Novel Cardiovascular Risk Markers in Cardiovascular Diseases

By

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Summary

Introduction

Cardiovascular disease is the leading cause of death worldwide, and the global prevalence of cardiovascular disease increased every year.

Cardiovascular disease (CVD) is a complex multifactorial disease. It is essential to understand the different biological and genetic factors that are associated with the development and progression of CVD in order to improve the diagnosis, prognosis and treatment of the condition and, thereby, reduce mortality and morbidity.

The use of cardiometabolic markers, including blood biomarkers such as troponin, and NT-proBNP, as well as non-invasive imaging markers, such as carotid intima media thickness and pulse wave arterial stiffness, might help to correctly identify patients with a CVD risk at an early stage. These markers may help practitioners understand and monitor disease progression, but whether they provide additional information to the conventional risk factors included in the risk prediction model or disease progression is not currently clear.

In contrast, other biomarkers could help provide insights into pathophysiological processes and help practitioners understand potential drug targets. MicroRNAs (miRNAs) are a family of small, noncoding RNA molecules that regulate gene expression by targeting specific messenger RNA. Dysregulation of specific miRNAs expression have been associated with cardiovascular diseases. Since the discovery of miRNAs in body fluids, including plasma, saliva and urine, a strong body of evidence has been published demonstrating the potential use of circulating miRNAs as markers for several diseases, including CVD.

This thesis studies the possible determinants for imaging markers of cardiovascular disease risk, including carotid intima media thickness and pulse wave arterial stiffness index and further investigates putative circulating miRNAs as novel biomarkers for cardiovascular diseases. The overarching aim is to provide insight

into novel biomarkers for monitoring and treatment of cardiovascular disease progression.

The associations of circulating miRNA expression with markers of cardiometabolic diseases: CAMERA trial

Candidate circulating miRNAs were selected on the basis of the findings in the literature review that showed that their dysregulation was associated with a change of cardiometabolic markers such as cardiac enzyme and imaging markers. A cross-sectional analysis was conducted to investigate the associations of circulating miRNAs, miR30c, miR103, miR133a, miR122 and miR146a with cardiometabolic markers, using 60 paired stored plasma samples at baseline and after 18 months from the CAMERA trial. Significant associations were observed for selected circulating miRNAs with biomarkers of cardiovascular risk in a population with coronary heart disease and insulin resistance. A significant association was found between the expression of miR103 with cardiac biomarkers, including troponin T and NT-pro-BNP, and miR122 with carotid intima media thickness. These associations supported previous observations that indicated an association between the upregulated miR122 expression and adverse lipid profile (Willeit *et al.*, 2017a), and the role of lipid in the progression of carotid intima media thickness that was shown in a study by (Huang *et al.*, 2016). In addition, upregulated miR103 expression in response to cardiac necrosis was observed in a study done by Wang et al. (2015a). Therefore, further research is needed to understand the specific role of miR103 in cardiac necrosis.

The effect of metformin on the expression of circulating miRNA: The Carotid Atherosclerosis: MEtformin for insulin ResistAnce (CAMERA) trial

Metformin is the first line therapy for Type 2 diabetes. The effect of metformin on the risk of cardiovascular diseases had been studied in two randomised controlled trials: the UKPDS and the HOME trial. Those studies showed that metformin reduced the risk of CVD through surrogates in patients with diabetes. In this study, the effect of metformin on the expression of these circulating miRNAs was explored using both the baseline samples and the 18-month plasma samples from the CAMERA randomised control trial (RCT). Randomisation to metformin failed to show any effect on the expression of circulating miR30c,

miR103, mi133a, miR122 and miR146a in a population without diabetes and with coronary heart disease, although the study was, perhaps, underpowered and the effect of metformin in the study in general was very modest. While no strong evidence for metformin having an effect on these miRNAs was observed, this was one of the first, if not the first, RCT to study miRNA biomarkers and provides a platform for future research in this area.

Association of cardiovascular risk factors with carotid intima media thickness and pulse wave arterial stiffness in UK Biobank; Cross sectional study

More established vascular biomarkers of CVD were studied for the purposes of this thesis, in a much larger study than previously possible, in order to understand whether measurement of the markers might be clinically useful. In this first study, using the UK Biobank (42,727 participants), the determinants of vascular imaging biomarkers (carotid intima media thickness and pulse wave arterial stiffness index) were examined using traditional cardiovascular risk factors including systolic and diastolic blood pressure, HbA1c, lipid markers and anthropometric measurements. A cross-sectional analysis was done to investigate the upstream determinants for carotid intima media thickness and pulse wave arterial stiffness. Generally, systolic blood pressure and age were the strongest independent risk factors for a high cIMT value. It was found that, for every one SD increase in age and systolic blood pressure, the mean cIMT increased by 0.357 and 0.115 SD, respectively. Systolic blood pressure and age were the strongest independent risk factors for high PWASI. It was found that, for every one SD increase in age and systolic blood pressure, the mean PWASI increased by 0.005 and 0.046 SD, respectively. Also, this study showed that the cIMT was higher in males than females, along with other cardiovascular risk factors. This study, therefore, highlights potentially important differences in what these biomarkers indicate about upstream cardiovascular risk. Although both appear to be influenced by established risk factors, in line with the findings of much of the rest of the literature, they are different. This means they may have different clinical utility in different settings.

Carotid intima media thickness and pulse wave arterial stiffness with association of cardiovascular events in UK Biobank: Prospective study

In this UK Biobank based study, the focus was on establishing whether carotid intima media thickness or pulse wave arterial stiffness index was associated with the incidence of cardiovascular diseases, stroke and CHD. In addition, we adjusted for the strongest established determinants based on findings of chapter 5 that include systolic blood pressure and age for cIMT, age and systolic blood pressure for PWASI.

After adjustment, one SD increase of mean cIMT was associated with 50 % increase risk of incidence of CVD events. The incidence of CVD events increased in highest quantile (mean cIMT >748μm) by 3 folds compared to persons in the lowest quantiles (mean cIMT <588μm). While, per one SD change in PWASI, the risk of CVD events decreased by 13% after adjustment with age and gender over a median 3 years of follow up in United Kingdom. Therefore, it appears cIMT is the more convincing biomarker that reflects CVD risk in the short term.

Overall conclusion

This study has demonstrated the potential utility of circulating miRNAs as novel biomarkers for cardiovascular disease, providing new data for the most promising miRNA biomarkers, in the context of a large and well standardised study relative to much of the rest of the literature. Also, the study reports on the upstream risk factors that are important potential determinants of imaging biomarkers as well as on the association between these imaging markers and incidence of cardiovascular disease in the large UK Biobank population. The understanding of these biomarkers might help in the future to develop the way cardiovascular disease risk is managed in the clinical setting.

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Author's Declaration

I declare that this represents my own work. I was responsible for; extracting and analysing the data; interpretation of the results; and writing-up this thesis, apart from participants recruitment, sample collection and routine biochemical laboratory measurement of bloods from the CAMERA trial. The work represented in my thesis has not been previously submitted for any degree to the University of Glasgow or any other institutions.

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Abbreviations

AHA	American Heart Association
ALT	Alanine Aminotransferase
AMPK	Adenosine Monophosphate-Activated Protein Kinase
ASCOT	Anglo-Scandinavian Cardiac Outcomes Trial
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
BMI	Body Mass Index
BNP	Brain Natriuretic Peptide
BP	Blood Pressure
CAD	Coronary Artery Disease
CAMERA	Carotid Atherosclerosis: MEtformin for insulin ResistAnce
CHD	Coronary Heart Disease
CI	Confidence Interval
CIMT	Carotid Intima Media Thickness
COPD	Chronic Obstructive Pulmonary Disease
CORDIOPRE	CORonary Diet Intervention with Olive oil and cardiovascular PREVention Study
Ct	Threshold Cycle
dCT	Delta Threshold Cycle
CT	Computed X-ray Tomography
CK	Creatine Kinase
CpG	Cytosines followed by Guanine Residues
DAG	Directed Acyclic Graphs
DIABETES	Diabetes Mellitus
DBP	Diastolic Blood Pressure
DNA	Deoxyribonucleic Acid
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic Acid
ESRD	End-Stage Renal Disease
FH	Familial Hypercholesterolemia
GGT	γ-Glutamyl Transferase
GSH	Glutathione
GTP	Guanosine-5'-Triphosphate
HHR	Hazard Ratio Rate
HCV	Hepatitis C
HDL	High Density Lipoprotein
HF	Heart Failure
HOMA	Homeostatic Model Assessment
HR	Hypertension
ICD	International Classification of Diseases

IGF	Insulin-like Growth Factor
IFG	Impaired Fasting Glucose
IQR	Interquartile Range
IR	Insulin Resistance
LDL	Low Density Lipoprotein
LV	Left Ventricular
LVH	Left Ventricular Hypertrophy
MI	Medical Therapy
MRI	Magnetic Resonance Imaging
MTP	μ-Mono-ThioPyrophosphate
NAFLD	Non-Alcoholic Fatty Liver Disease
NHS	National Health Service
NICE	National Institute for Health and Clinical Excellence
NIDDM	Non-Insulin-Dependent Diabetes Mellitus
NIH	National Institutes of Health
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
NSTEMI	Non-ST-Elevation Myocardial Infarction
NTP	Nortriptyline
OPCS	Polycystic Ovary Syndrome
OR	Odds Ratio
PBMC	Peripheral Blood Mononuclear cell
PCR	Polymerase Chain Reaction
PWASI	Pulse Wave Arterial Stiffness Index
PWV	Pulse Wave Velocity
DPP	Dipeptidyl Peptidase
QC	Quality Control
QIA	Quantitative Image Analysis
QPCR	Quantitative Real-Time Polymerase Chain Reaction
RCT	Randomised Control Trial
RNA	Ribonucleic Acid
ROC	A receiver Operating Characteristic Curve
RQ	Relative Quantification
RT	Reverse Transcription
SNP	Single Nucleotide Polymorphism
SBP	Systolic Blood Pressure
SD	Standard Deviation
SPSS	Statistical Package for Social Sciences
TG	Triglyceride
TNF	Tumour Necrosis Factor
UK	United Kingdom
UKB	Uk BioBank
UKPDS	UK Prospective Diabetes Study
USA	United States of America
VSMC	Vascular Smooth Muscle Cells

Posters and publications

Abstracts for Poster Presentation

Hanan Al-Ghibiwi, David Preiss, Naveed Sattar, Paul Welsh, Jennifer Logue, Diabetes UK professional conference, 8 – 6 March 2019. Novel miRNA biomarkers in Cardiometabolic disease: Data from the CAMERA (Carotid Atherosclerosis: MEtformin for insulin ResistAnce) trial (Appendix 3).

Hanan Al-Ghibiwi, David Preiss, Naveed Sattar, Paul Welsh, Jennifer Logue. 26th European Congress on Obesity Diabetes UK professional conference, 28 April – 1 May 2019. Established and novel biomarkers in cardiometabolic disease: Data from the CAMERA (Carotid Atherosclerosis: MEtformin for insulin ResistAnce) trial (Appendix 4).

Publication

The main findings of this study are being prepared for a paper to be submitted to an international journal.

1 General Introduction

1.1 Cardiovascular diseases

1.1.1 Definition

Cardiovascular disease is an umbrella term that encompasses a range of disorders that affect heart, brain and vascular system. Cardiovascular diseases are typically directly caused by haemorrhage, thrombosis or stenosis, that often lead to narrowed/blocked blood vessels that supply heart (coronary heart diseases) or brain. This can cause myocardial infarction, angina or stroke (Bhf.org.uk, 2020a)

1.1.2 Epidemiology of cardiovascular diseases

Cardiovascular diseases are the leading cause of death globally and a major contributor to the reduction of quality of life. In 2017, cardiovascular diseases caused an estimated 17.8 million deaths worldwide, corresponding to 35.6 million years lived with disability and another 330 million years of life lost. In addition, more than three quarters of the deaths were in low- and middle-income countries (Collaborators, 2018). Non-modifiable risk factors for cardiovascular diseases include older age and male sex and modifiable risk factors include raised blood pressure, and lipids as well as obesity. Identifying those at highest risk of CVDs and ensuring they receive appropriate treatment can help to prevent premature deaths (National Institute for Health and care Excellent, 2017).

According to a British Heart Foundation report, in 2020 there are around 7.4 million people living with CVD in the UK. In the United Kingdom, 27% of all deaths are caused by heart and circulatory diseases. In Scotland in 2018, there were 17 thousand deaths caused by CVD and an estimated (700) thousand people living with CVD. The cost of heart and circulatory diseases is estimated to be 9 billion pound a year. Coronary heart disease (CHD) is, specifically, the leading cause of death worldwide with the number of deaths globally being estimated at 9.43 million deaths. In the UK, it leads to 64,000 deaths each year. Figure 1-1 below shows the number of deaths in 2016 (Bhf.org.uk, 2020b).

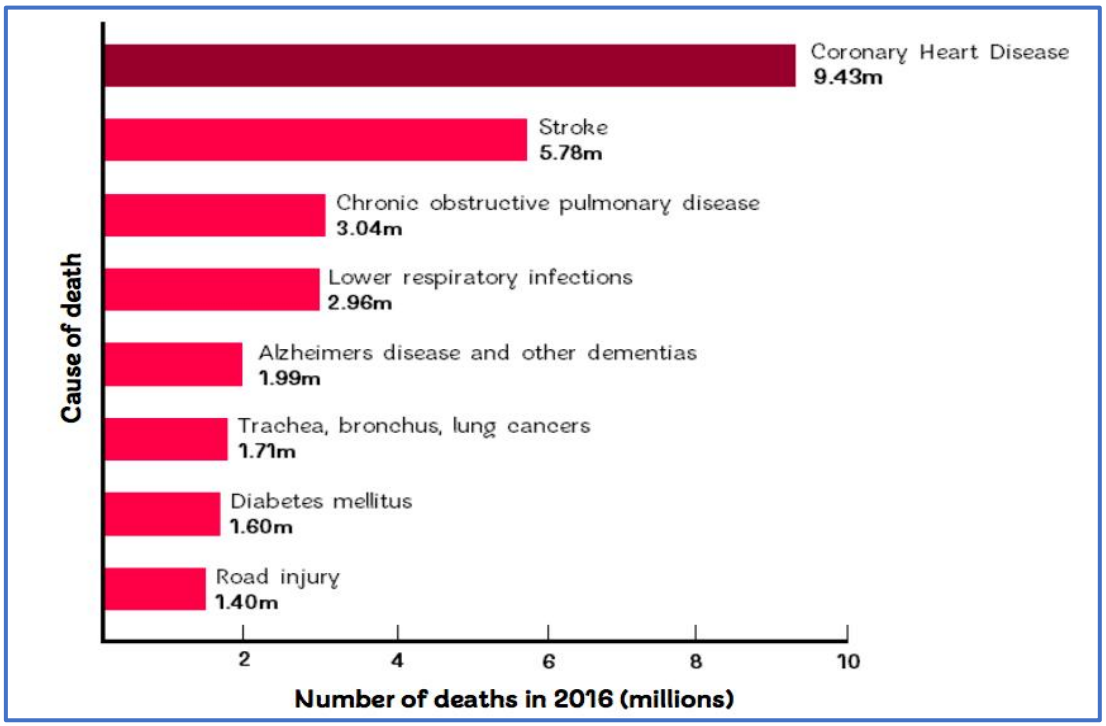


Figure 1-1: Graph shows the top 10 global causes of death (Bhf.org.uk, 2020b)

1.2 Risk factors for cardiovascular diseases

1.2.1 Modifiable risk factors

1.2.1.1Hypertension

Blood pressure defined as the pressure imposed by circulating blood on the walls of blood vessels. It is mainly determined by cardiac output and total peripheral resistance. Cardiac output and total peripheral resistance are controlled by homeostatic regulatory mechanisms, which are a complex system of physiological pathways, including neural, hormonal, vascular and renal mechanisms to regulate blood pressure (Taylor and Abdel-Rahman, 2009). Hypertension is defined as SBP values >_140 mmHg and/or diastolic BP (DBP) values >_90 mmHg. This is based on evidence from multiple RCTs that treatment of patients with these BP values is beneficial (Williams *et al.*, 2018).

American Heart Association defined high blood pressure as “High blood pressure (HBP or hypertension) is when the force of your blood pushing against the walls of your blood vessels, is consistently too high.” (American Heart Assocation, 2016).

High blood pressure is the important risk factor for cardiovascular disease in the UK. According to a recent British Heart Foundation Report (2020), an estimated 28% of adults living in the UK have high blood pressure and more than half of them are not receiving appropriate treatment. There are an estimated 6-8 million people living with uncontrolled hypertension (Bhf.org.uk, 2020c). A meta-analysis of randomised controlled trials carried out by Ettehad et al. (2016) found there was a strong association between reduction of blood pressure and reduced incidence of CVD. It showed that, for every 10 mm Hg reduction in systolic blood pressure, there was a 20% reduction in the risk of major cardiovascular disease events, a 17% reduction in the risk of coronary heart disease a 27% reduction in the risk of stroke, a 28% reduction in the risk of heart failure and a 13% reduction in the risk of all-cause mortality.

1.2.1.2High blood cholesterol

A high blood cholesterol level is a significant risk factor for atherosclerosis and consequently cardiovascular disease. It is estimated that 50% of adult in the UK are living with cholesterol levels above 5mmol/L and around 7-8 million of adults in the UK are currently in lipid lowering therapy such as statin. (*Heart statistics, British Heart Foundation*). Meta-analysis of randomised controlled trials showed a 1 mmol/L reduce in low density lipoprotein cholesterol (LDL-C) with statins was associated with 22% reduction of cardiovascular disease incidence (Adam *et al.*, 2017).

1.2.1.3 Diabetes

Type 2 diabetes is a chronic metabolic condition, that characterised by insufficient pancreatic insulin production and insulin resistance, lead to hyperglycaemia (NICE, 2019).

Around 4.7 million people are actually living with diabetes in the UK. It is estimated that as many as 740,000 people have undiagnosed type 2 diabetes. Around 90 % of those diagnosed are living with type 2 diabetes and 10 % have either type 1 or rarer types (Bhf.org.uk, 2020c). A cohort study based on 1.9 million individuals showed that around 17% of diabetic patients had cardiovascular diseases during the median follow up of 5.5 years. In addition, they found positive association between diabetes and peripheral arterial diseases (adjusted HR 2·98 [95% CI 2·76–3·22]), ischaemic stroke (1·72 [1·52–1·95]), stable angina (1·62 [1·49–1·77]), heart failure (1·56 [1·45–1·69]), and non-fatal myocardial infarction (1·54 [1·42–1·67]) (Shah *et al.*, 2015). Circulating hemoglobin A1c (HbA1c) levels are routinely used to determine average blood glucose concentrations over the preceding 3 months for screening of dysglycemia, including type 2 diabetes (National Institute for Health and care Excellent, 2019). A UK Biobank study for 12,894 participants, showed the hazard ratio (HR) for those with prediabetes and undiagnosed diabetes were at higher CVD risk; HR 1.83 and 2.26, respectively (Welsh *et al.*, 2020).

1.2.2 Lifestyle risk factors

1.2.2.1Smoking

Smoking is considered as a risk factor for CVD. Around 100,000 smokers in the UK die each year from smoking related causes. It is estimated that up to 20,000 deaths each year in the UK from CVD can be attributed to smoking (Bhf.org.uk, 2020c).

Smoking causes cardiovascular events such as the development of atherosclerosis by induction of a prothrombotic state, proinflammatory vascular response, and narrowing of vascular lumen, which lead to acute thrombosis. Evidence that shows a rapid decrease in the risk of myocardial infarction after smoking cessation supports the role of smoking in the progression of thrombosis (Office of the Surgeon, Office on and Health, 2004). Many observational studies have found evidence of a strong association between smoking and development of atherosclerosis, although trials are practically and ethically difficult. The association between coronary heart diseases and cigarette smoking could also be a result of the direct influence of nicotine on the conduction system and thrombosis. Bekheit and Fletcher (1976) studied 28 subjects who were habitual smokers for more than 10 years and found that smoking affects different sites of the conducting pathways of the human heart. They found that it shortens the effective refractory period of the A-V node and increases the velocity of conduction. This action is attributed to the effect of nicotine that causes adrenergic stimulation. A mendelian randomization study investigated the causality of the association between smoking and 14 different cardiovascular disease outcomes in 367,643 participants from Uk Biobank population. This mendelian randomization study found the genetic predisposition to aggressive smoking is associated with increases risk of a wide range of cardiovascular diseases, 361 SNPs were found to be associated with increased risk of heart failure, coronary heart diseases and pulmonary embolism (Larsson *et al.*, 2020).

1.2.2.2Obesity

Obesity is not typically included in cardiovascular risk scores used for CVD prevention, as it overlaps with existing risk factors. However, as a cause of CVD,

it should not be underestimated. For instance, it may cause elevated blood pressure and diabetes. Around 27 % of adults have a body mass index (BMI) of over 30 kg/m2 in the UK. In addition, it is estimated that 29 % of children in the UK are defined as obese or overweight (using BMI) (Bhf.org.uk, 2020c). One mendelian randomization study showed a strong causal association between adiposity, as indicated by body mass index and incidence of cardiovascular diseases, in up to 22,193 individuals, CHD risk causally increases 20% SD increase of BMI (Hägg *et al.*, 2015). In addition, a cross sectional study of 119,859 participants from UK Biobank population showed that higher BMI was associated with increased risk of CHD based on causal Mendelian randomization estimates (Lyall *et al.*, 2017).

1.2.2.3Physical activity

According to British Heart Foundation statistics, more than a third of adults in the UK (37 %) do not achieve the target recommended levels of physical activity of 2 hours and 50 minutes per week (Bhf.org.uk, 2020c).

Li and Siegrist (2012) carried out a meta-analysis that included 21 prospective cohort studies for individuals who were free of CVD at entry of study. They examined the association between physical activity and risk of CVD and found that a high level of leisure time physical activity reduced the risk of CVD by 20% to 30% in 650,000 adult individuals when compared to the level of risk of those with a low level of physical activity at leisure time followed up over a period ranging from 5 to 32 years. These effects are independent of the major cardiovascular risk factors which were considered as confounders. However, the Lifestyle Interventions and Independence for Elders (LIFE) randomized trial, showed nonsignificant difference in the incidence of cardiovascular diseases between intervention group (physical activity program)and control group, over 2.6 years follow up period (Newman *et al.*, 2016). Physical activity enhanced some potentially protective factors, such as decreased plaque progression in coronary arteries and lack of physical activity associated with some pathogenic effects on general vascular health such as reduced endothelial function, increases endogenous inflammation and coagulation factors (Mora *et al.*, 2007).

1.3 Atherosclerosis

Many of the above risk factors cause of CVD through atherosclerosis. Atherosclerosis is a chronic, inflammatory, progressive disease of the arteries with a long asymptomatic phase. A systemic process that can start to develop early during the second or third decade of life defined by lumen enlargement with wall thickening (remodelling) and a reduction of elastic fibers (stiffening) (Hamilton *et al.*, 2007).

1.3.1 Atherosclerosis pathophysiology

The wall of large arteries consists of three distinct layers: the intima, media and adventitia layer. The intima layer is the innermost layer that contains endothelial cells and connective tissue which covers the lumen surface of the arteries. The middle layer is called the media and consists of smooth muscles and connective tissues that are full of collagen and elastin. Smooth muscle cells are able to contract and relax. The outer layer, the adventitia, consists of fibroblast and fibrous tissue, which is mainly elastin and collagen (Taki *et al.*, 2017), (Hansson, 2005). These three layers are observable in a cross-sectional view of the artery, as shown graphically in (Figure 1-2).

The initial step of atherosclerosis occurs when LDL particles (atheroma) accumulate in the arterial intima. They are modified and oxidised by enzymes into proinflammatory particles, which induce the innate inflammatory system reaction. Fatty deposits may increase and build up in the cytoplasm of smooth muscle cells. These initial changes in the arterial wall occur at the branch points of arteries where intimal thickening occurs as an adaptive mechanism in response to normal hemodynamic stresses (Insull, 2009).

Inflammation starts when the smooth muscle cells secret chemokines and chemo-attractant and the endothelial cells become activated and secrete adhesion molecules, which work together to draw the inflammatory cells that include the monocytes, lymphocytes, mast cells, and neutrophils into the arterial wall. In addition, smooth muscle cells secret collagen, elastic fibres and proteoglycans into the extracellular matrix. Upon entry, monocytes transform into macrophages, digest LDL particles as multiple small inclusions and become foam cells. As a result

of this, the arterial walls thicken and harden. The degree of LDL accumulation is important for early-stage diagnosis of atherosclerosis. These lipid changes can be reversed (Insull, 2009). See Figure 1-3 for atherosclerosis progression.

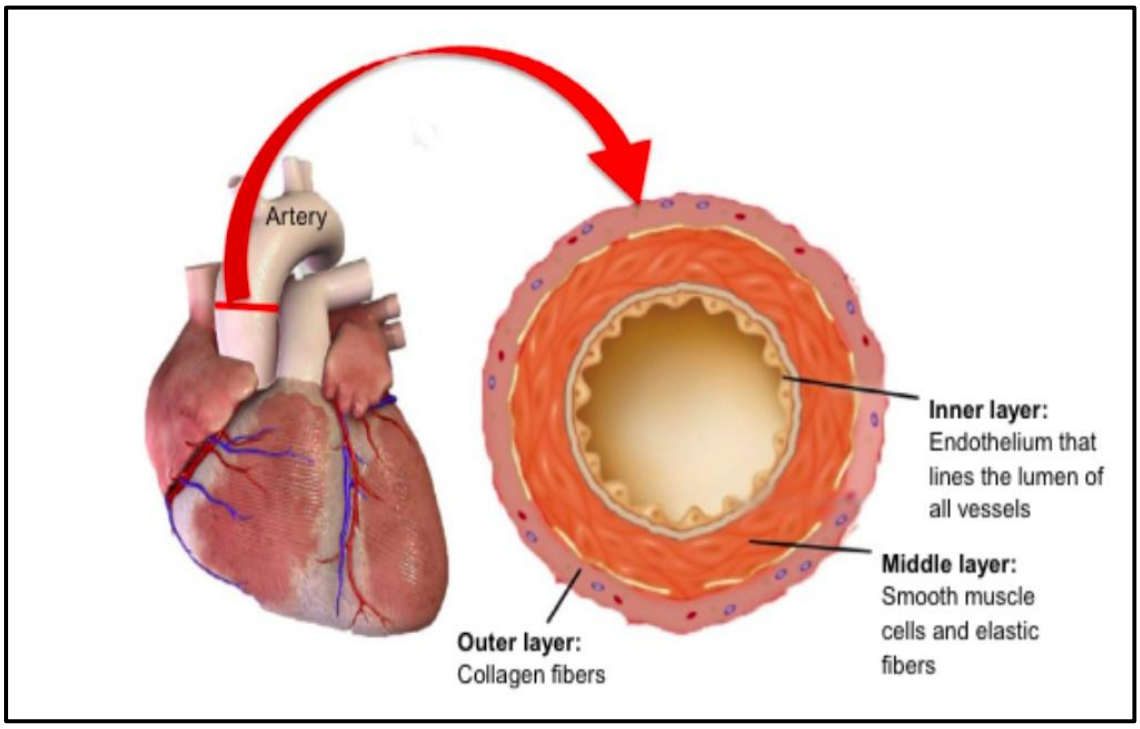


Figure 1-2 Cross section of carotid artery structure. (Taki *et al.*, 2017; *British Heart Foundation, Cardiovascular disease*)

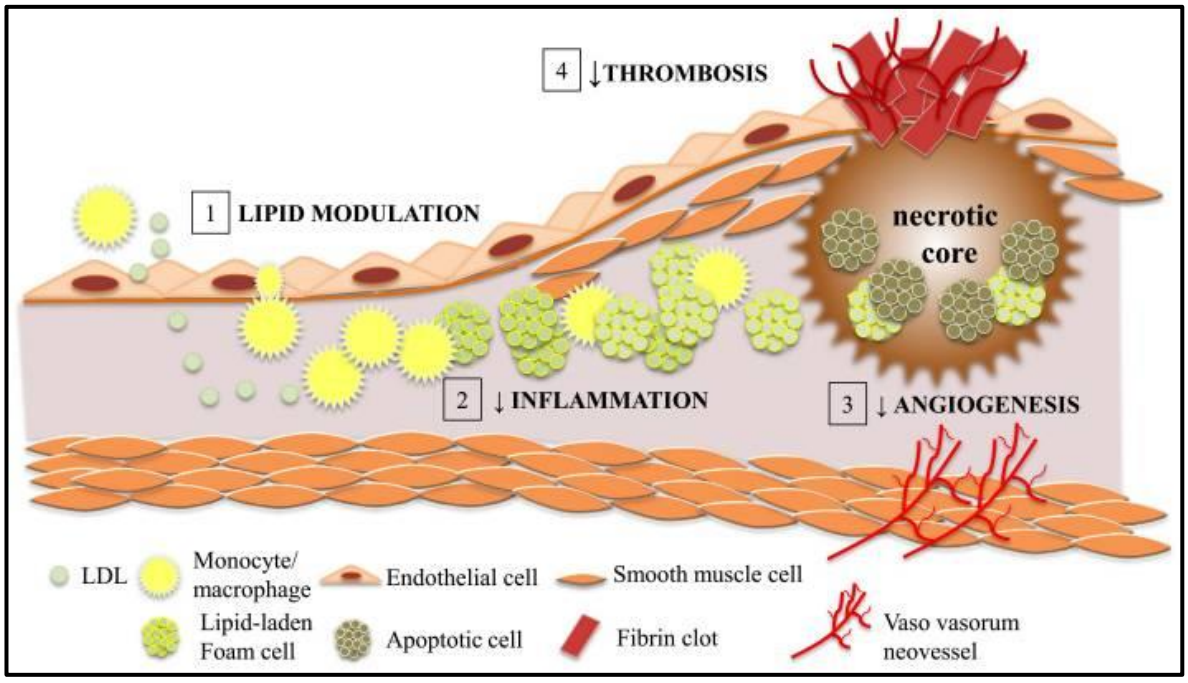


Figure 1-3 Progression of atherosclerosis and different therapeutic strategies. (Rhee and Wu, 2013)

1.3.2 Prevalence of subclinical atherosclerosis

The exact percentage of patients who has subclinical atherosclerosis is difficult to determine. It was reported that 64% of women and 50% of men died from sudden cardiac death in USA without any previous diagnosis of cardiac disease and most of were not classified as high risk patients according to the Framingham score (Toth, 2008). Table (1-1), shows different comparison of techniques for identification of subclinical atherosclerosis. The prevalence of subclinical atherosclerosis was 38.7% and 36% in men and women, respectively, during an evaluation of over 5000 adults aged >65 years who participated in a Cardiovascular Health Study (Kuller *et al.*, 1994b). A cardiovascular magnetic resonance imaging (MRI) showed that 38% of the women and 41% of the men had aortic atherosclerosis in 318 asymptomatic subjects who were randomly selected from a Framingham Offspring Study cohort study. It was also determined that plaque prevalence increases with age (Jaffer *et al.*, 2002).

Several studies have shown that the prevalence of subclinical atherosclerosis as measured by carotid ultrasound and/or CT was high in different population groups who had no prior manifestation of CHD, such as in postmenopausal women (Sutton-Tyrrell *et al.*, 2002) and white Americans in comparison with black Americans (Lee *et al.*, 2003). It is clear that subclinical atherosclerosis was associated with metabolic syndrome and diabetes (Ingelsson *et al.*, 2007). Therefore, early diagnosis of these diseases is important to allow the implementation of primordial prevention that may delay the progression of the diseases.

Characteristic	Coronary angiography	Intravascular ultrasound	B-mode ultrasound	Magnetic resonance imaging	Electron-beam computed tomograph
Invasive	Yes	Yes	No	No	No
Primary measure	Stenosis	Plaque volume and composition	Intimal-medial thickness	Plaque volume and composition	Coronary artery calcification
Plaque composition	No	Yes	No	Yes	No
Plaque burden	No	Yes	No	Yes	Yes
Plaque vulnerability	No	Yes	No	Yes	No

Table 1-1 Comparison of techniques for identification of subclinical atherosclerosis, (Toth, 2008)

1.3.3 Clinical prediction of CVD

Prevention of cardiovascular diseases requires timely identification of high risk individuals to target effective lifestyle, dietary or drug treatment prevention, this is often achieved by using CVD risk prediction models that include ASSIGN (Scotland) (A national clinical Guideline, 2017), $QRISK_3$ (UK) and the pooled cohort equation (USA) (Arnett *et al.*, 2019). In the UK, national guidelines recommend using a risk prediction model ($QRISK_2$ or $QRISK_3$) (Hippisley-Cox *et al.*, 2008) to determine whether to prescribe statin therapy for patient who has 10% or more CVD risk (NICE, 2016).

However, external validation studies of different CVD risk prediction models show that no models have "perfect" calibration or discrimination, and these models can over-or under-predict CVD risk. There is fact that a trade-off between two performance characteristics (discrimination and calibration), and a model typically cannot be perfect in both (Diamond, 1992). Therefore, we treat people who do not need to be treated and people who are not high risk go on to get CVD anyway. This is called the Rose prevention Paradox (Thompson, 2018).

An important line of enquiry is whether we can identify new risk factors for CVD for the purposes of improving prediction and identifying new drug targets. That is where biomarker research has become very important.

1.4 Biomarkers

1.4.1 The definition and types of biomarkers

In 2001, the new definition of a biomarker was standardised by the Working Group of the National Institutes of Health (NIH) as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.” ('Biomarkers Definitions Working Group, Biomarkers and surrogate endpoints: preferred definitions and conceptual framework,' 2001).

A biomarker can be measured using biofluids (such as a blood and urine) or a tissue test. It may be a measurement obtained from a person (blood pressure or ECG) or it may be an imaging test (CT scan or echocardiogram). A biomarker should indicate a unique and important characteristic of the disease. Biomarkers can be used as indicators of a disease trait (risk marker or risk factor), disease rate (progression), or disease state (preclinical or clinical) (Fox and Growdon, 2004). Accordingly, there are different classifications of biomarkers, namely: antecedent biomarkers which identify the risk for developing a disease; diagnostic biomarkers; staging biomarkers which categorise the severity of the disease; and, prognostic biomarkers which predict the future course of a disease, including disease recurrence, response to therapy and monitoring the efficacy of therapy ('Biomarkers Definitions Working Group, Biomarkers and surrogate endpoints: preferred definitions and conceptual framework,' 2001).

1.4.2 An ideal biomarkers characteristic

The aim of biomarkers is to increase the ability of clinicians to accurately manage the patient. A biomarker may help to differentiate patients with an acute myocardial infarction (MI) from those with unstable angina by measuring cardiac enzymes (e.g. troponin I or T). In a patient with an established acute MI A, a biomarker may be used in different indicators, for example, in an ECG ST-segment elevation indicating the need for thrombolysis; to identify the extent of myocardial damage (e.g. troponin); to identify the severity of underlying coronary disease (e.g. coronary angiography). The B-type natriuretic peptide [BNP] is a biomarker used for heart failure diagnosis (Ponikowski *et al.*, 2016). New biomarkers will accepted for use in clinics only if they are accurate, reproducible, easy to read and interpret by clinicians and have a high sensitivity and specificity for outcome independent of other established risk factors (Morrow *et al.*, 2003).

1.4.3 Structural cardiovascular diseases (CVD) biomarkers

There are circulating, structural, functional, and genomic markers for cardiovascular diseases. The list of biomarkers in the Table (1-2) below provides a brief summary of some key biomarkers (Vasan, 2006).

Table 1-2 Current biomarkers for cardiovascular diseases

Arterial Vulnerability		**Blood Vulnerability**	**Myocardial Vulnerability**
Serological biomarkers	• Abnormal lipid profile • Inflammation e**g:** IL-6 IL-18	• Fibrinogen • Increased coagulation factors • Decreased fibrinolysis	• Troponins • Natriuretic peptides
Structural biomarkers	• Carotid IMT • Coronary artery calcium		• LVH • LV dysfunction
Functional biomarkers	• Blood pressure • Endothelial dysfunction • Arterial stiffness • Ankle-brachial index		• Exercise stress test/stress echo • PET

PET, positron emission tomography**, IL,** interleukin; **IMT**, intimal-medial thickness, **LV,** left ventricle; **LVH,** LV hypertrophy

1.5.1.1Arterial stiffness

Lack of elasticity in the artery is an anatomical weakness, vascular stiffness occurs as a consequence of a complex interaction between dynamic and stable cellular and structural changes of the vascular walls. These vascular alterations develop as a result of different hemodynamic forces (Gimbrone and García-Cardeña, 2013) and other external factors such as salts, hormones and glucose, as well as common diseases, such as hypertension and diabetes. They can also result simply from the act of aging itself. They have a synergistic role in vascular stiffness. Figure 1-4 shows the different factors that cause stiffening of the vascular artery and the location of such stiffness.

Arterial stiffness is recognised widely (Tanaka, 2017) as an independent CVD risk factor. Although the best method of measuring it clinically remains debatable, the carotid-femoral pulse wave velocity (PWV), is considered a gold standard for arterial stiffness assessment (Van Bortel *et al.*, 2012). However, the carotid-femoral pulse wave velocity is indirect measure that reflect arterial stiffness by using mathematical equation and involved a number of assumptions (constant blood viscosity) (Tanaka, 2017).

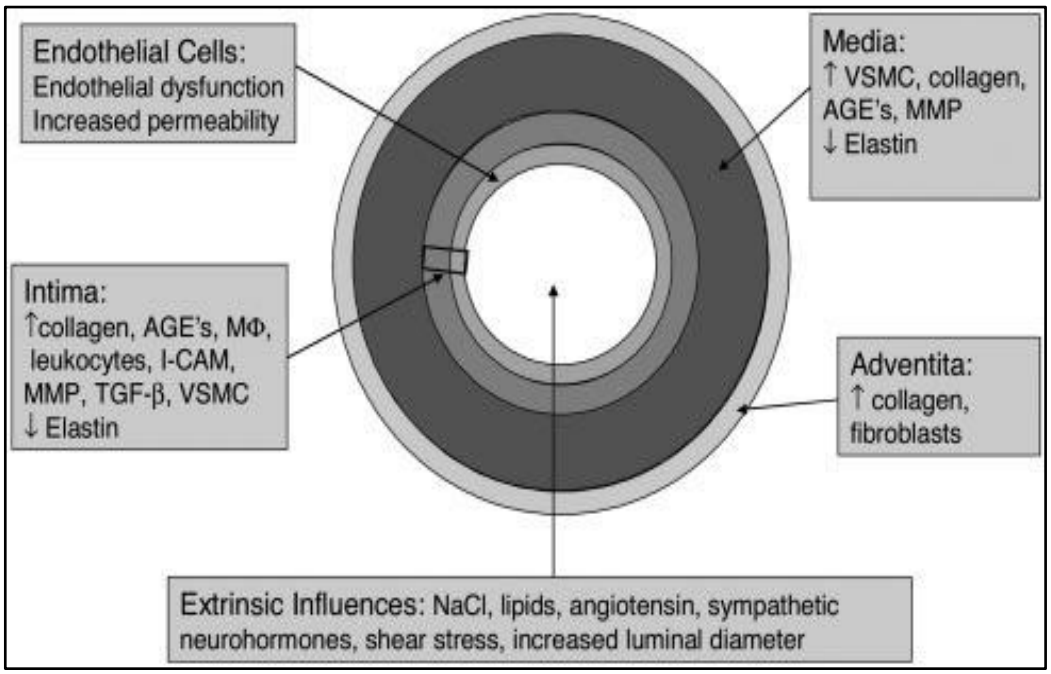


Figure 1-4: Summary of different factors that increase arterial stiffness, (Zieman, Melenovsky and Kass, 2005)

AGE's: Advanced Glycation End Products**, MMP:** Matrix MetalloProteinase, **I-CAM:** Intercellular Adhesion Molecule 1, **TGF β:** Transforming Growth Factor beta**, VSMC:** Vascular Smooth Muscle Cell

1.5.1.1.1 Structural and cellular components of arterial stiffness

The stability and compliance of the vascular wall are dependent on the relative amounts of its two scaffolding proteins: collagen and elastin. The elastin and collagen amounts are normally stable through a slow dynamic process of synthesis and degradation. Stimulation of the inflammatory mediators leads to an imbalance of these proteins and this leads to an increase in the production of abnormal collagen and a decrease in normal elastin, which contributes to vascular stiffness (Johnson *et al.*, 2001). The higher luminal pressure and hypertension are responsible for the change in the structural composition of the vascular arterial wall that leads to increasing arterial stiffness. These alterations in the vascular wall involve an increase in the synthesis of collagen and a destruction of elastin as a result of sustained hypertension (Xu *et al.*, 2000).

In addition to structural alteration, arterial stiffness is strongly affected by cellular changes that include endothelial cell signalling and vascular smooth muscle cells tone. The vascular endothelium plays a crucial role in vascular haemostasis. Endothelial cells provide haemostasis by secreting several mediators, such as anticoagulant factors and antiplatelet that help to maintain blood fluidity and prevent thrombosis (Rajendran *et al.*, 2013) as well as regulate vascular tone and diameter via the production of endothelin, prostaglandin and nitric oxide (Galley and Webster, 2004). Therefore, endothelial dysfunction is recognised as an important cellular factor of the development and progression of atherosclerosis. Also, vascular endothelial dysfunction is an independent risk factor for cardiovascular events. Additionally, it provides important prognostic data alongside the classical risk factors. Endothelial dysfunction is seen in patients with a family history of CVD without other classical risk factors (Widmer and Lerman, 2014). In addition, the progression of endothelial dysfunction is dependent upon the duration, intensity and number of proven risk factors for the individual subjects (Poredos and Kek, 2000).

Endothelium dysfunction occurs as a result of imbalance between the production and consumption of nitric oxide (NO), increasing consumption and reducing production (Matz *et al.*, 2000). Nitric oxide expression may be reduced (Lyons *et al.*, 1997). A decrease in the bioavailability of NO by the action of the natural nitric oxide synthase inhibitor (NOS), asymmetrical dimethylarginine, has been

linked to vascular stiffening (Miyazaki *et al.*, 1999). Also, the bioavailability of nitric oxide is reduced by the activation of the reactive oxygen species caused by oxidative stress that is associated with CVD risk factors such as diabetes, smoking, hypertension, dyslipidaemia, obesity and aging (Taddei *et al.*, 2001). Figure 1-5 shows the progression of atherosclerosis mediated by oxidative stress and endothelium dysfunction starting from CVD risk factors. The early detection of endothelial dysfunction is a critical step to prevent the development of atherosclerosis and cardiovascular disease, because this dysfunction could be an initial reversible step in the process of atherosclerosis. Chronic exposure to oxidative stress and cardiovascular risk factors lead to an overwhelming of the vascular endothelium that is followed by endothelium dysfunction which causes the impairment of vascular homeostasis that leads to upregulation of inflammatory cytokines and adhesin molecules and decreases the anti-inflammatory and anti-oxidant effect (Libby, Ridker and Maseri, 2002).

VSMC tone can be modified by mechano-stimulation, in part because of cell stretch and changes in calcium signalling, and by paracrine mediators such as angiotensin II, endothelin, oxidant stress, and nitric oxide.

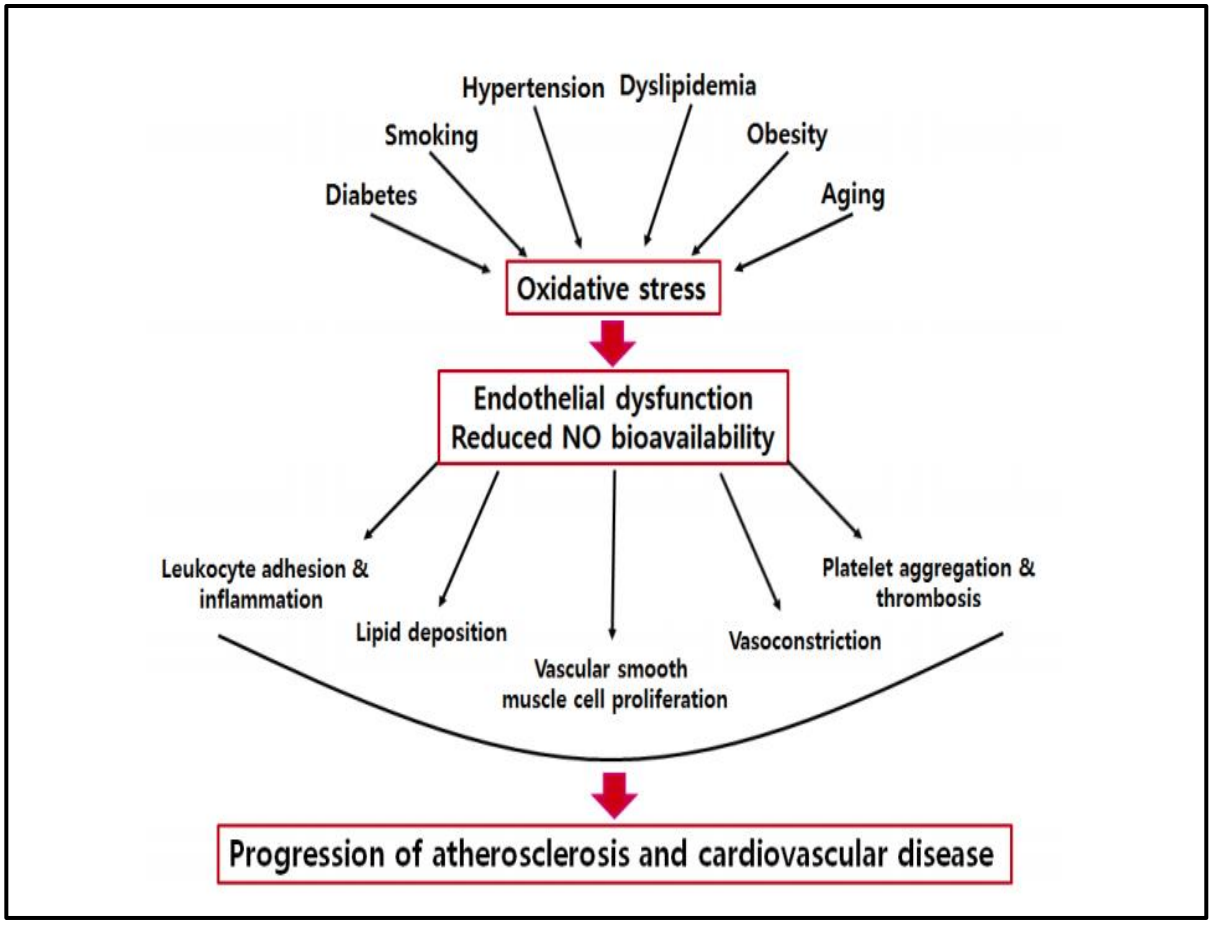


Figure 1-5 The development of atherosclerosis and cardiovascular diseases mediated by oxidative stress and endothelial dysfunction.

(Park and Park, 2015)

1.5.1.1.2 Arterial stiffness pathophysiology

The terms of elasticity, compliance and stiffness are used interchangeably to describe the change in volume and dimensions in the artery in response to the change in transmural pressure. However, the term distensibility has a different meaning, because it relates to a fractional change in calibre when compared to the original calibre (Hayoz *et al.*, 1992).

Over time, the walls of large arteries, such as the aorta, suffer from a loss of elastin fibres and accumulation of collagen fibres that lead to arterial stiffening (Wagenseil and Mecham, 2012). Stiffening that occurs in the aorta and large conduit arterials leads to increased velocity of the pulse wave and increases pulse pressure (increased systolic pressure vs decreased diastolic blood pressure) (Beltran *et al.*, 2001). In addition, stiffening of the reflection sites at the branch points of arteries will lead to a change in the decay rate and time of reflection of the pressure wave (Finkelstein *et al.*, 1985). The main components of the aorta and large arteries are elastin and collagen, which are the main functional determinants of these arteries. The collagen in these arteries decreases their compliance against pressure (i.e. as the transmural pressure increases, the aorta and large arteries become stiffer because of the contribution of the stiff collagen fibres) (Bank *et al.*, 1996). In the microcirculation, the compliance and calibre of the arteries that contain reflecting sites or sites of vascular resistance are dependent on smooth muscle tone. Endothelium NO penetrates the layer of these thin arteries and plays a pivotal role in determining the tone and stiffness of the arteries (Vaughn, Kuo and Liao, 1998).

1.5.1.1.3 Possible mechanism linking arterial stiffness and atherosclerosis

The mechanisms that link arterial stiffness with atherosclerosis have not been well-explained. However, there are several suggested hypotheses. Increased arterial stiffness enhances the elevation of blood pressure and vascular remodelling (Dao *et al.*, 2005). Also, increased luminal pressure and shear stress stimulate the production of collagen and accelerates the formation of atheroma by increasing the deposition of collagen on the arterial wall (Zieman, Melenovsky and Kass, 2005). In addition, increased arterial stiffness enhances the pulsatile component of BP, leading to the progression of atherosclerosis ((Vokó *et al.*, 1999). Arterial stiffening and plaque formation partly share the same systemic

pathophysiology process that causes the deposition of the extracellular matrix in vascular walls (Lee and Oh, 2010). Also, arterial stiffness and the formation of atheroma share common risk factors such as hypertension, diabetes mellitus and dyslipidaemia. This may be another important mechanism that links PWV and atherosclerosis (Cavalcante *et al.*, 2011).

Mechanical and chemical stress such as inflammation, hypertension and advanced glycation end products should also be considered in coronary atherosclerosis. In the stiffened artery, systolic BP increases and diastolic BP decreases (Cavalcante *et al.*, 2011). Increased systolic blood pressure and pulse pressure lead to an increase in ventricular workload and then ventricular hypertrophy as well as reduced diastolic blood pressure that is associated with coronary perfusion reduction (Dart and Kingwell, 2001). Moreover, arterial endothelial dysfunction, which is one of the important events in atherogenesis, also increases pulse pressure through elevated arterial stiffness that may lead to endothelial dysfunction. A study by Beigel et al. (2010) found an inverse association between pulse pressure and endothelial function in 525 consecutive subjects with no heart diseases independent of major CVD risk factors. The pulse pressure is the ratio between stroke volume and compliance of arterial circulation (Dart and Kingwell, 2001). Age is a determinant of pulse pressure, so elevated pulse pressure with aging is related to a fall in arterial circulation and a loss of arterial elasticity (Alfie *et al.*, 1999). Also, pulsatile flow is considered as a critical determinant for increasing oxidative stress in endothelial cells as well as endothelial dysfunction, independent of other blood pressure components (Silacci *et al.*, 2001). The possible mechanisms linking arterial stiffness and atherosclerosis are shown in (Figure 1-6).

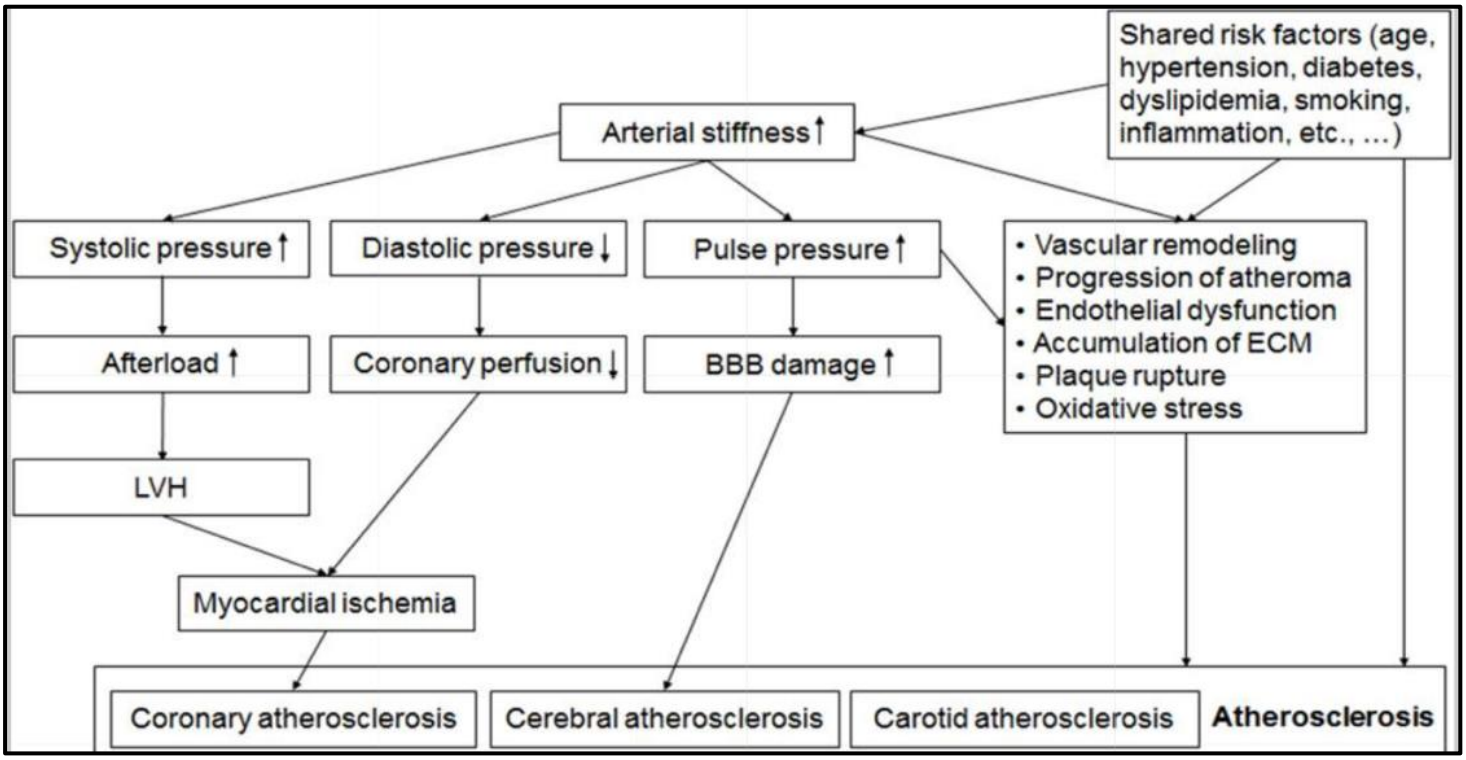


Figure 1-6 Possible mechanism connecting arterial stiffness and atherosclerosis (Kim and Kim, 2019)

LVH: Left Ventricular Hypertrophy, **BBB:** Blood Brain Barrier, **ECM:** Extracellular Matrix Accumulation

1.5.1.2Carotid intima media thickness

1.5.1.2.1 Possible mechanism linking cIMT and atherosclerosis

Carotid intima media thickness (cIMT) represents the process of intimal hyperplasia within the artery and can be thought of underlying mechanism for atherosclerosis disease initiation and progression. Atherosclerosis itself is a focal thickening caused by a plaque that extends into the lumen of a blood vessel, Figure (1-7). Therefore, the possible mechanisms that linking thickening of carotid arteries with development of atherosclerosis may explained by the association of cIMT with traditional CV risk factors and novel risk factors. Commonly, cIMT is used as a surrogate endpoint that linking early exposure to CVD risk factors to subclinical atherosclerotic disease (Meyer *et al.*, 2006) (Peña *et al.*, 2006), (Zanchetti A1, 2001) (Lorenz *et al.*, 2018). Carotid IMT represents a good biomarker for CVD risk (Okamura *et al.*, 2013).

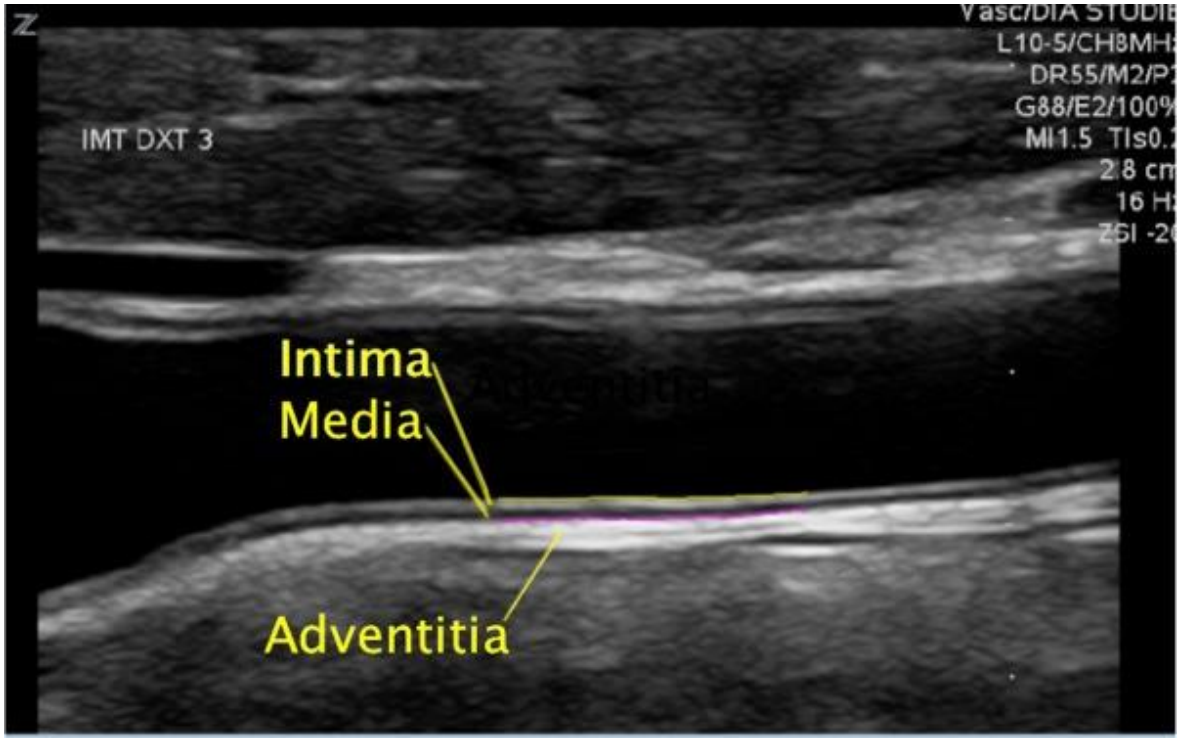


Figure 1-7 Three separate layers of carotid artery:intima,media and adventitia (Gaarder and Seierstad, 2015)

1.5.1.2.2 The association between cIMT and blood pressure

High blood pressure is the major predictor factor for carotid intima media thickness (Ren *et al.*, 2015). Systolic blood pressure seems to have a pathological mechanism that is indirectly associated with cIMT variation through a hemodynamic pathway. Moreover, the thickness of the carotid intima media on the left side and the number of plaques is stronger than the thickness of the carotid artery and the number of plaques on the right side (Chen *et al.*, 2015). In hypertensive patients, the increase in cIMT was recognised as a marker of arterial remodelling that is associated with a long history of uncontrolled hypertension rather than early atherosclerosis (Bianga Katchunga *et al.*, 2016). A study based on a sample of African hypertensive men showed that cIMT was inversely associated with the antioxidant glutathione (GSH) level, indicating that cIMT might have a role in the attenuation of GSH levels in the progression of subclinical atherosclerosis (Schutte *et al.*, 2009). Therefore, hypertension should be properly controlled to prevent atherosclerosis.

1.5.1.2.3 The association between cIMT and dyslipidaemia

An unhealthy lifestyle in young adulthood is associated with increased risks for subclinical atherosclerosis in middle age (Spring *et al.*, 2014). Dyslipidaemia is an important predictor of the development of atherosclerosis and has been shown to be associated with cIMT variation (Acevedo *et al.*, 2011). Bosevski et al carried out a study to define the factors that affected cIMT in a population of patients with type 2 diabetes (Bosevski, Georgievska-Ismail and Tosev, 2011). The regression analysis carried out in this study showed that age, blood creatinine and non-high-density lipoprotein cholesterol levels were independent predictors for mean and maximum cIMT.

Patients with familial hypercholesterolemia (FH) are particularly at high risk of premature CV disease. Hypercholesterolemic children and patients with FH are associated with higher cIMT values (Narverud *et al.*, 2014). The results of this meta-analysis confirm the evidence of early atherosclerosis development in children with FH. Furthermore, high concentrations of oxidized LDL (Paim *et al.*, 2013) and circulating LDL (Burgess *et al.*, 2012) were associated with the increased progression of cIMT. Low HDL levels were significantly associated with increased cIMT levels (Ayer *et al.*, 2009). Apolipoproteins are also important risk factors for

atherosclerosis. Among them, apolipoprotein B (apoB) (Dessein *et al.*, 2013) and ApoB/ApoA1 (Gruszfeld *et al.*, 2015) were independent predictors for cIMT. Furthermore, the absence or very low levels of erythrocyte-bound apoB were associated with clinical and subclinical atherosclerosis (Klop *et al.*, 2013). Lower LDL cholesterol interventions were shown to significantly reduce or slow the progression of cIMT (Fang *et al.*, 2015).

1.5.1.2.4 The association between cIMT and glycemia

High glucose and glycated haemoglobin (HbA1C) levels are also important risk factors for atherosclerosis related cardiovascular diseases (Welsh *et al.*, 2020).The HOMA-IR index, insulin levels, total IGF-1 level (Kim *et al.*, 2011) and insulin resistance (Kalra *et al.*, 2008) were found to be positively correlated with cIMT. In hyperglycaemic patients, impaired fasting glucose and type 2 diabetes are the major independent predictors for cIMT progression (Tropeano *et al.*, 2004). Insulin resistance was found to have a role in impaired reduction of cIMT and to lead to the early development of atherosclerosis in obese adolescents (Sanches Pde *et al.*, 2012). Shimizu et al. (2015) found that a lower level of HbA1c is a determinant of carotid atherosclerosis in rural community-dwelling elderly Japanese men (Shimizu *et al.*, 2015). However, a 6-month intensive life style modification intervention led to improved glycaemic control and delayed the progression of cIMT in diabetic patients (Kim *et al.*, 2011). In addition, hyperuricemia is another independent determinant of the risk of cardiovascular diseases. It was found that hyperuricemia was negatively associated with subclinical carotid atherosclerosis in men (Shimizu *et al.*, 2014). Therefore, a timely intervention control for high glucose and uric acid level may slow the early occurrence of atherosclerosis.

1.5.1.2.5 The association between cIMT and obesity

Several obesity anthropometric parameters are used to assess the association between obesity and atherosclerosis-related diseases. Higher cIMT has been found to be associated with anthropometric parameters, including body mass index, waist circumference (Ren *et al.*, 2015) and waist to hip ratio (Ge *et al.*, 2014). In addition, visceral adipose tissue has been shown to be associated with cIMT and atherosclerosis (Lee *et al.*, 2012). Also, a higher volume of epicardial (Kocaman *et al.*, 2013) and peri-aortic root fat (Petit *et al.*, 2010) were found to be independent determinants of increasing cIMT. Moreover, recent studies have

shown that the mean cIMT changed across different obesity phenotypes and the positive association between cIMT, carotid plaque and carotid atherosclerosis were shown in only the metabolically abnormal obese subtype (Kim *et al.*, 2015).

1.5.1.2.6 The association between cIMT and rheumatoid arthritis and inflammatory diseases

Growing evidence shows that patients with RA, systemic lupus erythematosus (SLE), Behcet disease (BD) and psoriatic arthritis (PsA) are associated with more risk of CV and subclinical atherosclerotic diseases. RA is associated with an increased risk of subclinical atherosclerosis and is considered an independent risk factor for increased IMT (Ristić *et al.*, 2010). However, a contradictory finding indicates that RA is not associated with cIM thickening (Kesse-Guyot *et al.*, 2010). The mechanisms of RA-associated with carotid intima media thickness include increased levels of oxidised LDL (Peluso *et al.*, 2019), pulse wave arterial stiffness (Lo Gullo *et al.*, 2015), increased levels of inflammation markers, such as IL-17, CD34+ cells (Lo Gullo *et al.*, 2015) and CRP (Mohan *et al.*, 2014) as well as vitamin D (Lo Gullo *et al.*, 2015) and NO (Ristić *et al.*, 2015). Additionally, psoriasis is associated with elevated cIMT and increased risk of subclinical atherosclerosis (Eder *et al.*, 2013).The causes of atherosclerosis in psoriasis include markers that cause acceleration of atherosclerosis such as oxidise LDL and NO (Ristić *et al.*, 2015) as well as endothelial dysfunction (Troitzsch *et al.*, 2012).

The influence of inflammatory mediators, immunological parameters and lipid peroxidation on cIMT remains unknown. Some evidence suggests levels of lipid peroxidation (Letonja, Nikolajevic Starcevic and Petrovič, 2012) and inflammatory cytokines, such as CRP (Klop *et al.*, 2013) are associated with elevated cIMT. In addition, studies on the parameters of haemocytes showed that the blood leukocyte count is an independent risk factor for early arterial damage and is associated with subclinical carotid atherosclerosis (Loimaala *et al.*, 2006). The neutrophil-to-lymphocyte ratio is positively associated with cIMT in the whole study population and the neutrophil-to-lymphocyte ratio has been found to be higher in patients with coronary artery disease (Cingoz *et al.*, 2014).

1.5.2 Novel blood-based cardiovascular diseases biomarkers

1.5.2.1MicroRNAs

MiRNAs are small, single-stranded, non-coding RNAs molecules. They are ~22 nucleotides in length and are synthesised by two RNase III proteins, Drosha and Dicer (Ha and Kim, 2014). MiRNAs were originally discovered in *Caenorhabditis elegans* and are found in most eukaryotes, including humans (Lee, Feinbaum and Ambros, 1993). It is expected that miRNAs represent 1-5% of the human genome and they are responsible for 30% of protein- coding genes (Stanczyk *et al.*, 2008).

To date, 2300 mature microRNAs have been identified within human genome, and 1115 of those miRNAs are currently annotated in miRbase V_{22} (Alles *et al.*, 2019). However, little is known about the specific targets and biological function of miRNA molecules so far, though it is known that miRNAs play a critical role in the regulation of gene expression that control different cellular and metabolic pathways (Ambros, 2004). Far from being passive debris or metabolites from cellular processes, at least some miRNA seems to be biologically active and potential therapeutic target (Hanna, Hossain and Kocerha, 2019).

1.5.2.2The biogenesis of miRNAs

MiRNA biogenesis starts with miRNA transcription by RNA polymerase II (Pol II). MiRNA precursors are found as clusters within different regions in the genome. Approximately half of known miRNAs are located within clusters of genes (intergenic) regions and introns protein-coding genes (intronic). These regions are known as junk DNA, because there is no known role for them (Lagos-Quintana *et al.*, 2003).The biogenesis of miRNA is classified into canonical and noncanonical pathways (Figure 1-8).

1.5.2.2.1 The canonical pathway of miRNA biogenesis

The canonical pathway is the dominant pathway for miRNAs biogenesis. In this pathway, primary miRNAs (pri-miRNA) are transcribed from their genes and then cleave to release pre-miRNAs that are typically ~55–70 nt in length. The process occurs the action of the microprocessor complex, through consisting of nuclear

heterodimers which are the RNA binding proteins DiGeorge Syndrome Critical Region 8 (DGCR8) and Drosha and the ribonuclease III enzyme (Dicer) (Denli *et al.*, 2004).

Once pre-miRNAs are generated, they are exported to the cytoplasm by a RanGTP/ exportin 5 dependent mechanism (Lund *et al.*, 2004). In the cytoplasm, pre-miRNas are identified and processed into their mature ~22-nt form miRNA duplex by the RNase III endonuclease Dicer (Grishok *et al.*, 2001). Both strands of mature miRNA duplex can be entered into the Argonaute (AGO) proteins in an ATP-dependent manner (Yoda *et al.*, 2010). Once pre-RISC (RNA-induced silencing complex) is formed, the two strands of the miRNA duplex are separated with AGO protein, with only one strand remaining (the guide strand) and the other strand destroyed (the passenger strand) (Ha and Kim, 2014). The AGO protein complex that contains single-stranded mature miRNAs are called mature RISC (RNA-induced silencing complex). For most miRNAs, only the guide strand is enabled to recognize and repress the expression of target genes (Schwarz *et al.*, 2003). The outcome of this recognition is mediating either endonucleolytic cleavage of the targeted messenger RNA (Llave *et al.*, 2002) or translation control by interference with protein synthesis by an unclear mechanism (Zeng, Wagner and Cullen, 2002).

1.5.2.2.2 Non-canonical miRNA biogenesis pathways

A number of non-canonical miRNA biogenesis pathways have been elucidated to date (Babiarz *et al.*, 2008). The non-canonical miRNA biogenesis can be classified into Drosha/DGCR8-independent and Dicer-independent pathways. The microprocessor (Drosha and DGCR8) is not involved in the processing of some miRNA, including mirtrons. Mirtron is a Pre-miRNA that is produced from the intron of mRNA. Theses introns are processed during splicing in the nucleus to produce miRNA hairpins that are directly suited for Dicer cleavage. Mirtron bypasses the microprocessor pathway and merges with the conical miRNA pathway at the Exportin-5-boudn transporter step and then is cleaved by Dicer in the cytoplasm (Ruby, Jan and Bartel, 2007).

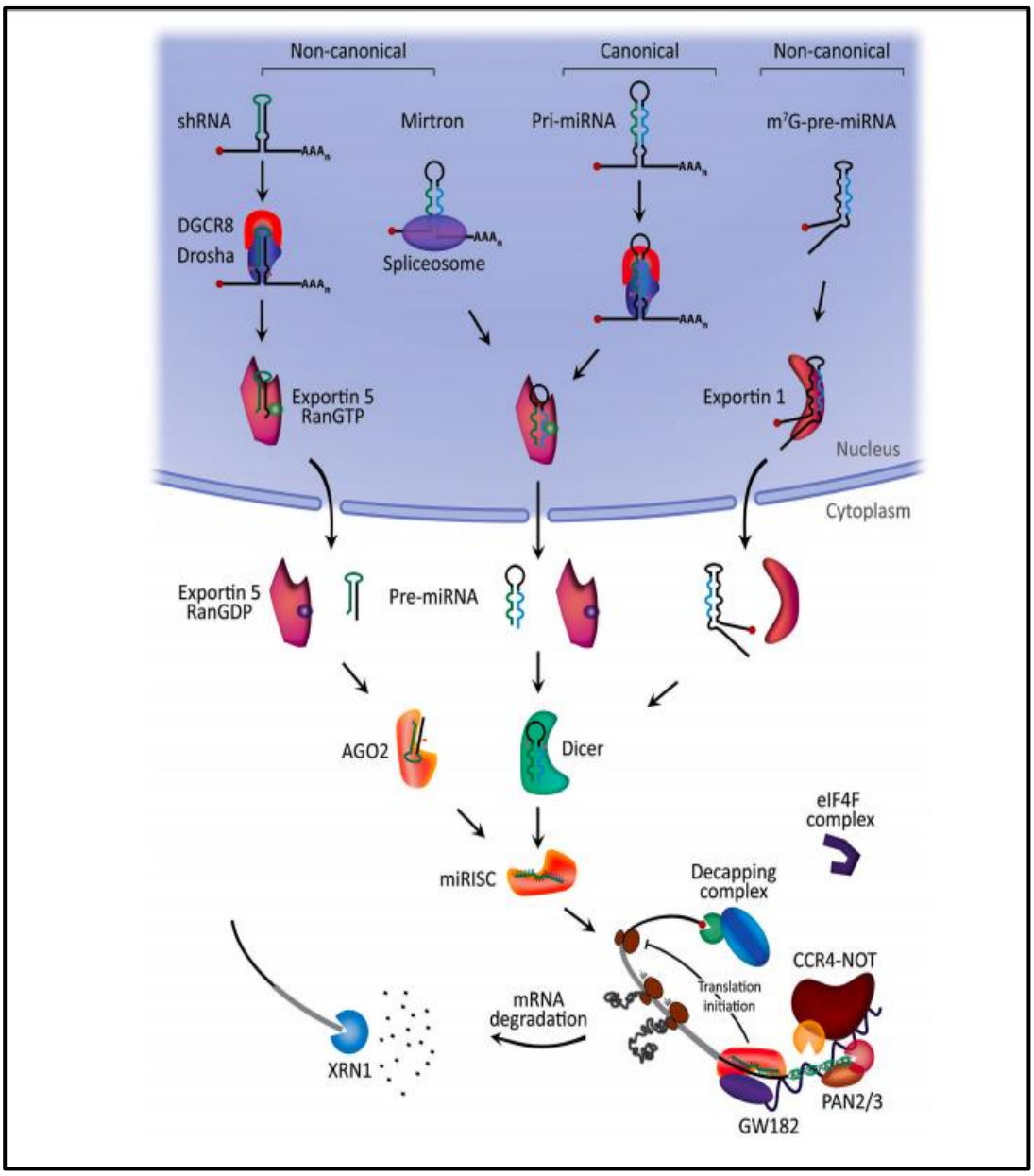


Figure 1-8 The conical and non-conical miRNAs biogenesis pathways (O'Brien *et al.*, 2018)

1.5.2.3 Circulating miRNAs

The number of intracellular miRNAs that have been detected is high, but the number of extracellular miRNAs, known as circulating miRNAs, is even higher. Recent studies have shown that circulating miRNAs are not only found in plasma or serum, but are present in other biofluids, including saliva, urine, breast milk, colostrum and tears (Mitchell *et al.*, 2008a). Moreover, the release of circulating miRNAs from injured and narcotic cells is not a passive release but shows a specific pattern of expression in biofluid in relation to different disease pathophysiology (Mar-Aguilar *et al.*, 2013).

Extracellular miRNAs are stable in biofluids and can survive in harsh conditions such as boiling, high or low pH, multiple freeze-thaw cycles and prolonged storage. Also, circulating miRNAs are resistant to degradation by RNase enzymes, suggesting that circulating miRNAs have protective mechanisms to bypass RNase activity in the extracellular environment (Gilad *et al.*, 2008).

The possible ways to release and transport circulating miRNAs are through exosomes, microvesicles, apoptotic bodies, high density lipoprotein and a protein complex. Accumulating evidence suggests that exosomes act as intracellular communicators to transfer their content, especially miRNAs, from one cell to another (Simpson, Kalra and Mathivanan, 2012). Microvesicles, like exosomes, can carry a variety of molecules, including miRNAs. Zernecke et al. (2009) showed that apoptotic bodies from endothelial cells were enriched with miR126 and the administration of these apoptotic bodies leads to a reduction of atherosclerosis in mice (Zernecke *et al.*, 2009). Vickers *at al.* (2011) found that a high number of miRNAs are associating with HDL derived from blood plasma.

In addition to the above mentioned mechanisms that lead to miRNAs release, numerous studies have shown that circulating miRNAs could be released through conjugation with a protein complex, particularly Argonaut 2(Ago2)

1.5.2.4 Circulating miRNA as biomarkers in cardiometabolic diseases

In recent years, numerous studies have shown that circulating miRNAs significantly alter cardiometabolic diseases. Some may be used as novel biomarkers for early diagnosis, prognosis or as therapeutic targets for cardiometabolic diseases. Several circulating miRNAs have been shown to be associated with the risk factors involved in cardiovascular diseases, such as diabetes, hypertension and dyslipidaemia.

Fichtlscherer et al, identified five circulating levels of miRNA that were reduced in patients with coronary artery disease compared with healthy controls (miR-126, miR-17, miR-92a, the inflammation-associated miR-155 and muscle– enriched miR-145). In contrast, cardiac muscle– enriched microRNAs (miR-133a, miR-208a) were higher in patients with coronary artery disease (Fichtlscherer *et al.*, 2010a).

De Rosa *et al*, found that circulating level of muscle enriched mIR133a, miR208a and miR499 were significantly higher in the serum of patients with troponin positive acute coronary syndrome than in patients with coronary artery disease (De Rosa *et al.*, 2011).

Devaux et al, studied circulating cardiac miRNAs (miR-208 and miR -499) in plasma samples of 87 healthy controls, 113 NSTEMI (non-ST elevation MI) and 397 STEMI (ST-elevation MI) patients. They found a higher expression of miR208 and miR499 in MI patients when compared with the controls and a strong correlation between traditional cardiac markers (CK and cTnT) and miR499 and miR208 in MI patients (Devaux *et al.*, 2012).

Li et al, investigated the expression of selected cardiac miRNAs (miR1, miR133a, miR208b, and miR499) in 67 plasma samples of acute myocardial infarction patients and 32 plasma samples from healthy controls. They found higher expressions of miR1, miR133a, miR208b, and miR499 in MI patients when compared with the controls and the receiver operating characteristic curve analyses showed no superiority of those miRNAs to cardiac troponin T for diagnosis of acute myocardial infarction (Li *et al.*, 2013b).

Jansen *et al*, studied ten miRNAs (miR-126, miR-222, miR-let7d, miR-21, miR-20a, miR-27a, miR-92a, miR-17, miR-130, and miR-199a) involved in the regulation of vascular performance that was quantified in plasma and circulating micro-vesicles (MVs) of 181 patients with stable coronary artery disease. They found that a higher expression of miR126 and miR199a in circulating MVs was significantly associated with a reduced risk of major adverse CV events (Jansen *et al.*, 2014a).

Ali Sheikh et al, investigated the diagnostic value of circulating miR765 and miR149 as diagnostic biomarkers for coronary heart disease in 69 CAD and 20 healthy subjects. They found that expression of circulating miR765 was higher in patients with CAD and expression of miR149 was lower in CAD compared with healthy control subjects. Therefore, circulating miR765 and miR149 might uses as non-invasive diagnostic biomarkers for CAD (Ali Sheikh *et al.*, 2015).

Zhang et al, found that plasma microRNA that is associated with diabetes induced atherosclerosis in 285 diabetic atherosclerosis patients compared with healthy control subjects. They found that miR-21, miR-218 and miR-211 were higher in the diabetic atherosclerosis group compared with healthy control subjects (Zhang *et al.*, 2016).

Zhang et al, explored the association between cardia specific miR-208 and severity of CHD. They found a higher expression level of miR208 in 290 CHD patients compared with controls and that the Gensini score was significantly associated with miR208 expression(Zhang *et al.*, 2017),.

Ruggeri et al,investigated the circulating miRNAs associated with doxorubicin induced cardiotoxicity in animal models. They found that miR-1-3p and miR-499a-5p showed a marked decrease in the doxorubicin group compared with controls. In addition, miR-127-3p, miR-133a-3p, and miR-215-5p showed a significant downregulation in the doxorubicin group compared with the control groups(Ruggeri *et al.*, 2018).

O'Sullivan et al, identified the circulating miRNAs that have diagnostic capability for determining in-stent restenosis (ISR) in 78 cohort CAD patients (39 with no ISR and 39 with ISR). They found that miR-93- 5p was a strong independent predictor

of ISR after correction for Framingham Heart Study risk factors (O'Sullivan *et al.*, 2019).

Kumar et al, determined the novel circulating miRNAs as biomarkers in patients with CAD. They found that miR133b and miR21 had a strong association with the severity of CAD. Also, miR133b had the ability to significantly differentiate subjects with ST elevation MI from non-STEMI (Kumar *et al.*, 2020).

To date, the list of circulating miRNAs that is associated with cardiovascular risk factors is still expanding. Results of a full literature search for miRNA biomarkers of CVD are reported in chapter 3, Table (3-1).

Despite the challenges and difficulties associated with studies of circulating microRNAs, it is important to determine miRNAs dysregulation, as it allows scientists to understand the cellular and molecular mechanism of development and progression of CVDs. Studies on circulating miRNAs could help discover new and accurate diagnostic and prognostic circulating markers, as well as a new therapeutic way to apply to different stages of CVDs.

1.6 Aim and objectives of this thesis

This thesis aims to investigate the causes and consequences of differences in novel structural and blood-based CVD biomarkers. The thesis aims to investigate the upstream determinants for the non-invasive imaging markers, includes; pulse wave arterial stiffness index and carotid intima media thickness. It then aims to specifically investigate the association of PWASI and cIMT with incidence of CVD to gain a better understanding of how these markers are related, and how they are different. For blood-based biomarkers, the thesis aims to identify putative circulating miRNAs as novel biomarkers for cardiometabolic disease, based on targets identified in pre-existing literature. To fulfil these aims, I will used two studies with different designs to meet specific objectives

1. The Carotid Atherosclerosis: MEtformin for insulin ResistAnce (CAMERA) study (Preiss *et al.*, 2014) a randomised, placebo controlled double-blinded trial. Plasma samples stored from the study will be used to gain novel insights into blood-based CVD biomarkers with the objectives:

a) To investigate the association of the expression of targeted circulating miRNAs with:

i. Cardiac biomarkers including troponin, NT-proBNP and cIMT.

ii. Metabolic biomarkers including insulin, Homeostasis model assessment of insulin resistance (HOMA-IR) and HbA1c.

iii. Lipid markers including HDL, LDL and triglycerides.

iv. Liver enzymes including gamma glutamyl transferase (GGT) and alanine aminotransferase (ALT).

b) To investigate the effect of metformin on the expression of targeted circulating miRNAs.

2. The UK Biobank study, a large-scale prospective epidemiological study. Data from this study will be used to gain novel insights into structural CVD biomarkers

I. To investigate the association of cIMT and PWASI with:

a) Simple demographic patterns and upstream CVD risk factors and assess whether these risk factors are independently associated with cIMT and PWASI.

II. To compare and contrast cIMT and PWASI as biomarkers of CVD outcomes, specifically:

a) The association between imaging markers (cIMT and PWASI) as categorical and continuous variables with incidence of cardiovascular disease in the UK Biobank population, independent of traditional cardiovascular risk factors.

2 General Methods

2.1 Introduction

This chapter describes all the general methods and experimental procedures that were used to produce this thesis.

2.2 Studies used in this thesis

2.2.1 The CAMERA trial

The Carotid Atherosclerosis: MEtformin for insulin ResistAnce (CAMERA) study was a single centre randomised, placebo-controlled double-blind trial carried out at the Glasgow Clinical Research Centre (Glasgow, UK) between 2009 and 2012 (Preiss *et al.*, 2014). The study was designed to examine the effect of metformin on carotid intima media thickness progression in individuals with cardiovascular diseases but without diabetes. The use of this study to investigate miRNA is post-hoc.

2.2.1.1 Cardiometabolic risk factors and expression of miRNAs measurements

A systematic literature review was conducted as a basis for making evidence-based decisions regarding which circulating miRNAs to include in this study, as shown in Table (3-1).In this thesis, I undertook measurements of all miRNAs of paired plasma samples (at baseline and at 18 months) from a random sample of 60 participants that were collected over an 18-month period by the authors of the CAMERA trial, as shown in Figure (3-1). In this study, the quantitative real-time polymerase chain reaction method was used to measure the expression of circulating miRNAs after extracting them from plasma samples, as described in Sections 2-3 and 2-4.

All other biochemical markers and the cIMT outcome measurement had been conducted as part of the CAMERA trial prior to the thesis. The data of cardiometabolic risk factors including troponin, cIMT and other biochemistry measures for the paired samples of 60 participants were provided by the authors of the CAMERA trial. These measures were conducted by laboratory technicians in the CAMERA trial. I performed the analysis in accordance with the objectives set

out in Chapters 3 and 4, as described in (2-5). More details for this study are shown in Chapters 3 and 4.

2.2.2 The UK Biobank study

The UK Biobank is a unique large-scale prospective epidemiological study, with open access data for all bona fide researchers. It aims to improve the prevention, treatment and diagnosis of a wide range of disorders and life-threatening diseases, including cardiovascular diseases, diabetes, arthritis and cancer. Baseline assessment recruited more than 500,000 men and women aged between 40 and 69 years in the UK from 2006-2010 (Sudlow *et al.*, 2015). The imaging study began in May 2014 and aimed to recall 100,000 participants for detailed imaging scans of vital organs (brain, heart, abdomen, bones, carotid artery, and body composition), as well as to repeat the baseline measurements. The study included a heart and body MRI, a brain MRI scan, a neck artery ultrasound scan and dual energy X-ray absorptiometry (DXA). All the baseline and imaging study data were used in this study.

2.2.2.1 Outcome and predictors variables that included in this study

All measurements of outcomes, carotid intima media thickness and pulse wave arterial stiffness index (cIMT and PWASI), and predictor variables used in this study (age, sex, ethnicity, blood pressure, anthropometric measurements, biochemical markers, diabetes, blood pressure, smoking, statin use and Townsend deprivation index) were carried out by UK Biobank staff members at assessment visits (2010-2014) for predictor variables and at imaging visits (2014-2018) for cIMT and PWASI, as described in Section (5-4-5). The cross-sectional study (Chapter 5) and prospective study (Chapter 6) were specifically applied and approved under UKB project number 43707 (UK Biobank, 2019).

An access application was submitted to the UK Biobank website with the author as the project principal investigator (https://www.ukbiobank.ac.uk/enable-your-research/approved-research/traditional-and-novel-biomarkers-of-adverse-cardiometabolic-imaging-and-associations-with-outcomes), and approval was

received in June/2018. In February/2019, UK Biobank released the data. See (Appendix 3). Details of the study are presented in Chapter 5.

2.3 MiRNA isolation and quantification

The miRNA measurement protocols were not well established in the laboratory prior to commencing this research. The author therefore researched and collaborated with internal partners (including fellow PhD student Tahani AlRamah, and Dr Lorraine Work's team at the University of Glasgow) to develop a working method for the miRNA measurement at increased scale and throughput, using CAMERA samples.

Fellow PhD student Tahani AlRamah had used this developed method to investigate the association between certain miRNAs including (miR222, miR221, miR192, miR193b, miR144, and miR155) and cardiometabolic markers in CAMERA sample, and she found this method was working for those circulating miRNAs, in this study, we investigated this method in different set of circulating miRNAs in the same sample.

Part of the attraction when using CAMERA for miRNA was that:

A) The study was conducted locally as a single-centre trial, with blood processing performed rapidly (within four hours) of blood-letting. This minimizes pre-analytical variability.

B) Data from the study could be used cross-sectionally and prospectively to investigate the effect of a very common clinical intervention drug.

2.3.1 MiRNA extraction from plasma samples

2.3.1.1Extraction

MiRNA was extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany Cat. No. 217004) as per the *manufacturer's instructions*. The MiRNeasy Mini Kit is designed for the purification of total RNA, including miRNA and other small RNA molecules. The MiRNeasy Mini Kit contains QIAzol Lysis Reagent, which is a

monophasic solution of phenol/guanidine thiocyanate and silica membrane-based purification of total RNA.

All extraction steps were carried out under a fume hood, and on top of ice using certified nuclease-free equipment. The two samples from each participant (baseline and 18 months) were extracted within a single run. For each participant, 300 µL of plasma sample was divided into two 1.5 mL Eppendorf tubes (150 µL each tube). In each tube, 750 µL (five volumes) of QIAzol lysis reagent (Qiagen, Hilden, Germany Cat. No.79306) was added to purify the RNA. The mixture was then mixed by vortex until all the precipitate disappeared. After which, the homogenate was placed at room temperature in the fume hood for five minutes for complete dissociation of nucleoprotein complexes. Following this, chloroform (150 µL) was added to all tubes containing the homogenate, which were then vigorously mixed by vortex for 15 seconds and kept at room temperature for three minutes. After centrifugation for 15 minutes (8000 G, 4°C), two layers were formed: the upper clear aqueous phase and lower pink layer. The upper aqueous phase was separated into two new 1.5 mL Eppendorf tubes (250 µL each), making a total of four tubes for each participant (Figure2-1). Following this, 375 µL of 100% ethanol (1.5 volume) was added to each tube. One RNeasy Mini spin column was prepared and placed into a 2 ml collecting tube for each sample. All four tubes of the same sample were added to the same column. The samples were added one by one, and mixed thoroughly by pipetting. They were then collected (up to 700 µL) into the column, and centrifuged (8000 G) for four minutes at room temperature before discarding flow-through. This process was repeated three more times to complete all the remaining samples. The buffers in the kit were prepared following the manufacturer's instructions. RWT buffer (700 µl) was added to the RNeasy mini spin column and centrifuged (8000 G) for two minutes at room temperature. Flow-through was then discarded. The column was washed again with 700 µL RPE buffer and centrifuged (8000 G) for two minutes at room temperature, before discarding the flow through. The RNeasy Mini spin column was washed using 500 µL of 75% ethanol and centrifuged (8000 G) for two minutes at room temperature. The spin column was placed into a new 2 mL collection tube and centrifuged (8000 G) for five minutes to dry the column. The spin column was then transferred into a new 1.5 mL collection tube. RNase-free water (20 µL) was added directly into the spin column and centrifuged (8000 G) for two minutes at

room temperature. The same flow through was used again and the previous step was repeated twice more to concentrate the RNA. The sample was kept on ice until the RNA concentration had been read using nanodrop RNA quantification

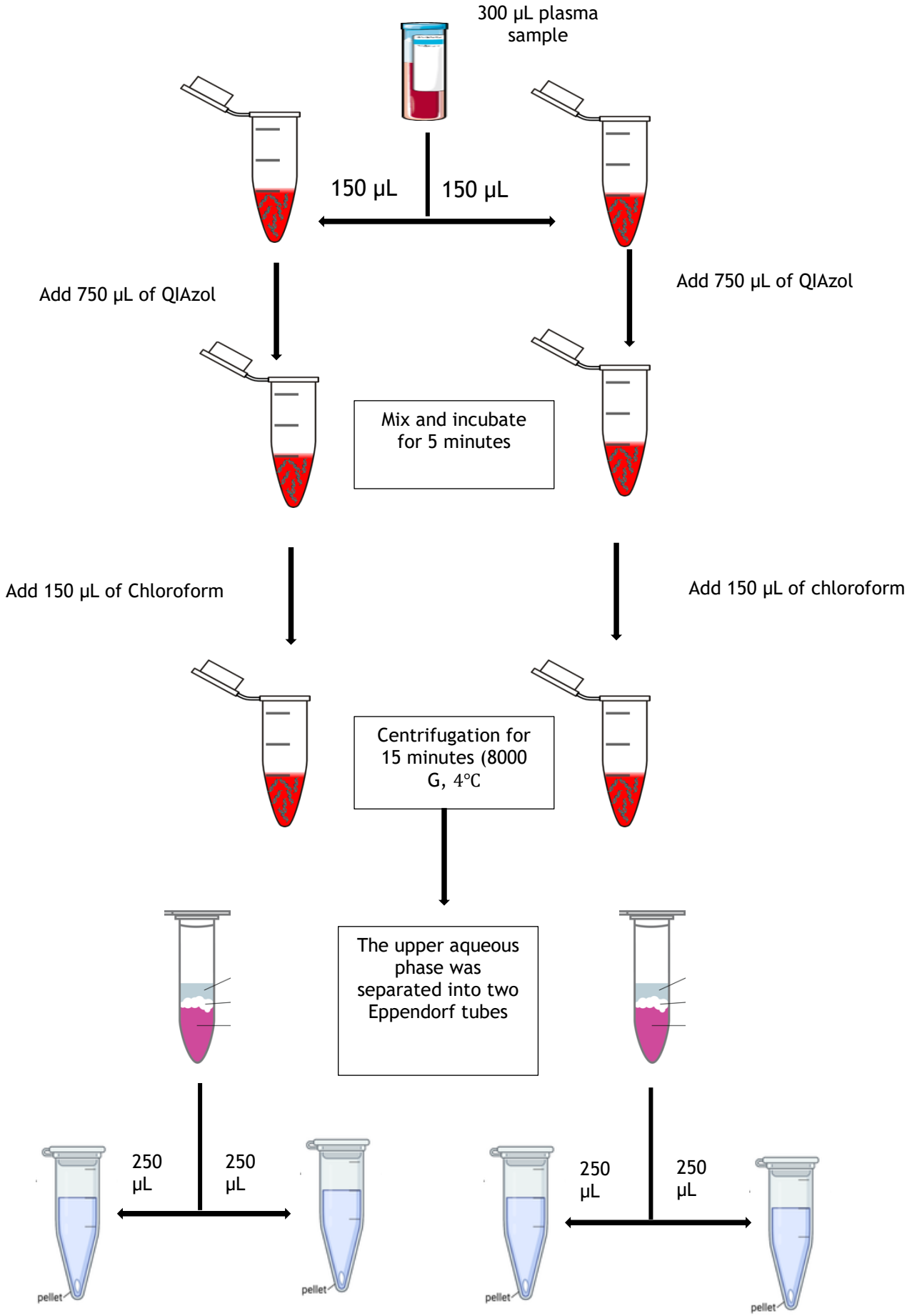


Figure 2-1 miRNA extraction from plasma samples

2.3.2 RNA quantification

The quality and concentration of RNA within each sample was determined with a NanoDrop ND-1000 micro-volume ultraviolet-visible spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts v3.7), using ND-100 software and following the manufacturer's instructions. The pedestal surface was cleaned and reconditioned before starting the measurements session. Nuclease-free water (2 µL) was pipetted onto the lower measurement pedestal, lowered onto the sampling arm and measured as blank. The next stage was to pipette 2 µL of an aliquot of the nucleic acid from each sample before recording the concentration with the purity ratios (260/280, 260/230). Measurements were repeated twice, and an average measurement taken, before storing the samples at $-80℃$.

2.3.3 MiRNA reverse transcription

For miRNA analysis, complementary DNA was prepared from RNA using the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, no.4366596) as per manufacturer's guide. Primers for each gene of interest were used to prepare the master mix.

2.3.3.1Samples preparation

A total of 9 ng (2ng/µl) of RNA is needed for each miRNA. Nuclease free water was used to make the dilution to reach a fixed volume of 5 µL from total RNA for RT process.

RNA must be diluted to 2 ng / µL. For each miRNA, a total of 9 ng of RNA is needed. Therefore, 9 ng x 8 miRNA=72. I rounded this up to 80 ng to account for potential loss of material while pipetting. To calculate volume of RNA needed, I used the following formula:

$$\frac{80}{\text{Total Conc.of RNA}} = \text{X µl (amount of RNA)}$$

For each miRNA, a total volume of 5 µL of RNA is needed. Thus, for 8 miRNAs 40 µL in total was needed. 40 µL $- X =$ amount of RNAse free water to make up the 40 µL.

2.3.3.2Master mix preparation

For each miRNA primer, one master mix was prepared. The CDNA Synthesis Master Mix reaction tube contains dNTP (100 mM), multiscribe, 10X RT buffer, RNase inhibitor, nuclease free water and the specific miRNA primers allocated (5X, RT). The master mix was prepared and stored over ice, Table (2-1).

Table 2-1 Master Mix Preparation

	For one sample	**For 1 ~ 8 samples**
dNTP (100 mM)	0.075 µL	0.75 µL
Multiscribe	0.5 µL	5 µL
10×RT Buffer	0.75 µL	7.5 µL
RNase inhibitor	0.095 µL	0.95 µL
RNase free water	0.08 µL	0.8 µL
Primer (5X;RT)	1.5 µL	15 µL

2.3.3.3Protocol

The practical work was carried out on the top of ice. Diluted RNA samples (4.5µl) were added to 96 well plate as well as 3 µl of master mix. The 96-well plate was briefly vortexed and centrifuged after each addition to ensure adequate mixing. The plate was sealed with adhesive sealing sheets and labelled with number of sample and date. The plate ran on polymerase chain reaction (PCR) block for RT reaction. Reverse transcription (RT) was performed with the following temperature programme:16°C for 30 mins, 42 °C for 30 mins, 85°C for 5 mins, 4°C for 30 mins and then held at 12°C. These conditions allow for primer annealing, DNA polymerization, and enzyme deactivation respectively. The plate was kept in -20°C until used for QPCR.

2.3.4 Quantitative real-time polymerase chain reaction (qRTPCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed for miRNA analysis by using Taqman® (2x) universal PCR master mix (Applied Biosystems, Foster City, California). Fluorescently tagged Taqman® primers (20X) for the miRNAs of interest were used according to protocol. All samples were run in duplicate, including negative and quality control samples.

2.3.4.1Plate preparation

The 96-well PCR plates were taken out at −20℃ and kept on ice until thawing occurred. Following this, all samples were diluted by adding 7.5 µL of RNase free water to each well and centrifuging the plate at 4℃ to bring everything down.

2.3.4.2Master mix preparation

The calculation of master mix for each primer was made according to the Table shown in (Appendix2). Primer (TM,20x), universal PCR mister mix (20x) and RNase free water were added in the same reaction tube and labelled by the master mix of gene of interest.

