10 and assayed from the starting passage number to P30. New cells (that were not subjected to freezing with DMSO) (Red) remain balanced until P15 with 1/3 replicates showing AEI until P30, at which point 2/3 replicates are imbalanced. Previously frozen down stocks (Blue) show complete imbalance when broken out initially with a trend towards achieving balance, which is reached by P30. *=Previously frozen down. AEI is represented by 0.4>rF>0.6, cells where considered stable if no AEI was induced. Each dot on the above figure represents an averaged rF value from three triplicate wells.

3.4.6 Alterations to AEI at rs2277680 arising from long term cell culture of GM07348

An analysis of the epigenetic consequences of long term cell culture for AEI was also examined in GM0734 cells. As previously mentioned an AEI comparison between new stocks (Red) and

frozen stocks (Blue) of cells from P1 to P30 was undertaken. In new stocks, AEI remains stable up to P20. Imbalance is introduced in later passages, although complete imbalance in all replicates (all replicates falling outside AEI 0.4-0.6) was not recorded.

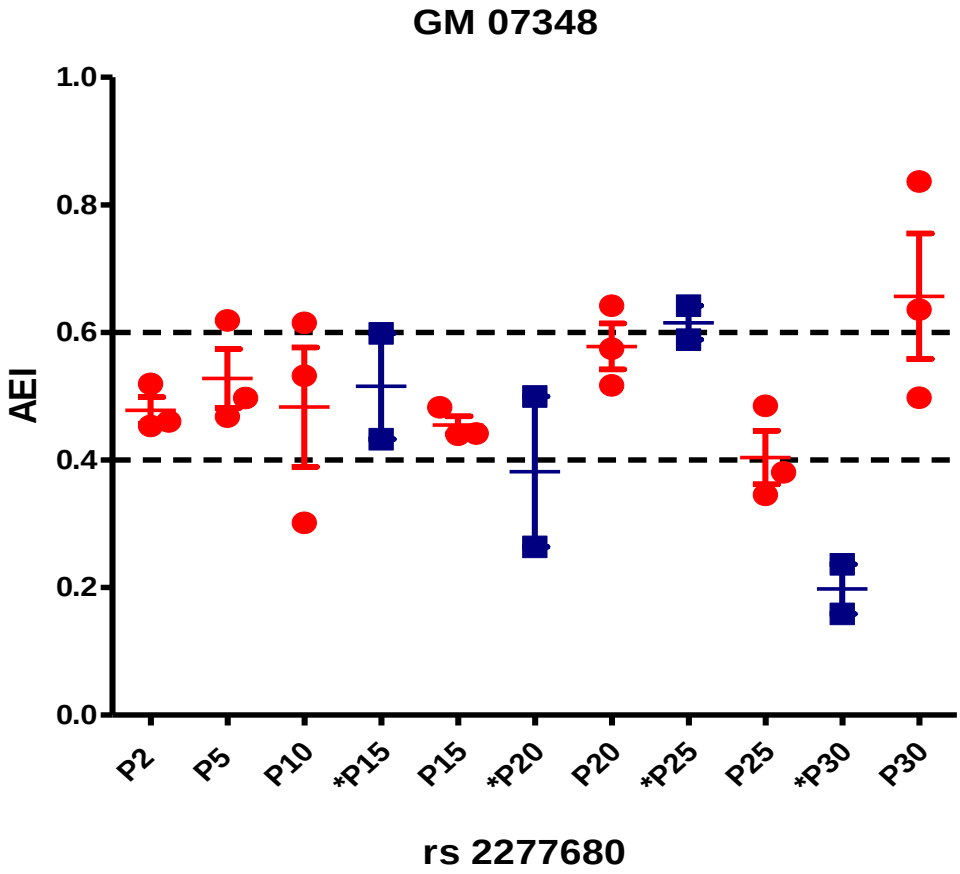

Figure 3.8: The effect of long-term cell culture on AEI at rs2277680 in GM07348.

Allelic expression in new stocks of cells (Red) remains stable from P1 to P30 with at least 1/3 replicates showing balance throughout all passages. Previously frozen down stocks (Blue) fluctuate between balance and imbalance. Complete imbalance (AEI in all replicates) is seen in all previously frozen stocks as P30. *=Previously frozen down. AEI is represented by 0.4>rF>0.6, cells where considered stable if no AEI was induced. Each dot on the above figure represents an averaged rF value from 3 triplicate wells

3.4.7 LD Heat-Map of CXCL16

All of the SNPs surrounding rs2277680 were downloaded from HapMap. 12 different SNPs were analysed, two of which were excluded as they were monomorphic. Data indicate that rs2277680 is inherited as part of a haploblock, an area of high linkage disequilibrium (LD), defined using Haploview. However, the upstream SNPs in the promoter region, the most likely to contain regulatory motifs, are not in strong LD with rs2277680. Therefore, there may have been recombination events between the assayed SNP (rs2277680) and the regulatory upstream variant that is driving the allelic expression imbalance (*cis*- effect). One consequence of this is that the same *cis*-effect may have the opposite effect on AEI in different cell lines, depending on which haplotypes are present.

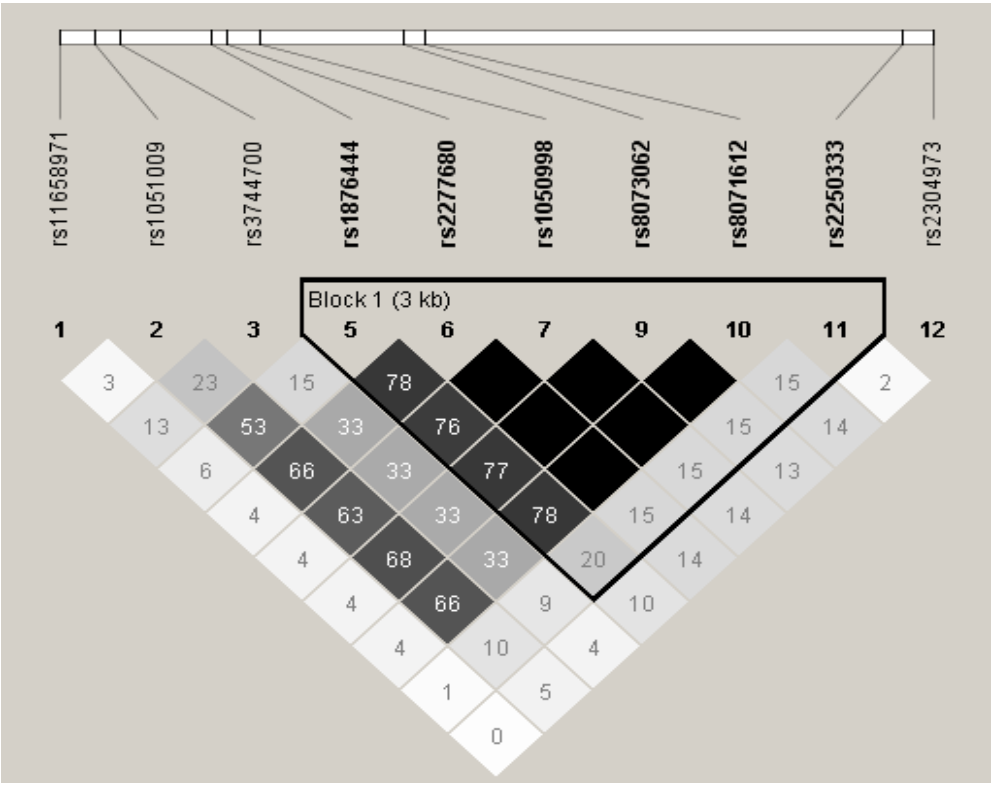

Figure 3.9: LD Heat Map-CXCL16.

This figure highlights that rs2277680 is inherited as part of haploblock. All SNPs within this block are in high LD (Black squares) and are inherited together. Black= strong correlation, in LD. White= no correlation, not in LD.

Data shown above is representative of LD values R-squared using Haploview. Rs2277680 is not in LD with SNPs located further upstream (in the promoter region), which are most likely regulatory motifs. Alterations in AEI may be the result of a recombination event between the assayed SNP (rs2277680) and the regulatory upstream variant that is driving the allelic expression imbalance (*cis*- effect).

3.5 Discussion

The purpose of this study was to investigate the presence of AEI in two CEPH cell lines GM12234 and GM07348, with the aim of identifying whether the epigenetic modifiers SFN, DAC, PA, Curcumin and SAHA could alter the degree allelic expression at rs2277680. CXCL16 is strongly associated with atherosclerosis, and such AEI assays have the potential to indicate the presence of *cis*-acting variants at specific loci and identify the influence alterations to such variants may have on allelic expression. As there is no information about rs2277680 this study provides data into AEI at this marker SNP.

A previous study (Gahan, 2014) showed that heterozygous cell lines GM12234 and GM07348 were not imbalanced, with each allele being equally expressed falling within the range 0.4 - 0.6, but could be induced into a state of imbalance (AEI) upon treatment with DMSO, SFN (10 µM), and TNFα 100 ng/µl in combination with SFN (10 µM) (Figure 3.1). This study also investigated AEI in ACS patients, noting that five out of 22 heterozygous individuals demonstrated evidence of AEI (Gahan, 2014). 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* had an AEI score of above 0.6, which correlated with data obtained from CEPH cell lines suggesting that one allele is associated with altered gene expression.

Following from this, the two cell lines; GM12234 and GM07348 underwent further experimentation in an attempt to expand on these initial findings. Data supports the initial observation (Gahan, 2014) in confirming the ability of DMSO to induce AEI in both GM12234 and GM07348 balanced cell lines (Figure 3.3). However, the direction of the imbalance is altered in the case of GM07348 (rF<0.4). GM12234 mirrors the initial observation in relation to the direction of AEI (Figure 3.3). The activity of SFN in this investigation is not as influential on AEI as initially thought, with treatment only inducing AEI in GM07348. The direction of which is the opposite of that previously observed in Gahan (2014) (Figure 3.1 and 3.3). However, both studies do suggest a strong effect for DMSO on AEI.

This study increased the incubation period with SFN, DMSO and TNFα 100 ng/µl and SFN from 6 to 24 h however; effects on AEI remained strongest following 6 h treatment. Additional ETAs were also assessed; PA, SAHA, DAC and their reconstitution agents DMSO, EtOH and methanol (Figure 3.5). It was proven necessary to test both ETA and reconstitution agent as DMSO was shown to greatly influence AEI (Gahan, 2014). Interestingly, DMSO formed the chemical starting point in the later development of SAHA (Vorinostat) (Marks and Breslow, 2007).

The allele driving the imbalance observed in this study remains elusive and is likely to vary between cell line and treatment based on results described above (Section 3.4). GM07348 appears to be more inclined to have AEI <0.4. The direction of imbalance in GM12234 tends to act in a concentration dependent manner, 5 µM ETA induces AEI < 0.4, concentrations of 10 µM induce AEI > 0.6 (Figure 3.5, A). This led to the question of whether the affected allele up- or down-regulated. This was investigated via RT-qPCR in samples identified to be in AEI (Figure 3.4 and 3.5). GM12234 treatments which induced AEI acted to increased *CXCL16* expression (Figure 3.6, A) with the exception of SFN (10 µM) 6 h and DMSO (10 µM) 24 h,

which acted to reduce expression from the imbalanced allele although not significantly. The opposite effect can be noted for GM07348, treatment with ETAs which induced AEI downregulated *CXCL16* expression significantly (Figure 3.6), with the exception of EtOH (5 µM) 24 h and TNFα 100 ng/µl+SFN 6 h.

