



The association between adiposity and platelet function: implications for cardiovascular disease

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A dissertation submitted to the University of Bristol in accordance with the requirements for
award of the degree of PhD Population Health Sciences in the Faculty of Life Sciences

Physiology, Pharmacology and Neuroscience / Population Health Sciences, March 2022,

Word Count: 12,345

Abstract

Higher body mass index (BMI) is a risk factor for thrombosis. Platelets are essential for haemostasis but contribute to thrombosis when activated pathologically. I aimed to assess the effect of BMI on platelet function and identify potential mechanisms which may mediate this effect by focusing on circulating plasma proteins.

In **Chapter 3** I used data from INTERVAL, a UK study of blood donors to explore the effect of BMI on platelet traits measured by the Sysmex XN-1000 haematology analyser. Observational and Mendelian randomization (MR) analyses in up to 33,388 participants suggested higher BMI raises immature platelet count (IPC), a measure of newly produced platelets. A follow-up analysis using a pre-cardiac surgery cohort (COPTIC) indicated that IPC was positively associated with whole blood platelet aggregation induced by platelet agonists (N=655).

Following on from these findings, a pilot patient study was initiated to develop laboratory methods to explore the effect of BMI on platelet function and signalling. This is presented in **Chapter 4**. Patients due to undergo bariatric surgery ($BMI >40 \text{ kg/m}^2$), alongside age and sex matched controls ($BMI 18.5-25 \text{ kg/m}^2$), donated blood samples (N=4). Platelet function experiments were carried out on blood samples, and a platelet proteome analysis was performed on isolated platelets using mass spectrometry. Established methods need to replicated in a larger sample size.

Due to the chronic inflammatory effect of higher BMI, **Chapter 5** explored the effect of the chemokines macrophage-derived chemokine (MDC) and thymus and activation-regulated chemokine (TARC) on platelet function and signalling. MDC and TARC were able to potentiate platelet activation. Two sample MR provided causal estimates for the effect of higher circulating levels of MDC and TARC on disease. This analysis provided evidence that higher

levels of MDC may contribute to venous thromboembolism.

As circulating proteins were shown to activate platelets, the effect of BMI on the overall circulating profile was explored in **Chapter 6**. This analysis was conducted on INTERVAL participants with proteomics measured by SomaLogic (N=2737). Here, observational and MR analyses were implemented to obtain causal estimates. Higher BMI was linked to effects on levels of proteins involved in appetite regulation, inflammation and sex hormones. Proteins which were most affected by BMI were enriched for genes involved in cardiovascular disease.

A weight loss randomized control trial (RCT) called DiRECT was also implemented to evaluate the effect of BMI on the plasma proteome (N=146) in **Chapter 7**. Participants had their BMI and plasma proteome measured by SomaLogic at baseline and after one year of Type 2 Diabetes guideline care (control) or a total diet replacement (TDR) (intervention). Linear regression was used to explore effects as well as an instrumental variable analysis, using the treatment group as an instrument for BMI change. A reduction in BMI had a broad effect on the plasma proteome: proteomic changes were enriched for metabolic disease.

The final results chapter (**Chapter 8**) combined the results of the two previous chapters in attempt to identify consistent and divergent proteomic features across MR and weight loss RCT study designs. This chapter indicated that proteins which are altered in MR can be reversed with a reduction in BMI. There was evidence that BMI affects levels of proteins which may be indicative of a change in platelet function.

Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Signed

Dated

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Abbreviations

Table 1: Full list of abbreviations

Abbreviation	Full name
ACD	Acid citrate dextrose
ACTB	Actin
ADH	Alcohol dehydrogenase
ADH4	Alcohol dehydrogenase 4
ADP	Adenosine diphosphate
APMAP	Adipocyte plasma membrane-associated protein
AU	Arbitrary Units
AUC	Area under the curve
BAT	Brown adipose tissue
BCA	Bicinchoninic acid
BMI	Body mass index
BSA	Bovine serum albumin
CAD	Coronary artery disease
CBPM	Carboxypeptidase M
CCR4	C-C motif chemokine receptor 4
CHAD	Chondroadherin
CHD	Coronary heart disease
CRP	C-reactive protein/collagen-related peptide
DAPT	Dual antiplatelet therapy

