Adiposity, Lipids and Risk for Myocardial Infarction in the Maltese Population

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Author's Declaration

I hereby declare that this report is my own work. I have acknowledged the main texts and references where appropriate, all of which have exercised a formative influence on this dissertation.

Year 2011-2020

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Philip Dingli

To my wife and children for their continuous support

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Abstract

Background

Myocardial infarction (MI) is a complex disease influenced by both genetic and environmental factors.

Aim

The Maltese Acute Myocardial Infarction (MAMI) Study is a case-control study that was set up to identify genetic and environmental factors relevant to MI in the Maltese population. Hyperlipidaemia and adiposity as risk factors for myocardial infarction in the Maltese population will be focused on. Furthermore, this study will investigate and apply alternative approaches using high throughput sequencing, extreme phenotypes and biological pathway approaches to determine their use in acquiring more knowledge on the genetic factors of MI.

Methods

The study includes 423 cases with a first MI between 2011 and 2013, 465 controls that were sex and age-matched in ten year age groups to the cases and 210 relatives of cases. Data on all participants was collected after written informed consent through an extensive interviewer-led questionnaire, measurements and testing and through medical history and records.

Results

This case-control study identified several important genetic and environmental factors particularly pertinent to the Maltese population. The association of adiposity with risk of MI varied significantly depending on how adiposity was classified, with BMI, the most widely used clinical measure of adiposity, underestimating risk. Waist-hip ratio was more strongly associated with risk of myocardial infarction. Similarly, total cholesterol to high density

cholesterol ratio and the Non high density cholesterol levels were more strongly associated with risk of MI than the most commonly used clinical measures of dyslipidaemia. The study also gives important insights into how dietary habits in a central Mediterranean climate are influencing the risk of MI with consumption of soft drinks (even diet soft drinks) and bread being strong risk factors whilst nuts, legumes, fruit and red vegetables being protective. The modulation of risk by combinations of different environmental risk factors is exemplified in an analysis performed on smoking and alcohol. The risk associated with *APOE* and *PTPN1* were minimal in the Maltese population. Using an extreme phenotype approach and high throughput sequencing a novel frameshift variant in *LDLR* likely to cause familial hypercholesterolaemia was identified and polymorphisms in *APOB* that increase the risk of MI in the Maltese population are described.

Conclusions

Using the extreme phenotype approach and high throughput sequencing, pathways related to the control of adipogenesis were examined and the findings presented highlight the importance of studying combinations of polymorphisms and using a systems biology approach to analyse the risk of MI in complex pathways.

List of Publications and Presentations from this Work to Date

Dingli, P., Attard, R, Cassar, K., Doggen, C., Farrugia, R., & Bezzina Wettinger, S. (2014). Persistently Elevated Risk of Myocardial Infarction After Smoking Cessation. Do Lipids Play a Role? Poster presentation in: The 2nd World Congress on Clinical Lipidology, 5th-7th December 2014, Vienna.

Dingli, P., Attard, R., Doggen, C., Vassallo, J., Cassar, K., Farrugia, R., & Bezzina Wettinger, S. (2015). Waist-hip-ratio a Better Indicator of Risk of Myocardial Infarction than BMI in a Mediterranean Southern European population. Atherosclerosis, 241, e169.

Attard, R., Dingli, P., Doggen, C., Cassar, K., Farrugia, R., & Bezzina Wettinger, S. (2015). Gender Differences and the Risk of Myocardial Infarction. Atherosclerosis, 241, e145-e146.

Dingli, P., Attard, R., Cassar, K., Farrugia, R., & Bezzina Wettinger, S. Next Generation Sequencing of Familial Hypercholesterolaemia-related Genes in a Mediterranean European Cohort. ESHG 2016, Barcelona, Spain.

The Effect of Smoking, Alcohol and Coffee Consumption on Inflammation and the Risk of Myocardial Infarction. Abstract published in: Malta Journal of Health Science, 2016: 3, 12.

Attard, R., Dingli, P., Doggen, CJM., Cassar, K., Farrugia, R., & Bezzina Wettinger, S. (2017). The Impact of Passive and Active Smoking on Inflammation, Lipid Profile and the Risk of Myocardial Infarction. Openheart;4(2):e000620.

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List of Abbreviations

ABCG	Adenosine triphosphate-binding cassette sub-family G
ANGPTL3	Angiopoietin-like 3
ANGPTL4	Angiopoietin-like 4
APC	Adenomatous polyposis coli gene product
ApoA	Apolipoprotein A
APOA1	Apolipoprotein A-I
ApoB	Apolipoprotein B
APOB	Apolipoprotein B
ApoB-100	Apolipoprotein B 100
ApoE	Apolipoprotein E
ARH	Autosomal recessive hypercholesterolaemia
AVG	Average
BAT	Brown adipose tissue
BMI	Body mass index
BMP-4	bone morphogenetic protein 4
CAD	Coronary artery disease
CamKII	calmodulin-dependent protein kinase II
Cam-Kinase II	calmodulin to activate calmodulin-dependent protein kinase II
CBP	CREB binding protein
CEBPB	CCAAT/enhancer-binding protein β
CEBPD	CCAAT/enhancer-binding protein β δ (CEBPD)
CETP	Cholesteryl ester transfer protein

CHD	Coronary heart disease
CI	Confidence interval
CK1	Casein kinase 1
CRD	Cysteine rich domain
CREB	cAMP response element-binding
CRP	C-reactive protein
CV	Cardiovascular
CYP7A1	Cytochrome P450 Family 7 Subfamily A Member 1
Daam-1	Dishevelled Associated Activator of Morphogenesis 1
DIX	Dishevelled-axin
DKK	Dickkopf
DM	Diabetes Mellitus
DVL	Dishevelled
DVL-1	Dishevelled
EC	Endothelial cells
FDG-PET	Fluoro-D-glucose positron emission tomography
FH	Familial hypercholesterolaemia
FRET	Fluorescent resonant energy transfer
Fz	Frizzled
Fz	Frizzled receptor
FZD	Frizzled
GSK3	Glycogen synthase kinase 3
HDL-C	High density lipoprotein cholesterol
HDLR	HDL ratio (TC/HDL-C)
HMG-CoA	3-hydroxy-3-methylglutaryl co-enzyme A

HMGCR	3-hydroxy-3-methylglutaryl-CoA Reductase
HR	Hazard ratio
Hs-CRP	High sensitivity CRP
HT	Hypertension
HWE	Hardy Weinburg equilibrium
IDL	Intermediate density lipoprotein
IFN-γ	Interferon -gamma
IGV	Integrative genomics viewer
IL6	Interleukin-6
IQR	Interquartile range
IRS-1	Insulin receptor substrate-1
IRS-1	Insulin receptor substrate-1
IRS-1	Insulin receptor substrate
JNK	Jun kinase
JUPITER	Justification for the Use of statins in Prevention: an Intervention Trial Evaluating Rosuvastatin
KASP	Kompetitive allele specific PCR
LBP	Lipopolysaccharide binding protein
LDL	Low-density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LDL-C	Low density lipoprotein cholesterol
LDLR	Low-density lipoprotein receptor
LDLR	LDL receptor
LDLRAP	LDL receptor adaptor protein
LGR4	Leucine-rich repeat-containing G-protein coupled receptor 4

LPA	Lipoprotein(a)
LRP1	Low density lipoprotein receptor-related protein 1
LRP4	Low density lipoprotein receptor-related protein 4
LRP5 and 6	Lipoprotein related proteins 5 and 6
MCP-1	Monocyte chemoattractant protein-1
MCP-1	Monocyte chemoattractant protein
MDH	Mater Dei Hospital
MR	Mendelian Randomisation
MI	Myocardial Infarction
NGS	Next generation sequencing
NHDL-C	Non-HDL cholesterol
NPC1L1	Niemann-Pick C1-Like 1
OR	Odds ratio
PCI	Percutaneous coronary intervention
PAR	Population attributable risk
PCR	Polymerase chain reaction
PCP	Planar cell polarity
PCSK9	Proprotein convertase subtilisin / kexin type 9
PDGF-R-α	Platelet-derived growth factor receptor A
PGC-1-α	PPAR-gamma coactivator 1-alpha
PGC-1-β	PPAR- gamma coactivator 1-beta
PKC	Protein kinase C
PLTP	Phospholipid transfer protein
PPAR	Peroxisome proliferator-activated receptor
PPAR-gamma	Peroxisome proliferator-activated receptor gamma

RHOA	RAS homologue gene-family member A
RHOA	RAS homologue gene-family member A
ROCK	Rho associated kinase
ROR2	Tyrosine-protein kinase transmembrane receptor ROR2
RR	Relative risk
SCARB1	Scavenger receptor class B type 1
SFA	Saturated fatty acids
sFRP	soluble Frizzled related proteins
sFRP-5	Secreted frizzled-related protein 5
SNPs	Single nucleotide polymorphisms
STARD3	StAR Related Lipid Transfer Domain Containing 3
sWAT	Superficial white adipose tissue
TC	Total cholesterol
TCF	T-cell factor
TCF7L2	Transcription factor 7-like 2
TG	Triglycerides
TNFα	Tissue necrosis factor alpha
UCP1	Uncoupling protein 1
VLDL	Very low-density lipoproteins
VSMC	Vascular smooth muscle cells
vWAT	Visceral white adipose tissue
WAT	White adipose tissue
WC	Waist circumference
WHR	Waist-to-hip ratio
WHtR	Waist-height ratio

WIF-1	Wnt-Inhibitory factor-1
WISP-2	WNT1-inducible-signaling pathway protein 2
Wnt	Wingless-Type Murine Mammary Tumour Virus Integration Site (Wnt)
ZNF423	Zinc finger 423 expression

Aims of this Study

1. To study hyperlipidaemia and adiposity as risk factors for myocardial infarction in the Maltese population.

2. To study aspects of the influence of genetic and environmental risk factors on lipid levels, adiposity and on risk for myocardial infarction.

3. To investigate and apply alternative approaches using high throughput sequencing, extreme phenotypes and biological pathway approaches to determine their use in acquiring more knowledge on the genetic factors of MI.

Chapter 1. Literature Review

Section 1.1. Cardiovascular Disease Statistics

Cardiovascular (CVD) disease is the topmost cause of death in the European Union. Malta is no exception with CVD being responsible for 38.8% of deaths in 2015. The standardised death rate attributed to CVD was 386.9 / 100,000 for Malta and 381.4 / 100,000 for all 28 European countries in 2015 (Eurostat 2018). Worldwide obesity has tripled since 1975 with 39% of adults aged 18 years and over being overweight and 13% being obese (11% of men and 15% of women) in 2016. The prevalence of overweight and obesity individuals between 5-19 years of age has risen from 4% in 1975 to 18% in 2016 giving rise to concerns regarding an impending global obesity epidemic (WHO 2018a). The situation is similar in Europe with 50% of all men and women being overweight and the situation is thought to be worse in Southern European countries (Brandt and Erixon 2013). Tackling obesity has been highlighted as a key strategy to decrease cardiovascular disease (Webber et al. 2014). An in-depth understanding of how obesity contributes to the risk of cardiovascular disease is a key step in setting up successful policies.

Section 1.2. Pathogenesis of Atherosclerosis

CVD is caused by the process of atherosclerosis in large and medium sized muscular arteries including the coronary arteries. The disease is characterized by a progressive thickening of the intima where inflammatory cells, lipids and extracellular matrix accumulate. This can either lead to narrowing of the artery or to an acute thrombotic occlusion of the affected artery when the lesion ruptures. Atheroma consist of a core region containing foam cells and extracellular lipid droplets, and a cap composed of smooth muscle cells and a collagen rich matrix. Vascular lesions start to develop in the second decade of life but usually become clinically relevant in middle-aged and elderly persons. Myocardial infarction is generally caused by plaque rupture,

thrombosis and vasoconstriction at the site of an atherosclerotic lesion in coronary arteries, resulting in necrosis of heart muscle tissue (Vinay Kumar, Abul K. Abbas 2014).

Endothelial dysfunction is the first step in the development of an atherosclerotic plaque. Endothelial cells are not just an inert arterial lining. The vascular endothelium is complex, it is involved in permeability, it provides a non-thrombogenic surface, it regulates vascular tone and transendothelial flow and it is an inhibitor of vascular smooth muscle cell growth and migration (Leopold and Loscalzo 2000). When exposed to laminar blood flow, endothelial cells (EC) play an important role in the hemodynamic regulation by releasing vasoactive mediators including nitric oxide. Mechanical and chemical damage to endothelial cells results in the expression of inflammatory and prothrombotic genes resulting in the recruitment of inflammatory cells to the subendothelial layer (Heusch et al. 2014).

Endothelial dysfunction may be initiated by a variety of insults including hypercholesterolaemia, insulin resistance, obesity, smoking and hypertension, all of which cause endothelial activation (Libby et al. 2010). Low density lipoproteins (LDL), very low density lipoproteins (VLDL) and intermediate-density lipoproteins, are retained in the intima due to endothelial activation (Libby et al. 2002; Stocker and Keaney 2004). Lipids trapped in the arterial wall undergo oxidative modification (Stocker and Keaney 2004) resulting in further activation of endothelial cells (Hansson 2005).

Activated endothelium expresses a variety of adhesion molecules and chemokines stimulating recruitment and migration of T-cells and monocytes (Hansson 2005; Libby et al. 2010). A dysfunctional endothelium leads to the adhesion, rolling, and finally migration of monocytes to the subendothelial layer, where they differentiate into tissue macrophages. Within the intima, macrophages up-regulate the expression of scavenger and toll-like receptors. Scavenger receptors allow macrophages to phagocytose oxidized LDL, leading to foam cell

formation (Figure 1-1). Toll-like receptors initiate a signal cascade that leads to increased inflammation and tissue damage (Libby et al. 2010). Macrophages can also release matrix-degrading enzymes which can weaken the fibrous cap that forms over the lesion, giving rise to plaque rupture and acute atherothrombotic events (Libby et al. 2002; Cochain and Zernecke 2017).

Migration of vascular smooth muscle cells (VSMC) from the media towards the intima is another important component of atherosclerotic plaque development. VSMC are activated by a complex mechanism, probably involving the inflammatory mediators released from atherosclerotic lesion, which results in proliferation and migration. This is accompanied by a switch from a contractile towards a synthetic phenotype, characterized by increased production of cytokines and extracellular matrix and a reduction in contractile protein. Synthetic VSMCs extracellular matrix production leads to thickening of the intima (Figure 1-1) (Owens et al., 2004; Mill and George, 2012). Vascular smooth muscle cell apoptosis leads to thinning of the plaque and plaque vulnerability (Owens et al. 2004).

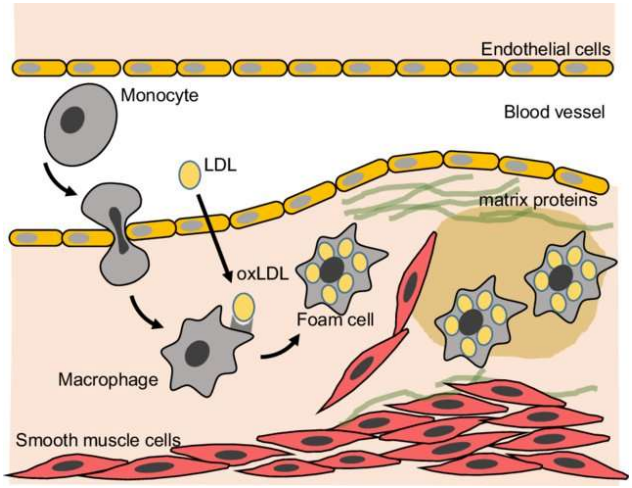

Figure 1-1 The pathogenesis of atherosclerotic plaque. Migration of macrophages to the intima and endocytosis of oxidised LDL results in foam cell formation, migration of smooth muscle cells to the intima, deposition of matrix proteins and the formation of a lipid core (Barr 2018).

Vascular calcification is a key process involved in the development of atherosclerosis (Demer and Tintut, 2008; Vervloet and Cozzolino, 2017). It is not a passive process but consists of regulated biomineralisation similar to bone formation. Calcification is widely used as a clinical indicator of atherosclerosis and is associated with increased cardiovascular morbidity and mortality. Approximately 85% of atherosclerotic plaques causing coronary thrombosis are calcified (Abedin et al. 2004).

Metalloproteinase and cysteine proteases produced by macrophages, T-cells and mast cells decrease the strength of the cap, increasing the risk of rupture. Furthermore, interferon-γ (IFN-γ) produced by T lymphocytes inhibits production of collagen by smooth muscle cells further decreasing the strength of the fibrous cap (Hansson 2005). Rupture of the fibrous cap results in thrombosis within the coronary artery. Thrombosis results in local formation of serotonin, thromboxane A_2, and thrombin which cause local and downstream vasoconstriction increasing myocardial hypoxia (Libby et al. 2010).

Myocardial infarction (MI) is one of the most frequent acute cardiovascular events. It occurs as a consequence of the progression of an atherosclerotic plaque towards an unstable phenotype, where the fibrous cap ruptures and releases the content of the plaque into the circulation (Bentzon et al. 2014)(Figure 1-2).

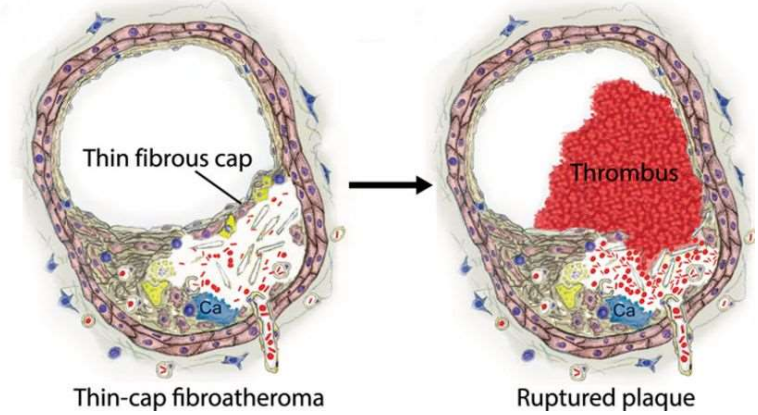

Figure 1-2 Rupture of thin-cap fibroatheroma with extravasation of lipid rich core into coronary artery and resultant thrombus formation Bentzon et al. 2014.

However, myocardial infarction can also be the result of plaque erosion with distal embolization or coronary vasospasm (Regitz-Zagrosek et al. 2016). Myocardial infarction however can also be due to mechanisms not involving plaque rupture including low blood pressure, high and low pulse rates and anaemia. The latter mechanisms of MI that do not involve plaque rupture are designated as type II MI, while MI due to plaque rupture is designated as type I MI (Thygesen et al. 2007). In any case, the affected region of the heart is deprived of oxygen and nutrients, resulting in cell loss in that area. Despite the fact that stem cells have been identified in the heart, the repair of the infarct area does not result in the significant regeneration of the lost cardiac muscle but results in a scar (Laflamme and Murry 2011).

Section 1.3. The Traditional Risk Factors

Nine potentially modifiable risk factors were found to account for over 90% of the population attributable risk (PAR) of first acute myocardial infarction (MI) in the INTERHEART study (Yusuf et al. 2004). The PAR is the incidence of the disease in the population that would be eliminated if exposure were eliminated (Rothman and Greenland 1998). INTERHEART studied 14,637 cases and 12,461 controls in 52 countries. The risk factors identified were smoking, dyslipidaemia, hypertension, diabetes, abdominal obesity, psychosocial factors, diet, alcohol consumption and physical activity (Yusuf et al. 2004). It is important to note that the population attributable risk has no upper limit, so undiscovered risk factors may have PAR scores of >10% (Smulders et al. 2008). The most important risk factors in INTERHEART were found to be a raised Apolipoprotein B100 (ApoB) / Apolipoprotein A1 (ApoA1) ratio and smoking (Yusuf et al. 2004). The majority of patients present with at last 1 modifiable risk

factor while in one study looking at over 500,000 MI admissions between 1994-2006 only 14.4% had no risk factors identified. While obesity alone without any of the other traditional risk factors was rarely seen, there was a direct association between obesity and increasing number of risk factors (Canto et al. 2011).

Section 1.3.1. Obesity

Obesity, which was identified as a major risk factor in the INTERHEART study (Yusuf et al. 2004) is a major problem in Malta. A staggering 69.75% of Maltese are overweight or obese with the prevalence of obesity being the highest between the 35-44 year age group (Cuschieri et al. 2016). The estimated cost of obesity-related costs for Malta in 2016 was 36.3 million euros, 97 euros on a per capita basis, with obesity absorbing 8.1% of the annual public recurrent health expenditure, representing 0.4% of Malta's GDP (Valenzia 2017).

The INTERHEART study puts into doubt our reliance on body mass index (BMI) to define obesity and concluded that waist-hip ratio is a better index of risk for MI then BMI (Yusuf et al. 2005). The OR for the highest quintile compared to the lowest quintile of Waist-hip Ratio (WHR) was 1.33 (99% CI, 1.16-1.53, p-value 0.0001) following multivariate analysis (Yusuf et al. 2005). In the INTERHEART study though, WHR was not measured as recommended by the World Health Organisation (WHO) and the method used can be inaccurate in obese individuals (Wilding and Finer 2006).

Adipose tissue is a metabolically dynamic organ that is made up of adipocytes, adipocyte precursors, mesenchymal cells, vascular tissue and immune cells. Humans have two main types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT). Pre-adipocytes within the adipose tissue can differentiate into mature adipocytes throughout life allowing adipose tissue to carry out hyperplastic expansion in response to storage requirements.

Mature adipose tissue can also expand in size to allow increased storage (Hypertrophy) (Gray and Vidal-Puig 2007).

WAT's function is primarily energy storage while BAT has a role in neonates to combat coldness via the process of adaptive non-shivering thermogenesis. WAT store and release energy as fatty acids in response to systemic demands whereas brown adipocytes burn fatty acids and glucose to produce heat (Cannon and Nedergaard 2004). BAT thermogenesis is mediated via β-adrenergic activation of lipolysis and degradation of fatty acids via uncoupling protein 1 (UCP1) (Lowell and Spiegelman 2000; Inokuma et al. 2006). Human adults also possess functional brown adipose tissue (Cypess et al. 2009; van Marken Lichtenbelt et al. 2009; Saito et al. 2009). The mass of BAT in adults shows a negative correlation with adiposity and BMI (Cypess et al. 2009; Saito et al. 2009). Directly measuring BAT in vivo is difficult and usually performed using fluoro-D-glucose positron emission tomography (FDG-PET) or MRI (Riis-Vestergaard et al. 2018).

WAT is subdivided into two major subdivisions visceral white adipose tissue (vWAT) and superficial white adipose tissue (sWAT) depots based on their location inside or outside the abdominal cavity (Cinti 2005). These two subsets of WAT have different development lineage, gene expression, adipokine profiles, metabolic characteristics and different contributions to cardiometabolic disease (Emdin et al. 2017; Chen and Wang 2018). High vWAT mass has been associated with inflammation and macrophage accumulation (Weisberg S et al. 2003), dyslipidaemia (Hwang et al. 2016), diabetes (Kelley et al. 2000; Wang et al. 2005) and cardiovascular disease (Gruzdeva et al. 2018). Gluteofemoral sWAT, on the other hand, appears to be protective against the metabolic syndrome (Manolopoulos et al. 2010).

Section 1.3.2. Lipids

The ApoB/ApoA1 ratio was the most important risk factor in all geographic regions representing 50% of the population attributable risk (Yusuf et al. 2004; Nordestgaard et al. 2010). The top vs the lowest decile of ApoB/ApoA1 ratio gave an odds ratio (OR) of 4.73 (99% CI, 3.93-5.69) (Yusuf et al. 2004). However ApoB/ApoA1 levels are not routinely measured in many clinical labs and there are no recognised cut-offs which make their use in clinical practice limited (McQueen et al. 2008).

An alternative to ApoB/ApoA to assess the ratio of atherogenic and atheroprotective cholesterol transport includes the use of lipoprotein ratios or 'atherogenic indices'. These indices may provide better risk prediction than lipid variables seen in isolation (Millán et al. 2009). The HDL Ratio (HDLR) is the ratio of Total cholesterol/ HDL cholesterol. It has been shown in large observational studies including the Framingham (Castelli et al. 1986), LRCP (Grover et al. 1994), PROCAM (Assmann et al. 1998) and in combinations of the large population cohorts (Kinosian et al. 1994) to be a better coronary risk predictor than using total cholesterol, LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C) alone. The ApoB/ApoA1 ratio was shown by the INTERHEART study to be a better risk marker then ratios of total cholesterol (TC) /HDL-C; LDL-C and non-HDL cholesterol (NHDL-C). This may be due to the fact that calculation of Apo B includes cholesterol-depleted LDL particles which are usually underestimated when measuring LDL (McQueen et al. 2008). HDLR was not found to be inferior to ApoB/ApoA measurements in a 15 year follow up of 3,322 Framingham inhabitants (Ingelsson et al. 2007) and in the EPIC-Norfolk study (van der Steeg et al. 2007).

Despite this evidence risk calculators such as the HEARTSCORE only take into consideration total cholesterol and HDL in their risk estimations based on epidemiological data (Mach et al.

2019). Furthermore, many clinicians rely solely on LDL and TC to evaluate risk of myocardial infarction due to lipids (Farukhi and Mora 2018).

Section 1.4. Statins in the Treatment of Coronary Artery Disease

Studies including the Scandinavian Simvastatin Survival Study (4S) showed lipid lowering therapy in the form of 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors (statins) improved outcomes when used as secondary prevention (Scandinavian Simvastatin Survival Study Group 1994). Other studies including the cholesterol and recurrent events (CARE) study (Sacks et al. 1996) and the Heart Protection Study (Collins et al. 2002) confirmed these findings even in patients with moderately raised cholesterol levels. The Air Force Coronary Atherosclerosis Prevention Study/Texas Coronary Atherosclerosis Prevention Study (AF-CAPS/TexCAPS) showed that lipid lowering therapy was beneficial even in primary prevention setting (Downs et al. 1998). The benefit of lipid lowering has been shown to be effective in the long term by the Lipid study who followed up patients for 16 years (Hague et al. 2016). The lower the LDL is pushed the better the results appear to be (Cannon et al. 2004).

The benefits of statins are not solely due to their lipid lowering action. Statins also reduce C-reactive protein (CRP) levels (van de Ree et al. 2003) and the levels of pro-inflammatory cytokines (Ascer et al. 2004) and induce a favourable redox state in the vascular wall (Antoniades et al. 2011). Statins have been shown to improve endothelial function (Järvisalo et al. 1999) and to reduce T-cell activation, macrophage infiltration and vascular wall inflammation (Schonbeck and Libby 2004). Overall, statins promote plaque stability (Cipollone et al. 2003).

The important link between inflammation and cardiovascular events was illustrated in the JUPITER trial. The JUPITER trial (Justification for the Use of statins in Prevention: an Intervention Trial Evaluating Rosuvastatin) showed that in patients with elevated high sensitivity -CRP (hs-CRP), statins resulted in a lower risk of cardiovascular events compared to placebo however it was impossible to conclude that altering the inflammatory state was responsible for the clinical benefit. Fortunately, in the case of statins the anti-inflammatory effect does not increase the risk of systemic infection (Ridker et al. 2008).

Despite the effective lipid lowering effect of statins and the various pleotropic effects the majority of MI survivors go on to have recurrent events indicating the need to target cardiovascular disease on alternative fronts (Libby 2005).

Section 1.5. Genetics of Coronary Artery Disease

The genetic basis of coronary artery disease is a complex field. Both monogenic and polygenic pathways can lead to coronary artery disease. A monogenic trait is a characteristic that is produced by a single gene or a single allele. A polygenic trait is a characteristic controlled by two or more genes that are located at different areas on different chromosomes. Knowledge about the genetic basis of coronary artery disease (CAD) can help identify high risk individuals in which primary preventive strategies can be instituted early and can also be the basis for understanding unknown pathways and developing novel treatment strategies (Assimes and Roberts 2016).

Familial hypercholesterolaemia (FH) is an autosomal dominant cause of elevated low-density lipoprotein cholesterol (LDL-C) levels. Heterozygotes have total cholesterol (TC) in the range of 8-15mmol/L and usually develop CAD by the age of 55 in men, 60 in women. In Caucasian populations the frequency of heterozygous FH is 1/500 while that of homozygous FH is

1/1,000,000. LDL receptor (*LDLR*) variants account for 90% of FH cases and variants in the LDLR-binding domain of ApoB account for 5% of cases. Proprotein convertase subtilisin/kexin type 9 (*PCSK9*) attaches to the LDLR resulting in LDLR breakdown and decreased LDLR on the cell surface. Gain of function *PCSK9* variants account for 1% of FH cases (Nordestgaard et al. 2013). Autosomal recessive FH is caused by variants on the LDL receptor adaptor protein (*LDLRAP*) (Cuchel et al. 2014).

Compound heterozygote variants in either the *LDLR* or *LDLRAP* can give a clinical picture similar to homozygous FH. Up to 50% of subjects exhibiting clinical features of FH will not have known variants (Talmud et al. 2013). This may be due to novel monogenic or polygenic variants. Relatives of FH patients may have a genetic diagnosis of FH without exhibiting the full phenotypic picture. This may be due to the presence of other protective genes or due to lifestyle measures (Nordestgaard et al. 2013).

Monogenic disorders of lipid metabolism explain only a minority of cases of myocardial infarction. In a study of early onset MI subjects in the US, only 1.7% of subjects had an FH variant (Khera et al. 2018). Most cases of hyperlipidaemia have a complex aetiology and several other genes are thought to influence lipid levels though to a lesser extent.

A meta-analysis of 46 lipid genome wide association studies comprising >100,000 individuals of European descent identified 95 loci associated with lipids ($p < 5 \times 10^{-8}$). Some of these loci overlap with genes involved in Mendelian lipid disorders. Another two, represent the well-established therapeutic targets 3-Hydroxy-3-Methylglutaryl-CoA Reductase (*HMGCR)* and Niemann-Pick C1-Like 1 (*NPC1L1*) (Teslovich et al. 2010). Single nucleotide polymorphisms (SNPs) associated with low density lipoprotein cholesterol (LDL-C) and High-density lipoprotein cholesterol (HDL-C) were found in the lipoprotein regulator genes Apolipoprotein E (*APOE*) and Cholesteryl ester transfer protein (*CETP*) respectively. Other loci harbour genes

known to influence lipid metabolism including Lipoprotein(a) (*LPA)*, Phospholipid transfer protein (*PLTP*), Angiopoietin-like 3 (*ANGPTL3*), Angiopoietin-like 4 (*ANGPTL4*), Scavenger receptor class B type 1 (*SCARB1*), Cytochrome P450 Family 7 Subfamily A Member 1 (*CYP7A1*), StAR Related Lipid Transfer Domain Containing 3 (STARD3), Low density lipoprotein receptor-related protein 1 (*LRP1*),), Low density lipoprotein receptor-related protein 4 (*LRP4*), Apolipoprotein A-I (*APOA1*), Apolipoprotein B (*APOB*) and low-density lipoprotein receptor (*LDLR*) (Teslovich et al., 2010; Musunuru, & Kathiresan, 2010). Common variants in these loci combine to express the dyslipidaemic phenotype. Some of these loci have sex specific effects (Teslovich et al. 2010). These 95 loci explain only about 10-12% of the total variance in blood-lipid levels meaning that most of the genetic variance remains unexplained (Shuldiner and Pollin 2010).

Protein tyrosine phosphatase 1B (PTP1B) is encoded for by protein tyrosine phosphatase, non-receptor type 1 (*PTPN1*) located on chromosome 20q13.13. Variation within the coding region is rare. A variant in the 3' untranslated region of *PTPN1*, a 1484 InsG has been associated with higher serum triglycerides (TG) and total cholesterol TC/HDL-C ratios (HDLR) and higher BMI in non-diabetic subjects (Di Paola et al., 2002; Mosapour et al., 2013). Bezzina Wettinger et al, (2014) has shown that *PTPN1* 1484InsG heterozygotes who were smokers had a lower risk of MI compared to wildtype individuals who were smokers in the Dutch population [age-adjusted OR 2.5 (95% CI 1.5-4.1) and 3.7 (95% CI 2.9-4.9) respectively, both relative to *PTPN1* 1484InsG wildtype individuals who were non-smokers] (Bezzina Wettinger et al. 2014).

Apolipoprotein E (*APOE*) is another important gene in lipid metabolism which exists in three main isoform E2, E3, E4 (Eichner 2002). The role of Apo-E in lipid metabolism has been studied extensively with E4 alleles (phenotypes E4/4 and E4/3) being found to be associated with higher LDL-C and E2 alleles (E3/2, E2/2) being associated with lower LDL-C levels. The

latter may be associated with increased plasma triglycerides and lipoprotein remnants (Davignon 2005). Individuals with the E3 allele have been shown to have increased levels of triglycerides in subjects with abdominal obesity (Zarkesh et al. 2012). Besides lipid homeostasis, the E2 and E4 alleles have also been associated with abdominal obesity in diabetics (Tabatabaei-Malazy et al. 2012).

Section 1.6. Challenge of Studying Genetics of Coronary Artery Disease

There are three main features of complex diseases which complicate the study of their genetic basis. First of all, complex diseases including CAD vary in severity of symptoms and age of onset creating difficulties defining the phenotype and selecting the best population to study. Secondly, CAD has a range of aetiological mechanisms that lead to the same outcome involving various biological pathways which can interact with each other. Finally, complex diseases are more likely to be caused by several and even numerous genes, each with a small overall contribution and relative risk. These genes may be protective or deleterious and the interaction between the numerous protective and deleterious variants is complex (Tabor et al. 2002).

Section 1.7. Heritability of Coronary Artery Disease

Family aggregation studies were the first to indicate a non-mendelian heritable component to CAD. In these studies rates of CAD among subjects with a positive family history were compared to those with no family history. This highlighted 1.5-2.4 fold higher rates of CAD in the individuals with a family history when adjusting for age, sex and for known traditional risk factors. Furthermore, the stronger the family history and the earlier the age of onset, the

higher the risk in first-degree relatives (Friedlander 1994; Schneider et al. 2006; Chow et al. 2011; Nielsen et al. 2013).

Twin and adoptee studies made it clear that in addition to the genetic component, family-related risk may be confounded by exposure to family-related environmental risk factors such as tendency to smoke, diet, and likelihood to carry out physical activity (de Faire and Pedersen 1994). Comparing concordance rates of disease between monozygotic and dizygotic twins in large Danish and Swedish registries the estimated heritability of CAD has been calculated to be between 40-60% (de Faire and Pedersen 1994; Wienke et al. 2001; Zdravkovic et al. 2002).

Familial hypercholesterolaemia is a good example of how genetics has revolutionised the management of dyslipidaemia. The genes responsible for familial hypercholesterolaemia were all described using linkage analysis (Assimes and Roberts 2016). The identification of these genes has led to the development of genetic testing and earlier treatment of FH cases. Inactivating variants in *PCSK9* which resulted in decreased levels of LDL and reduced the risk of CAD (Cohen et al. 2006) resulted in the development of monoclonal antibodies that inhibit PCSK9. The PCSK9 inhibitors decrease LDL by >50% while being safe and well tolerated (Bandyopadhyay et al. 2018). The discovery of *PCSK9* as a cause of familial hypercholesterolaemia and the understanding of its mechanism of action has led to the rapid development of a new class of lipid lowering drugs that have proven to be highly effective and a welcome addition to statins in clinical practice (Shapiro et al. 2018).

Apart from FH the use of family studies to identify other monogenic causes of CAD has not yielded convincing results and have been limited by lack of reproducibility (Wang et al. 2003; Mani et al. 2007a; Lieb et al. 2008; Keramati et al. 2014variantStudying families with extreme phenotypes such as early onset CAD in the absence of traditional risk factors may still be a possible avenue to discover new loci (Khera and Kathiresan 2017).

Candidate gene studies looking for lipid related genes have been largely disappointing. In a meta-analysis of 379 candidate gene studies addressing 36 genetic associations with coronary artery disease, the findings were found to be inconsistent (Khera and Kathiresan 2017). The first study to report an association often described a stronger effect than was seen in subsequent studies (Ioannidis et al. 2001). Association studies were often carried out using large population-based collections comparing affected and unaffected individuals (Case-control studies). These studies were often underpowered, resulting in problems with replication and high rates of false positive results due to the modest effect of the gene studied. (Assimes and Roberts 2016). Discrepancies in candidate gene studies can be due to differences in the population studied and also due to biological differences in relative risk in different populations. Non-replication might also be due to the small magnitude of relative risks that are likely to be detected in candidate-gene studies of complex diseases. Furthermore, confounding bias and misclassification are more likely to obscure small-to-moderate relative risks than large relative risks (Tabor et al. 2002). Different linkage disequilibrium patterns between populations can also explain the lack of reproducibility seen with the candidate gene approach (Tabor et al. 2002).

The genome wide association era brought with it a number of putative genes proposed to be associated with CAD and its risk factors. A locus described by this approach was that at Chromosome 9p21. The increase in risk depends on the number of copies of the risk allele, with a 25% increased risk for CAD with 1 copy and 50% for 2 copies. Higher risk per allele has been observed amongst individuals with early-onset CAD. The risk mediated by Chromosome 9p21 appears to be independent of traditional risk factors. While these findings were replicated in European and east Asian cohorts, it has not been replicated in African Americans (Helgadottir et al. 2007; McPherson et al. 2007). More recently the risk has been shown to be closer to 20% per allele in the CARDIoGRAMplusc4D, 1,000 genome study meta-

analysis. (Consortium et al. 2015). The mechanism of action of Chromosome 9p21 is unclear. Chromosome 9p21 risk variants alter the expression of the non-coding RNA ANRIL, thereby altering the activity of two nearby cyclin-dependent kinase inhibitors (CDKN2A and CDKN2B) that are involved in regulating the cell cycle and cellular proliferation (Jarinova et al. 2009)(Holdt et al. 2010). The protein products of these genes $P15^{INK4A}$ and $P16^{INK4a}$ alter the function of macrophages and vascular smooth muscle cells present within vessel wall plaques (Hannou et al. 2015).

Due to the multigenic nature of CAD with multiple low penetrance variants, initially GWAS failed to detect reproducible susceptibility loci (Risch 2000). However, with the formation of large international GWAS consortia, 163 loci that are associated with CAD have been identified. Most of these variants are common variants with a mean allele frequency greater than 5%. However, combined, these loci may explain up to 40% of CAD heritability and account for 40% of all cases. Common variants are responsbile for a greater proportion of CAD heritability than rare variants. These variants are often outside coding or promoter regions leaving in doubt the exact mechanisms of how they result in CAD. These common risk alleles explain the widespread predisposition of humans to atherosclerosis but familial clustering of CAD (The CADIoGRAMplusC4D Consortium 2013; Nelson et al. 2017; Erdmann et al. 2018)

Genome wide association studies may be missing important associations by not incorporating information about biological pathways and intermediate phenotypes. Since many genes display redundancy and the possibility of one protein to compensate for deficiency in another, analysing in haplotypes and in combinations of SNPS based on biological pathways may be a better approach.

Besides GWAS for common variants, GWAS for rare variants or Rare Variant Association studies have been performed (Khera and Kathiresan 2017). These studies have uncovered association between genes in triglyceride-rich lipoprotein metabolism to CAD. Damaging variants in lipoprotein lipase are associated with increased levels of circulating triglycerides and CAD (Khera et al. 2017). Apolipoprotein A5 (ApoA5) which encodes a protein that enhances LPL activity has also been associated with increased CAD risk (Do et al. 2015). Rare variants in Apolipoprotein C3 (ApoC3) and Angiopoietin Like 4 (ANGPTL4) that produce protein products that inhibit LPL are associated with decreased triglyceride-rich lipoproteins and decreased CAD risk (Crosby et al. 2014; Jorgensen et al. 2014; Dewey et al. 2016; Stitziel et al. 2016). Pharmacological therapies are currently being developed to mimic the protective variants in these genes in which inhibition of the related protein would be predicted to reduce risk.

Although most of the associations found by GWAS present only a small increased risk of CAD which may not be clinically relevant, the approach has played an important role in the discovery of new mechanisms in CAD. One such example is the role of Sortilin-in LDL catabolism. The *SORT1* locus was associated with increased LDL-C and a higher risk of MI. Functional studies then confirmed that sortilin, the protein product of *SORT1* is involved in ApoB and LDL catabolism (Musunuru et al. 2011). Similarly a variant located in an intronic region of disintegrin and metalloproteinase with thrombospondin motifs-7 (*ADAMTS7*) was associated with an increased risk of CAD (Reilly et al. 2011). This gene was subsequently found to be involved in proteolysis and remodelling of the vasculature (Pu et al. 2013). This finding could provide a new therapeutic target for the treatment of CAD.

The advent of large-scale genotyping of common variants has facilitated the development of Mendelian randomization (MR) studies. MR studies are an approch to evaluate the causal relationship between a genetic variant and a disease of interest (Tan et al. 2020). Mendelian

randomisation research may be less affected by confounding or reverse causation than conventional observational studies. MR uses a common genetic polymorphism with well-understood effects as a proxy for increased or decreased exposure to a modifiable phenotype to determine whether clinical interventions on the risk factor are likely to lead to changes in a disease outcome or to prove causation. This kind of study has been developed to help determine whether the link between a risk factor and outcome is causal. MR data suggest that homocysteine, fibrinogen, secretory phospholipase IIA, lipoprotein- associated PLA2, high sensitivity C reactive protein, and high density lipoprotein (HDL) are not casually associated with CAD (Jansen et al. 2014). These observations are supported by the failure of RCTs that investigated folic acid supplementation, secretory phospholipase IIA and lipoprotein associated PLA2 inhibitors, and, most recently cholesterol ester transfer protein inhibitors (Yang et al. 2012; Keene et al. 2014; SJ et al. 2014; The Stability Investigators 2014). However, it is clear that a single gene rarely has a single effect. In general, in this field a candidate gene will affect multiple biological pathways. If these pathways also independently affect the disease being studied the results of MR studies may not be reliable. Linkage disequilibrium and epigenetic effects may also affect the validity of MR results in, as yet, unknown ways. Most importantly for coronary artery disease, in conditions were there is also a strong environmental effect, the environmental effect can strongly alter the genetic influence also affecting the validity of MR studies. (Dimitrakopoulou et al. 2017). So, while MR results give interesting insight and have proven particularly useful in the determination of drug effects they cannot be relied on completely and absolutely to define causality.

Section 1.8. Whole Exome and Genome Sequencing Studies of Coronary Artery Disease

Next generation sequencing platforms have enabled large scale exome sequencing and whole genome sequencing. In 2010 the National Heart, Lung and Blood institute funded several whole exome sequencing studies in individuals experiencing CAD (Fu et al. 2013). Discoveries to date using WES and exome chip genotyping related to CAD have almost exclusively pointed to previously established genes involve in lipoprotein metabolism (Assimes and Roberts 2016). These findings combined with the results of MR studies have reinvigorated the development of triglyceride-rich lipoprotein lowering agents (Do et al. 2013; Kathiresan 2015).

In other smaller studies, WES has recently highlighted that rare nonsynonymous variants in Spectrin Beta Non-Erythrocyte 5 (SPTBN5), Nidogen 2 (NID2), and ADAMTSL4 may protect against CAD and implicated a 2-base insertion in RecQ Like Helicase 5 (RECQL5) as a cause of early onset MI in a Chinese Han family (Abramowitz et al. 2016; Xie et al. 2016). A whole exome sequencing study comparing 5,000 cases with early onset CAD to CAD-free controls found gain of function variants in LDLR and inactivating variants in *PCSK9* (Do et al. 2015). In an Icelandic cohort in which whole genome sequencing was performed a protective 12 bp deletion that leads to the inactivation of ASGR1 (which encodes asialoglycoprotein receptor) has been identified. Heterozygous carriers of this variant had reduced LDL and triglyceride levels and a decreased risk of CAD (Nioi et al. 2016).

The possibility to carry out whole genome sequencing has given rise to the possibility to identify all the rare and common genetic variants in an individual and high throughput technology has enabled the possibility of identifying individuals that are at high risk of myocardial infarction due to common variants with low OR's previously identified by GWAS.

Such an approach could identify subjects that had a similar risk of MI to FH patients offering the opportunity of early intervention. In one such approach using 6,630,150 polymorphisms performed on samples from the UK Biobank 8% of individuals were found to have an OR of ≥3.0, 2.3% of individuals were found to have an OR of ≥4.0. While 0.5% of individuals were found to have an OR ≥5.0. Such an approach could identify 20-fold more people at a comparable or greater risk than if they had familial hypercholesterolaemia (Khera et al. 2018). In a cohort of early myocardial infarction patients in the United States, 17.3% of subjects had a high polygenic score with a 3.7-fold (95% CI, 3.1-4.6; $p<0.001$) increased risk (Khera et al. 2019).

Genetic prediction scores can assess risk from the time of birth, well before risk factors become apparent. However, it is clear from the study in early MI patients (Khera et al. 2019) that the majority of subjects were not in the high polygenic risk score group and may possibly have been reassured by this method. Such an approach may not be applicable to other populations as the frequency of variant alleles, linkage disequilibrium patterns and haplotype patterns and effects varies in different population. Furthermore, the interactions of these genes with environmental and lifestyle factors will also vary.

A systems biology approach studying extreme phenotypes and taking into consideration regulatory networks still have a role to play and may give complementary data to that offered by large polygenic risk scores.

Section 1.9. Gene-Gene Interactions

A large proportion of disease susceptibility is explained by differences in an individual's genetic makeup. However, susceptibility to complex diseases such as cardiovascular disease varies more than can be explained by known risk alleles identified using candidate gene and

GWAS studies. Explaining this missing heritability is important to further understand genetic susceptibility to complex disease. Reasons underlying this missing heritability include the possibility that there are a large number of variants of smaller effect that are yet to be found and that haven't been identified due to poor phenotyping in large GWAS studies. Other explanations include that there may be high-risk variants that are very rare and that have not been identified. Furthermore gene-gene interactions and gene-environment interactions may play a central role in explaining missing heritability (Manolio et al. 2009)

Genetic interactions between loci is termed epistasis (Ilona Miko 2008). Epistasis can occur at multiple levels including: protein-protein, protein-DNA, protein-RNA (Mackay 2014). Attempts have been made to study gene-gene interactions for many disorders including lipids. These studies were performed using well-studied genome-wide associations, known protein-protein interactions and pathway information. In the case of lipids, an interaction between 3-Hydroxy-3-Methylglutaryl-CoA Reductase (*HMGCR*) and a locus near Lipase C (*LIPC*) was found (Ma et al. 2012). The difficulties encountered studying two loci are much larger when attempting to introduce higher-order SNP interactions that are known to exist, and which are also influenced by interacting pathways and non-genetic exposures.

The study of epistasis in the high throughput sequencing era is complicated by the number of combinations present when studying 1 million SNPs or more. Modelling combinations is hampered by the multiple assumptions made due to lack of biological knowledge regarding these pathways especially when it is unknown whether effects are dominant, additive or multiplicative (Ritchie and Van Steen 2018).

Gene- Gene interactions are often described in terms of:

- Functional epistasis that addresses molecular interactions

- Compositional epistasis which refers to the blocking of one allelic effect by another allele at a different locus
- Statistical epistasis which is defined as the statistical deviation from the additive effects of two loci on the phenotype

There are many issues which complicate the study of epistasis such as phenocopies and locus heterogeneity that affect the relationship between genotype and phenotype (Wan et al. 2010).

The problem with studying more than a 2 loci model is the number of parameters contained and the extremely large data sets that are required to accurately estimate these parameters (Cordell 2009). Whole genome analysis studies are ideal to study the common disease-common variant hypothesis. Whole genome analysis studies present a computational and statistical challenge due to multiple-testing burden resulting in many highly statistically significant results that are there only by chance and which are not overcome solely by large sample sizes and the Bonferroni-correction. Despite advances in technology incorrectly-called and SNPs which are not called, are still a source of error and certain genotypes for a given SNP are more likely to fail than others with any technology (Purcell et al. 2007).

Studying gene-gene interactions in the study of coronary artery disease have to date shown no large effect interactions amongst common variants (Mendonça et al. 2009; Musameh et al. 2015). New approaches are required to help tackle the complexity of studying multiple interactions and the large quantity of data produced by new sequencing methods such as high throughput sequencing.

Section 1.10. Environmental Factors

Environmental factors can have a strong influence on the effect of a particular gene. A particular trait may only be expressed in the presence of an environmental component. Not taking into consideration such environmental influences can result in underestimation or overestimation of observed effects. Stratifying for known environmental factors such as smoking, statin use, and diet are therefore very important. Such considerations are usually not performed in large GWAS, MR or Polygenic risk score studies and could explain some conflicting data (Lobo 2008).

Section 1.11. Conclusion

The events leading to myocardial infarction are complex and poorly understood. The interaction of genetic and environmental factors which vary throughout an individual's life and which vary from population to population make atherosclerosis and myocardial infarction a complex condition to study. Environmental risk factors including various measures of adiposity, diet, smoking and alcohol will be analysed to assess which are most important in the Maltese population. Genetic variants in *ApoE*, *PTPN1* and *FH* related genes will also be analysed in detail to assess their importance in the Maltese population. However, a simple approach, considering the effect of single exposures or genetic variants on the risk of MI is unlikely to add to our current knowledge. An approach which takes into account the complex interactions of environmental exposures is required. For this reason, combinations of exposures will be studied in an attempt to clarify the association between known risk factors and the risk of MI. This will be demonstrated in the interactions of smoking and alcohol. Similarly, an approach which takes into consideration the complex genetic interactions taking place and gene-environment interactions is required. A systems biology approach, looking at

genetic variants in adiposity related pathways will be focused upon in an attempt to overcome the limitations of GWAS, MR and Polygenic risk scores described above. Furthermore, studying the effect of combinations of polymorphisms on risk for MI and key intermediate phenotypes will be performed in an attempt to shed light on the intricacies of the pathogenesis of myocardial infarction.

Chapter 2. Methodology

Section 2.1. Introduction to the MAMI Study

The Maltese Acute Myocardial Infarction (MAMI) Study was set up to study epidemiological and genetic risk factors for MI in the Maltese population. It is a collaboration between the University of Malta and the Malta Department of Health. A total of 1098 research subjects were recruited to this study between the 10th of June 2011 and the 30th of April 2013. Three categories of research subjects were recruited; cases, controls and relatives of cases. All participants were selected to be of Maltese ethnicity with Maltese parents and grandparents.

Cases were research subjects admitted with a first MI to Mater Dei Hospital (MDH) or Gozo General Hospital in the study period. Males older than 70 years of age and females older than 75 years of age were excluded. The Universal definition of myocardial infarction was used to define cases (Thygesen et al. 2007). Only subjects with a type I MI were recruited. This was defined as a rise/ or fall of troponin with at least one value above the 99th percentile of the upper reference limit (URL) together with at least one of the following:

- Symptoms of ischaemia – chest pain, epigastric pain, shortness of breath
- ECG changes indicative of new ischaemia (new ST-T changes or new left bundle branch block)
- Development of pathological Q waves on the ECG
- Regional wall motion abnormalities on echocardiography or ventriculography

In total 423 cases were recruited with a type I MI. Controls, sex- and frequency-matched to the cases in five-year age group, were recruited from a randomly generated list of addresses of Maltese individuals issued by the National Statistics Office. They were initially invited using an invitation letter, which was followed up by a phone call. Individuals with previous MI, left bundle branch block, coronary artery bypass, angioplasty and/or coronary stenting were excluded.

Cases were invited twice to the study, once at the time of hospital admission with MI and again at least six months after, to allow the acute effects of the infarction to subside. A total of 301 cases (71.2%) attended the follow-up visit. Cases were asked to invite their relatives to participate. Since they are known to be at an increased risk of MI, relatives help in the search for the causative risk factors of MI. Upon completion of recruitment of relatives, additional controls were recruited to frequency match with the age of relatives so that difference between relatives and controls could be investigated. A total of 210 relatives and 465 controls were recruited as part of the MAMI Study. Recruitment of research subjects, conduction of questionnaires and physical measurements were performed as part of this thesis.

Out of 423 cases recruited to the study, previous cardiac interventions had been performed in 9.0% (n=38), 13.2% (n=5) had undergone percutaneous coronary intervention (PCI); 36.8% (n=14) had previous CABG and 21.1% (n=8) had a previous angioplasty, 21.1% (n=8) had both PCI and angioplasty, 2.6% (n=1) had CABG and angioplasty and 5.3% (n=2) had PCI, angioplasty and CABG.

A total of 29 cases gave their consent to participate but did not complete their admission questionnaire. An additional 68 cases could not be moved from bed during conduction of their admission questionnaire and so measurements of waist, hip, height and weight were not recorded at the time of admission.

Using the Genetic Association Study (GAS) Power Calculator (Skol et al. 2006) to compute statistical power using an aditive model assuming an incidence of MI of 8% (Mozaffarian et al. 2015) there is a 72% power to determine a 1.5 fold effect for an allele that has a frequency of 0.05 and a 100% power to determine a 2.0 fold effect for an allele that has a frequency of 0.05. If the allele frequency is 0.1 this study has a power of 96% to determine a 1.5 fold effect

and 100% power to determine a 2.0 fold effect. No adjustments are necessary for multiple testing (Rothman 1990).

Section 2.2. Data collection – Questionnaire, Physical Measurements, Medical Records

A 126-question interviewer-led questionnaire was carried out (Appendix 2). Questions related to marital status, education and work, smoking and alcohol consumption, tea and coffee consumption, sleeping habits, medical history, family tree, stress, travel, food consumption, physical activity and a menstrual history were asked in English or Maltese depending on the subject's preference. These questions were selected to assess both known and potential risk factors for MI. The questionnaire was then transcribed to digital format using the LimeSurvey.

The participants' medical history was collected by a review of the centralised medical records held at Mater Dei Hospital. Laboratory test results spanning back 6 years were also collected. Lists of medications were also documented.

Measurements of blood pressure with a digital sphygmomanometer, pulse, oxygen saturation, weight, height, waist and hip circumference and observations regarding Achilles tendonitis, tendon xanthomas, xanthelasmas, corneal arcus and peripheral oedema were taken during the interview. Waist circumference was taken at the approximate midpoint between the lower margin of the last palpable rib and the top of the iliac crest while the hip circumference was measured at the widest portion of the buttocks, both as per WHO protocol (World Health Organization 2008). Detailed standard operating procedures (SOPs) for these measurements are found in the MAMI study manual. An ECG was performed on all research subjects to exclude a previous MI in controls and to confirm MI in cases.

Section 2.3. Blood Letting

Blood letting was carried out from the cubital vein after a 12 hour fasting whilst allowing water consumption. To avoid effects of inflammatory factors on blood measurements, blood collection was postponed by: 6 weeks in case of inflammation, infection; 8 weeks in cases of vaccination and 6 weeks after medical, dental interventions or minor injuries. None of the research subjects had blood transfusions/donation in the previous 4 months. Research participants were given instructions to follow prior to blood collection to avoid preanalytic effects on the sample drawn.

Section 2.4. Blood Processing

Serum and EDTA blood samples taken by caring physician during the course of the cases admission were retrieved from the laboratory and tested for lipid profile and HbA1c. The remaining sample was aliquoted and stored at -80^{o}C. The banked whole blood samples of cases who did not come for their follow-up visit were used for DNA extraction.

Morning fasting blood samples were collected from all cases at follow-up, controls and relatives. HbA1c, complete blood count with differential counts, TSH, free T4, folate, Vitamin B12, cortisol, lipid profile, urea, electrolytes, liver function tests, fasting blood glucose, uric acid, creatinine and calcium were assayed at the Pathology labs of MDH immediatley following blood collection.

At the site of venepuncture, aliquots of citrated and EDTA whole blood were put in lysis buffer and stored immediately at -80^{o}C.

CBC and differential analysis were performed from EDTA whole blood on a Sysmex XT-400I (Sysmex America, Inc., US). Fasting lipid profiles were obtained by homogenous enzymatic

colorimetry (Roche-Hitachi Cobas 311). The TC to HDL-C ratio was calculated and is referred to as the HDL-ratio (HDLR). When TG levels were lower than 4.52 mmol/L, LDL-C was calculated using the Friedewald-calculation. LDL-C was measured directly by homogeneous enzymatic colorimetry when TG exceeded 4.52 mmol/L. HbA1c and abnormal haemoglobin levels were measured by fully automated high-pressure liquid chromatography (Bio-Rad, Variant II). Quantitative determination of fasting blood glucose, liver function tests, aspartate aminotransferase, urea, electrolytes, calcium, uric acid and creatinine were done on serum sample on a Roche/Hitachi Cobas c311 system at the Pathology labs of MDH. Isoelectric focusing for haemoglobin variant analysis was performed at the Laboratory of Molecular Genetics using the RESOLVE ® Neonatal Heamoglobin Test Kit (PerkinElmer, Massachusetts, US). *PTPN1* mRNA levels were measured using Multiplex Ligation-dependent Probe Aplification (MLPA) with the relative abundance of mRNA being expressed as a normalized ratio of the peak area divided by peak area of a control gene (MRC-Holland MLPA ®, Amsterdam, The Netherlands).

Samples for DNA, RNA, protein and additional biochemical analysis were processed, distributed into 80 aliquots in micronic tubes (Lelystad, The Netherlands) and stored at -80ºC within 90 minutes of blood collection. Upon completion of the whole collection serum samples stored at -80ºC were tested for cortisol, gastrin, *H. pylori* IgG antibody, lipopolysaccharide binding protein (LBP), IL-6 protein levels, insulin, C-peptide and hs-CRP on an Immulite 2000 immunoassay system (Siemens, U.S.).

Section 2.5. Quality Control

Parameters from controls were compared to data from the EHIS2010 and MONICA 1984 study (McElduff, Dobson, Jamrozik, & Hobbs, 2000) to ensure that there was no obvious bias in the recruitment method chosen for the selection of controls (Attard 2015).

Section 2.6. Statistical Analysis

Statistical analysis was performed using SPSS version 25. Means (range) for age and medians and interquartile ranges (IQR) were calculated to assess differences in continuous variables between categories. Frequencies were estimated for categorical data and assessed using Chi-square testing. Visual analysis of the distribution of variables showed skewed data for most variables and consequently non-parametric testing was performed. Association of risk factors with intermediate phenotype was assessed using the Mann-Whitney test. Correlation between risk factors were assesed using Spearman's test of correlation. P-values of ≤ 0.05 were arbitrarily considered as significant, while p-values ≤ 0.1 were still considered interesting (Baker 2016; Moyé 2016; Wasserstein and Lazar 2016).

Odds ratios (ORs) for myocardial infarction were calculated using logistic regression and are presented crude (ORs), Age Adjusted ORs (AgeOR) and after multivariate adjustment for common risk factors including diabetes, hypertension, alcohol consumption, smoking (AdjOR). The 95% confidence interval was calculated from the logistic regression model. ORs for continuous variables were calculated using tertiles and quartiles. Analyses were performed separately for men and women since effects vary by sex. The number of women in certain categories was small leading to confidence intervals including 1.0 and so conclusions could not always be drawn. Restrictions were also performed for age by dividing the research subjects into <60 and ≥ 60 years of age. Restrictions were also performed for lipid lowering

drug use and for measures of adiposity. Attributable risk and Population attributable risk were estimated for a high waist-hip ratio in both sexes.

Genetic polymorphisms were tested for Hardy-Weinberg equilibrium in controls. Allele and genotype frequencies were estimated and crude ORs and adjusted for age were calculated. Stratification analyses was performed in order to assess gene-gene interactions and gene-environment interactions. ORs for genotype were not adjusted for conventional risk factors for MI since these risk factors do not influence the genotype.

Section 2.7. Definitions

Diabetes was defined as self reported diabetes or subjects taking oral hypoglycaemic agents or research subjects with an Hba1c of ≥6.5. Impaired fasting glucose or impaired glucose tolerance were not tested for.

Hypercholesterolaemia was taken as self reported hypercholesterolaemia or subjects taking lipid lowering agents.

Hypertension was taken as self reported hypertension or subjects taking antihypertensive medication.

HOMA-IR was calculated using the following formula (Insulin IU/mL x fasting glucose)/22.5

HOMAC was calculated using the following formula Cpeptide nmol/Lx fasting glucose)/22.5.

Body Mass Index (BMI) was calculated using the formula: weight in kg/ height in m^2. BMI less than 18.50 was defined as underweight, 18.50 - 24.99 as normal, 25.00 - 29.99 as overweight, greater than or equal to 30.00 as obese.

Waist-hip ratio was calculated by dividing the average waist circumference by the average hip circumference. In men a waist-hip ratio less than 0.90 was defined as normal, while a wasit-hip ratio greater or equal to 0.90 was defined as high. In women a waist-hip ratio less than 0.85 was defined as normal and a waist-hip ratio greater or equal to 0.85 was defined as high.

Waist-height ratio was calculated by dividing the average waist circumference by height. A wasit height ratio less than or eqaul to 0.5 was defined as normal while a waist-height ratio greater than 0.5 was defined as high.for both sexes.

Waist circumference was considered normal if less than or equal to 0.80cm in women and if less than or equal to 0.94cm in men (World Health Organization 2008).

Section 2.8. General Limitations

The limitations of the analysis performed include that for most exposures, these were based on a snapshot at one particular point in time and may not reflect accurately the lifelong exposure to a particular risk factor. The results must be interpreted keeping in mind a lack of power for some analysis which were restricted by gender especially when analysis for combined effects was performed. However lack of power is expected to result in false negative results. Any associations that have been detected were detected because there was sufficient power for that particular analysis.

Section 2.9. Characteristics of the MAMI Study Participants

The general characteristics of the MAMI study participants have been previously described (Attard et al. 2017) (Table 2-1).

Table 2-1 Characteristic of the MAMI study participants (Attard et al. 2017).

	Cases with MI* N=394	Controls N=465
Men (%)	316 (80.2)	327 (70.3)
Mean age years (range)	59 (30-75)	55 (20-77)
Smokers, n (%)	146 (37.1)†	105 (22.6)
Ex-smokers, n (%)	145 (36.8)	161 (34.6)
Regular alcohol drinkers§ n (%)	232 (58.9)†	311 (66.9)
Reported diabetes n (%)	110 (28.2)†	53 (11.9)
Reported hypertension n (%)	184 (47.2)†	160 (35.6)
Reported hypercholesterolemia n (%)	179 (47.0)†	160 (36.9)
Overweight (BMI 25-30 kg/m^2)‖ n(%)	136 (41.7)	197 (42.6)
Obese (BMI >30 kg/m^2)‖ n (%)	149 (45.7)†	173 (37.4)

*A total of 423 cases gave consent to participate in the study, however 29 cases did not complete their admission questionnaire so questionnaire data about them is missing.

§Regular alcohol drinkers were defined as research subjects who consumed at least one unit of alcoholic beverage, including beer, wine, and spirits, per week in a year. ‖BMI cut-offs were as defined by WHO.

†p-values <0.01

Section 2.10. DNA Extraction

DNA extraction was performed using a modified salting-out procedure (Miller, Dykes, & Polesky, 1988). The quality and quantity of DNA was assessed by gel electrophoresis and Qubit respectively. DNA samples were diluted to a working solution of 1:10 and stored at 4ºC. The stock DNA samples were stored in TE pH 8.0 at 4ºC.

Section 2.11. Genotyping

Section 2.11.1. *PTPN1* Polymerase Chain Reaction

PTPN1 1484insG was genotyped by polymerase chain reaction (PCR) followed by restriction enzyme digest. The PCR mastermix consists of Quick-Load Taq 2 x NEB mastermix (New England Biolabs, Inc. USA), and forward and reverse primer. Approximetly 20-30ng of DNA were added to the PCR mix (Table 2-2). A negative conrol was included in each plate.

The PCR was run on an Eppendort mastercycler (Eppendorf, Hamburg, Germany) using the settings as specified in Table 2-3.

Table 2-2 *PTPN1* 1484InsG PCR master mix

PCR mastermix	x1 (mls)	x102 (mls)
Quick-Load Taq 2 x NEB mastermix (New England Biolabs, Inc. USA)	5	510
Sterile water	3.8	387.6
Forward primer 50mM	0.1	10.2
Reverse primer 50mM	0.1	10.2

Table 2-3 *PTPN1* 1484insG PCR settings

Temperature	Duration	Cycles
95ºC	5 min	
95ºC	30 sec	
56ºC	30 sec	x30 cycles
68ºC	50 sec	
68ºC	5 min	
4ºC	10 min	

A total of 5µL of PCR product were mixed with 1µL of loading dye and separated on a 3% agarose gel electrophoresis at 80V for 20 mins.

Section 2.11.2. *PTPN1* Restriction Digest

PCR products for the *PTPN1* 1484insG were digested with SacII (New England Biolabs, Inc USA) overnight at 37ºC (Table 2-4) . The volume of PCR product required for the digest was 3ul. *PTPN1* digest mastermix was prepared as per Table 2-4. Digests were separated by electorphoresis on a 3% agarose gel at 110V, constant voltage, for 3-5 hours and visualized by ethidium bromide staining. A restriction map and typical separation are shown in Figure 2-1.

Table 2-4 *PTPN1* 1484insG digest master mix.

Mastermix	x1 (mls)	x102 (mls)
Sterile water	10.2	1040.4
NEB4 (New England Biolabs, Inc USA)	1.5	153
BSA (New England Biolabs, Inc USA)	0.15	15.3
Sac II (New England Biolabs, Inc USA)	0.15	15.3

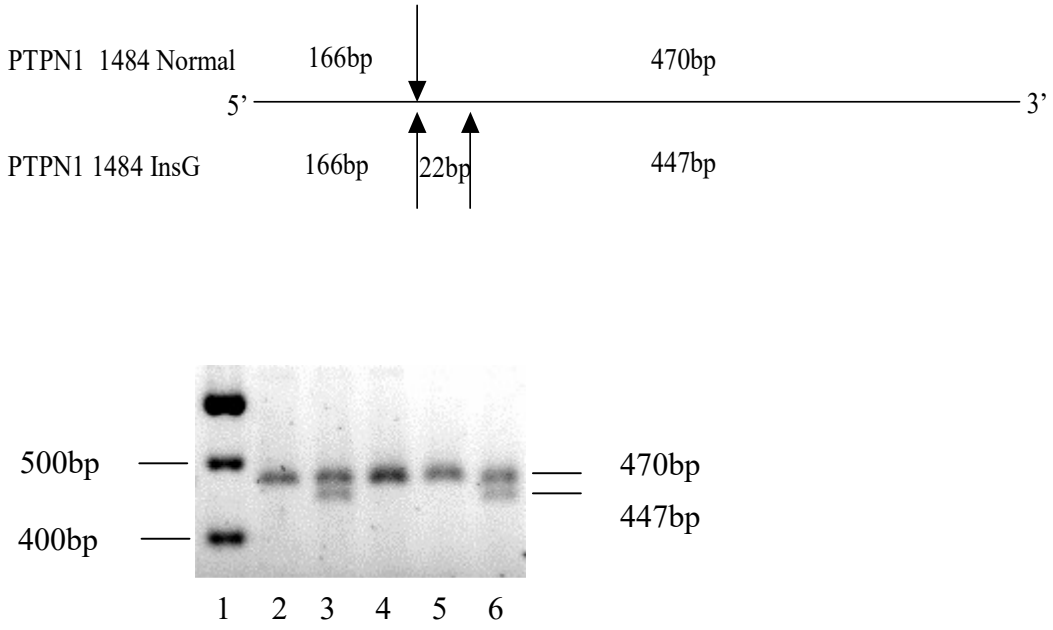

Figure 2-1 SacII restriction map for the detection of the PTPN1 1484 InsG polymorphism. The arrows show the enzyme restriction sites. Below, a representative separation of products of restriction enzyme digestion on agarose. Lane 1, size marker, 100bp ladder, lanes 2, 4 and 5: homozygous wildtype for PTPN1 1484, lanes 3 and 6: PTPN1 1484 InsG heterozygotes.

Section 2.12. Selection of Genes for Familial Hypercholesterolaemia

Dutch Lipid Network Scores (DLCNS) (Nordestgaard et al. 2013) were calculated for each research subject in the MAMI study. In order to choose research subjects with the highest probability of having FH, the highest LDL-C level the research subject was documented as having in his medical history was taken into account in order to overcome the effect of statins and lifestyle. The research subject's relatives were analysed for premature CVD and high LDL-C in order to select research subjects with the highest probability of having a genetic cause of hyperlipidaemia. Research subject number 12 was not part of the MAMI collection

but he was included in this analysis due to his high baseline LDL-C and history of premature CVD.

Section 2.13. High Throughput Sequencing

Section 2.13.1. Library preparation

The genes known to be involved in FH (*LDLR*, *APOB*, *PCSK9*, *LDLRAP1*) were sequenced using high-throughput sequencing (HTS) technology. Target-enriched DNA libraries were generated following the Agilent SureSelect^{XT} Target Enrichment System for Illumina Paired-End Sequencing Library protocol (Version B.2, Agilent Technologies Inc, Santa Clara, USA) using a custom-designed DNA bait library (Agilent SureSelect^{XT} Custom 0.5-2.9Mb, Part number 5190-4817, Agilent Technologies Inc, Santa Clara, USA). All the selected DNA samples were purified using the Vivacon® 500 ultrafiltration spin columns (Sartorius stedim, BioTech GmBH, Germany). DNA samples were desalted by washing 3 times with nuclease-free water removing 99% of the initial salt concentration. The desalted purified DNA samples were stored at 4^{o}C. The concentration and purity of the samples was assessed using the Nanodrop 2000 UV spectrophotometer (Thermo Scientific, USA). All DNA samples were of good quality, with a 260/280 absorption ratio between 1.8 and 2.0. The DNA concentration was used to determine whether the low input 200ng protocol or the standard 3μg protocol was followed, as recommended by the Agilent. The genomic DNA was sheared using the Covaris S220 series (Covaris, Inc., Massachusetts, USA) using SonoLab 7.2 software, as specified in Table 2-5 below.

Table 2-5 Covaris S220 Series settings used for the generation of DNA libraries of base-pair lengths of 150bp

Covaris Shearing Parameter	Parameter Value
Duty Factor	10%
Peak Incident Power	175
Cycles per burst	200
Treatment Time	360 seconds
Bath Temperature	4 to 8°C

All purifications steps carried out during library preparation were performed using the Agencourt® AMPure® XP beads (Part number: A63881, Lot number: 14633000, Beckman Coulter, Inc., USA).

End-repair of the sheared DNA fragments was carried out to ensure that the double-stranded library fragments do not have any overhanging strands and contain an end 5'phosphate and 3'hydroxyl group. Adenylation of the 3' ends of the blunted fragments was carried out, followed by adaptor ligation. The adaptor-ligated libraries were then amplified PCR in order to achieve the required pre-capture library concentration, using the thermal profile as indicated in Table 2-6.

Table 2-6 PCR thermal profile for the amplification of the pre-capture library.

	Temperature	Time	Number of cycles
Initial denaturation	98ºC	2 minutes	
Denaturation	98ºC	30 seconds	7 cycles
Annealing	65ºC	30 seconds	
Extension	72ºC	1 minute	
Final extension	72ºC	10 minutes	
Hold	4ºC		

DNA fragments containing target candidate gene regions were then captured through hybridization to complementary biotinylated RNA oligonucleotide 'baits' during an overnight incubation reaction. Dynabeads™ MyOne™ Streptavidin T1 magnetic beads were then used which selectively capture the biotinylated bead-bound library fragments, allowing the non-targeted portion of the genome to be washed away during a subsequent purification step. Post-capture amplification of the target-enriched sample libraries was carried out using the PCR thermal profile described in Table 2-7. Unique, synthetic oligonucleotide index sequences were ligated to the ends of each amplified library fragment sequence during post-capture amplification.

Table 2-7 PCR thermal profile for the amplification of bead-bound target-enriched DNA samples.

	Temperature	Time	Number of cycles
Initial denaturation	98ºC	2 minutes	
Denaturation	98ºC	30 seconds	
Annealing	57ºC	30 seconds	12 cycles
Extension	72ºC	1 minute	
Final extension	72ºC	10 minutes	
Hold	4ºC		

The quality and quantity of the index-tagged, target-enriched libraries were assessed using the Agilent High Sensitivity DNA kit (Agilent Technologies Inc., Waldbronn, Germany) and Agilent DNA 1000 kit (Agilent technologies Inc., Waldbronn, Germany) on the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, USA). The electropherograms of the indexed and adapter-ligated DNA libraries were confirmed to have peak fragment sizes of around 350 bp (Figure 2-2).

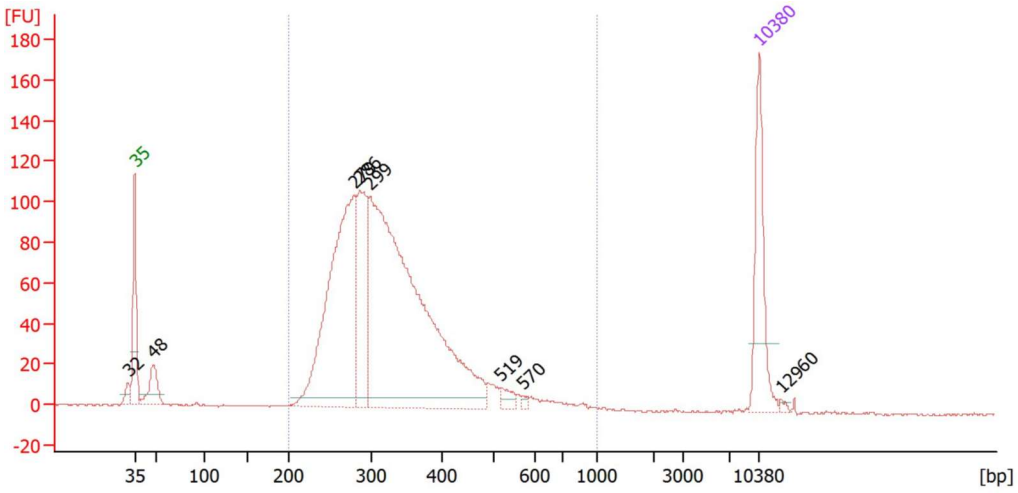

Figure 2-2 Electropherogram of index and adaptor ligated DNA with peak at around 350bp

The index-tagged, target-enriched, paired-end DNA libraries were subsequently sequenced on an Illumina HiSeq 4000 NGS platform at BGI Co Ltd (Shenzen, China). Two FASTQ files were generated for each sample, one for each of the paired forward and reverse reads. The quality of the raw reads were assessed using the FastQC v.11.5 (Andrews 2010). Data analysis of the raw read data was carried out using NextGene® software version 2.4.2.3 (SoftGenetics LLC, USA).

Section 2.13.2. High Throughput Sequencing Data Analysis

The NextGene HTS data processing pipeline was used, which first involves the conversion of the FASTQ files to a FASTA format. Quality trimming of the sequence reads is also carried out during this step. Reads which have either a median base quality Phred score below 20, a read length below 50 bases, or more than 3 uncalled bases were filtered out from the data sets. The ends of read sequences which had 3 or more consecutive bases with a base quality Phred score below or equal to 16 were trimmed back to the first base with a base quality Phred score above the 16 threshold. Illumina adapter sequences at the 3' ends of the reads were also trimmed out since these may interfere with alignment and downstream variant analysis.

The FASTA read sequences were then aligned to Genome Reference Consortium (GRC) human genome assembly build GRCh37 (patch 10), and variant calling and annotation carried out using the NextGene software program. Over 98% of targeted region of the genome had more than a 20-fold coverage in all the samples.

Variant prioritisation was carried out in Microsoft Excel (Microsoft Corporation, USA). A minimum coverage threshold of 20x was applied to all variants in the known FH genes. The candidate/shortlisted variants were genotyped by Kompetitive allele-specific PCR assay (KASP^{TM}) in the whole MAMI sample collection at LGC labs (LGC Genomics GmbH, Berlin, Germany). KASP^{TM} genotyping assays enable accurate bi-allelic discrimination of known SNPs and InDels. The selected variants were confirmed to be in Hardy-Weinberg equilibrium and OR for MI together with their confidence intervals were estimated using logistic regression in SPSS. The association between SNPs and their effect on LDL-C, NHDL-C and TG were analysed in male controls and subsequently in male controls off statins using Mann-Whitney test. Analysis in women was limited as numbers were too small. Some polymorphisms were studied in combinations and associations with intermediate phenotype were analysed using the Man-Whitney test.

Section 2.14. KASP™ Genotyping

PCR-based KASP™ genotyping was performed at LGC labs (LGC Genomics GmbH, Berlin, Germany) on all the MAMI samples for selected variants in FH and WNT related genes and also for *APOE*. KASP uses a universal reporting system simplifies assay design and eliminates time-intensive probe design steps and requires only 10ng DNA per sample per SNP. The KASP assay consists of KASP assay mix which consists of 2 allele specific primers and 1 reverse primer, the KASP mastermix which contains universal fluorescent probes, Taq

polymerase and Dntp's in an optimised buffer solution and the sample DNA. In the 1st round of PCR one of the allele-specific primers matches the target SNP and with the common reverse primer, amplifies the target region. In the 2nd round of PCR the complement of allele-specific tail sequence is generated. In further round of PCR, levels of allele-specific tail increase. The fluor labelled part of the fluorescent resonant energy transfer (FRET) cassette is complimentary to new tail sequences and binds, releasing the fluor from the quencher to generate a fluorescent signal. In this manner bi-allelic discrimination of known SNPs and InDelS was performed. KASP™ genotyping data was analysed on exported excel sheets (LGC 2019).

Section 2.15. Analysis of the WNT/β-Catenin Pathway's Role in Adipocyte Dysfunction

In order to study the role of the WNT/β-catenin pathway's role in adipocyte dysfunction and ultimately the potential involvement in MI, an extreme phenotype approach was chosen together with a systems biology approach. Sampling extremes of the phenotypic distribution may offer advantages for identification of true causative events in adipocyte dysfunction. The extreme phenotype was chosen based on ORs obtained during analyses in chapter 3 (Adiposity and MI). Genes that code for the various proteins in the WNT pathway were selected for HTS sequencing using KEGG pathways and a literature review. HTS sequencing of the genes in the WNT pathway was performed and differences in genetic variants between the two extreme groups were analysed. Genetic variants that were rare, where in an important gene or more common in one extreme phenotype when compared to the other, were selected for KASP™ genotyping in the whole MAMI Study collection. Further details on selection of extreme phenotypes can be found in Chapter 10.

ORs for Myocardial infarction adjusted for age were estimated for all the polymorphisms. Median levels and interquartile range of intermediate phenotypes were calculated and using the Mann-Whitney test, p-values were estimated for continuous variables. Continuous variables analysed included WHR, triglycerides, NHDL-C and HDL-C. Markers of liver inflammation measured included γ-GT and ALT. HOMA-IR and HOMA-C were measured as markers of insulin resistance and hs-CRP was also analysed. The percentage of diabetic and non-diabetic individuals was also calculated. A p-value of 0.1 was selected to indicate a possible effect.