Due to the observed disparity between results obtained in this study and the initial observations (Gahan 2014) it was necessary to determine the epigenetic stability of these cell lines in culture and to investigate whether alterations in AEI could be influenced by passage number and freezing, in addition to treatment with ETAs. Data suggest that transcription from the alleles of heterozygous CEPH cell lines remains balanced until higher passage numbers (Figure 3.8, 3.7) at which point imbalance takes over and their activity becomes less predictable. P30 is generally accepted as the upper limit of passage number for certain cells in culture, as further culturing may result in substantial variation of molecular profiles, limiting their *in vivo* applications and reproducibility (Calles et al., 2006; O'Driscoll et al., 2006; Wenger et al., 2004).

It should also be noted that freezing cells in DMSO may epigenetically alter transcriptional states and AEI, as can be seen in Figure 3.7 (Blue), suggesting that when cells are initially thawed from DMSO containing media, they are imbalanced. However, with increasing time in a cell culture environment, this imbalance tends to rectify itself based on Figure 3.7. DMSO has previously been shown to cause growth arrest and terminal differentiation of transformed cells and therefore freezing down cells in such for long periods may dramatically alter the cells molecular profile (Marks and Breslow, 2007).

Many associations have been noted between SNPs and disease phenotypes, yet a critical research question remains as to whether all cells contribute equally to the observed

phenotype. Molecular events in one or a few cells are known to have consequences at a multicellular level, as each cell can exhibit substantial phenotypic individuality driven by the local cell population context, such as cell density and non-uniform mechanical stress (Snijder et al., 2009; Werfel et al., 2013). Such single cell effects can be observed in cancer whereby macroscopic tumours can arise from a single cell that has escaped proliferation controls (Rockman, 2012; Yvert, 2014). In order to determine whether observed variation is due to AEI within single cells, or whether individual cells randomly silence one allele or another and the observed differences are due to the subsequent turnover in these cell numbers, it may be necessary in the future to observe expression signatures at a single cell level. The techniques used in this study do not enable us to directly distinguish between these possibilities. However, similarities between IL-18 AEI patterns in clinical samples and CEPH cell lines (Gahan 2014) favours the former option, single cells silencing one allele completely. Furthermore, as ETA treatments can influence AEI in a reverse direction over a short time scale (6 h) it would be unlikely to be due to cell turnover.

3.6 Limitations and Future Work

For this experiment CEPH cell lines where used, these EBV-transformed lymphoblastoid cell lines from multigenerational normal, healthy human volunteers 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). These cells may differ from primary cells from healthy subjects. In addition, lymphoblastoid cells such as the CEPH cells lines are particularly sensitive to DMSO, which may not be the case, to the same extent, in other cell lineages(Marks and Breslow, 2007).

Further studies for this chapter would include the determination of the duration of imbalance induced following treatment with DMSO and the amount of time necessary for balance to be re-established, if at all. Furthermore, methylation analysis of the gDNA of the samples used to determine epigenetic stability over time may help elucidate changing methylation patterns within CEPH cell lines over time.

3.7 Concluding Remarks

This study suggests than AEI can be induced following treatment with various ETAs, with DMSO showing a particularly strong induction, thus, proposing an epigenetic mechanism for AEI. DMSO in this study was shown to be a potent inducer of AEI in CEPH cell lines, GM12234 and GM07348, with the favoured allele varying between cell lines. ETOH another common reconstitution agent was also shown to induce AEI suggesting possible epigenetic activity. The AEI observed is not likely to be monoallelic but biallelic with most cells continuing to express both alleles, albeit at different levels as a result of treatment with ETAs. Monoallelic expression is unlikely as AEI can be observed following 6 h treatment which is too fast to be attributed to cell turnover alone, unless massive cell death has occurred.

CEPH cell lines where chosen for this study as opposed to primary cell lines as they have been genotyped by the HapMap consortium as part of the effort to locate specific regions which share genetic variations within and between populations (http://www.hapmap.org). Both the genotype data and gene expression data are publicly available (ftp://ftp.ncbi.nlm.nih.gov/hapmap/) and thus SNPs can be easily identified which is necessary for AEI studies.

In addition to this, a time series was performed in order to investigate the stability of AEI over time during cell culture. These results suggest that AEI is predominately stable, with considerable noise, over cell culture but become less predictable with increasing passage number. This study also raises the question as the effectiveness and reproducibility of using cells which have been frozen in DMSO and its potential for altering epigenetic marks within samples.

Chapter 4 : miRNAs as Predictive Markers for Stroke Recurrence

4.1 Introduction

A stroke is a sudden interruption of blood flow to the brain. This may be the result of a blockage caused by a clot or the bursting of a blood vessel, leading to a delay in the blood supply reaching the brain, depriving it of oxygen and essential nutrients.

Stroke is third leading cause of death and the leading cause of serious long-term disability in the US (www.strokecenter.org). It is estimated that one person in the US dies from stroke every 40 seconds (www.strokecenter.org). In Ireland, the Irish Heart Foundation conclude that one in every five people will suffer a stroke in their lifetime. Following an initial stroke, the likelihood of having a second stroke (recurrent) is extremely high with 25 % of women and 42% of men experiencing a second event within five years of the first event (stroke) (Sacco et al., 1982). Recurrent strokes are associated with a higher mortality and disability rate due to existing damage from the first event (www.strokecenter.org).

4.2 Stroke and Associated Outcomes

Ischemic stroke accounts for over 80 % of strokes and is caused by blockage of an artery supplying blood to the brain (Feigin et al., 2003). This results in a series of events including energy depletion, cell death, free radical formation and inflammation (Dirnagl et al., 1999; van der Worp and van Gijn, 2007). An acute ischemic stroke (AIS) occurs when an artery supplying the brain becomes occluded, leading to brain tissue death and focal neurological deficits (Mozaffarian et al., 2015; Prabhakaran et al., 2015). A clot which forms in the main artery supplying blood to the brain is known as cerebral thrombosis. A cerebral embolism occurs if this clot becomes dislodged and is carried to the brain. Finally, a Lacunar stroke occurs following “Occlusion of a single small perforating artery supplying the subcortical area of the

brain" (Wardlaw, 2005). Clots form in arteries which have already been narrowed by atherosclerosis, with atherosclerosis and cardio-embolism being described as the leading causes of brain ischemia (van der Worp and van Gijn, 2007). The remaining 20 % of strokes, according to the Irish Heart Foundation are haemorrhagic strokes, caused by bleeding into the brain from burst blood vessels (Feigin et al., 2003). A transient ischemic attack (TIA) can be described as a mini stroke and is often considered a warning sign of a bigger stroke to come (Purroy et al., 2007; Strömberg et al., 2015). TIAs only temporarily deprive the brain of a blood supply.

Disabilities arising from stroke include paralysis, weakness, loss of sensation on one side of body, difficulty swallowing and communicating, loss of cognitive function, and loss of vision (www.stroke.ie). About 80 % of people who survive a stroke will suffer paralysis on one side of the body (www.stroke.ie). This is can either be partial or complete depending on the seriousness of the stroke (Hendricks et al., 2002). The disabilities associated with stroke are a heavy burden on health systems worldwide, costing approximately 70 billion dollars a year. Therefore, the identification of markers which may predict stroke and subsequent recurrent strokes is a healthcare priority for the future (Mozaffarian et al., 2015; Prabhakaran et al., 2015).

4.2.1 Stroke Recurrence

Patients surviving stroke are known to be at a significantly increased risk for further strokes (Boysen and Truelsen, 2000). The risk of recurrent stroke is highest in the first few days following the onset of initial symptoms (Salem et al., 2011; Wu et al., 2007). It has been suggested that including TIA in predicting the risk of stroke recurrence may lead to an

overestimation of the reported risk , with a greater risk being noted 30 days after an initial event (Coull et al., 2004; Mohan et al., 2011).

Carotid endarterectomy (CEA) is one of the most effective options available to decrease the likelihood of stroke recurrence, and it is best carried out two weeks after the onset of initial symptoms (Giles and Rothwell, 2007; Hankey, 1996; Rothwell et al., 2004; Salem et al., 2012; Wolf et al., 1999; Wu et al., 2007). The large number of recurrent stroke events prior to CEA mandated an investigation for markers of plaque instability that could predict rupture events and identify the most at-risk patients. In order to assess this Salem *et al* harvested the plaques of 158 patients undergoing CEA and independently scored them for histological markers of plaque instability (Salem et al., 2012). Of these patients those which had recurrent events following admission to hospital had significantly larger lipid cores (P=0.004) and low grey scale medium (GSM) when compared against patients who did not experience recurrence (Salem et al., 2012). Stroke recurrence prediction was further investigated by Marnane *et al* using ^{18}F-Flurodeoxyhlucose (FDG) positron emission tomography (Marnane et al., 2012). Radiolabelled FDG was used, as it has been shown in previous studies to accumulate in carotid atherosclerotic lesions, particularly in sites with high inflammatory activity, and it is known to correlate to the extent of macrophage infiltration (Marnane et al., 2012; Ogawa et al., 2004; Rudd et al., 2002; Tawakol et al., 2006). Marnane *et al* investigated the relationship between inflammation related FDG uptake and stroke recurrence, in a patient cohort which included recent stroke (approximately 6.5 days), TIA, retinal embolism and ipsilateral carotid stenosis (Marnane et al., 2012). 13 out of 60 patients had recurrence within 90 days and of these FDG uptake was greater when compared against non-recurrent patients. The authors concluded that inflammation related FDG uptake is associated with early stroke recurrence, and that

FDG-PET can be used as a means of identification of high risk patients for stoke recurrence (Marnane et al., 2012; Strömberg et al., 2015). In a follow up study, Marnane and colleagues next sought to investigate the relationship between early stroke recurrence and the histological features of plaque inflammation and instability in resected carotid plaques, in order to determine the underlying pathophysiobiology of high early stroke recurrence risk (Marnane et al., 2014). Features of inflammation and instability investigated in this study included fibrous cap disruption, neovascularisation, macrophage infiltration and fibrous content. Carotid endarterectomy tissue was examined from patients with greater than 50 % ipsilateral stenosis. Plaques were analysed using a histopathological algorithm OPS (Oxford Plaque Study system) (J. K. Lovett et al., 2004; J.K. Lovett et al., 2004). Stroke recurrence before carotid endarterectomy was ascertained at seven, 28, and 90 days after initial symptoms. 44 patients met the criteria, of which 12/44 (27%) had recurrence after initial stroke/TIA but before CEA. When this patient subset was compared against patients who did not have recurrence 32/44 (72%), it was noted that stroke recurrence was associated with dense macrophage infiltration (P=0.002), extensive fibrous cap disruption (P=0.004), neovascularisation (P=0.04) and low fibrous content (P=0.003), highlighting that plaque inflammation and other vulnerability features were associated with the highest risk of stroke recurrence and may represent targets for future therapies.

Although the aforementioned studies have outlined methods for identifying patients most at risk of stroke recurrence, many are not feasible in a hospital based environment due to the demands on resources and the limited time frame necessary to produce test results that will promote successful interventions. Thus, this mandates an investigation for a marker of stroke recurrence that can be easily assayed by a molecular test, mitigating the indicated problems.

This chapter endeavoured to evaluate the potential of miR210 and miR145 to act as efficient markers in predicting stroke recurrence in a cohort of patients which meet the criteria outlined above (Marnane et al., 2014).