2.3.4.3Protocol

All practical work was done on top of ice. A MicroAmp™ optical 384-well reaction plate with a barcode was prepared and labelled with the sample name and date. Complementary DNA (1.4 µL) was pipetted onto the side of the well and centrifuged. Following this, qPCR master mix (8.6 µL) was added to each well, centrifuged and sealed with an optical adhesive lid.

The Quantstudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, California V1.3) was used for measuring the fluorescence of the quantitative real-time polymerase chain reaction (qRT-PCR). The fluorescence intensity measured was directly proportional to the amount of PCR product after each amplification cycle. A significant increase in fluorescence intensity was observed when there was a higher starting copy number of the nucleic acid target. The temperature cycle conditions for miRNA expression were 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds, followed by 60°C for 1 minute. For each miRNA, four runs of qRT-PCR were carried out, including the quality control sample (QC) and negative control sample (both in duplicate). For quantification analysis, the comparative cycle threshold (CT) method was used as a semi-quantitative read-out (Livak and Schmittgen, 2001a).

2.4 Normalisation and control for qRT-PCR Data

The quantification of miRNA expression can be carried out using one of two methods: absolute quantification or relative quantification method. Real-time quantitative PCR (q-RT-PCR) is a common method to measure target miRNAs. MiRNAs are generally measured relative to a reference sample; a relative method that requires a reference. This approach is appropriate for examining physiological changes in miRNA expression levels. However, it also makes it challenging to compare studies directly due to technical differences in relative expression. Absolute quantification using better statistical analysis is preferable for a comprehensive assessment of gene expression levels. Absolute quantification is still not in common use.

The reliability and fidelity of the relative quantification method of q-RT-PCR data can be improved by normalization with a constant reference gene to correct the

technical noises between samples and quantify the true biological change in miRNA expression which is associated with phenomenon or disease of interest. The potential sources of technical variability include (1) the amount of starting material and extraction process; (2) multiple freeze-thaw cycles; (3) the RNA extraction method and its efficiency; (4) the quality and quantity of miRNA; (5) the enzymatic efficiencies; (6) the variation between samples in total RNA and the concentration of miRNA in plasma or serum. There is a lack of agreement for a standardized normalizing strategy for quantifying the miRNA in plasma or serum.

Exogenous and/or endogenous miRNAs are usually used to normalize the expression of circulating miRNA to control both biological and isolation pre-analytical variability. Endogenous miRNAs are miRNA found naturally in the tissue of interest, whereas exogenous miRNAs are introduced from external sources for the purposes of the experiment. In both cases, it is expected that the control will act as a constant within the experimental setting. The optimal approach is to use a theoretical constantly expressed housekeeping gene, because this would be affected by the same source of variability as the target biomarker of interest. The endogenous method has been widely used for measuring the expression of mRNA and miRNA extracted from tissues and cells. Several invariant endogenous gene have been discovered in solid tissues or cells such as miRNA-16 and let-7 in breast cancer using breast cancer tissues (Di Leva, Garofalo and Croce, 2014). However, using this method on plasma/serum samples is challenging because very few studies are available. Endogenous controls are frequently selected based on empirical observations, with little biological insight, making it difficult to define what “should” be constant endogenously. Different studies reported different miRNA reference genes (Moldovan *et al.*, 2014). For choosing an appropriate endogenous control, it is important to first check whether the selected miRNA expression is stable between the samples before using it as a normaliser.

One solution that has been suggested to solve this problem is the spike in *Caenorhabditis elegans* miRNAs (cel-miR-39, cel-miR-54 and cel-miR-238), which do not have homologous sequences in humans (Mitchell *et al.*, 2008b). Some researchers have used all three (McDonald *et al.*, 2011b), while others only used one spike as a control in their samples (Widera *et al.*, 2011), (Turchinovich *et al.*, 2011).

To verify whether the qPCR experiment passes initial quality control, the convention is that technical replicates of the controls and samples must be within 0.5 cycles (with one cycle representing a doubling of material). This is achieved by calculating the standard deviation (SD) of a triplicate measurement of the same sample. The SD should not exceed 0.25 (Institute of Research in Immunology and Cancer, University of Montreal, n.d.).

(http://genomique.iric.ca/resources/files/Understanding_qPCR_results).

In addition, a random plasma sample was used as a quality control sample to try to control for variation between runs. Mean and standard deviation (SD) were calculated for exogenous control and for the QC sample. In addition, the coefficient of variation (CV) for both intra and inter assays were calculated for all the two controls. Although calculating CV is not generally used for evaluating precision of qRT-PCR results, this study attempted to quantify methodological error.

2.4.1 Plasma samples

2.4.1.1MiRNA exogenous control

C. elegans miRNAs was chosen as an external control because of a lack of sequence homology to human miRNAs. The synthetic versions of the *C. elegans* miRNAs (Qiagen, Hilden, Germany Cat. No. 219610) were used and supplied as lyophilized powder in 10 *p* mol per tube. It was spiked in into serum after the addition of the denaturing solution (Qlazol) to inhibit RNases and before the extraction to control the extraction process, reverse transcriptions and RT qpcr process. A trial from 12 study samples was run to test the reliability and stability *C. elegans* miRNAs as a normalizer.

Stock Solution

Was prepared by adding 300 µL nuclease free water vortexed, spun. The final concentration was 2 x 1010 copies/µL stock. The stock control was aliquoted into 30 µL /tube and stored at -80°C.

Working Control

4 µL of the stock control solution was mixed with 16 µL nuclease free water to produce the First Step Solution (4 x 109 copies/µL). First Step Solution (2 µL) was further diluted with nuclease free water (48 µL) to produce a 1.6 x 108 copies/µL.

2.4.1.2MiRNA endogenous control

MiR520d-5p was chosen as possible candidate gene for plasma miRNAs studies due to it having the most constant Ct and the lowest SD among all tested endogenous housekeeping genes (Rice *et al.*, 2015).

For this study, a trial run using the same eight random normal samples used in testing miR39 was carried out to test the potential use of miR520d-5p as an endogenous control gene. The results of the pilot of quality control procedures showed low (Ct > 40) and variably quantifiable expression of miR520d-5p between the samples. Therefore, miR520d-5p was not used as an endogenous control gene in this study (Alramah, 2020). Several other candidates were likewise discarded (miR-103, miR-331-3p, and RNU44).

2.5 Statistical analysis

2.5.1 Cohort demographics and classical risk factors

The normality of continuous variables was assessed by visual inspection of histogram or normal Q-Q plots. The descriptive statistics for continuous variables were presented as mean ± SD for normally distributed variables and as medians with interquartile ranges (IQR) for non-normally distributed. Any skewed variable was normalized by log transformation to use parametric test for analysis. Note that throughout the thesis, use of a "±" denotes a standard deviation after a mean, and use of a (range) after a number denotes a median and interquartile range. The descriptive statistics for categorical variables were presented as numbers and percentages. An independent t-test was used to compare the means of continuous variables between groups. The statistical analysis was conducted using IBM SPSS (Statistical Package for Social Sciences) Windows version 22 (SPSS Inc. Chicago, USA) and Microsoft Excel 2016. The criterion for statistical significance was $p < 0.05$. As this is a hypothesis-generating exploratory study, no formal correction was employed for multiple comparisons. Pearson correlation

coefficient (r) was used to assess the statistical relationship between mean cycle threshed (Ct) for each miRNA and cardiometabolic markers using baseline samples. A p- value < 0.05 was considered as statistically significant for correlation.

2.5.2 RT-qPCR data analysis

The comparative cycle threshold (Ct) was used for quantification analysis of miRNA in plasma. Ct (threshold cycle) is the number of cycles in PCR reaction in real time required to exponentially accumulate a fluorescent signal to surpass a threshold (i.e. exceeds the background level). The CT value is inversely proportional to the amount of target miRNA in the samples (i.e., the lower Ct value the higher the amount of target nucleic acid in the sample). As the number of molecules doubles in each cycle of PCR reaction, quantification is therefore naturally reported on a $\log_2$ scale (Figure2-2). The Ct values were expressed as mean and SD for triplicate measures for samples and controls; the concentration of target miRNA in the samples was of an approximately normal distribution, being naturally expressed on a Log ($_2$) scale. The triplicate is valid when the SD is smaller than 0.5. Measurements were repeated if the SD was over 0.5.

The quality of the RT-qPCR results was assessed by precision and repeatability (intra and inter-assay variability). Coefficient of variation (CV) between triplicates and between samples for exogenous control (mir-39 spiked-in control) were measured, as was the coefficient of variation (CV) of the repeatedly measured quality control sample. The CV is not generally used for evaluating the precision of qRT-PCR results, but this was an attempt to calculate error in a manner accessible to most laboratory scientists. In most quality control settings in routine biochemistry, intra-assay CVs of <10% and inter-assay CVs of < 15% are generally acceptable

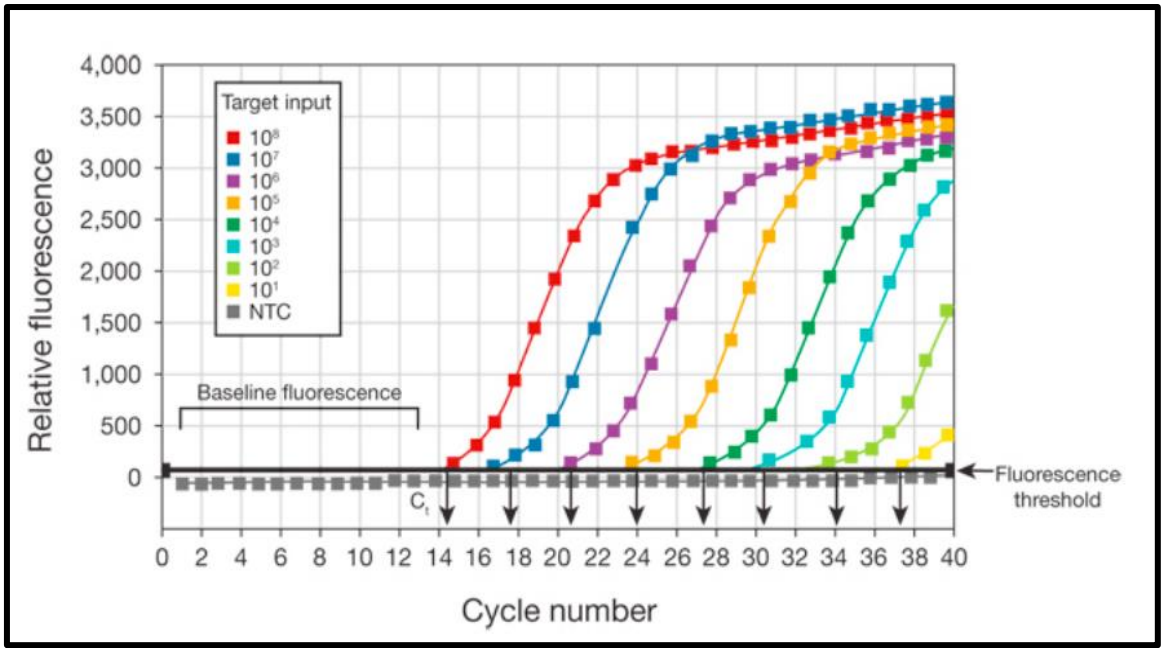


Figure 2-2 QPCR amplification curve

Amplification curves are created when fluorescent signal from each sample is plotted against cycle number, therefore, amplification curves represent the accumulation of products over the duration of real time PCR experiment

2.5.3 The coefficient of variation for each miRNAs in the entire study

Table 2-2 shows the results of CV% for CT of each miRNAs in 60 participants at baseline and at 18 months. The coefficient of variation (CV) was calculated to assess the reproducibility for each miRNA among the random samples that were included from the CAMERA trial. The inter -assay CV for all miRNAs was less than 15%, which is in the typically acceptable limit.

Table 2-2: Inter-assay CV of each miRNAs (CT) for entire study

miRNA	Baseline			18 months		
	Mean	SD	CV%	Mean	SD	CV%
miRNA-30c	31.34	1.31	4.2%	30.72	1.32	4.3%
miRNA-103	32.15	3	9%	31.26	3	9%
miRNA-133a	34.33	1.7	5%	34.5	2	5.5%
miRNA-122	32	1.5	4.7%	32	1.5	5%
miRNA-146a	27	2	7%	27	2	7.5%

CV: Coefficient of Variation, **SD:** Standard Deviation

2.5.4 Relative quantification method (RQ)

The relative quantification (RQ) method was used to compare paired observations at different timepoints. In this study, the relative or comparative quantification was used to measure the relative expression of target miRNAs to the exogenous control gene in paired samples at two different time points at baseline and at 18 months. First, delta Ct (ΔCt) was used to calculate the difference in expression between the target gene and the external spike in the control gene in the same sample. The ΔΔCt was then calculated using ΔCt for each sample (ΔCt baseline - ΔCt after 18 months). Doubling of the target miRNA during each cycle of PCR was assumed in this equation in order to calculate the relative quantification (fold of change). RQ was calculated using the formula ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001b) (Marabita *et al.*, 2016).

3 Cross-section association between the expression of miRNA and markers of cardiometabolic disease: CAMERA Trial

3.1 Introduction

Optimal prevention and intervention strategies require an understanding of the antecedents of disease, as well as an accurate classification and identification system for patients at risk of cardiometabolic diseases. This classification system can help physicians to target intensive treatment as a primary prevention strategy for high-risk patients to avoid developing cardiometabolic disease. A commonly established approach and probably the most widely used, globally, is the equation derived from the American Framingham cohort study. Many modified versions of the Framingham risk assessment equation are available, including the QRISK3 model. In addition, the American Heart Association (AHA) and the American College of Cardiology (ACC) developed the Atherosclerotic Cardiovascular Disease (ASCVD) risk score calculator, which is used in clinical practice to make decisions related to classifying and treating high-risk populations. In the 1990s, it became clear that there were other than traditional risk factors, such as genetic risk factors and markers of inflammation, that could affect cardiovascular risk; thus, there is an urgent need for improvement of the risk scoring.

Cardiovascular disease (CVD) is a complex and multifactorial disorder, it is critical to understand the different biological and genetical causes in order to improve diagnosis, prognosis, and treatment response, and thereby decrease CVD mortality and morbidity. Using cardiometabolic markers including troponin, NT-proBNP, insulin markers, lipid markers, and carotid intima-media thickness (cIMT) in addition to the conventional risk factors included in the risk prediction model might be useful; thus, study of the possible determinants for those markers is needed to reduce CVD incidence.

Carotid intima-media thickness ultrasound is a non-invasive technique used to evaluate subclinical cardiovascular diseases. Elevated cIMT has been shown to be correlated with the modifiable traditional risk factors, including obesity, dyslipidemia, high blood glucose, high blood pressure, and smoking (Chambless *et al.*, 1997; Rundek *et al.*, 2013); early detection and modification of these risk factors may have a significant impact on the prevention of cardiovascular diseases. It has been shown that traditional risk factors account for only <50% of atherosclerotic plaque variance (Lefkowitz and Willerson, 2001; Spence *et al.*,

1999). Therefore, it is believed that there are other factors contributing to atherosclerosis, in addition to the traditional risk factors.

3.1.1 High-sensitivity cardiac troponin T

Troponin T (TnT) is a tropomyosin binding subunit of troponin complex, which is located in thin filament anchoring subunit in cardiac and skeletal muscles (Jin, 2016). Cardiac troponin is theoretically only present in the blood as a result of damage to cardiomyocytes, since skeletal troponin has distinct genetic origin. Cardiac troponin T has been used for many as the serological biomarkers of choice for diagnosis and prognosis of acute myocardial infarction, because of their high sensitivity and specificity. High sensitivity cardiac troponin assays allow detection of cardiac troponin in the circulation down to very low levels, classically not of relevance, but such levels may be indicative of ongoing myocardial damage (Collet *et al.*, 2020). One study by Willeit et al. (2017c) showed that 80% of 154,052 participants with no clinically manifest myocardial injury had a detectable troponin level using high sensitivity assays. A positive association between cardiac troponin and CVD outcomes was also found when analysing 4,402 individuals with no CVD at baseline from the PROSPER study (Pravastatin in Elderly Individuals at Risk of Vascular Disease Study) (Willeit *et al.*, 2017b; Shepherd *et al.*, 2002). Further work has confirmed a strong association of high sensitivity troponin assays with incidence of CVD, leading to the potential that they might be used as a surrogate markers for CVD (Welsh *et al.*, 2019).

3.1.2 B-type natriuretic peptide (BNP)

B-type natriuretic peptide is a cardiac neurohormone secreted from the ventricular musculature in response to volume and pressure overload. It, and more usually, its inactive metabolite N-terminal pro B-type Natriuretic peptide (NT-proBNP) is used clinically in the diagnosis of heart failure at high levels above 450pg/ml (Mueller *et al.*, 2019). The bioactive natriuretic peptides, in general, have a natriuretic and vasodilatory effect that regulate blood pressure and fluid balance by suppress the renin-angiotensin-aldosterone system (Cardarelli and Lumicao, 2003). As well as a clinical role in heart failure diagnosis, it has been found that low level elevations in NT-proBNP concentration assessment strongly

predict first-onset heart failure, stroke, and coronary heart disease among the population with no history of cardiovascular diseases (Willeit *et al.*, 2016).

Therefore, both high-sensitivity cardiac troponin and NT-proBNP are widely accepted cardiovascular biomarkers in clinical practice. Both are recognised to be useful indicators of adverse prognosis among patients with stable cardiovascular disease and for subclinical cardiovascular disease in the general population (Zeller *et al.*, 2014; Ford *et al.*, 2016; Redfield *et al.*, 2002; Azevedo, Bettencourt and Barros, 2007).

Recently, increasing evidence has been presented for the use of circulating miRNA, which is small and single conserved non-coding strand of RNA as a biomarker for complex diseases such as cancer (Powrozek *et al.*, 2017), infectious diseases (Bihrer *et al.*, 2011), and cardiometabolic disease (Zhao, Zhao and Zhao, 2015).

Several studies have shown that dysregulation of circulating miRNAs expression may be related to the underlying causes of cardiometabolic diseases. A study by Ellis et al. (2013) found down-regulation of four circulating miRNAs: miR103, miR-142-3p, miR-30b, and miR-342-3p –in 32 heart failure patients, compared with 15 COPD patients and 14 healthy controls (Ellis *et al.*, 2013). Another study, by layde R et al. (2019), found that six circulating miRNAs: miR-125b-5p, miR-146a-5p, miR-328-3p, miR-191-5p, miR-103a-3p, and miR-30b-5p correlated with both oxidised LDL and cIMT (Layde R. Paim, 2019). These studies were cohort studies for individuals with the disease of interest, comparing with controls, and the researchers selected circulating miRNAs based on the differential expression of circulating miRNA in a microarray assay. These observational studies may have been at risk from unpredictable confounding factors, in contrast to randomised control trials. Our study is the first study to investigate the cross-sectional association between cardiometabolic markers and the expression of circulating miRNAs across randomised control trials of patients with coronary artery disease.

3.2 CAMERA trial

The Carotid Atherosclerosis: MEtformin for Insulin ResistAnce (CAMERA) study was a single-centre randomised, placebo-controlled, double-blinded trial carried out

at the Glasgow Clinical Research Centre (Glasgow, UK) between the years 2009 and 2012 (Preiss *et al.*, 2014). The study was designed to examine the effect of metformin on carotid intima media thickness progression in individuals with cardiovascular diseases but without diabetes. The primary outcome was progression of carotid intima media thickness after 18 months from metformin treatment. A secondary outcome was change of cardiovascular surrogate markers, such as HOMA-IR, insulin, triglyceride, cholesterol, and HDL. The study was able to detect that metformin had no effect on cIMT and no or little effect on several surrogate cardiovascular disease markers in high cardiovascular risk patients.

Study had included participants aged between 35–75 years on statin with, a history of proven coronary heart disease (defined as previous acute coronary syndrome, coronary artery bypass surgery, or angiographically proven coronary heart disease), and large waist circumference as per International Diabetes Foundation criteria (≥80 cm in women, ≥94 cm in men) .

Exclusion criteria were participants with type 2 diabetes or those with HbA1C ≥ 7.0, or a fasting blood glucose level ≥ 7.0 mmol/l at screening (people with a fasting plasma glucose level lower than 7.0 mmol/L at screening had an oral glucose tolerance test); and those with a post-challenge glucose level of ≥ 11.1 mmo/L were excluded. In addition, pregnant and lactating women at screening; premenopausal women not taking contraception, either oral or hormone replacement; those who had acute coronary syndrome within the previous 3 months; or with class 3 or 4 heart failure categorised according to the New York Heart Association, were excluded. Other exclusion criteria were people with uncontrolled angina; hepatic impairment; renal impairment with an estimated glomerular filtration rate < 45 mL/min per 1.73 m2 at screening; hypersensitivity to metformin; acute illness (severe infection, dehydration, acute cardiac failure, shock); and suspected tissue hypoxia.

The study was carried out in accordance with the ethical principles set out in the Declaration of Helsinki and good clinical practice guidelines and was approved by the Medicines and Healthcare Products Regulatory Agency and West Glasgow Research Ethics Committee. Written informed consent was obtained from all patients to use their blood samples for research purposes, and patients were

followed-up for 18 months. Eligible patients (a total of 173 participants) were randomly assigned without stratification by computer to treatment with metformin (850 mg twice daily) or matching placebo (1:1). The randomisation sequence was generated independently by the Robertson Centre for Biostatistics. All patients, trial staff, investigator, and statisticians were blinded to treatment allocation.

In the first week of the trial, participants were counselled to take one study tablet, either 850 mg of metformin or a matching placebo, once daily for one week. The dose was then increased to two tablets daily, one with the morning meal and one with the evening meal. The study medications were masked and supplied in numbered containers. Compliance was assessed by tablet count (defined as taking >80% of study medication during the study). A total of 29 patients reduced their tablet intake to one tablet daily (19 participants in the metformin group and 10 participants in the placebo group). Four participants assigned to the placebo and 11 participants assigned to metformin were excluded from the analysis because they did not attend the final visit, meaning a total sample of 157 participants. A total of 60 participants were randomly included in this study, as shown in Figure (3-1). In the CAMERA trial, more than 80% of patients had an adherence rate over 90%. Compliance was evaluated by tablet counting. An analysis only for those participants who completed the study duration (18 months in duration), showed that they had an adherence rate of more than 90%.

3.2.1 Power calculation

Assuming the minimum relevant difference to detect between metformin and placebo groups is 1 delta threshold cycle (dCT) (a 2-fold change) with a dCT SD of 1.3 in each group, a sample size of 30 in both groups gives 81% power with an alpha of 0.05. The sample also has 81% power to detect a correlation of r=0.35. The sample size (60) patients of this study is powered enough to detect if there is any difference in dCT of miRNAs between two groups and to investigate if there is correlation between expression of miRNAs and cardiometabolic risk factors.

Samples from the CAMERA trial were used to conduct this study for several reasons. First, it was a randomised controlled trail with well-defined exclusion and inclusion criteria. In addition, it has a well standardised method for sample

collection, processing, and storage. Finally, high-risk participants (previous CHD) represented the best candidates for investigating the expression of circulating miRNAs as surrogate cardiac markers.

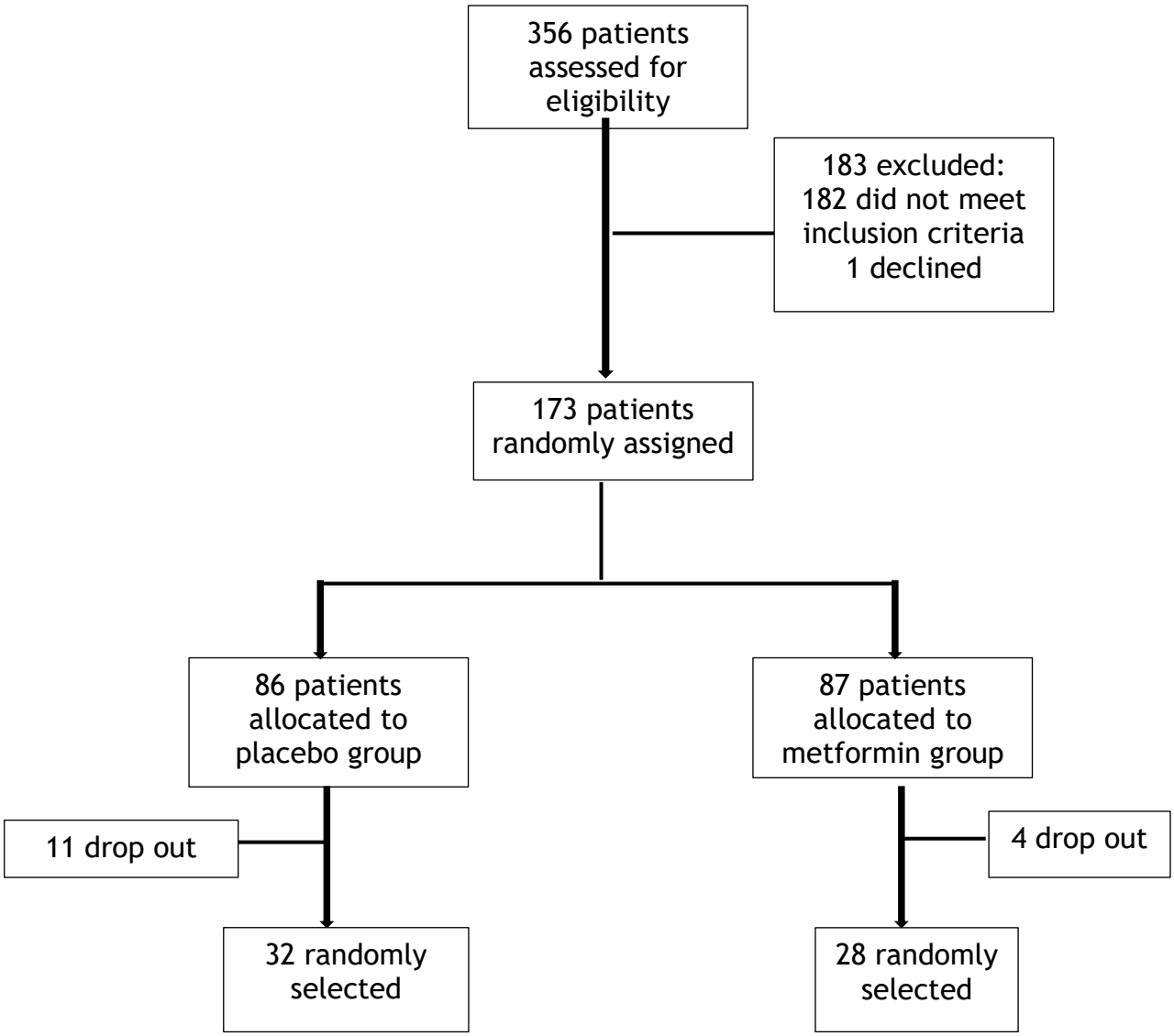


Figure 3-1 CAMERA trial profile

Summary of inclusion of baseline blood samples in the miRNA study

3.2.2 MiRNA biomarker candidate selection for inclusion in the study: a hypothesis-driven approach

Several miRNAs were identified as associated with cardiometabolic diseases, either circulating miRNAs or tissue miRNAs. A small number of circulating miRNAs have been replicated across several studies. There are several possible reasons for this: 1) large and different circulating miRNAs have dysregulated across different diseases; 2) there is no standardised protocol for circulating miRNAs isolation, quantification, and normalisation; 3) comparison between studies is difficult because of the different methods, sample collection, and sample type used across studies; 4) most studies have used a small sample size, which increase type 2 errors.

In order to make an evidence-based decision as to which circulating miRNAs to include in this study, a hypothesis-driven approach based on previously available evidence and not an exploratory approach was used. The reason for this was that this method requires less time, and is more focused and efficient, especially for a biological study. The decision was made by performing a pseudo-systematic literature search of articles published in English from 2000 to 2018 using the PubMed database. The keywords used for searching the database were: “miRNA”, “human”, “plasma”, “cardiovascular diseases”, “biomarkers”, and “metabolic”. The yielded article abstracts were reviewed, focusing on studies of miRNA in blood samples. A candidate list of circulating miRNAs was selected, and a pragmatic decision was made to include five miRNAs – miR30c, miR103, miR133a, miR122 and miR146a (Table 3-1) subjectively based on them having the greatest existing evidence for change of expression of these circulating miRNAs in cardiometabolic diseases, and the frequency of their citation in the literature.

Table 3-1: Summary of studies that support the selection of specific miRNAs to included in our study

Reference	Type of study	Group	Sample	miRNAs tested	Findings
Takahashi et al. (2010)	Prospective-randomised investigator-blinded study	A total of 66 patients with CAD and 33 subjects without CAD	PBMCs	miR146a/b	Levels of miR-146a/b were higher in the CAD group than in the non-CAD group (P<0.01)
D'Alessandra, et al. (2010)	Cohort study	17 healthy control and 33 patients with STEMI	Plasma and serum	34 miRNAs	miR-1, miR133a, miR499-5 were upregulated miR122 and miR375 were downregulated
Widera, et al.(2011)	Cohort study	444 patients with ACS patients for 6 months follow up	Plasma	Six miRNAs miR-1, miR133a, miR133b, miR208a, miR208b and miR499	miR-1, miR133a, miR133b and miR208b were independently associated with hsTnT levels of miR1, miR133a and miR208d were higher in MI patients compared with UA patients
Trajkovski M and Hausser et al.(2011)	Experimental study	Obese mice model and obese induced mice model	Liver biopsies	miR103 miR107	Upregulation of miR103 and miR107 in liver biopsy in both models
	Cross sectional	Healthy control (n=6) Chronic hepatitis B(n=6) Chronic hepatitis C (n =7) Alcoholic steatohepatitis (n=4)			The expression of miR103 and miR107 was higher in individuals with liver diseases There was a positive association between expression of miR103 and HOMA index

CAD: coronary artery disease, **ASO:** antisense oligonucleotide, **STEMI:** ST elevation myocardial infarction, **ACS:** acute coronary syndrome. **hsTnT:** high sensitivity troponin-T, **UA:** unstable angina, **AMI:** acute myocardial infarction

Reference	Type of study	Group	Sample	miRNAs Tested	Findings
Wang R, et al .(2011)	Cross sectional	28 control subjects and 51 AMI	Plasma	miR133 miR328	A 4.4 fold higher level of miR133a in plasma in AMI patients compared with control subjects A positive correlation between miR133a and troponin
Eitel,et al .(2012)	Prospective study	216 consecutive patients with STEMI undergoing primary angioplasty less than 12 hours after symptom onset.	Serum	miR!33a	Increased miR133a level in patients with MI
Wang et al. (2013)	First cohort study	1-13 patients of AMI and 27 healthy volunteers	Plasma	miR133a	level of miR133a was higher in AMI patients and in CHD patients compared to control group Positive correlation between circulating miR133a and troponin
	Second cohort study	2-22 CHD and 8 non-CHD			
	Third cohort study	3-246 patients with acute chest pain			

HF: heart failure, **COPD:** chronic obstructive pulmonary diseases, **STEMI:** ST elevation myocardial infarction, **AMI:** acute myocardial infarction, **CHD:** chronic heart disease

References	Type of study	Group	Sample	miRNAs Tested	Findings
Ellis et al. (2013)	Cohort study	32 HF and 15 COPD patients, as well as 14 healthy controls	Plasma	miR103	Decreased level of miR103 in HF compared with healthy control
Soh, et al. (2013)	Experimental study	Western diet fed mice	Different tissues (liver, kidney, heart)	miR30c	miR30c reduced lipid synthesis and hyperlipidaemia as well as atherosclerosis
Wang,et al, (2015)	Discovery study	56 obese patients 56 normal weight controls	Plasma	34 miRNAs	miR636 upregulated miR933 upregulated miR122 upregulated miR574-5p upregulated 30 miRNAs were downregulated
	Validating study	107 normal weight controls 123 obese patients		miR122	Positive association between circulating miR122 and insulin, glucose and HOMA_IR
Raitoharju, et al. (2016)	Cohort study	724 normal liver subjects, 147 fatty liver subjects.	Plasma	miR122	Increased miR122 level with ultra-sonographically detected fatty liver in individuals with and without excess alcohol consumption.
Willleit,et al. (2017)	Cross sectional	The Bruneck study (n=810) ASCOT (*n*= 155)	Plasma	miR122	miR122 level was strongly associated with higher levels of liver enzymes, adiposity, insulin resistance, inflammation and adiposity

CHD: Coronary Heart Disease, **SCH:** Sub-clinical Hypothyroidism, **ASCOT:** Anglo-Scandinavian Cardiac Outcomes Trial

Reference	Type of study	Group	Sample	miRNAs Tested	Findings
Zhou.et al. (2017)	Cohort study	49 CHD patients, and 13 healthy volunteers	Plasma	62 miRNAs	The expression of CHD/healthy: miR30c upregulated miR5091 upregulated miR125b-5p downregulated miR501-3p downregulated miR31-5p downregulated
	Validating study	135 CHD patients and 140 controls	Plasma	5 miRNAs	The expression of CHD/healthy: miR30c upregulated miR5091 upregulated miR125b-5p downregulated miR501-3p downregulated miR31-5p downregulated
Quan,et al. (2018)	Cohort study	60 patients with (CHD+SCH) patients ,73 patients with CHD and 59 healthy controls subjects	Plasma	miR46a	The miR146 was higher in CHD+SCH subjects than other groups miR146a was positively associated with the severity of CHD

3.3 Hypothesis

The expression levels of the targeted circulating miRNAs (miR30c, miR103, miR133a, miR122 and miR146a) are associated with cardiometabolic markers including cardiac markers, glycaemic markers, and lipid markers.

3.4 Aims

1. To investigate cross-sectional associations of the five selected circulating miRNAs using baseline plasma samples from the CAMERA trial with:

   a) Cardiac biomarkers including troponin, NT-proBNP, and cIMT.

   b) Metabolic biomarkers including insulin, homeostasis model assessment of insulin resistance (HOMA-IR), and HbA1c.

   c) Lipid markers including HDL, LDL, and triglycerides.

   d) Liver enzymes including gamma glutamyl transferase (GGT) and alanine aminotransferase (ALT).

3.5 Method

3.5.1 Samples

All the baseline ethylenediaminetetraacetic acid (EDTA) levels for the 60 paired stored plasma samples from the CAMERA trial were used in this study to measure the expression of selected miRNAs.

3.5.2 MicroRNAs isolation and quantification

Details on total RNA extraction and miRNA quantification can be found in general method section in chapter 2. To summarise, total RNA was isolated from plasma samples; this was achieved using a commercially available kit which uses a column-based extraction method. Then, reverse transcription was performed on isolated RNA with specific primers for the selected miRNAs (miR30c, miR103, miR133a, miR122 and miR146a) to generate complementary DNA (cDNA). The qRT-PCR experiments were used to measure the expression of the selected miRNAs by using cDNA. All protocols followed the manufacturer's instructions.

3.5.3 Normalisation and quality control for qRT-PCR results

The two methods for miRNA normalisation (endogenous and exogenous controls) were tested in our study samples. Moreover, a random plasma sample was used as a quality control (QC) sample to try to control for variation between runs.

3.5.3.1Endogenous control

A trial run was conducted using eight random plasma samples to test the possibility of using mir-520d-5p as a reliable endogenous control gene (Alramah.,2020).

3.5.3.2Exogenous control

Atrial run was done using the same eight random samples that used for testing endogenous control to test the efficiency of miR-39 as an exogenous control. MiR39 was used as exogenous control gene to reduce the effect of intra- and inter-kinetic variability in RT-qPCR (sample-to-sample and run-to-run variations). The

inter- and intra-CV of miR39 for entire study were calculated, as shown in Table (3-2).

3.5.3.3Quality control samples

A quality control (QC) sample was included in each run (four runs per miRNA) to evaluate the repeatability and precision of the RT-qPCR results. Inter-assay variation of the QC sample for each miRNA was calculated as shown in Table (3-3).

3.5.4 Measurement of biomarkers

All the selected biomarkers (biochemical markers) that were fully measured in the CAMERA trial, were used in this study to investigate the association between cardiometabolic markers and the expression of selected miRNAs. All the data regarding the selected biomarkers for the paired samples of 60 participants were obtained from the CAMERA trial to meet the aims of this study.

High-sensitivity troponin T was analysed from stored plasma samples by laboratory technicians, by using an automated clinically validated analyser (Roche Diagnostics; Burgess Hill, UK) and manufacturer reagents and quality control material; for high-sensitivity troponin T, the high control coefficient of variation was 5.7% and the low control coefficient of variation was 8.3%. In the CAMERA trial, one hundred and twenty two small metabolites were measured using quantitative nuclear magnetic resonance spectroscopy using the commercial Nightingale platform (Nightingale Health, Helsinki, Finland)(Preiss *et al.*, 2016). Only metabolites with significant associations with miRNA exposures are reported here.

3.5.5 Measurement of carotid intima media thickness

The measurements of carotid intima media thickness for all the participants were completed in the CAMERA trial. In this study, this data was used to investigate the association between cIMT measurements and miRNAs expression. The imaging protocol included B-mode ultrasound image acquisition of the right and left far walls of the distal 10 mm of the common carotid arteries, using an Acuson Sequoia

C512 (Siemens Medical Solutions; Erlangen, Germany) with an L8 5–12 MHz linear array broadband transducer, and electrocardiogram gating (Touboul *et al.*, 2004). All images were obtained by one doctor from a single ultrasound machine. Participants were in a recumbent position with the head tilted 10° to the contralateral side. Three digital clips were recorded from three positions, the lateral, posterolateral, and anterolateral angles, from each side of the neck. Depending on image quality, up to 18 cIMT images (in end-diastolic frame) for analysis of mean cIMT were available for each visit. The overall mean cIMT was calculated from the average left side cIMT and right side cIMT, which were calculated separately. Images were analysed by a single investigator using semiautomated artery measurement software18 or a combination of semiautomated. Pretrial intra-individual cIMT reproducibility was 7%.

3.5.6 Statistical analysis

3.5.6.1Summary statistics

The selected miRNAs (miR30c, miR103, miR133a, miR122 and miR146a) were normalized to cel-mir39 and expressed as dCT. All continuous classical risk factors were tested for normality by visual inspection (histogram and normal Q-Q plot) Univariate analysis was used to summarise and describe the data. Categorical data was summarised using counts and percentage, while continuous data was summarised using mean and SD for normally distributed variables and median and ± inter quartile range for non-normally distributed variables. Non-parametric variables were normalised by log transformation to use parametric tests. Comparison between mean of a continuous variable between two groups were evaluated using independent sample t-test and chi-square test for categorical variables.

3.5.6.2Correlation and regression

Bivariate analysis by calculating the Pearson correlation coefficient was applied to measure the strength and direction of the correlations between quantitative variables. The assumptions of applied Pearson correlation were checked. The normality of the continuous data was tested visually (normal Q-Q plot and histogram), while linearity of the continuous data was checked visually via scatter

plot. Pearson correlation (r) and univariable linear regression was used to investigate the association of classical risk factors with the five miRNAs and the association of cardiometabolic markers with miRNAs using baseline samples. Multiple linear regression analysis was used to examine the association of novel cardiometabolic with miRNAs (outcome) after being adjusted for possible prespecified confounders (age, sex and systolic blood pressure), as well as a further model adjusting for confounders identified in the univariable regression. The assumptions of multiple linear regression were checked. The linearity and normality of residuals was checked. I identified confounders were interested in adjusting for a priori, namely age, sex and systolic blood pressure because they are independent risk factors for cIMT variation. I elected to only show the significant association with miRNAs for clarity; miRNAs that had no unadjusted association with cardiometabolic markers were deleted from relevant regression tables. Because dCT values were on a $\log_2$ scale, back transformation of the beta coefficient yielded the ratio of the geometric means (i.e., a percentage change) in the miRNA for a unit change in the predictor.

3.6 Result

3.6.1 Normalisation and quality control for qRT-PCR results

3.6.1.1Endogenous control

As a result of low and inconsistent expression of miR-520-5p between samples (Ct>40) of the pilot study, miR520d-5p was not suitable endogenous control gene.

3.6.1.2Exogenous control

Table 3-2 shows the inter- and intra-CV for the entire study, and the results of the expression of miR39 between triplicates (SD = 0.2, CV = 0.7) and between samples (SD = 2, CV = 5.6). The acceptable assay for CV is (<15%).

3.6.1.3Quality control (QC)

Table 3-3 shows the results of the quality control sample for each miRNA. The QC sample showed no variation and stable between runs (CV < 2%). These results confirm that the variation detected between samples is due to real biological differences and not because of analytical variation.

Table 3-2: CT-miR39 intra- and inter-assay CV% for entire study

Inter-CV		Intra-CV between triplicate	
Mean of CT miR39	28.90	**Mean**	28.907
SD	1.62	**SD**	0.189
CV	0.056	**CV**	0.007
CV%	5.6	**CV%**	0.7

CV: Coefficient of Variation**, SD:** Standard Deviation

Table 3-3: Inter- assay CV for QC sample

miRNA	Mean of CT	SD	Inter assay CV
miRNA-30c	32.24	0.41	1.29
miRNA-103	27.28	0.52	1.91
miRNA-133a	35.45	0.81	2.28
miRNA-122	34.10	0.56	1.67
miRNA-146a	32.14	0.64	1.99

CV: Coefficient of Variation, **SD:** Standard Deviation

3.6.2 Patients demographics

Table 3-4 summarises the clinical characteristics and expression of miRNAs at the baseline of the 60 patients involved in the study. There was no difference between the two groups in baseline characteristics. Mean age was 60 ±8 years in the metformin arm and 63±9 years in the placebo arm.

There was no difference among the biochemical characteristics in the two groups at baseline; in terms of cardiac, insulin, and lipid markers. We observed that miR146a had lower dCT (-2.109,(± 2.3)) and miR133 had higher dCT(5.66,(±-1.87)), comparing with other selected miRs.

Table 3-4: Baseline characteristics for 60 patients

Demographic characteristics	Placebo group (n=32)	Metformin group (n=28)	P-value
Age (years)	63(±9)	60(±8)	0.25
Gender			
Men	23(71.9%)	21(75%)	0.785
Women	9(28.1%)	7(25%)	
Smoking history			
Current	10(31%)	13(46%)	0.471
Ex-smoker	14(44%)	9(32%)	
Never	8(25%)	6(22%)	
Blood pressure (mmHg)*	139/80(20/13)	140/81(20/9)	0.967
Anthropometric characteristics			
Body mass index (Kg /m^2)*	29.58(4.4)	29.28(3.1)	0.766
Biochemical characteristics (fasting)			
HOMA-IR	3.12 (±2.67)	2.45 (±0.9)	0.212
HbA1c (mmol/mol)	5.59 (±0.26)	5.65 (±0.27)	0.398
Triglyceride (mmol/L)	1.9 (±1)	1.7 (±0.7)	0.297
Total cholesterol (mmol/L)	4.3 (±1)	4.3 (±0.8)	0.969
LDL (mmol/L)	2.33 (±0.9)	2.35 (±0.84)	0.939
HDL (mmol/L)	1.14 (±0.2)	1.18 (±0.35)	0.548
Mean cIMT (mm)	0.69 (±0.11)	0.69 (±0.11)	0.89
Troponin (Pg/ml)	8.8(±4.6)	8.86(±3.39)	0.958
NT-proBNP (Pg/ml)	206(±372)	218(±317)	0.896
miRNA expression (dCT)			
MiR30c	2.4 (± 1.7)	2.6 (±1.71)	0.66
MiR103	2.99 (± 2.8)	3.71 (±2.8)	0.32
MiR133a	5.66 (± 2.3)	5.34 (±1.5)	0.54
MiR122	3.144 (± 1.87)	3.351 (±1.8)	0.66
MiR146a	-2.109 (± 2.3)	-1.58 (±-1.87)	0.336

*non-normal-distribution data, median and (inter-quartile range) were used.

HOMA-IR: homoeostatic model assessment insulin resistance, **LDL:** low density lipoprotein, **HDL:** high density lipoprotein, **NT-proBNP**: N-terminal pro b-type natriuretic peptide, **dCT:** delta cycle threshold (CTmiRNA-CTmiR39).

3.6.3 General characteristics of study population grouped by sex

Since sex is an important factor that affects the risk of cardiometabolic diseases, I grouped the study population by gender (males n= 44 and females n= 16), as shown in Table (3-5). The mean age of males (61 ± 8.6) was not different from the females (61 ± 9.91).

There were differences between sexes in HOMA-IR, with males having higher HOMA_IR value than females ($p < 0.006$). There was a difference in HDL between the two groups. Females had higher HDL (males = 1.13 mmol/L ± 0.315 and females = 1.25 mmol/L ± 0.14, $p = < 0.049$). in addition, there was difference between two groups in troponin concentration, males had higher troponin concentration than females (males = 9.5 pg/mL ± 4.13 and females = 6.7 pg/mL ± 3.10, $p = < 0.014$).

Fewer studies focus on the sex difference suggested that miRNAs may be differentially expressed between males and females (Sharma and Eghbali, 2014) (Ameling *et al.*, 2015). In this study, there was no differential expression of circulating miRNAs between the two genders.

Table 3-5: Characteristics of study population grouped by sex

Demographic characteristics	Male (n=44)	Female (n=16)	*P-value*
Age (years)	61 (±8.6)	61 (±9.91)	0.914
Smoking history			
Current	14 (31%)	9 (56%)	0.133
Ex-smoker	20 (45%)	3 (18%)	
Never	10 (22%)	4 (25%)	
Blood pressure (mmHg)*	139/80 (20/12)	140/80 (21/6)	0.911
Anthropometric characteristics			
Body mass index (Kg /m^2)*	29.8(3.6)	28.40(4.26)	0.248
Biochemical characteristics (fasting)			
HOMA-IR	3.11 (±2.30)	1.99 (±0.64)	**0.006**
HbA1c (mmol/mol)	5.63 (±0.29)	5.56 (±0.17)	0.394
Triglyceride (mmol/L)	1.77 (±1)	1.84 (±0.75)	0.805
Total cholesterol (mmol/L)	4.2 (±1)	4.5 (±1)	0.376
LDL (mmol/L)	2.33 (±0.8)	2.38 (±1)	0.810
HDL (mmol/L)	1.13 (±0.315)	1.25 (±0.14)	**0.049**
Mean cIMT (mm)	0.71 (±0.12)	0.65 (±0.09)	0.079
Troponin (Pg/ml)	9.5(±4.13)	6.7(±3.10)	**0.014**
NT-proBNP (Pg/ml)	238(±397)	142(±94)	0.348
miRNA expression (dct)			
MiR30c	2.4 (± 1.7)	2.77 (±1.83)	0.518
MiR103	3.31 (± 2.64)	3.36 (±3.35)	0.952
MiR133a	5.39 (± 2.08)	5.83 (±1.81)	0.433
MiR122	3.09 (± 1.99)	3.63 (±1.34)	0.243
MiR146a	-1.96 (± 2.22)	-1.58 (±-1.85)	0.503

*non-normal-distribution data, median and (inter-quartile range) were used.

HOMA-IR: homoeostatic model assessment insulin resistance, **LDL:** low density lipoprotein, **H**DL: high density lipoprotein, **NT-proBNP**: N-terminal pro b-type natriuretic peptide, **dCT:** delta cycle threshold (CTmiRNA-CTmiR39).

3.6.4 Correlation between circulating miRNAs expression and cardiometabolic markers

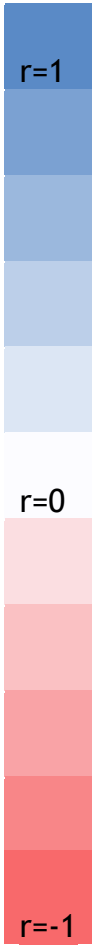
To identify the association between the selected circulating miRNAs (miR30c, miR103, miR133a, miR122, and miR146a) and cardiometabolic markers in the population with cardiovascular diseases and insulin resistance, a cross-sectional analysis using Pearson's correlation was performed. The results are shown in Table 3-6 (for cardiac markers), Table 3-7 (for insulin markers), Table 3-8 (for lipid markers), and Table 3-9 (for liver enzymes).

3.6.4.1 Circulating miRNAs and cardiac markers

Several circulating miRNAs have been determined in cardiovascular diseases and found to be strongly associated with cardiac biomarkers, such as troponin, NT-pro-BNP, and cIMT. The association between the expression of selected miRNAs and cardiac markers was investigated and the results are shown in Table 3-6. All of the miRNAs were generally positively correlated with each other. The mean of cIMT showed a positive correlation with the expression of miR122 (r = -0.298, P<0.05). The expression of miR103 was positively associated with concentration of troponin and NT-proBNP (r = -0.296, r = -0.127, respectively, P<0.05).

Table 3-6: Pearson correlation (r) of cardiac markers with miRNAs expression at baseline. As low dCT indicates high levels of the corresponding miRNA, positive r values of miRNA with cIMT, troponin T, and NT-proBNP indicate an inverse relationship, and vice-versa

miRNAs	dCT miR30c	dCTmiR103	dCT-miR133a	dCT-miR122	dCT-miR146a	cIMT	Troponin	NT-pro-BNP
dCT-miR30c	1							
dCT-miR103	0.328*	1						
dCT-miR133a	0.542**	0.115	1					
dCT-miR122	0.634**	0.183	0.428**	1				
dCT-miR146a	0.679**	0.239	0.445**	0.632**	1			
cIMT	-0.22	-0.225	-0.172	*-0.298	-0.092	1		
Troponin-T	-0.1	*-0.296	0.19	-0.079	-0.099	0.272	1	
NT-pro-BNP	0.024	*-0.127	0.104	0.094	-0.074	0.151	0.25	1



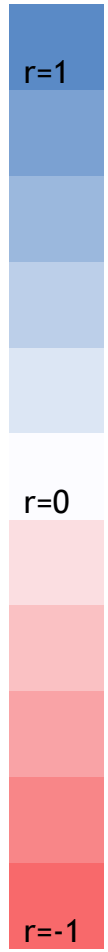
cIMT: carotid intima media thickness, **NT-pro-BNP:** N terminal pro-hormone B-type natriuretic peptide, **dCT:** delta cycle threshold (CTmiRNA-CTmiR39). *Correlation is significant < 0.05, **Correlation is significant <0.01

3.6.4.2Circulating miRNAs with metabolic markers

Table 3-7 shows the Pearson association between the expression of candidate miRNAs and metabolic markers. The expression of miR103 was inversely associated with insulin and HOMA-IR (r = 0.295, r = 0.275, P<0.05, respectively). HbA1c showed a positive association with the expression of miR30c and miR122 (r = -0.302, r = -0.279, P<0.05, respectively).

Table 3-7: Pearson's correlation for insulin sensitivity markers with miRNAs expression at baseline. As low dCT indicates high levels of the corresponding miRNA, positive r values of miRNA with HOMA_IR,I nsulin, Gllucose and HbA1cindicate an inverse relationship, and vice-versa.

miRNAs	dCT-miR30c	dCT-miR103	dCT-miR133a	dCT-miR122	dCT-miR146a	HOMA-IR	Insulin	Glucose	HbA1c
dCT-miR30c	1								
dCT-miR103	0.328*	1							
dCT-miR133a	0.542**	0.115	1						
dCT-miR122	0.634**	0.183	0.428**	1					
dCT-miR146a	0.679**	0.239	0.445**	0.632**	1				
HOMA-IR	0.137	0.275*	-0.02	-0.097	0.092	1			
Insulin	0.142	0.295*	-0.024	-0.089	0.078	0.985**	1		
Glucose	0.05	0.043	0.01	-0.108	0.11	0.599**	0.451**	1	
HbA1c	-0.302*	-0.162	-0.137	-0.279*	-0.201	0.274*	0.256	0.242	1



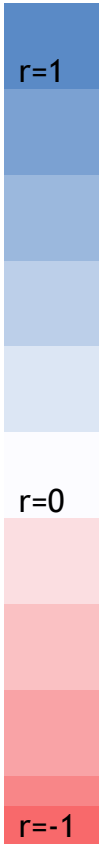
HOMA-IR: Homeostatic Model Assessment of Insulin Resistance, **HbA1c:** Haemoglobin A1c, **dCT:** delta cycle threshold (CTmiRNA-CTmiR39).
*Correlation is significant < 0.05, **Correlation is significant <0.01

3.6.4.3Circulating miRNAs and lipid markers

Table 3-8 presents the correlation between lipid markers and the expression of selected miRNAs. The expression of miR103 was positively associated with concentration of triglyceride in very large HDL (r = -0.275, P<0.05). The concentration of cholesterol in LDL particles showed a positive association with the expression of miR133a (r = -0.305, P<0.05).

Table 3-8: Pearson's correlation of lipid markers with miRNAs expression at baseline. As low dCT indicates high levels of the corresponding miRNA, positive r values of miRNA with xl-HDL_TG and LDL-C indicate an inverse relationship, and vice-versa.

miRNAs	**dCT-miR30c**	**dCT miR103**	**dCTmiR133a**	**dCTmiR122**	**dCTmiR146a**	**xl-HDL-TG**	**LDL-C**
dCT-miR30c	1						
dCT-miR103	0.328*	1					
dCT-miR133a	0.542**	0.115	1				
dCT-miR122	0.634**	0.183	0.428**	1			
dCT-miR146a	0.679**	0.239	0.445**	0.632**	1		
XL-HDL-TG	-0.088	-0.275*	0.039	-0.079	-0.14	1	
LDL-C	-0.056	-0.044	-0.305*	-0.212	-0.095	0.274*	1



Xl-HDL-TG: triglyceride in very large high-density lipoprotein, **LDL-C:** total cholesterol in low density lipoprotein, **dCT:** delta cycle threshold (CTmiRNA-CTmiR39). *Correlation is significant < 0.05, **Correlation is significant <0.01

3.6.4.4Circulating miRNAs and liver enzymes

Table 3.9 shows the correlation between the expression of miRNAs and liver enzymes (ALT, GGT). There was a positive association between the expression of miR122 and ALT (*r* =- 0. 267, *P*<0.05) and there was no any association between the selected miRNAs expression and the GGT enzyme.

Table 3-9: Pearson's correlation of liver enzymes with miRNAs expression at baseline. As low dCT indicates high levels of the corresponding miRNA, positive r values of miRNA with GCT and ALT indicate an inverse relationship, and vice-versa.

miRNAs	dCT-miR30c	dCTmiR103	dCT-miR133a	dCT-miR122	dCTmiR146a	GGT1	ALT1
dCT-miR30c	1						
dCT-miR103	0.328*	1					
dCT-miR133a	0.542*	0.115	1				
dCT-miR122	0.634*	0.183	0.428**	1			
dCT-miR146a	0.679*	0.239	0.445**	0.632**	1		
GGT	0.103	0.03	0.225	-0.026	0.046	1	
ALT	0.026	0.041	-0.132	-0.267*	-0.005	0.37**	1

GGT: Gamma-glutamyl transferase, **ALT:** Alanine aminotransferase, **dct:** delta cycle threshold (CTmiRNA-CTmiR39). *Correlation is significant < 0.05, **Correlation is significant <0.01

3.6.5 Univariable linear regression analysis

To identify the classical cardiometabolic biomarkers associated with each circulating miRNAs, univariable regression analysis was carried out, using cardiometabolic markers as exposures and expression of circulating miRNAs as outcomes.

A simple linear regression model showed potential cofounders between expression of circulating miRNAs and classical cardiometabolic biomarkers, Table (3-10). Note that in these models a positive beta coefficient represents an inverse association (and vice versa) due to the nature of the delta-CT units (smaller numbers represent higher miRNA expression). The linear regression model showed that the a priori cofounders including age, sex and systolic blood pressure were not associated with expression of any miRNA. However, linear regression analysis showed a negative association of miR103 expression with insulin sensitivity markers including HOMA-IR (β=0.301) and insulin (β=0.309). Also, HbA1c had a positive association with miR122 expression (β=-0.269), and a positive association with miR30c expression (β=-0.299). In addition, a positive association of miR122 expression and ALT enzymes (β=-0.267).

Table 3-10: Potential confounders with outcomes of miRNAs expression at baseline

Predictors	dCT-miR30c		dCT-miR103		dCT-miR133a		dCT-miR122		dCT-miR146a	
	Standardised β coefficient	**R^2%**	**Standardised β coefficient**	**R^2%**	**Standardised β coefficient**	**R^2%**	**Standardised β coefficient**	**R^2%**	**Standardised β coefficient**	**R^2%**
Age (year)	-0133	1.8%	-0.230	5%	0.06	0.4%	-0.118	1.4%	-0.116	1.3%
Sex	0.089	0. 8%	0.008	0	0.097	0.9%	0.129	1.7%	0.081	0.7%
Smoking	-0.169	2%	-0.008	0	-0.134	1.8%	-0.217	4.7%	-0.057	0.3%
Systolic blood pressure (mmHg)	0.066	0.4%	-0.141	2%	0.147	2.2%	-0.095	0.9%	0.053	0.3%
Body mass index (Kg /m^2)*	-0.11	1%	0.208	4%	-0.191	3.7%	-0.026	0.1%	-0.029	0.1%
HOMA-IR	0.046	0.2%	**0.301***	9%	-0.003	0	-0.138	1.9%	0.038	0.1%
HbA1c (mmol/mol)	**-0.299***	9%	-0.161	2.6%	-0.128	1.6%	**-0.269***	7.3%	-0.193	3.7%
Insulin (per 1 pmol/L)	0.038	0.1%	**0.309***	9.6%	-0.022	0	-0.153	2.3%	0.016	0
Triglyceride (mmol/L)	0.070	0.5%	-0.036	0.1%	0.072	0.5%	-0.012	0	0.108	1.2%
Total cholesterol (mmol/L)	0.068	0.5%	-0.130	1.7%	-0.121	1.5%	-0.061	0.4%	-0.052	0.3%
LDL (mmol/L)	0.029	0.1%	-0.126	1.6%	-0.199	4%	-0.069	0.5%	-0.086	0.7%
HDL (mmol/L)	-0.029	0.1%	-0.091	0.8%	0.078	0.6%	0.015	0	-0.112	1.3%
Ln- ALT (per 10 U/L	0.026	0.1%	0.041	0.2%	-0.132	1.7%	**-0.267**	**7.1%**	-0.36	0

Standardised beta coefficient displayed to allow direct comparison in terms of strength of associations. 95% CI omitted for clarity., **R^2:** coefficient of determination

*p<0.05

3.6.6 Multiple linear regression

Multiple linear regression analysis was used to examine the association of novel cardiometabolic biomarker with expression of miRNAs after being adjusted for possible covariate factors; the confounding independent variables included age, sex, and systolic blood pressure as well as HOMA-IR for miR103, and HbA1C for miR122 and miR30c. Each covariate that had a significant linear association with the expression of miRNA ($P<0.05$) was subsequently entered into a multivariable analysis.

Table 3-11 shows the association of the expression of circulating miR103 and miR122 with cardiac markers (troponin, NT-proBNP and cIMT). In these models, beta coefficients are represented by a % change, and a positive value indicates a positive association. In unadjusted models, the expression of miR103 was positively associated with higher concentration of troponin and NT-proBNP: for every 1pg/ml increase in troponin and NT-proBNP, the expression of circulating miR103 was increased by 9% and 10%, respectively. Adjustment for age, gender, systolic blood pressure and HOMA-IR did not substantially attenuate these associations. However, adjustment for HOMA-IR particularly improved the model fit from 11.7% to 20%.

Similarly, higher mean cIMT was positively associated with the expression of miR122: for every 1 μm increase in cIMT, the expression of circulating miR122 was increased by 8 % in the unadjusted model. Adjustment for age, gender, and systolic blood pressure did not attenuate the association. However additional adjustment for HbA1c made the association somewhat stronger; for every 1 μm increase in cIMT, the expression of circulating miR122 was increased by 8.5%. Overall model fit was also improved by addition of HbA1C to the model, increasing R^2 from 9.4% to 12.8%.

Table 3-12 shows the association of the expression of circulating miR103 and miR30c with markers of insulin sensitivity (insulin, HOMA_IR, and HbA1c). In the models adjusted for age, gender, and systolic blood pressure, higher HOMA-IR and insulin were inversely associated with the expression of circulating miR103. For a 1 unit increase of HOMA-IR and 1 pmol/L increase of insulin, the expression of

circulating miR103 decreased by 14% and 11%, respectively. However, additional adjustment for HOMA-IR, made the association somewhat weaker; for every 1 pmol/L increase in insulin, the expression of circulating miR103 was decreased by 9.8%. Overall model fit was also reduced by addition of HOMA-IR to the model, decreasing R^2 from 14.7% to 6.8%.

In adjusted models, the expression of circulating miR30c and miR122 were positively associated with of HBA1c, for every 1 mmol/mol increase of HbA1c, the expression of miR30c increased by 3% and the expression of miR122 increased by 3%.

Table 3-13 shows the association of the expression of circulating miR103 and miR133a with lipid markers. In the adjusted model, the expression of circulating miR103 was positively associated with triglycerides in very large HDL particles. For every 1 log unit (mmol/L) increase of triglycerides in very large HDL particles, the expression of miR103 increased by 4%. However additional adjustment for HOMA-IR made the association somewhat stronger; for every 1 log unit (mmol/L) increase of triglycerides in very large HDL particles, the expression of circulating miR103 was increased by 7.6%. Overall model fit was also improved by addition of HOMA-IR to the model, increasing R^2 from 15% to 29%.

Similarly, in the adjusted model, the expression of circulating miR133a was positively associated with cholesterol in LDL particles. For every 1 log unit (mmol/L) increase of cholesterol in LDL particles, the expression of miR133a increased by 4% (P<0.05).

Table 3-14 shows the association of the expression of circulating miR122 with liver enzymes. In the model adjusted for age, gender, and systolic blood pressure, there was a positive association between expression of circulating miR122. For every log unit (10 U/L) increase in ALT, the expression of circulating miR122 increased by 8%. However additional adjustment for HbA1c made the association somewhat weaker; for every 1 log unit (10 U/L) increase in ALT, the expression of circulating miR122 was increased by 4.1%. Overall model fit was also reduced by addition of HbA1c to the model, decreasing R^2 from 12% to 7.7%.

Table 3-11: linear regression model showing the assocation of novel cardiometabolic biomarkers with miRNAs expression at baseline

Predictors	Change in miR103				
	Model	β–coefficient (% change)	CI 95%		Overall R^2%
			Lower	Upper	
Troponin (per 1 pg/ml)	1	8.62	7.65	9.72	9.4%
	2	8.71	7.58	10	11.6%
	3	8.71	7.57	10	11.7%
	4	8.81	8.6	8.9	20%
NT-proBNP (per 1 pg/ml)	1	9.98	9.97	10	7.3%
	2	9.98	9.79	10	9.9%
	3	9.98	9.97	10	9.9%
	4	9.98	9.97	10	10%
Change in miR122					
Mean cIMT (per 1 μm)	1	8.13	0.025	10	8.9%
	2	8.14	0.015	10	9.4%
	3	8.16	0.015	11	9.4%
	4	8.5	0.026	21	12.8%

Model 1: No adjustment
Model 2: Adjustment for age and sex
Model 3: Adjustment for age, sex and systolic blood pressure
Model 4: Adjustment for age, sex and systolic blood pressure as well as HOMA-IR for miR103, and HbA1C for miR122

Table 3-12: Linear regression model showing the associations of metabolic biomarkers vs miRs expression at baseline

Predictors	Change in miR103				
	Model	β–coefficient (% change)	CI 95%		Overall R^2%
			Lower	Upper	
HOMA-IR (per 1 unit)	1	13	10.48	17	9.1%
	2	13.5	10.54	17.29	14.6%
	3	13.8	10.47	17.01	14.8%
Insulin (per 1 pmol/L)	1	10.84	10.15	12.59	9.6%
	2	10.84	10.13	11.59	14.5%
	3	10.85	10.13	11.61	14.7%
	4	9.8	5.57	17	6.8%
Change in miR30c					
HbA1c (per 1 mmol/mol)	1	2.51	0.81	7.81	9%
	2	2.76	0.84	9.075	9.8%
	3	2.96	0.91	9.5	11%
Change in miR122					
HbA1c (per 1 mmol/mol)	1	2.77	0.83	9.18	7.3%
	2	3.12	0.89	10.95	8.6%
	3	3.09	0.89	9.12	9%

Model 1: No adjustment

Model 2: Adjustment for age and sex

Model 3: Adjustment for age, sex and systolic blood pressure

Model 4: Adjustment for age, sex and systolic blood pressure as well as HOMA-IR for miR103, and HbA1C for miR122

Table 3-13: Linear regression model showing the associations of lipid biomarkers vs miRs expression at baseline

Predictors	Change in miR103				
	Model	β–coefficient (% change)	CI 95%		Overall R^2%
			Lower	Upper	
Ln-XL-HDL-TG (per 1 mmol/L)	1	4.79	2.40	9.55	7.5%
	2	4.35	2.18	8.66	14.8%
	3	4.27	2.13	8.58	15.3%
	4	7.6	1.79	8	29%
	Change in miR133a				
Ln-LDL-C (per 1mmol/L)	1	3.69	1.633	8.36	9.2%
	2	3.77	1.63	8.72	9.9%
	3	3.84	1.65	8.89	11%

Model 1: No adjustment

Model 2: Adjustment for age and sex

Model 3: Adjustment for age, sex and systolic blood pressure

Model 4: Adjustment for age, sex and systolic blood pressure as well as HOMA-IR for miR103, and HbA1C for miR122

Table 3-14: Linear regression model showing the associations of liver enzyme vs miR122 expression at baseline

Predictors	Change in miR122				
	Model	β–coefficient (% change)	CI 95%		Overall R^2%
			Lower	Upper	
Ln- ALT1 (per 10 U/L increase)	1	8.31	2.064	9.59	7.1%
	2	7.91	1.52	8.44	12%
	3	8.29	9.61	2.03	12%
	4	4.1	1.7	9.8	7.7%

Model 1: No adjustment

Model 2: Adjustment for age and sex

Model 3: Adjustment for age, sex and systolic blood pressure

Model 4: Adjustment for age, sex and systolic blood pressure as well as HOMA-IR for miR103, and HbA1C for miR122

3.7 Discussion

This section discusses the results of the cross-sectional association of the expression of circulating miRNAs (miR30c, miR103, miR133a, miR122, and miR146a) and cardiometabolic markers: cardiac markers, metabolic markers, lipid markers, and liver enzymes. It then considers these findings in relation to the previous literature.

3.7.1 The association between circulating miRNAs expression and cardiometabolic markers

3.7.1.1Cardiac markers

In this study, the expression of miR103 was associated with cardiac biomarkers including (troponin T and NT-pro-BNP) and miR122 was associated with carotid intima media thickness. In multiple regression analysis, the association between miR103 and troponin T was not attenuated after adjustment for age, gender, systolic blood pressure and HOMA-IR. However, further adjustment for HbA1c made the association between cIMT and miR122 somewhat stronger.

Coronary heart disease has generally been described as ischemic coagulation necrosis in the muscle fibres of the heart. Wang et al. (2015a) reported that miR103 was upregulated in cardiac cells after exposure to necrotic factor (H_2O_2), but the specific function in the heart was unclear (Wang *et al.*, 2015a). In addition, it was reported in another study that miR103 is induced and upregulated in response to hypoxia (Kulshreshtha *et al.*, 2007). Our samples were obtained from patients with coronary heart disease, so this association between necrosis and a higher level of miR103 may explain the higher level of circulating miR103 in our study population. However, incompatible with our findings is a study by Ellis et al. (2013) that observed a weaker negative association between the level of circulating miR103 and NT-proBNP and hs-troponin. The authors found downregulation of miR103 in plasma in heart failure patients compared with non-heart failure patients and a healthy control group (Ellis *et al.*, 2013). The difference between our data and the previous study might be due to the following reasons: (1) the difference in study population and disease phenotype; our study

population was patients with coronary heart diseases while in this study, the study population was patients with heart failure (2) may reflect the mechanism of miRNA secretion into the circulation (Creemers, Tijsen and Pinto, 2012); (3) miR103 is known to be expressed in other organs, including the heart, and plasma level may reflect the pathologies of all organs.

It is generally accepted that atherosclerosis is the major risk factor for developing coronary artery disease, and abnormality of lipid metabolism is one of the most predisposing factors for development of atherosclerosis. It has been shown that 70% of patients with premature CAD have abnormal lipoprotein metabolism (Genest *et al.*, 1992).

Carotid intima media thickness is a measure of the thickness of the intima and media layers of the carotid artery and is used to detect early subclinical atherosclerosis. In addition, cIMT is associated with the risk of a coronary artery disease event. Our findings have shown that there is a positive association between the expressions of miR122 and cIMT independent of age, sex, systolic blood pressure and HbA1c, but there are no cohort studies that have demonstrated this association between cIMT and expression of miR122. These observations could be explained by the evidence that shows the role of miR122 in lipid metabolism and how lipids might be linked to cIMI, although, there is no association was found between expression of miR122 and lipid levels in this study. Therefore, we speculate that LDL level is act as intermediated between higher expression of miR122 and thickness of carotid arteries.

In the Bruneck study of 810 participants, a strong positive association was found between the expression of miR122 in plasma and adverse lipid profile (higher triglycerides and lower HDL cholesterol) (Willeit *et al.*, 2017a; Stegemann *et al.*, 2014). In addition, a study Gao et al. (2012) found that the levels of miR122 in plasma were higher in hyperlipidemia patients compared with a control group, and the levels of miR122 were positively correlated with LDL, and inversely correlated with HDL (Gao *et al.*, 2012). Moreover, in large cohort studies, an association between lipid markers and cIMT has been shown. Haung et al. (2016) found a positive association between oxidised LDL and cIMT (Huang *et al.*, 2016), and the Whitehall II and IMPROVE studies found a positive association between

LDL and cIMT and a negative association between HDL and cIMT, after adjusting for age, sex, smoking, diabetes, and statin use (Marmot and Brunner, 2005; Baldassarre *et al.*, 2010). A prospective study of 583 hospital employees without lipid-lowering medications, and no previous incidence of cardiovascular diseases, showed that total cholesterol and internal markers for cholesterol synthesis were positively associated with cIMT (Weingartner *et al.*, 2010).

Previous studies have shown that there is a strong association between lipid abnormality and a higher level of miR122 expression, and an association between lipid abnormality and cIMT. Therefore, increased intima media thickness may explain by increased expression of miR122 that is associated with lipid abnormality. This hypothesis needs more studies to be proven.

Furthermore, we observed that adjustment for age, sex, systolic blood pressure and HOMA-IR did not substantially attenuate the association between expression of circulating miR103 and troponin. Although miR103 was associated with HOMA-IR this indicate that HOMA-IR and insulin are not major determinants for troponin due to the lack of confounding. In a study by Welsh et al, the association between cardiac troponin T and troponin I and cardiovascular risk factors in 19,501 individuals in the Generation Scotland Scottish Family Health Study, they found troponin to be weakly cross-sectionally associated with diabetes; after adjustment for age and sex there was no association of troponin T with diabetes (Welsh *et al.*, 2018). Another study has found no association between HOMA-IR and troponin T in patients with metabolic syndrome (r=-0.11, p-0.30) (Hitsumoto and Shirai, 2015).

3.7.1.2Metabolic markers

This cross-sectional study found an association between three miRNAs (miR30c, miR122, and miR103) and metabolic markers. Our findings showed a positive association between the expression of both circulating miRNAs (miR30c and miR122) and HbA1c, and an inverse association between the expression of circulating miR103 and HOMA-IR and insulin independent of age, sex and systolic blood pressure.

Our study further found that circulating miR30c was upregulated in patients with high HbA1c. This is inconsistent with the findings of a study by Chen et al. (2017) that investigated the hypothesis that miR30c is involved in the pathogenesis of diabetic cardiomyopathy, where it was found that the level of circulating miR-30c was reduced in patients with diabetes and chronic heart disease, and that circulating miR-30c levels had a negative association with plasma glucose level in patients with chronic heart disease and diabetes (Chen *et al.*, 2017). This study measured plasma miR30c in 28 healthy controls, 26 diabetic patients, 22 patients with chronic heart failure, and 15 patients with diabetes and chronic heart failure, and used U_6 as an internal control in contrast to our study that used c-elegans miR39 spike in control, and our study population were patients with coronary heart diseases with large waist circumference and insulin resistance.

The links between dyslipidemia, insulin resistance, and obesity are established. Any abnormality in lipid metabolism is considered an early manifestation of insulin resistance. Several studies have investigated the role of miR-30c in adipogenesis and lipid metabolism. One mechanistic study (Soh *et al.*, 2013b) showed that miR30c reduced microsomal triglyceride transfer protein (MTP) by breakdown of mRNA at the post-transcriptional level. MTP is a chaperone and induces lipoprotein secretion (Hussain *et al.*, 2012). In addition, Soh et al. (2013) showed that hepatic miR-30c decreased lipid formation and lipoprotein release and reduced atherosclerosis in an animal model.

The role of miR122 in insulin resistance and obesity has recently been investigated. For example, Wang et al. (2015b) found a positive association between circulating miR122 and insulin sensitivity, and a significant difference in circulating miR122 between 107 participants of normal weight and 123 obese patients. In addition, they found a positive association between circulating miR122 and HOMA-IR and glucose (Wang *et al.*, 2015b). Similarly, our data demonstrated a positive association between circulating miR122 and HbA1c. However, Ortega et al. (2013) found different expression of circulating miR122 between moderately and morbidly obese patients, and a significant decrease of circulating miR122 after weight loss surgery (Ortega *et al.*, 2013). A population-based study by Willeit et al. (2017) used a multidimensional approach to investigate the metabolic parameters that are associated with miR122 and measured the expression of

miRNA in more than 2,000 human blood samples (from the 1995 and 2000 evaluation in the Bruneck study and ASCOT). The authors reported that circulating miR122 levels were elevated in people with metabolic syndrome or T2D and strongly associated with major lipids. They also reported that circulating miR122 was reduced with statin therapy (Willeit *et al.*, 2017a). In this study showed positive association between miR122 expression and HbA1c, our study sample are patients with large waist circumference.

In addition, Xu et al. (2015) investigated the association between the expression of circulating miR103 and insulin resistance parameters in patients with non-alcoholic fatty liver disease. In contrast with our findings, these authors found that miR103 was positively correlated with HOMA-IR and independent factors in obese patients with NAFLD and overweight patients (Xu *et al.*, 2015). Xie et al. (2009b) showed that miR103 and miR143 were upregulated in vivo and in vitro during adipogenesis. The expression of miR103 was downregulated after treatment with TNF, indicating that cytokines lead to reduced adipogenesis in obesity. These results imply that obesity and insulin resistance status leads to loss of miRNA function, required in adipogenesis, and suggest a mechanism for how obesity may induce insulin resistance (Xie, Sun and Lodish, 2009).

The difference between the tissue expression of miR103 and circulating miR103 indicates that changes in levels of circulating miRNA do not necessarily reflect a change in miRNA expression inside the cells. In addition, the difference in phenotype between population and patient characteristics seems to be important in deregulation of the expression of miRNA associated with a disease condition, such as insulin resistance. Moreover, current evidence indicates that the blood miRNA profile may alter under different pathological conditions, such as cancer (Chen *et al.*, 2008), CVD (Jansen *et al.*, 2014b), and type 2 DM (Kantharidis *et al.*, 2011).

In our study, we did not measure the tissue level of miRNAs, but we speculate that the difference in miRNAs expression in serum may correlate with miRNAs expression in tissues. More research is required to study whether the alteration of circulating miRNA expression correlates with their alteration in tissue pairs. Differential expression of miRNAs between tissue and serum may be due to the

different identification strategies, population characteristics, and normalisation methods. However, the results of these studies are difficult to compare, which limits their value in studying miRNA expression within tissues and serum between studies. In addition, when studying the expression of circulating miRNAs, it could be difficult to find the right and sense correlation between different patient cohorts.

3.7.1.3Lipid markers

Recently, accumulative evidence from in vivo and in vitro studies has shown the role of miRNAs in lipid metabolism. In animal model studies, after treating with anti-miR-122, several genes involved in fatty acid synthesis and oxidation were changed (Elmen *et al.*, 2008; Esau *et al.*, 2006). Furthermore, inhibition of miR-122 in high-fat fed mice led to reduced cholesterol synthesis and stimulation of fatty acid oxidation, as well as hepatic steatosis (Esau *et al.*, 2006). MiR370 is a second miRNA that has recently been shown to have a role in lipid metabolism; miR370 decreases fatty acid oxidation by targeting carnitine palmitoyl transferase (Cpt1a), the enzyme that is responsible for transferring long-chain fatty acids across membranes by binding them to carnitine (Iliopoulos *et al.*, 2010). In addition, miR-143, miR-27, and miR-335 have been found to have a role in lipid metabolism and adipocyte differentiation (Esau *et al.*, 2004; Kajimoto, Naraba and Iwai, 2006; Lin *et al.*, 2009). Esau et al. (2004) showed that inhibition of miR143 led to downregulation of ERK5, thereby inhibiting adipocytes differentiation. By contrast, miR27 overexpression led to inhibition of PPARγ and C/EBPα, the two master regulators of adipogenesis, and reduced adipocyte differentiation (Lin *et al.*, 2009). High expression of miR335 is associated with triglyceride cholesterol and white adipose tissues.

In the present study, we found an association between the expression of miR103 and miR133 with lipid markers. The expression of circulating miR103 was positively associated with triglycerides in very large HDL particles. Similarly, in the adjusted model, the expression of circulating miR133a was positively associated with cholesterol in LDL particles.

MiR-133 is a myo-miRNA and is enriched in skeletal and cardiac muscle, where it is involved in cell differentiation (Chen *et al.*, 2006), development (Liu *et al.*,

2008), and specification (Ivey *et al.*, 2008). It has been shown that miR133 is downregulated during cardiac hypertrophy, suggesting that it may have a role in the underlying pathogenesis (van Rooij *et al.*, 2006; Care *et al.*, 2007). Our study found an association between lipid markers and the expression levels of miR133, indicating that miR133 may have a role in lipid metabolism. In agreement with our study, an in vivo study by Trajkovski et al. (2012) revealed the role of miR133 in brown adipose tissue, showing that downregulation of miR133 caused an increase in brown adipocyte differentiation (Trajkovski *et al.*, 2012). Another study supporting our finding is that carried out by de Gonzalo et al. (2017), who studied 72 participants with un-complicated type 2 diabetes, hypothesising that circulating miRNAs could be used to monitor myocardial neutral lipid contents in type 2 diabetes. They found a direct association between circulating miR-133a levels and plasma triglyceride concentration, and a near-significant association between miR-133a and HDL-C. Moreover, a significant association between myocardial neutral lipid content and circulating level of miR133 in an animal model of high-fat diet-induced insulin resistance (de Gonzalo-Calvo *et al.*, 2017)

Studies indicated that circulating miR133 levels can be considered an active response to stressful conditions, as well as markers for myocardial ischemia, as previously proposed by other authors (Kuwabara *et al.*, 2011). Several studies have demonstrated that hypoxia induces the uptake and accumulation of neutral lipids in side cardiac cells (Vickers *et al.*, 2011) (Cal *et al.*, 2012). Thus, the association observed between ischemia and the circulating miRNAs could be related, at least in part, to the substitute ischemia-induced accumulation of neutral lipid in the myocardium, as our study showed positive association between expression of miR133 and LDL levels in patients with coronary heart diseases. Further studies are necessary to explore this hypothesis.

Regarding the association between miR103 expression and lipid markers, a study by Xu et al. (2015) showed a positive association between circulating miR103, triglycerides (r=0.77), and HOMA-IR (r=088). The study included 50 participants who had non-alcoholic fatty liver disease and 30 age-matched healthy controls. These findings suggest that higher expression of circulating miR103 exists in patients with lipid abnormality and insulin resistance (Xu *et al.*, 2015). The authors further found that ectopic miR103 accelerated adipogenesis in obese

animals and humans (Xie, Lim and Lodish, 2009). Our results showed that insulin-resistant patients with higher levels of miR103 expression in serum and miR103 were positively correlated with triglycerides. Taken together, these results indicate that higher expression of miR103 and higher levels of triglycerides are associated with HOMA-IR.

3.7.1.4Liver enzymes

In this study, we found a positive association between expression of miR122 and liver enzyme (ALT), and the association between miR122 expression and liver enzymes has been discussed in several studies. Tissue-profiling studies have shown that miR122 represents 70% of hepatic miRNAs (Lagos-Quintana *et al.*, 2002). MiR122 has a role in lipid metabolism regulation and fatty acid homeostasis (Fernandez-Hernando *et al.*, 2011). Inhibition of miR-122 in mouse and chimpanzee models leads to reduction of plasma cholesterol concentrations and hepatic cholesterol accumulations (Moore *et al.*, 2010). In this context, we observed a positive association between alanine transaminase enzyme and miR122 among CAMERA trial patients who had both obesity and insulin resistance.

Alanine transaminase (ALT) is a routinely used biomarker for liver injury; this biomarker is not specific and has limited prognostic ability. In line with our findings, a study by Laterza et al. (2013) showed a positive correlation between the expression of miR122 in plasma and ALT enzyme in 15 healthy individuals and 30 patients with liver disease (Laterza *et al.*, 2013). This study was a case control study of healthy control and liver disease patients, in contrast to our study, which is a cross-sectional study of 60 patients with obesity and insulin resistance. Moreover, researchers have investigated the difference in expression of miR122 in obese patients, and found higher levels of circulating miR122 in moderately obese and morbidly obese groups than in normal weight subjects (Wang *et al.*, 2015b).

There is a significant relationship between insulin resistance, diabetes, and obesity with liver disease. Many studies have revealed an association between the NAFLD and obesity. For example, Bellentani et al. (2000) showed that central obesity is a risk factor for developing liver steatosis, regardless of alcohol intake (Bellentani *et al.*, 2000). Several cohort studies have observed increased risk of hepatocellular carcinoma among the diabetic population, suggesting that diabetes

usually precedes the development of liver disease (Adami *et al.*, 1991) (Sasaki *et al.*, 1997). An increased level of miR122 in parallel with ALT enzyme level has also been demonstrated in animal models. In animal models of drug-induced hepatic injury, miR122 increases by >6000-fold compared with only an 80-fold increase of ALT enzyme, indicating that circulating miR122 may be used as a sensitive biomarker in liver damage (Wang *et al.*, 2009). Further investigations should be carried out in humans to identify whether the reliability and sensitivity of circulating miR122 is better than those of ALT to confirm its clinical applicability.

Further research is required to characterise miR122 in populations with specific metabolic disorders. Given the growing evidence regarding miRNAs as highly specific and sensitive biomarkers, it seems likely that miRNAs will also be suitable in metabolic disorders. Larger patient cohorts with a distinct metabolic disorder, correlation of miRNA measurement with biopsy findings, and sequential monitoring are needed to further characterise the utility of circulating miR122.

3.8 Conclusion

In conclusion, the present study shows an association between the selected circulating miRNAs and cardiometabolic markers among patients with coronary heart disease, but without diabetes. However, further subsequent studies are required to investigate the role of these selected miRNAs as reproducible biomarkers among patients with coronary heart disease. Reproducible biomarker is biomarker that able to produce the same result when used repeatedly in the same circumstance (Nelson, 2005). The study has found broadly expected cross-sectional associations between the five selected circulating miRNAs (miR30c, miR103, miR133a, miR122, and miR146a) with biomarkers of cardiometabolic risk in a population with coronary heart disease but without diabetes. The findings of this study support the possibility of using circulating miRNAs as surrogate biomarkers for cardiometabolic diseases, as these circulating miRNAs may be a more sensitive way to predict the development of cardiometabolic diseases. However, there a long process is needed to prove the value of circulating miRNAs as diagnostic, prognostic, or therapeutic markers. Comparing between studies is difficult due to the lack of consistency in normalisation and the non-standardisation of preanalytical and analytical procedures. One issue reducing the

utility of circulating miRNAs as biomarkers is poor data reproducibility arising from inconsistency between studies (Sato *et al.*, 2009).

3.9 Limitations

The establishment of a reliable and accurate signature of circulating miRNAs for cardiometabolic disease diagnosis, prognosis, and treatment is challenging at every step, from sample collection to miRNAs quantification and data analysis (Witwer, 2015b).

Our study findings should be interpreted in the context of other pre-analytical and analytical sources of variation that may influence the expression of circulating miRNAs. There are a number of limitations that must be considered when analysing the findings of the current study. The plasma samples were taken from a single centre with a small sample size; a multi-centre study with a large sample size is needed. The sample size of this study is small, especially in the context of circulating miRNAs identification and validation. Several circulating miRNAs have relatively low abundance in plasma or serum, which hampers their accurate detection and quantification by RT-qpcr (Hamam *et al.*, 2017). Another important issue is sample collection and processing; the likelihood of haemolysis in plasma is greater than in serum. Recent evidence suggests that the significant difference in circulating miRNAs expression in plasma might be due to the haemolysis of blood cell or platelet-specific microRNAs during plasma sample collection from patients (Pritchard *et al.*, 2012). Notably, circulating miRNA was found in large quantities in serum compared to in plasma (Wang *et al.*, 2012b), suggesting the potential interaction of circulating miRNA with platelets and white blood cells during plasma collection. Moreover, plasma contains anticoagulants, such as heparin, which are considered potent inhibitors of downstream PCR reactions. Another important limitation is the lack of agreed suitable internal control of circulating miRNAs for normalisation of the expression levels, which can change according to pathological and physiological status. Therefore, we used other approaches for normalisation, including an equal amount of plasma and spiked in the synthetic C. elegans miRNA. These normalisation approaches have found to be more reliable and accurate methods of data normalisation than endogenous circulating miRNAs (Li and Kowdley, 2012a). In addition, as a cross-sectional study, the hypotheses

fail to infer causality because a temporal sequence cannot be established. The potential mechanisms linking the circulating miRNAs with cardiometabolic markers were not elucidated in this study, and this will require further preclinical studies in the future.

4 The effect of metformin on the expression of circulating miRNAs

4.1 Introduction

Metformin is an oral anti-diabetic drug of the biguanide class. Metformin originates from the French lilac plant, Galega officinalis, a plant ancestor of metformin which is rich in guanidine and is known to treat symptoms of type 2 diabetes (Witters, 2001). Metformin reduces blood glucose production by inhibiting hepatic glycogenolysis (Hundal *et al.*, 2000), increases peripheral glucose use (Kumar and Dey, 2002) and enhance insulin sensitivity by its positive effect on insulin receptor expression and tyrosine kinase activity (Gunton *et al.*, 2003). In addition, metformin has been found to increase circulating glucagon such as peptide-1(GLP-1),which is an insulinotropic hormone, by increasing secretion of GLP-1 (Mannucci *et al.*, 2001) or by inhibiting dipeptidyl peptidase-4 (DPP4) (Green *et al.*, 2006), which is the enzyme responsible for de-activating GLP-1 in both tissues and circulation. Glucagon-like peptide 1 (GLP-1), an insulinotropic hormone, has been shown to induce weight loss and inhibit food intake in humans with type 2 diabetes (Gutzwiller *et al.*, 1999) and without type 2 diabetes (Naslund *et al.*, 1998).

Metformin is given as a first line therapy for type 2 diabetes in line with the treatment guidelines of the American Association of Clinical Endocrinologists, the European Association for the Study of Diabetes, the American Diabetes Association, NICE and SIGN (Nathan *et al.*, 2009),(Garber *et al.*, 2019). Not only has metformin been found to have a positive effect on blood glucose levels, it has also been found to improve general metabolic control, plasma triglyceride levels and body mass index and also reduce the risk of macrovascular complications arising from diabetes (Vella *et al.*, 2010).

The effect of metformin on the risk of cardiovascular diseases had been studied in two randomised controlled trials. The United Kingdom Prospective Diabetes Study group (UKPDS) found a 36% reduction in all-cause mortality in obese patients with type 2 diabetes on metformin compared with patients on insulin or sulfonylureas ('Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group,' 1998). The HOME trial showed that metformin reduced the risk of developing a cardiovascular endpoint in patients

with type 2 diabetes by 40% (Kooy *et al.*, 2009). Also, Lexis et al. (2014a) reported that chronic treatment with metformin is associated with a significant reduction in the risk of myocardial infarction in diabetic patients when compared with non-metformin-based strategies.

There are many questions about the precise molecular mechanism of metformin as an antidiabetic and cardioprotective agent. Researchers have shown that metformin inhibits gluconeogenic gene expression and glucose production in cultured hepatocytes through its action on AMPK (AMP-activated protein kinase) (Cao *et al.*, 2014). However, some studies have found that metformin has a different effect on AMPK independent pathways, so the precise mechanism of metformin as an antidiabetic agent is not fully understood (Foretz *et al.*, 2014). In addition, metformin has been found to reduce myocardial complications associated with type 2 diabetes through its action on AMPK signalling pathways targeting endothelial, cardiomyocyte, and fibroblast dysfunctions (Foretz *et al.*, 2014). Moreover, studies have shown that metformin reduces myocardial ischemia and reperfusion injury, independent from its glucose-lowering effect. This cardio-protection effect of metformin is mediated by activation of the AMPK pathway, the Reperfusion Injury Salvage Kinase (RISK) pathway and by increased adenosine receptor stimulation.

MiRNA is a small noncoding RNA that contains 21-25 nucleotides which regulate gene expression at the post transcription level. MiRNA plays a role in all biological processes including gene regulation, proliferation, metabolism and apoptosis (Bartel, 2009). The association between miRNA dysfunction and the development of a number of clinically important diseases, ranging from myocardial infarction to autoimmune disease, have been well documented in the literature (Li and Kowdley, 2012b) (See chapter 1 for more details).

The potential value of miRNAs as molecular biomarkers for the prognosis, diagnosis, and prediction of the therapeutic response and disease outcome is well documented in cancer (Yang *et al.*, 2019). In terms of the selected circulating MiRNAs, there are numerous studies regarding the association of the circulating MiRNAs with cardiometabolic diseases. One study showed that miR103 and miR146a are significantly reduced in patients with type 2 diabetes (Zhu and Leung,

2015). James Soh et al. found that hepatic overexpression of miRNA-30c has been associated with low secretion of cholesterol in plasma by targeting microsomal triglyceride transfer protein (MTP). They also found that miRNA-30c lowered the plasma cholesterol by decreasing production of triglyceride-rich apoB-containing lipoproteins, secondary to lower MTP expression (Soh *et al.*, 2013a). Furthermore, Xiang Dong Li et al. found that the expression of miR122 in peripheral blood mononuclear cells (PBMCs) was higher in the acute myocardial infarction patients than unstable angina patients and they found a positive association between miR122 and total cholesterol in CAD and control participants. (Li *et al.*, 2017). Moreover, the role of miR122 in lipid synthesis was studied by Esau C et al., who found that inhibition of miR122 in a diet induced obesity mouse model led to reduced plasma cholesterol levels, increased hepatic fatty acid oxidation and reduced synthesis rate of hepatic cholesterol and fatty acid (Esau *et al.*, 2006). In addition, the significant positive association between coronary heart disease and circulating miR146a was confirmed in a study involving 73 participants with CHD. Using an ROC (Receiver Operating Characteristic) analysis, the researchers also found that the miR146a was a significant predictor for coronary heart disease (Quan *et al.*, 2018).

Having established the cross-sectional associations of the selected circulating miRNAs with cardiac biomarkers (chapter 3), the next logical question to answer was whether an intervention with metformin would influence the expression of the targeted circulating miRNAs. A CAMERA trial showed that metformin did not affect carotid intima media thickness (cIMT) and other cardiovascular biomarkers, but there was a notable reduction in the diabetic parameters in the metformin group after 18 months. According to the author's previous analysis, the targeted miRNA showed variable association with cardiac and glycaemic biomarkers.

4.2 Hypothesis

Randomisation to metformin will alter the expression of targeted miRNAs (miR30c, miR103, miR133a, miR122, miR146a). Assuming metformin is cardioprotective, so after the cross-sectional data it is hypothesised that miR103 and miR122 are the most likely candidate biomarkers (associated with cIMT and cardiac enzymes) to be influenced by metformin.

4.3 Aims

- To explore the effect of randomisation to metformin on circulating miRNAs (miR30c, miR103, miR133a, miR122 and miR146a) using samples from the CAMERA RCT.

Measurements were performed on blood samples previously collected from a randomised controlled trial on the impact of metformin on non-diabetic patients with coronary heart disease (the CAMERA study) in which participants were randomly assigned (1:1) by computer to either the metformin group (850 mg twice daily) or a matching placebo group.

4.4 Method

4.4.1 Samples

Plasma samples from the CAMERA trial were used to carry out this study. A total of 60 participants without diabetes and with proven coronary heart diseases were included in this study (placebo=32, metformin=28), as shown in Figure (3-1). Participants were blinded and randomly assigned to the treatment allocation of either metformin or placebo (1:1) and followed for 18 months. Two EDTA stored plasma samples were used for each participant; one plasma sample was taken at baseline and the second sample was taken at 18 months after treatment. A total of 120 paired samples were thawed and processed simultaneously.

4.4.2 Total RNA extraction and miRNA Detection and analysis by qRT-PCR

The paired samples for each participant (baseline and 18 months) were extracted, detected and miRNAs were quantified in a single assay to reduce interindividual variability. More details on RNA extraction, normalisation and quantification of miRNAs are given in the general method chapter.

4.4.3 Statistical analysis

The selected miRNAs were normalised to cel-miR-39 and expressed as dCT. The normality of continuous variables was assessed by a visual inspection of the histogram or normal Q-Q plots. The descriptive statistics for continuous variables were presented as mean± SD for normally distributed variables and as medians with interquartile ranges (IQR) for non-normally distributed variables. Any skewed variable was normalised by log transformation to enable us using a parametric test for analysis. An independent t-test was used to compare the mean of continuous normal distributed variables and a Mann–Whitney test was used for non-normal distribution data. The descriptive statistics for the categorical variable were presented as numbers and percentages and the statistical tests used to compare percentages between the categorical variables were the 2- proportion test and the x^2 test. The relative quantification method (RQ) was used to compare the

effect of metformin on the expression of the targeted miRNAs. This method allows the quantification of the change in the gene expression level of a target gene across two samples, in this case the baseline and 18-month samples. The results were expressed as fold change. To confirm and extend the results of the RQ analysis, a mixed design two-way repeated measures ANOVA analysis was performed.

Assuming the minimum relevant difference to detect between metformin and placebo groups is 1dCT (a 2-fold change) with a dCT SD of 1.2 in each group, a sample size of 28 and 32 in each group gives 89% power with an alpha of 0.05.

The statistical analysis was conducted using IBM SPSS (Statistical Package for Social Sciences) windows version 22 (SPSS Inc. Chicago, USA) and Microsoft Excel 2016. The result is considered to be statistically significant if the P value is less than 0.05.

4.5 Result

4.5.1 Demographic and haemodynamic characteristics for 60 participants at baseline

60 subjects were included in this study: 28 in the metformin group and 32 in the placebo group, as shown in Figure (3-1). Table 4-1 summarises the clinical characteristics and the expression of miRNAs for the 60 patients involved at the baseline phase.

It was important to check that the random sample of CAMERA participants still represented a randomised experiment. There was no difference between the two groups in terms of baseline characteristics. The mean age was (60 ±8) years in the metformin arm and (63±9) years in the placebo arm. There was the same proportion of men and women in the two arms. The percentage of men in the sample was 75% and 72% in the metformin and placebo arms respectively, while the percentage of women was 25% and 28% in the metformin and placebo arms respectively. There was no substantial difference in the biochemical characteristics of the two groups at the baseline in terms of cardiac, insulin and lipid markers. There was also no significant difference in the expression of circulating miRNAs between the two groups at the baseline. It was observed that miR146a had higher expression, while miR133a had lower expression in plasma when compared with other selected miRs. For the placebo and metformin groups, the dCT for miR146a was (-2.109 (±2.3)) and (-1.58(±1.87)), respectively. The dCT for miR133a was (5.66(±2.3)) and (5.34(± 1.5)) for the placebo and metformin groups, respectively.