Combinations of two polymorphisms were then analysed. These combinations were selected taking into consideration genes with related function or overlapping function, genes whose protein products form complexes, proteins that interact physically, and genes that act together in a particular pathway. Combinations were also selected based on the observed effects on intermediate phenotypes. Odds ratios for MI of these combinations of polymorphisms were estimated.

Section 2.16. Ethical Considerations

The MAMI study was approved by the research ethics committee of the University of Malta (MD 32/2010 and FHS 021/2014) (Appendix 1). Signed informed consent was obtained from all research subjects after a detailed explanation of the study protocol and after time was given for questions. Double coding was used to ensure research subject anonymity. Samples being analysed within the hospital laboratory system were coded rather than having the research subjects hospital identification number. Samples that were subequently selected for HTS were given a separate code.

Section 2.17. General Methological Approaches

The methodology of this thesis can be broadly divided into four main segments (Figure 2-3). The initial chapters deal with the epidemiology of obesity, lipids, diet, smoking and alcohol and their association with MI in the Maltese population. Subsequently, analysis of the effects of individual SNPs (ex. *ApoE* and *PTPN1)* on intermediate phenotypes and the risk of myocardial infarction in the whole study collection was performed. The third segment involves an extreme phenotype approach in the search for variants in FH related genes. The last chapter combines the extreme phenotype appraoch with biological pathway analysis in adiposity.

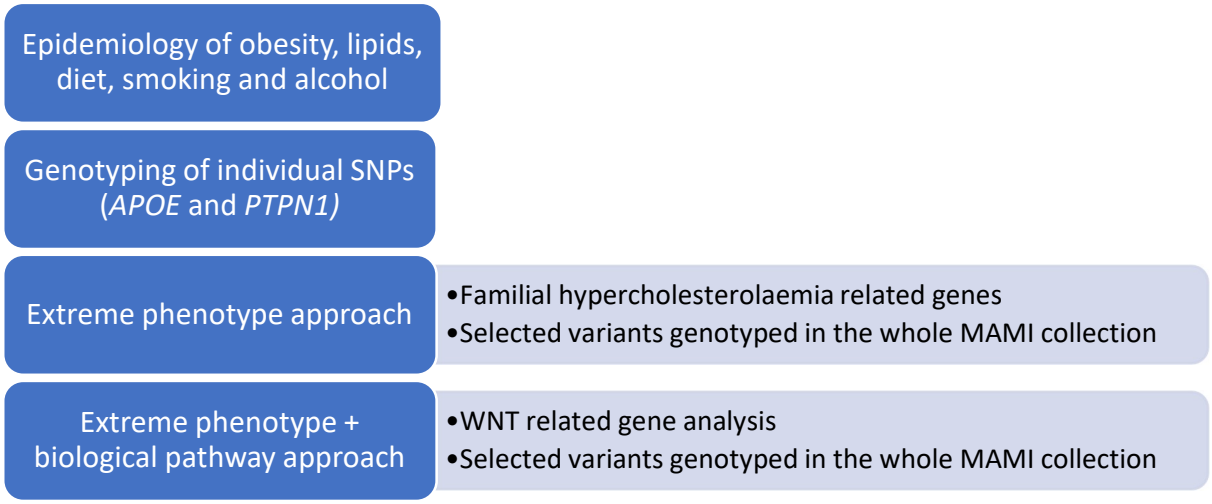

Figure 2-3 Summary of general methodological approaches used.

Chapter 3. Adiposity and Myocardial Infarction in the Maltese Population

Section 3.1. Adiposity and Myocardial Infarction

Obesity is often defined as a condition of abnormal or excessive fat accumulation in adipose tissue to the extent that health may be impaired (WHO 2000). Obesity is a rising epidemic and is linked to cardiovascular disease, diabetes, musculoskeletal disorders and some cancers (endometrial, breast and colon) (WHO 2015). Obesity has also been associated with a modestly increased risk of all-cause mortality (McGee 2005).

Worldwide obesity has doubled since 1980 and besides being a problem in high income countries, it is also becoming a problem in low and middle income regions (WHO 2015). With the increasing prevalence of childhood obesity (Grech, 2007), adult obesity is projected to become a full-fledged epidemic (WHO 2015). Due to its complications obesity comes at a significant cost to any health service (Pasco et al. 2012). Body mass index (BMI) is the most commonly used measure of adiposity in clinical practice, mostly due to practicality. BMI however ignores differences in body composition and the contribution of body fat to overall body weight. It overestimates adiposity in muscular body builds and underestimates adiposity in the elderly. BMI may also underestimate adiposity in young men (aged 20–29 years) in whom body fat contributes more, and lean tissue less, to body weight than in other age groups related to their sedentary lifestyle and unhealthy nutrition (Pasco et al. 2014).

Besides not discriminating between fat tissue and lean mass BMI also does not distinguish between different body distributions of fat (Snijder et al. 2006; Mason et al. 2008). The distribution of fat around the viscera and subcutaneously are linked to different clinical sequelae. Abdominal obesity has been shown to be associated with cardiovascular morbidity and mortality (Carmienke et al. 2013). Going beyond BMI and looking specifically at abdominal obesity may therefore have advantages.

Abdominal obesity measures such as waist circumference (WC) and waist-hip ratio (WHR) were shown to more accurately measure body fat distribution (Pasco et al. 2014). Both WC and WHR are closely related to adverse metabolic profiles. Both measures are independent predictors of mortality. Some have argued that WC, WHR and waist-height ratio (WHtR) may be more precise than BMI in assessing obesity-related health burden, including total mortality, risk of type 2 diabetes and cardiovascular disease (Carmienke et al. 2013). These concerns have not been taken up widely in clinical practice and in research areas that do not focus on obesity but study it as a confounder.

Which measure of obesity is best is far from clear cut. A recent meta-analysis of individual data showed that BMI and abdominal parameters had similar strengths of association with cardiovascular disease risk. For all-cause mortality, large observational studies reported controversial results regarding whether abdominal adiposity measures are more strongly predictive of mortality than BMI (Carmienke et al. 2013). The INTERHEART study, a large, international case control study of MI demonstrated that WHR was probably the best measure of adiposity for MI (Yusuf et al. 2005).

Rather than focusing on one measure, mortality prediction may be improved by combining BMI and an abdominal obesity measure such as WHR or WC. Which measure of adiposity is the best predictor for MI in the Maltese population has not been previously studied. Clinically there is a reliance on BMI, which may be inadequate to identify and target individuals at the highest risk of developing cardiovascular disease.

Section 3.2. Results

Section 3.2.1. Prevalence of Obesity in the Maltese Population

The prevalence of obesity amongst female controls was high with the majority being categorised as overweight (32.8%) or obese (33.6%) and only 32.1% of female control subjects having a normal BMI, 2 control subjects (1.5% were underweight). In male controls only 14% of subjects had a normal BMI with 38.5% being obese and 47.4% being overweight (Table 3-1).

Table 3-1 BMI in the female and male controls.

BMI Category	Women n (%)	Men n (%)
Obese	46 (33.6)	125 (38.5)
Overweight	45 (32.8)	154 (47.4)
Normal	44 (32.1)	46 (14.2)
Underweight	2 (1.5)	

Section 3.2.2. Association of BMI with Myocardial Infarction

BMI categories as defined by the WHO were not associated with the risk of myocardial infarction after multivariate adjustment in both women and men (Table 3-2). Due to the high prevalence of obesity and overweight individuals even in controls, an assessment using tertiles for BMI was performed. BMI tertiles were based on sex specific BMI distribution in the MAMI control population. Using BMI tertiles, BMI was not associated with MI after adjustment for age, (Table 3-3)

Table 3-2 Association of BMI with MI using WHO categories in men and women. UnAdjOR – Unadjusted odds ratio, AgeOR – Age Adjusted odds ratio, MultiAdjOR – Multivariate adjustment for Age, DM, reported HT, Smoking, Hypercholesterolaemia. Underweight women excluded from analysis

Sex	WHO BMI Category	Case n (%)	Control n (%)	UnAdjOR (95% CI)	AgeOR (95% CI)	MultiAdj (95% CI)
Women	Obese	37 (52.9)	46 (34.1)	2.4 (1.1-4.9)	1.5 (0.7-3.4)	0.9 (0.4-2.2)
	Overweight	18 (25.7)	45 (33.3)	1.2 (0.5-2.6)	0.9 (0.4-2.2)	0.9 (0.4-2.2)
	Normal	15 (21.4)	44 (32.5)	1.0	1.0	1.0
Men	Obese	111 (434)	125 (38.5)	1.6 (0.9-2.7)	1.5 (0.9-2.6)	1.3 (0.7 -2.4)
	Overweight	119 (46.5)	154 (47.4)	1.4 (0.8-2.3)	1.3 (0.8-2.2)	1.2 (0.7-2.2)
	Normal	26 (10.2)	46 (14.2)	1.0	1.0	1.0

Table 3-3 BMI distributed in tertiles based on sex specific distribution in controls and association with MI. UnAdjOR – Unadjusted odds ratio, AgeOR – Age Adjusted odds ratio, MultiAdjOR – Multivariate adjustment for Age, DM, reported HT, Smoking, hyperlipidaemia.

Sex	BMI Category	Case n (%)	Control n (%)	UnadjOR	AgeOR (95% CI)	MultiAdj (95% CI)
Women	Highest BMI	37 (52.9)	45 (32.8)	2.5 (1.2-5.2)	1.6 (0.7-3.4)	0.9 (0.4-2.3)
	Middle BMI	18 (25.7)	46 (33.6)	1.2 (0.5-2.7)	0.9 (0.4-2.1)	0.9 (0.4-2.2)
	Lowest BMI	15 (20.5)	46 (33.6)	1.0	1.0	1.0
Men	Highest BMI	95 (37.1)	109 (33.6)	1.3 (0.9-2.0)	1.4 (0.9 -2.0)	1.3 (0.8-2.0)
	Middle BMI	91 (35.5)	107 (33.0)	1.3 (0.9-2.0)	1.3 (0.9-2.0)	1.3 (0.8-2.0)
	Lowest BMI	70 (27.3)	108 (33.3)	1.0	1.0	1.0

Section 3.2.3. WHtR and Association with MI

An abnormal WHtR category was strongly associated with MI in both men (OR 4.4, 95% CI, 1.9-10.1) and women (OR 6.1, 95% CI, 1.7-22.5) after adjusting for age (Table 3-4).

Table 3-4 Association of WHtR with myocardial infarction. UnAdjOR – Unadjusted odds ratio, AgeOR – Age Adjusted odds ratio, MultiAdjOR – Multivariate adjustment for Age, DM, reported HT, Smoking, Hyperlipidaemia.

Sex	Waist -height -ratio category	Case n (%)	Control n (%)	UnadjOR (95%CI)	AgeOR (95% CI)	MultiAdj (95% CI)
Women	High	65 (95.6)	91 (66.9)	10.7 (3.2-36.0)	6.1 (1.7-22.5)	5.6 (1.5-20.9)
	Normal	3 (4.4)	45 (33.1)	1.0	1.0	1.0
Men	High	244 (98.4)	286 (90.8)	4.6 (2.0-10.6)	4.4 (1.9-10.1)	3.6 (1.5-8.6)
	Normal	4 (1.6)	29 (9.2)	1.0	1.0	1.0

WHtR analysed in tertiles was also showed a strong association with MI in both men and women. In the age adjusted analysis the highest tertile had an OR of 11.5 in women and an OR of 4.1 in men. The mid-tertile also showed a strong association in both sexes (Table 3-5).

Table 3-5 Association of waist-height ratio distributed in tertiles with myocardial infarction. UnAdjOR – Unadjusted odds ratio, AgeOR – Age Adjusted odds ratio, MultiAdjOR – Multivariate adjustment for Age, DM, reported HT, Smoking, hypercholesterolaemia.

Sex	WhtR tertile	Case n (%)	Control n (%)	UnAdjOR (95% CI)	AgeOR (95% CI)	MultiAdj (95% CI)
Women	Highest tertile (>0.586)	55 (80.9)	45 (33.3)	18.3 (5.3-62.9)	11.5 (3.1 -43.1)	9.1 (2.4-35.1)
	Mid tertile (0.502-0.586)	10 (14.7)	45 (33.3)	3.3 (0.9-12.9)	2.4 (0.6-9.9)	2.9 (0.7-11.9)
	Lowest tertile (<0.502)	3 (4.4)	45 (33.3)	1.0	1.0	1.0
Men	Highest tertile (>0.604)	136 (54.2)	107 (33.1)	4.1 (2.6-6.6)	4.1 (2.5. -6.5)	3.6 (2.1-6.0)
	Mid tertile (0.548-0.604)	82 (32.7)	108 (33.4)	2.5 (1.5-4.0)	2.4 (1.5-4.0)	2.1 (1.2-3.6)
	Lowest tertile (<0.548)	33 (13.1)	108 (33.4)	1.0	1.0	1.0

Section 3.2.4. Association of WHR with MI

Using WHO cut-offs to define high and normal WHR, a high WHR was also associated with MI in both sexes after adjustment for age with an OR of 8.5 (95%CI 4.1-17.8) in women and an OR of 8.1 in men (95% CI, 4.1-16.2) for the high waist-hip ratio group after adjustment for age(Table 3-6).

WHR tertiles were also strongly associated with risk for MI, with women in the highest tertile having an OR of 13.3 (95% CI, 3.8-46.7) and 9.1 (95% CI 5.1-16.1) in men after adjustment for age. The mid-tertile was only associated with MI in men with an Age adjusted OR of 4.7 (95% CI 2.6-8.6) (Table 3-7).

Table 3-6 Association of waist-hip ratio using WHO cut-offs with myocardial infarction. UnAdjOR – Unadjusted odds ratio, AgeOR – Age Adjusted odds ratio, MultiAdjOR – Multivariate adjustment for Age, DM, HT, Smoking, TC.

Sex	WHO WHR category	Case n (%)	Control n (%)	UnAdjOR (95% CI)	AgeOR (95% CI)	MultiAdj (95% CI)
Women	High WHR	57 (82.6)	41 (30.1)	11.0 (5.3-22.7)	8.5 (4.1-17.8)	7.0 (3.1-15.5)
	Normal WHR	12 (17.4)	95 (69.9)	1.0	1.0	1.0
Men	High WHR	245 (96.1)	242 (74.9)	8.2 (4.2 -16.2)	8.1 (4.1 -16.2)	7.5 (3.6-15.7)
	Normal WHR	10 (3.9)	81 (25.1)	1.0	1.0	1.0

The attributable risk of a high WHR in men is 87% while the population attributable risk was estimated at 98% in the Maltese male population. Similar results were observed in women It is important to note that the population attributable risk does not add up to 100 (Rowe et al. 2004)

Table 3-7 Association of waist-hip ratio analysed in tertiles based on sex specific distribution in controls. UnAdjOR – Unadjusted odds ratio, AgeOR – Age Adjusted odds ratio, MultiAdjOR – Multivariate adjustment for Age, DM, HT, Smoking, TC.

Sex	WHR tertile	Case n (%)	Control n (%)	UnadjOR (95% CI)	AgeOR (95% CI)	MultiAdjOR (95%CI)
Women	Highest tertile	59 (85.5)	44 (32.8)	20.1 (5.9-69.0)	13.3 (3.8-46.7)	16.7 (3.7-76.0)
	Mid tertile	7 (10.1)	45 (33.6)	2.3 (0.6-9.6)	1.7 (0.4-7.1)	3.1 (0.6-16.2)
	Lowest tertile	3 (4.3)	45 (33.6)	1.0	1.0	1.0
Men	Highest tertile	155 (61.3)	108 (33.3)	9.1 (5.2-16.1)	9.1 (5.1-16.1)	8.2 (4.4-15.4)
	Mid tertile	81 (32.0)	108 (33.3)	4.8 (2.6-8.6)	4.7 (2.6-8.6)	4.9 (2.6-9.3)
	Lowest tertile	17 (6.7)	108 (33.3)	1.0	1.0	1.0

Section 3.2.5. Association of Central Obesity with MI

Central obesity based on WHO cut-offs for waist circumference measurements was also strongly associated with myocardial infarction in both men and women even after adjustment for age with an OR of 7.4 (95% CI, 2.7-20.8) for the 'substantially increased' group in women and 3.1 (95% CI, 2.0-4.6) after adjustment for age. The increased risk group of central obesity showed a pattern towards being associated with MI in women and was associated with MI in men with an OR of 2.6 (95% CI, 1.7-4.1) (Table 3-8).

Waist circumference tertiles similarly showed a stepwise increased association with MI from the lowest tertile to the highest tertile in men. There was a pattern towards the mid-tertile for waist circumference being associated with risk of MI in women and was associated in men (OR 2.2 [95%CI 1.4-3.5]) (Table 3-9).

Table 3-8 Association of central obesity using WHO cut-off with myocardial infarction. UnAdjOR – Unadjusted odds ratio, AgeOR – Age Adjusted odds ratio, MultiAdjOR – Multivariate adjustment for Age, DM, HT, Smoking, hyperlipidaemia.

Sex	Waist circumference WHO classification	Case n (%)	Control n (%)	UnAdjOR (95% CI)	Age OR (95%CI)	MultiAdjOR (95% CI)
Women	Substantially increased	56 (81.2)	53 (38.7)	11.4 (4.2-30.7)	7.4 (2.7-20.8)	5.6 (1.9-16.5)
	Increased	8 (11.6)	30 (21.9)	2.9 (0.9-9.6)	2.3 (0.7-2.8)	2.6 (0.7-9.3)
	Normal	5 (7.2)	54 (39.4)	1.0	1.0	1.0
Men	Substantially increased	122 (47.8)	106 (32.8)	3.1 (2.1-4.7)	3.1 (2.0-4.6)	2.7 (1.7-4.3)
	Increased	83 (32.5)	82 (25.4)	2.7 (1.8-4.3)	2.6 (1.7-4.1)	2.3 (1.4-3.7)
	Normal	50 (19.6)	135 (41.8)	1.0	1.0	1.0

Table 3-9 Association of waist circumference analysed in tertiles based on sex specific distribution in controls with myocardial infarction. UnAdjOR – Unadjusted odds ratio, AgeOR – Age Adjusted odds ratio, MultiAdjOR – Multivariate adjustment for Age, DM, HT, Smoking, hyperlipidaemia.

Sex	Waist circumference	Case n(%)	Control n(%)	UnAdjOR (95% CI)	AgeOR (95% CI)	MultiAdjOR(95%CI)
	Highest tertile (>91.96cm)	49 (71.0)	45 (32.8)	10.2 (3.7-28.0)	6.5 (2.3-18.4)	4.6 (1.5-14.1)
Women	Mid tertile (79.11-91.95cm)	15 (21.7)	45 (32.8)	3.1 (1.1-9.3)	2.4 (0.8-7.3)	2.8 (0.9-8.9)
	Lowest tertile (<79.10cm)	5 (7.2)	47 (34.3)	1.0	1.0	1.0
Men	Highest tertile (>101.98cm)	127 (49.6)	108 (33.3)	3.2 (2.1-5.0)	3.1 (2.0-4.9)	2.7 (1.6-4.4)
	Mid tertile (92.01-101.98cm)	89 (34.8)	107 (33.0)	2.3 (1.4-3.6)	2.2 (1.4-3.5)	1.8 (1.1-3.0)
	Lowest tertile (<92.0cm)	40 (15.6)	109 (33.6)	1.0	1.0	1.0

Section 3.2.6. Association of Hip Circumference with Risk of MI

Hip circumference was only analysed in tertiles as there are no standard cut-off points. A large hip circumference was strongly associated with MI in the unadjusted assessment in women but not in men. After adjusting for age in women, hip circumference was not associated with MI (Table 3-10).

Table 3-10. Association of hip circumference analysed in tertiles based on sex specific distribution in controls and MI. UnAdjOR – Unadjusted odds ratio, AgeOR – Age Adjusted odds ratio, MultiAdjOR – Multivariate adjustment for Age, DM, HT, Smoking, TC.

Sex	Hip Circumference	Case n (%)	Control n (%)	UnAdjOR (95% CI)	AgeOR(95% CI)	MultiAdjOR(95% CI)
Women	Highest tertile (>108.11cm)	31 (44.9)	44 (32.1)	3.0 (1.3-6.6)	2.1 (0.9-4.9)	1.2 (0.4-3.3)
	Mid tertile (99.21-108.10cm)	27(39.1)	47 (34.3)	2.4 (1.1-5.4)	1.9 (0.8-4.5)	1.7 (0.7-4.5)
	Lowest tertile (<99.2cm)	11 (15.9)	46 (33.6)	1.0	1.0	1.0
Men	Highest tertile (>0.984)	86 (33.6)	107 (33.0)	1.1 (0.7-1.6)	1.1 (0.8-1.7)	1.1 (0.7-1.7)
	Mid tertile (0.916-0.983cm)	89 (34.8)	106 (32.7)	1.2 (0.8-1.7	1.2 (0.8-1.8)	0.9 (0.6-1.4)
	Lowest tertile (<0.916cm)	81 (31.6)	111 (34.3)	1.0	1.0	1.0

Section 3.2.7. Correlations between Measures of Adiposity and CVD Risk Factor Variables

WHR and WHtR showed moderately strong correlations with Hba1C in women. Lipid variables were weakly correlated to measures of adiposity. Hba1c and the lipid variables were only weakly correlated to measures of adiposity in men (Table 3-11, Table 3-12).

Table 3-11 Correlation of anthropometric measures with lipid variables in women using Spearman's correlation, p-value in brackets. BMI-body mass index, avg-average, TC -total cholesterol, HDL-C -high density lipoprotein cholesterol, NHDL-C-Non-high density lipoprotein cholesterol, HDLR -HDL ratio TG-Triglycerides (n=133, *n=136)

Lipid variable	BMI	Waist-hip Ratio	Waist Height Ratio	Avg Waist n=133	Avg Hip n=133
HbA1c	0.513 (<0.001)*	0.482 (<.001)*	0.558 (<0.001)*	0.495 (<0.001)*	0.344 (<0.001)*
TC	0.200 (0.021)	0.148 (0.088)	0.211 (0.015)	0.183 (0.035)	0.127 (0.144)
HDL-C	-0.323(<0.001)	-0.303 (<0.001)	-0.324 (<0.001)	-0.391 (<0.001)	-0.318 (<0.001)
NHDL-C	0.295 (0.001)	0.244 (0.005)	0.305 (<0.001)	0.307 (<0.001)	0.234 (0.007)
TG	0.396 (<0.001)	0.296 (0.001)	0.376 (<0.001)	0.387 (<0.001)	0.325 (<0.001)
HDLR	0.365 (<0.001)	0.318 (<0.001)	0.373 (<0.001)	0.416 (<0.001)	0.330 (<0.001)
LDL-C	0.225 (0.009)	0.179 (0.040)	0.236 (0.006)	0.240 (0.005)	0.181 (0.037)

Table 3-12 Correlation of anthropometric measures with lipid variables in men using Spearman's coefficient, p-value in brackets. BMI-body mass index, avg-average, TC -total cholesterol, HDL-C -high density lipoprotein cholesterol, NHDL-C-Non-high density lipoprotein cholesterol, HDLR -HDL ratio TG-Triglycerides (n=320, **n=323, *n=322, ^n=321)

Variable	BMI	Waist-hip Ratio	Waist Height Ratio	Avg Waist	Avg Hip
HbA1c	0.198 (<0.001)**	0.282 (<0.001)*	0.334 (<0.001)*	0.235 (<0.001)*	0.041 (0.462)*
TC	0.113 (0.044)^	0.042 (0.449)	0.107 (0.056)	0.077 (0.172)	0.073 (0.193)
HDL-C	-0.302 (<0.001)^	-0.227 (<0.001)	-0.295 (<0.001)	-0.286 (<0.001)	-0.178 (0.001)
NHDL-C	0.214 (<0.001)^	0.124 (0.027)	0.207 (<0.001)	0.169 (0.002)	0.117 (0.036)
TG	0.334 (<0.001)^	0.364 (<0.001)	0.396 (0.001)	0.351 (<0.001)	0.156 (0.02)
HDLR	0.322 (<0.001)^	0.228 (<0.001)	0.320 (<0.001)	0.287 (<0.001)	0.177 (0.001)
LDL-C	0.130 (0.019)^	0.8405328 (0.613)	0.106 (0.058)	0.0 (0.135)	0.087 (0.121)

Section 3.2.8. Stratified Analysis of BMI and WHR and Association with MI

BMI categories were further sub-divided into high and low WHR groups. In this stratified analysis risk was only associated with the high WHR groups while being in the obese and overweight groups with a normal WHR was protective for myocardial infarction (Table 3-13).

Table 3-13 Stratified analysis for WHR and BMI in men. UnAdjOR – Unadjusted odds ratio, AgeOR – Age Adjusted odds ratio, MultiAdjOR – Multivariate adjustment for Age, DM, HT, Smoking, TC.

Stratification of WHR with BMI	Case n (%)	Control n (%)	AgeOR (95% CI)	MultiAdjOR*
High WHR-Obese	108 (43.2)	110 (34.1)	3.6 (1.5-8.7)	3.6 (1.4-9.7)
Normal WHR-Obese	1 (0.4)	14 (4.3)	0.3 (0.03-2.4)	0.2 (0.03-2.7)
High WHR-Overweight	112 (45.6)	113 (35.0)	3.7 (1.5-9.0)	3.7 (1.4-9.8)
Normal WHR-Overweight	1 (0.4)	41 (13.7)	0.1 (0.01-0.8)	0.1 (0.02-1.2)
High WHR-Normal BMI	19 (7.6)	19 (5.9)	3.6 (1.3-10.6)	4.9 (1.5-15.6)
Normal WHR-Normal BMI	7 (2.8)	26 (8.0)	1	1

Section 3.2.9. Proportion of Subjects Identified with Cardiovascular Risk Factors Based on Established /Proposed Cut-offs for BMI, WC, WhtR.

The obese category (using BMI categories) in women had the highest percentage of controls with diabetes when compared to high WHR, high WHtR and High WC categories (Table 3-14). In Men the high WC group had the highest percentage of controls with DM (26.4%) followed by the obese category (22.4%) and lastly the high WHR and WHtR groups. A similar pattern was observed for HT. There was no appreciable difference between prevalence of hypercholesterolaemia in men using the various measures of adiposity. In women however the

largest prevalence of hypercholesterolaemia was also found in the overweight and the middle category for waist circumference albeit the numbers in each category were small. LDL was also highest in the overweight group (57.8%) compared to the obese (52.8%) and normal BMI (31.0). The high WHtR group had the highest prevalence of hypercholesterolaemia (74.2%) followed by high WC (68.6%). High WHR had a similar prevalence of hypercholesterolaemia to the obese group. A similar pattern was observed for LDL except that in the high and mid WC there was a similar prevalence of elevated LDL. These patterns were not observed in men where there was no appreciable difference in the prevalence of hypercholesterolaemia in the high-risk category for each measure of adiposity.

The prevalence of low HDL was highest in the high-risk category of each group being highest in prevalence in the high WHR group in women and the high BMI group and high WC groups in males. Hypertriglyceridemia was highest in the high WHR group in women and the obese group in men. The prevalence of the metabolic syndrome was highest in the low group for WC in women and in the obese group in men. The prevalence of low physical activity was similar across all adiposity variables in both men and women (Table 3-14).

Table 3-14 Proportion of controls identified with cardiovascular risk factors based on established cut-offs for BMI, WC Waist height ratio, waist-hip ratio, n -number, percentage in brackets. Lipid variable cut-offs defined by EHES (European Health Examination Survey). TC- Total cholesterol >5.0mmol/L, LDL-C-low density lipoprotein cholesterol >3.33mmol/L , HDL-C- High density lipoprotein cholesterol <1.55mmol/L, TG- triglycerides >2.33mmol/L, HDLR -HDL ratio >4.55, MS -metabolic syndrome, low physical activity (1-499.99 METS/day).

Sex	Measure of adiposity	mean age (25-75th percentile)	Smoking n (%)	Ex-Smokers n (%)	DM n (%)	HT n (%)	TC n (%)	LDL-C n (%)	HDL-C n (%)	HDLR n (%)	TG n (%)	MS n (%)	low physical activity n (%)
Women	Obese	61 (56-68)	3 (6.5)	13 (28.3)	14 (31.1)	30 (66.7)	28 (63.8)	23 (52.3)	25 (56.8)	8 (18.2)	4 (9.1)	29 (63.0)	15(40.5)
	Overweight	54.9 (47.5-67.0)	9 (20.0)	10 (22.2)	2 (4.4)	14 (31.1)	36 (80.0)	26 (57.8)	17 (37.8)	8 (17.8)	1 (2.2)	26 (57.8)	16 (40.0)
	Normal BMI	47.2 (33-61.5)	12 (27.3)	7 (15.9)	2 (4.5)	3 (6.8)	27 (64.3)	13 (31.0)	13 (31.0)	2 (4.8)	1 (2.4)	6 (13.6)	3 (33.3)
	High WHR	64.0 (57.5-70.5)	8 (19.0)	12 (28.6)	11 (26.8)	21 (50.0)	25 (62.5)	21 (52.5)	25 (62.5)	9 (22.5)	4 (10.0)	31 (73.8)	13 (37.1)
	Low WHR	65.0 (54.8-66.8)	17 (17.9)	18 (18.9)	7 (7.4)	26 (27.7)	66 (71.1)	41 (44.1)	30 (32.3)	9 (9.7)	2 (2.2)	30 (31.6)	37 (44.0)
	High WhtR	66 (57.0-70)	12 (13.2)	21(23.1)	15 (16.7)	43 (47.8)	66 (74.2)	50 (56.2)	44 (49.4)	16 (18.0)	5 (5.6)	59 (64.8)	29 (37.2)
	Low WhtR	43 (28-53.5)	13 (28.9)	9 (20.0)	3 (6.7)	4 (8.9)	24 (55.8)	11 (25.6)	11 (25.6)	2 (4.7)	1 (2.3)	2 (4.4)	20 (50)
	High Waist Cat	61.4 (56.0-70.0)	7 (13.2)	15(28.3)	14 (26.9)	32 (61.5)	35 (68.6)	29 (56.9)	30 (58.8)	13 (25.5)	4 (7.8)	36 (67.9)	16 (38.1)
	Medium Waist	59.0 (48.8-68.8)	4 (13.3)	3 (13.3)	1 (3.3)	10 (33.3)	25 (83.3)	17 (56.7)	11 (36.7)	3 (10.0)	1 (3.3)	25 (83.3)	11 (39.3)
	Low Waist Cat	43.5 (29.8-62.0)	14 (25.9)	11 (20.4)	3 (5.6)	5 (9.3)	31 (59.6)	16 (30.8)	14 (26.9)	2 (3.8)	1 (1.9)	54 (100)	23 (46.9)

Table 3.14 continued	Measure of adiposity	mean age (25-75th percentile)	Smoking	Ex-Smokers	DM	HT	Chol	LDL	HDL	HDLR	TRIG	MS	low physical activity
Men	Obese	55.8 (50.5-63.0)	33 (26.4)	51 (40.8)	28 (22.4)	68 (56.7)	86 (69.9)	71 (57.7)	111 (90.2)	61 (49.6)	26 (21.1)	87 (69.6)	43 (38.7)
	Overweight	59.5 (51.8-67.0)	27 (17.5)	74 (48.1)	26 (17.0)	62 (41.6)	91 (59.9)	78 (51.3)	45 (29.6)	40 (26.3)	13 (8.6)	52 (33.8)	39 (28.1)
	Normal BMI	57.0 (39.8-61.3)	19 (41.3)	6 (13.0)	2 (4.3)	7 (15.9)	28 (60.9)	19 (41.3)	26 (56.5)	5 (10.9)	1 (2.2)	2 (4.4)	17 (42.5)
	High WHR	57.5 (52.0-65.0)	61 (25.2)	104 (43.0)	48 (19.9)	113 (48.5)	156 (65.5)	127 (53.4)	190 (79.8)	90 (37.8)	36 (15.1)	132 (54.5)	72 (34.1)
	Low WHR	53.0 (40.8-62.0)	17 (20.7)	27 (32.9)	8 (9.8)	24 (30.4)	49 (59.8)	41 (50.0)	53 (64.6)	16 (19.5)	4 (4.9)	9 (11.0)	26 (33.3)
	High WHtRe	59.0 (52.0 - 66.0)	66 (23.2)	123 (43.2)	55 (19.4)	130 (47.4)	185 (65.8)	153 (54.4)	219 (77.9)	103 (36.7)	39 (13.9)	141 (49.5)	82 (32.5)
	Low WHtRe	53.0 (47.0 - 57.0)	12 (31.6)	8 (31.1)	1 (2.6)	7 (18.9)	20 (52.6)	15 (39.5)	23 (60.5)	3 (7.9)	1 (2.6)	38 (100)	15 (41.7)
	High Waist Cat	57.5 (52.0-62.3)	27 (25.5)	44 (41.5)	28 (26.4)	62 (60.8)	70 (67.3)	57 (54.8)	92 (88.5)	49 (47.1)	21 (20.2)	77 (72.6)	40 (43.0)
	Medium Waist	61.0 (52.8 -65.5)	22 (26.8)	38 (46.3)	10 (12.2)	31 (39.2)	54 (66.7)	47 (58.0)	60 (74.1)	28 (34.6)	12 (14.8)	62 (75.6)	22 (28.9)
	Low Waist Cat	58.0 (42.0 - 66.0)	29 (21.5)	48 (35.6)	18 (13.3)	44 (33.8)	80 (59.7)	63 (47.0)	91 (67.9)	29 (21.6)	7 (5.2)	1 (0.7)	36 (30.0)

Section 3.2.10. Association of HOMA-IR and WHR with MI

HOMA-IR above the 50th percentile was a risk factor for MI in both men, AgeOR 2.5 (1.7-3.6) and women, AgeOR 4.0 (1.6-9.8 (even after the exclusion of diabetic subjects from the analysis) (Table 3-15 and Table 3-16) .

Table 3-15 OR for MI in all men and Non-diabetic men taking the 50th percentile (1.61) as a cut-off point for HOMA-IR. AdjOR (95%CI) = adjusted for age, BMI, hypercholesterolemia, diabetes, hypertension, smoking, alcohol consumption and binge drinking, AdjOR' (95%CI) = AdjOR and further adjusted for statin use.

Men overall					
HOMA-IR	Cases (n=233)	Controls (n=311)	AgeOR (95%CI)	AdjOR (95%CI)	AdjOR' (95%CI)
>1.61	175 (75.1)	169 (54.3)	2.5 (1.7-3.6)	1.7 (1.1-2.7)	1.7 (1.1-2.7)
<=1.61	58 (24.9)	142 (45.7)	1.0	1.0	1.0
Non-diabetic men					
HOMA-IR	Cases (N=147)	Controls (N=251)	AgeOR (95%CI)	AdjOR (95%CI)	AdjOR' (95%CI)
>1.61	105 (71.4)	124 (49.4)	2.5 (1.6-3.9)	1.8 (1.1-3.0)	1.8 (1.1-3.0)
<=1.61	42 (28.6)	127 (50.6)	1.0	1.0	1.0

Table 3-16 OR for MI in all women and Non-diabetic women taking the 50th percentile (1.20) as a cut-off point.

Women overall					
HOMA-IR	Cases (n=55)	Controls (n=130)	AgeOR (95%CI)	AdjOR (95%CI)	AdjOR' (95%CI)
>1.20	48 (87.3)	70 (53.8)	4.0 (1.6-9.8)	3.8 (1.2-12.1)	
<=1.20	7 (12.7)	60 (46.2)	1.0	1.0	1.0
Non-diabetic women					
HOMA-IR	Cases (N=30)	Controls (N=110)	AgeOR (95%CI)	AdjOR (95%CI)	AdjOR' (95%CI)
>1.20	23 (76.7)	53 (48.2)	2.4 (0.9-6.3)	3.0 (0.8-10.7)	2.6 (0.7-9.5)
<=1.20	7 (23.3)	57 (51.8)	1.0	1.0	1.0

A positive correlation between HOMA-IR and WHR in both men and women was found amongst controls (Table 3-17).

Table 3-17 Spearman's coefficient between HOMA-IR and WHR in men and women, amongst controls only.

Men	N	Correlation coefficient	p-value
Overall	324	0.474	<0.001
Non-diabetics	249	0.468	<0.001
Women			
Overall	130	0.427	<0.001
Non-diabetics	110	0.399	<0.001

Men with high WHR (>0.90) and high HOMA-IR (using both the 50th and the 25th percentile cut-off as defined in non-diabetic controls) had a very strong increased risk of MI (AgeOR 24.9 [95% CI 5.9-104.7] (Table 3-18 Table 3-19)

Table 3-18 Risk of MI with combinations of WHR and HOMA-IR using 50th percentile as cut-off for HOMA-IR in men. AdjOR (95%CI) = adjusted for age, BMI, hypercholesterolemia, diabetes, hypertension, smoking, alcohol consumption and binge drinking, AdjOR' (95%CI) = AdjOR and further adjusted for statin use.

Men overall					
WHR and HOMA-IR	Cases (N=181)	Controls (N=308)	AgeOR (95%CI)	AdjOR (95%CI)	AdjOR' (95%CI)
high high	129 (71.3)	145 (47.1)	24.9 (5.9-104.7)	19.8 (4.5-86.2)	19.8 (4.5-86.3)
high low	46 (25.4)	81 (26.3)	15.8 (3.6-68.3)	16.1 (3.6-72.2)	16.1 (3.6-72.2)
low high	4 (2.2)	24 (7.8)	4.7 (0.8-27.6)	2.4 (0.3-18.0)	2.4 (0.3-17.9)
low low	2 (1.1)	58 (18.8)	1.0	1.0	1.0

Table 3-19 Risk of MI with combinations of WHR and HOMA-IR using 25th percentile as cut-off for HOMA-IR in men. AdjOR (95%CI) = adjusted for age, BMI, hypercholesterolemia, diabetes, hypertension, smoking, alcohol consumption and binge drinking, AdjOR' (95%CI) = AdjOR and further adjusted for statin use.

WHR and HOMA-IR	Cases (n=182)	Controls (n=308)	AgeOR (95%CI)	AdjOR (95%CI)	AdjOR' (95%CI)
high high	165 (90.7)	194 (63.0)	29.6 (4.0-220.7)	20.7 (2.7-157.0)	20.7 (2.7-157.3)
high low	11 (6.0)	33 (10.7)	11.6 (1.4-96.0)	10.4 (1.2-87.4)	10.3 (1.2-87.3)
low high	5 (2.7)	45 (14.6)	3.9 (0.4-35.2)	1.8 (0.2-18.4)	1.8 (0.2-18.4)
low low	1 (0.5)	36 (11.7)	1.0	1.0	1.0

Section 3.3. Discussion

The MAMI study shows that the prevalence of obesity has remained stable from data of the Monica study of 1984 (Cacciottolo 1990) and has also increased from the European health examination survey pilot study of 2010 (Department of Health Information and Research 2010) in women (Figure 3.1). In men the prevalence of obesity has almost doubled from 1984 to 2011. The prevalence of overweight subjects has remained stable and there was a decrease in subjects with normal weight (Figure 3-2). The changes in prevalence of obesity could be due to differences in recruitment methods between the studies however they probably reflect real changes in the prevalence of obesity with time and appear to be following a pattern which has been observed in other countries (Cuschieri et al. 2016). During the MAMI study acquiring a representative control population was considered of paramount importance. Research subjects were selected randomly and if they did not respond to a mail invitation they were called and given ample opportunity to attend for the interview and examination.

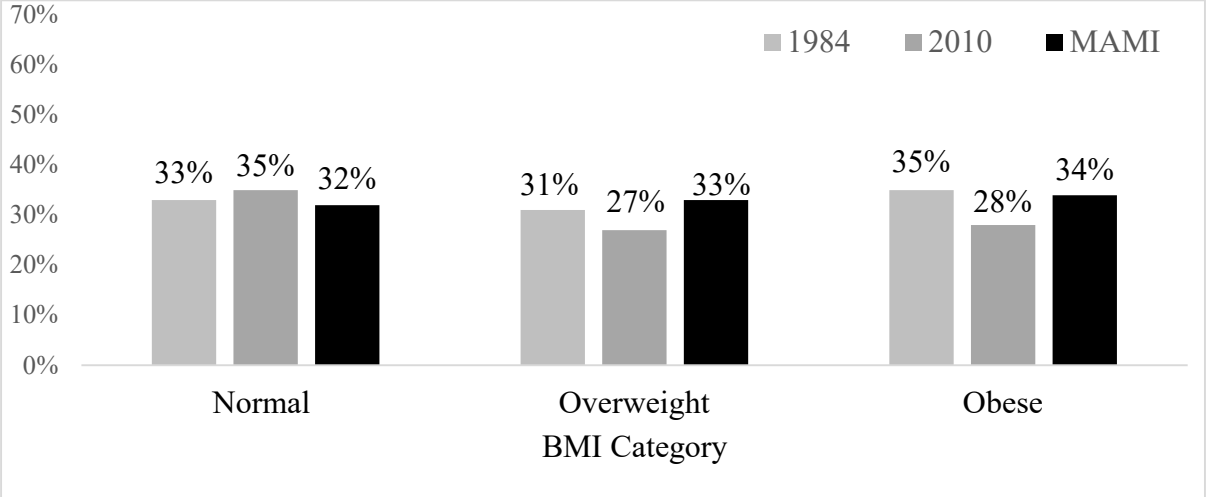

Figure 3-1. MI in women in the Monica study (1984), the European health examination survey (2010) and in the MAMI study.

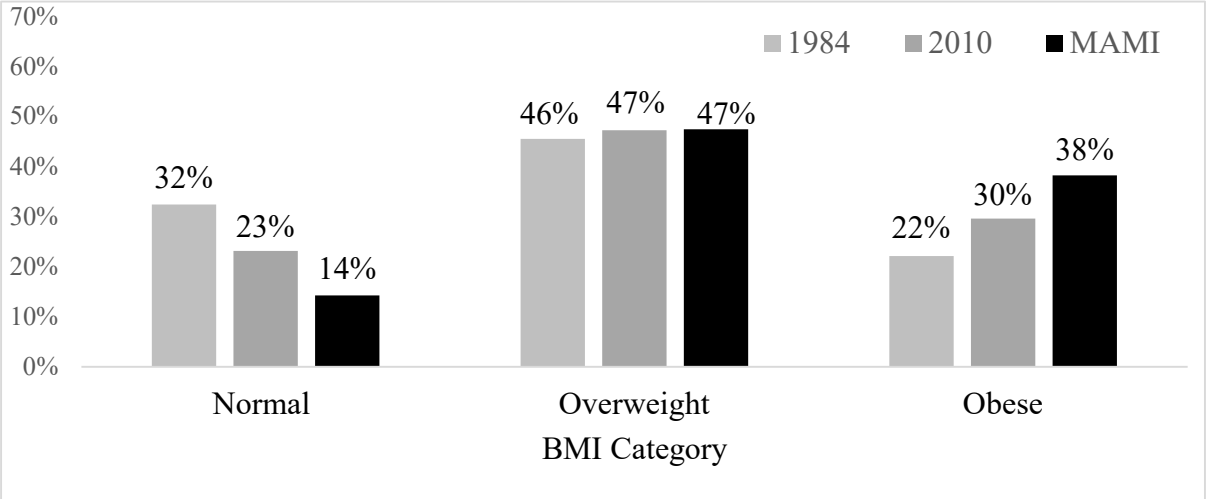

Figure 3-2. BMI in men in the Monica study (1984), the European health examination survey (2010) and in the MAMI study.

BMI has been linked to CAD and intermediate phenotypes of elevated LDL, elevated remnant lipoprotein cholesterol and elevated blood pressure and impaired glucose control (Varbo et al. 2015; Dale et al. 2017). However obesity defined by BMI alone fails to distinguish fat from lean mass and does not take into account fat distribution (Neeland et al. 2018). Patients with similar body weight or BMI values have been shown to display markedly different co-morbidities and levels of health risk (González-

Muniesa et al. 2017). A metanalysis of abdominal obesity indices comparing BMI, WC, Waist-hip ratio and waist-height ratio showed that BMI was the least accurate predictor for detecting cardiovascular risk factors in both men and women (Lee et al. 2008). Supporting this, BMI has never emerged as a component of the Framingham or Pooled cohort equation cardiovascular risk scores because it did not demonstrate any advantage over traditional risk factors (Goff et al. 2014). In the INTERHEART study, a large case control study of 27,098 patients from 52 countries also showed that BMI was a poor predictor of myocardial infarction. Overall the OR for BMI was 1.10 (95% CI, 1.07-1.13). This risk varied very little between different ethnicities (Yusuf et al. 2005).

BMI was not associated with MI in the MAMI study. The adjusted odds ratio for obesity in women was 0.9 (95% CI, 0.4-2.2) while the adjusted odds ratio for obesity in men was 1.3 (95% CI, 0.7-2.4). Analysing BMI in tertiles in both women and men showed no change in the odds ratio showing that BMI was a poor measure of adiposity to determine the risk of myocardial infarction.

In the INTERHEART study waist-hip ratio was studied in quintiles. The top quintile of WHR compared with the lowest quintile had an OR of 1.90 (95% CI, 1.69-1.85) in women and an OR of 1.73 (95% CI, 1.62-1.85) in men (Yusuf et al. 2005). The OR observed in the MAMI study were higher than those described in the INTERHEART study. In women the adjusted risk was of 5.6 (95%CI, 1.5 – 20.9) while in men the adjusted risk was of 3.6 (95% CI, 1.5-8.6). A metanalysis of WHR as a predictor of myocardial infarction showed that the risk of MI associated with a high waist-hip ratio was higher in women, with an OR of 4.63, 95% CI 3.28–6.53, than in men, who had an OR 2.71, 95% CI 2.15–3.41 (Cao et al. 2018). Analysis using tertiles could help stratify patients especially in the male cohort. In the female cohort the mid-tertile for

waist-hip ratio did not show a strong increase in risk of myocardial infarction while the mid-tertile in men showed an OR of 4.7 (95%CI, 2.5-8.9).

The ORs for WHR observed in the MAMI study are higher than those described in any of the studies tackling waist-hip ratio and the risk of myocardial infarction. Possible causes could include that Malta has a high rate of childhood and adolescent obesity (Grech and Farrugia Sant'Angelo 2009). This may mean that obesity is having an effect over a prolonged period compared to obesity that has its onset in adulthood. Therefore, individuals with childhood obesity have a longer period exposed to obesity.

Waist-height ratio in the MAMI study was also superior to BMI as a risk factor for myocardial infarction. Similar to the case for waist-hip ratio, odds ratios were higher in women than in men. Analysing waist-height ratio in tertiles identified a stepwise increase in risk in men but not in women (Table 3-7). The superiority of waist-height ratio to BMI in the prediction of MI has been demonstrated in a metanalysis which showed that waist-height ratio was 9% better than BMI in men and women in the prediction of myocardial infarction (Ashwell et al. 2012).

Waist circumference alone is often proposed to avoid multiple measurements and calculations (Ashwell et al. 2012). From the MAMI study results, waist-hip ratio and waist-height ratio are both superior to the use of waist circumference or central obesity alone. Using the ratios in tertiles could give the added benefit of stratifying subjects into categories of increasing risk, especially in men.

Interestingly, a stratified analysis for WHR and BMI clearly showed that within each BMI group the risk of myocardial infarction was only present in those with a high waist-hip ratio. Subjects with a normal WHR who were obese or overweight showed no increased risk (Table 1.10). This could reflect differences in the distribution of fat

between the subcutaneous and visceral areas. This clearly indicates that waist-hip ratio can be used to distinguish between the different metabolic phenotypes within each BMI group. Furthermore, when WHR was stratified with HOMA-IR there was a large difference in the risk between those that had a high WHR and normal HOMA-IR less than the 25th percentile and those that had a high WHR with a HOMA-IR higher than the 25th percentile. High HOMA-IR is a marker of insulin resistance which is an indication of adipocyte dysfunction. The high WHR-low HOMA-IR, high WHR-high HOMA-IR will be used as extreme phenotypes to study the pathways involved in adipocyte dysfunction.

Section 3.4. Limitations

Although measures of adiposity include WHR, WHtR and WC appear to be associated with MI the data analysed includes only a snapshot of their body habitus at one point in time. So, although it appears that exposures including elevated WHR, WhtR and WC are linked to the acute event of MI, no conclusions can be drawn to the link between these measures of adiposity and the initiation/development of atherosclerosis. Such a link would require information regarding lifetime body habitus. Another limitation is that no direct measure of body composition was performed. Percentage body fat and percentage lean body mass probably vary considerably within any WHR, WHtR, WC group. Since WHR, WHtR are only crude surrogate markers for visceral obesity, the true effect of visceral obesity on metabolic abnormalities and risk of MI may have been underestimated. Another important limitation is bias caused by unknown confounding factors or confounding factors which are difficult to analyse including diet, physical activity and long period of sedentary positions. In order to confirm a causal

relationship, the conclusions drawn above should be verified by replication in a prospective cohort study.

Section 3.5. Conclusion

The MAMI study results clearly show that although BMI is an easy and well-established measure of adiposity in the Maltese population it is not associated with the risk of myocardial infarction. Waist-hip ratio and waist-height ratio are the measures of adiposity with are most strongly associated with MI in both men and women. Our findings suggest that using BMI in the Maltese population as a risk factor for MI is inadequate and that WHR should be used instead.

Chapter 4. Lipids and the Risk of Myocardial Infarction

Section 4.1. Lipids and Risk of Myocardial Infarction

Although atherogenesis is a multifactorial process, lipoprotein metabolism is a key risk factor representing around 50% of the population-attributable risk of developing cardiovascular disease (Yusuf et al. 2004). TC and LDL-C represents the primary target in ATP III guidelines (Lackner and Peetz 2002) and in the European guidelines (Agewall et al. 2016b) for the management of dyslipidaemia. Using LDL-C as a target, cholesterol lowering drugs have attained a 25% relative risk reduction for cardiovascular events with a number need to treat of 30 (Baigent et al. 2005).

However, risk assessment based on TC and LDL-C alone are sub-optimal and patients continue to have cardiovascular events despite achieving significant LDL-C reductions. Many alternatives to using TC and LDL-C have been proposed (Superko and King, 2008; LaRosa, He and Vupputuri, 1999).

One such alternative is using NHDL-C which is the sum of the cholesterol concentration in all proatherogenic lipoproteins (VLDL, IDL, and LDL particles). In normolipidemic conditions, the majority of atherogenic cholesterol is present in the LDL particle which results in a very tight correlation between LDL-C and NHDL-C. The concordance between LDL-C and NHDL-C decreases when TG-rich lipoproteins accumulate in the circulation, a condition characterized by increased plasma levels of triglycerides. Another alternative is directly measuring the apolipoproteins in these particles (ApoB) and ApoA in HDL-C and using ApoB/ApoA as an indicator of the balance between atherogenic and atheroprotective cholesterol transport (Abatea, Vega and Grundy, 1993; The Lipid Research Clinics Program Epidemiology Committee, 1979).

The INTERHEART trial showed that ApoB/ApoA, in the unfasted state was superior to TC and LDL-C for estimating the risk of myocardial infarction (Yusuf et al. 2004). The use of ApoA or ApoB measurements are not widely used in clinical practice due to general unavailability in modern laboratories and due to the absence of reference values. Furthermore there use is not backed by large studies that confirm their superiority over LDL-C and TC in risk assessment (Kastelein et al. 2008).

An alternative to ApoB/ApoA to assess the ratio of atherogenic and atheroprotective cholesterol transport includes the use of lipoprotein ratios or 'atherogenic indices'. These indices may provide better risk prediction than lipid variables seen in isolation (Millán et al. 2009). These indices include TC/HDL-C, LDL-C/HDL-C, TG/HDL-C and NHDL-C/HDL-C (Kastelein et al. 2008).

TC/HDL-C is also known as the atherogenic index, Castelli Index or HDL ratio (HDLR). The predictive value of the index is higher than that of its individual components and is considered more sensitive and specific index of cardiovascular risk than TC (Millán *et al.*, 2009; Kastelein *et al.*, 2008). Another ratio, the LDL-C/HDL-C ratio is very similar to HDLR, possibly due to the fact that two thirds of plasma cholesterol is found in LDL. The LDL-C/HDL-C index relies on an accurate calculation of LDL-C which may not be possible with triglyceride levels >3.36mmol/L. Furthermore very-low-density lipoprotein (VLDL) can undergo cholesterol enrichment in individuals with high triglyceride levels. In these cases the LDL-C/HDL-C ratio will underestimate risk (Millán et al. 2009). Other ratios including NHDL-C/HDLC and LDL-C/HDL-C ratios have been investigated but their use did not offer any clinical benefits over HDLR (Rader et al. 2003).

The HDLR has been shown in large observational studies including the Framingham (Castelli et al. 1986) , LRCP (Grover et al. 1994) , PROCAM (Assmann et al. 1998)

and in combinations of the large population cohorts (Kinosian et al. 1994) to be a better coronary risk predictor than independently used TC, LDL-C and HDL-C. HDLR was not found to be inferior to ApoB/ApoA measurements in a 15 year follow up of 3,322 Framingham inhabitants (Ingelsson et al. 2007) and in the EPIC-Norfolk study (van der Steeg et al. 2007).

The prevalence of dyslipidaemia in the Maltese population will be analysed in the MAMI study. Furthermore, the association of various lipoprotein variables with myocardial infarction will be investigated to assess whether TC and LDL-C are the most highly associated or whether alternative indices should be considered.

Section 4.2. Results

Section 4.2.1. Lipids and Sex

Lipid variable levels varied with sex in controls. Median levels of NHDL-C, TG, HDLR were higher in men than in women. While on the other hand, median levels of HDL-C were higher in women than in men. TC and LDL-C levels were similar in both sexes. Due to sex differences subsequent analysis will be restricted by gender (Table 4-1).

Section 4.2.2. Lipids and Age

Amongst controls, lipid levels varied with age in both men and women. In men TC, LDL-C, NHDL-C and TG were lowest in the 20 to 29 year age group and increased with age before plateauing and decreasing in those older than 60. HDL-C and HDLR varied little with age (Figure 4-1). In women. TC, LDL-C, NHDL-C, TG and HDLR increased with age in controls. There was no appreciable difference with age in HDL-C (Figure 4-2).

Table 4-1 Effect of sex on median lipid levels in controls from the MAMI Study. p-values estimated using two-tailed Mann-Whitney test. HDLR ratio is TC/HDL-C

Lipid variable	Men	Women	
	Median (IQR) n=323	Median (IQR) n=134	p- value
Total cholesterol (mmol/L)	5.36 (4.72-6.04)	5.49 (4.72-6.1)	0.49
LDL cholesterol (mmol/L)	3.41 (2.76-3.99)	(2.63-3.83)	0.11
NHDL cholesterol (mmol/L)	4.04 (3.29-4.77)	3.78 (3.03-4.51)	0.01
HDL cholesterol (mmol/L)	1.31 (1.12-1.54)	1.67 (1.37-1.95)	< 0.001
Triglycerides (mmol/L)	1.21 (0.85-1.75)	1.05 (0.71-1.45)	< 0.001
HDLR	3.97 (3.3-4.97)	3.31 (2.60-4.01)	< 0.001

When comparing male controls with an age < 50 years to those with an age ≥ 50 years median levels of TC, NHDL-C, and TG were elevated in the older age group. There was no difference between these groups for LDL-C, HDL-C and HDLR (Table 4-2). When comparing male controls with an age < 60 to those with an age ≥ 60 years median levels of total cholesterol, LDL-C, HDL-C, NHDL-C, HDLR and triglycerides did not vary (Table 4-3).

Table 4-2 Effect of age on median levels of lipid variables in male controls younger than 50 years of age and in male controls of age 50 years or older. The p-values are calculated using the two-tailed Mann-Whitney test.

Lipid variable	< 50 years Median (mmol/L) n=72	≥ 50 years Median (mmol/l) n=251	p-value
Total cholesterol	5.22	5.44	0.02
LDL cholesterol	3.30	3.42	0.26
HDL cholesterol	1.31	1.33	0.93
NHDL cholesterol	3.77	4.10	0.05
HDLR	3.86	4.00	0.22
Triglycerides	0.967	1.29	0.001

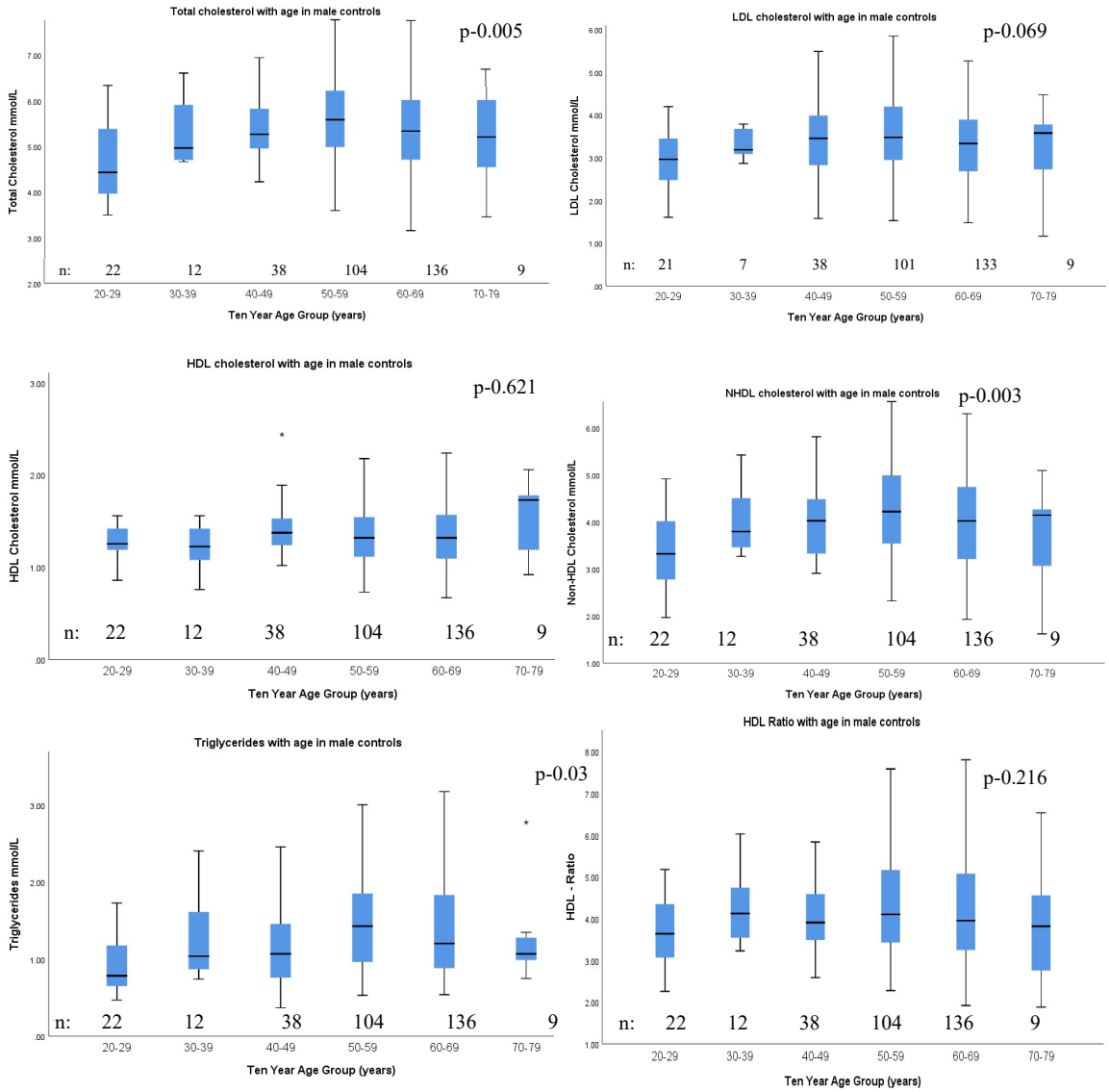

Figure 4-1 Boxplots of total cholesterol, Low density lipoprotein cholesterol, High density lipoprotein cholesterol, Non high density lipoprotein cholesterol, Triglyceride levels and HDLR (total cholesterol/ high density lipoprotein cholesterol) ratio in male controls with age excluding outliers. The boundary of the box closest to zero indicates the 25th percentile, a black line within the box marks the median, the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 10th and 90th percentiles. Numbers of included participants are shown at the bottom within the figure. p-value for the whole group is presented at the right top corner.

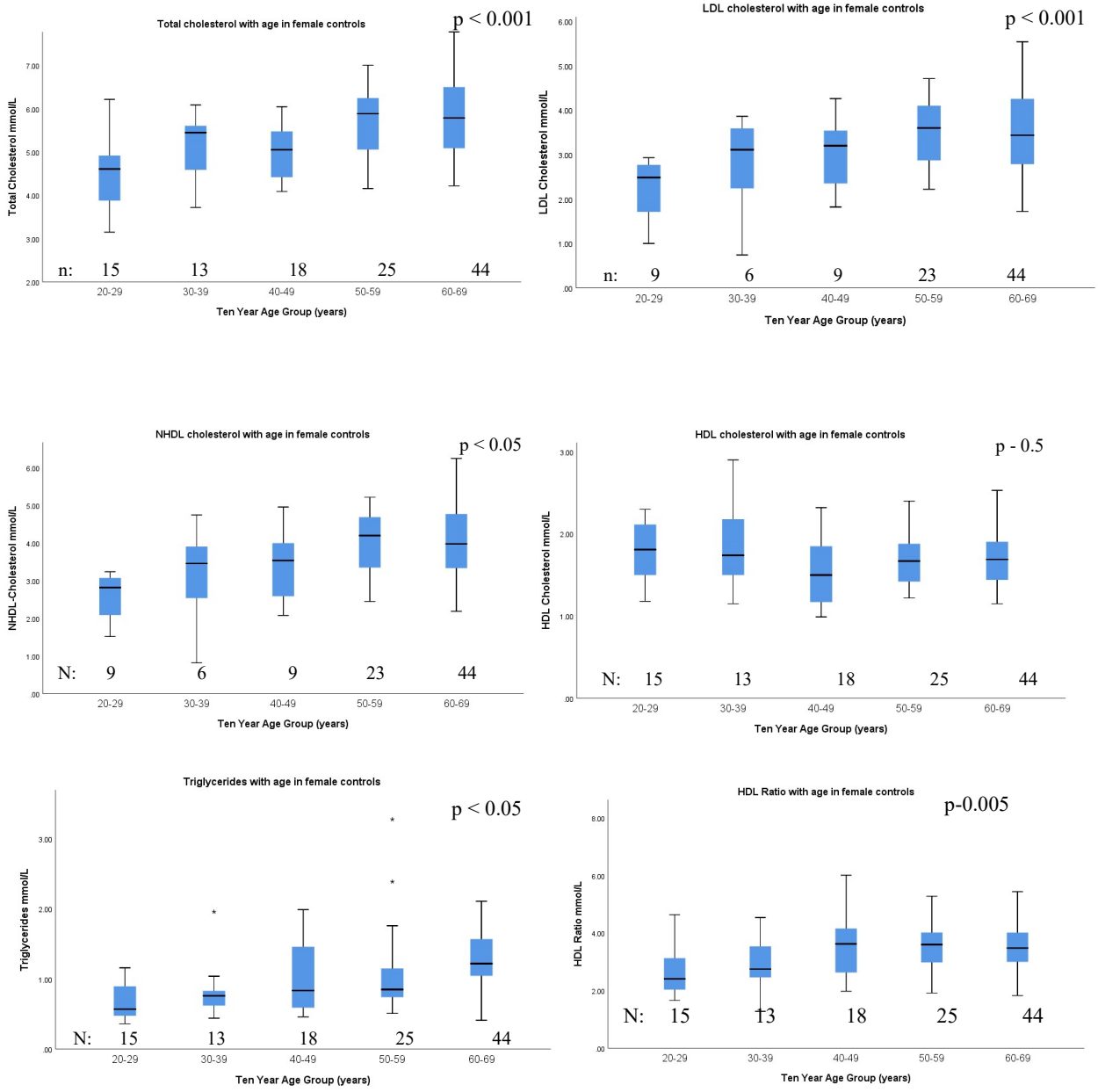

Figure 4-2 Boxplots of lipid variables in female controls with age excluding outliers. The boundary of the box closest to zero indicates the 25th percentile, a black line within the box marks the median, the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 10th and 90th percentiles. Numbers of included participants are shown at the bottom within the figure. P-value for the whole group is presented at the right top corner.

Table 4-3 Effect of age on median levels of lipid variables in men younger than 60 years of age and in men of age 60 years or older, data on controls only. The p-values are calculated using the two-tailed Mann-Whitney test.

	< 60 years	≥ 60 years	
Lipid variable	Median (IQR) mmol/L	Median (IQR) mmol/L	p-value
Total cholesterol	5.39 (4.74-6.04)	5.34 (4.71-6.04)	0.51
LDL cholesterol	3.41 (2.90-4.06)	3.34 (2.68-3.89)	0.25
HDL cholesterol	1.31 (1.13-1.51)	1.33 (1.10-1.58)	0.81
NHDL cholesterol	4.04 (3.35-4.76)	4.03 (3.18-4.77)	0.42
HDLR	4.00 (3.40-4.86)	3.90 (3.21-5.04)	0.51
Triglycerides	1.23	1.21	0.30

When analysing female controls < 50 years compared to women ≥ 50 years median TC, LDL-C, NHDL C, HDLR and TG were higher in the older female group. HDL-C varied little with age in female controls (Table 4-4).

Table 4-4 Effect of age on median levels of lipid variables in female controls. The p-values are calculated using the two-tailed Mann-Whitney test.

	< 50 years Median (mmol/l) n=46	≥ 50 years Median mmol/l n=87	p-value
Total cholesterol	4.78	5.80	< 0.01
LDL cholesterol	2.84	3.47	< 0.01
HDL cholesterol	1.65	1.67	0.96
NHDL cholesterol	3.23	4.06	< 0.01
HDLR	2.93	3.49	0.004
Triglycerides	0.75	1.11	< 0.001

Median levels of total cholesterol, LDL-C, NHDL-C, HDLR and TG were still higher in the older age group for women using 60 years of age as the cut-off in controls. HDL-C varied little with age in female controls (Table 4-5).

Table 4-5 Effect of age on median levels of lipid variables in female controls. The p-values are calculated using the two-tailed Mann-Whitney test

Lipid variable	Women < 60 n Median (IQR) mmol/L	Women ≥ 60 Median (IQR) mmol/l	p-value
Total cholesterol	5.20 (4.58-5.89)	5.79 (5.11-6.58)	< 0.001
LDL cholesterol	3.17 (2.37-3.82)	3.46 (2.79-4.21)	< 0.001
HDL cholesterol	1.67 (1.39-2.07)	1.68 (1.36-1.92)	0.98
NHDL cholesterol	3.43 (2.59-4.19)	3.99 (3.29-4.79)	0.001
HDLR	3.24 (2.43-3.94)	3.47 (2.86-4.43)	0.06
Triglycerides	0.79 (0.59-1.12)	1.19 (0.98-1.57)	< 0.001

A Spearman's rank-order correlation was run to assess the relationship between the various lipid variables and age in both male and female controls. Preliminary analysis showed the relationship to be monotonic as assessed by visual inspection of a scatterplot. There was a weak correlation between the various lipid variables and age in both male and female controls (Table 4-6, Table 4-7). The weak correlation remained even when male controls were stratified into < 50, ≥ 50 age groups (Table 4-8, Table 4-9). A similar weak correlation was also observed when women were stratified into two groups < 50, ≥ 50 age groups and also when stratified into < 60, ≥ 60 age groups (Data not shown).

Table 4-6 Spearman's correlation between various lipid variables and age in male controls (n=323).

	Spearman's coefficient	p-value
Total cholesterol	0.005	0.926
LDL cholesterol	-0.029	0.601
HDL cholesterol	0.038	0.494
NHDL cholesterol	0.038	0.494
HDLR	-0.031	0.585
Triglycerides	0.053	0.342

Table 4-7 Spearman's correlation between various lipid variables and age in female controls, n=134.

	Spearman's rho	p-value
Total cholesterol	0.415	< 0.001
LDL cholesterol	0.326	< 0.001
HDL-cholesterol	-0.047	0.593
NHDL cholesterol	0.387	< 0.001
HDLR	0.262	0.002
Triglycerides	0.435	< 0.001

Table 4-8 Spearman's correlation between various lipid variables and age in male controls < 50 years of age n=72

	Spearman's rho	p-value
Total cholesterol	0.303	0.010
LDL cholesterol	0.224	0.070
HDL cholesterol	0.146	0.220
NHDL cholesterol	0.227	0.019
HDLR	0.121	0.310
Triglycerides	0.214	0.070

Table 4-9 Spearman's correlation between various lipid variables and age in male controls ≥ 50 years of age n=251.

	Spearman's rho	p-value
Total cholesterol	-0.164	0.009
LDL cholesterol	-0.130	0.042
HDL cholesterol	0.060	0.345
NHDL cholesterol	-0.171	0.007
HDLR	-0.141	0.026
Triglycerides	-0.163	0.010

Section 4.2.3. Lipids and Risk of Myocardial Infarction

Lipid levels had an influence on the risk of myocardial infarction. In men, TG and HDLR were associated with the highest risk for myocardial infarction followed by NHDL-C and TC. LDL-C showed the weakest association with risk of myocardial infarction. High HDL-C levels were associated with a protective effect with respect to myocardial infarction. These analyses were performed in tertiles based on sex specific

distribution in controls, restricting the analysis to men not taking statins produced similar results to those seen in all men (Table 4-10). Restricting the analysis to men on statins resulted in a widening of the confidence intervals with only the risk of high triglycerides not crossing the midline (Table A1-1). In women high NHDL-C, TG and HDLR were associated with a higher risk of myocardial infarction. High HDL-C was protective for MI. TC and LDL-C were not associated with myocardial infarction in women (Table 4-11).

Table 4-10 Risk of myocardial infarction in men not on statins comparing highest vs lowest tertile of lipid variable. n = Cases and Controls in the highest tertile, % is the percentage in cases and controls, age adjusted odds ratio (Age OR) of risk of myocardial infarction and 95% confidence interval shown in brackets. AdjOR is OR adjusted for age, hypertension, diabetes, smoking, alcohol consumption and BMI. Lipids are taken fasted in controls compared to on admission in cases.

Lipid variable	Cases n (%)	Controls n (%)	Age OR (95% CI)	AdjOR (95% CI)
Total cholesterol	116 (50.0)	96 (36.4)	1.7 (1.1-2.6)	1.8 (1.1-3.1)
HDL-C	25 (10.8)	85 (32.2)	0.2 (0.1-0.3)	0.2 (0.1-0.3)
LDL-C	102 (45.9)	97 (36.7)	1.3 (0.8-2.0)	1.7 (1.0-2.9)
NHDL-C	131 (56.7)	95 (36.0)	2.5 (1.5-3.9)	2.9 (1.6-5.0)
Triglycerides	150 (64.7)	84 (31.8)	6.7 (4.0 -11.4)	5.5 (2.9 -10.1)
HDLR	160 (69.3)	92 (34.8)	5.5 (3.3 -9.3)	5.8 (3.1-10.8)

Table 4-11 Risk of myocardial infarction in women comparing highest vs lowest tertile of lipid variable. N = Cases and Controls in the highest tertile, % is the percentage in cases and controls, age adjusted odds ratio (Age OR) of risk of myocardial infarction and 95% confidence interval shown in brackets. AdjOR is OR adjusted for age, hypertension, diabetes, smoking, alcohol consumption, statin use and BMI. Lipids are taken fasted in controls compared to on admission in cases.

Lipid variable	Cases n (%)	Controls n (%)	Age OR (95% CI)	AdjOR (95% CI)
Total cholesterol	28 (38.9)	44 (32.8)	0.7 (0.3-1.5)	1.3 (0.5-3.1)
HDL-C	11 (14.9)	42 (31.3)	0.2 (0.1-0.5)	0.2 (0.1-0.5)
LDL-C	30 (41.1)	44 (32.8)	1.0 (0.5-2.2)	1.9 (0.7-5.0)
NHDL-C	31 (41.9)	44 (32.8)	1.6 (0.7-3.7)	3.9 (1.3-11.7)
Triglycerides	53 (71.6)	44 (32.8)	4.9 (1.9-12.4)	5.0 (1.7-14.4)
HDLR	47 (51.6)	44 (32.8)	3.6 (1.6-8.1)	3.6 (1.4-9.0)

The odds ratios did not vary when restricting to women off statins. The limited number of women on statins did not allow further analysis (Table A1-2).

Section 4.2.4. Self-Reported Hypercholesterolaemia

Self-reported hypercholesterolaemia rates varied between cases, controls and relatives. The highest rate of self-reported hypercholesterolaemia, 45.5% (n= 179) was observed amongst cases. Controls and relatives had similar rates of self-reported hypercholesterolaemia (34.4% and 32.4% respectively). The proportion of each group on lipid lowering agents was comparable although it was highest in cases (23.4%), followed by relatives (21.3%) and controls (18.3%) (Table 4-12).

Table 4-12 Self-reported hypercholesterolaemia and cholesterol lowering agent use among cases, controls and relatives. P-value calculated using chi-square.

	Cases n (%)	Controls n (%)	Relatives n (%)	p-value
Self-reported hypercholesterolaemia	179 (45.4)	160 (34.4)	68 (32.4)	0.001
On cholesterol lowering agent	92 (23.4)	85 (18.3)	46 (21.9)	0.17

Section 4.2.5. Diabetes and Lipids

In male controls TC, LDL-C, NHDL-C was higher in research subjects without diabetes compared to subjects with diabetes. HDL-C, HDLR and TG did not vary when comparing control subjects without diabetes to those with diabetes. There was no difference in TG, LDL-C, HDL-C, HDLR and TG between research subjects with controlled and uncontrolled diabetes, always amongst male controls. NHDL-C was higher in the group with uncontrolled diabetes compared to the group with controlled diabetes (Table 4-13).

Table 4-13 Lipid Variables with diabetes in male controls. P-value estimated for the group using Kruskal Wallis test. IQR -Interquartile range.

Lipid variable	No Diabetes (n=270)	Controlled Diabetes (n=10)	Uncontrolled Diabetes (n=28)	p -value
% statin use	15	31	51	
	Median mmol /L (IQR)	Median mmol/L (IQR)	Median mmol/l (IQR)	
Total cholesterol	5.4 (4.9-6.1)	4.8 (4.0-5.8)	4.5 (4.1-5.6)	< 0.001
LDL cholesterol	3.5 (2.9-3.5)	2.6 (1.9-2.6)	2.6 (2.1-3.3)	< 0.001
HDL cholesterol	1.3 (1.1-1.6)	1.3 (1.1-1.3)	1.2 (1.1-1.5)	0.676
NHDL cholesterol	4.1 (3.4-4.8)	2.9 (2.7-2.9)	3.3 (2.9-4.4)	0.001
HDLR	4.0 (3.3-5.0)	3.5 (2.5-4.4)	3.7 (3.1-4.5)	0.132
Triglycerides	1.2 (0.9-1.74)	1.3 (0.6-1.3)	1.4 (1.0-1.9)	0.580

Similar patterns were noticed in women but the number of women with controlled or uncontrolled diabetes was very small (Table A1-3).

Section 4.2.6. Lipids and Measures of Adiposity

Adiposity meaured using BMI in female controls showed no difference in total cholesterol between normal and overweight/obese subjects although there was a pattern towards higher levels in the obese category compared to the overweight. There was a pattern towards higher LDL-C in male controls with obesity. NHDL-C, HDLR and TG increased with increasing obesity ($p < 0.001$). HDL-C was lower in subjects with obesity compared with subjects who are overweight and normal weight ($p < 0.001$) (Table 4-14).

Table 4-14 Lipid Variables with body mass index in male controls. P-value estimated for the group using Kruskal Wallis test. IQR-Interquartile range.

Lipid variable	Normal BMI (n=46)	Overweight (n=152)	Obese (n=123)	p-value
	Median mmol/L (IQR)	Median mmol/l IQR	Median mmol/L (IQR)	
Total cholesterol	5.2 (4.7-5.8)	5.3 (4.7-6.0)	5.5 (4.9-6.2)	0.174
LDL cholesterol	3.2 (2.8-3.5)	3.4 (2.7-4.0)	3.5 (2.9-4.2)	0.071
HDL cholesterol	1.4 (1.2-1.7)	1.4 (1.2-1.6)	1.2 (1.1-1.4)	< 0.001
NHDL cholesterol	3.6 (3.3-4.2)	4.0 (3.1-4.6)	4.3 (3.6-5.0)	0.001
HDLR	3.5 (2.9-4.0)	3.8 (3.1-4.6)	4.5 (3.7-5.5)	< 0.001
Triglycerides	1.0 (0.8-1.5)	1.1 (0.7-1.6)	1.5 (1.1-2.2)	< 0.001

When analysis was restricted to men off statins where was no difference in TC between the groups. LDL-C, NHLD-C and TG were all higher in the obese group compared to the normal BMI group. HDL-C was lower in the Obese group compared to the normal BMI group (Table 4-15).

Table 4-15 Lipid Variables with body mass index in male controls off statins. P-value estimated for the group using Kruskal Wallis test. IQR-Interquartile range.

Lipid variable male controls off statins	Normal BMI (43)	Overweight (120)	Obese (n=99)	p-value
	Median (IQR) mmol/l	Median (IQR) mmol/l	Median (mmol/L)	
Total cholesterol	5.32 (4.67-5.87)	5.47 (4.74-6.06)	5.52 (5.02-6.37)	0.10
LDL cholesterol	3.27 (2.87-3.57)	3.50 (2.84-4.06)	3.67 (3.09-4.34)	0.01
HDL cholesterol	1.43 (1.22-1.73)	1.36 (1.17-1.62)	1.20 (1.05-1.45)	< 0.001
NHDL cholesterol	3.72 (3.25-4.29)	4.08 (3.35-4.78)	4.39 (3.77-5.05)	< 0.001
HDLR	3.59 (2.95-4.01)	3.95 (3.18-4.61)	4.66 (3.82-5.60)	< 0.001
Triglycerides	0.96 (0.75-1.48)	1.12 (0.75-1.57)	1.45 (1.07-2.18)	< 0.001

In female controls similar patterns were noted for the relationship of lipid variables with BMI. TC, LDL-C , NHDL-C and HDLR were higher in subjects who were overweight and obese compared to normal BMI subjects ($p < 0.05$). There was no difference in these variables when comparing overweight to obese subjects. HDL-C decreased with higher BMI in a step wise fashion (p-0.012) while TG increased in a stepwise fashion with BMI ($p < 0.001$) (Table 4-16).