4.3 miRNA Biomarkers and Interventions

Since their identification in 1993 (Lee et al., 1993; Wightman et al., 1993) miRNA biogenesis and function have been widely investigated. MiRNAs are promising disease biomarkers and therapeutic targets as they regulate more than 60 % of human genes and are known to be implicated in a wide range of biological activities including proliferation, differentiation and apoptosis, making them key players in disease diagnosis, prediction and treatment (Corcoran et al., 2011; Li and Kowdley, 2012; Ng et al., 2012; Png et al., 2012; Rayner et al., 2011; Taganov et al., 2006; Zhang et al., 2012). Regulation of gene expression via miRNAs has been shown to participate in differentiation and development but also in processes which require dynamic responses (Asirvatham et al., 2008b; Bhattacharyya et al., 2006; Chiou, 2007; van Rooij et al., 2007). The miRNA-gene interaction facilitates the recruitment of chromatin modifying proteins to nuclear sites, resulting in heterochromatic silencing (Grewal and Moazed, 2003).

4.3 miRNA therapeutic options

Research investigating a potential link between miRNA expression profiles, plaque development, and rupture is ongoing. The potential use of miRNAs in therapy includes organ targeted RNAi using viral vectors or synthetic RNA and therapeutic strategies on the basis of modulation of miRNA function (Poller et al., 2013). One of the first successful applications of RNAi therapeutics for CVD in a clinical setting was that of the RNAi drug ALN-PCS on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) (Abifadel et al., 2003; Zhao

et al., 2006; Fitzgerald et al., 2014). PCSK9 binds the receptor for LDL-C. Upon binding the receptor can no longer remove LDL-C from the blood, suggesting that inhibition of PCSK9 may help reduce cholesterol levels. Treatment with ALN-PCS resulted in a mean 70 % reduction in circulating PCSK9 plasma protein and a mean 40 % reduction in LDL cholesterol from baseline relative to placebo (Fitzgerald et al., 2014). Furthermore, anti-miR activity has been demonstrated for miR122 in the treatment of hepatitis C infection via the targeting of miR122 necessary for viral replication, fatty acid and cholesterol metabolism, through the use of a LNA-modified oligonucleotide SPC3649a (Li and Kowdley, 2012). This anti-Mir was delivered intravenously and resulted in depletion of mature miR122 and a dose dependent lowering of cholesterol, in addition to long lasting suppression with no viral resistance or side effects (Elmén et al., 2008, 2008; Lanford et al., 2010). Anti miR133 therapy has also been shown to reduce the progression of atherosclerosis and improve HDL function in Ldlr $^{-/-}$ mice (Rotllan et al., 2013).

Nonetheless, challenges remain. These include specificity, as the 3' UTR of a single mRNA can be targeted by multiple miRNAs. MiRNAs also exert many different actions dependent on cell type. Hence miRNA modulation therapies require precise cellular targeting and suitable delivery methods. For instance, miR144-3p has been shown to accelerate plaque formation through the post transcriptional regulation of ABCA1. ABCA1 has a critical role in cellular cholesterol efflux and the formation of HDL, inhibition of which through targeting miR144-3p has been shown in THP-1 cells and in ApoE$^{-/-}$ mice to increase inflammatory cytokine secretion and accelerate plaque formation (Hu et al., 2014a). This highlights how essential it is for appropriate miRNA targeting in atherosclerosis, as one miRNA can influence multiple pathways.

4.4 miRNAs and Plaque Rupture

miRNA expression profiles are likely to differ between atherosclerotic plaques and control arteries, and may provide useful markers and targets in atherosclerosis (Cipollone et al., 2011; Hao et al., 2014; Menghini et al., 2014, 2013, Raitoharju et al., 2013, 2011; Wierda et al., 2010).

The Tampere Vascular Study analysed miRNA expression profiles in human atherosclerotic plaques compared to non-atherosclerotic left internal thoracic arteries. They examined plaques from the aortic, femoral and carotid arteries using microarray analysis which was verified using RT-qPCR. They identified miR21, miR210, miR34a and miR146a/b to be upregulated in human atherosclerotic plaques and in return their target mRNA sequences to be down regulated (Raitoharju, Lyytikainen et al. 2011). The genes down regulated included genes involved in the regulation of signal transduction, transcription and vesicular transport. Those which where upregulated are thought to be involved key processes of atherosclerosis (Wierda et al., 2010; Raitoharju et al., 2011).

Following from this, Cipollone *et al* investigated whether a unique miRNA signature was associated with plaque instability in humans (Cipollone, Felicioni et al. 2011). The study aimed to identify miRNA expression profiles indicative of plaque instability in asymptomatic samples versus symptomatic samples. A total of 41 miRNAs were evaluated and of these they identified five miRNAs, miR100, miR127, miR145, miR133a and miR133b that had differential expression levels. Expression of these miRNAs was greater in symptomatic patients with ischemic stroke, suggesting a role for these miRNAs in plaque instability and rupture and as predictive biomarkers. They noted that >71 % of the patients affected by stroke had miRNA expression levels higher than asymptomatic patients and that many of the affected miRNA regulated

genes are involved in the modulation of inflammation (Cipollone et al., 2011). Further findings implicate miRNAs such as miR24 and miR21 in the regulation of matrix metalloproteinase (MMPs), known to play a part in fibrous cap thinning and in turn plaque rupture (Di Gregoli et al., 2014; Fan et al., 2014).

Circulating miRNAs have also attracted considerable attention as potential biomarkers, as they can be detected in circulating blood and potentially used for the risk stratification of patients (Deiuliis et al., 2014). Fichtlscherer *et al* identified four miRNAs whose expression levels were reduced in the plasma of patients with CAD compared to healthy controls; miR126, miR17, miR92a, and miR155. Muscle enriched miRNAs were also found to be more highly expressed in patients with CAD compared to volunteers (Fichtlscherer et al., 2010; Stellos and Dimmeler, 2014). Furthermore, the HUNT study assessed the use of circulating miRNAs to predict future fatal MI in healthy participants (Bye et al., 2016). This study was carried out over a ten year observation period with fatal AMI acting as the endpoint. The study quantified 179 miRNAs by RT-qPCR in the serum of participants (aged 40-70) that either suffered fatal AMI within 10 years or remained healthy (Bye et al., 2016). They identified a total of 12 miRNAs that differed in expression between cases and controls, of which 10 differed significantly (Bye et al., 2016). Gender dimorphisms were also apparent as miR424-5p and miR26a-5p were associated with increased risk in men and women respectively. They noted that the best model for predicting AMI consisted of miR106a-5p, miR424-5p, let-7g-5p, miR144-3p and miR660-5p providing 77.6 % correct classification for both genders, and 74.1% and 81.8% for men and women, respectively.

These studies clearly identify a correlation between miRNA expression and plaque instability. However, it is not known what is responsible for the differential miRNA expression between stable and non-stable plaques. It should be noted that shear stress alters the miRNA expression profile of a plaque, with low shear stress having greater influence on the induction of differential miRNA gene expression (Kumar et al., 2014). The studies described above have investigated different outcomes of atherosclerosis and therefore caution must be exerted when making comparisons between data sets. This chapter investigates stroke recurrence as an outcome of atherosclerosis. Consideration must also be given to whether patients were receiving statins or other cholesterol modifying drugs as this could alter/contribute to the miRNA expression observed.

4.5 MiR210 and Atherosclerosis

The expression of miR210 has been shown to be differentially expressed in the blood of CVD patients when compared to patients unaffected by CVD (Raitoharju et al., 2013). MiR210 is a hypoxia responsive miRNA regulated by HIF-1 (hypoxia inducible factor 1)(Chen et al., 2015). Although common in cancer, hypoxia is also a prominent feature of atherosclerosis, and has been shown to increase lesion progression by promoting lipid accumulation, inflammation and ATP depletion (Raitoharju et al., 2013). Neovascularisation in response to hypoxia has been associated with plaques prone to rupture (Raitoharju et al., 2013). Increased expression of miR210 has been noted in the intima layer of arterial plaques from patients with atherosclerosis, with parallel upregulation being noted in the serum of these patients (Li et al., 2011).

MiR210 also plays a multitude of roles in brain ischemia and has been reported to induce angiogenesis and neurogenesis essential for brain tissue repair and remodelling following

injury (Zeng et al., 2014). Furthermore, levels of blood miR210 have been suggested as a novel biomarker in acute cerebral ischemia (Zeng et al., 2014). Levels of blood miR210 were measured three, seven and 14 days following stroke via RT-qPCR and compared to healthy controls. Levels of miR210 were significantly lower in the blood of patients seven and 14 days after stroke, with increased levels of miR210 in stroke patients being associated with a more favourable outcome (Zeng et al., 2014).

Bio-informatic analysis of the gene promoter of miR210 has revealed the presence of CpG rich regions, and the potential for epigenetic regulation of miR210 (Chen et al., 2015). There is evidence to suggest that treatment with oxLDL acts to decrease methylation of miR210 at its promoter, leading to miR210 upregulation, suggesting that HIF-1 promoter activation of miR210 could be blocked by methylation (Chen et al., 2015). This study also identified hypomethylation on the miR210 promoter, particularly in the HIF-1 binding site of carotid atherosclerosis, stroke and cancer patients (Chen et al., 2015).

4.6 MiR145 and Atherosclerosis

MiR145 expression has been shown to differ in the blood of individuals affected and unaffected by CVD (Raitoharju et al., 2013). In the serum and plasma of individuals with CAD, miR145 expression was found to be down regulated when compared to levels in healthy volunteers (Fichtlscherer et al., 2010). However, miR145 was found to be upregulated in symptomatic carotid plaques when compared to asymptomatic plaques and was also significantly more expressed in the atherosclerotic plaques of hypertensive patients than in control plaques (Cipollone et al., 2005; Maitrias et al., 2015; Santovito et al., 2012). In addition to this, miR145 was found to be significantly upregulated in the serum of AIS patients 24 h after stroke onset, a pattern which correlated to high levels of plasma high sensitivity CRP (Jia

et al., 2015). This suggests that miR145 performs a multifaceted role at different time points in CVD progression, that can negatively or positively influence outcome.

MiR145 is highly expressed in SMCs and is reported to be the most abundant miRNA in normal vascular walls (Cheng et al., 2009a; Y. Wei et al., 2013). Murine studies identified the greatest miR145 expression to be located in the aorta and coronary vessels, with its expression being proportional to the number of VSMCs in all organs studied (Boettger et al., 2009a; Elia et al., 2009; Rensen et al., 2007).

MiR145 is known to play a role in development of the vasculature and has been shown to direct SMC fate, with downregulation of miR145 promoting atherosclerotic lesion formation (Cordes et al., 2009; Y. Wei et al., 2013). Overexpression of miR145 in VSMCs has been described as a novel *in vivo* target to limit atherosclerotic plaque morphology and cellular composition, shifting the balance toward plaque stability (Albinsson and Swärd, 2013; Lovren et al., 2012). In cell lines, miR145 enriched vesicles have been shown to convey atheroprotective signalling from ECs to VSMCs (Hergenreider et al., 2012; Raitoharju et al., 2013).

It is apparent that the relationship between miR145 and the various types of atherosclerosis related disease subtypes is highly complex, and dependent on multiple variables but nonetheless miR145 is a promising therapeutic target due to its prominent role in disease development. It should be noted that miR145 is highly conserved and lies in close proximity to miR143 on chromosome five (Rangrez et al., 2011) and consequently they are transcribed together (Boettger et al., 2009b; Elia et al., 2009; Xin et al., 2009).