Table 4-1: Baseline demographic and haemodynamic characteristics for 60 patients

Demographic characteristics	**Placebo group (n=32)**	**Metformin group (n=28)**	***P-value***
Age (years)	63(±9)	60(±8)	0.250
Gender			
Men	23(71.9%)	21(75%)	0.785
Women	9(28.1%)	7(25%)	
Smoking history			
Current	10(31%)	13(46%)	0.471
Ex-smoker	14(44%)	9(32%)	
Never	8(25%)	6(22%)	
Blood pressure (mmHg)	139/80(20/13)	140/81(20/9)	0.967
Anthropometric characteristics			
Body mass index (Kg /m^2)*	29.58(4.4)	29.28(3.1)	0.766
Biochemical Characteristics (Fasting)			
HOMA-IR	3.12 (±2.67)	2.45 (±0.9)	0.212
HbA1c (mmol/mol)	5.59 (±0.26)	5.65 (±0.27)	0.398
Triglyceride (mmol/L)	1.9 (±1)	1.7 (±0.7)	0.297
Total Cholesterol (mmol/L)	4.3 (±1)	4.3 (±0.8)	0.969
LDL (mmol/L)	2.33 (±0.9)	2.35 (±0.84)	0.939
HDL (mmol/L)	1.14 (±0.2)	1.18 (±0.35)	0.548
Mean cIMT (mm)	0.69 (±0.11)	0.69 (±0.11)	0.89
miRNA Expression (mean of dCT)			
miR30c	2.4 (± 1.7)	2.6 (±1.71)	0.66
miR103	2.99 (± 2.8)	3.71 (±2.8)	0.32
miR133a	5.66 (± 2.3)	5.34 (±1.5)	0.54
miR122	3.144 (± 1.87)	3.351 (±1.8)	0.66
miR146a	-2.109 (± 2.3)	-1.58 (±-1.87)	0.336

*non-normal-distribution data, median and (inter-quartile range) were used.
HOMA-IR: homoeostatic model assessment insulin resistance, **LDL:** low density lipoprotein, **HDL**: high density lipoprotein, **dCT**: delta cycle threshold (ctmiRA-ctmiR39).

4.5.2 Demographic and haemodynamic characteristics for 60 participants at 18 months

Table 4-2 summarises the clinical characteristics and the expression of miRNAs for the 60 patients at 18 months.

It is showed that, there was no difference between two groups at 18 months of follow-up. There was no difference in the biochemical characteristics of the two groups after 18 months in terms of cardiac, insulin and lipid markers. There was also no significant difference in the expression of circulating miRNAs between the two groups at 18 months. It was observed that miR146a had higher expression, while miR133a had lower expression in plasma when compared with other selected miRs. For the placebo and metformin group, the dCT for miR146a was (-1.89 (±2.5)) and (-1.23(±2.11)), respectively. The dCT for miR133a was (5.54(±2.73)) and (5.48(± 1.97)) for the placebo and metformin groups, respectively.

Table 4-2: Demographic and haemodynamic characteristics for 60 patients at 18 months

Demographic characteristics	**Placebo group (n=32)**	**Metformin group (n=28)**	***P-value***
Age (years)	63(±9)	60(±8)	0.250
Gender			
Men	23(71.9%)	21(75%)	0.785
Women	9(28.1%)	7(25%)	
Smoking history			
Current	9 (50%)	9 (50%)	0.26
Ex-smoker	17 (63%)	10 (37%)	
Never	6 (37.5%)	10 (62%)	
Blood pressure (mmHg)	138/79 (24/14)	145/83 (21/14)	0.314
Anthropometric characteristics			
Body mass index (Kg /m^2)*	30 (5.3)	28 (4.16)	0.159
Biochemical Characteristics (Fasting)			
HOMA-IR	3.80 (±3.6)	2.41 (±2.11)	0.074
HbA1c (mmol/mol)	5.58 (±0.40)	5.56 (±0.34)	0.78
Triglyceride (mmol/L)	1.67(±0.93)	1.50(±0.74)	0.451
Total Cholesterol (mmol/L)	4.26 (±1.3)	4.23(±0.97)	0.919
LDL (mmol/L)	1.21(±0.26)	1.24(±0.39)	0.7
HDL (mmol/L)	1.21(±0.26)	1.24(±0.39)	0.7
Mean cIMT (mm)	0.723(±0.13	0.733(±0.14)	0.7
miRNA Expression (mean of dCT)			
miR30c	1.58 (± 2.56)	1.91 (± 2.29)	0.6
miR103	2.02 (± 3.20)	2.57 (± 3.12)	0.50
miR133a	5.54 (± 2.73)	5.48 (± 1.97)	0.92
miR122	3.01 (± 2.23)	3.04 (± 2.34)	0.96
miR146a	-1.89 (± 2.5)	-1.23 (± 2.11)	0.27

*non-normal-distribution data, median and (inter-quartile range) were used.
HOMA-IR: homoeostatic model assessment insulin resistance, **LDL:** low density lipoprotein, **HDL:** high density lipoprotein, **dCT:** delta cycle threshold (ctmiRA-ctmiR39).

4.5.4 The effect of metformin on the expression of circulating miRNAs

Table 4-3 shows the achieved circulating miRNA level in the metformin and the placebo treatment groups after 18 months. There was no difference in the expression of targeted circulating miRNAs between the placebo and metformin groups at 18 months ($P>0.05$).

Table 4-4 shows the one sample t test of dCT at 18 months versus no change in each group (metformin and placebo). There was an increase in the expression of all circulating selected miRNAs in both the groups (metformin and placebo) over time, except for miR146a which showed a decrease of expression over time ($P<0.05$).

Corrections were then made for any baseline imbalance that may have affected the results. The change of expression of selected circulating miRNAs after 18 months of metformin treatment for both groups are shown in Figure 4-1 for miR30c, Figure 4-2 for miR103, Figure 4-3 for miR133a, Figure 4-4 for miR122 and Figure 4-5 for miR146a. The boxplot graphs show no differences in the circulating miRNAs expression (ddCT) between the metformin and the placebo groups ($P>0.05$) (Figure 4-6).

Table 4-3: The achieved level of circulating miRNAs after 18 months randomisation to metformin or placebo treatments

Targeted circulating miRNAs	Placebo group (n=32)	Metformin group (n=28)	*P*-value
miRNA Expression (dCT)			
miR30c	1.58 (± 2.56)	1.91 (±2.29)	0.600
miR103	2.02 (± 3.2)	2.57 (±3.12)	0.505
miR133a	5.54 (± 2.73)	5.48 (±1.9)	0.920
miR122	3.02 (± 2.23)	3.04 (±2.3)	0.966
miR146a	-1.89 (± 2.5)	-1.23 (±-2.11)	0.279

dCt: delta cycle threshold.

Table 4-4: The one sample t test for targeted circulating miRNAs after 18 months randomisation to metformin or placebo treatments

Targeted circulating miRNAs	Placebo group (n=32)			*P*-value	Metformin group (n=28)			*P*-value
	Mean difference(dCT)	95% CI			Mean difference(dCT)	95% CI		
		Lower	Upper			Lower	Upper	
miR30c	1.58	0.65	2.50	0.001	1.91	1.02	2.80	<0.001
miR103	2.02	0.86	3.17	0.001	2.57	1.36	3.78	<0.001
miR133a	5.54	4.55	6.53	<0.001	5.48	4.71	6.24	<0.001
miR122	3.01	2.21	3.82	<0.001	3.04	2.13	3.95	<0.001
miR146a	-1.89	-2.804	-0.988	<0.001	-1.23	-2.05	-0.414	0.005

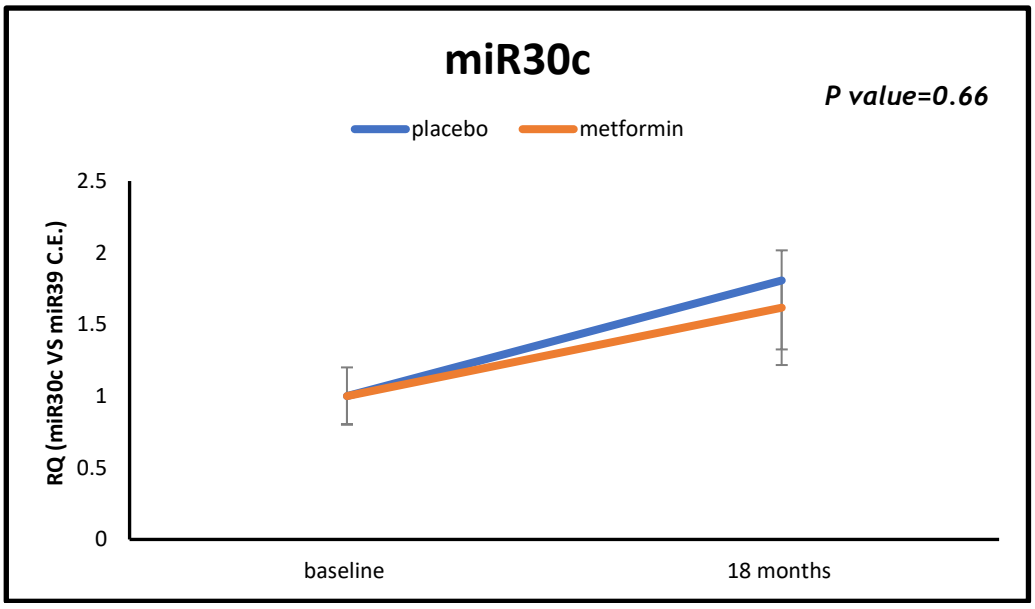


Figure 4-1-1 Line graph showing the RQ method for change of miR30c expression between the placebo and the metformin groups over the 18 months

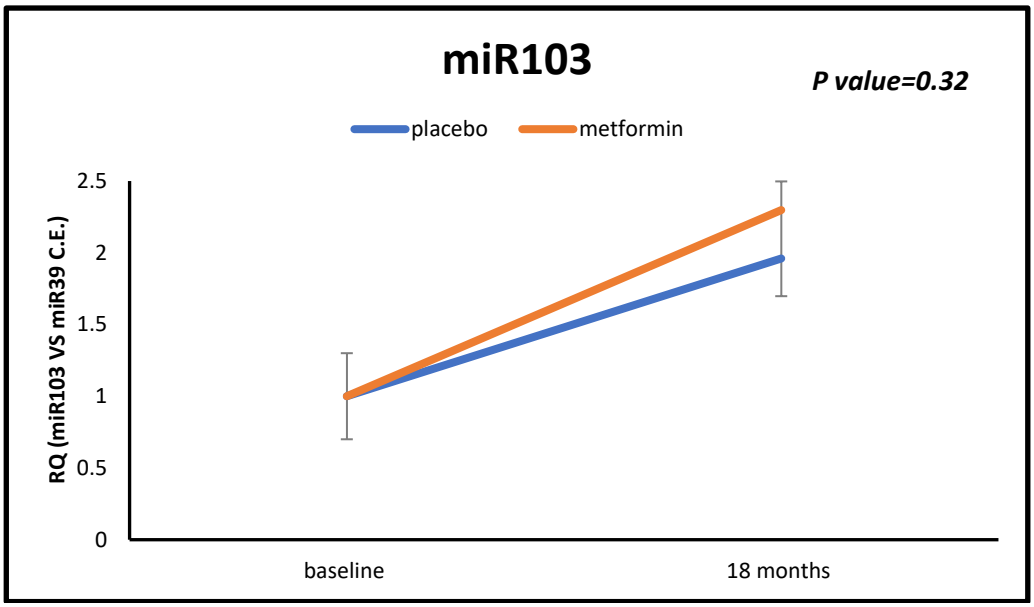


Figure 4-1-2 Line graph showing the RQ method for change of miR103 expression between the placebo and the metformin groups over the 18 months

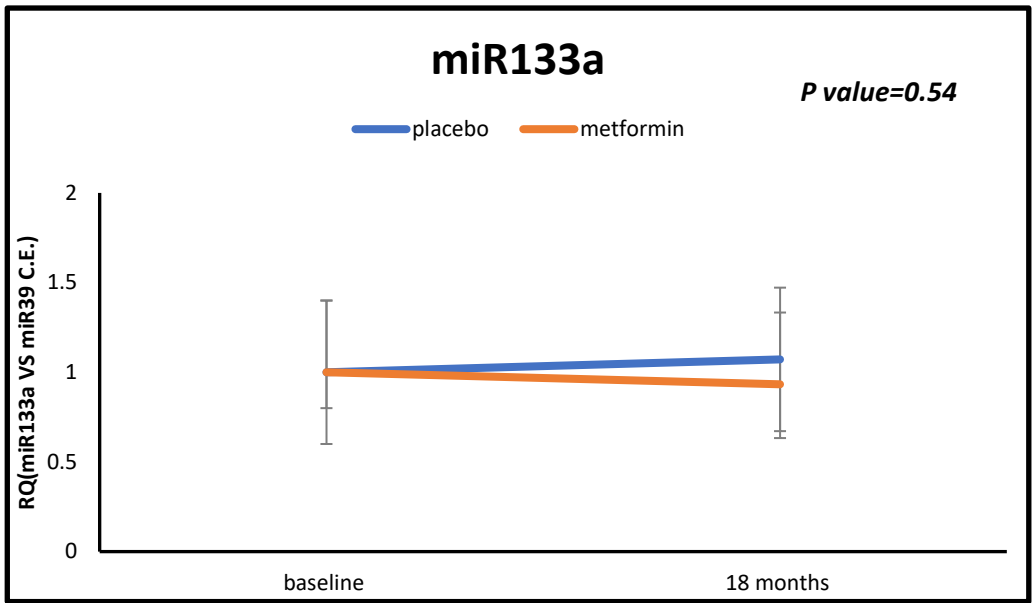


Figure 4-1-3 Line graph showing the RQ method for change of miR133a expression between the placebo and the metformin groups over the 18 months.

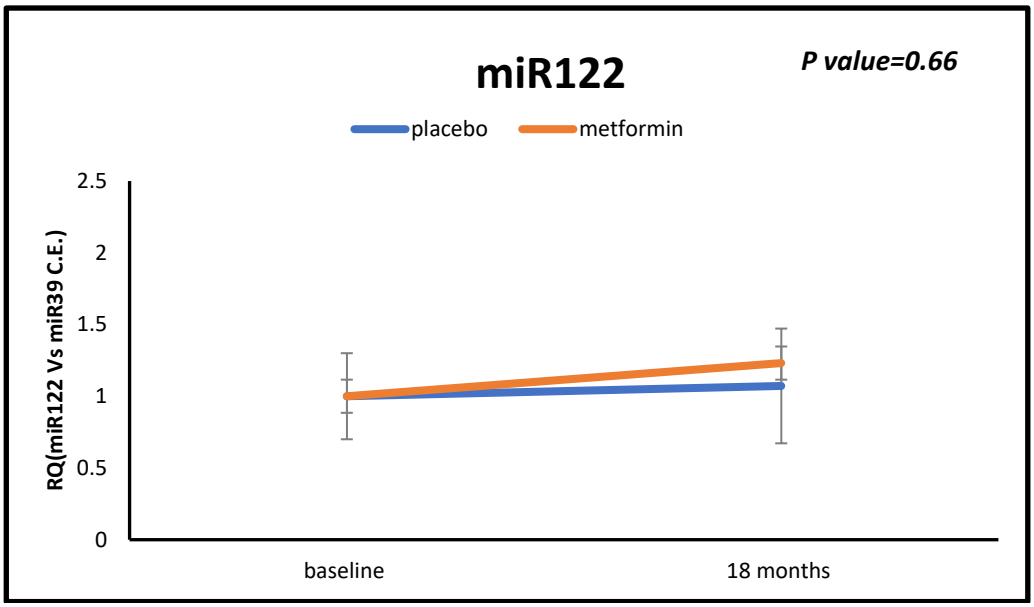


Figure 4-1-4 Line graph showing the RQ method for change of miR122 expression between the placebo and the metformin groups over the 18 months.

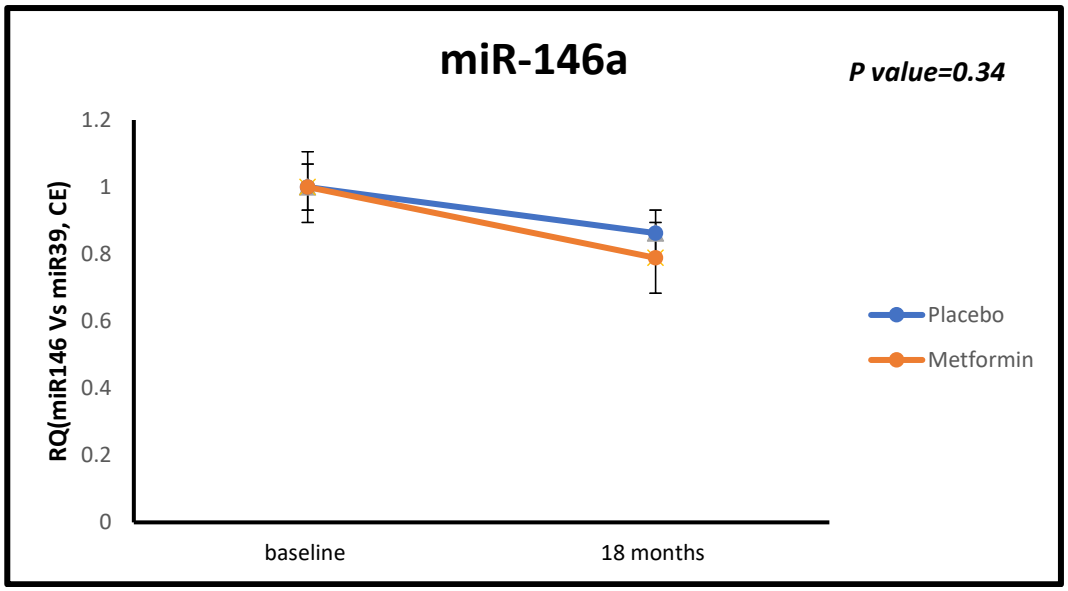


Figure 4-1-5 Line graph showing the RQ method for change of miR146 expression between the placebo and the metformin groups over the 18 months.

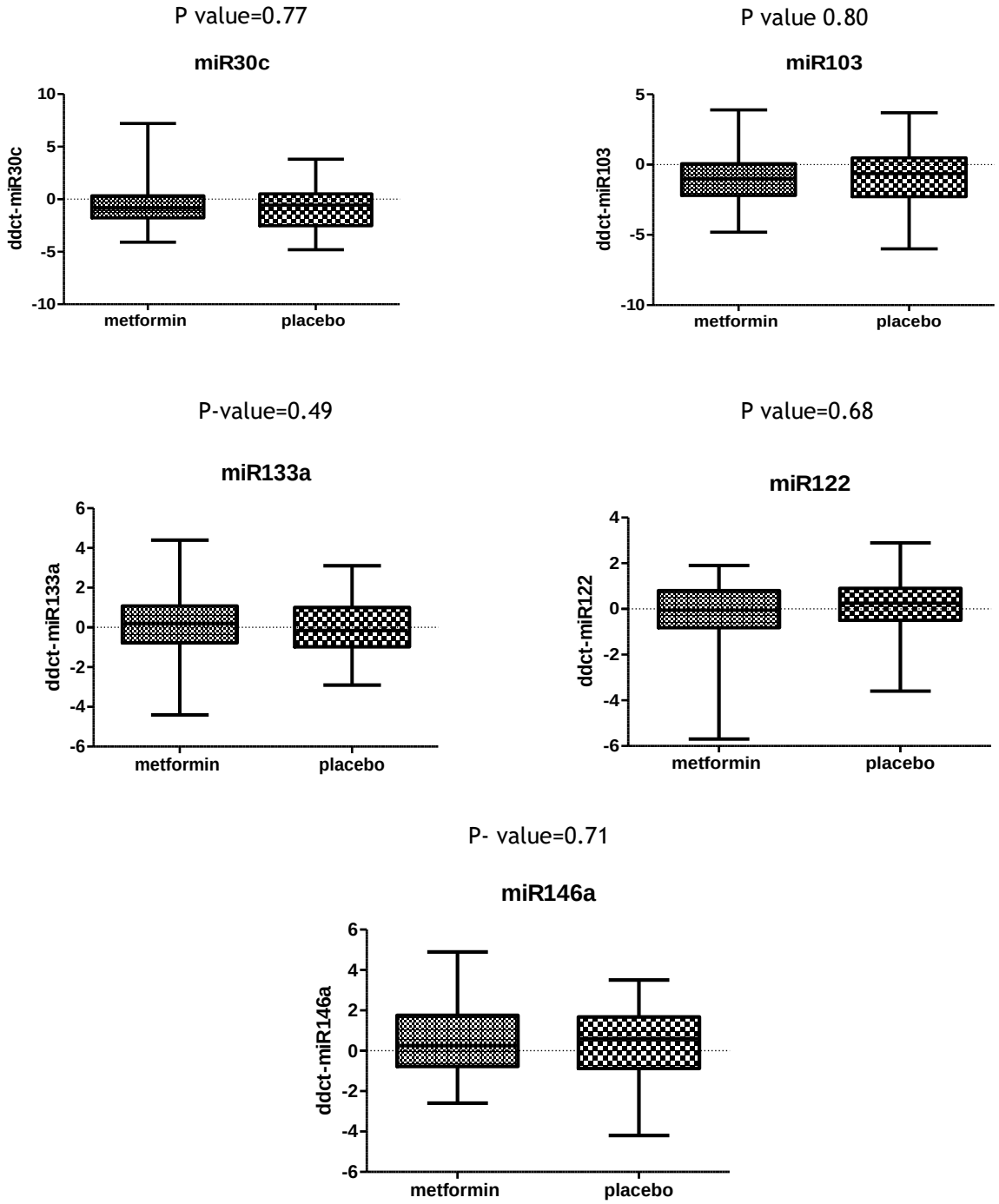


Figure 4 1-6 Relative miRNA expression (using ddct calcaultion) of miR103, miR30c, miR133a, miR122 and miR146a in plasma samples of the placebo and the metformin groups after 18 months. Threshold cycle difference (dct) of miRNA in placebo group at baseline = (ct of miRNA at baseline - ct of miR39 at baseline), dct of miRNA in placebo group at 18 months = (ct of miRNA at 18 months - ct of miR39 at 18 months), the relative expression of miRNA (ddct) in placebo group = (dct at baseline - dct at 18 months), the same for metformin group

4.5.5 The effect of time and randomisation on the expression of circulating miRNAs

To confirm and expand on the RQ analysis, a two-way repeated measures ANOVA analysis was carried out to determine the effect of time (baseline and 18 months) on the selected circulating miRNAs expression and to investigate the difference between the two treatment groups (placebo and metformin). The results are shown in Table 4-5.

Two circulating miRNAs had an increase (lower ΔCt) in their expression across time: miR30c and miR103. This increase was shown in both the groups, both when taken separately and when combined (placebo and metformin). There was no evidence that metformin randomisation had a different effect from the placebo for any miRNA across time.

Table 4-5: Mixed design two-way repeated measures ANOVA time by treatment analysis for circulating miRNAs

Randomised Treatment		Time	Mean	95% Confidence Interval	
				Lower Bound	Upper Bound
miR30c	Metformin	1	2.63	1.96	3.29
		2	1.91	1.02	2.80
	Placebo	1	2.24	1.54	2.93
		2	1.45	0.40	2.50
Time, *P-value*		**0.007**			
Interaction, *P-value*		**0.895**			
miR103	Metformin	1	3.71	2.62	4.81
		2	2.57	1.36	3.78
	Placebo	1	2.80	1.68	3.92
		2	1.86	0.65	3.06
Time, *P-value*		**0.001**			
Interaction, *P-value*		**0.712**			
miR133a	Metformin	1	5.34	4.74	5.93
		2	5.48	4.71	6.24
	Placebo	1	5.45	4.52	6.38
		2	5.21	4.16	6.25
Time, *P-value*		**0.780**			
Interaction, P-*value*		**0.321**			
miR122	Metformin	1	3.35	2.63	4.06
		2	3.04	2.13	3.95
	Placebo	1	2.99	2.27	3.72
		2	2.66	1.83	3.49
Time, *P-value*		**0.197**			
Interaction, *P-value*		**0.969**			
miR146a	Metformin	1	-1.57	-2.30	-0.84
		2	-1.23	-2.05	-0.41
	Placebo	1	-2.19	-3.13	-1.26
		2	-2.09	-3.09	-1.09
Time, *P-value*		**0.325**			
Interaction, *P-value*		**0.601**			

Time 1 = baseline, Time 2 = 18 months, ***P*-value for time** indicates evidence against the null hypothesis that time (in both treatment groups combined) has no effect on the expression of miRNAs. **Interaction *P*-value** indicates evidence against the null hypothesis that metformin has the same effect as placebo across time.

4.6 Discussion

The data presented in the previous chapter indicates that, using the baseline samples of the CAMERA trial, circulating miR30c, miR103, miR133a, miR122 and miR146a showed a cross-sectional association with cardiometabolic markers. This chapter presents data that indicates that randomisation to metformin, a drug known to have an effect on glycaemia and often hypothesised to have a beneficial effect on CVD risk markers (Lexis *et al.*, 2014), had no effect on the same circulating miRNAs. The dosage of metformin was at the upper end of the standard starting dose for type 2 diabetes (850mg twice daily) (Ltd., 2020).

The 60 paired samples included in this study were from participants who had taken a study medication and completed the follow-up period (18 months). In the CAMERA trial, all the analysis was performed on participants who had a baseline and one cIMT measurement at 18 months, with no major or minor protocol deviations. Minor protocol deviations included compliance of less than 50% and stopping the study medication before the end of the trial (Preiss *et al.*, 2014).

Adherence of the participants was generally high (section 3.2), and after randomly selecting participants who had a paired baseline and 18-month samples, we did not include participants with low adherence due to side effects, who may subsequently have withdrawn from the study. Therefore, it is likely the impact of metformin on these miRNAs is minimal, rather than this being an issue arising from the study design

4.6.1 The effect of metformin on the expression of circulating miRNAs

The results show that the randomisation to metformin had no effect on the expression of circulating miR30c, miR103, miR133a, miR122 and miR146a. This was indicated in the analysis using both achieved ΔCt levels and the RQ method traditionally performed in miRNA studies.

The effect of randomisation to metformin on the expression of the selected circulating miRNAs has been reported in previous studies. İbrahim Demirsoy et al.,

determine the expression level of 86 different circulating miRNAs include (miR30c, miR133a and miR146a) for the same participants after three months' treatment with metformin (Demirsoy *et al.*, 2018). The study includes 47 patients (20 female and 27 male) that were diagnosed with type 2 diabetes. In contrast to the findings of this study, the researchers had reported downregulation of the expression level of 13 miRNAs after three months of metformin treatment, one of them being miRNA146a (*P*=0.04, dCT,0.55). The discrepancy in the results may be due to difference in study population, sample size, study design, normalisation method of plasma miRNAs and duration of treatment. The study included 47 participants who were followed for three months following metformin treatment, while this study was a randomised placebo-controlled trial that included 32 participants in the placebo group and 28 participants in the metformin group who were followed for 18 months after metformin treatment. In addition, the global mean normalisation method was used by Demirsoy and his collagues to normalise plasma miRNA, while this study used exogenous control.

Vikas Ghai and his colleagues identified the circulating miRNAs associated with type 2 diabetes and the way they were affected by metformin treatment. The plasma samples were obtained from a subset of the Danish MetaHIT cohort (Pedersen *et al.*, 2016). The study included 80 individuals: 39 controls, 10 T2DM (non-metformin treated), and 31 T2DM (treated with metformin). The researchers used miR-451a-5p and miR-486-5p as normalisers; miR-451a-5p and miR-486-5p were invariant miRNAs that had low coefficient variance across samples. In contrast with the results of this study, they found that the circulating miR122 expression showed down regulation in plasma and the extracellular vesicles depleted a fraction between the diabetic participants taking metformin treatment and the control participants (log fold of change=0.65, *P* for miR122 in plasma =0.02), (log fold of change=0.71, *P* for miR122 in extracellular= 0.01). However, in line with the results of this study, they found no difference between the expression level of miR30c in plasma between the control participants and the diabetic participants with metformin treatment. In addition, they found no difference in the expression of circulating miR146a in plasma but there was an expression difference of miR146a in extracellular vesicles that depleted a fraction between the healthy participants and the diabetic participants treated with metformin (Ghai *et al.*, 2019).

Giuseppina Catanzaro investigated the circulating miRNAs in elderly patients after treatment with metformin and sitagliptin. The study was based on 40 elderly patients with type 2 diabetes who were on a maximum dose of metformin and had poor glycaemic control (HbA1c levels ranging from 7.5% to 9%). They identified the circulating miRNAs after 3 months and 15 months from treatment with sitagliptin and metformin. The researchers found that miR30c was significantly upregulated in the patients who responded to antihyperglycemic therapy according to the HA1c value, following 15 months of sitagliptin and metformin treatment (Giuseppina Catanzaro, 2018). MiR30c was recently shown to have a nephroprotective (Zhao *et al.*, 2017) and cardioprotective effect (Raut *et al.*, 2016) on diabetic patients. This contrasts with the results obtained by this study that showed no significant change in the expression of miR30c after 15 months of metformin treatment. The difference in the results could be due to the difference in study population, duration of treatment, or dose regimen of metformin. Catanzaro (2018) investigated elderly patients with diabetes who were taking metformin and sitagliptin, while this study is based on patients with coronary artery disease with insulin insensitivity. It could be that the sitagliptin explains the changes occurring on the expression of the circulating miR30c.

In contrast with the findings of this study regarding the effect of metformin on the circulating miR146a, Emanuela Mensà and his colleagues analysed the circulating level of miR146a in 144 participants (61 participants with metformin treatment and 83 participants without metformin treatment with T2DM) and found a higher level of circulating level of miR146 in patients treated with metformin, but no difference of circulating miR146a between patients on mono and combination treatment regimens (Mensà *et al.*, 2019). Moiseeva et al. reported that metformin decreased the production of pro-inflammatory cytokines during cell senescence or in response to lipopolysaccharide (LPS) by inhibiting NF-κB pathways (Moiseeva *et al.*, 2013). Furthermore, Yangmei Xie showed a negative correlation between miR146a and inflammatory mediators, including COX-2, TNF-α, and IL-1ß in the T2DM group (Xie *et al.*, 2018). This suggests that metformin might decrease the production of inflammatory mediators by increasing circulating miR146a in the diabetic population. Further research is required to prove this hypothesis.

4.6.2 The effect of time and randomisation on the expression of circulating miRNAs

This study showed an increasing expression of circulating miRNAs, that is, miR30c, miR103, miR133a, miR122 and a decreasing expression of circulating miR146a with time in both groups (metformin and placebo) as shown in Table 4-3. A sample t test also indicated a difference in the expression of selected miRNAs following 18 months of placebo and metformin treatments.

As previously mentioned, circulating miRNAs are remarkably stable in serum and plasma, show a resistance to cleavage by RNase activity and remain stable in harsh conditions such as extreme pH, long-time storage and repetitive freeze-thaws cycles (Glinge *et al.*, 2017). Pamela R et al. investigated the effect of long-term storage on the expression levels of 8 circulating miRNAs from 10 subjects from the population-based cohort study KORA. They found that the miR451a levels in plasma samples that were stored for 17 years were elevated when compared to those in plasma samples stored for 2 and 9 years. This difference in miRNAs expression after a period of time could be an indicator of contamination of plasma with miRNAs and cellular materials from haemolysis and apoptotic at the time of processing of the sample. The results could also be explained by the differences at the time of the sample collection during the CAMERA trial.

4.7 Conclusion

Randomisation to metformin failed to show any change in the expression of circulating miRNAs miR30c, miR103, miR133a, miR122 and miR146a in participants who had coronary heart diseases with insulin insensitivity. The contradictory findings between studies could possibly be due to differences in the study population, study design, size of samples, normalisation methods, dose regimen of metformin and duration of treatment.

4.8 Strengths and limitations

This study has several strengths. First, the samples were taken from the CAMERA trial, which is a randomised placebo controlled double blind trial that is

considered to be the gold standard. It was a well-designed study, that investigated the evidence of causation between interventions and outcomes. Second, the single centre recruitment allowed for consistency in sample collection and handling. Finally, the study had well defined inclusion and exclusion criteria.

The limitations of the study included the relatively small size of the sample for a randomised experiment. This may mean that limited power biases our results to the null. However, given the difficulties of measuring miRNA in a standardised fashion, this is still one of the largest randomised studies of which the author is aware. Data normalisation still remains one of the most important and difficult problems associated with qPCR analysis. So far, there are no common and univocal reference control gene finds for plasma miRNAs normalisation. It is clear that different normalisation methods give different results, presenting a high risk of generating confusion (Faraldi *et al.*, 2018). The other potential limitation was that the participants were followed up for 18 months rather than 24 months, so it was not possible to depict the pattern of circulating miRNAs expression at different time points after taking metformin (at 18 months and at 24 months). The main study (CAMERA trial) intended to follow up participants for 24 months to investigate the effect of metformin on the progression of cIMT. However, the follow-up period was reduced to 18 months for the current study, as this was considered an optimal compromise between power and adherence (fewer patients completed the 24-month visit in the trial). In retrospect, the CAMERA trial did not reject the null hypothesis that metformin randomisation has no impact on the progression of cIMT. Therefore, longer follow-up may have increased power to observe such an effect, and likewise increased power to observe an effect of metformin on circulating miRNA expression.

The study drug had a very moderate effect on the metabolic profile of the selected patients. In addition, one cannot rule out the possibility that pre-analytical variables (sample handling) may have had an impact on miRNA expression due to the changes evident during different study visits. However, all samples were handled in accordance with a trial SOP.

This study provided no evidence that metformin had a different effect to the placebo on any circulating miRNAs during the period of the trial. In this study, the

lack of change is presumed to be a valid and true observation and free from non-differential misclassification bias.

Misclassification bias is an important issue that must be considered during the data interpretation of this study. Non-differential misclassification is a critical contributor to bias in epidemiological studies and is a type of information bias, which is a deviation of estimate of association from a true association; the result will be biased towards the null (Rothman KJ, 1998). This type of bias occurs due to a lack of accurate measures or misclassification of key study variables, making the two comparator groups seem more alike than they would be with a more reliable measurement.

This study included an accurate measure of cardiometabolic markers carried out in the CAMERA trial across the placebo and metformin groups, and data was obtained from a well-designed RCT. In addition, in this study, the precise measurements of circulating miRNAs were done by using RT-qPCR with spike in control that was added before extraction to provide a more reliable estimate of all technical variability that may be introduced during any step in quantification of miRNAs. In addition, the precision of miRNAs measurements was assessed by CV%, as shown in Table (2-2). The CV% for CT is less than 15%, which is in the typically acceptable limit. Moreover, the associations between miRNAs and cardiometabolic markers seen in (Chapter 3) improve the reliability of the data; for example, there was no uniform lack of associations of miRNA with all outcomes of interest. This finding that metformin had no effect on the expression of circulating miRNAs is a true estimate and probably not primarily due to misclassification bias, although it may reflect low statistical power. More work is required on the validation and verification of miRNA assays for quality control before they could be translated into clinical use.

5 Association of cardiovascular risk factors with carotid intima media thickness and pulse wave arterial stiffness in UK Biobank; Cross-sectional study

5.1 Introduction

Atherosclerosis is the underlying cause of many ischemic cardiovascular diseases, and has a long asymptomatic phase that begins in early childhood (Henry C McGill Jr, 2000). Carotid intima media thickness and arterial stiffness are considered as non-invasive measurements of the anatomical structure and elastic properties of different arteries, and both are related to development of atherosclerosis, as discussed in chapter 1. Carotid intima media thickness is a marker for asymptomatic and subclinical atherosclerotic vascular diseases (Bauer *et al.*, 2012). Intimal or medial hypertrophy increases carotid intima media thickness, and therefore leads to progression in the development of symptomatic cardiovascular diseases.

5.1.1 Carotid intima media thickness

The first meta-analysis to investigate the association between increased carotid intima media thickness and the incidence of future cardiovascular diseases showed that higher carotid intima media thickness is a strong independent predictor of future vascular disease (Lorenz *et al.*, 2007). This meta-analysis identified eight observational studies involving 37,197 participants, who were followed up for a mean of 5.5 years. The future risk of MI was found to increase by 10% to 15% and the risk of stoke by 13% to 18% for every 0.1mm increase in IMT. Therefore, the early detection of subclinical atherosclerosis by measuring carotid intima media thickness might help to detect and prevent the onset of cardiovascular disease. The 2010 American College of Cardiology Foundation/American Heart Association (ACCF/AHA) guidelines recommended the use of cIMT at a Class IIa level for the assessment of cardiovascular risk in asymptomatic patients with an intermediate risk of CVD (Greenland *et al.*, 2010).

Traditional modifiable cardiovascular risk factors, including high blood pressure, high blood sugar, high low density lipoprotein, obesity, and cigarette smoking, have long been shown to be positively correlated with high cIMT (Chambless *et al.*, 2002). More recent studies have illustrated the potential upstream causes of intima thickening. Chiesa *et al.* (2019) studied cross-sectional associations

between a range of CVD risk factors (blood pressure, body composition, lipid, insulin, glucose, inflammatory markers, socioeconomic circumstances and lifestyle behaviours) and cIMT. They found that free fat mass and systolic blood pressure were independently associated with increased cIMT. These findings were derived from a young healthy population, and the sample size was around 5,000 participants (Chiesa *et al.*, 2019). Other studies have found an association between CVD risk factors and cIMT in special populations. Wu et al (2017) investigated the association between cIMT and cardiovascular profile in 1,579 residents aged 40-74 in northern Taiwan (Wu *et al.*, 2017). The contribution of lipid markers and BMI on cIMT was more significant in males than females. In addition, a cross-sectional study enrolling 68 patients on maintenance haemodialysis found a correlation between age, c-reactive protein and cIMT (Kuswardhani *et al.*, 2018).

Furthermore, an association has been shown between carotid intima media thickness and traditional risk factors, including insulin resistance, dyslipidemia and metabolic syndrome (Hedblad *et al.*, 2000),(Crouse *et al.*, 2007),(Iglseder *et al.*, 2005). However, traditional cardiovascular risk factors made a minor contribution to cIMT variance, meaning that there are other markers that may be regarded as independent markers for increasing cIMT. Studies have shown that there are other biological markers involved in the inflammation process of intimal hyperplasia, which may be considered as independent predictors for increasing cIMT. An association has been found between cIMT and other biological inflammatory markers such as c-reactive protein, fibrinogen. apoprotein-B and oxidized LDL (Baldassarre *et al.*, 2008), (Ernst and Resch, 1993), (Okamura *et al.*, 2013), (Hulthe and Fagerberg, 2002).

Most of available publications investigated the association between cIMT and a limited number of factors of the cardiovascular risk profile, with a small sample sizes that focused on either a special clinical population or specific age groups.

5.1.2 Arterial stiffness index

Arterial stiffness describes the decreasing capability of arteries to expand or contract in response to changes in pressure. The movement of the pressure wave

along the arterial tree (pulse wave velocity) is controlled by the elasticity of the intrinsic wall of the arteries. Pulse wave velocity increases in stiffer arteries and is considered to be an independent predictor of cardiovascular disease, representing a gold standard for arterial stiffness measurements (Members *et al.*, 2013). In addition, central arteries, such as the aortic artery, stiffen progressively with age. Increase stiffening of the aorta and other central arteries is a potential risk factor for CVD mortality and morbidity. Gatzka et al. found that coronary artery disease patients had significantly stiffer aortas than did asymptomatic controls (Gatzka *et al.*, 1998). The association between arterial stiffness and atherosclerosis has been examined in different studies, which have produced conflicting results (Maarek *et al.*, 1987), (Nakashima and Tanikawa, 1971). As discussed in chapter 1, the association between atherosclerosis and increasing stiffening of the arteries is complex and may involve several different mechanicals, metabolic, hemodynamic and enzymatic mechanisms that interact with each other. Age and blood pressure have been found to be principal determinants for arterial stiffness (McEniery *et al.*, 2010), with other traditional risk factors making an insignificant or marginal contribution (Cecelja and Chowienczyk, 2009). This age-dependent increasing stiffness of the central arteries is independent of mean blood pressure or other risk factors (Relf *et al.*, 1986). In addition, high arterial stiffness is closely linked with a high risk of hypertension, chronic kidney disease and stroke (Mancia G, 2013). The causality between arterial stiffness and hypertension is discussed in chapter 1.

In order to prevent CVD, it is important to more clearly understand the upstream causes of vascular disease. Most existing studies of the determinants of cIMT and PWASI are based on a limited sample size that focused on either special clinical populations or a specific age group. Furthermore, most studies have a limited ability to measure confounders as well as the heterogenicity noted between cIMT and PWASI measurements. In addition, non-invasive measures of arterial stiffness vary between studies, and different segments of cIMT have been examined in different studies. This variation in the pre-existing research makes it difficult to draw a robust conclusion.

The UK Biobank is an important resource that provides detailed measurements of cIMT and PWISI and other cardiovascular diseases risk factors. This study therefore sought to examine a wide range of CVD risk factors and thickening of the carotid artery, as well as increases in the pulse wave arterial stiffness index in participants who do not have cardiovascular disease. UK Biobank is an epidemiological resource based on a UK population that includes around 500,000 participants.

5.2 Hypotheses

This study aimed to address gaps in the literature by investigating the determinants of carotid intima media thickness and pulse wave arterial stiffness using a single large cross-sectional study with standardized measurements for both surrogates.

The hypotheses of this chapter are:

1. The classical risk factors include age, hypertension, diabetes and high lipid concentrations, explaining the variation in the non-invasive markers of vascular alteration, namely carotid intima media thickness and pulse wave arterial stiffness index. The distribution of those risk factors differs between high and low cIMT and PWASI.

2. That systolic blood pressure and age are the strongest predictor for cIMT and PWASI variation (based on the published literature to date).

5.3 Aims

This cross-sectional study will investigate the following specific aims:

1. To investigate the simple demographic patterns of cIMT and PWASI in UK Biobank.

2. To identify which upstream CVD risk factors that are independently associated with cIMT and PWASI.

3. To identify the strongest variables that explain the variation of cIMT and PWASI in the UK Biobank population.

5.4 Methods

5.4.1 Study design

This study was a cross sectional observational study using data from UK Biobank. It included all participants who had measured carotid intima media thickness and pulse wave arterial stiffness at imaging visit.

5.4.2 UK Biobank

UK Biobank is a prospective cohort study recruiting more than 500,000 men and women aged between 40–69 years in the UK between 2006-2010 (Sudlow *et al.*, 2015). This large scale prospective epidemiological study was established by the Welcome Trust, Medical Research Council, Department of Health, Scottish Government, and the Northwest Regional Development Agency and it was supported by the National Health Service (NHS). Also, it received funding from the Welsh Government, British Heart Foundation, Cancer Research UK, and Diabetes UK. The aim of the study was to improve, prevent, diagnose, and treat a wide range of illnesses, including cancer, heart diseases, and diabetes. Participants were registered with NHS primary care practices, spanning England, Scotland and Wales. Potential participants were identified through the NHS patient register according to being aged 40-69 and living near to an assessment centre. Participants attended one of the 22 recruitment centres across the UK, where they performed the baseline physical measurements and they gave blood and urines samples for genetic and biomarkers analysis, as well as completing a touch-screen, self-completed questionnaire and a computer-assisted personal interview covering a wide range of social and lifestyle information as well as medical conditions. Figure (5-1) shows the different components of the UK Biobank resources. Ethical permission was granted by the North West Haydock Research Ethics Committee in the UK (REC reference: 11/ NW/03820). All participants gave written informed consent before enrolment following the principles of the Declaration of Helsinki.

The present study was specifically applied for and approved under UKB project number 43707 (UK Biobank, 2019).

5.4.3 Data availability

5.4.3.1Baseline assessment data

The assessment visit included obtaining electronic signed consent, a touch screen questionnaire, a computer assisted nurse-led interview, blood pressure and arterial stiffness measurements and physical measurements, including height, hip and waist circumference, weight and bio-impedance measurements. Samples of blood, urine and saliva were collected with multiple aliquots stored in UK Biobank's lab for any future research.

5.4.3.2Additional data assessments to enhance phenotyping

UK Biobank is established an additional phenotyping assessment of all participants or large number of participants. A baseline assessment was completed for all of the cohort by the end of 2010, and the assessment was repeated for around 20,000 participants by 2013 (Clarke *et al.*, 1999). Biochemical assays for baseline samples for the whole cohort was completed by the end of 2018. A dietary web questionnaire providing an estimated nutrient intake for around 201,000 participants (Liu *et al.*, 2011). In addition, participants were invited to provide information not identified from health record linkages via Web questionnaires about any future exposures and health outcomes (Sudlow *et al.*, 2015).

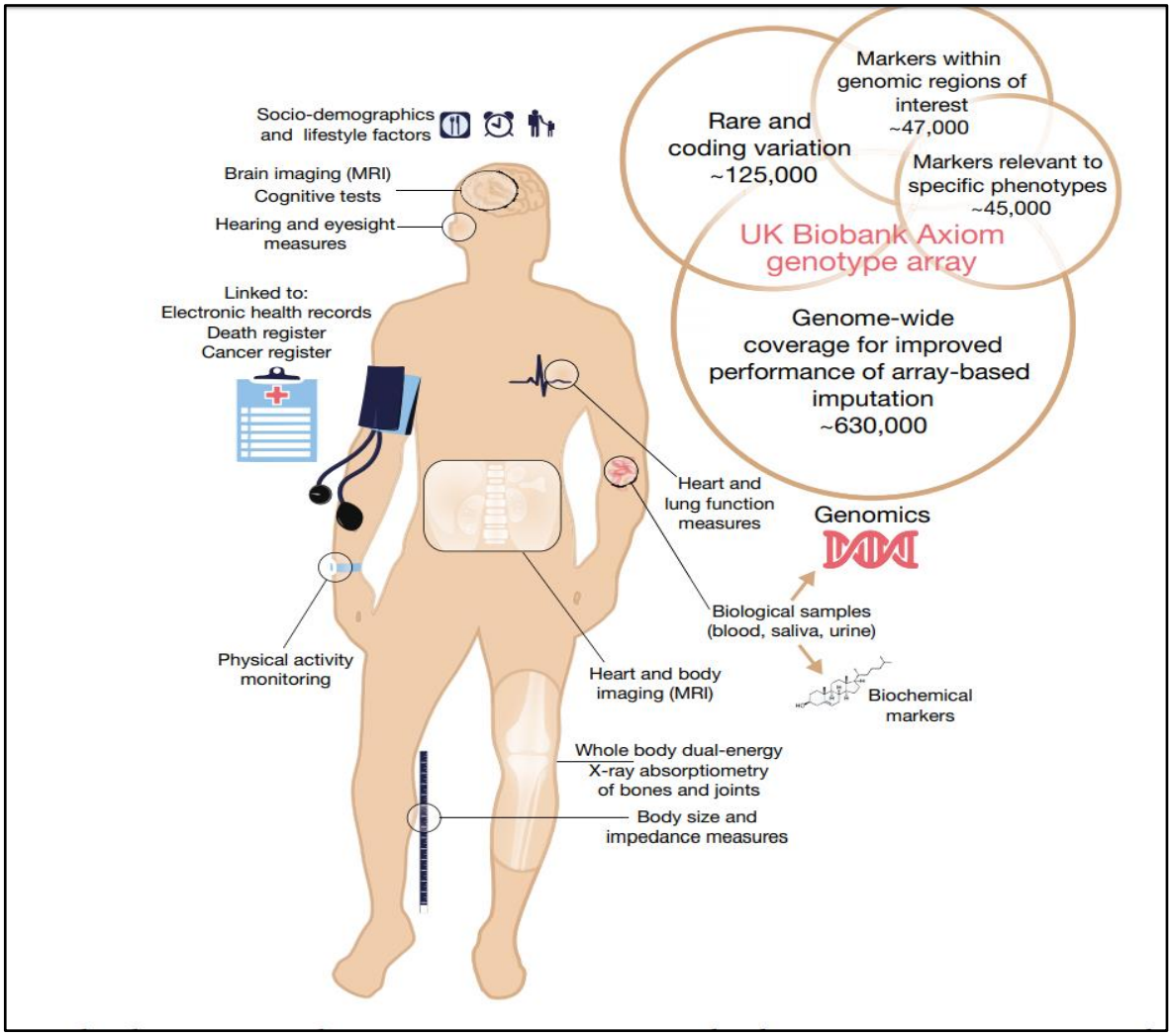


Figure 5-1 Scheme describes the different components of the UK Biobank resource

(Bycroft *et al.*, 2018)

5.4.4 UK-Biobank imaging visit

The imaging study was designed to achieve the target of 100,000 participants. beginning in May 2014, with participants invited to participate in a follow-up imaging assessment. The imaging phenotypes were selected based on their relevance to the widest range of diseases and hypotheses, and detailed imaging scans of vital organs (brain, heart, abdomen, bones, carotid artery, and body composition) were taken as well as a repeat of the baseline measurements. The study included a cardiac and whole-body MRI, a brain MRI scan, a neck artery ultrasound scan and dual energy X-ray absorptiometry (DXA).

Invitation letters were sent to people living within reasonable travelling distance of one of the imaging assessment centres. This invitation was not dependent on any other information that had been already gathered about participants. Participants were not eligible to take a part in the study if they had any metal or electrical implants (e.g. pacemakers) in their body, if they had had recent surgery (within the previous six weeks), or if they had any medical problems that would make it difficult to perform the scans (for example severe hearing or breathing problems, or tremors).

Carotid IMT phenotyping started in 2015, as a pilot phase, during which 2,272 individuals imaged at 18 centres with manual quality control being conducted. Subsequently, all imaging centres started to recruit participants and use automated measurements. Recruitment for imaging is ongoing, but as of 2019, ultrasound measurements of cIMT had been taken from 502,507 participants.

5.4.5 Definition of outcomes and predictors

5.4.5.1CIMT measure

Carotid intima media thickness was measured using a CardioHealth Station (Panasonic Biomedical Sales Europe BV, Leicestershire, UK). The participants were asked to lie down with their head related to the horizontal at 45 ^{o}, supported by a triangular pillow. The right side was scanned first, followed by the left side. A 2D scan was first done on the transverse plane (short axis) from below the carotid

bifurcation to below the jaw. A 2D scan was then performed for the longitudinal plane (long axis). The carotid intima media thickness was measured at two predefined angles for each carotid, giving a total of four cIMT readings (right 150^{o}, right 120^{o}, left 210^{o}, and left 240^{o}) at the long axis. The distal common carotid was scanned, and the flow divider between the external and internal carotid artery located. The far wall of common carotid was tracked within the box and after several cardiac cycles, the image auto-freezes in end diastole. A mean, maximum and minimum of the cIMT tracking was measured and recorded for each angle of acquisition for each carotid. Average mean common cIMT was calculated per individual using the maximum set of carotid angles, near or far wall measurements and left or right-side measurements (Biobank., 2015).

The measurement of IMT at one site may differ from measurements taken from another IMT site because of the focal nature of the atherosclerosis development in the artery (George Howard, 1994). Therefore, measurements of carotid intima media thickness from a single site may decrease the sensitivity of detection of the atherosclerosis change in the artery. Some studies evaluate cIMT from a single angle (Price *et al.*, 2007), while others provide images from multiple angles. Imaging cIMT from multiple angles is considered better way of evaluating the three dimensions of the carotid artery (Peters *et al.*, 2012). This study examined the mean cIMT at each angle - left, right and combined angles. Figure 5-2 shows the flow chart of UK Biobank of carotid ultrasound protocol.

The abnormal and normal carotid intima media thickness categories were defined according to cIMT of >0.9 mm and<0.9 mm, respectively (Price *et al.*, 2007) The boundaries of IMT quartiles were cIMT of<0 58mm, cIMT (0.59-0.66), cIMT (0.66-0.75), and cIMT>0.75.

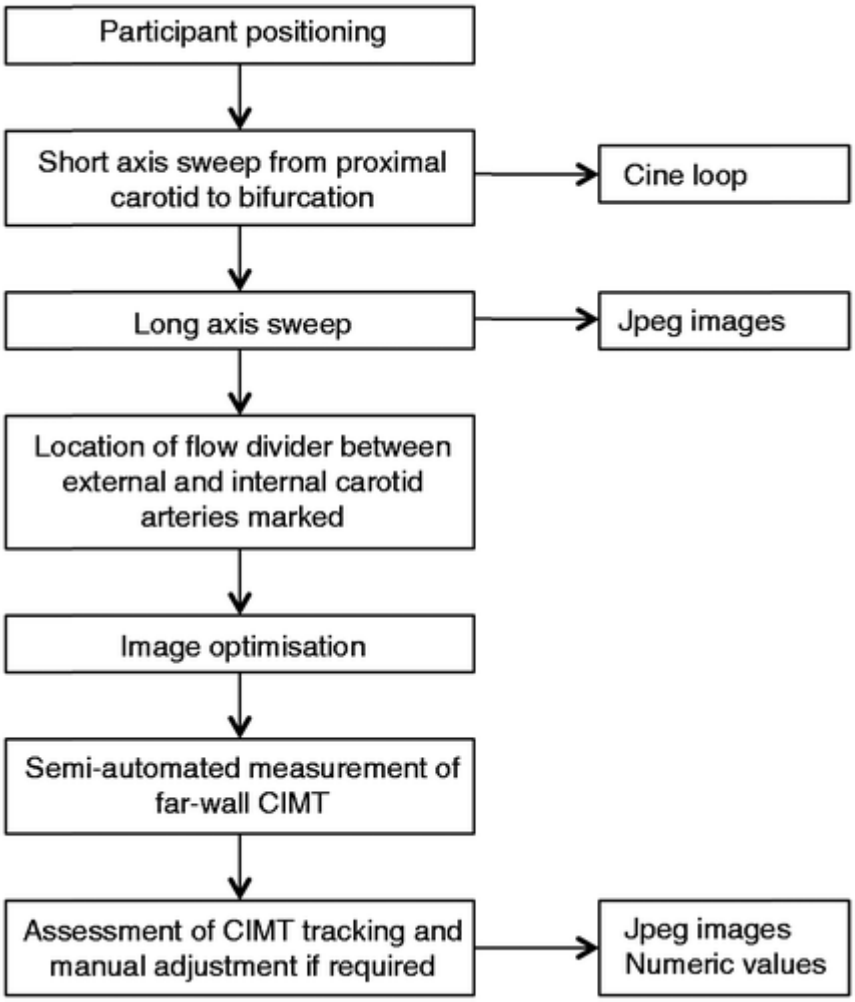


Figure 5-2 flow chart of UK Biobank carotid ultrasound protocol

5.4.5.2PWASI measurement

Pulse wave velocity for arterial stiffness index assessment was measured in the assessment centre using the PulseTrace PCA2 (CareFusion, San Diego, CA) in 42726 participants, from 2014 until 2019. The PulseTrace PCA2 uses an infrared sensor clipped to the end of the index finger to obtain pulse waveform, and the reading is ready after around 10- to 15-seconds. In case of an invisible waveform on the screen of the PulseTrace PCA2 device, or if the waveform did not stable for around one minute after clipping the sensor at the end of the index finger, the measurements were repeated in another finger, such as larger finger or on the thumb. The shape of the waveform is related directly to the time that pulse wave takes to travel through the arterial tree and to be reflected back to the finger. A Seca 22 height measure was used by UK Biobank staff to measure the standing height (shoeless) and was manually entered into the assessment centre software. The software immediately alerted the UK Biobank staff member if there were any impossible or implausible values and asked them to correct it. The ASI in m/s was obtained by dividing height (meters) by the time between the peaks of the pulse waveform. This method has been approved by comparing it with carotid-femoral PWV in 3 independent studies, which showed that both measurements were highly associated, and ASI is independent operator that no training is needed, and it is a simple, rapid and inexpensive technique (Alty *et al.*, 2007) (Millasseau *et al.*, 2002) (Sollinger *et al.*, 2006).

5.4.5.3Demographic and haemodynamic parameters

5.4.5.3.1 Age, sex and ethnicity

The date of birth, age and sex were obtained by UK Biobank staff ahead of the assessment visit from local NHS Primary Care Trust Registries. Ethnic group was collected via patient report during the touchscreen questionnaire during the assessment visit. In this variable, ethnicity was categorised into eight groups (white, black, Asian, mixed, Chinese, other ethnic groups, do not know, and prefer not to answer). In this study, ethnicity group was recorded as white, south Asian, black, and others. Participants with “Don’t know” and “Prefer not to answer” responses were excluded from our analysis.

5.4.5.3.2 Blood pressure

Blood pressure measurement was conducted at the third station of the assessment centre visit. Systolic and diastolic blood pressure was measured both manually and automatically. The readings for blood pressure were taken twice during the imaging visit at the assessment centre by qualified nurses. An Omron 705 IT electronic blood pressure monitor was used to measure blood pressure (OMRON Healthcare Europe B.V. Kruisweg 577 2132 NA Hoofddorp). There was only an initial and first repeat assessment visit for blood biochemistry and no assessment for blood components at imaging visit that measured PWASI and cIMT, and this is one of study limitations.

We defined blood pressure preferentially using the automated measurement, or the manual measurement where automated measurements was not available.

5.4.5.3.3 Anthropometric measurements

Body size measures, including height, weight, waist, and hip circumference, were taken during the assessments at the assessment centre by UK Biobank staff. Weight was measured using the Tanita BC-418 AM body composition analyser and height using the Seca 202 height measure. UK Biobank staff asked participants to remove heavy clothes and shoes and stand on the footpads of the body composition analyser. Body mass index was calculated by dividing weight by the square of height. Waist and hip circumferences were measured using a Wessex non-stretchable sprung tape measure.

5.4.5.3.4 Biochemical markers

UK Biobank measured selected biochemical markers in biological samples, including serum, urine and saliva. These samples were collected at baseline (2006-2010) for all 500,000 participants. There was a dedicated ISO accredited biochemistry facility built and staffed specifically for this purpose, and measurements were conducted using rigorous quality control and standard operating procedures. Around 34 biomarkers were selected to be analysed because they represented risk factors for diseases and were considered diagnostic biomarkers. Blood samples were also taken at the imaging visit, but these have

not been analysed to date. For this reason, the present study uses biochemistry measurements for each participant from 2006-2010, which is at the study baseline assessment and was several years before the imaging visit. This is discussed as a limitation of the study in section 5-8.

5.4.5.3.5 Diabetes diagnosed by doctor

For the present study, participants were identified as having diabetes if they reported yes in diabetes diagnosed by doctor variable. During the imaging visit (2014-2018), participants completed a touch screen questionnaire with the question “has a doctor ever told that you have diabetes?”. This was supplemented by information from the nurse interview of self-reported type 1 or type 2 diabetes or use of insulin drugs.

5.4.5.3.6 Blood pressure medications

Participants were identified as having hypertension if they reported taking blood pressure medications. During the imaging visit (2014-2018), participants were asked (via touchscreen questionnaire) "Do you regularly take any of the following medications? The choices included (cholesterol lowering medications, blood pressure medication, and insulin.

5.4.5.3.7 Smoking

During the imaging visit (2014-2018), participants completed a touch screen questionnaire. Two questions were "Do you smoke tobacco now?”, and the second question was “In the past, how often have you smoked tobacco?”. Two new variables were derived by Professor Martin Tobin at the University of Leicester based on "Current tobacco smoking" and "Past tobacco smoking", coded as Never-smoker and Ever-smoker.

5.4.5.3.8 Statins

Participants indicating that they took regular cholesterol lowering medications were asked by interviewer for specific details of the class of medication.

5.4.5.3.9 Townsend deprivation index

The Townsend deprivation index is a measure of material deprivation within a population that was first introduced by sociologist Peter Townsend in 1987. It is measured by including four variables which are unemployment, non-car ownership, nonhome ownership, and household overcrowding, of the population of each area, and a score is given accordingly. The higher the Townsend index score, the greater the degree of deprivation in this area. Townsend deprivation index was measured for each participant joining UK Biobank. The score was calculated using the preceding national census output areas and their postcode.

5.4.5.3.10 Baseline CVD

Baseline CVD was defined as cardiovascular disease (coronary heart disease, angina, stroke or transient ischaemic attack) self-reported by touchscreen or at the nurse led participant interview. For the present cross-sectional study, this was used as part of the exclusion criteria.

5.4.6 Accessing UK-Biobank data

We send an application for data-access to the UK-Biobank in June 2018. We got application approval in February 2019, and the UK Biobank team subsequently released the data.

5.4.7 Exclusion and inclusion criteria

The analysis included all participants who had any valid cIMT measurements at all angles and excluded participants who had one or more mean cIMT measurements missing. Participants with pre-existing cardiovascular disease were excluded. For PWASI, the analysis in this study included all participants for whom PWASI data was obtained at the imaging visit and excluded participants who had missing data. Participants who pre-existing cardiovascular disease were excluded (Figure.5-3).

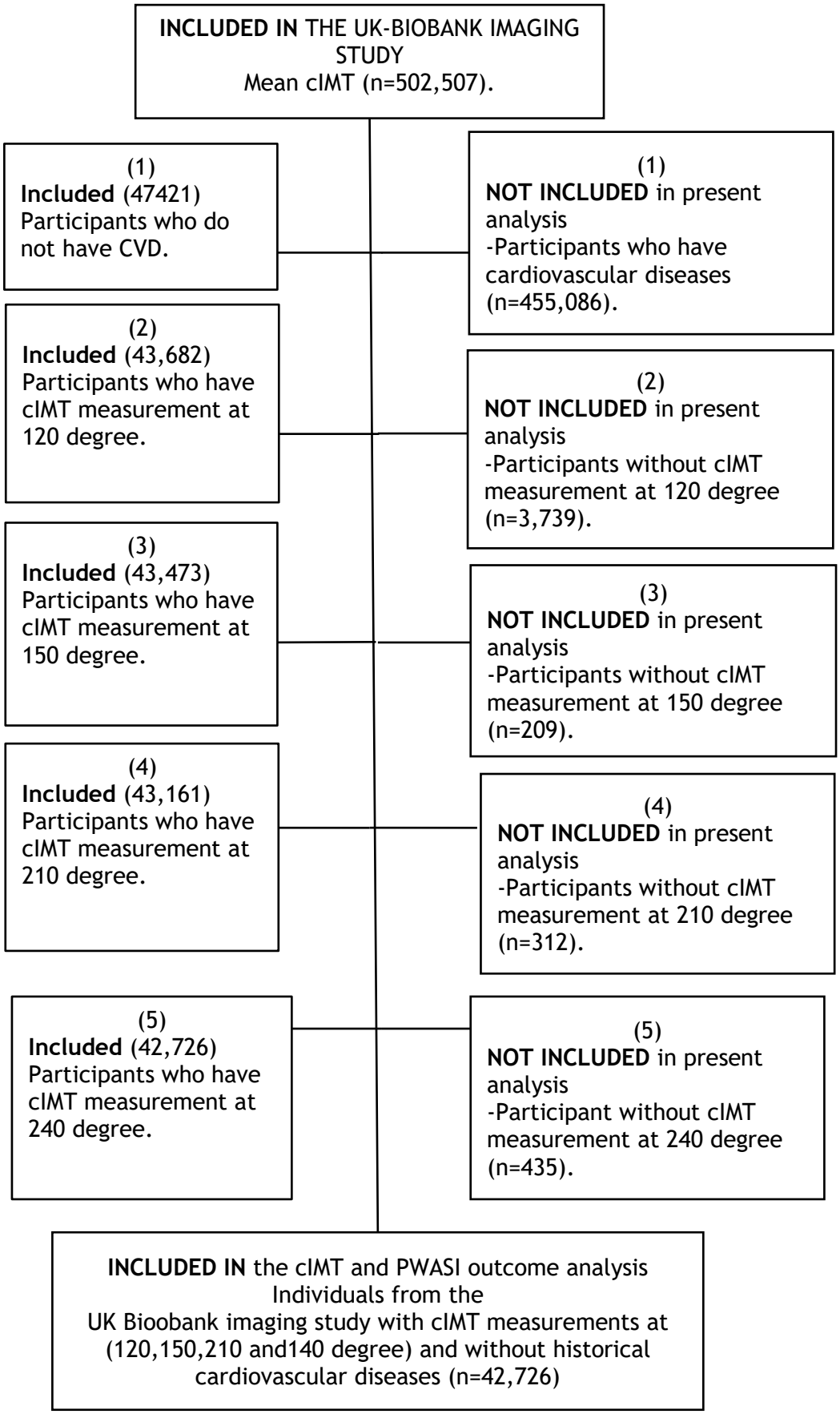


Figure 5-3 Diagram for inclusion of participants has measurements of cIMT and PWASI

5.4.8 Statistical analysis

5.4.8.1Statistical packages

IBM SPSS statistical version 24.

5.4.8.2Summary of statistics

All analyses were pre-specified with an analysis plan, and a specific application was made to the UK Biobank to undertake this work. Categorical data was summarised using counts and percentages, while continuous data was summarized using mean and standard deviation for normally distributed data, and median, IQR and range for non-normally distributed data.

5.4.8.3Comparison of categorical variables

The association between two or more categorical groups was assessed using a chi-square (x^2) test for two proportions.

5.4.8.4Comparison for continuous variables

An independent t-test was applied to compare parametric continuous data. In order to examine the assumptions for the test, the normality was firstly checked visually by histogram. Secondly, the equality of variance between groups was examined by Levenes' test. An assumption of equal variance is considered sustainable if that larger SD is less than twice the smaller SD. The log transformation method was applied for skewed data. Normality and t-test were then used after transformation. If normality is not met after log-transformation, the non-parametric Wilcoxon-Mann-Whitney test was used. To compare more than one group of continuous data, one-way ANOVA was applied for normally-distributed data, and otherwise the Kruskal-Wallis test was applied.

5.4.8.5Pearson correlation

A simple Pearson's correlation was calculated to examine the strength and linear relationship between continuous variables and mean of cIMT at all angle degrees, and mean PWASI. Variables with significant correlations with outcomes were subsequently included in simple linear regression analysis. If association were seen, then they were included in a multiple linear regression analysis to predict the outcomes (cIMT and PWASI).

5.4.8.6Regression

Univariate and multivariate analysis were used to evaluate and adjust the effect of cIMT and PWASI determinants on the outcome. As the dependent variable was a continuous variable, linear regression was used. The assumptions of constant variance of the residuals, normally distributed residuals, and the linearity of the association were examined by visual inspection of diagnostic plots. The independent variables were a mix of binary categorical variables with more than two groups and continuous variables. Each predictor was entered into a simple linear regression analysis; variables that were associated with the outcome significantly ($p<0.05$) in the univariate analysis were subsequently entered into the multiple linear regression. If two variables were significantly correlated, one (the weakest) was excluded from the multiple model as these variables had an association with the outcome due to their relationships with each other. In building multivariable models, first, all categorical and continuous variables significantly associated with cIMT in univariable analysis were subjected to multicollinearity diagnosis, and the variable that had variance inflation factors (VIF) of more than 2 was excluded from the model. No correction was made for multiple testing in regression analysis (i.e. significant test $p<0.05$).

5.5 Result

5.5.1 Carotid intima media thickness

5.5.1.1Characteristics of study population stratified by sex

Table 5-1 summarises the demographic and clinical characteristics of study subjects classified by sex. The study population consists of (42726) participants, of whom 20,290 were men and 22,436 were women. The mean age was 64.5 (±7.7) for men and 63.2 (±7.5) for women.

In this study, males had a higher mean value of cIMT (45.2μm higher) and pulse wave arterial stiffness index (1.12 m/s higher). In addition, males had poorer CVD risk profiles than females including age (1.3 years older), systolic blood pressure (6.3 mmHg higher), diastolic blood pressure (3.6 mmHg higher), glucose (0.35 mmol/L higher), HbA1c (0.47 mmol/mol higher), apolipoprotein-B (0.02g/L higher), triglycerides (0.49mmol/L higher), waist circumference (11.33 cm higher) and BMI (0.85 kg/m2 higher).

However, females had a higher hip circumference (0.19 cm higher) and higher lipid profile values include total cholesterol (0.26mmol/L higher), LDL (0.02 mmol/L higher), HDL (0.33mmol/L higher), and apolipoprotein-A (0.21g/L higher). For liver enzymes, males had a higher value of alanine aminotransferase (7 U/L higher) and aspartate aminotransferase (3.5 U/L higher).

The prevalence of diabetes in this study population was significantly higher in males (7%) compares to females (3%). Also, the prevalence of hypertension in this study population was significantly higher in males (66%) compares to females (52%). Furthermore, 77% of study population had not received statin therapy, and there was significantly higher percentage of males (32%) who had received statin therapy compared to females (15%). Regarding smoking, 63% of the study population never had smoked. There was significantly higher percentage of males (37%) who were former smokers compared to females (31%), and a significantly higher percentage of males (4%) were current smoker compared to females (3%). In addition, around 97% of study population was white, with the proportion of

white subjects greater in females more than males (0.2% higher), and the proportion of males who were of south Asian ethnicity was greater than females (0.6% higher).

Table 5-1:Demographic characteristics of the study population stratified by sex

Continuous variable	Total Cohort (Number) Mean± SD	Females (Number) Mean± SD	Males (Number) Mean± SD	*P*-value
Non-invasive measurement of atherosclerosis				
Mean cIMT (mean CIMT (120,150), mean CIMT (210,240)) (μm)	(N=42726) 685±125	(n=22436) 663 ±110	(n=20290) 708 ±135	<0.001
Pulse wave arterial stiffness index* (s/m)	(N=35863) 9.45(7.45,11)	(n=18526) 8.88(7,10.76)	(n=17337) 10 (8,11.89)	<0.001
Demographic data				
Age (year)	(N=42726) 63.8±7.6	(n=22436) 63.2±7.5	(n=20290) 64.5±7.7	<0.001
Townsend deprivation index at recruitment	(N=42684) -1.90±2.72	(n=22413) -1.86±2.72	(n=20271) -1.94±2.73	0.002
Anthropometric measurements				
Waist circumference (cm)	(N=41442) 87.9±12.5	(n=21740) 82.5±11.6	(n=19702) 93.8±10.4	<0.001
Hip circumference (cm)	(N=41443) 100.64±8.62	(n=21740) 100.73±9.73	(n=19703) 100.54 ±7.20	0.027
BMI (kg/m^2)	(N=41318) 26.39±4.29	(n=21671) 25.99±4.67	(n=19647) 26.84±3.79	<0.001
Blood pressure measurements				
Systolic blood pressure (mmHg)	(N=37664) 138 ±18.5	(n=19700) 135±19	(n=17964) 141±17.3	<0.001
Diastolic blood pressure(mmHg)	(N=37664) 78.7±10	(n=19700) 77±9.9	(n=17964) 80.6±9.8	<0.001
Diabetic measurements				
Glucose* (mmol/L)	(N=36427) 4.88 (4.56,5.22)	(n=18985) 4.86 (4.55,5.19)	(n=17442) 4.90 (4.90,5.26)	<0.001
HbA1c (mmol/mol)	(N=39676) 34.9±5	(n=20760) 34.75±4.63	(n=18916) 35.22 ±5.55	<0.001
Enzyme measurements				
Alanine aminotransferase* (U/L)	(N=39958) 19.59 (15,26)	(n=20981) 16.74 (13.36,21.66)	(n=18977) 23.40 (18.29,31.26)	<0.001
Aspartate aminotransferase* (U/L)	(N=39804) 24 (20.80,28.4)	(n=20903) 22.50 (19.70,26.10)	(n=18901) 26 (22.60,30.50)	<0.001
Lipid measurements				
Total cholesterol (mmol/L)	(N=39965) 5.73±1	(n=20981) 5.86±1.07	(n=18984) 5.60 ±1.08	<0.001

Continuous variables		Total Cohort (Number) Mean± SD	Female (Number) Mean± SD	Male (Number) Mean± SD	*P*-value
Lipid measurements					
HDL cholesterol (mmol/L)		(N=36464) 1.48±0.38	(n=19006) 1.64±0.37	(n=17458) 1.31±0.30	<0.001
LDL cholesterol (mmol/L)		(N=39875) 3.58±0.83	(n=20932) 3.59±0.82	(n=118943) 3.57±0.83	0.014
Triglyceride (mmol/L)		(N=39935) 1.64±0.96	(n=20970) 1.41±0.76)	(n=18965) 1.9±1	<0.001
Apolipoprotein-A (g/L.)		(N=36267) 1.55±0.26	(n=18822) 1.65±0.26	(n=17445) 1.44±0.22	<0.001
Apolipoprotein-B (g/L.)		(N=39775) 1.03±0.23	(n=20915) 1.02±0.23	(n=18860) 1.04±0.23	<0.001
Inflammatory measurements					
C-reactive protein* (mg/L)		(N=39880) 1.06 (0.54,2.13)	(n=20941) 1.06 (0.53,2.26)	(n=18939) 1.06 (0.56,1.99)	0.041
Vitamins					
Vitamin D*(nmol/L.)		(N=38399) 48 (33.8,63.3)	(n=19997) 47.60 (33.6,63.1)	(n=18420) 48.30 (34,63.50)	0.011
Categorical variables		**Total Cohort**	**Female**	**Male**	***P*-value**
		Count (%)	**Count (%)**	**Count (%)**	
Diabetes at imaging from self-reporting or taking medications	No	40567 (94.9%)	21662 (96.6%)	18905 (93.2%)	<0.001
	Yes	2159 (5.1%)	774 (3.4%)	1385 (6.8%)	
Hypertension, measured or on anti-hypertensives	No	17702 (41.4%)	10859 (48.4%)	6843 (33.7%)	<0.001
	Yes	25024 (58.6%)	11577 (51.6%)	13447 (66.3%)	
Smoking	Never	26513 (62.7%)	14647 (66%)	11866 (59%)	<0.001
	Former	14299 (33.8%)	6901 (31.1%)	7398 (36.8%)	
	Current	1481 (3.5%)	648 (2.9%)	833 (4.1%)	
Ethnicity	White	41341 (97%)	21738 (97.1%)	19603 (96.9%)	<0.001
	Black	281 (0.7%)	147 (0.7%)	134 (0.7%)	
	South Asian	383 (0.9%)	142 (0.6%)	241 (1.2%)	
	Other	613 (1.4%)	366 (1.6%)	247 (1.2%)	
Statin use	No	32907 (77%)	19086 (85.1%)	13821 (68.1%)	<0.001
	Yes	9819 (23%)	3350 (14.9%)	6469 (31.9%)	

Data are expressed as Mean ± SD for normal distribution data and median (interquartile range) for non -distribution data, * non-normal distribution data

5.5.1.2CIMT measurements

A total of 47,726 participants had a valid measure of cIMT. There were significant differences in cIMT between males and females at all angles, although the average thickness at different angles was similar within the same sex. Therefore, in line with previous studies, it was decided to select the mean of all angles as the outcome for this study. Table (5-2) summarizes the results

Data from this study confirmed a well-documented significant difference in mean cIMT between males and females. Males generally had a higher value of mean cIMT than females. The mean cIMT value for males was 45.5 µm, 54.7 µm and 36.3µm for all angles, combined left angles, and combined right angles respectively, which was higher than that of females. The cIMT of males was significantly thicker than those of females. The mean of cIMT in males and females was 0.708 and 0.663 mm respectively. In addition, males represented 70% and females 29% of the abnormal cIMT group. A histogram representing the data is shown in (Figure 5-4).

Table 5-2:Carotid intima media thickness measurements stratifed by gender

Continuous variables	**Total Cohort N ≈ 42726**	**Females N ≈22436**	**Males N ≈20290**	***P value***
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	
Mean cIMT 120 (right), µm	682.6±144.4	663.4±128.8	703.8±157.2	<0.001
Mean cIMT 150 (right), µm	675.6±140.8	660.3±126.8	692.5±153	<0.001
Mean cIMT (120,150) (right), µm	679.1±133.6	661.9±119.2	698.2±145.6	<0.001
Mean cIMT 210 (left), µm	690±153	666±135.9	716.8±165.8	<0.001
Mean cIMT 240 (left), µm	691.7±157.5	663.8±138.6	722.5±171	<0.001
Mean cIMT (210,240) (left), µm	690.9±146.4	664.9±128.8	719.6±158.7	<0.001
Mean cIMT (mean cIMT (120,150), mean cIMT (210,240)), µm	685±125	663.4±110.9	708.9±135.1	<0.001

cIMT: carotid intima media thickness, **SD:** standard deviation.

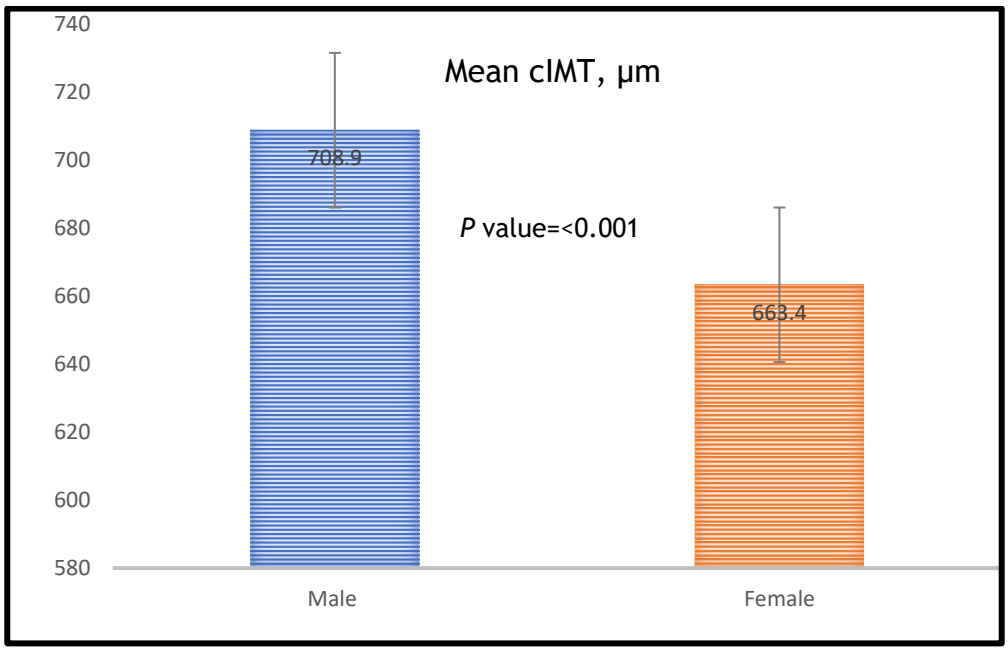


Figure 5-4 Mean cIMT difference between males and females

5.5.1.3 Characteristics of study population stratified by normal and abnormal cIMT

The distribution of the demographic and clinical characteristics of the study population according to normal and abnormal cIMT is shown in Table (5-3). The dataset was classified into normal and abnormal cIMT values. Normal cIMT was defined as mean cIMT was less than 0.9 mm, while abnormal cIMT was defined as a mean cIMT was more than 0.9 mm. In this study, the number of subjects who had a cIMT value of less than 0.9mm was 40,140, while the number of subjects who had a cIMT value of more than 0.9mm was 2,586.

The data showed that subjects with abnormal cIMT values had significantly poorer CVD risk profiles than subjects with normal cIMT values.

This included age (5.7 year older), systolic blood pressure (11.1 mmHg higher), glucose (0.06mmol/L higher), HbA1c (1.12 mmol/mol higher), waist circumference (4.74 cm higher), hip circumference (0.81cm higher) and BMI (0.75 kg/m^2 higher). As well, lipid parameters were higher, including apolipoprotein-B (0.06g/L higher), triglycerides (0.25mmol/L higher), total cholesterol (0.14mmol/L higher) and LDL (0.18 mmol/L higher). Subjects with normal cIMT values had higher HDL cholesterol (0.1 mmol/L higher) and higher concentration from apolipoprotein-A (0.06 g/L higher).

For liver enzymes, subjects with abnormal cIMT values had a higher concentrations of liver enzyme including alanine aminotransferase (2.3 U/L higher) and aspartate aminotransferase (1.3 U/L higher). In addition, they had higher concentrations of C reactive protein (0.23 mg/L higher) and vitamin D (1.2 nmol/L higher)

The prevalence of diabetes in this study population was significantly higher in abnormal cIMT (8.4%) compared to normal cIMT (4.8%). In addition, the prevalence of hypertension in this study population was significantly higher in abnormal cIMT (79%) compared to normal cIMT (57.3%). Furthermore, a significantly higher percentage of subjects in the abnormal cIMT group (34.8%) had received statin therapy compared to the normal cIMT group (22.2%).

In terms of smoking, a higher percentage of subjects in the normal group had never smoked (63%) compared to the abnormal group (52%). There was a higher percentage of former smokers (44%) in the abnormal group compared to the normal group (33%) and a significantly higher percentage of current smokers (4%) in the abnormal group compared to the normal group (3.5%). In addition, 97% of participants in the normal and abnormal cIMT categories were of white ethnicity. There was a higher percentage of males (71%) compared to females (29%) in the abnormal cIMT group, and a higher percentage of females (55%) compared to males (45%) in the normal cIMT group

Table 5-3: Demographic and clinical characteristics of study population stratified by normal and abnormal cIMT

Continuous variables	Normal mean cIMT<900 µm Number (mean± SD)	Abnormal mean cIMT>900µm Number (mean± SD)	*P value*
Non-invasive measurement of atherosclerosis			
Mean CIMT (mean CIMT (120,150), mean CIMT (210,240)) (µm)	(**n**=40140) 665.6±100	(**n**=2586) 985.3±85.6	<0.001
Pulse wave arterial stiffness index	(**n**=33726) 9.41(7.43,11.34)	(N=2137) 10(7.89,12.03)	<0.001
Demographic data			
Age (year)	(**n**=40140) 63.5±7.6	(**n**=2586) 69.2±6.3	<0.001
Townsend deprivation index at recruitment	(**n**=40100) -1.89 ±2.73	(**n**=2584) -2.04±2.69	0.004
Anthropometric measurements			
Waist circumference (cm)	(**n**=38948) 87.67±12.47	(**n**=2494) 92.41±12	<0.001
Hip circumference(cm)	(**n**=38949) 100.59±8.65	(**n**=2494) 101.40±8.03	<0.001
BMI (kg/m^2)	(**n**=38832) 26.35±4.29	(**n**=2486) 27.11±4.21	<0.001
Blood pressure measurements			
Systolic blood pressure (mmHg)	(**n**=35431) 137 ±18.3	(**n**=2233) 148 ±19	<0.001
Diastolic blood pressure(mmHg)	(**n**=35431) 78.7±10	**(n**=2233) 78.7±10.1	0.89
Diabetic measurements			
Glucose (mmol/L)	(**n**=34221) 4.88 (4.56,5.22)	(**n**=2206) 4.94(4.61,5.29)	<0.001
HbA1c (mmol/mol)	(N=37282) 35±5	(**n**=2394) 36.12±5.49	<0.001
Enzymes measurements			
Alanine aminotransferase (U/L)	(**n**=37555) 19.44 (14.9,26.4)	(**n**=2403) 21.74 (16.89,28.51)	<0.001
Aspartate aminotransferase (U/L)	(**n**=37410) 24 (20.8,28.3)	(**n**=2394) 25.30 (21.9,29.7)	<0.001
Lipid measurements			
Total cholesterol (mmol/L)	(**n**=37563) 5.72±1	(**n**=2402) 5.86±1.14	<0.001

Data are expressed as (mean ± SD) for normal-distribution data and *non-normal distribution data are expressed as number, median (IQR), statistical tests are independent t test for normal; distribution data and Wilcoxon rank-sum test for non-normal distribution data.

Continuous variable		Normal mean cIMT<900 µm (Number) mean± SD	Abnormal mean cIMT>900 µm (Number) mean±, SD	*P-value*
Lipid measurements				
HDL cholesterol (mmol/L)		(**n**=34258) 1.48±0.38	(**n**=2206) 1.38±0.35	<0.001
LDL cholesterol (mmol/L)		(**n**=37475) 3.57±0.82	(**n**=2400) 3.75±0.86	<0.001
Triglyceride (mmol/L)		(**n**=37534) 1.37(0.98,1.99)	(**n**=2401) 1.62(1.14,2.28)	<0.001
Apolipoprotein-A (g/L.)		(**n**=34066) 1.55±0.27	(**n**=2201) 1.49±0.25	<0.001
Apolipoprotein-B (g/L.)		(**n**=37376) 1.03±0.23	(**n**=2399) 1.09±0.23	<0.001
Inflammatory markers				
C-reactive protein (mg/L)		(**n**=37482) 1.05(0.54,2.11)	(**n**=2398) 1.28(0.68,2.45)	<0.001
Vitamins				
Vitamin D (nmol/L.)		(**n**=36132) 48 (33.70,63.20)	(**n**=2267) 49.30 (34.90,64.30)	0.006
Categorical variables		**Normal mean count, %**	**Abnormal mean count, %**	***P-value***
Diabetes	No	3819 (95.2%)	2369 (91.6%)	<0.001
	Yes	1942 (4.8%)	217 (8.4%)	
Hypertension	No	17159 (42.7%)	423 (21 %)	<0.001
	Yes	22981 (57.3%)	2043 (79%)	
Smoking	Never	25194 (63.4%)	1319 (51.9%)	<0.001
	Former	13177 (33.1%)	1122 (44.1%)	
	Current	1380 (3.5%)	101 (4%)	
Ethnicity	White	38837 (97%)	2504 (97.2%)	0.009
	Black	256 (0.6%)	25 (1%)	
	South Asian	358 (0.9%)	25 (1%)	
	Other	592 (1.5%)	21 (0.8%)	
Statin	No	31222 (77.8%)	1685 (65.2%)	<0.001
	Yes	8918 (22.2%)	901 (34.8%)	
Sex	Male	10319 (45%)	927 **(**71%)	<0.001
	Females	12405 (55%)	381 (29%)	

Data for categorical variables are expressed as account (percentage), Statistical tests are 2-proportion test and χ^2 test

5.5.1.4 Characteristics of the study population stratified according to cIMT quartiles

Table (5-4) and Table (5-5) summarise the demographic and risk factor characteristics as a function of cIMT quantiles. The 25th, 50th, 75th percentiles of the summary cIMT measure were 594, 665, 757, respectively; these cut-off points were used to categorize the cIMT quintiles. This study showed that in general, the cardiovascular risk profile worsened across the cIMT quantiles from lowest to highest. Specifically, the highest cIMT quantiles group had a worse CVD risk factors profile compared to the lowest cIMT quantile including, age (9 year older), systolic blood pressure (15 mmHg higher), diastolic blood pressure (0.9 mmHg higher), glucose(0.09 mmol/L higher), HbA1c (1.78 mmol/mol higher), hip circumference(1 cm higher),waist circumference (5 cm higher) and BMI (1 kg/m^2 higher). In addition, subjects in highest cIMT quantile groups had a poorer lipid profile compared to the lowest cIMT quantile group. This included apolipoprotein-B (0.07g/L higher), apolipoprotein-A (0.05g/L lower), triglycerides (0.27mmol/L higher), total cholesterol (0.23mmol/L higher), LDL (0.26 mmol/L higher) and HDL (0.08 mmol/L lower).

Regarding liver enzymes, subjects in highest cIMT quantile group had higher value of alanine aminotransferase (2.96U/L higher) and aspartate aminotransferase (1.8 U/L higher) compared to the lowest cIMT quantile group. Also, they had a higher C-reactive protein value (1.23 mg/L higher) and vitamin D value (2.7 nmol/L higher).

The prevalence of diabetes in this study population was significantly higher in a highest cIMT quantile group (6.7%) compared to lowest cIMT quantile group (3.5%). Also, the prevalence of hypertension in this study population was significantly higher in a highest cIMT quantile group (74%) compared to lowest cIMT quantile group (42.7%). Furthermore, there was significantly higher percentage of subjects who had received statin therapy in highest cIMT quantile group (31.7%) compared to lowest cIMT quantile group (14.8%).In comparison with lower cIMT quantile group, There was significantly lower proportion of subjects who never smoked (56.6%) in highest cIMT quantile group compared to lowest cIMT quantile group (68%), while, there was higher

proportion of subjects who were former smokers in highest quantile group (39.8%) compared to lowest quantile group (28.2%). The majority of participants were white in each of the cIMT quantiles. In addition, there was a higher percentage of males (75.2%) compared to females (24.8%) in the highest quantile group. Also, there was a higher percentage of females (61.7%) compared to males (38.3%) in the lowest quantile group.

Table 5-4: Demographic and clinical characteristics of the study population (continuous variables) according to cIMT quartiles

Continuous variables	**mean cIMT (150.5-594) µm Number (mean± SD)**	**mean cIMT (594.25-665) µm Number (mean± SD)**	**mean cIMT (665.25-756.75) µm Number (mean± SD)**	**mean cIMT (757-2126.25) µm Number (mean± SD)**	***P value***
Non-invasive measurement of atherosclerosis					
Mean cIMT (mean cIMT (120,150), mean cIMT (210,240)) µm	(**n**=10701), 546 ±35.1	(**n**=10663), 629 ±20	(**n**=10692), 708±26	(**n**=10670), 856±91	<0.001
Pulse wave arterial stiffness index*	(**n**=9058), 9.03(7.26,10.86)	(**n**=8982), 9.35(7.38,11.20)	(**n**=8910), 9.61(7.56,11.54)	(**n**=8913),9.83(7.74,11.86)	<0.001
Demographic data					
Age (year)	(**n**=10701),59.3±7	(**n**=10663), 62.7±7.1	(**n**=10692), 65.5±7	(**n**=10670), 67.9±6.6	<0.001
Townsend deprivation index	(**n**=10686), -1.77±2.78	(**n**=10650), -1.86±2.72	(**n**=10685), -1.97±2.70	(**n**=10663), -1.98±2.69	<0.001
Anthropometric measurements					
Waist circumference (cm)	(**n**=10417), 85.5±12.25	(**n**=10368), 86.8±12.12	(**n**=10358), 88.3±12.55	(**n**=10299), 91±12.19	<0.001
Hip circumference (cm)	(**n**=10418), 99.89±8.75	(**n**=10368),100.47±8.62	(**n**=10358), 100.88±8.68	(**n**=10299), 101.8±8.36	<0.001
BMI (kg/m2)	(**n**=10385), 25.9±4.29	(**n**=10344), 26.3±4.25	(**n**=10324), 26.5±4.32	(**n**=10265), 26.9±4.25	<0.001
Blood pressure measurements					
Systolic blood pressure (mmHg)	(**n**=9522), 130±16.4	(**n**=9466), 136±17.4	(**n**=9351), 140±18	(**n**=9325), 146±18.9	<0.001
Diastolic blood pressure	(**n**=9522), 78±9.7	(**n**=9466), 78.9±10	(**n**=9351), 78.9±10	(**n**=9325), 78.9±10	<0.001
Diabetic measurements					
Glucose *(mmol/mol)	(**n**=9069), 4.83(4.53,5.16)	(**n**=9128),4.87(4.55,5.21)	(**n**=9096), 4.89(4.58, 5.23)	(**n**=9134), 4.92(4.60,5.28)	<0.001
HbA1c (mmol/mol)	(**n**=9931), 34±4.41	(**n**=9877), 34.77±4.87	(**n**=9962), 35.22±5.20	(**n**=9906), 35.78±5.67	<0.001

Data are expressed as number (mean ± SD) for normal distribution, *non-normal distribution data are expressed as number, median (IQR), Statistical tests are one-way ANOVA for normal-distribution, and the Kruskal-Wallis test for non-distribution data,

Continuous variable	Mean cIMT <588µm Number (mean± SD)	Mean cIMT (588-658) µm Number (mean± SD)	Mean cIMT (658-748) µm Number (mean± D)	Mean cIMT >748µm Number (mean± SD)	*P- value*
Enzyme measurements					
Alanine aminotransferase* (U/L)	(**n**=9987), 18.31(14, 25.22)	(**n**=9979),18.9 (14.67,25.86)	(**n**=10004), 19.98(15.36,26.83)	(**n**=9988), 21(16.43,28.09)	<0.001
Aspartate aminotransferase* (U/L)	(**n**=9938), 23.2(20.10,27.50)	(**n**=9943), 23.80(20.5,28)	(**n**=9966), 24.40(21,28.50)	(**n**=9957), 25(21.70,29.20)	<0.001
Lipid measurements					
Total cholesterol (mmol/L)	(**n**=9988), 5.6±1.04	(**n**=9983), 5.7±1.07	(**n**=10006), 5.78±1.07	(**n**=9988), 5.83±1.12	<0.001
HDL cholesterol (mmol/L)	(**n**=9080), 1.50±0.37	(**n**=9136), 1.51±0.38	(**n**=9105),1.49±0.38	(**n**=9143), 1.42±0.36	<0.001
LDL cholesterol (mmol/L)	(**n**=9969), 3.47±0.80	(**n**=9962), 3.56±0.82	(**n**=9980), 3.61±0.82	(**n**=9964), 3.73±0.85	<0.001
Triglyceride*	(**n** ≈9980), 1.26 (0.91, 1.86)	(**n** ≈9975), 1.34 (0.95,1.92)	(**n**≈10001),1.42 (1.02,2.04)	(**n**=9979), 1.53 (1.10, 2.19)	<0.001
Apolipoprotein-A(g/L)	(**n**=9024), 1.56±0.26	(**n**= 9086), 1.56±0.27	(**n**=9055), 1.56±0.27	(**n**=9102), 1.51±0.25	<0.001
Apolipoprotein-B (g/L)	(**n**=9933), 1±0.22	(**n**=9927), 1.02±0.23	(**n**=9956), 1.04±0.23	(**n**=9959), 1.07±0.23	<0.001
Inflammatory markers					
C-reactive protein (mg/L)*	(**n**=9969), 0.95(0.48, 1.94)	(**n**=9960), 1.02(0.53,2.06)	(**n**=9983), 1.11(0.57,2.20)	(**n**=9968), 2.18(1.2,3.63)	<0.001
Vitamins					
Vitamin D*(nmol/L)	(**n**=9680), 46.20 (32.30, 62.10)	(**n**=9611), 48.20(33.80,63.20)	(**n**=9589), 48.80(34.60,63.50)	(**n**=9519), 48.90(34.40,64.10)	<0.001

Data are expresses as number (mean ± SD) for normal distribution, *non-normal distribution data are expressed as number, median (IQR), Statistical tests are one-way ANOVA for normal-distribution, and the Kruskal-wallis test for non-distribution data,

Table 5-5: Demographic and clinical characteristic of study population (categorical variables) according to cIMT quartiles.

Categorical Variable		**Mean cIMT <588µm Number (mean± SD)**	**Mean cIMT (588-658) µm Number (mean± SD)**	**Mean CIMT (658-748) µm Number (mean± D)**	**Mean cIMT >748µm Number (mean± SD)**	***P- value***
Diabetes	No	10327(96.5%)	10198(95.6%)	10089(94.4%)	9953(93.3%)	<0.001
	Yes	374(3.5%)	465(4.4%)	603(5.6%)	717(6.7%)	
Hypertension	No	6130 (57.3%)	4839(45.4%)	3946(36.9%)	2787(26.1 %)	<0.001
	Yes	4571(42.7%)	5824(54.6%)	6746(63.1%)	7883(73.9%)	
Smoking	Never	7237(68.1%)	6802(64.4%)	6519(61.6%)	5955(56.6%)	<0.001
	Former	3002(28.2%)	3386(32.1%)	3728(35.2%)	4183(39.8%)	
	Current	388(3.7%)	376(3.6%)	336(3.2%)	381(3.6%)	
Statin	No	9122(85.2%)	8498(79.7%)	8003(74.9%)	7284(68.3%)	<0.001
	Yes	1579(14.8%)	2165(20.3%)	2689(25.1%)	3386(31.7%)	
Ethnicity	White	10280 (96.3%)	10316(97%)	10395(97.5%)	10350(97.3%)	<0.001
	Black	52 (0.5%)	67(0.6%)	68(0.6%)	94(0.9%)	
	South Asian	134 (1.3%)	99(0.9%)	78(0.7%)	72(0.7%)	
	Other	211 (2%)	156(1.5%)	124(1.2%)	122(1.1%)	
sex	Female	4305 (61.7%)	4715 (58%)	3658 (43.1%)	108 (24.8%)	<0.001
	Male	2673 (38.3%)	3414 (42%)	4831 (56.9%)	328 (75.2%)	

Categorical data are expressed as account (percentage), chi-χ^2 test, for categorical data

5.5.1.5Relationship of mean cIMT and cardiometabolic risk parameters in all study subjects

5.5.1.5.1 Correlations

This section investigates the association between mean cIMT and CVD risk factors. Bivariate Pearson correlation was carried out among all continuous variables. Mean cIMT showed a significant positive linear association with HbA1c (r=0.122), diastolic blood pressure (r=0.026), cholesterol (r=0.076), triglyceride (r=0.129), LDL (r=0.104) and liver enzymes such as alanine aminotransferase (r=0.103), aspartate aminotransferase (r=0.097), as well as c-reactive protein (r=0.080), apolipoprotein B (r=0.124), vitamin D (r=0.0414), and anthropometric measurements (waist, r=0.165, hip, r=0.059, BMI, r=0.084). However, there was a negative linear relationship between cIMT and other CVD risk factors, including HDL (r=-0.093), apoprotein A (r=-0.068), and Townsend deprivation index (r=-0.031). Age and systolic blood pressure showed the strongest correlation with mean cIMT (r=0.412, r=0.307) respectively compared to other CVD risk factors. The results of the correlation between mean cIMT and CVD risk factors are shown in Table (5-6).