Table 4-16 Lipid Variables with body mass index in female controls. P-value estimated for the group using Kruskal Wallis test. IQR – Interquartile range.

Lipid variable	Normal BMI Median (IQR) mmol/l n=42	Overweight Median (IQR) mmol/l n=45	Obese Median (IQR) mmol/L n=44	p-value
Total cholesterol	5.2 (4.6-6.0)	5.7 (5.0-6.2)	5.7 (4.7-6.2)	0.039
LDL cholesterol	2.9 (2.4-3.5)	3.5 (2.8-4.1)	3.5 (2.5-3.9)	0.013
HDL cholesterol	1.8 (1.5-2.2)	1.7 (1.4-1.9)	1.5 (1.2-3.9)	0.012
NHDL cholesterol	3.4 (2.8-4.0)	3.9 (3.3-4.8)	4.0 (3.0-4.7)	0.004
HDLR	3.0 (2.3-3.4)	3.6 (3.0-4.0)	3.5 (2.9-4.5)	0.001
Triglycerides	0.8 (0.6-1.1)	1.1 (0.7-1.4)	1.3 (1.0-1.7)	0.001

The effect of adiposity on lipid variables was also anlysed using waist-hip ratio as the measure of adiposity. In male controls there was no difference in TC and LDL-C beween subjects with a normal WHR and those with a high WHR while there was a pattern towards higher NHDL-C. HDL-C was significantly lower in subjects with a high waist-hip ratio (p-0.006). Triglycerides and HDLR were significantly higher in subjects with a high WHR compared to those with a normal WHR ($p < 0.001$) (Table 4-17).

Table 4-17 Lipid Variables with waist-hip ratio according to WHO cut-off in male controls. P-value estimated for the group using Kruskal Wallis test. IQR – interquartile range. WHR- Waist-hip ratio

Lipid variable	Normal WHR Median (IQR) mmol/l n=81	High WHR Median (IQR) mmol/L n=238	p-value
Total cholesterol	5.3 (4.7-5.9)	5.4 (4.7-6.1)	0.332
LDL cholesterol	3.4 (2.7-3.9)	3.4 (2.8-4.0)	0.522
HDL cholesterol	1.4 (1.2-1.7)	1.3 (1.1-1.5)	0.006
NHDL cholesterol	3.7 (3.2-4.6)	4.1 (3.4-4.8)	0.061
HDLR	3.6 (3.0-4.4)	4.0 (3.4-5.0)	0.003
Triglycerides	0.9 (0.7-1.5)	1.3 (1.0-1.9)	< 0.001

Similar patterns were observed when WHR and lipids were analysed in tertiles (Table 4-18). Similar patterns were observed in female control subjects when waist-hip ratio categories were divided according to WHO cutoffs (Table 4-19). However when waist-hip ratio was divided into tertiles there was a difference in all the lipid variables between the different waist-hip ratio tertiles (Table 4-20).

Table 4-18 Lipid Variables with waist-hip ratio analysed in tertiles in male controls. P-value estimated for the group using Kruskal Wallis test. IQR – interquartile range. WHR- Waist-hip ratio

Lipid variable	WHR lowest tertile Median (IQR) mmol/L n=108	WHR middle tertile Median (IQR) mmol/L n=107	WHR highest tertile Median (IQR) mmol/L n=105	p-value
Total cholesterol	5.28 (4.71-5.91)	5.47 (4.81-6.13)	5.38 (4.66-6.27)	0.412
LDL cholesterol	3.31 (2.75-3.85)	3.38 (2.86-4.04)	3.42 (2.65-4.09)	0.627
HDL cholesterol	1.40 (1.18-1.63)	1.40 (1.12-1.58)	1.21 (1.07-1.43)	< 0.001
NHDL cholesterol	3.76 (3.18-4.53)	4.05 (3.42-4.73)	4.23 (3.37-4.92)	0.070
HDLR	3.69 (3.08-4.48)	3.96 (3.34-4.70)	4.41 (3.58-5.30)	< 0.001
Triglycerides	0.97 (0.71-1.57)	1.13 (0.85-1.62)	1.56 (1.20-2.07)	< 0.001

Table 4-19 Lipid Variables with waist-hip ratio in female controls. P-value estimated for the group using Kruskal Wallis test. IQR – interquartile range.

Lipid Variable	Normal waist-hip ratio Median (IQR) mmol/L n=93	High waist-hip ratio Median (IQR) mmol/L n=39	p value
Total cholesterol	5.46 (4.78-6.06)	5.73 (4.71-6.13)	0.84
LDL cholesterol	3.25 (2.71-3.72)	3.42 (2.54-4.13)	0.56
HDL cholesterol	1.71 (1.49-2.04)	1.49 (1.27-1.83)	0.01
NHDL cholesterol	3.70 (3.05-4.29)	3.99 (3.03-4.79)	0.25
HDLR	3.14 (2.53-3.78)	3.52 (2.94-4.54)	0.03
Triglycerides	0.94 (0.70-1.33)	1.11 (0.79-1.70)	0.06

Table 4-20 Lipid Variables with waist-hip ratio analysed in tertiles in female controls. P-value estimated for the group using Kruskal Wallis test. IQR – interquartile range.

Lipid variable	WHR lowest tertile n=43		WHR middle tertile n=45		WHR highest tertile n=42		p-value
	Median (mmol/l)	IQR (mmol/L)	Median (mmol/l)	IQR (mmol/L)	Median (mmol/L)	IQR (mmol/L)	
Total cholesterol	5.12	4.61-5.69	5.76	5.11-6.36	5.58	4.68-6.13	0.027
LDL cholesterol	2.89	2.39-3.46	3.51	2.84-4.11	3.35	2.51-4.12	0.022
HDL cholesterol	1.85	1.54-2.12	1.67	1.43-1.95	1.38	1.26-1.80	0.001
NHDL cholesterol	3.24	2.69-3.91	4.01	3.37-4.75	3.40	3.1-4.77	0.004
HDLR	2.96	2.29-3.55	3.38	2.78-4.13	3.55	3.08-4.49	0.001
Triglycerides	0.82	0.60-1.09	1.09	0.755-1.56	1.15	0.83-1.77	0.001

The was a correlation between lipid variables and waist-hip ratio as measured by Spearman's correlation in both men (Table 4-21) and women (Table 4-22).

Table 4-21 Correlation between lipid variables and waist-hip ratio in male controls. n=320.

	WHR	
	Spearman's rho	p-value
Total cholesterol	0.042	0.449
LDL cholesterol	0.028	0.613
HDL cholesterol	-.227	< 0.001
NHDL cholesterol	0.124	0.027
HDLR	0.228	< 0.001
Triglycerides	0.364	< 0.001

Table 4-22 Correlation between lipid variables and waist-hip ratio in female controls. n=133.

	Spearman's rho	p-value
Total cholesterol	0.148	0.088
LDL cholesterol	0.179	0.040
HDL cholesterol	-.303	< 0.001
NHDL cholesterol	0.244	0.005
HDLR	0.318	< 0.001
Triglycerides	0.296	0.001

Section 4.2.7. Lipids and Alcohol Consumption

The effects of alcohol on lipid variables is discussed in Chapter 6.

Section 4.2.8. Lipids Physical Activity

Physical activity was divided based on metabolic equivalents (METs) per week into low physical activity, moderate physicial activity and high physical ativity groups. TG decreased with increasing levels of physical activity in male controls (p-0.018). There was no difference in TC, LDL-C, HDL-C, NHDL-C and HDLR with varying levels of physical activity in male controls (Table 4-23).

Table 4-23 Lipid variables and physical activity in male controls. The p-value was estimated using Mann-Whitney test. IQR- Interquartile range.

Lipid variable in male controls	Low physical activity (n=100)	Moderate physical activity (n=56)	High physical activity (n=131)	p- value
	Median IQR (mmol/L)	Median IQR (mmol/L)	Median IQR (mmol/L)	
Total cholesterol	5.3 (4.8-6.2)	5.4(4.8-6.2)	5.4 (4.7-6.0)	0.73
LDL cholesterol	3.4 (2.8-3.9)	3.4 (2.7-4.0)	3.4 (2.8-4.1)	0.97
HDL cholesterol	1.3 (1.1-1.5)	1.3 (1.1-1.5)	1.4 (1.1-1.6)	0.50
NHDL cholesterol	4.0 (3.3-4.7)	4.1 (3.4-4.9)	4.0 (3.2-4.8)	0.64
HDLR	3.9 (3.3-5.0)	4.1 (3.6-5.0)	4.0 (3.1-4.8)	0.45
Triglycerides	1.3 (0.9-1.8)	1.5 (1.0-2.1)	1.1 (0.8-1.7)	0.02

In female controls the change in TG was not observed and there was no change in TC, LDL-C, NHDLC and HDLR. HDL-C however was decreased slightly in the high physical activity group (p-0.03) (Table 4.24).

Table 4-24 Lipid variables and physical activity in female controls. P-value estimated using Mann-Whitney test. IQR- Interquartile range.

Lipid variable in male controls	Low physical activity (n=49)	Moderate physical activity (n=)	High physical activity (n=131)	p-value
	Median IQR (mmol/L)	Median IQR (mmol/L)	Median IQR (mmol/L)	
Total cholesterol	5.45 (4.8-6.1)	5.8 (5.4-6.2)	5.4 (4.7-6.0	0.467
LDL cholesterol	3.4 (2.7-3.8)	3.6 (3.0-4.0)	3.4 (2.8-4.1)	0.140
HDL cholesterol	1.5 (1.3-1.9)	1.5 (1.3-1.8)	1.4 (1.1-1.6)	0.03
NHDL cholesterol	3.8 (3.1-4.6)	4.1 (3.5-4.7)	4.0 (3.2-4.8)	0.140
HDLR	3.5 (2.6-4.1)	3.8 (3.2-4.4)	3.9 (3.1-4.8)	0.07
Triglycerides	1.0 (0.7-1.3)	1.1 (0.8-1.8)	1.1 (0.8-1.7)	0.210

Section 4.2.9. Lipids and Education

Controls were divided according to their level of education into primary, secondary, post-secondary, tertiary and post-tertiary. There was a pattern towards lower levels of total cholesterol, LDL and triglycerides with increasing levels of education. There was a pattern towards rising HDL-C with higher levels of education. NHDL-C and HDLR decreased significantly with level of education (p-0.035) (Table 4-25). Similar

observations were noted in female controls (Table 4-26). These observations remained valid in the < 60 year age group in men.

Table 4-25 Lipid variables and education level in male controls. P-value estimated using Mann-Whitney test. IQR- Interquartile range.

Lipid variable in male controls	Primary education (n=40) Median (IQR) mmol/L	Secondary education (n=130) Median (IQR) mmol/L	Post-Secondary (n=91) Median (IQR) mmol/L	Tertiary (n=41) Median (IQR) mmol/L	Post tertiary (n=20) Median (IQR) mmol/L	p-value
Total cholesterol	5.6 (4.8-6.2)	5.4 (4.9-6.2)	5.3 (4.7-6.0)	5.3 (4.4-5.9)	5.3 (4.4-5.7)	0.22
LDL cholesterol	3.7 (2.9-4.2)	3.4 (2.9-4.2)	3.2 (2.7-3.9	3.1 (2.5-3.8)	3.4 (2.6-3.7)	0.12
HDL cholesterol	1.2 (1.1-1.6)	1.3 (1.1-1.5)	1.4 (1.2-1.6)	1.3 (1.1-1.7)	1.4 (1.2-1.5)	0.15
NHDL cholesterol	4.3 (3.4-4.9)	4.2 (3.5-4.9)	3.9 (3.2-4.6)	3.7 (3.0-4.6)	4.0 (3.1-4.3)	0.04
HDLR	4.3 (3.5-5.5)	4.3 (3.5-5.2)	3.8 (3.1-4.6)	3.8 (3.0-4.5)	3.7 (3.4-4.1)	0.01
Triglycerides	1.2 (1.0-1.9)	1.4 (1.0-1.9)	1.1 (0.8-1.7	1.3 (0.9-1.7)	1.0 (0.8-1.4)	0.12

Table 4-26 Lipid variables and education level in Female controls. P-value estimated using Mann-Whitney test. IQR- Interquartile range

Lipid variable in female controls	Primary education (n=29) Median (IQR) mmol/L	Secondary education (n=60) Median (IQR) mmol/L	Post-Secondary (n=26) Median (IQR) mmol/L	Tertiary (n=16) Median (IQR) mmol/L	Post tertiary (n=3) Median (IQR) mmol/L	p-value
Total cholesterol	5.7 (5.2-6.3)	5.6 (4.8-6.2)	5.1 (4.4-5.7)	5.2 (4.6-6.2)	4.2 (3.2-4.2)	0.01
LDL cholesterol	3.5(2.9-4.1)	3.4 (2.7-4.1)	3.0 (2.3-3.6)	2.9 (2.5-3.7)	2.1 (1.3-2.1)	0.07
HDL cholesterol	1.7 (1.3-1.9)	1.6 (1.4-1.8)	1.7 (1.4-2.3)	1.9 (1.4-2.2)	1.9 (1.6-2.3)	0.33
NHDL- cholesterol	4.0 (3.6-4.7)	4.0 (3.2-4.8)	3.4 (2.6-4.0)	3.2 (2.8-4.7)	2.3 (1.6-	0.003
HDLR	3.5 (3.1-4.1)	3.5 (2.6-4.4)	3.1 (2.2-3.7)	3.1 (2.3-3.6)	2.1 (2.0-	0.01
Triglycerides	1.2 (1.0-1.6)	1.1 (0.7-1.5)	0.9 (0.7-1.1)	0.8 (0.5-1.3)	0.6 (0.6-	0.003

Section 4.3. Discussion

In 1984, 48% of the population aged 25 to 64 had measured blood serum cholesterol that was high while a further 30% of the population had measured serum blood cholesterol that was borderline high. By 2010, the proportion of the population aged 25 to 64 with a high level of serum cholesterol had gone down by 26%. Looking at controls in the MAMI study that were between 25-64 years of age, 33.1% had a

desirable total cholesterol, 44.6% had a borderline high cholesterol while 22.3% had a high total cholesterol. Compared to EHES 2010 results there was a decrease in individuals having a desirable TC, and an increase in the subjects having a borderline high level while the percentage of individuals with a high TC level has remained the same (Figure 4-3).

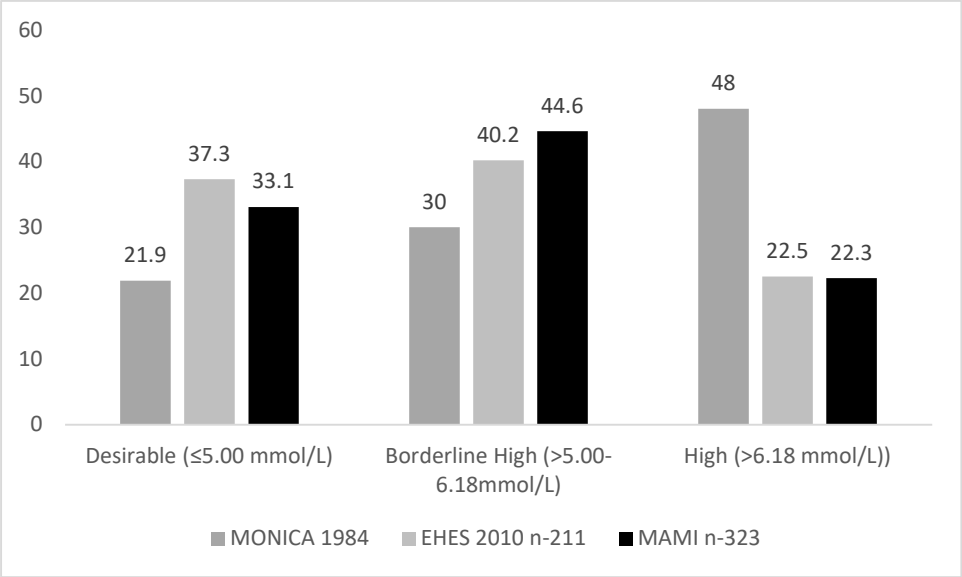

Figure 4-3 Total cholesterol levels distributed by EHES classification. Comparing MONICA study data to EHES pilot study data and MAMI control subject data.

In men on the other hand there has been a decrease in the percentage with desirable TC and an increase in borderline high and high TC compared to EHES pilot study data (Figure 4-4). In women the percentage of individuals with desirable TC has remained stable. There has been a decrease in percentage of women with high TC and a proportionate increase in those with borderline high TC (Figure 4-5).

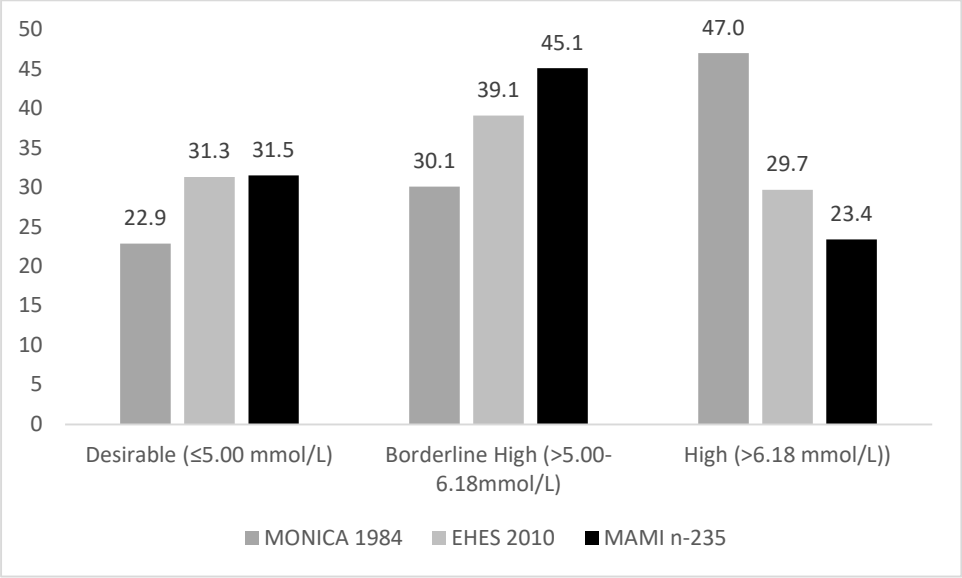

Figure 4-4 Total cholesterol levels distributed by EHES classification in men. Comparing MONICA study data to EHES pilot study data and MAMI control subject data.

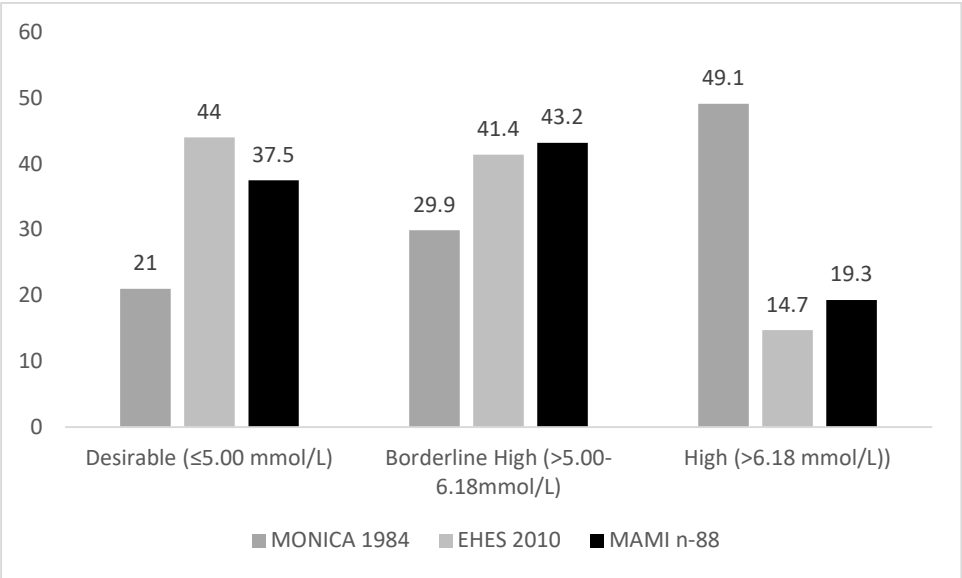

Figure 4-5 Total cholesterol levels distributed by EHES classification in Women. Comparing MONICA study data to EHES pilot study data and MAMI control subject data.

LDL-C levels have increased in the general population. The percentage of individuals in the borderline high, high and very high levels have all increased with a proportionate decrease of individuals in the optimal level (Figure 4-6).

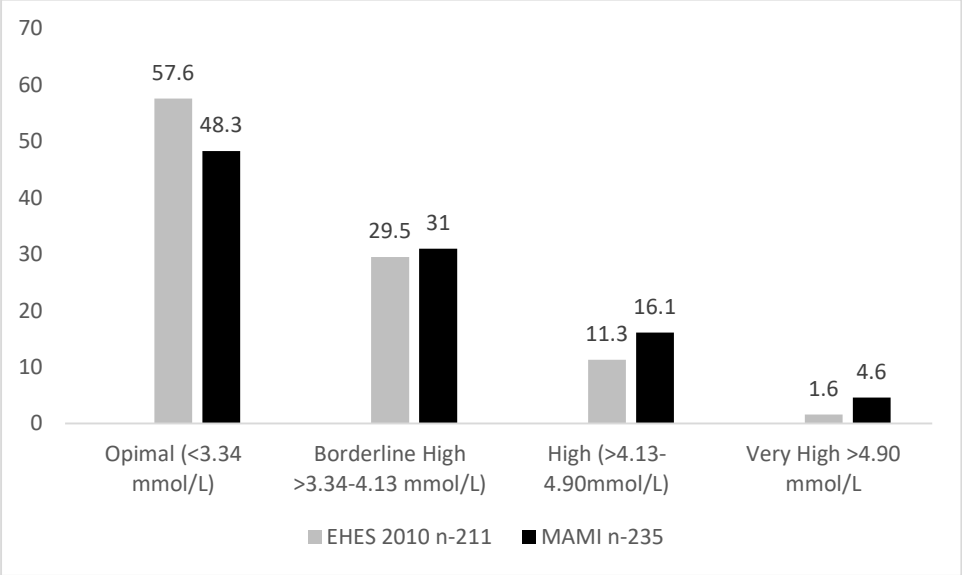

Figure 4-6 Low density lipoprotein cholesterol distributed by EHES classification comparing 2010 EHES pilot study data to MAMI study.

Lipids are clearly linked to risk of myocardial infarction in the MAMI study. Furthermore, the results highlight the importance of not relying solely on TC and LDL-C for risk stratification but using alternatives which are more strongly associated such as NHDL-C and HDLR. Differences in age and sex were present and further analysis were performed stratifying for age and sex. BMI appeared to be a better to predict adiposity related dyslipidaemia then waist-hip ratio. Physical activity had little effect on lipid variables while important changes in lipids were noticed with higher levels of education.

There were important differences between sexes when analysing lipid variables. In contrast to international cohorts' premenopausal women had similar levels of total

cholesterol and LDL cholesterol then age matched men. As reported in other cohorts HDL-C was higher in women while triglycerides were lower. When comparing lipid levels by age group, lipid variables in the older and younger age groups were more different in women than in men possibly due to the effects of menopause, sex-specific hormones and other sex-specific mechanisms. Besides different lipoprotein concentrations premenopausal women are also known to have a higher average LDL size and larger HDL particle size, shifts which can be considered anti-atherogenic (Wang et al. 2011).

Serum lipids are related to age. Early cross sectional studies and prospective cohorts (Ferrara et al. 1997) have shown that TC and LDL-C gradually increase after adolescence until the age of 60-65 years in men and 70-75 years in women and subsequently start to decline (Schaefer et al. 1994; Kuzuya et al. 2002; Upmeier et al. 2011). The decrease in LDL cholesterol may be partly due to selective survival of individuals with lower cholesterol levels (Schaefer et al. 1994). HDL-C and TG change less during adulthood but in cross-sectional reports HDL-C tends to increase in older individuals (Schaefer et al. 1994; Kuzuya et al. 2002; Upmeier et al. 2011). Findings of the MAMI study are consistent with these studies.

Similar to the findings in the INTERHEART study NHDL-C had a higher Odds Ratio for MI than TC. LDL-C was not measured in the INTERHEART study as they directly measured ApoB levels (McQueen et al. 2008). In the MAMI study LDL-C was only weakly associated with the risk of myocardial infarction in the men and women. In the MAMI study TC/HDL-C ratio (HDLR) was the most strongly associated with MI in men but in women NHL-C had the highest odds ratio. In view of these results reliance on TC and LDL-C for risk estimation in the Maltese population may to be inadequate and secondary targets including NHDL-C and HDLR should be considered.

Diabetes is associated with typical lipid variable changes termed diabetic dyslipidaemia which consists of a triad of raised TG, reduced HDL-C and excess small dense LDL-C (Ozder 2014). These changes are brought about by insulin resistance or insulin deficiency with secondary effects on lipid metabolism. In contrast, in the MAMI Study TC, LDL-C, NHDL-C were higher in research subjects without diabetes compared to those with diabetes. This is because statin use was more prevalent in subjects with controlled diabetes and (31%) and uncontrolled diabetes (51%) then in controls (15%). Thus, research subjects with diabetes had lower levels of TC, LDL-C and non-HDL-C than research subjects without diabetes. There was no difference between subjects with no diabetes and diabetes with regards to HDL-Cholesterol, HDLR and TG.

Obesity has been associated with various lipid abnormalities including elevated TC, TG and lower HDL-C. These changes are brought about by an increase in VLDL production and decreased VLDL clearance due to obesity associated insulin resistance. Insulin resistance leads to increased free fatty acids in the circulation with subsequent increased production of VLDL, while decreased clearance is due to decreased lipoprotein lipase activity in skeletal muscle. Decreased VLDL clearance is also caused by insulin mediated decreased LDL-C receptor activity. The elevated fasting triglycerides usually associated with obesity bring about triglyceride enrichment of LDL-C and HDL-C particles resulting in an increase in small dense LDL particles and a decrease in HDL_2 (a protective subfraction of HDL). Insulin resistance also brings about a decrease in ApoB-100 (apolipoprotein B-100) degradation bringing about an increase in VLDL, IDL and LDL-C (Miller et al. 2005). Elevated TC, LDL-C, NHDL-C, HDLR and TG and decreased HDL-C were observed in overweight and obese men and women. When analysing using WHO cut-offs the expected adiposity-associated dyslipidaemia was not as clear especially in women. There was no difference in TC

and LDL-C between normal and high waist-hip-ratio subjects. HDL-C was lower while non-HDLC, HDLR and triglycerides were higher in the high waist-hip ratio individuals.

Physical activity has well known effects on lipid variables. In longitudinal studies HDL rises and triglycerides decrease with physical activity. Whether this is a direct effect or whether this occurs due to improvements in BMI is still debated (Monda et al. 2009). Interestingly in the male MAMI control group only triglycerides showed an inverse association with physical activity and there was no association with HDL-C. In women the HDL-C was lower but there was no difference in triglyceride levels with physical activity.

Education was found to have an effect on lipid levels in both male and female controls. NHDL-C and HDLR improved with education level in both sexes. TC, LDL-C, HDL-C and TG did not vary significantly with education level in men However in female controls improvements were also noted in TC, LDL-C and TG. Such pronounced differences were not present in other studies. (Lara and Amigo 2018).

The Odds Ratios for triglycerides in the MAMI study suffer from the limitation that levels in controls were taken in the fasting state while those in cases were non-fasting. The cases were non-fasted since the first samples collected during their admission were collected and were often in the non-fasting state. Furthermore, it is important to note that there may be important changes in the levels of all lipid variables after a myocardial infarction with the change depending on how long from the event the blood was taken. Combined data from various studies shows a mean fall in total cholesterol from 9%-11% from baseline over 14 days whereas triglycerides were noted to rise 18% from baseline in the first 12 weeks (Barth et al. 2010). However there is considerable heterogeneity in the literature with other authors quoting minimal changes in lipid levels within the first 4 days (Pitt et al. 2008). Further limitations include recall bias

with regards to physical activity and changes in physical activity with seasonality. During the questionnaire only a snapshot regarding recent activity was recorded and this may not necessarily reflect an individual's habits over his lifetime.

Section 4.4. Conclusion

The MAMI study results show that NHDL-C and HDLR are more strongly associated with risk of MI than TC and LDL-C in the Maltese population. This could have implications on what variable should be given most prominence clinically while trying to assess an individual's risk of myocardial infarction. Furthermore, the results show how lipid levels are affected by multiple factors including sex, adiposity physical activity which could all act as strong confounding factors in studies on the effect of lipid levels and their association with myocardial infarction.

Chapter 5. Diet and MI

Section 5.1. Introduction

Dietary intake is a complex modifiable risk factor for coronary artery disease and MI (Iqbal et al. 2008). Initially the focus was on low fat diets, which although successful in decreasing serum cholesterol levels did not convincingly decrease total or cardiovascular mortality. In view of these failures, novel approaches focusing on increasing beneficial food groups embodied in the 'Mediterranean diet' have proven successful in primary and secondary prevention trials (Dalen and Devries 2014). The study of diet in relation to any long-term outcome is very complex. Besides the fact that dietary patterns differ between different populations, they also differ by sex and on an individual level they differ over time depending on seasonality and mood (Owens et al. 1996). The study of diet is further complicated by portion size, the number of times a specific food is consumed per day, total energy intake, the metabolic status of the individual and the level of exertion the individual is performing. Furthermore, the fact that micronutrients interact and that food patterns may lead to clustering result in residual confounding which is often difficult to eliminate. Above all these concerns, studies of diet and exercise are strongly affected by recall bias (Iqbal et al. 2008). The analysis of dietary influences on coronary artery disease and myocardial infarction is complicated further by co-morbidities including smoking, diabetes mellitus and hypercholesterolaemia and social influences such as level of education. (Iqbal et al. 2008; Schoenfeld and Ioannidis 2013).

The study of diet can concentrate on micronutrients (saturated fats), on food groups (number of apples, green vegetables) or on dietary patterns (ex. Oriental, western, prudent) (Iqbal et al. 2008). The INTERHEART study identified 3 major dietary patterns, namely oriental, western and prudent. The western diet is rich in fried foods, salty snacks and meat intake while a prudent diet is rich in fruit and vegetable intake

(Iqbal et al. 2008). In the INTERHEART study, there was no association between western dietary pattern intake and ApoB/ApoA1 ratio or HbA1c, while there was an inverse relationship between the western dietary pattern and waist-hip ratio and systolic blood pressure. The western diet was only weakly associated with increased MI risk, where the western diet only had an adverse role in the highest quartile of intake. On the other hand, there was a weak positive relationship between prudent dietary pattern and ApoB/ApoA1, HbA1c and systolic blood pressure. There was also a weak inverse association with waist-hip ratio and increasing quartiles of prudent dietary intake. Most importantly a prudent dietary pattern was associated with a lower risk of acute myocardial infarction. (Iqbal et al. 2008). Similarly a Mediterranean diet consisting of high intake of fruits, vegetables, legumes, wholegrain products, fish and unsaturated fatty acids (derived mainly from olive oil) showed a 10% reduction in CV incidence and mortality in a metanalysis of prospective cohort studies (Sofi et al. 2010). The PREDIMED study, the largest dietary intervention trial to assess the effects of the Mediterranean diet on CV prevention defined a Mediterranean diet as highlighted in Table 5-1 (Estruch et al. 2013).

Before the success of the PREDIMED study most studies concentrated on individual food types rather than food patterns. The negative relationship of saturated fatty acids to coronary heart disease has been largely exaggerated (Temple 2018) and that it is only processed red meats which are associated with an increased risk of MI. Consumption of red meat was not associated with CAD while each serving of processed meat was associated with a 42% increased risk of CAD in a metanalysis of nine prospective cohorts (Micha et al. 2010).

Table 5-1 The Mediterranean diet as defined by the PREDIMED study (Estruch et al. 2013)

Recommended/Discouraged	Food	Goal
Recommended	Olive Oil	≥ 4 tbsp / day
	Tree nuts and peanuts	≥ 3 servings / wk
	Fresh fruits	≥ 3 servings / day
	Vegetables	≥ 2 servings / day
	Fish (especially fatty fish)	≥ 3 servings / wk
	Legumes	≥ 3 servings / wk
	Sofrito	≥ 2 servings / wk
	White meat	Instead of red meat
	Wine with meals	≥ 7 glasses / wk
Discouraged		
	Soda drinks	< 1 drink/ day
	Commercial bakery goods, sweets and pastries	< 3 servings/wk
	Spread fats	< 1 serving / day
	Red and processed meats	<1 serving / day

Despite this, recommendations exist to avoid red meat consumption based on the expected effects of saturated fat in meat on plasma LDL and total cholesterol levels even though there is evidence that red meat does not affect TC, LDL, ApoA1 or ApoB, or blood pressure (Temple 2018). However, substituting red meat with high-quality

plant protein (legumes, soy, nuts) as opposed to low quality carbohydrates, leads to favourable changes in blood lipids and lipoproteins due to the fact that they contain higher proportions of polyunsaturated fatty acids and fibre (Guasch-Ferré et al. 2019). Unprocessed red meat has also been associated with higher intake of heme iron, which is positively associated with oxidative stress and inflammation (Azadbakht and Esmaillzadeh 2009). The higher risk with processed meats is thought to be due to higher sodium and nitrate preservative levels (Micha et al. 2010).

Another important protein source is poultry, which has been considered a healthy alternative for unprocessed red meat in terms of CAD risk. Moderate consumption of poultry was beneficial with regards to cardiovascular mortality compared to low consumption in a large prospective study (Sinha et al. 2009). In most studies an inverse association between poultry intake and total cardiovascular mortality is found, often in a dose dependent manner (Sauvaget et al. 2003; Takata et al. 2013; Park et al. 2017).

Fish are another important source of protein and omega-3-fatty acids. Prospective cohort studies have shown that eating fish at least once a week reduces risk of CAD by 16% (RR 0.84 (95% CI 0.75-0.95). More benefit was seen with moderate fish intake of 2-4 servings/week RR 0.79 (95% CI 0.67-0.92). However, there was no benefit beyond 4 servings/week (Zheng et al. 2012).

The relationship of egg consumption to cardiovascular disease has been particularly difficult to elucidate with a series of recent conflicting studies. The most recent of these studies pooled data from 6 prospective cohorts with a median follow up of 17.5 years and showed that each additional half an egg consumed per day was significantly associated with higher risk of incident CVD(adjusted HR, 1.06, adjusted ARD 1.11%) and all-cause mortality (adjusted HR, 1.08, adjusted Absolute risk difference, 1.93%) (Zhong et al. 2019). Prior to this, a Chinese study following up half a million

individuals prospectively, compared egg consumers with non-consumers. Daily egg consumption was associated with an 18% lower risk of CVD (Qin et al. 2018). Although the quality of prospective data with regards to egg consumption and CVD continues to improve, conflicting data indicates that further clarification is required.

Similarly, confusing are the risks and recommendations related to dairy product intake. Dietary guidelines recommend reducing whole fat dairy product intake as they are a source of saturated fats (Mach et al. 2019). However, evidence for this recommendation is limited. The PURE study prospectively looked at the impact of whole fat and low-fat dairy on cardiovascular disease. Surprisingly, higher intake of dairy products (> servings/day compared to no intake) showed no association with myocardial infarction and was associated with lower total mortality and non-cardiovascular mortality whereas no clinical outcome was reported for butter and cheese (Dehghan et al. 2018).

Fruits and vegetables were key components of the prudent dietary pattern in the INTERHEART study which showed a beneficial effect on risk of MI (Iqbal et al. 2008). Besides this case-control study other prospective cohort studies have shown a protective effect of fruit and vegetable consumption on CVD. A metanalysis of prospective studies reported a risk reduction with higher amounts of fruit and vegetable consumption (Gan et al. 2015). These findings clearly indicate that fruit and vegetable intake are significantly associated with lower risk of CHD. Similarly, a meta-analysis of prospective studies of consumption of 30 grams of nuts per day was inversely associated with coronary artery disease and overall cardiovascular disease and all-cause mortality (Luo et al. 2014).

The impact of carbohydrates on the risk of CHD has been controversial. Replacing dietary saturated fatty acids with carbohydrates lowers the TC, the LDL-C and the

HDL-C resulting in a negligible change in the ratio of TC to HDLC (Mensink et al. 2003) with little impact on the risk of CHD (Mozaffarian et al. 2010). However replacing SFA with carbohydrates increases plasma triglycerides which may increase cardiovascular risk (Mensink et al. 2003). One must distinguish between refined starches which are positively associated with the risk of CHD (HR 1.10, CI 1.00-1.21) and carbohydrates from whole grains which was associated with a 9% lower risk of CHD (HR: 0.91, CI 0.85-0.98) (Li et al. 2015).

Soft drinks are rich in unrefined carbohydrates and can contribute to a significant amount of the daily caloric intake (Agewall et al. 2016a). A metanalysis of prospective cohort studies showed a higher risk of stroke and MI, RR 1.22 (95% CI 1.14-1.30) with incremental sugar sweetened beverage consumption (Narain et al. 2016).

A controversial source of carbohydrate is chocolate. A recent metanalysis of prospective data showed that chocolate intake is associated with a decreased risk of CHD, stroke and even diabetes and that eating < 6 servings/ week is optimal (Gao et al. 2014).

The impact of diet is population specific and there is limited published data about the diet and MI in the Maltese population. In order to investigate the role of diet on the risk of MI in the Maltese population the MAMI study included an investigator-led dietary food frequency questionnaire.

Section 5.2. Results

Section 5.2.1. Meat, Poultry and Fish Consumption

Red meat consumption greater than once weekly was associated with an increased risk of myocardial infarction after multivariate adjustment in men. This risk was not

observed for lower frequency of red-meat consumption. Pork consumption greater than once weekly showed no increased risk after multivariate adjustment (Figure 5-1). Chicken consumption in men did not increase risk of MI even when consumed greater than 3 times weekly. Fish consumption greater than twice weekly also did not show any effect on risk of MI after multivariate adjustment. Similar patterns were observed in women.

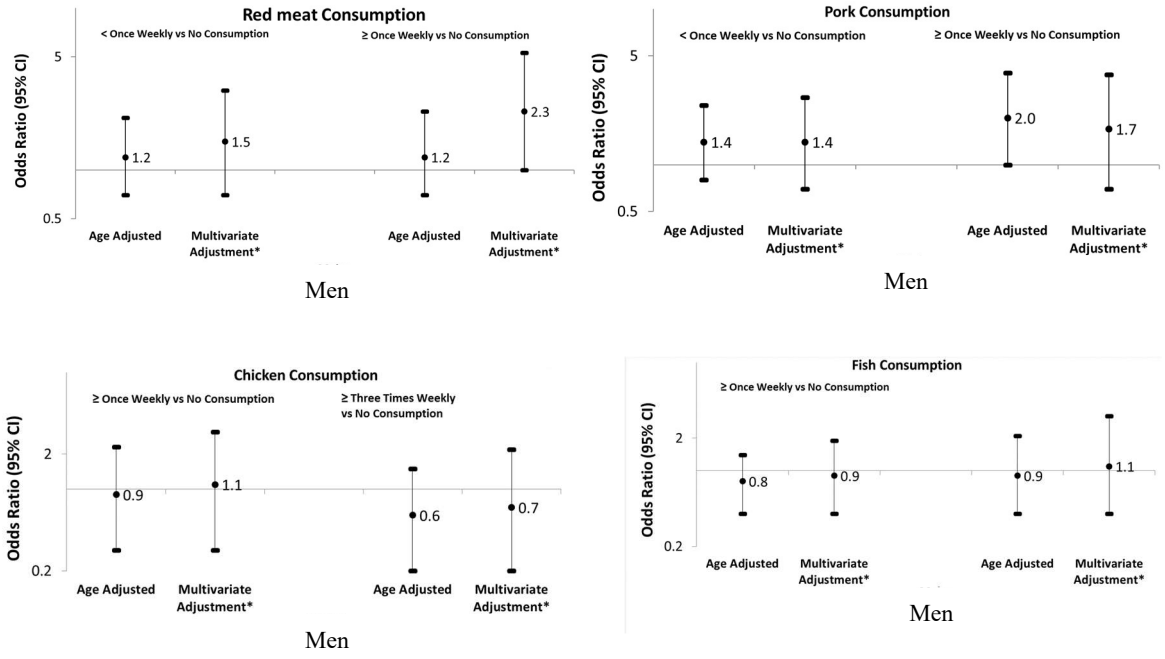

Figure 5-1 Risk for MI associated with consumption of red meat, pork, chicken and fish in men from the MAMI Study. The dot shows the Odds Ratio whilst the whiskers represent the upper and lower 95% Confidence Interval. *Multivariate adjustment for Age, DM, BMI, Smoking, Hypertension, Physical activity, TC and Alcohol.

Section 5.2.2. Soft Drink Consumption

Soft drink consumption was associated with an increased risk of myocardial infarction if more than 14 glasses per week were consumed AdjOR 5.0 (95%CI 1.0-11.0) (Figure 5-2). Diet soft drink consumption was also associated with an increased risk of MI when more than 14 glasses were consumed, AdjOR 1.6 (95%CI, 1.2-10) (Figure 5-2).

Fruit juice consumption also showed a pattern toward increased risk when one glass or more per day was consumed (Figure 5-2).

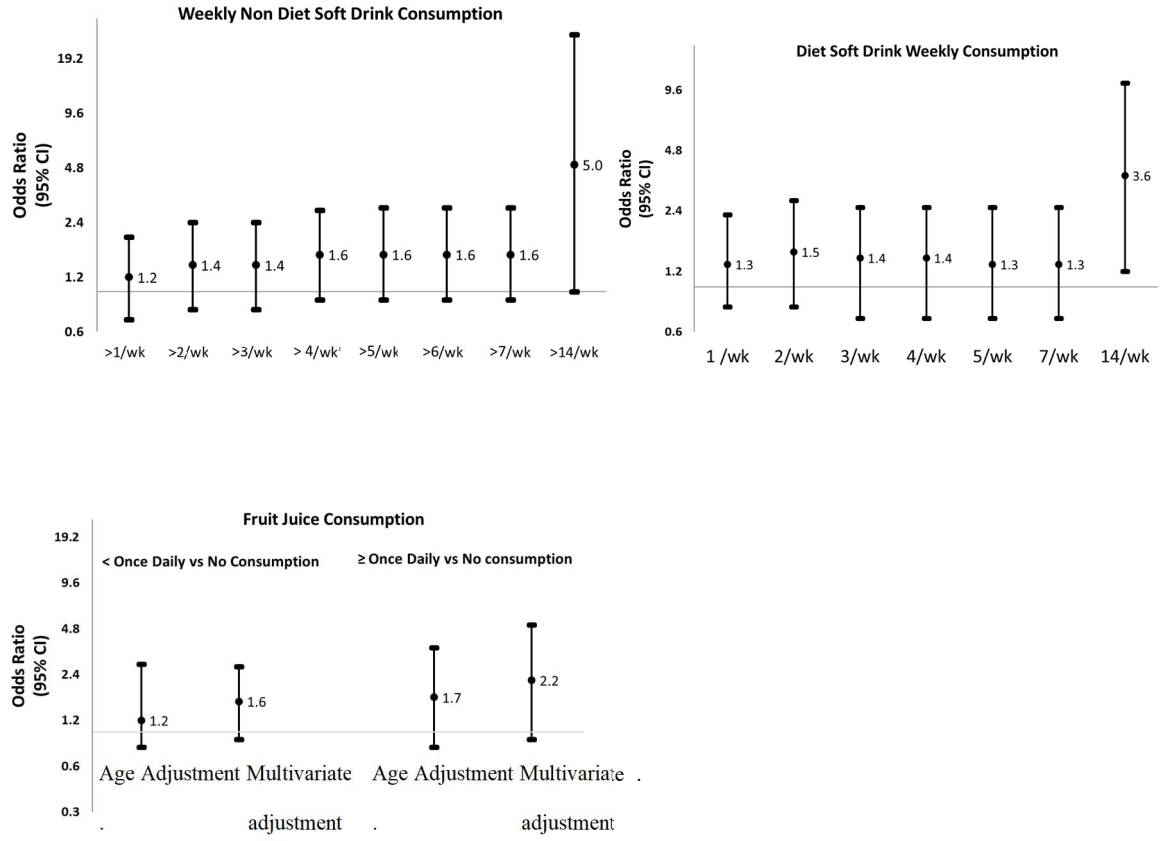

Figure 5-2 Odds ratios and 95% CI for myocardial infarction due to consumption of non-diet soft drink, diet soft drink and fruit juice in men. *Multivariate adjustment for DM, BMI, Smoking, HT, physical activity, total cholesterol, alcohol.

Section 5.2.3. Nut and Vegetable Consumption

Nut consumption greater than twice weekly had beneficial effects when compared to subjects who did not consume nuts and decreased risk of myocardial infarction by half (Figure 5-3). Fruit consumption higher than 3 portions/day was also associated with a decrease in the risk of myocardial infarction (Figure 5-3). Consumption of red

vegetables and legumes was also associated with a decreased risk of myocardial infarction (Figure 5-3).

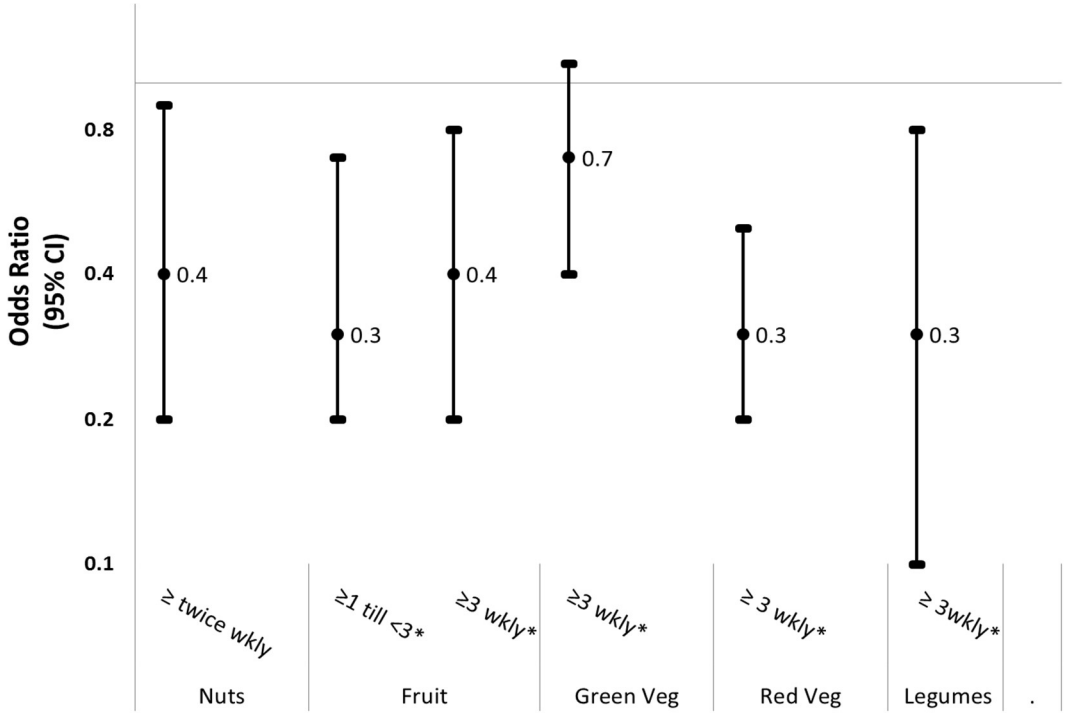

Figure 5-3 Odds ratios and 95% CI for MI for consumption of Nuts (including peanuts), fruits, and vegetables legumes in men. Nuts (including peanuts), fruit, vegetables and legumes are protective for MI in men. Data relative to no consumption of the respective food. *Odds ratios adjusted for age, TC, BMI, DM, smoking, hypertension, physical activity and alcohol.

Section 5.2.4. Dairy Product Consumption

Dairy products consumed more than once daily tended to be associated with an increased risk of MI. Frequent egg consumption, even 4 or more a day was not associated with an increased risk compared to no egg consumption. Butter was associated with an increased risk of MI while margarine was not associated with an increased risk relative to no consumption of the respective food (Figure 5-4).

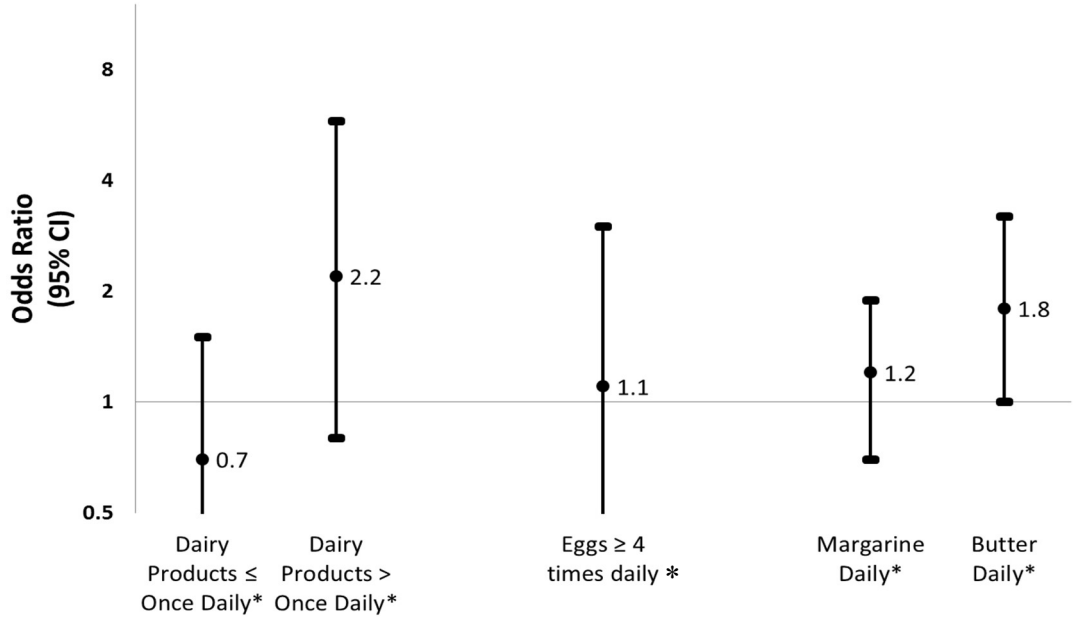

Figure 5-4 Consumption of milk, eggs, margarine and butter and risk of MI. *Odds ratios adjusted for age, TC, BMI, DM, smoking, hypertension, physical activity and alcohol

Section 5.2.5. Bread Consumption

Refined bread consumption more than twice daily was associated with an increased risk for myocardial infarction in men. Even with twice daily consumption of wholemeal bread there was a pattern towards increased risk relative to no consumption of the respective food (Figure 5-5).

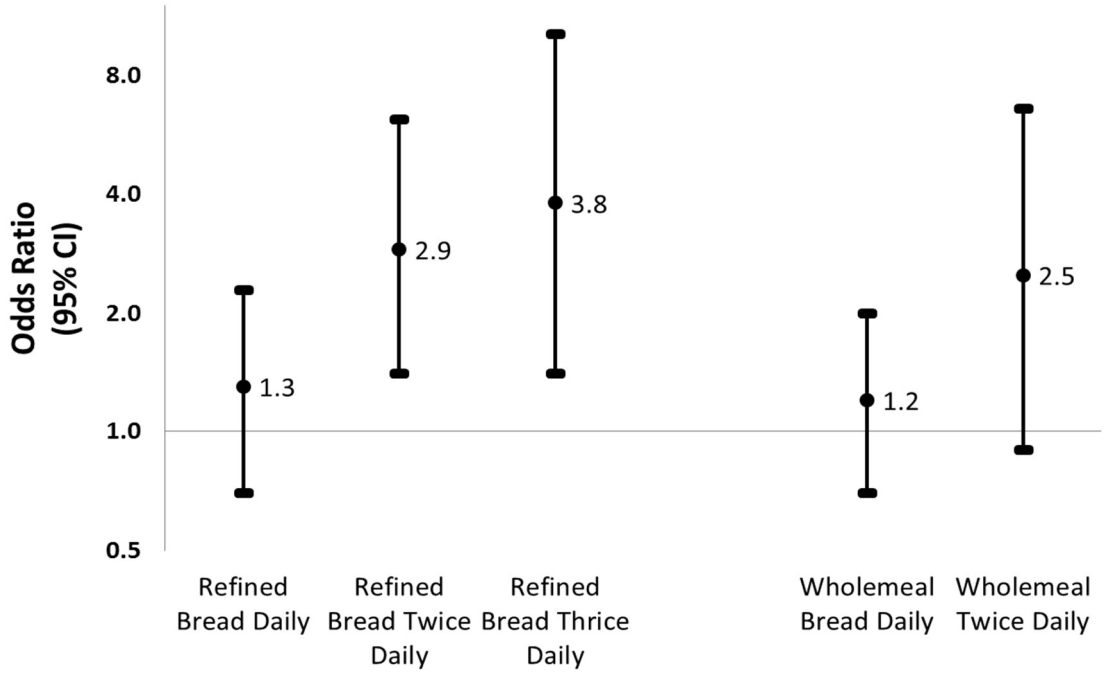

Figure 5-5 Bread consumption and risk of MI. Refined bread consumption more than twice daily was associated with an increased risk for myocardial infarction in men. Even with twice daily consumption of wholemeal bread there was a pattern towards increased risk. Data relative to no consumption of the respective food. *Odds ratios adjusted for age, TC, BMI, DM, smoking, hypertension, physical activity and alcohol.

Section 5.2.6. Fast Food Consumption

There was a pattern towards increasing risk with once weekly consumption of fast food which increased with higher frequencies of consumption relative to no consumption (Figure 5-6).

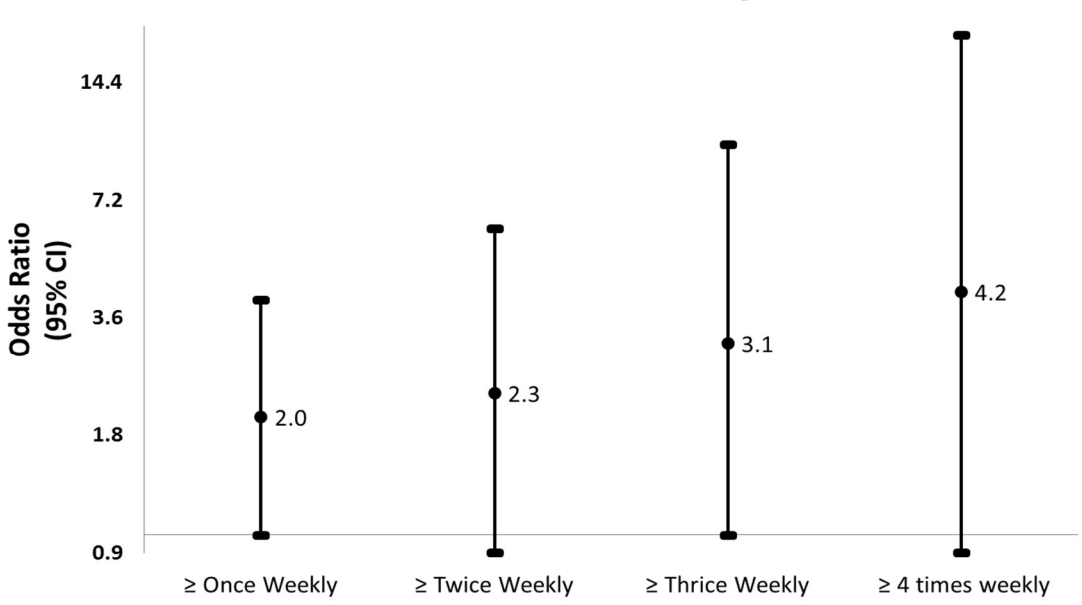

Figure 5-6 Fast food consumption and risk of MI. There was a pattern towards increasing risk with once weekly consumption of fast food which increased with higher frequencies of consumption. Data relative to no consumption. *Odds ratios adjusted for age, TC, BMI, DM, smoking, hypertension, physical activity and alcohol.

Section 5.2.7. Pasta Consumption

Not eating pasta was associated with an increased risk of MI compared to twice weekly consumption (Figure 5-7).

Section 5.2.8. Chocolate Consumption

Dark chocolate consumption greater than once daily showed a pattern toward decreased risk while milk chocolate consumption greater than once daily showed a pattern towards a slight increase in risk (Figure 5-8).

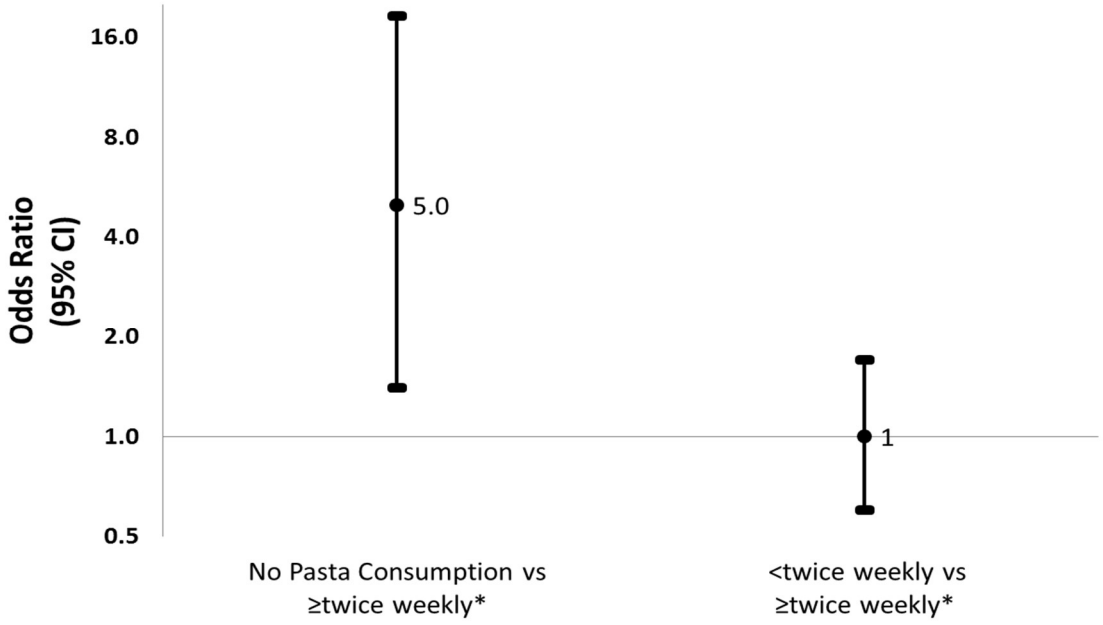

Figure 5-7 Pasta consumption and risk of MI. Not eating pasta was associated with an increased risk of MI compared to twice weekly consumption in men. *Odds ratios adjusted for age, TC, BMI, DM, smoking, hypertension, physical activity and alcohol.

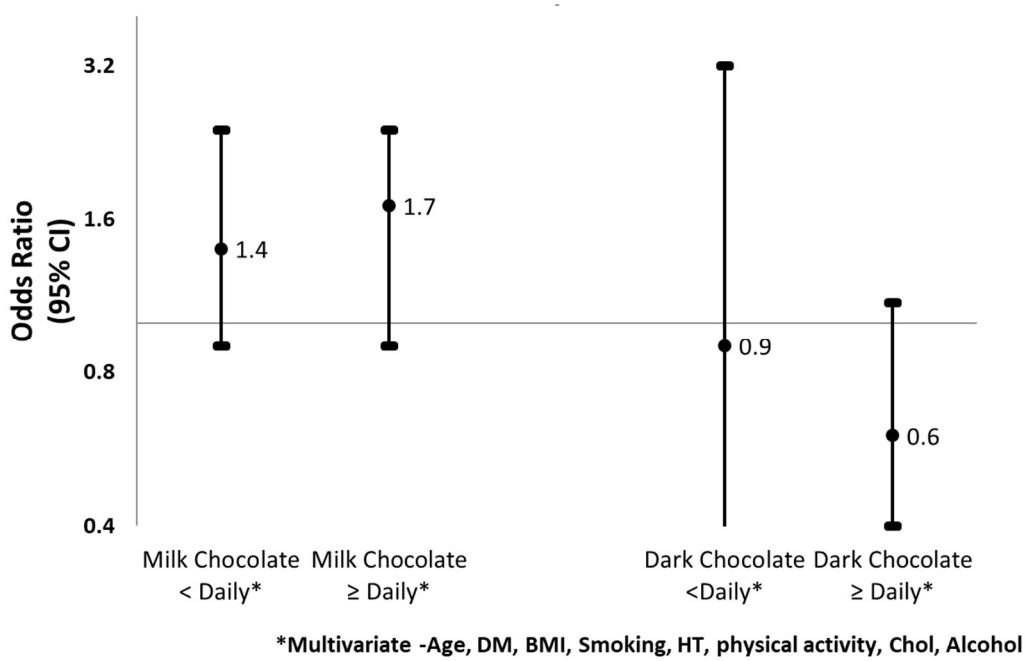

Figure 5-8 Chocolate consumption and risk of MI. Not eating pasta was associated with an increased risk of MI compared to twice weekly consumption in men. *Odds ratios adjusted for age, TC, BMI, DM, smoking, hypertension, physical activity and alcohol.

Section 5.3. Discussion

The MAMI study diet analysis results provide new important insights related to diet in the Maltese population.

The risk seen in the MAMI study with red meat consumption more than once weekly does not reflect the latest prospective cohort data. The risk could be related to differences in preparing the meat between different populations or to the quality of meat supply. Furthermore, it could be related to patterns of consumption in the Maltese population where in general there is consumption of bread or the preparation of rich salty sauces to accompany the meat, besides Maltese tending to have large portion sizes compared to other European populations. There was a pattern towards chicken being protective which is in keeping with studies that showed benefit of regular poultry ingestion. The positive effect associated with poultry consumption can be explained by its high content of carnosine which acts as a free radical scavenger which has antioxidative activity (Matsumura et al. 2002) and also by the fact that carnosine leads to accelerated metabolism of stress hormones including cortisol and noradrenaline (Prokopieva et al. 2016).

Fish consumption did not show any benefit in the MAMI study when consumed more than once weekly. However, there was limited number of subjects that consumed fish greater than once weekly. High fish consumption in the order of greater than 3 servings/ week has been associated with lower risk of myocardial infarction (Wallin et al. 2018) while other studies, including German cohorts, where fish consumption was lower (16.4g/day) did not show any decreased risk (Kühn et al. 2013). Furthermore, any benefits of fish consumption may have been offset by the concomitant ingestion of bread and rich sauces.

Soft drink or diet soft drink consumption more than twice daily showed an association with MI. Fruit juice consumption was also associated with an increased risk of MI . Soft drinks have been associated with an increased risk for myocardial infarction (RR 1.13, 95% CI 1.02-1.24) in a metanalysis (Narain et al. 2016). Diet soft drinks have also been associated with a higher risk of vascular events (HR = 1.43, 95% CI = 1.06-1.94) (Gardener et al. 2012). The risk associated with these kinds of sugar sweetened beverages is thought to be due to their high caloric load fuelling the metabolic syndrome and its components and consequent inflammatory responses. The mechanisms behind diet soft drinks are less clear but are thought to be mediated by effects on adiposity and elevations in fasting glucose (Gardener et al. 2012). The MAMI study results reflect a southern Mediterranean cohort with hot humid environment persisting for a large portion of the year which could lead to drinking of higher volumes of fluids when compared to other populations.

The effect of nut, fruit and vegetable consumption on risk of myocardial infarction was comparable to that found in international studies. Even relatively low rates of consumption of nuts greater than twice weekly and fruits greater than once weekly showed a beneficial effect. An important difference was observed between green vegetables and red vegetables. While there was only a pattern towards green vegetables being protective, red vegetables showed a significant decrease in risk 0.3 (95% CI, 0.2-0.4). The difference in benefit between green and red vegetables could be due to the fact that yellow-orange and red vegetables contain carotenoids and anthocyanidin which are postulated to decrease oxidative stress and inflammation by downregulating IL-8, prostaglandin E2 or heme oxygenase and superoxide dismutase. There have been conflicting reports with regards to yellow-orange and red vegetables with some reports showing an inverse association between intake of yellow-orange-red vegetables and

CHD while other studies have reported no effect (Blekkenhorst et al. 2018). The Framingham Offspring study showed an inverse relationship between the consumption of tomato products and CVD, HR 0.90 (95% CI , 0.83-0.99) for every one serve increase per day (Jacques et al. 2013). Tomatoes are rich in lycopene, a dietary carotenoid. The bioavailability and absorption of lycopene are relatively low and vary according to the preparation of the lycopene containing food and on the fat content of the meal (Lenore Arab and Susan Steck 2000). Legumes were also beneficial in the MAMI study when consumed 3 times weekly or more, OR 0.3 (95% CI 0.1-0.8). Dietary proteins such as legumes are thought to be beneficial when substituting high saturated fatty acid containing meats however in a metanalysis of legume consumption there was only a pattern towards a decrease in cardiovascular disease mortality, RR 0.96 (0.86-1.06) (Li et al. 2017).

The data with regards to milk, butter and egg consumption was not in keeping with the latest metanalyses. Egg consumption as a source of protein on its own or as part of more complex dishes is very common in the Maltese diet. Egg consumptions of 4 per day or greater showed no increased risk of myocardial infarction. The assessment of egg consumption is hampered by the multiple ways that eggs can be prepared and consumed. Dairy products showed a pattern towards increased risk if consumed more than once daily. Margarine was associated with a lower risk when compared to butter.

Bread consumption is an important part of Maltese cuisine and the Maltese diet, with the traditional Maltese loaf being a staple part of the diet accompanying most meals (Sammut 2006). A clear dose-dependent increase in risk was observed with both refined and wholemeal bread consumption. Three servings of refined bread per day was associated with MI, adjusted OR 3.8 (95%CI 1.2-8.4), while there was only a pattern toward increased risk with wholemeal preparations. No studies have looked

specifically at bread consumption and risk of myocardial infarction. Due to its importance in Maltese diet high bread consumption could be a key driver of increased risk. In recent years a number of Maltese bread suppliers have added partially hydrogenated vegetable oils to local bread (Bezzina Wettinger, personal observation). These are well known to increase risk for MI (Ascherio et al. 1994) Partially hydrogenated vegetable oils have been banned in some European countries (WHO 2018b). The risk in bread could very well be due to this factor.

Fast food in the MAMI study was defined as 'fried food and fast food'. This could include a wide variety of foods including pizza, pastizzi, burgers and chips. There was a clear dose-dependent pattern towards increased risk of MI with even once weekly consumption showing a pattern towards higher risk compared to no fast food consumption.

The absence of pasta from the diet was associated with increased risk in the MAMI study. This could possibly be due to lycopene rich sauces which often accompany local pasta dishes. It contradicts the observations of increased risk seen with other carbohydrate food products including soft drinks and refined bread, though it could very well be that the very high sugar content of the former, and the partially hydrogenated oils in the latter are responsible for the high risks observed with these products.

Dairy product consumption showed a pattern towards increased risk while butter showed an increased risk of myocardial infarction. Dark chocolate showed a pattern towards decreased risk. This is largely in keeping with metanalysis on chocolate consumption (Yuan et al. 2017).

Section 5.4. Limitations

Limitations of this section of the study include a strong potential for recall bias. As dietary habits were recorded at only one point in time, they may not reflect that subjects' habits in their entirety especially due to differences in diet related to seasons. There was also little information regarding the mode of cooking which can have a large impact on the nutrient profile of the food. Another limitation is that portion sizes were not recorded, and the subject was only asked regarding number of times/day that they ate a particular food product. Furthermore, it is often difficult to disentangle complex dishes into the categories chosen in our interview.

Section 5.5. Conclusion

The study of diet in the Maltese population in relation to myocardial infarction although fraught with limitations still provides important insights. From our findings it appears that health promotion initiatives should focus on decreasing soft drink and diet soft drink consumption, bread consumption and fast foods. The current focus of health promotion agencies on decreasing red-meat, dairy products, egg consumption and chocolate consumption is not supported by our findings. The focus from our findings should be on increasing nut, fruits, red vegetable and legume consumption and to remove trans-fatty acids from food products.

Chapter 6. Smoking, Alcohol, Lipids and Risk of Myocardial Infarction

Section 6.1. Smoking, Alcohol, Lipids and Myocardial Infarction

Smoking is one of the most important preventable risk factors for multiple diseases and for premature death (Burns 2003). The INTERHEART study showed that current smoking had a three-fold increased risk of MI compared with never smokers, with the risk being larger in younger than in older individuals and with the risk increasing by 5-6% for every cigarette smoked (Teo et al. 2006).

Passive smoking has also been shown to increase the risk of MI with a graded increase in risk associated with the extent of exposure to second hand smoke (Teo et al. 2006). The risk associated with passive cigarette smoking appears to be less than that for active smokers. A metanalysis on passive exposure to smoking found a relative risk (RR) of 1.16 (95% CI, 1.11-1.24) with exposure in the home and RR of 1.11 (95% CI , 1.00-1.23) for exposure in the workplace (He et al. 1999). On the other hand, living with a smoker was shown to increase the risk of myocardial infarction by 23% (Rossi et al. 2011). However, conflicting evidence exists on the dose-dependent relationship between passive smoking exposure and risk of MI (He et al. 1999).

Cigarette smoking is linked to both stable coronary disease, acute coronary syndromes and sudden death. Cigarette smoking has been linked to progression of atherosclerotic plaques and to the formation of new lesions. Endothelial dysfunction, inflammation, modification of lipids and thrombosis are all affected by smoking and play a role in atherosclerosis (Ambrose and Barua 2004).

Smokers have been shown to have higher TC, TG and LDL-C levels and lower HDL-C levels (Craig et al. 1989; Jain and Ducatman 2018). Smokers have higher concentrations of highly atherogenic small-dense LDL particles and oxidative

modification of LDL is increased in smokers (Gepner et al. 2011). Oxidized LDL are taken up by macrophages to form foam-cells which develop into atherosclerotic plaques (Heitzer et al. 1996). Nicotine may lead to the secretion of catecholamines, cortisol and growth hormones resulting in the activation of adenyl cyclase in adipose tissue. This results in the lipolysis of stored triglycerides and release of free fatty acids with increased hepatic synthesis of triglycerides and VLDL (Jain and Ducatman 2018).

Despite the risk of MI decreasing after stopping smoking, ex-smokers are known to have an increased risk of MI when compared to never smokers. In the INTERHEART study the residual risk of smoking was still present after 20 years (Teo et al. 2006). Despite the weight gain that is often associated with smoking cessation, HDL-C levels and quality are known to improve and LDL-C levels or size do not appear to be affected (Gepner et al. 2011). However, conflicting literature exists about the impact of smoking cessation on the lipid profile (Maeda et al. 2003).

The effect of smoking in the Maltese population has been previously studied (Attard 2015; Attard et al. 2017). Current and ex-smoking both showing an association with increased risk of MI with current smokers having an AdjOR of 2.7 (95% CI, 1.7-4.2) and ex-smokers having an AdjOR of 1.6 (95% CI, 1.0-2.4) compared to non-smokers (Table 6-1). These findings are consistent with findings of large international studies on the risks associated with myocardial infarction (Teo et al. 2006).

A smoking frequency of less than 10 pack-years was not associated with an increased risk of MI in men AdjOR 0.5 (95% CI, 0.2-1.6). There was a pattern towards increased risk when smoking between 10-30 pack-years AdjOR 1.9 (95% CI, 0.8-4.7) while a smoking frequency greater than 30 pack-years was associated with an AdjOR of 6.9 (95%, CI 3.7-12.8) compared to non-smokers (Table 6-2) (Attard 2015; Attard et al. 2017).

Table 6-1 Risk of MI in relation to smoking status in men. ORs were adjusted for age (Age OR) and for age, sex, regular alcohol consumption, reported hypertension, hypercholesterolaemia, diabetes and BMI (AdjOR) (Attard 2015; Attard et al. 2017).

	Cases (n=394)	Controls (n=465)	Age OR (95% CI)	AdjOR (95% CI)
Current smokers	146 (37.1)	105 (22.6)	3.1 (2.2-4.5)	2.7 (1.7-4.2)
Ex-smokers	145 (36.8)	161 (34.6)	1.7 (1.2-2.4)	1.6 (1.0-2.4)
Non-smokers	103 (26.1)	199 (42.8)	1.0	1.0

Table 6-2 Risk of MI in men according to smoking frequency. ORs were adjusted for age (AgeAdjOR) and for age, sex, regular alcohol consumption, reported hypertension, hypercholesterolaemia, diabetes and BMI (AdjOR) (Attard 2015; Attard et al. 2017).

	Cases (n-231)	Controls (n=284)	AgeAdjOR (95% CI)	AdjOR (95% CI)
> 30 pack-years	103 (44.6)	29 (10.2)	7.6 (4.7-12.4)	6.9 (3.7-12.8)
10-30 pack years	17 (7.4)	19 (6.7)	2.3 (1.1-4.8)	1.9 (0.8-4.7)
< 10 pack-years	8 (3.5)	37 (13.0)	0.6 (0.3-1.5)	0.5 (0.2-1.6)
Non-smokers	103 (44.6)	199 (70.1)	1.0	1.0

Exposure to passive smoking had an Adjusted OR of 3.2 (95% CI, 1.7-6.3) when exposure occurred both at home and in public when compared to those not exposed to passive smoking. There was only a pattern towards risk in those exposed only at home or only in the public setting (Table 6-3) (Attard 2015; Attard et al. 2017).

Table 6-3 Risk of MI in men according to passive smoking exposure in different settings. ORs were adjusted for age (AgeAdjOR) and for age, sex, regular alcohol consumption, reported hypertension, hypercholesterolaemia, diabetes and BMI (AdjOR) (Attard 2015; Attard et al. 2017).

	Cases (n=248)	Controls (n=360)	Age OR (95% CI)	AdjOR (95% CI)
Exposure to passive smoking at home and in public	43 (17.3	37 (10.3)	1.9 (1.1-3.3)	3.2 (1.7-6.3)
Exposure to passive smoking only at home	12 (4.8)	10 (2.8)	1.7 (0.7-4.3)	2.0 (0.7-5.6)
Exposure to passive smoking only in public setting	122 (49.2)	202 (56.1)	1.0 (0.7-1.4)	1.2 (0.7-2.0)
Not exposed to passive smoking	71 (28.6)	111 (30.8)	1.0	1.0

Low to moderate alcohol consumption has been associated with a decreased risk of CAD and MI (Ronksley et al. 2011; Romelsjö et al. 2012). On the other hand, heavy alcohol intake and binge drinking have been associated with CAD and MI (Keichl et al. 1998; Leong et al. 2014). Alcohol consumption increases HDL-C and changes the concentration and composition of lipoproteins, plasma proteins and enzymes involved in lipoprotein metabolism including cholesteryl ester transfer protein, phospholipid transfer protein, lecithin, cholesterol acyltransferase, lipoprotein lipase, hepatic lipase, paraoxonase-1 and phospholipases. Alcohol intake also results in post translational modifications of lipoprotein particles: low sialic acid content in apolipoprotein components of lipoprotein particles (e.g. HDL apo E and apo J) and acetaldehyde modification of apolipoproteins (Hannuksela et al. 2002).

Results from the MAMI study have shown that regular alcohol drinkers were protected against MI with an adjusted OR of 0.4 (95% CI, 0.3-0.6). (Attard 2015; Attard et al. 2017) (Table 6-4a). This protective effect was seen in daily alcohol consumers and also those that consumed alcohol less regularly up till once a month or less (Table 6-4b) regardless of the drinking intensity (Table 6-4c) (Attard 2015; Attard et al. 2017).

On the other hand, a strong risk of MI was present in daily binge drinkers (defined as ≥ 6 alcoholic beverages on one occasion) (OR 5.0, 95% CI 1.7-14.8), compared with regular drinkers who do not binge drink. Binge drinking did not increase risk if it occurred weekly or less frequently (Table 6-5).

Table 6-4 Risk of MI due to (a) current drinking and (b) different frequency of alcohol consumption, during the last year before interview, (c) intensity of weekly consumption. ORs were adjusted for age (Age OR (95%CI)) and for age, sex, diabetes, hypercholesterolemia, reported hypertension, smoking and BMI [AdjOR (95%CI)] (Attard 2015; Attard et al. 2017).

(a) Risk of MI due to current drinking				
	Cases (n=394)	Controls (n=465)	Age OR (95%CI)	AdjOR (95%CI)
Current drinkers	287 (72.8)	402 (86.5)	0.5 (0.3-0.7)	0.4 (0.3-0.6)
Non-drinkers	107 (27.2)	63 (13.5)	1.0	1.0
(b) Frequency of alcohol consumption				
	Cases (N=394)	Controls (N=465)	Age OR (95%CI)	AdjOR (95%CI)
Daily	64 (16.2)	81 (17.4)	0.5 (0.3-0.7)	0.5 (0.3-0.8)
4-6/week	18 (4.6)	21 (4.5)	0.6 (0.3-1.1)	0.3 (0.1-0.8)
2-3/week	54 (13.7)	66 (14.2)	0.6 (0.3-0.9)	0.3 (0.2-0.6)
2-4/month	67 (17.0)	129 (27.7)	0.3 (0.2-0.5)	0.3 (0.2-0.5)
Once a month or less often	84 (21.3)	105 (22.6)	0.5 (0.3-0.8)	0.5 (0.3-0.8)
Non-drinkers	107 (27.2)	63 (13.5)	1.0	1.0

(c) Intensity of alcohol consumption				
Number of weekly alcoholic drinking units	Cases* (N=390)	Controls (N=465)	Age OR (95%CI)	AdjOR (95%CI)
>10 drinking units per week	35 (9.0)	67 (14.4)	0.6 (0.4-1.0)	0.5 (0.3-0.8)
1-10 drinking units per week	67 (17.2)	59 (12.7)	1.7 (1.1-2.5)	1.5 (0.9-2.5)
Non-regular drinkers	288 (73.8)	348 (74.8)	1.0	1.0

Table 6-5. Risk of MI due to binge drinking. Analysis was done only amongst individuals (men and women) who reported to consume alcoholic beverages during the last year before the interview (non-drinkers - 107 cases and 63 controls, were excluded from this analysis). *1 case did not specify the frequency of binge drinking. **3 controls did not specify the frequency of binge drinking. ORs were adjusted for age [Age OR (95%CI)] and for age, gender, diabetes, hypercholesterolemia, reported hypertension, smoking and BMI [AdjOR (95%CI)] and further for type of alcoholic beverage consumed [AdjOR' (95%CI)].