4.7 Aims and Objectives

- Evaluate the potential use of miR210 and miR145 in predicting the likelihood of stroke recurrence in carotid endarterectomies from patients that have been clinically categorised in areas such as plaque macrophage count, evidence of plaque ruptutre and overall plaque stabilitity in addition to other categories.

- Determine whether correlations can be made between the levels of miR210 and miR145 expression and other markers of plaque instability including macrophage infiltration, fibrous cap disruption and neovascularisation.

- Assess whether miR210 and miR145 have any potential use as biomarkers for stroke recurrence

4.8 Materials and Methods

4.8.1 Carotid endarectomy Samples

Carotid endarterectomy samples were obtained from the Dublin Carotid Atherosclerosis Stroke Study (DUCASS) with permission from the Mater Hospital (Marnane et al., 2014). Ethics committee approval was obtained from all participating institutions, and all participants provided informed consent. For an in depth description of the patient recruitment criteria, tissue processing and histological analysis of these patients, see Marnane et al. (2014).

4.8.2 Clinical Characteristics

44 patients met the pre-specified inclusion criteria set out by Marnane *et al* and were used in the miRNA analysis described below. Of the 44 patients, the index event which occurred within 28 days of study recruitment varied. TIA accounted for 21/44 (47%) index events, ischemic stroke 13/44 (29%) and retinal artery embolism for 10/44 (22%). Of these 44 patients, 27/44 (61%) sought medical attention following the first event and 17/44 (38%) sought medical attention for a recurrent event (TIA/Stroke). All patients with clinical evidence of recurrence had brain diffusion weight MRI to confirm a recurrent event, which was identified by acute ischemic change (Marnane et al., 2014).

4.8.3 miRNA Extraction

Carotid endarterectomy plaques which had been collected from the 44 patients used in the Marnane *et al* study and were frozen in liquid nitrogen and stored an -80 °C. These samples were manually homogenised and miRNA extraction was performed using RNAzol (Sigma Aldrich, St. Louis, Missouri, US) as per manufacturer's guidelines.

4.8.4 cDNA synthesis for miRNA analysis

cDNA was synthesised using the Universal cDNA synthesis kit II (Exiqon, Denmark) as per manufacturer's instructions.

Reagents	1 Reaction	Program
5x Reaction Buffer	2 µl	42 °C 60 min
H_2O	4.5 µl	95 °C 5 min
Enzyme Mix	1 µl	
RNA Spike In	0.5 µl	4 °C End/Store
RNA(5ng/µL)	2 µl	
Final Volume	**10 µl**	

Table 4.1: Reagents used for cDNA synthesis

4.8.5 miRNA quantitative real-time PCR

MiRNA quantitative real-time PCR reactions were performed using ExiLENT SYBR green master mix (Exiqon, Denmark). The PCR master mix was protected from light. cDNA was diluted 1:80 in nuclease-free water. The 5s ribosomal unit was used as an endogenous control. All reactions were performed in triplicate.

Reagents	1 Reaction	Program
PCR Master Mix	5 µl	95 °C
PCR Primer Mix	1 µl	X40 cycles- 95 °C 10 s,
Diluted cDNA Template	4 µ l	60 °C 1 min
Final Volume	**10 µl**	

Table 4.2: Reagents used for miRNA RT-qPCR

4.8.6 Statistical Analysis

Statistical analysis for all data gathered from the extraction and quantification of miR210 and miR145 in the patient subset described above was performed using Prism GraphPad version 5 and R-Studio (https://www.rstudio.com/). The threshold for significance was $p<0.05$. Fold change expression values for all miRNAs were log-transformed to create a normal distribution for parametric analysis (Avissar et al., 2009; Pradervand et al., 2009). Unpaired t-test with

Welch's correction was used for Figure 4.1, 4.2 and 4.3. Spearman's non-parametric test was used for Figure 4.4-4.8

4.9 Results

4.9.1 MiR210 and miR145 expression levels in Carotid Endarterectomy samples from recurrent and non-recurrent patients

Carotid endarterectomies taken from patients profiled by Marnane *et al*, (2014) (Appendix Figure B) were used to determine if any association could be detected between the level of expression of miR210 and miR145 and stroke recurrence. In order to create a normal distribution for parametric analysis the fold change expression values for miR210 and miR145 were log-transformed. Analysis was performed using an unpaired t-test with Welch correction (Figure 4.1, 4.2, 4.3). These data suggest that the levels of miR210 and miR145 are decreased in patients who suffered a recurrent event stroke/TIA after initial stroke/TIA, but before carotid endarterectomy (CEA) (Figure 4.1).

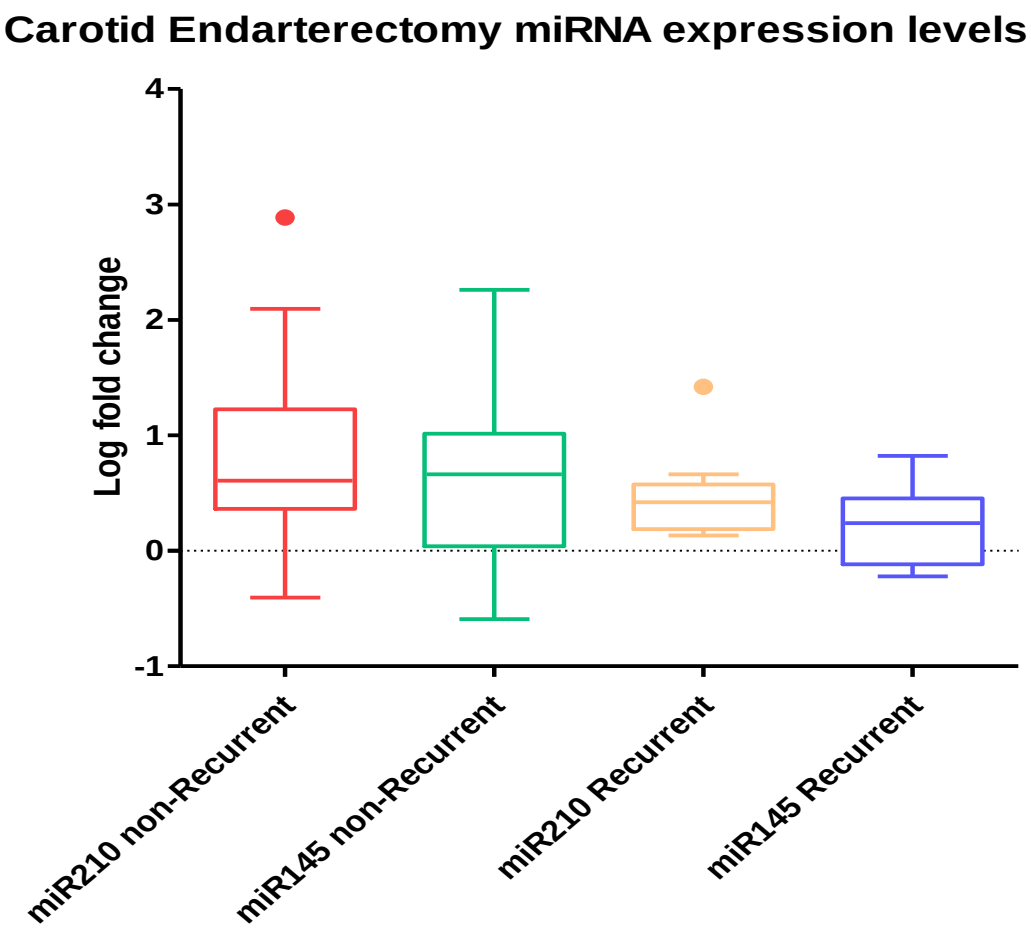

Figure 4.1: Expression levels of miR210 and miR145 in Carotid endarterectomy samples.

Carotid endarterectomies from patients profiled by Marnane *et al.*, 2014 were analysed by RT-qPCR for expression levels of miR210 and miR145. Fold change expression values for all miRNAs were log-transformed to create a normal distribution for parametric analysis. Increased levels of miR210 and miR145 can be found in non-recurrent patients when compared against recurrent patients. Recurrent patients were identified as patients who had stroke recurrence after initial stroke/TIA but before carotid endarterectomy. Red and green = non-recurrent, yellow and blue = recurrent.

4.9.2 Expression Levels of miR210 in recurrent versus non-recurrent Patients

Levels of miR210 were reduced in patients who suffered from a recurrent event before CEA (Figure 4.2). This reduction was not significant (P=0.0869).

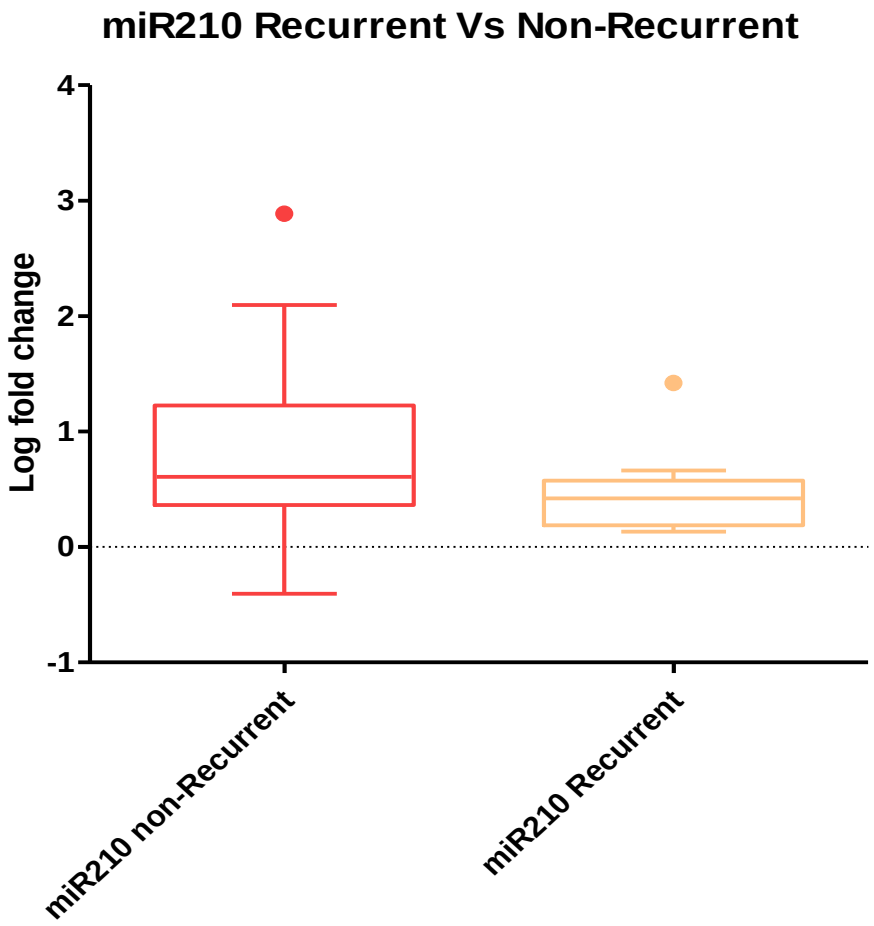

Figure 4.2: Expression levels of miR210 in recurrent and non-recurrent Carotid endarterectomy samples. Increased levels of miR210 can be found in non-recurrent patients when compared against recurrent patients, this was however not statistically significant when analysed using an unpaired t-test with Welch correction (P=0.0869). The data was considered significant if $p<0.05$. Recurrent patients had stroke recurrence after initial stroke/TIA but before carotid endarterectomy.

4.9.3 Levels of miR145 in Recurrent versus non-Recurrent Patients

Levels of miR145 expression were significantly reduced in patients who suffered from a recurrent event before CEA (P=0.030) (Figure 4.3).