5.5.1.5.2 CIMT, single factor analysis in the whole cohort

To identify the predictors of cIMT in all subjects, univariable regression analysis was carried out for all variables that met statistical significance in bivariate Pearson correlation (Table 5-6). The predictors that showed a significant association with mean cIMT including age (ß=+0.413, *P*=<0.001), systolic blood pressure (ß=+0.307, *P*=<0.001), male (ß=+0.18, *P*=<0.001), and body mass index (ß=+0.092, P=<0.001). In addition, the mean of cIMT had a significant positive association with other cardiometabolic parameters, including anthropometric measurement (waist circumference: ß=+0.194, *P*=<0.001, hip circumference: ß=+0.084, *P*=<0.001), glucose (ß=+0.059, *P*=<0.001), HbA1c (ß=+0.127, *P*=<0.001). Moreover, mean cIMT was significantly positive associated with lipid markers, including LDL cholesterol (ß=+0.104, *P*=<0.001), triglycerides (ß=+0.129, *P*=<0.001) and apolipoprotein-B (ß=+0.124, *P*=<0.001). However, it was inversely associated with HDL (ß=-0.092, *P*=<0.001), and apolipoprotein-A (ß=-0.067, P=<0.001). cIMT also had an association with liver function makers; mean cIMT

was significantly associated with liver enzymes (Alt: ß=+0.103, P=<0.001, AST: ß=+0.097, P=<0.001). In addition, mean cIMT was significantly positive associated with vitamin D and C-reactive protein (ß=+0.08, P=<0.001), (ß=+0.031, P=<0.001) respectively.

When ranked by standardised beta coefficients, the most important univariable determinants for cIMT were age (B=+0.413), Figure (5-5), shows a scatterplot of linear regression of age with mean carotid intima media thickness. Then, systolic blood pressure (B=+0.307), Figure (5-6) shows a scatterplot of linear regression of systolic blood pressure with mean carotid intima media thickness. After that, male (B=+0.18) and waist circumference (B=+0.164). The results are shown in Table (5-7) for univariable analysis for CVD risk factors and cIMT.

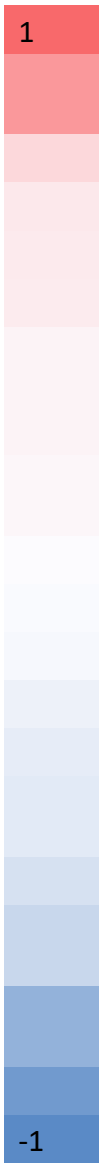
5.5.1.5.3 Multiple regression model of cIMT with cardiovascular risk factors

This section identified the covariables associated with carotid intima media thickness in adjusted models (multivariate), as shown in Table (5-8). The stepwise model ended with these factors: age, gender, systolic blood pressure, diabetes, hip circumference, vitamin-D, apolipoprotein-A, apolipoprotein-B, hypertension and smoking. Following this, waist circumference was dropped due to collinearity.

After performing a stepwise multiple regression model, the risk factors identified as a strongest predictive factors for cIMT were: age (for every 1 SD increases in age, the mean cIMT increases by (0.357μm) SD), systolic blood pressure (for every 1 SD increase in systolic blood pressure, the mean cIMT increases by (0.115 μm) SD) and sex (mean cIMT increases by (0.124 μm) SD in male subjects compared with females). Overall, this model, including age, gender, systolic blood pressure, diabetes, hip circumference, vitamin-D, apolipoprotein-A, apolipoprotein-B, hypertension, and smoking, explained 22% of the variance in cIMT in the UK Biobank population.

Table 5-6: Heatmap representation of the Pearson correlation coefficients of mean cIMT with clinical characteristics in the whole cohort

Continuous variables	Mean cIMT	
	Pearson correlation (r)	*P-value*
Age (year)	0.4129	<0.001
Systolic blood pressure (mmHg)	0.3074	<0.001
Waist circumference (cm)	0.1648	<0.001
Log-Triglycerides(mmol/L)	0.129	<0.001
Apolipoprotein-B (g/L.)	0.1248	<0.001
HbA1c (mmol/mol)	0.1221	<0.001
LDL cholesterol (mmol/L)	0.1041	<0.001
Log-Alanine aminotransferase (U/L)	0.1035	<0.001
Log-Aspartate aminotransferase (U/L)	0.0974	<0.001
BMI (kg/m^2)	0.0847	<0.001
Log-C-reactive protein(mg/L)	0.0801	<0.001
Total cholesterol (mmol/L)	0.0766	<0.001
log-Glucose (mmol/L)	0.0663	<0.001
Hip circumference (cm)	0.059	<0.001
Pulse wave velocity at baseline (m/s)	0.055	<0.001
Log Vitamin D*(nmol/L.)	0.0414	<0.001
Diastolic blood pressure (mmHg)	0.0264	<0.001
Townsend deprivation index at recruitment	-0.0309	<0.001
Apolipoprotein-A(g/L.)	-0.0675	<0.001
HDL cholesterol (mmol/L)	-0.0925	<0.001



r: Pearson correlation coefficient. **BMI**: body mass index, **HbA1c:** glycated haemoglobin, **LDL:** low density lipoprotein, **HDL:** high density lipoprotein

Table 5-7: The univariable linear regression analyses for variables characterising participants in the whole cohort (independent variables) and cIMT

Continuous variables	Un-Standardized Beta coefficient				Standardised (b) coefficient	*P*-value	R^2%
	Regression estimate		95% CI				
			lower	Upper			
Age (year)	I	252	243	261	0.413	<0.001	17%
	B	6.773	6.631	6.915			
Anthropometric measurements							
Waist circumference(cm)	I	539	531	548	0.164	<0.001	16%
	B	1.648	1.553	1.743			
Hip circumference(cm)	I	598	584	612	0.058	<0.001	0.3%
	B	0.854	0.715	0.993			
BMI (kg/m2)	I	619	612	627	0.084	<0.001	0.7%
	B	2.465	2.185	2.745			
Blood pressure measurements							
Systolic blood pressure (mmHg)	I	398	389	407	0.307	<0.001	9.4%
	B	2.06	1.997	2.126			
Diastolic blood pressure(mmHg)	I	658	64	668	0.026	<0.001	0.07%
	B	0.327	0.201	0.452			
Diabetic measurements							
Log-Glucose*(mmol/L)	I	597	583	610	0.066	<0.001	0.4%
	B	55.21	46.67	63.74			
HbA1c (mmol/mol)	I	580	571	588	0.122	<0.001	1.4%
	B	2.999	2.75	3.23			
Enzymes measurements							
Log-Alanine aminotransferase* (U/L)	I	598	590	606	0.103	<0.001	1%
	B	28.69	25.98	31.39			
Log-Aspartate aminotransferase* (U/L)	I	537	522	551	0.097	<0.001	0.9%
	B	46.14	41.51	50.77			
Lipid measurements							
Total cholesterol (mmol/L)	I	634	627	640	0.076	<0.001	0.5%
	B	8.86	7.73	9.99			
HDL cholesterol (mmol/L)	I	730	725	735	-0.092	<0.001	0.8%
	B	-30.70	-34.10	-27.315			
LDL cholesterol (mmol/L)	I	628	623	633	0.104	<0.001	1%
	B	15.73	14.25	17.20			

I: Intercept, **B**: beta coefficients, per unit increase of predictor, Standardized (b) coefficient: mean change in cIMT (µm) per 1 SD increase in predictor variable

Continuous variables		Un-Standardized (B)coefficient			Standardized (b) coefficients	*P value*	R^2%
		Regression estimate	95% CI				
			Lower	Upper			
Lipid measurements							
Apolipoprotein-A(g/L.)	I	73	727	742	-0.067	<0.001	0.4%
	B	-31.94	-36.79	-27.084			
Apolipoprotein-B (g/L.)	I	614	608	620	0.124	<0.001	1.5%
	B	68.240	62.906	73.574			
Log-Triglycerides(mmol/L)	I	673	672	675	0.129	<0.00	1.6%
	B	70	69	72			
Other markers							
Log-C-reactive-protein (mg/L)*	I	683	682	68	0.080	<0.001	0.6%
	B	9.847	8.644	11.051			
Log-Vitamin D*(nmol/L.)	I	682	680	683	-0.031	<0.001	0.1%
	B	-1.420	-1.855	-0.984			

Categorical variables		Un-standardized (B)coefficient and 95% CI			Standardized (b) coefficients	*P value*	R^2%
		RE	95% CI				
			Lower	Upper			
Diabetes	Reference	683	681	684.37	0.0634	<0.001	0.4%
	Yes	36.232	30.82	41.63			
Hypertension	Reference	650	648.37	651.95	0.234	<0.001	5.4%
	Yes	59.46	57.12	61.80			
Smoking	Reference	675.42	673.93	676.91	0.099	<0.001	0.96%
	Former	26.13	23.61	28.66			
	Current	10.28	3.78	28.66	0.0151	0.002	
Ethnicity	Reference	685.43	684.22	686.63	0.0162	0.001	0.14%
	Black	25.06	10.40	39.72			
	South Asian	-28.35	-40.93	-15.78	-0.0214	<0.001	
	Other	-27.31	-37.28	-17.35	-0.026	<0.001	
Statin	Reference	674	673.38	676.05	0.150	<0.001	2.2%
	Yes	44.69	41.90	47.48			
Gender	Reference	663	661.76	664.98	0.18	<0.001	3.3%
	Male	45.52	43.18	47.85			

I: Intercept, **B**: beta coefficients, per unit increase of predictor, Standardized (b)coefficient: mean change in cIMT (µm) per 1 SD increase in predictor variable, **RE**: regression estimate

Table 5-8: Stepwise regression analysis for variables characterising participants (independent variables) with cIMT

Variables	Unstandardized (B) Coefficients (95.0% Confidence Interval for B)			Standardized (b) Coefficients	*P-value*
	B	95.0% Confidence Interval			
		Lower	Upper		
Reference	153	125	181		<0.001
sex	25.50	21.04	27.04	0.124	<0.001
Age (year)	5.87	5.66	6.09	0.357	<0.001
Systolic blood pressure (mmHg)	0.77	0.678	0.862	0.115	<0.001
Diabetes	10.97	3.50	18.45	0.019	0.004
Hip circumferences (cm)	0.744	0.544	0.945	0.049	<0.001
Apolipoprotein-B(g/L)	32.99	25.27	40.71	0.055	<0.001
Hypertension	8.76	5.11	12.40	0.034	<0.001
Apolipoprotein-A (g/L)	-25.224	-31.970	-18.47	-0.053	<0.001
Smoking					
Former	10.96	8.33	13.59	0.0418	<0.001
Current	21.49	14.77	28.20	0.0317	<0.001
Vitamin D	0.169	0.093	0.245	0.029	<0.001
R^2	**0.216**				

Stepwise (Criteria: Probability of-F-to enter <=0.050, Probability of-F-to-remove >=0.100).

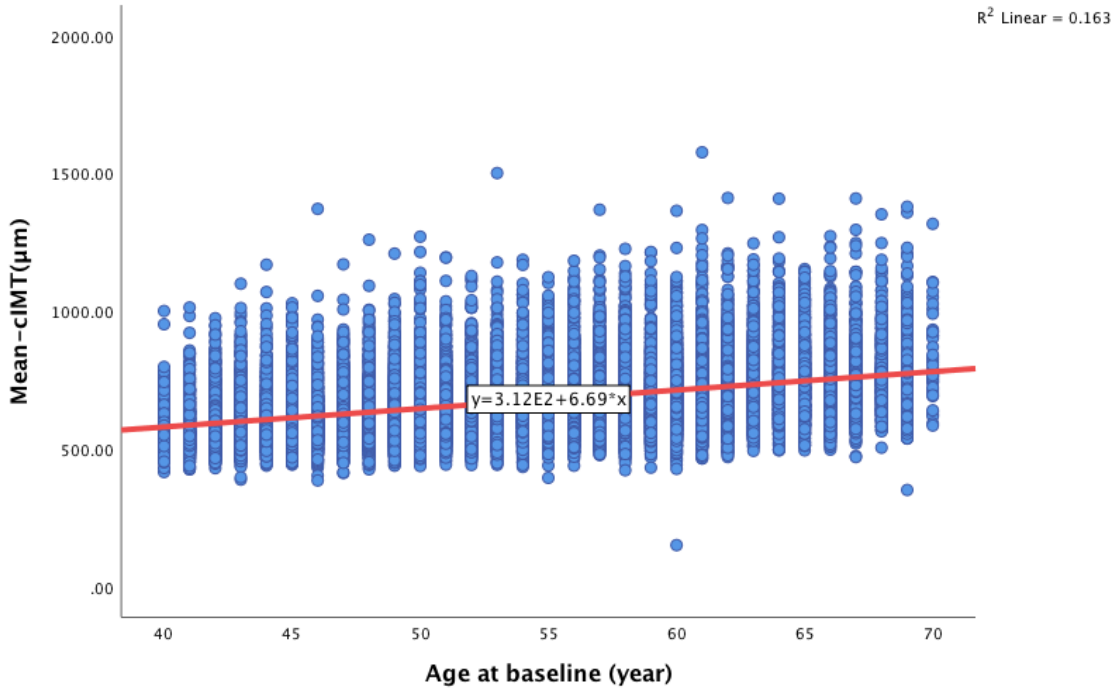


Figure 5-5 Scatterplot of linear regression of age with mean carotid intima media thickness

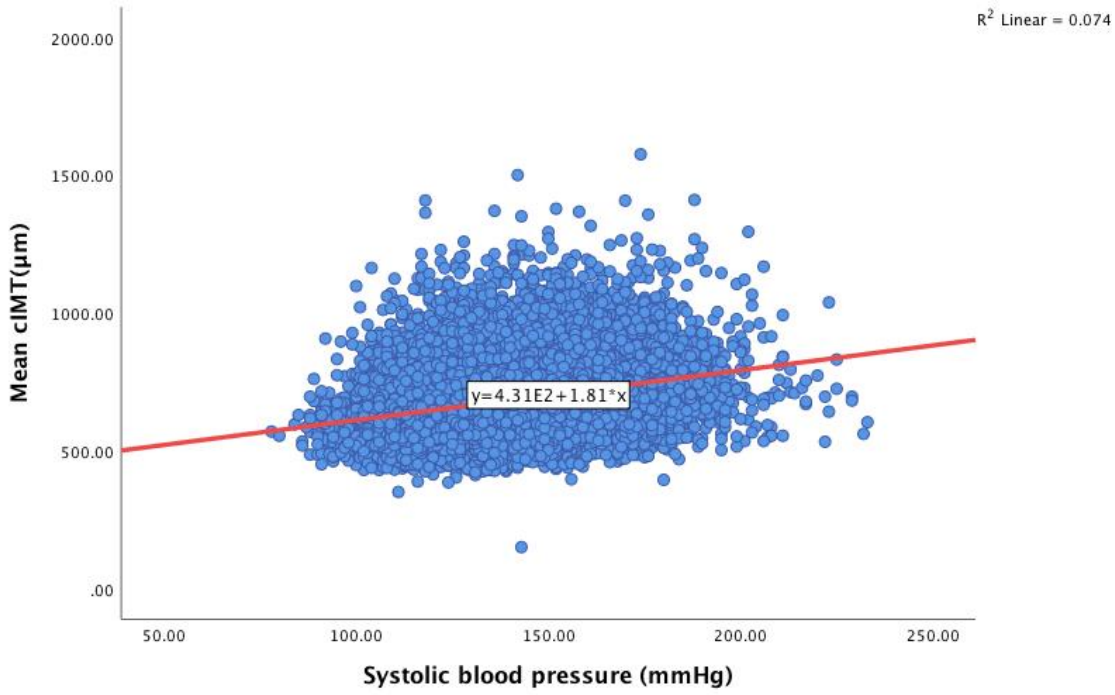


Figure 5-6 Scatterplot of linear regression of systolic blood pressure with mean carotid intima media thickness

5.5.1.6 Relationship of mean cIMT and cardiometabolic risk parameters in males and females

5.5.1.6.1 CIMT, multiple linear regression in males and females

This section identified the covariables associated with carotid intima media thickness in adjusted modes (multivariate) in males and females as shown in Table (5-9). Firstly, all CVD risk factors age, systolic blood pressure, diastolic blood pressure, body mass index, diabetes, waist circumference, hip circumference, glucose, liver enzymes, LDL, HDL, total cholesterol, triglycerides, apolipoprotein A, apolipoprotein B, vitamin D hypertension, ethnicity and statins were subjected to multicollinearity diagnosis, and variables that had variance inflation factors (VIF) of more than 2 were excluded from the model. Therefore, the stepwise model in females included age, systolic blood pressure, LDL, hip circumference, hypertension, apolipoprotein-A, smoking, and aspartate aminotransferase. The stepwise model in males included age, systolic blood pressure, BMI, diabetes, apolipoprotein-A, apolipoprotein-B, smoking, and triglycerides.

Overall, the stepwise model that included the above variables in males and females explained 16% of the variance in cIMT in males, and 21% of the variance in cIMT in females (the coefficient of determination in males was R^2=0.16 and R^2=0.21 in females). In the stepwise model, mean cIMT increased by 5.82 μm for every one-year age increase in females (0.06μm/ years higher than males). In addition, the mean cIMT increased by 0.67 μm for every 1mm Hg increase in systolic blood pressure (0.1018 mm Hg higher than males). In males, Apolipoprotein-A was a cIMT protective factors, as the mean cIMT decreases by 34.5 SD for every 1 SD increase in apolipoprotein-A. While, in females, the mean cIMT decreased by 20 SD for every 1 SD increase in apolipoprotein-A.

Predictors	Females ≈ 16,178					Predictors	Males ≈ 14,529				
	B	(95%CI)		b	*P-value*		B	(95%CI)		b	P-*value*
		Lower	Upper					Lower	Upper		
Intercept	207.72	175.46	239.98		<0.001	Intercept	187	151	222	0.339	<0.001
Age	5.82	5.54	6.10	0.39	<0.001	Age	6.02	5.68	6.36		<0.001
Systolic blood pressure	0.676	0526	0.825	0.118	<0.001	Systolic blood pressure	0.778	0.625	0.930	0.100	<0.001
Hip circumferences	0.579	0.368	0.791	0.048	<0.001						
LDL	4.37	2.0	6.70	0.033	<0.001	Body mass index	2.47	1.73	3.21	0.068	<0.001
						Diabetes	12.15	1.37	22.9	0.022	0.027
Hypertension	6.58	0.818	12.35	0.029	0.025						
						Apolipoprotein-B	49.81	38.37	61.26	0.085	<0.001
Apolipoprotein-A	-19.97	-27.31	-12.62	-0.048	<0.001						
Smoking	7.91	4.79	11.04	0.043	<0.001	Apolipoprotein-A	-34.5	-46.6	-22.3	-0.056	<0.001
						Smoking	15.47	11.33	19.61	0.069	
Log-aspartate aminotransferase	-10.82	-17.25	-4.40	-0.022	0.001						
						Log-triglycerides	-2.82	-5.49	-0.143	-0.022	0.001
R^2	**0.21**					**R^2**	**0.165**				

Stepwise (Criteria: Probability of-F-to enter <=0.050, Probability of-F-to-remove >=0.100**. LDL:** low density lipoprotein

Table 5-9: The best-fit multiple regression models for mean cIMT in male and female subjects

5.5.2 Pulse wave arterial stiffness index

5.5.2.1Characteristics of the study population stratified by high and low pulse wave arterial stiffness index

The distribution of demographic and clinical characteristics of the study population by high and low pulse wave arterial stiffness index is summarized in Table (5-10). A high pulse wave arterial stiffness index exceeded the mean of pulse wave arterial stiffness index of the study population, and a low pulse wave arterial stiffness index was below the mean of the pulse wave arterial stiffness index of study population. The mean pulse wave arterial stiffness index in the study population was 9.7. In this study, the number of subjects who had a PWASI value of less than 9.7 was 15,802, while the number of subjects who had PWASI value of more than 9.7 was 26,924.

All means of adverse demographic and clinical characteristics of study population were significantly higher in subjects who had high pulse wave arterial stiffness index. CVD risk factors were significantly higher in the high pulse wave arterial stiffness group, including; age (1.5 years older), systolic blood pressure (2.7 mmHg higher), diastolic blood pressure (2.6 mmHg higher), glucose (0.02 mmol/L higher), HbA1c (0.4 mmol/mol higher), waist circumference (15.65 cm higher), hip circumference (1.18 cm higher) and BMI (0.75 kg/m^2 higher). The lipid profile was also higher: apolipoprotein-B (0.02g/L higher), triglycerides (0.16mmol/L higher), and LDL (0.03 mmol/L higher). However, subjects with low PWASI had a higher concentration of HDL cholesterol (0.05 mmol/L higher) and a higher concentration of apolipoprotein-A (0.04 g/L higher), while, there was no significant difference between two groups in terms of total cholesterol.

Regarding liver enzymes, subjects with high PWASI had a higher concentration of liver enzymes, including alanine aminotransferase (1.75 U/L higher) and aspartate aminotransferase (0.6 U/L higher). In addition, they had a higher concentration of C reactive protein (0.10 mg/L higher) but a lower concentration of vitamin D (1.1 nmol/L lower).

The prevalence rates of diabetes, hypertension, cigarette smoking and treatment with statins were higher in the high pulse wave arterial stiffness group. A total of 5.7% of high PWASI subjects had diabetes, compared to 4 % of low PWASI subjects had diabetes. In addition, the proportion of hypertensive subjects was higher by 16.3% in the high PWASI group than the low PWASI group. Regarding smoking, the percentage of current smokers in the high PWASI group was 3.9%, compared to 2.9% in the low PWASI group. Furthermore, the prevalence of treatment with statins was higher by 4.9% in the high PWASI group than the low PWASI group. The percentage of females was higher than percentage of males in the low and high PWASI group by (19.6% and 3% higher), respectively.

Table 5-10: Demographic and clinical characteristics of the study population stratified by high or low pulse wave arterial stiffness

Continuous Variables	Pulse wave arterial stiffness index*≤9.7	Pulse wave arterial stiffness index*>9.7	*P-value*
	Number (mean ±SD)	Number (mean ±SD)	
Non-invasive measurements of atherosclerosis			
Mean CIMT (mean CIMT (120,150), mean CIMT (210,240)) (µm)	(n=15802) 673.7±122.3	(n=26924) 691.6±126.2	<0.001
Demographic data			
Age (year)	(n=15802) 63.1±7.8	(n=20061) 64.3±7.5	<0.001
Townsend deprivation index at recruitment	(n=15787) -2±2.7	(n=26897) -2±2.7	0.16
Anthropometric measurements			
Waist circumference (cm)	(n=15799) 85.50±12.30	(n=25643) 101.15±8.55	<0.001
Hip circumference (cm)	(n=15801) 99.82±8.67	(n=25642) 101±8.55	<0.001
BMI (kg/m2)	(n=15775) 25.93±4.27	(n=25543) 26.68±4.28	<0.001
Blood pressure measurements			
Systolic blood pressure (mmHg)	(n=15773) 137±19	(n=21891) 139.7±18	<0.001
Diastolic blood pressure(mmHg)	(n=15773) 77.2±9.9	(n=21891) 79.8±10	<0.001
Diabetic measurements			
Glucose*(mmol/L)	(n=13438) 4.87(4.55, 5.20)	(n=22989) 4.89(4.57,5.24)	<0.001
HbA1c (mmol/mol)	(n=14637) 34.7±4.80	(n=25029) 35.1±5.26	<0.001
Enzymes measurements			
Alanine aminotransferase* (U/L)	(n=14753) 18.54(14.41,24.80)	(n=25205) 20.29(15.48,27.69)	<0.001
Aspartate aminotransferase* (U/L)	(n=14696) 23.7(20.50, 27.80)	(n=25108) 24.30(21,28.70)	<0.001
Lipid measurements			
Total cholesterol (mmol/L)	(n=14758) 5.73±1.08	(n=25207) 5.73±1.08	0.75
HDL cholesterol (mmol/L)	(n=13453) 1.5±0.38	(n=23011) 1.45±0.37	<0.001
LDL cholesterol (mmol/L)	(n=14720) 3.56±0.82	(n=25155) 3.59±0.83	0.004

Data are expressed as mean ± SD for normal distribution data, and as median and interquartile range non-normal distribution data. Statistical tests are independent t test for normal distribution data and Wilcoxon rank-sum test for non-normal distribution data
*non-normal distribution data

Continuous variable		Pulse wave arterial stiffness index*<9.7	Pulse wave arterial stiffness index*>9.7	*P-value*
		Number (mean± SD)	**Number (mean± SD)**	
Lipid measurements				
Triglyceride (mmol/L) *		(n=14741) 1.29(0.93,1.87)	(n=25194) 1.45(1.03,2.09)	<0.001
Apolipoprotein-A(g/L.)		(n=13378) 1.57±0.27	(n=22889 1.53±0.26	<0.001
Apolipoprotein-B (g/L.)		(n=14697) 1.02±0.23	(n=25078 1.04±0.23	<0.001
Inflammatory measurements				
C-reactive protein (mg/L)*		(n=14732) 1 (0.50,2.02)	(n=25148) 1.1 (0.57,2.19)	<0.001
Vitamins				
Vitamin D*(nmol/L.)		(n=14169) 48.70 (34.50,64.10)	(n=24230) 47.6 (33.30,62.70)	<0.001
Categorical variables		**Pulse wave arterial stiffness index*<9.7**	**Pulse wave arterial stiffness index*>9.7**	*P-value*
		Number (mean± SD)	**Number (mean± SD)**	
Diabetes	no	15175(96%)	25392(94.3%)	<0.001
	yes	627(4%)	1532(5.7%)	
Hypertension	no	8177(51.7%)	9525(35.4%)	<0.001
	yes	7625(48.3%)	17399(64.6%)	
Smoking	never	10267(65.6%)	16246(61%)	<0.001
	former	4938(31.4%)	9361(35.1%)	
	Current	449(2.9%)	1032(3.9%)	
Ethnicity	white	15261(96.8%)	26080(97.1%)	0.093
	black	121(%0.8)	160(0.6%)	
	south Asian	140(0.9%)	243(0.9%)	
	other	243(1.5%)	370(1.4%)	
Statins	no	12651(80.1%)	20256(75.2%)	<0.001
	yes	3151(19.9%)	6668(24.8%)	
Sex	Male	2018(40.2%)	9228(48.5%)	<0.001
	Female	2996(59.8%)	9790(51.5%)	

Data are expressed as mean ± SD, Data are expressed as accounts (percentage), statistical tests are 2- proportion test and χ^2 test

5.5.2.2Relationship between pulse wave arterial stiffness index and cardiometabolic risk factors

5.5.2.2.1 Correlation

In this section, the association between pulse wave arterial stiffness index and CVD risk factors for PWASI was investigated. Bivariate correlation was carried out for all continuous variables. Pearson correlation was used for the analysis of normal distribution data and log transformation of the non-normal distribution data was attempted. The data for the Pearson correlation for cIMT with continuous variables is shown in Table (5-11).

Mean PWASI showed a significant positive linear association with anthropometric measurements (waist, *r*=0.11, hip, *r*=0.054, BMI, *r*=0.064), diastolic blood pressure (*r*=0.11), systolic blood pressure (*r*=0.068), HbA1c (*r*=0.026), and liver enzymes, such as alanine aminotransferase (*r*=0.09) and aspartate aminotransferase (*r*=0.05), C-reactive protein (*r*=0.055), age (*r*=0.046) and lipid markers including apolipoprotein B (*r*=0.038), triglyceride (*r*=0.099), LDL cholesterol (*r*=0.0227). However, there was an inverse linear relationship between mean PWASI and HDL (*r*=-0.093) apolipoprotein-A (*r*=-0.068), and vitamin D (*r*=-0.0179).

Diastolic blood pressure and waist circumference showed the strongest correlation with mean PWASI (*r*=0.11, *r*=0.11 respectively) compared to other CVD risk factors.

5.5.2.2.2 Pulse wave arterial stiffness index, single factor analysis in the whole cohort

To identify the predictors of PWASI in all subjects, univariable regression analysis was carried out for all variables that met statistical significance in the bivariate Pearson correlation (Table 5-12). The predictors that showed a significant association with the mean PWASI include systolic blood pressure (ß=+0.068, *P*=<0.001), Figure (5-7) shows a scatterplot of linear regression of systolic blood pressure with pulse wave arterial stiffness index, age (ß=+0.046, *P*=<0.001), Figure (5-8) shows a scatterplot of linear regression of age with pulse wave arterial stiffness index, diastolic blood pressure (ß=+0.105, *P*=<0.001), and body mass index (ß=+0.064, P=<0.001). In addition, the mean of PWASI was significantly

positively associated with other cardiometabolic parameters, including anthropometric measurement (waist circumference: ß=+0.11, *P*=<0.001, hip circumference: ß=+0.054, *P*=<0.001), glucose (ß=+0.036, *P*=<0.001), HbA1c (ß=+0.025, *P*=<0.001). Moreover, the mean PWASI was significantly positively associated with lipid markers, including LDL cholesterol (ß=+0.0226, *P*=<0.001)., triglycerides (ß=+0.099, *P*=<0.001) and apolipoprotein-B (ß=+0.038, *P*=<0.001). However, it was inversely associated with HDL (ß=-0.093, *P*=<0.001), and apolipoprotein-A (ß=-0.068, P=<0.001). PWASI also had an association with liver function makers; mean PWASI was significantly associated with liver enzyme (ALT: ß=+0.09, P=<0.001, AST: ß=+0.050, P=<0.001). In addition, mean PWASI was inversely associated with vitamin D (ß=-0.0179 P=<0.001) and positively associated with c-reactive protein (ß=-0.055 P=<0.001).

When ranked by standardised beta coefficients, the most important univariable determinants for PWASI were diastolic blood pressure (B=+0.105), then, triglycerides (B=+0.099), male (B=+0.094) and HDL (B=-0.093).

5.5.2.2.3 Pulse wave arterial stiffness index, multiple factors analysis in entire population

In this section, we identified the covariables associated with Pulse wave arterial stiffness index in adjusted models (multivariate) as shown in Table (5-13). First, all categorical and continuous variables significantly associated with PWASI in the univariable analysis were subjected to multicollinearity diagnosis, and the variable that had variance inflation factors (VIF) more than 2 was excluded from the model. Therefore, the stepwise model ended by these factors; age, gender, systolic blood pressure, body mass index, HDL cholesterol, smoking, alanine aminotransferases and triglycerides. After performing a stepwise multiple regression model, the risk factors were identified as the predictive factors for PWASI were age (for every 1 SD increase in age, the mean PWASI increases by (0.005) SD) and male sex (mean PWASI increases by (0.083) SD in males subjects compared with females). Overall, the model, which includes age, gender, systolic blood pressure, body mass index, HDL cholesterol, smoking, alanine aminotransferase and triglycerides explained 10.6% of the variance in PWASI in the UK Biobank population.

Table 5-11: Heatmap representation of the Pearson correlation coefficients of PWASI with clinical characteristics in the whole cohort

Continuous variables	**Pulse wave arterial stiffness**	
	Pearson correlation (r)	***P value***
Waist circumference	0.11	<0.001
diastolic blood pressure (mmHg)	0.11	<0.001
log-Triglycerides(mmol/L)	0.0995	<0.001
Log-Alanine aminotransferase* (U/L)	0.09	<0.001
systolic blood pressure (mmHg)	0.068	<0.001
BMI (kg/m2)	0.064	<0.001
Log-C-reactive protein (mg/L)*	0.0554	<0.001
Hip circumference	0.054	<0.001
age (year)	0.046	<0.001
Log-Aspartate aminotransferase*(U/L)	0.05	<0.001
Apolipoprotein-B (g/L.)	0.038	<0.001
log-Glucose*(mmol/L)	0.036	<0.001
HbA1c (mmol/mol)	0.026	<0.001
LDL cholesterol (mmol/L)	0.0227	<0.001
Total cholesterol (mmol/L)	0.0029	0.599
Townsend deprivation index	-0.0027	0.607
Log Vitamin D*(nmol/L.)	-0.0179	0.0013
Apolipoprotein-A(g/L.)	-0.0684	<0.001
HDL cholesterol (mmol/L)	-0.093	<0.001

r Pearson correlation coefficient

BMI: body mass index, HbA1c: glycated haemoglobin, LDL: low density lipoprotein, HDL: high density lipoprotein

Table 5-12: Univariable linear regression analysis for variables characterising participants in the whole cohort (independent variables) and the pulse wave arterial stiffness index

Continuous variables		Un-standardized Beta coefficient and 95% CI			Standardized (b) coefficients	*P-value*	R^2
		Regression estimate	95% CI				
			Lower	Upper			
Age (years)	I	7.76	7.34	8.19	0.046	<0.001	0.0021
	B	0.30	0.023	0.036			
Townsend deprivation index at recruitment	I	9.64	9.58	9.71	-0.0027	0.603	0.001
	B	-0.005	-0.023	0.013			
Anthropometric measurements							
Waist circumference (cm)	I	5.82	5.46	6.18	0.11	<0.001	0.0122
	B	0.043	0.039	0.047			
Hip circumference (cm)	I	6.6	5.92	7.12	0.054	<0.001	0.0029
	B	0.031	0.025	0.037			
BMI (kg/m2)	I	7.7	7.3	8	0.064	<0.001	0.0042
	B	0.074	0.062	0.086			
Blood pressure measurements							
Systolic blood pressure (mmHg)	I	7.15	6.77	7.53	0.068	<0.001	0.0046
	B	0.018	0.015	0.020			
Diastolic blood pressure(mmHg)	I	5.59	5.19	5.99	0.105	<0.001	0.011
	B	0.051	0.046	0.056			
Diabetic measurements							
Log-Glucose*(mmol/L)	I	8.14	7.68	8.61	0.036	<0.001	0.0013
	B	0.93	0.65	1.22			
HbA1c (mmol/mol)	I	8.78	8.41	9.15	0.025	<0.001	0.0007
	B	0.025	0.014	0.035			
Enzymes measurements							
Log-Alanine aminotransferase * (U/L)	I	7.32	7.05	7.60	0.090	<0.001	0.0083
	B	0.76	0.67	0.85			
Log-Aspartate aminotransferase * (U/L)	I	7.30	6.80	7.80	0.050	<0.001	0.0025
	B	0.72	0.57	0.88			
Lipid measurements							
Total cholesterol (mmol/L)	I	9.58	9.36	9.80	0.0028	0.599	<0.001
	B	0.010	-0.027	0.047			
HDL cholesterol (mmol/L)	I	11.06	10.89	11.24	-0.093	<0.001	0.0086
	B	-0.96	-1.08	-0.85			

I: Intercept, **B:** Beta coefficients, per unit increase of predictor, **Standardized (b) coefficient**: mean change in PWASI per 1 SD increase in predictor variable

Continuous variables		Un-standardized Beta coefficient			Standardized (b) coefficients	*P* value	R^2
		RE	95% CI				
			Lower	Upper			
LDL cholesterol (mmol/L)	I	9.26	9.08	9.45	0.0226	<0.001	0.0005
	B	0.10	0.055	0.15			
Apolipoprotein-A(g/L.)	I	11.20	10.94	11.46	-0.068	<0.001	0.0047
	B	-1.01	-1.17	-0.84			
Apolipoprotein-B (g/L.)	I	8.98	8.79	9.17	0.038	<0.001	0.0014
	B	0.63	0.45	0.81			
Log-Triglycerides(mmol/L)	I	9.37	9.32	9.42	0.099	<0.001	0.0099
	B	0.74	0.66	0.82			
Log-C-reactive-protein (mg/L)*	I	9.60	9.57	9.64	0.055	<0.001	0.0031
	B	0.184	0.149	0.220			
Log-Vitamin D*(nmol/L.)	I	10.22	9.86	10.57	-0.0179	0.001	0.0003
	B	-0.15	-0.24	-0.058			

Categorical variables		Un-standardized B coefficient			Standardized (b) coefficients	*P* value	R^2
		B	95% CI				
			Lower	upper			
Diabetes	Reference	9.62	9.57	9.67	0.026	<0.001	0.0007
	Yes	0.60	0.36	0.83			
Hypertension	Reference	9.27	9.20	9.35	0.072	<0.001	0.0053
	Yes	0.712	0.611	0.814			
Smoking	Reference	9.56	9.50	9.63	0.019	<0.001	0.0008
	Former	0.207	0.098	0.316			
	Current	0.586	0.308	0.864	0.022		
Ethnicity	Reference	9.66	9.61	9.71	-0.009	0.086	0.0001
	Black	-0.56	-1.21	0.079			
	South Asian	-0.05	-0.59	0.48	-0.001	0.843	
	Other	-0.33	-0.76	0.103	-0.0078	0.135	
Statin	Reference	9.53	9.47	9.59	0.045	<0.001	0.002
	Yes	0.53	0.405	0.646			
Gender	Reference	9.20	9.13	9.27	0.094	<0.001	0.009
	Male	0.93	0.83	1.03			

Table 5-13: Stepwise linear regression analysis for variables characterising participants (independent variables) with pulse wave arterial stiffness

Variables	**Unstandardized (B) Coefficients (95.0% Confidence Interval for B)**			**Standardized (b) Coefficients**	***P value***
	B	**95.0% Confidence Interval**			
		Lower	**Upper**		
Reference	2.74	1.846	3.63	0.083	<0.001
Sex	0.871	0.708	1.03		<0.001
Age (year)	0.077	0.067	0.087	0.005	<0.001
Systolic blood pressure (mmHg)	0.008	0.004	0.012	0.046	<0.001
Body mass index (kg/m2)	0.024	0.005	0.043	0.031	<0.001
HDL cholesterol (mmol/L)	-0.49	-0.729	-0.250	-0.057	<0.001
Smoking	0.214	0.095	0.333	0.040	<0.001
Log-Alanine aminotransferase* (U/L)	0.012	0.007	0.018	0.052	<0.001
Log-triglycerides (mmol/L)	0.207	0.121	0.292	0.062	<0.001
R^2	**0.106**				

Stepwise (Criteria: Probability of-F-to enter <=0.050, Probability of-F-to-remove >=0.100). **HDL:** high density lipoprotein

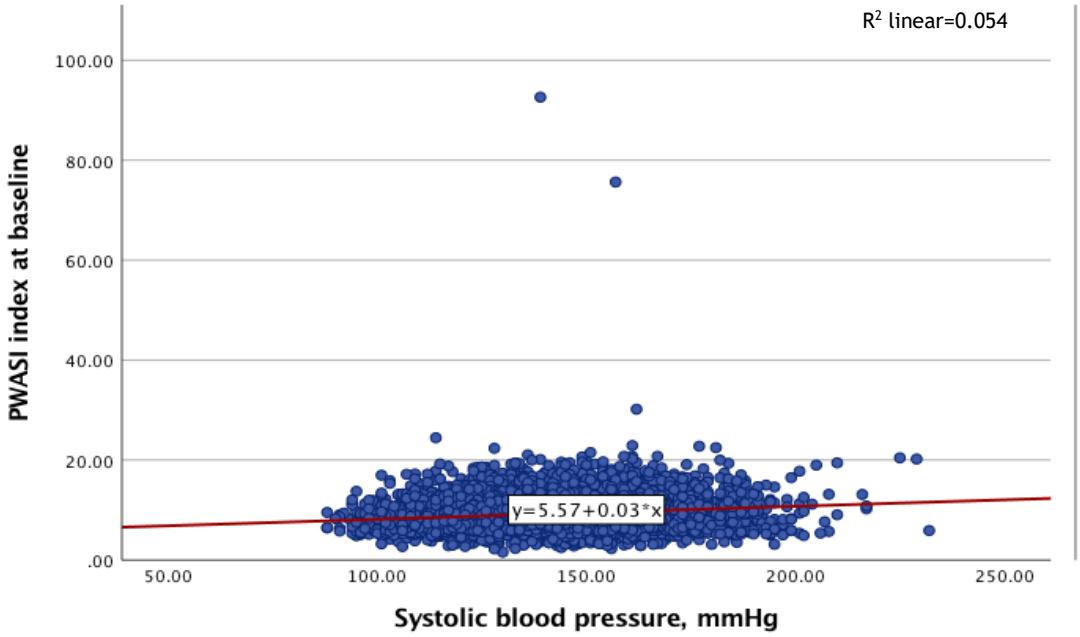


Figure 5-7 Scatterplot of linear regression of systolic blood pressure with pusle wave arterial stiffness index

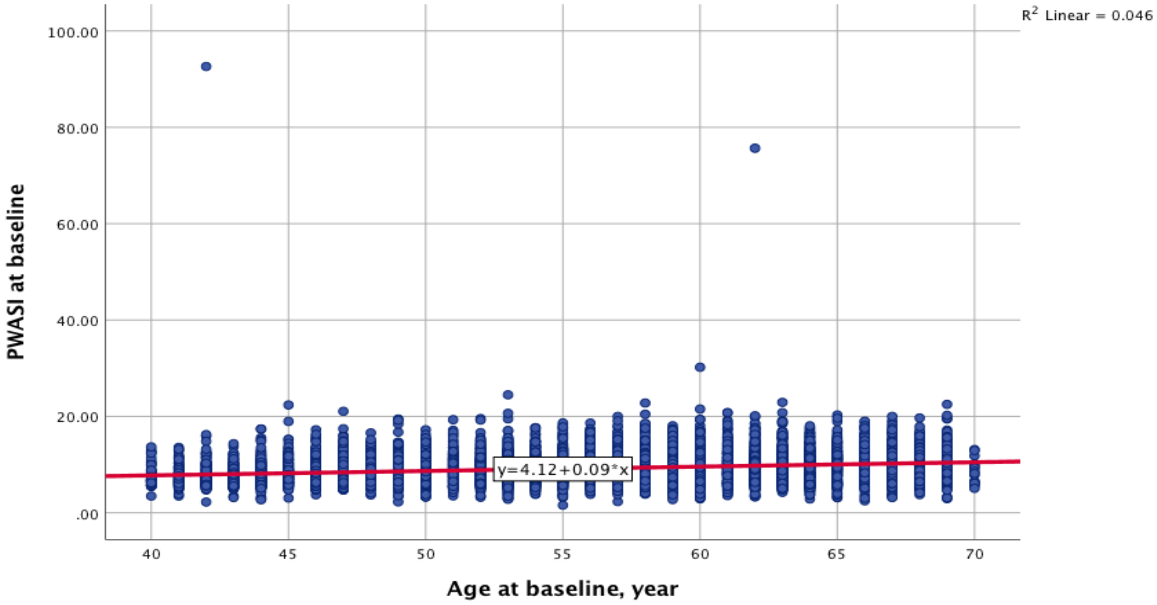


Figure 5-8 Scatterplot of linear regression of age with pusle wave arterial stiffness index

5.5.2.3 Relationship of PWASI and cardiometabolic risk parameters in males and females

5.5.2.3.1 PWASI, multiple linear regression in males and females

This section identifies the covariables associated with carotid intima media thickness in adjusted modes (multivariate) in males and females, as shown in Table (5-14). Firstly, all CVD risk factors, namely age, systolic blood pressure, diastolic blood pressure, body mass index, diabetes, waist circumference, hip circumference, glucose, liver enzymes, LDL, HDL, total cholesterol, triglycerides, apolipoprotein A, apolipoprotein B, vitamin D hypertension, ethnicity and statins were subjected to multicollinearity diagnosis, and variables that had variance inflation factors (VIF) of more than 2 were excluded from the model. Therefore, the stepwise model in females employed the following variables: age, systolic blood pressure, body mass index, HDL, and alanine aminotransferase. The stepwise model in males was ended by the following variables; age, systolic blood pressure, triglycerides, HDL, alanine aminotransferase and smoking.

Overall, the stepwise model that included the above variables in males and females explained 6% of the variance in PWASI in males, and 5% of the variance in PWASI in females (the coefficient of determination for males was R^2=0.069 and for females R^2=0.052). In the stepwise model, PWASI increases by 0.200 for every one-year increase in males (0.193 years higher than females). In addition, for every one SD increase in systolic blood pressure, PWASI increases by 0.047 SD and 0.052 SD in females and males respectively. The protective factors for PWASI in this model was HDL, as PWASI decreased by 0.066 for every 1 mmol/L increases in HDL in both females and males.

Predictors	Females ≈ 13,697					Predictors	Males ≈ 13,271				
	Unstandardized B Coefficients			Standardized (b) Coefficients	*P-value*		Unstandardized B Coefficients			Standardized (b) Coefficients	*P-value*
	Beta	95.0%					Beta	95.0%			
		Lower	Upper					Lower	Upper		
Reference	3.34	2.26	4.43	0.007	<0.001	Reference	3.513	2.27	4.74	0.200	<0.001
Age (year)	0.072	0.058	0.085			Age (year)	0.088	0.073	0.0103		
Systolic blood pressure (mmHg)	0.007	0.002	0.012	0.047	0.007	Systolic blood pressure (mmHg)	0.010	0.003	0.017	0.052	0.003
HDL cholesterol (mmol/L)	-0.519	-0.788	-0.251	-0.066	<0.001	log-Triglycerides(mmol/L)	0.18	0.073	0.304	0.061	0.001
Log-Alanine aminotransferase* (U/L)	0.008	0.001	0.015	0.036	0.029	HDL cholesterol (mmol/L)	-0.74	-1.147	-0.334	-0.066	0.001
Body mass index (kg/m^2)	0.035	0.013	0.058	0.011	0.002	Log-Alanine aminotransferase* (U/L)	0.020	0.011	0.028	0.079	0.024
						Smoking	0.274	0.098	0.451	0.051	0.002
R^2	**0.052**					**R^2**	**0.069**				

Table 5-14: Stepwise linear regression analysis for independent variables with PWASI between males and females.

5.6 Discussion

This study evaluated the determinants of the carotid intima media thickness and pulse wave arterial stiffness index, which are considered indicators of vascular structural or functional change.

This cross-sectional study found that the strongest determinants for cIMT were age and systolic blood pressure, which, in combination with the other risk factors shown in Table (5-8), explained 21.6 % of cIMT variation.

With regard to PWASI, the strongest determinants were male sex, and systolic blood pressure which in combination with the other risk factors shown in Table (5-13), explained 10.6% of PWASI variation.

5.6.1 The determinants for cIMT variation

5.6.1.1Age and gender as independent predictors for cIMT

Firstly, this study showed that cIMT values increased linearly with age in all study participants and in subgroup analysis. Mean cIMT increases were approximately 5.13 μm/yr in men and 4.87 μm/yr in women (4.96 μm/yr in both sexes). This is in keeping with the work of Sinning et al which found that mean cIMT in men was higher than in women, and the determinants of cIMT were age and gender dependent. Furthermore, the prevalence of cardiometabolic risk factors increased with age and were significantly higher in men than in women, with exception of LDL, which is higher in women than in men. This study include 4,814 participants (2,433 men and 2,381 women) from the Gutenberg-Heart Study, aged 35 to 74 years (Sinning *et al.*, 2011).

The results of this study are also in line with data from a large US based study: Atherosclerosis Risk in Communities. The cross-sectional association of mean IMT as a function of age in 4952 individuals was estimated, and data study showed that cIMT increases with age in all carotid segments (Howard *et al.*, 1993). In addition, a study by Maria Łoboz-Rudnicka,(Loboz-Rudnicka *et al.*, 2016) found that mean cIMT increased with age in both men and women, and cIMT values were significantly higher in men compared to women, which is

most pronounced over the age of 45 years. The changing of hormone profiles at the perimenopausal period might partially explained the changing in women's vascular system in this stage.

5.6.1.2Systolic blood pressure as an independent predictor

The second strongest determinant of cIMT in the current study was systolic blood pressure. The study showed that there was a linear association between high systolic blood pressure and increased cIMT values in all study participants and in both genders in single and multiple analysis. In addition, the study found a higher mean systolic blood pressure in individuals who had a mean cIMT in excess of 900μm. Diastolic blood pressure was only weakly associated with cIMT in univariable models and was not included in the stepwise model.

In line with the findings of this study, the STANISALS cohort study, which is a single, cross sectional, population based study, based on the Nancy region of France, involving 696 adult participants, found the risk of having cIMT >900 μm linearly increased with increased systolic blood pressure, and there was a weaker association between diastolic blood pressure and cIMT (Ferreira *et al.*, 2016). Another population-based cross-sectional study among low-income adults in rural China, that involving 2,643 normal participants, of whom 549 participants were from an impaired fasting glucose group and 533 were diabetic, reported a significant association between higher cIMT values and higher systolic blood pressure: diastolic blood pressure was found to be a protective factor form increasing cIMT in multiple analysis (Gao *et al.*, 2017). Likewise, another study showed a significant positive association between systolic blood pressure and cIMT, but this association was not reported with diastolic blood pressure (Zanchetti A1, 2001). These findings have been also documented in other studies (Mannami *et al.*, 1997), and suggest that SBP may induce a higher pressure overload and thus more hyperplasia and hypertrophy than DBP. Moreover, some authors debated that SBP may be more important than DBP as a risk factor for cardiovascular diseases and atherosclerosis.(Lakka *et al.*, 1999; Mannami *et al.*, 1997).

Several studies have discussed the role of hypertension in structural remodelling characterised by carotid intima media thickness. Roman et al

examined the presence of structural changes in carotid artery in 43 hypertensive patients and 43 control subjects, and they found that high systolic blood pressure was a stronger predictor for increased cIMT (Roman *et al.*, 1992). Another study detect a significant and independent positive association between carotid IMT, systolic blood pressure and age, as well as an association between cIMT and cardiac remodelling, this study is a sub-study from a large multicentre cross-sectional study, the ISMIR (Ispessimento Medio-Intimale e Rischio Cardiovascolare), involving 198 asymptomatic, never treated, essential hypertensives and 67 healthy subjects (Di Bello *et al.*, 2009). Our data supports the theory that targeted organ damage mainly as a result of increased hemodynamic load (Lorenz *et al.*, 2007).

Artery wall thickening and cardiac remodelling appear to be dependent on hemodynamic change (Suurkula M, 1994), and several mechanisms could explain the association between them. Elevation of systolic blood pressure is usually accompanied with reduced arterial compliance, leading to increased heart afterload, which may cause cardiac hypertrophy (Ku DN, 1985). As showed in the results of this study, systolic blood pressure was significantly higher (147±19) for individual who had abnormal cIMT (>900μm). The STANISLAS cohort study provided strong evidence for cardiac damage caused by higher SBP in hypertensive and non-hypertensive individuals. The risk of having cIMT >900μm increased gradually from <5% in individuals with SBP <110 mm Hg to >20% in individuals with SBP>140 mm Hg (Ferreira *et al.*, 2016).In contrast to our findings, The Kuopio Ischaemic Heart Disease Risk Factor Study found that hypertension was not associated with the presence of increased IMT in 720 men (SALONEN and SALONEN, 1990). However, a subsequent data from the same author did detect a significant association between systolic blood pressure and increases cIMT in 1,224 men.(Salonen and Salonen, 1991b). It is likely that selection criteria (based on age or blood pressure restrictions) has some impact on the strength of observed associations. UK Biobank has the advantage of comprising a wide range of ages and blood pressures among the broadly healthy participants.

5.6.1.3Lipid markers as independent predictors for cIMT

Numerous prospective studies have reported the association of elevated lipid markers including total cholesterol, triglycerides and LDL cholesterol with increased cIMT, and the protective association of HDL cholesterol against increased cIMT. this study showed that total cholesterol, LDL cholesterol, and triglycerides were significantly higher in subjects with mean cIMT value of more than 900µm, while HDL was significantly higher in subjects with normal cIMT (<900µm). However, LDL cholesterol, total cholesterol and triglycerides explained less than 1% of the variance in cIMT. In line with findings of this study, the study done by Rosvall et al, showed a positive association between risk factors, include age, LDL cholesterol and SBP with increased progression of cIMT values, while HDL cholesterol showed an inverse association with it. The study population involved 3426 middle aged Swedish men and women (Rosvall *et al.*, 2015; John R. Crouse and Investigators, 1996; Crouse *et al.*, 1996)In contrast to the findings of this study, Rundek et al, found that total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides had no significant effect on cIMT in (n=1,790) stroke-free individuals, this study is a part from Northern Manhattan Study, which is prospective, population-based study (Rundek *et al.*, 2013). Study by Loboz,et al, reported an insignificant association between lipid abnormality and increased cIMT (Loboz-Rudnicka *et al.*, 2016). These inconsistent findings might be attributed to the difference in sample size between studies and the difference in the prevalence rate of lipid abnormality among different population, as well as, 77% of study population of UK Biobank were on statin therapy at baseline. Additionally, it might be due to the variation in the underlying mechanisms of cIMT thickening among different populations (Gijsberts *et al.*, 2015).

In support with the causal association between lipid abnormality and increase cIMT values, clinical trials of lipid lowering agent, especially statins, have reported significant decreased in the progression of carotid intima media thickness after statin therapy. The METEOR trial showed that rosuvastatin significantly decreased the progression of carotid intima media thickness in low risk participants with subclinical atherosclerosis. The METEOR trial is Randomized, double-blind, placebo-controlled study of 984 participants (mean age,57 years) with CAD and modest cIMT thickening. (Crouse *et al.*, 2007).

5.6.1.4Metabolic markers as independent predictors for cIMT

The association between increased cIMT and obesity parameters, including BMI and waist circumference, is well documented in the literature. The current study showed a significant association between mean cIMT and obesity parameters, including BMI and hip circumference in a multivariate analysis of the total study population. In multiple analysis of subgroups, hip circumference was the only determinant for predicting increased cIMT in females, while in males, BMI and hip circumference were determinants for abnormal cIMT.

In contrast with the findings of this study, a study by Łoboz-Rudnicka et al (2016) found that BMI and waist circumference were independent determinants for cIMT in females and there was no association between BMI, waist circumference and cIMT in males. The study group consisted of only 256 participants, including 134 females.

Diabetic patients are at greater risk of having higher cIMT. The association between increased cIMT and diabetes parameters, including glucose and HbA1c, is well documented in the literatures. A single linear regression analysis in the present study showed a significant association between diabetic parameters and cIMT in all population and in the sex subgroups analysed separately. However, in multiple regression analysis, diabetic parameters did not prove to be independent determinants of cIMT in all population. However, diabetes was found to be an independent determinant of cIMT in males, but not in females.

In line with our findings of this study, Salonen et al investigated the association between cIMT and cardiovascular diseases risk factors in a population-based ultrasonography study. Diabetes was found to be a significant predictor for cIMT progression. This study involved (1224) Eastern Finnish men aged between (42-60) years, which a sample form the Kuopio Ischaemic Heart Disease Risk Factor Study (KIHD) (Salonen and Salonen, 1991b). Another study demonstrated that cIMT values are significantly higher in type 1DM compared with controls. This study was investigated prospectively 310 patients with type 1DM and 40 healthy controls, aged <40 years (Frost and Beischer, 1998).The placebo-controlled subgroup analysis of the STOP_NIDDM trial showed that treatment

subjects with impaired glucose tolerance with the α-glucosidase inhibitor acarbose, a compound that decrease postprandial hyperglycaemia, had a significant reduction on the progression of cIMT compared with placebo group. A total of 132 participants were randomized to placebo (n=66) or (n=66) acarbose for 3 years (Hanefeld *et al.*, 2004). Moreover, the insulin secretagogues, repaglindine and glyburide, led to significant decrease in HbA1c , as well as significant regression of cIMT (Esposito *et al.*, 2004).

Quantitative evidence was provided in 2010 by Einarson, who published a meta-analysis of data from 15,592 patients (8250 normal controls, 3013 impaired glucose, 4329 diabetics) in 11 studies. A small but statistically significant link was found between postprandial glucose levels and cIMT, and both are associated with adverse cardiovascular outcome (Einarson, Hunchuck and Hemels, 2010).

The RIAD study is case- control cross-sectional study showed that impaired fasting glucose in a combination with impaired glucose tolerance are significantly associated with increases thickening of the arterial wall compared with normoglycemia adjusted for age and sex, the study involved 104 in control group and 104 in case group (Hanefeld *et al.*, 1999). However, there are contradictory findings in relation to the association between fasting glucose and cIMT in the literature. A cross-sectional study of 160 nondiabetic Chinese subjects, among them 33 had normal glucose tolerance, 13 had impaired fasting glucose, 80 had impaired glucose tolerance and 34 had both IFG and IGT, found that IMT values in the NGT group were lower than in the other groups (Zhang *et al.*, 2006); neither did a study of a group of patients with metabolism disorder in Germany (Hanefeld *et al.*, 1999). This indicates the effect of ethnicity in this type of association.

Behavioural change related to diet and physical activity, including lifestyle changes, and weight control, influence the progression and development of atherosclerosis. Numerous risk factors examined in large epidemiological studies, such as the Multiple Risk Factor Intervention Trial ('Multiple Risk Factor Intervention Trial: Risk Factor Changes and Mortality Results,' 1982), Honolulu Heart Program (Reed, MacLean and Hayashi, 1987), The Seven Countries Study

(Keys, 1997) have demonstrated that lifestyle modifications can affect coronary artery disease mortality and morbidity. Maria et al investigated the long-term effects of a Mediterranean diet on the carotid intima media thickness progression in patients with type 2 diabetes, the study was a parallel two arms, single centre trial. A total of 215 participants randomized to Mediterranean diet (n=108) or a low-fat diet (n=107). They found that the regression rate of cIMT was higher in the Mediterranean diet compared with low-fat diet (Maiorino *et al.*, 2017).

5.6.1.5Smoking and cIMT

Cigarette smoking increases burden of atherosclerosis independently and acts with other risk factors to increases risk for cardiovascular diseases. (Burns, 2003). Tobacco smoking has a direct impact on essential components for development of atherosclerosis including vasomotor dysfunction, inflammation and lipid oxidation (Ambrose and Barua, 2004).

In this study, smoking increased cIMT by 0.099 to 0.0418 SD compared to non-smoking in single and multiple analysis of all populations. In the subgroup analysis, smoking was an independent predictor of increased cIMT in males and females. In line with the findings of this study, data reported by Fitch et al. (2011) found that pack year smoking history was a significant predictor of cIMT, controlling for other cardiovascular risk factors (Fitch *et al.*, 2011). A population-based cross-sectional study of 3,789 participants in China showed that cIMT was 9.82×10^{-3} mm higher in current smokers compared to never smokers (Liu *et al.*, 2017). A community-based study that investigated the factors determining gender and age differences in IMT in ethnic Chinese found smoking was of borderline significance as an independent predictor for increased cIMT (Su *et al.*, 2012). In addition, a large cross-sectional study of 1,504 smokers that aimed to identify the predictors of carotid atherosclerosis found that pack year smoking was associated with increased cIMT. Whilst the previous study is consistent with the findings of the current study, data regarding smoking in previous studies referred to the smoking burden (pack-year) and active smokers of a wide age range(18-79) were studied (Johnson *et al.*, 2010).

5.6.1.6Novel determinants for carotid intima media thickness

In addition to the traditional risk factors that influence cIMT, some novel factors are associated with vascular structural and functional changes. This study reported that traditional risk factors, namely age, systolic blood pressure, BMI, LDL, and waist circumference, explained 28% of the variance in cIMT. However, less traditional factors, including apolipoprotein A and apolipoprotein B, explained additional 2% of the cIMT variance. Age and systolic blood pressure accounted for most of the variance in cIMT (about 26%). These results are consistent with previous findings from the Framingham Offspring cohort. The traditional risk factors in the Framingham score explained 28.6% of the variability of common carotid IMT and 27.5% of the variability of internal carotid artery IMT, with age, gender and systolic blood pressure considered the strongest predictors of IMT variability in common and internal carotid arteries (Polak *et al.*, 2010). Other findings from the Cardiovascular Health Study showed that cholesterol level, hypertension , diabetes, age, sex and smoking explained 17% of cIMT variance (O'Leary *et al.*, 1996).

The Prospective study of Multi-Ethnic Study of Atherosclerosis participants showed a significant association between apolipoprotein B and cIMT progression independent of typical CV risk factors. This study was a sub-cohort analysis for (1,925) of MESA participants (Steffen *et al.*, 2017). In the Women's healthy lifestyle project, Apo-B was significantly associated with internal cIMT, independent of age and menopausal status, the study involved (453) healthy women that enrolled in a dietary and physical activity randomized clinical trial (Schott *et al.*, 2004). Contrary to above findings regarding the association between ApoB and cIMT progression, a study by Maki et al that investigated the association between baseline lipoprotein cholesterol, triglycerides and apolipoprotein B level and progression of cIMT in (110) participants from the control arm of clinical trial. They showed that over an 18 month time periods there was no association between ApoB and the progression of cIMT (Maki *et al.*, 2012).

Despite the discrepancy between the population characteristics of these studies, and the difference in cIMT protocols, traditional risk factors explained

21.6% of cIMT variance. Age, sex and systolic blood pressure are the most important contributory factors, and other factors make a smaller contribution.

5.6.2 The determinants for PWASI

5.6.2.1Systolic blood pressure and age association with PWASI

Pulse wave arterial stiffness is another indicator for vascular structural and functional change, reflecting a measure of vascular function rather than structure. This study showed that higher pulse wave arterial stiffness was significantly associated with cardiovascular risk factors. The data in this study showed that arterial stiffness was strongly positively associated with hypertension parameters, including systolic blood pressure and diastolic blood pressure (B=0.068 and 0.105 respectively) in univariate analyses. In addition, the classical risk factors accounted for 10% of PWASI variation.

There are conflicting results in the literatures regarding the association between both stiffness measures include the augmentation index and pulse wave velocity, and blood pressure, augmentation index is a measure of systemic arterial stiffness derived from the ascending aortic pressure waveform (Wilkinson *et al.*, 2000). A study Wilkinson et al examined differences in the determinants for arterial stiffness between two different age groups (>50, <50). A total of (442) participants were identified from a joint database of community -based volunteers and patients that attending an open access cardiovascular risk assessment clinical at the Wales Heart Research Institute, University of Wales College of Medicine, Cardiff. They found diastolic blood pressure to be independently associated with arterial stiffness in the younger group, but not in the older group. In this study, an augmentation index was used as a measure of arterial stiffness (Wilkinson *et al.*, 2001). Other studies have found that an augmentation index was associated with both systolic and diastolic blood pressure. This study incudes 50 asymptomatic men who had a previous history of cardiovascular diseases or diabetes (Kelly *et al.*, 2001).

In contrast to our findings, study by Nurnberger *et al*, found that diastolic blood pressure was strongly associated with two stiffness markers, namely aortic

pulse wave velocity and augmentation index. However, again the study was limited by small size since the study population included only 77 healthy young males (Nürnberger *et al.*, 2003). In addition, it has been reported that arterial stiffness has been found to increase with the degree of loading (Shadwick, 1999) and systolic blood pressure is not significantly affected by the degree of loading. In large community based study of 45,092 participants, Haojia Chen and his colleagues found significant association between systolic blood pressure and brachial- ankle pulse wave velocity (Chen *et al.*, 2021).

The differences in findings across studies may be attributed to the huge variability of study population, such as the presence of different disorders and drug regimens, broad age groups and gender distribution.

~~A~~ge plays a significant role in the change of vascular function and structure. For example, a widening of pulse pressure is associated with increased stroke volume in younger people, but after the age of 50 widening of pulse pressure was associated with reduced arterial compliance (Alfie *et al.*, 1999). In addition, this study showed that age was independent predictor for arterial stiffness index. In line with these findings, study by Soo Kyung Cho and his colleagues investigated the impact of age on arterial stiffness in newly untreated hypertension patients. The 144 patients were divided into two groups younger (age<50 yr, n=71) and older patients (age>50 yr, n=73). Systolic blood pressure, pulse pressure, augmentation pressure and augmentation index were higher in older groups (Cho *et al.*, 2015). This suggests that hypertension has a role in the structural alteration of vascular walls, such as increases collagen content and decreased elastin content, which may accelerate with age (Kass *et al.*, 2001). In addition, an acute reduction of blood pressure does not normalize elevated pulse wave velocity in hypertension (Stewart *et al.*, 2006).

5.6.2.2Lipid markers as independent predictors for PWASI

The data showed a significant association between HDL, LDL, triglycerides and arterial stiffness (B=-0.093, B=0.0226, B=0.099 respectively) in univariate analysis, however, HDL and triglycerides were the only lipid markers which remained as independent associated with arterial stiffness in multiple

regression analysis with adjustment for confounding factors. This finding is in agreement with a study by Wen and co-workers, which examined the association between lipid ratio and pulse wave arterial stiffness index that estimated by branchial ankle pulse wave velocity in 1015 Chinese men aged between 18 to 44 years. Triglycerides/ high density lipoprotein cholesterol (HDL-C), total cholesterol/HDL-C, non-HDL_C/HDL-C and triglycerides increases the risk of arterial stiffness after adjustment of cofounders factors (Wen *et al.*, 2017). Recently, Urbina and colleagues showed that triglycerides/ high density lipoprotein cholesterol (HDL-C) was significant determinant of arterial stiffness in adolescents and young adults aged between 10 and 26 years(Urbina *et al.*, 2013).

The mechanism of how TG/HDL might cause arterial stiffness could be explained as follows: in theory, oxygen may be generated in response to elevated serum triglycerides, and this may induce insulin resistance, thus affect several proatherogenic signalling pathways (Lamarche *et al.*, 1997). In addition, a high triglycerides are associated with abnormal vascular smooth muscle and endothelial vasodilatory response (Lupattelli *et al.*, 2000). In contrast, HDL_C is considered as antiatherogenic and offers protection from atherosclerosis by inhibiting the oxidation of lipoproteins (Shih *et al.*, 2000).

Overall, the data in this study indicates that although classical CVD risk factors are associated with PWASI, the association (R2=10%) is weak; indeed, it is much weaker than a similar model for cIMT (R2=21.6%). Indeed, the fit of the model for both vascular markers is not particularly strong. It is important to consider why this is the case. As the imaging assessment study took place four years after the baseline assessment, and some time had thus elapsed between the imaging visit and the assessment visit, it may be that results for both cIMT and PWASI are biased to the null, underestimating the true association of risk factors with the vascular measures.

In the UK Biobank, a baseline assessment was completed for all of the cohort by the end of 2010, and the assessment had been repeated for around 20,000 participants by 2013. Biochemical assays for the baseline samples for the entire cohort were completed by the end of 2018. Consequently, the classical risk

factors for the participants did not succeed in adequately identifying the variation in imaging markers four years after taking the biochemical measurements at the assessment visit. This may indicate the need to study the association between those risk factors and other novel risk factors and cIMT and PWASI at the same time point.

Oscar et al. investigated the association between PWASI and cIMT, and the differences and similarities of determinants for these outcomes in a cohort of 320 Atahualpa participants aged ≥60 years. The study showed a significant association between pulse wave velocity, which is used as a surrogate of arterial stiffness and cIMT. However, there was a difference in the determinants of those outcomes. The variables significantly associated with high PWV were increasing age, female gender, high blood pressure and a low level of physical activity. The variables significantly associated with abnormal cIMT include male gender, increasing age, high blood pressure and poor diet **(Del Brutto *et al.*, 2020)**. Therefore, external data supports the notion that classical risk factors that determine cIMT and PWASI are likely to be different. The risk factors that we included in the model of our study (a priori) may just naturally be better determinants of cIMT; for instance, not including physical activity may bias the model against PWASI.

5.7 Conclusion

The present study is the first to report an association between a wide range of cardiometabolic risk factors, carotid intima media thickness and pulse wave arterial stiffness index in around 500,000 participants from the UK Biobank population without any previous history of cardiovascular disease.

In this study, age and systolic blood pressure were the strongest determinants of cIMT variation in the UK Biobank population in multiple-factor analysis. These findings may suggest that cIMT is not a direct marker of atherosclerosis, but rather an adaptive alteration that takes place in the arterial walls with age and includes the hypertrophy of the media in response to high blood pressure. However, this topic requires further research. Also, Age and systolic blood pressure were the strongest determinants of PWASI variation in the UK Biobank

population in multiple factor analysis. Therefore, controlling these risk factors may help to understand the underlying causes of vascular walls changes, and consequently reduce the risk of cardiovascular diseases.

5.8 Strengths and limitations

The major strengths of this study are its large sample size; it was a well-designed cohort study that included a several markers, which enable the researchers to study the association between a wide range of CVD risk factors and outcomes. In addition, all measurements have been taken in standardized and unified way in all participants of UK Biobank. However, some limitations to the findings are acknowledged. There was only an initial and first repeat assessment visit for blood biochemistry and blood pressure, and no bloods were taken at the imaging visit. Therefore, on average 4 years passed between these measurements. Changes to blood biochemistry (such as lipids) and blood pressure in the intervening time period may have contributed to the small percentage that classical factors have accounted for imaging markers (cIMT and PWASI) variation. However, as the effects of classical risk factors would be cumulative over time, and in general they would be stable or worsen with age, it is reasonable to have hypothesised that these factors would have contributed to vascular markers of cardiovascular disease measured, on average, 4 years later. This Biobank study included participants aged between 40-70 years, and the findings may therefore not be generalizable to other populations. As the study is cross sectional, causality cannot be inferred. Despite the adjustment of many confounders to minimize the potential cofounding bias, we cannot rule out the effect of unmeasured one. The selection of determinants to be investigated might be limited with respect to other socioeconomic or sociocultural characteristics. However, the aim was to include biological related risk factors for atherosclerosis, which were available in this study. The work requires external validation and will get more power as a greater number of participants undergo imaging visit.

6 Carotid intima media thickness and pulse wave arterial stiffness with association of cardiovascular events in UK Biobank: Prospective study

6.1 Introduction

Aging and atherosclerosis in the vascular system causes progressive structural and functional changes in large arteries. The results of these changes are increasing stiffness of the arteries and increasing carotid intima media thickness as discussed in chapter1. Modifiable and causal risk factors for cardiovascular diseases including dyslipidemia, smoking, high blood pressure, diabetes are account for most of heart attack in both genders (Yusuf *et al.*, 2004). However, some risk factors, contrary to exception, that causing atherosclerotic CVD has limited predictive value, so they do not has an ability to classify people as high risk for incidence CVD (Wormser *et al.*, 2011). Most of cardiovascular events, including stroke and myocardial infarction, occur in individuals who would be misclassified as intermediate or low risk by traditionally based methods such as Framingham risk scores (Lauer, 2007). Consequently, they do not offer appropriate therapy for the prevention of CVD. In addition, different studies have shown that more than 75% of all hard coronary heart events happen in individuals who had been misclassified at low or intermediate risk, such as the Women's Health Study (Ridker *et al.*, 2007), the Framingham Heart Study (Wilson *et al.*, 2008), the Physicians' Health Study (Ridker *et al.*, 2008) and the Northwick Park Heart Study (Shah *et al.*, 2009).

Subclinical atherosclerosis is a silent disease and develops over a long period of time. Therefore, non-invasive screening of arterial walls rather than simply assessing the risk factors, may be a better approach to detecting individuals at risk of incidence CVD events (Shah, 2010). This offers clinicians a unique opportunity for early detection and personalized prevention for CVD.

There is discrepancy in results between publications considering cIMT as a surrogate marker for cardiovascular diseases. Several studies have shown that increasing carotid intima media thickness was associated with cardiovascular diseases. The Kuoppio Ischaemic Heart Disease study found that a 0.1 incremental increase of cIMT was associated with 11% increase of myocardial infarction risk (Salonen and Salonen, 1991a). In the following years, many large studies were conducted to confirm the association between cIMT and incidence cardiovascular events including The Cardiovascular Health Study (O'Leary *et*

al., 1992), the Atherosclerosis Risk In Communities study (Chambless *et al.*, 1997), the Rotterdam Study (Bots *et al.*, 1997), the Malm€o Diet and Cancer Study (Rosvall *et al.*, 2005), the Carotid Atherosclerosis Progression Study (Lorenz *et al.*, 2010). All of studies produced consistent results and showed a strong association between cIMT and incidence of cardiovascular disease. However, two meta-analyses produced conflicting results regarding the value of carotid IMT in CVD risk prediction. One meta analyses showed that for every 0.1-mm increase in carotid IMT, the relative risks of CVD events increased by 1.15 (Lorenz *et al.*, 2010), while Den Ruijter et al found that there was no benefit to adding carotid IMT to traditional risk prediction model for CVD events prediction (Den Ruijter *et al.*, 2012). These conflicting observations are also reflected in diverging guideline recommendations. The measurements of carotid IMT for CVD risk assessment in intermediate risk asymptomatic adults was recommended as a class IIa recommendation by the 2010 American Heart Association/American College of Cardiology (AHA/ACC) guidelines (Greenland *et al.*, 2010). Ultrasound scanning of the carotid arteries to detect atherosclerosis was recommended by the European Society of Hypertension/European Society of Cardiology and classified as a class IIa recommendation with level of evidence B.1 (Mancia *et al.*, 2013). The Mannheim Carotid Intima-Media Thickness and Plaque Consensus from an update the advisory board of the "Watching the Risk" symposium declared that the measurements of carotid IMT for the initial detection of CVD risk in asymptomatic patients, was recommended if they were determined intermediate risk or if other risk factors existed (Greenland *et al.*, 2010). However, the AHA/ACC guidelines, in 2013 did not recommend the use of carotid IMT in clinical practice (Goff *et al.*, 2014)

In addition, traditional risk factors may underestimate the risk of CVD for individuals who are in a specific risk group, such as person having asymptomatic hypertension (Mancia *et al.*, 2007). Therefore, imaging biomarkers, such as PWASI, may have an advantage over circulating biomarkers in detecting CVD in certain risk group (Wang, 2011). Furthermore, a number of studies found that arterial stiffness had an ability to predict the risk of future fatal and non-fatal CVD and mortality (Vlachopoulos, Aznaouridis and Stefanadis, 2010). Arterial stiffness is used as risk stratification in several

populations and, recently, the European Society of Hypertension/European Society of Cardiology guidelines for the management of arterial hypertension suggested assessing of arterial stiffness by measuring aortic pulse wave velocity, to identify subclinical target organ damage (Mancia *et al.*, 2014).

It is important to correctly identify individuals at risk of cardiovascular diseases in order to optimise the prevention and treatment therapy. There are divergencies in the findings of studies in the literature and recommendations in the guidelines regarding the use of imaging markers, including PWASI and carotid IMT, as surrogate markers for the risk assessment of CVD.

These discrepancies among the results are probably caused by heterogeneity in the study design, especially difference in the measurement methods that were used to assess PWASI and carotid IMT in cross studies and the differences in population size. In addition, most studies yielded positive results, so there may have been publication bias. This is why we used the UK Biobank resources that have a consistent recruitment strategy and consistent measurements for all risk factors and imaging markers for all the participants.

We therefore sought to examine the association between imaging markers (PWASI and carotid IMT) and cardiovascular disease events independent of cardiovascular risk factors using data from UK Biobank.

6.2 Hypothesis

The non-invasive imaging markers including PWASI and carotid IMT, have an association with cardiovascular diseases events in the UK Biobank population independent of traditional risk factors.

- The highest quintile for cIMT is associated with highest expected hazard for cardiovascular diseases.

- The highest tertile for PWASI is associated with highest expected hazard for cardiovascular diseases.

6.3 Aims

This prospective based population study has the following specific aims:

- To investigate the association between imaging markers (cIMT and PWASI) as categorical and continuous variables with incidence of cardiovascular diseases in the UK Biobank population independently of traditional cardiovascular risk factors.

- To investigate the association between imaging markers (cIMT and PWASI) as categorical and continuous variables with incidence of stroke and coronary heart diseases in the UK Biobank population independently of traditional cardiovascular risk factors.

6.4 Method

6.4.1 Study design

This study is a prospective study using data from the UK Biobank. The measurements of exposures (cIMT and PWASI) were taken at the imaging assessment visit in 2014.

6.4.2 UK Biobank study population

The details of the UK Biobank study were given in chapter 5. All the participants provided informed consent and the study was conducted in accordance with the Helsinki Declaration. It was approved by the North West Haydock Research Ethics Committee in the UK (REC reference: 11/ NW/03820). This study was approved under UKB project number 43707 (UK Biobank, 2019).

6.4.3 UK Biobank imaging visit

The details of the imaging visit were summarised in chapter 5.

6.4.4 UK Biobank long term follow up

Participants follow up information was obtained from medical records. Permission was obtained from all the participants at enrolment to access all of their past and future medical and health related records. Access to these records was required to obtain follow up information about the cause of mortality and other health events. Follow up is via linkages with routinely collected national databases, including primary care, cancer registration, death registration, hospital out-patient episodes and hospital inpatients episodes. The NHS number in England and Wales and the Community Health Index number in Scotland was used to continually track the participants' health records during the follow up process. Other identifiers, such as name, date of birth, address of general practice, email address and mobile number were also obtained to ensure that the participants would not be lost during the follow up. The UK Biobank uses the International Classification of Disease (ICD) and Office of Population Censuses and Surveys Classification of Interventions and

Procedures (OPCS) to increase biological specificity and decrease misclassification.

6.4.5 Follow up duration

The follow up for cardiovascular cases was from inclusion into the study until November 30, 2016 for participants from Scotland and until January 31, 2018 for participants from England and Wales.

6.4.6 Exposures and outcomes definitions

The details for cIMT and PWASI measures in UK Biobank were discussed in chapter 5.

We included 42,726 participants described in chapter 5, in order to maintain sufficient power. As recruitment to the imaging visit (during or after 2014) was at a later follow-up time than the UK Biobank baseline visit, we excluded participants where the imaging visit occurred after the end of available follow-up time in the HES and death records.

This approach yielded n=19629 participants who had completed follow up information at the end of follow up period and had an available cIMT measurement that were included in our analysis. The number of participants with an available measurement for PWASI was n=18,205.

Diagnoses of cardiovascular events were extracted using the "Spell and Episode" category from the Hospital Episode Statistics records after the imaging visit at 2014 and during the follow up period. This category contains main and secondary diagnoses, coded according to the International Classification of Diseases 10th Revision (ICD-10), recorded following hospital inpatient stay. Follow-up for new-onset CVD, CHD and stroke, was between the imaging visit and November 30, 2016 for participants from Scotland and until January 31, 2018 for participants from England and Wales, the median and interquartile range for follow up period is 1.4 (1.82) year. In addition, participants follow up for death due to cardiovascular diseases as primary cause started at imaging visit and was censored at the time of last observation.

The information about cause of death was obtained from the National Health Service Information Centre. See Table (6-1) for the diseases definitions that used.

Table 6-1: Disease definitions for incidence cardiovascular disease used in the prospective UK Biobank study

Variables	**ICD10**
Cardiovascular disease	Death: I00-I99 Hospitalisation: I20-25, I60-69, G45
Coronary heart diseases	Death: I20-I25 Hospitalisation: I20-25
Stroke	Death: I60-I69 Hospitalisation: I60-69, G45

6.4.7 Demographic and haemodynamic parameters

More details of demographic and haemodynamic parameters were described in chapter 5.

6.4.8 Confounder variables

Potential confounders were selected for adjustment based on directed acyclic graphs (DAG) taking into account prior knowledge regarding their associations with carotid intima media thickness and pulse wave arterial stiffness index that were discussed in chapter (5). According to the resulting DAG, age, gender, systolic blood pressure, and ethnicity were potential antecedents of cIMT and incidence cardiovascular disease, hence we included these variables as potential confounders in the multivariate model (Figure 6-1). In addition, we found age, gender, ethnicity and systolic blood pressure were potential antecedents of pulse wave arterial stiffness and incidence cardiovascular disease as shown in (Figure 6-2). In addition, we adjusted for cardiovascular diseases that occurred at between the start of the study and the imaging visit.

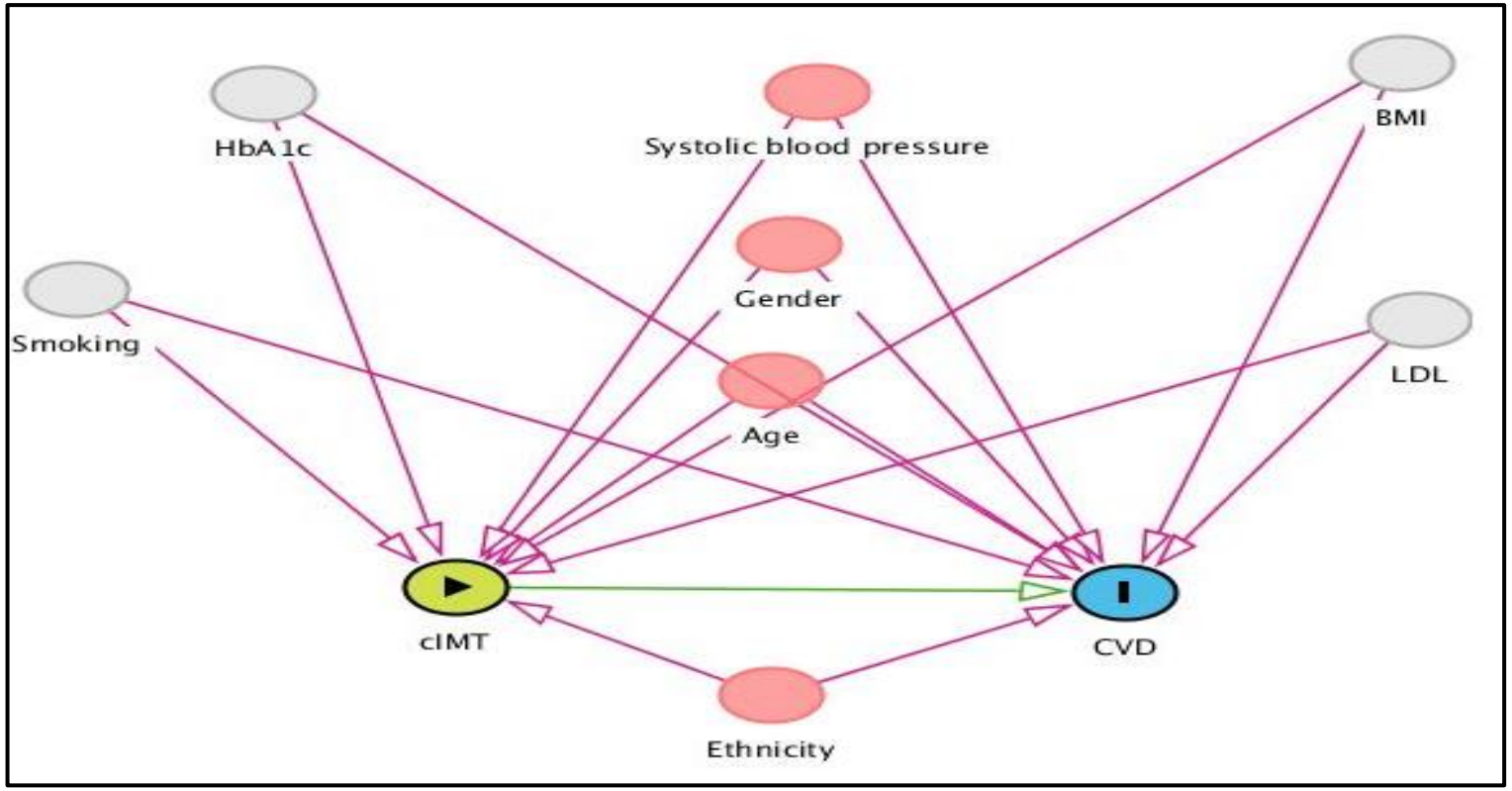


Figure 6-1 Causal directed acyclic graph linking cIMT (the exposure) to CVD (the outcome)

exposure, outcome, ancestor of exposure, ancestor of exposure and outcome, unobserved, biasing path, causal path. Adjustment for cofounders (ancestors of both the exposure and outcome), age, gender, systolic blood pressure, ethnicity. These 4 co-variates were identified as the minimal sufficient adjustment sets to estimate the total effect of cIMT in predicting CVD.

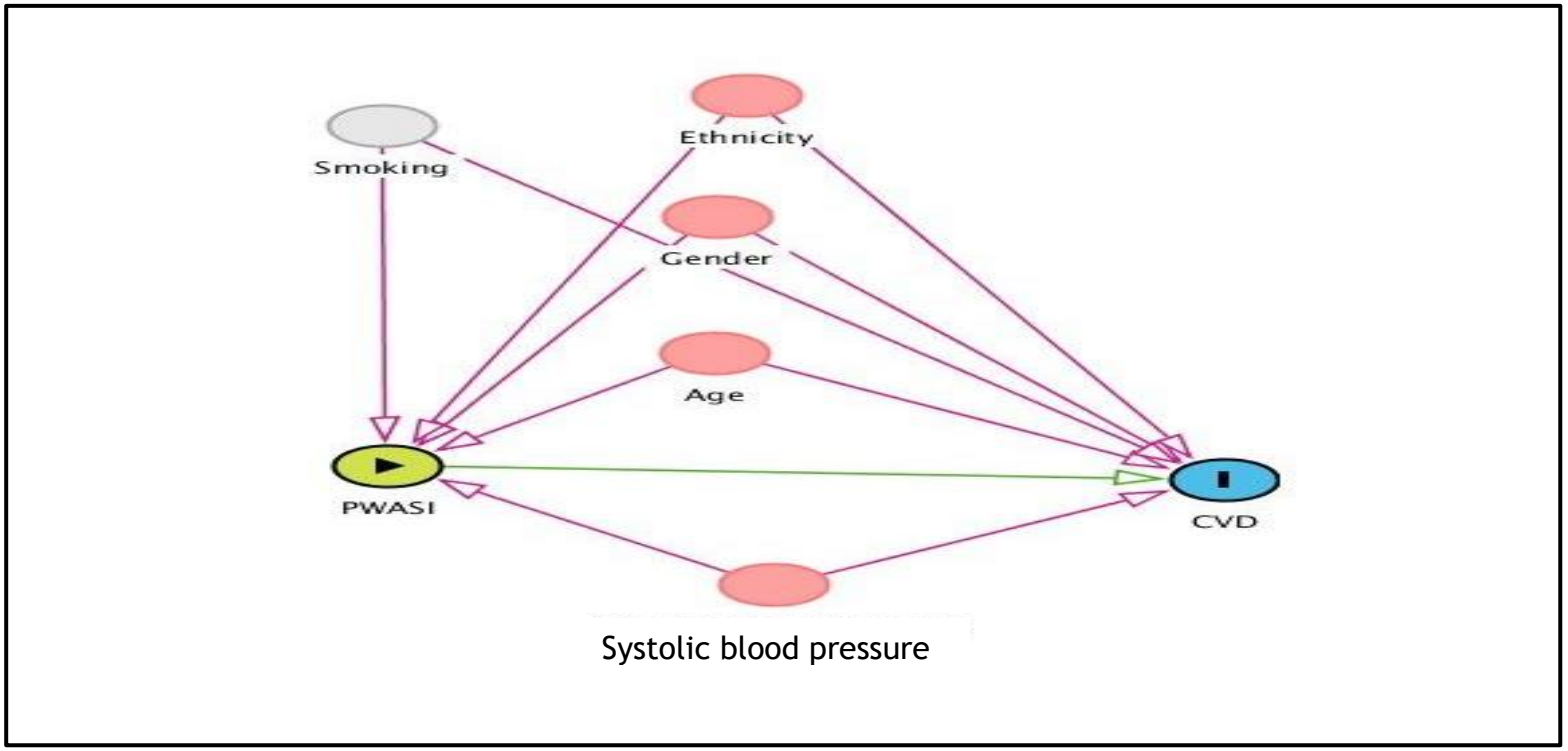


Figure 6-2 Causal directed acyclic graph linking PWASI (the exposure) to CVD (the outcome)

exposure, outcome, ancestor of exposure, ancestor of exposure and outcome, unobserved, biasing path, causal path. Adjustment for cofounders (ancestors of both the exposure and outcome), age, gender, systolic blood pressure, ethnicity. These 4 co-variates were identified as the minimal sufficient adjustment sets to estimate the total effect of PWASI in predicting CVD.

6.4.9 Statistical analysis

6.4.9.1Statistical package

IBM SPSS statistical version 24

6.4.9.2Summary of statistical methods

All analyses were pre-specified with an analysis plan, and a specific application to UK Biobank to undertake this work. Categorical data was summarised using counts and percentage, while continuous data was summarized using mean and standard deviation for normally distributed data and median, IQR for non-normally distributed data. Comparisons were made across groups by incidence CVD status at the end of follow-up.

6.4.9.3Comparison of categorical variables

The categories for cIMT were <588μm, 588-658 μm, 658-748 μm and >748μm; the first quartile was considered as the reference category.

The categories for PWASI were <8.5, 8.5-10.7, >10.7; the first tertile was considered as the reference category. I decided to present PWASI as a tertile analysis as, after categorising PWASI as quartiles, the analysis estimates became unstable due to small event numbers. However, both analyses for PWASI and cIMT are also presented as continuous analyses (per standard deviation) to facilitate comparison.

The association between two or more categorical groups was assessed using a chi-square (x2) test and test for two proportions.

6.4.9.4Comparison for continuous variables

Independent t-test was applied for comparison between two parametric continuous variables. The assumptions for the test were examined. Firstly, the normality was checked visually by histogram. Secondly, the equality of variance between groups was examined by Levenes' test. The assumption of equal variance is considered sustainable if the larger SD is less than twice the

smaller SD. I used non-parametric Wilcoxon-Mann-Whitney test for non-normal distribution data.

6.4.9.5 The relationship between cIMT, PWASI and endpoint of cardiovascular diseases, stroke and coronary heart diseases

We used Cox regression analysis to calculate the unadjusted or adjusted hazard ratio rate (HR) and corresponding 95% confidence interval (CI) for cardiovascular diseases, stroke and coronary heart diseases. Univariate and multivariate Cox regression models were used. Participants who died of unrelated causes or who were lost to follow-up before an event were censored at the time of last observation. Continuous variables were entered into the model on a per-SD scale in order to facilitate comparability between different exposures.

For imaging markers adjusted models, model (2) was adjusted with age and sex, model (3) was adjusted with age, sex and systolic blood pressure, model (4) was adjusted with age, sex, systolic blood pressure and ethnicity and model (5) was adjusted with age, sex, systolic blood pressure, ethnicity and cardiovascular diseases at baseline. These selections were driven by the important determinants of cIMT and PWASI in chapter 5. These overlap largely with general CVD risk factors. A confounder, however, must be associated with both the outcome and the exposure, otherwise adjustment will have no effect. Quartiles of carotid intima media thickness and tertiles of pulse wave arterial stiffness and the continuous standardised version of these variables were used in these analyses. The standardised exposures allow direct comparison of the hazard ratios. A power calculation was conducted for the Cox models based on standardised continuous biomarkers; the statistically most powerful analysis. Based on a sample size of 19,625 participants, 275 CVD events, and required power of 80%, we had power to detect a hazard ratio of 1.18 for every standard deviation increase in the continuous biomarker.

6.5 Results

6.5.1 Demographic and hemodynamic characteristics in each group of different outcomes

The available follow-up time was median 1.4 years (interquartile range 1.82) A total of 272 experienced an incidence cardiovascular disease event during the follow up period: 235 experienced CHD, and 42 experienced ischaemic stoke.

Table (6-2) shows the demographic and haemodynamic characteristics in each group of different outcomes. The mean age of the participants who had cardiovascular diseases was 66 years, and 69.1 percent were men. White ethnicity made up 97.5 percent of the participants. The mean cIMT was (728 ±137) μm and pulse wave arterial stiffness index was (9.48 ±3.32). Coronary heart diseases accounted for 85%, while stroke accounted for 15% of cardiovascular disease events. There was no difference between participants who had a stroke and coronary heart disease in clinical characteristics except in for triglycerides, and statin treatment. Participants with coronary heart disease at the end of follow up had significantly higher triglycerides (0.3 mmol/L higher, *P*=0.046). In addition, there was a sex difference in participants who has cardiovascular diseases; males had higher incidence of CVD during follow up period than females.

Table 6-2: Demographic and haemodynamic characteristics in each group of different outcomes

Continuous Variables	**CVD ≈277**		**CHD ≈235**	**Stroke ≈42**	***P-value***
Non-invasive measurement of atherosclerosis					
Pulse wave arterial stiffness index	(272),9.48±3.32		(227),9.2±2.8	(37),11±5	0.051
Mean cIMT (μm)	(275),728±137		(230),732±143	(37),715±99	0.358
Demographic data					
Age (year)	(275),66 ±6.4		(230),66 ±6.5	(37),67±6	0.64
Anthropometric measurements					
BMI (kg/m^2)	(274),28±4.53		(229),28±4.6	(37),27±3.75	0.243
Blood pressure measurements					
Systolic blood pressure (mmHg)	(272),141 ±19.43		(227),140±19.7	(37),143±18	0.515
Diastolic blood pressure(mmHg)	(272),78.5±10.9		(232),78±11	(37), 80±12	0.305
Diabetic measurements					
Glucose (mmol/L)	(235),5.2±1.8		(198),5.3±1.9	(30),4.9±0.67	0.279
HbA1c (mmol/mol)	(257),36.9±5.58		(214),37±5.88	(35),36.1±3.4	0.394
Lipid measurements					
Total cholesterol (mmol/L)	(257),5.49±1.33		(215),5.5±1.4	(34),5.6±1.1	0.552
HDL cholesterol (mmol/L)	(236),1.29±0.305		(199),1.3±0.31	(30),1.35±0.36	0.377
LDL cholesterol (mmol/L)	(257),3.45±0.96		(215),3.5±1	(34),3.5±0.83	0.379
Triglyceride (mmol/L)	(257),1.98±1.29		(215),2 ±1.36	(34),1.7±0.7	0.046
C-reactive protien(mg/L)*	(254),1.26(1.23)		(213),1.25(2.32)	(34),1.24(1.85)	0.669
Apolipoprotein-A(g/L.)	(236),1.45±0.24		(199),1.45±0.24	(30),1.5±0.26	0.550
Apolipoprotein-B (g/L.)	(252),1.077±0.25		(210),1±0.25	(34),1±0.21	0.511
Categorical variables	**Count, (%)**				***P-value***
Male	190 (69.1%)		166 (70.6%)	23 (54.8%)	0.029
Female	85 (30.9%)		69 (29.4%)	19 (45.2%)	
Diabetes	No	248(90.2%)	207 (90%)	34 (92%)	0.573
	Yes	27 (9.8%)	23 (10%)	3 (8.1%)	
Hypertension	No	61 (22.2%)	46 (20%)	12 (32.4%)	0.096
	Yes	214 (77.8%)	184 (80%)	25 (67.6%)	
Smoking	No	251 (91.3%)	221 (94%)	33 (89.2%)	0.412
	Yes	24 (8.7%)	19 (8.3%)	4 (10.8%)	
Statin	No	78 (28.45%)	91 (39.6%)	22 (60%)	0.028
	Yes	51 (18.5%)	139 (60.4%)	15 (41%)	
Ethnicity	Whit e	268 (97.5%)	223 (97%)	37 (100%)	0.962
	Black	1 (0.4%)	1 (0.4%)	0	
	south Asian	5 (01.8%)	5 (2.2%)	0	
	Other	1 (0.4%)	1 (0.4%)	0	

(number), mean ± SD for normal distribution data**, *P-value***: to compare the means of two independent groups (participants with CHD and participants with stroke)

6.5.2 Predictors of cardiovascular events

The univariable exposures associated with cardiovascular events are presented in Table (6-3). The potential predictors include age, BMI, HbA1c, triglyceride, C-reactive protein, glucose, systolic blood pressure, LDL cholesterol, total cholesterol, apolipoprotein-A and HDL cholesterol were significantly predictive for incidence of cardiovascular diseases events. Neither diastolic blood pressure (*P*=0.599) nor apolipoprotein B (*P*=0.171) were significant predictors of cardiovascular events.

The strongest predictor of increasing risk for cardiovascular events in this population was age; a one SD increase in age (~ 6.4 years) was associated with a 95% increase in expected hazard for cardiovascular diseases (95% confidence interval (CI), 69% to 126%; *P*<0.001). A one SD (~ 4.5Kg) increase in body mass index was associated with a 38% increase in expected hazard for cardiovascular diseases (95% confidence interval (CI), 24% to 54%; *P*<0.001). Also, a one SD (~5.6mmol/mol) increase in HbA1c was associated with 37% increase in expected hazard for cardiovascular diseases (95% confidence interval (CI), 25% to 50%; P<0.001). In addition, a one SD (~19 mmHg) increase in systolic blood pressure was associated with 17% increase in expected hazard for cardiovascular diseases (95% confidence interval (CI), 4% to 32%; P=0.009). HDL cholesterol was protective against incidence of cardiovascular disease; a one SD (0.305mmol/L) increase of HDL was associated with a 38% decrease of expected hazard for cardiovascular diseases (95% confidence interval (CI), 30% to 45%; P=<0.001). The expected hazard for incidence of cardiovascular disease events was 145% higher in males as compared to females. Also, the expected hazard for incidence of cardiovascular disease events in participants with hypertension and diabetes were 209% and 121% higher as compared to non-hypertensive and non-diabetic participants respectively. Figure (6-3), shows the hazard ratio and 95% confidence intervals associated with the potential predictors considered in the univariable analyses with time to the incident of cardiovascular diseases.