DAVID	Database for annotation, visualization and integrated discovery
DiRECT	Diabetes REmission Clinical Trial
DTT	Dithiothreitol
DVT	Deep vein thrombosis
DXA	Dual-energy X-ray Absorptiometry
EC50	Half maximal effective concentration
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal regulated kinase
FAAA	Fumarylacetoacetate
FABP	Fatty acid binding protein
FABP	Fatty acid binding protein
FDR	False discovery rate
FHS	Framingham Heart Study
FSC	Forward scatter
GAD	Genetic association database
GAM	Generalized additive model
GHR	Growth hormone receptor
GIANT	Genetic Investigation of Anthromopetric Traits
GLSMR	GAM and linear stratified Mendelian randomisation
GO	Gene ontology
GP	Glycoprotein
GPCR	G protein coupled receptor
GRK6	G protein-coupled receptor kinase 6
GRS	Genetic risk score
GWAS	Genome wide association study
H-IPF	High fluorescence immature platelet fraction
HbA1c	Glycated haemoglobin
HDL	High density lipoprotein

IGF-1	Insulin-like growth factor 1
IGFBP	Insulin-like growth factor binding protein
IHD	Ischaemic heart disease
IL-6	Interleukin-6
IL6R	Interleukin-6 receptor
INHBC	Inhibin β C chain
IP3	Inositol trisphosphate
IPC	Immature platelet count
IPF	Immature platelet fraction
IQR	Interquartile range
IRAS	Integrated Research Application System
IVW	Inverse variance weighted
LACE	Localised average causal effect
LDL	Low density lipoprotein
LTA	Light transmittance aggregometry
MACE	Major adverse cardiovascular event
MAPK	Mitogen-activated protein kinase
MDC	Macrophage derived chemokine
MEA	Multiple electrode aggregometry
MFI	Median fluorescence intensity
MMP-2	Metalloproteinase-2
MPO	Myeloperoxidase
MPV	Mean platelet volume
MR	Mendelian randomization
MS	Mass spectrometry
MS	Metabolic syndrome
NHSBT	NHS Blood and Transplant
NHSBT	National Health Service Blood and Transplant
NLMR	Nonlinear Mendelian randomisation

NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
OLS	Ordinary least squares
P-LCR	Platelet large cell ratio
PAFAH	Platelet-activating factor acetylhydrolase
PAR-1	Protease-activated receptor 1
PAR1-AP/TRAP-6	PAR1-activating peptide
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCI	Percutaneous coronary intervention
PCT	Plateletcrit
PDGF	Platelet-derived growth factor
PDVF	Poly(vinylidene fluoride)
PDW	Platelet distribution width
PE	Pulmonary embolism
PEAR1	Platelet endothelial aggregation receptor 1
pEC50	The negative logarithm of the EC50
PECAM-1	Platelet endothelial cell adhesion molecule-1
PFA	Paraformaldehyde
PGE1	Prostaglandin E1
PGI2	Prostaglandin I2
PI3K	Phosphoinositide-3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PLT (F)	Platelet count (PLT-F channel)
PLT (I)	Platelet count (impedance channel)
PPP	Platelet poor plasma

pQTL	Protein quantitative trait loci
PROTACs	Proteolysis targeting chimeras
PRP	Platelet rich plasma
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
PTPRU	Receptor-type tyrosine-protein phosphatase U
QC	Quality control
RCT	Randomised control trial
RFU	Relative fluorescent units
RIPA	Radioimmunoprecipitation assay
ROCK	Rho-associated protein kinase
RPM	Revolutions per minute
S100A9/MRP-14	Myeloid-related protein-14
SCAR5	Scavenger receptor class A member 5
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFL	Side fluorescence
SHBG	Sex hormone binding globulin
SNP	Single nucleotide polymorphism
SOMAmer	Slow Off-rate Modified Aptamers
SSC	Side scatter
T2D	Type 2 Diabetes mellitus
TARC	Thymus and activation regulated chemokine
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
TLS	Total least squares
TMT-MS	Tandem mass tag mass spectrometry
TNF-alpha	Tumour necrosis factor-alpha

TPO	Thrombopoietin
TSLS/2SLS	Two stage least squares
TxA2	Thromboxane A2
VASP	Vasodilator-stimulator phosphoprotein
VLED	Very low energy diet
VTE	Venous thromboembolism
WAT	White adipose tissue
WHRadjBMI	Waist-hip ratio adjusted for BMI

Chapter 1

General information

1.1 Format

This chapter will provide general information about the PhD. This includes details regarding collaborations, publications, conference presentations and awards and prizes.