Frequency of Binge drinking					
Frequency of binge drinking:	Cases* (n=286)	Controls** (n=399)	Age OR (95%CI)	AdjOR (95%CI)	AdjOR' (95%CI)
Daily	19 (6.6)	5 (1.3)	6.5 (2.3-17.8)	5.0 (1.6-15.0)	3.3 (1.0-11.1)
Weekly	39 (13.6)	34 (8.5)	2.0 (1.2-3.3)	1.1 (0.6-2.1)	1.0 (0.6-1.9)
Monthly	21 (7.3)	24 (6.0)	1.7 (0.9-3.3)	1.0 (0.4-2.2)	1.0 (0.5-2.2)
Less often than once a month	61 (21.2)	102 (25.6)	1.1 (0.7-1.6)	0.8 (0.5-1.3)	0.8 (0.5-1.2)
Regular drinkers who do not binge	146 (51.0)	234 (58.6)	1.0	1.0	1.0

Without taking into consideration the quantities, weekly wine consumption protected against MI (AdjOR 0.5, 95%CI 0.3-0.9), spirits gave an AdjOR of 0.6 (95%CI 0.1-3.1) and beer increased the risk of MI by 3.5-fold (95%CI 1.4-8.8) (Table 6-6a). These risks were modified by the drinking intensity (Table 6-6b). Individuals who consumed 1-9 units of wine per week were protected against MI (AdjOR 0.5, 95% CI 0.3-0.8) while the AdjOR for drinking ≥ 20 units of wine weekly was 1.4 (95% CI 0.5-3.7), compared with non-regular drinkers (Table 6-6b). High weekly consumption of beer (≥ 20 weekly units of beer) was associated with a 4.3-fold (95%CI, 1.6-11.4) increased risk of MI, while 1-9 units of beer per week may convey a protective effect [AdjOR 0.5 (95%CI 0.2-1.3)], compared to those who do not drink regularly (Table 6-6b). The effect of increasing weekly consumption of spirits on the risk of MI could not be calculated due to the small number of individuals who reported weekly spirit consumption (Attard 2015; Attard et al. 2017).

Amongst binge drinkers, cases tended to binge on beer while controls tended to binge on wine. Amongst cases, 44.4% of binge drinkers binged on beer, 11.1% on beer and spirits, 11.1% on beer and wine, 27.8% on beer, wine and spirits and 5.6% binged on spirits only, whereas a total of 50.0% of binge drinking controls binged on wine while another 50.0% binged on beer and wine (Attard 2015; Attard et al. 2017).

Knowledge regarding the effects of alcohol on risk of MI in the presence of other conventional risk factors for MI such as smoking is limited (Tavani et al. 2001). Although it has been suggested that the association between MI and alcohol consumption may be a function of drinking patterns (Chikritzhs et al. 2009) very few studies have investigated this while taking into consideration the type of alcoholic beverage consumed.

The risk of active and passive smoking and the influence of different types of alcoholic beverages and frequency of alcohol consumption on the risk of MI was investigated in the Maltese population. The contribution of lipid variables was analysed especially with regards to the residual risk of MI after stopping smoking.

Table 6-6 Risk of MI in relation to (a) type and (b) number of weekly drinking units of specific types of alcoholic beverages compared with individuals who did not report to consume weekly alcoholic beverages. ORs adjusted for age [Age OR (95%CI)] and for age, sex, diabetes, hypercholesterolemia, reported hypertension, smoking and BMI [AdjOR (95%CI)], *4 cases who were regular alcohol drinkers did not specify the type and number of weekly alcohol consumption (Attard 2015; Attard et al. 2017).

(a) Type of alcoholic beverages consumed every week				
Type of alcoholic beverage	Cases* (n=390)	Controls (n=465)	Age OR (95%CI)	AdjOR (95%CI)
Wine only	28 (7.2)	65 (14.0)	0.5 (0.3-0.8)	0.5 (0.3-0.9)
Beer only	26 (6.7)	7 (1.5)	4.7 (2.0-11.2)	3.5 (1.4-8.8)
Spirits only	4 (1.0)	6 (1.3)	0.9 (0.2-3.1)	0.6 (0.1-3.1)
Beer + Wine	19 (4.9)	18 (3.9)	1.3 (0.7-2.6)	0.8 (0.3-1.8)
Spirits + Wine	7 (1.8)	5 (1.1)	1.5 (0.5-4.8)	1.3 (0.3-6.1)
Beer + Spirits	8 (2.1)	2 (0.4)	4.6 (1.0-22.0)	5.1 (0.6-46.1)
Beer + Wine + Spirits	10 (2.6)	14 (3.0)	0.8 (0.3-1.8)	0.6 (0.2-1.7)
Non-regular drinkers	288 (73.8)	348 (74.8)	1.0	1.0
(b) Weekly number of drinking units of different alcoholic beverages				
Amongst individuals who consume wine per week				
	Cases (n=352)	Controls (n=450)	Age OR (95%CI)	AdjOR (95%CI)
≥20 unit/week	12 (3.4)	11 (2.4)	1.1 (0.5-2.6)	1.4 (0.5-3.7)
10-19 units/week	14 (4.0)	20 (4.4)	0.8 (0.4-1.6)	0.6 (0.2-1.6)
1-9 units/week	38 (10.8)	71 (15.8)	0.6 (0.4-1.0)	0.5 (0.3-0.8)
Non-regular drinkers	288 (81.8)	348 (77.3)	1.0	1.0
Amongst individuals who consume beer per week				
	Cases (n=354)	Controls (n=389)	Age OR (95%CI)	AdjOR (95%CI)
≥20 units/week	25 (7.1)	6 (1.5)	5.2 (2.1-12.8)	4.3 (1.6-11.4)
10-19 units/week	20 (5.6)	10 (2.6)	2.3 (1.0-5.0)	1.9 (0.8-4.7)
1-9 units/week	21 (5.9)	25 (6.4)	1.1 (0.6-1.9)	0.5 (0.2-1.3)
Non-regular drinkers	288 (81.4)	348 (89.5)	1.0	1.0
Amongst individuals who consume spirits per week				
	Cases (n=318)	Controls (n=375)	Age OR (95%CI)	AdjOR (95%CI)
≥20 units/week	6 (1.9)	1 (0.3)	/	/
10-19 units/week	4 (1.3)	6 (1.6)	0.8 (0.2-2.8)	0.4 (0.1-1.8)
1-9 units/week	20 (6.3)	20 (5.3)	1.1 (0.6-2.1)	0.9 (0.4-2.2)
Non-regular drinkers	288 (90.6)	348 (92.8)	1.0	1.0

Section 6.2. Results

Male controls smokers, compared to non-smokers, had higher levels of triglycerides (median 1.46mmol/L vs 1.29mmol/L, p-value < 0.01), total cholesterol (median 4.44mmol/L vs 3.88 mmol/L, p-value < 0.05) and hs-CRP (median 2.05mg/L vs 1.28 mg/L respectively, p-value < 0.05). HDL was lower in smokers compared to non-smokers (median 1.20mmol/L vs 1.35mmol/L, p-value < 0.05). These differences were not present when comparing ex-smokers to non-smokers except for triglycerides (median 1.29mmol/L vs 1.07 mmol/L, p-value < 0.05) (Table 6-7).

Table 6-7 Mean age, median levels and interquartile ranges of biochemical parameters and BMI in male controls by smoking status (ex-smokers only include those that have abstained for > 15 years). *p < 0.05, ** p < 0.01 calculated using two-tailed Mann-Whitney test compared to non-smokers.

	Smokers (n=80)	Ex-smokers (n=131)	Non-smokers (n=116)
Age (years), mean (range)	53 (23-70)	59 (20-73)	53 (20-70)
hs-CRP (mg/L)	2.05 (4.80-6.05) *	1.69 (0.78-3.00)	1.28 (0.64-2.53)
Total cholesterol (mmol/L)	5.38 (4.80-6.05)	5.48 (4.71-6.13)	5.25 (4.69-5.83)
LDL cholesterol (mmol/L)	3.44 (2.85-4.04)	3.42 (2.68-4.11)	3.33 (2.81-3.86)
HDL cholesterol (mmol/L	1.20 (1.08-1.42) *	1.35 (1.12-1.61)	1.35 (1.16-1.54)
Triglycerides (mmol/L)	1.46 (1.06-1.88) **	1.29 (0.8-1.86) *	1.07 (0.75-1.55)
Total cholesterol/HDL	4.44 (3.49-5.22) *	3.96 (3.26-4.95	3.88 (3.20-4.62)
NHDL cholesterol (mmol/L)	4.19 (3.39-4.86)	4.09 (3.24-4.83)	3.91 (3.24-4.50)
BMI (kg/m^2)	28.4 (25.0-32.0)	29.1 (27.2-32.2)	28.1 (25.8-32.2)

These changes occurred in a step wise manner with increasing frequency of smoking but there was no difference in the < 10 pack-year individuals compared with non-smokers (Table 6-8).

Table 6-8 Mean age, median levels and interquartile ranges of hs-CRP, lipid variables and BMI in male controls by pack years compared to non-smokers. *p < 0.05, ** p < 0.01 calculated using two-tailed Mann-Whitney test compared to non-smokers.

	> 30 pack years (n=24)	10-30 pack-years (n=13)	< 10 pack years (n=27)	Non-smokers (n=116)
Age (years), mean (range)	59 (47-70) *	53 (34-69)	44 (23-68) *	53 (20-70)
Total cholesterol (mmol/L)	5.78 (5.06-6.01) *	5.02 (4.74-6.98)	5.14 (4.41-5.85)	5.25 (4.69-5.83)
LDL cholesterol (mmol/L)	3.79 (3.10-3.98) *	3.21 (3.03-4.59)	3.03 (2.51-3.75	3.33 (2.81-3.86)
HDL cholesterol (mmol/L	1.20 (0.96-1.44) *	1.19 (1.08-1.27) *	1.25 (1.16-1.56)	1.35 (1.16-1.54)
Triglycerides (mmol/L)	1.78 (1.26-2.27) **	1.63 (1.13-2.04) **	1.15 (0.79-1.65)	1.07 (0.75-1.55)
Total cholesterol/HDL	4.98 (3.76-6.05) **	4.34 (3.89-5.59) *	3.78 (3.24-4.77)	3.88 (3.20-4.62)
NHDL cholesterol (mmol/L)	4.50 (3.99-4.86) **	3.99 (3.47-5.73)	3.68 (3.13-4.54)	3.91 (3.24-4.50)
hs-CRP (mg/L)	3.14 (1.46-4.29) **	2.53 (1.89-4.03) **	0.82 (0.53-1.86)	1.28 (0.64-2.53)
BMI (kg/m^2)	27.7 (24.8-31.5)	30.0 (25.3-36.3)	28.2 (24.3-31.8)	28.1 (25.8-32.2)

Section 6.2.2. Association of Passive Smoking with Lipid Variables, hs-CRP and BMI

Exposure to passive smoking also had an effect on total cholesterol, triglycerides, Total cholesterol: HDL-C and NHDL-C while having no effect on LDL-C and HDL-C (Table 6-9).

Section 6.2.3. Association of Lipid variables, hs-CRP and BMI with Years from Smoking Cessation

Serum triglycerides remained elevated after stopping smoking for up to 15 years. After 15 years the serum triglyceride levels were comparable to non-smokers. The remaining lipid variables and hs-CRP did not show the same pattern (Table 6-10).

Table 6-9. Mean age, median levels and interquartile ranges of hs-CRP, lipid variables and BMI in male controls exposed to passive smoking compared to individuals not exposed to passive smoking. *$p < 0.05$, ** $p < 0.01$ calculated using two-tailed Mann-Whitney test compared to non-smokers.

	Exposed to passive smoking at home and in public (n=26)	Not exposed to passive smoking (n=65)
Age (years), mean (range)	59 (43-70)	54 (20-71)
Total cholesterol (mmol/L)	5.73 (4.91-6.57) *	5.23 (4.40-5.77)
LDL cholesterol (mmol/L)	3.37 (2.33-4.41)	3.17 (2.58-3.72)
HDL cholesterol (mmol/L	1.35 (1.18-1.58)	1.45 (1.22-1.69)
Triglycerides (mmol/L)	1.50 (0.99-2.40) *	1.03 (0.73-1.40)
Total cholesterol/HDL	4.23 (3.34-4.93) *	3.61 (2.93-4.03)
NHDL cholesterol (mmol/L)	4.36 (3.39-5.18) *	3.70 (2.98-4.33)
hs-CRP (mg/L)	1.20 (0.69-3.02)	1.33 (0.72-2.34)
BMI (kg/m^2)	29.8 (27.9-32.6)	28.7 (26.1-32.1)

Table 6-10. Change in Age, hs-CRP, Lipid variables and BMI with years from smoking cessation and in non-smoking controls *p < 0.05, **p < 0.01, p-values calculated according to the two-tailed Mann-Whitney tests, compared to non-smokers.

	1-4 years (n=14)	5-10 years (n=15)	10-15 years (n=21)	> 15 years (n=75)	Non-smokers (n=116)
Age (years), mean (range)	54 (26-68)	57 (28-70)	58 (32-70) *	62 (40-73) *	53 (20-70)
Total cholesterol (mmol/L)	5.37 (4.43-6.28)	5.78 5.07-6.37)	5.97 (5.26-6.32) *	5.41 (4.55-5.94)	5.25 (4.69-5.83)
LDL cholesterol (mmol/L)	3.41 (2.42-4.20)	3.79 (2.91-4.04)	3.75 (3.12-4.24)	3.22 (2.55-4.09)	3.33 (2.81-3.86)
HDL cholesterol (mmol/L	1.31 (1.11-1.47)	1.31 (1.06-1.58)	1.48 (1.11-1.65)	1.34 (1.12-1.63)	1.35 (1.16-1.54)
Triglycerides (mmol/L)	1.70 (1.04-2.33) *	1.74 (1.20-2.36) **	1.52 (1.41-2.09) **	1.07 (0.84-1.60)	1.07 (0.75-1.55)
Total cholesterol/HDL	4.16 (3.42-4.79)	3.96 (3.06-5.50)	3.75(3.12-4.24)	3.87 (3.11-4.70)	3.88 (3.20-4.62)
NHDL cholesterol (mmol/L)	3.88 (3.28-4.95)	4,40 (3.18-5.28)	4.43 (3.95-5.05) *	3.86 (3.07-4.81)	3.91 (3.24-4.50)
hs-CRP (mg/L)	1.77 (1.44-4.54)	1.56 (0.72-2.81)	2.19 (0.76-4.30)	1.68 (0.78-2.74)	1.28 (0.64-2.53)
BMI (kg/m^2)	30.1 (28.1-35.1) *	30.5 (27.8-37.2) **	28.7 (26.8-31.2)	28.7 (26.6-31.6)	28.1 (25.8-32.2)

Section 6.2.4. The Modifying Effect of Alcohol on the Risk of MI Associated with Smoking

Regular alcohol drinking reduced risk of MI equally across smokers, non-smokers and ex-smokers. Smokers who were non-regular alcohol drinkers had a 2.7-fold (95% CI 1.4-5.3) increased risk of MI. This risk decreased to 1.9-fold (95%CI 1.1-3.4) in smokers who consumed regular alcoholic beverages. This reduction in risk of MI was also evident for ex-smokers [AdjOR 1.8 (95% CI 0.9-3.4) who were non-regular drinkers vs. AdjOR 1.0 (95%CI 0.6-1.6), if they drank alcohol regularly], relative to non-smokers who were non-regular drinkers. An AdjOR of 0.7 (95% CI 0.4-1.2) was observed amongst non-smokers who consumed alcohol regularly (Table 6-11).

Table 6-11 The combined effect of smoking and alcohol consumption on the risk of MI. The AdjOR for MI associated with regular drinking for each smoking category is also shown. ORs were adjusted for age [Age OR (95%CI)] and for age, sex, diabetes, hypercholesterolemia, reported hypertension, and BMI [AdjOR (95%CI)].

Smoking status	Regular drinkers	Cases (n=394)	Controls (n=465)	Age OR (95%CI)	AdjOR (95%CI) Relative to non-smokers and non-regular drinkers	AdjOR (95%CI) Relative to non-regular drinkers of each smoking category
Smokers	Yes	89 (22.6)	75 (16.1)	2.2 (1.4-3.5)	1.9 (1.1-3.4)	0.6 (0.3-1.2)
	No	57 (14.5)	30 (6.5)	3.2 (1.8-5.6)	2.7 (1.4-5.3)	1.0
Ex-smokers	Yes	98 (24.9)	126 (27.1)	1.2 (0.8-1.8)	1.0 (0.6-1.6)	0.5 (0.3-0.9)
	No	47 (11.9)	35 (7.5)	2.0 (1.2-3.5)	1.8 (0.9-3.4)	1.0
Non-smokers	Yes	45 (11.40	110 (23.7)	0.6 (0.4-1.1)	0.7 (0.4-1.2)	0.7 (0.4-1.2)
	No	58 (14.7)	89 (19.1)	1.0	1.0	1.0

Section 6.2.5. The Impact of Alcohol Consumption on Lipid variables and Hs-CRP

Current alcohol drinkers had higher median TC, NHDL-C and triglycerideslevels compared to the non-drinkers (Table 6-12a). Taking the frequency of consumption into account, revealed that current drinkers had higher median levels of TC and NHDL-C compared with non-drinkers if consumption was more frequent than monthly, and higher HDL-cholesterol if consumption was more frequent than 2-3 times per week (Table 6-12b). Those who consumed alcoholic beverages 4-6 times per week or more had a lower BMI compared with non-drinkers. No significant difference in mean age was present between the different categories. No changes in lipid profile parameters were evident with binge drinking compared with regular drinkers who do not binge drink (Table 6-13). The number of daily binge drinkers was too small to analyse median levels. Median BMI decreased and median TC and HDL-cholesterol increased with increasing weekly alcohol drinking units reaching statistical significance for individuals drinking >10 drinking units per week compared with those who do not consume alcohol regularly (Table 6-12c). Current drinkers had lower median hs-CRP levels than non-drinkers (1.68 mg/L vs. 2.47 mg/L, p-value < 0.1) (Table 6-12a). The lowest median hs-CRP was found amongst those who consume alcoholic beverages 4-6 times per week (Table 6-12b).

Weekly binge drinking or less often had no effect on median hs-CRP levels compared to regular drinkers who did not binge drink (Table 6-13). The number of male controls who binged daily was too small to draw conclusions on patterns in hs-CRP. The intensity of alcohol consumption (weekly drinking units) had no effect on median hs-CRP levels (Table 6-12c).

Table 6-12 Median levels and interquartile range of biochemical parameters and BMI amongst male controls (a) current drinkers vs. non-drinkers and with (b) different frequency and (c) quantities of weekly alcohol consumption in this last year before the interview. Mean and range are reported for age. The p-values calculated using the two-tailed Mann-Whitney tests, compare each category with (a) non-drinkers, (b) non-drinkers in the last year prior to recruitment, (c) non-regular drinkers. *p-value < 0.1; **p-value < 0.05; ***p-value < 0.01. BMI, body mass index; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein, LDL, low-density lipoprotein, HDL ratio, TC/HDL-C.

(a) Effect of current alcohol consumption		
	Current drinkers (n=293)	Non-drinkers (n=34)
Age (years), mean (range)	55 (20-73)	56 (20-69)
Total cholesterol (mmol/L)	5.40 (4.75-6.06) **	4.91 (4.31-5.70)
HDL-cholesterol (mmol/L)	1.33 (1.12-1.56)	1.24 (1.12-1.45)
Non-HDL cholesterol (mmol/L)	4.06 (3.32-4.78) *	3.86 (3.04-4.33)
HDL ratio	3.99 (3.29-5.01)	3.86 (3.48-4.66)
Triglycerides (mmol/L)	1.26 (0.87-1.79) *	0.98 (0.78-1.51)
LDL-cholesterol (mmol/L)	3.41 (2.80-4.04)	3.20 (2.62-3.85)
hs-CRP (mg/L)	1.68 (0.77-3.00) *	2.47 (0.96-3.78)
BMI (kg/m^2)	28.44 (26.16-32.08)	29.85 (27.97-32.23)

Table 6-12 Continued (b) Effect of frequency of alcohol consumption

	Daily (N=67)	4-6 per week (N=16)	2-3 per week (N=53)	2-4 per month (N=89)	Monthly or less often (N=68)	Non-drinkers (N=34)
Age (years), mean (range)	61 (38-71)	54 (23-70)	54 (20-70)	53 (23-70)	55 (20-73)	56 (20-69)
BMI (kg/m^2)	27.92 (24.95-30.37) ***	28.75 (24.55-29.75) **	28.73 (25.72-31.98)	28.52 (26.54-32.38)	29.73 (26.49-33.78)	29.85 (27.97-32.23)
hs-CRP (mg/L)	1.63 (1.09-2.64)	1.17 (0.58-2.59) *	1.78 (0.75-3.30)	1.87 (0.73-2.90) *	1.71 (0.76-3.90)	2.47 (0.96-3.78)
Total cholesterol (mmol/L)	5.57 (4.81-6.16) **	5.67 (5.33-6.47) **	5.52 (4.83-6.15) **	5.39 (4.80-6.02) **	5.25 (4.53-5.80)	4.91 (4.31-5.70)
HDL-cholesterol (mmol/L)	1.39 (1.20-1.63) **	1.48 (1.20-1.81) **	1.43 (1.12-1.70) *	1.29 (1.11-1.45)	1.22 (1.07-1.50)	1.24 (1.12-1.45)
Non-HDL cholesterol (mmol/L)	4.04 (3.18-4.77)	4.32 (3.65-4.92) *	4.04 (3.44-4.84) *	4.10 (3.29-4.86) *	4.03 (3.18-4.73)	3.86 (3.04-4.33)
HDL ratio	3.83 (3.11-4.88)	3.88 (3.24-4.44)	3.91 (3.50-4.72)	4.16 (3.34-5.17)	4.18 (3.35-5.13)	3.86 (3.48-4.66)
Triglycerides (mmol/L)	1.23 (0.85-1.80)	1.40 (1.09-1.94) *	1.13 (0.78-1.87)	1.30 (0.87-1.76) *	1.27 (0.95-1.96) *	0.98 (0.78-1.51)
LDL-cholesterol (mmol/L)	3.48 (2.71-3.93)	3.43 (2.98-4.11)	3.48 (2.77-4.28)	3.44 (2.84-4.12)	3.32 (2.67-3.70)	3.20 (2.62-3.85)

(c) Effect of quantity of weekly alcohol consumption

	>10 drinks per week (N=45)	1-10 drinkers per week (N=46)	Non-regular drinkers (N=235)
Age (years), mean (range)	59 (27-70) **	56 (26-71)	55 (20-73)
BMI (kg/m^2)	27.90 (24.99-30.45) **	28.08 (25.91-29.84) **	29.17 (26.49-32.39)
hs-CRP (mg/L)	1.56 (0.74-2.47)	1.40 (0.82-2.69)	1.82 (0.80-3.44)
Total Cholesterol (mmol/L)	5.76 (5.09-6.29) ***	5.52 (4.83-6.46)	5.26 (4.67-5.95)
HDL-cholesterol (mmol/L)	1.47 (1.21-1.81) ***	1.40 (1.12-1.69)	1.28 (1.11-1.49)
Non-HDL cholesterol (mmol/L)	4.28 (3.61-4.81)	4.16 (3.21-4.98)	3.98 (3.25-4.69)
HDL ratio	3.93 (3.17-4.98)	3.92 (3.08-4.92)	3.99 (3.42-5.01)
Triglycerides (mmol/L)	1.41 (0.97-2.14)	1.14 (0.73-1.74)	1.20 (0.86-1.74)
LDL-cholesterol (mmol/L)	3.66 (3.08-3.93)	3.48 (2.79-4.29)	3.32 (2.74-3.90)

Table 6-13 Median levels and interquartile range of biochemical parameters and BMI amongst male controls with different frequencies of binge drinking in the last year before the interview. Mean and range are reported for age. The p-values, calculated according to the two-tailed Mann-Whitney tests, compare smokers who are regular drinkers vs. smokers who are non-regular drinkers. *p-value < 0.1; **p-value < 0.05; ***p-value < 0.01. BMI, body mass index; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein.

Binge drinking					
	Daily (n=4)	Weekly (n=34)	Monthly (n=20)	Less often than once a month (n=83)	Regular drinkers that do not binge drink (n=150)
Age (years), mean (range)	49 (38-55) **	55 (20-70)	46 (23-70) ***	54 (20-70) **	58 (26-73)
BMI (kg/m^2)	27.06 (23.24-34.62)	28.45 (25.68-33.18)	27.64 (25.27-30.20)	29.26-26-57-31.82)	28.37(25.89-32.18)
hs-CRP (mg/L)	3.56 (1.74-4.34)	1.93 (1.24-3.49)	1.30 (0.49-2.26)	1.66 (0.80-3.20)	1.66 (0.71-2.72)
Total Cholesterol (mmol/L)	5.61 (4.96-5.85)	5.27 (4.69-5.95)	5.39 (4.57-5.66)	5.62 (4.73-6.30)	5.38 (4.78-5.99)
HDL-cholesterol (mmol/L)	1.26 (1.07-1.69)	1.25 (1.09-1.49)	1.28 (1.13-1.45)	1.39 (1.12-1.55)	1.36 (1.11-1.60)
Non-HDL cholesterol (mmol/L)	4.08 (3.72-4.61)	3.93 (3.53-4.52)	3.73 (3.12-4.59)	4.16 (3.30-5.05)	4.06 (3.25-4.81)
HDLR ratio	4.07 (3.43-5.30)	4.09 (3.49-4.97)	3.94 (3.26-4.56)	4.00 (3.29-5.05)	3.94 (3.14-5.04)
Triglycerides (mmol/L)	1.63 (1.21-1.97)	1.38 (0.91-2.04)	1.15 (0.73-1.73)	1.21 (0.87-1.74)	1.20 (0.85-1.78)
LDL-cholesterol (mmol/L)	3.34 (3.15-3.74)	3.31 (2.67-3.85)	3.19 (2.71-3.78)	3.53 (2.79-4.24)	3.41 (2.83-4.01)

All lipid profile parameters were higher amongst non-smokers who were current drinkers compared with non-smokers who are non-regular drinkers, reaching a statistically significant difference for TC, NHDL-C and LDL-C (Table 6-14a). These remained significant when analysis was restricted to individuals of 50 years or older (Table 6-14b). Current smokers who were non-regular drinkers had significantly higher median levels of TC, NHDL-C, TC:HDL-cholesterol ratio, triglycerides and LDL-C and lower HDL-cholesterol levels compared with non-smokers who were non-regular drinkers (Table 6-14a). Since the mean age of current smokers who were non-regular drinkers was significantly higher than that of non-smokers non-regular drinkers (59 years vs. 52 years, p-value <0.05), data was restricted to individuals of 50 years or older (Table 6-14b). The observed differences in the lipid profile were still present upon restriction (Table 6-14b). Current smokers who were also current drinkers had significantly lower median levels of total-cholesterol, NHDL-C, TC:HDL-cholesterol ratio, triglycerides, LDL-C and higher HDL-cholesterol levels compared with current smokers who were non-regular drinkers (Table 6-14a). These patterns remained after restricting the data to those older than 50 years (Table 6-14b). Whilst there were improvements in the lipid profile amongst smokers who were also current drinkers compared to smokers who were non-regular drinkers, current drinking was associated with a worse lipid profile in non-smokers despite that smokers tended to drink alcohol more frequently (Table A1-4). All the patterns remained even after excluding statin users from the analysis as well as after excluding diabetics. Amongst non-smokers median hs-CRP levels were similar in regular drinkers and in those that do not consume alcohol regularly (Table 6-14a).

Table 6-14 Median levels and interquartile range of biochemical parameters and BMI amongst male controls with different smoking and alcohol consumption combinations (a) overall, and (b) limiting the analysis to ≥ 50 years of age. Mean and range are reported for age. The p-values calculated according to the two-tailed Mann-Whitney tests, compare each category with non-smokers who are non-regular drinkers. *p-value < 0.1; **p-value < 0.05; ***p-value < 0.01. BMI, body mass index; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C reactive protein; LDL, low-density lipoprotein.

(a) Overall men				
	Current drinkers who smoke (n=63)	Non-regular drinkers who smoke (n=17)	Current drinkers non-smokers (n=71)	Non-regular drinkers non-smokers (n=45)
Age (years), mean (range)	52 (23-69)	59 (50-70) **	53 (20-70)	52 (20-69)
Total Cholesterol (mmol/L)	5.32 (4.73-5.91) *	5.8 (5.16-6.59) ***	5.36 (4.97-6.03) **	4.95 (4.40-5.71)
HDL-cholesterol (mmol/L)	1.22 (1.12-1.42)	1.11 (1.00-1.39) ***	1.36 (1.19-1.55)	1.30 (1.11-1.53)
Non-HDL (mmol/L)	3.98 (3.30-4.81) **	4.46 (4.13-5.16) ***	4.02 (3.41-4.71) **	3.56 (3.07-4.32)
HDL- Ratio	4.23 (3.34-5.05) *	5.13 (4.35-6.27) ***	3.98 (3.22-4.72)	3.64 (3.17-4.45)
Triglycerides (mmol/L)	1.31 (0.97-1.82) ***	1.75 (1.20-2.16) ***	1.16 (0.79-1.57)	0.86 (0.74-1.43)
LDL-cholesterol (mmol/L)	3.21 (2.76-3.92)	3.67 (3.30-4.38) ***	3.42 (2.98-3.98) **	3.13 (2.62-3.66)
BMI (kg/m^2)	27.92 (24.53-32.04)	29.31 (27.10-31.70)	28.19 (25.67-32.10)	28.14 (25.96-32.39)
hs-CRP (mg/L)	1.96 (0.72-4.23)	3.32 (1.66-5.11) ***	1.61 (0.68-3.00)	1.09 (0.73-2.48)
(b) In men ≥50 years of age				
	Current drinkers who smoke (n=42)	Non-regular drinkers who smoke (n=17)	Current drinkers non-smokers (n=49)	Non-regular drinkers non-smokers (n=32)
Age (years), mean (range)	61 (50-69) *	59 (50-70)	60 (50-70)	58 (50-69)
Total Cholesterol (mmol/L)	5.46 (4.87-6.04) *	5.80 (5.16-6.59) **	5.48 (5.00-6.24) **	4.97 (4.40-5.77)
HDL-cholesterol (mmol/L)	1.33 (1.16-1.56)	1.11 (1.00-1.39)	1.36 (1.09-1.56)	1.25 (1.03-1.53)
Non-HDL (mmol/L)	4.07 (3.29-4.85)	4.46 (4.13-5.16) ***	4.06 (3.68-5.07) *	3.57 (3.07-4.32)
HDLR ratio	3.98 (3.29-5.02)	5.13 (4.35-6.27) ***	4.10 (3.19-5.13)	3.71 (3.16-4.64)
Triglycerides (mmol/L)	1.54 (1.07-2.04) **	1.75 (1.20-2.16) ***	1.21 (0.79-1.63)	1.12 (0.77-1.70)
LDL-cholesterol (mmol/L)	3.41 (2.78-3.92)	3.67 (3.30-4.38) **	3.50 (3.15-4.21) **	3.10 (2.56-3.66)
BMI (kg/m^2)	28.87 (24.53-32.19)	29.31 (27.10-31.70)	28.81 (26.10-32.04)	28.41 (26.43-32.78)
hs-CRP (mg/L)	2.19 (1.05-4.56)	3.32 (1.66-5.11) **	1.51 (0.81-2.56)	1.51 (0.84-2.85)

Amongst current smokers, regular drinking was associated with lower median levels of hs-CRP (1.96 mg/L vs. 3.32 mg/L amongst those that do not drink regularly, p-value < 0.1) (Table 6-15a). Hs-CRP levels increase with age (Tang et al. 2018). Since smokers who were regular drinkers were younger than smokers who did not consume alcohol regularly, the analysis was restricted to individuals of 50 years and older to rule out any effects of age on median hs-CRP levels.

Table 6-15 Median levels and interquartile range of biochemical parameters and BMI amongst male controls who smoke and are regular drinkers versus smokers who are non-regular drinkers, (a) overall and (b) limiting the analysis to ≥ 50 years of age. Mean and range are reported for age. p-values calculated according to the two-tailed Mann-Whitney tests, compare smokers who are regular drinkers vs. smokers who are non-regular drinkers. *p-value < 0.1; **p-value < 0.05; ***p-value < 0.01. BMI, body mass index; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein.

(a) Overall men		
	Current drinkers who smoke (N=63)	Non-regular drinkers who smoke (N=17)
Age (years), mean (range)	52 (23-69)	59 (50-70)
BMI (kg/m^2)	27.92 (24.53-32.04)	29.31 (27.10-31.70)
hs-CRP (mg/L)	1.96 (0.72-4.23) *	3.32 (1.66-5.11)
Total cholesterol (mmol/L)	5.32 (4.73-5.91) *	5.80 (5.16-6.59)
HDL-cholesterol (mmol/L)	1.22 (1.12-1.42) *	1.11 (1.00-1.39)
Non-HDL (mmol/L)	3.98 (3.30-4.81) **	4.46 (4.13-5.16)
HDLR ratio	4.23 (3.34-5.05) **	5.13 (4.35-6.27)
Triglycerides (mmol/L)	1.31 (0.97-1.82) *	1.75 (1.20-2.16)
LDL-cholesterol (mmol/L)	3.21 (2.76-3.92) *	3.67 (3.30-4.38)

(b) In men ≥50 years of age		
	Current drinkers who smoke (N=42)	Non-regular drinkers who smoke (N=17)
Age (years), mean (range)	61 (50-69)	59 (50-70)
Total cholesterol (mmol/L)	5.46 (4.87-6.04)	5.80 (5.16-6.59)
HDL-cholesterol (mmol/L)	1.33 (1.16-1.56) **	1.11 (1.00-1.39)
Non-HDL (mmol/L)	4.07 (3.29-4.85) *	4.46 (4.13-5.16)
HDL ratio	3.98 (3.29-5.02) **	5.13 (4.35-6.27)
Triglycerides (mmol/L)	1.54 (1.07-2.04)	1.75 (1.20-2.16)
LDL-cholesterol (mmol/L)	3.41 (2.78-3.92) *	3.67 (3.30-4.38)
BMI (kg/m^2)	28.87 (24.53-32.19)	29.31 (27.10-31.70)
hs-CRP (mg/L)	2.19 (1.05-4.56)	3.32 (1.66-5.11)

Section 6.3. Discussion

In the MAMI study, the risk of MI decreased gradually after smoking cessation with no detectable risk beyond 5 years. This contrasts with persistently elevated risk up to 20 years after smoking cessation seen in other studies (Teo et al. 2006). This is probably due to the limited number of subjects in the MAMI study or may also be due to drastic changes in lifestyle in ex-smokers. The fact that smokers have accelerated atherogenesis throughout their young adult life increases the risk of plaque rupture later in life even after the subject has been smoke-free for years. Residual risk after stopping smoking is expected to persist for decades as the process of plaque regression is slow despite any lifestyle changes that are carried out.

In the MAMI study, although hs-CRP, TC, LDL and HDL all normalised after smoking cessation triglyceride concentrations remained elevated for up to 15 years after the subjects quit date. This could be linked to the residual risk of myocardial infarction years after stopping smoking.

Low to moderate alcohol consumption protects against MI despite it being associated with higher median levels of TC, NHDL-C and TG. Its effect on the risk of MI depends greatly on the pattern, frequency and intensity of alcohol consumption, and the type of alcoholic beverage. Daily binge drinking exerted a strong deleterious effect on the risk of MI. Consumption of 1-10 drinking units per week was protective, while weekly consumption of more than 10 drinking units increased risk of MI. An altered lipid profile was found in those who consumed more than 10 drinking units per week. Whereas weekly consumption of wine was protective, weekly consumption of beer was associated with a 3.5-fold increased risk of MI. Alcohol decreased the risk of MI irrespective of smoking status. Current smokers who were also current alcohol drinkers had significantly lower levels of hs-CRP, TC, NHDL-C, HDLR, TG and LDL-C and higher levels of HDL-C compared with smokers who were non-regular drinkers. On the other

hand in non-smokers, current drinkers had higher TC, NHDL-C and LDL-C compared with the non-regular drinkers. These opposing effects of alcohol consumption on the lipid profile between smokers and non-smokers were not due to differences in the frequency of alcohol consumption, statin use or diabetes.

The protective effect of low-to-moderate alcohol consumption and the deleterious effect of binge drinking were confirmed in this study (Romelsjo et al. 2012; Ronksley et al. 2011; Leong et al. 2014; Biyik and Ergene 2007). Although studies on alcohol and risk of MI are numerous, few were designed to investigate the effect of pattern, type and intensity of alcoholic beverages consumed on the risk of MI. Similar to previous observations (Cleophas 1999; Schroder et al. 2007), small doses of wine and beer were found to offer the same protective effect on the risk of MI in this study. However, while wine remained protective with increasing weekly doses up to a consumption of 10-19 units, concordant with findings reported by others (Schroder et al. 2007), beer conveyed a deleterious effect with increasing consumption, reaching a 4.3-fold increased risk of MI amongst individuals consuming more than 19 weekly units of beer. Findings on the benefits of beer on the cardiovascular system are conflicting ranging from no effect (Salonen et al. 1983; Bobak et al. 2000), lower risk and no dose-response effect (Di Castelnuovo et al. 2002), to a J-shaped relationship for increasing beer consumption (Costanzo et al. 2011). These conflicting findings are possibly due to the amount of ethanol present in different types of beer and due to failure to account for the different types of alcoholic beverages consumed concurrently. In this study the deleterious effect of beer on the risk of MI was lower amongst individuals who consumed both wine and beer. These findings suggest that the non-alcoholic component of different types of beverages and not the alcohol content alone affect the risk of MI. This highlights the importance of the type of alcoholic beverage and not only considering the grams of alcohol in beverages. Only a small number of individuals reported to consume weekly spirits and so conclusions on its effect on the risk of MI could not

be drawn from this study. Others have suggested that high spirit intake increases risk of MI (Schroder et al. 2007).

Conflicting literature on the effect of moderate alcohol intake on the lipid profile ranges from reduction in TG and increased HDL-C (Vu et al. 2016), increase in both TG and HDL-C (Ruidavets et al. 2002), no effect (Van de Wiel 2011) to increase in TG and lower HDL-C (Park and Kim 2012). In this study, current drinking was associated with higher median levels of TC, NHDL-C and TG. These effects were only evident in those who consumed alcohol 2-4 times per month or more often. Alcohol consumption was associated with a rise in median HDL-C levels if consumption was 2-3 times per week or more frequent. Apart for the frequency of consumption, the intensity also influenced the lipid profile parameters. Higher median levels of TC and HDL-C were observed in individuals who consumed more than 10 weekly drinking units. Binge drinking had no effect on the lipid profile. The different effects on the lipid profile posed by the frequency and intensity of alcohol consumption observed in this study, offer an explanation for some of the conflicting results present in the literature.

We have previously reported the effect of smoking on lipid profile and inflammation (Attard et al. 2017). Alcohol and smoking often occur together (Grucza and Bierut 2006) but research on the effect of interactions between smoking and alcohol consumption on the risk of MI are limited (Lussier-Cacan et al. 2002). In the MAMI study alcohol decreased risk of MI, independent of smoking status. This is similar to what has been observed in other studies (Tavani et al. 2001). Smoking status modified the effects of alcohol on the lipid profile. While amongst non-smokers, current drinking was assocated with a worse lipid profile (higher median levels of TC, NHDL-C and LDL-C), amongst smokers, current drinking improved the lipid profile (lower median levels of TC, NHDL-C, HDLR, TG and LDL-C and higher median levels of HDL-C). A decrease in TC and LDL-C and higher HDL-C levels have previously been reported amongst smokers who consume alcoholic beverages compared to smokers who are

non-drinkers (Wakabayashi 2008). In the MAMI study the difference in the lipid profile parameters between current alcohol drinkers who smoke and non-smokers was independent of the frequency of alcohol consumption, as despite having a higher frequency of alcohol consumption (associated with an abnormal lipid profile), alcohol use in smokers still showed an improved lipid profile compared with non-smokers.

The protective effect of alcohol consumption on the risk of MI amongst non-smokers can be mediated via other mechanisms including differences in the apolipoprotein composition and/or via epigenetic modifications which were not investigated in this study. It is known that alcohol drinkers have a differential methylation pattern associated with the expression of a number of genes involved in the immune response pathway (Liu et al. 2018). The lower levels of hs-CRP amongst current alcohol drinkers indicate that at least part of the protective effect of moderate alcohol consumption on the risk of MI may be mediated by the anti-inflammatory effect of moderate drinking. These findings are concordant with observations from other studies (De Lorgeril et al. 2004; Kesteloot 2004). This lowering action of moderate alcohol consumption on median hs-CRP levels was evident only in smokers.

A limitation of this study is that no information was collected on the type of wine consumed and so different effects of red and white wine on the risk of MI cannot be investigated. We could not measure the individual effects of beer, wine and spirit consumption on the lipid profile and inflammation since upon stratification the number of research subjects in certain groups was small. Alcohol units were calculated based on data reported by participants thus the volume of a bottle of beer, glass of wine and shot of spirit is approximate and the alcohol strength of different brands also differs. Differences in the observations amongst non-smokers in this study and in that of Wakabayashi, who reported no difference in TC, lower LDL-C and higher HDL-C in non-smokers who consumed alcohol compared with non-smokers non-drinkers (Wakabayashi 2008), may be due to the use of a different reference category (non-

drinkers vs. non-regular drinkers as used in this study) as well as other differences including the type of beverages, pattern of consumption and population differences, such as genetic differences in alcohol-metabolizing enzymes (Harada et al. 1981).

Section 6.4. Limitations

It is possible that active smoking was under-reported by the study participants as active smoking was not verified or checked using CO or cotinine levels. This would lead to a misclassification of active smokers to non-smokers. Passive smoking was defined as exposure to passive smoking in a closed environment. However, it is possible that research subjects also reported exposure to second-hand smoke occurring in an open public space. Furthermore, exposure to passive smoking may have occurred years ago and the effects of this exposure may have cleared. These limitations could explain the differences in risk of MI associated with passive smoking in a home and public setting. Recall bias is also a limitation and cases may be more reluctant to admit to smoking than controls. This could result in the actual risk of MI for active smokers being even higher. Misclassification of ex-drinkers and occasional drinkers as non-drinkers, or failure to account for amount, type and frequency of alcohol consumption, and to adjust for other conventional risk factors may lead to conflicting results.

Section 6.5. Conclusions

Active and passive smoking are important risk factors for myocardial infarction in the Maltese population with lipid and inflammatory pathways being important mediators of this risk. Elevated triglycerides that persisted up to 15 years after smoking cessation may be an important component of the residual risk of smoking on MI after quitting. Findings presented here

strongly indicate that the risk of MI may be influenced by factors which may convey a different effect when found in combination than when present alone. These results corroborate findings of other authors and highlight the need for alcohol consumption to be considered when determining the cardiovascular risk score along with other conventional risk factors of MI. Smoking influences the relationship between alcohol consumption, lipid profile, inflammation and risk of MI. Given the high incidence of smoking and alcohol consumption, further studies on the modifying effect of alcohol are warranted. The anti-inflammatory effect of alcohol explains at least part of its protective effect against risk of MI. The pattern, intensity and type of alcoholic beverages consumed effect risk of MI and not just the alcohol content thus studies should take these factors into account besides the amount of alcohol consumed. These observations highlight the complexity of MI and the need of analysing the risk of MI in the context of different combinations of environmental backgrounds.

Chapter 7. Apolipoprotein E and Cardiovascular Disease

Section 7.1. Apolipoprotein E and Risk of MI

Apolipoprotein E (Apo-E) is a key protein in lipoprotein metabolism which has been associated with an increased risk of myocardial infarction. Apo-E is a serum glycoprotein which is found in circulating chylomicrons, chylomicron remnants, very low density lipoproteins, intermediate density lipoproteins and high-density lipoproteins (Hallman et al. 1991). *APOE* is a ligand for cell surface receptors including the LDL receptor, the LDL receptor related protein and the very low density lipoprotein receptor (Lambert et al. 2000). These receptors mediate the clearance of VLDL, intermediate density lipoproteins and chylomicron remnants from plasma (Xu et al. 2016).

The gene for Apo-E (*APOE*) is located on chromosome 19q13.2 and has three functionally distinct isoforms E2, E3 and E4 which are caused by amino acid changes at position 112 (rs429358) and position 158 (rs7412) (Mahley 2016). *APOE2* has cysteines at both sites, *APOE4* has arginines at both sides and *APOE3* has a cysteine at position 112 and arginine at position 158 (Table 7-1). These alleles give rise to three homozygous (E2/2, E3/3, EE4/4) and three heterozygous (E3/E2, E4/2, and E4/3) genotypes (Table 7-1). The *APOE* genotype has a strong effect on the level of its gene product; E2 is associated with higher concentrations of Apo-E in the circulation while E4 is associated with lower concentrations (Eichner et al. 2002).

Table 7-1 Apolipoprotein E genotypes

Name	rs429358	rs7412
E2	T	T
E3	T	C
E4	C	C

Apo-E production takes place predominantly in the liver (75%) and secondly in the brain. Macrophages are also able to synthesise Apo-E (Mahley 2016). Apo-E is a 34-kDa protein of 299 amino acids with a single glycosylation site at threonine-194 and has two structural

domains separated by a hinge region. The amino terminal domain contains the low-density lipoprotein (LDL) receptor binding region and the carboxyl-terminal domain contains the lipid binding region. The tertiary structure consists of four helices arranged in antiparallel fashion. The carboxyl-terminal domain has amphipathic α-helices that bind to lipids. Amino acid changes at residue 112 in the amino-terminal domain alter the lipoprotein preference by interacting with amino acids 240-260 in the carboxyl-terminal domain where lipid binding occurs (Mahley 2016).

Apo-E plays an important part in lipid metabolism. TG from fatty meals are transported in the lymphatic system and blood by chylomicrons. Chylomicrons derived from the intestine and very low-density lipoproteins (VLDL) from liver cells undergo lipolysis in the circulation and become remnant lipoprotein particles. Apo-E on the remnant lipoprotein particles binds to LDL receptors, primarily in the liver, hence initiating the removal of the particles from the circulation. Some VLDL remnants are cleared rapidly whereas others undergo further lipolysis and are converted into intermediate density lipoprotein (IDL) and finally to LDL. While *APOE4* and *APOE3* bind with approximately equal affinity to lipoprotein receptors, *APOE2* binds with less than 2 percent of this strength. Therefore, compared with carriers of the E3 or E4 allele, carriers of the E2 allele are slower to clear dietary fat from their blood (Eichner 2002).

Since *APOE2* exhibits impaired binding to the receptor and an inability to promote clearance of triglyceride-rich lipoprotein remnant particle; *APOE2* is associated with raised triglyceride levels. *APOE4*, on the other hand, seems to accelerate the clearance of VLDL and remnant lipoproteins with an increase in LDL levels due to down-regulation of LDL receptors or due to a competition between remnant lipoproteins and LDL for a place on the receptors (Pitas et al. 1987). The presence of at least one *APOE2* allele has been associated with lower levels of TC than in individuals who are *APOE3* homozygous. Having at least one *APOE4* allele has been

associated with higher levels of total plasma cholesterol than *APOE3* homozygous individuals (Hallman et al. 1991).

Besides differences in Apo-E structure due to the genotype, variation in *APOE* mRNA levels play a part in the level of risk, through effects on protein levels. Variations in APOE mRNA levels are brought about by polymorphisms in the *APOE* regulatory region (ex. -219GT). Polymorphisms in this region have been associated with a higher risk of myocardial infarction and premature myocardial infarction (Lambert et al. 2000).

Apart from the effect on Apo-E levels and lipoprotein levels, *APOE* genotypes may modify risk due to differences in macrophage-derived Apo-E with effects on oxidative stress and arterial wall cholesterol balance (Kruth et al. 1994).

The variance in LDL-C attributable to *APOE* varies between 1 - 8.3 percent (Eichner 2002). *APOE* is thought to contribute more to normal cholesterol variability than any other gene identified (Eichner 2002). A meta-analysis including 11,804 CHD patients and 17,713 controls, identified a significantly increased risk for CHD among carriers of the *APOEE3/4* and E4/4 genotypes compared with carriers of the E3/3 genotype. Overall no significant evidence was found between the variant genotypes of *APOE* E2/3, E2/4, and E2/2 and CHD risk however there was a decreased risk in Caucasians with the E2 allele variant (Xu et al. 2016).

Section 7.2. Results

Section 7.2.1. *APOE* Allele Determination by KASP

Polymorphisms on the *APOE* gene at positions 112 (rs.429358) and 158 (rs.7412) in the MAMI study samples were determined using KASP genotyping. The alleles were in Hardy Weinberg

equilibrium in controls. E3/E3 was the commonest genotype in both case and controls followed by E3/E4 and E2/E3 (Table 7-2).

Table 7-2 *APOE* Genotype in women and men

	Women		Men	
Genotype	Cases (n=63) n (%)	Controls (n=130) n (%)	Cases (n=264) n (%)	Controls (n=309) n (%)
E2/E2	0 (0)	1 (0.8)	1 (0.4)	1 (0.3)
E4/E4	0 (0)	0 (0)	4 (1.5)	6 (1.9)
E2/E4	1 (1.6)	1 (0.8)	5 (1.9)	5 (1.6)
E2/E3	3 (4.8)	19 (14.6)	22 (8.3)	33 (10.7)
E3/E4	12 (19.0)	24 (18.5)	36 (13.6)	49 (15.9)
E3/E3	47 (74.6)	85 (65.4)	196 (74.2)	215 (69.6)

Section 7.2.2. Lipid Level Variability due to *APOE* Genotype

APOE genotype had minor effect on lipid levels in male controls who were not on cholesterol lowering agents. E2/E3 genotype had a lower TC level compared to the E3/E3 genotype (p-value 0.008). APOE genotype had no effect on HDL-C, LDL-C, NHDL-C or triglyceride levels (Figure 7-1). However, when subjects with E3/E3 genotype were compared to subjects containing at least one E4 allele and at least one E3 allele no effect on cholesterol level was appreciable. Observations in women are not presented here as the number of women in each category was small.

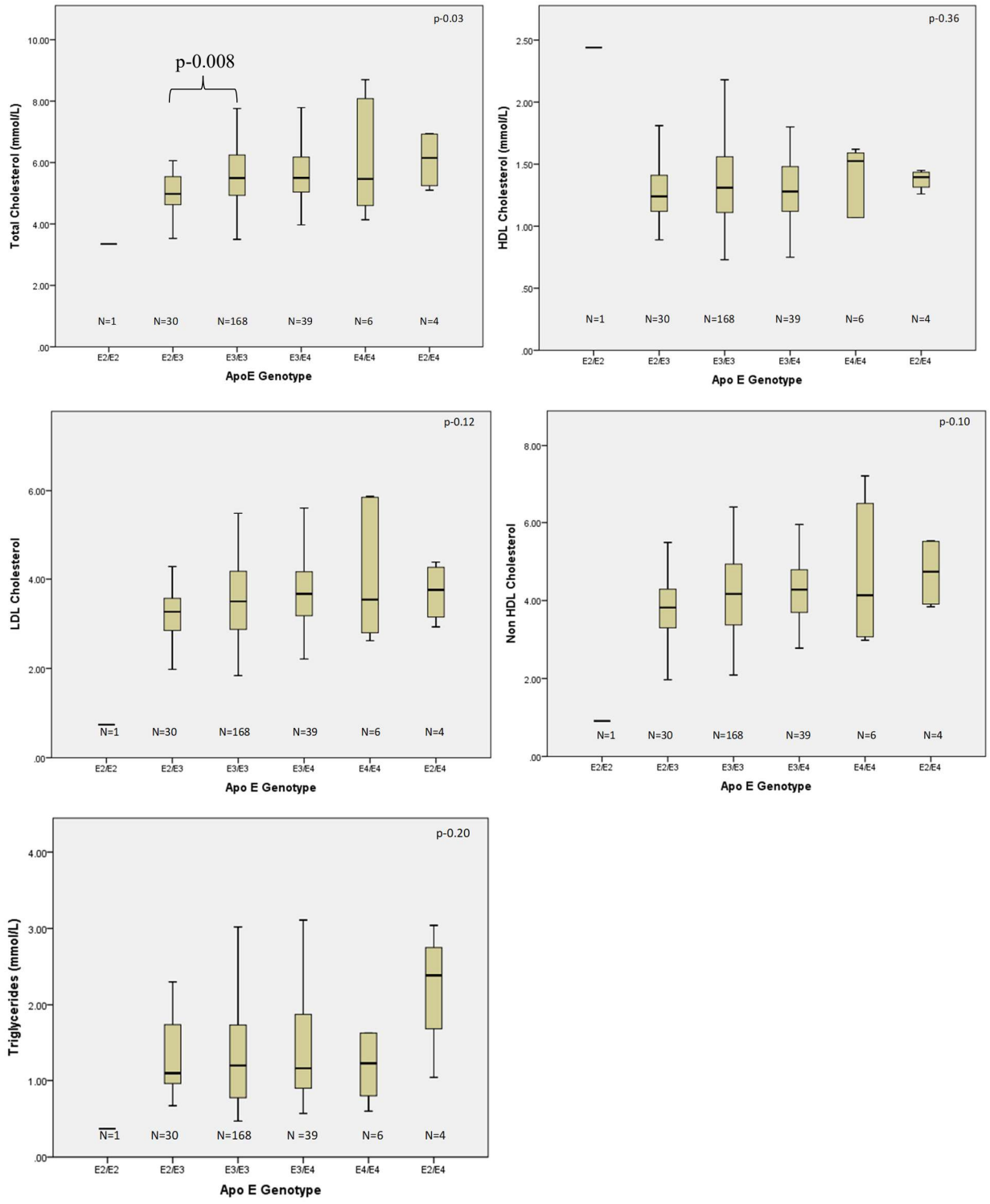

Figure 7-1 Boxplots showing effect of *APOE* genotype on lipid levels in male controls not on cholesterol lowering agents. Box represents 25th and 75th percentile, black line in middle of box represents the median while whiskers extend to 10th and 90th percentile. p-value for all groups in top right hand corner.

Section 7.2.3. Influence of APOE on Measures of Adiposity

The effect of *APOE4* and *APOE2* genotype were compared to the reference group *APOE3/E3*. *APOE* genotype did not influence waist-hip ratio in male controls who were not on lipid lowering agents (Figure 7-2) or in female controls who were not on lipid lowering agents (Figure 7-3).

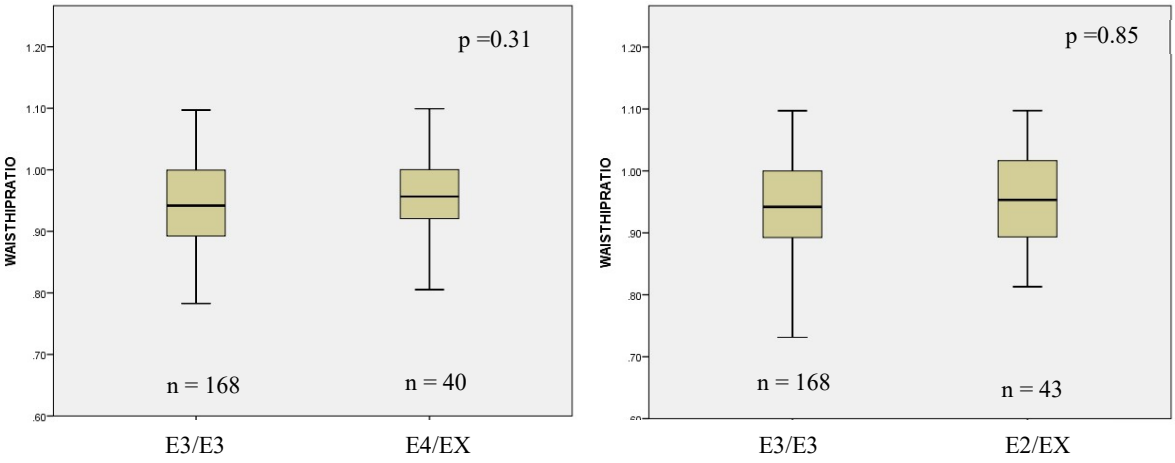

Figure 7-2 Effect of *APOE* on Waist-hip ratio in male controls not on lipid lowering agents. Box represents 25th and 75th percentile, black line in middle of box represents the median while whiskers extend to 10th and 90th percentile.

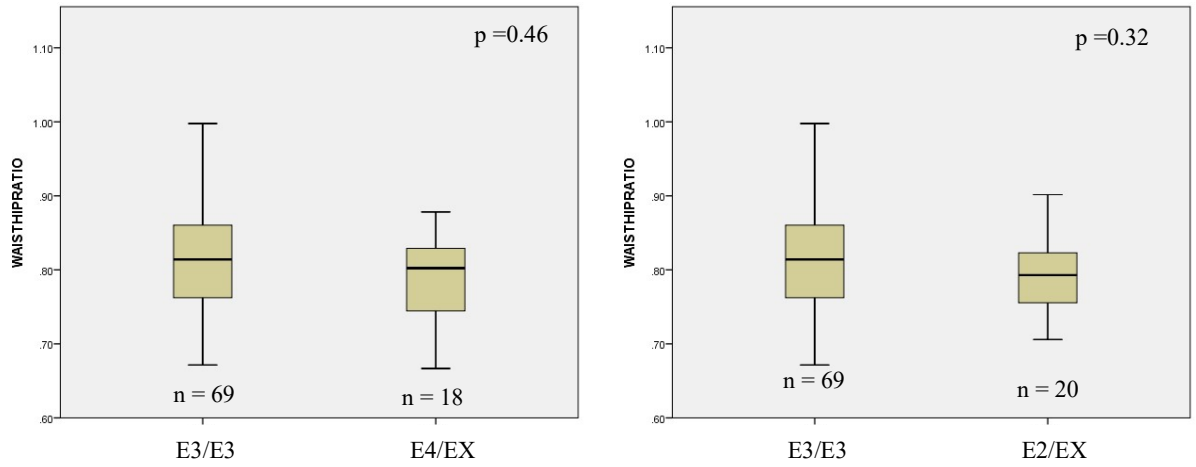

Figure 7-3 Effect of *APOE* genotype on Waist-hip ratio in female controls who were not on lipid lowering agents. Box represents 25th and 75th percentile, black line in middle of box represents the median while whiskers extend to 10th and 90th percentile.

APOE genotype did not have any effect on BMI in male controls not on lipid lowering agents (Figure 7-4) or in female controls who were not on lipid lowering agents (Figure 7-5).

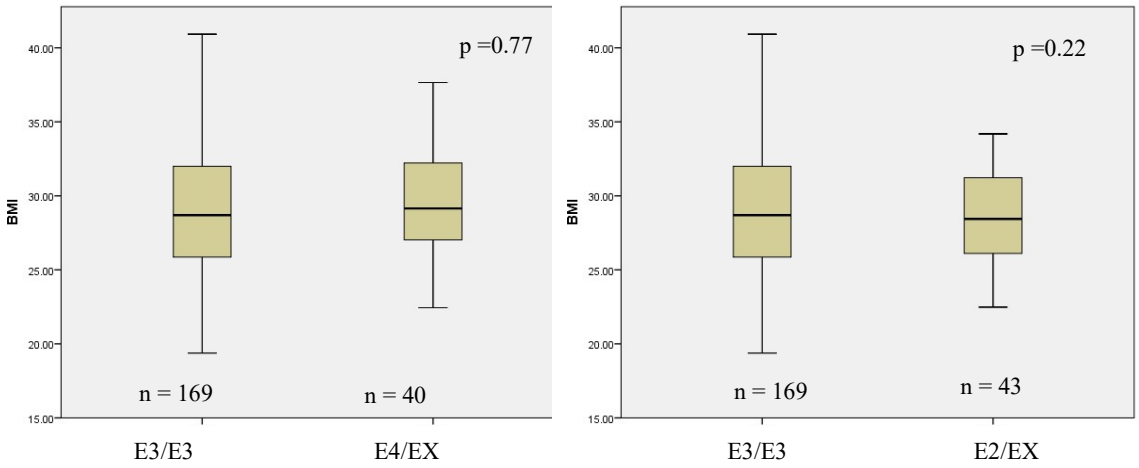

Figure 7-4 Effect of *APOE* genotype on male controls wo were not on lipid lowering agents. Box represents 25th and 75th percentile, black line in middle of box represents the median while whiskers extend to 10th and 90th percentile.

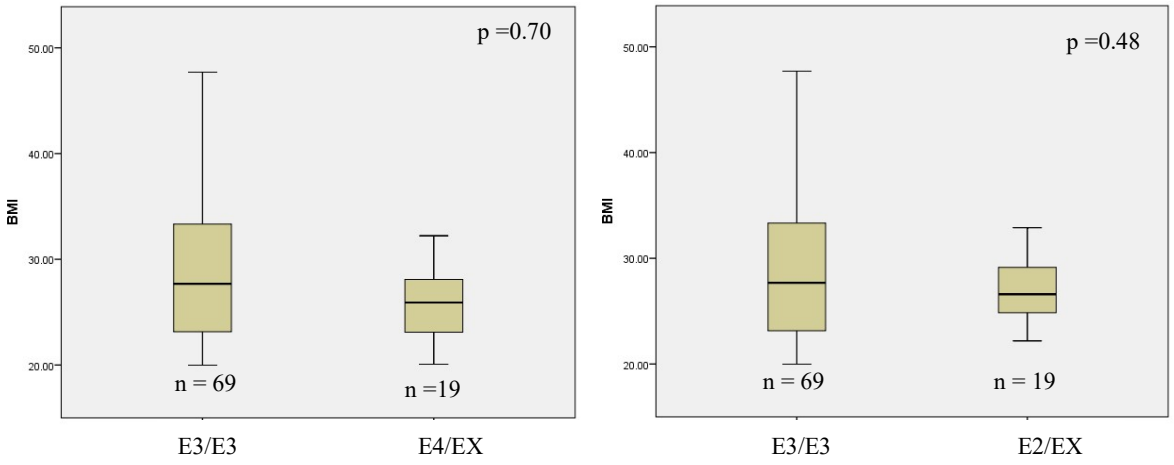

Figure 7-5 Effect of *APOE* genotype on BMI in female controls who were not on lipid lowering agents. Box represents 25th and 75th percentile, black line in middle of box represents the median while whiskers extend to 10th and 90th percentile.

Section 7.2.4. Influence of *APOE* on Insulin Sensitivity

Compared with the E3/E3 genotype having at least one E4 allele had no effect on median HOMA-IR levels in neither men (Figure 7-6) nor women (Figure 7-7). While having at least one E2 allele did not affect HOMA-IR median level in men, women with this genotype had lower median levels of HOMA-IR (Figure 7-7), all relative to those having the E3/E3 genotype. This analysis was restricted to individuals off statins and who are not taking exogenous insulin.

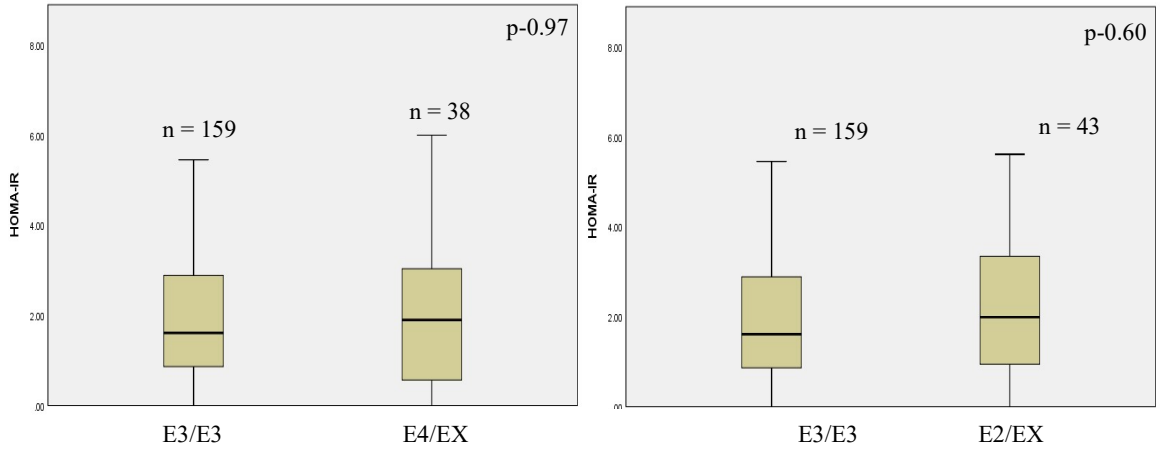

Figure 7-6 Boxplot comparing individuals with the *APOE3/E3* genotype with those having at least one E4 allele and those having at least one E2 allele. Analysis restricted to male controls, off statins and off insulin. Box represents 25th and 75th percentile, black line in middle of box represents the median while whiskers extend to 10th and 90th percentile.

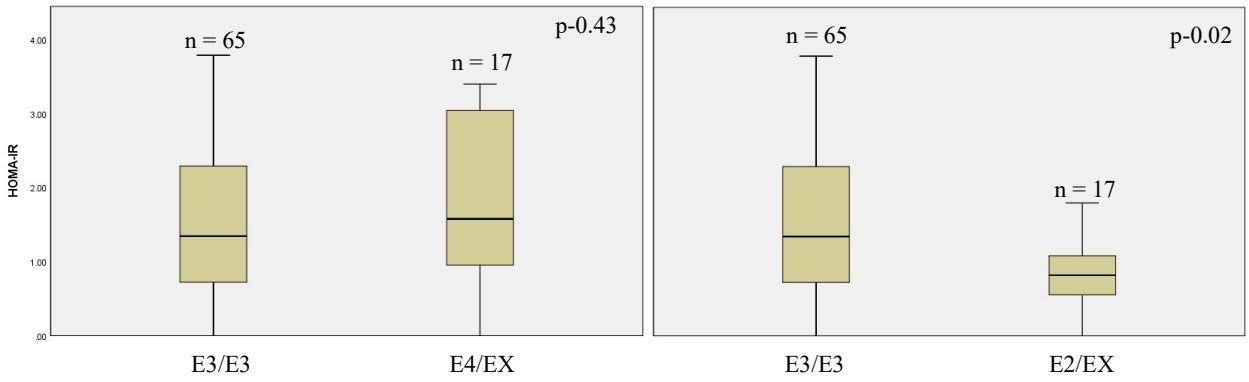

Figure 7-7 Boxplot comparing individuals with the *APOE3/E3* genotype with those having at least one E4 allele and those having at least one E4 allele. Analysis was restricted to female controls, off statins and off exogenous insulin. Box represents 25th and 75th percentile, black line in middle of box represents the median while whiskers extend to 10th and 90th percentile.

Section 7.2.5. Influence of *APOE* on Measure of Inflammation

There was no difference in hs-CRP levels between the E3/E3 genotype and subjects that had at least one E4 allele in either sex. There was a lower hs-CRP in individuals that had at least one E2 allele compared to the E3/E3 genotype both in men (Figure 7-8) and women (Figure7.9)

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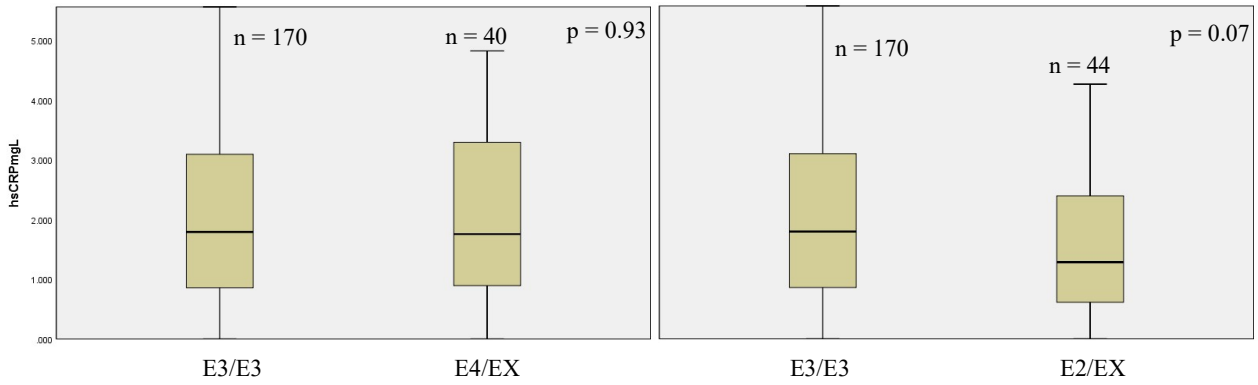

Figure 7-8 Boxplots showing the median and interquartile ranges of hs-CRP amongst male controls off statins having the E3/E3 genotype versus those having at least one E4 allele and those having at least one E2 allele. Box represents 25th and 75th percentile, black line in middle of box represents the median while whiskers extend to 10th and 90th percentile

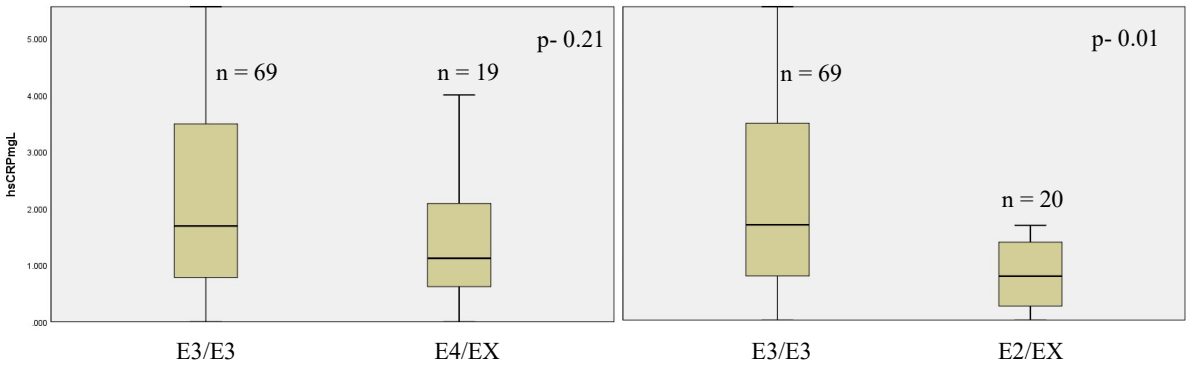

Figure 7-9 Boxplots showing the median and interquartile ranges of hs-CRP amongst female controls off statins having the E3/E3 genotype versus those having at least one E4 allele and those having at least one E2 allele. Box represents 25th and 75th percentile, black line in middle of box represents the median while whiskers extend to 10th and 90th percentile

Section 7.2.6. *APOE* Genotype and Risk of MI

No particular effect of the *APOE* genotype on the risk for MI was observed in men who were not on lipid lowering treatment (Table 7-3). ORs are not reported for categories containing less than 10 research subjects. When stratified by the *APOE* genotype the number of females in each category was too small to draw conclusions on the possible effect of this genotype on risk for MI.

Table 7-3 Age adjusted Odds Ratios for *APOE* Genotypes for men not on hypocholesterolaemic treatment. *APOE3/E3* is the reference category.

APOE genotype	Cases n (%)	Controls n (%)	AgeOR (95% CI)
APOE2/E2	1 (0.5)	1 (0.4)	
APOE4/E4	2 (1.0)	6 (2.4)	
APOE2/E4	4 (2.0)	4 (1.6)	
APOE2/E3	20 (9.8)	30 (12.0)	0.8 (0.4-1.4)
APOE3/E4	30 (14.6)	39 (15.6)	0.9 (0.5-1.6)
APOE3/E3	148 (72.2)	170 (68.0)	1.0

Section 7.3. Discussion

The distribution of *APOE* alleles was similar to that described in other populations (Xu et al. 2016). In this study the E3/E3 genotype was the most prevalent followed by the E3/E4, E2/E3, E2/E4, E4/E4 and E2/E2, as observed by others (Maxwell *et al.*, 2013; Xu *et al.*, 2016). There was no difference in the genotype frequencies between MAMI study cases and controls. Similarly the *APOE4* genotype was also not found in higher frequencies in CAD patients in Tunisian, Kuwaiti, Polish and Finnish populations (Bahri *et al.*, 2008; Akanji *et al.*, 2007; Żak *et al.*, 2005; (Lehtinen et al. 1995), while the *APOE2* genotype was less frequent amongst CHD patients (Xu et al. 2016). In this study we did not observe any particular risk for MI due to the different *APOE* genotypes. Findings on the effect of the *APOE* genotype on the risk of CVD are conflicting, ranging from increased risk for CHD among carriers of the *APOE3/4* and E4/4

genotypes, a decreased risk with the E2 allele variant (Xu et al. 2016) to no effect of *APOE* genotypes on risk of MI (Sapkota et al. 2015), when compared to the E3/E3 genotype.

APOE genotype had a minor effect on lipid levels in the Maltese population, with only men having the E2/E3 genotype demonstrating a lower median levels of TC, compared to those having the E3/E3 genotype. No effect was observed on median levels of HDL-C, LDL-C, NHDL-C or TG amongst men off statins. Effects of the *APOE* genotype on the lipid profile parameters are conflicting. *APOE2* has been associated with both raised levels of TC and TG (Xu et al. 2016) and also with lower levels of TC (Hallman et al. 1991), compared with the *APOE3/E3* genotype. While we have not observed significant effects on the lipid profile associated with the *APOE4* genotype, other have associated this genotype with raised LDL-C (Xu et al. 2016) and higher levels of TC (Hallman et al. 1991).

While the *APOE* genotype had no effect on median levels of neither BMI nor WHR in both sexes, the presence of at least one E2 allele was associated with lower insulin resistance amongst women. This effect was not observed amongst men. Such observations are similar to those of Sapkota et al. 2015, who found no association between *APOE* genotype and diabetes (Sapkota et al. 2015) but contrast with those of others who reported an association between the E4 allele with type 2 DM (Chaudhary et al. 2012).

While the presence of a least one E4 allele has no effect of hs-CRP levels, at least one E2 allele was associated with lower levels of hs-CRP in both men and women, compared with the E3/E3 genotype. Apo-E plays a central role in inflammation. Contrary to our results the presence of the *APOE4* isoform has been associated with lower concentrations of hs- CRP in a Finnish study (Kahri et al. 2006).

Section 7.4. Conclusion

The effect of the *APOE* genotype varies in different populations. In this study, while no particular risk of MI was associated with the different *APOE* genotypes, and no effects on measurements of adiposity were observed, the E2/E3 genotype was associated with lower median levels of total cholesterol. In addition the presence of at least one E2 allele lead to lower insulin resistance in women and lower hs-CRP levels in both men and women. The conflicting finds on the association between *APOE* genotype and risk for MI along with its effect on intermediate phenotypes, highlight the need for the genetics of lipids and of MI to be viewed more holistically in the context of genes within biological pathways and also in the context of influences of conventional risk factors on the risk imparted by genotypes (gene-environmental interactions).

Chapter 8. *PTPN1* and its Effects on Lipids, Adiposity and MI

Section 8.1. *PTPN1* and its Association with Lipids, Adiposity and Diabetes

Protein tyrosine phosphatase 1B (PTP1B) is a cytosolic phosphatase located in the endoplasmic reticulum which dephosphorylates the tyrosyl residues of several proteins, thereby disactivating them (Frangioni et al. 1992). PTP1B is encoded for by protein tyrosine phosphatase non-receptor type 1 (*PTPN1*). *PTPN1* is located on chromosome 20q13.13 which consists of 10 exons spanning 74 kilobases (kb) (Palmer et al. 2004).

PTP1B has a wide array of functions. Besides being a negative regulator of insulin signalling (Byon et al. 1998) it influences: cell adhesion (Liang et al., 2005; Xu et al., 2002); leptin and growth hormone effects by dephosphorylating JAK2 and TYK2 (Gu et al., 2003; Kaszubska et al., 2002; Zabolotny et al., 2002; Myers et al., 2001); dephosphorylation of epidermal growth factor receptor, platelet-derived growth hormone receptors and insulin-like growth factor type I receptor (D A Buckley et al., 2002 ; Dubé et al., 2004; Haj et al., 2002; Liu and Chernoff, 1997); it regulates interferon signalling in fibroblasts (Myers et al. 2001) and is an important modulator of myeloid differentiation and macrophage activation in vivo (Heinonen et al. 2006).

The PTP1B protein consists of an N-terminal catalytic domain, C-terminal catalytic domain and proline rich regions. The N-terminal domain regulates the activity of the protein through reversible oxidation of the Cys215 residue and through serine and threonine phosphorylation (Yip et al. 2010). The proline rich regions are important in protein-protein interactions (Yip et al. 2010). The C-terminal end of the protein contains 35 residues which are hydrophobic and serve to target the enzyme to the surface of the endoplasmic reticulum membrane (Frangioni et al. 1992). On the C -terminal side of the PTP1B protein there is a regulatory segment which consists of 115 residues where phosphorylation occurs by serine/threonine (Ser/Thr) kinases (Liu and Chernoff, 1997; Flint *e*t al., 1993).

PTPN1 has been associated with important cardiovascular risk factors. Since the Chromosome 20q13.13 locus has been implicated in type 2 diabetes linkage studies it is considered a candidate gene for type 2 diabetes (Klupa *et al.*, 2000; Permutt *et al.*, 2001). Two out of 33 *PTPN1* haplotypes were associated with hypertension in Chinese and Japanese populations (Olivier et al. 2004). Individual *PTPN1* SNPs were also associated with LDL-C, VLDL cholesterol, TC and measures of obesity (Olivier et al. 2004).

Most SNPs described in *PTPN1* are present in the non-coding regions (Lek et al. 2016), while polymorphisms in the coding region are rare (Di Paola *et al.*, 2002b; Mok *et al.*, 2002).

The *PTPN1* 1484insG polymorphism in the 3' untranslated region (UTR) has been found to increase the PTP1B mRNA stability (Di Paola et al. 2002b). It results in overexpression of the PTP1B protein. The 3' UTR can regulate the stability of the mRNA by binding to specific proteins which typically occur mostly in adenylate/uridylate (AU) rich regions (Conne et al. 2000). The allele frequency of the *PTPN1* 1484insG allele variant varies from 4.1% to 9.6% in different populations (Di Paola et al., 2002b; Meshkani et al., 2007; Dahlman et al., 2004; Bento et al., 2004). The *PTPN1* 1484insG has been associated with higher serum triglycerides and TC/HDL ratios and higher BMI in non-diabetic research subjects (Di Paola et al., 2002; Mosapour et al., 2013).

In the Study of Myocardial Infarction Leiden (SMILE), PTPN1 mRNA levels and BMI were inversely correlated. The effect of *PTPN1* ins 1484G genotype on risk of MI differed in different age groups. In men 50 years or older it was protective with an OR of 0.7 (95% CI 0.5-1.1), this effect was stronger in smokers than in non-smokers. Smokers who were heterozygotes for the *PTPN1* 1484InsG had a lower risk of MI then those with the wild type genotype [age-adjusted OR 2.5 (95% CI 1.5-4.1) and 3.7 (95% CI 2.9-4.9) respectively, relative to *PTPN1* 1484InsG wild type individuals who were non- smokers. In men younger than 50

years of age *PTPNI* 1484inG was possibly deleterious with an age adjusted OR of 1.4 (95% CI, 0.7-2.7). Heterozygotes for the polymorphism were also found to have lower levels of HDL-C and higher levels of TG (Bezzina Wettinger et al. 2014).