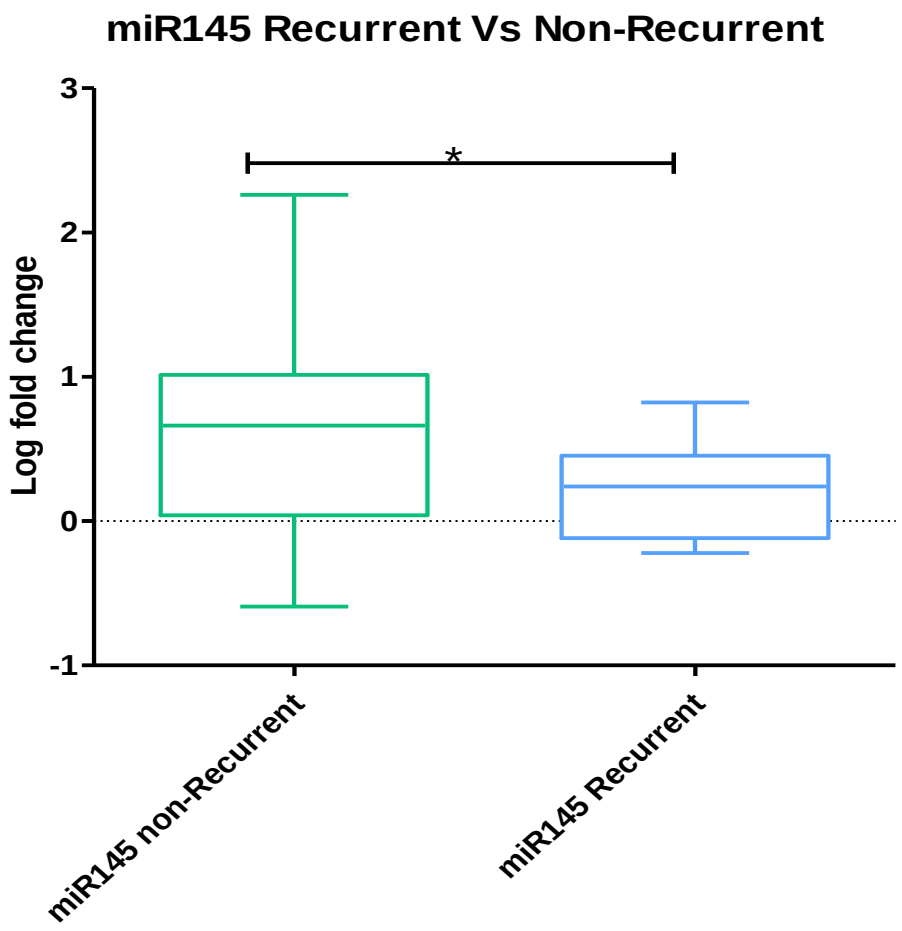

Figure 4.3: Expression levels of miR145 in recurrent and non-recurrent Carotid endarterectomy samples.
Levels of miR145 where significantly increased in non-recurrent patients when compared against those who had stroke recurrence after initial stroke/TIA but before carotid endarterectomy (P=0.0360). The comparison was considered significant if $p<0.05$ (Unpaired t-test with Welch correction). Recurrent patients had stroke recurrence after initial stroke/TIA but before carotid endarterectomy.

4.9.4 MiR145 and miR210 expression and plaque macrophage count

Based on the above observations an investigation was carried out to determine whether miR145 and miR210 expression levels were associated with any markers of plaque instability profiled by Marnane *et al* (2014) (Appendix Figure A). miRNA expression level data were compared against matched histological data using R-Studio. A Spearman's non-parametric test identified a significant correlation between miR145 (P=0.0023) and miR210 (P=0.04307) expression levels with plaque macrophage CD68 counts (Figure 4.4). Despite a degree of variation between samples, there is a trend between lower miR145 and miR210 expression and greater plaque macrophage count.

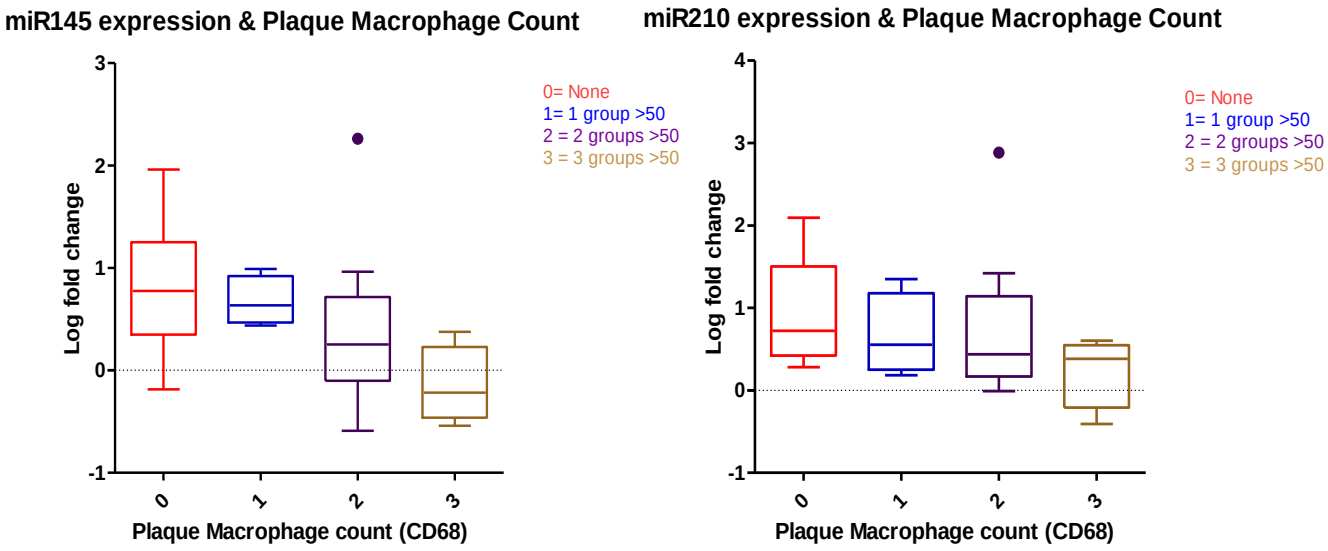

Figure 4.4: Association between miR145 and miR210 expression and Plaque Macrophage Count.

Data indicate a correlation between miR145 and mir210 expression and plaque macrophage count (P=0.0023 and P=0.04307, respectively) in both recurrent and non-recurrent patients identified by Marnane *et al*. Data were analysed using Spearman's non-parametric test and the threshold for significance was $p < 0.05$. 0 = No macrophages found in plaque, 1 = 1 group of >50 macrophages in the plaque, 2 = 2 groups >50 macrophage in the plaque, 3 = 3 groups with >50 macrophages counted in the plaque.

4.9.5 MiR145 expression and cap macrophage count

A similar association was identified between miR145 levels and the number of macrophages in the cap of the plaque (Figure 4.5) (P=0.0018). A trend can be noted between decreased miR145 expression and increased macrophage count in the plaque cap.

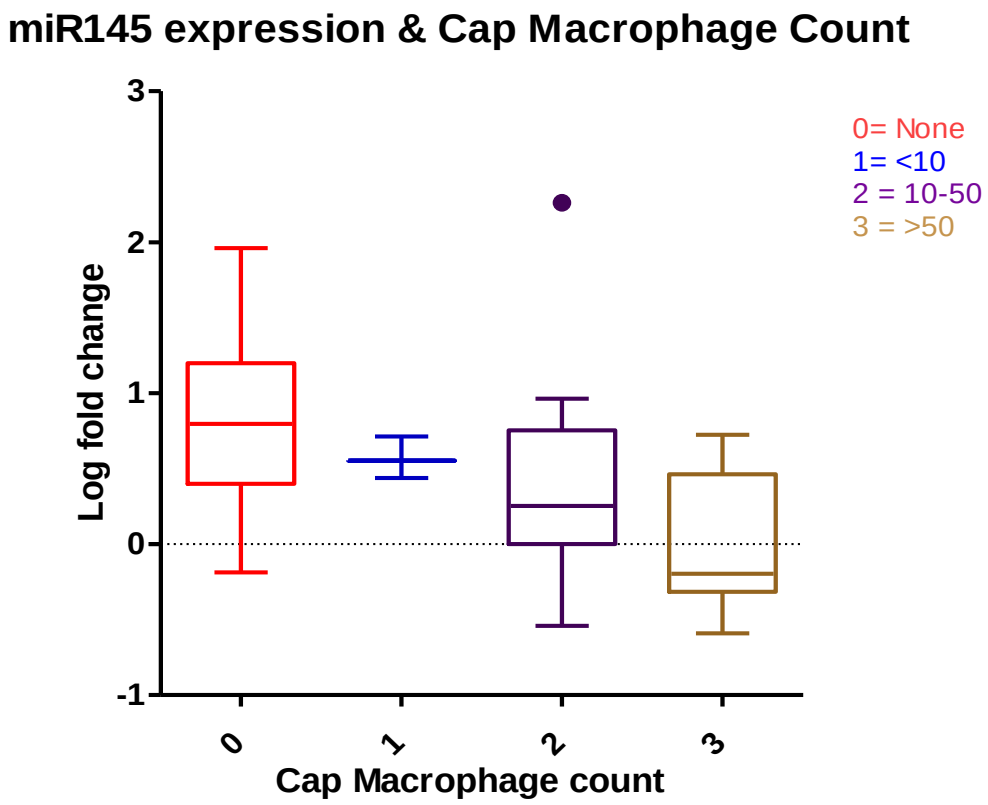

Figure 4.5: Association between miR145 expression and Cap Macrophage Count.

Data indicate a correlation between miR145 expression and cap macrophage count (P=0.0018) in both recurrent and non-recurrent patients identified by Marnane *et al.* Data were analysed using Spearman's non-parametric test and the threshold for significance was p<0.05. 0 = No macrophages found in the cap, 1 = <10 macrophages counted, 2 = 10-50 macrophages counted in the cap, 3 = >50 macrophages counted in the cap.

4.9.6 MiR145 and Brown Histiocyte presence

When histological data obtained from Marnane *et al* (2014) was compared with matched miR145 and miR210 expression levels from this study, an association between miR145 expression levels and the presence or absence of brown histiocytes in recurrent and non-recurrent patients was uncovered (Figure 4.6)(P=0.0207). Their presence was classified by Marnane *et al* as indicative of previous intraplaque haemorrhage (IPH). A trend can be noted between decreased miR145 expression and the presence of brown histiocytes.

Figure 4.6: Association between miR145 expression levels and brown histiocyte count.

An association was noted between miR145 and the presence of absence of brown histiocytes in recurrent and non-recurrent patients (P=0.0207). Data were analysed using Spearman's non-parametric test and the threshold for significance was $p<0.05$. Brown histiocytes where identified using haematoxylin and eosin stain. 0 = No, 1 = Yes.

4.9.6 Association between miR145 expression and Cap Rupture

An association was also found between miR145 expression and cap rupture in recurrent and non-recurrent patients (Figure 4.7) (P=0.0258). Cap rupture was identified as a luminal thrombus with lipid nanocapsules (LNC). Patients with cap rupture were identified in this study to have decreased miR145 expression levels, suggesting a relationship between decreased miR145 levels and cap rupture.