Table 6-3:Individual Predictors of Cardiovascular Events

Variables	HR (95%)			*P-value*
	EXP(B)	95%		
		Lower	Upper	
Age (year) per SD	1.95	1.69	2.26	<0.001
BMI (kg/m^2) per SD	1.38	1.24	1.54	<0.001
HbA1c (mmol/mol) per SD	1.37	1.25	1.50	<0.001
Trigliceride (mmol/L) per SD	1.37	1.22	1.54	<0.001
C-reactive protein (mg/L) per SD	1.20	1.073	1.35	0.002
Glucose (mmol /L) per SD	1.17	1.064	1.30	0.002
Systolic blood pressure (mmHg) per SD	1.17	1.04	1.32	0.009
Diastolic blood pressure(mmHg) per SD	0.96	0.86	1.09	0.599
Apolipoprotein-B (g/L.) per SD	0.918	0.813	1.03	0.171
LDL cholesterol (mmol/L) per SD	0.825	0.734	0.929	0.001
Total cholesterol (mmol/L) per SD	0.774	0.688	0.87	<0.001
Apolipoprotein-A(g/L) per SD	0.707	0.622	0.804	<0.001
HDL cholesterol (mmol/L) per SD	0.618	0.543	0.703	<0.001
Variables	**HR (95%)**			***P-value***
	EXP(B)	**95%**		
		Lower	**Upper**	
Gender (male)	2.45	1.89	3.16	<0.001
Diabetes (yes)	2.21	1.49	3.29	<0.001
Hypertension (yes)	3.09	2.33	4.11	<0.001
Smoking (yes)	1.39	0.918	1.45	0.08
Statin (yes)	5.77	4.54	7.33	<0.001

The HR for each potential predictor variable is estimated from a proportional hazard model including only that variable in the model, Hazard ratios are expressed per 1 SD (log-transformed) increase for continuous risk factors

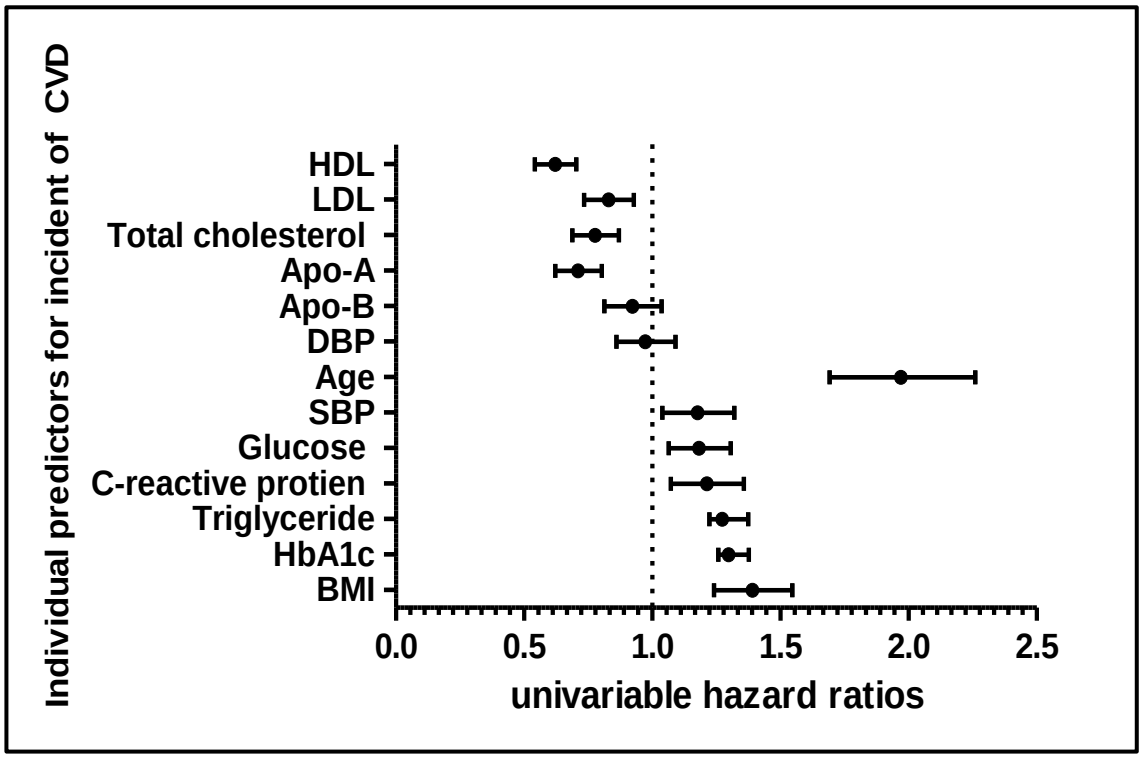


Figure 6-3 A forest plot showing the hazard ratio and 95% confidence intervals associated with variables considered in the univariable analyses with time to the incident of cardiovascular diseases as the outcome

6.5.3 CIMT and PWASI predicts cardiovascular disease

In a Cox proportional hazards regression (Table 6-4), the yearly incidence of cardiovascular disease events increased with increasing quantile for mean carotid intima media thickness, with persons in the highest quantile having a risk that was 3 times as great as that of persons in the lowest quantiles. A total of 40 CVD events in first quantile, 61 CVD events in second quantile, 64 CVD events in third quantile and 110 CVD events in last quantile. However, after adjustment for sex, age, systolic blood pressure, ethnicity and cardiovascular disease at baseline, the mean carotid intima media thickness in the highest quantile only remained significantly predictive for cardiovascular diseases compared to the lowest quantile, a patient in highest quantile (cIMT>748 μm) having 50% higher hazard estimate compared to the lowest quantile (cIMT<588 μm). Figure (6-4), shows the hazard ratio of cardiovascular diseases events in different quantile of cIMT.

A Cox proportion hazard model using mean carotid intima media thickness as continuous variable showed a strong association with incidence of cardiovascular diseases; one SD (≈0.13mm) increase of mean carotid intima media thickness was associated with 50 % increase incidence of cardiovascular disease events. This association was attenuated but still significant after adjustment for age, gender, systolic blood pressure, ethnicity and cardiovascular disease at baseline. Figure (6.5), shows the unadjusted cumulative survival ratio for of cardiovascular diseases events as outcome, according to cIMT quantiles. Figure (6.6), shows the hazard ratio for incident of cardiovascular diseases events for each increase in carotid intima media thickness.

In Cox proportional hazard regression (Table 6-5), PWASI significantly predicted increasing risk of cardiovascular disease in the highest tertile; a patient in the highest PWASI tertile (PWASI >10.7) having a 33% higher hazard estimate for CVD compared with the lowest tertile (PWASI<8.5) after adjustment for age, gender, systolic blood pressure and ethnicity. Figure (6.7), shows the unadjusted cumulative survival ratio for cardiovascular diseases, according to PWASI tertile. Figure (6.8), shows the hazard ratio for incident of

cardiovascular disease events outcome for each increase in pulse wave arterial stiffness index.

Table 6-4: HRs of CVD as a function of mean cIMT expressed as quartiles and as a continuous Variables

Variable	No of events/ no at risk	HR (95%)				
		Model (1)	Model (2)	Model (3)	Model (4)	Model (5)
Mean-cIMT (µm)		EXP(B)	EXP(B)	EXP(B)	EXP(B)	EXP(B)
mean cIMT <588µm	40/4968	1	1	1	1	1
mean cIMT (588-658) µm	61/4922	1.57* (1.05,2.35)	1.22 (0.819,1.83)	1.21 (0.80,1.81)	1.21 (0.80,1.81)	1.10 (0.71,1.69)
mean cIMT (658-748) µm	64/4801	1.72* (1.16,2.56)	1.07 (0.718,1.62)	1.09 (0.725,1.64)	1.09 (0.725,1.64)	0.928 (0.59,1.45)
mean cIMT >748µm	110/4934	3.00** (2.09,4.31)	1.43* (0.97,2.12)	1.50* (1.01,2.22)	1.50* (1.014,2.22)	1.49* (0.99,2.25)
Per 1 SD increase	275/19625	1.50** (1.34,1.67)	1.16* (1.02,1.31)	1.18* (1.04,1.34)	1.18* (1.04,1.34)	1.18* (1.04,1.34)

Hazard ratios (HR) with 95% confidence interval (CI) estimated using cIMT per SD change in µm is shown per model for incidence CVD
Model 1: unadjusted
Model 2: adjusted with age and gender
Model 3: adjusted with age, gender and systolic blood pressure
Model 4: adjusted with age, gender, systolic blood pressure and ethnicity
Model 5: adjusted with age, gender, systolic blood pressure, ethnicity and cardiovascular diseases
1 SD of cIMT~ 0.13mm
*P<0.05, **P<0.001

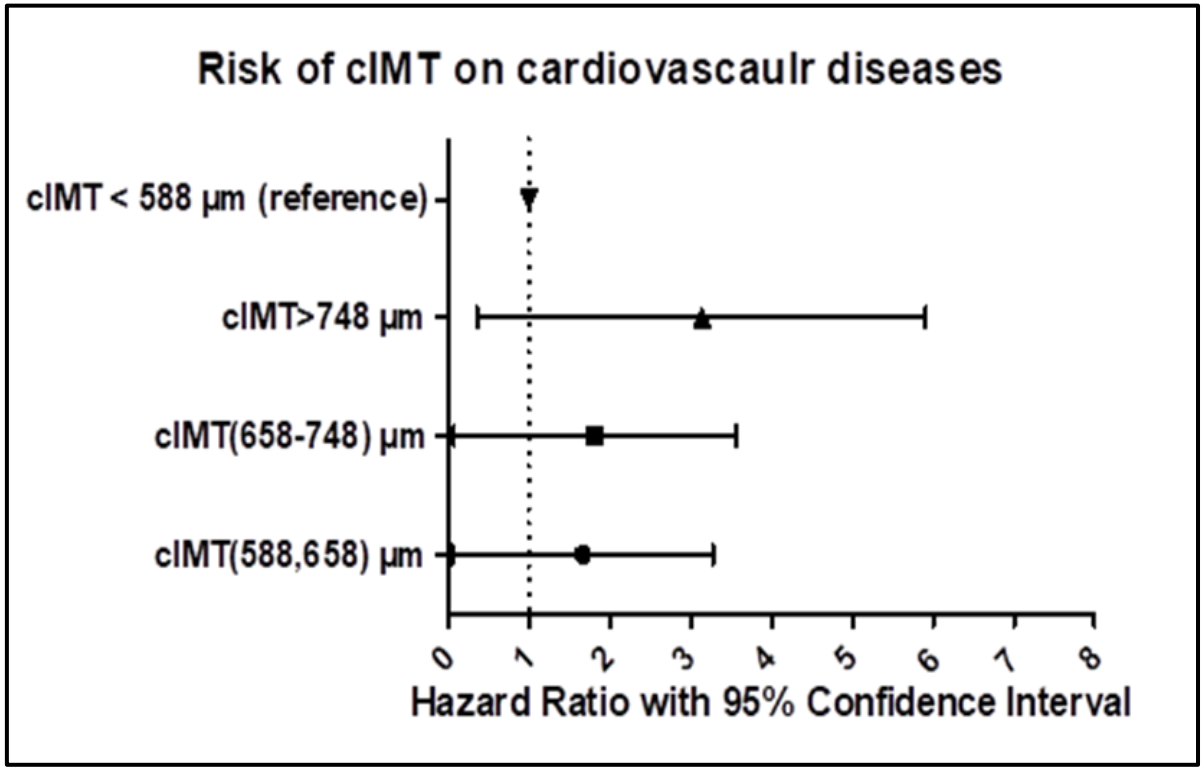


Figure 6-4 HRR of cardiovascular disease events in cIMT quartiles

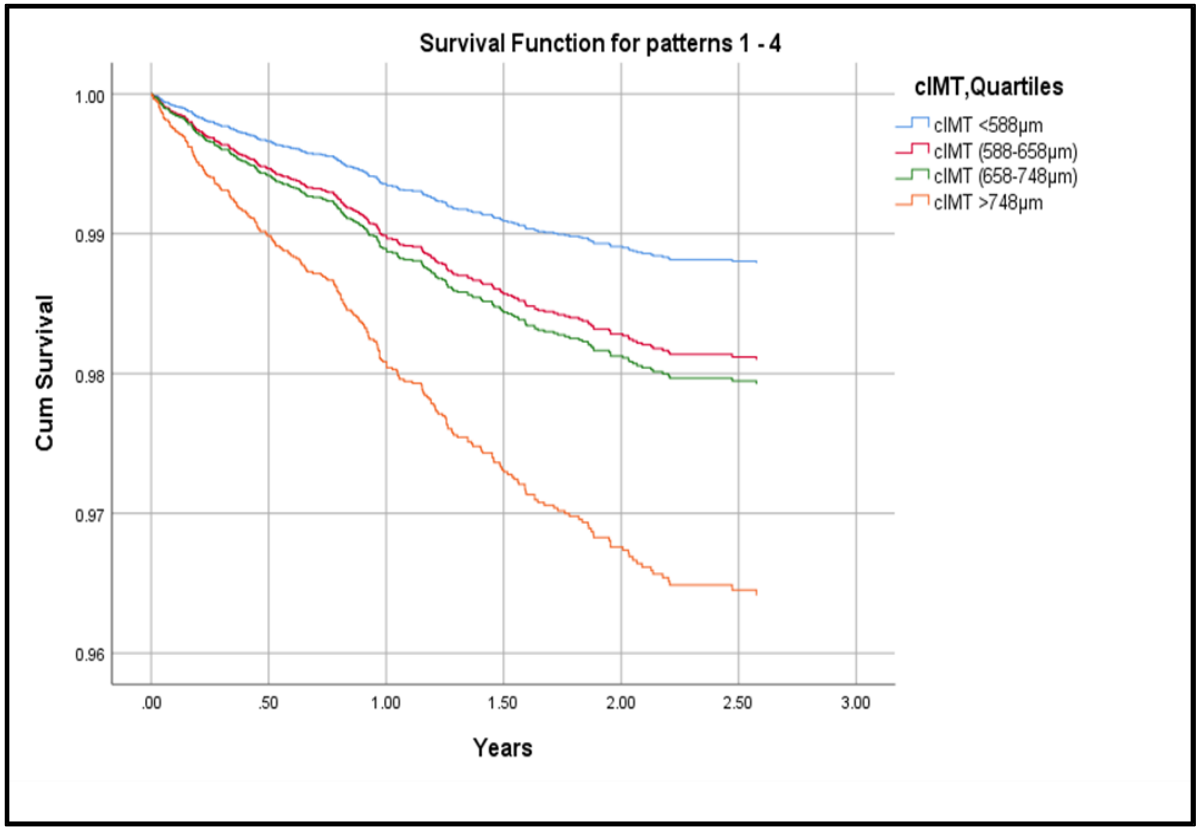


Figure 6-5 Unadjusted cumulative survival ratio for the end point of cardiovascular diseases, according to cIMT quartile

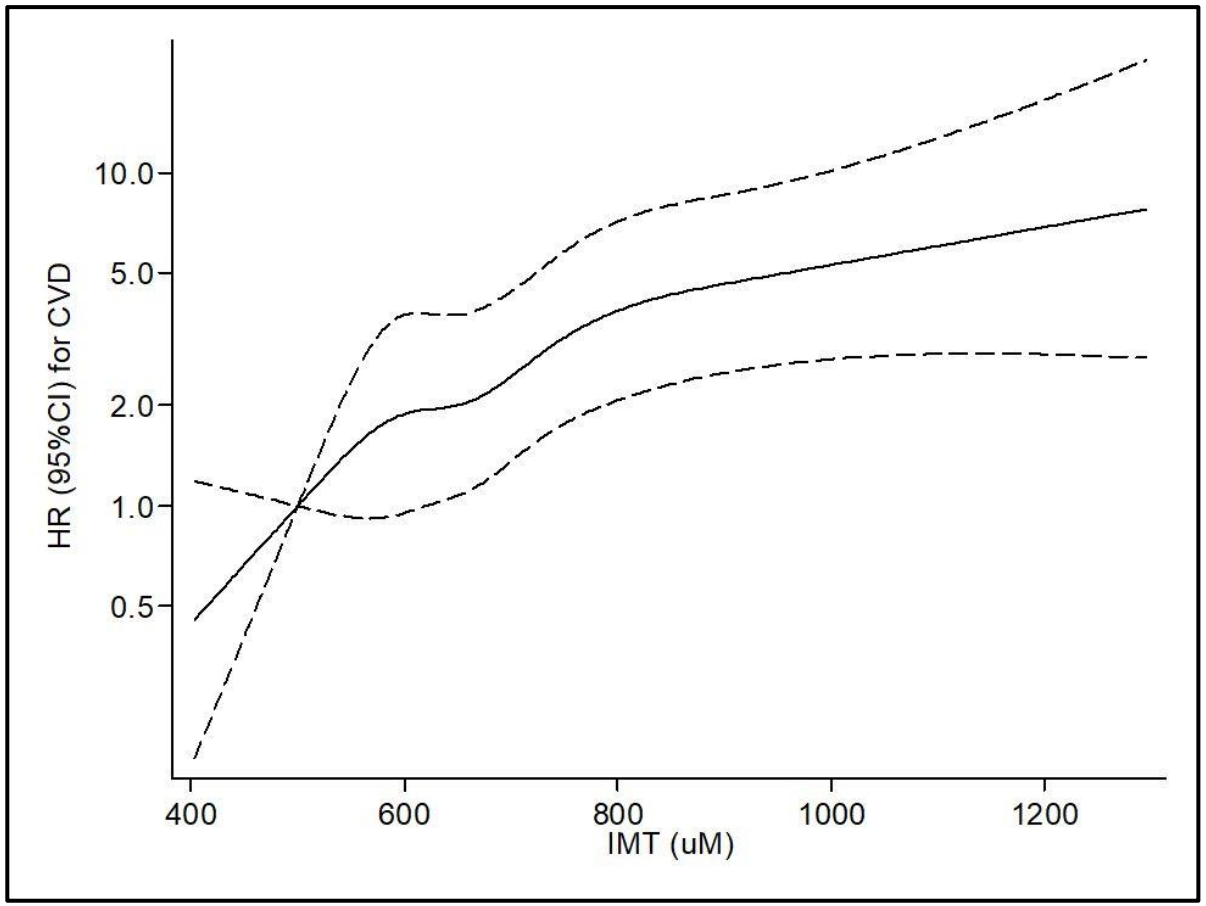


Figure 6-6 Hazard ratio (univariate) (95%CI) for incident of cardiovascular diseases events for each increase in carotid intima media thickness

Table 6-5: HRs of CVD as a function of PWASI Expressed as tertiles and as a Continuous Variables

Variable	No of events/ no at risk	HR (95%)				
		Model (1)	Model (2)	Model (3)	Model (4)	Model (5)
PWASI		EXP(B)	EXP(B)	EXP(B)	EXP(B)	EXP(B)
PWASI<8.5	96/6053	1	1	1	1	1
PWASI (8.5-10.7)	94/6237	1.04 (0.782,1.38)	0.943 (0.708,1.25)	0.967 (0.722,1.25)	0.968 (0.723,1.28)	0.979 (0.725,1.29)
PWASI >10.7	82/6527	0.930 (0.692,1.24)	0.697* (0.517,0.941)	0.730* (0.534,0.987)	1.31* (0.993,1.80)	0.714* (0.525,0.967)
Per 1 SD increase of PWASI	272/18817	0.943 (0.841,1.05)	0.875* (0.785,0.975)	0.886* (0.792,0.977)	0.887* (0.792,0.988)	0.889* (0.798,0.989)

Hazard ratios (HR) with 95% confidence interval (CI) estimated using PWASI per SD change in m/s is shown per model for incidence CVD
Model 1: unadjusted
Model 2: adjusted with age and gender
Model 3: adjusted with age, gender and systolic blood pressure
Model 4: adjusted with age, gender, systolic blood pressure and ethnicity
Model 5: adjusted with age, gender, systolic blood pressure, ethnicity and cardiovascular diseases
*P<0.05, **P<0.001

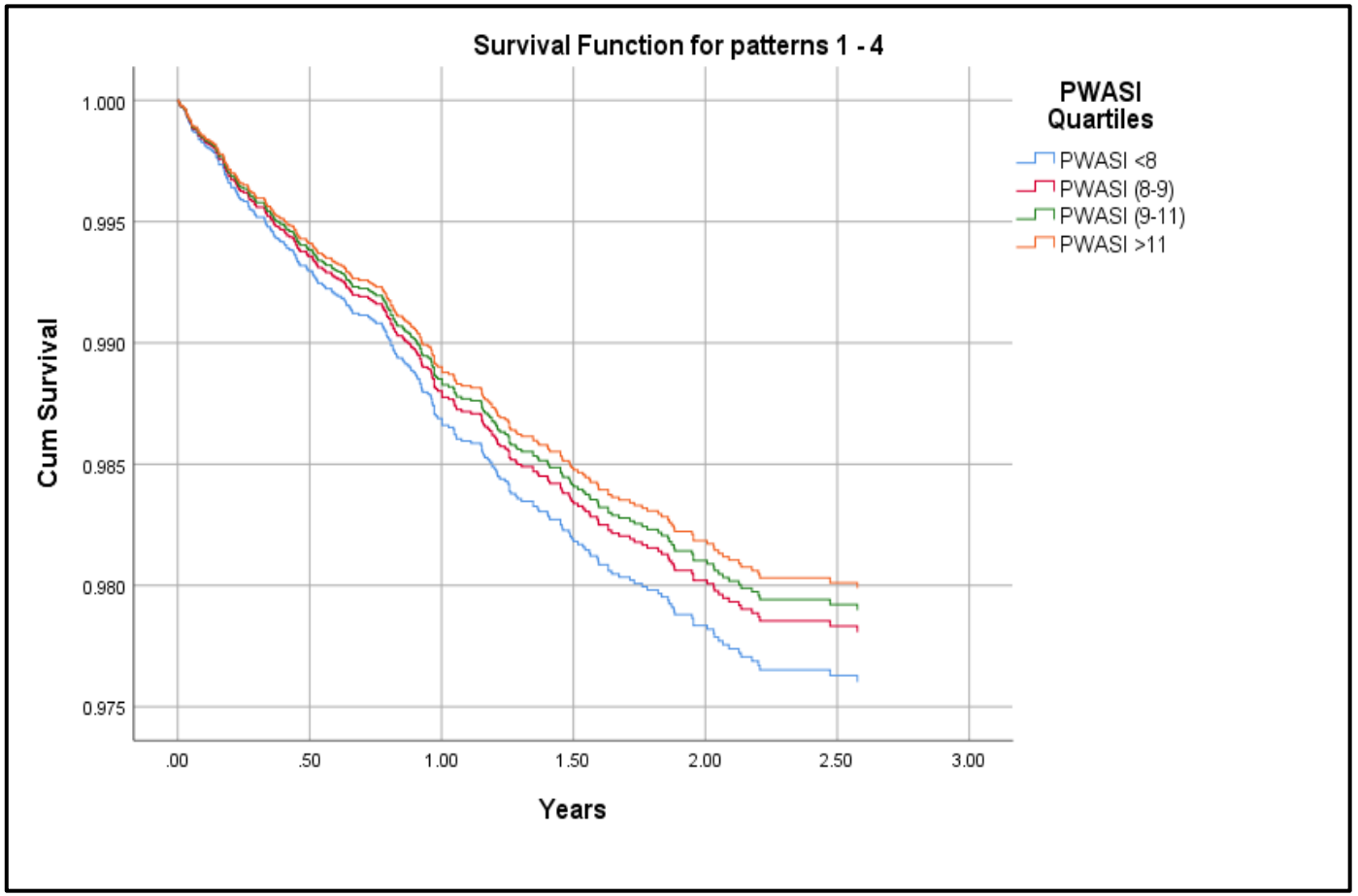


Figure 6-7 Unadjusted cumulative survival ratio for the end point of cardiovascular diseases, according to PWASI tertile

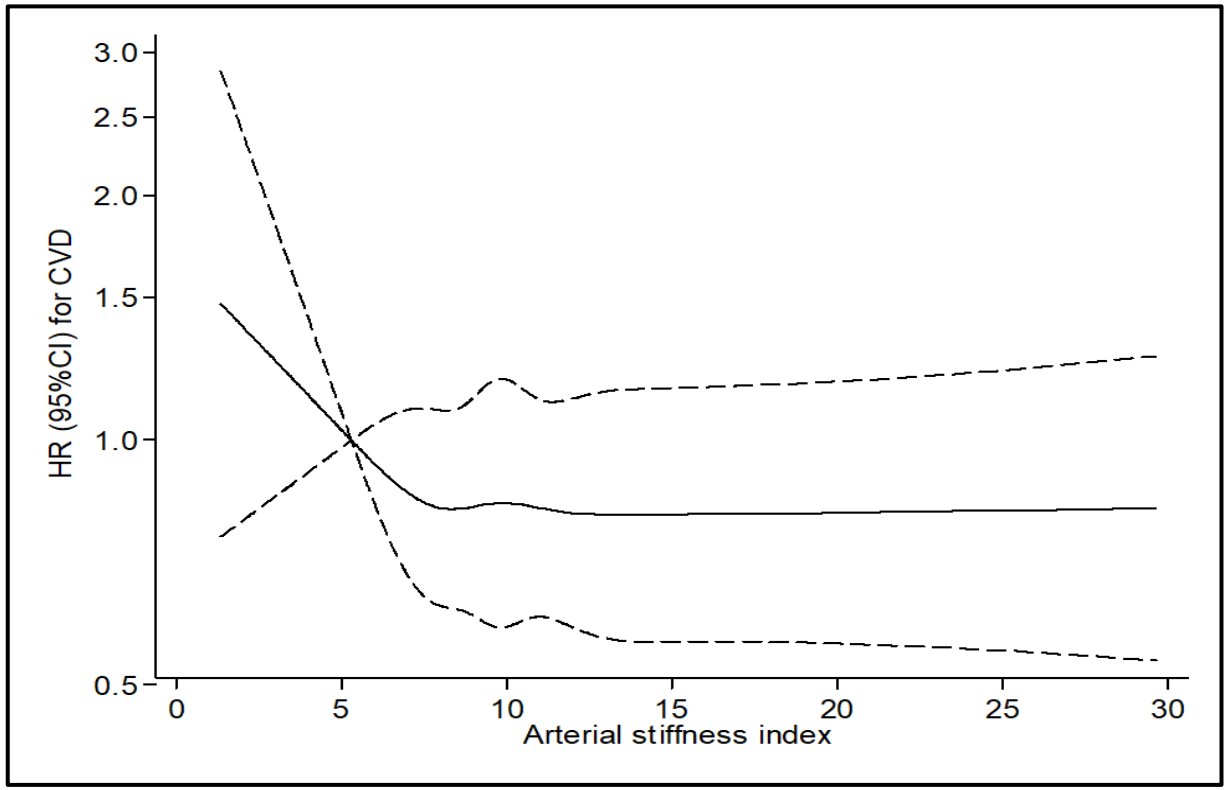


Figure 6-8 Hazard ratio (univariate) for incident of cardiovascular disease events outcome for each increase in pulse wave arterial stiffness index

6.5.4 cIMT and PWASI predicts coronary heart diseases and stroke

The results of Cox proportional hazard models for coronary heart diseases and stroke are shown in Tables 6-6 to 6-9.

Table 6-6 showed a hazard estimate of CHD as a function of mean cIMT expressed as quartiles and as continuous variables. For the Cox proportional hazard model using carotid intima media thickness as a continuous variable, there was a strong association with incidence of coronary heart diseases in univariate analyses and after adjustment for cardiovascular risk factors. In univariable analysis, one SD increase of mean carotid intima media thickness was associated with 50 % increase risk for incidence of CHD. The association was attenuated after adjustment with CVD risk factors, but it remained significant association. Categorizing carotid intima media thickness into subintervals showed a graded significant relationship with incidence of coronary heart disease, but this significant association turned to insignificant after adjustment with cardiovascular risk factors. In the un-adjusted model, the expected hazard for CHD for the highest cIMT quantile (cIMT>748μm) was 3 times higher than the expected hazard of CHD at the lowest cIMT quantile (cIMT<588μm). Participants at highest cIMT quantile (cIMT>748μm) had significantly increased risk for incidence CHD in the univariable model and after adjustment, compared with lowest quantile.

Table (6-7) shows the hazard estimate of stroke as a function of mean cIMT (expressed as quartiles and a continuous variable). For the Cox proportional hazard model using carotid intima media thickness as continuous variable, in univariate analysis, an increment of 1 SD in carotid intima media thickness was associated with 50% increase in risk, and this association turned to insignificant after adjustment with cardiovascular risk factors. For cIMT quantiles, in the univariable model, the hazard estimate of stroke for patients in the third cIMT quantile (658-748 μm) was 3 times higher compared with patients at first cIMT quantile (<588μm). This associated turned insignificant after adjustment with CVD risk factors. Cox proportional hazard models using pulse wave arterial stiffness (as categorical variables) showed PWASI at third PWASI tertile (>10.7)

significantly predicted coronary heart disease after adjustment with cardiovascular risk factors. In addition, using PWASI as a continuous variable showed that PWASI significantly predicted coronary heart disease in the univariate analyses and adjustment models (Table 6-8). However, there was no association between pulse wave arterial stiffness and risk of stroke in univariate or multivariable models, either using PWASI as categorical or continuous measure (Table 6-9).

Table 6-6: HRs of CHD as a function of mean cIMT Expressed as Quartiles and as a Continuous Variables

Variable	No of events/ no at risk	HR (95%)				
		Model (1)	Model (2)	Model (3)	Model (4)	Model (5)
Mean-cIMT (µm)		EXP(B)	EXP(B)	EXP(B)	EXP(B)	EXP(B)
mean cIMT <588µm	37/4968	1	1	1	1	1
mean cIMT (588-658) µm	51/4922	1.57* (1.05,2.35)	1.22 (0.819,1.83)	1.21 (0.806,1.81)	1.21 (0.806,1.81)	1.20 (0.805,1.81)
mean cIMT (658-748) µm	47/4801	1.72* (1.16,2.56)	1.07 (0.718,1.62)	1.093 (0.725,1.64)	1.09 (0.725,1.64)	1.14 (0.761,1.72)
mean cIMT >748µm	100/4934	3** (2.09,4.31)	1.43* (0.975,2.12)	1.50* (1.01,2.22)	1.50* (1.01,2.22)	1.50* (1.01,2.22)
Per 1 SD increase	235/19625	1.50** (1.34,1.67)	1.16* (1.02,1.31)	1.18* (1.04,1.34)	1.18* (1.04,1.34)	1.18* (1.04,1.34)

Hazard ratios (HR) with 95% confidence interval (CI) estimated using cIMT per SD change in µm is shown per model for incidence CHD
Model 1: unadjusted
Model 2: adjusted with age and gender
Model 3: adjusted with age, gender and systolic blood pressure
Model 4: adjusted with age, gender, systolic blood pressure and ethnicity
Model 5: adjusted with age, gender, systolic blood pressure, ethnicity and cardiovascular diseases
1 SD of cIMT~ 0.14 mm
*P<0.05, **P<0.001

Table 6-7: HRs of stroke as a function of mean cIMT Expressed as Quartiles and as a Continuous Variables

Variable	No of events/ no at risk	HR (95%)				
		Model (1)	Model (2)	Model (3)	Model (4)	Model (5)
Mean-cIMT (µm)		EXP(B)	EXP(B)	EXP(B)	EXP(B)	EXP(B)
mean cIMT <588µm	5/4968	1	1	1	1	1
mean cIMT (588-658) µm	9/4922	1.85 (0.623,5.54)	1.32 (0.440,4)	1.31 (0.436,3.97)	1.31 (0.436,3.97)	1.31 (0.436,3.97)
mean cIMT (658-748) µm	16/4801	3.44* (1.26,9.39)	1.94 (0.692,5.47)	1.91 (0.678,5.40)	1.91 (0.677,5.39)	1.93 (0.685,5.45)
mean cIMT >748µm	12/4934	2.58 (0.909,7.32)	1.15 (0.382,3.46)	1.11 (0.366,3.39)	1.11 (0.366,3.38)	1.11 (0.366,3.38)
Per 1 SD increase	42/19402	1.38* (1.03,1.83)	1.04 (0.752,1.44)	1.04 (0.751,1.44)	1.04 (0.751,1.44)	1.04 (0.754,1.45)

Hazard ratios (HR) with 95% confidence interval (CI) estimated using cIMT per SD change in µm is shown per model for incidence stroke
Model 1: unadjusted
Model 2: adjusted with age and gender
Model 3: adjusted with age, gender and systolic blood pressure
Model 4: adjusted with age, gender, systolic blood pressure and ethnicity
Model 5: adjusted with age, gender, systolic blood pressure, ethnicity and cardiovascular diseases
1 SD of cIMT~ 0.09 mm
*P<0.05, **P<0.001

Table 6-8: HRs of CHD as a function of PWASI Expressed as tertiles and as a Continuous Variables

Variable	No of events/ no at risk	HR (95%)				
		Model (1)	Model (2)	Model (3)	Model (4)	Model (5)
PWASI		EXP(B)	EXP(B)	EXP(B)	EXP(B)	EXP(B)
PWASI<8.5	85/6053	1	1	1	1	1
PWASI (8.5-10.5)	84/6464	1.04 (0.776,1.41)	0.941 (0.695,1.27)	0.968 (0.714,1.32)	0.972 (0.716,1.32)	0.977 (0.718,1.33)
PWASI >10.7	63/6527	0.806 (0.582,1.11)	0.594* (0.427,0.827)	0.625* (0.447,0.883)	0.628* (0.445,0.879)	0.618* (0.442,0.864)
Per 1 SD increase	232/18817	0.878* (0.779,0.989)	0.817** (0.730,0.914)	0.829** (0.738,0.928)	0.830* (0.739,0.931)	0.837* (0.749,0.934)

Hazard ratios (HR) with 95% confidence interval (CI) estimated using PWASI per SD change in m/s is shown per model for incidence CHD
Model 1: unadjusted
Model 2: adjusted with age and gender
Model 3: adjusted with age, gender and systolic blood pressure
Model 4: adjusted with age, gender, systolic blood pressure and ethnicity
Model 5: adjusted with age, gender, systolic blood pressure, ethnicity and cardiovascular diseases
*P<0.05, **P<0.001

Table 6-9: HRs of stroke as a function of PWASI Expressed as tertiles and as a Continuous Variables

Variable	No of events/ no at risk	HR (95%)				
		Model (1)	Model (2)	Model (3)	Model (4)	Model (5)
PWASI		EXP(B)	EXP(B)	EXP(B)	EXP(B)	EXP(B)
PWASI<8.5	15/6053	1	1	1	1	1
PWASI (8.5-10.5)	10/6237	0.706 (0.317,1.57)	0.697 (0.312,1.55)	0.698 (0.313,1.56)	0.695 (0.311,1.56)	0.697 (0.311,1.56)
PWASI >10.7	17/6537	1.23 (0.614,2.47)	1.059 (0.520,2.15)	1.05 (0.516,2.165)	1.048 (0.511,2.14)	1.05 (0.512,2.15)
PWASI, Per 1 SD increase	42/18817	1.31 (0.943,1.83)	1.24 (0.902,1.71)	1.22 (0.900,1.71)	1.23 (0.897,1.71)	1.23 (0.897,1.71)

Hazard ratios (HR) with 95% confidence interval (CI) estimated using PWASI per SD change in m/s is shown per model for incidence stroke
Model 1: unadjusted
Model 2: adjusted with age and gender
Model 3: adjusted with age, gender and systolic blood pressure
Model 4: adjusted with age, gender, systolic blood pressure and ethnicity
Model 5: adjusted with age, gender, systolic blood pressure, ethnicity and cardiovascular
*P<0.05, **P<0.001

6.6 Discussion

Our study demonstrates an association between imaging markers, that include cIMT and PWASI, and incidence of cardiovascular disease within a median of 1.4 year follow-up period, in the UK Biobank population. We found that cIMT as a continuous variable is strongly predictive of cardiovascular disease events, including coronary heart disease, and the prediction remained significant after adjustment with age, gender, systolic blood pressure and history of cardiovascular diseases in the Cox regression multivariable model. In addition, the highest quartile for cIMT remained significantly predictive for incidence cardiovascular events, include coronary heart diseases, after adjustment for age, gender and systolic blood pressure. However, for stroke outcome the significant association with cIMT as a continuous variable turned insignificant after adjustment with age, gender, systolic blood pressure and history of cardiovascular disease. Furthermore, there was insignificant association between PWASI as continuous variable and incidence of cardiovascular disease events including coronary heart diseases. The highest tertile of PWASI remained significantly associated with the incidence of cardiovascular disease events include coronary heart diseases after adjustment for age, gender, and systolic blood pressure.

6.6.1 Carotid intima media thickness predicts cardiovascular diseases

We found in our study that the age and gender adjusted HR was 16% increased for CHD for every 0.13 mm increased of mean carotid intima media thickness. These data confirm the results of previous large-scale population-based studies. The Kuopio Heart Disease Risk Factor Study (KIHD) is a population based study that investigated the association between CHD risk factors and carotid atherosclerosis in 3,000 middle aged eastern Finnish men (Salonen, 1988). In February, 1987, Salonen and Salonen, investigated 1,288 men who were a random sample from the KIHD study and they found that intimal medial thickening of the common arteries was associated with a 2.17 fold increased risk of acute coronary syndrome in 257 who had intimal medial thickening compared with 608 who had no structural changes in carotid arteries (Salonen and Salonen, 1991a). The Rotterdam Elderly

Study was a longitudinal population based study that investigated the determinants of the incidence of diseases and disability in elderly involving 11,854 participants aged between 55 or older (Hofman *et al.*, 1991). Bots and their colleagues performed a case-control study in a subgroup from the Rotterdam Elderly Study (n=7983) and they found the odds ratio for MI was 1.43 and the odd ratio for stroke was 1.41 for every 0.163 mm increase of common carotid intima media thickness in median, over a follow up period of 2.7 years. In addition, the significant association between carotid intima media thickness with cardiovascular diseases turned to nonsignificant after adjustment for the following risk factors (age, sex, body mass index, smoking, total cholesterol, HDL cholesterol, hypertension, systolic blood pressure, diabetes mellitus and previous cardiovascular diseases) (Bots *et al.*, 1997). In the Cholesterol Lowering Atherosclerosis Trial, a randomized arterial imaging trial that studied the effect of lipid lowering on progression of atherosclerosis (Blankenhorn *et al.*, 1987) Hodis and co-workers found a significant association between carotid Intime media thickness and incidence of coronary artery diseases in 146 non-smoking men aged between 40-59, who all had cardiovascular diseases at baseline, which was a sample from the Cholesterol Lowering Atherosclerosis Trial. After a long follow up period (8.8 years), they found for each 0.03 mm increase of common carotid intima media thickness there was 2.2 relative risk of nonfatal myocardial infarction or coronary death. (Hodis *et al.*, 1998). In addition, Kuller and co-workers found that participants with subclinical diseases has more risk for cardiovascular morbidity than participants without subclinical diseases (Kuller *et al.*, 1995). Subclinical disease was defined by a combination of major electrocardiogram abnormalities, low ejection fraction, increase carotid or internal carotid artery wall thickness or stenosis, decrease ankle-brachial blood pressure, and the positive responses to the Rose questionnaire angina or intermittent claudication (Kuller *et al.*, 1994a). Another study by Yoshihiro Kokubo showed that carotid artery IMT, measured by using carotid artery ultrasonography, is a predictor of new onset of CVD events in 4724 participants aged 50 and 70 years without cardiovascular disease at baseline in the general Japanese population (Kokubo *et al.*, 2018a).

In terms of carotid intima media thickness quartiles, our study showed that the highest quartile (>0.748mm) was significantly associated with incidence

cardiovascular disease. In line with our findings, Salonen and Salonen showed that the common carotid intima media thickness measurement greater than 1.0 mm was predictive of acute myocardial infarction over a 1 month to 3 years period (Salonen and Salonen, 1991a). Also, Belcaro and colleagues, found that one time measurement of intima media thickness greater than 1.0 mm predicted cardiovascular events over a 6 year period; they examined a randomly selected population sample of 2000 healthy and asymptomatic subjects (Belcaro *et al.*, 1996). In previous studies, early atherosclerosis intima-media thickness values less than 1.0 mm were considered as reference the category. Despite, variance in the definition of the end point between studies, our findings of association between carotid intima media thickness and risk of coronary events are consistent with findings from these previous studies.

Two other studies showed the association between carotid artery intima media thickness at different sites and incidence of myocardial infarction in the future. Chambless *et al*. found an association between carotid intima media thickness and myocardial infarction in the Atherosclerosis Risk in Communities Study (ARIC), a study of (15,790) men and women, aged between 45 and 64 years. Their analyses were based on common carotid intima media thickness, carotid bifurcation and internal carotid intima–media thickness. The results showed a stronger association between common carotid intima media thickness and myocardial infarction than other locations in follow up period 4-7 years. Relative risks of woman were 1·32 (95% CI 1·13–1·54), 1·23 (95% CI, 1·15–1·31) and 1·15 (95% CI, 1·08–1·23) and relative risks for men were 1·92 (95% CI, 1·66–2·22), 1·40 (95% CI, 1·29–1·53) and 1·28 (95% CI, 1·18–1·39) for every one SD increase of common carotid, carotid bifurcation and internal carotid intima–media thickness, respectively. The association with cardiovascular events remained statistically significant after adjustment with cardiovascular risk factors which included age, cigarette pack-years, systolic pressure, HDL cholesterol and HDL cholesterol/ LDL cholesterol ratio, and was apparent at higher IMT quartiles (Chambless *et al.*, 1997).

However, the Cardiovascular Health Study, a prospective study of 5201 participants aged between 65 years and older, did not show the difference in strength of the association between different sites of measurements. O'Leary *et al*. found that for every 0.2 mm increase of maximal common carotid intima media

thickness, the age and gender adjusted HR was 1.33 for MI and .137 for stroke. In addition, the association between cIMT and myocardial infarction remained significant even after adjustment with cardiovascular risk factors which includes; age, sex, systolic and diastolic blood pressure, presence or absence of atrial fibrillation, pack-years of smoking, and history of diabetes; there were 4464 participants included in this analysis in median follow up 7 years (O'Leary *et al.*, 1999). These differences may be due to the difference sites of cIMT measurements as well as the number of risk factors included in the analyses, in addition to the longer follow up period compared with our study.

Our findings are in line with above mentioned studies. Despite the considerable difference in the protocols used in IMT measurements, the corresponding risks of incidence cardiovascular disease are very similar. However, the direct quantitative comparison between results are not possible, due to the difference between studies in exposure measurements, presentation and endpoint definition, as well as the location of the carotid artery measured.

As it is known that the correlation between established cardiovascular risk factors and CVD are partly explained by their effect on the process of atherosclerosis, so it was expected that additional adjustment of cardiovascular risk factors would reduce such significant associations. These risk factors were considered as intermediate or explanatory variables rather than confounding variables.

In contrast to our findings, a study by Wannarong et al that was conducted to assess the prediction of cardiovascular events by progression of total plaque area and carotid intima media thickness among 349 participants who attended a vascular prevention clinic. This study showed that regression of carotid intima media thickness rather than progression was associated with cardiovascular events over a median follow up period 3.17 year (Wannarong *et al.*, 2013). The difference in the findings is likely to be explained by the short follow up period and small sample size compared to our study, as well as the measurement of IMT at far wall of common carotid where there is minimal plaque formation and should not be considered as truly represent of atherosclerosis (Spence, 2014). Also, the IMT at the far wall of the common carotid is more closely correlated to hypertensive hypertrophy, and it is not necessarily reflecting the atherosclerosis process (Finn,

Kolodgie and Virmani, 2010). In our study, we examined carotid intima media thickness at different angels to truly represent the focal nature of atherosclerosis.

6.6.2 Pulse wave arterial stiffness predicts cardiovascular diseases

We also found an association between independent measures of vascular aging, pulse wave arterial stiffness index, and the incidence of cardiovascular events include coronary heart diseases, in 18,817 of the UK Biobank population. In our study, a negative association was shown between incidence cardiovascular disease events, include CHD, and PWASI, whereby the risk decreases by 13% per SD change, after adjustment with age and gender. This was based on a mean follow up period of two years in Scotland and 4 years in Wales and England; 272 participants experienced CVD events. They had shown that arterial stiffness index was an independent predictor for a new onset of cardiovascular disease events and mortality in 18,190, the risk of CVD events increased by 6% per SD change in median of 6 years of follow up (Said *et al.*, 2018b).

A study by Mitchell et al found that higher aortic PWV was associated with 48% increase in CVD risk over a median follow up period of 7.8 years. They examined 2232 participants from the Framingham Heart Study and 151 participants experienced an event (Mitchell *et al.*, 2010).

A meta-analysis of 17 longitudinal studies evaluated the predictive value of aortic pulse wave velocity (PWV) for future CVD events and followed up 15,877 participants for a mean of 7.7 years. It showed that increases in aortic PWV by 1 SD were associated with a 47% increase in cardiovascular events (Vlachopoulos, Aznaouridis and Stefanadis, 2010). Our study showed an unexpected result regarding the inverse association between PWASI and incidence CVD. This association only became evident after adjusting for baseline CVD; without adjusting for baseline CVD the association was borderline positive (model 4). This difference between our results and previous results, may be due in part to the relatively short follow up period, the length of follow up period between studies and difference in the study designs. The median length of follow up period of this study is 3 years, while the length follow-up period for other studies was more than

7 years. In addition, a very recent publication in UK Biobank explores the association between PWASI and CVD in a larger dataset; (Said *et al.*, 2018a) our study was designed to investigate cIMT and PWASI in the same size of population at the same visit. The study by Said et al included 141,000 participants and 18,000 CVD events over 3 years, leveraging a larger cohort size by not comparing two biomarkers side by side. They report that after adjustment a one standard deviation increase in PWASI is associated with a HR for CVD of 1.04 (95%CI 1.03-1.06). Therefore, although the association is positive, it is very weak. Our study makes it clear that cIMT is likely to be the stronger biomarker for CVD risk, and the external study supports this view.

Moreover, the risk for developing cardiovascular disease events is higher in patients with another cardiovascular risk factor. Our results showed a 17% increase risk for cardiovascular disease events for every (19mm Hg) increase of systolic blood pressure, in agreement with a study by Blacher et al that found a 17 % increased risk of CVD per 10 mm Hg of pulse pressure (Blacher *et al.*, 2000). Also, our data showed that older age was associated with a 95% increased risk for cardiovascular disease, in line with findings of a cohort study of 1574 men that showed that age was a significant predictor for cardiovascular events (HR,2.14 per 10 years) (Anderson *et al.*, 2011).

6.7 Conclusion

This prospective study presents preliminary evidence of associations between vascular imaging markers and incidence of cardiovascular diseases, these show the importance of non-invasive screening of the arterial walls to classify individuals who at risk of incidence CVD. This gives clinicians a chance for early detection and personalized prevention for CVD.

6.8 Strengths and limitations

This study has several strengths. This study was the first to investigate whether the non-invasive vascular markers (PWASI and cIMT) are associated with the incidence cardiovascular disease in the UK Biobank population, with a median

follow up period of 1.5 years. In addition, the large sample size allowed us to detect a small effect size at a statistically significant level.

Several important limitations of our study should be considered. We acknowledge that an observational study cannot definitively prove that there is a causal association underlying the link between imaging markers and risk of cardiovascular diseases events, and our results should be interpreted with caution. One important limitation here is the limited duration of disease follow-up. As a result, this study may not include CVD events for some participants, that may occur after the end of the follow up period, thus reducing statistical power. Cardiovascular disease events caused by changes in vascular health, as described in Table (6-1), may require a longer follow up period while waiting for events to occur, so the association might be underestimated. A frequently used time horizon for incident CVD is >10 years (Kokubo *et al.*, 2018b). A second limitation is the methodological differences of arterial stiffness (aortic PWV versus ASI) and cIMT measurement, which are likely to play a role in the discrepancies found between ours and previous studies. Arterial stiffness index derived by finger photoplethysmography is influenced by the elasticity of the large central arteries and the properties of the central and peripheral reflection sites, (Sollinger *et al.*, 2006; D'Agostino *et al.*, 2008). Moreover, the reflection sites are different between aortic and peripheral arteries; the pulse wave velocity measured in the aortic artery is usually lower than the peripheral PWV because of the nearer reflection sites to peripheral arteries (Lantelme *et al.*, 2002). In addition, there is a difference between brachial PP and central PP. The brachial artery was used as a measure for systemic arterial stiffness instead of central PP, which is located at the carotid artery. Central PP is generally lower than brachial PP because larger number of reflection sites are located on peripheral arteries compared with central arteries. Also, the peripheral arteries of younger individuals are usually stiffer than central arteries, so that may lead to an increased difference between brachial PP and central PP and might resulted in an overestimation of the stiffness of their arterial tree (Laurent *et al.*, 2006). These differences make results from our and previous studies more difficult to directly compare. Although measurement errors cannot be avoided, well-trained nurses in UK Biobank performed the examinations. In addition, it is possible that high-risk participants may have dropped out during

follow-up period of cardiovascular events so the association may be underestimated.

Moreover, we cannot rule out the effect of residual confounding by duration or severity of associated risk factors, or unknown risk factors. We evaluated a UK population aged from 40 to 70 year therefore, our findings may not be generalisable to younger individuals. The follow-up period was relatively short, meaning that the number of outcome events was low. This is likely to be the main reason for insignificant HRs for stroke after full adjustment for risk factors, see Table (6-7) and (6-9). The number of stroke events was small, thereby reducing the statistical power.

7 General discussion

7.1 Summary

This thesis explored the upstream determinants of imaging markers, specifically PWASI and cIMT, and the connection between traditional CVD risk factors and the imaging markers. It also assessed the possibility of using circulating miRNA as potential biomarkers for cardiovascular diseases. To fulfil these aims, I used two studies with different designs and different population phenotypes, namely:

1- The Carotid Atherosclerosis: MEtformin for insulin ResistAnce (CAMERA) study

   I. To investigate the association of the expression of targeted circulating miRNAs with cardiac biomarkers, metabolic biomarkers, lipids, and LFTs

   II. To investigate the effect of metformin on the expression of targeted circulating miRNAs.

2- The UK Biobank, a large-scale prospective epidemiological study

   I. To investigate the association of cIMT and PWASI with simple demographic patterns and upstream CVD risk factors and assess whether these risk factors are independently associated with cIMT and PWASI.

   II. To compare and contrast cIMT and PWASI as biomarkers of CVD outcomes, specifically: the association between imaging markers (cIMT and PWASI) as categorical and continuous variables with incidence cardiovascular disease in the UK Biobank population, independent of traditional cardiovascular risk factors.

7.2 Major findings

7.2.1 Suitability of the miRNA methodology for samples from randomised controlled trial

This thesis presents data investigating circulating miRNA biomarkers, and their associations with risk factors from plasma samples that were taken from a RCT. Circulating miRNAs exhibit promising potential diagnostic and prognostic markers or therapeutic targets. To allow clinical applications, larger scale studies are required to further test their sensitivity, specificity, validity and reproducibility as biomarkers for clinical use and to reduce or prevent type 1 or 2 errors.

Unfortunately, all the protocols that are currently used for the detection of circulating miRNA in body fluids include several steps, starting from extraction to the detection of target genes via QPCR or microarrays. In addition, these detection methods are expensive, time consuming, and require expertise which is not easy to apply in a large study. The development of a robust, simple and sensitive method for detection of circulating miRNAs is a necessity before it is possible to use them as useful biomarkers.

In the last few years, efforts have been made in the development of novel assay methodologies for the detection of circulating miRNA in body fluids. One of these methods is isothermal amplification that uses a constant temperature instead of a thermal cycle (Zanoli and Spoto, 2013). Another method is the use of aptamers, which are single stranded oligonucleotides that have sensitivity and specificity in identifying target miRNAs in plasma without the need of miRNA extraction, reverse transcription, amplification, and normalisation (Lu *et al.*, 2019).

7.2.2 Circulating miRNAs as biomarkers

The emergence of circulating miRNAs as novel minimally invasive biomarkers is a relatively a new and promising area of research. Circulating miRNAs have unique characteristics that make them potential biomarker candidates in different clinical applications, such as disease diagnosis, monitoring treatment therapy and predicting the recurrence of diseases. These characteristics include their stability in harsh conditions, their ease of detection and isolation from body fluids and the

same signatures of miRNAs in blood in both sexes, well as individuals of different ages (Hunter *et al.*, 2008). MiRNAs were thought to be exclusively intracellular until recent studies showed that miRNAs are also extracellular as they are present in a cell-free circulating form in plasma or serum and other body fluids. The mechanisms for the release of these miRNAs in the extracellular environment is not clear. MiRNAs circulate in different forms, which can be roughly subdivided into vesicle-associated (exosomes, microvesicles, and apoptotic bodies) (Valadi *et al.*, 2007), (Zernecke *et al.*, 2009), (Sohel, 2016) and non-vesicle associated, with RNA-binding proteins (Arroyo *et al.*, 2011) or high-density lipoprotein complexes (HDL). The biology underlying the implications of different release and transport mechanisms is not currently clear, but interest in the use of simple free-miRNA in the blood streams has burgeoned.

Recently, a growing body of evidence suggests that circulating miRNAs could act as non-invasive diagnostic, prognostic or therapeutic biomarkers for a wide variety of diseases such as Parkinson's disease (Roser *et al.*, 2018), infectious diseases (Correia *et al.*, 2017), cancers (Filipów and Łaczmański, 2019), diabetes (Guay and Regazzi, 2013) and cardiovascular diseases (Akodad, Mericskay and Roubille, 2016). For example, one of the most widely studied miRNAs to date is miR-122. It is highly expressed in liver tissue and has been considered as a promising biomarker of hepatocellular injury, hepatic biology, and liver diseases (Musaddaq et al., 2019). However, most circulating microRNAs studies are based on a limited sample size, different normalisation strategies and different population characteristics such as a certain age group or gender. As a result, there have been discrepancies between the results of different studies and comparison between studies is not possible. Therefore, there are no circulating miRNAs, including the ones mentioned in this thesis, that have been successfully established as optimal biomarkers in clinical applications.

In the first study (chapter 3), we developed a literature search to target specific miRNA in our studies. In these studies, the selected circulating miRNAs (miR30c, miR103, miR133a, miR122 and miR146a) showed significant associations with markers of cardiometabolic risk in populations with coronary heart diseases and insulin resistance.

Among the five selected circulating miRNAs, miR103 showed an especially positive association with cardiac markers, including troponin T and NT-pro-BNP. There was also a positive association of miR122 with carotid intima media thickness, thus highlighting the potential role of miR103 and miR122 as mediators in the development of cardiac diseases. These miRNAs appear to contribute to the development of atherosclerosis which was supported by the findings of previous studies. Kulshreshtha et al. (2007) have shown that miR103 was upregulated in response to necrotic factors (H_2O_2) and that hypoxia is a key factor in the development of atherosclerosis. In addition, Willeit et al. (2017a) found a strong association between the expression of miR122 and adverse lipid profile, as it is known that cholesterol is a key component in plaque formation. Other miRNAs were less successful biomarkers and, thus, our targeted approach was able to validate and expand on existing data. Our more speculative exploration of the effect of metformin on miRNA was null, likely due, at least in part, to the small size and low power of the study as well as the limited effect of metformin in the trial itself. However, our data do show proof in principle that the use of miRNA as surrogate biomarkers in trials should at least be further explored.

The results of this study showed the association of circulating miRNAs and surrogate markers for cardiovascular diseases. The results are supported by an expanding literature around the potential role of those miRNAs in development and progression of atherosclerosis. Finally, the associations of specific miRNA with cardiometabolic markers were specific for certain cardiac risk factors and was therefore not solely due to non-specific general associations. However, proper standardisation of all preanalytical and analytical procedures, as well as proper evaluation of individual and environmental factors are required before large scale epidemiological studies can be undertaken, and the results translated into clinical practice.

7.2.3 Determinants of carotid intima media thickness

In the UK Biobank-based study of healthy middle-aged people, we observed a mean cIMT thickness of around 680uM (Table 5.2). In comparison to other studies (including the CAMERA trial; mean 720 uM) this appears to be broadly as expected given age and health.

It is well established that incidence of cardiovascular diseases is higher in men than in women. Men and women share most clinical risk factors, but the significance and the relative weighting of these factors usually differs in relation to the two genders (Maas and Appelman, 2010). In addition, studies show higher severity and incidence of atherosclerosis in men than women in all age groups (Villablanca, Jayachandran and Banka, 2010). Carotid intima media thickness is a key marker of early atherosclerosis development (O'Leary and Polak, 2002). The second study (chapter 5) was designed to investigate the predictors for cIMT variation using the UK Biobank population. This study had confirmed the findings of previous studies carried out with the large UK Biobank population that showed that cIMT was around 45uM higher in men than women (Sinning *et al.*, 2011) (Howard *et al.*, 1993) The thickness of the vasculature structure also increased with age. Therefore, these findings emphasise the need of prevention of atherosclerosis according to age and sex.

High systolic blood pressure is one of the most important factors for the progression and the acceleration of growth of atherosclerotic plaques. Higher cIMT is an early sign for atherosclerosis (Rathsman *et al.*, 2012). In addition, the positive association between systolic blood and cIMT was well confirmed in previous studies (Ferreira *et al.*, 2016) (Ribeiro *et al.*, 2017). This study (chapter 5) showed that systolic blood pressure is an independent predictor for cIMT variation in the large UK Biobank population, as well as diastolic blood pressure (to a lesser extent). It is suggested that persistent high blood pressure plays a role in the hyperplasia of carotid arteries and consequently the development of atherosclerosis. Many studies have shown the role of high systolic blood pressure in the development of carotid hyperplasia (Casiglia *et al.*, 2000) (Arnett *et al.*, 2000). Other determinants included lipid, glucose and BMI. While none of these determinants were truly surprising, they do expand on existing knowledge by using a study of a very rare large size and thus providing precise estimates. Furthermore, the study validates the cIMT measurement in the UK Biobank and we can, therefore, utilise this biomarker in the UK Biobank population with confidence. Overall, the R^2 for cIMT determined by traditional CVD risk factors was >0.216 in men and women.

7.2.4 Determinants of PWASI

If cIMT is a measure of vascular anatomical changes, PWASI, in contrast, is a measure of vascular function. It is much easier to measure than cIMT and, therefore, potentially clinically the more attractive biomarker. However, our data suggests it probably has weaker associations with established CVD risk factors. The role of systolic blood pressure in progression of arterial stiffness is well documented in the literature (O'Rourke, 1990). However, a UK Biobank study, using data from the baseline timepoint (distinct from the imaging timepoint used here), showed that midlife diastolic blood pressure was the strongest determinant for arterial stiffness progression (Webb, 2020). In addition, several studies have shown the effect of stiffness of the arterial wall on the progression of atherosclerosis (Popele *et al.*, 2001). Therefore, the understanding of determinants of arterial wall stiffness will help clinicians to control those factors before progression to atherosclerosis and cardiovascular diseases. Our findings showed that systolic blood pressure was the most important determinant for increasing PWASI in participants in the UK Biobank study. Other determinants included smoking and low HDL-cholesterol. Other less well-established risk factors included BMI and glycaemia, as well as inflammation (as measured by CRP) in men. However, overall, the R^2 for PWASI determined by traditional CVD risk factors was <10% in men and women. It may, therefore, be a less robust biomarker of the impact of risk factors on the vasculature.

7.2.5 Carotid intima media thickness and PWASI as biomarkers for CVD incidence

Following our understanding of the upstream determinants of carotid intima media thickness variation in the UK Biobank population in a cross-sectional study, it is essential to understand the link between this imaging marker and new onset CVD events in the UK Biobank population. In this study (chapter 6) it was shown that cIMT is associated with future CVD events after adjusting for classical risk factors (HR=1.18). The associations observed were consistent with those reported by meta-analyses of prospective longitudinal cohort studies (Lorenz *et al.*, 2012). However, we demonstrated no strong evidence of an association of PWASI with incidence CVD. This is in contrast to previous studies (Said *et al.*, 2018b), (Mitchell

et al., 2010). However, our work in the previous chapter and the use of the same timepoint and group of participants to study both biomarkers makes our results in this regard very convincing. CIMT appears to be a better candidate surrogate biomarker for use in CVD risk assessment and in clinical trials than PWASI.

7.3 Future perspectives

7.3.1 Challenges in using circulating miRNAs as biomarkers

The alteration of circulating miRNAs expression is associated with several cardiovascular diseases, suggesting that they may act as excellent biomarkers for prediction and prognosis, and provide therapeutic targets for cardiovascular diseases. Despite the numerous advantages of circulating miRNAs as biomarkers, there are still many issues that limit their reliability as a biomarker in clinical application. The biological complexity of the cardiovascular disease pathogenesis is a major challenge in the discovery and validation of miRNAs.

7.3.1.1Preanalytical and analytical variables

The quantification of circulating miRNAs can be performed using different biofluids, such as plasma, serum and whole blood. The choice of starting material can significantly affect the expression of the selected miRNAs, as each biofluid has a distinct profile of miRNAs (Weber *et al.*, 2010). Three cohort studies showed different expressions of miR-1 in blood samples. The discrepancy between results could be explained by the fact that each cohort study used a different blood fraction (plasma or serum) and a different type of anticoagulants for the two groups that used plasma samples (Liu *et al.*, 2015), (Zhong *et al.*, 2014), (Li *et al.*, 2013a).

In addition, the selection of anticoagulant (EDTA, heparin, citrate) used for plasma preparation may affect the level of expression of miRNAs. Heparin is also known to inhibit real-time quantitative polymerase chain reactions. Furthermore, several studies have found a lower expression of circulating miRNAs derived from citrate-treated plasma comparing to those obtained from EDTA-treated plasma for the same participants (Fichtlscherer *et al.*, 2010b), (Moldovan *et al.*, 2013).

Another pre-analytical variable is centrifugation conditions (including g-force, time, and temperature), a critical factor that may have an impact on the yield of miRNAs in serum or plasma. Mitchel et al. and Wang et al. used different centrifugation protocols for plasma and serum isolation. Mitchel et al. found that the expression of miRNAs in plasma and serum was highly correlated (Mitchell *et al.*, 2008b). Another study that used a two-step centrifugation protocol found a higher expression of miRNA in serum compared to plasma (Wang *et al.*, 2012a).

Moreover, several studies showed that the yield of circulating miRNAs in plasma or serum can be changed by erythrocyte-specific miRNAs after haemolysis during the blood collection process (McDonald *et al.*, 2011a), (Kirschner *et al.*, 2011). The other preanalytical variables that may have an impact on the expression of miRNAs in biofluids include sample storage, handling, and repetitive freeze-thaw cycles. A study by Glinge et al. found that the expression of miR-1 and miR2 reduced after multiple freeze-thaw cycles (Glinge *et al.*, 2017). In addition, many research groups found that different extraction methods provide a different quantity and quality of miRNA (Moret *et al.*, 2013).

7.3.1.2Individual related factors

In addition to the potential technical biases hindering the reliability of miRNAs as a biomarker, other critical variables could have a substantial impact on the accurate expression of circulating miRNA in biofluids. Several factors are linked to intrinsic inter-individual variability and disease. A large body of evidence suggests that individual variability, such as age (Sredni *et al.*, 2011) gender (Duttagupta *et al.*, 2011) and ethnic group (Huang *et al.*, 2011) could contribute to the change of expression of the profile of miRNAs in biofluids. In addition, many studies have shown varying effects of medication on the expression of circulating miRNAs. Boeckel et al. showed that the administration of heparin before the blood collection process has an impact on miRNAs results. (Boeckel *et al.*, 2013). Moreover, anti-platelet treatment (Willeit *et al.*, 2013), and angiotensin converting enzyme inhibitors also have an effect on the expression level for specific miRNAs (Weber *et al.*, 2011). Among the individual-related factors, dietary constituents, such as vitamin A, vitamin D and curcumin, can be considered as potential confounders in the expression of circulating miRNAs (Becker and Lockwood, 2013). Furthermore, lipid intake before blood sampling

can lead to variable results due to the interference of lipids with miRNAs extraction (Kroh *et al.*, 2010). Blood collection time is another issue that must be considered during the identification and quantification of miRNAs in biofluids. There are differences between fasting and non-fasting patients in terms of blood components, such as lipoproteins and HDL, which carry specific miRNAs (Cheng, 2015). Therefore, the blood collection time is an important factor that must be considered in order to accurately quantify the expression of miRNAs.

Another issue regarding the use of circulating miRNAs as biomarkers is the low tissue and disease specificity for several miRNAs. A study by Witwer et al. showed that there is an increasing level of circulating miRNA-141 in pregnant women and patients with prostate cancer and coronary artery disease. They proposed that the secretion of miR-141 is from epithelial tissue and breast, colon and lung tissue (Witwer, 2015a).

Hormonal changes in the female body throughout the menstrual cycle can also alter the expression of miRNAs in plasma (McBride *et al.*, 2012). Some miRNAs were found to fluctuate in response to circadian rhythm. One study found that a third of detected miRNA displayed a rhythmic pattern in two different phases during a 24-hour period in a group of 24 healthy subjects (Heegaard *et al.*, 2016).

Lifestyle is another factor that affects the expression of miRNAs. One study reported that serum miR625 is significantly lower in lung cancer patients who smoke compared to those who do not smoke (Roth *et al.*, 2012). A study by de Gonzalo-Calvo showed that different doses of acute aerobic exercise induced a different pattern of miRNA profile (de Gonzalo-Calvo *et al.*, 2015).

In addition, the variation of miRNAs expression does not occur only in a diseased state; instead, the individual physiological state may also lead to variation in the expression of circulating miRNAs. In a disease state, such as CVD, a greater number of circulating miRNAs is released into the circulation compared to in healthy individuals (Sohel, 2016). A study by Faraldi et al showed that some endogenous miRNAs used as internal controls might change under certain pathological conditions and therefore would not be suitable as internal control miRNA (Faraldi *et al.*, 2019). In addition, studies showed that a large proportion of screened miRNAs was differentially expressed in healthy individuals, and their

expression varied according to storage length, sampling method and the age of participants (Keller *et al.*, 2017).

Therefore, it is crucial to carefully examine potential miRNA biomarkers by understanding the biological function that underlies the mechanism of release, transport and uptake, and to determine how to further upscale the biochemical laboratory measurement of miRNAs before any breakthrough in terms of the application of circulating miRNAs as promising biomarkers in clinical practice. Figure (7-1) shows the factors that might affect the utility of circulating miRNAs as biomarkers. The standardized strategy from sample collection to data analysis should be applied to avoid any variation in results between studies. Figure (7-2) shows the factors contributing to differences in the expression of circulating miRNAs.

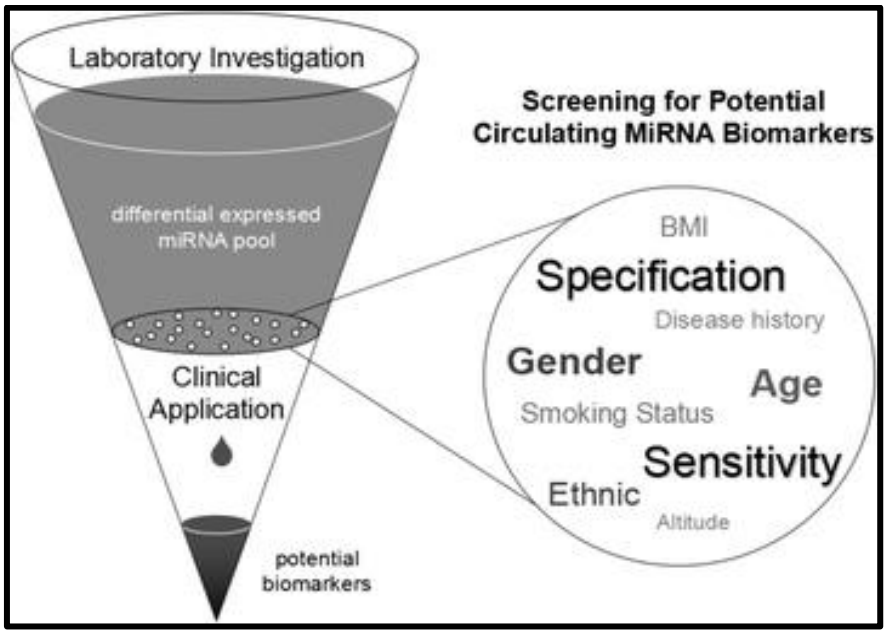


Figure 7-1 Screening for potential circulating miRNAs biomarkers (Wang *et al.*, 2018)

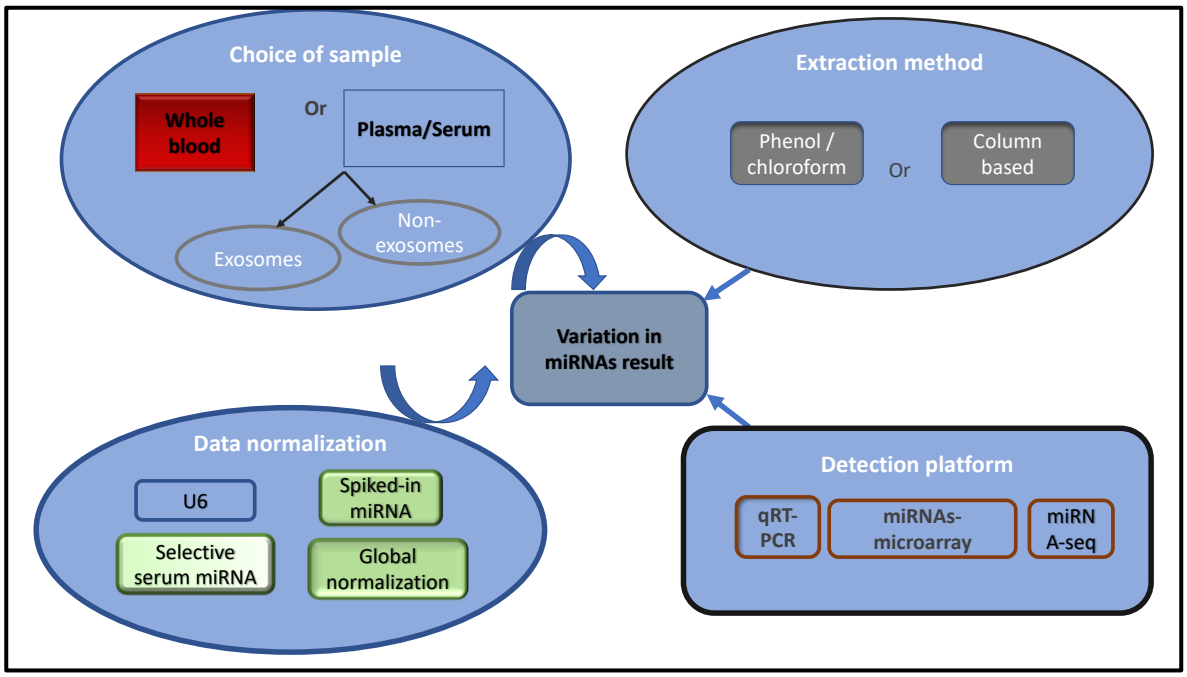


Figure 7-2: Factors contributing differneces in the expression of circualting miRNAs (Sohel, 2016)

7.3.2 UK Biobank study

From the findings that we presented in this thesis; we plan to expand my analysis of UK Biobank study to further confirm my findings. The UK Biobank imaging study aims to recruit 100,000 participants and is estimated to finish by 2023 (Littlejohns *et al.*, 2020). This is will allow us to include more participants in our study that have a biochemical assay at imaging visit and will further extend follow-up and improve power. We will be able to examine the association between CVD risk factors and imaging markers, cIMT and PWASI, using the same time point (imaging visit). After that, we will determine, using prediction metrics such as the C-Index, which vascular measurement most improves existing CVD risk scores that are used clinically.

7.4 Conclusion

This work has showed the potential utility of circulating miRNA as biomarkers of cardiovascular disease. It also demonstrated the upstream determinants for vascular markers including cIMT and PWASI in a large UK Biobank study. The understanding of these factors might help clinician for primordial prevention of development and progression of subclinical atherosclerosis which is considered as early indicator of atherosclerosis.

Appendices

7.4.1 Appendix 1: Poster for diabetes, Liverpool, UK, 2019

University of Glasgow

British Heart Foundation

جامعة الملك سعود King Saud University

Novel miRNA biomarkers in cardiometabolic disease: Data from the Carotid Atherosclerosis Metformin for Insulin Resistance (CAMERA) trial

Hanan AL-Ghibiwi[1], David Preiss[2], Naveed Sattar[1], Jennifer Logue[1], Paul Welsh[1]

[1]Institute of Cardiovascular and Medical Sciences, College of Medical,Veterinary and Life Science, University of Glasgow, Glasgow, UK .[2]Clinical Trial Service Unit and Epidemiological Studies Unit, University of Oxford, UK

Background and aim

Background: Several studies have shown that circulating miRNAs are closely related to the cardiometabolic diseases. The suitability and diagnostic value of circulating miRNAs in detection the cardiometabolic diseases have been inconsistence in literature.

Aim: To explore in the cross sectional association between selected circulating miRNAs and risk factors of cardiometabolic disease at baselines and to investigate the effect of metformin on the expression of these miRNAs.

Method

- The CAMERA trial was a randomised, placebo-controlled double-blinded trial including 173 participants aged 35-75 years, with established coronary heart disease, but without diabetes.
- Participants were randomly assigned to metformin or placebo (1:1) and followed for 18 months.
- MiRNAs were extracted from 60 paired stored samples (taken at baseline and after 18 months of treatment).
- The expressions of miR30c, 103, 133a, 122, 146a were measured using real time quantitative polymerase chain reaction (RT-qPCR).
- Spike-in of cel-miR-39 was used as normalizer.
- Pearson's correlation was used to correlate the cycle threshold (CT) of selected miRNAs with classical cardiometabolic risk factors including carotid intima media thickness(cIMT), HbA1c and body mass index.
- The$2^{-\Delta\Delta CT}$ method to investigate the randomised effect of metformin.

Table (1): Mir-39 intra and inter-assay CV% for the entire study

Mir-39 intra and inter-assay CV% for the entire study			
Inter CV%		**Intra CV%**	
Mean	28.907	**Mean**	28.907
SD	1.625	**SD**	0.189
CV%	5.622	**CV%**	0.653329898

Result

In cross sectional analysis at baseline:
- MiR-122 and miR103 showed positive association with mean CIMT and troponin respectively. (Table 3 & 4).
- No miRNA was associated with other cardiac markers such as CRP and NT-pro-BNP.
- MiR103,miR30c and miR122 showed strong associations with insulin sensitivity and adjustment for sex and age not attenuate these associations (Table 5and 6).

The Effect of Randomisation to Metformin on Circulating miRNAs:
- Baseline characteristics were well balanced between randomised groups, including for miRNA (Table 1).
- Over 18 months, metformin showed no effect on the expression of mir-221, 222, 144, 155, 192 and 193b(figure 2)

Table (2):Baseline characteristics for 60 patients

	Placebo group (n=32)	Metformin group (n=28)
Demographic characteristics		
Age (years)	63(9)	60(8)
Gender		
Men	23(72%)	21(75%)
Women	9(28%)	7(25%)
Smoking history		
Current	10(31%)	13(46%)
Ex-smoker	14(44%)	9(32%)
Never	8(25%)	6(22%)
Blood pressure (mmHg)	139/80(20/13)	140/81(20/9)
Anthropometric characteristics		
Body mass index (Kg /m^2)	29.58(4.4)	29.28(3.1)
Biochemical Characteristics (Fasting)		
HOMA-IR	3.12(2.67)	2.45 (0.9)
HbA1c (mmol/mol)	5.59(0.26)	5.65(0.27)
Insulin (pmol/l)*	10.38(6.91)	9.79(4.75)
CRP(mg/L)*	2(2.33)	1.8(4.40)
Troponin(pg/ml)	8.8(4.6)	8.8(3.4)
NT-proBNP(pg/ml)*	95.90(139.65)	90(204.55)
Mean cIMT	0.69(0.11)	0.69(0.11)
miRNA Expression (delta ct)		
MiR30c	2.4(1.8)	2.6(1.71)
MiR103	2.99(2.8)	3.71(2.8)
MiR133a	5.66(2.3)	5.34(1.5)
MiR122	3.144(1.87)	3.351(1.8)
MiR146a	-2.109(2.3)	-1.576(1.87)

*** Not normally distributed variables, median (inter-quartile range) were used.**

Table (3):Pearson correlation of CAD biomarkers vs miRNAs expression at baseline

Biomarker/miRNAs		dct30c	dct103	dct133a	dct122	dct146a
Ln-CRP(mg/L)	**r**	-0.022	-0.091	0.068	0.185	0.117
Ln-Mean cIMT		-0.220	-0.225	-0.172	*-0.298	-0.092
Ln-Troponin(pg/ml)		-0.102	*-0.296	0.190	-0.079	-0.099
Ln-NT-proBNP(pg/ml)		0.024	-0.127	0.104	0.094	-0.074

CRP:C reactive protein, Mean cIMT : mean carotid intima media thickness, NT-proBNP:N-terminal pro-B-type natriuretic peptide

Table (4):Linear regression model showing the associations of CAD biomarkers vs miR103 and miR122 expression at baseline

MiRNA (Outcome)	Predictor	Model (1) B (95% CI) p-value	Model (2) B (95% CI) p-value
dCT-miR103	Troponin (pg/ml)	-0.213 (-0.386, -0.040) 0.017	-0.199 (-0.399, 000) =0.05
	NT-proBNP (pg/ml)	-0.002 (-0.004, 0.00) 0.037	-0.002 (-0.004, 0.00) 0.095
dCT-miR122	Mean cIMT	-4.666 (-8.598, -0.733) 0.021	-0.296 (-9.326, .047) 0.052

Models demonstrate the association of one SD increase in risk factors with miRNA, Model 1: unadjusted Model 2: adjustment for age and sex

Table (5):Pearson correlation of CAD biomarkers vs miRNAs expression at baseline

Biomarker/miRNAs		dct30c	dct103	dct133a	dct122	dct146a
Ln-HOMA-IR	**r**	0.137	0.275*	-0.020	-0.097	0.092
Ln-INS (pmol/L)		0.142	0.295*	-0.024	-0.089	0.078
Ln-Glucose (mmol/mol)		0.050	0.043	0.010	-0.108	0.110
Ln-HbA1c		-0.302*	-0.162	-0.137	-0.279*	-0.201

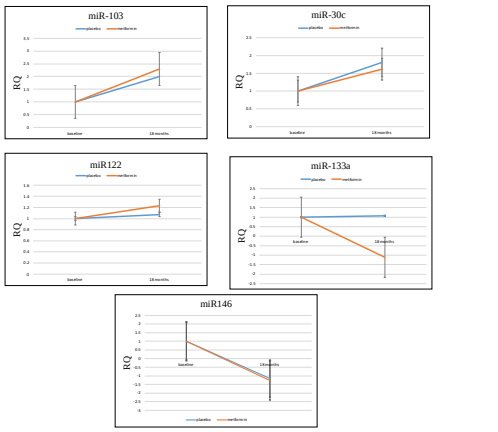
dCt: delta Ct, INS: insulin, HOMA-IR: homeostatic model assessment for insulin resistance, *r*: Pearson Correlation Coefficient.

Table (6):Linear regression model showing the associations of CAD biomarkers vs miR103 and miR122 expression at baseline

MiRNA	Predictor	Model (1), B (95% CI) p-value	Model (2), B (95% CI) p-value
dCT-miR103	HOMA-IR	0.417 (0.067,0.767) <0.05	0.433 (0.077,0.790) 0.018
	Insulin (pmol/L)	0.117 (0.022,0.213) <0.05	0.117 (0.020,0.214) <0.05
dCT-miR30c	HbA1c (%)	-1.931 (-3.549, -0.313) <0.05	-1.789 (-3.479, -0.099) <0.05
dCT-miR122	HbA1c (%)	-1.849 (-3.585, -0.112) <0.05	-1.677 (-3.486,0.132) 0.07

Models demonstrate the association of one SD increase in risk factors with miRNA, Model 1: unadjusted Model 2: adjustment for age and sex

Figure (1): Line Graph Comparing the RQ change for each miRNA between Placebo and Metformin Groups.



Conclusion

❖ Our finding supports the potential use of miRNAs as biomarkers for cardiometabolic risk assessment.
-Cross-sectional associations of targeted miRNAs with biomarkers of cardiometabolic risk (broadly consistent with literature).
-No effect of metformin randomisation (but a was null intervention for primary endpoint).

Appendix 2: Poster for 26th European Congress on Obesity (ECO2019), Glasgow, UK,2019

Established and novel biomarkers in cardiometabolic disease
Data from the Carotid Atherosclerosis Metformin for Insulin Resistance (CAMERA) trial

Hanan AL-Ghibiwi[1], David Preiss[2], Naveed Sattar[1], Jennifer Logue[1], Paul Welsh[1]

[1]Institute of Cardiovascular and Medical Sciences, College of Medical,Veterinary and Life Science, University of Glasgow, Glasgow, UK .[2] Clinical Trial Service Unit and Epidemiological Studies Unit, University of Oxford, UK

Background and aim

Background: There is emerging evidence that some microRNAs (miRNA) may play a role in the development of insulin resistance and type 2 diabetes. In particular, circulating miRNAs miR-30c,miR103,miR122 have been reported to have associations with an causal effects on insulin resistance in various models.

Aim: To explore in the cross sectional association between selected circulating miRNAs and risk factors of cardiometabolic disease and to investigate the effect of metformin on the expression of these miRNAs.

Method

- The Carotid Atherosclerosis: MEtformin for insulin ResistAnce randomised trial [(1)] (CAMERA) was a randomised, placebo-controlled double-blinded trial including 173 participants aged 35-75 years, with established coronary heart disease, but without diabetes.
- Participants were randomly assigned to metformin or placebo (1:1) and followed for 18 months.
- MiRNAs were extracted from 60 paired stored samples (taken at baseline and after 18 months of treatment).
- The expressions of miR30c, 103, 133a, 122, 146a were measured using real time quantitative polymerase chain reaction (RT-qPCR).
- Spike-in of cel-miR-39 was used as normalizer.
- Pearson's correlation was used to correlate the cycle threshold (CT) of selected miRNAs with classical cardiometabolic risk factors including carotid intima media thickness(cIMT), HbA1c and body mass index.
- The $2^{-\Delta\Delta CT}$ method was used to investigate the randomised effect of metformin.

Table (1): Mir-39 intra and inter-assay CV% for the entire study

Mir-39 intra and inter-assay CV% for the entire study			
Inter-assay variability		**Intra assay variability**	
Mean	28.9	**Mean**	28.9
SD	1.6	**SD**	0.2
CV%	5.6	**CV%**	0.65

Result

In cross sectional analysis at baseline:
- Baseline characteristics of the randomly selected subsample were well balanced (Table 2)
- MiR-122 & miR103 had a positive association with CIMT and troponin after adjusting for age and sex. (Table 3 & 4)
- No miRNA was strongly associated with CRP or NT-pro-BNP, but there was a positive trend for miRNA103.
- MiR103,miR30c and miR122 showed strong associations with insulin sensitivity even after adjustment for sex and age (Table 5 and 6).

The Effect of Randomisation to Metformin on Circulating miRNAs:
- Over 18 months, metformin showed no effect on the expression of mir-221, 222, 144, 155, 192 and 193b (figure 1)

Table (2):Baseline characteristics for 60 patients

	Placebo group (n=32)	Metformin group (n=28)
Demographic characteristics		
Age (years)	63(9)	60(8)
Gender		
Men	23(72%)	21(75%)
Women	9(28%)	7(25%)
Smoking history		
Current	10(31%)	13(46%)
Ex-smoker	14(44%)	9(32%)
Never	8(25%)	6(22%)
Blood pressure (mmHg)	139/80(20/13)	140/81(20/9)
Anthropometric characteristics		
Body mass index (Kg /m²)	29.58(4.4)	29.28(3.1)
Biochemical Characteristics (Fasting)		
HOMA-IR	3.12(2.67)	2.45 (0.9)
HbA1c (mmol/mol)	5.59(0.26)	5.65(0.27)
Insulin (pmol/l)*	10.38(6.91)	9.79(4.75)
CRP(mg/L)*	2(2.33)	1.8(4.40)
Troponin(pg/ml)	8.8(4.6)	8.8(3.4)
NT-proBNP(pg/ml)*	95.90(139.65)	90(204.55)
Mean cIMT	0.69(0.11)	0.69(0.11)
miRNA Expression (delta ct)		
MiR30c	2.4(1.8)	2.6(1.71)
MiR103	2.99(2.8)	3.71(2.8)
MiR133a	5.66(2.3)	5.34(1.5)
MiR122	3.144(1.87)	3.351(1.8)
MiR146a	-2.109(2.3)	-1.576(1.87)

*** Not normally distributed variables, median (inter-quartile range) were used.**

Table (3):Pearson correlation of CAD biomarkers vs miRNAs expression at baseline

Biomarker/miRNAs		dct30c	dct103	dct133a	dct122	dct146a
Ln-CRP(mg/L)	**r**	-0.022	-0.091	0.068	0.185	0.117
Ln-Mean cIMT		-0.220	-0.225	-0.172	*-0.298	-0.092
Ln-Troponin(pg/ml)		-0.102	*-0.296	0.190	-0.079	-0.099
NT-proBNP(pg/ml)		-0.080	*-0.270	0.075	-0.001	-0.106

CRP:C reactive protein, Mean cIMT : mean carotid intima media thickness, NT-proBNP:N-terminal pro-B-type natriuretic peptide

Table (4):Linear regression model showing the associations of CAD biomarkers vs miR103 and miR122 expression at baseline

MiRNA (Outcome)	Predictor	Model (1) B (95% CI) p-value	Model (2) B (95% CI) p-value
dCT-miR103	Ln-Troponin (pg/ml)	-0.296 (-3.409,-0.281) 0.022	-0.300 (-3.694,-0.05) 0.044
	NT-proBNP (pg/ml)	-0.270 (-0.004, 0.00) 0.037	-0.235 (-0.004, 0.00) 0.084
dCT-miR122	Ln-Mean cIMT	-0.298 (-6.040,-0.520) 0.021	-0.291 (-6.435,0.032) 0.052

Models demonstrate the association of one SD increase in risk factors with miRNA, Model 1: unadjusted Model 2: adjustment for age and sex

Table (5):Pearson correlation of CAD biomarkers vs miRNAs expression at baseline

Biomarker/miRNAs		dct30c	dct103	dct133a	dct122	dct146a
Ln-HOMA-IR	**r**	0.137	0.275*	-0.020	-0.097	0.092
Ln-INS (pmol/L)		0.142	0.295*	-0.024	-0.089	0.078
Ln-Glucose (mmol/mol)		0.050	0.043	0.010	-0.108	0.110
Ln-HbA1c		-0.302*	-0.162	-0.137	-0.279*	-0.201

dCt: delta Ct, INS: insulin, HOMA-IR: homeostatic model assessment for insulin resistance,

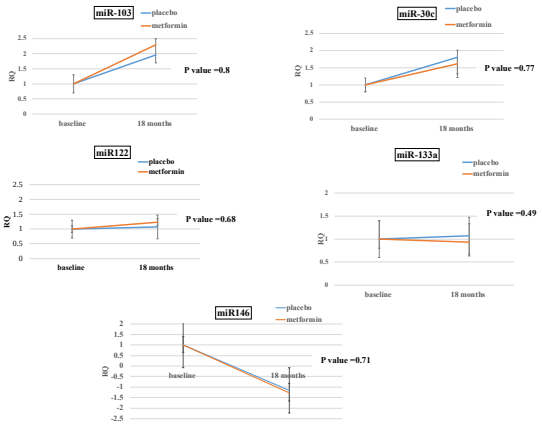
***r*: Pearson Correlation Coefficient.**

Table (6):Linear regression model showing the associations of CAD biomarkers vs miR103 and miR122 expression at baseline

MiRNA	Predictor	Model (1), B (95% CI) p-value	Model (2), B (95% CI) p-value
dCT-miR103	Ln-HOMA-IR	0.275 (0.105,2.861) 0.035	0.290 (0.142,2.994) 0.032
	Ln-Insulin (pmol/L)	0.295 (0.253,3.308) 0.023	0.293 (0.201,3.333) 0.028
dCT-miR30c	Ln-HbA1c (%)	-0.302 (-20.257,-1.894) 0.019	-0.282 (-19.882,-0.764) 0.035
dCT-miR122	Ln-HbA1c (%)	-0.279 (-20.718,-1.042) 0.031	-0.255 (-20.158,0.261) 0.056

Models demonstrate the association of one SD increase in risk factors with miRNA, Model 1: unadjusted Model 2: adjustment for age and sex

Figure (1): Line Graph Comparing the RQ change for each miRNA between Placebo and Metformin Groups.



Conclusion

- Cross-sectional associations of targeted miRNAs with biomarkers of cardiometabolic risk
  - broadly consistent with literature
  - much larger sample size in a well conducted single centre trial study
- No effect of metformin randomisation (but a was null intervention for primary endpoint)
- Our findings broadly support the potential use of miRNAs as biomarkers of cardiometabolic risk

Appendix 3: Material Transfer Agreement with the Applicant for data and/or samples

Dear Ms Al-Ghibiwi,

UK Biobank is pleased to approve your Application Reference Number 43707 to use the UK Biobank Resource. Execution of this Material Transfer Agreement (**MTA)** and payment of the Access Charges are the final steps before access is granted. UK Biobank's approval of this Application is valid for 90 days, after which the Applicant Principal Investigator (PI) will need to re-apply for access. The content of UK Biobank's standard MTA, and the conditions contained within it, are non-negotiable.

Parties

This is an agreement between UK Biobank Limited on the one hand and the Applicant Institution (**University of Glasgow**) on the other hand. The Applicant PI is not a party to the MTA, however, UK Biobank requires that the Applicant PI acknowledges that the provisions of this MTA have been "read and understood" by the Applicant PI so that they are fully aware of their Institution's obligations to both UK Biobank and to UK Biobank's participants. The Applicant Institution will be responsible for the conduct of any and all of the Applicant Researchers involved in this Research Project.

Structure of agreement

The MTA will become effective on receipt by UK Biobank of:

1. (i) A copy of this MTA Agreement executed by an authorised signatory of the Applicant Institution and confirmed as "read and understood" by the Applicant PI; and
2. (ii) Cleared funds covering the Access Charges from the Applicant Institution.

UK Biobank will then promptly send a dated confirmatory email. Provision of samples and/or data

Annex A summarises the data and/or samples that UK Biobank will make available to the Applicant in accordance with their approved Application Reference Number 43707. The timeframe and methodology by which the data and/or samples will be dispatched is also set out in Annex A.

Payment

The Access Charges which are payable are set out in Annex B. This also serves as an invoice on which VAT will be included (as appropriate). The derivation of these Access Charges is

also set out in Annex B. This payment should be submitted in cleared funds to Barclays Bank PLC, Account name: UK Biobank Limited, Account number: 33069427 and Sort code: 20-24-09.

Standard terms and schedules

This Agreement incorporates the attached terms and conditions (including any documents and/or materials that are referred to in them), the Annexes and where applicable the contents of the Preliminary and Main Application Forms with Reference Number 43707.

Yours faithfully

For and on behalf of UK Biobank/Effective Date (Jonathan Sellors / Company Solicitor)

26/3/2019

Accepted and agreed

For and on behalf of Applicant Institution
(Please sign and print your name and positPiaoun)l G Ellis

Senior Contracts
Manager

Read and Understood by the Applicant Principal Investigator (Please sign and print your name and position)

Hanan ALlghibiwi, PhD
student

ACCESS_031_A v1.3

16th February 2015

List of references

A national clinical Guideline (2017) *Risk estimation and the prevention of cardiovascular disease*. Available at: https://www.sign.ac.uk/assets/sign149.pdf (Accessed: 22/ September/2020.

Acevedo, M., Krämer, V., Tagle, R., Arnaiz, P., Corbalán, R., Berríos, X. and Navarrete, C. (2011) '[Cardiovascular risk factors among young subjects with high carotid intima media thickness]', *Rev Med Chil*, 139(10), pp. 1322-9.

Adam, S., Soran, H., Kwok, S., Hoong Ho, J. and Durrington, P. N. (2017) 'Meta-analysis of the effect of intensive cholesterol reduction on numbers needed to treat to prevent cardiovascular events in 75,439 participants in 10 randomised controlled clinical trials', *Atherosclerosis Supplements*, 28, pp. e2-e3.

Adami, H. O., McLaughlin, J., Ekbom, A., Berne, C., Silverman, D., Hacker, D. and Persson, I. (1991) 'Cancer risk in patients with diabetes mellitus', *Cancer Causes Control*, 2(5), pp. 307-14.

Akodad, M., Mericskay, M. and Roubille, F. (2016) 'Micro-RNAs as promising biomarkers in cardiac diseases', *Annals of translational medicine*, 4(24), pp. 551-551.

Alfie, J., Waisman, G. D., Galarza, C. R. and Cámera, M. I. (1999) 'Contribution of Stroke Volume to the Change in Pulse Pressure Pattern With Age', *Hypertension*, 34(4), pp. 808-812.

Ali Sheikh, M. S., Xia, K., Li, F., Deng, X., Salma, U., Deng, H., Wei Wei, L., Yang, T.-L. and Peng, J. (2015) 'Circulating miR-765 and miR-149: Potential Noninvasive Diagnostic Biomarkers for Geriatric Coronary Artery Disease Patients', *BioMed Research International*, 2015, pp. 740301.

Alles, J., Fehlmann, T., Fischer, U., Backes, C., Galata, V., Minet, M., Hart, M., Abu-Halima, M., Grässer, F. A., Lenhof, H.-P., Keller, A. and Meese, E. (2019) 'An estimate of the total number of true human miRNAs', *Nucleic Acids Research*, 47(7), pp. 3353-3364.

Alramah, T. Y. M. 2020. Novel metabolic risk markers in obesity and type two Diabetes mellitus. university of Glasgow.

Alty, S. R., Angarita-Jaimes, N., Millasseau, S. C. and Chowienczyk, P. J. (2007) 'Predicting Arterial Stiffness From the Digital Volume Pulse Waveform', *IEEE Transactions on Biomedical Engineering*, 54(12), pp. 2268-2275.

Ambros, V. (2004) 'The functions of animal microRNAs', *Nature*, 431(7006), pp. 350-355.

Ambrose, J. A. and Barua, R. S. (2004) 'The pathophysiology of cigarette smoking and cardiovascular disease: an update', *J Am Coll Cardiol*, 43(10), pp. 1731-7.

Ameling, S., Kacprowski, T., Chilukoti, R. K., Malsch, C., Liebscher, V., Suhre, K., Pietzner, M., Friedrich, N., Homuth, G., Hammer, E. and Völker, U. (2015) 'Associations of circulating plasma microRNAs with age, body mass index and sex in a population-based study', *BMC medical genomics*, 8, pp. 61-61.

American Heart Assocation (2016) *High Blood Pressure*. Available at: https://www.heart.org/en/health-topics/high-blood-pressure/the-facts-about-high-blood-pressure/what-is-high-blood-pressure#:~:text=High%20blood%20pressure%20(HBP%20or,vessels%2C%20is%20consistently%20too%20high. (Accessed: 22 / September /2020.

Anderson, T. J., Charbonneau, F., Title, L. M., Buithieu, J., Rose, M. S., Conradson, H., Hildebrand, K., Fung, M., Verma, S. and Lonn, E. M. (2011) 'Microvascular function predicts cardiovascular events in primary prevention: long-term results from the Firefighters and Their Endothelium (FATE) study', *Circulation*, 123(2), pp. 163-9.