However, conflicting results have been observed on the association between the PTPN1 1484insG allele variant and diabetes, insulin resistance (Florez *et al.*, 2005; Dahlman *et al.*, 2004; Permutt *et al.*, 2001; Bento et al. 2004), hypertension (Echwald *et al.*, 2002; Dahlman *et al.*, 2004; Meshkani et al. 2007; Olivier et al. 2004), and measures of obesity (Echwald *et al.*, 2002; Dahlman *et al.*, 2004; Olivier et al. 2004). These conflicting findings can be due to several factors including different definitions or tests of conditions and intermediate phenotypes in difference studies, insufficient numbers of participants, confounding, population specific linkage disequilibrium patterns amongst others.

The effects of age, smoking, adiposity, statin use and the PTPN1 1484InsG genotype on the mRNA expression of the *PTPN1* gene was evaluated. Furthermore, the relationship between PTPN1 mRNA expression and lipid profile and BMI and the effect of the PTPN1 1484InsG polymorphism on risk of MI were investigated.

Section 8.2 *PTPN1* Results

Section 8.2.1. *PTPN1* 1484insG Allele Frequency and Odds-Ratios for MI

The PTPN1 1484InsG polymorphism was in Hardy-Weinberg equilibrium among controls. The allele frequency in the control and case population was 5.8% and 6.4% respectively. There were only 3 homozygous alternate individuals amongst 329 cases and 444 controls (1 case and 2 controls). The age adjusted OR for MI for heterozygous compared to wild type men was 1.1 (95% CI, 0.7-1.9) (Table 8-1). This did not change when analysis was restricted by 60 years

of age (Table 8-1). Additional restriction by statin use, showed that amongst male non-statin users the heterozygous genotype may be increasing the risk for MI only in the elderly [AgeOR 2.0 (95%CI 0.7-5.7)] with no particular risk for MI observed in the young [AgeOR 1.2 (95%CI 0.6-2.3)] (Table 8-2). Similar results were observed for women (Appendix Table A1-6). Restrictions by age were not possible in women due to small numbers.

Table 8-1 Age adjusted OR for *PTPN1* 1484insG polymorphism in men, overall and stratified for men <60 years and ≥60 years of age. HA-Homozygous alternate, Het-heterozygous, WT-Wildtype.

Category	Genotype	Cases n (%)	Control n (%)	OR (95% CI)	Age -Adjusted OR (95% CI)
All men	HA	1 (0.4)	2 (0.6)		
	Het	31 (12.0)	34 (10.9)	0.6 (0.1-6.8)	1.1 (0.7-1.9)
	WT	227 (87.6)	277 (88.5)	1.0	1.0
Allele frequency		6.4%	5.8%		
Men < 60 years					
	HA	1 (0.7)	1 (0.6)		
	Het	20 (13.5)	21 (12.3)	1.2 (0.1-18.9)	1.1 (0.1-17.1)
	WT	127 (85.8)	149 (87.1)	1.0	1.0
Allele frequency		7.4%	6.7%		
Men ≥ 60 years					
	HA	0 (0.0)	1 (0.7)		
	Het	11 (9.9)	13 (9.2)	1.1 (0.5-2.5)	1.1 (0.5-2.5)
	WT	100 (90.1)	128 (90.1)	1	1
Allele frequency,		4.6%	5.3%		

Results were also similar after restrictions for smoking, BMI, diabetes but analysis was restricted due to limited numbers in each group (results not shown).

Table 8-2 Age adjusted OR for PTPN1 heterozygotes in men, overall and stratified by 60 years of age and statin use.. HA-Homozygous alternate, Het-heterozygous, WT-Wildtype.

Category	*PTPN1* 1484 ins G Genotype	Cases n (%)	Control n (%)	OR (95% CI)	Age -Adjusted OR (95% CI)
Men off statins > 60	HA				
	Het	10 (11.2)	6 (5.9)	2.0 (0.7-5.8)	2.0 (0.7-5.7)
	WT	79 (88.8)	95 (94.1)	1	1
Men on statins > 60 years					
	HA	0 (0.0)	1 (2.4)		
	Het	1 (4.5)	7 (17.1)	.2 (0.03-2.0)	0.2 (0.02-1.7)
	WT	21 (95.5)	33 (80.5)	1	1
Men < 60 years off statins					
	HA	1 (0.9)	1 (0.7)		
	Het	16 (14.4)	20 (13.1)	1.1 (0.6-2.3)	1.2 (0.6-2.3)
	WT	94 (84.7)	132 (86.3)	1	1
Men < 60 on statins					
	Het	4 (10.8)	1 (5.6)	2.1 (0.2-19.9)	2.0 (0.2-19.6)
	WT	33 (89.2)	17 (94.4)		

Section 8.2.2. *PTPN1* 1484insG Genotype and Lipid Levels

There were no differences in median levels of TC, LDL-C, HDL-C, NHDL-C, HDLR and TG in male controls with the different *PTPN1* 1484InsG genotypes (Table 8-3). Similar results were obtained in female controls and when restricting by smoking, BMI, diabetes and statin use (data not shown). There was no difference in lipid levels even after restricting to those without diabetes (data not shown).

Section 8.2.3. *PTPN1* 1484insG and Measures of Adiposity

PTPN1 1484insG also did not appear to be associated with average waist, waist-hip ratio, waist height ratio and BMI in the male controls (Table 8-4).

Table 8-3 Changes in lipid profile with *PTPN1* 1484 Ins G Genotype in male controls. *p-value for difference between wildtype (WT) and heterozygous (Het) categories only, excluding homozygous alternative (HA). TC, Total Cholesterol, HDL-C, HDL-high density lipoprotein Cholesterol, LDL-Cholesterol, NDHL-C, Non HDL Cholesterol, HDLR, HDL/TC ratio, TG, Triglycerides.

Genotype	n	TC mmol/l Median (IQR)	HDL -C mmol/l Median (IQR)	LDL -C mmol/l Median (IQR))	NHDL -C mmol/l Median (IQR)	HDLR Median (IQR)	TG mmol/l Median (IQR)
HA	1	5.11	1.39	3.18	3.72	3.70	1.18
Het	33	5.53 (4.79 -6.01)	1.36 (1.02-1.36)	3.45 (2.75-4.12)	4.13 (3.16-4.91)	4.01 (3.26-5.35)	1.30 (0.92 -1.83)
WT	271	5.34 (4.71-6.02)	1.31 (1.12-1.55)	3.34 (2.74-3.92)	4.02 (3.30-4.72)	3.96 (3.30-4.97)	1.20 (0.85-1.76)
p-value		0.43	0.85	0.68	0.62	0.80	0.44

Table 8-4 Measures of adiposity and *PTPN1* 1484 Ins G genotype in male controls. *p-value for difference between heterozygotes (Het) and wildtype (WT). HA – Homozygous alternative

Genotype	N Controls	Avg Waist (cm) (IQR)	Waist-hip Ratio (IQR)	Waist Height Ratio (IQR)	BMI (IQR)
WT	273	96.00 (89.00-104.28)	0.95 (0.90-1.00)	0.57 (0.53-0.62)	28.73 (26.73-31.97)
Het	34	96.13 (90.34-105.28)	0.93 (0.89-0.99)	0.56 (0.63-0.62)	28.71 (26.33-32.50)
HA	2	108.0	1.06	0.67	33.04
p-value		0.99	0.22	0.63	0.75

Section 8.2.4. *PTPN1* mRNA Levels and Lipid Levels

There was no significant difference in lipid values between the different *PTPN1* mRNA tertiles in male controls (Table 8-5). Similar results were obtained in women (Results not shown)

Table 8-5 *PTPN1* mRNA tertiles and relationship to lipid variables in male controls. TC, Total Cholesterol, HDL-C, HDL-high density lipoprotein Cholesterol, LDL-Cholesterol, NDHL-C, Non HDL Cholesterol, HDLR, HDL/TC ratio, TG, Triglycerides.

PTPN1 mRNA Tertile	n	TC mmol/L Median (IQR)	LDL-C mmol/L Median (IQR)	HDL-C mmol/L Median (IQR)	NHDL-C mmol/L Median (IQR)	HDLR median (IQR)	TG mmol/L median (IQR)
Lowest	105	5.44 (4.73-6.25)	3.46 (2.71-4.18)	1.39 (1.11-1.63)	4.19 (3.14-4.88)	3.96 (3.11-4.00)	1.22 (0.79-1.77)
Mid t	105	5.32 (4.59-5.99)	3.39 (2.75-3.94)	1.34 (1.1-1.51)	3.96 (3.48-.95)	3.99 (3.48-4.95)	1.22 (0.94-1.75)
Highest	105	5.39 (4.74-5.92	3.38 (2.81-3.86)	1.31 (1.13-1.54)	4.04 (3.29-4.67)	3.94 (3.29-4.89)	1.21 (0.77-1.81)
p-value – all tertiles		0.14	0.35	0.52	0.12	0.36	0.39

Section 8.2.5. *PTPN1* mRNA Levels and Measures of Adiposity

There was no difference in measures of adiposity with *PTPN1* mRNA tertiles in male controls (Table 8-6). Similar results were obtained in female controls (Data not shown).

Table 8-6 *PTPN1* mRNA level tertiles and measures of adiposity in male controls

PTPN1 Tertile	n	Average Waist cm Median (IQR)	Average Hip Median (IQR)	Waist-hip Ratio Median (IQR)	Waist Height Ratio Median (IQR)	BMI
Lowest	106	95.75 (90.00-95.75)	100.75 (97.00-105.81)	0.95 (0.90 -1.00)	0.57 (0.53-0.62)	28.74 (16.65-31.51)
Mid	106	93.35 (87.99-103-14)	99.43 (99.00-106.29)	0.95 (0.90-1.00)	0.57 (0.52-0.61)	28.10 (25.49-32.07)
Highest	106	98.60 (89.13-106.10)	102.15 (96.83-107.83)	0.94 (0.90-0.94)	0.58 (0.53 -0.63)	29.30 (26.37-32.33)
P-value all tertiles		0.51	0.31	0.98	0.48	0.37

In women that were not on statins *PTPN1*mRNA tertile was associated with a difference in measures of adiposity with the lowest tertile of *PTPN1*mRNA showing higher average waist, waist-hip ratio, waist height ratio and BMI (Table 8.7). There was no difference in lipid

variables based on mRNA tertiles in women on statins (Data not shown). There was no increased risk of MI based on *PTPN1* tertile in women not on statins (Data not shown) . This was not observed in when all female controls were analysed together or in men (Data not shown).

Table 8-7 *PTPN1*mRNA levels and measures of adiposity in female controls not on statins.

PTPN1 mRNA tertile	n	Average waist Median (IQR)	Waist-Hip Ratio Median (IQR)	Waist-height- ratio Median (IQR)	BMI Median (IQR)
Lowest	33	85.80 (80.51-96.68)	0.823 (0.80-0.86)	0.54 (0.52-0.63)	29.14 (25.05-34.26)
Mid	37	80.65 (74.53-92.34)	0.79 (0.74-0.85)	0.51(0.47-0.58)	26.23 (23.15-29.48)
Highest	39	78.78 (70.80-93.25)	0.80 (0.75-0.86)	0.50 (0.44-0.59)	23.40 (22.14-30.16)
p-value		0.03	0.05	0.02	0.05

Section 8.2.6. Correlation of *PTPN1* mRNA Levels, Lipid Levels and Measures of Adiposity

PTPN1 mRNA did not correlate with lipid levels or measures of adiposity (Table 8-8).

Table 8-8. Spearman's correlation of *PTPN1*mRNA level with lipid and measures of adiposity in male controls, n=316

Variable	Spearman's rho	p-value
Total cholesterol	-0.45	0.43
LDL cholesterol	-0.22	0.71
HDL cholesterol	-0.16	0.78
Non-HDLC	-0.38	0.50
HDLR	-0.02	0.79
Triglycerides	-0.05	0.36
Average waist	-0.07	0.25
Waist-hip ratio	-0.53	0.35
BMI	-0.09	0.09

Section 8.2.7. *PTPN1* mRNA levels and 1484insG Genotype and Prevalence of Diabetes

There was no significant difference in prevalence of diabetes between different *PTPN1*mRNA tertiles using Chi square test (X^2 = 0.250, df=2, p-value-0.907) in male controls (Table 8-9). Similar results were obtained in female controls (results not shown)

Table 8-9 Prevalence of diabetes with *PTPN1* 1484 ins G mRNA tertile in male controls.

PTPN1 mRNA tertile	Diabetes n (%)	No Diabetes n (%)
Lowest tertile	12 (31.6%)	88 (32.8%)
Mid tertile	12 (31.6%)	92 (34.3%)
Highest tertile	14 (36.8%)	88 (32.8%)
	(X^2 = 0.250, df=2, p-value-0.91).	

There was also no difference in the prevalence of diabetes with *PTPN1* genotype using the Fisher exact test in male controls (Table 8-10). A similar result was obtained in female controls.

Table 8-10 Effect of *PTPN1* 1484 ins G genotype on prevalence of diabetes

PTPN1 genotype	Diabetes n (%)	No Diabetes n (%)
Heterozygote	3 (8.8%)	31 (91.2%)
Wildtype	35 (13.4%)	227 (86.6%)
	p-value-0.59	

Section 8.2.8. *PTPN1* HTS

The *PTPN1* on HTS had 2 synonymous polymorphisms rs2230604 and rs2230605 on NGS sequencing of *PTPN1*

Section 8.3. Discussion

PTPN1 1484InsG and *PTPN1* levels were not associated with risk of MI, with measures of adiposity, lipid levels or diabetes in men. However, in women that were not on statins *PTPN1*mRNA tertile was associated with a difference in measures of adiposity with the lowest tertile of *PTPN1*mRNA showing higher average waist, waist-hip ratio, waist height ratio and BMI but was not associated with an increased risk of MI. There was no difference in lipid variables based on mRNA tertiles in women on statins. This observation was not observed in when all female controls were analysed together. Possible reasons for this could be statins and *PTPN1* could be interacting in such a way that the effect of the low *PTPN1* mRNA levels is only seen when subjects are not taking statins.

There are many reasons to explain the lack of reproducibility of association studies involving the *PTPN1* gene. *PTPN1* may be influenced by a large number of genetic and environmental factors and their interactions and the genetic and environmental factors will differ between populations (Li and Meyre 2012). Furthermore, the general effect of *PTPN1* 1484insG polymorphism's general effects are small and would require a large study populations to detect an effect.

Failure to replicate the results observed in the SMILE study may also be due to true heterogeneity between populations. There is often a different magnitude of effect between small and large studies. It is quoted that more than 10,000 individuals are needed to overcome genuine genetic heterogeneity since risk due to genetic polymorphisms are often with OR values of <1.50 (Ioannidis et al. 2003). Confounding can occur if cases and controls are drawn from different backgrounds (Colhoun et al. 2003). In some association studies, when no expected main effect is observed, post-hoc subgroup analyses in order to find a positive association may be spurious (Colhoun et al. 2003).

Another factor which may help explain lack of reproducibility is that linkage disequilibrium varies with the ethnic background. Disease associated SNPs from GWAS are usually proxy markers and not causal. Alternatively some of these SNPs may be causal but only associated in an ethnic-specific manner (Gambaro et al. 2000). Due to this there is a risk that negative replication may lead to the rejection of a true association in that specific population.

Besides genetic heterogeneity one must be cautious about phenotypic heterogeneity which may also explain lack of replication. Even analysing a variable in a different way, for example in a quantitative as opposed to a categorical manner may give rise to conflicting results. (Groves et al. 2006).

Two synonymous polymorphisms rs2230604 and rs2230605 were found on NGS sequencing of *PTPN1*. .Despite being synonymous rs2230604 has been associated with increased BMI and higher HDL levels in Chinese children (Mo et al., 2010) and with cellular responses to the influenza vaccine (Ovsyannikova et al., 2014) but was not associated with diabetes in South Indians (Bodhini et al., 2011). A metanalysis performed in 2016 showed that rs2230604 was reversely correlated with T2DM (Wang, Cui and Lan., 2016). This polymorphism was present in 3 of the samples. rs2230605 on the other hand was associated with increased risk of diabetes susceptibility in a recent metanalysis (Wang, Cui and Lan., 2016).

Section 8.4. Conclusion

PTPN1 1484insG genotype and *PTPN1* levels are not associated with risk of MI, lipid levels, diabetes and measures of adiposity in men. However, the lowest *PTPN1* mRNA level terile were associated with higher WHR, WHtR and BMI in women that were not on statins. An important conclusion that emerges from this study and from reviewing other conflicting studies

on individual SNPs is that a more integrated approach is required to analyse combinations of polymorphisms, together with lifestyle/environmental influences when possible.

Chapter 9. Familial Hypercholesterolaemia

Section 9.1. Familial Hypercholesterolaemia

Familial hypercholesterolaemia (FH) is a genetic cause of high LDL-C levels which increases risk of CAD 13-fold if not treated (Nordestgaard et al. 2013). Familial hypercholesterolaemia is underdiagnosed and undertreated despite having an easily measurable biomarker (LDL-C) and well tolerated, widely available treatment (Defesche et al. 2017). Little is known regarding the prevalence of FH in the Maltese population in whom the genetics of FH has not yet been studied.

FH is an important cause of premature CAD, heterozygotes usually develop CAD by the age of 55 years in men and 60 years in women. TC in heterozygotes can be expected to be between 8-15mmol/L. Homozygotes have TC levels between 12-30mmol/L and may die before they reach 20 years of age if they are not identified and treated early. TG and HDL-C levels are usually unremarkable (Nordestgaard et al. 2013). In Caucasian populations, heterozygosity for FH is present in 1/500 while 1/1,000,000 are thought to be homozygous. However, in the absence of studies on prevalence in the general population these estimates may be incorrect and a higher prevalence may be found in populations prone to founder effect (Nordestgaard et al. 2013). The Maltese population is thought to be one of the populations that exhibits the founder effect and this could result in a higher frequency of pathogenic variants that are rare elsewhere (Farrugia et al. 2007; Livingstone 2009).

FH occurs due to defects in genes involved in LDL endocytosis resulting in higher plasma concentrations due to decreased uptake of LDL from the circulation into the liver. Endocytosis of LDL usually occurs in the peripheral circulation via the LDL receptor (LDLR) or the LDL receptor adaptor protein (LDLRAP). After endocytosis of LDL, the LDL receptors are usually recycled and transported back to the cell surface. If proprotein convertase subtilisin/kexin type 9 (PCSK9) attaches to the LDLR it results in LDLR breakdown and decreased LDLR on the

cell surface. Excess LDL-C in the circulation results in uptake by non-hepatic cells including macrophages in the arterial intima leading to increased plaque formation. FH due to variants in *LDLR*, *APOB* or *PCSK9* have an autosomal dominant pattern of inheritance (Defesche et al. 2017). Loss of function variants in *LDLR* constitute >90% of cases of FH, variants in the LDLR binding domain of ApoB to which LDLR usually binds constitute 5% of cases while gain of function of PCSK9 resulting in increased clearance of LDL receptors by the cell account for 1 % of cases (Talmud et al. 2013). Rarely variants in *APOE* and *STAP1* (signal transduction adaptor protein-1) have also been associated with autosomal dominant FH (Defesche et al. 2017)

Variants in *LDLRAP1* are a cause of autosomal recessive hypercholesterolaemia (ARH) (Eden et al. 2001; Garcia et al. 2001), which is rare, but is more prevalent in Sardinia (Arca et al. 2002). ARH has also been described with variants in adenosine triphosphate-binding cassette sub-family G (ABCG), ABCG5 and ABCG8 and lysosomal acid lipase but these cases are extremely rare (Berge et al. 2000; Lu et al. 2001; Lee et al. 2012; Defesche et al. 2017).

Compound heterozygous variants in either the *LDLR* or *LDLRAP1* can give rise to a phenotype resembling homozygous FH (Cuchel et al. 2014). Some rare subjects are double heterozygotes, with variants in two alleles of two different genes leading to a phenotype that is intermediate between heterozygous and homozygous FH (Cuchel et al. 2014).

Relatives of patients having the same variant as a case with FH may not have a clinical diagnosis, but their LDL levels will still be higher than relatives without the variant. On the other hand, patients with a genetic diagnosis that do not fulfil the clinical criteria may have other protective genes or may be controlling their levels using lifestyle measures (Nordestgaard et al. 2013).

The *LDLR* gene spans 45 kilobases, has 18 exons (Brown and Goldstein 1974), and maps to the short arm of chromosome 19 at 19p13.1-p13.3 (Lindgren et al. 1985). The 860-amino acid LDL receptor protein removes LDL from plasma (Austin et al. 2004b). To date 3559 variants have been described in the LDLR gene (Leigh et al. 2017). The *APOB* gene contains 29 exons and spans 43 kilobases and is located on chromosome 2p24.1 (Knott et al. 1985; Law et al. 1985). The protein product of ApoB is 4536 amino acids long and serves as a ligand for the LDL receptor protein (Innerarity et al. 1990). PCSK9 was localized to chromosome 1p32 and has 12 exons (Abifadel et al. 2008a).

The Dutch Lipid Network Criteria (DLNC) is a validated set of criteria based on the patient's family history of premature CVD in first degree relatives, their own CVD history, their untreated lipid levels and physical signs such as the presence of tendon xanthomata or arcus cornealis, prior to the age of 45 years. The subsequent score categorizes patients by the likelihood of (FH) diagnosis (Austin et al. 2004a).

FH-causing variants can be detected in 20-30% of patients with possible FH and 60-80% of patients with definite FH (Graham et al. 2005; Humphries et al. 2006; Taylor et al. 2010; Futema et al. 2014). A large proportion of patients with FH have the clinical features of FH but will not be found to have one of the known variants. These cases may involve variants in genes which have not yet been associated with FH or may have polygenic causes which do not include the key genes described above (Nordestgaard et al. 2013).

Additional loci which affect the concentration of LDL-C have been identified using GWAS studies and their clinical relevance proven in genetic risk scores (Teslovich et al. 2010; Talmud et al. 2013). In founder populations a small number of variants are expected to account for the majority of molecular diagnoses. For example 3 specific *LDLR* variants account for almost 80% of FH cases in the Finnish population while 4 *LDLR* variants account for nearly 50% of patients with FH in the Netherlands and 81.5% of Lebanese FH patients are explained by a

single *LDLR* variant p.Cys68X (Abifadel et al. 2009; Lahtinen et al. 2015), in non-founder populations a molecular variant is only identified in 50% of individuals with clinically diagnosed FH (Talmud et al. 2013).

There is no data regarding FH in the Maltese population. There is no published data regarding clinical scoring using DLNC or other commonly used scores in Maltese subjects. Furthermore, there is no data regarding variants causing elevated LDL-C in the Maltese population. By using the DLCN criteria to identify high risk subjects and carrying out high throughput sequencing (HTS) of causative genes, genetic causes for elevated LDL-C will be studied in the Maltese population. This will help assess the degree of influence of monogenic or polygenic causes of elevated cholesterol levels in the MAMI Study.

Section 9.2. Results

Section 9.2.1. DLCN Scores in the MAMI Study

The distribution of the DLCN scores for controls, cases and relatives in the MAMI study can be found in Table 9-1. Only 1% of cases had probable FH using the DLCN score while no controls had probable FH. None of the research subjects had definite FH.

Table 9-1 Distribution of DLCN scores in the MAMI study in controls, cases and relatives. None of the research subjects had definite FH.

	Unlikely FH	Possible FH	Probable FH
Control	89 (84%)	17 (16%)	0
Case	165 (70%)	67 (29%)	3 (1%)
Relative	37 (69%)	17 (31%)	0

Section 9.2.2. Research Subjects chosen for HTS FH Analysis

A total of 10 research subjects were chosen for HTS of the *LDLRAP1*, *LDLR*, *APOB* and *PSCK9* genes from the MAMI study. These subjects were chosen based on the DLCN score,

their lifetime LDL levels and family history of high LDL or premature CVD. An 11th subject was chosen since he had a history of premature CVD having sustained his first MI at the age of 32 and a second MI at 33 years of age requiring a coronary artery bypass. Clinical characteristics of the research subjects chosen can be found in Table 9-2.

Table 9-2 Clinical characteristics of the subjects chosen for NGS to assess FH related genes.

Sample	Age (yrs.)	Sex	LDL-C (mmol/L)	Lipid lowering therapy	Family History CHD/High LDL	Premature CVD	Corneal arcus
1	40	Female	4.05	No	No	Yes	Yes
2	60	Male	7.05	No	No	No	No
3	47	Male	6.35	No	Yes	Yes	No
4	53	Male	7.65	No	No	Yes	No
5	39	Male	3.90	No	No	Yes	Yes
6	55	Male	9.25	NA	NA	Yes	NA
7	56	Male	6.31	Yes	Yes	No	No
8	73	Male	4.86	No	Yes	No	No
9	61	Male	5.4	No	Yes	No	No
10	60	Female	5.4	Yes	Yes	No	No
11	63	Male	9.97	Yes	No	Yes	No

Section 9.2.3. Selection of Polymorphisms related to FH

A summary of the SNPs found in *LDLR*, *LDLRAP*, *APOB*, *PCKS9* is provided in Table 9-3. Variants in Table 9-4 were ultimately not selected because they were either very common variants or they were in LD with variants that were selected. The variant p.I4314VI was not amongst those tested further, however it should be in the future since it is a rare variant.

Table 9-3 Summary of SNPs found *in LDLR, LDLRAP, APOB* and *PSCK9* in the 11 samples selected.

Variant type		Gene			
		APOB	*LDLR*	*LDLRAP1*	*PCSK9*
Noncoding variants		27	45	41	59
	Splice	0	0	0	0
	3'UTR	2	2	8	1
	5'UTR	0	0	0	1
	Intronic	25	43	33	57
Coding variants		16	10	4	7
	Synonymous	2	7	1	2
	Missense	13	1	3	4
	Frameshift	0	2	0	0
	In-Frame	1	0	0	1
	Stop-gained	0	0	0	0
	Stop-lost	0	0	0	0
Total		43	55	45	66

Table 9-4 Polymorphisms that were initially shortlisted but finally not selected for KASP genotyping for the 11 samples selected for FH analysis. *ClinVar (NCBI 2019a) last accessed 9.19

Function	Zygosity	Gene	rs number	Amino Acid Change	Comments	ClinVar*
Missense	Heterozygous	APOB	rs1042034	p.S4338SN	Very common	Benign
Missense	Heterozygous	APOB	rs72654423	p.I4314VI	Rare Should be studied	Conflicting
Missense	Heterozygous	*APOB*	rs386512865	p.E4181EK	Very common	Likely benign
					Associated with elevated oxLDL but not with MI	
Missense	Heterozygous	*APOB*	rs676210	p.P2739LP	Very common	Benign
Missense	Heterozygous	*APOB*	.	p.R2644SR	LD with V730I	
Missense	Heterozygous	*APOB*	rs679899	p.A618VA	Too common	Benign
Missense	Homozygous	*APOB*	rs1367117	p.T98I	Too common	Benign
In-Frame	Heterozygous	*APOB*		p.L12_L14del	Too common.	Uncertain

Genetic variants that were selected for further analysis included: one frameshift variant and one missense variant were identified in *LDLR,* one in-frame variant and two missense variants were identified in *PCSK9*, four missense variants in *APOB* and 2 missense variants in *LDLRAP1* (Table 9-5). The allele frequency of these variants can be found in Table 9-6. These variants were not consistently associated with FH in the literature.

Table 9-5 Selected FH candidate SNPs *Zygosity: 1. homozygous wild type, 2. heterozygous, 3. homozygous mutant. *Frequency in Vietnamese Genetic Variation Database **Genetic variation in the Estonian population.

rs number	NA	rs11669576	rs35574083	rs11583680	rs505151	rs12713681	rs533617	rs12691202	rs72653059	rs121908326	rs41291058
Gene	LDLR	LDLR	PCSK9	PCSK9	PCSK9	APOB	APOB	APOB	APOB	LDLRAP1	LDLRAP1
Polymorphism	P608Lfs p.F609delinsFGfs	1171G>A	65_66insCTG	158C>CT	2009G>A	6895G>CG	5768A>AG	2188G>A	c607A>AC	605>AC	712C>CT
Amino acid change		A391T		A53V	G670E	D2299H	H1923R	V730I	I203V	S202H	R238RW
Variant type	Frameshift	Missense	In-frame	Missense	Missense	Missense	Missense	Missense	Missense	Missense	Missense
Zygosity* Sample 1	1	1	1	1	3	1	1	1	1	1	1
Sample 2	1	1	1	1	2	1	1	1	1	1	1
Sample 3	1	1	2	2	3	1	1	1	1	1	1
Sample 4	1	1	2	1	3	1	1	1	1	1	1
Sample 5	1	1	2	1	3	1	1	1	1	1	1
Sample 6	1	2	1	1	3	1	1	2	1	1	1
Sample 7	1	1	1	1	2	1	1	1	1	1	1
Sample 8	1	1	2	2	3	2	2	1	2	1	2
Sample9	1	1	2	2	3	1	1	1	1	1	1
Sample 10	1	1	1	1	3	1	1	2	1	1	1
Sample 11	2	1	2	2	3	1	1	1	1	2	1
GnomAD European	Not described	A=0.048	0.10*	T=0.134	A=0.960	C=0.999	C=0.0324	T=0.0271	C=0.000	A=0.0005	T=0.051
GnomAD African	Not described	A=0.181	0.096**	T=0.007	A=0.737	C=0.978	C=0.008	T=0.0059	C=0.012	A=0.004	T=0.008

Table 9-6 Allele frequency in cases and controls (overall, not restricted by sex)

Gene/ rs number	Cases	Controls
LDLR rs11669576	0.06	0.05
PCSK9 rs11583680	0.15	0.13
PCSK9 rs505151	0.04	0.04
APOB rs12713681	0.008	0.016
APOB rs533617	0.04	0.02
APOB rs12691202	0.04	0.03
APOB rs72653059	0.006	0.01
LDLRAP1 rs41291058	0.06	0.06

Section 9.2.4. *LDLR* Frameshift Variant Analysis

Two frameshift variants in the LDLR gene at position 19: 11227650 (p.P608Lfs) and 19:11227655 (p.F609delinsFGfs) were called in sample 11. Both were in a heterozygous state. An image of the reads involving the frameshift variants on the Integrative Genomics Viewer (IGV) can be found in Figure 9-1. (Robinson et al. 2011)

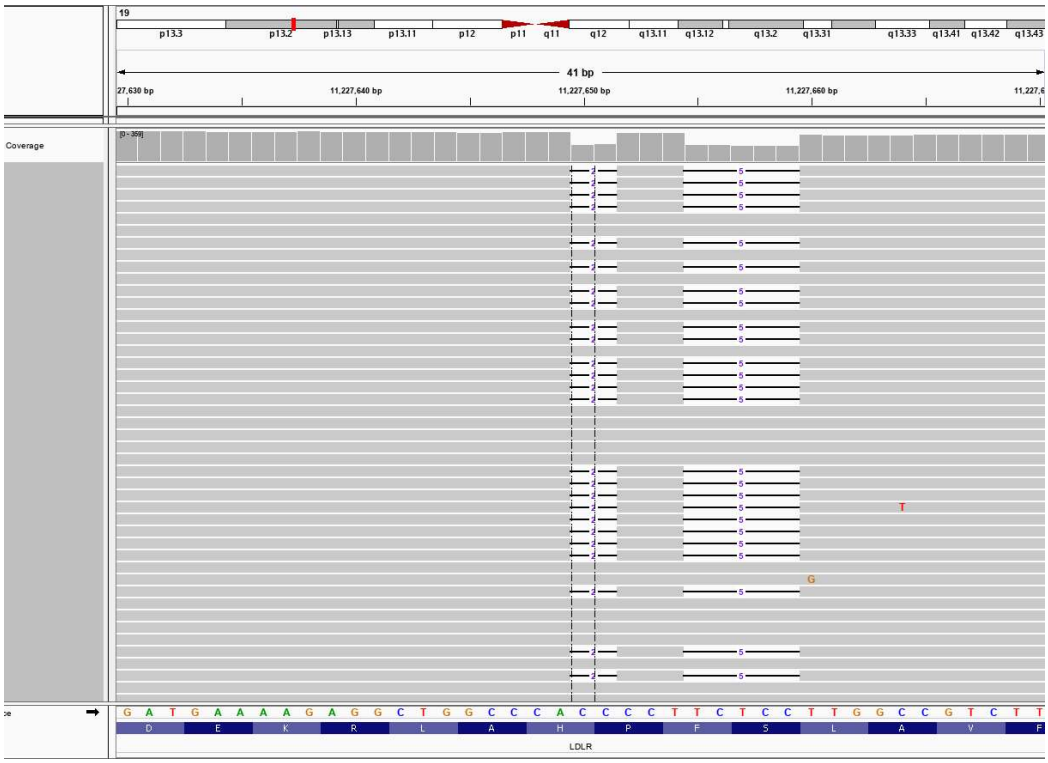

Figure 9-1 Integrative genomics viewer (IGV) image of frameshift variant in *LDLR* gene of sample 11.

The nucleotide sequence was looked up on Ensembl Browser Homo Sapiens GRCh37. The transcript selected was the longest canonical gene transcript ENST00000558518.1. EMBL-EBI EMBOSS Transeq tool was used to translate the nucleotide sequence to the protein amino acid sequence. The wildtype nucleotide sequence of Exon 12 with bases deleted by the frameshift variants denoted in red are depicted in Figure 9-2. The full gene has 18 exons. The amino acid bases deleted by the frameshift variants are highlighted in grey in Figure 9-3.

Exon 12. Genomic location 11227535-11227674

ATCTCCTCAGTGGCCGCCTCTACTGGGTTGACTCCAAACTTCACTCCATCTCAAG
CATCGATGTCAACGGGGGCAACCGGAAGACCATCTTGGAGGATGAAAAGAGGCT
GGCCCACCCCTTCTCCTTGGCCGTCTTTGAG

Figure 9-2 Wildtype nucleotide sequence with bases deleted by the frameshift variants highlighted in grey. Gene accession number ENSG00000130164

Amino acid 1 - 661

MGPWGWKLRWTVALLLAAAGTAVGDRCERNEFQCQDGKCISYKWVCDGSAECQDGSDESQETCLSVTCKSGDF
SCGGRVNRCIPQFWRCDGQVDCDNGSDEQGCPPKTCSQDEFRCHDGKCISRQFVCDSDRDCLDGSDEASCPVLTC
GPASFQCNSSTCIPQLWACDNDPDCEDGSDEWPQRCRGLYVFQGDSSPCSAFEFHCLSGECIHSSWRCDGGPDCKD
KSDEENCAVATCRPDEFQCSDGNCIHGSRQCDREYDCKDMSDEVGCVNVTLCEGPNKFKCHSGECITLDKVCNMA
RDCRDWSDEPIKECGTNECLDNNGGCSHVCNDLKIGYECLCPDGFQLVAQRRCEDIDECQDPDTCSQLCVNLEGG
YKCQCEEGFQLDPHTKACKAVGSIAYLFFTNRHEVRKMTLDRSEYTSLIPNLRNVVALDTEVASNRIYWSDLSQRM
ICSTQLDRAHGVSSYDTVISRDIQAPDGLAVDWIHSNIYWTDSVLGTVSVADTKGVKRKTLFRENGSKPRAIVVDP
VHGFMYWTDWGTPAKIKKGGLNGVDIYSLVTENIQWPNGITLDLLSGRLYWVDSKLHSISSIDVNGGNRKTILEDE
KRLAHLWPSLRTKYFGQISSTKPFSVPTASQVPMSTCWLKTYCPQRIWFSSTTSPSQEE*

Figure 9-3 Variant protein sequence. The amino acids highlighted in grey show the protein sequence obtained with the frameshift variant. The asterix denotes the first stop codon in the variant sequence. Protein accession number ENST00000558518.1

Section 9.2.5. *PCSK9* CTG Repeat Analysis

The in-frame variant (rs35574083) identified in PCSK9 is a Leu insertion in a stretch of 9 Leucine amino acids (CTG bases). It was viewed with the IGV viewer. A sample of the output for 3 samples can be seen in Figure 9-4.

Figure 9-4 Integrative genomics viewer output showing an extra CTG repeat in PCSK9 relative to the Reference Human Genome.

All 7 FH samples with the in-frame variant have a single insertion of CTG, which results in an additional Leucine amino acid being inserted into the sequence. These individuals have 10 Leucine amino acids instead of the 9 Leucine amino acids usually present in the wildtype.

Section 9.2.6 Selection of genetic variants for KASP analysis

Following a literature search rs41291058, rs11583680, rs505151, rs11669576, rs12713681, rs533617, rs12691202, rs72653059 were genotyped in all the MAMI Study samples using KASP. The polymorphisms rs35574083 and rs121908326 were not genotyped due to KASP failure.

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Section 9.2.7. Effect on Lipids of Selected Polymorphisms

The effect of each variant on lipid profile was assessed in male controls. Heterozygotes for rs11669576 in LDLR had increased median LDL-C in male controls (3.74mmol/L) vs wildtype individuals (median 3.33mmol/L, p value 0.03). There was also a pattern towards increased triglycerides in heterozygous individuals (median 1.51 mmol/L in HET, 1.20 mmol/L in WT, p-value 0.07). Heterozygotes for this polymorphism also had increased NHDL-C levels (median 4.50 mmol/l in heterozygotes, 3.97mmol/L in WT individuals, p value 0.01) (Table 9-7). The same patterns were observed after restricting to male controls off statins although numbers were not sufficient to reach statistical significance (Table 9-8).

Median triglyceride levels were lower in heterozygotes for rs533617 in ApoB compared to that in the group with wildtype genotype for this SNP (median 0.87mmol/L for heterozygous genotype and 1.26mmol/L for WT genotype, p value 0.03) (Table 9-7). This association was present even in male controls off statins (Table 9-8). Heterozygotes had lower median NHDL-C levels reaching statistical significance in the group off statins (median in heterozygotes-3.61mmol/L vs 4.17 mmol/L in WT, p value 0.04) (Table 9-7).Analysis in females was also limited by small numbers and showed no significant associations (Data not shown). Effects on remainder of lipid profile and on Hs-CRP can be seen in appendix A1-7 till A1-16.

Table 9-7 Change in lipid variables with selected FH related polymorphisms in male controls. LDL-C, Low density lipoprotein cholesterol; TG, triglycerides; NHDL-C, non-high density lipoprotein cholesterol; IQR, Interquartile range* Highlights significant p-values.

Gene (rs number) Amino acid change	Genotype	N (%)	LDL-C (mmol/L) (IQR)	p-value	TG (mmol/L) (IQR)	p-value	NHDL-C (mmol/L) (IQR)	p-value
*LDLR (*rs11669576)	HET	27 (8.6)	3.74 (3.22-4.21)	0.03 *	1.51 (1.07-1.88)	0.07 *	4.50 (4.00-4.97)	0.01 *
A391T	WT	288 (91.4)	3.33 (2.72-3.92)		1.20 (0.84-1.74)		3.97 (3.24-4.72)	
PCSK9 (rs11583680)	HA	5 (1.6)	2.86	0.34	1.70 (0.98-2.47)	0.38	3.36 (3.07-4.90)	0.64
A53V	HET	76 (24.3)	3.40 (2.61-4.07)	0.73	1.18 (0.86-1.78)	0.96	3.99 (3.13-4.82)	0.88
	WT	232 (74.1)	3.39 (2.81-3.95)		1.24 (0.85-1.76)		4.04 (3.30-4.72)	
PCSK9 (rs505151)	HA	1 (0.3)	3.85		2.04		4.78	
G67OE	HET	21 (6.7)	3.26 (2.73-3.97)	0.85	0.93 (0.76-1.84)	0.23	3.98 (3.11-4.60)	0.57
	WT	293 (93.0)	3.39 (2.74-4.05)		1.25 (0.87-1.75)		4.04 (3.29-4.80)	
APOB (rs12713681)	HET	7 (2.2)	3.75 (3.17-5.31)	0.14	1.30 (1.12-1.54)	0.74	4.40 (3.66-6.01)	0.23
D2299H	WT	311(97.8)	3.38 (2.74-4.00)		1.25 (0.87-1.76)		4.03 (3.25-4.77)	
APOB (rs533617)	HA	1 (0.3)	4.12		1.63		4.86	
H1923R	HET	12 (3.8)	3.18 (2.87-3.60)	0.43	0.87 (0.68-1.25)	0.03 *	3.67 (3.19-4.33)	0.20
	WT	306 (95.9)	3.40 (2.74-4.04)		1.26 (0.87-1.79)		4.05 (3.29-4.79)	
ApoB (rs12691202)	HET	15 (4.7)	3.35 (2.56-3.78)	0.78	1.16 (1.00-2.45)	0.44	4.01 (3.59-4.48)	0.82
V730I	WT	303 (95.3)	3.39 (2.74-4.04)		1.26 (0.85-1.76)		4.04 (3.25-4.79)	
ApoB (rs72653059)	HET	7 (2.2)	3.75 (3.17-5.31)	0.15	1.30 (1.12-1.54)	0.72	4.40 (3.66-6.01)	0.23
I203V	WT	310 (97.8)	3.39 (2.74-4.00)		1.23 (0.85-1.75)		4.04 (3.27-4.77)	
LDLRAP1 (rs41291058)	HA	1 (0.3)	4.38		2.46		5.50	
R238W	HET	42 (13.3)	3.39 (2.68-3.79)	0.53	1.20 (0.85-1.97)	1.00	3.96 (3.11-4.66)	0.61
	WT	273 (86.4)	2.74 (2.74-4.05)		1.25 (0.85-1.74)		4.04 (3.30-4.78)	

Table 9-8 Change in lipid variables with selected FH related polymorphisms in male controls off statins. LDL-C, Low density lipoprotein cholesterol; TG, triglycerides; NHDL-C, non-high density lipoprotein cholesterol; IQR, Interquartile range * Highlights significant p-values.

Gene (rs number) Amino acid change	Genotype	N (%)	LDL-C (mmol/L) (IQR)	p-value	TG (mmol/L) (IQR)	p-value	NHDL-C (mmol/L) (IQR)	p-value
*LDLR (*rs11669576)	HET	20 (7.9)	3.80 (3.24-4.23)	0.14	1.45 (1.06-1.88)	0.20	4.51 (3.83-5.08)	0.08*
A391T	WT	234 (92.1)	3.44 (2.87-4.13)		1.18 (0.82-1.74)		4.08 (3.37-4.86)	
PCSK9 (rs11583680)	HA	5 (2.0)	2.86 (2.50-3.78)		1.70 (0.98-2.47)		3.36 (3.07-4.90)	
A53V	HET	64 (25.2)	3.53 (2.81-4.18)	0.89	1.15 (0.81-1.74)	0.78	4.27 (3.57-4.92)	0.98
	WT	185 (72.8)	3.47 (2.94-4.13)		1.21 (0.84-1.74)		4.10(3.40-4.85)	
PCSK9 (rs505151)	HA	1 (0.4)	3.85		2.04		4.78	
G67OE	HET	18 (7.1)	3.32 (2.75-4.04)	0.46	0.95(0071-1.89)	0.35	4.02 (3.07-4.73)	0.38
	WT	236 (92.5)	3.47 (2.93-4.19)		1.19(0.86-1.73)		4.14 (3.43-4.87)	
APOB (rs12713681)	HET	6 (2.3)	3.81	0.14	1.03	0.67	4.40	
D2299H	WT	251 (97.7)	3.46 (2.89-4.15)		1.20 (0.84-1.74)		4.13 (3.39-4.86)	0.22
APOB (rs533617)	HA	1 (0.4)	4.12		1.63		4.86	
H1923R	HET	11 (4.3)	3.14 (2.85-3.41)	0.12	0.87 (0.67-1.18)	0.01*	3.61 (3.18-4.10)	0.04*
	WT	246 (95.3)	3.50 (2.92-4.18)		1.23 (0.86-1.76)		4.17 (3.44-4.88)	
APOB (rs12691202)	HET	11 (4.3)	3.66 (3.14-4.24)	0.51	1.12 (0.99-1.22)	0.49	4.17 (3.59-4.77)	0.83
V730I	WT	246 (95.7)	3.46 (2.89-4.15)		1.24 (0.84-1.74)		4.13 (3.38-4.86)	
APOB (rs72653059)	HET	6 (2.3)	3.81 (3.31-5.39)	0.14	1.30 (1.03-1.84)	0.66	4.40 (3.77-6.22)	0.22
I203V	WT	251 (97.7)	3.46 (2.89-4.15)		1.19 (0.84-1.74)		4.13 (3.39-4.86)	
LDLRAP1 (rs41291058)	HA	1 (0.4)	4.38		2.46		5.50	
R238W	HET	34 (13.3)	3.44 (2.80-3.87)	0.46	1.15 (0.80-1.91)	0.84	3.99 (3.12-4.76)	0.46
	WT	220 (86.3)	3.46 (2.96-4.17)		1.21 (0.84-1.73)		4.14 (3.45-4.86)	

Section 9.2.8. Odds Ratios of Selected FH Polymorphisms

Odds ratios were calculated using logistic regression restricted by sex and by use of lipid-lowering therapy. A pattern towards a two-fold increase in risk was observed for the *APOB* polymorphism G2188A (rs12691202) in males off statins though the 95% CI includes one (0.9-4.1) (Table 9-9). Some of the odds ratios changed upon restriction by age (below and above 60 years in men (Table 9-10). The *LDLR* G1171A and the *APOB* G2188A polymorphisms tended to be associated with risk for MI only in the higher age group, though the confidence intervals include 1 and therefore this cannot be stated with certainty. However, there is no risk for these SNPs in the younger age group. Heterozygosity for the polymorphism in *APOB* A5768G was associated with a 3.7-fold increased risk of MI (95%CI 1.0-14.6) only in the older age group. Heterozygosity for *LDLRAP1* C712T was associated with a decreased risk of MI [Adjusted OR 0.4 (95%CI 0.2-0.9)]. Once again, this effect was only evident in the older age group. There were also no associations with MI in women off statins (Data not shown).

Table 9-9 Age adjusted OR for men off statins. HET-heterozygous, WT-Wildtype, HA-homozygous alternate.

Gene (rs number) Amino acid change	Genotype	Cases	Controls	AgeAdjOR(95%CI)
LDLR (rs11669576)	HET	24 (11.2)	20 (7.9)	1.5 (0.8-2.8)
G1171	WT	190 (88.8)	234 (92.1)	1
PCSK9 (rs11583680)	HA	4 (1.9)	5 (2.0)	0.9 (0.2-3.6)
C158T	HET	53 (25.0)	64 (25.2)	1.0 (0.6-1.5)
	WT	155 (73.1)	185 (72.8)	1
PCSK9 (rs505151)	HA	0 (0.0)	1(0.4)	
G2009A	HET	18 (8.5)	18(7.1)	1.2 (0.6-2.5)
	WT	194 (91.5)	236 (92.5)	1
APOB (rs12713681)	HET	1 (0.5)	6 (2.3)	0.2 (0.0-1.5)
G6895C	WT	213 (99.5)	251 (97.7)	1
APOB (rs533617)	HA	0 (0.0)	1 (0.4)	
A5768G	HET	13 (6.2)	11 (4.3)	1.5 (0.7-3.4)
	WT	198 (93.8)	246 (95.3)	1
APOB (rs12691202)	HET	18 (8.5)	11 (4.3)	1.9 (0.9-4.1)
G2188A	WT	194 (91.5)	246 (95.7)	1
APOB (rs72653059)	HET	1 (0.5)	6 (2.3)	0.2 (0.02-1.5)
I203V	WT	212 (99.5)	251 (97.7)	
LDLRAP1 (rs41291058)	HA	0 (0.0)	1 (0.4)	
C712T	HET	18 (8.5)	34 (13.3)	1.0 (0.6-1.5)
	WT	193 (91.5)	220 (86.3)	1

Table 9-10 Odds ratios adjusted for age and statin use AdjOR) in male controls overall, <60 years of age and ≥ 60 years of age. HET-heterozygous, WT-wildtype, HA- homozygous alternate.

		Overall			<60			≥60		
Gene (rs number)	Genotype	Cases n (%)	Controls n (%)	AdjOR (95%CI)	Cases n (%)	Controls n (%)	AdjOR (95% CI)	Cases n (%)	Controls n (%)	AdjOR (95%CI)
LDLR (rs11669576)	HET	34 (12.3)	27 (8.6)	1.5 (0.9-2.5)	16 (10.5)	15 (8.8)	1.1 (0.5-2.5)	18 (14.6)	12 (8.3)	2.0 (0.9-4.4)
	WT	242 (87.7)	288 (91.4)	1.0	137 (89.5)	156 (91.2)	1.0	105 (85.4)	132 (91.7)	1.0
PCSK9 (rs11583680)	HA	7 (2.6)	5 (1.6)	1.6 (0.5-5.1)	5 (3.3)	3 (1.8)	1.6 (0.4-7.2)	2 (1.6)	2 (1.4)	1.3 (0.2-9.6)
	HET	62 (22.6)	76 (24.3)	0.9 (0.6-1.4)	31 (20.4)	42 (24.9)	0.8 (0.5-1.4)	31 (25.4)	34 (23.6)	1.0 (0.6-1.9)
	WT	205 (74.8)	232 (74.1)	1.0	116 (76.3)	124 (73.4)	1.0	89 (73.0)	108 (75.0)	1.0
PCSK9 (rs505151)	HA	0	1 (0.3)	/	0	1 (0.6)	/	0	0	/
	HET	23 (8.4)	21 (6.7)	1.3 (0.7-2.4)	11 (7.2)	12 (7.0)	1.2 (0.5-2.8)	12 (9.8)	9 (6.3)	1.6 (0.7-4.1)
	WT	252 (91.6)	293 (93.0)	1.0	142 (92.8)	159 (92.4)	1.0	110 (90.2)	134 (93.7)	1.0
APOB (rs12713681)	HET	1 (0.4)	7 (2.2)	0.2 (0.1-1.3)	0	4 (2.3)	/	1 (0.8)	3 (2.1)	0.4 (0.1-4.1)
	WT	276 (99.6)	311 (97.8)	1.0	154 (100)	168 (97.7)	1.0	122 (99.2)	143 (97.9)	1.0
APOB (rs533617)	HA	0	1 (0.3)	\	0	1 (0.6)	\	0	0	\
	HET	17 (6.2)	12 (3.8)	1.7 (0.8-3.7)	9 (5.9)	9 (5.2)	1.2 (0.4-3.1)	8 (6.7)	3 (2.1)	3.7 (1.0-14.6)
	WT	255 (93.8)	306 (95.9)	1.0	144 (94.1)	163 (94.2)	1.0	111 (93.3)	143 (97.9)	1.0
APOB (rs12691202)	HET	20 (7.3)	15 (4.7)	1.6 (0.8-3.1)	10 (6.5)	9 (5.2)	1.0 (0.4-2.7)	10 (8.3)	6 (4.1)	2.0 (0.7-5.7)
	WT	253 (92.7)	303 (95.3)	1.0	143 (93.5)	163 (94.8)	1.0	110 (91.7)	140 (95.9)	1.0
APOB (rs72653059)	HET	1 (0.4)	7 (2.2)	0.2 (0.1-1.3)	0	4 (2.3)	\	1 (0.8)	3 (2.1)	0.4 (0.1-4.2)
	WT	274 (99.6)	310 (97.8)	1.0	154 (100)	168 (97.7)	1.0	120 (99.2)	142 (97.9)	1.0
LDLRAP (rs41291058)	HA	0	1 (0.3)	/	0	1 (0.6)	/	0	0	/
	HET	26 (9.5)	42 (13.3)	0.7 (0.4-1.1)	18 (11.7)	20 (11.7)	0.9 (0.4-1.8)	8 (6.7)	22 (15.2)	0.4 (0.2-0.9)
	WT	248 (90.5)	273 (86.4)	1.0	136 (88.3)	150 (87.7)	1.0	112 (93.3)	123 (84.8)	1.0

Section 9.2.9. Combinations of *LDLR* rs11669576 and for *APOB* rs12691202

Two cases in the MAMI study were heterozygous for *LDLR* polymorphism rs11669576 and for *APOB* rs12691202. Two cases in the MAMI study had this combination. Their lipid profile can be observed in Table 9-11.

Table 9-11 Characteristics of two individuals that were heterozygous for both *LDLR* polymorphism rs11669576 and for *APOB* rs12691202. TC: Total cholesterol; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol.

Study status	Sex	Age	TC (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)	NHDL-C (mmol/L)	HDLR	TRIG (mmol/L)
case	male	67	5.95	4.07	1.03	4.92	5.78	1.88
case	male	53	7.65	5.65	0.84	6.81	9.11	2.56

The effect of having one of these polymorphisms on the lipid profile compared to being wildtype for both was studied (Table 9-12). NHDL-C was higher in male control subjects who were not on statins that were heterozygous for *LDLR* rs11669576 and wildtype for *APOB* rs12691202 when compared to subjects that were wildtype for both polymorphisms although numbers in these groups are small (Table 9-12). Interestingly, the lipid profile of those heterozygous for *APOB* rs12691202 and wildtype for *LDLR* rs11669576 was not different from the group that was wildtype for both genetic variants.

A stratified analysis was conducted to determine the odds ratios calculated for the combination of *LDLR* polymorphism rs11669576 and *APOB* polymorphism rs12691202 in males. The odds ratios for the separate SNPs are both slightly above 1 (1.4 and 1.6 respectively) but the confidence intervals include 1 and therefore the result would need to be confirmed in larger numbers (Table 9-13)

Table 9-12 Combination of *LDLR* polymorphism rs11669576 with *APOB* polymorphism rs12691202 in male controls off statins. HET-heterozygous, WT-wildtype. TC: Total cholesterol; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol; NHDL-C: Non-High density lipoprotein cholesterol: TRIG; Triglycerides.

	LDLR (rs11669576) HET *APOB* (rs12691202) WT n-10	*LDLR* (rs11669576) WT *APOB* (rs12691202) HET n-20	*LDLR* (rs11669576) WT *APOB* (rs12691202) WT n-223
TC (mmol/L)	5.62 (5.28-6.34)	5.30 (4.72-6.01)	5.41 (4.80-6.10)
LDL-C(mmol/L)	3.80 (3.24-4.23)	3.59 (3.12-3.90)	3.42 (2.86-4.14)
HDL-C (mmol/L)	1.31 (1.12-1.46)	1.34 (1.11-1.53)	1.31 (1.11-1.55)
NHDL-C (mmol/L)	4.51 (3.83-5.08) *	4.07 (3.55-4.55)	4.07 (3.36-4.86)
HDLR	4.54 (3.88-5.16)	4.17 (3.83-4.55)	4.00 (3.36-5.05)
TRIG (mmol/L)	1.45 (1.06-1.88)	1.12 (0.93-1.18)	1.20 (0.81-1.74)

Table 9-13 Odds ratios for combinations of *LDLR* polymorphism rs11669576 and *APOB* polymorphism rs12691202.

LDLR rs11669576	*APOB* rs12691202	Cases (n=270)	Controls (n=314)	AgeOR (95%CI)
HET	HET	2 (0.7)	0	/
HET	WT	31 (11.5)	27 (8.6)	1.4 (0.8-2.5)
WT	HET	18 (6.7)	14 (4.5)	1.6 (0.8-3.2)
WT	WT	219	273 (86.9)	1

Section 9.2.10. *APOB* rs12713681 and rs533617 Combinations

One subject from the selected FH subjects for NGS was heterozygous for polymorphisms in *APOB*: rs12713681 and rs533617. Another subject in the MAMI collection was also heterozygous for both these variants. Both were not on statins. Their lipid profile can be seen in Table 9-14.

Table 9-14 Characteristics of individuals that were heterozygous for both rs12713681 and rs533617 in *APOB*. TC: Total cholesterol; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol; NHDL-C: Non-High density lipoprotein cholesterol: TRIG; Triglycerides.

Study status	Sex	Age	TC (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)	NHDL-C (mmol/L)	TRIG (mmol/L)
control	male	64	5.07	3.86	0.67	4.4	1.18
case	female	73	6.8	4.73	1.72	5.08	0.78

Male controls off statins that were heterozygous for *APOB* rs533617 and wildtype for rs12713681 had lower LDL-C levels, NHDL-C levels, HDLR levels and TG levels and higher HDL-C levels than subjects that were wildtype for both polymorphisms, however only 10 subjects had this particular combination (Table 9-15).

Table 9-15 Lipid profile amongst male controls non-statin users for combinations of two polymorphisms present in *APOB* : rs12713681 and rs533617. P-value < 0.01***, < 0.05**, < 0.1*. TC: Total cholesterol; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol; NHDL-C: Non-High density lipoprotein cholesterol: TRIG; Triglycerides. HA-Homozygous alternate, HET-heterozygous, WT – Wildtype.

	rs533617 HA rs12713681 WT (n=1)	rs533617 HET rs12713681 HET (n=1)	rs533617 HET rs12713681 WT (n=10)	rs533617 WT rs12713681 HET (n=5)	rs533617 WT rs12713681 WT (n=238)
TC (mmol/L)	6.06	5.07	5.005 (4.66-5.475)	5.83 (4.905-7.475)	5.47 (4.81-6.2225)
LDL-C (mmol/L)	4.12	3.86	3.055 (2.815-3.32) *	3.75 (3.26-5.46)	3.495 (2.895-4.1725)
HDL-C (mmol/L)	1.2	0.67***	1.7 (1.305-1.905) **	1.15 (0.98-1.42)	1.31 (1.1175-1.53)
NHDL-C (mmol/L)	4.86	4.4	3.49 (3.1375-3.8225) **	4.4 (3.685-6.435)	4.165 (3.4175-4.8725)
HDLR	5.05	7.57**	3.185 (2.5875-3.95) ***	4.75 (3.785-7.305)	4.11 (3.475-5.1225)
TRIG (mmol/L)	1.63	1.18	0.84 (0.6675-1.0375) **	1.42 (0.93-2.14)	1.225 (0.8575-1.76)
hs-CRP (mg/L)	6.28	2.25	1.0445 (0.619-4.4675)	2.28 (1.229-3.955)	1.695 (0.8005-3)

Age adjusted odds ratios for the *APOB* combinations of rs12713681 and rs533617 showing a pattern towards risk in men that are heterozygous for *APOB* rs533617 and wildtype for *APOB* rs12713681can be seen in Table 9-16.

Table 9-16 Age adjusted odds ratios for the *APOB* combinations of rs12713681 and rs533617 in men. HA-homozygous alternate, HET-heterozygous, WT-wildtype.

rs533617	rs12713681	Cases (n=270)	Controls (n=318)	AgeOR (95%CI)
HA	WT	0	1 (0.3)	\
HET	HET	0	1 (0.3)	\
HET	WT	17 (6.3)	11 (3.5)	1.9 (0.9-4.1)
WT	HET	1 (0.4)	6 (1.9)	0.2 (0.1-1.6)
WT	WT	252 (93.3)	299 (94.0)	1.0

In men ≥ 60 years of age the OR for being heterozygous for rs533617 and wildtype for rs12713681 rose to 5.8 (95% CI, 1.2-28.5) in men (Table 9-17).

Table 9-17 Age adjusted odds ratios (AgeOR) and odds ratios adjusted for age and statins for the *APOB* combinations of rs12713681 and rs533617 in men ≥ 60 years of age. HA-homozygous alternate, HET-heterozygous, WT-wildtype.

rs533617	rs12713681	Cases (n=118)	Controls (n=146)	AgeOR (95%CI)	age and statins OR (95%CI)
HET	HET	0	1 (0.7)	\	\
HET	WT	8 (6.8)	2 (1.4)	5.5 (1.1-26.8)	5.8 (1.2-28.5)
WT	HET	1 (0.8)	2 (1.4)	0.8 (0.1-8.5)	0.8 (0.1-8.8)
WT	WT	109 (92.4)	141 (96.6)	1	1

Section 9.3. Discussion

Two adjacent frameshift variants at position 19: 11227650 (p.P608Lfs) and 19:11227655 (p.F609delinsFGfs) on the *LDLR* gene were found in subject 11. This individual is likely to have FH since he had an LDL-C of above 9 mmol/L and had an MI at the age of 32. These frameshift variants have not been described in the literature. Another frameshift variant localised very close to the p.P608Lfs frameshift variant has been previously described (p.Asn688Glnfs [rs137853965]) and is denoted as pathogenic on ClinVar (last updated April 2019) increasing the probability that the novel frameshift variant in research subject 11 is also pathogenic (National Center for Biotechnology Information. 2019). The presence of this frameshift variant should be confirmed with sanger sequencing to exclude the possibility that this was a sequencing or mismapping error. The likelihood that this was a sequencing error however is low since the coverage was around 300 and both wildtype and frameshift alleles had good coverage.

A second polymorphism identified in *LDLR,* rs11669576 (A291T) was present in another of the 11 subjects. Heterozygosity for this polymorphism in the MAMI study was associated with higher LDL-C and NHDL-C and a pattern towards higher triglyceride levels in male controls compared to subjects that were wildtype for this polymorphism. The LDLR A291T variant also tended to be associated with an increased risk of MI, with male heterozygotes having an OR of 1.5 (95% CI, 0.9-2.5) after adjustment for age and statin use. When restricting to males ≥60 years of age the OR reached 2.0 (95% CI, 0.9-4.9) after adjustment for age and statin use. This variant is reported as probably benign (NCBI 2019a) on ClinVar (last evaluated on Aug, 2019) however the findings here indicate that this variant could possibly play a role in raising LDL-C locally. This finding requires replication both in a Maltese cohort and in other populations too as it cannot be excluded that the functional effect is due to a variant in linkage disequilibrium (LD) with this variant in the Maltese population. However this would not be a

variant in the exonic regions of LDLR as the 8 samples in the HTS datasets that had this variant did not exhibit LD with some other exonic LDLR variant. It is indeed likely that this is a functional variant with modest effect that requires reassessment on clinical databases.

The rs533617 (H1923R) polymorphism identified in *APOB* was associated with lower TG in male controls and lower NHDL-C in male controls off statins. Despite this, males, ≥60 years of age, that were heterozygous for this polymorphism had an OR of 3.7 (95%CI, 1.0-14.6) after adjustment for age and statin use, however numbers were small (only 11 samples were heterozygous for this polymorphism in this age group). Rs533617 is denoted as having conflicting interpretations of pathogenicity on ClinVar (last evaluated in Aug, 2017). In the candidate FH samples selected for HTS only one sample was heterozygous for this missense variant. That same sample was also heterozygous for two other ApoB variants: pD2299H (rs1271368) and p.I203V (rs72653059) which were found to be in complete LD in this study since 8 research subjects (7 controls and 1 case) who are heterozygous for rs12713681 are also heterozygous for rs72653059. LD effects with these variants cannot be excluded however neither of these two variants had effects on lipid profile. The OR of these variants is low, but numbers are small and the confidence interval is very wide and includes 1. rs12713681 (D2299H) is denoted as having a conflicting interpretation of pathogenicity on ClinVar (NCBI 2019b).

Another variant in *APOB,* rs12691202 (V730I) showed a pattern towards increased risk in the male subjects off lipid lowering agents, OR 1.9 (95% CI, 0.9-4.1) after age adjustment in males. Two of the selected research subjects were heterozygous for this polymorphism. The variant has no effect on lipid profile, but it is associated with increased hs-CRP levels indicating that this variant might influence inflammation. Apo-B has been reported to inhibit or attenuate the response of Toll-like receptors to bacterial lipopolysaccharide, resulting in lower levels of inflammatory molecules (Bas et al. 2010; van Bergenhenegouwen et al. 2016). Therefore,

some *APOB* variants might be less efficient in attenuating this effect resulting in a stronger inflammatory response. However, the two candidate FH individuals that had this variant also had a rare variant, *APOB* R2644S which is not reported in dbSNP. This combination was present in one other sample in the 101 HTS datasets. Two other samples in the HTS datasets had rs12691202 but not the ApoB R2644S indicating that there is incomplete LD. rs12691202 is denoted as having conflicting interpretations of pathogenicity on ClinVar with 7 reports indicating it is benign, 2 as likely benign and 3 indicating uncertain significance. The *APOB* R2644S polymorphism needs to be assessed in the MAMI Study to determine which of these two variants is the functional one, or whether a combination of both is causing functional effects.

The remaining polymorphisms did not show any associations with lipid variables or with risk of MI. *APOB* p.I203V or rs72653059, which is listed as being likely benign on ClinVar (NCBI 2019d) was heterozygous in 1 research subject.

The in-frame variant (rs35574083) identified in PCSK9 consisted of a repetitive stretch of 9 Leucine amino acids (CTG bases). Since this repetitive stretch only spans 27 bases, Illumina reads of 100bp can easily stretch across the repetitive region, with the read ends being uniquely mapped on either side of this region. In ClinVar there are conflicting interpretations of pathogenicity. The rs35574083 insertion polymorphism occurs in a nine-leucine repeat sequence located at the signal peptide of PCSK9 (Slimani et al. 2015). Alleles of rs35574083 with an in-frame insertion of one leucine, as has been observed in six of the selected FH candidates have been associated with loss of function variants in *PCSK9* and a decrease in LDL-C levels and an increase in HDL-C levels (Yue et al. 2006; Abifadel et al. 2009). In-frame insertion causing the insertion of two leucines have been associated with higher total and LDL-cholesterol in French Canadian patients with familial combined hyperlipidaemia (Abifadel et al. 2008b). This might explain why the functionality of the variant described as

rs35574083 is not yet clarified. It is important to distinguish if there is one or two Leucines inserted. In the current study the effect of this in-frame insertion was not ascertained due to KASP failure.

rs11583680 (A53V) in *PCSK9* is likely benign on ClinVar. There is a 10-fold difference in frequency between Europeans and Africans for this polymorphism. This polymorphism was assessed along with others in a Mendelian Randomisation study based on its association with LDL cholesterol in the Global Lipids Genetics Consortium (Wolffenbuttel et al. 2013; Schmidt et al. 2017). In this study with a sample size of 27,194 research subjects the mean difference in circulating LDL was only -0.02mmol/L (95% CI, 0.03-0.02)(Schmidt et al. 2017). This polymorphism was found to be protective for CHD with OR of 0.97 (95% CI, 0.94-1.00, p value 0.03) when its effects were estimated on 60,801 CHD cases and 123504 controls from the CARDIoGRAMplusC4D Consortium but had no effect on stroke (Hopewell et al. 2018). At such large sample sizes every small difference reaches statistical significance, even though it might not be biologically relevant. In the current study this polymorphism had no major effect on lipid profile or on risk for MI which is in agreement with previous studies.

The *PCSK9* rs505151 has been previously reported to be a gain-of -function variant. This variant located in exon 12, results in an amino acid substitution from glutamate to glycine at position 670 (Ding and Kullo 2008). In a meta-analysis on rs505151 using a dominant model, the G allele of rs505151 was associated with significantly increased cardiovascular risk (OR 1.50, 95%, CI: 1.19-1.89, P=0.0067, I^2-48%), increased serum LDL-C levels and increased TG concentrations (Qiu et al. 2017). In the current study the OR for heterozygotes was 1.6 (95%CI, 0.7-4.1) in the older age group (above 60 years). Although this conforms to previous findings, sample size is too small to reliably conclude that it influences risk. Although two of the selected candidate FH research subjects were heterozygous for this variant, since median lipid levels on 18 male controls off statins are not different from the wildtype, it is unlikely that this variant

has a strong effect, or it might be counteracted by other factors not studied here, genetic or otherwise.

The variant rs121908326 (S202H) in *LDLRAP1* has been associated with autosomal recessive familial hypercholesterolaemia in a Lebanese subject (Garcia et al. 2001) but is currently interpreted as being of uncertain significance on ClinVar (National Center for Biotechnology Information. 2019). The frequency in the African population is 0.004 (Sherry et al. 2001). One individual was heterozygous for this variant in the 11 selected FH candidates, but in reality, there is another SNP on the other allele and therefore it is a compound heterozygote with *LDLRAP1* S202H on one allele and *LDLRAP1* S202P on the other allele. Out of 103 adult samples sequenced using HTS (whole exome or same custom-made panel) in the Malta NGS Project, only one other sample had the *LDLRAP1* S202H variation and was heterozygous for it. Though the lipid profile of this research subject is not available this 48 year old woman did not report to have hypercholesterolaemia and is not on statins. In any case, *LDLRAP1* is autosomal recessive so one potentially functional allele would not be expected to result in an altered phenotype. The S202P variant is reported as benign in ClinVar (last assessed in Sep 2016). The *LDLRAP1* S202H variant was not assessed in the all the samples of the MAMI Study due to KASP failure. It should be assessed with a methodology that distinguishes the S202H and the S202P variants. Although it cannot be excluded that these two variants together could cause FH, the same sample that had these two SNPs also had the frameshift in *LDLR* which is likely to be deleterious. The addition of these two SNPs may have led to a more severe phenotype.

The rs41291058 (R238W) polymorphism in *LDLRAP1* has 5 listings as benign and 1 of uncertain significance in ClinVar. It is marked as possibly damaging on Polyphen and deleterious on SIFT. It has not been reported in the LOVD-Leiden Open Variation Database. It has also been described in FH patients in a Latvian cohort (Radovica et al. 2014). This gene

exhibits autosomal recessive inheritance. Two individuals were homozygous for this variant and there lipid profile can be observed in Table 9-18.

Table 9-18 Lipid profile in two individuals who were homo Alt for the *LDLRAP1* rs41291058

	Sex	age	TC mmol/L	LDL-C mmol/L	HDLC mmol/L	HDLR	NHDL-C Mmol/L	TG	Statins
Control	male	44	6.95	4.38	1.45	4.79	5.5	2.46	no
Case	female	70	5.44	3.18	1.13	4.81	4.31	2.49	no

Some combinations of genotypes that were observed in the collection were analysed. Male controls, off statins that were heterozygotes for *LDLR* polymorphism rs11669576 (A391T) who were wildtype for *APOB* polymorphism rs12691202 (V730I) had higher NHDL-C levels relative to individuals wildtype for both indicating that the *LDLR* polymorphism alone can raise NHDL-C levels. Though the odds ratios for these variants alone was above 1 the confidence interval spans one and therefore larger studies are determined to show the effect on risk for MI. Only two research subjects in the MAMI Study had both variants together. They were both cases and they had elevated TC and LDL-C.

Heterozygotes for *APOB* rs533617 (H1923R) who were wildtype for *APOB* rs12713681 had decreased levels of LDL-C, decreased levels of NHDL-C, decreased HDL ratios and decreased triglycerides with small increase in HDL-C. While these variations are similar to those observed for rs533617 alone the risk with this combination is greatly increased in males, off statins, ≥60 years of age with an OR of 5.8 (95% CI, 1.2-2.8). If these SNPs are indeed causing an effect on risk of MI it is not through lipid levels, nor through effects on inflammation as hs-CRP was not higher in this sub-group. It is possible that there are differences in the composition of the LDL, IDL and VLDL particles.

To summarise, the approach adopted here helped identify a number of variants that influence lipid profile and risk of myocardial infarction. A frameshift variant in *LDLR*, alone or in

combination with variants in *LDLRAP1* is a likely cause of FH and warrants further study. A second *LDLR* variant, p.A391T, was associated with increased levels of deleterious components of the lipid profile. It could be acting in combination with *APOB* p.V730I (rs12691202), which was found to be associated with increased hsCRP. Alternatively (and perhaps more likely) since this latter variant is in partial LD with a novel *APOB* variant p.R2644S, the latter could be the causative variant. The samples of the MAMI Study need to be tested for this variant. The rs41291058 (R238W) polymorphism in *LDLRAP1* might also be a functional variant in the homozygous state. Two of the selected candidate FH samples had *PCSK9* p.G670E which has been previously reported to be a gain-of-function variant. Although in the MAMI Study samples this variant was not associated with elevated lipid levels, it could be that other common variants in *PCSK9* (such as rs 11584680 and the in-frame insertion of a single Leucine could be mitigating its effect. The *APOB* p.H1923R variant observed in one of the selected candidates tended to be associated with an almost 2-fold increased risk of MI despite being associated with lower cholesterol and TG. Another rare variant ApoB p.I4314V was present in one of the 11 selected research subjects. This variant should also be tested in all the MAMI Study samples.

Four of the eleven selected candidate FH research subjects did not have an identifiable candidate variant. This is not unexpected as other novel or candidate genes for this condition, or polygenic causes may also be involved.It is well known that up to 60% of cases of hypercholesterolaemia suspected of having monogenic FH do not have a monogenic variant but rather the raised LDL is due to a polygenic cause (Talmud et al. 2013).

Section 9.4. Limitations

The number of samples selected for NGS of suspected FH was limited to 11, including more samples would have made it more likely to find additional variants responsible for FH locally. Proper calculation of the DLCN score was hampered by absence of genetic results which is a key component of the DLCN SCORE. Furthermore, restrictions and stratifications possible in the analyses were limited due to the size of the MAMI study. Analysis in women was also limited by small numbers. Only coding and splice site regions have been sequenced. PCSK9 variants have been found in only 2% of cases of familial hypercholesterolaemia but unlike in the case of LDLR and APOB polymorphisms numerous non-synonymous variants in PCSK9 have an effect on serum cholesterol levels in the general population (Wu and Li 2014).

Section 9.5. Conclusions

Two combined frameshift variants on *LDLR* (P608Lfs and F609delinsFGfs) are a likely causative novel variant for FH. Heterozygosity for rs11669576 in *LDLR* may also be contributing to increased LDL-C and NHDL-C levels. The rs533617 polymorphism in ApoB appears to be increasing the risk of MI, but the mechanism is not via increased lipid levels. Hypercholesterolaemia locally deserves further study both to finalise the findings made here and to determine the role of additional genes and/or the polygenic ethiologies.

Chapter 10. Wingless-Type Murine Mammary Tumor Virus Integration Site (Wnt) Pathway and Cardiovascular Disease

Section 10.1. Adipose Tissue Regulation

Adipose tissue is a metabolically dynamic organ that is made up of adipocytes, adipocyte precursors, mesenchymal cells, vascular tissue and immune cells. Wnt signalling plays a key role in adipocyte regulation and could be an important candidate pathway related to myocardial infarction because besides its involvement with obesity it is involved in diabetes and in various stages of atherosclerosis and vascular inflammation (Gay and Towler 2017).

Humans have two main types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is subdivided into two major subdivisions visceral white adipose tissue (vWAT) and superficial white adipose tissue (sWAT) depots based on their location inside or outside the abdominal cavity (Cinti 2005). These two subsets of WAT have different development lineage, gene expression, adipokine profiles, metabolic characteristics and different contributions to cardiometabolic disease (Emdin et al. 2017; Chen and Wang 2018). Pre-adipocytes within the adipose tissue can differentiate into mature adipocytes throughout life allowing adipose tissue to carry out hyperplastic expansion in response to storage requirements. Mature adipose tissue can also expand in size to allow increased storage via hypertrophy (Gray and Vidal-Puig 2007). The inability of superficial white adipose tissue (sWAT) to expand sufficiently in the setting of a positive energy balance is thought to underpin the link of obesity with metabolic complications (Gray and Vidal-Puig 2007).

WAT function is primarily energy storage while BAT has a role in neonates to combat coldness via the process of adaptive non-shivering thermogenesis. WAT stores and releases energy as fatty acids in response to systemic demands whereas brown adipocytes burn fatty acids and glucose to produce heat (Cannon and Nedergaard 2004). High vWAT mass has been associated with inflammation and macrophage accumulation (Weisberg S et al. 2003), dyslipidaemia (Hwang et al. 2016), diabetes (Kelley et al. 2000; Wang et al. 2005) and

cardiovascular disease (Gruzdeva et al. 2018). Gluteofemoral sWAT on the other hand appears to be protective against the metabolic syndrome (Manolopoulos et al. 2010). Wnt signalling appears to play a role in the developmental and functional differences between sWAT and vWAT (Chen and Wang 2018).

BAT appears to be beneficial and its regulation involves the Wnt pathway. BAT thermogenesis is mediated via β-adrenergic activation of lipolysis and degradation of fatty acids via uncoupling protein 1 (UCP1) (Lowell and Spiegelman 2000; Inokuma et al. 2006). Human adults possess functional brown adipose tissue (Cypess et al. 2009; van Marken Lichtenbelt et al. 2009; Saito et al. 2009). The mass of BAT in adults shows a negative correlation with adiposity and BMI (Cypess et al. 2009; Saito et al. 2009). Beige adipocytes (Brite adipocytes) are UCP-1 expressing, multilocular, mitochondrial rich cells within WAT and like BAT have the capacity to convert energy into heat (Cypess et al. 2009) and may be protective against obesity (Seale et al. 2011; Vitali et al. 2012; Shabalina et al. 2013). Beige adipocytes can be induced in a process called browning which involves cell fate switch in response to transcription factors (Wang and Seale 2016). Besides inhibition of Notch and JAK signalling, Wnt/β catenin signalling also has a role in the development of beige adipocytes (Moisan et al. 2015; Lo et al. 2016). In response to cold or β3 adrenergic receptor stimulation, progenitors in both sWAT and vWAT adipocyte tissue are induced to asymmetric division, preadipocyte formation and finally beige adipocyte formation possibly via the activation of Wnt/β-catenin signalling (Chen and Wang 2018). Activation of Wnt/β catenin through Wnt-10b or Wnt-10a overexpression inhibits brown adipogenesis by downregulating peroxisome proliferator-activated receptor gamma (*PPARG*) and CCAAT/enhancer-binding protein alpha (*CEBPA)*. Furthermore in mature brown adipocytes Wnt-10b can stimulate conversion of brown adipocytes to white adipocytes (Kang et al. 2005). Understanding the regulation of BAT and

the regulation of the process of browning could play an important part in the management of obesity in the future.

Excessive accumulation of WAT is the defining characteristic of obesity. WAT can expand by an increase in adipocyte size (hypertrophy) and adipocyte number (hyperplasia). Obese individuals have more adipocytes than lean individuals and this increased number is maintained even after weight loss. The number of fat cells is thought to be set during childhood and adolescence. Approximately 10% of fat cells are renewed annually at all ages and at all levels of body mass index (Spalding et al. 2008). Several circulating factors, including insulin and glucocorticoids are capable of inducing adipogenesis via CCAAT/enhancer-binding protein β (*CEBPB*) and δ (*CEBPD*) which in turn induce the expression of *CEBPA* and *PPARG*. Activation of *PPARG* is lipogenic, promoting the uptake of free fatty acids from serum into adipose tissue to produce triglycerides in the adipose cells and also increases the expandability of adipose tissue. *PPARG* activation improves insulin sensitivity and is involved in the mechanism of action of thiazolidinediones, an oral hypoglycaemic agent (Gray and Vidal-Puig 2007).