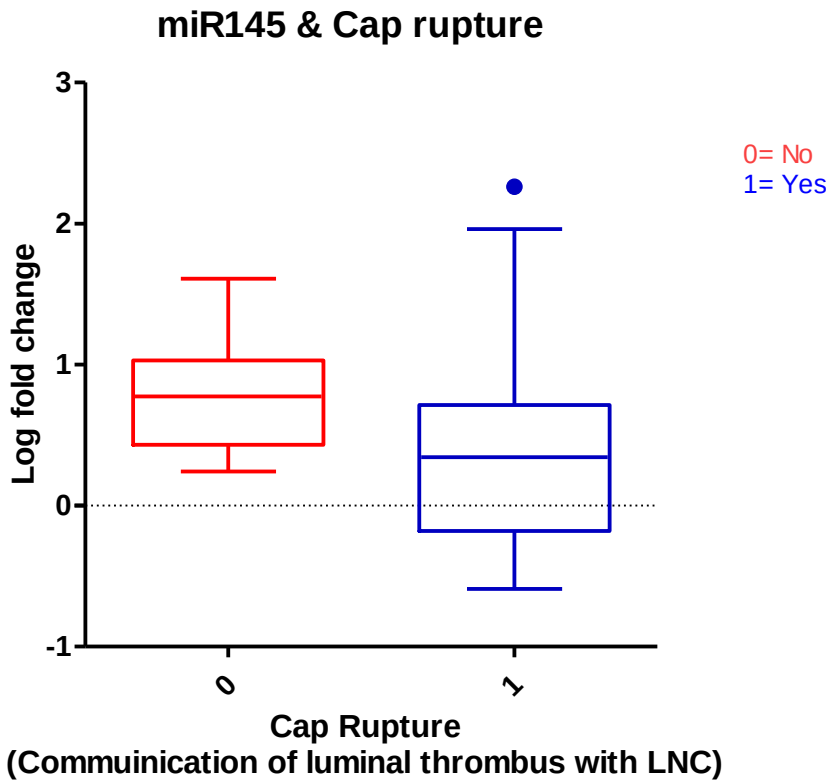

Figure 4.7: Association between miR145 expression levels and Cap Rupture.

An association was noted between miR145 and cap rupture in recurrent and non-recurrent patients (P=0.0258). Data were analysed using Spearman's non-parametric test and the threshold for significance was $p<0.05$. LNC= Lipid Nanocapsules. 0 = No, 1 = Yes.

4.9.7 Association between miR145 and Plaque stability

Plaque stability in the patient cohort used in this study and by Marnane *et al* (2014) was determined according to the Oxford Plaque Study Categories (J. K. Lovett et al., 2004; J.K. Lovett et al., 2004). Values range from one (stable) to four (Unstable with thrombus or rupture). Using Spearman's non-parametric test an association was found between miR145 and plaque stability. Patients with the most severe plaque phenotype (unstable with thrombus or rupture) tended to have the lowest levels miR145 expression (Figure 4.8) (P=0.0447).

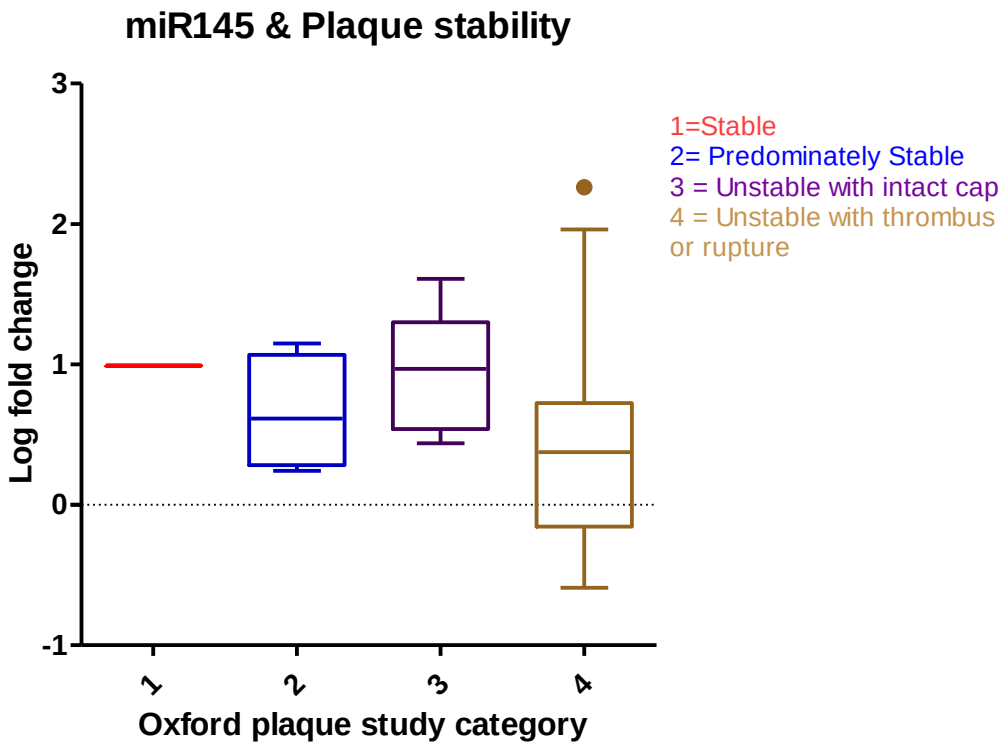

Figure 4.8: Association between miR145 expression levels and plaque stability.

An association was noted between miR145 expression levels and plaque stability, categorised using Oxford plaque study categories (J. K. Lovett et al., 2004; J.K. Lovett et al., 2004) in recurrent and non-recurrent patients (P=0.0447). Data was analysed using Spearman's non-parametric test and the threshold for significance $p<0.05$. 1 = Stable, 2 = Predominately Stable, 3 = Unstable with Intact Cap, 4 = Unstable with thrombus or Rupture.

4.9.8 Non-Significant associations of miR145

In addition, the above analysis was also carried out on other markers of plaque instability such as cap erosion with evidence i.e. luminal thrombus with absence of endothelium, degree of cap disruption, evidence of previous plaque rupture, lipid core size, calcification, fibrous tissue and the thickness of the fibrous cap, all of which have no associations with miR210 or miR145 expression levels (Table 4.3), P- value threshold P<0.05

Plaque Instability Feature, graded via Histological Analysis	**miR145 (P-value as determined by Spearman non-parametric analysis)**	**miR210 (P-value as determined by Spearman non-parametric analysis)**
Plaque Macrophage count	P<0.05 (Figure 4.4)	P<0.05 (Figure 4.4)
Cap Macrophage count	P<0.05 (Figure 4.5)	0.06232
Cap Erosion	0.671	0.7238
Evidence of Previous Plaque Rupture	0.4629	0.3577
Lipid Core Size	0.05621	0.09475
Calcification	0.5761	0.5196
Fibrous tissue	0.1893	0.1916
Fibrous cap thickness	0.263	0.432
Cap rupture	P<0.05 (Figure 4.7)	0.316
Plaque Stability	P<0.05 (Figure 4.8)	0.3548
Brown Histiocyte	P<0.05 (Figure 4.6)	0.1994

Table 4.5: Non-Significant associations of miR145 and miR210 and plaque instability features

4.10 Discussion

The main objective of this study was to determine the potential use of miR210 and miR145 in predicting stroke recurrence and whether correlations could be made between the levels of miR210 and miR145 with other markers of plaque instability/vulnerability, including markers of macrophage infiltration, fibrous cap disruption and neovascularisation.

This study made use of carotid endarterectomies from patients with >50 % ipsilateral stenosis used in the Dublin Carotid Atherosclerosis Stroke Study (Marnane et al., 2014). Results from this study noted a "nine-fold increase in the risk of stroke recurrence before CEA associated with abundant macrophage content. High plaque macrophage content independently predicted the risk of early stroke recurrence" (Marnane et al., 2014). Areas of macrophage infiltration were identified using CD68 antibody stain (Marnane et al., 2014). CD68 is transmembrane glycoprotein which binds LDL and is expressed by human monocytes and macrophages (Holness and Simmons, 1993; van der Kooij et al., 1997).

In order to transform to a normal distribution, fold change expression values for miR210 and miR145 were log-transformed for parametric analysis (Avissar et al., 2009; Pradervand et al., 2009). MiR145 exhibited a significant decrease in expression in patients with stroke recurrence compared to non-recurrent patients (P=0.0360) (Figure 4.3). In the same patient subset levels of miR210 were compared in recurrent and non-recurrent patients. MiR210 expression, while elevated in patients without recurrence, did not reach significance (Figure 4.2).

Further analysis of the relationship between miR210, miR145 and other markers of inflammation and plaque instability was carried out. No significant associations were noted

between miR145 and miR210 with calcification, fibrous tissue content, lipid core size and plaque erosion (Table 4.5). A significant association was found between miR210 and plaque macrophage count (Figure 4.4) (P=0.04307). A significant association was also found between miR145 with plaque and cap macrophages as counted by CD68 staining (Figure 4.4 and 4.5) (P= 0.0018, 0.0023), along with the presence of brown histiocytes indicative of intraplaque haemorrhage (IPH) in this instance (Figure 4.6) (P=0.0207). Data from this study suggest that reduced miR145 is associated with greater plaque macrophage infiltration (>500) and cap macrophage infiltrations (>50), as identified by CD68 staining (Figure 4.4 and 4.5). Patients with little or no macrophage infiltration in both the cap and plaque had the highest levels of miR145 expression. Decreased miR145 levels were also associated with cap rupture and plaque instability (Figure 4.7 and 4.8) (P=0.058, 0.0447).

An observation noted by Vengrenyuk *et al* (2015) may provide insight into the relationship between miR145 and CD68 macrophages. This study noted that cholesterol loading of aortic SMC acted to induce a macrophage-like state via the down regulation of the miR143/145-myocardin axis and an increase in the expression of macrophage markers such as CD68 in VSMC (Vengrenyuk et al., 2015). This acquisition of macrophage-like traits by VSMC was supported by the determination that 30-40 % of cells identified as positive for macrophage markers were in fact of VSMC origin in human plaques (Allahverdian et al., 2014; Andreeva et al., 1997).

MiR145 is essential for determining VSMC fate and function, along with the acquisition of a contractile phenotype. Consequently, downregulation of miR145 would prevent proper VSMC functioning (Rangrez et al., 2011). MiR145 is synergistically regulated by Myocardin and serum

response factor (SRF) (Rangrez et al., 2011). Myocardin is a smooth muscle and cardiac muscle-specific transcription factor and transcriptional co-activator of SRF (Wang et al., 2003).

The study conducted by Vengrenyuk concluded that cholesterol loading acts to decrease miR145 expression and in turn SRF/Myocardin induced expression of VSMC genes such as smooth muscle actin (SMα), upregulating genes associated with a macrophage phenotype, such as CD68 (Vengrenyuk et al., 2015). The reverse is also true: cholesterol unloading restored miR145 functionality and decreased CD68 levels, restoring VSMC characteristics, thus identifying an inverse relationship between miR145 and CD68 macrophages in a high cholesterol environment, as is commonly noted in atherosclerosis.

A direct association of miR145 had been shown with CVD in both human and experimental animal models (Cheng et al., 2009b; Cordes et al., 2009; Elia et al., 2009; Fichtlscherer et al., 2010; Rangrez et al., 2011). MiR145 expression has been identified as being "downregulated to a nearly undetectable levels in the atherosclerotic lesions containing neointimal hyperplasia" (Cordes et al., 2009). A decrease in miR145/143 expression was also noted in patients with aortic aneurism compared to control group (Elia et al., 2009). Coinciding with this, a reduction in the expression of miR145 in the blood of patients with CAD has also been identified (Fichtlscherer et al., 2010). Cheng *et al* demonstrated that restoration of down regulated miR145 is sufficient to inhibit neointimal lesion formation in rat carotid arteries after angioplasty, with overexpression acting to increase VSMC differentiation markers (Cheng et al., 2009a; Rangrez et al., 2011).