Arnett, D. K., Blumenthal, R. S., Albert, M. A., Buroker, A. B., Goldberger, Z. D., Hahn, E. J., Himmelfarb, C. D., Khera, A., Lloyd-Jones, D., McEvoy, J. W., Michos, E. D., Miedema, M. D., Muñoz, D., Smith, S. C., Virani, S. S., Williams, K. A., Yeboah, J. and Ziaeian, B. (2019) '2019 ACC/AHA Guideline on the Primary Prevention of Cardiovascular Disease', *Journal of the American College of Cardiology*, 74(10), pp. e177.

Arnett, D. K., Boland, L. L., Evans, G. W., Riley, W., Barnes, R., Tyroler, H. A., Heiss, G. and Investigators, t. A. (2000) 'Hypertension and arterial stiffness: The atherosclerosis risk in communities study*', *American Journal of Hypertension*, 13(4), pp. 317-323.

A national clinical Guideline (2017) *Risk estimation and the prevention of cardiovascular disease*. Available at: https://www.sign.ac.uk/assets/sign149.pdf (Accessed: 22/ September/2020.

Acevedo, M., Krämer, V., Tagle, R., Arnaiz, P., Corbalán, R., Berríos, X. and Navarrete, C. (2011) '[Cardiovascular risk factors among young subjects with high carotid intima media thickness]', *Rev Med Chil*, 139(10), pp. 1322-9.

Adam, S., Soran, H., Kwok, S., Hoong Ho, J. and Durrington, P. N. (2017) 'Meta-analysis of the effect of intensive cholesterol reduction on numbers needed to treat to prevent cardiovascular events in 75,439 participants in 10 randomised controlled clinical trials', *Atherosclerosis Supplements*, 28, pp. e2-e3.

Adami, H. O., McLaughlin, J., Ekbom, A., Berne, C., Silverman, D., Hacker, D. and Persson, I. (1991) 'Cancer risk in patients with diabetes mellitus', *Cancer Causes Control*, 2(5), pp. 307-14.

Akodad, M., Mericskay, M. and Roubille, F. (2016) 'Micro-RNAs as promising biomarkers in cardiac diseases', *Annals of translational medicine*, 4(24), pp. 551-551.

Alfie, J., Waisman, G. D., Galarza, C. R. and Cámera, M. I. (1999) 'Contribution of Stroke Volume to the Change in Pulse Pressure Pattern With Age', *Hypertension*, 34(4), pp. 808-812.

Ali Sheikh, M. S., Xia, K., Li, F., Deng, X., Salma, U., Deng, H., Wei Wei, L., Yang, T.-L. and Peng, J. (2015) 'Circulating miR-765 and miR-149: Potential Noninvasive Diagnostic Biomarkers for Geriatric Coronary Artery Disease Patients', *BioMed Research International*, 2015, pp. 740301.

Alles, J., Fehlmann, T., Fischer, U., Backes, C., Galata, V., Minet, M., Hart, M., Abu-Halima, M., Grässer, F. A., Lenhof, H.-P., Keller, A. and Meese, E. (2019) 'An estimate of the total number of true human miRNAs', *Nucleic Acids Research*, 47(7), pp. 3353-3364.

Alramah, T. Y. M. 2020. Novel metabolic risk markers in obesity and type two Diabetes mellitus. university of Glasgow.

Alty, S. R., Angarita-Jaimes, N., Millasseau, S. C. and Chowienczyk, P. J. (2007) 'Predicting Arterial Stiffness From the Digital Volume Pulse Waveform', *IEEE Transactions on Biomedical Engineering*, 54(12), pp. 2268-2275.

Ambros, V. (2004) 'The functions of animal microRNAs', *Nature*, 431(7006), pp. 350-355.

Ambrose, J. A. and Barua, R. S. (2004) 'The pathophysiology of cigarette smoking and cardiovascular disease: an update', *J Am Coll Cardiol*, 43(10), pp. 1731-7.

Ameling, S., Kacprowski, T., Chilukoti, R. K., Malsch, C., Liebscher, V., Suhre, K., Pietzner, M., Friedrich, N., Homuth, G., Hammer, E. and Völker, U. (2015) 'Associations of circulating plasma microRNAs with age, body mass index and sex in a population-based study', *BMC medical genomics*, 8, pp. 61-61.

American Heart Assocation (2016) *High Blood Pressure*. Available at: https://www.heart.org/en/health-topics/high-blood-pressure/the-facts-about-high-blood-pressure/what-is-high-blood-pressure#:~:text=High%20blood%20pressure%20(HBP%20or,vessels%2C%20is%20consistently%20too%20high. (Accessed: 22 / September /2020.

Anderson, T. J., Charbonneau, F., Title, L. M., Buithieu, J., Rose, M. S., Conradson, H., Hildebrand, K., Fung, M., Verma, S. and Lonn, E. M. (2011)

'Microvascular function predicts cardiovascular events in primary prevention: long-term results from the Firefighters and Their Endothelium (FATE) study', *Circulation*, 123(2), pp. 163-9.

Arnett, D. K., Blumenthal, R. S., Albert, M. A., Buroker, A. B., Goldberger, Z. D., Hahn, E. J., Himmelfarb, C. D., Khera, A., Lloyd-Jones, D., McEvoy, J. W., Michos, E. D., Miedema, M. D., Muñoz, D., Smith, S. C., Virani, S. S., Williams, K. A., Yeboah, J. and Ziaeian, B. (2019) '2019 ACC/AHA Guideline on the Primary Prevention of Cardiovascular Disease', *Journal of the American College of Cardiology*, 74(10), pp. e177.

Arnett, D. K., Boland, L. L., Evans, G. W., Riley, W., Barnes, R., Tyroler, H. A., Heiss, G. and Investigators, t. A. (2000) 'Hypertension and arterial stiffness: The atherosclerosis risk in communities study*', *American Journal of Hypertension*, 13(4), pp. 317-323.

Arroyo, J. D., Chevillet, J. R., Kroh, E. M., Ruf, I. K., Pritchard, C. C., Gibson, D. F., Mitchell, P. S., Bennett, C. F., Pogosova-Agadjanyan, E. L., Stirewalt, D. L., Tait, J. F. and Tewari, M. (2011) 'Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma', *Proc Natl Acad Sci U S A*, 108(12), pp. 5003-8.

Ayer, J. G., Harmer, J. A., Nakhla, S., Xuan, W., Ng, M. K. C., Raitakari, O. T., Marks, G. B. and Celermajer, D. S. (2009) 'HDL-Cholesterol, Blood Pressure, and Asymmetric Dimethylarginine Are Significantly Associated With Arterial Wall Thickness in Children', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 29(6), pp. 943-949.

Azevedo, A., Bettencourt, P. and Barros, H. (2007) 'Demographic, clinical and echocardiographic determinants of B-type natriuretic peptide plasma concentration. A population-based study', *Rev Port Cardiol*, 26(2), pp. 105-13.

Babiarz, J. E., Ruby, J. G., Wang, Y., Bartel, D. P. and Blelloch, R. (2008) 'Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs', *Genes & development*, 22(20), pp. 2773-2785.

Baldassarre, D., De Jong, A., Amato, M., Werba, J. P., Castelnuovo, S., Frigerio, B., Veglia, F., Tremoli, E. and Sirtori, C. R. (2008) 'Carotid intima-media thickness and markers of inflammation, endothelial damage and hemostasis', *Ann Med*, 40(1), pp. 21-44.

Baldassarre, D., Nyyssonen, K., Rauramaa, R., de Faire, U., Hamsten, A., Smit, A. J., Mannarino, E., Humphries, S. E., Giral, P., Grossi, E., Veglia, F., Paoletti, R. and Tremoli, E. (2010) 'Cross-sectional analysis of baseline data to identify the major determinants of carotid intima-media thickness in a European population: the IMPROVE study', *Eur Heart J*, 31(5), pp. 614-22.

Bank, A. J., Wang, H., Holte, J. E., Mullen, K., Shammas, R. and Kubo, S. H. (1996) 'Contribution of collagen, elastin, and smooth muscle to in vivo human brachial artery wall stress and elastic modulus', *Circulation*, 94(12), pp. 3263-70.

Bartel, D. P. (2009) 'MicroRNAs: Target Recognition and Regulatory Functions', *Cell*, 136(2), pp. 215-233.

Bauer, M., Caviezel, S., Teynor, A., Erbel, R., Mahabadi, A. A. and Schmidt-Trucksass, A. (2012) 'Carotid intima-media thickness as a biomarker of subclinical atherosclerosis', *Swiss Med Wkly*, 142, pp. w13705.

Becker, N. and Lockwood, C. M. (2013) 'Pre-analytical variables in miRNA analysis', *Clin Biochem*, 46(10-11), pp. 861-8.

Belcaro, G., Nicolaides, A. N., Laurora, G., Cesarone, M. R., Sanctis, M. D., Incandela, L. and Barsotti, A. (1996) 'Ultrasound Morphology Classification of the Arterial Wall and Cardiovascular Events in a 6-Year Follow-up Study', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 16(7), pp. 851-856.

Bellentani, S., Saccoccio, G., Masutti, F., Crocè, L. S., Brandi, G., Sasso, F., Cristanini, G. and Tiribelli, C. (2000) 'Prevalence of and risk factors for hepatic steatosis in Northern Italy', *Ann Intern Med*, 132(2), pp. 112-7.

Beltran, A., McVeigh, G., Morgan, D., Glasser, S. P., Neutel, J. M., Weber, M., Finkelstein, S. M. and Cohn, J. N. (2001) 'Arterial compliance abnormalities in isolated systolic hypertension', *Am J Hypertens*, 14(10), pp. 1007-11.

Bhf.org.uk (2020a) *British Heart Foundation -Cardiovascular disease*. Available at: https://www.bhf.org.uk/informationsupport/conditions/cardiovascular-heart-disease (Accessed: 2020.

Bhf.org.uk (2020b) *British Heart Foundation -Heart statistics*. Available at: https://www.bhf.org.uk/what-we-do/our-research/heart-statistics 2020).

Bhf.org.uk (2020c) *Heart statistics, British Heart Foundation*. Available at: https://www.bhf.org.uk/what-we-do/our-research/heart-statistics.

Bianga Katchunga, P., Mongane Irenge, J., Canwa, R., Matabaro, A., Nsimire, S. and Kabunga, Q. (2016) '[Carotid intima-media thickness in the Congolese hypertensive in South Kivu]', *Ann Cardiol Angeiol (Paris)*, 65(2), pp. 64-70.

Bihrer, V., Waidmann, O., Friedrich-Rust, M., Forestier, N., Susser, S., Haupenthal, J., Welker, M., Shi, Y., Peveling-Oberhag, J., Polta, A., von Wagner, M., Radeke, H. H., Sarrazin, C., Trojan, J., Zeuzem, S., Kronenberger, B. and Piiper, A. (2011) 'Serum microRNA-21 as marker for necroinflammation in hepatitis C patients with and without hepatocellular carcinoma', *PLoS One*, 6(10), pp. e26971.

Biobank., U. (2015) *Imaging modality: carodtif Ultrasound*. Available at: (http://biobank.ndph.ox.ac.uk/showcase/showcase/docs/carult_explan_doc.pdf) (Accessed: 1/1/2020 2020).

'Biomarkers Definitions Working Group, Biomarkers and surrogate endpoints: preferred definitions and conceptual framework', (2001) *Clin Pharmacol Ther*, 69(3), pp. 89-95.

Blacher, J., Staessen, J. A., Girerd, X., Gasowski, J., Thijs, L., Liu, L., Wang, J. G., Fagard, R. H. and Safar, M. E. (2000) 'Pulse pressure not mean pressure determines cardiovascular risk in older hypertensive patients', *Arch Intern Med*, 160(8), pp. 1085-9.

Blankenhorn, D. H., Johnson, R. L., Nessim, S. A., Azen, S. P., Sanmarco, M. E. and Selzer, R. H. (1987) 'The Cholesterol Lowering Atherosclerosis Study (CLAS): design, methods, and baseline results', *Control Clin Trials*, 8(4), pp. 356-87.

Boeckel, J.-N., Thomé, C. E., Leistner, D., Zeiher, A. M., Fichtlscherer, S. and Dimmeler, S. (2013) 'Heparin Selectively Affects the Quantification of MicroRNAs in Human Blood Samples', *Clinical Chemistry*, 59(7), pp. 1125-1127.

Bosevski, M., Georgievska-Ismail, L. and Tosev, S. (2011) 'Carotid IMT in type 2 diabetic patients: a survey on factors of influence', *Prilozi*, 32(2), pp. 289-97.

Bots, M. L., Hoes, A. W., Koudstaal, P. J., Hofman, A. and Grobbee, D. E. (1997) 'Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam Study', *Circulation*, 96(5), pp. 1432-7.

British Heart Foundation, *Cardiovascular disease*. Available at: https://www.bhf.org.uk/informationsupport/conditions/cardiovascular-heart-disease (Accessed: 19th July 2020).

Burgess, J. L., Kurzius-Spencer, M., Gerkin, R. D., Fleming, J. L., Peate, W. F. and Allison, M. (2012) 'Risk Factors for Subclinical Atherosclerosis in Firefighters', *Journal of Occupational and Environmental Medicine*, 54(3), pp. 328-335.

Burns, D. M. (2003) 'Epidemiology of smoking-induced cardiovascular disease', *Prog Cardiovasc Dis*, 46(1), pp. 11-29.

Bycroft, C., Freeman, C., Petkova, D., Band, G., Elliott, L. T., Sharp, K., Motyer, A., Vukcevic, D., Delaneau, O., O'Connell, J., Cortes, A., Welsh, S., Young, A., Effingham, M., McVean, G., Leslie, S., Allen, N., Donnelly, P. and Marchini, J. (2018) 'The UK Biobank resource with deep phenotyping and genomic data', *Nature*, 562(7726), pp. 203-209.

Cal, R., Castellano, J., Revuelta-López, E., Aledo, R., Barriga, M., Farré, J., Vilahur, G., Nasarre, L., Hove-Madsen, L., Badimon, L. and Llorente-Cortés, V. (2012) 'Low-density lipoprotein receptor-related protein 1 mediates hypoxia-induced very low density lipoprotein-cholesteryl ester uptake and accumulation in cardiomyocytes', *Cardiovasc Res*, 94(3), pp. 469-79.

Cao, J., Meng, S., Chang, E., Beckwith-Fickas, K., Xiong, L., Cole, R. N., Radovick, S., Wondisford, F. E. and He, L. (2014) 'Low concentrations of metformin suppress glucose production in hepatocytes through AMP-activated protein kinase (AMPK)', *J Biol Chem*, 289(30), pp. 20435-46.

Cardarelli, R. and Lumicao, T. G., Jr. (2003) 'B-type natriuretic peptide: a review of its diagnostic, prognostic, and therapeutic monitoring value in heart failure for primary care physicians', *J Am Board Fam Pract*, 16(4), pp. 327-33.

Care, A., Catalucci, D., Felicetti, F., Bonci, D., Addario, A., Gallo, P., Bang, M. L., Segnalini, P., Gu, Y., Dalton, N. D., Elia, L., Latronico, M. V., Hoydal, M., Autore, C., Russo, M. A., Dorn, G. W., 2nd, Ellingsen, O., Ruiz-Lozano, P., Peterson, K. L., Croce, C. M., Peschle, C. and Condorelli, G. (2007) 'MicroRNA-133 controls cardiac hypertrophy', *Nat Med*, 13(5), pp. 613-8.

Casiglia, E., Palatini, P., Da Ros, S., Pagliara, V., Puato, M., Dorigatti, F. and Pauletto, P. (2000) 'Effect of blood pressure and physical activity on carotid artery intima-media thickness in stage 1 hypertensives and controls', *American Journal of Hypertension*, 13(12), pp. 1256-1262.

Cavalcante, J. L., Lima, J. A., Redheuil, A. and Al-Mallah, M. H. (2011) 'Aortic stiffness: current understanding and future directions', *J Am Coll Cardiol*, 57(14), pp. 1511-22.

Cecelja, M. and Chowienczyk, P. (2009) 'Dissociation of aortic pulse wave velocity with risk factors for cardiovascular disease other than hypertension: A systematic review', *Hypertension*, 54(6), pp. 1328-1336.

Chambless, L. E., Folsom, A. R., Davis, V., Sharrett, R., Heiss, G., Sorlie, P., Szklo, M., Howard, G. and Evans, G. W. (2002) 'Risk Factors for Progression of Common Carotid Atherosclerosis: The Atherosclerosis Risk in Communities Study, 1987–1998', *American Journal of Epidemiology*, 155(1), pp. 38-47.

Chambless, L. E., Heiss, G., Folsom, A. R., Rosamond, W., Szklo, M., Sharrett, A. R. and Clegg, L. X. (1997) 'Association of coronary heart disease incidence with carotid arterial wall thickness and major risk factors: the Atherosclerosis Risk in Communities (ARIC) Study, 1987-1993', *Am J Epidemiol*, 146(6), pp. 483-94.

Chen, C., Yang, S., Li, H., Yin, Z., Fan, J., Zhao, Y., Gong, W., Yan, M. and Wang, D. W. (2017) 'Mir30c Is Involved in Diabetic Cardiomyopathy through Regulation of Cardiac Autophagy via BECN1', *Mol Ther Nucleic Acids*, 7, pp. 127-139.

Chen, H., Chen, Y., Wu, W., Cai, Z., Chen, Z., Yan, X. and Wu, S. (2021) 'Total cholesterol, arterial stiffness, and systolic blood pressure: a mediation analysis', *Scientific Reports*, 11(1), pp. 1330.

Chen, J. F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., Conlon, F. L. and Wang, D. Z. (2006) 'The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation', *Nat Genet*, 38(2), pp. 228-33.

Chen, X., Ba, Y., Ma, L., Cai, X., Yin, Y., Wang, K., Guo, J., Zhang, Y., Chen, J., Guo, X., Li, Q., Li, X., Wang, W., Zhang, Y., Wang, J., Jiang, X., Xiang, Y., Xu, C., Zheng, P., Zhang, J., Li, R., Zhang, H., Shang, X., Gong, T., Ning, G., Wang, J., Zen, K., Zhang, J. and Zhang, C.-Y. (2008) 'Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases', *Cell Research*, 18(10), pp. 997-1006.

Chen, Y., Xiong, H., Wu, D., Pirbhulal, S., Tian, X., Zhang, R., Lu, M., Wu, W. and Huang, W. (2015) 'Relationship of short-term blood pressure variability with carotid intima-media thickness in hypertensive patients', *Biomed Eng Online*, 14, pp. 71.

Cheng, G. (2015) 'Circulating miRNAs: Roles in cancer diagnosis, prognosis and therapy', *Advanced Drug Delivery Reviews*, 81, pp. 75-93.

Chiesa, S. T., Charakida, M., Georgiopoulos, G., Dangardt, F., Wade, K. H., Rapala, A., Bhowruth, D. J., Nguyen, H. C., Muthurangu, V., Shroff, R., Davey Smith, G., Lawlor, D. A., Sattar, N., Timpson, N. J., Hughes, A. D. and Deanfield, J. E. (2019) 'Determinants of Intima-Media Thickness in the Young', *JACC: Cardiovascular Imaging*, pp. 3219.

Cho, S. K., Cho, S. K., Kim, K. H., Cho, J. Y., Yoon, H. J., Yoon, N. S., Hong, Y. J., Park, H. W., Kim, J. H., Ahn, Y., Jeong, M. H., Cho, J. G. and Park, J. C. (2015) 'Effects of age on arterial stiffness and blood pressure variables in patients with newly diagnosed untreated hypertension', *Korean circulation journal*, 45(1), pp. 44-50.

Cingoz, F., Iyisoy, A., Demirkol, S., Sahin, M. A., Balta, S., Celik, T., Unlu, M., Arslan, Z., Cakar, M., Kucuk, U., Demirbas, S. and Kocak, N. (2014) 'Carotid Intima-Media Thickness in Patients With Slow Coronary Flow and Its Association With Neutrophil-to-Lymphocyte Ratio:A Preliminary Report', *Clinical and Applied Thrombosis/Hemostasis*, 20(4), pp. 393-399.

Clarke, R., Shipley, M., Lewington, S., Youngman, L., Collins, R., Marmot, M. and Peto, R. (1999) 'Underestimation of risk associations due to regression dilution in long-term follow-up of prospective studies', *Am J Epidemiol*, 150(4), pp. 341-53.

Coffey, S., Lewandowski, A. J., Garratt, S., Meijer, R., Lynum, S., Bedi, R., Paterson, J., Yaqub, M., Noble, J. A., Neubauer, S., Petersen, S. E., Allen, N., Sudlow, C., Collins, R., Matthews, P. M. and Leeson, P. (2017) 'Protocol and

quality assurance for carotid imaging in 100,000 participants of UK Biobank: development and assessment', *European Journal of Preventive Cardiology*, 24(17), pp. 1799-1806.

Collaborators, G. B. D. C. o. D. (2018) 'Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980-2017: a systematic analysis for the Global Burden of Disease Study 2017', *Lancet (London, England)*, 392(10159), pp. 1736-1788.

Collet, J.-P., Thiele, H., Barbato, E., Barthélémy, O., Bauersachs, J., Bhatt, D. L., Dendale, P., Dorobantu, M., Edvardsen, T., Folliguet, T., Gale, C. P., Gilard, M., Jobs, A., Jüni, P., Lambrinou, E., Lewis, B. S., Mehilli, J., Meliga, E., Merkely, B., Mueller, C., Roffi, M., Rutten, F. H., Sibbing, D., Siontis, G. C. M. and Group, E. S. D. (2020) '2020 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: The Task Force for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC)', *European Heart Journal*, 42(14), pp. 1289-1367.

Correia, C. N., Nalpas, N. C., McLoughlin, K. E., Browne, J. A., Gordon, S. V., MacHugh, D. E. and Shaughnessy, R. G. (2017) 'Circulating microRNAs as Potential Biomarkers of Infectious Disease', *Frontiers in Immunology*, 8(118).

Creemers, E. E., Tijsen, A. J. and Pinto, Y. M. (2012) 'Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease?', *Circ Res*, 110(3), pp. 483-95.

Crouse, J. R., 3rd, Raichlen, J. S., Riley, W. A., Evans, G. W., Palmer, M. K., O'Leary, D. H., Grobbee, D. E. and Bots, M. L. (2007) 'Effect of rosuvastatin on progression of carotid intima-media thickness in low-risk individuals with subclinical atherosclerosis: the METEOR Trial', *Jama*, 297(12), pp. 1344-53.

Crouse, J. R., Goldbourt, U., Evans, G., Pinsky, J., Sharrett, A. R., Sorlie, P., Riley, W. and Heiss, G. (1996) 'Risk Factors and Segment-Specific Carotid Arterial Enlargement in the Atherosclerosis Risk in Communities (ARIC) Cohort', *Stroke*, 27(1), pp. 69-75.

D'Agostino, R. B., Sr., Vasan, R. S., Pencina, M. J., Wolf, P. A., Cobain, M., Massaro, J. M. and Kannel, W. B. (2008) 'General cardiovascular risk profile for use in primary care: the Framingham Heart Study', *Circulation*, 117(6), pp. 743-53.

Dao, H. H., Essalihi, R., Bouvet, C. and Moreau, P. (2005) 'Evolution and modulation of age-related medial elastocalcinosis: impact on large artery stiffness and isolated systolic hypertension', *Cardiovasc Res*, 66(2), pp. 307-17.

Dart, A. M. and Kingwell, B. A. (2001) 'Pulse pressure--a review of mechanisms and clinical relevance', *J Am Coll Cardiol*, 37(4), pp. 975-84.

de Gonzalo-Calvo, D., Dávalos, A., Montero, A., García-González, Á., Tyshkovska, I., González-Medina, A., Soares, S. M., Martínez-Camblor, P., Casas-Agustench, P., Rabadán, M., Díaz-Martínez Á, E., Úbeda, N. and Iglesias-Gutiérrez, E. (2015) 'Circulating inflammatory miRNA signature in response to different doses of aerobic exercise', *J Appl Physiol (1985)*, 119(2), pp. 124-34.

de Gonzalo-Calvo, D., van der Meer, R. W., Rijzewijk, L. J., Smit, J. W. A., Revuelta-Lopez, E., Nasarre, L., Escola-Gil, J. C., Lamb, H. J. and Llorente-Cortes, V. (2017) 'Serum microRNA-1 and microRNA-133a levels reflect myocardial steatosis in uncomplicated type 2 diabetes', *Scientific Reports*, 7(1), pp. 47.

De Rosa, S., Fichtlscherer, S., Lehmann, R., Assmus, B., Dimmeler, S. and Zeiher, A. M. (2011) 'Transcoronary concentration gradients of circulating microRNAs', *Circulation*, 124(18), pp. 1936-44.

Del Brutto, O. H., Del Brutto, V. J., Mera, R. M., Costa, A. F., Peñaherrera, R., Peñaherrera, E., Matcha, G., Generale, L. M., Torpey, A. P., Peralta, L. D., Hill, J. P., Rundek, T., Romano, J. G. and Sedler, M. J. (2020) 'The association between aortic arterial stiffness, carotid intima-media thickness and carotid plaques in community-dwelling older adults: A population-based study', *Vascular*, 28(4), pp. 405-412.

Demirsoy, İ. H., Ertural, D. Y., Balci, Ş., Çınkır, Ü., Sezer, K., Tamer, L. and Aras, N. (2018) 'Profiles of Circulating MiRNAs Following Metformin Treatment in Patients with Type 2 Diabetes', *Journal of medical biochemistry*, 37(4), pp. 499-506.

Den Ruijter, H. M., Peters, S. A., Anderson, T. J., Britton, A. R., Dekker, J. M., Eijkemans, M. J., Engstrom, G., Evans, G. W., de Graaf, J., Grobbee, D. E., Hedblad, B., Hofman, A., Holewijn, S., Ikeda, A., Kavousi, M., Kitagawa, K., Kitamura, A., Koffijberg, H., Lonn, E. M., Lorenz, M. W., Mathiesen, E. B., Nijpels, G., Okazaki, S., O'Leary, D. H., Polak, J. F., Price, J. F., Robertson, C., Rembold, C. M., Rosvall, M., Rundek, T., Salonen, J. T., Sitzer, M., Stehouwer, C. D., Witteman, J. C., Moons, K. G. and Bots, M. L. (2012) 'Common carotid intima-media thickness measurements in cardiovascular risk prediction: a meta-analysis', *Jama*, 308(8), pp. 796-803.

Denli, A. M., Tops, B. B. J., Plasterk, R. H. A., Ketting, R. F. and Hannon, G. J. (2004) 'Processing of primary microRNAs by the Microprocessor complex', *Nature*, 432(7014), pp. 231-235.

Dessein, P. H., Woodiwiss, A. J., Norton, G. R. and Solomon, A. (2013) 'Rheumatoid arthritis is associated with reduced adiposity but not with unfavorable major cardiovascular risk factor profiles and enhanced carotid atherosclerosis in black Africans from a developing population: a cross-sectional study', *Arthritis Res Ther*, 15(4), pp. R96.

Devaux, Y., Vausort, M., Goretti, E., Nazarov, P. V., Azuaje, F., Gilson, G., Corsten, M. F., Schroen, B., Lair, M. L., Heymans, S. and Wagner, D. R. (2012) 'Use of circulating microRNAs to diagnose acute myocardial infarction', *Clin Chem*, 58(3), pp. 559-67.

Di Bello, V., Carerj, S., Perticone, F., Benedetto, F., Palombo, C., Talini, E., Giannini, D., La Carrubba, S., Antonini-Canterin, F., Di Salvo, G., Bellieni, G., Pezzano, A., Romano, M. F. and Balbarini, A. (2009) 'Carotid intima-media thickness in asymptomatic patients with arterial hypertension without clinical cardiovascular disease: relation with left ventricular geometry and mass and coexisting risk factors', *Angiology*, 60(6), pp. 705-13.

Di Leva, G., Garofalo, M. and Croce, C. M. (2014) 'MicroRNAs in cancer', *Annual review of pathology*, 9, pp. 287-314.

Diamond, G. A. (1992) 'What price perfection? Calibration and discrimination of clinical prediction models', *J Clin Epidemiol*, 45(1), pp. 85-9.

Duttagupta, R., Jiang, R., Gollub, J., Getts, R. C. and Jones, K. W. (2011) 'Impact of Cellular miRNAs on Circulating miRNA Biomarker Signatures', *PLOS ONE*, 6(6), pp. e20769.

Eder, L., Jayakar, J., Shanmugarajah, S., Thavaneswaran, A., Pereira, D., Chandran, V., Rosen, C. F. and Gladman, D. D. (2013) 'The burden of carotid artery plaques is higher in patients with psoriatic arthritis compared with those with psoriasis alone', *Ann Rheum Dis*, 72(5), pp. 715-20.

'Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group', (1998) *Lancet*, 352(9131), pp. 854-65.

Einarson, T. R., Hunchuck, J. and Hemels, M. (2010) 'Relationship between blood glucose and carotid intima media thickness: A meta-analysis', *Cardiovascular diabetology*, 9, pp. 37-37.

Ellis, K. L., Cameron, V. A., Troughton, R. W., Frampton, C. M., Ellmers, L. J. and Richards, A. M. (2013) 'Circulating microRNAs as candidate markers to distinguish heart failure in breathless patients', *European Journal of Heart Failure*, 15(10), pp. 1138-1147.

Elmen, J., Lindow, M., Schutz, S., Lawrence, M., Petri, A., Obad, S., Lindholm, M., Hedtjarn, M., Hansen, H. F., Berger, U., Gullans, S., Kearney, P., Sarnow, P., Straarup, E. M. and Kauppinen, S. (2008) 'LNA-mediated microRNA silencing in non-human primates', *Nature*, 452(7189), pp. 896-9.

Ernst, E. and Resch, K. L. (1993) 'Fibrinogen as a cardiovascular risk factor: a meta-analysis and review of the literature', *Ann Intern Med*, 118(12), pp. 956-63.

Esau, C., Davis, S., Murray, S. F., Yu, X. X., Pandey, S. K., Pear, M., Watts, L., Booten, S. L., Graham, M., McKay, R., Subramaniam, A., Propp, S., Lollo, B. A., Freier, S., Bennett, C. F., Bhanot, S. and Monia, B. P. (2006) 'miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting', *Cell Metab*, 3(2), pp. 87-98.

Esau, C., Kang, X., Peralta, E., Hanson, E., Marcusson, E. G., Ravichandran, L. V., Sun, Y., Koo, S., Perera, R. J., Jain, R., Dean, N. M., Freier, S. M., Bennett, C. F., Lollo, B. and Griffey, R. (2004) 'MicroRNA-143 regulates adipocyte differentiation', *J Biol Chem*, 279(50), pp. 52361-5.

Esposito, K., Giugliano, D., Nappo, F. and Marfella, R. (2004) 'Regression of carotid atherosclerosis by control of postprandial hyperglycemia in type 2 diabetes mellitus', *Circulation*, 110(2), pp. 214-9.

Fang, N., Han, W., Gong, D., Zou, C. and Fan, Y. (2015) 'Atorvastatin Treatment for Carotid Intima-Media Thickness in Chinese Patients With Type 2 Diabetes: A Meta-Analysis', *Medicine (Baltimore)*, 94(44), pp. e1920.

Faraldi, M., Gomarasca, M., Banfi, G. and Lombardi, G. (2018) 'Free Circulating miRNAs Measurement in Clinical Settings: The Still Unsolved Issue of the Normalization', *Advances in clinical chemistry*, 87, pp. 113-139.

Faraldi, M., Gomarasca, M., Sansoni, V., Perego, S., Banfi, G. and Lombardi, G. (2019) 'Normalization strategies differently affect circulating miRNA profile associated with the training status', *Scientific Reports*, 9(1), pp. 1584.

Fernandez-Hernando, C., Suarez, Y., Rayner, K. J. and Moore, K. J. (2011) 'MicroRNAs in lipid metabolism', *Curr Opin Lipidol*, 22(2), pp. 86-92.

Ferreira, J. P., Girerd, N., Bozec, E., Machu, J. L., Boivin, J. M., London, G. M., Zannad, F. and Rossignol, P. (2016) 'Intima-Media Thickness Is Linearly and Continuously Associated With Systolic Blood Pressure in a Population-Based Cohort (STANISLAS Cohort Study)', *J Am Heart Assoc*, 5(6).

Fichtlscherer, S., De Rosa, S., Fox, H., Schwietz, T., Fischer, A., Liebetrau, C., Weber, M., Hamm, C. W., Röxe, T., Müller-Ardogan, M., Bonauer, A., Zeiher, A. M. and Dimmeler, S. (2010a) 'Circulating microRNAs in patients with coronary artery disease', *Circ Res*, 107(5), pp. 677-84.

Fichtlscherer, S., Rosa, S. D., Fox, H., Schwietz, T., Fischer, A., Liebetrau, C., Weber, M., Hamm, C. W., Röxe, T., Müller-Ardogan, M., Bonauer, A., Zeiher, A. M. and Dimmeler, S. (2010b) 'Circulating MicroRNAs in Patients With Coronary Artery Disease', *Circulation Research*, 107(5), pp. 677-684.

Filipów, S. and Łaczmański, Ł. (2019) 'Blood Circulating miRNAs as Cancer Biomarkers for Diagnosis and Surgical Treatment Response', *Frontiers in Genetics*, 10(169).

Finkelstein, S. M., Cohn, J. N., Collins, V. R., Carlyle, P. F. and Shelley, W. J. (1985) 'Vascular hemodynamic impedance in congestive heart failure', *The American Journal of Cardiology*, 55(4), pp. 423-427.

Finn, A. V., Kolodgie, F. D. and Virmani, R. (2010) 'Correlation between carotid intimal/medial thickness and atherosclerosis: a point of view from pathology', *Arterioscler Thromb Vasc Biol*, 30(2), pp. 177-81.

Fitch, K. V., Stavrou, E., Looby, S. E., Hemphill, L., Jaff, M. R. and Grinspoon, S. K. (2011) 'Associations of cardiovascular risk factors with two surrogate markers of subclinical atherosclerosis: Endothelial function and carotid intima media thickness', *Atherosclerosis*, 217(2), pp. 437-440.

Ford, I., Shah, A. S., Zhang, R., McAllister, D. A., Strachan, F. E., Caslake, M., Newby, D. E., Packard, C. J. and Mills, N. L. (2016) 'High-Sensitivity Cardiac Troponin, Statin Therapy, and Risk of Coronary Heart Disease', *J Am Coll Cardiol*, 68(25), pp. 2719-2728.

Foretz, M., Guigas, B., Bertrand, L., Pollak, M. and Viollet, B. (2014) 'Metformin: from mechanisms of action to therapies', *Cell Metab*, 20(6), pp. 953-66.

Fox, N. and Growdon, J. H. (2004) 'Biomarkers and surrogates', *NeuroRX*, 1(2), pp. 181-181.

Frost, D. and Beischer, W. (1998) 'Determinants of carotid artery wall thickening in young patients with Type 1 diabetes mellitus', *Diabet Med*, 15(10), pp. 851-7.

Gaarder, M. and Seierstad, T. (2015) 'Measurements of carotid intima media thickness in non-invasive high-frequency ultrasound images: the effect of dynamic range setting', *Cardiovascular Ultrasound*, 13(1), pp. 5.

Galley, H. F. and Webster, N. R. (2004) 'Physiology of the endothelium', *Br J Anaesth*, 93(1), pp. 105-13.

Gao, L., Bai, L., Shi, M., Ni, J., Lu, H., Wu, Y., Tu, J., Ning, X., Wang, J. and Li, Y. (2017) 'Association between carotid intima-media thickness and fasting blood glucose level: A population-based cross-sectional study among low-income adults in rural China', *Journal of diabetes investigation*, 8(6), pp. 788-797.

Gao, W., He, H.-W., Wang, Z.-M., Zhao, H., Lian, X.-Q., Wang, Y.-S., Zhu, J., Yan, J.-J., Zhang, D.-G., Yang, Z.-J. and Wang, L.-S. (2012) 'Plasma levels of lipometabolism-related miR-122 and miR-370 are increased in patients with hyperlipidemia and associated with coronary artery disease', *Lipids in health and disease*, 11, pp. 55-55.

Garber, A. J., Abrahamson, M. J., Barzilay, J. I., Blonde, L., Bloomgarden, Z. T., Bush, M. A., Dagogo-Jack, S., DeFronzo, R. A., Einhorn, D., Fonseca, V. A., Garber, J. R., Garvey, W. T., Grunberger, G., Handelsman, Y., Hirsch, I. B., Jellinger, P. S., McGill, J. B., Mechanick, J. I., Rosenblit, P. D. and Umpierrez, G. E. (2019) 'CONSENSUS STATEMENT BY THE AMERICAN ASSOCIATION OF CLINICAL ENDOCRINOLOGISTS AND AMERICAN COLLEGE OF ENDOCRINOLOGY ON THE COMPREHENSIVE TYPE 2 DIABETES MANAGEMENT ALGORITHM - 2019 EXECUTIVE SUMMARY', *Endocr Pract*, 25(1), pp. 69-100.

Gatzka, C. D., Cameron, J. D., Kingwell, B. A. and Dart, A. M. (1998) 'Relation Between Coronary Artery Disease, Aortic Stiffness, and Left Ventricular Structure in a Population Sample', *Hypertension*, 32(3), pp. 575-578.

Ge, W., Parvez, F., Wu, F., Islam, T., Ahmed, A., Shaheen, I., Sarwar, G., Demmer, R. T., Desvarieux, M., Ahsan, H. and Chen, Y. (2014) 'Association between anthropometric measures of obesity and subclinical atherosclerosis in Bangladesh', *Atherosclerosis*, 232(1), pp. 234-41.

Genest, J. J., Jr., Martin-Munley, S. S., McNamara, J. R., Ordovas, J. M., Jenner, J., Myers, R. H., Silberman, S. R., Wilson, P. W., Salem, D. N. and Schaefer, E. J. (1992) 'Familial lipoprotein disorders in patients with premature coronary artery disease', *Circulation*, 85(6), pp. 2025-33.

George Howard, D. G. L. B., MD; Gregory W. Evans, MS; John R. Crouse III, MD; Ward Riley, PhD; Donna Arnett, PhD; Regina de Lacy, MS; Gerardo Heiss, MD, PhD; for the ARIC Investigators (1994) 'Relations of intimal-medial thickness among sites within the carotid artery as evaluated by B-mode ultrasound. ARIC Investigators. Atherosclerosis Risk in Communities', *stroke*, 25(8), pp. 1581-7.

Ghai, V., Kim, T.-K., Etheridge, A., Nielsen, T., Hansen, T., Pedersen, O., Galas, D. and Wang, K. (2019) 'Extracellular Vesicle Encapsulated MicroRNAs in Patients with Type 2 Diabetes Are Affected by Metformin Treatment', *Journal of clinical medicine*, 8(5), pp. 617.

Gijsberts, C. M., Groenewegen, K. A., Hoefer, I. E., Eijkemans, M. J. C., Asselbergs, F. W., Anderson, T. J., Britton, A. R., Dekker, J. M., Engström, G., Evans, G. W., de Graaf, J., Grobbee, D. E., Hedblad, B., Holewijn, S., Ikeda, A., Kitagawa, K., Kitamura, A., de Kleijn, D. P. V., Lonn, E. M., Lorenz, M. W., Mathiesen, E. B., Nijpels, G., Okazaki, S., O'Leary, D. H., Pasterkamp, G., Peters, S. A. E., Polak, J. F., Price, J. F., Robertson, C., Rembold, C. M., Rosvall, M., Rundek, T., Salonen, J. T., Sitzer, M., Stehouwer, C. D. A., Bots, M. L. and den Ruijter, H. M. (2015) 'Race/Ethnic Differences in the Associations of the Framingham Risk Factors with Carotid IMT and Cardiovascular Events', *PloS one*, 10(7), pp. e0132321-e0132321.

Gilad, S., Meiri, E., Yogev, Y., Benjamin, S., Lebanony, D., Yerushalmi, N., Benjamin, H., Kushnir, M., Cholakh, H., Melamed, N., Bentwich, Z., Hod, M., Goren, Y. and Chajut, A. (2008) 'Serum microRNAs are promising novel biomarkers', *PLoS One*, 3(9), pp. e3148.

Gimbrone, M. A., Jr. and García-Cardeña, G. (2013) 'Vascular endothelium, hemodynamics, and the pathobiology of atherosclerosis', *Cardiovascular pathology : the official journal of the Society for Cardiovascular Pathology*, 22(1), pp. 9-15.

Giuseppina Catanzaro, Z. M. B., 1 Martina Chiacchiarini ,2 Luana Abballe ,1 Claudia Sabato ,2 Alessandra Vacca ,1 Paola Borgiani,3 Francesco Dotta ,4 Manfredi Tesauro ,5 Agnese Po ,2 and Elisabetta Ferretti (2018) 'Circulating MicroRNAs in Elderly Type 2 Diabetic Patients', *International Journal of Endocrinology*, pp. 11.

Glinge, C., Clauss, S., Boddum, K., Jabbari, R., Jabbari, J., Risgaard, B., Tomsits, P., Hildebrand, B., Kääb, S., Wakili, R., Jespersen, T. and Tfelt-Hansen, J. (2017) 'Stability of Circulating Blood-Based MicroRNAs - Pre-Analytic Methodological Considerations', *PLoS One*, 12(2), pp. e0167969.

Goff, D. C., Jr., Lloyd-Jones, D. M., Bennett, G., Coady, S., D'Agostino, R. B., Sr., Gibbons, R., Greenland, P., Lackland, D. T., Levy, D., O'Donnell, C. J., Robinson, J. G., Schwartz, J. S., Shero, S. T., Smith, S. C., Jr., Sorlie, P., Stone, N. J. and Wilson, P. W. F. (2014) '2013 ACC/AHA guideline on the assessment of cardiovascular risk: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines', *J Am Coll Cardiol*, 63(25 Pt B), pp. 2935-2959.

Green, B. D., Irwin, N., Duffy, N. A., Gault, V. A., O'Harte F, P. and Flatt, P. R. (2006) 'Inhibition of dipeptidyl peptidase-IV activity by metformin enhances the antidiabetic effects of glucagon-like peptide-1', *Eur J Pharmacol*, 547(1-3), pp. 192-9.

Greenland, P., Alpert, J. S., Beller, G. A., Benjamin, E. J., Budoff, M. J., Fayad, Z. A., Foster, E., Hlatky, M. A., Hodgson, J. M., Kushner, F. G., Lauer, M. S., Shaw, L. J., Smith, S. C., Jr., Taylor, A. J., Weintraub, W. S., Wenger, N. K., Jacobs, A. K., Smith, S. C., Jr., Anderson, J. L., Albert, N., Buller, C. E., Creager, M. A., Ettinger, S. M., Guyton, R. A., Halperin, J. L., Hochman, J. S., Kushner, F. G., Nishimura, R., Ohman, E. M., Page, R. L., Stevenson, W. G., Tarkington, L. G. and Yancy, C. W. (2010) '2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines', *J Am Coll Cardiol*, 56(25), pp. e50-103.

Grishok, A., Pasquinelli, A. E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D. L., Fire, A., Ruvkun, G. and Mello, C. C. (2001) 'Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing', *Cell*, 106(1), pp. 23-34.

Gruszfeld, D., Weber, M., Nowakowska-Rysz, M., Janas, R., Kozlik-Feldmann, R., Xhonneux, A., Carlier, C., Riva, E., Verduci, E., Closa-Monasterolo, R., Escribano, J., Dobrzanska, A. and Koletzko, B. (2015) 'Protein intake in infancy and carotid intima media thickness at 5 years--a secondary analysis from a randomized trial', *Ann Nutr Metab*, 66(1), pp. 51-9.

Guay, C. and Regazzi, R. (2013) 'Circulating microRNAs as novel biomarkers for diabetes mellitus', *Nat Rev Endocrinol*, 9(9), pp. 513-21.

Gunton, J. E., Delhanty, P. J., Takahashi, S. and Baxter, R. C. (2003) 'Metformin rapidly increases insulin receptor activation in human liver and signals preferentially through insulin-receptor substrate-2', *J Clin Endocrinol Metab*, 88(3), pp. 1323-32.

Gutzwiller, J. P., Goke, B., Drewe, J., Hildebrand, P., Ketterer, S., Handschin, D., Winterhalder, R., Conen, D. and Beglinger, C. (1999) 'Glucagon-like peptide-1: a potent regulator of food intake in humans', *Gut*, 44(1), pp. 81-6.

Ha, M. and Kim, V. N. (2014) 'Regulation of microRNA biogenesis', *Nature Reviews Molecular Cell Biology*, 15(8), pp. 509-524.

Hägg, S., Fall, T., Ploner, A., Mägi, R., Fischer, K., Draisma, H. H., Kals, M., de Vries, P. S., Dehghan, A., Willems, S. M., Sarin, A. P., Kristiansson, K., Nuotio, M. L., Havulinna, A. S., de Bruijn, R. F., Ikram, M. A., Kuningas, M., Stricker, B. H., Franco, O. H., Benyamin, B., Gieger, C., Hall, A. S., Huikari, V., Jula, A., Järvelin, M. R., Kaakinen, M., Kaprio, J., Kobl, M., Mangino, M., Nelson, C. P., Palotie, A., Samani, N. J., Spector, T. D., Strachan, D. P., Tobin, M. D., Whitfield, J. B., Uitterlinden, A. G., Salomaa, V., Syvänen, A. C., Kuulasmaa, K., Magnusson, P. K., Esko, T., Hofman, A., de Geus, E. J., Lind, L., Giedraitis, V., Perola, M., Evans, A., Ferrières, J., Virtamo, J., Kee, F., Tregouet, D. A., Arveiler, D., Amouyel, P., Gianfagna, F., Brambilla, P., Ripatti, S., van Duijn, C. M., Metspalu, A., Prokopenko, I., McCarthy, M. I., Pedersen, N. L. and Ingelsson, E. (2015) 'Adiposity as a cause of cardiovascular disease: a Mendelian randomization study', *Int J Epidemiol*, 44(2), pp. 578-86.

Hamam, R., Hamam, D., Alsaleh, K. A., Kassem, M., Zaher, W., Alfayez, M., Aldahmash, A. and Alajez, N. M. (2017) 'Circulating microRNAs in breast cancer: novel diagnostic and prognostic biomarkers', *Cell Death Dis*, 8(9), pp. e3045.

Hamilton, P. K., Lockhart, C. J., Quinn, C. E. and McVeigh, G. E. (2007) 'Arterial stiffness: clinical relevance, measurement and treatment', *Clin Sci (Lond)*, 113(4), pp. 157-70.

Hanefeld, M., Chiasson, J. L., Koehler, C., Henkel, E., Schaper, F. and Temelkova-Kurktschiev, T. (2004) 'Acarbose slows progression of intima-media thickness of the carotid arteries in subjects with impaired glucose tolerance', *Stroke*, 35(5), pp. 1073-8.

Hanefeld, M., Temelkova-Kurktschiev, T., Schaper, F., Henkel, E., Siegert, G. and Koehler, C. (1999) 'Impaired fasting glucose is not a risk factor for atherosclerosis', *Diabet Med*, 16(3), pp. 212-8.

Hanna, J., Hossain, G. S. and Kocerha, J. (2019) 'The Potential for microRNA Therapeutics and Clinical Research', *Frontiers in Genetics*, 10(478).

Hansson, G. K. (2005) 'Inflammation, Atherosclerosis, and Coronary Artery Disease', *New England Journal of Medicine*, 352(16), pp. 1685-1695.

Hayoz, D., Rutschmann, B., Perret, F., Niederberger, M., Tardy, Y., Mooser, V., Nussberger, J., Waeber, B. and Brunner, H. R. (1992) 'Conduit artery compliance and distensibility are not necessarily reduced in hypertension', *Hypertension*, 20(1), pp. 1-6.

Heart statistics, *British Heart Foundation*. Available at: https://www.bhf.org.uk/what-we-do/our-research/heart-statistics (Accessed: 20 2020).

Hedblad, B., Nilsson, P., Janzon, L. and Berglund, G. (2000) 'Relation between insulin resistance and carotid intima-media thickness and stenosis in non-diabetic subjects. Results from a cross-sectional study in Malmo, Sweden', *Diabet Med*, 17(4), pp. 299-307.

Heegaard, N. H. H., Carlsen, A. L., Lilje, B., Ng, K. L., Rønne, M. E., Jørgensen, H. L., Sennels, H. and Fahrenkrug, J. (2016) 'Diurnal Variations of Human Circulating Cell-Free Micro-RNA', *PloS one*, 11(8), pp. e0160577-e0160577.

Henry C McGill Jr, C. A. M., Edward E Herderick, Gray T Malcom, Richard E Tracy (2000) 'Origin of atherosclerosis in childhood and adolescence', *American Society for Clinical Nutrition*, 72, pp. 1307s-15s.

Hippisley-Cox, J., Coupland, C., Vinogradova, Y., Robson, J., Minhas, R., Sheikh, A. and Brindle, P. (2008) 'Predicting cardiovascular risk in England and Wales: prospective derivation and validation of QRISK2', *BMJ*, 336(7659), pp. 1475-1482.

Hitsumoto, T. and Shirai, K. (2015) 'Factors affecting high-sensitivity cardiac troponin T elevation in Japanese metabolic syndrome patients', *Diabetes, metabolic syndrome and obesity : targets and therapy*, 8, pp. 157-162.

Hodis, H. N., Mack, W. J., LaBree, L., Selzer, R. H., Liu, C. R., Liu, C. H. and Azen, S. P. (1998) 'The role of carotid arterial intima-media thickness in predicting clinical coronary events', *Ann Intern Med*, 128(4), pp. 262-9.

Hofman, A., Grobbee, D. E., De Jong, P. T. V. M. and Van den Ouweland, F. A. (1991) 'Determinants of disease and disability in the elderly: The Rotterdam elderly study', *European Journal of Epidemiology*, 7(4), pp. 403-422.

Howard, G., Sharrett, A. R., Heiss, G., Evans, G. W., Chambless, L. E., Riley, W. A. and Burke, G. L. (1993) 'Carotid artery intimal-medial thickness distribution in general populations as evaluated by B-mode ultrasound. ARIC Investigators', *Stroke*, 24(9), pp. 1297-304.

Huang, R. S., Gamazon, E. R., Ziliak, D., Wen, Y., Im, H. K., Zhang, W., Wing, C., Duan, S., Bleibel, W. K., Cox, N. J. and Dolan, M. E. (2011) 'Population

differences in microRNA expression and biological implications', *RNA Biol*, 8(4), pp. 692-701.

Huang, Y. Q., Cai, A. P., Chen, J. Y., Huang, C., Li, J. and Feng, Y. Q. (2016) 'The Relationship of Plasma miR-29a and Oxidized Low Density Lipoprotein with Atherosclerosis', *Cell Physiol Biochem*, 40(6), pp. 1521-1528.

Hulthe, J. and Fagerberg, B. (2002) 'Circulating oxidized LDL is associated with subclinical atherosclerosis development and inflammatory cytokines (AIR Study)', *Arterioscler Thromb Vasc Biol*, 22(7), pp. 1162-7.

Hundal, R. S., Krssak, M., Dufour, S., Laurent, D., Lebon, V., Chandramouli, V., Inzucchi, S. E., Schumann, W. C., Petersen, K. F., Landau, B. R. and Shulman, G. I. (2000) 'Mechanism by which metformin reduces glucose production in type 2 diabetes', *Diabetes*, 49(12), pp. 2063-9.

Hunter, M. P., Ismail, N., Zhang, X., Aguda, B. D., Lee, E. J., Yu, L., Xiao, T., Schafer, J., Lee, M. L., Schmittgen, T. D., Nana-Sinkam, S. P., Jarjoura, D. and Marsh, C. B. (2008) 'Detection of microRNA expression in human peripheral blood microvesicles', *PLoS One*, 3(11), pp. e3694.

Hussain, M. M., Rava, P., Walsh, M., Rana, M. and Iqbal, J. (2012) 'Multiple functions of microsomal triglyceride transfer protein', *Nutrition & metabolism*, 9, pp. 14-14.

Iglseder, B., Cip, P., Malaimare, L., Ladurner, G. and Paulweber, B. (2005) 'The Metabolic Syndrome Is a Stronger Risk Factor for Early Carotid Atherosclerosis in Women Than in Men', *Stroke*, 36(6), pp. 1212-1217.

Iliopoulos, D., Drosatos, K., Hiyama, Y., Goldberg, I. J. and Zannis, V. I. (2010) 'MicroRNA-370 controls the expression of microRNA-122 and Cpt1alpha and affects lipid metabolism', *Journal of lipid research*, 51(6), pp. 1513-1523.

Ingelsson, E., Sullivan, L. M., Murabito, J. M., Fox, C. S., Benjamin, E. J., Polak, J. F., Meigs, J. B., Keyes, M. J., O'Donnell, C. J., Wang, T. J., D'Agostino, R. B., Sr., Wolf, P. A. and Vasan, R. S. (2007) 'Prevalence and prognostic impact of subclinical cardiovascular disease in individuals with the metabolic syndrome and diabetes', *Diabetes*, 56(6), pp. 1718-26.

Insull, W., Jr. (2009) 'The pathology of atherosclerosis: plaque development and plaque responses to medical treatment', *Am J Med*, 122(1 Suppl), pp. S3-s14.

Ivey, K. N., Muth, A., Arnold, J., King, F. W., Yeh, R. F., Fish, J. E., Hsiao, E. C., Schwartz, R. J., Conklin, B. R., Bernstein, H. S. and Srivastava, D. (2008) 'MicroRNA regulation of cell lineages in mouse and human embryonic stem cells', *Cell Stem Cell*, 2(3), pp. 219-29.

Jaffer, F. A., O'Donnell, C. J., Larson, M. G., Chan, S. K., Kissinger, K. V., Kupka, M. J., Salton, C., Botnar, R. M., Levy, D. and Manning, W. J. (2002) 'Age and sex distribution of subclinical aortic atherosclerosis: a magnetic resonance imaging examination of the Framingham Heart Study', *Arterioscler Thromb Vasc Biol*, 22(5), pp. 849-54.

Jansen, F., Yang, X., Proebsting, S., Hoelscher, M., Przybilla, D., Baumann, K., Schmitz, T., Dolf, A., Endl, E., Franklin, B. S., Sinning, J.-M., Vasa-Nicotera, M., Nickenig, G. and Werner, N. (2014a) 'MicroRNA expression in circulating microvesicles predicts cardiovascular events in patients with coronary artery disease', *Journal of the American Heart Association*, 3(6), pp. e001249-e001249.

Jansen, F., Yang, X., Proebsting, S., Hoelscher, M., Przybilla, D., Baumann, K., Schmitz, T., Dolf, A., Endl, E., Franklin, B. S., Sinning, J. M., Vasa-Nicotera, M., Nickenig, G. and Werner, N. (2014b) 'MicroRNA expression in circulating microvesicles predicts cardiovascular events in patients with coronary artery disease', *J Am Heart Assoc*, 3(6), pp. e001249.

Jin, J.-P. (2016) 'Chapter One - Evolution, Regulation, and Function of N-terminal Variable Region of Troponin T: Modulation of Muscle Contractility and Beyond', in Jeon, K.W. (ed.) *International Review of Cell and Molecular Biology*: Academic Press, pp. 1-28.

John R. Crouse , U. G., Greg Evans , Joan Pinsky , A. Richey Sharrett , Paul Sorlie , Ward Riley , Gerardo Heiss and Investigators, a. f. t. A. (1996) 'Risk Factors and Segment-Specific Carotid Arterial Enlargement in the Atherosclerosis Risk in Communities (ARIC) Cohort', *stroke*, 27, pp. 69-75.

Johnson, C. P., Baugh, R., Wilson, C. A. and Burns, J. (2001) 'Age related changes in the tunica media of the vertebral artery: implications for the assessment of vessels injured by trauma', *J Clin Pathol*, 54(2), pp. 139-45.

Johnson, H. M., Piper, M. E., Jorenby, D. E., Fiore, M. C., Baker, T. B. and Stein, J. H. (2010) 'Risk factors for subclinical carotid atherosclerosis among current smokers', *Prev Cardiol*, 13(4), pp. 166-71.

Kajimoto, K., Naraba, H. and Iwai, N. (2006) 'MicroRNA and 3T3-L1 pre-adipocyte differentiation', *Rna*, 12(9), pp. 1626-32.

Kalra, L., Iveson, E., Rambaran, C., Sherwood, R., Chowienczyk, P., Ritter, J., Shah, A. and Forrester, T. (2008) 'An international matched cohort study of the contribution of metabolic impairments to subclinical atherosclerosis in United Kingdom and Jamaican African-Caribbeans', *Atherosclerosis*, 199(1), pp. 95-101.

Kantharidis, P., Wang, B., Carew, R. M. and Lan, H. Y. (2011) 'Diabetes complications: the microRNA perspective', *Diabetes*, 60(7), pp. 1832-1837.

Kass, D. A., Shapiro, E. P., Kawaguchi, M., Capriotti, A. R., Scuteri, A., deGroof, R. C. and Lakatta, E. G. (2001) 'Improved Arterial Compliance by a Novel Advanced Glycation End-Product Crosslink Breaker', *Circulation*, 104(13), pp. 1464-1470.

Keller, A., Rounge, T., Backes, C., Ludwig, N., Gislefoss, R., Leidinger, P., Langseth, H. and Meese, E. (2017) 'Sources to variability in circulating human miRNA signatures', *RNA biology*, 14(12), pp. 1791-1798.

Kelly, R. P., Millasseau, S. C., Ritter, J. M. and Chowienczyk, P. J. (2001) 'Vasoactive Drugs Influence Aortic Augmentation Index Independently of Pulse-Wave Velocity in Healthy Men', *Hypertension*, 37(6), pp. 1429-1433.

Kesse-Guyot, E., Vergnaud, A. C., Fezeu, L., Zureik, M., Blacher, J., Péneau, S., Hercberg, S., Galan, P. and Czernichow, S. (2010) 'Associations between dietary patterns and arterial stiffness, carotid artery intima-media thickness and atherosclerosis', *Eur J Cardiovasc Prev Rehabil*, 17(6), pp. 718-24.

Keys, A. (1997) 'Coronary heart disease in seven countries. 1970', *Nutrition*, 13(3), pp. 250-2; discussion 249, 253.

Kim, H.-N., Kim, S.-H., Eun, Y.-M. and Song, S.-W. (2015) 'Obesity with metabolic abnormality is associated with the presence of carotid atherosclerosis in Korean men: a cross-sectional study', *Diabetology & Metabolic Syndrome*, 7(1), pp. 68.

Kim, H. L. and Kim, S. H. (2019) 'Pulse Wave Velocity in Atherosclerosis', *Front Cardiovasc Med*, 6, pp. 41.

Kim, J. H., Kim, S. Y., Jung, E. S., Jung, S. W., Koo, J. S., Kim, J. H., Yeon, J. E., Kwon, S. Y., Lee, S. W., Byun, K. S. and Lee, C. H. (2011) 'Carotid intima-media thickness is increased not only in non-alcoholic fatty liver disease patients but also in alcoholic fatty liver patients', *Digestion*, 84(2), pp. 149-55.

Kirschner, M. B., Kao, S. C., Edelman, J. J., Armstrong, N. J., Vallely, M. P., van Zandwijk, N. and Reid, G. (2011) 'Haemolysis during sample preparation alters microRNA content of plasma', *PLoS One*, 6(9), pp. e24145.

Klop, B., van de Geijn, G.-J. M., Bovenberg, S. A., van der Meulen, N., Elte, J. W. F., Birnie, E., Njo, T. L., Janssen, H. W., van Miltenburg, A., Jukema, J. W. and Cabezas, M. C. (2013) 'Erythrocyte-bound apolipoprotein B in relation to atherosclerosis, serum lipids and ABO blood group', *PloS one*, 8(9), pp. e75573-e75573.

Kocaman, S. A., Durakoğlugil, M. E., Çetin, M., Erdoğan, T., Ergül, E. and Çanga, A. (2013) 'The independent relationship of epicardial adipose tissue with carotid intima-media thickness and endothelial functions: the association

of pulse wave velocity with the active facilitated arterial conduction concept', *Blood pressure monitoring*, 18(2), pp. 85-93.

Kokubo, Y., Watanabe, M., Higashiyama, A., Nakao, Y. M., Nakamura, F. and Miyamoto, Y. (2018a) 'Impact of Intima-Media Thickness Progression in the Common Carotid Arteries on the Risk of Incident Cardiovascular Disease in the Suita Study', *J Am Heart Assoc*, 7(11).

Kokubo, Y., Watanabe, M., Higashiyama, A., Nakao, Y. M., Nakamura, F. and Miyamoto, Y. (2018b) 'Impact of Intima-Media Thickness Progression in the Common Carotid Arteries on the Risk of Incident Cardiovascular Disease in the Suita Study', *Journal of the American Heart Association*, 7(11), pp. e007720.

Kooy, A., de Jager, J., Lehert, P., Bets, D., Wulffele, M. G., Donker, A. J. and Stehouwer, C. D. (2009) 'Long-term effects of metformin on metabolism and microvascular and macrovascular disease in patients with type 2 diabetes mellitus', *Arch Intern Med*, 169(6), pp. 616-25.

Kroh, E. M., Parkin, R. K., Mitchell, P. S. and Tewari, M. (2010) 'Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR)', *Methods*, 50(4), pp. 298-301.

Ku DN, G. D., Zarins CK, Glagov S (1985) 'Pulsatile flow and atherosclerosis in the human carotid bifurcation: positive plaque location and low oscillating shear stress', *Arteriosclerosis*, 5, pp. 293-302.

Kuller, L., Borhani, N., Furberg, C., Gardin, J., Manolio, T., O'Leary, D., Psaty, B. and Robbins, J. (1994a) 'Prevalence of subclinical atherosclerosis and cardiovascular disease and association with risk factors in the Cardiovascular Health Study', *Am J Epidemiol*, 139(12), pp. 1164-79.

Kuller, L., Borthani, N., Furberg, C., Gardin, J., Manolio, T., O'Leary, D., Psaty, B. and Robbins, J. (1994b) 'Prevalence of Subclinical Atherosclerosis and Cardiovascular Disease and Association with Risk Factors in the Cardiovascular Health Study', *American Journal of Epidemiology*, 139(12), pp. 1164-1179.

Kuller, L. H., Shemanski, L., Psaty, B. M., Borhani, N. O., Gardin, J., Haan, M. N., O'Leary, D. H., Savage, P. J., Tell, G. S. and Tracy, R. (1995) 'Subclinical Disease as an Independent Risk Factor for Cardiovascular Disease', *Circulation*, 92(4), pp. 720-726.

Kulshreshtha, R., Ferracin, M., Wojcik, S. E., Garzon, R., Alder, H., Agosto-Perez, F. J., Davuluri, R., Liu, C. G., Croce, C. M., Negrini, M., Calin, G. A. and Ivan, M. (2007) 'A microRNA signature of hypoxia', *Mol Cell Biol*, 27(5), pp. 1859-67.

Kumar, D., Narang, R., Sreenivas, V., Rastogi, V., Bhatia, J., Saluja, D. and Srivastava, K. (2020) 'Circulatory miR-133b and miR-21 as Novel Biomarkers in

Early Prediction and Diagnosis of Coronary Artery Disease', *Genes*, 11(2), pp. 164.

Kumar, N. and Dey, C. S. (2002) 'Metformin enhances insulin signalling in insulin-dependent and-independent pathways in insulin resistant muscle cells', *Br J Pharmacol*, 137(3), pp. 329-36.

Kuswardhani, R. T., Wiradharma, K. G., Kandarini, Y., Widiana, G. R. and Martadiani, E. D. (2018) 'Factors associated with carotid intima-media thickness in patients on maintenance hemodialysis', *International journal of general medicine*, 12, pp. 1-6.

Kuwabara, Y., Ono, K., Horie, T., Nishi, H., Nagao, K., Kinoshita, M., Watanabe, S., Baba, O., Kojima, Y., Shizuta, S., Imai, M., Tamura, T., Kita, T. and Kimura, T. (2011) 'Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage', *Circ Cardiovasc Genet*, 4(4), pp. 446-54.

Lagos-Quintana, M., Rauhut, R., Meyer, J., Borkhardt, A. and Tuschl, T. (2003) 'New microRNAs from mouse and human', *Rna*, 9(2), pp. 175-9.

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W. and Tuschl, T. (2002) 'Identification of tissue-specific microRNAs from mouse', *Curr Biol*, 12(9), pp. 735-9.

Lakka, T. A., Salonen, R., Kaplan, G. A. and Salonen, J. T. (1999) 'Blood pressure and the progression of carotid atherosclerosis in middle-aged men', *Hypertension*, 34(1), pp. 51-6.

Lamarche, B. t., Tchernof, A., Moorjani, S., Cantin, B., Dagenais, G. R., Lupien, P. J. and Despre´s, J.-P. (1997) 'Small, Dense Low-Density Lipoprotein Particles as a Predictor of the Risk of Ischemic Heart Disease in Men', *Circulation*, 95(1), pp. 69-75.

Lantelme, P., Mestre, C., Lievre, M., Gressard, A. and Milon, H. (2002) 'Heart rate: an important confounder of pulse wave velocity assessment', *Hypertension*, 39(6), pp. 1083-7.

Larsson, S. C., Mason, A. M., Bäck, M., Klarin, D., Damrauer, S. M., Program, M. V., Michaëlsson, K. and Burgess, S. (2020) 'Genetic predisposition to smoking in relation to 14 cardiovascular diseases', *European Heart Journal*.

Laterza, O. F., Scott, M. G., Garrett-Engele, P. W., Korenblat, K. M. and Lockwood, C. M. (2013) 'Circulating miR-122 as a potential biomarker of liver disease', *Biomark Med*, 7(2), pp. 205-10.

Lauer, M. S. (2007) 'Primary prevention of atherosclerotic cardiovascular disease: the high public burden of low individual risk', *Jama*, 297(12), pp. 1376-8.

Laurent, S., Cockcroft, J., Van Bortel, L., Boutouyrie, P., Giannattasio, C., Hayoz, D., Pannier, B., Vlachopoulos, C., Wilkinson, I. and Struijker-Boudier, H. (2006) 'Expert consensus document on arterial stiffness: methodological issues and clinical applications', *Eur Heart J*, 27(21), pp. 2588-605.

Layde R. Paim, R. S., Guilherme de Rossi, José R. Matos-Souza, Anselmo de A. (2019) 'Circulating microRNAs, Vascular Risk, and Physical Activity in Spinal Cord-Injured Subjects', *Journal of Neurotrauma*, 36(6), pp. 845-852.

Lee, H. Y. and Oh, B. H. (2010) 'Aging and arterial stiffness', *Circ J*, 74(11), pp. 2257-62.

Lee, M. J., Shin, D. H., Kim, S. J., Oh, H. J., Yoo, D. E., Kim, J.-K., Park, J. T., Han, S. H., Kang, S.-W., Choi, K. H. and Yoo, T.-H. (2012) 'Visceral Fat Thickness Is Associated With Carotid Atherosclerosis in Peritoneal Dialysis Patients', *Obesity*, 20(6), pp. 1301-1307.

Lee, R. C., Feinbaum, R. L. and Ambros, V. (1993) 'The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14', *Cell*, 75(5), pp. 843-54.

Lee, T. C., O'Malley, P. G., Feuerstein, I. and Taylor, A. J. (2003) 'The prevalence and severity of coronary artery calcification on coronary artery computed tomography in black and white subjects', *J Am Coll Cardiol*, 41(1), pp. 39-44.

Lefkowitz, R. J. and Willerson, J. T. (2001) 'Prospects for cardiovascular research', *JAMA*, 285(5), pp. 581-7.

Letonja, M., Nikolajevic Starcevic, J. and Petrovič, D. (2012) 'Association of manganese superoxide dismutase and glutathione S-transferases genotypes with carotid atherosclerosis in patients with diabetes mellitus type 2', *International angiology : a journal of the International Union of Angiology*, 31, pp. 33-41.

Lexis, C. P. H., Wieringa, W. G., Hiemstra, B., van Deursen, V. M., Lipsic, E., van der Harst, P., van Veldhuisen, D. J. and van der Horst, I. C. C. (2014) 'Chronic Metformin Treatment is Associated with Reduced Myocardial Infarct Size in Diabetic Patients with ST-segment Elevation Myocardial Infarction', *Cardiovascular Drugs and Therapy*, 28(2), pp. 163-171.

Li, C., Fang, Z., Jiang, T., Zhang, Q., Liu, C., Zhang, C. and Xiang, Y. (2013a) 'Serum microRNAs profile from genome-wide serves as a fingerprint for diagnosis of acute myocardial infarction and angina pectoris', *BMC Med Genomics*, 6, pp. 16.

Li, X.-D., Yang, Y.-J., Wang, L.-Y., Qiao, S.-B., Lu, X.-F., Wu, Y.-J., Xu, B., Li, H.-F. and Gu, D.-F. (2017) 'Elevated plasma miRNA-122, -140-3p, -720, -2861, and -3149 during early period of acute coronary syndrome are derived from

peripheral blood mononuclear cells', *PloS one*, 12(9), pp. e0184256-e0184256.

Li, Y.-Q., Zhang, M.-F., Wen, H.-Y., Hu, C.-L., Liu, R., Wei, H.-Y., Ai, C.-M., Wang, G., Liao, X.-X. and Li, X. (2013b) 'Comparing the diagnostic values of circulating microRNAs and cardiac troponin T in patients with acute myocardial infarction', *Clinics (Sao Paulo, Brazil)*, 68(1), pp. 75-80.

Li, Y. and Kowdley, K. V. (2012a) 'Method for microRNA isolation from clinical serum samples', *Anal Biochem*, 431(1), pp. 69-75.

Li, Y. and Kowdley, K. V. (2012b) 'MicroRNAs in common human diseases', *Genomics Proteomics Bioinformatics*, 10(5), pp. 246-53.

Libby, P., Ridker, P. M. and Maseri, A. (2002) 'Inflammation and Atherosclerosis', *Circulation*, 105(9), pp. 1135-1143.

Lin, Q., Gao, Z., Alarcon, R. M., Ye, J. and Yun, Z. (2009) 'A role of miR-27 in the regulation of adipogenesis', *Febs j*, 276(8), pp. 2348-58.

Littlejohns, T. J., Holliday, J., Gibson, L. M., Garratt, S., Oesingmann, N., Alfaro-Almagro, F., Bell, J. D., Boultwood, C., Collins, R., Conroy, M. C., Crabtree, N., Doherty, N., Frangi, A. F., Harvey, N. C., Leeson, P., Miller, K. L., Neubauer, S., Petersen, S. E., Sellors, J., Sheard, S., Smith, S. M., Sudlow, C. L. M., Matthews, P. M. and Allen, N. E. (2020) 'The UK Biobank imaging enhancement of 100,000 participants: rationale, data collection, management and future directions', *Nature Communications*, 11(1), pp. 2624.

Liu, B., Ni, J., Shi, M., Bai, L., Zhan, C., Lu, H., Wu, Y., Tu, J., Ning, X., Hao, J. and Wang, J. (2017) 'Carotid Intima-media Thickness and its Association with Conventional Risk Factors in Low-income Adults: A Population-based Cross-Sectional Study in China', *Scientific reports*, 7, pp. 41500-41500.

Liu, B., Young, H., Crowe, F. L., Benson, V. S., Spencer, E. A., Key, T. J., Appleby, P. N. and Beral, V. (2011) 'Development and evaluation of the Oxford WebQ, a low-cost, web-based method for assessment of previous 24 h dietary intakes in large-scale prospective studies', *Public Health Nutr*, 14(11), pp. 1998-2005.

Liu, N., Bezprozvannaya, S., Williams, A. H., Qi, X., Richardson, J. A., Bassel-Duby, R. and Olson, E. N. (2008) 'microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart', *Genes Dev*, 22(23), pp. 3242-54.

Liu, X., Fan, Z., Zhao, T., Cao, W., Zhang, L., Li, H., Xie, Q., Tian, Y. and Wang, B. (2015) 'Plasma miR-1, miR-208, miR-499 as potential predictive biomarkers for acute myocardial infarction: An independent study of Han population', *Exp Gerontol*, 72, pp. 230-8.

Livak, K. J. and Schmittgen, T. D. (2001a) 'Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method', *Methods*, 25(4), pp. 402-408.

Livak, K. J. and Schmittgen, T. D. (2001b) 'Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method', *Methods*, 25(4), pp. 402-8.

Llave, C., Xie, Z., Kasschau, K. D. and Carrington, J. C. (2002) 'Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA', *Science*, 297(5589), pp. 2053-6.

Lo Gullo, A., Mandraffino, G., Bagnato, G., Aragona, C. O., Imbalzano, E., D'Ascola, A., Rotondo, F., Cinquegrani, A., Mormina, E., Saitta, C., Versace, A. G., Sardo, M. A., Lo Gullo, R., Loddo, S. and Saitta, A. (2015) 'Vitamin D Status in Rheumatoid Arthritis: Inflammation, Arterial Stiffness and Circulating Progenitor Cell Number', *PloS one*, 10(8), pp. e0134602-e0134602.

Loboz-Rudnicka, M., Jaroch, J., Bociaga, Z., Rzyczkowska, B., Uchmanowicz, I., Polanski, J., Dudek, K., Szuba, A. and Loboz-Grudzien, K. (2016) 'Impact of cardiovascular risk factors on carotid intima-media thickness: sex differences', *Clin Interv Aging*, 11, pp. 721-31.

Loimaala, A., Rontu, R., Vuori, I., Mercuri, M., Lehtimäki, T., Nenonen, A. and Bond, M. G. (2006) 'Blood leukocyte count is a risk factor for intima-media thickening and subclinical carotid atherosclerosis in middle-aged men', *Atherosclerosis*, 188(2), pp. 363-9.

Lorenz, M. W., Gao, L., Ziegelbauer, K., Norata, G. D., Empana, J. P., Schmidtmann, I., Lin, H.-J., McLachlan, S., Bokemark, L., Ronkainen, K., Amato, M., Schminke, U., Srinivasan, S. R., Lind, L., Okazaki, S., Stehouwer, C. D. A., Willeit, P., Polak, J. F., Steinmetz, H., Sander, D., Poppert, H., Desvarieux, M., Ikram, M. A., Johnsen, S. H., Staub, D., Sirtori, C. R., Iglseder, B., Beloqui, O., Engström, G., Friera, A., Rozza, F., Xie, W., Parraga, G., Grigore, L., Plichart, M., Blankenberg, S., Su, T.-C., Schmidt, C., Tuomainen, T.-P., Veglia, F., Völzke, H., Nijpels, G., Willeit, J., Sacco, R. L., Franco, O. H., Uthoff, H., Hedblad, B., Suarez, C., Izzo, R., Zhao, D., Wannarong, T., Catapano, A., Ducimetiere, P., Espinola-Klein, C., Chien, K.-L., Price, J. F., Bergström, G., Kauhanen, J., Tremoli, E., Dörr, M., Berenson, G., Kitagawa, K., Dekker, J. M., Kiechl, S., Sitzer, M., Bickel, H., Rundek, T., Hofman, A., Mathiesen, E. B., Castelnuovo, S., Landecho, M. F., Rosvall, M., Gabriel, R., de Luca, N., Liu, J., Baldassarre, D., Kavousi, M., de Groot, E., Bots, M. L., Yanez, D. N., Thompson, S. G. and group, P.-I. s. (2018) 'Predictive value for cardiovascular events of common carotid intima media thickness and its rate of change in individuals at high cardiovascular risk - Results from the PROG-IMT collaboration', *PloS one*, 13(4), pp. e0191172-e0191172.

Lorenz, M. W., Markus, H. S., Bots, M. L., Rosvall, M. and Sitzer, M. (2007) 'Prediction of clinical cardiovascular events with carotid intima-media

thickness: a systematic review and meta-analysis', *Circulation*, 115(4), pp. 459-67.

Lorenz, M. W., Polak, J. F., Kavousi, M., Mathiesen, E. B., Völzke, H., Tuomainen, T.-P., Sander, D., Plichart, M., Catapano, A. L., Robertson, C. M., Kiechl, S., Rundek, T., Desvarieux, M., Lind, L., Schmid, C., DasMahapatra, P., Gao, L., Ziegelbauer, K., Bots, M. L. and Thompson, S. G. (2012) 'Carotid intima-media thickness progression to predict cardiovascular events in the general population (the PROG-IMT collaborative project): a meta-analysis of individual participant data', *The Lancet*, 379(9831), pp. 2053-2062.

Lorenz, M. W., Schaefer, C., Steinmetz, H. and Sitzer, M. (2010) 'Is carotid intima media thickness useful for individual prediction of cardiovascular risk? Ten-year results from the Carotid Atherosclerosis Progression Study (CAPS)', *Eur Heart J*, 31(16), pp. 2041-8.

Ltd., A. P.-M. (2020) *Metformin hydrochloride 850 mg film-coated tablets*. Available at: https://www.medicines.org.uk/emc/product/7348/smpc#gref (Accessed: 8 May 2021).

Lu, Q., Ma, Z., Ding, Y., Bedarida, T., Chen, L., Xie, Z., Song, P. and Zou, M.-H. (2019) 'Circulating miR-103a-3p contributes to angiotensin II-induced renal inflammation and fibrosis via a SNRK/NF-κB/p65 regulatory axis', *Nature Communications*, 10(1), pp. 2145.

Lund, E., Güttinger, S., Calado, A., Dahlberg, J. E. and Kutay, U. (2004) 'Nuclear export of microRNA precursors', *Science*, 303(5654), pp. 95-8.

Lupattelli, G., Lombardini, R., Schillaci, G., Ciuffetti, G., Marchesi, S., Siepi, D. and Mannarino, E. (2000) 'Flow-mediated vasoactivity and circulating adhesion molecules in hypertriglyceridemia: Association with small, dense LDL cholesterol particles', *American Heart Journal*, 140(3), pp. 521-526.

Lyall, D. M., Celis-Morales, C., Ward, J., Iliodromiti, S., Anderson, J. J., Gill, J. M. R., Smith, D. J., Ntuk, U. E., Mackay, D. F., Holmes, M. V., Sattar, N. and Pell, J. P. (2017) 'Association of Body Mass Index With Cardiometabolic Disease in the UK Biobank: A Mendelian Randomization Study', *JAMA Cardiol*, 2(8), pp. 882-889.

Lyons, D., Roy, S., Patel, M., Benjamin, N. and Swift, C. G. (1997) 'Impaired nitric oxide-mediated vasodilatation and total body nitric oxide production in healthy old age', *Clin Sci (Lond)*, 93(6), pp. 519-25.

Maarek, B., Simon, A. C., Levenson, J., Pithois-Merli, I. and Bouthier, J. (1987) 'Heterogeneity of the atherosclerotic process in systemic hypertension poorly controlled by drug treatment', *Am J Cardiol*, 59(5), pp. 414-7.

Maas, A. H. E. M. and Appelman, Y. E. A. (2010) 'Gender differences in coronary heart disease', *Netherlands heart journal : monthly journal of the Netherlands*

Society of Cardiology and the Netherlands Heart Foundation, 18(12), pp. 598-602.

Maiorino, M. I., Bellastella, G., Petrizzo, M., Gicchino, M., Caputo, M., Giugliano, D. and Esposito, K. (2017) 'Effect of a Mediterranean diet on endothelial progenitor cells and carotid intima-media thickness in type 2 diabetes: Follow-up of a randomized trial', *Eur J Prev Cardiol*, 24(4), pp. 399-408.

Maki, K. C., Dicklin, M. R., Davidson, M. H., Mize, P. D. and Kulkarni, K. R. (2012) 'Indicators of the atherogenic lipoprotein phenotype measured with density gradient ultracentrifugation predict changes in carotid intima-media thickness in men and women', *Vasc Health Risk Manag*, 8, pp. 31-8.

Mancia, G., De Backer, G., Dominiczak, A., Cifkova, R., Fagard, R., Germano, G., Grassi, G., Heagerty, A. M., Kjeldsen, S. E., Laurent, S., Narkiewicz, K., Ruilope, L., Rynkiewicz, A., Schmieder, R. E., Boudier, H. A., Zanchetti, A., Vahanian, A., Camm, J., De Caterina, R., Dean, V., Dickstein, K., Filippatos, G., Funck-Brentano, C., Hellemans, I., Kristensen, S. D., McGregor, K., Sechtem, U., Silber, S., Tendera, M., Widimsky, P., Zamorano, J. L., Erdine, S., Kiowski, W., Agabiti-Rosei, E., Ambrosioni, E., Lindholm, L. H., Viigimaa, M., Adamopoulos, S., Agabiti-Rosei, E., Ambrosioni, E., Bertomeu, V., Clement, D., Erdine, S., Farsang, C., Gaita, D., Lip, G., Mallion, J. M., Manolis, A. J., Nilsson, P. M., O'Brien, E., Ponikowski, P., Redon, J., Ruschitzka, F., Tamargo, J., van Zwieten, P., Waeber, B. and Williams, B. (2007) '2007 Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC)', *J Hypertens*, 25(6), pp. 1105-87.

Mancia G, e. a. (2013) '2013 ESH/ESC Guidelines for the management of arterial hypertension: the Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC)', *J Hypertens*, 31(7), pp. 1281-357.

Mancia, G., Fagard, R., Narkiewicz, K., Redon, J., Zanchetti, A., Bohm, M., Christiaens, T., Cifkova, R., De Backer, G., Dominiczak, A., Galderisi, M., Grobbee, D. E., Jaarsma, T., Kirchhof, P., Kjeldsen, S. E., Laurent, S., Manolis, A. J., Nilsson, P. M., Ruilope, L. M., Schmieder, R. E., Sirnes, P. A., Sleight, P., Viigimaa, M., Waeber, B. and Zannad, F. (2013) '2013 ESH/ESC Guidelines for the management of arterial hypertension: the Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC)', *J Hypertens*, 31(7), pp. 1281-357.

Mancia, G., Fagard, R., Narkiewicz, K., Redon, J., Zanchetti, A., Böhm, M., Christiaens, T., Cifkova, R., De Backer, G., Dominiczak, A., Galderisi, M., Grobbee, D. E., Jaarsma, T., Kirchhof, P., Kjeldsen, S. E., Laurent, S., Manolis, A. J., Nilsson, P. M., Ruilope, L. M., Schmieder, R. E., Sirnes, P. A., Sleight, P., Viigimaa, M., Waeber, B. and Zannad, F. (2014) '2013 ESH/ESC Practice Guidelines for the Management of Arterial Hypertension', *Blood Press*, 23(1), pp. 3-16.

Mannami, T., Konishi, M., Baba, S., Nishi, N. and Terao, A. (1997) 'Prevalence of asymptomatic carotid atherosclerotic lesions detected by high-resolution ultrasonography and its relation to cardiovascular risk factors in the general population of a Japanese city: the Suita study', *Stroke*, 28(3), pp. 518-25.

Mannucci, E., Ognibene, A., Cremasco, F., Bardini, G., Mencucci, A., Pierazzuoli, E., Ciani, S., Messeri, G. and Rotella, C. M. (2001) 'Effect of metformin on glucagon-like peptide 1 (GLP-1) and leptin levels in obese nondiabetic subjects', *Diabetes Care*, 24(3), pp. 489-94.

Mar-Aguilar, F., Mendoza-Ramírez, J. A., Malagón-Santiago, I., Espino-Silva, P. K., Santuario-Facio, S. K., Ruiz-Flores, P., Rodríguez-Padilla, C. and Reséndez-Pérez, D. (2013) 'Serum Circulating microRNA Profiling for Identification of Potential Breast Cancer Biomarkers', *Disease Markers*, 34, pp. 259454.

Marabita, F., de Candia, P., Torri, A., Tegnér, J., Abrignani, S. and Rossi, R. L. (2016) 'Normalization of circulating microRNA expression data obtained by quantitative real-time RT-PCR', *Brief Bioinform*, 17(2), pp. 204-12.

Marmot, M. and Brunner, E. (2005) 'Cohort Profile: the Whitehall II study', *Int J Epidemiol*, 34(2), pp. 251-6.

Matz, R. L., Schott, C., Stoclet, J. C. and Andriantsitohaina, R. (2000) 'Age-related endothelial dysfunction with respect to nitric oxide, endothelium-derived hyperpolarizing factor and cyclooxygenase products', *Physiol Res*, 49(1), pp. 11-8.

McBride, D., Carré, W., Sontakke, S. D., Hogg, C. O., Law, A., Donadeu, F. X. and Clinton, M. (2012) 'Identification of miRNAs associated with the follicular-luteal transition in the ruminant ovary', *REPRODUCTION*, 144(2), pp. 221-233.

McDonald, J. S., Milosevic, D., Reddi, H. V., Grebe, S. K. and Algeciras-Schimnich, A. (2011a) 'Analysis of circulating microRNA: preanalytical and analytical challenges', *Clin Chem*, 57(6), pp. 833-40.

McDonald, J. S., Milosevic, D., Reddi, H. V., Grebe, S. K. and Algeciras-Schimnich, A. (2011b) 'Analysis of Circulating MicroRNA: Preanalytical and Analytical Challenges', *Clinical Chemistry*, 57(6), pp. 833-840.

McEniery, C. M., Yasmin, Maki-Petaja, K. M., McDonnell, B. J., Munnery, M., Hickson, S. S., Franklin, S. S., Cockcroft, J. R. and Wilkinson, I. B. (2010) 'The impact of cardiovascular risk factors on aortic stiffness and wave reflections depends on age: The Anglo-Cardiff Collaborative Trial (ACCT III)', *Hypertension*, 56(4), pp. 591-597.

Members, A. T. F. and Mancia, G. and Fagard, R. and Narkiewicz, K. and Redon, J. and Zanchetti, A. and Böhm, M. and Christiaens, T. and Cifkova, R. and De Backer, G. and Dominiczak, A. and Galderisi, M. and Grobbee, D. E. and Jaarsma, T. and Kirchhof, P. and Kjeldsen, S. E. and Laurent, S. and Manolis,

A. J. and Nilsson, P. M. and Ruilope, L. M. and Schmieder, R. E. and Sirnes, P. A. and Sleight, P. and Viigimaa, M. and Waeber, B. and Zannad, F. and Council, E. S. and Redon, J. and Dominiczak, A. and Narkiewicz, K. and Nilsson, P. M. and Burnier, M. and Viigimaa, M. and Ambrosioni, E. and Caufield, M. and Coca, A. and Olsen, M. H. and Schmieder, R. E. and Tsioufis, C. and van de Borne, P. and Guidelines, E. C. f. P. and Zamorano, J. L. and Achenbach, S. and Baumgartner, H. and Bax, J. J. and Bueno, H. and Dean, V. and Deaton, C. and Erol, C. and Fagard, R. and Ferrari, R. and Hasdai, D. and Hoes, A. W. and Kirchhof, P. and Knuuti, J. and Kolh, P. and Lancellotti, P. and Linhart, A. and Nihoyannopoulos, P. and Piepoli, M. F. and Ponikowski, P. and Sirnes, P. A. and Tamargo, J. L. and Tendera, M. and Torbicki, A. and Wijns, W. and Windecker, S. and Reviewers, D. and Clement, D. L. and Coca, A. and Gillebert, T. C. and Tendera, M. and Rosei, E. A. and Ambrosioni, E. and Anker, S. D. and Bauersachs, J. and Hitij, J. B. and Caulfield, M. and De Buyzere, M. and De Geest, S. and Derumeaux, G. A. and Erdine, S. and Farsang, C. and Funck-Brentano, C. and Gerc, V. and Germano, G. and Gielen, S. and Haller, H. and Hoes, A. W. and Jordan, J. and Kahan, T. and Komajda, M. and Lovic, D. and Mahrholdt, H. and Olsen, M. H. and Ostergren, J. and Parati, G. and Perk, J. and Polonia, J. and Popescu, B. A. and Reiner, Ž. and Rydén, L. and Sirenko, Y. and Stanton, A. and Struijker-Boudier, H. and Tsioufis, C. and van de Borne, P. and Vlachopoulos, C. and Volpe, M. and Wood, D. A. (2013) '2013 ESH/ESC Guidelines for the management of arterial hypertension: The Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC)', *European Heart Journal*, 34(28), pp. 2159-2219.

Mensà, E., Giuliani, A., Matacchione, G., Gurău, F., Bonfigli, A. R., Romagnoli, F., De Luca, M., Sabbatinelli, J. and Olivieri, F. (2019) 'Circulating miR-146a in healthy aging and type 2 diabetes: Age- and gender-specific trajectories', *Mechanisms of Ageing and Development*, 180, pp. 1-10.

Meyer, A. A., Kundt, G., Steiner, M., Schuff-Werner, P. and Kienast, W. (2006) 'Impaired flow-mediated vasodilation, carotid artery intima-media thickening, and elevated endothelial plasma markers in obese children: the impact of cardiovascular risk factors', *Pediatrics*, 117(5), pp. 1560-7.

Millasseau, S. C., Kelly, R. P., Ritter, J. M. and Chowienczyk, P. J. (2002) 'Determination of age-related increases in large artery stiffness by digital pulse contour analysis', *Clin Sci (Lond)*, 103(4), pp. 371-7.

Mitchell, G. F., Hwang, S. J., Vasan, R. S., Larson, M. G., Pencina, M. J., Hamburg, N. M., Vita, J. A., Levy, D. and Benjamin, E. J. (2010) 'Arterial stiffness and cardiovascular events: the Framingham Heart Study', *Circulation*, 121(4), pp. 505-11.

Mitchell, P. S., Parkin, R. K., Kroh, E. M., Fritz, B. R., Wyman, S. K., Pogosova-Agadjanyan, E. L., Peterson, A., Noteboom, J., O'Briant, K. C., Allen, A., Lin, D. W., Urban, N., Drescher, C. W., Knudsen, B. S., Stirewalt, D. L., Gentleman, R., Vessella, R. L., Nelson, P. S., Martin, D. B. and Tewari, M. (2008a) 'Circulating microRNAs as stable blood-based markers for cancer detection', *Proc Natl Acad Sci U S A*, 105(30), pp. 10513-8.

Mitchell, P. S., Parkin, R. K., Kroh, E. M., Fritz, B. R., Wyman, S. K., Pogosova-Agadjanyan, E. L., Peterson, A., Noteboom, J., O'Briant, K. C., Allen, A., Lin, D. W., Urban, N., Drescher, C. W., Knudsen, B. S., Stirewalt, D. L., Gentleman, R., Vessella, R. L., Nelson, P. S., Martin, D. B. and Tewari, M. (2008b) 'Circulating microRNAs as stable blood-based markers for cancer detection', *Proceedings of the National Academy of Sciences*, 105(30), pp. 10513-10518.

Miyazaki, H., Matsuoka, H., Cooke, J. P., Usui, M., Ueda, S., Okuda, S. and Imaizumi, T. (1999) 'Endogenous Nitric Oxide Synthase Inhibitor', *Circulation*, 99(9), pp. 1141-1146.

Mohan, A., Sada, S., Kumar, B. S., Sarma, K. V. S., Devi, B. V., Rao, P. V. L. N. S., Rajasekhar, D. and Katyarmal, D. T. (2014) 'Subclinical atherosclerosis in patients with rheumatoid arthritis by utilizing carotid intima-media thickness as a surrogate marker', *The Indian journal of medical research*, 140(3), pp. 379-386.

Moiseeva, O., Deschênes-Simard, X., St-Germain, E., Igelmann, S., Huot, G., Cadar, A. E., Bourdeau, V., Pollak, M. N. and Ferbeyre, G. (2013) 'Metformin inhibits the senescence-associated secretory phenotype by interfering with IKK/NF-κB activation', *Aging Cell*, 12(3), pp. 489-98.

Moldovan, L., Batte, K., Wang, Y., Wisler, J. and Piper, M. (2013) 'Analyzing the circulating microRNAs in exosomes/extracellular vesicles from serum or plasma by qRT-PCR', *Methods Mol Biol*, 1024, pp. 129-45.

Moldovan, L., Batte, K. E., Trgovcich, J., Wisler, J., Marsh, C. B. and Piper, M. (2014) 'Methodological challenges in utilizing miRNAs as circulating biomarkers', *J Cell Mol Med*, 18(3), pp. 371-90.

Moore, K. J., Rayner, K. J., Suárez, Y. and Fernández-Hernando, C. (2010) 'microRNAs and cholesterol metabolism', *Trends in endocrinology and metabolism: TEM*, 21(12), pp. 699-706.

Mora, S., Cook, N., Buring, J. E., Ridker, P. M. and Lee, I. M. (2007) 'Physical activity and reduced risk of cardiovascular events: potential mediating mechanisms', *Circulation*, 116(19), pp. 2110-8.

Moret, I., Sánchez-Izquierdo, D., Iborra, M., Tortosa, L., Navarro-Puche, A., Nos, P., Cervera, J. and Beltrán, B. (2013) 'Assessing an improved protocol for plasma microRNA extraction', *PLoS One*, 8(12), pp. e82753.

Morrow, D. A., de Lemos, J. A., Sabatine, M. S. and Antman, E. M. (2003) 'The search for a biomarker of cardiac ischemia', *Clin Chem*, 49(4), pp. 537-9.

Mueller, C., McDonald, K., de Boer, R. A., Maisel, A., Cleland, J. G. F., Kozhuharov, N., Coats, A. J. S., Metra, M., Mebazaa, A., Ruschitzka, F., Lainscak, M., Filippatos, G., Seferovic, P. M., Meijers, W. C., Bayes-Genis, A., Mueller, T., Richards, M., Januzzi Jr, J. L. and Cardiology, o. b. o. t. H. F. A.

o. t. E. S. o. (2019) 'Heart Failure Association of the European Society of Cardiology practical guidance on the use of natriuretic peptide concentrations', *European Journal of Heart Failure*, 21(6), pp. 715-731.

'Multiple Risk Factor Intervention Trial: Risk Factor Changes and Mortality Results', (1982) *JAMA*, 248(12), pp. 1465-1477.

Nakashima, T. and Tanikawa, J. (1971) 'A study of human aortic distensibility with relation to atherosclerosis and aging', *Angiology*, 22(8), pp. 477-90.

Narverud, I., Retterstøl, K., Iversen, P. O., Halvorsen, B., Ueland, T., Ulven, S. M., Ose, L., Aukrust, P., Veierød, M. B. and Holven, K. B. (2014) 'Markers of atherosclerotic development in children with familial hypercholesterolemia: A literature review', *Atherosclerosis*, 235(2), pp. 299-309.

Naslund, E., Gutniak, M., Skogar, S., Rossner, S. and Hellstrom, P. M. (1998) 'Glucagon-like peptide 1 increases the period of postprandial satiety and slows gastric emptying in obese men', *Am J Clin Nutr*, 68(3), pp. 525-30.

Nathan, D. M., Buse, J. B., Davidson, M. B., Ferrannini, E., Holman, R. R., Sherwin, R. and Zinman, B. (2009) 'Medical Management of Hyperglycemia in Type 2 Diabetes: A Consensus Algorithm for the Initiation and Adjustment of Therapy', *A consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes*, 32(1), pp. 193-203.

National Institute for Health and care Excellent (2017) *Cardiovascular disease: risk assessment and reduction, including lipid modification*. Available at: https://www.nice.org.uk/guidance/cg181 (Accessed: 22 / September/ 2020.

National Institute for Health and care Excellent (2019) *Type 2 diabetes in adults: management | Guidance and guidelines|* Available at: https://www.nice.org.uk/guidance/ng28/chapter/1-recommendations (Accessed: 22/ september/2020.

Nelson, M. (2005) 'DIETARY INTAKE MEASUREMENT | Validation', in Caballero, B. (ed.) *Encyclopedia of Human Nutrition (Second Edition)*. Oxford: Elsevier, pp. 16-26.

Newman, A. B., Dodson, J. A., Church, T. S., Buford, T. W., Fielding, R. A., Kritchevsky, S., Beavers, D., Pahor, M., Stafford, R. S., Szady, A. D., Ambrosius, W. T., McDermott, M. M. and Group, f. t. L. S. (2016) 'Cardiovascular Events in a Physical Activity Intervention Compared With a Successful Aging Intervention: The LIFE Study Randomized Trial', *JAMA Cardiology*, 1(5), pp. 568-574.

NICE (2016) *Cardiovascular disease: risk assessment and reduction, including lipid modification*. Available at: https://www.nice.org.uk/guidance/cg181/chapter/1-recommendations (Accessed: 23 / September/2020.

NICE (2019) *Type 2 diabetes in adults: management*. Available at: https://www.nice.org.uk/guidance/ng28/chapter/Introduction (Accessed: 23/ September/2020.