Peroxisome proliferator-activated receptor (PPAR) proteins belong to the steroid hormone receptor superfamily and combine with the retinoid X receptors to form heterodimers that regulate genes involved in lipid and glucose metabolism, adipocyte differentiation, fatty acid transport, carcinogenesis and inflammation (Delerive et al. 2001; Dubuquoy et al. 2002). PPARs exist in three different forms as PPAR-alpha (PPAR-α), PPAR-beta/delta (PPAR-β/δ) and PPAR-gamma (PPAR-γ), which are encoded by the genes *PPARA*, *PPARD* and *PPARG*. (Petr et al. 2018). PPAR-α and PPAR-β/δ are present mainly in the liver and in tissues with high levels of fatty acid oxidation such as skeletal muscle, cardiac muscle and the kidneys. PPAR-γ is predominantly active in adipocytes (Petr et al. 2018).

PPAR-gamma coactivator 1-alpha (PGC-1-α) encoded for by the *PPARGC1A* gene, is a transcriptional coactivator of the PPAR superfamily. This protein interacts with PPAR-γ enabling its interaction with many others transcriptional factors. PGC-1-α is involved in mitochondrial biogenesis, glucose utilization, fatty acid oxidation, thermogenesis, gluconeogenesis and insulin signalling (Franks et al. 2014). PPAR- gamma coactivator 1-beta (PGC-1-β), encoded by the *PPARGC1B* gene, together with the *PPARGC1A* gene, encodes homologous proteins that, through nuclear transcription factor coactivation, regulate adipogenesis, insulin signalling, lipolysis, mitochondrial biogenesis, angiogenesis and hepatic gluconeogenesis (Franks et al. 2014). PPAR-γ activation can increase β-Catenin degradation, inhibiting the Wnt pathway. PPARγ and Wnt/β Catenin pathways appear to be mutually antagonistic (Christodoulides et al. 2008).

Wnt/β Catenin signalling is also important in the hyperplasia/hypertrophy response in both vWAT and sWAT (Fig 10-1). In response to high fat diet stimulation, vWAT adipocyte precursors hyperplastically expand in response to Wnt/β-catenin signalling up till a point where hyperplasia stops and the cells remain quiescent until they receive another signal to reactivate. Platelet-derived growth factor receptor A (PDGF-R-α) activation inhibits hyperplastic expansion (possibly) via suppressing Wnt/β-catenin signalling and subsequently triggers hypertrophy of existing mature adipocytes. Hypertrophied adipocytes may secrete some adipokines to promote the subsequent lipid filling of these activated adipocytes (Marcelin et al. 2017). The response is different in sWAT. In sWAT Wnt/β-catenin signalling promotes sWAT mature adipocyte hypertrophy. As hypertrophied adipocytes reach their maximal size and secrete adipokines, adipocyte precursors are then induced to undergo hyperplastic expansion and new adipocytes will form to meet the demand of increasing lipid storage (Fig 10-1) (Wang et al. 2013b).

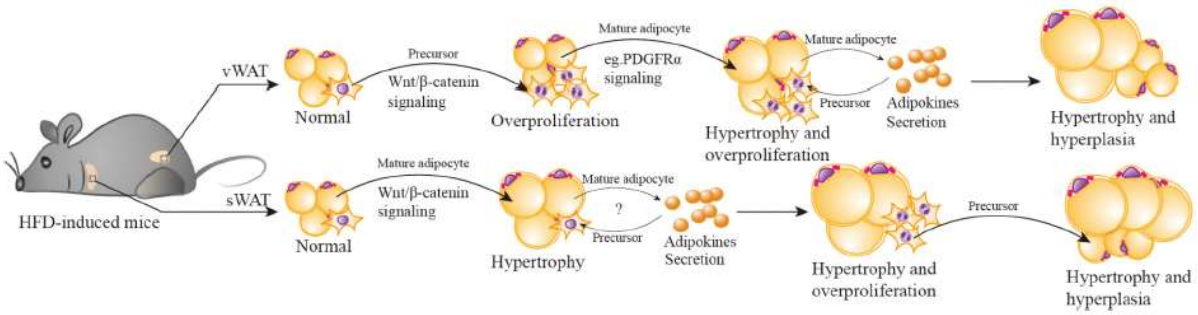

Figure 10-1 Wnt/b-catenin signalling in adipocyte hypertrophy. In visceral adipose, adipocyte precursors are activated by Wnt/b-catenin signalling, leading to "over proliferation". Following this process adipocyte precursors remain quiescent until they receive another signal to reactivate. The PDGFRa pathway inhibits "overproliferated" adipocyte precursor adipogenesis via suppressing Wnt/b-catenin signalling and then triggers hypertrophy of existing mature adipocytes. Hypertrophied adipocytes may secrete some adipokines to promote the subsequent lipid filling of these activated adipocyte precursors. In superficial WAT(sWAT) high fat diet activates the Wnt/b-catenin signalling to promote sWAT mature adipocytes hypertrophy. As hypertrophied adipocytes reach their maximal size and secrete adipokines or emit other signals, adipocyte precursors are then induced to "over proliferation" and new adipocytes will form to meet the demand of increasing lipid storage (Chen and Wang 2018)

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Section 10.2. Adipose Tissue Dysfunction

When adipocyte expansion is unable to keep up with the energy storing demands due to excessive intake, adipose tissue dysfunction occurs. In dysfunctional adipose tissue proinflammatory cytokines tissue necrosis factor alpha (TNFα), interleukin-6 (IL6), interleukin-8 (IL8) and monocyte chemoattractant protein (MCP-1) are secreted by hypertrophied adipocytes (Jernås et al. 2006), leading to serine phosphorylation of insulin receptor substrate-1 (IRS-1) which blocks insulin signalling. These proinflammatory cytokines also recruit macrophages and T-cells (Hirosumi et al. 2002; Huh et al. 2014). Circulating free fatty acids start to accumulate in non-adipose tissue including the liver, skeletal muscle, β-cells and heart. Ectopic lipid accumulation can affect the metabolic processes of these organs in a

process called lipotoxicity (Unger 2005). This process is thought to be responsible for the insulin resistance associated with obesity (Cnop et al. 2005; Zhao et al. 2006). The maximum capacity that adipose tissue can store before ectopic lipid accumulation and metabolic complications occur varies between individuals and is likely genetically determined. Up to 20% of the morbidly obese do not display metabolic complications, displaying normal insulin resistance. These individuals have been called the metabolically healthy obese. They tend to have early onset obesity with higher total adipocyte number and lower levels of visceral fat (Karelis et al. 2004).

Obesity-induced insulin resistance is linked to a cluster of metabolic abnormalities including dyslipidaemia, non-alcoholic fatty liver disease, hypertension, coronary heart disease and stroke (Longo et al. 2019). Increased visceral/intra-abdominal fat accumulation (central obesity) is the strongest predictor of adipose tissue dysfunction, ectopic fat accumulation, lipotoxicity and insulin resistance independent of BMI (Tchernof and Després 2013; Zhang et al. 2015). Increased abdominal sWAT as opposed to peripheral adiposity is also a risk factor for establishing metabolic diseases (Porter et al. 2009; Karpe and Pinnick 2015). Understanding why certain individuals do not develop the complications of obesity despite being morbidly obese could help identify beneficial therapeutic pathways which can be harnessed to limit the development of adipocyte dysfunction.

Section 10.3. The Wnt Pathways

Wnt appears to be an important regulator of adipocyte function in both sWAT, vWAT and also in BAT. A good understanding of this pathway is therefore necessary to understand adipocyte regulation.

The Wnt/β-Catenin signalling pathway is activated by Frizzled receptor (Fz) and its co-receptor, low -density lipoprotein receptor related protein 6 (LRP-6) or LRP-5. Once Wnt binds to frizzled co-receptor complex, the signal is transduced to the cytoplasmic protein dishevelled (DVL-1) (Mill and George 2012). At this level the cascade branches into 3 major signal transduction pathways: The canonical or Wnt/ β-Catenin pathway; the noncanonical planar cell polarity pathway and the Ca^{2+} dependent pathways (Wnt/Ca^{2+}) pathway (Mill and George 2012).

Without Wnt ligand, cytoplasmic β-catenin protein is degraded by the action of the Axin complex. The axin complex is composed of the scaffolding protein Axin, the tumor suppressor adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3)(Macdonald et al. 2009). CK1 and GSK3 phosphorylate the amino terminal region of β-catenin resulting in β-catenin ubiquitination and proteasomal degradation. This prevents β-catenin from reaching the nucleus (He et al. 2004; Gordon and Nusse 2006).

In the canonical Wnt pathway, the binding of Wnt ligand (which in the case of adipose tissue is secreted from neighbouring cells) to its receptor results in stabilization of cytoplasmic β-catenin through inhibition of the β-catenin degradation complex. β -catenin is then free to enter the nucleus and activate Wnt-regulated genes through its interaction with TCF (T-cell factor) family transcription factors and concomitant recruitment of coactivators (He et al. 2004). The β-catenin-TCF/LEF complex needs to recruit transcriptional co-activator to be functional, including the cAMP response element-binding (CREB) binding protein (CBP)(Hecht 2000). This complex acts as a transcription activator or repressor of Wnt target genes (Gordon and Nusse 2006; Macdonald et al. 2009; Nusse R 2019). Wnt target genes encode Myc, cyclin D1 and PPARδ (Clevers 2006). In the absence of Wnt, Wnt target genes are usually repressed by the DNA-bound (LEF/TCF), groucho, and histone deacetylases (HDAC) family of proteins (Macdonald et al. 2009) (Figure 10-2) .

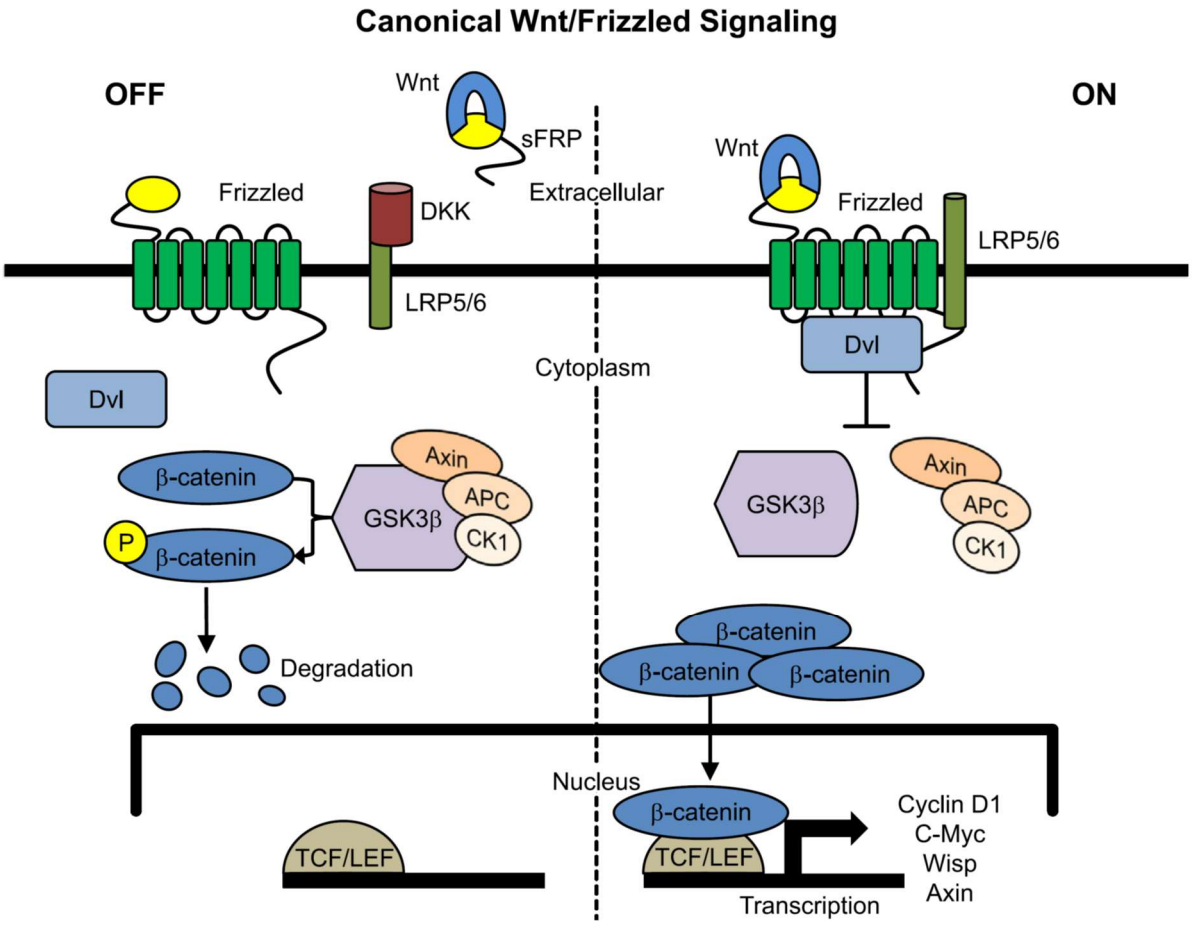

Figure 10-2 Canonical Wnt Signalling. In the presence of the Wnt ligand, the Wnt proteins bind to receptors of the Frizzled and LRP families (LRP5/6) on the cell surface. This results in activation of dishevelled which inhibits GSK3 and disrupts the APC/Axin/GSK3 complex. The stabilised β-catenin translocates to the nucleus and forms a complex with lymphoid enhancer-factor (LEF)/T-cell factor (TCF) family of transcription factors (Blankesteijn and Hermans 2015)

In the planar cell polarity (PCP), non-canonical Wnt pathway, signalling through the Fz receptors is mediated by Dishevelled. Dishevelled associates with actin to activate two independent pathways, the RAS homologue gene-family member A (*RHOA*) mediated pathway and the Ras-related C3 botulinum toxin substrate 1 *(RAC1)* mediated pathway. Activation of *RHOA* requires Dishevelled Associated Activator of Morphogenesis 1 (Daam-1) and leads to activation of Rho associated kinase (*ROCK1*). *RAC1* activation is independent of Daam-1 and stimulates Jun kinase (JNK). Both pathways lead to remodelling of the

cytoskeleton which can control lateral asymmetry and control changes in cell adhesion, motility and polarity. The PCP Wnt pathway also leads to calcium mobilization within the cell (Veeman et al. 2003) (Figure 10-2).

In the Wnt/Ca^{2+} pathway, Wnt proteins function via cell surface receptors to stimulate an increase in intracellular Ca^{2+} via two distinct pathways. The first involves phospholipase C mediated production of inositol triphosphate and diacylglycerol. These interact with calmodulin to activate calmodulin-dependent protein kinase II (Cam-Kinase II) (De 2011). The mobilized calcium ions from the endoplasmic reticulum also interact with 1,2 diacylglycerol to activate protein kinase C (PKC) (Sheldahl et al. 1999). Calmodulin dependent protein kinase II and protein kinase C then activate nuclear transcription factors. This leads to nuclear factor kappa-β, cAMP-responsive element binding and nuclear factor associated with T cells transcription factors translocating to the nucleus and promoting an osteogenic gene profile (Albanese et al. 2018). The second pathway involves cyclic GMP -selective phosphodiesterase and p38-MAPK (Foulquier et al. 2018) (Figure 10-3).

Canonical and Non-canonical signalling pathways are interconnected and cross regulate each other (Cherry and Adler 2011; Kestler and Kühl 2011). Wnt-1, Wnt-3a, Wnt-8 and Wnt-8b are involved in canonical Wnt signalling while Wnt-4 and Wnt-5a interact via non-canonical Wnt signalling, but there is significant crosstalk between the pathways (De 2011). Wnt-5a can inhibit Wnt-3a induced canonical signalling in a dose-dependent manner via Tyrosine-protein kinase transmembrane receptor ROR2 (ROR2) by downregulating β-catenin-induced receptor gene expression (Mikels and Nusse 2006). Furthermore, although Wnt-5a is usually considered non-canonical it can also activate or inhibit the canonical pathway depending on receptor availability showing that it serves dual or opposing roles and highlighting the complexities of Wnt mediated pathways (Mikels and Nusse 2006).

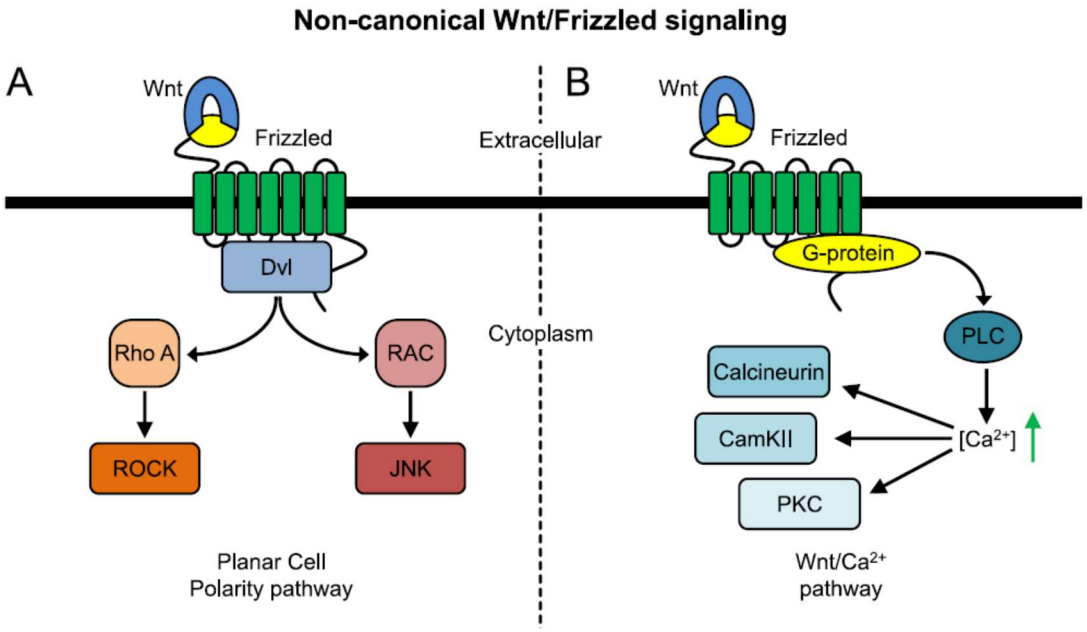

Figure 10-3 Non-Canonical Wnt signalling via two distinct pathways, the planar cell polarity (PCP) and Wnt-Ca2^{++} dependent pathway. PCP signalling through the Fz receptors is mediated by Dishevelled, which interacts with RAS homologue gene-family member A (*RHOA*) and Ras-related C3 botulinum toxin substrate 1 *(RAC1)*. Activation of *RHOA* leads to activation of Rho associated kinase (*ROCK1*). *RAC* activation stimulates Jun kinase (JNK). In the Wnt/Ca^{++} pathway, Wnt proteins stimulate an increase in intracellular Ca^{++} via phospholipase C (PLC). These interact with calmodulin to activate calmodulin-dependent protein kinase II (Cam-Kinase II). The mobilized calcium ions from the endoplasmic reticulum also interact with 1,2 diacylglycerol to activate protein kinase C (PKC). Calmodulin dependent protein kinase II and protein kinase C then activate nuclear transcription factors (De 2011).

Adding to this complexity, Wnt signalling can be modulated by several classes of endogenous proteins including the soluble Frizzled related proteins (sFRP), the Dickkopf family of proteins, Wnt-Inhibitory factor-1 (WIF-1) and Cerberus (Blankesteijn and Hermans 2015). In such a complex pathway studying polymorphisms in isolation is unlikely to yield results. Using the knowledge of biological pathways to identify important combinations of polymorphisms may hold the key to identifying clinically relevant polymorphisms in polygenic diseases such as obesity.

Section 10.4. Wnt Signalling and Adipose Tissue Regulation

The canonical Wnt/β-Catenin pathway is very important for the process of adipogenesis. The differentiation of preadipocytes into adipocytes requires suppression of canonical Wnt signalling (Blüher 2009). On the other hand, activation of Canonical Wnt/β Catenin promotes the differentiation of mesenchymal precursor cells into myocytes and osteocytes. Besides inhibiting commitment to the adipogenic lineage suppression of Wnt/ β-Catenin is also important for terminal differentiation by inhibiting the expression of *PPARG* and *CEBPA*. In response to adipogenic stimuli CCAAT/enhancer-binding protein β (C/EBP beta) and C/EBP delta inhibit the canonical Wnt pathway leading to adipocyte hyperplasia and hypertrophy (Figure 10-4).

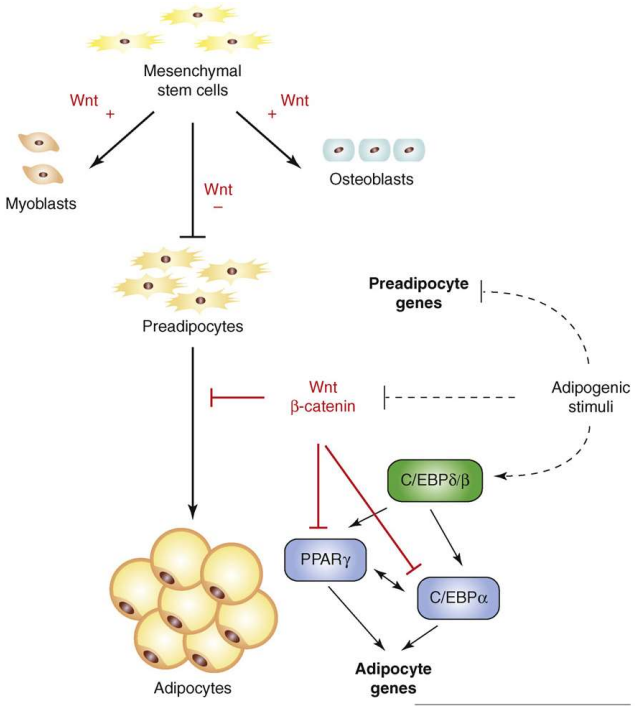

Figure 10-4 Suppression of WNT/b-catenin signalling promotes differentiation of mesenchymal precursor cells into commitment to the adipocytic lineage and terminal differentiation. WNT signalling restrains adipocyte differentiation by inhibiting the expression of *PPARG* and *CEBPA*, the central regulators of adipogenesis. These transcription factors are induced directly by *CEBPB* and *CEBPD* in response to adipogenic stimuli (Christodoulides et al. 2008).

The Non-canonical Wnt signalling pathway is also involved in adipogenesis. Wnt-5b has a functional role in adipogenesis. Wnt-5a is a paralog of Wnt-5b protein in which 80% of amino acids are identical (Katoh and Katoh 2005). Wnt-5a and Wnt-5b overexpression promotes adipogenesis by antagonizing the Wnt-3a mediated Wnt/β-Catenin pathway. Wnt-5b overexpression is a potent stimulator of adipogenesis via inhibition of canonical Wnt signalling and increased PPARγ expression (van Tienen et al. 2009). Therefore, the non-canonical WNT pathway appears to promote adipogenesis by antagonising the canonical WNT pathway.

Other factors in the Wnt signalling pathways appear to play an important role in adipogenesis and obesity. Overexpression of Dkk-1 and LRP-5/6 can promote human adipogenesis by antagonising the Wnt/β-Catenin pathway in the commitment stage. Higher levels of Dkk-1 have been associated with greater, overall, central and ectopic skeletal muscle adiposity (Ali et al. 2019). Secreted spondin proteins 1-4 which potentiate Wnt signalling may also play a role in regulation of obesity-induced insulin resistance (Kang et al. 2019). MicroRNA including miR-205-5p also appears to regulate adipogenesis by controlling dishevelled-3 expression and subsequently inhibiting the Wnt/β-Catenin pathway (He et al. 2015). TCF7L2, an important diabetes candidate gene, is also involved in adipogenesis but its precise role has still not been determined (Chen et al. 2018). *SIRT1* has also been shown to inhibit adipogenesis via the Wnt/β Catenin pathway (Zhou et al. 2016). Wnt-1 also known as WNT1-inducible-signaling pathway protein 2 (WISP-2) is a suppressor adipokine for adipogenic commitment which is inhibited directly by proadipogenic bone morphogenetic protein 4 (BMP-4). BMP-4 can induce zinc finger 423 expression (*ZNF423*) which promotes the commitment phase of adipogenesis (Grünberg et al. 2018). Wnt-4/β-Catenin promotes adipogenesis and is modulated by glucagon like peptide-1, an important regulator of the incretin system and a therapeutic target in diabetes (Liu et al. 2016a). It is clear that Wnt plays a central role in the regulation of adipose tissue.

Wnt signalling plays an important role in the interaction of adipocytes and inflammatory cells in patients with obesity and insulin resistance (Laudes 2011a; Laudes 2011b). Wnt-5a appears to be proinflammatory and in vWAT has been shown to contribute to elevated IL-6 levels (Zuriaga et al. 2017). Adipose tissue secretes Secreted frizzled-related protein 5 (sFRP-5), an anti-inflammatory adipokine, Wnt antagonist, reduces the chronic inflammatory state and insulin resistance associated with obesity (Ouchi et al. 2010). Furthermore, sFRP-5 is capable of stimulating adipocyte differentiation via the inhibition of the Wnt/β-catenin signalling pathway (Liu et al. 2018). sFRP-5 also mediates epigenetic silencing of the Wnt signalling pathway in white adipose tissue promoting adipogenesis (Christodoulides et al. 2008), sFRP-5 binds to Wnt-5a to prevent JNK activation downstream of the Wnt signalling pathway (Ouchi et al. 2010). This results in a decrease in the production pro-inflammatory cytokines and antagonism of IRS-1 SER^{307} phosphorylation which results in improved insulin sensitivity and anti-diabetic effects (Lu et al. 2013; Oh and Olefsky 2014; Palsgaard et al. 2012).

In lean and healthy subject where the number of macrophages in adipose tissue is low, adipocytes secrete anti-adipogenic Wnt-5a, which is neutralised by Sfrp-5 released by healthy adipocytes. In obese and type 2 diabetic subjects, the number of macrophages is increased and the adipocytes from such subjects secrete less Sfrp-5, resulting in increase in Wnt-5a activity in adipose tissue. This in turn induces insulin resistance of mature adipocytes via JNK interfering with insulin receptor substrate (IRS-1) and also impairs generation of novel adipocytes by inhibiting adipogenesis of mesenchymal precursor cells (Laudes 2011b).

The Wnt pathways are central to the regulation of adipose hypertrophy and hyperplasia and also to the interaction of adipose tissue with inflammatory cells making this an important pathway in the study of adipocyte dysfunction.

Section 10.5. Wnt Signalling and Risk Factors for Coronary Artery Disease

Wnt has been linked to various risk factors of coronary artery disease. Variants in *LRP6* have been associated with high LDL, high triglycerides, hypertension, diabetes, osteoporosis and early onset coronary artery disease (Mani et al. 2007a). Loss of function *LRP5* variants have been linked to impaired glucose tolerance (Jin 2008) while gain of function variants in *LRP5* have been associated with high bone mass and increased lower body fat accumulation (Loh et al. 2015). Variants in the Wnt-Fz-LRP-5/6 complex co-receptor leucine-rich repeat-containing G-protein coupled receptor 4 (LGR4) has been associated with osteoporosis, electrolyte disturbance and lowered body weight in an Icelandic population (Styrkarsdottir et al. 2013) and with increased risk of obesity and metabolic disorders in Chinese individuals (Chen and Wang 2018). Variants in TCF7L2 have been associated with type 2 diabetes and failure to loose weight during lifestyle interventions (Grant et al. 2006; Haupt et al. 2010). Wnt also plays a role in cardiac remodelling following a myocardial infarct. Wnt-3a induces apoptosis via the canonical pathway, SFRPs can compete for the frizzled receptor and protect against activation of the Wnt pathway and reduces infarction expansion preserving cardiac function (Laeremans et al. 2011). Besides its role in adipocyte dysfunction, the Wnt pathway also appears to be linked directly to various risks factors which result in coronary artery disease making the analysis of this pathway particularly interesting for the MAMI study.

Section 10.6. Wnt Signalling in Atherogenesis

Wnt/β Catenin and Wnt/Ca^{2++} signalling is involved at multiple stages in the pathogenesis of atherosclerosis. Elevated levels of Wnt-5a and Dkk-1 have been found in the circulation of

subjects with atherosclerosis (Kim et al. 2011; Bhatt and Malgor 2014) and Dkk-1 levels have been proposed as an independent marker of adverse cardiac events (Wang et al. 2013a).

Section 10.6.1. Endothelial Activation

Endothelial dysfunction is one the primary steps in atherosclerosis and one of the steps in which Wnt signalling is involved. There have been large inconsistencies with regards to Wnt signalling in the endothelial cell, possibly due to large variations in expression patterns between different sources of endothelial cells, however, Wnt signalling has been implicated in both endothelial proliferation and endothelial inflammation (Blankesteijn and Hermans 2015).

Wnt-5a expression with activation of C-Jun-amino-terminal kinase-interacting protein (JNK) has been associated with low-flow-mediated dilatation, an indication of endothelial dysfunction in diabetic patients (Bretón-Romero et al. 2016). In vitro, Wnt-5a can promote both type 1 endothelial activation by upregulating the non-canonical Wnt/ Ca^{2++}pathway and inhibition of the canonical Wnt pathway (Ueland et al. 2009; Ihm et al. 2010). Wnt-5a can also mediate type 2 endothelial activation through Cyclooxygenase 2 (Dejana 2010). Wnt-5a may induce inflammation by activating *NFKB* via an IKK-dependent mechanism, acting in parallel to the Ca^{2+} dependent Wnt signalling pathway (Marinou et al. 2012). Canonical Wnt-3a can also mediate endothelial dysfunction via JNK-mediated phosphorylation of $p66^{shc}$ (a redox regulatory protein) at Ser36. The phosphorylation of $p66^{shc}$ could be inhibited by Dkk-1 (Vikram et al. 2014). Dkk-1 has also been implicated in platelet-induced endothelial activation. During platelet activation Dkk-1 is released and enhances β-Catenin phosphorylation in endothelial cells. Neutralising antibodies against Dkk-1 decreased platelet-induced cytokine production in endothelial cells (Ueland et al. 2009). Dkk-1 can therefore be considered pro-atherosclerotic with its release from platelets inhibiting the Wnt/β-Catenin

pathway in ECs leading to endothelial dysfunction, the precursor of atherosclerosis (Foulquier et al. 2018).

Section 10.6.2. Wnt Signalling in Macrophages

Monocyte adherence to the endothelium with transmigration into the sub endothelium to transform into foam cells is a key step in the initiation of atherosclerosis (Witztum 1994). WNT signalling is involved in key activation processes of atherosclerosis via complex interactions. Wnt signalling via the canonical pathway enhances monocyte adhesion to endothelial cells (Bienz 2005; Lee et al. 2006). Wnt-5a is secreted by activated human macrophages as part of an inflammatory response. Macrophage activation occurs secondary to toll-like receptor activation and *NFKB* activation (Blumenthal et al. 2006). Wnt-5a is also secreted by vascular endothelial cells during systemic inflammatory diseases (Skaria and Schoedon 2017). Oxidized LDL can induce Wnt-5a mRNA expression in human monocyte-derived macrophages (Bhatt and Malgor 2014), Wnt-5a can trigger multiple signalling pathways depending on the availability of receptors and Wnt antagonists (Kikuchi et al. 2012).

WNT signalling has both inflammatory and anti-inflammatory effects. Wnt-5a co-localises with Toll-like receptor 4 (TLR4) in macrophage-rich atherosclerotic plaques (Christman et al. 2008; Dejana 2010). Furthermore, macrophage activation by TLR4 and Lipopolysaccharide binding protein (LPS) leads to increased expression of Wnt-5a in human macrophages (Blumenthal et al. 2006; Christman et al. 2008). Wnt-5a can interact with Fz-5 to induce the expression of pro-inflammatory cytokines (Blankesteijn and Hermans 2015). On the other hand, Wnt-3a, a ligand of Fz-1, has an anti-inflammatory effect via suppression of GSK3 and downregulation of nuclear factor-kappa-B gene transcription (Schaale et al. 2011). Canonical Wnt signalling and LRP-5 have been directly implicated in foam cell formation (Borrell-Pagès

et al. 2011; Borrell-Pagès et al. 2014). Wnt-5a also appears to regulate reverse cholesterol transportation in the macrophage. Wnt-5a reduced cholesterol accumulation in macrophages by regulating the mRNA expression of Caveolin-1 and ATP binding cassette transporter A1 (Qin et al. 2014). Wnt-3a on the other hand had an anti-inflammatory effect by suppressing GSK3β (Schaale et al. 2011).

LRP-5 plays an important role in plaque maturation. *LRP5* expression is enhanced in advanced plaques compared to early plaques, and cholesterol accumulation in macrophages and macrophage migration can both be decreased by silencing *LRP5*. (Borrell-Pagès et al. 2011). Osteopontin and BMP-2, which are both involved in the progression of the atherosclerotic lesion, are both upregulated by LRP-5 on oxidized LDL exposure (Foulquier et al. 2018). Interestingly besides its involvement in plaque formation, Wnt has also been implicated in plaque regression (Ramsey et al. 2014). WNT signalling is involved in various aspects of atherosclerosis, in both pro- and anti-atherosclerotic ways, exposure to LDL or ox-LDL can shift this balance.

Section 10.6.3. Wnt Signalling and Cadherin: Beta-Catenin Complex in Vascular Smooth Muscle Cells

Wnt signalling is essential to vascular smooth muscle cell involvement in atherosclerosis. β-Catenin has a stimulating effect on vascular smooth muscle cell proliferation. Beta-Catenin/TCF signalling can upregulate the expression of several pro-proliferative genes such as cyclin D1 and decrease the level of the cell cycle inhibitor p21 (Quasnichka et al. 2006; Blankesteijn and Hermans 2015) in response to vascular wall injury (Slater et al. 2004). Canonical Wnt signalling via Wnt-1, Wnt-2, Wnt-3a can modulate arterial smooth muscle cell proliferation by the expression of cyclin D (Wang et al. 2004; Takahashi et al. 2016; Williams

et al. 2016). Inhibitors of the canonical Wnt signalling pathway such as Sclerostin, on the other hand, may help prevent atherosclerosis (Krishna et al. 2017). Wnt-4 has also been implicated as an endogenous activator of VSMC proliferation (Tsaousi et al. 2011). Wnt inhibitory factor and Sfpr-1 expression both inhibited VSMC proliferation (Ezan et al. 2004; Tsaousi et al. 2011).

Vascular smooth muscle is also regulated by β-Catenin: cadherin mediated pathways. Cadherins are key molecules in cell-to-cell adhesion. Stimulation of vascular smooth muscle is associated with the dismantling of cadherin junctions and nuclear translocation of β-catenin (Quasnichka et al. 2006; Corada et al. 2010). The cadherin: catenin complex regulates adhesion and cell signalling and modulates vascular smooth muscle cell apoptosis, migration and proliferation (Dwivedi et al. 2009). Inhibition of R-Cadherin increases the expression of β -Catenin which increases cyclin D1 which is a Wnt target gene. This results in vascular smooth muscle cell proliferation (Slater et al. 2004). N-cadherin is also involved in the regulation of vascular smooth muscle cell proliferation with loss of N-cadherin resulting in an increase in β -catenin signalling and modulation of cyclin D1 and p21 expression (Quasnichka et al. 2006). Upregulation of Wnt-4 results in WNT/ β-catenin signalling in vascular smooth muscle cell proliferation during intimal thickening (Tsaousi et al. 2011). The Wnt inhibitor, secreted frizzled related protein-1 (sFRP-1) can reduce vascular smooth muscle cell proliferation (Ezan et al. 2004) and the transmembrane coreceptor for Wnt proteins, low-density lipoprotein receptor-related protein (LRP)6, promotes VSMC proliferation (Wang et al. 2004). Wnt signalling therefore has an important role in remodelling of the atherosclerotic plaque via control of vascular smooth muscle cells.

Section 10.6.4. Wnt Signalling in Vascular Calcification

Vascular calcification which is a feature of mature plaques is associated with upregulation of Wnt signalling via a mechanism involving BMP-2 (Boström et al. 1993). BMP-2 regulates osteogenic and chondro-osteogenic transcription factors including Msx homeobox 2 (Msx2) which upregulates Wnt ligands including Wnt-3a and Wnt-7a in vascular myofibroblasts and the endothelium and downregulates inhibitors including DKK-1 (Towler et al. 2006; Shao et al. 2007). The Canonical Wnt pathway that is well established in the regulation of osteogenesis therefore also promotes vascular calcification. Pathogenic stimuli which can upregulate vascular BMP expression include oxidized lipids, hypertension and hyperglycaemia (Cheng et al. 2003; Zhang et al. 2003; Cola et al. 2004). In addition to the upregulation of Wnt ligands there is also downregulation of the enzyme catalase that provides cellular resistance against oxidative stress, a target gene of the transcription factor Forkhead box (FOXO) (Boström et al. 2011). Wnt-5a expression has been demonstrated in human atherosclerotic tissue in macrophage rich regions and in smooth muscle cells (Christman et al. 2008). Through the calcium dependent Wnt signalling pathway, Wnt5a activates histone methyltransferase SET domain bifurcated 1 which supresses peroxisome proliferator-activated receptor gamma (PPARγ) function and mediates osteoblastic differentiation of mesenchymal stem cells (Takada et al. 2007). PPARγ is expressed in adipose tissue, endothelial cells, vascular smooth muscle cells and macrophages (Fokko and Plutzky 2007; Hamblin et al. 2009). It plays a key role in lipid metabolism and is also thought to modulate inflammation, macrophage differentiation and atherosclerosis (Berger and Moller 2002). PPARγ has inhibitory effects on vascular smooth muscle and endothelial cell proliferation and migration (Ricote et al. 1999; Puddu et al. 2003) and monocyte recruitment to atherosclerotic plaques (Marx et al. 1998). PPARγ is considered to be anti-atherosclerotic either due to effects on lipid and blood glucose levels or by increasing plaque stability (Albanese et al. 2018).

Advanced glycation end products (AGE's) and their receptors RAGEs can promote calcification via Wnt/β-Catenin signalling. RAGES may promote the differentiation of arterial smooth muscle cells into osteogenic cells through the Wnt/β catenin pathway (Liu et al. 2016b). Activation of Wnt signalling by Wnt-3a induces Runx2 expression which influences osteoblast differentiation. DKK1 inhibits Runx2 induction and calcium deposition in vascular smooth muscle cells (Cai et al. 2016). WNT also appears to enhance the expression of the matrix metalloproteinases (MMP), MMP-2 and MMP-9 which promote vascular smooth muscle calcification (Freise et al. 2016).

Pericytes are thought to play a key role in vascular calcification. Pericytes are pluripotent mesenchymal progenitor cells (Kirton et al. 2006) that have the ability to transform into chondrocytes, osteocytes or adipocytes (Doherty et al. 1998; Farrington-Rock et al. 2004). Pericytes can be recruited to the intima, media and sites of arterial calcification (Andreeva et al. 1998; Collett and Canfield 2005). Activation of Wnt/β-Catenin/T cell factor signalling mediated by transforming growth factor-β3 results in a chondrogenic response and inhibition of adipogenic differentiation of pericytes. This indicates that the Wnt β-Catenin pathway plays an important role in plaque maturation (Kirton et al. 2007).

Although most Wnt signalling leads to vascular calcification, Wnt-16 was found to decrease TGFβ-induced chondrogenic transformation of vascular smooth muscle cells (Beazley et al. 2015). LRP-6 also appears to limit vascular calcification with the protective effects of LRP-6 probably being secondary to downregulation of β-catenin independent FZD signalling (Cheng et al. 2015).

The Wnt/βCatenin pathways portray a particularly complex picture. The pathways influence adipose tissue regulation, the interaction of adipocytes with inflammatory cells and atherosclerosis at all stages and in multiple manners. There is cross talk between the different

Wnt pathways, cross talk with other important pathways and evidence of high levels of redundancy, as would be expected in such a key regulatory pathway. Due to these issues studying single SNPs is less likely to yield valuable observations and may in fact underly the conflicting data in the genetics of complex diseases. A more holistic pathway approach is required such as studying the effects of gene-gene interactions by studying combinations within the pathway and observing effects on intermediate phenotypes.

Section 10.7. Further Methods

It is well known that not all obese individuals develop the metabolic complications of obesity. The reasons for this are largely unknown (Primeau et al. 2011). Identifying what triggers the metabolic complications of obesity could be important for developing therapeutic targets for the management of obesity. In Chapter 3 it was demonstrated that a high WHR was more closely associated with risk of MI then BMI. Furthermore, it was demonstrated that a high HOMA-IR, a marker of insulin resistance and adipocyte dysfunction, together with a high WHR was strongly associated with MI compared to a high WHR with normal HOMA-IR. High HOMA-IR combined with a High WHR was therefore selected as an extreme phenotype likely to best represent a group with adipocyte dysfunction.

Seven samples of male controls with a high WHR (WHR >0.9) and no insulin resistance (HOMA-IR <=0.85, lower than the 10th decile) and 8 samples of males cases with a high WHR (WHR>0.9 and in the highest decile for insulin resistance (HOMA-IR>=5.34, higher than the 90th percentile) were selected for HTS of the WNT pathway genes. An attempt was made to select cases with family members who also had a WHR>0.9 and HOMA-IR>=5.34.

The WNT pathway is a complex pathway that is central to the control of adipogenesis and also to atherosclerosis. This pathway is not only involved in the regulation of both visceral and

superficial WAT but also that of BAT. Therefore, it was hypothesised that this pathway could be involved in determining if obese individuals developed metabolic complications of obesity or not. Since the WNT pathway is a highly conserved pathway with multiple levels of redundancy and which interacts with several other pathways it was also hypothesised that using a pathway approach based on the known biology of these pathways would show associations that are not apparent when single polymorphisms are studied in isolation. The pathway approach was combined with high throughput sequencing to select genetic variants which were then assessed alone and in specific combinations for effects on intermediate phenotypes and also on the risk of MI.

Genes for HTS related to the WNT pathways and the regulation of adipogenesis were selected after a literature review. Variants in these genes where analysed in the 15 research subjects described above. Variants that were more frequent in one phenotype than in the other, were rare or were present in an important gene were selected for KASP genotyping in the entire MAMI study collection.

Genetic variants that were rare, were in an important gene or more common in one extreme phenotype when compared to the other were selected for KASP genotyping in the whole MAMI Study collection.

Section 10.8. Results

Section 10.8.1. Selection of genes pertaining to adipocyte regulation

After studying KEGG pathways for WNT and other related pathways that are involved in adipocyte differentiation and the process of beiging, the following genes were selected for high throughput sequencing in 7 male controls with high WHR and low HOMA-IR and 8 male cases with high WHR and high HOMA-IR (Table 10-1).

Table 10-1 Genes pertaining to the WNT pathways, adipocyte regulation (including, the BMP pathway) and the process of browning selected for high throughput sequencing.

Gene	Role
ACACA	Adipocyte Differentiation
ACVR1	BMP Pathway
ACVR1B	BMP Pathway
ACVR1C	BMP Pathway
ACVR2A	BMP Pathway
ACVR2B	BMP Pathway
ACVRLK3	BMP Pathway
ADD1	Adipocyte Differentiation
ADRB3	BEIGE
AKT1	Adipocyte Differentiation
AKT2	Adipocyte Differentiation
AKT3	Adipocyte Differentiation
ALDH1A1	BEIGE
APC	WNT
APC2	WNT canonical
ARNTL	Adipocyte Differentiation
ATF2	WHITE TO BROWN
AXIN1	WNT canonical
AXIN2	WNT
BAMBI	WNT canonical
BMP15	BMP Pathway
BMP2A	BMP Pathway
BMP2A	BMP Pathway
BMP2B	BMP Pathway
BMP4	BMP Pathway
BMP6	BMP Pathway
BMP7	BEIGE
BMP8b	BEIGE
BMP9	BMP Pathway
BMPR	BEIGE
BMPR1B	BMP Pathway
BMPR2	BMP Pathway
BRINP1	BEIGE
CACYBP	WNT canonical
CAMK2A	WNT CA++
CANGL2	WNT PCP
CCND1	WNT canonical
CDK1	Adipocyte Differentiation
CDK4	Adipocyte Differentiation
CDK4	Adipocyte Differentiation
CDK6	Adipocyte Differentiation
CDK6	Adipocyte Differentiation

Table 10-1 cont.

Gene	Role
CEBPA	BMP Pathway
CEBPB	BMP Pathway
CEBPG	BMP Pathway
CER1	WNT canonical
CHD8	WNT canonical
CHRD	BMP Pathway
CIDEA	BEIGE
CRBP1	WNT canonical
CREBBP	WNT canonical
CSNK1A1	WNT
CSNK1A1L	WNT canonical
CSNK1E	WNT
CSNK2A1	WNT canonical
CTBP1	BEIGE
CTBP2	BEIGE
CTNNB1	WNT
CTNNBIP1	WNT
CUL1	WNT canonical
CXXC4	WNT canonical
DAAM1	WNT PCP
DDIT3	Adipocyte Differentiation
DKK1	WNT
DKK3	WNT
DLK1	Adipocyte Differentiation
DVL1	WNT
DVL2	WNT
E2F1	Adipocyte Differentiation
E2F2	Adipocyte Differentiation
E2F3	Adipocyte Differentiation
E2F4	Adipocyte Differentiation
EBF1	Adipocyte Differentiation
EBF2	Adipocyte Differentiation
EGR2	Adipocyte Differentiation
EIF4EBP1	Adipocyte Differentiation
EP300	WNT
ETO	Adipocyte Differentiation
FABP4	Adipocyte Differentiation
FAS	Adipocyte Differentiation
FBXW11	WNT canonical
FGF1	Adipocyte Differentiation
fgf10	Adipocyte Differentiation
FGF21	BEIGE
FGFR1	Adipocyte Differentiation
FGFR2C	Adipocyte Differentiation
FNDC5	BEIGE

Table 10-1 cont.

Gene	Role
FOSL1	WNT canonical
FOXA2	Adipocyte Differentiation
FOXC2	BEIGE
FOXO1	Adipocyte Differentiation
FRAT1	WNT canonical
FRAT1	WNT
FZD1	WNT
FZD10	WNT canonical
FZD2	WNT
FZD3	WNT
FZD4	WNT
FZD5	WNT
FZD6	WNT
FZD7	WNT
FZD8	WNT
FZD9	WNT
GATA2	Adipocyte Differentiation
GATA3	Adipocyte Differentiation
GDF6	BMP Pathway
GDF7	BMP Pathway
GENE	PATHWAY
GPC4	WNT PCP
GSK3B	WNT canonical
GSK3B	WNT
HCRT	BEIGE
HDAC9	Adipocyte Differentiation
HES-1	Adipocyte Differentiation
HOXC8	BEIGE
IGF-1	Adipocyte Differentiation
IKBKE	BEIGE
INHBA	BMP Pathway
IRF1	Adipocyte Differentiation
IRF2	Adipocyte Differentiation
IRF3	Adipocyte Differentiation
IRF4	Adipocyte Differentiation
IRF5	Adipocyte Differentiation
IRF6	Adipocyte Differentiation
IRF7	Adipocyte Differentiation
IRF8	Adipocyte Differentiation
IRF9	Adipocyte Differentiation
IRS1	Adipocyte Differentiation
IRS2	Adipocyte Differentiation
IRS3	Adipocyte Differentiation
IRS4	Adipocyte Differentiation
JUN	WNT canonical

Table 10-1 cont.

Gene	Role
KL	BEIGE
KLF15	Adipocyte Differentiation
KLF2	Adipocyte Differentiation
KLF5	Adipocyte Differentiation
KLF6	Adipocyte Differentiation
KLF7	Adipocyte Differentiation
LDLRP	WNT canonical
LEF1	WNT canonical
LEF1	WNT
LOX	BMP Pathway
LPL	Adipocyte Differentiation
LRP5	WNT
LRP6	WNT
LRRK2	WNT
MADH1	BMP Pathway
MADH5	BMP Pathway
MADH5	BMP Pathway
MAP3K7	WNT canonical
MAPK14	BMP Pathway
MED1	Adipocyte Differentiation
MMP7	WNT canonical
MTOR	Adipocyte Differentiation
MUC1	WNT
MYC	WNT canonical
NCOA1	BEIGE
NCOA2	BEIGE
NCOR1	Adipocyte Differentiation
NCOR2	Adipocyte Differentiation
NDN	Adipocyte Differentiation
NEK2	WNT
NFATC1	WNT CA++
NKD1	WNT canonical
NLK	WNT canonical
NOCA3	Beiging
NPR1	Beiging
NPR3	Beiging
NR1D1	Adipocyte differentiation
NRIH2	Adipocyte differentiation
NRIH3	Adipocyte differentiation
NRIP1	Beiging
OP1	Bmp pathway
OP2	Bmp pathway
Orexin	Beiging
OXR1	Beiging
PDGFRA	Beiging

Table 10-1 cont.

Gene	Role
PDK1	Adipocyte differentiation
PDK2	Adipocyte differentiation
PDK3	Adipocyte differentiation
PDK4	Adipocyte differentiation
Pik3ca	Adipocyte differentiation
Pik3cb	Adipocyte differentiation
Pik3cd	Adipocyte differentiation
PIK3R1	Wnt
Pik3r2	Adipocyte differentiation
Pik3r3	Adipocyte differentiation
PKA	Beiging
PLCB1	Wnt ca++
PLK1	Wnt
PORCN	Wnt
PPARD	WNT canonical
PPARG	Bmp pathway
PPARGC1A	White to brown differentiation
PPARGC1B	Adipocyte differentiation
PPARGC1B	Beiging
PPP3CA	Wnt ca++
PPPARGC1A	Beiging
PPRCI	Adipocyte differentiation
PRDM16	Brown differentiation
PRKACA	WNT canonical
Prkar1a	Beiging
PRKCA	Wnt ca++
PRKG1	Beige
PRKM1	Bmp pathway
PRKM3	Bmp pathway
PRKM8	Wnt
PRKMK3	Bmp pathway
PRKMK6	Bmp pathway
PSEN1	WNT canonical
PTGS2	Beige
PTK2	Bmp pathway
PTPN1	Wnt
PTPRF	Wnt
PTPRK	Wnt
PTPRU	Wnt
RAC1	Wnt
RANBP9	Wnt
RARA	Bmp pathway
RARB	Bmp pathway
RARG	Bmp pathway
RB1	Adipocyte differentiation

Table 10-1 cont.

Gene	Role
RBL1	Adipocyte differentiation
RBL2	Adipocyte differentiation
RBX1	WNT canonical
RHOA	Wnt
ROCK2	Wnt
ROCK2	Wnt
RUVBL1	WNT canonical
RXRA	Adipocyte differentiation
S6K1	Adipocyte differentiation
SCD	Adipocyte differentiation
SENP2	WNT canonical
SFRP1	WNT canonical
SFRP4	Wnt
SHN2	Bmp pathway
SIAH1	WNT canonical
SIRT1	Adipocyte differentiation
SKP1	WNT canonical
SKP2	Wnt
SMAD3	WNT canonical
SMAD4	WNT canonical
SMAD6	Bmp pathway
SMAD7	Bmp pathway
SMARKA2	Adipocyte differentiation
SMARKA4	Adipocyte differentiation
SMURF1	Bmp pathway
SMURF2	Bmp pathway
SOCS1	Adipocyte differentiation
SOST	WNT canonical
SOX17	WNT canonical
SREBP1	Adipocyte differentiation
STAT5A	Adipocyte differentiation
STAT5B	Adipocyte differentiation
STRAP	Wnt
SYM1	Bmp pathway
SYNE1	Wnt
TAF8	Adipocyte differentiation
TBL1X	WNT canonical
TBX1	Beiging
TBX15	Beiging
TCF7	Wnt
TCF7L1	Wnt
TLR10	Regulation of obesity
TGFB1	Bmp pathway
THRA	Beiging
THRB	Beiging

Table 10-1 cont.

Gene	Role
TP53	WNT canonical
TRPV4	Beiging
UCP1	Present in brown adipose
VEGF	Beiging
VEGFR2	Beiging
WIF1	WNT canonical
WISP2	Bmp pathway
WNT1	Wnt
WNT10A	Wnt
WNT11	Wnt
WNT16	WNT canonical
WNT2	Wnt
WNT2B	Wnt
WNT3	Wnt
WNT3A	Wnt
WNT4	Wnt
WNT5	Wnt ca++
WNT6	Wnt
WNT7A	Wnt
WNT7B	Wnt
WNT8A	Wnt
WWTR1	Adipocyte differentiation
ZFP423	Bmp pathway
ZIC1	Present in brown adipose

Polymorphisms in these genes were analysed and chosen for KASP genotyping if they were rare, if they were in important genes, if were noted to be pathogenic or if they were predominantly present in one of the groups. Polymorphisms in selected genes can be seen in Table 10-2

Table 10-2 Polymorphisms related to WNT or adipose tissue control

Gene	Coding polymorphism					
	Missense	Frameshift	In-frame	Stop-gained	Stop lost	Total
FZD	1					1
LRP5	2	1				3
WNT10A	2					2
WNT6	1					1
WNT16	2					2
KDR	3					3
KL	2					2
MAPK11	1					1
NFATC1	4		1			5
IRS1	2		1			3
IRS2	1					1
UCP1	2					2
BMP4	2					2
CHRD	2					2
ADD1	3					3
PPARGC1A	3					3
PPARGC1B	3					3
LPL	2					2
E2F2	3					3
TLR10	10					10
CREBBP	2					2
CRTC2	2					2

Section 10.8.2. Polymorphisms related to the WNT pathway

The polymorphisms related to the Wnt pathway and adipocyte differentiation which were chosen for further analysis can be found in (Table 10-3).

Table 10-3 . Selected polymorphisms in Wnt pathway and adipocyte differentiation related genes. ClinVar accessed 9.19.

Gene	rs number	Chromosome	DNA change	Amino Acid change	Consequence	Frequency	ClinVar
FZD5	rs35994626	2	G>A	Pro216Leu	Missense	0.048	Not reported
LRP5	rs4988321	11	G>A	Val667Met	Missense	0.03791	Likely benign
LRP5	rs3736228	11	C>T	Ala1330Val	Missense	0.1338	Benign
MAPK11	rs33932986	22	C>T	Gln275Leu	Missense	0.01867	Not reported
NFATC1	rs1051978	18	C>A	Pro55Thr	Missense	0.09701	Not reported
GATA4	rs3729856	8	A>G	Ser377Gly	Missense	0.09583	Benign
WNT10A	rs121908120	2	T>A	Phe228Ile	Missense	0.013	
WNT6	rs141494427	2	C>G	Pro155Arg	Missense	0.05560	Not reported
WNT16	rs2707466	7	C>T	Thr253Arg	Missense	0.4422	Not reported
IRS1	rs1801278	2	C>T	Gly971Arg	Missense	0.05211	
IRS2	rs1805097	13	C>T	Gly1057Asp	Missense	0.3504	Diabetes Type II
KDR	rs2305948	4	C>T	Val297Ile	Missense	0.1048	No interpretation
KL	rs9536314	13	T>G	Phe352Val	Missense	0.141	Likely benign
KL	rs9527025	13	G>C	Cys370Ser	Missense	0.1418	Likely benign
UCP1	rs2270565	4	T>A	Met229Leu	Missense	0.089	Not reported
UCP1	rs45539933	4	C>T	Ala64Thr	Missense	0.08782	Not reported
BMP4	rs17563	14	A>G	Val152Ala	Missense	0.454	Benign/ Likely
CHRD	rs16858780	3	A>C	Met630Leu	Missense	0.23059	Not reported
ADD1	rs4961	4	G>T	Gly460Trp	Missense	0.2027	Drug response
ADD1	rs146536048	4	G>A	Val559Met	Missense	0.00341	Not reported
PPARGC1A	rs8192678	4	C>T	G1444A	Missense	0.30984	Not reported
PPARGC1A	rs3736265	4	G>A	C1835T	Missense	0.08595	Not reported

Table 10-3 cont.

PPARGC1B	rs45520937	5	G>A	G794A	Missense	0.08262	Not reported
PPARGC1B	rs7732671	5	G>C	Ala203Pro	Missense		
PPARA	rs1800206	22	C>G	Leu162Val	Missense	0.0434	Hyperbetalipoprotei
LPL	rs1801177	8	G>A	Asp36Asn	Missense	0.014	Likely benign
E2F2	rs114788023	1	C>T	Asp338Asn	Missense	0.004	
TLR10	rs11466653	4	A>G	Met326Thr	Missense	0.06434	Papillary thyroid Ca
CREBBP	rs61753381	16	G>T	Leu551Ile	Missense	0.01029	Conflicting evidence
CRTC2	rs11264680	1	T>C	Met 147Val	Missense	0.47	Not reported

Section 10.8.3. Risk of MI with selected polymorphisms related to the Wnt Pathway and Adipocyte Regulation

The risk of MI with each of the polymorphisms was analysed in the whole MAMI collection and separately by sex (Table 10-4). All the polymorphisms selected and tested except the rs1801177 polymorphism in LPL, rs61753381 in CREBBP, rs3729856 in GATA4 were in Hardy Weinberg Equilibrium (HWE) in controls.

There was a weak pattern towards a protective effect with heterozygosity for rs121908120 in *WNT10A* in men AdjOR 0.7 (95% CI, 0.4 -1.3) and in both sexes combined AdjOR 0.8 (95% CI, 0.5-1.3). The rs8192678 polymorphism in *PPARGC1A* was deleterious in homozygous individuals when both sexes were analysed combined, AdjOR 1.9 (95% CI, 1.4-2.7), this effect was not observed when the sexes were analysed separately or in heterozygous subjects. Heterozygosity for the rs2305948 polymorphism in *KDR* was protective, AdjOR 0.5 (95% CI, 0.2-1.0) in women but not in men or in both sexes analysed together. There was a pattern towards a protective effect in heterozygous men with the rs4988321 polymorphism in *LRP5*, AdjOR 0.7 (95%CI, 0.4-1.1) and in both sexesanalysed together, AdjOR 0.71 (95% CI, 0.5-1.1). A similar protective effect was observed with the rs3736228 polymorphism also in *LRP5*. The rs4988321 and rs3736228 in *LRP5* are in linkage disequilibrium (LD). The rs9536314 and rs9527025 in *KL* and rs2270565 and rs45539933 in *UCP1* were also noted to be in complete LD. Women who were heterozygous for the rs2270565 or the rs45539933 showed a pattern towards being protected from MI. This effect was not present in men. Heterozygotes for the rs33932986 polymorphism in *MAPKII* in men and both sexes analysed together had a tendency towards decreased risk of MI, AdjOR 0.8 (95% CI, 0.5-1.2) in men. Women who were heterozygous for the rs16858780 in *CHRD* showed a pattern towards a strong decreased risk of MI, AdjOR 0.5 (95%CI 0.2-1.1). This effect was not present in both sexes analysed together or in men. Female heterozygotes for the *ADD1* polymorphism RS4961 also showed

a pattern towards having a decreased risk of MI, AdjOR 0.6 (95%CI 0.3-1.2). This effect was not observed in men. (Table 10-4).

Table 10-4 Odds Ratio for risk of Myocardial Infarction for genetic variants in the WNT Pathway selected using an extreme phenotype approach

rs number	*Gene*	Genotype	Men			Women		
			n cases (%)	n controls (%)	Age adjusted OR (95% CI)	n cases(%)	n controls (%)	Age Adjusted OR (95% CI)
rs1801177	*LPL*							
		HA	1 (0.4)	2 (0.6)		0 (0)	0(0)	
		Het	22 (7.9)	21 (6.6)	1.2 (0.6-2.2)	6 (9.1)	5 (3.8)	3.0 (0.8-11.8)
		WT	254 (91.7)	294 (92.7)	1	60 (90.9)	127 (96.2)	1
rs 114788023	*E2F2*							
		HA	2 (0.7)	0(0)				
		Het	6 (2.2)	12 (3.8)	0.6 (0.2-1.6)	2 (3.0)	1 (0.7)	-
		WT	266 (97.1)	307 (96.2)	1	64 (97.0)	133 (99.3)	1
rs 35994626	*FZD5*							
		HA	2 (0.7)	3 (1.0)		0 (0)	0(0)	
		Het	44 (15.9)	42 (13.4)	1.3 (0.8-2.0)	13 (20.3)	17 (12.8)	1.63 (0.7-3.7)
		WT	230 (83.3)	268 (85.6)	1	51 (79.7)	117 (87.2)	1
rs121908120	*WNT10A*							
		HA	0 (0)	0 (0)		0(0)	0(0)	
		Het	23 (8.4)	35 (11.0)	0.7 (0.4 -1.3)	6 (9.2)	13 (9.7)	1.1(0.4-3.3)
		WT	251 (91.6)	282 (89.0)	1	59 (90.8)	121 (90.3)	1
rs1801278	*IRS1*							
		HA	5 (1.8)	6(1.9)	1.1 (0.3-3.5)	2 (3.1)	2(1.5)	
		Het	75(27.2)	77(24.2)	1.2 (0.8-1.7)	16 (24.6)	32 (23.9)	1.1 (0.5-2.3)
		WT	196 (71.0)	235(73.9)	1	27 (72.3)	100 (74.6)	1
rs8192678	*PPARGC1A*							
		HA	60 (21.9)	68 (22.0)	1.1 (0.69-1.7)	8 (12.3)	22 (16.5)	0.6 (0.2-1.5)
		Het	138 (50.4)	146 (47.2)	1.2 (0.79-1.7)	24 (52.3)	73 (54.9)	0.8 (0.4-1.7)
		WT	76 (27.7)	95 (30.7)	1	23 (35.4)	38 (28.6)	
rs11466653	*TLR 10*							
		HA	2 (0.7)	3 (0.9)		0 (0)	0(0)	
		Het	34 (12.6)	47 (14.8)	0.8 (0.5-1.3)	11 (17.2)	22 (16.4)	1.3 (0.6-3.0)
		WT	234 (86.7)	267 (84.2)	1	53 (82.8)	112(83.6)	1

Table 10-4 cont.

rs number	*Gene*	Genotype	Men			Women		
			n cases (%)	n controls (%)	Age adjusted OR (95% CI)	n cases(%)	n controls (%)	Age Adjusted OR (95% CI)
rs2305948	*KDR*							
		HA	5 (1.8)	1 (0.3)		0 (0)	3 (2.2)	
		Het	51 (18.4)	53 (16.8)	1.1 (0.7-1.7)	9 (13.8)	32 (23.9)	0.5 (0.2-1.0)
		WT	221 (79.8)	262 (82.9)	1.0	56 (86.2)	99 (73.9)	1.0
rs9536314	*KL*							
		HA	8 (2.9)	5 (1.6)	2.0 (0.6-6.3)	2 (3.1)	4 (3.0)	
		Het	76 (27.7)	86 (27.4)	1.0 (0.7-1.5)	18 (27.7)	33 (24.6)	1.2 (0.6-2.4)
		WT	190 (69.3)	223 (71.0)	1.0	45 (69.2)	97 (72.4)	1.0
rs9527025	*KL*							
		HA	8 (3.0)	5(1.6)	2.0 (0.7-6.4)	2 (3.1)	4 (2.0)	
		Het	74 (27.3)	85 (27.0)	1.0 (0.71-1.5)	18 (28.1)	33 (24.4)	1.2 (0.6-2.4)
		WT	189 (69.7)	225 (71.4)	1.0	44 (68.8)	98 (72.6)	1.0
rs4988321	*LRP5*							
		HA	1 (0.4)	0 (0)		1 (1.5)	2 (1.5)	
		Het	34 (12.4)	55 (17.4)	0.7 (0.4-1.1)	7 (10.6)	15 (11.5)	1.1 (0.4-3.2)
		WT	239 (87.2)	262 (82.6)	1.0	58 (87.9)	114 (87.00	1.0
rs3736228	*LRP5*							
		HA	5 (1.8)	2 (0.6)		1 (1.5)	4 (3.0)	
		Het	56(20.5)	81 (25.9)	0.7 (0.5-1.1)	11 (16.7)	27 (10.5)	0.8 (0.4-1.9)
		WT	212 (77.7)	230 (73.5)	1.0	54 (81.8)	101 (76.5)	1.0
rs61753381	*CREBBP*							
		HA	1 (0.4)	3 (0.9)		0 (0)	0 (0)	
		Het	26(9.6)	21 (6.6)	1.5 (0.8-2.7)	5 (7.8)	6 (4.5)	2.2 (0.6-8.2)
		WT	243 (90.0)	295 (92.5)	1.0	59 (92.2)	128 (95.5)	1.0
rs1051978	*NFATC1*							
		HA	0(0)	2 (0.6)		0(0)	0(0)	
		Het	17 (6.2)	21 (6.7)	0.9 (0.5-1.7)	5 (7.8)	13 (9.8)	1.0 (0.3-3.0)
		WT	258 (93.8)	290 (92.7)	1.0	59 (92.2)	120 (90.2)	1.0

Table 10-4 cont.

rs number	*Gene*	Genotype	Men			Women		
			n cases (%)	n controls (%)	Age adjusted OR (95% CI)	n cases(%)	n controls (%)	Age Adjusted OR (95% CI)
rs2270565	*Ucp1*							
		HA	2 (0.7)	5 (1.6)		0 (0)	1(0.8)	
		Het	57 (20.7)	57 (17.9)	1.2 (0.8-1.8)	7 (10.8)	29 (21.8)	0.42 (0.2-1.1)
		WT	216 (78.5)	257 (80.6)	1.0	58 (89.2)	103 (77.4)	1.0
rs45539933	*Ucp1*							
		HA	0(0)	5 (1.6)		0 (0)	1 (0.8)	
		Het	58 (21.6)	56 (17.8)	1.2 (0.8-1.9)	8 (12.3)	27 (20.8)	0.5 (0.2-1.3)
		WT	210 (78.4)	253 (80.6)	1.0	57 (87.7)	102 (78.5)	1.0
rs3729856	*GATA4*							
		HA	1 (0.4)	5 (1.6)		0 (0)	2 (1.5)	
		Het	40(14.8)	42 (13.4)	1.2 (0.7-1.8)	9 (14.1)	17(12.8)	1.4 (0.5-3.7)
		WT	229 (84.8)	267 (85.0)	1.0	55 (85.9)	114 (85.7)	1.0
rs 17563	*BMP4*							
		HA	50 (17.8)	66 (20.8)	0.9 (0.5-1.4)	7 (10.8)	16 (12.0)	0.7 (0.24-2.13)
		Het	134 (49.1)	150 (47.2)	1.0 (0.7-1.5)	38 (58.5)	74 (55.6)	1.1 (0.54-2.21)
		WT	89 (32.6)	102 (32.1)	1.0	20 (30.8)	43 (32.3)	1.0
rs33932986	*MAPK11*							
		HA	0 (0)	4 (1.3)		0(0)	1(0.8)	
		Het	40 (14.9)	58 (18.4)	0.8 (0.5-1.2)	11(16.9)	31 (23.3)	0.7 (0.3-1.7)
		WT	229 (85.1)	254 (80.4)	1.0	54 (83.1)	101 (75.9)	1.0
rs141494427	*WNT6*							
		HA	2 (0.7)	2 (0.6)		0 (0)	3 (2.3)	
		Het	47 (17.4)	50 (15.9)	1.1 (0.7-1.7)	9 (13.8)	18 (13.6)	1.0 (0.4-2.4)
		WT	221 (81.9)	262 (83.4)	1.0	56 (86.2)	111 (84.1)	1.0
rs2707466	*WNT16*							
		HA	53 (19.3)	72 (22.8)	0.83 (0.5-1.3)	10 (15.2)	29 (21.8)	0.6 (0.2-1.5)
		Het	143 (52.0)	155 (49.1)	1.02 (0.7-1.5)	39 (59.1)	74 (55.6)	0.67 (0.3-1.5)
		WT	79 (28.7)	89 (28.2)	1.0	17 (25.8)	30 (22.6)	1.0
rs1805097	*IRS2*							
		HA	32 (12.5)	40 (12.8)	1.1 (0.6-1.8)	6 (10.2)	16 (12.2)	0.9 (0.3-2.9)
		Het	118 (45.9)	135 (43.3)	1.1 (0.8-1.6)	33 (55.9)	60 (45.8)	1.4 (0.7-2.9)
		WT	107 (41.6)	137 (43.9)	1.0	20 (33.9)	55 (42.0)	1.0

Table 10-4 cont.

Rs number	Gene	Genotype	Men N cases (%)	N controls (%)	Age Adjusted OR	Women N cases (%)	N control (%)	Age Adjusted OR
rs16858780	*CHRD*							
		HA	6 (2.2)	7 (2.2)	1.1 (0.4-3.2)	1 (1.6)	2 (1.5)	0.9 (0.1-13.3)
		Het	70 (25.6)	70 (22.2)	1.0 (0.8-1.7)	11 (17.2)	34 (25.2)	0.5 (0.2-1.1)
		WT	197 (72.2)	238 (75.6)	1.0	52 (81.3)	99 (73.3)	1.0
rs4961	*ADD1*							
		HA	8 (3.0)	8(2.6)	1.3 (0.5-3.5)	0(0)	6 (4.5)	
		Het	69 (25.6)	75 (24.4)	1.1 (0.7-1.6)	15 (23.4)	44 (33.1)	0.6 (0.3-1.2)
		WT	193 (71.5)	225 (73.1)	1.0	49 (76.6)	83 (62.4)	1.0
rs146536048	*ADD1*							
		Het	11 (4.0)	9 (2.8)	1.4 (0.6-3.5)	2 (3.1)	4 (3.0)	0.8 (1.1-4.3)
		WT	266 (96.0)	307 (97.2)	1.0	63 (96.9)	129 (97.0)	1.0
rs8192678	*PPARGC1A*							
		HA	60 (22.0)	67 (21.2)	1.1 (0.7-1.8)	7 (11.3)	22 (16.5)	0.5 (0.2-1.5)
		Het	139 (50.9)	153 (48.4)	0.5 (0.8-1.7)	33 (53.2)	72 (54.1)	0.9 (0.4-1.7)
		WT	74 (27.1)	96 (30.4)	1.0	22 (35.5)	39 (29.3)	1.0
rs3736265	*PPARGC1A*	C1835T						
		HA	2 (0.7)	0 (0)				
		Het	21 (7.8)	29 (9.1)	0.8 (0.4-1.5)	3 (4.8)	10 (7.4)	0.5 (0.1-2.1)
		WT	246 (91.4)	289 (90.9)	1.0	60 (95.2)	125 (92.6)	1.0
rs45520937	*PPARGC1B*							
		HA	0(0)	1(0.3)				
		Het	9 (3.3)	13 (4.1)	0.8 (0.3-1.9)			
		WT	265 (96.7)	303 (95.6)	1.0			
rs11264680	*CRTC2*							
		HA	65 (24.0)	56 (17.9)	1.4 (0.9-2.2)	17 (27.0)	27 (20.3)	1.6 (0.6-3.9)
		Het	130 (18.0)	163 (52.1)	1.0 (0.7-1.4)	32 (50.8)	62 (46.6)	1.4 (0.7-3.1)
		WT	76 (28.0)	94 (30.0)	1.0	14 (22.2)	44 (33.1)	1.0
rs1800206	*PPARA*							
		HA	0 (0)	1 (0.3)		0 (0)	1(0.8)	
		Het	24 (8.8)	24 (7.6)	1.2 (0.7-2.1)	4 (6.4)	14(10.6)	0.7 (0.2-2.3)
		WT	250 (91.2)	192 (92.1)	1.0	59 (93.7)	117 (8.6)	1.0

Section 10.8.4. Intermediate Phenotypes with Selected Polymorphisms in the WNT Pathway

Results for median levels of several phenotypes related to obesity and insulin resistance are shown for each genotype studied in Table 10-5, Table 10-6.

Heterozygotes for the rs1801177 polymorphism in *LPL* had higher median triglyceride levels (1.61 mmol/L) when compared to the wildtype (1.21 mmol/L) subjects in male controls (p-value 0.04). There were no differences in NHDL-C or HDL-C levels noted with this polymorphism (Table 10-5). There was also no difference in markers of liver inflammation (γ-GT and ALT) or in surrogate markers of insulin resistance or hs-CRP (Table 10-6).

Male control subjects who were Het for rs114788023 in *E2F2* showed a pattern towards lower median levels of γ-GT (19.50mmol/l) compared with the wildtype (median level 29.00mmol/l), p-value 0.08. There was also less insulin resistance in heterozygotes of this polymorphism with lower median levels of HOMA-C (0.14 for vs 0.11 for heterozygotes, p-value 0.02) and HOMA-IR (1.92 for wildtype vs 0.73 for heterozygotes, p value 0.002). There was no difference in WHR or lipid variables (Table 10-6).

Controls that were heterozygous for rs1801278 in *IRS1* showed higher median levels of HOMA-IR (2.28) compared to wildtype individuals (HOMA-IR-1.58), p-value 0.02. Homozygotes for this polymorphism had HOMA-IR of 2.72. There was no difference in HOMA-C though or in the percentage of diabetic subjects. There was also no difference in ALT and γ-GT, hs-CRP or lipid variable levels (Table 10-6)

The homozygous alternate genotype for the rs8192678 polymorphism in *PPARGCIA* had lower median HDL-C levels (1.22mmol/l vs wildtype subjects 1.31mmol/L, p-value 0.10), lower γGT (median 27.00 vs 31.00 mmol/L in wildtype, p-value 0.05) and lower hs-CRP (median 1.56 mmol/L vs 2.25mmol/L in wildtype, p value 0.05). These patterns were also observed in

heterozygotes for this polymorphism for hs-CRP and γGT but not for HDL (Table 10-8, Table 10-9). Male control subjects that were heterozygous for the rs2305948 polymorphism in *KDR* had a lower median WHR (0.93, IQR 0.89-0.97) compared to wildtype controls where the median WHR was 0.95 (IQR 0.90-1.01), p-value 0.02. No changes in lipid variable levels, markers of liver inflammation, or in surrogate markers of insulin resistance and hs-CRP were noted with this polymorphism (Table 10-6). Male controls with the homozygous alternate polymorphism in rs9536314 in *KL* and also those with the heterozygous genotype had a lower median triglyceride level (0.73mmol/L in homozygous alternate and 1.15mmol/L in heterozygotes) compared to those with the wildtype genotype for this polymorphism (median triglyceride level 1.30mmol/L), p-values 0.04 and 0.09 respectively. The same results were observed for the rs9527025, another polymorphism in the *KL gene*, with median triglyceride level being lower amongst individuals with the homozygous alternate genotype (0.73mmol/L) and heterozygous genotype (1.14mmol/L) compared with wildtype individuals, (1.30mmol/L) p-values 0.04 and 0.07 respectively. There was no difference in WHR, NHDL-C, HDL-C, markers of liver inflammation, nor in surrogate markers of insulin resistance and hs-CRP with these polymorphisms (Table 10-6). Heterozygotes for rs4988321in *LRP5* showed higher levels of insulin resistance as shown by both HOMA-C (0.18 in heterozygotes and 0.14 in wildtype, p value 0.02) and HOMA-IR (2.39 in heterozygotes and 1.75 in wildtype, p value 0.03) and lower HDL median levels compared with the wildtype individuals (1.20 vs 1.34mmol/L, p-value 0.06). There were no differences in other lipid variables, percentage of diabetic individuals, hs-CRP or measures of liver inflammation (Table 10-6). Although heterozygotes for the rs3736228, also in *LRP5*, which is in partial LD with rs4988321, tended to have lower median levels of HDL (1.24mmol/L) and higher median levels of HOMA-IR (2.17) compared with the wildtype genotype (HDL 1.34mmol/L and HOMA-IR 1.65), differences did not reach statistical significance.

Heterozygotes for rs61753381 in *CREBBP* had higher levels of hs-CRP (Median 2.67mmol/L) compared to wildtype individuals (Median 1.68mmol/L, p-value 0.02). There was also a higher percentage of diabetic individuals with this polymorphism (20.0% vs 12.1% amongst wildtype) however there was no difference in HOMA-IR and HOMA-C (Table 10-6). There were no differences in lipid variables, or measures of liver inflammation with this variant.

Male controls heterozygous for the rs45539933 variant in *UCP1* had lower triglyceride levels (median triglyceride 1.09mmol/l) compared to those with the wildtype genotype (median triglycerides 1.27mmol/L), p value 0.05. Individuals with the homozygous alternate genotype had higher median ALT compared with the wildtype (40.00 vs. 22.00, p-value 0.02). There was no difference in WHR, NHDL-C, HDL-C levels, in markers of insulin resistance or in hs-CRP levels with this variant. A similar difference was noted between heterozygous and wildtype genotypes of rs2270565 in *UCP1* (triglycerides 1.12 vs. 1.27mmol/L, p-value 0.06 and ALT 40.00 vs. 22.00, respectively, p-value 0.02) since these polymorphism are in LD (Table 10-5).

Amongst controls, men heterozygous for rs3729856 in *GATA4* gene, had lower levels of insulin resistance as measured by HOMA-C (median value 0.12 in heterozygotes vs. 0.14 in wildtype, p-value 0.08). There was no difference in HOMA-IR or in the percentage of diabetic subjects. Heterozygotes for this variant also had lower hs-CRP levels compared with the homozygous wildtype (1.43 vs. 1.75mg/L). No other differences were observed in neither the markers of liver inflammation nor in lipid variables. This polymorphism was not in Hardy-Weinberg equilibrium in controls.

Male control subjects that were heterozygous for *WNT6* rs141494427 showed a pattern towards higher NHDL-C levels (median 4.28mmol/L vs 3.99mmol/L).

Male control subjects that were homozygous alternate for rs2707466 in *WNT16* had lower median NHDL-C levels (3.79mmol/L) than wildtype individuals (median NHDL-C-4.31mmol/l), p value 0.004. Heterozygotes for this polymorphism also had lower median NHDL-C levels (3.96mmol/L) than the wildtype group, p- value 0.014. There was no difference in WHR, triglyceride, HDL, hs-CRP levels, or in markers of liver inflammation and insulin resistance with this polymorphism (Table 10-6).

Male controls with the homozygous alternate genotype for rs1805097 in *IRS2* had lower NHDL-C levels (median 3.90mmol/l) than wildtype subjects (median 4.06mmol/l), p-value 0.09 and higher HDL levels (median 1.43mmol/l) compared to the wildtype group (median 1.28mmol/L), p-value 0.04 (Table 10-5). The homozygous alternative subjects for rs1805097 in *IRS2* also had lower levels of HOMA-C (0.11 vs 0.14 in wildtype, p- value 0.01) and HOMA-IR (median 1.31 vs 2.10 in wildtype, p value 0.03). There was no difference in the percentage of diabetic individuals amongst male controls, and no difference in median levels of triglycerides, WHR, hs-CRP, ALT and GGT (Table 10-6).