Furthermore, overexpression of miR145 in VSMCs has been said to limit atherosclerotic plaque morphology and cellular composition, shifting the balance toward plaque stability (Albinsson and Swärd, 2013; Lovren et al., 2012). This study suggests that decreased miR145 is associated with a more unstable plaque phenotype (Figure 4.8) and identifies a potential role for miR145 overexpression in maintaining plaque stability.

4.11 Limitations and Future Work

Strengths of this study include the longitudinal design, prospective follow up and emphasis on early stroke recurrence as carried out by Marnane et al. However, the sample size used was relatively small which may have reduced the statistical power to detect modest associations of recurrence. This study also benefitted from a patient cohort which had been fully clinically characterised and therefore enabled this study to better understand the role miRNA expression levels by viewing them with a plaque inflammation and stability context as identified by Marnane et al. It must also be noted that the definition of a cardiac event such as stroke or TIA can vary between studies, depending on the criteria used. In this study stroke was defined according to the World Health Organisation definition, with neuroimaging confirmation and the Oxfordshire clinical definition of TIA. In addition to this, variability may exist between studies as to what events are described as a recurrent event and therefore the patient subsets can vary, reducing the capacity to make direct comparisons between studies. It has been suggested that including TIA in predicting the risk of stroke recurrence may lead to an overestimation of the reported risk , with a greater risk being noted 30 days after an initial event (Coull et al., 2004; Mohan et al., 2011). The risk of stroke recurrence may also be influenced by differences in the initial management of acute stroke and secondary prevention methods taken.

The miRNAs investigated in this study, miR210 and miR145, were investigated based the current literature and their association with CAD (Raitoharju et al., 2013). Future investigations would involve RNA-sequencing (RNA-Seq) of miRNA expression in this patient subset to identify any additional differentially expressed miRNAs. Based on the results obtained in this study it may beneficial (within a clinical setting) to upregulate miR210 and miR145 expression in order to reduce the risk of stroke recurrence. This may be achieved through the use of synthetic oligonucleotides identical to the selected miR or adenovirus associated vectors which have been shown to restore miR145 expression levels following angioplasty (Cheng et al., 2009b; Garzon et al., 2010). The use of epigenetic targeting agents may also prove promising in the upregulation of selected miRNAs. On a final note, and as mentioned previously, caution must be exerted when targeting miRNAs, as one miRNA can be involved in multiple physiological process, thus requiring specialised targeting and delivery methods to limit off-target effects.

4.12 Concluding remarks

This is the first study to demonstrate an inverse relationship between miR145 and CD68 in stroke recurrence in a clinical data set, confirming the role of miR145 in CVD, and as a biomarker of decreased stroke recurrence, a potential therapeutic target and predictive marker. This observation provides a link between miRNA expression profiles and plaque vulnerability and gives an insight into the potential pathways responsible. This study also identified a similar relationship between decreased miR210 expression and recurrence risk. However, the pathways involved remain unknown. Elevated miR210 in non-recurrent patients may be indicative of ongoing tissue repair and remodelling following injury and may be associated with better outcome overall (Zeng et al., 2014).

Chapter 5: General Discussion

Cardiovascular disease remains the single largest cause of death in the western world and its incidence is on the rise globally. Atherosclerosis, characterised by the development of atheromatous plaque, can trigger luminal narrowing and upon rupture result in myocardial infarction or ischemic stroke (Phinikaridou et al., 2013). This thesis aimed to explore on a molecular level whether inflammatory genes commonly upregulated in atherosclerosis could be beneficially altered by epigenetic intervention and the potential use of miRNAs in predicting stroke recurrence and plaque instability.

Atherosclerosis is a highly complex disease. Its development is contributed to by various cell types, molecules and lipids at gene and protein level (Garrido-Urbani et al., 2014). Inflammation has been described as a master regulator of atherosclerosis, being involved in every stage of its development from initiation to rupture. During atherosclerosis the expression of chemokines and cytokines is altered in response to endothelial injury and subsequent inflammation, with inflammatory markers CXCL16, IL8 and IL18 being of particular interest in this thesis (Bäck et al., 2015; Galkina and Ley, 2009). These markers of inflammation are upregulated in atherosclerosis (Apostolakis et al., 2009; Minami et al., 2001; Sheikine and Hansson, 2006; Youssef et al., 2007). In Chapter 2, I investigated the impact of epigenetic intervention on the expression levels of *CXCL16*, *IL8* and *IL18.* Epigenetic intervention, in the form of SAHA and SFN (established HDACi), was analysed in the Ea.Hy926 and THP-1 cell lines following stimulation with TNFα.

Epigenetic intervention has already shown promise in the treatment of various cancers, with treatments for CTCL and MDS being approved by the FDA (Mack, 2010). SAHA (Vorinostat) has been approved for use in the treatment of CTCL, and is known to inhibit both HDACs and cell proliferation at nanomolar non-toxic concentrations (Heerboth et al., 2014). The low

concentrations required and low toxicity of ETAs like SAHA make them attractive therapeutic options. SFN, unlike SAHA, is not FDA approved but it is a dietary sourced HDACi commonly found in cruciferous vegetables (Myzak et al., 2007; Elbarbry and Elrody, 2011). SFN was used in this study to determine if dietary intervention through dietary epigenetic targeting agents can beneficially alter atherosclerotic gene expression, as this represents a cost effective and accessible option.

Data generated in Chapter 2 indicate that the effect of epigenetic targeting agents such as SAHA and SFN depends on the pre-existing inflammatory environment, which could limit their potential use in the clinic. The results presented in this thesis indicate that if progressed to clinical samples, those who may benefit most from such intervention would have low inflammation, as indicated by the effect of TNFα stimulation *in vitro*, and therefore are unlikely to be suffering from significant atherosclerosis. This suggests a more preventative role for SFN/SAHA rather than as a treatment option, as in some instances stimulation with TNFα following pretreatment with SFN/SAHA induced greater expression of inflammatory markers CXCL16, IL8 and IL18. This however, would not prove beneficial to patients suffering from atherosclerosis or any other inflammatory disease. The duration of exposure to TNFα and the concentration of ETA used may also influence gene expression. It has previously been noted that the pro/ anti- inflammatory effects of broad spectrum HDACis such as TSA and SAHA were separable over a concentration range, with differential effects being observed on macrophage inflammatory responses to LPS (Halili et al., 2010). This may also apply to the concentration and duration of TNFα exposure (Turner et al., 2010). A similar observation was noted by Van den Bossche *et al*., who noted that the activity of broad spectrum inhibitors of HDACs and HMTs can have both pro- and anti-inflammatory effects (Van den Bossche et al., 2014). Both

SFN and SAHA are pan-HDAC inhibitors and have shown a similar mode of action in this thesis with respect to pro and anti-inflammatory activity (Baier et al., 2014; Marks and Breslow, 2007).

Our observations suggest that, although there is great potential for the use of HDACi in regulating atherosclerosis gene expression, a greater understanding of HDACi targets is required, along with the elucidation of specific HDACs involved in atherosclerosis (Kroesen et al., 2014; Yoon and Eom, 2016). Identification of such HDAC targets will allow for the development of suitable delivery methods to limit off-target effects and increase specificity (Sutaria et al., 2012). This may be achieved through the use of HDL nanoparticle carriers, which have been shown to target and accumulate in mouse atherosclerotic plaques, and could be used in the delivery of HDACis (Duivenvoorden et al., 2014; Fitzgerald et al., 2014; Sutaria et al., 2012).

A patient's current medication must be taken into account, when considering HDAC interference therapies. For instance, statins are the primary choice of treatment in reducing cholesterol and are likely to control atherosclerosis inflammation by affecting histone modifications (Csoka and Szyf, 2009; Dje N'Guessan et al., 2009; Schiano et al., 2015). The amount of ETA used may also determine outcome: 10 μM of SFN and SAHA was used in this study based on previous literature in cell lines (Halili et al., 2010; Mathew et al., 2014; Oliveira et al., 2014).

A similar pattern in the response of miR210 and miR145 to pretreatment with HDACis SFN and SAHA, depending on concentration and duration of TNFα exposure is also observed. MiR210 is particularly susceptible to pretreatment with DMSO; its expression dramatically increased

following pretreatment in both EA.hy 926 and THP-1 cell lines. DMSO provided the pharmacological starting point for the development of SAHA, arising from its ability to induce growth arrest and terminal differentiation in transformed cells, and therefore is likely to epigenetically alter miRNA expression (Marks and Breslow, 2007). Data previously generated from the Cardiovascular Disease Research group also identified the ability of DMSO to induce AEI in previously balanced cell CEPH cell lines GM12234 and GM07348, which provided the basis for the investigations carried out in Chapter 3.

Chapter 3 aimed to confirm the findings made by Gahan, 2014 in relation to DMSO and ultimately determine whether there is an epigenetic influence on AEI. This was assessed using HDACis SFN and SAHA along with additional ETAs CURC, DAC and PA. As DMSO is the vehicle for SFN and SAHA and a known epigenetic modifier (Iwatani et al., 2006; Thaler et al., 2012), it was used alone as a vehicle control along with other reconstitution agents, methanol and ethanol.

Alterations in allelic expression have been proposed as a possible mechanism to explain the vast amount of complexity associated with CVD and the extent of patient variability, suggesting that the underlying mechanism affecting allelic expression is epigenetic (Sadee, 2009). This study investigated whether *cis*-acting variation as a result of epigenetic alterations could be modified to alter CVD disease susceptibility, through analysis of AEI at rs2277680 following treatment with various ETAs. It should also be noted that SNPs can be found within HDACs and HATs and can also contribute to disease susceptibility (Han et al., 2013; Lai et al., 2014). A SNP within HDAC9 has been shown to be associated with stroke in two independent studies (Smith, 2014; Cao et al., 2014). In CEPH cell lines, the present study identified that the allelic expression states at rs2277680 in two cell lines GM07348 and GM12234 could be

altered by ETAs, and suggested a targeted approach by which allelic states could be altered to induce the most favourable expression pattern.

Data indicate AEI can be induced following treatment with various ETAs, with DMSO showing a particularly strong induction, proposing an epigenetic mechanism for AEI (Sadee, 2009). The AEI observed is not likely to be total monoallelic expression as changes can be induced following 6 h incubation with ETA (Eckersley-Maslin and Spector, 2014). Biallelic expression is more likely, with most cells continuing to express both alleles, albeit at different levels as a result of treatment with ETA (Barlow, 2011; Gendrel et al., 2014; Jeffries et al., 2016; Nag et al., 2013).

The two CEPH cell lines assessed at rs2277680, a SNP located within CXCL16, varied greatly in response to treatment with ETAs in relation to the direction of AEI induced and also the amount *CXCl16* mRNA produced as a result of treatment. An AEI analysis in untreated cell lines found no imbalance at rs2277680. Treatment with DMSO acted to induce imbalance in both cell lines with a shorter incubation period of 6 h inducing more imbalance than 24 h in both cases. The direction of AEI induced however was different; GM12234 had AEI rF ≥ 0.6 and GM07348 AEI rF < 0.4, suggesting that different alleles are affected. This could be due to some recombination event between the assayed SNP and the upstream SNPs in the gene promotor, which are likely to be driving the AEI. Haplotype analysis showed that many of these upstream SNPs are not in strong linkage disequilibrium with rs2277680. ETAs such as PA, SAHA, DAC and their corresponding vehicle controls induced AEI in both cell lines. The degree of AEI induced varied depending on the concentration of ETA used, 5 µM or 10 µM. The direction of AEI was altered in a concentration dependent manner; GM12234 cells treated with 5 µM showed AEI rF ≤0.4 whilst 10 µM induced rF towards balance or >0.6 µM.