Nürnberger, J., Dammer, S., Opazo Saez, A., Philipp, T. and Schäfers, R. F. (2003) 'Diastolic blood pressure is an important determinant of augmentation index and pulse wave velocity in young, healthy males', *J Hum Hypertens*, 17(3), pp. 153-8.

O'Brien, J., Hayder, H., Zayed, Y. and Peng, C. (2018) 'Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation', *Frontiers in Endocrinology*, 9(402).

O'Leary, D. H. and Polak, J. F. (2002) 'Intima-media thickness: a tool for atherosclerosis imaging and event prediction', *Am J Cardiol*, 90(10c), pp. 18l-21l.

O'Leary, D. H., Polak, J. F., Kronmal, R. A., Kittner, S. J., Bond, M. G., Wolfson, S. K., Jr., Bommer, W., Price, T. R., Gardin, J. M. and Savage, P. J. (1992) 'Distribution and correlates of sonographically detected carotid artery disease in the Cardiovascular Health Study. The CHS Collaborative Research Group', *Stroke*, 23(12), pp. 1752-60.

O'Leary, D. H., Polak, J. F., Kronmal, R. A., Manolio, T. A., Burke, G. L. and Wolfson, S. K., Jr. (1999) 'Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group', *N Engl J Med*, 340(1), pp. 14-22.

O'Rourke, M. (1990) 'Arterial stiffness, systolic blood pressure, and logical treatment of arterial hypertension', *Hypertension*, 15(4), pp. 339-347.

O'Sullivan, J. F., Neylon, A., Fahy, E. F., Yang, P., McGorrian, C. and Blake, G. J. (2019) 'MiR-93-5p is a novel predictor of coronary in-stent restenosis', *Heart Asia*, 11(1), pp. e011134-e011134.

O'Leary, D. H., Polak, J. F., Kronmal, R. A., Savage, P. J., Borhani, N. O., Kittner, S. J., Tracy, R., Gardin, J. M., Price, T. R. and Furberg, C. D. (1996) 'Thickening of the Carotid Wall', *Stroke*, 27(2), pp. 224-231.

Office of the Surgeon, G., Office on, S. and Health (2004) 'Reports of the Surgeon General', *The Health Consequences of Smoking: A Report of the Surgeon General*. Atlanta (GA): Centers for Disease Control and Prevention (US).

Okamura, T., Sekikawa, A., Sawamura, T., Kadowaki, T., Barinas-Mitchell, E., Mackey, R. H., Kadota, A., Evans, R. W., Edmundowicz, D., Higashiyama, A., Nakamura, Y., Abbott, R. D., Miura, K., Fujiyoshi, A., Fujita, Y., Murakami, Y., Miyamatsu, N., Kakino, A., Maegawa, H., Murata, K., Horie, M., Mitsunami, K., Kashiwagi, A., Kuller, L. H. and Ueshima, H. (2013) 'LOX-1 ligands containing

apolipoprotein B and carotid intima-media thickness in middle-aged community-dwelling US Caucasian and Japanese men', *Atherosclerosis*, 229(1), pp. 240-5.

Ortega, F. J., Mercader, J. M., Catalán, V., Moreno-Navarrete, J. M., Pueyo, N., Sabater, M., Gómez-Ambrosi, J., Anglada, R., Fernández-Formoso, J. A., Ricart, W., Frühbeck, G. and Fernández-Real, J. M. (2013) 'Targeting the circulating microRNA signature of obesity', *Clin Chem*, 59(5), pp. 781-92.

Paim, L. R., Schreiber, R., Matos-Souza, J. R., Silva, A. A., Campos, L. F., Azevedo, E. R., Alonso, K., de Rossi, G., Etchebehere, M., Gorla, J. I., Cliquet, A. and Nadruz, W. (2013) 'Oxidized low-density lipoprotein, matrix-metalloproteinase-8 and carotid atherosclerosis in spinal cord injured subjects', *Atherosclerosis*, 231(2), pp. 341-345.

Park, K.-H. and Park, W. J. (2015) 'Endothelial Dysfunction: Clinical Implications in Cardiovascular Disease and Therapeutic Approaches', *Journal of Korean medical science*, 30(9), pp. 1213-1225.

Pedersen, H. K., Gudmundsdottir, V., Nielsen, H. B., Hyotylainen, T., Nielsen, T., Jensen, B. A., Forslund, K., Hildebrand, F., Prifti, E., Falony, G., Le Chatelier, E., Levenez, F., Doré, J., Mattila, I., Plichta, D. R., Pöhö, P., Hellgren, L. I., Arumugam, M., Sunagawa, S., Vieira-Silva, S., Jørgensen, T., Holm, J. B., Trošt, K., Kristiansen, K., Brix, S., Raes, J., Wang, J., Hansen, T., Bork, P., Brunak, S., Oresic, M., Ehrlich, S. D. and Pedersen, O. (2016) 'Human gut microbes impact host serum metabolome and insulin sensitivity', *Nature*, 535(7612), pp. 376-81.

Peluso, R., Caso, F., Tasso, M., Sabbatino, V., Lupoli, R., Dario Di Minno, M. N., Ursini, F., Costa, L. and Scarpa, R. (2019) 'Biomarkers of subclinical atherosclerosis in patients with psoriatic arthritis', *Open access rheumatology : research and reviews*, 11, pp. 143-156.

Peña, A. S., Wiltshire, E., MacKenzie, K., Gent, R., Piotto, L., Hirte, C. and Couper, J. (2006) 'Vascular Endothelial and Smooth Muscle Function Relates to Body Mass Index and Glucose in Obese and Nonobese Children', *The Journal of Clinical Endocrinology & Metabolism*, 91(11), pp. 4467-4471.

Peters, S. A., Lind, L., Palmer, M. K., Grobbee, D. E., Crouse, J. R., 3rd, O'Leary, D. H., Evans, G. W., Raichlen, J., Bots, M. L., den Ruijter, H. M. and group, M. s. (2012) 'Increased age, high body mass index and low HDL-C levels are related to an echolucent carotid intima-media: the METEOR study', *J Intern Med*, 272(3), pp. 257-66.

Petit, J. M., Guiu, B., Masson, D., Duvillard, L., Jooste, V., Buffier, P., Terriat, B., Bouillet, B., Brindisi, M. C., Loffroy, R., Robin, I., Hillon, P., Cercueil, J. P. and Verges, B. (2010) 'Specifically PNPLA3-mediated accumulation of liver fat in obese patients with type 2 diabetes', *J Clin Endocrinol Metab*, 95(12), pp. E430-6.

Polak, J. F., Pencina, M. J., Meisner, A., Pencina, K. M., Brown, L. S., Wolf, P. A. and D'Agostino, R. B., Sr. (2010) 'Associations of carotid artery intima-media thickness (IMT) with risk factors and prevalent cardiovascular disease: comparison of mean common carotid artery IMT with maximum internal carotid artery IMT', *J Ultrasound Med*, 29(12), pp. 1759-68.

Ponikowski, P., Voors, A. A., Anker, S. D., Bueno, H., Cleland, J. G. F., Coats, A. J. S., Falk, V., González-Juanatey, J. R., Harjola, V.-P., Jankowska, E. A., Jessup, M., Linde, C., Nihoyannopoulos, P., Parissis, J. T., Pieske, B., Riley, J. P., Rosano, G. M. C., Ruilope, L. M., Ruschitzka, F., Rutten, F. H., van der Meer, P. and Group, E. S. D. (2016) '2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC)Developed with the special contribution of the Heart Failure Association (HFA) of the ESC', *European Heart Journal*, 37(27), pp. 2129-2200.

Popele, N. M. v., Grobbee, D. E., Bots, M. L., Asmar, R., Topouchian, J., Reneman, R. S., Hoeks, A. P. G., Kuip, D. A. M. v. d., Hofman, A. and Witteman, J. C. M. (2001) 'Association Between Arterial Stiffness and Atherosclerosis', *Stroke*, 32(2), pp. 454-460.

Poredos, P. and Kek, A. (2000) 'Relation of blunted dilation of the brachial artery in insulin-dependent diabetes mellitus to microalbuminuria', *Am J Cardiol*, 86(3), pp. 364-7.

Powrozek, T., Kuznar-Kaminska, B., Dziedzic, M., Mlak, R., Batura-Gabryel, H., Sagan, D., Krawczyk, P., Milanowski, J. and Malecka-Massalska, T. (2017) 'The diagnostic role of plasma circulating precursors of miRNA-944 and miRNA-3662 for non-small cell lung cancer detection', *Pathol Res Pract*, 213(11), pp. 1384-1387.

Preiss, D., Lloyd, S. M., Ford, I., McMurray, J. J., Holman, R. R., Welsh, P., Fisher, M., Packard, C. J. and Sattar, N. (2014) 'Metformin for non-diabetic patients with coronary heart disease (the CAMERA study): a randomised controlled trial', *Lancet Diabetes Endocrinol*, 2(2), pp. 116-24.

Preiss, D., Rankin, N., Welsh, P., Holman, R. R., Kangas, A. J., Soininen, P., Würtz, P., Ala-Korpela, M. and Sattar, N. (2016) 'Effect of metformin therapy on circulating amino acids in a randomized trial: the CAMERA study', *Diabet Med*, 33(11), pp. 1569-1574.

Price, J. F., Tzoulaki, I., Lee, A. J. and Fowkes, F. G. (2007) 'Ankle brachial index and intima media thickness predict cardiovascular events similarly and increased prediction when combined', *J Clin Epidemiol*, 60(10), pp. 1067-75.

Pritchard, C. C., Kroh, E., Wood, B., Arroyo, J. D., Dougherty, K. J., Miyaji, M. M., Tait, J. F. and Tewari, M. (2012) 'Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies', *Cancer Prev Res (Phila)*, 5(3), pp. 492-497.

Quan, X., Ji, Y., Zhang, C., Guo, X., Zhang, Y., Jia, S., Ma, W., Fan, Y. and Wang, C. (2018) 'Circulating MiR-146a May be a Potential Biomarker of Coronary Heart Disease in Patients with Subclinical Hypothyroidism', *Cellular Physiology and Biochemistry*, 45(1), pp. 226-236.

Rajendran, P., Rengarajan, T., Thangavel, J., Nishigaki, Y., Sakthisekaran, D., Sethi, G. and Nishigaki, I. (2013) 'The vascular endothelium and human diseases', *International journal of biological sciences*, 9(10), pp. 1057-1069.

Rathsman, B., Rosfors, S., Sjöholm, A. and Nyström, T. (2012) 'Early signs of atherosclerosis are associated with insulin resistance in non-obese adolescent and young adults with type 1 diabetes', *Cardiovascular diabetology*, 11, pp. 145-145.

Raut, S. K., Singh, G. B., Rastogi, B., Saikia, U. N., Mittal, A., Dogra, N., Singh, S., Prasad, R. and Khullar, M. (2016) 'miR-30c and miR-181a synergistically modulate p53-p21 pathway in diabetes induced cardiac hypertrophy', *Molecular and Cellular Biochemistry*, 417(1), pp. 191-203.

Redfield, M. M., Rodeheffer, R. J., Jacobsen, S. J., Mahoney, D. W., Bailey, K. R. and Burnett, J. C. (2002) 'Plasma brain natriuretic peptide concentration: impact of age and gender', *Journal of the American College of Cardiology*, 40(5), pp. 976-982.

Reed, D. M., MacLean, C. J. and Hayashi, T. (1987) 'Predictors of atherosclerosis in the Honolulu Heart Program. I. Biologic, dietary, and lifestyle characteristics', *Am J Epidemiol*, 126(2), pp. 214-25.

Relf, I. R., Lo, C. S., Myers, K. A. and Wahlqvist, M. L. (1986) 'Risk factors for changes in aorto-iliac arterial compliance in healthy men', *Arteriosclerosis: An Official Journal of the American Heart Association, Inc.*, 6(1), pp. 105-108.

Ren, L., Cai, J., Liang, J., Li, W. and Sun, Z. (2015) 'Impact of Cardiovascular Risk Factors on Carotid Intima-Media Thickness and Degree of Severity: A Cross-Sectional Study', *PloS one*, 10(12), pp. e0144182-e0144182.

Rhee, J. W. and Wu, J. C. (2013) 'Advances in nanotechnology for the management of coronary artery disease', *Trends Cardiovasc Med*, 23(2), pp. 39-45.

Ribeiro, A. H., Lotufo, P. A., Fujita, A., Goulart, A. C., Chor, D., Mill, J. G., Bensenor, I. M. and Santos, I. S. (2017) 'Association Between Short-Term Systolic Blood Pressure Variability and Carotid Intima-Media Thickness in ELSA-Brasil Baseline', *American Journal of Hypertension*, 30(10), pp. 954-960.

Rice, J., Roberts, H., Rai, S. N. and Galandiuk, S. (2015) 'Housekeeping genes for studies of plasma microRNA: A need for more precise standardization', *Surgery*, 158(5), pp. 1345-51.

Ridker, P. M., Buring, J. E., Rifai, N. and Cook, N. R. (2007) 'Development and validation of improved algorithms for the assessment of global cardiovascular risk in women: the Reynolds Risk Score', *Jama*, 297(6), pp. 611-9.

Ridker, P. M., Paynter, N. P., Rifai, N., Gaziano, J. M. and Cook, N. R. (2008) 'C-reactive protein and parental history improve global cardiovascular risk prediction: the Reynolds Risk Score for men', *Circulation*, 118(22), pp. 2243-51, 4p following 2251.

Ristić, G. G., Lepić, T., Glisić, B., Stanisavljević, D., Vojvodić, D., Petronijević, M. and Stefanović, D. (2010) 'Rheumatoid arthritis is an independent risk factor for increased carotid intima-media thickness: impact of anti-inflammatory treatment', *Rheumatology (Oxford)*, 49(6), pp. 1076-81.

Ristić, G. G., Subota, V., Lepić, T., Stanisavljević, D., Glišić, B., Ristić, A. D., Petronijević, M. and Stefanović, D. Z. (2015) 'Subclinical Atherosclerosis in Patients with Rheumatoid Arthritis and Low Cardiovascular Risk: The Role of von Willebrand Factor Activity', *PLoS One*, 10(8), pp. e0130462.

Roman, M. J., Saba, P. S., Pini, R., Spitzer, M., Pickering, T. G., Rosen, S., Alderman, M. H. and Devereux, R. B. (1992) 'Parallel cardiac and vascular adaptation in hypertension', *Circulation*, 86(6), pp. 1909-18.

Roser, A. E., Caldi Gomes, L., Schünemann, J., Maass, F. and Lingor, P. (2018) 'Circulating miRNAs as Diagnostic Biomarkers for Parkinson's Disease', *Frontiers in Neuroscience*, 12(625).

Rosvall, M., Janzon, L., Berglund, G., Engstrom, G. and Hedblad, B. (2005) 'Incident coronary events and case fatality in relation to common carotid intima-media thickness', *J Intern Med*, 257(5), pp. 430-7.

Rosvall, M., Persson, M., Ostling, G., Nilsson, P. M., Melander, O., Hedblad, B. and Engstrom, G. (2015) 'Risk factors for the progression of carotid intima-media thickness over a 16-year follow-up period: the Malmo Diet and Cancer Study', *Atherosclerosis*, 239(2), pp. 615-21.

Roth, C., Stückrath, I., Pantel, K., Izbicki, J. R., Tachezy, M. and Schwarzenbach, H. (2012) 'Low levels of cell-free circulating miR-361-3p and miR-625* as blood-based markers for discriminating malignant from benign lung tumors', *PLoS One*, 7(6), pp. e38248.

Rothman KJ, G. S. (1998) *Modern Epidemiology*. Second Edition. edn. Philadelphia: Lippincott Williams and Wilkins.

Ruby, J. G., Jan, C. H. and Bartel, D. P. (2007) 'Intronic microRNA precursors that bypass Drosha processing', *Nature*, 448(7149), pp. 83-6.

Ruggeri, C., Gioffré, S., Chiesa, M., Buzzetti, M., Milano, G., Scopece, A., Castiglioni, L., Pontremoli, M., Sironi, L., Pompilio, G., Colombo, G. I. and

D'Alessandra, Y. (2018) 'A Specific Circulating MicroRNA Cluster Is Associated to Late Differential Cardiac Response to Doxorubicin-Induced Cardiotoxicity In Vivo', *Disease Markers*, 2018, pp. 8395651.

Rundek, T., Blanton, S. H., Bartels, S., Dong, C., Raval, A., Demmer, R. T., Cabral, D., Elkind, M. S., Sacco, R. L. and Desvarieux, M. (2013) 'Traditional risk factors are not major contributors to the variance in carotid intima-media thickness', *Stroke*, 44(8), pp. 2101-8.

Said, M. A., Eppinga, R. N., Lipsic, E., Verweij, N. and Harst, P. v. d. (2018a) 'Relationship of Arterial Stiffness Index and Pulse Pressure With Cardiovascular Disease and Mortality', *Journal of the American Heart Association*, 7(2), pp. e007621.

Said, M. A., Eppinga, R. N., Lipsic, E., Verweij, N. and van der Harst, P. (2018b) 'Relationship of Arterial Stiffness Index and Pulse Pressure With Cardiovascular Disease and Mortality', *Journal of the American Heart Association*, 7(2), pp. e007621.

Salonen, J. T. (1988) 'Is there a continuing need for longitudinal epidemiologic research? The Kuopio Ischaemic Heart Disease Risk Factor Study', *Ann Clin Res*, 20(1-2), pp. 46-50.

SALONEN, J. T. and SALONEN, R. (1990) 'Association of Serum Low Density Lipoprotein Cholesterol, Smoking and Hypertension with Different Manifestations of Atherosclerosis', *International Journal of Epidemiology*, 19(4), pp. 911-917.

Salonen, J. T. and Salonen, R. (1991a) 'Ultrasonographically assessed carotid morphology and the risk of coronary heart disease', *Arterioscler Thromb*, 11(5), pp. 1245-9.

Salonen, R. and Salonen, J. T. (1991b) 'Determinants of carotid intima-media thickness: a population-based ultrasonography study in eastern Finnish men', *J Intern Med*, 229(3), pp. 225-31.

Sanches Pde, L., Mello, M. T., Fonseca, F. A., Elias, N., Piano, A., Carnier, J., Tock, L., Oyama, L. M., Tufik, S. and Dâmaso, A. (2012) 'Insulin resistance can impair reduction on carotid intima-media thickness in obese adolescents', *Arq Bras Cardiol*, 99(4), pp. 892-8.

Sasaki, A., Uehara, M., Horiuchi, N., Hasegawa, K. and Shimizu, T. (1997) 'A 15-year follow-up study of patients with non-insulin-dependent diabetes mellitus (NIDDM) in Osaka, Japan. Factors predictive of the prognosis of diabetic patients', *Diabetes Res Clin Pract*, 36(1), pp. 41-7.

Sato, F., Tsuchiya, S., Terasawa, K. and Tsujimoto, G. (2009) 'Intra-platform repeatability and inter-platform comparability of microRNA microarray technology', *PLoS One*, 4(5), pp. e5540.

Schott, L. L., Wildman, R. P., Brockwell, S., Simkin-Silverman, L. R., Kuller, L. H. and Sutton-Tyrrell, K. (2004) 'Segment-specific effects of cardiovascular risk factors on carotid artery intima-medial thickness in women at midlife', *Arterioscler Thromb Vasc Biol*, 24(10), pp. 1951-6.

Schutte, R., Schutte, A. E., Huisman, H. W., van Rooyen, J. M., Malan, N. T., Péter, S., Fourie, C. M., van der Westhuizen, F. H., Louw, R., Botha, C. A. and Malan, L. (2009) 'Blood glutathione and subclinical atherosclerosis in African men: the SABPA Study', *Am J Hypertens*, 22(11), pp. 1154-9.

Schwarz, D. S., Hutvágner, G., Du, T., Xu, Z., Aronin, N. and Zamore, P. D. (2003) 'Asymmetry in the Assembly of the RNAi Enzyme Complex', *Cell*, 115(2), pp. 199-208.

Shadwick, R. E. (1999) 'Mechanical design in arteries', *Journal of Experimental Biology*, 202(23), pp. 3305-3313.

Shah, A. D., Langenberg, C., Rapsomaniki, E., Denaxas, S., Pujades-Rodriguez, M., Gale, C. P., Deanfield, J., Smeeth, L., Timmis, A. and Hemingway, H. (2015) 'Type 2 diabetes and incidence of cardiovascular diseases: a cohort study in 1·9 million people', *The Lancet Diabetes & Endocrinology*, 3(2), pp. 105-113.

Shah, P. K. (2010) 'Screening asymptomatic subjects for subclinical atherosclerosis: can we, does it matter, and should we?', *J Am Coll Cardiol*, 56(2), pp. 98-105.

Shah, T., Casas, J. P., Cooper, J. A., Tzoulaki, I., Sofat, R., McCormack, V., Smeeth, L., Deanfield, J. E., Lowe, G. D., Rumley, A., Fowkes, F. G., Humphries, S. E. and Hingorani, A. D. (2009) 'Critical appraisal of CRP measurement for the prediction of coronary heart disease events: new data and systematic review of 31 prospective cohorts', *Int J Epidemiol*, 38(1), pp. 217-31.

Sharma, S. and Eghbali, M. (2014) 'Influence of sex differences on microRNA gene regulation in disease', *Biol Sex Differ*, 5(1), pp. 3.

Shepherd, J., Blauw, G. J., Murphy, M. B., Bollen, E. L. E. M., Buckley, B. M., Cobbe, S. M., Ford, I., Gaw, A., Hyland, M., Jukema, J. W., Kamper, A. M., Macfarlane, P. W., Meinders, A. E., Norrie, J., Packard, C. J., Perry, I. J., Stott, D. J., Sweeney, B. J., Twomey, G. and Westendorp, R. G. J. (2002) 'Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial', *The Lancet*, 360(9346), pp. 1623-1630.

Shih, D. M., Xia, Y. R., Wang, X. P., Miller, E., Castellani, L. W., Subbanagounder, G., Cheroutre, H., Faull, K. F., Berliner, J. A., Witztum, J. L. and Lusis, A. J. (2000) 'Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis', *J Biol Chem*, 275(23), pp. 17527-35.

Shimizu, Y., Nakazato, M., Sato, S., Koyamatsu, J., Yamanashi, H., Nagayoshi, M., Kadota, K., Hayashida, N., Yamasaki, H., Kusano, Y., Takamura, N., Aoyagi, K. and Maeda, T. (2015) 'Association between hemoglobin A1c and carotid atherosclerosis in rural community-dwelling elderly Japanese men', *Journal of Physiological Anthropology*, 34(1), pp. 16.

Shimizu, Y., Sato, S., Koyamatsu, J., Yamanashi, H., Tamai, M., Kadota, K., Arima, K., Yamasaki, H., Takamura, N., Aoyagi, K. and Maeda, T. (2014) 'Subclinical carotid atherosclerosis and hyperuricemia in relation to renal impairment in a rural Japanese population: The Nagasaki Islands study', *Atherosclerosis*, 233(2), pp. 525-529.

Silacci, P., Desgeorges, A., Mazzolai, L., Chambaz, C. and Hayoz, D. (2001) 'Flow Pulsatility Is a Critical Determinant of Oxidative Stress in Endothelial Cells', *Hypertension*, 38(5), pp. 1162-1166.

Simpson, R. J., Kalra, H. and Mathivanan, S. (2012) 'ExoCarta as a resource for exosomal research', *Journal of extracellular vesicles*, 1, pp. 10.3402/jev.v1i0.18374.

Sinning, C., Wild, P. S., Echevarria, F. M., Wilde, S., Schnabel, R., Lubos, E., Herkenhoff, S., Bickel, C., Klimpe, S., Gori, T., Munzel, T. F., Blankenberg, S. and Espinola-Klein, C. (2011) 'Sex differences in early carotid atherosclerosis (from the community-based Gutenberg-Heart Study)', *Am J Cardiol*, 107(12), pp. 1841-7.

Soh, J., Iqbal, J., Queiroz, J., Fernandez-Hernando, C. and Hussain, M. M. (2013a) 'MicroRNA-30c reduces hyperlipidemia and atherosclerosis in mice by decreasing lipid synthesis and lipoprotein secretion', *Nat Med*, 19(7), pp. 892-900.

Soh, J., Iqbal, J., Queiroz, J., Fernandez-Hernando, C. and Hussain, M. M. (2013b) 'MicroRNA-30c reduces hyperlipidemia and atherosclerosis in mice by decreasing lipid synthesis and lipoprotein secretion', *Nature medicine*, 19(7), pp. 892-900.

Sohel, M. H. (2016) 'Extracellular/Circulating MicroRNAs: Release Mechanisms, Functions and Challenges', *Achievements in the Life Sciences*, 10(2), pp. 175-186.

Sollinger, D., Mohaupt, M. G., Wilhelm, A., Uehlinger, D., Frey, F. J. and Eisenberger, U. (2006) 'Arterial stiffness assessed by digital volume pulse correlates with comorbidity in patients with ESRD', *Am J Kidney Dis*, 48(3), pp. 456-63.

Spence, J. D. (2014) 'Time course of atherosclerosis regression', *Atherosclerosis*, 235(2), pp. 347-348.

Spence, J. D., Barnett, P. A., Bulman, D. E. and Hegele, R. A. (1999) 'An approach to ascertain probands with a non-traditional risk factor for carotid atherosclerosis', *Atherosclerosis*, 144(2), pp. 429-34.

Spring, B., Moller, A. C., Colangelo, L. A., Siddique, J., Roehrig, M., Daviglus, M. L., Polak, J. F., Reis, J. P., Sidney, S. and Liu, K. (2014) 'Healthy lifestyle change and subclinical atherosclerosis in young adults: Coronary Artery Risk Development in Young Adults (CARDIA) study', *Circulation*, 130(1), pp. 10-7.

Sredni, S., Gadd, S., Jafari, N. and Huang, C.-C. (2011) 'A Parallel Study of mRNA and microRNA Profiling of Peripheral Blood in Young Adult Women', *Frontiers in Genetics*, 2(49).

Stanczyk, J., Pedrioli, D. M., Brentano, F., Sanchez-Pernaute, O., Kolling, C., Gay, R. E., Detmar, M., Gay, S. and Kyburz, D. (2008) 'Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis', *Arthritis Rheum*, 58(4), pp. 1001-9.

Steffen, B. T., Guan, W., Remaley, A. T., Stein, J. H., Tattersall, M. C., Kaufman, J. and Tsai, M. Y. (2017) 'Apolipoprotein B is associated with carotid atherosclerosis progression independent of individual cholesterol measures in a 9-year prospective study of Multi-Ethnic Study of Atherosclerosis participants', *Journal of clinical lipidology*, 11(5), pp. 1181-1191.e1.

Stegemann, C., Pechlaner, R., Willeit, P., Langley, S. R., Mangino, M., Mayr, U., Menni, C., Moayyeri, A., Santer, P., Rungger, G., Spector, T. D., Willeit, J., Kiechl, S. and Mayr, M. (2014) 'Lipidomics profiling and risk of cardiovascular disease in the prospective population-based Bruneck study', *Circulation*, 129(18), pp. 1821-31.

Stewart, A. D., Jiang, B., Millasseau, S. C., Ritter, J. M. and Chowienczyk, P. J. (2006) 'Acute Reduction of Blood Pressure by Nitroglycerin Does Not Normalize Large Artery Stiffness in Essential Hypertension', *Hypertension*, 48(3), pp. 404-410.

Su, T.-C., Chien, K.-L., Jeng, J.-S., Chen, M.-F., Hsu, H.-C., Torng, P.-L., Sung, F.-C. and Lee, Y.-T. (2012) 'Age-and Gender-Associated Determinants of Carotid Intima-Media Thickness: A Community-Based Study', *Journal of Atherosclerosis and Thrombosis*, 19(9), pp. 872-880.

Sudlow, C., Gallacher, J., Allen, N., Beral, V., Burton, P., Danesh, J., Downey, P., Elliott, P., Green, J., Landray, M., Liu, B., Matthews, P., Ong, G., Pell, J., Silman, A., Young, A., Sprosen, T., Peakman, T. and Collins, R. (2015) 'UK biobank: an open access resource for identifying the causes of a wide range of complex diseases of middle and old age', *PLoS Med*, 12(3), pp. e1001779.

Sutton-Tyrrell, K., Kuller, L. H., Matthews, K. A., Holubkov, R., Patel, A., Edmundowicz, D. and Newman, A. (2002) 'Subclinical atherosclerosis in multiple vascular beds: an index of atherosclerotic burden evaluated in postmenopausal women', *Atherosclerosis*, 160(2), pp. 407-416.

Suurkula M, A. S., Fagerberg B, Wendelhang I, Widgren B, Wickstrand J (1994) 'Ultrasound evaluation of atherosclerotic manifestation in carotid artery in high- risk hypertensive patients. Risk Intervention Study ðRISÞ', *Arterioscler Thromb*, 14, pp. 1297-1304.

Taddei, S., Virdis, A., Ghiadoni, L., Salvetti, G., Bernini, G., Magagna, A. and Salvetti, A. (2001) 'Age-related reduction of NO availability and oxidative stress in humans', *Hypertension*, 38(2), pp. 274-9.

Taki, A., Kermani, A., Ranjbarnavazi, S. M. and Pourmodheji, A. (2017) 'Chapter 4 - Overview of Different Medical Imaging Techniques for the Identification of Coronary Atherosclerotic Plaques', in Balocco, S., Zuluaga, M.A., Zahnd, G., Lee, S.-L. and Demirci, S. (eds.) *Computing and Visualization for Intravascular Imaging and Computer-Assisted Stenting*: Academic Press, pp. 79-106.

Tanaka, H. (2017) 'Various Indices of Arterial Stiffness: Are They Closely Related or Distinctly Different?', *Pulse*, 5(1-4), pp. 1-6.

Taylor, D. A. and Abdel-Rahman, A. A. (2009) 'Novel Strategies and Targets for the Management of Hypertension', in Enna, S.J. and Williams, M. (eds.) *Advances in Pharmacology*: Academic Press, pp. 291-345.

Thompson, C. (2018) 'Rose's Prevention Paradox', *Journal of Applied Philosophy*, 35(2), pp. 242-256.

Toth, P. P. (2008) 'Subclinical atherosclerosis: what it is, what it means and what we can do about it', *International journal of clinical practice*, 62(8), pp. 1246-1254.

Touboul, P. J., Hennerici, M. G., Meairs, S., Adams, H., Amarenco, P., Desvarieux, M., Ebrahim, S., Fatar, M., Hernandez Hernandez, R., Kownator, S., Prati, P., Rundek, T., Taylor, A., Bornstein, N., Csiba, L., Vicaut, E., Woo, K. S. and Zannad, F. (2004) 'Mannheim intima-media thickness consensus', *Cerebrovasc Dis*, 18(4), pp. 346-9.

Trajkovski, M., Ahmed, K., Esau, C. C. and Stoffel, M. (2012) 'MyomiR-133 regulates brown fat differentiation through Prdm16', *Nat Cell Biol*, 14(12), pp. 1330-5.

Troitzsch, P., Paulista Markus, M. R., Dörr, M., Felix, S. B., Jünger, M., Schminke, U., Schmidt, C. O., Völzke, H., Baumeister, S. E. and Arnold, A. (2012) 'Psoriasis is associated with increased intima-media thickness--the Study of Health in Pomerania (SHIP)', *Atherosclerosis*, 225(2), pp. 486-90.

Tropeano, A. I., Boutouyrie, P., Katsahian, S., Laloux, B. and Laurent, S. (2004) 'Glucose level is a major determinant of carotid intima-media thickness in patients with hypertension and hyperglycemia', *J Hypertens*, 22(11), pp. 2153-60.

Turchinovich, A., Weiz, L., Langheinz, A. and Burwinkel, B. (2011) 'Characterization of extracellular circulating microRNA', *Nucleic acids research*, 39(16), pp. 7223-7233.

UK Biobank (2019) *Traditional and novel biomarkers of adverse cardiometabolic imaging, and associations with outcomes*. Available at: https://www.ukbiobank.ac.uk/2019/04/traditional-and-novel-biomarkers-of-adverse-cardiometabolic-imaging-and-associations-with-outcomes/.

Urbina, E. M., Khoury, P. R., McCoy, C. E., Dolan, L. M., Daniels, S. R. and Kimball, T. R. (2013) 'Triglyceride to HDL-C ratio and increased arterial stiffness in children, adolescents, and young adults', *Pediatrics*, 131(4), pp. e1082-90.

Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J. and Lötvall, J. O. (2007) 'Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells', *Nat Cell Biol*, 9(6), pp. 654-9.

Van Bortel, L. M., Laurent, S., Boutouyrie, P., Chowienczyk, P., Cruickshank, J. K., De Backer, T., Filipovsky, J., Huybrechts, S., Mattace-Raso, F. U. S., Protogerou, A. D., Schillaci, G., Segers, P., Vermeersch, S., Weber, T., on behalf of the Artery Society, t. E. S. o. H. W. G. o. V. S., Function and No, t. E. N. f. (2012) 'Expert consensus document on the measurement of aortic stiffness in daily practice using carotid-femoral pulse wave velocity', *Journal of Hypertension*, 30(3), pp. 445-448.

van Rooij, E., Sutherland, L. B., Liu, N., Williams, A. H., McAnally, J., Gerard, R. D., Richardson, J. A. and Olson, E. N. (2006) 'A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure', *Proc Natl Acad Sci U S A*, 103(48), pp. 18255-60.

Vasan, R. S. (2006) 'Biomarkers of Cardiovascular Disease', *Circulation*, 113(19), pp. 2335-2362.

Vaughn, M. W., Kuo, L. and Liao, J. C. (1998) 'Effective diffusion distance of nitric oxide in the microcirculation', *Am J Physiol*, 274(5), pp. H1705-14.

Vella, S., Buetow, L., Royle, P., Livingstone, S., Colhoun, H. M. and Petrie, J. R. (2010) 'The use of metformin in type 1 diabetes: a systematic review of efficacy', *Diabetologia*, 53(5), pp. 809-20.

Vickers, K. C., Palmisano, B. T., Shoucri, B. M., Shamburek, R. D. and Remaley, A. T. (2011) 'MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins', *Nature cell biology*, 13(4), pp. 423-433.

Villablanca, A. C., Jayachandran, M. and Banka, C. (2010) 'Atherosclerosis and sex hormones: current concepts', *Clin Sci (Lond)*, 119(12), pp. 493-513.

Vlachopoulos, C., Aznaouridis, K. and Stefanadis, C. (2010) 'Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis', *J Am Coll Cardiol*, 55(13), pp. 1318-27.

Vokó, Z., Bots, M. L., Hofman, A., Koudstaal, P. J., Witteman, J. C. M. and Breteler, M. M. B. (1999) 'J-Shaped Relation Between Blood Pressure and Stroke in Treated Hypertensives', *Hypertension*, 34(6), pp. 1181-1185.

Wagenseil, J. E. and Mecham, R. P. (2012) 'Elastin in large artery stiffness and hypertension', *Journal of cardiovascular translational research*, 5(3), pp. 264-273.

Wang, H., Peng, R., Wang, J., Qin, Z. and Xue, L. (2018) 'Circulating microRNAs as potential cancer biomarkers: the advantage and disadvantage', *Clinical Epigenetics*, 10(1), pp. 59.

Wang, J. X., Zhang, X. J., Li, Q., Wang, K., Wang, Y., Jiao, J. Q., Feng, C., Teng, S., Zhou, L. Y., Gong, Y., Zhou, Z. X., Liu, J., Wang, J. L. and Li, P. F. (2015a) 'MicroRNA-103/107 Regulate Programmed Necrosis and Myocardial Ischemia/Reperfusion Injury Through Targeting FADD', *Circ Res*, 117(4), pp. 352-63.

Wang, K., Yuan, Y., Cho, J.-H., McClarty, S., Baxter, D. and Galas, D. J. (2012a) 'Comparing the MicroRNA spectrum between serum and plasma', *PloS one*, 7(7), pp. e41561-e41561.

Wang, K., Yuan, Y., Cho, J. H., McClarty, S., Baxter, D. and Galas, D. J. (2012b) 'Comparing the MicroRNA spectrum between serum and plasma', *PLoS One*, 7(7), pp. e41561.

Wang, K., Zhang, S., Marzolf, B., Troisch, P., Brightman, A., Hu, Z., Hood, L. E. and Galas, D. J. (2009) 'Circulating microRNAs, potential biomarkers for drug-induced liver injury', *Proceedings of the National Academy of Sciences of the United States of America*, 106(11), pp. 4402-4407.

Wang, R., Hong, J., Cao, Y., Shi, J., Gu, W., Ning, G., Zhang, Y. and Wang, W. (2015b) 'Elevated circulating microRNA-122 is associated with obesity and insulin resistance in young adults', *Eur J Endocrinol*, 172(3), pp. 291-300.

Wang, T. J. (2011) 'Assessing the role of circulating, genetic, and imaging biomarkers in cardiovascular risk prediction', *Circulation*, 123(5), pp. 551-65.

Wannarong, T., Parraga, G., Buchanan, D., Fenster, A., House, A. A., Hackam, D. G. and Spence, J. D. (2013) 'Progression of Carotid Plaque Volume Predicts Cardiovascular Events', *Stroke*, 44(7), pp. 1859-1865.

Webb, A. J. S. (2020) 'Progression of Arterial Stiffness is Associated With Midlife Diastolic Blood Pressure and Transition to Late-Life Hypertensive Phenotypes', *Journal of the American Heart Association*, 9(1), pp. e014547.

Weber, J. A., Baxter, D. H., Zhang, S., Huang, D. Y., Huang, K. H., Lee, M. J., Galas, D. J. and Wang, K. (2010) 'The microRNA spectrum in 12 body fluids', *Clin Chem*, 56(11), pp. 1733-41.

Weber, M., Baker, M. B., Patel, R. S., Quyyumi, A. A., Bao, G. and Searles, C. D. (2011) 'MicroRNA Expression Profile in CAD Patients and the Impact of ACEI/ARB', *Cardiol Res Pract*, 2011, pp. 532915.

Weingartner, O., Pinsdorf, T., Rogacev, K. S., Blomer, L., Grenner, Y., Graber, S., Ulrich, C., Girndt, M., Bohm, M., Fliser, D., Laufs, U., Lutjohann, D. and Heine, G. H. (2010) 'The relationships of markers of cholesterol homeostasis with carotid intima-media thickness', *PLoS One*, 5(10), pp. e13467.

Welsh, C., Welsh, P., Celis-Morales, C. A., Mark, P. B., Mackay, D., Ghouri, N., Ho, F. K., Ferguson, L. D., Brown, R., Lewsey, J., Cleland, J. G., Gray, S. R., Lyall, D. M., Anderson, J. J., Jhund, P. S., Pell, J. P., McGuire, D. K., Gill, J. M. R. and Sattar, N. (2020) 'Glycated Hemoglobin, Prediabetes, and the Links to Cardiovascular Disease: Data From UK Biobank', *Diabetes Care*, 43(2), pp. 440-445.

Welsh, P., Preiss, D., Hayward, C., Shah, A. S. V., McAllister, D., Briggs, A., Boachie, C., McConnachie, A., Padmanabhan, S., Welsh, C., Woodward, M., Campbell, A., Porteous, D., Mills, N. L. and Sattar, N. (2019) 'Cardiac Troponin T and Troponin I in the General Population', *Circulation*, 139(24), pp. 2754-2764.

Welsh, P., Preiss, D., Shah, A. S. V., McAllister, D., Briggs, A., Boachie, C., McConnachie, A., Hayward, C., Padmanabhan, S., Welsh, C., Woodward, M., Campbell, A., Porteous, D., Mills, N. L. and Sattar, N. (2018) 'Comparison between High-Sensitivity Cardiac Troponin T and Cardiac Troponin I in a Large General Population Cohort', *Clinical chemistry*, 64(11), pp. 1607-1616.

Wen, J., Zhong, Y., Kuang, C., Liao, J., Chen, Z. and Yang, Q. (2017) 'Lipoprotein ratios are better than conventional lipid parameters in predicting arterial stiffness in young men', *The Journal of Clinical Hypertension*, 19(8), pp. 771-776.

Widera, C., Gupta, S. K., Lorenzen, J. M., Bang, C., Bauersachs, J., Bethmann, K., Kempf, T., Wollert, K. C. and Thum, T. (2011) 'Diagnostic and prognostic impact of six circulating microRNAs in acute coronary syndrome', *Journal of Molecular and Cellular Cardiology*, 51(5), pp. 872-875.

Widmer, R. J. and Lerman, A. (2014) 'Endothelial dysfunction and cardiovascular disease', *Global cardiology science & practice*, 2014(3), pp. 291-308.

Wilkinson, I. B., Franklin, S. S., Hall, I. R., Tyrrell, S. and Cockcroft, J. R. (2001) 'Pressure amplification explains why pulse pressure is unrelated to risk in young subjects', *Hypertension*, 38(6), pp. 1461-6.

Wilkinson, I. B., MacCallum, H., Flint, L., Cockcroft, J. R., Newby, D. E. and Webb, D. J. (2000) 'The influence of heart rate on augmentation index and central arterial pressure in humans', *The Journal of physiology*, 525 Pt 1(Pt 1), pp. 263-270.

Willeit, P., Kaptoge, S., Welsh, P., Butterworth, A. S., Chowdhury, R., Spackman, S. A., Pennells, L., Gao, P., Burgess, S., Freitag, D. F., Sweeting, M., Wood, A. M., Cook, N. R., Judd, S., Trompet, S., Nambi, V., Olsen, M. H., Everett, B. M., Kee, F., Ärnlöv, J., Salomaa, V., Levy, D., Kauhanen, J., Laukkanen, J. A., Kavousi, M., Ninomiya, T., Casas, J.-P., Daniels, L. B., Lind, L., Kistorp, C. N., Rosenberg, J., Mueller, T., Rubattu, S., Panagiotakos, D. B., Franco, O. H., de Lemos, J. A., Luchner, A., Kizer, J. R., Kiechl, S., Salonen, J. T., Wannamethee, S. G., de Boer, R. A., Nordestgaard, B. G., Andersson, J., Jørgensen, T., Melander, O., Ballantyne, C. M., DeFilippi, C., Ridker, P. M., Cushman, M., Rosamond, W. D., Thompson, S. G., Gudnason, V., Sattar, N., Danesh, J. and Di Angelantonio, E. (2016) 'Natriuretic peptides and integrated risk assessment for cardiovascular disease: an individual-participant-data meta-analysis', *The Lancet Diabetes & Endocrinology*, 4(10), pp. 840-849.

Willeit, P., Skroblin, P., Moschen, A. R., Yin, X., Kaudewitz, D., Zampetaki, A., Barwari, T., Whitehead, M., Ramirez, C. M., Goedeke, L., Rotllan, N., Bonora, E., Hughes, A. D., Santer, P., Fernandez-Hernando, C., Tilg, H., Willeit, J., Kiechl, S. and Mayr, M. (2017a) 'Circulating MicroRNA-122 Is Associated With the Risk of New-Onset Metabolic Syndrome and Type 2 Diabetes', *Diabetes*, 66(2), pp. 347-357.

Willeit, P., Welsh, P., Evans, J. D. W., Tschiderer, L., Boachie, C., Jukema, J. W., Ford, I., Trompet, S., Stott, D. J., Kearney, P. M., Mooijaart, S. P., Kiechl, S., Di Angelantonio, E. and Sattar, N. (2017b) 'High-Sensitivity Cardiac Troponin Concentration and Risk of First-Ever Cardiovascular Outcomes in 154,052 Participants', *J Am Coll Cardiol*, 70(5), pp. 558-568.

Willeit, P., Zampetaki, A., Dudek, K., Kaudewitz, D., King, A., Kirkby, N. S., Crosby-Nwaobi, R., Prokopi, M., Drozdov, I., Langley, S. R., Sivaprasad, S., Markus, H. S., Mitchell, J. A., Warner, T. D., Kiechl, S. and Mayr, M. (2013) 'Circulating microRNAs as novel biomarkers for platelet activation', *Circ Res*, 112(4), pp. 595-600.

Williams, B., Mancia, G., Spiering, W., Agabiti Rosei, E., Azizi, M., Burnier, M., Clement, D. L., Coca, A., de Simone, G., Dominiczak, A., Kahan, T., Mahfoud, F., Redon, J., Ruilope, L., Zanchetti, A., Kerins, M., Kjeldsen, S. E., Kreutz, R., Laurent, S., Lip, G. Y. H., McManus, R., Narkiewicz, K., Ruschitzka, F., Schmieder, R. E., Shlyakhto, E., Tsioufis, C., Aboyans, V., Desormais, I. and Group, E. S. D. (2018) '2018 ESC/ESH Guidelines for the management of arterial hypertension: The Task Force for the management of arterial hypertension of the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH)', *European Heart Journal*, 39(33), pp. 3021-3104.

Wilson, P. W., Pencina, M., Jacques, P., Selhub, J., D'Agostino, R., Sr. and O'Donnell, C. J. (2008) 'C-reactive protein and reclassification of

cardiovascular risk in the Framingham Heart Study', *Circ Cardiovasc Qual Outcomes*, 1(2), pp. 92-7.

Witters, L. A. (2001) 'The blooming of the French lilac', *The Journal of clinical investigation*, 108(8), pp. 1105-1107.

Witwer, K. W. (2015a) 'Circulating MicroRNA Biomarker Studies: Pitfalls and Potential Solutions', *Clinical Chemistry*, 61(1), pp. 56-63.

Witwer, K. W. (2015b) 'Circulating microRNA biomarker studies: pitfalls and potential solutions', *Clin Chem*, 61(1), pp. 56-63.

Wormser, D., Kaptoge, S., Di Angelantonio, E., Wood, A. M., Pennells, L., Thompson, A., Sarwar, N., Kizer, J. R., Lawlor, D. A., Nordestgaard, B. G., Ridker, P., Salomaa, V., Stevens, J., Woodward, M., Sattar, N., Collins, R., Thompson, S. G., Whitlock, G. and Danesh, J. (2011) 'Separate and combined associations of body-mass index and abdominal adiposity with cardiovascular disease: collaborative analysis of 58 prospective studies', *Lancet*, 377(9771), pp. 1085-95.

Wu, T. W., Hung, C. L., Liu, C. C., Wu, Y. J., Wang, L. Y. and Yeh, H. I. (2017) 'Associations of Cardiovascular Risk Factors with Carotid Intima-Media Thickness in Middle-Age Adults and Elders', *J Atheroscler Thromb*, 24(7), pp. 677-686.

Xie, H., Lim, B. and Lodish, H. F. (2009) 'MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity', *Diabetes*, 58(5), pp. 1050-1057.

Xie, H., Sun, L. and Lodish, H. F. (2009) 'Targeting microRNAs in obesity', *Expert opinion on therapeutic targets*, 13(10), pp. 1227-1238.

Xie, Y., Chu, A., Feng, Y., Chen, L., Shao, Y., Luo, Q., Deng, X., Wu, M., Shi, X. and Chen, Y. (2018) 'MicroRNA-146a: A Comprehensive Indicator of Inflammation and Oxidative Stress Status Induced in the Brain of Chronic T2DM Rats', *Frontiers in pharmacology*, 9, pp. 478-478.

Xu, C., Zarins, C. K., Pannaraj, P. S., Bassiouny, H. S. and Glagov, S. (2000) 'Hypercholesterolemia Superimposed by Experimental Hypertension Induces Differential Distribution of Collagen and Elastin', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 20(12), pp. 2566-2572.

Xu, Q., Li, Y., Shang, Y.-F., Wang, H.-L. and Yao, M.-X. (2015) 'miRNA-103: molecular link between insulin resistance and nonalcoholic fatty liver disease', *World journal of gastroenterology*, 21(2), pp. 511-516.

Yang, F.-r., Li, H.-j., Li, T.-t., Zhao, Y.-f., Liu, Z.-k. and Li, X.-r. (2019) 'Prognostic Value of MicroRNA-15a in Human Cancers: A Meta-Analysis and Bioinformatics', *BioMed Research International*, 2019, pp. 2063823.

Yoda, M., Kawamata, T., Paroo, Z., Ye, X., Iwasaki, S., Liu, Q. and Tomari, Y. (2010) 'ATP-dependent human RISC assembly pathways', *Nat Struct Mol Biol*, 17(1), pp. 17-23.

Yusuf, S., Hawken, S., Ounpuu, S., Dans, T., Avezum, A., Lanas, F., McQueen, M., Budaj, A., Pais, P., Varigos, J. and Lisheng, L. (2004) 'Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study', *Lancet*, 364(9438), pp. 937-52.

Zanchetti A1, C. G., Bond MG, Gallus GV, Veglia F, Ventura A, Mancia G, Baggio G, Sampieri L, Rubba P, Collatina S, Serrotti E. (2001) 'Systolic and pulse blood pressures (but not diastolic blood pressure and serum cholesterol) are associated with alterations in carotid intima-media thickness in the moderately hypercholesterolaemic hypertensive patients of the Plaque Hypertension Lipid Lowering Italian Study. PHYLLIS study group', *J Hypertens*, 19, pp. 79-88.

Zanoli, L. M. and Spoto, G. (2013) 'Isothermal amplification methods for the detection of nucleic acids in microfluidic devices', *Biosensors (Basel)*, 3(1), pp. 18-43.

Zeller, T., Tunstall-Pedoe, H., Saarela, O., Ojeda, F., Schnabel, R. B., Tuovinen, T., Woodward, M., Struthers, A., Hughes, M., Kee, F., Salomaa, V., Kuulasmaa, K., Blankenberg, S. and Investigators, M. (2014) 'High population prevalence of cardiac troponin I measured by a high-sensitivity assay and cardiovascular risk estimation: the MORGAM Biomarker Project Scottish Cohort', *Eur Heart J*, 35(5), pp. 271-81.

Zeng, Y., Wagner, E. J. and Cullen, B. R. (2002) 'Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells', *Mol Cell*, 9(6), pp. 1327-33.

Zernecke, A., Bidzhekov, K., Noels, H., Shagdarsuren, E., Gan, L., Denecke, B., Hristov, M., Köppel, T., Jahantigh, M. N., Lutgens, E., Wang, S., Olson, E. N., Schober, A. and Weber, C. (2009) 'Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection', *Sci Signal*, 2(100), pp. ra81.

Zhang, J.-Y., Gong, Y.-L., Li, C.-J., Qi, Q., Zhang, Q.-M. and Yu, D.-M. (2016) 'Circulating MiRNA biomarkers serve as a fingerprint for diabetic atherosclerosis', *American journal of translational research*, 8(6), pp. 2650-2658.

Zhang, Y., Li, H. H., Yang, R., Yang, B. J. and Gao, Z. Y. (2017) 'Association between circulating microRNA-208a and severity of coronary heart disease', *Scand J Clin Lab Invest*, 77(5), pp. 379-384.

Zhang, Y. F., Hong, J., Zhan, W. W., Li, X. Y., Gu, W. Q., Yang, Y. S., Xu, M. and Ning, G. (2006) 'Hyperglycaemia after glucose loading is a major predictor of preclinical atherosclerosis in nondiabetic subjects', *Clin Endocrinol (Oxf)*, 64(2), pp. 153-7.

Zhao, W., Zhao, S. P. and Zhao, Y. H. (2015) 'MicroRNA-143/-145 in Cardiovascular Diseases', *Biomed Res Int*, 2015, pp. 531740.

Zhao, Y., Yin, Z., Li, H., Fan, J., Yang, S., Chen, C. and Wang, D. W. (2017) 'MiR-30c protects diabetic nephropathy by suppressing epithelial-to-mesenchymal transition in db/db mice', *Aging Cell*, 16(2), pp. 387-400.

Zhong, J., He, Y., Chen, W., Shui, X., Chen, C. and Lei, W. (2014) 'Circulating microRNA-19a as a potential novel biomarker for diagnosis of acute myocardial infarction', *International journal of molecular sciences*, 15(11), pp. 20355-20364.

Zhu, H. and Leung, S. W. (2015) 'Identification of microRNA biomarkers in type 2 diabetes: a meta-analysis of controlled profiling studies', *Diabetologia*, 58(5), pp. 900-11.

Zieman, S. J., Melenovsky, V. and Kass, D. A. (2005) 'Mechanisms, Pathophysiology, and Therapy of Arterial Stiffness', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 25(5), pp. 932-943.

Ayer, J. G., Harmer, J. A., Nakhla, S., Xuan, W., Ng, M. K. C., Raitakari, O. T., Marks, G. B. and Celermajer, D. S. (2009) 'HDL-Cholesterol, Blood Pressure, and Asymmetric Dimethylarginine Are Significantly Associated With Arterial Wall Thickness in Children', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 29(6), pp. 943-949.

Azevedo, A., Bettencourt, P. and Barros, H. (2007) 'Demographic, clinical and echocardiographic determinants of B-type natriuretic peptide plasma concentration. A population-based study', *Rev Port Cardiol*, 26(2), pp. 105-13.

Babiarz, J. E., Ruby, J. G., Wang, Y., Bartel, D. P. and Blelloch, R. (2008) 'Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs', *Genes & development*, 22(20), pp. 2773-2785.

Baldassarre, D., De Jong, A., Amato, M., Werba, J. P., Castelnuovo, S., Frigerio, B., Veglia, F., Tremoli, E. and Sirtori, C. R. (2008) 'Carotid intima-media thickness and markers of inflammation, endothelial damage and hemostasis', *Ann Med*, 40(1), pp. 21-44.

Baldassarre, D., Nyyssonen, K., Rauramaa, R., de Faire, U., Hamsten, A., Smit, A. J., Mannarino, E., Humphries, S. E., Giral, P., Grossi, E., Veglia, F., Paoletti, R. and Tremoli, E. (2010) 'Cross-sectional analysis of baseline data to identify the major determinants of carotid intima-media thickness in a European population: the IMPROVE study', *Eur Heart J*, 31(5), pp. 614-22.

Bank, A. J., Wang, H., Holte, J. E., Mullen, K., Shammas, R. and Kubo, S. H. (1996) 'Contribution of collagen, elastin, and smooth muscle to in vivo human brachial artery wall stress and elastic modulus', *Circulation*, 94(12), pp. 3263-70.

Bartel, D. P. (2009) 'MicroRNAs: Target Recognition and Regulatory Functions', *Cell*, 136(2), pp. 215-233.

Bauer, M., Caviezel, S., Teynor, A., Erbel, R., Mahabadi, A. A. and Schmidt-Trucksass, A. (2012) 'Carotid intima-media thickness as a biomarker of subclinical atherosclerosis', *Swiss Med Wkly*, 142, pp. w13705.

Becker, N. and Lockwood, C. M. (2013) 'Pre-analytical variables in miRNA analysis', *Clin Biochem*, 46(10-11), pp. 861-8.

Belcaro, G., Nicolaides, A. N., Laurora, G., Cesarone, M. R., Sanctis, M. D., Incandela, L. and Barsotti, A. (1996) 'Ultrasound Morphology Classification of the Arterial Wall and Cardiovascular Events in a 6-Year Follow-up Study', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 16(7), pp. 851-856.

Bellentani, S., Saccoccio, G., Masutti, F., Crocè, L. S., Brandi, G., Sasso, F., Cristanini, G. and Tiribelli, C. (2000) 'Prevalence of and risk factors for hepatic steatosis in Northern Italy', *Ann Intern Med*, 132(2), pp. 112-7.

Beltran, A., McVeigh, G., Morgan, D., Glasser, S. P., Neutel, J. M., Weber, M., Finkelstein, S. M. and Cohn, J. N. (2001) 'Arterial compliance abnormalities in isolated systolic hypertension', *Am J Hypertens*, 14(10), pp. 1007-11.

Bhf.org.uk (2020a) *British Heart Foundation -Cardiovascular disease*. Available at: https://www.bhf.org.uk/informationsupport/conditions/cardiovascular-heart-disease (Accessed: 2020.

Bhf.org.uk (2020b) *British Heart Foundation -Heart statistics*. Available at: https://www.bhf.org.uk/what-we-do/our-research/heart-statistics 2020).

Bhf.org.uk (2020c) *Heart statistics, British Heart Foundation*. Available at: https://www.bhf.org.uk/what-we-do/our-research/heart-statistics.

Bianga Katchunga, P., Mongane Irenge, J., Canwa, R., Matabaro, A., Nsimire, S. and Kabunga, Q. (2016) '[Carotid intima-media thickness in the Congolese hypertensive in South Kivu]', *Ann Cardiol Angeiol (Paris)*, 65(2), pp. 64-70.

Bihrer, V., Waidmann, O., Friedrich-Rust, M., Forestier, N., Susser, S., Haupenthal, J., Welker, M., Shi, Y., Peveling-Oberhag, J., Polta, A., von

Wagner, M., Radeke, H. H., Sarrazin, C., Trojan, J., Zeuzem, S., Kronenberger, B. and Piiper, A. (2011) 'Serum microRNA-21 as marker for necroinflammation in hepatitis C patients with and without hepatocellular carcinoma', *PLoS One*, 6(10), pp. e26971.

Biobank., U. (2015) *Imaging modality: carodtif Ultrasound*. Available at: (http://biobank.ndph.ox.ac.uk/showcase/showcase/docs/carult_explan_doc.pdf) (Accessed: 1/1/2020 2020).

'Biomarkers Definitions Working Group, Biomarkers and surrogate endpoints: preferred definitions and conceptual framework', (2001) *Clin Pharmacol Ther*, 69(3), pp. 89-95.

Blacher, J., Staessen, J. A., Girerd, X., Gasowski, J., Thijs, L., Liu, L., Wang, J. G., Fagard, R. H. and Safar, M. E. (2000) 'Pulse pressure not mean pressure determines cardiovascular risk in older hypertensive patients', *Arch Intern Med*, 160(8), pp. 1085-9.

Blankenhorn, D. H., Johnson, R. L., Nessim, S. A., Azen, S. P., Sanmarco, M. E. and Selzer, R. H. (1987) 'The Cholesterol Lowering Atherosclerosis Study (CLAS): design, methods, and baseline results', *Control Clin Trials*, 8(4), pp. 356-87.

Boeckel, J.-N., Thomé, C. E., Leistner, D., Zeiher, A. M., Fichtlscherer, S. and Dimmeler, S. (2013) 'Heparin Selectively Affects the Quantification of MicroRNAs in Human Blood Samples', *Clinical Chemistry*, 59(7), pp. 1125-1127.

Bosevski, M., Georgievska-Ismail, L. and Tosev, S. (2011) 'Carotid IMT in type 2 diabetic patients: a survey on factors of influence', *Prilozi*, 32(2), pp. 289-97.

Bots, M. L., Hoes, A. W., Koudstaal, P. J., Hofman, A. and Grobbee, D. E. (1997) 'Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam Study', *Circulation*, 96(5), pp. 1432-7.

British Heart Foundation, *Cardiovascular disease*. Available at: https://www.bhf.org.uk/informationsupport/conditions/cardiovascular-heart-disease (Accessed: 19th July 2020).

Burgess, J. L., Kurzius-Spencer, M., Gerkin, R. D., Fleming, J. L., Peate, W. F. and Allison, M. (2012) 'Risk Factors for Subclinical Atherosclerosis in Firefighters', *Journal of Occupational and Environmental Medicine*, 54(3), pp. 328-335.

Burns, D. M. (2003) 'Epidemiology of smoking-induced cardiovascular disease', *Prog Cardiovasc Dis*, 46(1), pp. 11-29.

Bycroft, C., Freeman, C., Petkova, D., Band, G., Elliott, L. T., Sharp, K., Motyer, A., Vukcevic, D., Delaneau, O., O'Connell, J., Cortes, A., Welsh, S., Young, A., Effingham, M., McVean, G., Leslie, S., Allen, N., Donnelly, P. and Marchini, J. (2018) 'The UK Biobank resource with deep phenotyping and genomic data', *Nature*, 562(7726), pp. 203-209.

Cal, R., Castellano, J., Revuelta-López, E., Aledo, R., Barriga, M., Farré, J., Vilahur, G., Nasarre, L., Hove-Madsen, L., Badimon, L. and Llorente-Cortés, V. (2012) 'Low-density lipoprotein receptor-related protein 1 mediates hypoxia-induced very low density lipoprotein-cholesteryl ester uptake and accumulation in cardiomyocytes', *Cardiovasc Res*, 94(3), pp. 469-79.

Cao, J., Meng, S., Chang, E., Beckwith-Fickas, K., Xiong, L., Cole, R. N., Radovick, S., Wondisford, F. E. and He, L. (2014) 'Low concentrations of metformin suppress glucose production in hepatocytes through AMP-activated protein kinase (AMPK)', *J Biol Chem*, 289(30), pp. 20435-46.

Cardarelli, R. and Lumicao, T. G., Jr. (2003) 'B-type natriuretic peptide: a review of its diagnostic, prognostic, and therapeutic monitoring value in heart failure for primary care physicians', *J Am Board Fam Pract*, 16(4), pp. 327-33.

Care, A., Catalucci, D., Felicetti, F., Bonci, D., Addario, A., Gallo, P., Bang, M. L., Segnalini, P., Gu, Y., Dalton, N. D., Elia, L., Latronico, M. V., Hoydal, M., Autore, C., Russo, M. A., Dorn, G. W., 2nd, Ellingsen, O., Ruiz-Lozano, P., Peterson, K. L., Croce, C. M., Peschle, C. and Condorelli, G. (2007) 'MicroRNA-133 controls cardiac hypertrophy', *Nat Med*, 13(5), pp. 613-8.

Casiglia, E., Palatini, P., Da Ros, S., Pagliara, V., Puato, M., Dorigatti, F. and Pauletto, P. (2000) 'Effect of blood pressure and physical activity on carotid artery intima-media thickness in stage 1 hypertensives and controls', *American Journal of Hypertension*, 13(12), pp. 1256-1262.

Cavalcante, J. L., Lima, J. A., Redheuil, A. and Al-Mallah, M. H. (2011) 'Aortic stiffness: current understanding and future directions', *J Am Coll Cardiol*, 57(14), pp. 1511-22.

Cecelja, M. and Chowienczyk, P. (2009) 'Dissociation of aortic pulse wave velocity with risk factors for cardiovascular disease other than hypertension: A systematic review', *Hypertension*, 54(6), pp. 1328-1336.

Chambless, L. E., Folsom, A. R., Davis, V., Sharrett, R., Heiss, G., Sorlie, P., Szklo, M., Howard, G. and Evans, G. W. (2002) 'Risk Factors for Progression of Common Carotid Atherosclerosis: The Atherosclerosis Risk in Communities Study, 1987–1998', *American Journal of Epidemiology*, 155(1), pp. 38-47.

Chambless, L. E., Heiss, G., Folsom, A. R., Rosamond, W., Szklo, M., Sharrett, A. R. and Clegg, L. X. (1997) 'Association of coronary heart disease incidence with carotid arterial wall thickness and major risk factors: the Atherosclerosis Risk in Communities (ARIC) Study, 1987-1993', *Am J Epidemiol*, 146(6), pp. 483-94.

Chen, C., Yang, S., Li, H., Yin, Z., Fan, J., Zhao, Y., Gong, W., Yan, M. and Wang, D. W. (2017) 'Mir30c Is Involved in Diabetic Cardiomyopathy through Regulation of Cardiac Autophagy via BECN1', *Mol Ther Nucleic Acids*, 7, pp. 127-139.

Chen, H., Chen, Y., Wu, W., Cai, Z., Chen, Z., Yan, X. and Wu, S. (2021) 'Total cholesterol, arterial stiffness, and systolic blood pressure: a mediation analysis', *Scientific Reports*, 11(1), pp. 1330.

Chen, J. F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., Conlon, F. L. and Wang, D. Z. (2006) 'The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation', *Nat Genet*, 38(2), pp. 228-33.

Chen, X., Ba, Y., Ma, L., Cai, X., Yin, Y., Wang, K., Guo, J., Zhang, Y., Chen, J., Guo, X., Li, Q., Li, X., Wang, W., Zhang, Y., Wang, J., Jiang, X., Xiang, Y., Xu, C., Zheng, P., Zhang, J., Li, R., Zhang, H., Shang, X., Gong, T., Ning, G., Wang, J., Zen, K., Zhang, J. and Zhang, C.-Y. (2008) 'Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases', *Cell Research*, 18(10), pp. 997-1006.

Chen, Y., Xiong, H., Wu, D., Pirbhulal, S., Tian, X., Zhang, R., Lu, M., Wu, W. and Huang, W. (2015) 'Relationship of short-term blood pressure variability with carotid intima-media thickness in hypertensive patients', *Biomed Eng Online*, 14, pp. 71.

Cheng, G. (2015) 'Circulating miRNAs: Roles in cancer diagnosis, prognosis and therapy', *Advanced Drug Delivery Reviews*, 81, pp. 75-93.

Chiesa, S. T., Charakida, M., Georgiopoulos, G., Dangardt, F., Wade, K. H., Rapala, A., Bhowruth, D. J., Nguyen, H. C., Muthurangu, V., Shroff, R., Davey Smith, G., Lawlor, D. A., Sattar, N., Timpson, N. J., Hughes, A. D. and Deanfield, J. E. (2019) 'Determinants of Intima-Media Thickness in the Young', *JACC: Cardiovascular Imaging*, pp. 3219.

Cho, S. K., Cho, S. K., Kim, K. H., Cho, J. Y., Yoon, H. J., Yoon, N. S., Hong, Y. J., Park, H. W., Kim, J. H., Ahn, Y., Jeong, M. H., Cho, J. G. and Park, J. C. (2015) 'Effects of age on arterial stiffness and blood pressure variables in patients with newly diagnosed untreated hypertension', *Korean circulation journal*, 45(1), pp. 44-50.

Cingoz, F., Iyisoy, A., Demirkol, S., Sahin, M. A., Balta, S., Celik, T., Unlu, M., Arslan, Z., Cakar, M., Kucuk, U., Demirbas, S. and Kocak, N. (2014)

'Carotid Intima-Media Thickness in Patients With Slow Coronary Flow and Its Association With Neutrophil-to-Lymphocyte Ratio:A Preliminary Report', *Clinical and Applied Thrombosis/Hemostasis*, 20(4), pp. 393-399.

Clarke, R., Shipley, M., Lewington, S., Youngman, L., Collins, R., Marmot, M. and Peto, R. (1999) 'Underestimation of risk associations due to regression dilution in long-term follow-up of prospective studies', *Am J Epidemiol*, 150(4), pp. 341-53.

Coffey, S., Lewandowski, A. J., Garratt, S., Meijer, R., Lynum, S., Bedi, R., Paterson, J., Yaqub, M., Noble, J. A., Neubauer, S., Petersen, S. E., Allen, N., Sudlow, C., Collins, R., Matthews, P. M. and Leeson, P. (2017) 'Protocol and quality assurance for carotid imaging in 100,000 participants of UK Biobank: development and assessment', *European Journal of Preventive Cardiology*, 24(17), pp. 1799-1806.

Collaborators, G. B. D. C. o. D. (2018) 'Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980-2017: a systematic analysis for the Global Burden of Disease Study 2017', *Lancet (London, England)*, 392(10159), pp. 1736-1788.

Collet, J.-P., Thiele, H., Barbato, E., Barthélémy, O., Bauersachs, J., Bhatt, D. L., Dendale, P., Dorobantu, M., Edvardsen, T., Folliguet, T., Gale, C. P., Gilard, M., Jobs, A., Jüni, P., Lambrinou, E., Lewis, B. S., Mehilli, J., Meliga, E., Merkely, B., Mueller, C., Roffi, M., Rutten, F. H., Sibbing, D., Siontis, G. C. M. and Group, E. S. D. (2020) '2020 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: The Task Force for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC)', *European Heart Journal*, 42(14), pp. 1289-1367.

Correia, C. N., Nalpas, N. C., McLoughlin, K. E., Browne, J. A., Gordon, S. V., MacHugh, D. E. and Shaughnessy, R. G. (2017) 'Circulating microRNAs as Potential Biomarkers of Infectious Disease', *Frontiers in Immunology*, 8(118).

Creemers, E. E., Tijsen, A. J. and Pinto, Y. M. (2012) 'Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease?', *Circ Res*, 110(3), pp. 483-95.

Crouse, J. R., 3rd, Raichlen, J. S., Riley, W. A., Evans, G. W., Palmer, M. K., O'Leary, D. H., Grobbee, D. E. and Bots, M. L. (2007) 'Effect of rosuvastatin on progression of carotid intima-media thickness in low-risk individuals with subclinical atherosclerosis: the METEOR Trial', *Jama*, 297(12), pp. 1344-53.

Crouse, J. R., Goldbourt, U., Evans, G., Pinsky, J., Sharrett, A. R., Sorlie, P., Riley, W. and Heiss, G. (1996) 'Risk Factors and Segment-Specific Carotid Arterial Enlargement in the Atherosclerosis Risk in Communities (ARIC) Cohort', *Stroke*, 27(1), pp. 69-75.

D'Agostino, R. B., Sr., Vasan, R. S., Pencina, M. J., Wolf, P. A., Cobain, M., Massaro, J. M. and Kannel, W. B. (2008) 'General cardiovascular risk profile for use in primary care: the Framingham Heart Study', *Circulation*, 117(6), pp. 743-53.

Dao, H. H., Essalihi, R., Bouvet, C. and Moreau, P. (2005) 'Evolution and modulation of age-related medial elastocalcinosis: impact on large artery stiffness and isolated systolic hypertension', *Cardiovasc Res*, 66(2), pp. 307-17.

Dart, A. M. and Kingwell, B. A. (2001) 'Pulse pressure--a review of mechanisms and clinical relevance', *J Am Coll Cardiol*, 37(4), pp. 975-84.

de Gonzalo-Calvo, D., Dávalos, A., Montero, A., García-González, Á., Tyshkovska, I., González-Medina, A., Soares, S. M., Martínez-Camblor, P., Casas-Agustench, P., Rabadán, M., Díaz-Martínez Á, E., Úbeda, N. and Iglesias-Gutiérrez, E. (2015) 'Circulating inflammatory miRNA signature in response to different doses of aerobic exercise', *J Appl Physiol (1985)*, 119(2), pp. 124-34.

de Gonzalo-Calvo, D., van der Meer, R. W., Rijzewijk, L. J., Smit, J. W. A., Revuelta-Lopez, E., Nasarre, L., Escola-Gil, J. C., Lamb, H. J. and Llorente-Cortes, V. (2017) 'Serum microRNA-1 and microRNA-133a levels reflect myocardial steatosis in uncomplicated type 2 diabetes', *Scientific Reports*, 7(1), pp. 47.

De Rosa, S., Fichtlscherer, S., Lehmann, R., Assmus, B., Dimmeler, S. and Zeiher, A. M. (2011) 'Transcoronary concentration gradients of circulating microRNAs', *Circulation*, 124(18), pp. 1936-44.

Del Brutto, O. H., Del Brutto, V. J., Mera, R. M., Costa, A. F., Peñaherrera, R., Peñaherrera, E., Matcha, G., Generale, L. M., Torpey, A. P., Peralta, L. D., Hill, J. P., Rundek, T., Romano, J. G. and Sedler, M. J. (2020) 'The association between aortic arterial stiffness, carotid intima-media thickness and carotid plaques in community-dwelling older adults: A population-based study', *Vascular*, 28(4), pp. 405-412.

Demirsoy, İ. H., Ertural, D. Y., Balci, Ş., Çınkır, Ü., Sezer, K., Tamer, L. and Aras, N. (2018) 'Profiles of Circulating MiRNAs Following Metformin Treatment in Patients with Type 2 Diabetes', *Journal of medical biochemistry*, 37(4), pp. 499-506.

Den Ruijter, H. M., Peters, S. A., Anderson, T. J., Britton, A. R., Dekker, J. M., Eijkemans, M. J., Engstrom, G., Evans, G. W., de Graaf, J., Grobbee, D. E., Hedblad, B., Hofman, A., Holewijn, S., Ikeda, A., Kavousi, M.,

Kitagawa, K., Kitamura, A., Koffijberg, H., Lonn, E. M., Lorenz, M. W., Mathiesen, E. B., Nijpels, G., Okazaki, S., O'Leary, D. H., Polak, J. F., Price, J. F., Robertson, C., Rembold, C. M., Rosvall, M., Rundek, T., Salonen, J. T., Sitzer, M., Stehouwer, C. D., Witteman, J. C., Moons, K. G. and Bots, M. L. (2012) 'Common carotid intima-media thickness measurements in cardiovascular risk prediction: a meta-analysis', *Jama*, 308(8), pp. 796-803.

Denli, A. M., Tops, B. B. J., Plasterk, R. H. A., Ketting, R. F. and Hannon, G. J. (2004) 'Processing of primary microRNAs by the Microprocessor complex', *Nature*, 432(7014), pp. 231-235.

Dessein, P. H., Woodiwiss, A. J., Norton, G. R. and Solomon, A. (2013) 'Rheumatoid arthritis is associated with reduced adiposity but not with unfavorable major cardiovascular risk factor profiles and enhanced carotid atherosclerosis in black Africans from a developing population: a cross-sectional study', *Arthritis Res Ther*, 15(4), pp. R96.

Devaux, Y., Vausort, M., Goretti, E., Nazarov, P. V., Azuaje, F., Gilson, G., Corsten, M. F., Schroen, B., Lair, M. L., Heymans, S. and Wagner, D. R. (2012) 'Use of circulating microRNAs to diagnose acute myocardial infarction', *Clin Chem*, 58(3), pp. 559-67.

Di Bello, V., Carerj, S., Perticone, F., Benedetto, F., Palombo, C., Talini, E., Giannini, D., La Carrubba, S., Antonini-Canterin, F., Di Salvo, G., Bellieni, G., Pezzano, A., Romano, M. F. and Balbarini, A. (2009) 'Carotid intima-media thickness in asymptomatic patients with arterial hypertension without clinical cardiovascular disease: relation with left ventricular geometry and mass and coexisting risk factors', *Angiology*, 60(6), pp. 705-13.

Di Leva, G., Garofalo, M. and Croce, C. M. (2014) 'MicroRNAs in cancer', *Annual review of pathology*, 9, pp. 287-314.

Diamond, G. A. (1992) 'What price perfection? Calibration and discrimination of clinical prediction models', *J Clin Epidemiol*, 45(1), pp. 85-9.

Duttagupta, R., Jiang, R., Gollub, J., Getts, R. C. and Jones, K. W. (2011) 'Impact of Cellular miRNAs on Circulating miRNA Biomarker Signatures', *PLOS ONE*, 6(6), pp. e20769.

Eder, L., Jayakar, J., Shanmugarajah, S., Thavaneswaran, A., Pereira, D., Chandran, V., Rosen, C. F. and Gladman, D. D. (2013) 'The burden of carotid artery plaques is higher in patients with psoriatic arthritis compared with those with psoriasis alone', *Ann Rheum Dis*, 72(5), pp. 715-20.

'Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group', (1998) *Lancet*, 352(9131), pp. 854-65.

Einarson, T. R., Hunchuck, J. and Hemels, M. (2010) 'Relationship between blood glucose and carotid intima media thickness: A meta-analysis', *Cardiovascular diabetology*, 9, pp. 37-37.

Ellis, K. L., Cameron, V. A., Troughton, R. W., Frampton, C. M., Ellmers, L. J. and Richards, A. M. (2013) 'Circulating microRNAs as candidate markers to distinguish heart failure in breathless patients', *European Journal of Heart Failure*, 15(10), pp. 1138-1147.

Elmen, J., Lindow, M., Schutz, S., Lawrence, M., Petri, A., Obad, S., Lindholm, M., Hedtjarn, M., Hansen, H. F., Berger, U., Gullans, S., Kearney, P., Sarnow, P., Straarup, E. M. and Kauppinen, S. (2008) 'LNA-mediated microRNA silencing in non-human primates', *Nature*, 452(7189), pp. 896-9.

Ernst, E. and Resch, K. L. (1993) 'Fibrinogen as a cardiovascular risk factor: a meta-analysis and review of the literature', *Ann Intern Med*, 118(12), pp. 956-63.

Esau, C., Davis, S., Murray, S. F., Yu, X. X., Pandey, S. K., Pear, M., Watts, L., Booten, S. L., Graham, M., McKay, R., Subramaniam, A., Propp, S., Lollo, B. A., Freier, S., Bennett, C. F., Bhanot, S. and Monia, B. P. (2006) 'miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting', *Cell Metab*, 3(2), pp. 87-98.

Esau, C., Kang, X., Peralta, E., Hanson, E., Marcusson, E. G., Ravichandran, L. V., Sun, Y., Koo, S., Perera, R. J., Jain, R., Dean, N. M., Freier, S. M., Bennett, C. F., Lollo, B. and Griffey, R. (2004) 'MicroRNA-143 regulates adipocyte differentiation', *J Biol Chem*, 279(50), pp. 52361-5.

Esposito, K., Giugliano, D., Nappo, F. and Marfella, R. (2004) 'Regression of carotid atherosclerosis by control of postprandial hyperglycemia in type 2 diabetes mellitus', *Circulation*, 110(2), pp. 214-9.

Fang, N., Han, W., Gong, D., Zou, C. and Fan, Y. (2015) 'Atorvastatin Treatment for Carotid Intima-Media Thickness in Chinese Patients With Type 2 Diabetes: A Meta-Analysis', *Medicine (Baltimore)*, 94(44), pp. e1920.

Faraldi, M., Gomarasca, M., Banfi, G. and Lombardi, G. (2018) 'Free Circulating miRNAs Measurement in Clinical Settings: The Still Unsolved Issue of the Normalization', *Advances in clinical chemistry*, 87, pp. 113-139.

Faraldi, M., Gomarasca, M., Sansoni, V., Perego, S., Banfi, G. and Lombardi, G. (2019) 'Normalization strategies differently affect circulating miRNA profile associated with the training status', *Scientific Reports*, 9(1), pp. 1584.

Fernandez-Hernando, C., Suarez, Y., Rayner, K. J. and Moore, K. J. (2011) 'MicroRNAs in lipid metabolism', *Curr Opin Lipidol*, 22(2), pp. 86-92.

Ferreira, J. P., Girerd, N., Bozec, E., Machu, J. L., Boivin, J. M., London, G. M., Zannad, F. and Rossignol, P. (2016) 'Intima-Media Thickness Is Linearly and Continuously Associated With Systolic Blood Pressure in a Population-Based Cohort (STANISLAS Cohort Study)', *J Am Heart Assoc*, 5(6).

Fichtlscherer, S., De Rosa, S., Fox, H., Schwietz, T., Fischer, A., Liebetrau, C., Weber, M., Hamm, C. W., Röxe, T., Müller-Ardogan, M., Bonauer, A., Zeiher, A. M. and Dimmeler, S. (2010a) 'Circulating microRNAs in patients with coronary artery disease', *Circ Res*, 107(5), pp. 677-84.

Fichtlscherer, S., Rosa, S. D., Fox, H., Schwietz, T., Fischer, A., Liebetrau, C., Weber, M., Hamm, C. W., Röxe, T., Müller-Ardogan, M., Bonauer, A., Zeiher, A. M. and Dimmeler, S. (2010b) 'Circulating MicroRNAs in Patients With Coronary Artery Disease', *Circulation Research*, 107(5), pp. 677-684.

Filipów, S. and Łaczmański, Ł. (2019) 'Blood Circulating miRNAs as Cancer Biomarkers for Diagnosis and Surgical Treatment Response', *Frontiers in Genetics*, 10(169).

Finkelstein, S. M., Cohn, J. N., Collins, V. R., Carlyle, P. F. and Shelley, W. J. (1985) 'Vascular hemodynamic impedance in congestive heart failure', *The American Journal of Cardiology*, 55(4), pp. 423-427.

Finn, A. V., Kolodgie, F. D. and Virmani, R. (2010) 'Correlation between carotid intimal/medial thickness and atherosclerosis: a point of view from pathology', *Arterioscler Thromb Vasc Biol*, 30(2), pp. 177-81.

Fitch, K. V., Stavrou, E., Looby, S. E., Hemphill, L., Jaff, M. R. and Grinspoon, S. K. (2011) 'Associations of cardiovascular risk factors with two surrogate markers of subclinical atherosclerosis: Endothelial function and carotid intima media thickness', *Atherosclerosis*, 217(2), pp. 437-440.

Ford, I., Shah, A. S., Zhang, R., McAllister, D. A., Strachan, F. E., Caslake, M., Newby, D. E., Packard, C. J. and Mills, N. L. (2016) 'High-Sensitivity Cardiac Troponin, Statin Therapy, and Risk of Coronary Heart Disease', *J Am Coll Cardiol*, 68(25), pp. 2719-2728.

Foretz, M., Guigas, B., Bertrand, L., Pollak, M. and Viollet, B. (2014) 'Metformin: from mechanisms of action to therapies', *Cell Metab*, 20(6), pp. 953-66.

Fox, N. and Growdon, J. H. (2004) 'Biomarkers and surrogates', *NeuroRX*, 1(2), pp. 181-181.

Frost, D. and Beischer, W. (1998) 'Determinants of carotid artery wall thickening in young patients with Type 1 diabetes mellitus', *Diabet Med*, 15(10), pp. 851-7.

Gaarder, M. and Seierstad, T. (2015) 'Measurements of carotid intima media thickness in non-invasive high-frequency ultrasound images: the effect of dynamic range setting', *Cardiovascular Ultrasound*, 13(1), pp. 5.

Galley, H. F. and Webster, N. R. (2004) 'Physiology of the endothelium', *Br J Anaesth*, 93(1), pp. 105-13.

Gao, L., Bai, L., Shi, M., Ni, J., Lu, H., Wu, Y., Tu, J., Ning, X., Wang, J. and Li, Y. (2017) 'Association between carotid intima-media thickness and fasting blood glucose level: A population-based cross-sectional study among low-income adults in rural China', *Journal of diabetes investigation*, 8(6), pp. 788-797.

Gao, W., He, H.-W., Wang, Z.-M., Zhao, H., Lian, X.-Q., Wang, Y.-S., Zhu, J., Yan, J.-J., Zhang, D.-G., Yang, Z.-J. and Wang, L.-S. (2012) 'Plasma levels of lipometabolism-related miR-122 and miR-370 are increased in patients with hyperlipidemia and associated with coronary artery disease', *Lipids in health and disease*, 11, pp. 55-55.

Garber, A. J., Abrahamson, M. J., Barzilay, J. I., Blonde, L., Bloomgarden, Z. T., Bush, M. A., Dagogo-Jack, S., DeFronzo, R. A., Einhorn, D., Fonseca, V. A., Garber, J. R., Garvey, W. T., Grunberger, G., Handelsman, Y., Hirsch, I. B., Jellinger, P. S., McGill, J. B., Mechanick, J. I., Rosenblit, P. D. and Umpierrez, G. E. (2019) 'CONSENSUS STATEMENT BY THE AMERICAN ASSOCIATION OF CLINICAL ENDOCRINOLOGISTS AND AMERICAN COLLEGE OF ENDOCRINOLOGY ON THE COMPREHENSIVE TYPE 2 DIABETES MANAGEMENT ALGORITHM - 2019 EXECUTIVE SUMMARY', *Endocr Pract*, 25(1), pp. 69-100.

Gatzka, C. D., Cameron, J. D., Kingwell, B. A. and Dart, A. M. (1998) 'Relation Between Coronary Artery Disease, Aortic Stiffness, and Left Ventricular Structure in a Population Sample', *Hypertension*, 32(3), pp. 575-578.

Ge, W., Parvez, F., Wu, F., Islam, T., Ahmed, A., Shaheen, I., Sarwar, G., Demmer, R. T., Desvarieux, M., Ahsan, H. and Chen, Y. (2014) 'Association between anthropometric measures of obesity and subclinical atherosclerosis in Bangladesh', *Atherosclerosis*, 232(1), pp. 234-41.

Genest, J. J., Jr., Martin-Munley, S. S., McNamara, J. R., Ordovas, J. M., Jenner, J., Myers, R. H., Silberman, S. R., Wilson, P. W., Salem, D. N. and Schaefer, E. J. (1992) 'Familial lipoprotein disorders in patients with premature coronary artery disease', *Circulation*, 85(6), pp. 2025-33.

George Howard, D. G. L. B., MD; Gregory W. Evans, MS; John R. Crouse III, MD; Ward Riley, PhD; Donna Arnett, PhD; Regina de Lacy, MS; Gerardo Heiss,

MD, PhD; for the ARIC Investigators (1994) 'Relations of intimal-medial thickness among sites within the carotid artery as evaluated by B-mode ultrasound. ARIC Investigators. Atherosclerosis Risk in Communities', *stroke*, 25(8), pp. 1581-7.

Ghai, V., Kim, T.-K., Etheridge, A., Nielsen, T., Hansen, T., Pedersen, O., Galas, D. and Wang, K. (2019) 'Extracellular Vesicle Encapsulated MicroRNAs in Patients with Type 2 Diabetes Are Affected by Metformin Treatment', *Journal of clinical medicine*, 8(5), pp. 617.

Gijsberts, C. M., Groenewegen, K. A., Hoefer, I. E., Eijkemans, M. J. C., Asselbergs, F. W., Anderson, T. J., Britton, A. R., Dekker, J. M., Engström, G., Evans, G. W., de Graaf, J., Grobbee, D. E., Hedblad, B., Holewijn, S., Ikeda, A., Kitagawa, K., Kitamura, A., de Kleijn, D. P. V., Lonn, E. M., Lorenz, M. W., Mathiesen, E. B., Nijpels, G., Okazaki, S., O'Leary, D. H., Pasterkamp, G., Peters, S. A. E., Polak, J. F., Price, J. F., Robertson, C., Rembold, C. M., Rosvall, M., Rundek, T., Salonen, J. T., Sitzer, M., Stehouwer, C. D. A., Bots, M. L. and den Ruijter, H. M. (2015) 'Race/Ethnic Differences in the Associations of the Framingham Risk Factors with Carotid IMT and Cardiovascular Events', *PloS one*, 10(7), pp. e0132321-e0132321.

Gilad, S., Meiri, E., Yogev, Y., Benjamin, S., Lebanony, D., Yerushalmi, N., Benjamin, H., Kushnir, M., Cholakh, H., Melamed, N., Bentwich, Z., Hod, M., Goren, Y. and Chajut, A. (2008) 'Serum microRNAs are promising novel biomarkers', *PLoS One*, 3(9), pp. e3148.

Gimbrone, M. A., Jr. and García-Cardeña, G. (2013) 'Vascular endothelium, hemodynamics, and the pathobiology of atherosclerosis', *Cardiovascular pathology : the official journal of the Society for Cardiovascular Pathology*, 22(1), pp. 9-15.

Giuseppina Catanzaro, Z. M. B., 1 Martina Chiacchiarini ,2 Luana Abballe ,1 Claudia Sabato ,2 Alessandra Vacca ,1 Paola Borgiani,3 Francesco Dotta ,4 Manfredi Tesauro ,5 Agnese Po ,2 and Elisabetta Ferretti (2018) 'Circulating MicroRNAs in Elderly Type 2 Diabetic Patients', *International Journal of Endocrinology*, pp. 11.

Glinge, C., Clauss, S., Boddum, K., Jabbari, R., Jabbari, J., Risgaard, B., Tomsits, P., Hildebrand, B., Kääb, S., Wakili, R., Jespersen, T. and Tfelt-Hansen, J. (2017) 'Stability of Circulating Blood-Based MicroRNAs - Pre-Analytic Methodological Considerations', *PLoS One*, 12(2), pp. e0167969.

Goff, D. C., Jr., Lloyd-Jones, D. M., Bennett, G., Coady, S., D'Agostino, R. B., Sr., Gibbons, R., Greenland, P., Lackland, D. T., Levy, D., O'Donnell, C. J., Robinson, J. G., Schwartz, J. S., Shero, S. T., Smith, S. C., Jr., Sorlie, P., Stone, N. J. and Wilson, P. W. F. (2014) '2013 ACC/AHA guideline on the assessment of cardiovascular risk: a report of the American College

of Cardiology/American Heart Association Task Force on Practice Guidelines', *J Am Coll Cardiol*, 63(25 Pt B), pp. 2935-2959.

Green, B. D., Irwin, N., Duffy, N. A., Gault, V. A., O'Harte F, P. and Flatt, P. R. (2006) 'Inhibition of dipeptidyl peptidase-IV activity by metformin enhances the antidiabetic effects of glucagon-like peptide-1', *Eur J Pharmacol*, 547(1-3), pp. 192-9.

Greenland, P., Alpert, J. S., Beller, G. A., Benjamin, E. J., Budoff, M. J., Fayad, Z. A., Foster, E., Hlatky, M. A., Hodgson, J. M., Kushner, F. G., Lauer, M. S., Shaw, L. J., Smith, S. C., Jr., Taylor, A. J., Weintraub, W. S., Wenger, N. K., Jacobs, A. K., Smith, S. C., Jr., Anderson, J. L., Albert, N., Buller, C. E., Creager, M. A., Ettinger, S. M., Guyton, R. A., Halperin, J. L., Hochman, J. S., Kushner, F. G., Nishimura, R., Ohman, E. M., Page, R. L., Stevenson, W. G., Tarkington, L. G. and Yancy, C. W. (2010) '2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines', *J Am Coll Cardiol*, 56(25), pp. e50-103.

Grishok, A., Pasquinelli, A. E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D. L., Fire, A., Ruvkun, G. and Mello, C. C. (2001) 'Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing', *Cell*, 106(1), pp. 23-34.

Gruszfeld, D., Weber, M., Nowakowska-Rysz, M., Janas, R., Kozlik-Feldmann, R., Xhonneux, A., Carlier, C., Riva, E., Verduci, E., Closa-Monasterolo, R., Escribano, J., Dobrzanska, A. and Koletzko, B. (2015) 'Protein intake in infancy and carotid intima media thickness at 5 years--a secondary analysis from a randomized trial', *Ann Nutr Metab*, 66(1), pp. 51-9.

Guay, C. and Regazzi, R. (2013) 'Circulating microRNAs as novel biomarkers for diabetes mellitus', *Nat Rev Endocrinol*, 9(9), pp. 513-21.

Gunton, J. E., Delhanty, P. J., Takahashi, S. and Baxter, R. C. (2003) 'Metformin rapidly increases insulin receptor activation in human liver and signals preferentially through insulin-receptor substrate-2', *J Clin Endocrinol Metab*, 88(3), pp. 1323-32.

Gutzwiller, J. P., Goke, B., Drewe, J., Hildebrand, P., Ketterer, S., Handschin, D., Winterhalder, R., Conen, D. and Beglinger, C. (1999) 'Glucagon-like peptide-1: a potent regulator of food intake in humans', *Gut*, 44(1), pp. 81-6.

Ha, M. and Kim, V. N. (2014) 'Regulation of microRNA biogenesis', *Nature Reviews Molecular Cell Biology*, 15(8), pp. 509-524.

Hägg, S., Fall, T., Ploner, A., Mägi, R., Fischer, K., Draisma, H. H., Kals, M., de Vries, P. S., Dehghan, A., Willems, S. M., Sarin, A. P., Kristiansson, K.,

Nuotio, M. L., Havulinna, A. S., de Bruijn, R. F., Ikram, M. A., Kuningas, M., Stricker, B. H., Franco, O. H., Benyamin, B., Gieger, C., Hall, A. S., Huikari, V., Jula, A., Järvelin, M. R., Kaakinen, M., Kaprio, J., Kobl, M., Mangino, M., Nelson, C. P., Palotie, A., Samani, N. J., Spector, T. D., Strachan, D. P., Tobin, M. D., Whitfield, J. B., Uitterlinden, A. G., Salomaa, V., Syvänen, A. C., Kuulasmaa, K., Magnusson, P. K., Esko, T., Hofman, A., de Geus, E. J., Lind, L., Giedraitis, V., Perola, M., Evans, A., Ferrières, J., Virtamo, J., Kee, F., Tregouet, D. A., Arveiler, D., Amouyel, P., Gianfagna, F., Brambilla, P., Ripatti, S., van Duijn, C. M., Metspalu, A., Prokopenko, I., McCarthy, M. I., Pedersen, N. L. and Ingelsson, E. (2015) 'Adiposity as a cause of cardiovascular disease: a Mendelian randomization study', *Int J Epidemiol*, 44(2), pp. 578-86.

Hamam, R., Hamam, D., Alsaleh, K. A., Kassem, M., Zaher, W., Alfayez, M., Aldahmash, A. and Alajez, N. M. (2017) 'Circulating microRNAs in breast cancer: novel diagnostic and prognostic biomarkers', *Cell Death Dis*, 8(9), pp. e3045.

Hamilton, P. K., Lockhart, C. J., Quinn, C. E. and McVeigh, G. E. (2007) 'Arterial stiffness: clinical relevance, measurement and treatment', *Clin Sci (Lond)*, 113(4), pp. 157-70.

Hanefeld, M., Chiasson, J. L., Koehler, C., Henkel, E., Schaper, F. and Temelkova-Kurktschiev, T. (2004) 'Acarbose slows progression of intima-media thickness of the carotid arteries in subjects with impaired glucose tolerance', *Stroke*, 35(5), pp. 1073-8.

Hanefeld, M., Temelkova-Kurktschiev, T., Schaper, F., Henkel, E., Siegert, G. and Koehler, C. (1999) 'Impaired fasting glucose is not a risk factor for atherosclerosis', *Diabet Med*, 16(3), pp. 212-8.

Hanna, J., Hossain, G. S. and Kocerha, J. (2019) 'The Potential for microRNA Therapeutics and Clinical Research', *Frontiers in Genetics*, 10(478).

Hansson, G. K. (2005) 'Inflammation, Atherosclerosis, and Coronary Artery Disease', *New England Journal of Medicine*, 352(16), pp. 1685-1695.

Hayoz, D., Rutschmann, B., Perret, F., Niederberger, M., Tardy, Y., Mooser, V., Nussberger, J., Waeber, B. and Brunner, H. R. (1992) 'Conduit artery compliance and distensibility are not necessarily reduced in hypertension', *Hypertension*, 20(1), pp. 1-6.

Heart statistics, *British Heart Foundation*. Available at: https://www.bhf.org.uk/what-we-do/our-research/heart-statistics (Accessed: 20 2020).

Hedblad, B., Nilsson, P., Janzon, L. and Berglund, G. (2000) 'Relation between insulin resistance and carotid intima-media thickness and stenosis in non-diabetic subjects. Results from a cross-sectional study in Malmo, Sweden', *Diabet Med*, 17(4), pp. 299-307.

Heegaard, N. H. H., Carlsen, A. L., Lilje, B., Ng, K. L., Rønne, M. E., Jørgensen, H. L., Sennels, H. and Fahrenkrug, J. (2016) 'Diurnal Variations of Human Circulating Cell-Free Micro-RNA', *PloS one*, 11(8), pp. e0160577-e0160577.

Henry C McGill Jr, C. A. M., Edward E Herderick, Gray T Malcom, Richard E Tracy (2000) 'Origin of atherosclerosis in childhood and adolescence', *American Society for Clinical Nutrition*, 72, pp. 1307s-15s.

Hippisley-Cox, J., Coupland, C., Vinogradova, Y., Robson, J., Minhas, R., Sheikh, A. and Brindle, P. (2008) 'Predicting cardiovascular risk in England and Wales: prospective derivation and validation of QRISK2', *BMJ*, 336(7659), pp. 1475-1482.

Hitsumoto, T. and Shirai, K. (2015) 'Factors affecting high-sensitivity cardiac troponin T elevation in Japanese metabolic syndrome patients', *Diabetes, metabolic syndrome and obesity : targets and therapy*, 8, pp. 157-162.

Hodis, H. N., Mack, W. J., LaBree, L., Selzer, R. H., Liu, C. R., Liu, C. H. and Azen, S. P. (1998) 'The role of carotid arterial intima-media thickness in predicting clinical coronary events', *Ann Intern Med*, 128(4), pp. 262-9.

Hofman, A., Grobbee, D. E., De Jong, P. T. V. M. and Van den Ouweland, F. A. (1991) 'Determinants of disease and disability in the elderly: The Rotterdam elderly study', *European Journal of Epidemiology*, 7(4), pp. 403-422.

Howard, G., Sharrett, A. R., Heiss, G., Evans, G. W., Chambless, L. E., Riley, W. A. and Burke, G. L. (1993) 'Carotid artery intimal-medial thickness distribution in general populations as evaluated by B-mode ultrasound. ARIC Investigators', *Stroke*, 24(9), pp. 1297-304.

Huang, R. S., Gamazon, E. R., Ziliak, D., Wen, Y., Im, H. K., Zhang, W., Wing, C., Duan, S., Bleibel, W. K., Cox, N. J. and Dolan, M. E. (2011) 'Population differences in microRNA expression and biological implications', *RNA Biol*, 8(4), pp. 692-701.