Male controls that were heterozygous for rs16858780 in *CHRD* had lower ALT levels than WT individuals (median 20.5mmol/L vs 23.00 mmol/L, p- value 0.01)

Table 10-5 Changes in WHR and lipid variables with different genotypes in selected polymorphisms in genes related to the WNT Pathway in male controls. TG: triglycerides; NDHL-C: Non-HDL cholesterol; HDL-C: High density lipoprotein cholesterol; IQR: interquartile range. *p-value < 0.10. ** p-value < 0.05. ***p-value < 0.01. HA-homozygous alternate, Het -heterozygous, WT- wildtype

rs number	*Gene*	Genotype	N	WHR Median (IQR) mmol/L	TG Median (IQR) mmol/L	NHDL-C median (IQR) mmol/L	HDL-C median (IQR) mmol/L
rs1801177	*LPL*	HA	2	0.90	1.24	4.05	1.20
		Het	21	0.96 (0.90-1.00)	1.61 (1.01-2.32) **	4.40 (3.42-5.22)	1.39 (1.05-1.57)
		WT	294	0.95 (0.90-1.00)	1.21 (0.85-1.74)	4.02 (3.25-4.72)	1.31 (1.12-1.54)
		p-value WT/HA		0.36	0.93	0.94	0.44
		p-value WT/Het		0.99	0.04	0.16	0.64
rs114788023	*E2F2*	Het	12	0.94 (0.88-0.99)	1.17 (0.79-2.02)	3.71 (3.35-5.01)	1.40 (1.13-1.71)
		WT	307	0.95 (0.90-1.00)	1.25 (0.86-1.76)	4.04 (3.27-4.77)	1.31 (1.11-1.54)
		p-value WT/Het		0.45	0.72	0.95	0.40
rs35994626	*FZD5*	HA	2	0.96	0.87	3.86	1.20 (1.07-1.55)
		Het	40	0.94 (0.89-1.00)	1.13 (0.82-1.90)	4.23 (3.47-4.81)	1.31 (1.07-1.55)
		WT	268	0.95 (0.90-1.00)	1.26 (0.85-1.76)	4.04 (3.25-4.78)	1.31 (1.11-1.55)
		p-value WT/HA		0.89	0.37	0.60	0.86
		p-value WT/Het		0.20	0.78	0.32	0.72
rs121908120	*WNT 10A*	Het	35	0.94 (0.89-1.00)	1.38 (0.98-1.74)	4.17 (3.18-5.02)	1.31 (1.09-1.59)
		WT	279	0.95 (0.90- 1.00)	1.22 (0.85-1.77)	4.02 (3.29-4.72)	1.31 (1.11-1.54)
		p-value WT/Het		0.59	0.61	0.44	0.78

Table 10-5 cont.

rs number	*Gene*	Genotype	N	WHR Median (IQR) mmol/L	TG Median (IQR) mmol/L	NHDL-C median (IQR) mmol/L	HDL-C median (IQR) mmol/L
rs1801278	*IRS1*	HA	6	0.99 (0.89-1.02)	1.57 (1.13-2.05)	4.38 (3.82-5.41)	1.19 (1.08-1.49)
		Het	76	0.97 (0.92-1.01)	1.30 (0.88-1.80)	3.94 (3.16-4.85)	1.32 (1.07-1.55)
		WT	233	0.94 (0.90-1.00)	1.20 (0.85-1.75)	4.05 (3.30-4.73)	1.31 (1.12-1.55)
		p-value WT/HA		0.54	0.31	0.22	0.42
		p-value WT/Het		0.13	0.73	0.62	0.57
rs8192678	*PPARGC1A*	Homozygous Alternate	67	0.96 (0.90-1.01)	1.33 (0.86-2.04)	4.00 (3.26-4.79)	1.22 (1.07-1.49)
		Het	144	0.94 (0.90-1.00)	1.24 (0.81-1.80)	4.02 (3.26-4.72)	1.34 (1.12-1.56)
		WT	95	0.95 (0.90-1.00)	1.19 (0.87-1.73)	4.06 (3.41-4.79)	1.31 (1.15-1.53)
		p-value WT/HA		0.40	0.51	0.61	0.10
		p-value WT/Het		0.76	0.88	0.55	0.90
rs11466653	*TLR 10*	HA	3	0.89 (0.81-0.89)	0.63*	3.85	1.48
		Het	46	0.95 (0.90-1.00)	1.30 (0.76-1.85)	4.14 (3.25-4.50)	1.30 (1.09-1.49)
		WT	265	0.95 (0.90-1.00)	1.25 (0.87-1.76)	4.02 (3.29-4.79)	1.31 (1.12-1.55)
		p-value WT/HA		0.36	0.05	0.79	0.40
		p-value WT/Het		0.63	0.80	0.92	0.46
rs2305948	*KDR*	HA	1	0.96	5.26**	4.81	0.73**
		Het	53	0.93 (0.89-0.97)	1.13 (0.81-1.70)	3.84 (3.29-4.50)	1.32 (1.09-1.62)
		WT	259	0.95 (0.90-1.01)	1.26 (0.87-1.76)	4.07 (3.25-4.78)	1.31 (1.12-1.54)
		p-value WT/HA		0.96	0.02	0.49	0.02
		p-value WT/Het		0.02	0.44	0.44	0.71

Table 10-5 cont.

rs number	*Gene*	Genotype	n	WHR Median (IQR)	TG Median (IQR)	NHDL-C median (IQR)	HDL-C median (IQR)
rs9536314	*KL*	HA	5	0.92 (0.88-0.98)	0.73 (0.68-1.17) *	3.60 (3.07-3.90)	1.19 (1.00-1.43)
		Het	86	0.94 (0.90-0.99)	1.15 (0.78-1.74)	4.04 (3.16-4.85)	1.36 (1.13-1.57)
		WT	220	0.95 (0.90-1.01)	1.30 (0.88-1.84)	4.03 (3.30-4.72)	1.30 (1.10-1.53)
		p-value WT/HA		0.36	0.04	0.15	0.45
		p-value WT/Het		0.26	0.09	0.65	0.19
rs9527025	*KL*	HA	5	0.92 (0.88-0.98)	0.73 (0.68-1.17) *	3.60 (3.07-3.89)	1.19 (1.00-1.43)
		Het	85	0.94 (0.90-0.99)	1.14 (0.77-1.72) *	3.97 (3.16-4.83)	1.37 (1.12-1.57)
		WT	222	0.95 (0.90-1.00)	1.30 (0.88-1.83)	4.04 (3.30-4.72)	1.30 (1.11-1.53)
		p-value WT/HA		0.36	0.04	0.14	0.43
		p-value WT/Het		0.25	0.07	0.55	0.24
rs4988321	*LRP5*	Het	55	0.95 (0.91-1.00)	1.40 (0.85-2.03)	4.05 (3.29-4.81)	1.20 (1.08-1.46) *
		WT	259	0.95 (0.90-1.00)	1.22 (0.86-1.74)	4.02 (3.27-4.72)	1.34 (1.12-1.55)
		p-value WT/Het		0.61	0.32	0.65	0.06
rs3736228	*LRP5*	HA	2	1.02	1.00	3.66	1.16
		Het	80	0.94 (0.90-1.00)	1.22 (0.85-1.78)	3.99 (3.25-4.79)	1.24 (1.08-1.57)
		WT	228	0.95 (0.90-1.00)	1.25 (0.87-1.75)	4.06 (3.30-4.74)	1.34 (1.13-1.55)
		p-value WT/HA		0.22	0.42	0.48	0.30
		p-value WT/Het		0.80	0.97	0.64	0.18
rs61753381	*CREBBP*	HA	3	1.00	1.20	2.59	1.57
		Het	21	0.94 (0.89-0.99)	1.62 (0.87-2.53)	4.73 (3.61-5.20)	1.33 (1.06-1.52)
		WT	292	0.95 (0.90-1.00)	1.23 (0.86-1.74)	4.03 (3.28-4.71)	1.31 (1.12-1.55)
		p-value WT/HA		0.31	0.98	0.10	0.45
		p-value WT/Het		0.64	0.14	0.14	0.66

Table 10-5 cont.

rs number	*Gene*	Genotype	n	WHR Median (IQR)	TG Median (IQR)	NHDL-C median (IQR)	HDL-C median (IQR)
rs1051978	*NFATC1*	HA	2	0.90	1.33	4.55	1.20
		Het	21	0.95 (0.92-0.99)	1.08 (0.70-2.07)	3.93 (2.89-4.44)	1.24 (1.06-1.51)
		WT	287	0.95 (0.90-1.00)	1.25 (0.87-1.74)	4.02 (3.30-4.74)	1.31 (1.12-1.55)
		p-value WT/HA		0.23	0.80	0.33	0.41
		p-value WT/Het		0.62	0.80	0.34	0.41
rs2270565	*UCP1*	HA	5	0.94 (0.88-0.96)	1.62 (0.75-2.81)	4.10 (2.65-4.27)	1.19 (0.86-2.02)
		Het	57	0.95 (0.90-1.01)	1.12 (0.72-1.58)	4.06 (3.33-4.52)	1.30 (1.12-1.54)
		WT	254	0.95 (0.90-1.00)	1.27 (0.87-1.82) *	4.02 (3.28-4.86)	1.31 (1.12-1.55)
		p-value WT/HA		0.38	0.81	0.34	0.96
		p-value WT/Het		0.53	0.06	0.66	0.53
rs45539933	*UCP1*	HA	5	0.94 (0.88-0.96)	1.62 (0.75-2.81)	4.10 (4.10-4.27)	1.19 (0.86-2.02)
		Het	56	0.95 (0.90-1.00)	1.09 (0.72-1.60) *	4.08 (3.36-4.54)	1.32 (1.12-1.54)
		WT	250	0.95 (0.90-1.00)	1.27 (0.88-1.82)	4.02 (3.26-4.85)	1.31 (1.11-1.54)
		p-value WT/HA		0.37	0.82	0.34	0.99
		p-value WT/Het		0.96	0.05	0.85	0.84
rs3729856	*GATA4*	HA	5	1.00	1.00 (0.70-2.60)	3.61 (2.78-4.67)	1.31 (1.11-1.47)
		Het	41	0.96	1.28 (0.80-1.81)	4.07 (3.36-4.63)	1.39 (1.19-1.53)
		WT	265	0.94	1.25 (0.87-1.76)	4.05 (3.26-4.81)	1.30 (1.10-1.54)
		p-value WT/HA		0.65	0.93	0.60	0.89
		p-value WT/Het		0.91	0.67	0.92	0.21
rs17563	*BMP4*	HA	66	0.93 (0.93-1.00)	1.20 (0.90-1.85)	4.05 (3.39-4.93)	1.36 (1.16-1.57)
		Het	148	0.95 (0.91-1.00)	1.25 (0.85-1.76)	4.12 (3.23-4.78)	1.30 (1.09-1.51)
		WT	101	0.95 (0.90-1.00)	1.26 (0.86-1.75)	3.94 (3.21-4.62)	1.29 (1.10-1.56)
		p-value WT/HA		0.72	0.67	0.29	0.29
		p-value WT/Het		0.60	0.99	0.56	0.66

Table 10-5 cont.

rs number	*Gene*	Genotype	n	WHR Median (IQR)	TG Median (IQR)	NHDL-C median (IQR)	HDL-C median (IQR)
rs33932986	*MAPK11*	HA	4	0.96 (0.91-0.99)	1.29(0.88-1.99)	3.75 (3.63-4.06)	1.51 (1.36-1.54)
		Het	58	0.95 (0.91-1.01)	1.26 (0.94-1.85)	4.07 (3.16-4.69)	1.30 (1.09-1.45)
		WT	251	0.94 (0.90-1.00)	1.22 (0.85-1.75)	4.02 (3.27-4.81)	1.31 (1.12-1.57)
p-value WT/HA				0.88	0.86	0.64	0.29
p-value WT/Het				0.56	0.63	0.98	0.25
rs121909120	*tcf4*						
		Het	35	0.94 (0.89-1.00)	1.38 (0.98-1.74)	4.17 (3.18-5.02)	1.31 (1.09-1.59)
		WT	279	0.95 (0.90-1.00)	1.22 (0.85-1.77)	4.02 (3.29-4.72)	1.31 (1.11-1.54)
		p-value WT/Het		0.59	0.61	0.44	0.78
rs141494427	*WNT 6*	HA	2	0.87	0.77	3.30	1.65
		Het	50	0.96 (0.92-1.01)	1.18 (0.84-1.56)	4.28 (3.68-4.74)	1.37 (1.37-1.54)
		WT	259	0.94 (0.90-1.00)	1.27 (0.87-1.82)	3.99 (3.23-4.80) *	1.31 (1.10-1.55)
		p-value WT/HA		0.17	0.11	0.28	0.31
		p-value WT/Het		0.27	0.55	0.08	0.47
rs2707466	*WNT 16*	HA	72	0.94 (0.88-1.00)	1.14 (0.83-1.76)	3.79 (3.17-4.33)	1.34 (1.34-1.55)
		Het	154	0.95 (0.90-1.01)	1.33 (0.87-1.82)	3.96 (3.18-4.77)	1.30 (1.12-1.54)
		WT	87	0.95 (0.90-1.00)	1.19 (0.88-1.74)	4.31 (3.64-5.01)	1.36 (1.11-1.55)
		p-value WT/HA		0.63	0.51	0.004	0.69
		p-value WT/Het		0.41	0.58	0.014	0.68
rs1805097	*IRS2*	HA	40	0.93 (0.87-0.98)	1.13 (0.74-1.62)	3.90 (2.95-4.54) *	1.43 (1.12-1.43) **
		Het	134	0.96 (0.91-1.01)	1.23 (0.84-1.75)	4.04 (3.43-4.87)	1.32 (1.12-1.53)
		WT	135	0.95 (0.90-1.00)	1.30 (0.89-1.79)	4.06	1.28 (1.09-1.53)
		p-value WT/HA		0.19	0.16	0.09	0.04
		p-value WT/Het		0.38	0.71	0.12	0.32

Table 10-5 cont.

rs number	*Gene*	Genotype	n	WHR Median (IQR)	TG Median (IQR)	NHDL-C median (IQR)	HDL-C median (IQR)
rs4961	*ADD1*	HA	8	0.93 (0.83-0.94) *	1.22 (0.83-1.88)	4.23 (3.02-4.71)	1.29 (1.05-1.56)
		Het	75	0.94 (0.91-0.99)	1.13 (0.88-1.59)	4.02 (3.51-4.64)	1.40 (1.11-1.60)
		WT	222	0.96 (0.90-1.01)	1.30 (0.85-1.85)	4.04 (3.25-3.83)	1.30 (1.11-1.54)
		p-value WT/HA		0.05	0.86	0.85	0.85
		p-value WT/Het		0.35	0.15	0.92	0.39
rs16858780	*CHRD*	HA	7	0.97 (0.95-1.00)	1.80 (1.29-2.29)	4.69 (3.51-5.01)	1.31 (1.11-1.41)
		Het	69	0.94 (0.90-1.00)	1.19 (0.87-1.78)	3.90 (3.32-4.66)	1.31 (1.07-1.57)
		WT	236	0.95 (0.90-1.00)	1.22 (0.85-1.74)	4.07 (3.23-4.79)	1.31 (1.12-1.54)
		p-value WT/HA		0.61	0.11	0.34	0.83
		p-value WT/Het		9.90	0.75	0.51	0.57
rs146536048	*ADD1*	Het	9	0.99 (0.95-1.01)	1.33 (0.73-1.93)	4.01 (2.96-4.54)	1.22 (1.03-1.68)
		WT	304	0.95 (0.90-1.00)	1.25 (088-1.76)	4.04 (3.29-4.78)	1.31 (1.12-1.54)
		p-value WT/HA		0.163	0.96	0.57	0.68
		p-value WT/Het					

Table 10-6 Median levels of intermediate phenotypes associated with adipocyte dysfunction and percentage diabetics, for genotypes with selected polymorphisms in genes from the WNT Pathway. IQR- interquartile range. * p-value < 0.10. ** p-value < 0.05. ***p-value < 0.01. HA-homozygous alternate, Het -heterozygous, WT-Wildtype.

rs number gene	Genotype p-value	n	γ-GT median (IQR)	ALT median (IQR)	HOMA-C median (IQR)	HOMA-IR median (IQR)	Diabetes N (%)	Hs-CRP median (IQR)
rs1801177	HA	2 (0.6)	25.50	24.50	0.19	2.79 (2.09-2.79)	1 (100)	7.30 (1.10-7.30)
LPL	Het	21 (6.6)	27.00 (21.00-34.50)	24.00 (20.50-22.50)	0.13 (0.10-0.15)	1.57 (0.59-2.28)	3 (14.3)	1.80 (1.13-3.03)
	WT	294 (92.7)	29.00 (20.00-41.00)	22.00 (17.00-31.00)	0.14 (0.20-0.21)	1.84 (0.87-3.20)	36 (12.9)	1.68 (0.78-3.21)
	p-value WT/HA		0.67	0.93	0.37	0.39		0.41
	p-value WT/Het		0.72	0.4	0.39	0.28		0.53
rs114788023	Het	12 (3.8)	19.50 (18.0-19.75)	20.00 (15.50-24.75)	0.11 (0.08-0.13)	0.73 (<0.001-1.33)	0 (0)	1.28 (0.61-1.75)
E2F2	WT	307 (96.2)	29.00 (20.00-41.00)	22.00 (17.00-31.00)	0.14 (0.10-0.21)	1.92 (0.90-3.20)	40 (13.6)	1.75 (0.82-3.25)
	p-value WT/Het		0.08	.253	0.022	0.002		0.20
rs35994626	HA	2	31.50	20.50	0.17	2.98	0 (0)	4.49
FZD5	Het	40	27.00 (18.00-36.50)	21.00 (14.50-27.50)	0.12 (0.09-0.20)	1.78 (0.78-3.23)	3 (7.5)	1.40 (0.59-2.69)
	WT	268	29.00 (20.75-41.00)	23.00 (17.00-30.25)	0.14 (0.10-0.21)	1.84 (0.89-2.94)	37 (14.5)	1.77 (0.82-3.26)
	p-value WT/HA		0.89	0.65	0.57	0.65		0.19
	p-value WT/Het		0.15	0.14	0.39	0.79		0.17
rs121908120	Het	35	29.00 (19.00-39.00)	21.00 (16.00-25.00)	0.14 (0.10-0.21)	2.09 (1.29-3.47)	6 (17.1)	0.18 (0.38-3.35)
WNT10A	WT	279	29.00 (20.00-41.25)	22.50 (17.00-31.25)	0.14 (0.10-1.21)	1.76 (0.85-2.95)	34 (12.7)	1.69 (0.82-3.11)
	p-value WT/HA		0.48	0.20	0.74	0.29		1.52
	p-value WT/Het							
Rs1801278	HA	6	37.00 (21.00-39.75)	26.50 (22.00-33.25)	0.15 (0.12-0.21)	2.72 (1.37-3.45)	1 (20.0)	2.40 (1.57-4.00)
IRS1	Het	76	28.50 (19.00-39.00)	22.00 (16.25-32.50)	0.15 (0.11-0.23)	2.28 (1.30-3.38)	10 (13.9)	1.82 (0.94-3.62)
	WT	233	29.00 (20.25-43.00)	(17.00-30.00)	0.14 (0.10-0.21)	1.58 (0.81-2.90)	29 (12.8)	1.66 (0.74-2.76)
	p-value WT/HA		0.59	0.26	0.57	0.30		0.23
	p-value WT/Het		0.71	0.97	0.14	0.02		0.18

Table 10-6 cont.

rs number gene p-value	Genotype	n	γ-GT median (IQR)	ALT median (IQR)	HOMA-C median (IQR)	HOMA-IR median (IQR)	Diabetes N (%)	Hs-CRP median (IQR)
rs8192678	HA	67	27.00(20.00-37.75)	21.00 (17.00-28.50)	0.13(0.10-0.20)	1.65 (0.78-2.90)	9 (13.8)	1.56 (0.80-2.67)
PPARGC1A	Het	144	29.00 (19.00-39.50)	22.00 (17.00-28.50)	0.15 (0.10-0.22)	1.94 (0.89-3.29)	18 (12.8)	1.61 (0.80-2.78)
	WT	95	31.00 (22.25-48.75)	24.00 (17.25-34.00)	0.13 (0.10-0.19)	1.61 (0.89-2.85)	9 (10.1)	2.25 (0.86-3.93)
	p-value WT/HA		0.06	0.30	0.47	0.73		0.09
	p-value WT/Het		0.05	0.11	0.35	0.52		0.05
rs11466653	HA	3	21.00	21.00	0.09	0.86	0 (0)	0.55
TLR10	Het	46	28.00 (19.00-45.00)	21.00 (16.00-30.00)	0.10-0.24	2.19 (0.97-3.29)	5 (10.9)	1.48 (0.69-2.72)
	WT	265	29.00 (20.00-40.00)	23.00 (17.00-31.00)		0.85 (0.85-2.94)	33 (13.0)	1.77 (0.82-3.25)
	p-value WT/HA		0.17	0.49	0.55	0.76		0.06
	p-value WT/Het		0.92	0.47	0.83	0.56		0.15
rs2305948	HA	1	21.00	13.00	0.08	0.002	0 (0)	1,52
KDR	Het	53	28.00 (18.00-39.75)	21.00 (17.00-33.75)	0.14 (0.10-0.21)	1.92 (0.65-3.60)	9 (17.6)	1.48 (0.80-2.38)
	WT	259	30.00 (20.00-41.00)	23.00 (17.00-30.00)	0.14 (0.10-0.21)	1.81 (0.88-2.97)	30 (12.0)	1.79 (0.79-3.37)
	p-value WT/HA		0.56	0.12	0.21	0.19		0.88
	p-value WT/Het		0.39	0.95	0.71	0.75		0.19
rs9536314	HA	5	31.00 (14.00-110.00)	29.00(17.50-33.50)	0.10 (0.09-0.20)	0.64 (0.31-3.02	0 (0)	1.90 (0.27-2.14)
KL	Het	86	29.00 (18.00-42.50)	23.00 (17.00-32.00)	0.14 (0.10-0.22)	1.93 (0.89-3.24)	13 (16.3)	1.59 (0.76-2.74)
	WT	220	29.00 (20.25-40.75)	22.00 (17.00-29.00)	0.14 (0.10-0.20)	1.83 (0.88-3.01)	26 (12.1)	1.70 (0.82-3.35)
	p-value WT/HA		0.64	0.46	0.40	0.29		0.34
	p-value WT/Het		0.68	0.50	0.70	0.92		0.42
rs9527025								
KL								
	HA	5	31.00 (14.00-110.00)	29.00 (17.50-33.50)	0.10 (0.09-0.20)	0.64 (0.31-3.02)	0 (0)	1.90 (0.27-2.14)
	Het	85	28.00 (18.00-42.75)	23.50 (17.00-32.00)	0.14 (0.10-0.22)	1.90 (0.89-3.26)	13 (!6.5)	1.51 (0.75-2.73)
	WT	222	29.00 (20.00-40.25)	22.00 (17.00-29.00)	0.14 (0.10-0.20)	1.82 (0.87-1.98)	26 (12.0)	1.70 (0.82-3.40)
	p-value WT/HA		0.65	0.44	0.40	0.29		0.34
	p-value WT/Het		0.72	0.35	0.67	0.90		0.39

Table 10-6 cont.

rs number gene p-value	Genotype	n	γ-GT median (IQR)	ALT median (IQR)	HOMA-C median (IQR)	HOMA-IR median (IQR)	Diabetes N (%)	Hs-CRP median (IQR)
rs4988321 *LRP5*								
	HA				HOMAC			
	Het	55	32.00 (21.00-45.00)	24.00 (17.00-33.00)	0.18 (0.10-0.26)	2.39 (1.10-4.15)	9 (16.4)	1.75 (0.75-2.84)
	WT	259	28.00 (19.00-39.25)	21.00 (17.00-30.00)	0.14 (0.10-0.20)	1.75 (0.85-2.90)	30 (12.1)	1.68 (0.81-3.32)
	p-value WT/HA							
	p-value WT/Het		0.20	0.13	0.02	0.03		0.89
rs3736228 *LRP5*								
	HA	2	19.00	30.50	0.25	4.94	0 (0)	9.83
	Het	80	30.00 (20.50-42.00)	23.00 (17.00-31.50)	0.16 (0.10-0.23)	2.17 (0.98-3.55)	9 (11.3)	1.78 (0.84-2.90)
	WT	228	29.00 (20.00-40.00)	22.00 (17.00-30.25)	0.14 (0.10-0.19)	1.65 (0.86-2.84)	30 (13.8)	1.66 (0.78-3.22)
	p-value WT/HA		0.16	0.22	0.10	0.07		0.03
	p-value WT/Het		0.79	0.90	0.16	0.10		0.62
rs61753381 *CREBBP*								
	HA	3	23.00	24.00	0.10	0.003	2 (66.7)	0.50
	Het	21	36.00 (21.00-51.00)	25.00 (18.00-33.00)	0.14 (0.10-0.25)	1.82 (0.74-4.05)	4 (20.0)	2.67 (1.43-5.47)
	WT	292	29.00 (20.00-40.00)	17.00-30.00)	0.14 (0.10-0.21)	1.85 (0.88-2.96)	34 (12.1)	1.68 (0.80-3.08)
	p-value WT/HA		0.68	0.84	0.45	0.30		0.06
	p-value WT/Het		0.15	0.35	0.55	0.60		0.02
rs1051978 *NFATC1*								
	HA	2	48.50	33.00	0.12	1.30	0 (0)	3.15
	Het	21	27.00 (19.50-50.50)	21.00 (16.50-35.00)	0.17 (0.08-0.24)	1.92 (0.79-2.92)	4 (21.1)	1.96 (1.37-2.46)
	WT	287	29.00 (20.00-40.00)	22.00 (17.00-30.00)	0.14 (0.10-0.21)	1.80 (0.86-2.98)	35 (12.6)	1.66 (0.77-3.22)
	p-value WT/HA		0.11	0.13	0.64	0.55		0.33
	p-value WT/Het		0.86	0.98	0.60	0.90		0.36

Table 10-6 cont.

rs number gene Genotype p-value	n	γ-GT median (IQR)	ALT median (IQR)	HOMA-C median (IQR)	HOMA-IR median (IQR)	Diabetes N (%)	Hs-CRP median (IQR)
rs2270565 *UCP1*							
HA	5	39.00 (28.00-71.00)	40.00 (29.00-43.50)	0.14 (0.09-0.21)	1.61	0 (0)	2.50 (1.35-5.00)
Het	57	28.00 (18.00-41.50)	23.00 (18.25-30.00)	0.14 (0.10-0.21)	1.98 (1.12-2.70)	9 (16.7)	2.10 (0.91-4.17)
WT	254	29.00 (20.00-40.25)	22.00 (17.00-29.25)	0.14 (0.10-0.21)	1.82 (0.83-3.06)	31 (12.7)	1.66 (0.78-2.95)
p-value WT/HA		0.11	0.02	0.94	0.98		0.27
p-value WT/Het		0.48	0.40	0.82	0.45		0.11
rs45539933 *UCP1*							
HA	5	39.00 (28.00-71.00)	40.00 (29.00-43.50)	0.14 (0.09-0.21)	1.61 (0.51-3.49)	0 (0)	2.50
Het	56	28.00 (18.00-40.00)	23.00 (18.00-30.00)	0.14 (0.10-019)	1.96 (1.10-2.57)	8 (15.1)	2.0 (0.88-4.12)
WT	250	29.00 (20.00-40.25)	22.00 (17.00-30.25)	0.14 (0.10-0.21)	1.82 (0.82-3.17)	30 (12.4)	1.67 (0.78-2.96)
p-value WT/HA		0.11	0.02	0.89	0.97		0.27
p-value WT/Het		0.40	0.55	0.93	0.64		0.17
rs3729856 *GATA4*							
HA	5	31.00 (17.00-46.50)	24.00 (17.00-33.50)	0.13 (0.08-0.34)	1.41 (0.57-4.19)	1 (25.0)	1.56 (1.05-6.93)
Het	41	31.00 (18.00-45.00)	22.50 (17.00-29.75)	0.12 (0.08-0.20)	1.47 (0.61-2.70)	4 (10.3)	1.43 (0.50-2.62)
WT	265	29.00 (20.00-40.00)	22.00 (17.00-30.00)	0.14 (0.10-0.21)	1.89 (0.90-3.07)	35 (13.6)	1.75 (0.83-3.35)
p-value WT/HA		0.93	0.77	0.84	0.91		0.92
p-value WT/Het		0.89	0.87	0.08	0.22		0.06
rs17563 *BMP4*							
HA	66	30.00 (21.00-40.50)	23.00 (17.00-31.50)	0.13 (0.10-0.23)	1.61 (0.88-3.27)	11 (17.5)	1.63 (0.75-3.07)
Het	148	30.00 (20.00-41.00)	24.00 (18.00-30.00)	0.14 (0.11-0.21)	1.92 (0.94-2.96)	14 (9.8)	1.89 (0.82-3.47)
WT	101	26.00 (18.75-40.25)	21.00 (17.00-30.50)	0.13 (0.09-0.21)	1.58 (0.77-2.96)	15 (15.3)	1.51 (0.70-3.02)
p-value WT/HA		0.33	0.72	0.54	0.71		0.70
p-value WT/Het		0.29	0.43	0.19	0.45		0.22

Table 10-6 cont.

rs number gene p-value	Genotype	n	γ-GT median (IQR)	ALT median (IQR)	HOMA-C median (IQR)	HOMA-IR median (IQR)	Diabetes N (%)	Hs-CRP median (IQR)
rs33932986 *MAPK11*								
	HA	4	59.00 (39.00-161.50)	30.50 (20.25-51.25)	0.19 (0.13-0.27)	2.87 (1.95-4.20)	0 (0)	3.46 (2.37-5.12)
	Het	58	27.00 (19.00-40.50)	23.00 (17.00-32.00)	0.15 (0.10-0.21)	2.24 (0.88-3.49)	8 (15.1)	1.69 (0.82-3.53)
	WT	251	29.00 (20.00-41.00)	22.00 (17.00-29.00)	0.13 (0.10-0.21)	1.65 (0.86-2.94)	31 (12.7)	1.67 (0.78-2.97)
	p-value WT/HA		0.02	0.19	0.27	0.14		0.06
	p-value WT/Het		0.048	0.62	0.27	0.25		0.53
rs121909120 *TCF4*	HA							
	Het	35	29.00 (18.00-39.00)	21.00 (16.00-25.00)	1.44 (0.10-0.21)	2.09 (1.29-3.47)	6 (17.1)	1.77 (0.38-3.35)
	WT	279	29.00 (20.00-41.25)	22.50 (17.00-31.25)	0.14 (0.10-0.21)	1.76 (0.85-2.95)	34 (12.7)	1.69 (0.82-3.12)
	p-value WT/HA							
	p-value WT/Het		0.48	0.20	0.74	0.29		0.52
rs141494427 *WNT6*								
	HA	2	12.50	13.50	0.08	0.002	0 (0)	0.92
	Het	50	30.00 (23.00-46.50)	23.00 (16.00-27.50)	0.14 (0.10-0.24)	2.19 (1.12-3.24)	2 (4.1)	1.77 (0.84-3.55)
	WT	259	29.00 (20.00-41.00)	22.00 (17.00-32.00)	0.14 (0.10-0.21)	1.75 (0.83-2.96)	37 (14.9)	1.68 (0.77-3.03)
	p-value WT/HA		0.02	0.05	0.07	0.07		0.25
	p-value WT/Het		0.36	0.59	0.49	0.24		0.23
rs2707466 *WNT16*								
	HA	72	26.50 (19.00-43.75)	21.00 (16.25-26.75)	0.14 (0.10-0.23)	1.61 (0.66-2.94()	10 (14.7)	1.79 (0.78-3.22)
	Het	154	30.00 (21.00-40.00)	23.00 (17.00-33.00)	(0.14-0.20)	1.91 (0.90-2.98)	22 (15.1)	1.68 (0.73-3.52)
	WT	87	29.00 (10.00-41.50)	22.00 (17.50-29.50)	0.14 (0.10-0.22)	1.77 (0.94-3.38)	8 (9.2)	1.68 (0.88-2.71)
	p-value WT/HA		0.53	0.40	0.71	0.29		0.85
	p-value WT/Het		0.94	0.44	0.80	0.82		0.64

Table 10-6 cont.

rs number gene	Genotype p-value	n	γ-GT median (IQR)	ALT median (IQR)	HOMA-C median (IQR)	HOMA-IR median (IQR)	Diabetes N (%)	Hs-CRP median (IQR)
rs1805097 *IRS2*								
	HA	40	28.00 (18.00-42.00)	21.00 (17.00-27.00)	0.11 (0.08-0.18)	1.31 (0.47-2.64)	4 (10.3)	1.27 (0.65-2.97)
	Het	134	30.00 (20.00-40.25)	23.50 (19.00-28.00)	0.14 (0.10-0.22)	1.60 (0.80-3.48)	19 (14.8)	1.91 (0.84-3.52)
	WT	135	29.00 (20.00-40.00)	22.00 (16.00-33.00)	0.14 (0.11-0.21)	2.10 (1.13-2.96)	16 (12.3)	1.67 (0.76-2.92)
	p-value WT/HA		0.55	0.58	0.01	0.03		0.29
	p-value WT/Het		0.86	0.67	0.50	0.26		0.52
rs4961 *ADD1*								
	HA	8	32.50 (20.25-53.75)	19.50 (13.75-26.50)	0.14 (0.10-0.17)	1.65 (<0.01-2.24)	0 (0)	2.69 (0.89-7.27)
	Het	75	30.00 (21.00-44.00)	24.00 (18.00-33.00)	0.14 (0.10-0.22)	1.88 (1.07-2.92)	3 (4.1)	1.77 (0.83-3.00)
	WT	222	28.00 (20.00-40.00)	22.00 (17.00-29.50)	0.14 (0.10-0.21)	1.90 (0.85-3.31)	36 (16.7)	1.68 (0.80-3.29)
	p-value WT/HA		0.45	0.35	0.43	0.30		0.29
	p-value WT/Het		0.41	0.24	0.64	0.92		0.97
rs16858780 *CHRD*					FBG gives 0.05			
	HA	7	33.00 (27.00-72.00)	24.00 (19.00-32.00)	0.14 (0.11-0.20)	2.63 (1.06-3.53)	0 (0)	1.56 (0.65-2.76)
	Het	69	28.00 (19.25-39.00)	20.50 (15.00-26.75)	0.14 (0.10-0.20)	1.86 (0.89-2.73)	12 (18.5)	1.68 (0.99-3.33)
	WT	236	29.00 (20.00-40.75)	23.00 (18.00-32.00)	0.14 (0.10-0.21)	1.83 (0.86-3.22)	27 (11.8)	1.72 (0.78-3.12)
	p-value WT/HA		0.33	1.00	0.85	0.54		0.70
	p-value WT/Het		0.40	0.01	0.69	0.70		0.63
rs146536048 *ADD1*								
	HA							
	Het	9	32.00 (24.00-40.00)	31.00 (22.00-44.50)	0.13 (0.11-0.17)	1.49 (0.89-2.25)	3 (33.3)	1.26 (0.91-3.48)
	WT	304	29.00 (20.00-41.00)	22.00 (17.00-30.00)	0.14 (0.10-0.21)	1.87 (0.88-3.20)	37 (12.7)	1.74 (0.80-3.20)
	p-value WT/HA							
	p-value WT/Het		0.57	0.04	0.61	0.43		0.98

Section 10.8.5. Combinations of WNT Related Polymorphisms and their Risk of Myocardial Infarction

Polymorphisms which had similar effects on intermediate phenotypes were studied in combination. Polymorphisms of proteins that interact biologically were also studied in combination. In total, 38 combinations of polymorphisms were selected and analysed. Odds ratios for these combinations can be found in Table 10-7.

In a combined analysis of the two polymorphisms in *LRP5,* rs4988321 and rs3736228, when both polymorphisms were heterozygous the Age adjusted OR was 0.6 (95% CI 0.4-1.0). In men ≥60 years of age, the age-adjusted OR was 0.4 (95% CI 0.2-0.8), relative to the homozygous wildtype for both the *LRP5* rs4988321 and rs3736228 polymorphisms (Table 10-7).

Combining rs4988321 in *FZD* with rs35994626 in LRP5 a protective effect was noted when the *FZD* polymorphism was homozygous wildtype and the *LRP5* was heterozygous, OR 0.6 (95%CI 0.4-1.0). Similar findings were found in men > 60 years of age [OR 0.4 (9%%CI 0.2-0.9)] and a pattern towards a protective effect was observed in men off statins [OR 0.7 (95%CI 0.4-1.2)] Table 10-10, all relative to the homozygous wildtype for both the rs4988321 in *FZD* and rs35994626 in LRP5.

Heterozygosity for rs8192678 in *PPARGCIA* together with homozygous wildtype genotype for rs17563 in *BMP4* was associated with an increased risk of MI, AgeAdjOR 1.9 (95% CI 1.0-3.9). This risk was also observed in men off statins AgeAdjOR 2.7 (95% CI 1.2-6.1). A similar risk was observed amongst men who were homozygous wildtype for the rs8192678 in *PPARGCIA* and heterozygous for the rs17563 in *BMP4* [OR 1.9 (95% CI 0.9-3.9)]. This risk was also observed in men off statins [OR 2.1 (95%CI 1.0-4.9)]. In men off statins the risk reached an AgeAdjOR of 3.6 (95% CI 1.2-10.9) in the category that was homozygous alternate

for both polymorphisms Table 10-7, always relative to the homozygous wildtype for both the rs8192678 in *PPARGCIA* and rs17563 in *BMP4.*

Table 10-7 Odds Ratios for combinations of polymorphisms selected based on effects on intermediate phenotypes and biological interaction, analysed by sex on and off statins. AgeAdjOR – Age Adjusted OR, HA – Homozygous alternate, HET -heterozygous, WT- Wildtype

Polymorphism / *Gene 1*	Genotype		All Men			Men ≥60 years			Men Off Statins		
Polymorphism / *Gene2*	Gene 1	Gene 2	n cases (%)	n controls (%)	AgeAdjOR (95%CI)	n cases (%)	n controls (%)	AgeAdjOR (95%CI)	n cases (%)	n-controls (%)	AgeAdjOR (95%CI)
rs1801278/ *IRS1*	HA	HA	0 (0.0)	1 (0.3)	/	/	/	/	0 (0.0)	1 (0.4)	/
rs1805097/ *IRS2*	HET	HA	9 (3.5)	8 (2.6)	1.4 (0.5-3.9)	4 (3.5)	5 (3.5)	1.2 (0.3-4.7)	7 (3.6)	6 (2.4)	1.6 (0.5-5.1)
	WT	HA	23 (9.0)	31 (10.6)	1.0 (0.5-1.8)	9 (7.9)	15 (10.6)	0.9 (0.4-2.2)	20 (10.2)	21 (8.4)	1.4 (0.7-2.9)
	HA	HET	4 (1.6)	3 (1.0)	/	1 (0.9)	1 (0.7)	/	3 (1.5)	3 (1.2)	/
	HET	HET	37 (14.5)	33 (10.6)	1.4 (0.8-2.5)	13 (11.4)	13 (9.2)	1.4 (0.6-3.4)	25 (12.7)	30 (12.0)	1.1 (0.6-2.1)
	WT	HET	76 (29.7)	99 (31.8)	1.0 (0.6-1.5)	35 (30.7)	46 (32.6)	1.1 (0.6-2.0)	60 (30.5)	78 (31.2)	1.0 (0.6-1.7)
	HA	WT	1 (0.4)	2 (0.6)	/	/	/	/	1 (0.5)	1 (0.4)	/
	HET	WT	26 (10.2)	34 (10.9)	1.0 (0.5-1.7)	17 (14.9)	12 (8.5)	2.0 (0.9-4.8)	20 (10.2)	28 (11.2)	0.9 (0.5-1.8)
	WT	WT	80 (31.3)	100 (32.2)	1.0	35 (30.7)	49 (34.8)	1.0	61 (31.0)	82 (32.8)	1.0
rs4988321/*LRP5*	HA	HA	1 (0.4)	0 (0)	/	/	/	/	1 (0.5)	0 (0.0)	/
rs3736228/*LRP5*	HET	HA	3 (1.1)	2 (0.6)	/	2 (1.7)	0(.0)	/	2 (1.0)	2(0.8)	/
	WT	HA	1 (0.4)	0(0)	/	1 (0.8)	0 (0.0)	/	1 (0.5)	0 (0.0)	/
	HET	HET	28 (10.4)	51 (16.3)	0.6 (0.4-1.0)	9 (7.5)	28 (19.6)	0.4 (0.2-0.8)	22 (10.5)	41 (16.2)	0.6 (0.34-1.04)
	WT	HET	27 (10.0)	30 (9.6)	1.0 (0.6-1.7)	15 (12.5)	12 (8.4)	1.4 (0.6-3.2)	23 (11.0)	25 (9.9)	1.0 (0.56-1.92)
	HET	WT	2 (0.7)	2 (0.6)	/	1 (0.8)	1 (0.7)	/	2(1.0)	0(0.0)	/
	WT	WT	208 (77.0)	228 (72.8)	1.0	92 (76.7)	102 (71.3)	1.0	158 (75.6)	185(73.1)	1.0
rs45539933/*UCP1*	HA	HA	/	/	/	0 (0.0)	0 (0.7)	/	0 (0.0)	4 (1.6)	/
rs2270565/*UCP1*	HET	HET	56 (21.1)	55 (17.5)	1.2 (0.8-1.9)	26 (21.7)	24 (16.8)	1.4 (0.7-2.5)	46 (22.3)	42 (16.5)	1.4 (0.9-2.9)
	WT	HET	0 (0)	1 (0.3)	/	0 (0.0)	1 (0.7)	/	/	/	/
	HET	WT	2 (0.8)	1 (0.3)	/	0 (0.0)	1 (0.7)	/	1 (0.5)	1 (0.4)	/
	WT	WT	208 (78.2)	251 (79.9)	1.0	94 (78.3)	116 (81.1)	1.0	159 (77.2)	207 (81.5)	1.0
rs9527025/*KL*	HA	HA	8 (3.0)	5 (1.6)	2.1 (0.7-6.5)	1 (1.7)	2 (1.4)	/	8 (3.9)	5 (1.6)	3.0 (0.9-10.5)
rs9536314/*KL*	HET	HET	73 (27.4)	85 (27.2)	1.0 (0.7-1.5)	34 (29.1)	37 (25.7)	1.2 (0.7-2.0)	60 (29.0)	65 (25.8)	1.2 (0.9-1.8)
	WT	WT	185 (69.5)	223 (71.2)	1	81 (69.2)	105 (72.9)	1.0	139 (67.1)	183 (72.6)	1.0

Table 10-7 cont.

Polymorphism / *Gene 1* Polymorphism / *Gene2*	Genotype Gene 1	Gene 2	All Men n cases (%)	All Men n controls (%)	All Men AgeAdjOR (95%CI)	Men ≥60 years n cases (%)	Men ≥60 years n controls (%)	Men ≥60 years AgeAdjOR (95%CI)	Men Off Statins n cases (%)	Men Off Statins n-controls (%)	Men Off Statins AgeAdjOR (95%CI)
rs35994626/*FZD*	WT	HA	1 (0.4)	0 (0.0)	/	/	/	/	1 (0.5)	0 (0.0)	/
rs4988321/ *LRP5*	HET	HET	5 (1.8)	3 (1.0)	1.8 (0.4-7.6)	2 (1.7)	2 (1.4)	1.1 (0.1-7.8)	4 (1.9)	3 (1.2)	1.4 (0.3-6.5)
	WT	HET	29 (10.6)	50 (16.1)	0.6 (0.4-1.0)	11 (9.2)	27 (18.8)	0.4 (0.2-0.9)	23 (10.9)	38 (15.1)	0.7 (0.4-1.2)
	HA	WT	2 (0.7)	3 (1.0)	/	1 (0.8)	2 (1.4)	/	1 (0.5)	3 (1.2)	/
	HET	WT	39 (14.3)	39 (12.5)	1.2 (0.7-1.9)	15 (12.5)	13 (9.0)	1.2 (0.6-2.7)	31 (14.7)	35 (13.9)	1.1 (0.6-1.9)
	WT	WT	197 (72.2)	216 (69.5)	1.0	91 (75.8)	100 (69.4)	1.0	151 (71.6)	173 (68.7)	1
rs121908120/ *WNT10A*	WT	HA	1 (0.4)	0 (0.0)	/	/	/	/	1 (0.5)	0(0.0)	/
rs4988321/*LRP5*	HET	HET	3 (1.1)	6 (1.9)	/	0 (0.0)	1 (0.7)	/	2 (0.9)	4 (1.6)	/
	WT	HET	31 (11.4)	49 (15.6)	0.7 (0.4-1.1)	13 (10.7)	28 (19.3)	0.5 (0.2-1.0)	25 (11.8)	39 (15.4)	0.7 (0.4-1.2)
	HET	WT	20 (7.3)	29 (9.2)	0.7 (0.4-1.3)	8 (6.6)	11 (7.6)	0.8 (0.3-2.0)	15 (7.1)	24 (9.4)	0.7 (0.4-1.4)
	WT	WT	218 (79.9)	231 (73.3)	1	100 (82.6)	105 (72.4)	1.0	169 (79.7)	187 (73.6)	1
rs35994626/*FZD5*	HET	HA	1 (0.4)	0 (0.0)	/	1 (0.8)	0 (0.0)	/	1 (0.5)	0(.0)	/
rs3736228/*LRP5*	WT	HA	4 (1.5)	2 (0.6)	/	2 (1.7)	0 (0.0)	/	3 (1.4)	2 (0.8)	/
	HET	HET	7 (2.6)	7 (2.3)	1.1 (0.4-3.2)	4 (3.3)	3 (2.1)		5 (2.4)	7 (2.8)	0.8 (0.3-2.6)
	WT	HET	49 (18.0)	71 (23.1)	0.7 (0.5-1.1)	21 (17.4)	37 (26.1)	0.6 (0.3-1.2)	41 (19.5)	56 (22.2)	0.8 (0.5-1.3)
	HA	WT	2 (0.7)	3 (1.0)		1 (0.8)	2 (1.4)	0.6 (0.1-6.6)	1 (0.5)	3 (1.2)	
	HET	WT	35 (12.9)	35 (11.4)	1.2 (0.7-1.9)	12 (9.9)	12 (8.5)	1.1 (0.5-2.6)	28 (13.3)	31 (12.4)	1.1 (0.6-2.0)
	WT	WT	174 (64.0)	190 (61.7)	1	80 (66.1)	88 (62.0)	1.0	131 (62.4)	150 (60.2)	1
rs121908120/*WNT10A*	WT	HA	5 (1.9)	2 (0.6)	/	3 (2.5)	0(0.0)	/	4 (1.9)	2 (0.8)	/
rs3736228/*LRP5*	HET	HET	5 (1.9)	14 (4.5)	0.4 (0.1-1.1)	1 (0.8)	6 (4.2)	/	4 (1.9)	10 (4.0)	0.4 (0.1-1.4)
	WT	HET	50 (18.5)	66 (21.2)	0.8 (0.5-1.2)	23 (19.0)	34 (23.9)	0.7 (0.4-1.4)	41 (19.6)	55 21.9)	0.8 (0.5-1.3)
	HET	WT	18 (6.7)	21 (6.8)	1.0 (0.5-1.9)	7 (5.8)	6 (4.2)	1.3 (0.4-4.0)	13 (6.2)	18 (7.2)	0.9 (0.4-1.8)
	WT	WT	192 (71.1)	208 (66.9)	1	87 (71.9)	96 (67.6)	1.0	147 (70.3)	166 (66.1)	1

Table 10-7 cont.

Polymorphism / *Gene 1* Polymorphism / *Gene2*	Genotype Gene 1	Gene 2	All Men n cases (%)	All Men n controls (%)	All Men AgeAdjOR (95%CI)	Men ≥60 years n cases (%)	Men ≥60 years n controls (%)	Men ≥60 years AgeAdjOR (95%CI)	Men Off Statins n cases (%)	Men Off Statins n-controls (%)	Men Off Statins AgeAdjOR (95%CI)
rs8192678/*PPARGC1A*	HA	HA	13 (4.8)	12 (3.9)	1.9 (0.7-4.7)	5 (4.2)	6 (4.3)	1.4(0.3-6.3)	13 (6.3)	8 (3.2)	3.6 (1.2-10.9)
rs17563/*BMP4*	HET	HA	23 (8.5)	28 (9.1)	1.4 (0.6-3.0)	11 (9.2)	14 (10.0)	1.3(0.4-4.1)	16 (7.7)	21 (8.3)	1.8 (0.7-4.5)
	WT	HA	14 (5.2)	24 (7.8)	1.0 (0.4-2.4)	6 (5.0)	11(7.9)	0.8(0.2-3.2)	12 (5.8)	21 (8.3)	1.4 (0.5-3.7)
	HA	HET	25 (9.3)	32 (10.4)	1.3 (0.6-2.8)	11(9.2)	17 (12.1)	1.0 (0.3-3.2)	18 (8.7)	26 (10.3)	1.7 (0.7-4.0)
	HET	HET	69 (25.6)	78 (25.2)	1.5 (0.8-2.8)	32 (26.7)	35 (25.0)	1.5 (0.5-4.0)	58 (27.9)	65 (25.8)	2.1 (1.0-4.4)
	WT	HET	39 (14.4)	35 (11.3)	1.9 (0.9-3.9)	18 (15.0)	14 (10.0)	2.0 (0.6-6.2)	27 (13.0)	31 (12.3)	2.1 (1.0-4.9)
	HA	WT	20 (7.4)	24 (7.8)	1.5 (0.6-3.2)	9 (7.5)	11 (7.9)	1.3 (0.4-4.7)	15 (7.2)	18 (7.1)	2.1 (0.8-5.5)
	HET	WT	46 (17.0)	40 (12.9)	1.9 (1.0-3.9)	20 (16.7)	19 (13.6)	1.7 (0.6-5.1)	36 (17.3)	31 (12.3)	2.7 (1.2-6.1)
	WT	WT	21 (7.8)	36 (11.7)	1.0	8 (6.7)	13 (9.3)	1.0	13 (6.3)	31 (12.3)	1.0
rs121908120/*WNT10A*	WT	HA	2 (0.7)	2 (0.6)	/	2 (1.7)	0(0.0)	/	1 (0.5)	2 (0.8)	/
rs141494427/*WNT6*	HET	HET	0 (0.0)	5 (1.6)	/	0 (0.0)	2 (1.4)	/	0 (0.0)	3 (1.2)	/
	WT	HET	47 (17.5)	45 (14.4)	1.2 (0.8-1.9)	20 (16.5)	22 (15.5)	1.1 (0.6-2.1)	39 (18.7)	37 (14.6)	1.3 (0.8-2.2)
	HET	WT	23 (8.6)	30 (9.6)	0.9 (0.5-1.6)	8 (6.6)	10 (7.0)	1.0 (0.4-2.6)	17 (8.1)	25 (9.9)	0.8 (0.4-1.6)
	WT	WT	197 (73.2)	231 (73.8)	1.0	91 (75.2)	108 (76.1)	1.0	152 (72.7)	186 (73.5)	1.0
rs2707466 /*WNT16*	HA	HA	1 (0.4)	0 (0.0)	/	1 (0.8)	0 (0.0)	/	1 (0.5)	0 (0.0)	/
rs35994626/*FZD5*	HET	HA	1 (0.4)	2 (0.6)	/	0 (0.0)	1 (0.7)	/	0 (0.0)	2 (0.8)	/
	WT	HA	0 (0.0)	1 (0.3)	/	0 (0.0)	1 (0.7)	/	0(0.0)	1 (0.4)	/
	HA	HET	10 (3.6)	14 (4.5)	0.8 (0.3-1.8)	3 (2.5)	5 (3.5)	/	6 (2.8)	14 (5.6)	0.5 (0.2-1.3)
	HET	HET	26 (9.5)	14 (4.5)	1.9 (0.9-4.0)	9(7.4)	5 (3.5)	1.8 (0.5-6.0)	23 (10.9)	12 (4.8)	2.0 (0.9-4.4)
	WT	HET	8 (2.9)	14 (4.5)	0.6 (0.2-1.6)	5 (4.1)	5 (3.5)	0.9 (0.2-5.4)	6 (2.8)	12 (4.8)	0.5 (0.2-1.6)
	HA	WT	42 (15.3)	57 (18.4)	0.8 (0.5-1.3)	18 (14.9)	26 (18.4)	0.7 (0.3-1.5)	29 (13.7)	47 (18.7)	0.6 (0.3-1.1)
	HET	WT	115 (42.0)	135 (43.5)	0.9 (0.6-1.3)	54 (44.6)	67 (47.5)	0.8 (0.4-1.5)	84 (39.8)	102 (40.5)	0.8 (0.5-1.3)
	WT	WT	71 (25.9)	73 (23.5)	1.0	31 (25.6)	31 (22.0)	1.0	62 (29.4)	62 (24.6)	1.0
rs2707466/WNT16	HA	HET	6 (2.2)	5 (1.6)	1.3 (0.4-4.6)	1 (0.8)	4 (2.8)	/	4 (1.9)	4 (1.6)	/
rs121908120/WNT10A	HET	HET	9 (3.3)	22 (7.0)	0.5 (0.2-1.1)	3 (2.5)	7 (4.9)	0.5 (0.1-2.1)	6 (2.8)	6 (6.3)	0.4 (0.2-1.1)
	WT	HET	8 (2.9)	8 (2.5)	1.2 (0.4-3.5)	4 (3.3)	1 (0.7)	/	7 (3.3)	8 (3.1)	1.1 (0.4-3.1)
	HA	WT	47 (17.2)	66 (21.0)	0.8 (0.5-1.4)	21 (17.2)	27 (19.0)	4.4 (0.5-41.3)	32 (15.1)	56 (22.0)	0.6 (0.4-1.1)
	HET	WT	133 (48.5)	132 (42.0)	1.1 (0.8-1.7)	61 (50.0)	67 (47.2)	0.9 (0.4-1.9)	102 (48.1)	102 (40.2)	1.1 (0.7-1.7)
	WT	WT	71 (25.9)	81 (25.8)	1.0	32 (26.2)	36 (25.4)	1.0 (0.6-1.8)	61 (28.8)	69 (26.8)	1.0

Table 10-7 cont.

Polymorphism / *Gene 1* Polymorphism / *Gene2*	Genotype Gene 1	Gene 2	All Men n cases (%)	All Men n controls (%)	All Men AgeAdjOR (95%CI)	Men ≥60 years n cases (%)	Men ≥60 years n controls (%)	Men ≥60 years AgeAdjOR (95%CI)	Men Off Statins n cases (%)	Men Off Statins n-controls (%)	Men Off Statins AgeAdjOR (95%CI)
rs2707466/*WNT16*	WT	HA	1 (0.4)	0 (0.0)	/	/	/	/	1 (0.5)	0 (0.0)	/
rs4988321/*LRP5*	HA	HET	9 (3.3)	12 (3.8)	0.7 (0.3-1.9)	5 (4.1)	5 (3.5)	0.9 (0.2-3.6)	6 (2.8)	10 (3.9)	0.5 (0.2-1.6)
	HET	HET	18 (6.6)	26 (8.3)	0.7 (0.4-1.4)	6 (5.0)	14 (9.8)	0.3 (0.1-1.0)	16 (7.5)	17 (6.7)	0.9 (0.4-1.9)
	WT	HET	7 (2.6)	16 (5.1)	0.4 (0.2-1.1)	2 (1.7)	9 (6.3)	0.2 (0.0-0.9)	5 (2.4)	15 (5.9)	0.3 (0.1-0.9)
	HA	WT	44 (16.1)	60 (19.1)	0.8 (0.5-1.3)	17 (14.0)	27 (18.9)	0.5 (0.2-1.2)	30 (14.2)	51 (20.1)	0.6 (0.3-1.0)
	HET	WT	124 (45.3)	127 (40.4)	1.0 (0.7-1.5)	57 (47.1)	60 (42.0)	0.8 (0.4-1.5)	92 (43.3)	100 (39.4)	0.9 (0.6-1.4)
	WT	WT	71 (25.9)	73 (23.2)	1.0	34 (28.1)	28 (19.6)	1.0	62 (29.2)	61 (24.0)	1.0
rs2707466 /*WNT16*	HA	HA	2 (0.7)	1 (0.3)	/	1 (0.8)	0 (0.0)	/	1 (0.5)	1 (0.4)	/
rs3736228/*LRP5*	HET	HA	2 (0.7)	0 (0.0)	/	2 (1.7)	0 (0.0)	/	2 (1.0)	0 (0.0)	/
	WT	HA	1 (0.4)	1 (0.3)	/	/	/	/	1 (0.5)	1 (0.4)	/
	HA	HET	8 (3.0)	17 (5.5)	0.5 (0.2-1.2)	4 (3.3)	10 (7.1)	0.4 (0.1-1.5)	6 (2.9)	15 (6.0)	0.35 (0.1-1.0)
	HET	HET	31 (11.4)	41 (13.2)	0.8 (0.4-1.4)	12 (9.9)	19 (13.6)	0.6 (0.2-1.5)	26 (12.4)	30 (12.0)	0.85 (0.4-1.6)
	WT	HET	16 (5.9)	22 (7.1)	0.7 (0.3-1.5)	8 (6.6)	10 (7.1)	0.8 (0.3-2.3)	13 (6.2)	20 (8.0)	0.57 (0.3-1.3)
	HA	WT	40 (14.8)	51 (16.5)	0.8 (0.5-1.4)	16 (13.2)	19 (13.6)	0.8 (0.4-2.0)	26 (12.4)	43 (17.1)	0.61 (0.3-1.1)
	HET	WT	109 (40.2)	112 (36.1)	1.0 (0.6-1.5)	50 (41.3)	55(39.3)	0.9 (0.5-1.7)	80 (38.3)	87 (34.7)	0.87 (0.5-1.4)
	WT	WT	62 (22.9)	65 (21.0)	1.0	28 (23.1)	27 (19.3)	1.0	54 (25.8)	54 (21.5)	1.0
rs8192678/*PPARG*	HET	HA	1 (0.4)	1 (0.3)	/	/	/	/	1 (0.5)	1 (0.4)	/
rs2270565/*UCP1*	WT	HA	1 (0.4)	4 (1.3)	/	0 (0.0)	1 (0.7)	/	1 (0.5)	3 (1.2)	/
	HA	HET	6 (2.2)	14 (4.5)	0.6 (0.2-1.5)	2 (1.7)	5 (3.6)	/	5 (2.4)	10 (4.0)	0.8 (0.3-2.7)
	HET	HET	35 (12.9)	26 (8.4)	1.6 (0.9-3.0)	18 (14.9)	14 (10.0)	1.6 (0.7-4.0)	31 (14.8)	17 (6.7)	2.6 (1.3-5.4)
	WT	HET	15 (5.5)	16 (5.2)	1.2 (0.5-2.5)	6(5.0)	6 (4.3)	1.2 (0.3-4.0)	9 (4.3)	14 (5.6)	1.0 (0.4-2.4)
	HA	WT	52 (19.1)	54 (17.5)	1.2 (0.7-2.0)	24 (19.8)	29(20.7)	1.0 (0.5-2.1)	42 (20.0)	42 (16.7)	1.5 (0.8-2.7)
	HET	WT	102 (37.5)	119 (38.5)	1.1 (0.7-1.6)	45(37.2)	54(38.6)	1.0 (0.5-2.0)	78 (37.1)	99 (39.3)	1.2 (0.7-1.9)
	WT	WT	60 (22.1)	75 (24.3)	1	26(21.5)	31(22.1)	1.0	43 (20.5)	66 (26.2)	1
rs8192678/*PPARG*	HA	HA	0 (0.0)	1 (0.3)	/	/	/	/	2 (1.0)	3 (1.2)	/
rs45539933/*UCP1*	HET	HA	2 (0.7)	3 (0.9)	/	0 (0.0)	1 (0.7)	/	/	/	/
	WT	HA	0 (0.0)	1 (0.3)	/	/	/	/	0 (0.0)	1 (0.4)	/
	HA	HET	5 (1.8)	9 (2.8)	0.7 (0.2-2.1)	2 (1.6)	3 (2.1)	/	5 (2.4)	6 (2.3)	1.1 (0.3-3.9)
	HET	HET	28 (10.3)	25 (7.9)	1.3 (0.7-2.4)	15 (12.3)	13 (9.0)	1.4 (0.6-3.4)	21 (10.0)	18 (7.0)	1.4 (0.7-3.0)
	WT	HET	22 (8.1)	23 (7.2)	1.2 (0.6-2.3)	9 (7.4)	9 (6.2)	1.2 (0.4-3.4)	19 (9.0)	18 (7.0)	1.6 (0.7-3.4)
	HA	WT	44 (16.2)	56 (17.6)	0.9 (0.6-1.5)	20 (16.4)	29 (20.0)	0.8 (0.4-1.7)	35 (16.7)	45 (17.4)	1.0 (0.6-1.9)
	HET	WT	103 (38.0)	122 (38.4)	1.0 (0.7-1.5)	47 (38.5)	54 (37.2)	1.0 (0.5-2.0)	81 (38.6)	105 (40.7)	1.1 (0.7-1.7)
	WT	WT	67 (24.7)	78 (24.5)	1.0	29 (23.8)	36 (24.8)	1.0	47 (22.4)	62 (24.0)	1.0

Table 10-7 cont.

Polymorphism / *Gene 1* Polymorphism / *Gene2*	Genotype Gene 1	Gene 2	All Men n cases (%)	All Men n controls (%)	All Men AgeAdjOR (95%CI)	Men ≥60 years n cases (%)	Men ≥60 years n controls (%)	Men ≥60 years AgeAdjOR (95%CI)	Men Off Statins n cases (%)	Men Off Statins n-controls (%)	Men Off Statins AgeAdjOR (95%CI)
rs17563 */BMP4*	HA	HA	42 (15.8)	56 (17.9)	0.9 (0.5-1.4)	19 (15.8)	29 (20.4)	0.8 (0.4-1.6)	34 (16.6)	45 (17.7)	1.0 (0.6-1.7)
rs45539933/*UCP1*	HET	HA	102 (38.3)	121 (38.7)	1.0	46 (38.3)	54 (38.0)	1.0	79 (38.5)	104 (40.9)	1.0
	WT	HA	65 (24.4)	75 (24.0)	1.0 (0.7-1.6)	29(24.2)	33(23.2)	1.1 (0.6-1.6)	46 (22.4)	58 (22.8)	1.0 (0.6-1.7)
	HA	HET	6 (2.3)	9 (2.9)	0.8 (0.3-2.3)	2(1.7)	3(2.1)		5 (2.4)	6 (2.4)	1.1 (0.3-3.7)
	HET	HET	29 (10.9)	24 (7.7)	1.4 (0.8-2.5)	15(12.5)	12(8.5)	1.5 (0.6-3.6)	22 (10.7)	18 (7.1)	1.5 (0.7-2.9)
	WT	HET	22 (8.3)	23 (7.3)	1.2 (0.6-2.2)	9(7.5)	10(7.0)	1.1 (0.4-2.9)	19 (9.3)	19 (7.5)	1.4 (0.7 -2.9)
	HA	WT	0 (0.0)	1 (0.3)							
	HET	WT	0 (0.0)	3 (1.0)		0(0.0)	1(0.7)		0 (0.0)	3 (1.2)	
	WT	WT	0 (0.0)	1 (0.3)					0(0.0)	1 (0.4)	
rs3736265/*PPARGC1A*	WT	HA	58 (22.0)	68 (22.1)	1.1 (0.7-1.7)	25 (21.2)	34 (24.5)	0.9 (0.4-1.8)	46 (22.8)	52 (20.6)	1.4 (0.8-2.4)
rs8192678/*PPARGC1A*	HET	HET	9 (3.4)	11 (3.6)	0.9 (0.4-2.4)	8 (6.8)	10 (7.2)	1.0 (0.3-2.8)	8 (4.0)	9 (3.6)	1.1 (0.4-3.2)
	WT	HET	124 (47.0)	134 (43.5)	1.2 (0.8-1.8)	54 (45.8)	57 (41.0)	1.1 (0.6-2.2)	97 (48.0)	108 (42.9)	1.4 (0.9-2.2)
	HA	WT	2 (0.8)	0 (0.0)	/	1 (0.8)	0 (0.0)	/	2 (1.0)	0 (0.0)	/
	HET	WT	10 (3.8)	17 (5.5)	0.7 (0.3-1.7)	5 (4.2)	9 (6.5)	0.7 (0.2-2.2)	6 (3.0)	15 (6.0)	0.6 (0.2-1.6)
	WT	WT	61 (23.1)	78 (25.3)	1.0	25 (21.2)	29 (20.9)	1.0	43 (21.3)	68 (27.0)	1.0
rs3736265/*PPARGC1A*	WT	HA	0 (0.0)	1 (0.3)	/	/	/	/	0(0.0)	1 (0.4)	/
rs45520937/*PPARGC1B*	HET	HET	1 (0.4)	0 (0.0)	/	1 (0.8)	0 (0.0)	/	1 (0.5)	0 (0.0)	/
	WT	HET	8 (3.0)	13 (4.1)	0.7 (0.3-1.7)	5 (4.1)	6 (4.1)	1.0 (0.3-3.3)	7 (3.4)	11 (4.3)	0.7 (0.3-2.0)
	HA	WT	2 (0.7)	0 (0.0)	/	1 (0.8)	0 (0.0)	/	2 (1.0)	0 (0.0)	/
	HET	WT	20 (7.4)	9.1 (274)	0.8 (0.4-1.4)	13 (10.7)	20 (13.8)	0.8 (0.4-1.6)	15 (7.2)	24 (9.3)	0.7 (0.3-1.4)
	WT	WT	238 (88.5)	274 (86.4)	1.0	101 (83.5)	119(82.1)	1.0	182 (87.9)	221 (86.0)	1.0
rs3736265 */PPARGC1A*	WT	HA	3 (1.1)	0 (0.0)	/	3 (2.5)	0 (0.0)	/	1 (0.5)	0 (0.0)	/
rs7732671/*PPRGC1B*	HA	HET	1 (0.4)	0 (0.0)	/	2 (1.7)	6 (4.2)	/	1 (0.5)	5 (2.0)	/
	HET	HET	3 (1.1)	7 (2.3)	0.4 (0.1-1.7)	14 (11.8)	21 (14.8)	0.4 (0.1-2.0)	2 (1.0)	5 (2.0)	0.4 (0.1-2.0)
	WT	HET	38 (14.2)	54 (17.4)	0.8 (0.5-1.3)	/	/	/	34 (16.5)	42 (16.7)	1.0 (0.6-1.7)
	HA	WT	1 (0.4)	0 (0.0)	/	1 (0.8)	0 (0.0)	0.8 (0.4-1.6)	1 (0.5)	0 (0.0)	/
	HET	WT	18 (6.7)	22 (7.1)	0.9 (0.5-1.7)	12 (10.1)	14 (9.9)	1.0 (0.4-2.3)	14 (6.8)	19 (7.6)	0.8 (0.4-1.7)
	WT	WT	203 (76.0)	227 (73.2)	1.0	87 (73.1)	101 (71.1)	1.0	153 (74.3)	185 (73.7)	1.0

Table 10-7 cont.

Polymorphism / *Gene 1* Polymorphism / *Gene2*	Genotype Gene 1	Gene 2	All Men n cases (%)	All Men n controls (%)	All Men AgeAdjOR (95%CI)	Men ≥60 years n cases (%)	Men ≥60 years n controls (%)	Men ≥60 years AgeAdjOR (95%CI)	Men Off Statins n cases (%)	Men Off Statins n-controls (%)	Men Off Statins AgeAdjOR (95%CI)
rs3736265 /*PPARGC1A*	WT	HA	0 (0.0)	1 (0.3)	/	0 (0.0)	1 (0.7)	/	/	/	/
rs 1800206/*PPARA*	HA	HET	1 (0.4)	0 (0.0)	/	0 (0.0)	3 (2.1)	/	1 (0.5)	0 (0.0)	/
	HET	HET	2 (0.7)	4 (1.3)	/	/	/	/	1 (0.5)	4 (1.6)	/
	WT	HET	19 (7.1)	20 (6.3)	1.1 (0.6-2.2)	7 (5.8)	7 (4.9)	1.2 (0.4-3.5)	14 (6.8)	17(6.6)	1.1 (0.5-2.2)
	HA	WT	1 (0.4)	0 (0.0)		1 (0.8)	0 (0.0)		1 (0.5)	0 (0.0)	/
	HET	WT	19 (7.1)	24 (7.6)	0.9 (0.5-1.7)	14 (11.6)	16 (11.1)	1.0 (0.5-2.2)	15 (7.3)	19 (7.4)	0.9 (0.4-1.8)
	WT	WT	226 (84.3)	268 (84.5)	1.0	99 (81.8)	117 (81.3)	1.0	174 (84.5)	217 (84.4)	1.0
rs3736265/ *PPARGC1A*	HA	HA	1 (0.4)	0 (0.0)	/	/	/	/	/	/	/
rs11264680/ *CRTC2*	HET	HA	5 (1.9)	2 (0.6)	/	3 (2.5)	2 (1.4)	/	1 (0.5)	0 (0.0)	/
	WT	HA	58 (21.7)	54 (17.3)	1.2 (0.7-2.0)	31 (25.8)	24 (16.8)	1.5 (0.7-3.0)	3 (1.5)	1(0.4)	/
	HA	HET	1 (0.4)	0 (0.0)	/	1 (0.8)	0 (0.0)	/	42 (20.5)	45 (17.8)	1.0 (0.6-1.8)
	HET	HET	12 (4.5)	14 (4.5)	1.0 (0.4-2.2)	8 (6.7)	8 (5.6)	1.1 (0.4-3.4)	1 (0.5)	0 (0.0)	/
	WT	HET	116 (43.3)	149 (47.6)	0.9 (0.6-1.3)	46 (38.3)	67 (46.9)	0.8 (0.4-1.5)	10 (4.9)	13 (5.1)	0.8 (0.3-2.0)
	HET	WT	4 (1.5)	13 (4.2)	0.3 (0.1-1.0)	3 (2.5)	10 (7.0)	0.4 (0.1-1.4)	3 (1.5)	10 (4.0)	0.3 (0.1-1.0)
	WT	WT	70 (26.2)	81 (25.9)	1.0	28 (23.3)	32 (22.4)	1.0	57 (27.8)	66 (26.1)	
rs3736265/*PPARGC1A*	WT	HA	1 (0.4)	3 (0.9)	/	1 (0.9)	2 (1.4)	/	1 (0.5)	1(0.4)	/
rs61753381/*CREBBP*	HET	HET	2 (0.0)	3 (0.9)	/	2 (1.7)	2 (1.4)	/	2 (1.0)	3 (1.2)	/
	WT	HET	24 (9.2)	18 (5.7)	1.7 (0.9-3.2)	11(9.4)	6 (4.1)	2.3 (0.8-6.6)	20 (10.0)	14 (5.4)	1.9 (0.9-3.9)
	HA	WT	2 (0.8)	0 (0.0)	/	/	/	/	2 (1.0)	0 (0.0)	/
	HET	WT	19 (7.3)	26 (8.2)	0.9 (0.5-1.6)	12 (10.3)	18 (12.4)	0.9 (0.4-1.9)	14 (7.0)	21 (8.1)	0.8 (0.4-1.7)
	WT	WT	213 (81.6)	268 (84.3)	1.0	90 (76.9)	117 (80.7)	1.0	162 (80.6)	219 (84.9)	1.0
rs8192678/*PPARGC1A*	HET	HA	0 (0.0)	1 (0.3)	/	/	/	/	0 (0.0)	1 (0.4)	/
rs45520937/*PPARGC1B*	HA	HET	1 (0.4)	4 (1.3)	/	0 (0.0)	3 (2.2)	/	0 (0.0)	3 (1.2)	/
	HET	HET	7 (2.6)	7 (2.3)	1.3 (0.4-3.8)	5 (4.2)	2 (1.4)	/	7 (3.4)	7 (2.8)	1.5 (0.5-4.6)
	WT	HET	1 (0.4)	1 (0.3)	/	1 (0.8)	0 (0.0)	/	1 (0.5)	1 (0.4)	/
	HA	WT	58 (21.6)	64 (20.8)	1.2 (0.7-1.9)	25 (21.2)	31 (22.3)	1.1 (0.5-2.2)	47 (22.7)	49 (19.4)	1.6 (0.9-2.7)
	HET	WT	130 (48.3)	137 (44.5)	1.2 (0.8-1.8)	57 (48.3)	65 (46.8)	1.1 (0.6-2.1)	102 (49.3)	109 (43.3)	1.5 (0.9-2.3)
	WT	WT	72 (26.8)	94 (30.5)	1.0	30 (25.4)	38 (27.3)	1.0	50 (24.2)	82 (32.5)	1.0

Table 10-7 cont.

Polymorphism / *Gene 1* Polymorphism / *Gene2*	Genotype Gene 1	Gene 2	All Men n cases (%)	All Men n controls (%)	All Men AgeAdjOR (95%CI)	Men ≥60 years n cases (%)	Men ≥60 years n controls (%)	Men ≥60 years AgeAdjOR (95%CI)	Men Off Statins n cases (%)	Men Off Statins n-controls (%)	Men Off Statins AgeAdjOR (95%CI)
rs8192678/*PPARGC1A*	HA	HA	1 (0.4)	0(0.0)	/	1 (0.9	0 (0.0)	/	/	/	/
rs7732671/*PPARGC1B*	HET	HA	2 (0.7)	0 (0.0)	/	2 (1.7)	0 (0.0)	/	1 (0.5)	0 (0.0)	/
	HA	HET	9 (3.3)	13 (4.3)	1.0 (0.4-2.4)	3 (2.6)	6 (4.4)	/	7 (3.4)	9 (3.7)	1.5 (0.5-4.4)
	HET	HET	20 (7.4)	30 (10.0)	0.9 (0.5 1.8)	8 (6.8)	10 (7.4)	1.0 (0.3-2.9)	19 (9.1)	26 (10.6)	1.3 (0.7-2.7)
	WT	HET	14 (5.2)	14 (4.7)	1.3 (0.6-3.0)	5 (4.3)	8 (5.9)	0.7 (0.2-2.6)	12 (5.8)	10 (4.1)	2.1 (0.8 -5.3)
	HA	WT	49 (18.2)	52 (17.3)	1.2 (0.7-2.1)	21 (17.9)	27 (19.9)	0.9 (0.4-2.1)	40 (19.2)	40 (16.3)	1.8 (1.0 -3.2)
	HET	WT	115 (42.8)	113 (37.5)	1.3 (0.8-2.0)	52 (44.4)	55 (40.4)	1.2 (0.6-2.2)	89 (42.8)	89 (26.2)	1.7 (1.1-2.8)
	WT	WT	59 (21.9)	79 (26.2)	1.0	25 (21.4)	30 (22.1)	1.0	40 (19.2)	72 (29.3)	1.0
RS8192678/ *PPARGC1A*	HET	HA	0 (0.0)	1 (0.3)	/	0 (0.0)	1 (0.7)	/	/	/	/
Rs1800206/*PPARA*	HA	HET	3 (1.1)	3 (1.0)	/	2 (1.7)	2 (1.4)	/	2 (1.0)	3 (1.2)	/
	HET	HET	15 (5.6)	10 (3.3)	1.8 (0.8-4.3)	5 (4.2)	4 (2.9)	/	11 (5.3)	9 (3.6)	1.8 (0.7-4.8)
	WT	HET	6 (2.2)	10 (3.3)	0.8 (0.3-2.2)	0 (0.0)	3 (2.2)	/	5 (2.4)	9 (3.6)	0.9 (0.3-2.9)
	HA	WT	55 (20.4)	65 (21.2)	1.0 (0.6-1.7)	23 (19.5)	32 (23.2)	0.8 (0.4-1.7)	44 (21.3)	49 (19.5)	1.4 (0.8-2.4)
	HET	WT	122 (45.4)	134 (43.6)	1.1 (0.7-1.7)	57 (48.3)	62 (44.9)	1.0 (0.6-2.0)	98 (47.3)	108 (43.0)	1.4 (0.9-2.2)
	WT	WT	68 (25.3)	84 (27.4)	1.0	31 (26.3)	34 (24.6)	1.0	47 (22.7)	73 (29.1)	1.0
rs8192678/PPARGC1A	HA	HA	17 (6.4)	16 (5.3)	1.2 (0.6-3.0)	10 (8.5)	10 (7.3)	2.1(0.6-7.9)	13 (6.4)	11 (4.5)	2.1 (0.7 -5.8)
rs11264680/CRTC2	HET	HA	24 (9.0)	27 (8.9)	1.1 (0.6-2.0)	12 (10.3)	11 (8.0)	2.4 (0.7-8.4)	17 (8.3)	24 (9.7)	1.3 (0.5-3.2)
	WT	HA	22 (8.3)	10 (3.3)	2.7 (1.2-6.1)	11(9.4)	3 (2.2)	7.6 (1.5-37.9)	15 (7.4)	9 (3.6)	3.3 (1.1-9.5)
	HA	HET	31 (11.7)	35 (11.6)	1.1 (0.6-2.0)	12 (10.3)	15 (10.9)	1.7 (0.6-5.0)	24 (11.8)	27 (10.9)	1.8 (0.8-4.2)
	HET	HET	16 (6.0)	33 (10.9)	0.6 (0.3-1.2)	28 (23.9)	36 (26.3)	1.7 (0.6-5.0)	52 (25.5)	55(22.3)	1.8 (0.8-3.8)
	WT	HET	35 (13.2)	51 (16.8)	0.8 (0.5-1.4)	14 (12.0)	22(16.1)	1.4 (0.4-4.4)	22 (10.8)	46 (18.6)	0.9 (0.4-2.1)
	HA	WT	11 (4.1)	16 (5.3)	0.8 (0.4-2.0)	3 (2.6)	8 (5.8)	0.8 (0.2-4.1)	10 (4.9)	13 (5.3)	1.5 (0.5-4.3)
	HET	WT	48 (18.0)	42 (13.9)	1.4 (0.8-2.3)	21 (17.9)	19 (13.9)	2.4 (0.8-7.6)	37 (18.1)	35 (14.2)	2.0 (0.9-4.4)
	WT	WT	62 (23.3)	73 (24.1)	1.0	6 (5.1)	13(9.5)	1.0	14 (6.9)	27 (10.9)	1.0
rs8192678/*PPARGC1A*	HET	HA	1 (0.4)	2 (0.6)	/	1 (0.9)	2 (1.4)	/	1 (0.5)	0 (0.0)	/
rs61753381/*CREBBP*	WT	HA	0 (0.0)	1 (0.3)	/	/	/	/	0 (0.0)	1 (0.4)	/
	HA	HET	6 (2.3)	6 (1.9)	1.3 (0.4-4.2)	2 (1.7)	2 (1.4)	/	5 (2.4)	4 (1.6)	/
	HET	HET	14 (5.3)	9 (2.9)	1.9 (0.8-4.7)	8 (6.8)	8 (4.3)	1.8 (0.6-6.0)	12 (5.9)	7 (2.8)	2.5 (0.9-6.9)
	WT	HET	5 (1.9)	5 (1.6)	1.4 (0.4-5.1)	2 (1.7)	0(0.0)	/	4 (2.0)	5 (2.0)	/
	HA	WT	51 (19.2)	62 (20.1)	1.1 (0.7-1.7)	22 (18.8)	32 (22.9)	0.9 (0.5-2.0)	41 (20.0)	48 (19.0)	1.4 (0.4-5.8)
	HET	WT	121 (45.4)	135 (43.7)	1.2 (0.8-1.7)	54 (46.2)	60 (42.9)	1.3 (0.7-2.3)	95 (46.3)	110 (43.7)	1.4 (0.9-2.2)
	WT	WT	68 (25.6)	89 (28.8)	1.0	28 (23.9)	38 (27.1)	1.0	47 (22.9)	77 (30.6)	1.0

Table 10-7 cont.