In cases where AEI was detected an investigation was carried out to determine whether the gene is up or down regulated. Samples which were shown to be AEI were analysed via RT-qPCR for CXCL16 mRNA expression differences. GM12234 samples, which were imbalanced, had increased *CXCL16* mRNA expression when compared to levels expressed by untreated samples which did not have AEI. GM07348 treatments which induced AEI acted in the opposite manner, decreasing *CXCL16* mRNA levels below levels observed in untreated samples which did not have AEI. This suggests that treatment with ETAs acts to alter the amount of transcript produced from one allele and may have therapeutic applications in the future (Johnson et al., 2008; Wang and Sadée, 2006). However, identification of the *cis*- acting variant which is driving imbalance remains to be elucidated. The AEI observed in this study is not likely to be monoallelic but biallelic with most cells continuing to express both alleles albeit at different levels as a result of treatment with ETAs. Monoallelic expression is unlikely as AEI can be observed after 6 h treatment which is too fast to be attributed to cell turnover, unless very substantial cell death has occurred. This was not observed.

In comparison to the initial observation made by Gahan, 2014 SFN only induced imbalance in GM07348 and in the opposite direction to what was initially observed. Furthermore, TNFα 100 ng/µl when combined with SFN did not induce AEI in the present study, the opposite of which was shown by Gahan, 2014. This led to an investigation questioning the epigenetic stability of CEPH cell lines over time. Data from this thesis suggests that allelic expression levels remain relatively balanced throughout various passage numbers in new stocks of CEPH cells. Cells that have been frozen down in DMSO and FCS display a much more scattered pattern of allelic expression. GM12234 cells when initially thawed were imbalanced. However, by passage 30 balance was restored. The opposite was noted for GM07348, when initially thawed no AEI was

detected, but by passage 30 both samples showed imbalance. Based on the described data, it is likely that disparity between data in this study and that of Gahan, 2014 may arise from the action of freezing down cells in DMSO, as cell lines used to determine the effect of ETAs were older stocks which were frozen in DMSO. A similar point could be made for stocks used by Gahan, 2014 as the passage number and period for which cells were frozen in DMSO remains unknown. This study raised the question as to the effectiveness and reproducibility of using cells which have been frozen down in DMSO and its potential for altering epigenetic marks within samples. Furthermore, it should also be noted that CEPH cell lines are EBV transformed. The insertion of EBV is random and may also influence AEI patterns within and between cell lines (Xiao et al., 2016), accounting for the differences observed between the data presented in this study and by Gahan, 2014.

Chapter 4 moved away from cell line based investigations to clinically investigate the association between miR210 and miR145 with stroke recurrence and histological markers of plaque instability and inflammation in patient samples. This study was carried out to determine the potential use of miR210 and miR145 as biomarkers for predicting stroke recurrence and identifying vulnerable plaques. Clinical samples used in this chapter were received from the Mater Hospital and have previously been used in a study which identified that “Plaque inflammation and unstable morphology are associated with early stroke recurrence in symptomatic carotid stenosis” (Marnane et al., 2014). These samples were used to evaluate the use of miR210 and miR145 previously investigated in cell lines in Chapter 2, in predicting stroke recurrence and the potential for a blood based test in predicting stroke recurrence. This study also enabled the identification of associations between miR210 and miR145 and markers of plaque instability.

This thesis suggests a potential use for miR145 in predicting stroke recurrence. MiR145 was shown to be significantly downregulated in recurrent patients when compared to non-recurrent patients. The exact mechanism behind this remains to be elucidated. However, miR145 may play a protective role, demonstrated by its elevated levels in non-recurrent patients. It is possible that miR145 expression levels dropped following the initial stroke but have since been restored preventing a second recurrent event. Patients in which miR145 expression levels remain low are more at risk of a second event as shown by data in this study. This is not unlikely, as overexpression of miR145 in VSMCs has been described as a novel *in vivo* target to limit atherosclerotic plaque morphology and cellular composition, shifting the balance toward plaque stability (Albinsson and Swärd, 2013; Lovren et al., 2012). Furthermore, in cell lines miR145 enriched vessels have been shown to convey atheroprotective signalling from ECs to VSMCs (Hergenreider et al., 2012; Raitoharju et al., 2013). Elevated miR145 expression levels in non-recurrent patients may also be indicative of neural repair, as miR145 has been shown to promote neural stem cell (NSC) differentiation and neurogenesis (Morgado et al., 2016), processes which are likely to occur following stroke. This ultimately suggests a protective role for miR145 in stroke, reduction of which may increase the likelihood of a recurrent event. It should be noted that in this study only symptomatic plaques were available from recurrent and non-recurrent patients and thus, caution should be exerted when making comparisons to other studies such as Cipollone *et al*, Matrias *et al* and Santovito *et al.* These studies reported elevated miR145 in symptomatic plaques when compared to asymptomatic plaques and in the plaques of hypertensive patients. However, a study by Jia *et al* suggests that miR145 performs a multifaceted role at different time points in CVD progression, which can negatively or positively influence outcome.

A comprehensive literature search has not revealed any reports of a link between miR145 and CD68 macrophage infiltration in a clinical dataset, suggesting that this is the first report to detect this. Such an association has previously been noted in a murine study by (Vengrenyuk et al., 2015). Data generated in this study suggest an association between miR145 expression in recurrent and non-recurrent patients with CD68 positive macrophage staining in the cap and plaque, as identified using Spearman's non-parametric correlation. In this clinical dataset, a trend can be seen between elevated macrophage numbers in the plaque and cap and decreased miR145 expression levels. The mechanism behind this observation may be explained by a hypothesis put forward by Vengrenyuk *et al,* suggesting that cholesterol loading of aortic SMC decreases miR145 expression and reduces the expression of genes involved in maintaining VSMC identity, whilst increasing the expression of genes of associated macrophage cell identity such as CD68 (Vengrenyuk et al., 2015). Additional associations where identified between miR145 and brown histiocyte (presence or absence), cap rupture and plaque stability. A similar trend can be noted in all data with decreased miR145 expression and the most unfavourable/severe category, were the presence of brown histiocytes indicative of intraplaque haemorrhage, cap rupture and the most unstable plaque phenotype.

5.1.1 Concluding Remarks

This project encompassed clinical and molecular data in an attempt to identify epigenetic regulation and dysregulation in CVD and future avenues which may be used in patient risk stratification and as treatment options. The potential use of dietary epigenetic intervention via SFN was determined in Chapter 2. This study suggests the use of SFN either in dietary or encapsulated form as a preventative option rather than therapy, exerting both pro and anti-inflammatory effects. Future studies are necessary to determine the exact HDAC targets of

SFN to increase specificity and optimise delivery methods. Chapter 3 highlighted the ability of AEI to be induced at rs2277680 in CEPH cell lines and evaluated the effect treatment with ETAs such as SFN and SAHA had on AEI via *cis* effects. The ability of AEI to change over time in cell culture was also investigated; indicating that CEPH cell lines remain primarily stable during culture but freezing in DMSO alters allelic expression profiles, at least temporarily. Finally this is the first study to demonstrate an inverse relationship between miR145 and CD68 in a clinical data set. A similar relationship was also noted between miR145 and histological features of vulnerable plaques such plaque stability, cap rupture and the intraplaque haemorrhage as identified by the presence of brown histiocytes. This study also identified a significant association between decreased miR145 expression and stroke recurrence.

Appendices

Histological Feature	Grade 1	Grade 2	Grade 3	Grade 4
Plaque macrophage infiltration*	None	Occasional or 1 group >50	2–5 groups of >50	>5 groups of >50 or 1 group >500
Plaque lymphocyte infiltration†	None	Occasional or 1 group >20	2–5 groups of >20	>5 groups of >20 or 1 group >100
Fibrous cap rupture	Intact cap	Probably intact, eg, artifactual break in cap from surgical incision	Probably ruptured, eg, site of rupture not clear, but thrombus seen adherent to lipid in lumen	Definitely ruptured
Extent of fibrous cap disruption	None	<25%	>25%	N/A
Luminal thrombus	None	Small	Large	N/A
Intraplaque hemorrhage	None	Present but less than grade 3	>2 mm in circumferential length and >0.5 mm in maximum width	N/A
Lipid-rich necrotic core	None	Present but less than grade 3	>50% of plaque thickness or >25% of cross section	N/A
Calcification	None	Stippling only	Calcified nodules	N/A
Fibrous tissue	Very little fibrous tissue	≈50% fibrous	Predominantly fibrous	N/A
Neovascularization	No microvessels present	≤10 microvessels per section	>10 microvessels per section	N/A
Foam cells	None	<50	≥50	N/A
Brown histiocytes	None	Present	N/A	N/A
Oxford Plaque Study composite assessment	Stable—predominantly fibrous plaque with thick, intact cap	Predominantly stable—some features of instability, eg, inflammation, but thick, intact cap	Unstable with intact cap—thin cap, large lipid core, but no definite rupture or surface thrombus	Unstable with ruptured cap—rupture or thrombus present

N/A indicates not applicable.
*Counts of macrophages identified using CD68 antibody stain and confirmed on hematoxylin and eosin sections.
†Counts of lymphocytes identified using CD3 antibody stain.
Modified from Oxford plaque study methods.[6]

Figure A: Semi quantitative grading scales for Plaque Histological Analysis (Marnane et al., 2014).

	Stroke Recurrence (n=12)	No Stroke Recurrence (n=32)	*P* Value
Age, y, mean (±SD)	68 (9.7)	69 (9.7)	0.75
Male sex, % (n)	91.7 (11)	68.8 (22)	0.24
Hypertension, % (n)	41.7 (5)	62.5 (20)	0.31
Hyperlipidemia, % (n)	66.7 (8)	68.8 (22)	1.0
Diabetes mellitus, % (n)	8.3 (1)	21.9 (7)	0.41
Smoker, % (n)	91.7 (11)	71.9 (23)	0.24
Peripheral vascular disease, % (n)	16.7 (2)	12.5 (4)	0.66
High-risk cardiac source, % (n)	33.3 (4)	28.1 (9)	0.73
Carotid stenosis ≥70%, % (n)	91.7 (11)	75 (24)	0.41
Statin preindex event, % (n)	50 (6)	40.6 (13)	0.58
Antiplatelet preindex event, % (n)	50 (6)	40.6 (13)	0.58
Statin <72 h of medical presentation, % (n)	100 (12)	75 (24)	0.08
Antiplatelet <72 h of medical presentation, % (n)	91.7 (11)	81.3 (26)	0.65
NIHSS <72 h of medical presentation, median (IQR)	2 (0–3)	0 (0–2)	0.08
Delay from presentation to carotid endarterectomy, d, median (IQR)	9 (4–11)	10 (8–19)	0.09

IQR indicates interquartile range; and NIHSS, National Institutes of Health Stroke Scale score, performed by a trained study physician.

Figure B: Clinical characteristics of patients with and without pre-endarterectomy stroke recurrence as defined as clinical, ipsilateral, nonprocedural ischemic stroke occurring between qualifying index event and endarterectomy (Marnane et al., 2014)

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