Huang, Y. Q., Cai, A. P., Chen, J. Y., Huang, C., Li, J. and Feng, Y. Q. (2016) 'The Relationship of Plasma miR-29a and Oxidized Low Density Lipoprotein with Atherosclerosis', *Cell Physiol Biochem*, 40(6), pp. 1521-1528.

Hulthe, J. and Fagerberg, B. (2002) 'Circulating oxidized LDL is associated with subclinical atherosclerosis development and inflammatory cytokines (AIR Study)', *Arterioscler Thromb Vasc Biol*, 22(7), pp. 1162-7.

Hundal, R. S., Krssak, M., Dufour, S., Laurent, D., Lebon, V., Chandramouli, V., Inzucchi, S. E., Schumann, W. C., Petersen, K. F., Landau, B. R. and

Shulman, G. I. (2000) 'Mechanism by which metformin reduces glucose production in type 2 diabetes', *Diabetes*, 49(12), pp. 2063-9.

Hunter, M. P., Ismail, N., Zhang, X., Aguda, B. D., Lee, E. J., Yu, L., Xiao, T., Schafer, J., Lee, M. L., Schmittgen, T. D., Nana-Sinkam, S. P., Jarjoura, D. and Marsh, C. B. (2008) 'Detection of microRNA expression in human peripheral blood microvesicles', *PLoS One*, 3(11), pp. e3694.

Hussain, M. M., Rava, P., Walsh, M., Rana, M. and Iqbal, J. (2012) 'Multiple functions of microsomal triglyceride transfer protein', *Nutrition & metabolism*, 9, pp. 14-14.

Iglseder, B., Cip, P., Malaimare, L., Ladurner, G. and Paulweber, B. (2005) 'The Metabolic Syndrome Is a Stronger Risk Factor for Early Carotid Atherosclerosis in Women Than in Men', *Stroke*, 36(6), pp. 1212-1217.

Iliopoulos, D., Drosatos, K., Hiyama, Y., Goldberg, I. J. and Zannis, V. I. (2010) 'MicroRNA-370 controls the expression of microRNA-122 and Cpt1alpha and affects lipid metabolism', *Journal of lipid research*, 51(6), pp. 1513-1523.

Ingelsson, E., Sullivan, L. M., Murabito, J. M., Fox, C. S., Benjamin, E. J., Polak, J. F., Meigs, J. B., Keyes, M. J., O'Donnell, C. J., Wang, T. J., D'Agostino, R. B., Sr., Wolf, P. A. and Vasan, R. S. (2007) 'Prevalence and prognostic impact of subclinical cardiovascular disease in individuals with the metabolic syndrome and diabetes', *Diabetes*, 56(6), pp. 1718-26.

Insull, W., Jr. (2009) 'The pathology of atherosclerosis: plaque development and plaque responses to medical treatment', *Am J Med*, 122(1 Suppl), pp. S3-s14.

Ivey, K. N., Muth, A., Arnold, J., King, F. W., Yeh, R. F., Fish, J. E., Hsiao, E. C., Schwartz, R. J., Conklin, B. R., Bernstein, H. S. and Srivastava, D. (2008) 'MicroRNA regulation of cell lineages in mouse and human embryonic stem cells', *Cell Stem Cell*, 2(3), pp. 219-29.

Jaffer, F. A., O'Donnell, C. J., Larson, M. G., Chan, S. K., Kissinger, K. V., Kupka, M. J., Salton, C., Botnar, R. M., Levy, D. and Manning, W. J. (2002) 'Age and sex distribution of subclinical aortic atherosclerosis: a magnetic resonance imaging examination of the Framingham Heart Study', *Arterioscler Thromb Vasc Biol*, 22(5), pp. 849-54.

Jansen, F., Yang, X., Proebsting, S., Hoelscher, M., Przybilla, D., Baumann, K., Schmitz, T., Dolf, A., Endl, E., Franklin, B. S., Sinning, J.-M., Vasa-Nicotera, M., Nickenig, G. and Werner, N. (2014a) 'MicroRNA expression in circulating microvesicles predicts cardiovascular events in patients with coronary artery disease', *Journal of the American Heart Association*, 3(6), pp. e001249-e001249.

Jansen, F., Yang, X., Proebsting, S., Hoelscher, M., Przybilla, D., Baumann, K., Schmitz, T., Dolf, A., Endl, E., Franklin, B. S., Sinning, J. M., Vasa-Nicotera, M., Nickenig, G. and Werner, N. (2014b) 'MicroRNA expression in circulating microvesicles predicts cardiovascular events in patients with coronary artery disease', *J Am Heart Assoc*, 3(6), pp. e001249.

Jin, J.-P. (2016) 'Chapter One - Evolution, Regulation, and Function of N-terminal Variable Region of Troponin T: Modulation of Muscle Contractility and Beyond', in Jeon, K.W. (ed.) *International Review of Cell and Molecular Biology*: Academic Press, pp. 1-28.

John R. Crouse , U. G., Greg Evans , Joan Pinsky , A. Richey Sharrett , Paul Sorlie , Ward Riley , Gerardo Heiss and Investigators, a. f. t. A. (1996) 'Risk Factors and Segment-Specific Carotid Arterial Enlargement in the Atherosclerosis Risk in Communities (ARIC) Cohort', *stroke*, 27, pp. 69-75.

Johnson, C. P., Baugh, R., Wilson, C. A. and Burns, J. (2001) 'Age related changes in the tunica media of the vertebral artery: implications for the assessment of vessels injured by trauma', *J Clin Pathol*, 54(2), pp. 139-45.

Johnson, H. M., Piper, M. E., Jorenby, D. E., Fiore, M. C., Baker, T. B. and Stein, J. H. (2010) 'Risk factors for subclinical carotid atherosclerosis among current smokers', *Prev Cardiol*, 13(4), pp. 166-71.

Kajimoto, K., Naraba, H. and Iwai, N. (2006) 'MicroRNA and 3T3-L1 pre-adipocyte differentiation', *Rna*, 12(9), pp. 1626-32.

Kalra, L., Iveson, E., Rambaran, C., Sherwood, R., Chowienczyk, P., Ritter, J., Shah, A. and Forrester, T. (2008) 'An international matched cohort study of the contribution of metabolic impairments to subclinical atherosclerosis in United Kingdom and Jamaican African-Caribbeans', *Atherosclerosis*, 199(1), pp. 95-101.

Kantharidis, P., Wang, B., Carew, R. M. and Lan, H. Y. (2011) 'Diabetes complications: the microRNA perspective', *Diabetes*, 60(7), pp. 1832-1837.

Kass, D. A., Shapiro, E. P., Kawaguchi, M., Capriotti, A. R., Scuteri, A., deGroof, R. C. and Lakatta, E. G. (2001) 'Improved Arterial Compliance by a Novel Advanced Glycation End-Product Crosslink Breaker', *Circulation*, 104(13), pp. 1464-1470.

Keller, A., Rounge, T., Backes, C., Ludwig, N., Gislefoss, R., Leidinger, P., Langseth, H. and Meese, E. (2017) 'Sources to variability in circulating human miRNA signatures', *RNA biology*, 14(12), pp. 1791-1798.

Kelly, R. P., Millasseau, S. C., Ritter, J. M. and Chowienczyk, P. J. (2001) 'Vasoactive Drugs Influence Aortic Augmentation Index Independently of Pulse-Wave Velocity in Healthy Men', *Hypertension*, 37(6), pp. 1429-1433.

Kesse-Guyot, E., Vergnaud, A. C., Fezeu, L., Zureik, M., Blacher, J., Péneau, S., Hercberg, S., Galan, P. and Czernichow, S. (2010) 'Associations between dietary patterns and arterial stiffness, carotid artery intima-media thickness and atherosclerosis', *Eur J Cardiovasc Prev Rehabil*, 17(6), pp. 718-24.

Keys, A. (1997) 'Coronary heart disease in seven countries. 1970', *Nutrition*, 13(3), pp. 250-2; discussion 249, 253.

Kim, H.-N., Kim, S.-H., Eun, Y.-M. and Song, S.-W. (2015) 'Obesity with metabolic abnormality is associated with the presence of carotid atherosclerosis in Korean men: a cross-sectional study', *Diabetology & Metabolic Syndrome*, 7(1), pp. 68.

Kim, H. L. and Kim, S. H. (2019) 'Pulse Wave Velocity in Atherosclerosis', *Front Cardiovasc Med*, 6, pp. 41.

Kim, J. H., Kim, S. Y., Jung, E. S., Jung, S. W., Koo, J. S., Kim, J. H., Yeon, J. E., Kwon, S. Y., Lee, S. W., Byun, K. S. and Lee, C. H. (2011) 'Carotid intima-media thickness is increased not only in non-alcoholic fatty liver disease patients but also in alcoholic fatty liver patients', *Digestion*, 84(2), pp. 149-55.

Kirschner, M. B., Kao, S. C., Edelman, J. J., Armstrong, N. J., Vallely, M. P., van Zandwijk, N. and Reid, G. (2011) 'Haemolysis during sample preparation alters microRNA content of plasma', *PLoS One*, 6(9), pp. e24145.

Klop, B., van de Geijn, G.-J. M., Bovenberg, S. A., van der Meulen, N., Elte, J. W. F., Birnie, E., Njo, T. L., Janssen, H. W., van Miltenburg, A., Jukema, J. W. and Cabezas, M. C. (2013) 'Erythrocyte-bound apolipoprotein B in relation to atherosclerosis, serum lipids and ABO blood group', *PloS one*, 8(9), pp. e75573-e75573.

Kocaman, S. A., Durakoğlugil, M. E., Çetin, M., Erdoğan, T., Ergül, E. and Çanga, A. (2013) 'The independent relationship of epicardial adipose tissue with carotid intima-media thickness and endothelial functions: the association of pulse wave velocity with the active facilitated arterial conduction concept', *Blood pressure monitoring*, 18(2), pp. 85-93.

Kokubo, Y., Watanabe, M., Higashiyama, A., Nakao, Y. M., Nakamura, F. and Miyamoto, Y. (2018a) 'Impact of Intima-Media Thickness Progression in the Common Carotid Arteries on the Risk of Incident Cardiovascular Disease in the Suita Study', *J Am Heart Assoc*, 7(11).

Kokubo, Y., Watanabe, M., Higashiyama, A., Nakao, Y. M., Nakamura, F. and Miyamoto, Y. (2018b) 'Impact of Intima-Media Thickness Progression in the Common Carotid Arteries on the Risk of Incident Cardiovascular Disease in the Suita Study', *Journal of the American Heart Association*, 7(11), pp. e007720.

Kooy, A., de Jager, J., Lehert, P., Bets, D., Wulffele, M. G., Donker, A. J. and Stehouwer, C. D. (2009) 'Long-term effects of metformin on metabolism and microvascular and macrovascular disease in patients with type 2 diabetes mellitus', *Arch Intern Med*, 169(6), pp. 616-25.

Kroh, E. M., Parkin, R. K., Mitchell, P. S. and Tewari, M. (2010) 'Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR)', *Methods*, 50(4), pp. 298-301.

Ku DN, G. D., Zarins CK, Glagov S (1985) 'Pulsatile flow and atherosclerosis in the human carotid bifurcation: positive plaque location and low oscillating shear stress', *Arteriosclerosis*, 5, pp. 293-302.

Kuller, L., Borhani, N., Furberg, C., Gardin, J., Manolio, T., O'Leary, D., Psaty, B. and Robbins, J. (1994a) 'Prevalence of subclinical atherosclerosis and cardiovascular disease and association with risk factors in the Cardiovascular Health Study', *Am J Epidemiol*, 139(12), pp. 1164-79.

Kuller, L., Borthani, N., Furberg, C., Gardin, J., Manolio, T., O'Leary, D., Psaty, B. and Robbins, J. (1994b) 'Prevalence of Subclinical Atherosclerosis and Cardiovascular Disease and Association with Risk Factors in the Cardiovascular Health Study', *American Journal of Epidemiology*, 139(12), pp. 1164-1179.

Kuller, L. H., Shemanski, L., Psaty, B. M., Borhani, N. O., Gardin, J., Haan, M. N., O'Leary, D. H., Savage, P. J., Tell, G. S. and Tracy, R. (1995) 'Subclinical Disease as an Independent Risk Factor for Cardiovascular Disease', *Circulation*, 92(4), pp. 720-726.

Kulshreshtha, R., Ferracin, M., Wojcik, S. E., Garzon, R., Alder, H., Agosto-Perez, F. J., Davuluri, R., Liu, C. G., Croce, C. M., Negrini, M., Calin, G. A. and Ivan, M. (2007) 'A microRNA signature of hypoxia', *Mol Cell Biol*, 27(5), pp. 1859-67.

Kumar, D., Narang, R., Sreenivas, V., Rastogi, V., Bhatia, J., Saluja, D. and Srivastava, K. (2020) 'Circulatory miR-133b and miR-21 as Novel Biomarkers in Early Prediction and Diagnosis of Coronary Artery Disease', *Genes*, 11(2), pp. 164.

Kumar, N. and Dey, C. S. (2002) 'Metformin enhances insulin signalling in insulin-dependent and-independent pathways in insulin resistant muscle cells', *Br J Pharmacol*, 137(3), pp. 329-36.

Kuswardhani, R. T., Wiradharma, K. G., Kandarini, Y., Widiana, G. R. and Martadiani, E. D. (2018) 'Factors associated with carotid intima-media thickness in patients on maintenance hemodialysis', *International journal of general medicine*, 12, pp. 1-6.

Kuwabara, Y., Ono, K., Horie, T., Nishi, H., Nagao, K., Kinoshita, M., Watanabe, S., Baba, O., Kojima, Y., Shizuta, S., Imai, M., Tamura, T., Kita, T. and Kimura, T. (2011) 'Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage', *Circ Cardiovasc Genet*, 4(4), pp. 446-54.

Lagos-Quintana, M., Rauhut, R., Meyer, J., Borkhardt, A. and Tuschl, T. (2003) 'New microRNAs from mouse and human', *Rna*, 9(2), pp. 175-9.

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W. and Tuschl, T. (2002) 'Identification of tissue-specific microRNAs from mouse', *Curr Biol*, 12(9), pp. 735-9.

Lakka, T. A., Salonen, R., Kaplan, G. A. and Salonen, J. T. (1999) 'Blood pressure and the progression of carotid atherosclerosis in middle-aged men', *Hypertension*, 34(1), pp. 51-6.

Lamarche, B., Tchernof, A., Moorjani, S., Cantin, B., Dagenais, G. R., Lupien, P. J. and Despre´s, J.-P. (1997) 'Small, Dense Low-Density Lipoprotein Particles as a Predictor of the Risk of Ischemic Heart Disease in Men', *Circulation*, 95(1), pp. 69-75.

Lantelme, P., Mestre, C., Lievre, M., Gressard, A. and Milon, H. (2002) 'Heart rate: an important confounder of pulse wave velocity assessment', *Hypertension*, 39(6), pp. 1083-7.

Larsson, S. C., Mason, A. M., Bäck, M., Klarin, D., Damrauer, S. M., Program, M. V., Michaëlsson, K. and Burgess, S. (2020) 'Genetic predisposition to smoking in relation to 14 cardiovascular diseases', *European Heart Journal*.

Laterza, O. F., Scott, M. G., Garrett-Engele, P. W., Korenblat, K. M. and Lockwood, C. M. (2013) 'Circulating miR-122 as a potential biomarker of liver disease', *Biomark Med*, 7(2), pp. 205-10.

Lauer, M. S. (2007) 'Primary prevention of atherosclerotic cardiovascular disease: the high public burden of low individual risk', *Jama*, 297(12), pp. 1376-8.

Laurent, S., Cockcroft, J., Van Bortel, L., Boutouyrie, P., Giannattasio, C., Hayoz, D., Pannier, B., Vlachopoulos, C., Wilkinson, I. and Struijker-Boudier, H. (2006) 'Expert consensus document on arterial stiffness: methodological issues and clinical applications', *Eur Heart J*, 27(21), pp. 2588-605.

Layde R. Paim, R. S., Guilherme de Rossi, José R. Matos-Souza, Anselmo de A. (2019) 'Circulating microRNAs, Vascular Risk, and Physical Activity in Spinal Cord-Injured Subjects', *Journal of Neurotrauma*, 36(6), pp. 845-852.

Lee, H. Y. and Oh, B. H. (2010) 'Aging and arterial stiffness', *Circ J*, 74(11), pp. 2257-62.

Lee, M. J., Shin, D. H., Kim, S. J., Oh, H. J., Yoo, D. E., Kim, J.-K., Park, J. T., Han, S. H., Kang, S.-W., Choi, K. H. and Yoo, T.-H. (2012) 'Visceral Fat Thickness Is Associated With Carotid Atherosclerosis in Peritoneal Dialysis Patients', *Obesity*, 20(6), pp. 1301-1307.

Lee, R. C., Feinbaum, R. L. and Ambros, V. (1993) 'The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14', *Cell*, 75(5), pp. 843-54.

Lee, T. C., O'Malley, P. G., Feuerstein, I. and Taylor, A. J. (2003) 'The prevalence and severity of coronary artery calcification on coronary artery computed tomography in black and white subjects', *J Am Coll Cardiol*, 41(1), pp. 39-44.

Lefkowitz, R. J. and Willerson, J. T. (2001) 'Prospects for cardiovascular research', *JAMA*, 285(5), pp. 581-7.

Letonja, M., Nikolajevic Starcevic, J. and Petrovič, D. (2012) 'Association of manganese superoxide dismutase and glutathione S-transferases genotypes with carotid atherosclerosis in patients with diabetes mellitus type 2', *International angiology : a journal of the International Union of Angiology*, 31, pp. 33-41.

Lexis, C. P. H., Wieringa, W. G., Hiemstra, B., van Deursen, V. M., Lipsic, E., van der Harst, P., van Veldhuisen, D. J. and van der Horst, I. C. C. (2014) 'Chronic Metformin Treatment is Associated with Reduced Myocardial Infarct Size in Diabetic Patients with ST-segment Elevation Myocardial Infarction', *Cardiovascular Drugs and Therapy*, 28(2), pp. 163-171.

Li, C., Fang, Z., Jiang, T., Zhang, Q., Liu, C., Zhang, C. and Xiang, Y. (2013a) 'Serum microRNAs profile from genome-wide serves as a fingerprint for diagnosis of acute myocardial infarction and angina pectoris', *BMC Med Genomics*, 6, pp. 16.

Li, X.-D., Yang, Y.-J., Wang, L.-Y., Qiao, S.-B., Lu, X.-F., Wu, Y.-J., Xu, B., Li, H.-F. and Gu, D.-F. (2017) 'Elevated plasma miRNA-122, -140-3p, -720, -2861, and -3149 during early period of acute coronary syndrome are derived from peripheral blood mononuclear cells', *PloS one*, 12(9), pp. e0184256-e0184256.

Li, Y. and Kowdley, K. V. (2012a) 'Method for microRNA isolation from clinical serum samples', *Anal Biochem*, 431(1), pp. 69-75.

Li, Y. and Kowdley, K. V. (2012b) 'MicroRNAs in common human diseases', *Genomics Proteomics Bioinformatics*, 10(5), pp. 246-53.

Li, Y.-Q., Zhang, M.-F., Wen, H.-Y., Hu, C.-L., Liu, R., Wei, H.-Y., Ai, C.-M., Wang, G., Liao, X.-X. and Li, X. (2013b) 'Comparing the diagnostic values of circulating microRNAs and cardiac troponin T in patients with acute myocardial infarction', *Clinics (Sao Paulo, Brazil)*, 68(1), pp. 75-80.

Libby, P., Ridker, P. M. and Maseri, A. (2002) 'Inflammation and Atherosclerosis', *Circulation*, 105(9), pp. 1135-1143.

Lin, Q., Gao, Z., Alarcon, R. M., Ye, J. and Yun, Z. (2009) 'A role of miR-27 in the regulation of adipogenesis', *Febs j*, 276(8), pp. 2348-58.

Littlejohns, T. J., Holliday, J., Gibson, L. M., Garratt, S., Oesingmann, N., Alfaro-Almagro, F., Bell, J. D., Boultwood, C., Collins, R., Conroy, M. C., Crabtree, N., Doherty, N., Frangi, A. F., Harvey, N. C., Leeson, P., Miller, K. L., Neubauer, S., Petersen, S. E., Sellors, J., Sheard, S., Smith, S. M., Sudlow, C. L. M., Matthews, P. M. and Allen, N. E. (2020) 'The UK Biobank imaging enhancement of 100,000 participants: rationale, data collection, management and future directions', *Nature Communications*, 11(1), pp. 2624.

Liu, B., Ni, J., Shi, M., Bai, L., Zhan, C., Lu, H., Wu, Y., Tu, J., Ning, X., Hao, J. and Wang, J. (2017) 'Carotid Intima-media Thickness and its Association with Conventional Risk Factors in Low-income Adults: A Population-based Cross-Sectional Study in China', *Scientific reports*, 7, pp. 41500-41500.

Liu, B., Young, H., Crowe, F. L., Benson, V. S., Spencer, E. A., Key, T. J., Appleby, P. N. and Beral, V. (2011) 'Development and evaluation of the Oxford WebQ, a low-cost, web-based method for assessment of previous 24 h dietary intakes in large-scale prospective studies', *Public Health Nutr*, 14(11), pp. 1998-2005.

Liu, N., Bezprozvannaya, S., Williams, A. H., Qi, X., Richardson, J. A., Bassel-Duby, R. and Olson, E. N. (2008) 'microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart', *Genes Dev*, 22(23), pp. 3242-54.

Liu, X., Fan, Z., Zhao, T., Cao, W., Zhang, L., Li, H., Xie, Q., Tian, Y. and Wang, B. (2015) 'Plasma miR-1, miR-208, miR-499 as potential predictive biomarkers for acute myocardial infarction: An independent study of Han population', *Exp Gerontol*, 72, pp. 230-8.

Livak, K. J. and Schmittgen, T. D. (2001a) 'Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method', *Methods*, 25(4), pp. 402-8.

Livak, K. J. and Schmittgen, T. D. (2001b) 'Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method', *Methods*, 25(4), pp. 402-408.

Llave, C., Xie, Z., Kasschau, K. D. and Carrington, J. C. (2002) 'Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA', *Science*, 297(5589), pp. 2053-6.

Lo Gullo, A., Mandraffino, G., Bagnato, G., Aragona, C. O., Imbalzano, E., D'Ascola, A., Rotondo, F., Cinquegrani, A., Mormina, E., Saitta, C., Versace, A. G., Sardo, M. A., Lo Gullo, R., Loddo, S. and Saitta, A. (2015) 'Vitamin D Status in Rheumatoid Arthritis: Inflammation, Arterial Stiffness and Circulating Progenitor Cell Number', *PloS one*, 10(8), pp. e0134602-e0134602.

Loboz-Rudnicka, M., Jaroch, J., Bociaga, Z., Rzyczkowska, B., Uchmanowicz, I., Polanski, J., Dudek, K., Szuba, A. and Loboz-Grudzien, K. (2016) 'Impact of cardiovascular risk factors on carotid intima-media thickness: sex differences', *Clin Interv Aging*, 11, pp. 721-31.

Loimaala, A., Rontu, R., Vuori, I., Mercuri, M., Lehtimäki, T., Nenonen, A. and Bond, M. G. (2006) 'Blood leukocyte count is a risk factor for intima-media thickening and subclinical carotid atherosclerosis in middle-aged men', *Atherosclerosis*, 188(2), pp. 363-9.

Lorenz, M. W., Gao, L., Ziegelbauer, K., Norata, G. D., Empana, J. P., Schmidtmann, I., Lin, H.-J., McLachlan, S., Bokemark, L., Ronkainen, K., Amato, M., Schminke, U., Srinivasan, S. R., Lind, L., Okazaki, S., Stehouwer, C. D. A., Willeit, P., Polak, J. F., Steinmetz, H., Sander, D., Poppert, H., Desvarieux, M., Ikram, M. A., Johnsen, S. H., Staub, D., Sirtori, C. R., Iglseder, B., Beloqui, O., Engström, G., Friera, A., Rozza, F., Xie, W., Parraga, G., Grigore, L., Plichart, M., Blankenberg, S., Su, T.-C., Schmidt, C., Tuomainen, T.-P., Veglia, F., Völzke, H., Nijpels, G., Willeit, J., Sacco, R. L., Franco, O. H., Uthoff, H., Hedblad, B., Suarez, C., Izzo, R., Zhao, D., Wannarong, T., Catapano, A., Ducimetiere, P., Espinola-Klein, C., Chien, K.-L., Price, J. F., Bergström, G., Kauhanen, J., Tremoli, E., Dörr, M., Berenson, G., Kitagawa, K., Dekker, J. M., Kiechl, S., Sitzer, M., Bickel, H., Rundek, T., Hofman, A., Mathiesen, E. B., Castelnuovo, S., Landecho, M. F., Rosvall, M., Gabriel, R., de Luca, N., Liu, J., Baldassarre, D., Kavousi, M., de Groot, E., Bots, M. L., Yanez, D. N., Thompson, S. G. and group, P.-I. s. (2018) 'Predictive value for cardiovascular events of common carotid intima media thickness and its rate of change in individuals at high cardiovascular risk - Results from the PROG-IMT collaboration', *PloS one*, 13(4), pp. e0191172-e0191172.

Lorenz, M. W., Markus, H. S., Bots, M. L., Rosvall, M. and Sitzer, M. (2007) 'Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis', *Circulation*, 115(4), pp. 459-67.

Lorenz, M. W., Polak, J. F., Kavousi, M., Mathiesen, E. B., Völzke, H., Tuomainen, T.-P., Sander, D., Plichart, M., Catapano, A. L., Robertson, C. M., Kiechl, S., Rundek, T., Desvarieux, M., Lind, L., Schmid, C., DasMahapatra, P., Gao, L., Ziegelbauer, K., Bots, M. L. and Thompson, S. G. (2012) 'Carotid intima-media thickness progression to predict cardiovascular events in the general population (the PROG-IMT collaborative project): a meta-analysis of individual participant data', *The Lancet*, 379(9831), pp. 2053-2062.

Lorenz, M. W., Schaefer, C., Steinmetz, H. and Sitzer, M. (2010) 'Is carotid intima media thickness useful for individual prediction of cardiovascular risk? Ten-year results from the Carotid Atherosclerosis Progression Study (CAPS)', *Eur Heart J*, 31(16), pp. 2041-8.

Ltd., A. P.-M. (2020) *Metformin hydrochloride 850 mg film-coated tablets*. Available at: https://www.medicines.org.uk/emc/product/7348/smpc#gref (Accessed: 8 May 2021).

Lu, Q., Ma, Z., Ding, Y., Bedarida, T., Chen, L., Xie, Z., Song, P. and Zou, M.-H. (2019) 'Circulating miR-103a-3p contributes to angiotensin II-induced renal inflammation and fibrosis via a SNRK/NF-κB/p65 regulatory axis', *Nature Communications*, 10(1), pp. 2145.

Lund, E., Güttinger, S., Calado, A., Dahlberg, J. E. and Kutay, U. (2004) 'Nuclear export of microRNA precursors', *Science*, 303(5654), pp. 95-8.

Lupattelli, G., Lombardini, R., Schillaci, G., Ciuffetti, G., Marchesi, S., Siepi, D. and Mannarino, E. (2000) 'Flow-mediated vasoactivity and circulating adhesion molecules in hypertriglyceridemia: Association with small, dense LDL cholesterol particles', *American Heart Journal*, 140(3), pp. 521-526.

Lyall, D. M., Celis-Morales, C., Ward, J., Iliodromiti, S., Anderson, J. J., Gill, J. M. R., Smith, D. J., Ntuk, U. E., Mackay, D. F., Holmes, M. V., Sattar, N. and Pell, J. P. (2017) 'Association of Body Mass Index With Cardiometabolic Disease in the UK Biobank: A Mendelian Randomization Study', *JAMA Cardiol*, 2(8), pp. 882-889.

Lyons, D., Roy, S., Patel, M., Benjamin, N. and Swift, C. G. (1997) 'Impaired nitric oxide-mediated vasodilatation and total body nitric oxide production in healthy old age', *Clin Sci (Lond)*, 93(6), pp. 519-25.

Maarek, B., Simon, A. C., Levenson, J., Pithois-Merli, I. and Bouthier, J. (1987) 'Heterogeneity of the atherosclerotic process in systemic hypertension poorly controlled by drug treatment', *Am J Cardiol*, 59(5), pp. 414-7.

Maas, A. H. E. M. and Appelman, Y. E. A. (2010) 'Gender differences in coronary heart disease', *Netherlands heart journal : monthly journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation*, 18(12), pp. 598-602.

Maiorino, M. I., Bellastella, G., Petrizzo, M., Gicchino, M., Caputo, M., Giugliano, D. and Esposito, K. (2017) 'Effect of a Mediterranean diet on endothelial progenitor cells and carotid intima-media thickness in type 2 diabetes: Follow-up of a randomized trial', *Eur J Prev Cardiol*, 24(4), pp. 399-408.

Maki, K. C., Dicklin, M. R., Davidson, M. H., Mize, P. D. and Kulkarni, K. R. (2012) 'Indicators of the atherogenic lipoprotein phenotype measured with density gradient ultracentrifugation predict changes in carotid intima-media thickness in men and women', *Vasc Health Risk Manag*, 8, pp. 31-8.

Mancia, G., De Backer, G., Dominiczak, A., Cifkova, R., Fagard, R., Germano, G., Grassi, G., Heagerty, A. M., Kjeldsen, S. E., Laurent, S., Narkiewicz, K., Ruilope, L., Rynkiewicz, A., Schmieder, R. E., Boudier, H. A., Zanchetti, A., Vahanian, A., Camm, J., De Caterina, R., Dean, V., Dickstein, K., Filippatos, G., Funck-Brentano, C., Hellemans, I., Kristensen, S. D., McGregor, K., Sechtem, U., Silber, S., Tendera, M., Widimsky, P., Zamorano, J. L., Erdine, S., Kiowski, W., Agabiti-Rosei, E., Ambrosioni, E., Lindholm, L. H., Viigimaa, M., Adamopoulos, S., Agabiti-Rosei, E., Ambrosioni, E., Bertomeu, V., Clement, D., Erdine, S., Farsang, C., Gaita, D., Lip, G., Mallion, J. M., Manolis, A. J., Nilsson, P. M., O'Brien, E., Ponikowski, P., Redon, J., Ruschitzka, F., Tamargo, J., van Zwieten, P., Waeber, B. and Williams, B. (2007) '2007 Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC)', *J Hypertens*, 25(6), pp. 1105-87.

Mancia G, e. a. (2013) '2013 ESH/ESC Guidelines for the management of arterial hypertension: the Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC)', *J Hypertens*, 31(7), pp. 1281-357.

Mancia, G., Fagard, R., Narkiewicz, K., Redon, J., Zanchetti, A., Bohm, M., Christiaens, T., Cifkova, R., De Backer, G., Dominiczak, A., Galderisi, M., Grobbee, D. E., Jaarsma, T., Kirchhof, P., Kjeldsen, S. E., Laurent, S., Manolis, A. J., Nilsson, P. M., Ruilope, L. M., Schmieder, R. E., Sirnes, P. A., Sleight, P., Viigimaa, M., Waeber, B. and Zannad, F. (2013) '2013 ESH/ESC Guidelines for the management of arterial hypertension: the Task Force for the management of arterial hypertension of the European

Society of Hypertension (ESH) and of the European Society of Cardiology (ESC)', *J Hypertens*, 31(7), pp. 1281-357.

Mancia, G., Fagard, R., Narkiewicz, K., Redon, J., Zanchetti, A., Böhm, M., Christiaens, T., Cifkova, R., De Backer, G., Dominiczak, A., Galderisi, M., Grobbee, D. E., Jaarsma, T., Kirchhof, P., Kjeldsen, S. E., Laurent, S., Manolis, A. J., Nilsson, P. M., Ruilope, L. M., Schmieder, R. E., Sirnes, P. A., Sleight, P., Viigimaa, M., Waeber, B. and Zannad, F. (2014) '2013 ESH/ESC Practice Guidelines for the Management of Arterial Hypertension', *Blood Press*, 23(1), pp. 3-16.

Mannami, T., Konishi, M., Baba, S., Nishi, N. and Terao, A. (1997) 'Prevalence of asymptomatic carotid atherosclerotic lesions detected by high-resolution ultrasonography and its relation to cardiovascular risk factors in the general population of a Japanese city: the Suita study', *Stroke*, 28(3), pp. 518-25.

Mannucci, E., Ognibene, A., Cremasco, F., Bardini, G., Mencucci, A., Pierazzuoli, E., Ciani, S., Messeri, G. and Rotella, C. M. (2001) 'Effect of metformin on glucagon-like peptide 1 (GLP-1) and leptin levels in obese nondiabetic subjects', *Diabetes Care*, 24(3), pp. 489-94.

Mar-Aguilar, F., Mendoza-Ramírez, J. A., Malagón-Santiago, I., Espino-Silva, P. K., Santuario-Facio, S. K., Ruiz-Flores, P., Rodríguez-Padilla, C. and Reséndez-Pérez, D. (2013) 'Serum Circulating microRNA Profiling for Identification of Potential Breast Cancer Biomarkers', *Disease Markers*, 34, pp. 259454.

Marabita, F., de Candia, P., Torri, A., Tegnér, J., Abrignani, S. and Rossi, R. L. (2016) 'Normalization of circulating microRNA expression data obtained by quantitative real-time RT-PCR', *Brief Bioinform*, 17(2), pp. 204-12.

Marmot, M. and Brunner, E. (2005) 'Cohort Profile: the Whitehall II study', *Int J Epidemiol*, 34(2), pp. 251-6.

Matz, R. L., Schott, C., Stoclet, J. C. and Andriantsitohaina, R. (2000) 'Age-related endothelial dysfunction with respect to nitric oxide, endothelium-derived hyperpolarizing factor and cyclooxygenase products', *Physiol Res*, 49(1), pp. 11-8.

McBride, D., Carré, W., Sontakke, S. D., Hogg, C. O., Law, A., Donadeu, F. X. and Clinton, M. (2012) 'Identification of miRNAs associated with the follicular–luteal transition in the ruminant ovary', *REPRODUCTION*, 144(2), pp. 221-233.

McDonald, J. S., Milosevic, D., Reddi, H. V., Grebe, S. K. and Algeciras-Schimnich, A. (2011a) 'Analysis of circulating microRNA: preanalytical and analytical challenges', *Clin Chem*, 57(6), pp. 833-40.

McDonald, J. S., Milosevic, D., Reddi, H. V., Grebe, S. K. and Algeciras-Schimnich, A. (2011b) 'Analysis of Circulating MicroRNA: Preanalytical and Analytical Challenges', *Clinical Chemistry*, 57(6), pp. 833-840.

McEniery, C. M., Yasmin, Maki-Petaja, K. M., McDonnell, B. J., Munnery, M., Hickson, S. S., Franklin, S. S., Cockcroft, J. R. and Wilkinson, I. B. (2010) 'The impact of cardiovascular risk factors on aortic stiffness and wave reflections depends on age: The Anglo-Cardiff Collaborative Trial (ACCT III)', *Hypertension*, 56(4), pp. 591-597.

Members, A. T. F. and Mancia, G. and Fagard, R. and Narkiewicz, K. and Redon, J. and Zanchetti, A. and Böhm, M. and Christiaens, T. and Cifkova, R. and De Backer, G. and Dominiczak, A. and Galderisi, M. and Grobbee, D. E. and Jaarsma, T. and Lovic, D. and Mahrholdt, H. and Olsen, M. H. and Ostergren, J. and Parati, G. and Perk, J. and Polonia, J. and Popescu, B. A. and Reiner, Ž. and Rydén, L. and Sirenko, Y. and Stanton, A. and Struijker-Boudier, H. and Tsioufis, C. and van de Borne, P. and Vlachopoulos, C. and Volpe, M. and Wood, D. A. (2013) '2013 ESH/ESC Guidelines for the management of arterial hypertension: The Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC)', *European Heart Journal*, 34(28), pp. 2159-2219.

Mensà, E., Giuliani, A., Matacchione, G., Gurău, F., Bonfigli, A. R., Romagnoli, F., De Luca, M., Sabbatinelli, J. and Olivieri, F. (2019) 'Circulating miR-146a in healthy aging and type 2 diabetes: Age- and gender-specific trajectories', *Mechanisms of Ageing and Development*, 180, pp. 1-10.

Meyer, A. A., Kundt, G., Steiner, M., Schuff-Werner, P. and Kienast, W. (2006) 'Impaired flow-mediated vasodilation, carotid artery intima-media thickening, and elevated endothelial plasma markers in obese children: the impact of cardiovascular risk factors', *Pediatrics*, 117(5), pp. 1560-7.

Millasseau, S. C., Kelly, R. P., Ritter, J. M. and Chowienczyk, P. J. (2002) 'Determination of age-related increases in large artery stiffness by digital pulse contour analysis', *Clin Sci (Lond)*, 103(4), pp. 371-7.

Mitchell, G. F., Hwang, S. J., Vasan, R. S., Larson, M. G., Pencina, M. J., Hamburg, N. M., Vita, J. A., Levy, D. and Benjamin, E. J. (2010) 'Arterial stiffness and cardiovascular events: the Framingham Heart Study', *Circulation*, 121(4), pp. 505-11.

Mitchell, P. S., Parkin, R. K., Kroh, E. M., Fritz, B. R., Wyman, S. K., Pogosova-Agadjanyan, E. L., Peterson, A., Noteboom, J., O'Briant, K. C., Allen, A., Lin, D. W., Urban, N., Drescher, C. W., Knudsen, B. S., Stirewalt, D. L., Gentleman, R., Vessella, R. L., Nelson, P. S., Martin, D. B. and Tewari, M. (2008a) 'Circulating microRNAs as stable blood-based markers for cancer detection', *Proceedings of the National Academy of Sciences*, 105(30), pp. 10513-10518.

Mitchell, P. S., Parkin, R. K., Kroh, E. M., Fritz, B. R., Wyman, S. K., Pogosova-Agadjanyan, E. L., Peterson, A., Noteboom, J., O'Briant, K. C., Allen, A., Lin, D. W., Urban, N., Drescher, C. W., Knudsen, B. S., Stirewalt, D. L., Gentleman, R., Vessella, R. L., Nelson, P. S., Martin, D. B. and Tewari, M. (2008b) 'Circulating microRNAs as stable blood-based markers for cancer detection', *Proc Natl Acad Sci U S A*, 105(30), pp. 10513-8.

Miyazaki, H., Matsuoka, H., Cooke, J. P., Usui, M., Ueda, S., Okuda, S. and Imaizumi, T. (1999) 'Endogenous Nitric Oxide Synthase Inhibitor', *Circulation*, 99(9), pp. 1141-1146.

Mohan, A., Sada, S., Kumar, B. S., Sarma, K. V. S., Devi, B. V., Rao, P. V. L. N. S., Rajasekhar, D. and Katyarmal, D. T. (2014) 'Subclinical atherosclerosis in patients with rheumatoid arthritis by utilizing carotid intima-media thickness as a surrogate marker', *The Indian journal of medical research*, 140(3), pp. 379-386.

Moiseeva, O., Deschênes-Simard, X., St-Germain, E., Igelmann, S., Huot, G., Cadar, A. E., Bourdeau, V., Pollak, M. N. and Ferbeyre, G. (2013) 'Metformin inhibits the senescence-associated secretory phenotype by interfering with IKK/NF-κB activation', *Aging Cell*, 12(3), pp. 489-98.

Moldovan, L., Batte, K., Wang, Y., Wisler, J. and Piper, M. (2013) 'Analyzing the circulating microRNAs in exosomes/extracellular vesicles from serum or plasma by qRT-PCR', *Methods Mol Biol*, 1024, pp. 129-45.

Moldovan, L., Batte, K. E., Trgovcich, J., Wisler, J., Marsh, C. B. and Piper, M. (2014) 'Methodological challenges in utilizing miRNAs as circulating biomarkers', *J Cell Mol Med*, 18(3), pp. 371-90.

Moore, K. J., Rayner, K. J., Suárez, Y. and Fernández-Hernando, C. (2010) 'microRNAs and cholesterol metabolism', *Trends in endocrinology and metabolism: TEM*, 21(12), pp. 699-706.

Mora, S., Cook, N., Buring, J. E., Ridker, P. M. and Lee, I. M. (2007) 'Physical activity and reduced risk of cardiovascular events: potential mediating mechanisms', *Circulation*, 116(19), pp. 2110-8.

Moret, I., Sánchez-Izquierdo, D., Iborra, M., Tortosa, L., Navarro-Puche, A., Nos, P., Cervera, J. and Beltrán, B. (2013) 'Assessing an improved protocol for plasma microRNA extraction', *PLoS One*, 8(12), pp. e82753.

Morrow, D. A., de Lemos, J. A., Sabatine, M. S. and Antman, E. M. (2003) 'The search for a biomarker of cardiac ischemia', *Clin Chem*, 49(4), pp. 537-9.

Mueller, C., McDonald, K., de Boer, R. A., Maisel, A., Cleland, J. G. F., Kozhuharov, N., Coats, A. J. S., Metra, M., Mebazaa, A., Ruschitzka, F.,

Lainscak, M., Filippatos, G., Seferovic, P. M., Meijers, W. C., Bayes-Genis, A., Mueller, T., Richards, M., Januzzi Jr, J. L. and Cardiology, o. b. o. t. H. F. A. o. t. E. S. o. (2019) 'Heart Failure Association of the European Society of Cardiology practical guidance on the use of natriuretic peptide concentrations', *European Journal of Heart Failure*, 21(6), pp. 715-731.

Musaddaq, G., Shahzad, N., Ashraf, M. A. and Arshad, M. I. (2019) 'Circulating liver-specific microRNAs as noninvasive diagnostic biomarkers of hepatic diseases in human', *Biomarkers*, 24(2), pp. 103-109.

'Multiple Risk Factor Intervention Trial: Risk Factor Changes and Mortality Results', (1982) *JAMA*, 248(12), pp. 1465-1477.

Nakashima, T. and Tanikawa, J. (1971) 'A study of human aortic distensibility with relation to atherosclerosis and aging', *Angiology*, 22(8), pp. 477-90.

Narverud, I., Retterstøl, K., Iversen, P. O., Halvorsen, B., Ueland, T., Ulven, S. M., Ose, L., Aukrust, P., Veierød, M. B. and Holven, K. B. (2014) 'Markers of atherosclerotic development in children with familial hypercholesterolemia: A literature review', *Atherosclerosis*, 235(2), pp. 299-309.

Naslund, E., Gutniak, M., Skogar, S., Rossner, S. and Hellstrom, P. M. (1998) 'Glucagon-like peptide 1 increases the period of postprandial satiety and slows gastric emptying in obese men', *Am J Clin Nutr*, 68(3), pp. 525-30.

Nathan, D. M., Buse, J. B., Davidson, M. B., Ferrannini, E., Holman, R. R., Sherwin, R. and Zinman, B. (2009) 'Medical Management of Hyperglycemia in Type 2 Diabetes: A Consensus Algorithm for the Initiation and Adjustment of Therapy', *A consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes*, 32(1), pp. 193-203.

National Institute for Health and care Excellent (2017) *Cardiovascular disease: risk assessment and reduction, including lipid modification*. Available at: https://www.nice.org.uk/guidance/cg181 (Accessed: 22 / September/ 2020.

National Institute for Health and care Excellent (2019) *Type 2 diabetes in adults: management | Guidance and guidelines|* Available at: https://www.nice.org.uk/guidance/ng28/chapter/1-recommendations (Accessed: 22/ september/2020.

Nelson, M. (2005) 'DIETARY INTAKE MEASUREMENT | Validation', in Caballero, B. (ed.) *Encyclopedia of Human Nutrition (Second Edition)*. Oxford: Elsevier, pp. 16-26.

Newman, A. B., Dodson, J. A., Church, T. S., Buford, T. W., Fielding, R. A., Kritchevsky, S., Beavers, D., Pahor, M., Stafford, R. S., Szady, A. D., Ambrosius, W. T., McDermott, M. M. and Group, f. t. L. S. (2016) 'Cardiovascular Events in a Physical Activity Intervention Compared With a Successful Aging Intervention: The LIFE Study Randomized Trial', *JAMA Cardiology*, 1(5), pp. 568-574.

NICE (2016) *Cardiovascular disease: risk assessment and reduction, including lipid modification*. Available at: https://www.nice.org.uk/guidance/cg181/chapter/1-recommendations (Accessed: 23 / September/2020.

NICE (2019) *Type 2 diabetes in adults: management*. Available at: https://www.nice.org.uk/guidance/ng28/chapter/Introduction (Accessed: 23/ September/2020.

Nürnberger, J., Dammer, S., Opazo Saez, A., Philipp, T. and Schäfers, R. F. (2003) 'Diastolic blood pressure is an important determinant of augmentation index and pulse wave velocity in young, healthy males', *J Hum Hypertens*, 17(3), pp. 153-8.

O'Brien, J., Hayder, H., Zayed, Y. and Peng, C. (2018) 'Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation', *Frontiers in Endocrinology*, 9(402).

O'Leary, D. H. and Polak, J. F. (2002) 'Intima-media thickness: a tool for atherosclerosis imaging and event prediction', *Am J Cardiol*, 90(10c), pp. 18l-21l.

O'Leary, D. H., Polak, J. F., Kronmal, R. A., Kittner, S. J., Bond, M. G., Wolfson, S. K., Jr., Bommer, W., Price, T. R., Gardin, J. M. and Savage, P. J. (1992) 'Distribution and correlates of sonographically detected carotid artery disease in the Cardiovascular Health Study. The CHS Collaborative Research Group', *Stroke*, 23(12), pp. 1752-60.

O'Leary, D. H., Polak, J. F., Kronmal, R. A., Manolio, T. A., Burke, G. L. and Wolfson, S. K., Jr. (1999) 'Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group', *N Engl J Med*, 340(1), pp. 14-22.

O'Rourke, M. (1990) 'Arterial stiffness, systolic blood pressure, and logical treatment of arterial hypertension', *Hypertension*, 15(4), pp. 339-347.

O'Sullivan, J. F., Neylon, A., Fahy, E. F., Yang, P., McGorrian, C. and Blake, G. J. (2019) 'MiR-93-5p is a novel predictor of coronary in-stent restenosis', *Heart Asia*, 11(1), pp. e011134-e011134.

O'Leary, D. H., Polak, J. F., Kronmal, R. A., Savage, P. J., Borhani, N. O., Kittner, S. J., Tracy, R., Gardin, J. M., Price, T. R. and Furberg, C. D. (1996) 'Thickening of the Carotid Wall', *Stroke*, 27(2), pp. 224-231.

Office of the Surgeon, G., Office on, S. and Health (2004) 'Reports of the Surgeon General', *The Health Consequences of Smoking: A Report of the Surgeon General*. Atlanta (GA): Centers for Disease Control and Prevention (US).

Okamura, T., Sekikawa, A., Sawamura, T., Kadowaki, T., Barinas-Mitchell, E., Mackey, R. H., Kadota, A., Evans, R. W., Edmundowicz, D., Higashiyama, A., Nakamura, Y., Abbott, R. D., Miura, K., Fujiyoshi, A., Fujita, Y., Murakami, Y., Miyamatsu, N., Kakino, A., Maegawa, H., Murata, K., Horie, M., Mitsunami, K., Kashiwagi, A., Kuller, L. H. and Ueshima, H. (2013) 'LOX-1 ligands containing apolipoprotein B and carotid intima-media thickness in middle-aged community-dwelling US Caucasian and Japanese men', *Atherosclerosis*, 229(1), pp. 240-5.

Ortega, F. J., Mercader, J. M., Catalán, V., Moreno-Navarrete, J. M., Pueyo, N., Sabater, M., Gómez-Ambrosi, J., Anglada, R., Fernández-Formoso, J. A., Ricart, W., Frühbeck, G. and Fernández-Real, J. M. (2013) 'Targeting the circulating microRNA signature of obesity', *Clin Chem*, 59(5), pp. 781-92.

Paim, L. R., Schreiber, R., Matos-Souza, J. R., Silva, A. A., Campos, L. F., Azevedo, E. R., Alonso, K., de Rossi, G., Etchebehere, M., Gorla, J. I., Cliquet, A. and Nadruz, W. (2013) 'Oxidized low-density lipoprotein, matrix-metalloproteinase-8 and carotid atherosclerosis in spinal cord injured subjects', *Atherosclerosis*, 231(2), pp. 341-345.

Park, K.-H. and Park, W. J. (2015) 'Endothelial Dysfunction: Clinical Implications in Cardiovascular Disease and Therapeutic Approaches', *Journal of Korean medical science*, 30(9), pp. 1213-1225.

Pedersen, H. K., Gudmundsdottir, V., Nielsen, H. B., Hyotylainen, T., Nielsen, T., Jensen, B. A., Forslund, K., Hildebrand, F., Prifti, E., Falony, G., Le Chatelier, E., Levenez, F., Doré, J., Mattila, I., Plichta, D. R., Pöhö, P., Hellgren, L. I., Arumugam, M., Sunagawa, S., Vieira-Silva, S., Jørgensen, T., Holm, J. B., Trošt, K., Kristiansen, K., Brix, S., Raes, J., Wang, J., Hansen, T., Bork, P., Brunak, S., Oresic, M., Ehrlich, S. D. and Pedersen, O. (2016) 'Human gut microbes impact host serum metabolome and insulin sensitivity', *Nature*, 535(7612), pp. 376-81.

Peluso, R., Caso, F., Tasso, M., Sabbatino, V., Lupoli, R., Dario Di Minno, M. N., Ursini, F., Costa, L. and Scarpa, R. (2019) 'Biomarkers of subclinical atherosclerosis in patients with psoriatic arthritis', *Open access rheumatology : research and reviews*, 11, pp. 143-156.

Peña, A. S., Wiltshire, E., MacKenzie, K., Gent, R., Piotto, L., Hirte, C. and Couper, J. (2006) 'Vascular Endothelial and Smooth Muscle Function

Relates to Body Mass Index and Glucose in Obese and Nonobese Children', *The Journal of Clinical Endocrinology & Metabolism*, 91(11), pp. 4467-4471.

Peters, S. A., Lind, L., Palmer, M. K., Grobbee, D. E., Crouse, J. R., 3rd, O'Leary, D. H., Evans, G. W., Raichlen, J., Bots, M. L., den Ruijter, H. M. and group, M. s. (2012) 'Increased age, high body mass index and low HDL-C levels are related to an echolucent carotid intima-media: the METEOR study', *J Intern Med*, 272(3), pp. 257-66.

Petit, J. M., Guiu, B., Masson, D., Duvillard, L., Jooste, V., Buffier, P., Terriat, B., Bouillet, B., Brindisi, M. C., Loffroy, R., Robin, I., Hillon, P., Cercueil, J. P. and Verges, B. (2010) 'Specifically PNPLA3-mediated accumulation of liver fat in obese patients with type 2 diabetes', *J Clin Endocrinol Metab*, 95(12), pp. E430-6.

Polak, J. F., Pencina, M. J., Meisner, A., Pencina, K. M., Brown, L. S., Wolf, P. A. and D'Agostino, R. B., Sr. (2010) 'Associations of carotid artery intima-media thickness (IMT) with risk factors and prevalent cardiovascular disease: comparison of mean common carotid artery IMT with maximum internal carotid artery IMT', *J Ultrasound Med*, 29(12), pp. 1759-68.

Ponikowski, P., Voors, A. A., Anker, S. D., Bueno, H., Cleland, J. G. F., Coats, A. J. S., Falk, V., González-Juanatey, J. R., Harjola, V.-P., Jankowska, E. A., Jessup, M., Linde, C., Nihoyannopoulos, P., Parissis, J. T., Pieske, B., Riley, J. P., Rosano, G. M. C., Ruilope, L. M., Ruschitzka, F., Rutten, F. H., van der Meer, P. and Group, E. S. D. (2016) '2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC)Developed with the special contribution of the Heart Failure Association (HFA) of the ESC', *European Heart Journal*, 37(27), pp. 2129-2200.

Popele, N. M. v., Grobbee, D. E., Bots, M. L., Asmar, R., Topouchian, J., Reneman, R. S., Hoeks, A. P. G., Kuip, D. A. M. v. d., Hofman, A. and Witteman, J. C. M. (2001) 'Association Between Arterial Stiffness and Atherosclerosis', *Stroke*, 32(2), pp. 454-460.

Poredos, P. and Kek, A. (2000) 'Relation of blunted dilation of the brachial artery in insulin-dependent diabetes mellitus to microalbuminuria', *Am J Cardiol*, 86(3), pp. 364-7.

Powrozek, T., Kuznar-Kaminska, B., Dziedzic, M., Mlak, R., Batura-Gabryel, H., Sagan, D., Krawczyk, P., Milanowski, J. and Malecka-Massalska, T. (2017) 'The diagnostic role of plasma circulating precursors of miRNA-944 and miRNA-3662 for non-small cell lung cancer detection', *Pathol Res Pract*, 213(11), pp. 1384-1387.

Preiss, D., Lloyd, S. M., Ford, I., McMurray, J. J., Holman, R. R., Welsh, P., Fisher, M., Packard, C. J. and Sattar, N. (2014) 'Metformin for non-

diabetic patients with coronary heart disease (the CAMERA study): a randomised controlled trial', *Lancet Diabetes Endocrinol*, 2(2), pp. 116-24.

Preiss, D., Rankin, N., Welsh, P., Holman, R. R., Kangas, A. J., Soininen, P., Würtz, P., Ala-Korpela, M. and Sattar, N. (2016) 'Effect of metformin therapy on circulating amino acids in a randomized trial: the CAMERA study', *Diabet Med*, 33(11), pp. 1569-1574.

Price, J. F., Tzoulaki, I., Lee, A. J. and Fowkes, F. G. (2007) 'Ankle brachial index and intima media thickness predict cardiovascular events similarly and increased prediction when combined', *J Clin Epidemiol*, 60(10), pp. 1067-75.

Pritchard, C. C., Kroh, E., Wood, B., Arroyo, J. D., Dougherty, K. J., Miyaji, M. M., Tait, J. F. and Tewari, M. (2012) 'Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies', *Cancer Prev Res (Phila)*, 5(3), pp. 492-497.

Quan, X., Ji, Y., Zhang, C., Guo, X., Zhang, Y., Jia, S., Ma, W., Fan, Y. and Wang, C. (2018) 'Circulating MiR-146a May be a Potential Biomarker of Coronary Heart Disease in Patients with Subclinical Hypothyroidism', *Cellular Physiology and Biochemistry*, 45(1), pp. 226-236.

Rajendran, P., Rengarajan, T., Thangavel, J., Nishigaki, Y., Sakthisekaran, D., Sethi, G. and Nishigaki, I. (2013) 'The vascular endothelium and human diseases', *International journal of biological sciences*, 9(10), pp. 1057-1069.

Rathsman, B., Rosfors, S., Sjöholm, A. and Nyström, T. (2012) 'Early signs of atherosclerosis are associated with insulin resistance in non-obese adolescent and young adults with type 1 diabetes', *Cardiovascular diabetology*, 11, pp. 145-145.

Raut, S. K., Singh, G. B., Rastogi, B., Saikia, U. N., Mittal, A., Dogra, N., Singh, S., Prasad, R. and Khullar, M. (2016) 'miR-30c and miR-181a synergistically modulate p53-p21 pathway in diabetes induced cardiac hypertrophy', *Molecular and Cellular Biochemistry*, 417(1), pp. 191-203.

Redfield, M. M., Rodeheffer, R. J., Jacobsen, S. J., Mahoney, D. W., Bailey, K. R. and Burnett, J. C. (2002) 'Plasma brain natriuretic peptide concentration: impact of age and gender', *Journal of the American College of Cardiology*, 40(5), pp. 976-982.

Reed, D. M., MacLean, C. J. and Hayashi, T. (1987) 'Predictors of atherosclerosis in the Honolulu Heart Program. I. Biologic, dietary, and lifestyle characteristics', *Am J Epidemiol*, 126(2), pp. 214-25.

Relf, I. R., Lo, C. S., Myers, K. A. and Wahlqvist, M. L. (1986) 'Risk factors for changes in aorto-iliac arterial compliance in healthy men', *Arteriosclerosis: An Official Journal of the American Heart Association, Inc.*, 6(1), pp. 105-108.

Ren, L., Cai, J., Liang, J., Li, W. and Sun, Z. (2015) 'Impact of Cardiovascular Risk Factors on Carotid Intima-Media Thickness and Degree of Severity: A Cross-Sectional Study', *PloS one*, 10(12), pp. e0144182-e0144182.

Rhee, J. W. and Wu, J. C. (2013) 'Advances in nanotechnology for the management of coronary artery disease', *Trends Cardiovasc Med*, 23(2), pp. 39-45.

Ribeiro, A. H., Lotufo, P. A., Fujita, A., Goulart, A. C., Chor, D., Mill, J. G., Bensenor, I. M. and Santos, I. S. (2017) 'Association Between Short-Term Systolic Blood Pressure Variability and Carotid Intima-Media Thickness in ELSA-Brasil Baseline', *American Journal of Hypertension*, 30(10), pp. 954-960.

Rice, J., Roberts, H., Rai, S. N. and Galandiuk, S. (2015) 'Housekeeping genes for studies of plasma microRNA: A need for more precise standardization', *Surgery*, 158(5), pp. 1345-51.

Ridker, P. M., Buring, J. E., Rifai, N. and Cook, N. R. (2007) 'Development and validation of improved algorithms for the assessment of global cardiovascular risk in women: the Reynolds Risk Score', *Jama*, 297(6), pp. 611-9.

Ridker, P. M., Paynter, N. P., Rifai, N., Gaziano, J. M. and Cook, N. R. (2008) 'C-reactive protein and parental history improve global cardiovascular risk prediction: the Reynolds Risk Score for men', *Circulation*, 118(22), pp. 2243-51, 4p following 2251.

Ristić, G. G., Lepić, T., Glisić, B., Stanisavljević, D., Vojvodić, D., Petronijević, M. and Stefanović, D. (2010) 'Rheumatoid arthritis is an independent risk factor for increased carotid intima-media thickness: impact of anti-inflammatory treatment', *Rheumatology (Oxford)*, 49(6), pp. 1076-81.

Ristić, G. G., Subota, V., Lepić, T., Stanisavljević, D., Glišić, B., Ristić, A. D., Petronijević, M. and Stefanović, D. Z. (2015) 'Subclinical Atherosclerosis in Patients with Rheumatoid Arthritis and Low Cardiovascular Risk: The Role of von Willebrand Factor Activity', *PLoS One*, 10(8), pp. e0130462.

Roman, M. J., Saba, P. S., Pini, R., Spitzer, M., Pickering, T. G., Rosen, S., Alderman, M. H. and Devereux, R. B. (1992) 'Parallel cardiac and vascular adaptation in hypertension', *Circulation*, 86(6), pp. 1909-18.

Roser, A. E., Caldi Gomes, L., Schünemann, J., Maass, F. and Lingor, P. (2018) 'Circulating miRNAs as Diagnostic Biomarkers for Parkinson's Disease', *Frontiers in Neuroscience*, 12(625).

Rosvall, M., Janzon, L., Berglund, G., Engstrom, G. and Hedblad, B. (2005) 'Incident coronary events and case fatality in relation to common carotid intima-media thickness', *J Intern Med*, 257(5), pp. 430-7.

Rosvall, M., Persson, M., Ostling, G., Nilsson, P. M., Melander, O., Hedblad, B. and Engstrom, G. (2015) 'Risk factors for the progression of carotid intima-media thickness over a 16-year follow-up period: the Malmo Diet and Cancer Study', *Atherosclerosis*, 239(2), pp. 615-21.

Roth, C., Stückrath, I., Pantel, K., Izbicki, J. R., Tachezy, M. and Schwarzenbach, H. (2012) 'Low levels of cell-free circulating miR-361-3p and miR-625* as blood-based markers for discriminating malignant from benign lung tumors', *PLoS One*, 7(6), pp. e38248.

Rothman KJ, G. S. (1998) *Modern Epidemiology*. Second Edition. edn. Philadelphia: Lippincott Williams and Wilkins.

Ruby, J. G., Jan, C. H. and Bartel, D. P. (2007) 'Intronic microRNA precursors that bypass Drosha processing', *Nature*, 448(7149), pp. 83-6.

Ruggeri, C., Gioffré, S., Chiesa, M., Buzzetti, M., Milano, G., Scopece, A., Castiglioni, L., Pontremoli, M., Sironi, L., Pompilio, G., Colombo, G. I. and D'Alessandra, Y. (2018) 'A Specific Circulating MicroRNA Cluster Is Associated to Late Differential Cardiac Response to Doxorubicin-Induced Cardiotoxicity In Vivo', *Disease Markers*, 2018, pp. 8395651.

Rundek, T., Blanton, S. H., Bartels, S., Dong, C., Raval, A., Demmer, R. T., Cabral, D., Elkind, M. S., Sacco, R. L. and Desvarieux, M. (2013) 'Traditional risk factors are not major contributors to the variance in carotid intima-media thickness', *Stroke*, 44(8), pp. 2101-8.

Said, M. A., Eppinga, R. N., Lipsic, E., Verweij, N. and Harst, P. v. d. (2018a) 'Relationship of Arterial Stiffness Index and Pulse Pressure With Cardiovascular Disease and Mortality', *Journal of the American Heart Association*, 7(2), pp. e007621.

Said, M. A., Eppinga, R. N., Lipsic, E., Verweij, N. and van der Harst, P. (2018b) 'Relationship of Arterial Stiffness Index and Pulse Pressure With Cardiovascular Disease and Mortality', *Journal of the American Heart Association*, 7(2), pp. e007621.

Salonen, J. T. (1988) 'Is there a continuing need for longitudinal epidemiologic research? The Kuopio Ischaemic Heart Disease Risk Factor Study', *Ann Clin Res*, 20(1-2), pp. 46-50.

SALONEN, J. T. and SALONEN, R. (1990) 'Association of Serum Low Density Lipoprotein Cholesterol, Smoking and Hypertension with Different Manifestations of Atherosclerosis', *International Journal of Epidemiology*, 19(4), pp. 911-917.

Salonen, J. T. and Salonen, R. (1991a) 'Ultrasonographically assessed carotid morphology and the risk of coronary heart disease', *Arterioscler Thromb*, 11(5), pp. 1245-9.

Salonen, R. and Salonen, J. T. (1991b) 'Determinants of carotid intima-media thickness: a population-based ultrasonography study in eastern Finnish men', *J Intern Med*, 229(3), pp. 225-31.

Sanches Pde, L., Mello, M. T., Fonseca, F. A., Elias, N., Piano, A., Carnier, J., Tock, L., Oyama, L. M., Tufik, S. and Dâmaso, A. (2012) 'Insulin resistance can impair reduction on carotid intima-media thickness in obese adolescents', *Arq Bras Cardiol*, 99(4), pp. 892-8.

Sasaki, A., Uehara, M., Horiuchi, N., Hasegawa, K. and Shimizu, T. (1997) 'A 15-year follow-up study of patients with non-insulin-dependent diabetes mellitus (NIDDM) in Osaka, Japan. Factors predictive of the prognosis of diabetic patients', *Diabetes Res Clin Pract*, 36(1), pp. 41-7.

Sato, F., Tsuchiya, S., Terasawa, K. and Tsujimoto, G. (2009) 'Intra-platform repeatability and inter-platform comparability of microRNA microarray technology', *PLoS One*, 4(5), pp. e5540.

Schott, L. L., Wildman, R. P., Brockwell, S., Simkin-Silverman, L. R., Kuller, L. H. and Sutton-Tyrrell, K. (2004) 'Segment-specific effects of cardiovascular risk factors on carotid artery intima-medial thickness in women at midlife', *Arterioscler Thromb Vasc Biol*, 24(10), pp. 1951-6.

Schutte, R., Schutte, A. E., Huisman, H. W., van Rooyen, J. M., Malan, N. T., Péter, S., Fourie, C. M., van der Westhuizen, F. H., Louw, R., Botha, C. A. and Malan, L. (2009) 'Blood glutathione and subclinical atherosclerosis in African men: the SABPA Study', *Am J Hypertens*, 22(11), pp. 1154-9.

Schwarz, D. S., Hutvágner, G., Du, T., Xu, Z., Aronin, N. and Zamore, P. D. (2003) 'Asymmetry in the Assembly of the RNAi Enzyme Complex', *Cell*, 115(2), pp. 199-208.

Shadwick, R. E. (1999) 'Mechanical design in arteries', *Journal of Experimental Biology*, 202(23), pp. 3305-3313.

Shah, A. D., Langenberg, C., Rapsomaniki, E., Denaxas, S., Pujades-Rodriguez, M., Gale, C. P., Deanfield, J., Smeeth, L., Timmis, A. and Hemingway, H. (2015) 'Type 2 diabetes and incidence of cardiovascular diseases: a

cohort study in 1·9 million people', *The Lancet Diabetes & Endocrinology*, 3(2), pp. 105-113.

Shah, P. K. (2010) 'Screening asymptomatic subjects for subclinical atherosclerosis: can we, does it matter, and should we?', *J Am Coll Cardiol*, 56(2), pp. 98-105.

Shah, T., Casas, J. P., Cooper, J. A., Tzoulaki, I., Sofat, R., McCormack, V., Smeeth, L., Deanfield, J. E., Lowe, G. D., Rumley, A., Fowkes, F. G., Humphries, S. E. and Hingorani, A. D. (2009) 'Critical appraisal of CRP measurement for the prediction of coronary heart disease events: new data and systematic review of 31 prospective cohorts', *Int J Epidemiol*, 38(1), pp. 217-31.

Sharma, S. and Eghbali, M. (2014) 'Influence of sex differences on microRNA gene regulation in disease', *Biol Sex Differ*, 5(1), pp. 3.

Shepherd, J., Blauw, G. J., Murphy, M. B., Bollen, E. L. E. M., Buckley, B. M., Cobbe, S. M., Ford, I., Gaw, A., Hyland, M., Jukema, J. W., Kamper, A. M., Macfarlane, P. W., Meinders, A. E., Norrie, J., Packard, C. J., Perry, I. J., Stott, D. J., Sweeney, B. J., Twomey, G. and Westendorp, R. G. J. (2002) 'Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial', *The Lancet*, 360(9346), pp. 1623-1630.

Shih, D. M., Xia, Y. R., Wang, X. P., Miller, E., Castellani, L. W., Subbanagounder, G., Cheroutre, H., Faull, K. F., Berliner, J. A., Witztum, J. L. and Lusis, A. J. (2000) 'Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis', *J Biol Chem*, 275(23), pp. 17527-35.

Shimizu, Y., Nakazato, M., Sato, S., Koyamatsu, J., Yamanashi, H., Nagayoshi, M., Kadota, K., Hayashida, N., Yamasaki, H., Kusano, Y., Takamura, N., Aoyagi, K. and Maeda, T. (2015) 'Association between hemoglobin A1c and carotid atherosclerosis in rural community-dwelling elderly Japanese men', *Journal of Physiological Anthropology*, 34(1), pp. 16.

Shimizu, Y., Sato, S., Koyamatsu, J., Yamanashi, H., Tamai, M., Kadota, K., Arima, K., Yamasaki, H., Takamura, N., Aoyagi, K. and Maeda, T. (2014) 'Subclinical carotid atherosclerosis and hyperuricemia in relation to renal impairment in a rural Japanese population: The Nagasaki Islands study', *Atherosclerosis*, 233(2), pp. 525-529.

Silacci, P., Desgeorges, A., Mazzolai, L., Chambaz, C. and Hayoz, D. (2001) 'Flow Pulsatility Is a Critical Determinant of Oxidative Stress in Endothelial Cells', *Hypertension*, 38(5), pp. 1162-1166.

Simpson, R. J., Kalra, H. and Mathivanan, S. (2012) 'ExoCarta as a resource for exosomal research', *Journal of extracellular vesicles*, 1, pp. 10.3402/jev.v1i0.18374.

Sinning, C., Wild, P. S., Echevarria, F. M., Wilde, S., Schnabel, R., Lubos, E., Herkenhoff, S., Bickel, C., Klimpe, S., Gori, T., Munzel, T. F., Blankenberg, S. and Espinola-Klein, C. (2011) 'Sex differences in early carotid atherosclerosis (from the community-based Gutenberg-Heart Study)', *Am J Cardiol*, 107(12), pp. 1841-7.

Soh, J., Iqbal, J., Queiroz, J., Fernandez-Hernando, C. and Hussain, M. M. (2013a) 'MicroRNA-30c reduces hyperlipidemia and atherosclerosis in mice by decreasing lipid synthesis and lipoprotein secretion', *Nature medicine*, 19(7), pp. 892-900.

Soh, J., Iqbal, J., Queiroz, J., Fernandez-Hernando, C. and Hussain, M. M. (2013b) 'MicroRNA-30c reduces hyperlipidemia and atherosclerosis in mice by decreasing lipid synthesis and lipoprotein secretion', *Nat Med*, 19(7), pp. 892-900.

Sohel, M. H. (2016) 'Extracellular/Circulating MicroRNAs: Release Mechanisms, Functions and Challenges', *Achievements in the Life Sciences*, 10(2), pp. 175-186.

Sollinger, D., Mohaupt, M. G., Wilhelm, A., Uehlinger, D., Frey, F. J. and Eisenberger, U. (2006) 'Arterial stiffness assessed by digital volume pulse correlates with comorbidity in patients with ESRD', *Am J Kidney Dis*, 48(3), pp. 456-63.

Spence, J. D. (2014) 'Time course of atherosclerosis regression', *Atherosclerosis*, 235(2), pp. 347-348.

Spence, J. D., Barnett, P. A., Bulman, D. E. and Hegele, R. A. (1999) 'An approach to ascertain probands with a non-traditional risk factor for carotid atherosclerosis', *Atherosclerosis*, 144(2), pp. 429-34.

Spring, B., Moller, A. C., Colangelo, L. A., Siddique, J., Roehrig, M., Daviglus, M. L., Polak, J. F., Reis, J. P., Sidney, S. and Liu, K. (2014) 'Healthy lifestyle change and subclinical atherosclerosis in young adults: Coronary Artery Risk Development in Young Adults (CARDIA) study', *Circulation*, 130(1), pp. 10-7.

Sredni, S., Gadd, S., Jafari, N. and Huang, C.-C. (2011) 'A Parallel Study of mRNA and microRNA Profiling of Peripheral Blood in Young Adult Women', *Frontiers in Genetics*, 2(49).

Stanczyk, J., Pedrioli, D. M., Brentano, F., Sanchez-Pernaute, O., Kolling, C., Gay, R. E., Detmar, M., Gay, S. and Kyburz, D. (2008) 'Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis', *Arthritis Rheum*, 58(4), pp. 1001-9.

Steffen, B. T., Guan, W., Remaley, A. T., Stein, J. H., Tattersall, M. C., Kaufman, J. and Tsai, M. Y. (2017) 'Apolipoprotein B is associated with

carotid atherosclerosis progression independent of individual cholesterol measures in a 9-year prospective study of Multi-Ethnic Study of Atherosclerosis participants', *Journal of clinical lipidology*, 11(5), pp. 1181-1191.e1.

Stegemann, C., Pechlaner, R., Willeit, P., Langley, S. R., Mangino, M., Mayr, U., Menni, C., Moayyeri, A., Santer, P., Rungger, G., Spector, T. D., Willeit, J., Kiechl, S. and Mayr, M. (2014) 'Lipidomics profiling and risk of cardiovascular disease in the prospective population-based Bruneck study', *Circulation*, 129(18), pp. 1821-31.

Stewart, A. D., Jiang, B., Millasseau, S. C., Ritter, J. M. and Chowienczyk, P. J. (2006) 'Acute Reduction of Blood Pressure by Nitroglycerin Does Not Normalize Large Artery Stiffness in Essential Hypertension', *Hypertension*, 48(3), pp. 404-410.

Su, T.-C., Chien, K.-L., Jeng, J.-S., Chen, M.-F., Hsu, H.-C., Torng, P.-L., Sung, F.-C. and Lee, Y.-T. (2012) 'Age-and Gender-Associated Determinants of Carotid Intima-Media Thickness: A Community-Based Study', *Journal of Atherosclerosis and Thrombosis*, 19(9), pp. 872-880.

Sudlow, C., Gallacher, J., Allen, N., Beral, V., Burton, P., Danesh, J., Downey, P., Elliott, P., Green, J., Landray, M., Liu, B., Matthews, P., Ong, G., Pell, J., Silman, A., Young, A., Sprosen, T., Peakman, T. and Collins, R. (2015) 'UK biobank: an open access resource for identifying the causes of a wide range of complex diseases of middle and old age', *PLoS Med*, 12(3), pp. e1001779.

Sutton-Tyrrell, K., Kuller, L. H., Matthews, K. A., Holubkov, R., Patel, A., Edmundowicz, D. and Newman, A. (2002) 'Subclinical atherosclerosis in multiple vascular beds: an index of atherosclerotic burden evaluated in postmenopausal women', *Atherosclerosis*, 160(2), pp. 407-416.

Suurkula M, A. S., Fagerberg B, Wendelhang I, Widgren B, Wickstrand J (1994) 'Ultrasound evaluation of atherosclerotic manifestation in carotid artery in high- risk hypertensive patients. Risk Intervention Study ðRISÞ', *Arterioscler Thromb*, 14, pp. 1297-1304.

Taddei, S., Virdis, A., Ghiadoni, L., Salvetti, G., Bernini, G., Magagna, A. and Salvetti, A. (2001) 'Age-related reduction of NO availability and oxidative stress in humans', *Hypertension*, 38(2), pp. 274-9.

Taki, A., Kermani, A., Ranjbarnavazi, S. M. and Pourmodheji, A. (2017) 'Chapter 4 - Overview of Different Medical Imaging Techniques for the Identification of Coronary Atherosclerotic Plaques', in Balocco, S., Zuluaga, M.A., Zahnd, G., Lee, S.-L. & Demirci, S. (eds.) *Computing and Visualization for Intravascular Imaging and Computer-Assisted Stenting*: Academic Press, pp. 79-106.

Tanaka, H. (2017) 'Various Indices of Arterial Stiffness: Are They Closely Related or Distinctly Different?', *Pulse*, 5(1-4), pp. 1-6.

Taylor, D. A. and Abdel-Rahman, A. A. (2009) 'Novel Strategies and Targets for the Management of Hypertension', in Enna, S.J. & Williams, M. (eds.) *Advances in Pharmacology*: Academic Press, pp. 291-345.

Thompson, C. (2018) 'Rose's Prevention Paradox', *Journal of Applied Philosophy*, 35(2), pp. 242-256.

Toth, P. P. (2008) 'Subclinical atherosclerosis: what it is, what it means and what we can do about it', *International journal of clinical practice*, 62(8), pp. 1246-1254.

Touboul, P. J., Hennerici, M. G., Meairs, S., Adams, H., Amarenco, P., Desvarieux, M., Ebrahim, S., Fatar, M., Hernandez Hernandez, R., Kownator, S., Prati, P., Rundek, T., Taylor, A., Bornstein, N., Csiba, L., Vicaut, E., Woo, K. S. and Zannad, F. (2004) 'Mannheim intima-media thickness consensus', *Cerebrovasc Dis*, 18(4), pp. 346-9.

Trajkovski, M., Ahmed, K., Esau, C. C. and Stoffel, M. (2012) 'MyomiR-133 regulates brown fat differentiation through Prdm16', *Nat Cell Biol*, 14(12), pp. 1330-5.

Troitzsch, P., Paulista Markus, M. R., Dörr, M., Felix, S. B., Jünger, M., Schminke, U., Schmidt, C. O., Völzke, H., Baumeister, S. E. and Arnold, A. (2012) 'Psoriasis is associated with increased intima-media thickness--the Study of Health in Pomerania (SHIP)', *Atherosclerosis*, 225(2), pp. 486-90.

Tropeano, A. I., Boutouyrie, P., Katsahian, S., Laloux, B. and Laurent, S. (2004) 'Glucose level is a major determinant of carotid intima-media thickness in patients with hypertension and hyperglycemia', *J Hypertens*, 22(11), pp. 2153-60.

Turchinovich, A., Weiz, L., Langheinz, A. and Burwinkel, B. (2011) 'Characterization of extracellular circulating microRNA', *Nucleic acids research*, 39(16), pp. 7223-7233.

UK Biobank (2019) *Traditional and novel biomarkers of adverse cardiometabolic imaging, and associations with outcomes*. Available at: https://www.ukbiobank.ac.uk/2019/04/traditional-and-novel-biomarkers-of-adverse-cardiometabolic-imaging-and-associations-with-outcomes/.

Urbina, E. M., Khoury, P. R., McCoy, C. E., Dolan, L. M., Daniels, S. R. and Kimball, T. R. (2013) 'Triglyceride to HDL-C ratio and increased arterial stiffness in children, adolescents, and young adults', *Pediatrics*, 131(4), pp. e1082-90.

Van Bortel, L. M., Laurent, S., Boutouyrie, P., Chowienczyk, P., Cruickshank, J. K., De Backer, T., Filipovsky, J., Huybrechts, S., Mattace-Raso, F. U. S., Protogerou, A. D., Schillaci, G., Segers, P., Vermeersch, S., Weber, T., on behalf of the Artery Society, t. E. S. o. H. W. G. o. V. S., Function and No, t. E. N. f. (2012) 'Expert consensus document on the measurement of aortic stiffness in daily practice using carotid-femoral pulse wave velocity', *Journal of Hypertension*, 30(3), pp. 445-448.

van Rooij, E., Sutherland, L. B., Liu, N., Williams, A. H., McAnally, J., Gerard, R. D., Richardson, J. A. and Olson, E. N. (2006) 'A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure', *Proc Natl Acad Sci U S A*, 103(48), pp. 18255-60.

Vasan, R. S. (2006) 'Biomarkers of Cardiovascular Disease', *Circulation*, 113(19), pp. 2335-2362.

Vaughn, M. W., Kuo, L. and Liao, J. C. (1998) 'Effective diffusion distance of nitric oxide in the microcirculation', *Am J Physiol*, 274(5), pp. H1705-14.

Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J. and Lötvall, J. O. (2007) 'Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells', *Nat Cell Biol*, 9(6), pp. 654-9.

Vella, S., Buetow, L., Royle, P., Livingstone, S., Colhoun, H. M. and Petrie, J. R. (2010) 'The use of metformin in type 1 diabetes: a systematic review of efficacy', *Diabetologia*, 53(5), pp. 809-20.

Vickers, K. C., Palmisano, B. T., Shoucri, B. M., Shamburek, R. D. and Remaley, A. T. (2011) 'MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins', *Nature cell biology*, 13(4), pp. 423-433.

Villablanca, A. C., Jayachandran, M. and Banka, C. (2010) 'Atherosclerosis and sex hormones: current concepts', *Clin Sci (Lond)*, 119(12), pp. 493-513.

Vlachopoulos, C., Aznaouridis, K. and Stefanadis, C. (2010) 'Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis', *J Am Coll Cardiol*, 55(13), pp. 1318-27.

Vokó, Z., Bots, M. L., Hofman, A., Koudstaal, P. J., Witteman, J. C. M. and Breteler, M. M. B. (1999) 'J-Shaped Relation Between Blood Pressure and Stroke in Treated Hypertensives', *Hypertension*, 34(6), pp. 1181-1185.

Wagenseil, J. E. and Mecham, R. P. (2012) 'Elastin in large artery stiffness and hypertension', *Journal of cardiovascular translational research*, 5(3), pp. 264-273.

Wang, H., Peng, R., Wang, J., Qin, Z. and Xue, L. (2018) 'Circulating microRNAs as potential cancer biomarkers: the advantage and disadvantage', *Clinical Epigenetics*, 10(1), pp. 59.

Wang, J. X., Zhang, X. J., Li, Q., Wang, K., Wang, Y., Jiao, J. Q., Feng, C., Teng, S., Zhou, L. Y., Gong, Y., Zhou, Z. X., Liu, J., Wang, J. L. and Li, P. F. (2015a) 'MicroRNA-103/107 Regulate Programmed Necrosis and Myocardial Ischemia/Reperfusion Injury Through Targeting FADD', *Circ Res*, 117(4), pp. 352-63.

Wang, K., Yuan, Y., Cho, J.-H., McClarty, S., Baxter, D. and Galas, D. J. (2012a) 'Comparing the MicroRNA spectrum between serum and plasma', *PloS one*, 7(7), pp. e41561-e41561.

Wang, K., Yuan, Y., Cho, J. H., McClarty, S., Baxter, D. and Galas, D. J. (2012b) 'Comparing the MicroRNA spectrum between serum and plasma', *PLoS One*, 7(7), pp. e41561.

Wang, K., Zhang, S., Marzolf, B., Troisch, P., Brightman, A., Hu, Z., Hood, L. E. and Galas, D. J. (2009) 'Circulating microRNAs, potential biomarkers for drug-induced liver injury', *Proceedings of the National Academy of Sciences of the United States of America*, 106(11), pp. 4402-4407.

Wang, R., Hong, J., Cao, Y., Shi, J., Gu, W., Ning, G., Zhang, Y. and Wang, W. (2015b) 'Elevated circulating microRNA-122 is associated with obesity and insulin resistance in young adults', *Eur J Endocrinol*, 172(3), pp. 291-300.

Wang, T. J. (2011) 'Assessing the role of circulating, genetic, and imaging biomarkers in cardiovascular risk prediction', *Circulation*, 123(5), pp. 551-65.

Wannarong, T., Parraga, G., Buchanan, D., Fenster, A., House, A. A., Hackam, D. G. and Spence, J. D. (2013) 'Progression of Carotid Plaque Volume Predicts Cardiovascular Events', *Stroke*, 44(7), pp. 1859-1865.

Webb, A. J. S. (2020) 'Progression of Arterial Stiffness is Associated With Midlife Diastolic Blood Pressure and Transition to Late-Life Hypertensive Phenotypes', *Journal of the American Heart Association*, 9(1), pp. e014547.

Weber, J. A., Baxter, D. H., Zhang, S., Huang, D. Y., Huang, K. H., Lee, M. J., Galas, D. J. and Wang, K. (2010) 'The microRNA spectrum in 12 body fluids', *Clin Chem*, 56(11), pp. 1733-41.

Weber, M., Baker, M. B., Patel, R. S., Quyyumi, A. A., Bao, G. and Searles, C. D. (2011) 'MicroRNA Expression Profile in CAD Patients and the Impact of ACEI/ARB', *Cardiol Res Pract*, 2011, pp. 532915.

Weingartner, O., Pinsdorf, T., Rogacev, K. S., Blomer, L., Grenner, Y., Graber, S., Ulrich, C., Girndt, M., Bohm, M., Fliser, D., Laufs, U., Lutjohann, D. and Heine, G. H. (2010) 'The relationships of markers of cholesterol homeostasis with carotid intima-media thickness', *PLoS One*, 5(10), pp. e13467.

Welsh, C., Welsh, P., Celis-Morales, C. A., Mark, P. B., Mackay, D., Ghouri, N., Ho, F. K., Ferguson, L. D., Brown, R., Lewsey, J., Cleland, J. G., Gray, S. R., Lyall, D. M., Anderson, J. J., Jhund, P. S., Pell, J. P., McGuire, D. K., Gill, J. M. R. and Sattar, N. (2020) 'Glycated Hemoglobin, Prediabetes, and the Links to Cardiovascular Disease: Data From UK Biobank', *Diabetes Care*, 43(2), pp. 440-445.

Welsh, P., Preiss, D., Hayward, C., Shah, A. S. V., McAllister, D., Briggs, A., Boachie, C., McConnachie, A., Padmanabhan, S., Welsh, C., Woodward, M., Campbell, A., Porteous, D., Mills, N. L. and Sattar, N. (2019) 'Cardiac Troponin T and Troponin I in the General Population', *Circulation*, 139(24), pp. 2754-2764.

Welsh, P., Preiss, D., Shah, A. S. V., McAllister, D., Briggs, A., Boachie, C., McConnachie, A., Hayward, C., Padmanabhan, S., Welsh, C., Woodward, M., Campbell, A., Porteous, D., Mills, N. L. and Sattar, N. (2018) 'Comparison between High-Sensitivity Cardiac Troponin T and Cardiac Troponin I in a Large General Population Cohort', *Clinical chemistry*, 64(11), pp. 1607-1616.

Wen, J., Zhong, Y., Kuang, C., Liao, J., Chen, Z. and Yang, Q. (2017) 'Lipoprotein ratios are better than conventional lipid parameters in predicting arterial stiffness in young men', *The Journal of Clinical Hypertension*, 19(8), pp. 771-776.

Widera, C., Gupta, S. K., Lorenzen, J. M., Bang, C., Bauersachs, J., Bethmann, K., Kempf, T., Wollert, K. C. and Thum, T. (2011) 'Diagnostic and prognostic impact of six circulating microRNAs in acute coronary syndrome', *Journal of Molecular and Cellular Cardiology*, 51(5), pp. 872-875.

Widmer, R. J. and Lerman, A. (2014) 'Endothelial dysfunction and cardiovascular disease', *Global cardiology science & practice*, 2014(3), pp. 291-308.

Wilkinson, I. B., Franklin, S. S., Hall, I. R., Tyrrell, S. and Cockcroft, J. R. (2001) 'Pressure amplification explains why pulse pressure is unrelated to risk in young subjects', *Hypertension*, 38(6), pp. 1461-6.

Wilkinson, I. B., MacCallum, H., Flint, L., Cockcroft, J. R., Newby, D. E. and Webb, D. J. (2000) 'The influence of heart rate on augmentation index and central arterial pressure in humans', *The Journal of physiology*, 525 Pt 1(Pt 1), pp. 263-270.

Willeit, P., Kaptoge, S., Welsh, P., Butterworth, A. S., Chowdhury, R., Spackman, S. A., Pennells, L., Gao, P., Burgess, S., Freitag, D. F., Sweeting, M., Wood, A. M., Cook, N. R., Judd, S., Trompet, S., Nambi, V., Olsen, M. H., Everett, B. M., Kee, F., Ärnlöv, J., Salomaa, V., Levy, D., Kauhanen, J., Laukkanen, J. A., Kavousi, M., Ninomiya, T., Casas, J.-P., Daniels, L. B., Lind, L., Kistorp, C. N., Rosenberg, J., Mueller, T., Rubattu, S., Panagiotakos, D. B., Franco, O. H., de Lemos, J. A., Luchner, A., Kizer, J. R., Kiechl, S., Salonen, J. T., Wannamethee, S. G., de Boer, R. A., Nordestgaard, B. G., Andersson, J., Jørgensen, T., Melander, O., Ballantyne, C. M., DeFilippi, C., Ridker, P. M., Cushman, M., Rosamond, W. D., Thompson, S. G., Gudnason, V., Sattar, N., Danesh, J. and Di Angelantonio, E. (2016) 'Natriuretic peptides and integrated risk assessment for cardiovascular disease: an individual-participant-data meta-analysis', *The Lancet Diabetes & Endocrinology*, 4(10), pp. 840-849.

Willeit, P., Skroblin, P., Moschen, A. R., Yin, X., Kaudewitz, D., Zampetaki, A., Barwari, T., Whitehead, M., Ramirez, C. M., Goedeke, L., Rotllan, N., Bonora, E., Hughes, A. D., Santer, P., Fernandez-Hernando, C., Tilg, H., Willeit, J., Kiechl, S. and Mayr, M. (2017a) 'Circulating MicroRNA-122 Is Associated With the Risk of New-Onset Metabolic Syndrome and Type 2 Diabetes', *Diabetes*, 66(2), pp. 347-357.

Willeit, P., Welsh, P., Evans, J. D. W., Tschiderer, L., Boachie, C., Jukema, J. W., Ford, I., Trompet, S., Stott, D. J., Kearney, P. M., Mooijaart, S. P., Kiechl, S., Di Angelantonio, E. and Sattar, N. (2017b) 'High-Sensitivity Cardiac Troponin Concentration and Risk of First-Ever Cardiovascular Outcomes in 154,052 Participants', *J Am Coll Cardiol*, 70(5), pp. 558-568.

Willeit, P., Zampetaki, A., Dudek, K., Kaudewitz, D., King, A., Kirkby, N. S., Crosby-Nwaobi, R., Prokopi, M., Drozdov, I., Langley, S. R., Sivaprasad, S., Markus, H. S., Mitchell, J. A., Warner, T. D., Kiechl, S. and Mayr, M. (2013) 'Circulating microRNAs as novel biomarkers for platelet activation', *Circ Res*, 112(4), pp. 595-600.

Williams, B., Mancia, G., Spiering, W., Agabiti Rosei, E., Azizi, M., Burnier, M., Clement, D. L., Coca, A., de Simone, G., Dominiczak, A., Kahan, T., Mahfoud, F., Redon, J., Ruilope, L., Zanchetti, A., Kerins, M., Kjeldsen, S. E., Kreutz, R., Laurent, S., Lip, G. Y. H., McManus, R., Narkiewicz, K., Ruschitzka, F., Schmieder, R. E., Shlyakhto, E., Tsioufis, C., Aboyans, V., Desormais, I. and Group, E. S. D. (2018) '2018 ESC/ESH Guidelines for the management of arterial hypertension: The Task Force for the management of arterial hypertension of the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH)', *European Heart Journal*, 39(33), pp. 3021-3104.

Wilson, P. W., Pencina, M., Jacques, P., Selhub, J., D'Agostino, R., Sr. and O'Donnell, C. J. (2008) 'C-reactive protein and reclassification of cardiovascular risk in the Framingham Heart Study', *Circ Cardiovasc Qual Outcomes*, 1(2), pp. 92-7.

Witters, L. A. (2001) 'The blooming of the French lilac', *The Journal of clinical investigation*, 108(8), pp. 1105-1107.

Witwer, K. W. (2015a) 'Circulating MicroRNA Biomarker Studies: Pitfalls and Potential Solutions', *Clinical Chemistry*, 61(1), pp. 56-63.

Witwer, K. W. (2015b) 'Circulating microRNA biomarker studies: pitfalls and potential solutions', *Clin Chem*, 61(1), pp. 56-63.

Wormser, D., Kaptoge, S., Di Angelantonio, E., Wood, A. M., Pennells, L., Thompson, A., Sarwar, N., Kizer, J. R., Lawlor, D. A., Nordestgaard, B. G., Ridker, P., Salomaa, V., Stevens, J., Woodward, M., Sattar, N., Collins, R., Thompson, S. G., Whitlock, G. and Danesh, J. (2011) 'Separate and combined associations of body-mass index and abdominal adiposity with cardiovascular disease: collaborative analysis of 58 prospective studies', *Lancet*, 377(9771), pp. 1085-95.

Wu, T. W., Hung, C. L., Liu, C. C., Wu, Y. J., Wang, L. Y. and Yeh, H. I. (2017) 'Associations of Cardiovascular Risk Factors with Carotid Intima-Media Thickness in Middle-Age Adults and Elders', *J Atheroscler Thromb*, 24(7), pp. 677-686.

Xie, H., Lim, B. and Lodish, H. F. (2009a) 'MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity', *Diabetes*, 58(5), pp. 1050-1057.

Xie, H., Sun, L. and Lodish, H. F. (2009b) 'Targeting microRNAs in obesity', *Expert opinion on therapeutic targets*, 13(10), pp. 1227-1238.

Xie, Y., Chu, A., Feng, Y., Chen, L., Shao, Y., Luo, Q., Deng, X., Wu, M., Shi, X. and Chen, Y. (2018) 'MicroRNA-146a: A Comprehensive Indicator of Inflammation and Oxidative Stress Status Induced in the Brain of Chronic T2DM Rats', *Frontiers in pharmacology*, 9, pp. 478-478.

Xu, C., Zarins, C. K., Pannaraj, P. S., Bassiouny, H. S. and Glagov, S. (2000) 'Hypercholesterolemia Superimposed by Experimental Hypertension Induces Differential Distribution of Collagen and Elastin', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 20(12), pp. 2566-2572.

Xu, Q., Li, Y., Shang, Y.-F., Wang, H.-L. and Yao, M.-X. (2015) 'miRNA-103: molecular link between insulin resistance and nonalcoholic fatty liver disease', *World journal of gastroenterology*, 21(2), pp. 511-516.

Yang, F.-r., Li, H.-j., Li, T.-t., Zhao, Y.-f., Liu, Z.-k. and Li, X.-r. (2019) 'Prognostic Value of MicroRNA-15a in Human Cancers: A Meta-Analysis and Bioinformatics', *BioMed Research International*, 2019, pp. 2063823.

Yoda, M., Kawamata, T., Paroo, Z., Ye, X., Iwasaki, S., Liu, Q. and Tomari, Y. (2010) 'ATP-dependent human RISC assembly pathways', *Nat Struct Mol Biol*, 17(1), pp. 17-23.

Yusuf, S., Hawken, S., Ounpuu, S., Dans, T., Avezum, A., Lanas, F., McQueen, M., Budaj, A., Pais, P., Varigos, J. and Lisheng, L. (2004) 'Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study', *Lancet*, 364(9438), pp. 937-52.

Zanchetti A1, C. G., Bond MG, Gallus GV, Veglia F, Ventura A, Mancia G, Baggio G, Sampieri L, Rubba P, Collatina S, Serrotti E. (2001) 'Systolic and pulse blood pressures (but not diastolic blood pressure and serum cholesterol) are associated with alterations in carotid intima-media thickness in the moderately hypercholesterolaemic hypertensive patients of the Plaque Hypertension Lipid Lowering Italian Study. PHYLLIS study group', *J Hypertens*, 19, pp. 79-88.

Zanoli, L. M. and Spoto, G. (2013) 'Isothermal amplification methods for the detection of nucleic acids in microfluidic devices', *Biosensors (Basel)*, 3(1), pp. 18-43.

Zeller, T., Tunstall-Pedoe, H., Saarela, O., Ojeda, F., Schnabel, R. B., Tuovinen, T., Woodward, M., Struthers, A., Hughes, M., Kee, F., Salomaa, V., Kuulasmaa, K., Blankenberg, S. and Investigators, M. (2014) 'High population prevalence of cardiac troponin I measured by a high-sensitivity assay and cardiovascular risk estimation: the MORGAM Biomarker Project Scottish Cohort', *Eur Heart J*, 35(5), pp. 271-81.

Zeng, Y., Wagner, E. J. and Cullen, B. R. (2002) 'Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells', *Mol Cell*, 9(6), pp. 1327-33.

Zernecke, A., Bidzhekov, K., Noels, H., Shagdarsuren, E., Gan, L., Denecke, B., Hristov, M., Köppel, T., Jahantigh, M. N., Lutgens, E., Wang, S., Olson, E. N., Schober, A. and Weber, C. (2009) 'Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection', *Sci Signal*, 2(100), pp. ra81.

Zhang, J.-Y., Gong, Y.-L., Li, C.-J., Qi, Q., Zhang, Q.-M. and Yu, D.-M. (2016) 'Circulating MiRNA biomarkers serve as a fingerprint for diabetic atherosclerosis', *American journal of translational research*, 8(6), pp. 2650-2658.

Zhang, Y., Li, H. H., Yang, R., Yang, B. J. and Gao, Z. Y. (2017) 'Association between circulating microRNA-208a and severity of coronary heart disease', *Scand J Clin Lab Invest*, 77(5), pp. 379-384.

Zhang, Y. F., Hong, J., Zhan, W. W., Li, X. Y., Gu, W. Q., Yang, Y. S., Xu, M. and Ning, G. (2006) 'Hyperglycaemia after glucose loading is a major predictor of preclinical atherosclerosis in nondiabetic subjects', *Clin Endocrinol (Oxf)*, 64(2), pp. 153-7.

Zhao, W., Zhao, S. P. and Zhao, Y. H. (2015) 'MicroRNA-143/-145 in Cardiovascular Diseases', *Biomed Res Int*, 2015, pp. 531740.

Zhao, Y., Yin, Z., Li, H., Fan, J., Yang, S., Chen, C. and Wang, D. W. (2017) 'MiR-30c protects diabetic nephropathy by suppressing epithelial-to-mesenchymal transition in db/db mice', *Aging Cell*, 16(2), pp. 387-400.

Zhong, J., He, Y., Chen, W., Shui, X., Chen, C. and Lei, W. (2014) 'Circulating microRNA-19a as a potential novel biomarker for diagnosis of acute myocardial infarction', *International journal of molecular sciences*, 15(11), pp. 20355-20364.

Zhu, H. and Leung, S. W. (2015) 'Identification of microRNA biomarkers in type 2 diabetes: a meta-analysis of controlled profiling studies', *Diabetologia*, 58(5), pp. 900-11.

Zieman, S. J., Melenovsky, V. and Kass, D. A. (2005) 'Mechanisms, Pathophysiology, and Therapy of Arterial Stiffness', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 25(5), pp. 932-943.