Polymorphism / *Gene 1* Polymorphism / *Gene2*	Genotype Gene 1	Gene 2	All Men n cases (%)	All Men n controls (%)	All Men AgeAdjOR (95%CI)	Men ≥60 years n cases (%)	Men ≥60 years n controls (%)	Men ≥60 years AgeAdjOR (95%CI)	Men Off Statins n cases (%)	Men Off Statins n-controls (%)	Men Off Statins AgeAdjOR (95%CI)
rs7732671/ *PPARGC1B*	WT	HA	0 (0.0)	1 (0.3)	/	//	/	/	0 (0.0)	1 (0.4)	/
rs45520937/*PPARGC1B*	HET	HET	0 (0.0)	2 (0.6)	/	0 (0.0)	1 (0.7)	/	0 (0.0)	1 (0.4)	/
	WT	HET	9 (3.3)	11 (3.5)	0.9 (0.4-2.2)	6 (5.0)	5 (3.5)	1.4 (0.4-4.8)	8 (3.8)	10 (4.0)	0.9 (0.3-2.3)
	HA	WT	3 (1.1)	0 (0.0)	/	3 (2.5)	0 (0.0)	/	/	/	/
	HET	WT	43 (15.8)	59 (19.0)	0.8 (0.5-1.3)	16 (13.4)	26 (18.3)	0.7 (0.4-1.4)	1 (0.5)	0(0.0)	/
	WT	WT	217 (79.8)	237 (76.5)	1.0	94 (79.0)	110 (77.5)	1.0	164 (77.7)	193 (76.9)	1.0
rs7732671/*PPARGC1B*	HET	HA	/	/	/						1.3 (0.3-5.5)
rs1800206/*PPARA*	WT	HA	0 (0.0)	1 (0.3)	/	0 (0.0)	1 (0.7)	/	/	/	/
	HA	HET	1 (0.4)	0 (0.0)	/	1 (0.8)	0 (0.0)	/	/	/	/
	HET	HET	4 (1.5)	5 (1.6)	/	0 (0.0)	3 (2.1)	/	4 (1.9)	4 (1.6)	/
	WT	HET	19 (7.0)	19 (6.1)	1.1 (0.6-2.1)	6 (5.0)	7 (5.0)	1.0 (0.3-3.1)	14 (6.6)	17 (6.8)	0.9 (0.4-2.0)
	HA	WT	2 (0.7)	0 (0.0)	/	2 (1.7)	0 (0.0)	/	1 (0.5)	0 (0.0)	/
	HET	WT	39 (14.3)	56 (18.1)	0.8 (0.5-1.2)	16 (13.4)	24 (17.0)	0.8 (0.4-1.5)	34 (16.1)	43 (17.2)	0.9 (0.5-1.6)
	WT	WT	207 (76.1)	228 (73.8)	1.0	94 (79.0)	106 (75.2)	1.0	158 (74.9)	186 (74.4)	1.0
rs732671/ *PPARGC1B*	HA	HA	/	/	/	/	/	/			
rs11264680/ *CRTC2*	HET	HA	12 (4.5)	12 (3.9)	1.2 (0.5-2.9)	4 (3.4)	6 (4.3)	/	10 (4.8)	9 (3.7)	1.4 (0.5-3.7)
	WT	HA	52 (19.3)	44 (14.4)	1.4 (0.8-2.3)	29 (24.6)	20 (14.3)	2.1 (1.0-4.4)	37 (17.8)	37 (15.0)	1.2 (0.7-2.2)
	HA	HET	2 (0.7)	0 (0.0)	/	2 (1.7)	0(0.0)	/	/	/	/
	HET	HET	17 (6.3)	31 (10.2)	0.6 (0.3-1.3)	7 (5.9)	16 (11.4)	0.6 (0.2-1.8)	14 (6.7)	24 (9.8)	0.7 (0.3-1.6)
	WT	HET	110 (40.9)	129 (42.3)	1.0 (0.7-1.6)	45 (38.1)	57 (40.7)	1.1 (0.6-2.2)	85 (40.9)	104 (42.3)	1.0 (0.6-1.7)
	HA	WT	1 (0.4)	0 (0.0)	/	1 (0.8)	0(0.0)	/	1 (0.5)	1 (0.0)	/
	HET	WT	14 (5.2)	17 (5.6)	1.0 (0.5-2.3)	5 (4.2)	5 (3.6)	1.5 (0.4-5.6)	14 (6.7)	13 (5.3)	1.6 (0.7-3.7)
	WT	WT	61 (22.7)	72 (23.6)	1.0	25 (21.2)	36 (25.7)	1.0	47 (22.6)	59 (24.0)	1.0
rs7732671/*PPARGC1B*	WT	HA	1 (0.4)	3 (1.0)	/	1 (0.9)	2 (1.4)	/	1 (0.5)	1 (0.4)	/
rs61753381/*CREBBP*	HET	HET	7 (2.6)	3 (1.0)	2.7 (0.7-10.5)	3 (2.6)	1 (0.7)	/	7 (3.4)	3 (1.2)	2.9 (0.7-11.4)
	WT	HET	19 (7.1)	18 (5.8)	1.2 (0.6 -2.3)	10 (8.6)	7 (4.9)	1.8 (0.6-4.8)	15 (7.2)	14 (5,6)	1.3 (0.6-2.8)
	HA	WT	3 (1.1)	0 (0.0)	/	3 (2.6)	0(0.0)	/	1 (0.0)	0 (0.0)	/
	HET	WT	36 (13.5)	58 (18.7)	0.7 (0.5-1.1)	13 (11.2)	26 (18.3)	0.6 (0.3-1.3)	31 (15.0)	44 (17.5)	0.9 (0.5-1.5)
	WT	WT	200 (75.2)	228 (73.5)	1.0	86 (74.1)	106 (74.6)	1.0	152 (73.4)	189 (75.3)	1.0

Table 10-7 cont.

Polymorphism / *Gene 1* Polymorphism / *Gene2*	Genotype Gene 1	Gene 2	All Men n cases (%)	All Men n controls (%)	All Men AgeAdjOR (95%CI)	Men ≥60 years n cases (%)	Men ≥60 years n controls (%)	Men ≥60 years AgeAdjOR (95%CI)	Men Off Statins n cases (%)	Men Off Statins n-controls (%)	Men Off Statins AgeAdjOR (95%CI)
rs1800206/*PPARA*	HET	HA	4 (1.5)	4 (1.3)	/	/	/	/	3 (1.4)	4 (1.6)	/
rs11264680/*CRTC2*	WT	HA	61 (22.6)	52 (16.7)	1.4 (0.8-2.2)	34 (28.3)	26 (18.3)	1.5 (0.7-3.1)	44 (21.2)	42 (16.7)	1.2 (0.7-5.0)
	HA	HET	0 (0.0)	1 (0.3)	/	0 (0.0)	1 (0.7)	/	/	/	/
	HET	HET	14 (5.2)	11 (3.5)	1.5 (0.6-3.5)	5 (4.2)	3 (2.1)	/	10 (4.8)	10 (4.0)	1.2(0.5-3.0)
	WT	HET	115 (42.6)	151 (48.4)	0.9 (0.6-1.3)	50 (41.7)	71 (50.0)	0.8 (0.4-1.5)	89 (42.8)	121 (48.0)	0.9 (0.5-1.3)
	HET	WT	6 (2.2)	9 (2.9)	0.7 (0.3-2.2)	2 (1.7)	7 (4.9)	0.3 (0.1-1.8)	5 (2.4)	7 (2.8)	0.8 (0.2-2.5)
	WT	WT	70 (25.9)	84 (26.9)	1.0	29(24.2)	34 (23.9)	1.0	57 (27.4)	68 (28.0)	1.0
rs1800206/PPARA	WT	HA	1 (0.4)	3 (0.9)	/	1 (0.9)	2 (1.4)	/	1 (0.5)	1 (0.4)	/
rs61753381/CREBBP	HET	HET	0 (0.0)	4 (1.3)	/	0 (0.0)	2 (1.4)	/	0 (0.0)	3 (1.2)	/
	WT	HET	26 (9.8)	17 (5.4)	1.9 (1.0-3.6)	13 (11.1)	6 (4.2)	2.8 (1.0-7.6)	22 (10.7)	14 (5.4)	2.0 (1.0-4.0)
rs1800206/*PPARA*	HA	WT	0 (0.0)	1 (0.3)	/	0 (0.0)	1 (0.7)	/	/	/	/
rs61753381/*CREBBP*	HET	WT	24 (9.0)	20 (6.3)	1.5 (0.8-2.8)	7 (6.0)	8 (5.6)	1.2 (0.4-3.3)	18 (8.7)	18 (7.0)	1.3(0.7-2.6)
	WT	WT	215 (80.8)	272 (85.8)	1.0	96 (82.1)	125 (86.8)	1.0	165 (80.1)	221 (86.0)	1.0
rs11264680/*CRTC2*	HA	HA	1 (0.4)	0 (0)	/	1 (0.9)	0(0.0)	/	1 (0.5)	0(0.0)	/
rs61753381/*CREBBP*	HET	HA	0 (0.0)	3 (1.0)	/	0 (0.0)	2 (1.4)	/	0 (0.0)	1 (0.4)	/
	HA	HET	10 (3.8)	5 (1.6)	2.7 (0.9-8.0)	5 (4.3)	1 (0.7)	/	8 (3.9)	5 (2.0)	1.9 (0.6-6.3)
	HET	HET	10 (3.8)	10 (3.2)	1.3 (0.5-3.3)	4 (3.4)	3 (2.1)	1.9 (0.4-9.0)	6 (3.0)	6 (2.4)	1.8 (0.6-5.4)
	WT	HET	6 (2.3)	6 (1.9)	1.2 (0.4-3.9)	4 (3.4)	4 (2.8)	1.4 (0.3-6.0)	37 (18.2)	41 (16.2)	1.1 (0.3-3.6)
	HA	WT	53 (20.2)	51 (16.3)	1.4 (0.9-2.1)	27 (23.3)	25 (17.5)	1.5 (0.7-3.1)	37 (18.2)	41 (16.2)	1.1 (0.3-3.6)
	HET	WT	114 (43.3)	150 (47.9)	1.1 (0.7-1.6)	48 (41.4)	70 (49.0)	1.0 (0.5-1.8)	88 (43.3)	124 (49.0)	0.9 (0.6-1.4)
	WT	WT	69 (26.2)	88 (28.1)	1.0	27 (23.3)	38 (26.6)	1.0	55 (27.1)	70 (27.7)	1

The combined genotype of homozygous wildtype for rs8192678 in *PPARGCIA* and homozygous alternate for rs11264680 in *CRTC2* was associated with an increased risk of MI, AgeAdjOR 2.7 (95%CI 1.2-6.1) which was even higher in men older than 60 years of age, AgeAdjOR of 7.6 (95% 1.5-37.9). Men off statins with this genotype combination had an OR of 3.3 (95% CI 1.1-9.5), all relative to the homozygous wildtype genotypes for both the rs8192678 in *PPARGCIA* and rs11264680 in *CRTC2* variants Table 10-7.

The combined genotype for heterozygous rs8192678 in *PPARGCIA* and homozygous wildtype for rs7732671 in *PPARGC1B* was associated with an increased risk of MI in men off statins AgeAdjOR 1.7 (95% CI 1.1-2.8). Homozygous alternate rs8192678 in *PPARGCIA* and homozygous wildtype for rs7732671 in *PPARGC1B* was also associated with a 1.8-fold (95%CI 1.0-3.2) risk for MI amongst men off statins (Table 10-7).

The combination of homozygous wildtype genotype for rs7732671 in PPARGC1B and homozygous alternate for the rs11264680 in *CRTC2* also showed an increased risk of MI in men over 60 years of age, AgeAdjOR 2.1 (95% CI 1.0-4.4), but not in men overall or in men off statins, relative to the homozygous wildtype genotype for both the rs7732671 in PPARGC1B and rs11264680 in *CRTC2* (Table 10-7).

The combination of homozygous wildtype for rs1800206 in *PPARA* and heterozygous for rs61753381 in *CREBBP* was associated with an increased risk of MI with an AgeAdjOR of 1.9 (95% CI 1.0-3.6) in Men overall, an AgeAdjOR of 2.8 (95%CI 1.0-7.6) in Men older than 60 years of age and an AgeAdjOR 2.0 (95% CI 1.0-4.0) in Men off statins, relative to the homozygous wildtype for these two variants (Table 10-7).

The combination of homozygous wildtype for rs2707466 in *WNT16* and heterozygous for the *LRP5* polymorphism rs4988321 protected against MI, AgeAdjOR 0.2 (95%CI 0.04-0.9) in Men ≥60 years of age and AgeAdjOR 0.3 (95%CI 0.1-0.9) in Men off statins. When both polymorphisms were

heterozygous there was also a decreased risk for MI in Men greater than 60 years of age [AgeAdjOR 0.3 (0.1-1.0)], all relative to the homozygous wildtype genotype for both the rs2707466 in *WNT16* and rs4988321 in *LRP5* (Table 10-7).

Section 10.9. Discussion

Odds ratios for isolated polymorphisms in genes related to the WNT/β Catenin pathway showed few effects on risk of MI, with a small magnitude when analysed separately. Only the rs2305948 polymorphism in *KDR* was protective with an AgeAdjOR 0.5 (95% CI, 0.2-1.0) in women but not in men. rs2305948 has been associated with clopidogrel resistance after myocardial infarction (Zhang et al. 2016) but has not been previously highlighted as an aetiological component in the development of MI.

Despite this there are several individual SNPs that did have an effect on intermediate phenotypes (Table 10-8,Table 10-9). Although a number of these may be due to chance, it is unlikely that all are due to chance. The polymorphism rs61753381 in *CREBBP* increases hs-CRP considerably (1.68mmol/L vs 2.67mmol/L) and the number of diabetics was higher in heterozygotes than in men who were WT for it (20% vs 12.1%). This could therefore be a good candidate for diabetes. These findings indicate that an extreme phenotype approach is very useful in identifying variants and genes involved in influencing such phenotypes.

Combinations selected on the basis of biological interactions or on common effects on intermediate phenotypes revealed stronger effects on the risk of MI. Analysing two polymorphisms (rs3736228 and rs4988321) in *LRP5* in conjunction showed that when both polymorphisms were heterozygous the age adjusted OR was 0.6 (95% CI, 0.4-1.0) in men, and in men > 60 years of age the Age adjusted OR was 0.4 (95% CI 0.2-0.8). *LRP5* is a close paralog to *LRP6* and both serve as co-receptors for Frizzled proteins for WNT ligands. *LRP6* has been associated with elevated LDL, elevated triglycerides,

hypertension, diabetes and low bone density but had no effect on HDL or BMI The combination of wildtype for rs8192678 in PPARGC1A and heterozygote for rs11264680 in CRTC2 was associated with a high OR of 7.6 (95% CI, 1.5-37.9) in men older than 60 years of age and of 3.3 (95% CI, 1.1-9.5) in men not on statins. A higher odds ratio in the older age groups might reflect the long process of development of atherosclerosis leading to MI. Another example is observed for the category of individuals with WT genotype for rs1800206 in *PPARA* and heterozygous genotype for rs61753381 in *CREBBP* where the OR is 1.9 (95% CI 1.0-3.6) in men overall and 2.8 (95% CI 1.0-7.6) in men over 60 years of age.

Environmental factors such as statins can mask effects of some genotypes. For example, in the combination of heterozygous genotype for rs8192678 in *PPARGC1A* and wildtype genotype for rs17563 in *BMP4* the risk of MI in men overall is 1.9 (95% CI, 1.0-3.9) whilst in men off statins the risk reaches 2.7 (95% CI, 1.2-6.1). Similarly in the category that is homozygous alternative for both variants, the risk in men overall is 1.9 (95%CI 0.7-4.7) whilst in men off statins it is 3.6 (95%CI 1.2-10.9) Various other examples of this can be observed (Table 10-7). It is therefore very important to be cautious when comparing literature from studies when statins were not in widespread use to those after statin use.

The effects on risk for MI is not always easy to explain considering the effects on some of the intermediate phenotypes observed. For example *KL* rs9536314 and rs9527025, two variants in LD are associated with a large decrease in triglycerides, but the effect on risk tends towards being deleterious for individuals homozygous for the two (AgeAdjOR 3.0 (95%CI 0.9-10.5) indicating that this variant must be producing its effects via other mechanisms.

The strength of the effects of the genetic variants on intermediate phenotypes varies considerably - for example heterozygotes for rs45539933 in UCP1 have a 14% decrease in median triglyceride

levels (1.09 vs 1.27 mmol/L) whereas heterozygotes for *LPL* rs1801177 have a 33% increase in TG (1.61 vs 1.21 mmol/L). Additionally, having one or two alleles of a genetic variant does not necessarily result in an additive effect on the intermediate phenotype it influences. For example, heterozygotes for the *KL* rs9536314 (in complete LD with rs9527025) variant have an 11.5% decrease in TG (1.15 vs 1.30 mmol/L) whilst homozygotes have a 44% decrease in TG levels (0.73 vs 1.15 mmol/L). In view of this, genetic scores that do not take into account the magnitude of effects and assign an increase in score of 1 for every allele that increases risk for example (Hegele et al. 2014), are too rudimentary and do not accurately reflect the situation. Genetic risk scores, if they are to be used to predict risk of complex conditions, need to consider the direction and the magnitude of the effects of alleles, and also the effect with one or two alleles of that variant.

Another point of note is that genes and genetic variants have effects on several intermediate phenotypes, sometimes with effects expected to act in the opposite direction on risk for MI. For example, *PPARGC1A* rs8192678 was associated with a decrease in GGT and hs-CRP, but also with a decrease in HDL levels. This is one major reason why Mendelian Randomization studies based on analysis of one intermediate phenotype cannot always be applied to predict if genetic variants are truly functional or not.

From this exercise it is clear that epistasis is difficult to detect and characterise using traditional parametric statistical methods such as linear and logistic regression because when interactions between multiple SNPs are studied, there are many genotype combinations that have few or no data points requiring exponentially larger sample sizes to estimate interaction effects. Statistical tools to detect gene – gene interactions for discrete traits include parametric tools such as logistic regression (Gilbert-Diamond and Moore, 2011) and non-parametric data mining strategies including multifactor dimensionality reduction (MDR). MDR was developed to overcome the problem of high-order interactions having may contingency-table cells with no observations that gave rise to large co-efficients and standard errors. With MDR, multilocus genotypes are pooled into high-risk and low-risk groups,

reducing the genotype predictors from n dimensions to one dimension (Ritchie *et al.*, 2001) . For quantitative traits linear regression can be used if large sample sizes are available or MDR can be used or a method called combinatorial partitioning method (CPM) that examines multiple genes, each containing multiple variable loci, to identify partitions of multilocus genotypes that predict interindividual variation in quantitative trait levels (Nelson *et al.*, 2001). In order to study the million of SNPs in genome-wide studies data mining and machine learning methods will need to be developed to study gene-gene interactions and their effect on outcomes (Moore and Ritchie, 2004).

Section 10.10. Limitations and Further Work

One of the major limitations is that not all the genes in the chosen pathway were analysed. Other genes in the pathway could have an important effect alone or in combination with the genes studied. The genes that were chosen were selected from a small group of individuals demonstrating an extreme phenotype of high WHR and High HOMA-IR, other phenotypes consistent with adipocyte dysfunction (elevated liver enzymes, high hs-CRP) could have been looked into and may have given different results. When combinations of polymorphisms were analysed some of the subgroups were small. Only men could be analysed due to smaller numbers of women. Nevertheless, despite these limitations, combinations of two variants in one or two genes appears to be a better approach than taking into account the effect of just one genetic variant. Environmental factors were not considered but could influence the genetic factors studied and could form the basis of future research. The development of dedicated software and bioinformatic tools to assist data analysis would greatly assist in analysing the vast amounts of data emanating from high throughput genetic analysis. Further research should focus on the effects of combinations of polymorphisms on intermediate phenotypes.

Section 10.11. Conclusion

These results illustrate the importance of studying polymorphisms using a pathway based approach. In a complex disease such as atherosclerosis and acute myocardial infarction where genes in conserved pathways are likely to exhibit high degrees of redundancy and have only small effects, studying polymorphisms that are associated with a biological pathway has highlighted novel potential associations between various members of the WNT/β catenin pathway and MI.

Chapter 11. General Conclusions

The MAMI study gives very important insights into the risk factors associated with myocardial infarction in the Maltese population. It has demonstrated the alarming prevalence of individuals being either overweight or obese with only 32% of women and 14% of men having a normal BMI. This reflects the growing prevalence of obesity worldwide (Blüher 2019). However, similar to large international studies (Yusuf et al. 2005) BMI, the most common measure of adiposity in clinical use was not associated with MI. On the other hand, waist-hip ratio was found to be strongly associated with the risk of myocardial infarction in both men and women. The MAMI study results support the use of waist-hip ratio in order to identify subjects at the highest risk of developing MI both in clinical practice and as a confounder in research especially since the analysis suggests that even within the overweight and obese categories, individuals that had a normal waist-hip ratio were not at increased risk of MI (Table 11-1).

Table 11-1. Difference in risk of MI in obese individuals depending on waist-hip ratio.

High Waist-Hip Ratio in Obese individuals	3.6 fold increased risk of MI
Normal Waist-Hip Ratio in Obese individuals	No increased risk of MI

The prevalence of hypercholesterolaemia was also demonstrated to be high in the Maltese population with only 33.1% of controls having a TC level within the desirable range (≤ 5.0mmol/L). Compared to data collected in 2010 during the European health examination survey the percentage of individuals with a desirable TC has decreased with a proportionate increase in the percentage of individuals with a borderline high level (>5.00-6.18mmol/L). The percentage of subjects with a high TC level (>6.18mmol/L) has remained stable. A similar worsening in LDL-C levels was also been observed with the percentage of individuals having a very high LDL-C level (>4.90mmol/L) increasing from 1.6% to 4.6% with similar increases being noticed in the high and borderline high groups. Despite easily available statins, although TC and LDL-C cholesterol were both associated with an increased risk of myocardial infarction with Adjusted ORs of 1.8 (95% CI, 1.1.-3.1) and 1.7 (95% CI, 1.0-2.9)

respectively (in men not on statins) these components of the lipid profile were not the most closely associated with the risk of MI despite being the most commonly used in clinical practice. In fact, the TC/ HDL-C ratio and NHDL-C were the most closely associated with MI (in men off statins) with OR of 5.8 (95% CI, 3.1-10.8) and 2.9 (95% CI, 1.6-5.0) respectively. Similar findings were present in women. Based on these findings TC/HDL-C ratio and NHDL-C, which are routinely reported as part of a standard lipid profile, should be incorporated more into clinical practice in order to identify subjects at the highest risk of developing MI. The superior association with MI of TC/HDL-C has been known for years (Millán et al. 2009) but has not been adopted clinically, possibly due to the absence of randomised controlled trials supporting its use (Table 11-2).

Table 11-2 Risk of MI with high risk dyslipidaemias identified in the MAMI study. TC – Total cholesterol; LDL-C, low density lipoprotein cholesterol; NHDL-C, non-high density lipoprotein cholesterol; HDLR, total cholesterol/ high density lipoprotein cholesterol ratio.

Highest tertile of TC	1.8 fold increased risk of MI
Highest tertile of LDL-C	1.7 fold increased risk of MI
Highest tertile of NHDL-C	3.0 fold increased risk of MI
Highest tertile for HDLR (TC/HDL-C ratio)	5.8 fold increased risk of MI

Other important findings from the MAMI study which have a direct impact on clinical practice relate to diet. Soft drink consumption in both diet ('light') and normal forms were strongly associated with the risk of myocardial infarction. This is particularly important considering the hot climate in Malta. Although avoiding soft drinks features regularly in the advice given by doctors to patients these strong associations support more drastic measures being taken to decrease intake of these beverages in the form of policies, taxes and health campaigns. Similarly bread consumption which is a crucial part of Maltese diet was found to be strongly associated with the risk of MI possibly due to the addition of partially hydrogenated vegetable oils. This can be tackled by legislation which is already present in many

countries. On the other hand, the protective effect of nuts, fruits, red vegetables and legumes on the risk of MI should also be highlighted and presented to the public as healthy alternatives. The MAMI study findings also support that the negative focus on unprocessed red meats and eggs should not remain the main issues in dietary advice especially since this takes the focus off more important dietary issues presented above (Table 11.3) .

Table 11-3 Main public Health and dietary advice from the MAMI study results. MI, myocardial infarction

Non-Diet soft drink consumption increases risk of MI 5-fold
Diet-soft drink consumption also increases risk of MI 3.6 fold
Refined bread consumption and fast food increases risk of MI in a dose dependent manner
Protective effect of nuts, fruits, red-vegetables, legumes >50% decreased risk
Daily egg consumption did not increase risk of myocardial infarction

The MAMI study findings also illustrate how individual risk factors modulate the risk of each other. The influence on the risk of MI by particular risk factors will be different when found in combination than when present alone. This is shown clearly in the case of alcohol and smoking where smokers who also drank alcohol had beneficial effects on inflammatory makers and lipids when compared to smokers who did not drink. Furthermore, the MAMI study shows the importance of not grouping all alcohol into one basket, as the risk differed with the type of alcohol consumed and with the patterns of ingestion. These observations are often overlooked in larger studies and may explain conflicting results that are often observed particularly since these lifestyle factors vary considerably across and even within populations due to culture.

Genetic factors related to adiposity, lipids and the risk of MI were also studied. *APOE* only had a small effect on TC with no effect on risk of MI. The *PTPN1* 1484InsG was also not associated with the risk of MI, measures of adiposity or lipid levels in men. On the other hand, in women *PTPN1* mRNA levels were associated with measures of adiposity. This contrasted with studies in other populations (Eichner 2002; Bezzina Wettinger et al. 2014) and highlights how results reported about genetic factors vary between different populations probably due to differences in linkage disequilibrium, phenotypic heterogeneity and differences in the environmental factors present.

Interesting results came from the analysis of familial hypercholesterolaemia related genes, *LDLR*, *APOB*, *PCSK9* and *LDLRAP1* using high throughput sequencing. For this analysis an extreme phenotype approach was utilised to select a small number of research subjects. Despite the small number of research subjects on which high throughput sequencing was performed two combined frameshift variants on *LDLR* (P608Lfs with F609delinsFGfs) were identified which are likely to be causative and have not been previously described. Furthermore, polymorphisms in *ApoB* were identified that increase the risk of MI but not via increased lipid levels.

The extreme phenotype approach was also used to analyse pathways influencing the development of the metabolic complications of obesity. The WNT pathway was selected based on literature, and sequenced with HTS in selected individuals with specific extreme phenotypes based on waist-hip-ratio and HOMA-IR. From this study a number of polymorphisms were observed to influence relevant intermediate phenotypes. This analysis also highlighted the importance of studying combinations of polymorphisms rather than separate polymorphisms in isolation . The reduced risk of MI in heterozygotes for rs4988321 and rs3736228 in *LRP5* who were wildtype for rs35994626 in *FZD* (AgeAdjOR 0.6, 95% CI 0.3-1.0) exemplifies this fact. In complex conditions the effects of several variants may be increasing or diluting each others' observed effects.

Limations of the study include that most exposures were based on a snapshot at one particular point in time and may not reflect accurately the lifelong exposure to a particular risk factor. This limitation is relevant to lipid levels, changes in adiposity distribution and especially diet. Documentation of dietary habits will also be strongly affected by recall bias, seasonal dietary changes and changes in diet over time. The results must be interpreted keeping in mind a lack of power for some analysis which were restricted by gender. The number of women in certain subgroups could lead to a lack of power in some analysis. However lack of power is expected to result in false negative results. Any associations that have been detected were detected because there was sufficient power for that particular analysis. HTS findings for FH and the WNT pathway could be affected by mismapping and especially in the case of the FH variants, should be confirmed by sanger sequencing. Although for many confounders adjustments by logistic regression analysis and analysis with restrictions were performed, it is possile that other unknown potential confounders were not accounted for. Where multiple comparisons were performed, no additional adjustments to the p-value were made to avoid missing biologically signficiant associations (Rothman 1990). The significance of our findings, especially were multiple comparisons were performed should be confirmed in replication studies and with other approaches.

There are many avenues for future research expanding upon the presented data. The analysis of dietary components can be expanded to include combinations of dietary components. The FH variants that were identified from the MAMI sutdy should be replicated in cases recruited from MDH lipid clinic to reproduce the findings of this study in a high risk population. Analysis of alternative biomarkers of adipocyte dysfunction and their association with described WNT pathway genetic variants should be undertaken. Furthermore, further analysis of the WNT pathway to include the effect of combinations of variants on intermediate phenotypes could also lead to interesting observations. The study of combinations of polymorphisms related to WNT and hyperlipidaemia on the whole MAMI study collection and further study of the interaction between environmental/ lifestyle factors and genetic variants will be persued further.

The results from the present study cast a doubt on the choice of some risk factors in routine clinical use and suggest alternatives that are more closely associated with MI. This study also highlights the interplay between combinations of different environmental factors and combinations of different genetic factors which act to determine the final clinical endpoint at an individual level. Finally, this study supports the use of an extreme phenotype and a systems biology approach to study these complex interactions.

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Appendix 1. Ethical Approvals for MAMI study

University of Malta
MEDICAL SCHOOL
Mater Dei Hospital, Tal-Qroqq MSD 2090

Ref No: 32/2010

17th June 2010

Dr Stephanie Bezzina Wettinger, PhD
Division of Applied Biomedical Science
Institute of health Care, University of Malta

Dear Dr Bezzina Wettinger

Please refer to your application submitted to the Research Ethics Committee in connection with your research entitled:

INFLAMMATION, ATHEROSCLEROSIS, AND MYOCARDIAL INFARCTION (MI) IN THE MALTESE POPULATION

The University Research Ethics Committee granted ethical approval for the above-mentioned Protocol.

Yours sincerely

Dr Bridget Ellul
f/Chairman
Research Ethics Committee

L-UNIVERSITÀ TA' MALTA
Msida - Malta
KUMITAT TA' L-UNIVERSITA'
GĦALL-ETIKA FIR-RIĊERKA

UNIVERSITY OF MALTA
Msida - Malta
UNIVERSITY RESEARCH
ETHICS COMMITTEE

1st June 2012

Dr Stephanie Bezzina Wettinger
Faculty of Health Sciences
University of Malta
Msida.

Dear Dr Bezzina Wettinger

I refer to your letter of April 26th 2012 regarding the changes you and your team are suggesting after carrying out the pilot study of the research entitled, 'Inflammation, Atherosclerosis and Myocardial Infarction in the Maltese Population' (Ref MD 32/2010), approved by UREC in June 2010.

I agree that the changes you propose are minor ones, and since that you have submitted all the necessary additional documentation and forms, I am writing to inform you that the changes are approved and that you can proceed with your research study.

With thanks for your cooperation, and with best wishes for your important research

Yours truly

Fr Paul Pace
Chairman

Appendix 2. Interviewer Led Questionnaire

Personal information

Name _____ **Surname** _____

ID Card № _____

Address _____

_____ **Post Code** _____

Telephone _____ **Mobile** _____

Email _____

Date of interview ☐☐ ☐☐ ☐☐☐☐ **Start time** ☐☐:☐☐

day month year *(00:00-23.59)*

_____ _____

Interviewer Code **Signature**

To be filled in by phlebotomist

Date blood sample drawn ☐☐ ☐☐ ☐☐☐☐ **Time** ☐☐:☐☐

day month year *(00:00-23.59)*

Time of last meal ☐☐:☐☐

(00:00-23.59)

_____ _____

Phlebotomist Code **Signature**

(if different from interviewer)

Personal Characteristics

I will first ask you about some personal details

PC1. **What is your date of birth?** ☐☐ ☐☐ ☐☐☐☐ or **Age**: ☐☐*years*

day *month* *year*

PC2. **Gender** (tick where appropriate)**:** ☐ Male ☐ Female

PC3. **Are your parents and grandparents Maltese?**

☐ No → ***do not*** *proceed with interview*

☐ Yes → *GO TO question*

☐ Don't know → ***do not*** *proceed with interview*

PC4. **Have you ever had any of the following?**

Angina (severe, suffocating chest pain)	☐ No	☐ Yes	☐ Don't know
Heart attack	☐ No	☐ Yes	☐ Don't know
A coronary stent	☐ No	☐ Yes	☐ Don't know
Angioplasty	☐ No	☐ Yes	☐ Don't know
Coronary by-pass	☐ No	☐ Yes	☐ Don't know

Note to interviewer: ***Do not*** *proceed with interview* ***only*** *if the research subject answered* ***Yes or Don't know*** *to any of the conditions/procedures in question PC4, unless research subject is a relative of a case – in which case you can proceed*

PC5. **What is your CURRENT legal marital status?**

☐ Single ☐ Married ☐ Separated; Divorced; Marriage Annulled

☐ Widowed ☐ Living with partner

CS5. **Was your marital status different at the time of your first heart attack?**

☐ No

☐ Yes → ***Please specify*** _____

☐ Don't know

PC6. **With whom do you live?**

☐ Live alone → *GO TO question **EW10***

☐ Live with other people → *GO TO question **PC8***

☐ Live in a nursing home → *GO TO question **PC8***

PC7. **How many people are you living with** (including you)**?** ☐☐

PC8. **How are these people related to you?** _____

_____

PC9. **How many of these are available for use in your CURRENT household?**

Mobiles ☐☐

Televisions ☐☐

Computers ☐☐

Cars ☐☐

PC10. **As a child, did the home you lived in longest have:**

☐ A bathroom

☐ Hot water

☐ Your own bedroom

☐ Use of a car

Education and Work Occupation

EW9. **What is the HIGHEST level of formal education have obtained?** Please include any vocational training.

☐ No formal education → *GO TO question **EW12***

☐ Primary/Junior education

☐ Secondary education/Senior school

☐ Post-secondary but non-tertiary education (e.g. sixth form, MCAST, Higher

Secondary School, Polytechnic, Vocational training (e.g. teaching college, nursing school))

☐ Tertiary undergraduate education

☐ Tertiary postgraduate education

EW10. **How old were you when you obtained this level of education?**

☐☐*years* or **Year:** ☐☐☐☐

EW11. **What was your employment status in the year before your heart attack?** (Tick as many as required)

Note to interviewer: *Read the following options to the research subject*

☐ Working for pay or profit (including unpaid work for a family business or holding, including a paid apprenticeship or paid traineeship, including currently not at work due to maternity, parental, sick leave or holidays)

☐ Unemployed **Since** ☐☐*years* or ☐☐*months*

☐ Unable to work because of sickness or disability

☐ Fulfilling domestic tasks that is looking after home and/or family

☐ Student, further training, unpaid work experience or traineeship

☐ In retirement → **When did you retire? Age:** ☐☐*years* or **Year:** ☐☐☐☐

☐ Doing voluntary work

☐ Doing other unpaid work including babysitting for relatives, or helping adult children in their work

EW12. **Have you ever worked for pay or profit?** ☐ No → *GO TO question **PA24***

☐ Yes

EW13. **What was the title of your main job if previously employed in the year BEFORE your first heart attack meaning the one at which you used to work the most hours?**

Job title: _____

Describe what you mainly did in your job: _____

_____

EW14. **How many hours per week did you work in the year before your first heart attack if previously employed** (including part-time jobs)**?** ☐☐☐*hours per week*

The following sets of questions are about working patterns for the job in the year before your first heart attack

EW17. **What was your pattern of work hours?**

☐ The same hours every day → ***please specify the time you used to start and finish work***
Start ☐☐:☐☐ End ☐☐:☐☐

(00:00-23.59) *(00:00-23.59)*

☐ On shift → ***please specify*** _____

☐ Different hours every day

☐ Same hours in both winter and summer

☐ The hours change in winter and summer. **How?** _____

EW18. **Do you do any type of work after you get back home** (include housework, shopping for food)**?**

☐ No

☐ Yes → ***Please specify work***_____

→ ***Specify number of hours per week*** ☐☐☐

CS. **Did you change occupation or working pattern after your first heart attack?**

☐ No

☐ Yes → ***specify:*** ☐ Reduced hours from _____ to _____

☐ Changed occupation from _____ to _____

☐ Other_____

PHYSICAL ACTIVITY

Now I am going to ask you about the time you spent being physically active in the past 7 days.

Please answer each question even if you do not consider yourself to be an active person. Think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the vigorous activities which require hard physical effort that you did in the last 7 days. Vigorous activities make you breathe much harder than normal and may include heavy lifting, digging, aerobics, or fast cycling. Think only about those physical activities that you did for at least 10 minutes at a time.

PA21. **During the past 7 days, on how many days did you do vigorous physical activities?**

☐*days* → *GO TO question*

☐ Don't know → *GO TO question*

PA22. **During the past 7 days, how much time did you spend in total doing vigorous physical activities?**

☐ Don't know ☐☐☐*min*

Now think about activities which take moderate physical effort that you did in the past 7 days.

Moderate physical activities make you breathe somewhat harder than normal and may include carrying light loads, cycling at a regular pace, or doubles tennis. Do not include walking. Again, think about only those physical activities that you did for at least 10 minutes at a time.

PA23. **During the past 7 days, on how many days did you do moderate physical activities?**

☐*days* → *GO TO question*

☐ Don't know → *GO TO question*

PA24. **During the past 7 days, how much time did you spend in total doing moderate physical activities?**

☐ Don't know☐☐☐*min*

Now think about the time you spent walking in the past 7 days.

This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.

PA25. **During the past 7 days, on how many days did you walk for at least 10 minutes at a time?**

☐*days* → *GO TO question*

☐ Don't know → *GO TO question*

PA26. **During the past 7 days, how much time did you spend walking in total?**

Note to Interviewer: *Mention **only if** required "give an approximate number"*

☐ Don't know☐☐☐*min*

The following questions refer to a typical week in the YEAR before your first heart attack

On how many days did you do vigorous physical activities?

☐*days* → *GO TO question*

☐ Don't know → *GO TO question*

How much time did you spend in total doing vigorous physical activities?

☐ Don't know☐☐☐*min*

On how many days did you do moderate physical activities?

☐*days* → *GO TO question*

☐ Don't know → *GO TO question*

How much time did you spend in total doing moderate physical activities?

☐ Don't know☐☐☐*min*

On how many days did you walk for at least 10 minutes at a time?

☐*days* → *GO TO question*

☐ Don't know → *GO TO question*

How much time did you spend walking in total?

Note to Interviewer: *Mention **only if** required "give an approximate number"*

☐ Don't know☐☐☐*min*

Smoking and Drinking

Now I will move onto some questions about smoking and alcohol consumption.

SK27. **Have you ever smoked or used any tobacco containing products?**

☐ No → *GO TO question*

☐ Yes → *GO TO question*

SK28. **How old were you when you started smoking?** **Age:** ☐☐*years*

SK29. **Do you still smoke?** ☐ Yes → *GO TO question*

☐ No → *GO TO question*

SK30. **When did you stop smoking?** **Age:** ☐☐*years*

SK31. **What do you smoke (or did you smoke if you stopped)?**

Filter cigarette	☐ Manufactured	☐☐☐ *number per day*
Non-filter cigarette	☐ Manufactured	☐☐☐ *number per day*
Cigars		☐☐☐ *number per day*
Filter cigarette	☐ Hand rolled	☐☐☐ *weight in grams per week*
Non-filter cigarette	☐ Hand rolled	☐☐☐ *weight in grams per week*
Pipe		☐☐☐*weight in grams per week*
Snuff (powdered tobacco)		☐☐☐*weight in grams per week*

CS. **What did you smoke BEFORE your first heart attack?**

Filter cigarette	☐ Manufactured	☐☐☐ *number per day*
Non-filter cigarette	☐ Manufactured	☐☐☐ *number per day*
Cigars		☐☐☐ *number per day*
Filter cigarette	☐ Hand rolled	☐☐☐ *weight in grams per week*
Non-filter cigarette	☐ Hand rolled	☐☐☐ *weight in grams per week*
Pipe		☐☐☐*weight in grams per week*
Snuff (powdered tobacco)		☐☐☐*weight in grams per week*

SK32. **Have any of the following people ever smoked regularly in your presence?**

☐Parents

☐Brothers/Sisters

☐Friends

☐Co-workers (in the same room)

☐Spouse/partner

☐Children

☐None

☐Others _____

SK33. **If the people who you live with smoke, how much do they smoke around you per day?**

	a. **Now** (after your first heart attack)	B. **Before** (your first heart attack)
Cigarettes/Cigars:	☐☐☐*# day*	☐☐☐*# day*
Tobacco:	☐☐☐*grams/day*	☐☐☐*grams/day*

SK34. **What has been your typical exposure to other peoples' smoke** (bars, restaurants, shopping areas, public transport, at work)**?** (SELECT ONLY ONE)

a. **Now** (after your first heart attack)		B. **Before** (your first heart attack)	
☐ Never	☐ Rarely	☐ Never	☐ Rarely
☐ Occasionally	☐ Everyday	☐ Occasionally	☐ Everyday

The following questions are about your use of alcoholic beverages during the past 12 months.

DK35. **During the past 12 months, how often have you had an alcoholic drink of any kind** (that is beer, wine, spirits, liqueurs or other alcoholic beverages)**?**

- ☐ Never
- ☐ Monthly or less
- ☐ 2 to 4 times a month
- ☐ 2 to 3 times a week
- ☐ 4 or 6 times a week
- ☐ Every day

DK36. **How often have you had an alcoholic drink of any kind** (that is beer, wine, spirits, liqueurs or other alcoholic beverages)**?**

a. **Now** (after your first heart attack)

- ☐ Never
- ☐ Monthly or less
- ☐ 2 to 4 times a month
- ☐ 2 to 3 times a week
- ☐ 4 or 6 times a week
- ☐ Every day

B. **Before** (your first heart attack)

- ☐ Never
- ☐ Monthly or less
- ☐ 2 to 4 times a month
- ☐ 2 to 3 times a week
- ☐ 4 or 6 times a week
- ☐ Every day

The following questions refer to the year BEFORE your first heart attack

DK37. **Did you drink beer?**

- ☐ Yes → *fill in table for beer below*
- ☐ No

DK38. **Did you drink wine?**

- ☐ Yes → *fill in table for wine below*
- ☐ No

DK39. **Did you drink spirits?**

- ☐ Yes → *fill in table for spirits below*
- ☐ No

DK40. **Did you drink liqueurs?**

- ☐ Yes → *fill in table for liqueurs below*
- ☐ No

DK41. **Did you drink other alcoholic beverages?**

☐ Yes → *fill in table for other alcoholic beverages below*

☐ No

DK42. **How many of the following alcoholic drinks did you have each day in a typical week?** Start with Monday and take one day at a time.

	Beer	**Wine**	**Spirits**	**Liqueurs**	**Other alcoholic beverages**
	# bottles	*# glasses*	*# tots*	*# tots*	*# drinks*
Monday	☐☐	☐☐	☐☐	☐☐	☐☐
Tuesday	☐☐	☐☐	☐☐	☐☐	☐☐
Wednesday	☐☐	☐☐	☐☐	☐☐	☐☐
Thursday	☐☐	☐☐	☐☐	☐☐	☐☐
Friday	☐☐	☐☐	☐☐	☐☐	☐☐
Saturday	☐☐	☐☐	☐☐	☐☐	☐☐
Sunday	☐☐	☐☐	☐☐	☐☐	☐☐

DK43. **In the year before your first heart attack, how often did you have 6 or more drinks on one occasion?**

☐ Never

☐ Less than monthly

☐ Monthly

☐ Weekly

☐ Daily or almost daily

Tea and Coffee Consumption

TC44. **How many cups of the following beverages do/did you drink each day?**

A. **NOW** (after your first heart attack)

Coffee ☐☐ *#cups* ☐ Instant ☐ Decaffeinated ☐ Filter

Tea ☐☐ *#cups* ☐ Black ☐ Other

B. **BEFORE** (your first heart attack)

Coffee ☐☐ *#cups* ☐ Instant ☐ Decaffeinated ☐ Filter

Tea ☐☐ *#cups* ☐ Black ☐ Other

TC45. **Do/Did you add milk to your tea or coffee?**

a. **Now** (after your first heart attack)

☐ Never ☐ Sometimes ☐ Always

B. **Before** (your first heart attack)

☐ Never ☐ Sometimes ☐ Always

TC46. **If you add(ed) milk, is/was it:**

a. **Now** (after your first heart attack)

☐ skimmed
☐ full fat
☐ condensed/tinned
☐ Semi-skimmed

B. **Before** (your first heart attack)

☐ skimmed
☐ full fat
☐ condensed/tinned
☐ Semi-skimmed

TC47. **Do/Did you add sugar to your tea or coffee?**

a. **Now** (after your heart attack)

☐ No

☐ Yes → **How many teaspoons?** ☐

B. **Before** (your heart attack)

☐ No

☐ Yes → **How many teaspoons?** ☐

Sleeping Patterns

Questions about your sleeping patterns

SP48. **How many hours do/did you usually sleep during your longest/nocturnal sleep?**

a. **Now** (after your heart attack)

☐☐*hrs*

B. **Before** (your first heart attack)

☐☐*hrs*

SP49. **Do/did you usually take naps/siestas?**

a. **Now** (after your first heart attack)

☐ No ☐ Yes

Length of average nap/siesta ☐☐☐*min*

Usual time of day ☐☐:☐☐

(00:00-23.59)

B. **Before** (your first heart attack)

☐ No ☐ Yes

Length of average nap/siesta ☐☐☐*min*

Usual time of day ☐☐:☐☐

(00:00-23.59)

SP50. **Do/did you snore?**

a. **Now** (after your first heart attack)

☐ No ☐ Yes ☐ Don't know

B. **Before** (your first heart attack)

☐ No ☐ Yes ☐ Don't know

SP51. **Do/did you suffer from sleep apnea** (pauses in breathing during sleep)**?**

a. **Now** (after your first heart attack)

☐ No
☐ Yes → *GO TO question*
☐ Don't Know

B. **Before** (your first heart attack)

☐ No
☐ Yes → *GO TO question*
☐ Don't Know

SP. **Do you use treatment?**

Specify: _____

SP. **Did you use treatment?**

Specify: _____

SP52. **Do/did you unintentionally dose off in the daytime?**

a. **Now** (after your first heart attack)

☐ No ☐ Yes

B. **Before** (your first heart attack)

☐ No ☐ Yes

Sickness and medications

Some questions about your health in general

SM53. **Did you experience any of the following BEFORE your first heart attack? If yes, at what age did you get it the first time?**

Painful gums ☐ No ☐ Yes ☐☐*years*
Loose Teeth ☐ No ☐ Yes ☐☐*years*
None ☐

SM54. **Have you ever had a bone fracture?**

☐ No → *GO TO question*
☐ Yes

SM55. **Which bone(s) did you facture?**	SM56. **When did you suffer this fracture?**	SM57. **How did you fracture your bone?**
	Age: ☐☐*years* or **Year:** ☐☐☐☐	☐ Trauma ☐ Fell from standing height? ☐ Fell from a height (e.g. ladder) ☐ Other, ***please specify***_____
	Age: ☐☐*years* or **Year:** ☐☐☐☐	☐ Trauma ☐ Fell from standing height? ☐ Fell from a height (e.g. ladder) ☐ Other, ***please specify***_____
	Age: ☐☐*years* or **Year:** ☐☐☐☐	☐ Trauma ☐ Fell from standing height? ☐ Fell from a height (e.g. ladder) ☐ Other, ***please specify***_____

SM58. **Have you had any surgical operations?** ☐ No

☐ Yes → *GO TO question*

SM59. **Which operation(s) was done on you?**	SM60. **When did you have this operation?**	SM61. **What type of anesthesia were you given?**
	Age: ☐☐*years* or **Year:** ☐☐☐☐	☐ Local ☐ General ☐ Spinal
	Age: ☐☐*years* or **Year:** ☐☐☐☐	☐ Local ☐ General ☐ Spinal
	Age: ☐☐*years* or **Year:** ☐☐☐☐	☐ Local ☐ General ☐ Spinal

CS. **Was one of these operations done in the previous 6 weeks before your first heart attack?**

☐ No

☐ Yes → ***Please specify which operation***_____

CS. **Did you have any infection(s) 6 weeks prior to your first heart attack?**

☐ No → *GO TO question*

☐ Yes, *specify*: _____

SM63. **What vaccinations have you taken in the past?**

☐ Polio	☐ Hepatitis B
☐ Chicken pox	☐ Tetanus
☐ Measles	☐ Pneumococcus
☐ Mumps	☐ Influenza
☐ Rubella	☐ H1N1 (swine flu)

SM64. **Do you use any medications regularly (include vitamins, supplements (eg Calcium)?**

☐ No ☐ Yes ☐ Don't know

If yes, ***specify*** ***dose*** ***and*** ***brand***

_____
_____
_____
_____
_____

Did you use or did you use weight loss tablets (e.g. Reductil)**?**

☐ No

☐ Yes → **Please specify**_____

☐ Don't know

Have you ever had any IV treatments (e.g. Iridia, Aclasta, Bon Viva)**?**

☐ No

☐ Yes → **Please specify**_____

☐ Don't know

Have you been prescribed insulin?

☐ No

☐ Yes → **At what age did you start?** ☐☐*years*

☐ Don't know

Have you ever required a long course of corticosteroids? I will now mention a list. See whether you recognize any of them (e.g. Prednison, Deltacortil, Ultraloin, Prednisolone).

☐ No → *GO TO question*

☐ Yes → ***specify the medication, the duration and the dose in table below***

☐ Don't know

Medication	**Dose**	**Duration**	**How old were you when you first used them or long ago did you use them?**
Specify: _____	☐ Lowest dose☐ Highest dose☐ Average dose	☐ <1month ☐ 1-3 months ☐ 3-6months ☐ >6months ☐ Other _____	**Age:** ☐☐*years* or **Years:** ☐☐

Have you ever used pain relief medications long-term? I will now mention a list. See whether you recognize any of them (e.g. Voltaran, Ponstan, Neurofen)

☐ No → *GO TO question*

☐ Yes → ***specify the medication, the duration and the dose in table below***

☐ Don't know

Medication	**Dose**	**Duration**	**How old were you when you first used them or long ago did you use them?**
Specify: _____	☐ Lowest dose☐ Highest dose☐ Average dose	☐ <1month ☐ 1-3 months ☐ 3-6months ☐ >6months ☐ Other _____	**Age:** ☐☐*years* or **Years:** ☐☐

Did you use the pain relief medication ☐daily, ☐ weeklyor when ☐you feel pain?

Have you ever used any of the following medicines? I will now mention a list. See whether you recognize any of them (e.g. Coxivs, Vioxx, Arcoxia, Celebrex)

☐ No → *GO TO question*

☐ Yes → ***specify the medicine, the duration and the dose in table below***

☐ Don't know

Medicine	**Dose**	**Duration**	**How old were you when you first used them or long ago did you use them?**

Specify: _____	☐ Lowest dose☐ Highest dose☐ Average dose	☐ <1month ☐ 1-3 months ☐ 3-6months ☐ >6months ☐ Other _____	**Age:** ☐☐*years* or **Years:** ☐☐

Medical and Family History

	MH70. **Have you been diagnosed with the following disorders/conditions or have the following procedures been performed?**			MH71. If answer is "yes" please **specify age of first occurrence**
	Yes	No	Don't know	Age in years
Asthma (allergic asthma included)	☐	☐	☐	☐☐
Chronic bronchitis, chronic obstructive pulmonary disease, Emphysema	☐	☐	☐	☐☐
Myocardial infarction (heart attack)	☐	☐	☐	☐☐
1st MI	☐	☐	☐	☐☐
2nd MI	☐	☐	☐	☐☐
3rd MI				
Heart failure (ilma fil-pulmun)	☐	☐	☐	☐☐
Pulmonary edema	☐	☐	☐	☐☐
Coronary heart disease (angina pectoris)	☐	☐	☐	☐☐
Angiogram	☐	☐	☐	☐☐
Angioplasty	☐	☐	☐	☐☐
Coronary by-pass (CABG)	☐	☐	☐	☐☐

Stent	☐	☐	☐	☐☐
High blood pressure (hypertension)	☐	☐	☐	☐☐
High Cholesterol	☐	☐	☐	☐☐
Diabetes	☐	☐	☐	☐☐
Stroke (cerebral haemorrhage, cerebral thrombosis)	☐	☐	☐	☐☐
Deep vein thrombosis	☐	☐	☐	☐☐
Embolism	☐	☐	☐	☐☐
Rheumatoid arthritis (inflammation of the joints)	☐	☐	☐	☐☐
Osteoarthritis (arthritis, joint degeneration)	☐	☐	☐	☐☐
	MH70. **Have you been diagnosed with the following disorders/conditions or have the following procedures been performed?**			MH71. If answer is "yes" please **specify age of first occurrence**
	Yes	No	Don't know	Age in years
Psoriatic arthritis	☐	☐	☐	☐☐
Systemic *lupus* erythematosus	☐	☐	☐	☐☐
Gout	☐	☐	☐	☐☐
Osteoporosis	☐	☐	☐	☐☐
Psoriasis	☐	☐	☐	☐☐
Allergy, such as rhinitis, eye inflammation, dermatitis, food allergy or other (allergic asthma excluded)	☐	☐	☐	☐☐
Eczema	☐	☐	☐	☐☐
Paget's disease	☐	☐	☐	☐☐
Stomach ulcer (gastric or duodenal ulcer)	☐	☐	☐	☐☐

Cancer (malignant tumour, also including leukaemia and lymphoma) *If yes specify:*	☐ ☐ ☐	☐☐
Parkinson's disease	☐ ☐ ☐	☐☐
Others: _____	☐ ☐ ☐	☐☐

Family Tree and Medical Conditions

FT72. **Family tree of participant:** (please indicate medical conditions of family members, age of onset, adoption status, mark dead members and note cause of death; for twins whether identical or fraternal)

FT74.**Extra remarks**_____

Stress and Stressful Experiences

SS75. **What level of stress do you feel?**

☐ Little/none ☐ Moderate ☐ High/Severe

SS76. **From the following, which best describes your attitude to life?**

☐ Calm ☐ Stressed ☐ Panicky ☐ Anxious ☐ Depressed

SS78. **Did you experience any of the following in the year before your first heart attack?**

Marital separation/Divorce	☐ No ☐ Yes
Major personal injury or illness	☐ No ☐ Yes
Retirement	☐ No ☐ Yes
Loss of job	☐ No ☐ Yes
Problems at work	☐ No ☐ Yes
Death/major illness of a close family member	☐ No ☐ Yes
Death of spouse/partner	☐ No ☐ Yes
Serious financial problems	☐ No ☐ Yes
Violence	☐ No ☐ Yes
Major intra-family conflict	☐ No ☐ Yes
Other major stress _____	☐ No ☐ Yes
*If yes **please specify***	
Major positive stress	
(e.g. wedding, birth)_____	☐ No ☐ Yes
*If yes **please specify***	

CS. **What were you doing when you got your first heart attack?** (e.g. engaged in heavy exercise, during sexual intercourse, angry or upset, travelling)

Please specify: _____

_____

Note to Interviewer: *If the reply to question is "travelling" proceed to question*

Did you travel recently before your first heart attack?

☐ No

☐ Yes → *GO TO question*

How did you travel? (e.g. by plane)

Please specify: _____

Did you travel for long hours with restricted movement?

☐ No

☐ Yes

☐ Other, ***please specify***_____

Note to interviewer: *The following question is addressed to women only*

CS. **Were you pregnant when you suffered your first heart attack?**

☐ No

☐ Yes → *GO TO question*

☐ Don't know

How many weeks pregnant were you? ☐☐*weeks*

Nutrition

NT81. **How often did you eat foods from each of the following categories before your first heart attack?**

		<1/month-Never	**Monthly** #times	**Weekly** #times	**Daily** #times
a	Red Meat	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
b	Pork	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
c	Poultry	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
d	Rabbit	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
e	Fish	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
f	Eggs	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
g	Wholemeal (bread, flour or cereals)	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
h	Refined/milled grains	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
i	Dairy products (yoghurt, cheese, ice cream, milk With cereal)	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
j	Deep fried foods/fast food	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
k	Olive oil	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
l	Salty foods/ snacks	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
m	Chocolate				
	• Dark	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
	• Milk	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
	• White	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
n	Desserts/sweet snacks				
o	Soft drinks	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
	• Diet	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
	• Non-diet	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
r	Cola drinks	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
s	Sweet drinks e.g. orange	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐

	concentrate				
t	Legumes (beans)	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
u	Nuts/seeds	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
v	Fruits/juice	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
w	Leafy green vegetables	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
	If eaten how are they usually prepared ☐raw ☐cooked crisp ☐cooked soft				
	Carrots, tomatoes, capsicurns	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
x	Other vegetables (raw)	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
y	Other vegetables (cooked)	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐
z	Liquid meals (for elderly)	☐	☐ ☐☐	☐ ☐☐	☐ ☐☐

CS82. **Have your eating habits changed after your first heart attack?**

☐ No

☐ Yes → ***please specify*** _____

_____

_____

_____

_____

_____

NT. **Were you breastfeed as a baby?** ☐ No ☐ Yes ☐ Don't know

NT. **Were you overweight at any age as a child?** (Below 16 years of age)

☐ No ☐ Yes ☐ Don't know

NT. **How much did you weigh when you were born?**

☐ Don't Know **Weight:** ☐☐*lbs* ☐☐*ounces*

Note to interviewer: *If the research subject does not know the weight as a baby or can't recall the weight proceed to question*

NT. **What size were you as a baby?**

☐ a small baby

☐ an average baby

☐ a big baby

Question for Men only

MO86. **Male pattern baldness** (interviewer will note) ☐No ☐Yes ☐Don't know

Note to interviewer: *If the research subject is male proceed to page*

Questions for Women only

WO87. **Have you ever used oral contraceptives? (Hormone pills to avoid getting pregnant)**

☐No → *GO TO question*

☐Yes → *GO TO question*

☐Don't Know

WO88. **For how long did you use oral contraceptives?**

A. **Age started:** ☐ ☐*years* B. **Age stopped:** ☐ ☐*years*

WO90. **Are you currently using them?** ☐No ☐Yes

WO89. **What brand(s) do/did you use?**

☐ Don't know or ***Please specify according to order of use***_____

_____

WO91. **Age of onset of menarche (first period):** ☐ ☐*yrs*

WO92. **Duration of Loss:** ☐☐*days*

WO93. **Cycle length (time between periods)** ☐☐*days*

WO94. **Have your menstrual periods stopped permanently?**

☐ No → *GO TO question*

☐ Yes → *GO TO question*

☐ Don't know

WO95. **At what age did the periods stop?** ☐☐*years*

WO96. **Why did the periods stop?**

☐ Menopause

☐ Oophorectomy

☐ Hysterectomy

☐ Radiation

☐ Other, ***please specify*** _____

WO97. **Have you ever used female hormone replacements?**

☐ No → *GO TO question*

☐ Yes → *GO TO question*

☐ Don't know

WO98. **For how long:** ☐☐*years*

WO99. **Are you still taking them?** ☐ No ☐ Yes

WO100. **Have you ever used the following type of hormone replacement?**

☐ Estrogen alone

Brand(s) _____

For how many years: ☐☐*years*

☐ Estrogen + Progesterone

Brand(s) _____

For how many years: ☐☐*years*

WO101. **Have you been diagnosed with Polycystic ovary syndrome?**

☐ No ☐ Yes ☐ Don't know

WO102. **Were you ever told that you had cysts on your ovaries?**

☐ No ☐ Yes ☐ Don't know

WO103. **Did you get pregnant easily?** ☐ No ☐ Yes ☐ Don't know

WO104. **Did you need fertility treatment?** ☐ No ☐ Yes ☐ Don't know

WO105. **How many times did you get pregnant?** *(Not counting any miscarriages)* ☐☐ *times*

WO106. **Did you have any miscarriages?**

☐ No → *GO TO question*

☐ Yes → *GO TO question*

WO. **How many miscarriages?** ☐☐

WO107. **Did you breastfeed your children?**

☐ No children/adopted ☐ No ☐ Yes

WO108. **Did you have high blood pressure during gestation?**

☐ No ☐ Yes ☐ Don't know

WO109. **Did you have high glucose levels during gestation?**

☐ No ☐ Yes ☐ Don't know

WO110. **Were proteins detected in the urine during gestation?**

☐ No ☐ Yes ☐ Don't know

WO. **Were you ever told that you had preclampsia/eclampsia (sudden rise in blood pressure) during gestation?** ☐ No ☐ Yes ☐ Don't know

WO. **Did you experience swelling of the hands and feet more than normal during gestation?**

☐ No ☐ Yes ☐ Don't know

WO111. **What was the birth weight of your first child:**

☐ Don't know **Weight:** ☐☐*lbs* ☐☐*ounces*

Note to interviewer: *If the research subject does not know the weight of the baby or can't recall the weight proceed to question*

NT. **What size was your first baby?**

☐ a small baby

☐ an average baby

☐ a big baby

The following section should be filled in by the interviewer

Time at end of interview:☐☐:☐☐

(00:00-23.59)

Overall score of quality of responses:

☐ Excellent ☐ Good ☐ Average ☐ Poor ☐ Very poor

Was the interview held with the research subject alone? ☐ No ☐ Yes

If no, how was the person accompanying the research subject related to him/her? _____

Was the research subject helped? ☐ No ☐ Yes

How many years have they known each other? ☐☐*years*

Physical Measurements

PM112. **Blood pressure** (after 10 min rest sitting or lying down)

#1 ☐☐☐ ☐☐☐

Systolic *Diastolic*

#2 ☐☐☐ ☐☐☐

Systolic *Diastolic*

PM113. **Heart Rate**

☐☐☐

beats/minute

PM114. **Waist** (cm)

#1 ☐☐☐.☐

#2 ☐☐☐.☐

PM115. **Hips** (cm)

#1 ☐☐☐.☐

#2 ☐☐☐.☐

PM116. **Weight** ☐☐☐.☐ *kg*

PM117. **Height** ☐☐☐.☐ *cm*

Appendix 3

Table A3-1 Risk of myocardial infarction in males on statins comparing highest vs lowest tertile of lipid variable. N = Cases and Controls in the highest tertile, % is the percentage in cases and controls, age adjusted odds ratio (Age OR) of risk of mycocardial infarction and 95% confidence interval shown in brackets. Adj OR is OR adjusted for age, hypertension, diabetes, smoking, alcohol consumption and BMI. Lipids are taken fasted in controls compared to on admission in cases

Lipid variable	Cases n (%)	Controls n (%)	Age OR (95% CI)	Adj OR (95% CI)
Total cholesterol	20 (31.7)	11 (18.6)	1.9 (0.7-4.7)	2.1 (0.7-6.4)
HDL-C	10 (15.9)	19 (32.2)	0.5 (0.2-1.2)	0.5 (0.2-1.6)
LDL-C	17 (27.0)	8 (13.6)	2.1 (0.8-5.9)	2.0 (0.6-6.7)
NHDL-C	23 (36.5)	12 (20.3)	2.0 (0.8-4.9)	2.0 (0.7-6.0)
Triglycerides	46 (73.0)	23 (39.0)	6.1 (1.8-21.2)	4.0 (1.0-15.7)
HDLR	32 (50.8)	15 (25.4)	2.2 (0.9-5.7)	2.7 (0.9-8.0)

Table A3-2. Risk of myocardial infarction in females not on statins comparing highest vs lowest tertile of lipid variable. N = Cases and Controls in the highest tertile, % is the percentage in cases and controls, age adjusted odds ratio (Age OR) of risk of mycocardial infarction and 95% confidence interval shown in brackets. Adj OR is OR adjusted for age, hypertension, diabetes, smoking, alcohol consumption and BMI. Lipids are taken fasted in controls compared to on admission in cases

Lipid variable	Cases n (%)	Controls n (%)	Age OR (95% CI)	Adj OR (95% CI)
Total cholesterol	22 (44.9)	37 (33.6)	1.0 (0.4-2.6)	1.5 (0.5-4.5)
HDL-C	8 (16.3)	37 (33.6)	0.2 (0.1-0.5)	0.2 (0.1-0.5)
LDL-C	23 (46.9)	39 (35.5)	1.3 (0.5-3.6)	2.2 (0.6-8.4)
NHDL-C	22 (44.9)	37 (33.6)	1.6 (0.6-4.7)	3.5(0.9-14.6)
Triglycerides	34 (69.4)	34 (30.9)	4.3 (1.6-12.3)	5.1 (1.6-16.5)
HDLR	32 (65.3)	36 (32.7)	3.9 (1.4-10.9)	3.6 (1.1-11.5)

Table A3-3 Lipid Variables with diabetes status in female controls. p-value estimated for the group using Mann Whitney test. IQR -Interquartile range

Lipid variable in female controls	No Diabetes (n=118)		Controlled Diabetes (n=5)		Uncontrolled Diabetes (n=8)		p value
	Median (mmol/L)	IQR (mmol/L)	Median (mmol/l)	IQR (mmol/L)	Median (mmol/l)	IQR (mmol/L)	
Total cholesterol	5.6	4.8-6.1	4.34	4.2-5.3	4.9	4.3-6.1	0.064
LDL cholesterol	3.3	2.7-3.9	2.3	2.0-2.9	2.7	2.4-4.1	0.064
HDL-Cholesterol	1.7	1.4-2.0	1.7	1.4-2.3	1.5	1.2-1.7	0.424
NHDLC	3.9	3.2-4.6	2.8	2.1-3.5	3.2	2.7-4.6	0.061
HDLR	3.4	2.7-4.1	2.9	2.1-3.3	3.5	2.5-4.0	0.256
Triglycerides	1.1	0.7-1.5	0.9	0.6-1.4	1.1	0.6-1.3	0.772

Table A3-4. Difference in frequency of alcohol consumption between regular drinkers who are current smokers and non-smokers in male controls (a) overall and (b) in those of 50 years or older.

Male controls who are Regular alcohol drinkers			
		Smokers	Non-smokers
Frequency of current drinking	Daily	23 (36.5)	16 (22.5)
	4 to 6 times per week	3 (4.8)	2 (2.8)
	2 to 3 times per week	14 (22.2)	15 (21.1)
	2 to 4 times per month	16 (25.4)	30 (42.3)
	Every month or less	3 (4.8)	6 (8.5)
	Non-drinkers	4 (6.3)	2 (2.8)
The chi-square statistic is 14.6895. The p-value is <0.1.			

Male controls >=50 years of age who are Regular alcohol drinkers			
		Smokers	Non-smokers
Frequency of current drinking	Daily	22 (52.4)	12 (24.5)
	4 to 6 times per week	1 (2.4)	2 (4.1)
	2 to 3 times per week	10 (23.8)	11 (22.4)
	2 to 4 times per month	4 (9.5)	19 (38.8)
	Every month or less	1 (2.4)	4 (8.2)
	Non-drinkers	4 (9.5)	1 (2.0)
The chi-square statistic is 23.4955. The *p*-value is <0.01.			

Table A3-5 Age adjusted Odds Ratios for APOE Genotypes for women not on hypocholesterolaemic treatment. APOE3/E3 is the reference category.

APOE genotype	Cases n (%)	Controls n (%)	AgeOR (95% CI)
APOE2/E2	0 (0.0)	1 (0.9)	
APOE4/E4	0 (0.0)	0 (0.0)	
APOE2/E4	1 (2.4)	1 (0.9)	1.7 (0.1-30.8)
APOE2/E3	3 (7.1)	18 (16.8)	0.3 (0.1-1.2)
APOE3/E4	6 (14.3)	18 (16.8)	0.8 (0.3-2.4)
APOE3/E3	32 (76.2)	69 (64.5)	1

Table A3-6 Age adjusted Odds Ratio for PTPN1 heterozygotes in women in the MAMI Study HA-Homozygous alternate, Het-heterozygous, WT -Wildtype

	Cases n (%)	Control n (%)	OR (95% CI)	Age -Adjusted OR (95%CI)
HA	0 (0.0)	0 (0.0)		
Het	3 (18..8)	8 (11.9)	1.7 (0.4-7.3)	1.4 (0.3-6.3)
WT	13 (81.3)	59 (88.1)		

Table A3-7 Effects of *LDLR* rs11669576 variant in male controls off statins . *p<0.05, ** p<0.01 calculated using two-tailed Mann-Whitney test

LDLR rs11669576		
	Homo wt (N=234)	Het (N=20)
CHOL	5.395 (4.795-6.0675)	5.62 (5.275-6.3375)
LDL-C	3.44 (2.87-4.1275)	3.795 (3.235-4.2325)
HDL-C	1.31 (1.1125-1.5475)	1.305 (1.115-1.46)
NHDL-C	4.08 (3.37-4.8575)	4.505* (3.825-5.08)
HDLR	4.01 (3.3925-5.0375)	4.535 (3.8775-5.1575)
TG	1.18 (0.815-1.7375)	1.445 (1.055-1.8775)
hs-CRP (mg/L)	1.655 (0.75875-2.8675)	2.59** (1.47-3.57)

Table A3-8 Effects PCSK9 rs11583680 variant in male controls off statins. . *p<0.05, ** p<0.01 calculated using two-tailed Mann-Whitney test

PCSK9 rs11583680			
	Homo wt (N=185)	Het (N=64)	Homo alt (N=64)
CHOL	5.395 (4.91-6.0675)	5.56 (4.74-6.31)	5.56 (4.74-6.31)
LDL-C	3.465 (2.94-4.1275)	3.53 (2.81-4.18)	3.53 (2.81-4.18)
HDL-C	1.31 (1.1225-1.5275)	1.28 (1.12-1.6)	1.28 (1.12-1.6)
NHD-CL	4.1 (3.395-4.8475)	4.27 (3.57-4.92)	4.27 (3.57-4.92)
HDLR	4.01 (3.48-5.1025)	4.23 (3.34-5.04)	4.23 (3.34-5.04)
TG	1.21 (0.84-1.74)	1.15 (0.81-1.74)	1.15 (0.81-1.74)
hs-CRP (mg/L)	1.65 (0.7795-3.225)	1.815 (0.8175-2.7325)	1.815 (0.8175-2.7325)

Table A3-9 Effects *PCSK9* rs505151 variant in male controls off statins. . *p<0.05, ** p<0.01 calculated using two-tailed Mann-Whitney test

PCSK9 rs505151			
	Homo wt (N=236)	Het (N=17)	Homo alt (N=1)
CHOL	5.46 (4.86-6.23)	5.35 (4.595-6.09)	5.8 (5.8-5.8)
LDL-C	3.47 (2.93-4.19)	3.32 (2.745-4.04)	3.85 (3.85-3.85)
HDL-C	1.31 (1.13-1.53)	1.28 (1.005-1.6)	1.02 (1.02-1.02)
NHDL-C	4.14 (3.43-4.87)	4.02 (3.065-4.73)	4.78 (4.78-4.78)
HDLR	4.08 (3.48-5.04)	3.96 (2.925-5.89)	5.69 (5.69-5.69)
TG	1.19 (0.86-1.73)	0.95 (0.705-1.885)	2.04 (2.04-2.04)
hs-CRP (mg/L)	1.7 (0.8005-3.0075)	1.43 (0.6915-2.87)	4.14 (4.14-4.14)

Table A3-10 Effects *APOB* rs12713681 variant in male controls off statins. . *p<0.05, ** p<0.01 calculated using two-tailed Mann-Whitney test

ApoB rs12713681		
	Homo wt (N=249)	Het (N=6)
CHOL	5.46 (4.81-6.15)	5.45 (4.9125-7.3175)
LDLC	3.46 (2.89-4.145)	3.805 (3.305-5.385)
HDLC	1.31 (1.12-1.54)	1.09* (0.865-1.415)
NHDL	4.13 (3.385-4.86)	4.4 (3.7725-6.2225)
HDLR	4.04 (3.42-5.04)	5.49** (3.9325-7.7725)
TRIG	1.2 (0.84-1.74)	1.3 (1.025-1.84)
hs-CRP (mg/L)	1.69 (0.8-3.08)	2.265 (1.7095-3.1875)

Table A3-11 Effects *APOB* rs533617 variant in male controls off statins. . *p<0.05, ** p<0.01 calculated using two-tailed Mann-Whitney test

ApoB rs533617			
	Homo wt (N=246)	Het (N=11)	Homo alt (N=1)
CHOL	5.47 (4.83-6.2275)	5.02 (4.69-5.41)	6.06 (6.06-6.06)
LDLC	3.5 (2.915-4.1775)	3.14 (2.85-3.41)	4.12 (4.12-4.12)
HDLC	1.31 (1.1125-1.53)	1.62* (1.29-1.89)	1.2 (1.2-1.2)
NHDL	4.165 (3.4375-4.8775)	3.61** (3.18-4.1)	4.86 (4.86-4.86)
HDLR	4.145 (3.48-5.1275)	3.29** (2.59-4.13)	5.05 (5.05-5.05)
TRIG	1.225 (0.8625-1.755)	0.87** (0.67-1.18)	1.63 (1.63-1.63)
hs-CRP (mg/L)	1.7 (0.79925-3)	1.12 (0.637-4.45)	6.28 (6.28-6.28)

Table A3-12 Effects *APOB* rs12691202 variant in male controls off statins. . *p<0.05, ** p<0.01 calculated using two-tailed Mann-Whitney test

ApoB rs12691202		
	Homo wt (N=246)	het (N=11)
CHOL	5.46 (4.83-6.205)	5.32 (4.72-6.14)
LDLC	3.455 (2.885-4.1475)	3.66 (3.14-4.24)
HDLC	1.31 (1.1125-1.54)	1.31 (1.13-1.49)
NHDL	4.13 (3.3825-4.86)	4.17 (3.59-4.77)
HDLR	4.065 (3.41-5.1025)	4.18 (3.91-4.75)
TRIG	1.24 (0.84-1.74)	1.12 (0.99-1.22)
hs-CRP (mg/L)	1.67 (0.78075-3)	2.7** (2.18-4.82)

Table A3-13 Effects *APOB* rs12713681 variant in male controls off statins. . *p<0.05, ** p<0.01 calculated using two-tailed Mann-Whitney test

ApoB rs12713681		
	Homo wt (N=249)	Het (N=6)
CHOL	5.46 (4.81-6.15)	5.45 (4.9125-7.3175)
LDLC	3.46 (2.89-4.145)	3.805 (3.305-5.385)
HDLC	1.31 (1.12-1.54)	1.09* (0.865-1.415)
NHDL	4.13 (3.385-4.86)	4.4 (3.7725-6.2225)
HDLR	4.04 (3.42-5.04)	5.49** (3.9325-7.7725)
TRIG	1.2 (0.84-1.74)	1.3 (1.025-1.84)
hs-CRP (mg/L)	1.69 (0.8-3.08)	2.265 (1.7095-3.1875)

Table A3-14 Effects *APOB* rs533617variant in male controls off statins. . *p<0.05, ** p<0.01 calculated using two-tailed Mann-Whitney test

ApoB rs533617			
	Homo wt (N=246)	Het (N=11)	Homo alt (N=1)
CHOL	5.47 (4.83-6.2275)	5.02 (4.69-5.41)	6.06 (6.06-6.06)
LDLC	3.5 (2.915-4.1775)	3.14 (2.85-3.41)	4.12 (4.12-4.12)
HDLC	1.31 (1.1125-1.53)	1.62* (1.29-1.89)	1.2 (1.2-1.2)
NHDL	4.165 (3.4375-4.8775)	3.61** (3.18-4.1)	4.86 (4.86-4.86)
HDLR	4.145 (3.48-5.1275)	3.29** (2.59-4.13)	5.05 (5.05-5.05)
TRIG	1.225 (0.8625-1.755)	0.87** (0.67-1.18)	1.63 (1.63-1.63)
hs-CRP (mg/L)	1.7 (0.79925-3)	1.12 (0.637-4.45)	6.28 (6.28-6.28)

Table A3-15 Effects *APOB* rs12691202 variant in male controls off statins. . *p<0.05, ** p<0.01 calculated using two-tailed Mann-Whitney test.

ApoB rs12691202		
	Homo wt (N=246)	het (N=11)
CHOL	5.46 (4.83-6.205)	5.32 (4.72-6.14)
LDLC	3.455 (2.885-4.1475)	3.66 (3.14-4.24)
HDLC	1.31 (1.1125-1.54)	1.31 (1.13-1.49)
NHDL	4.13 (3.3825-4.86)	4.17 (3.59-4.77)
HDLR	4.065 (3.41-5.1025)	4.18 (3.91-4.75)
TRIG	1.24 (0.84-1.74)	1.12 (0.99-1.22)
hs-CRP (mg/L)	1.67 (0.78075-3)	2.7** (2.18-4.82)

Table A3-16 Effects LDLRAP1 rs41291058 variant in male controls off statins. . *p<0.05, ** p<0.01 calculated using two-tailed Mann-Whitney test.

LDLRAP1 rs41291058			
	Homo wt (N=220)	Het (N=34)	Homo alt (N=1)
CHOL	5.475 (4.91-6.145)	5.355 (4.71-5.9925)	6.95 (6.95-6.95)
LDLC	3.46 (2.96-4.1725)	3.44 (2.795-3.87)	4.38 (4.38-4.38)
HDLC	1.31 (1.11-1.53)	1.31 (1.145-1.5625)	1.45 (1.45-1.45)
NHDL	4.135 (3.45-4.86)	3.99 (3.1175-4.755)	5.5 (5.5-5.5)
HDLR	4.08 (3.475-5.1225)	3.995 (3.275-4.8575)	4.79 (4.79-4.79)
TRIG	1.21 (0.84-1.7325)	1.145 (0.7975-1.905)	2.46 (2.46-2.46)
hs-CRP (mg/L)	1.655 (0.78575-2.9575)	2.23 (1.2925-4.1925)	1.24 (1.24-1.24)