1.2 Collaborations

This PhD project is part of the 4 year British Heart Foundation Integrative Cardiovascular Science Programme and is funded by the University of Bristol alumni. My PhD project has made full use of interdisciplinary techniques to answer the research questions. In 2019 I spent seven weeks at the Wellcome Sanger Institute near Cambridge, where I joined the human genetics team led by Professor Nicole Soranzo and worked closely with other academics including Professor Adam Butterworth from the University of Cambridge. I have created and implemented a clinical study where I wrote the approved ethics application. This study was in collaboration with Dr Dimitri Pournaras, a bariatric surgeon at Southmead Hospital. In light of the COVID-19 pandemic, I utilised the laboratory techniques developed to determine the effects of severe COVID-19 on platelet function by working with clinicians including Dr David Arnold and Dr Fergus Hamilton. Other collaborations include Assistant Professor Michael Holmes from the University of Oxford and Professor Naveed Sattar from the University of Glasgow. This work will continue as part of the British Heart Foundation Accelerator Award Transition Fellowship, where I have started a collaboration with Professor Karsten Suhre from Weill-Cornell Medicine, Qatar.

1.3 Publications

Goudswaard L. J., Corbin L. J., Burley K. L., Mumford A., Akbari P., Soranzo N., Butterworth A. S., Watkins N. A., Pournaras D. J., Harris J., Timpson N. J., Hers I. Higher body mass index raises immature platelet count: potential contribution to obesity-related thrombosis. (2022) Platelets <https://doi.org/10.1080/09537104.2021.2003317>

Goudswaard L. J., Bell J. A., Hughes D. A., Corbin L. J., Walter K., Davey Smith G., Soranzo N., Danesh J., Di Angelantonio E., Ouwehand W. H., Watkins N. A., Roberts D. J., Butterworth A. S., Hers I., Timpson N. J. Effects of adiposity on the human plasma proteome: Observational and Mendelian randomisation estimates. (2021) Int J Obes <https://doi.org/10.1038/s41366-021-00896-1>

Goudswaard L. J., Harrison S., Van De Klee D., Chaturvedi N., Lawlor D. A., Davey Smith G., Hughes A.D., Howe L. D. Blood pressure variability and night-time dipping assessed by 24-hour ambulatory monitoring: Cross-sectional association with cardiac structure in adolescents. (2021) PLOS ONE 16(6): e0253196. <https://doi.org/10.1371/journal.pone.0253196>

Liu, H., Jackson, M. L., **Goudswaard, L. J.**, Moore S. F., Hutchinson J. L, Hers I. Sphingosine-1-phosphate modulates PAR1-mediated human platelet activation in a concentration-dependent biphasic manner. (2021) Sci Rep 11, 15308. <https://doi.org/10.1038/s41598-021-94052-4>

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1.4 Publications in preparation

Goudswaard L. J., Williams C. M., Khalil J., Hamilton F., Arnold D., Milne A., Lewis P. A., Heesom K. K., Mundell S. J., Davidson A., Poole A. W., Hers I. Altered platelet proteome and impaired platelet integrin $\alpha_{IIb}\beta_3$ activation in hospitalised patients with COVID-19.

Constantinescu A. E., Bull C. J., **Goudswaard L. J.**, Zheng J., Elsworth B., Timpson N. J., Moore S. F., Hers I., Vincent E. E. Phenome-wide analysis to investigate deep vein thrombosis aetiology.

1.5 Presentations

Platelet Society meeting, 2018, Cambridge, UK. Title: Do the chemokines MDC and TARC contribute to obesity related platelet hyperactivity and cardiovascular disease? (Oral presentation).

IEU Scientific Advisory Board meeting, 2020, Bristol, UK. Title: Effect of adiposity on the human plasma proteome: a Mendelian randomisation study using individual level data (Poster).

International Genetic Epidemiological Society meeting, 2020, Virtual conference. Title: Effects of body mass index on the human proteome: a Mendelian randomization study using individual level data (Selected for short talk as one of the top ranked posters).

International Society of Thrombosis and Haemostasis meeting, 2020, Virtual conference. Title: Effects of body mass index on the human plasma proteome – potential drivers of obesity-related cardiovascular disease? (Poster).

Platelet society meeting, 2021, Virtual conference. Title: Altered platelet proteome, enhanced basal platelet procoagulant activity, and increased platelet-leukocyte interactions in patients with Covid-19. (Oral presentation)

Platelet society meeting, 2021, Virtual conference. Title: Estimating the causal association between body mass index and platelet properties: evidence for a positive association with immature platelet count (Poster)

British Heart Foundation PhD Student Conference, 2021, Virtual conference. Title: Estimating the association of body mass index and platelet properties: an observational and Mendelian randomization study (Poster)

International Society of Thrombosis and Haemostasis meeting, 2021, Virtual conference. Title: Altered platelet proteome and platelet-leukocyte interactions in patients with COVID-19 (Oral presentation, presented by Prof Hers)

International Society of Thrombosis and Haemostasis meeting, 2021, Virtual conference. Title: Estimating the causal association between body mass index and platelet properties: evidence for a positive association with immature platelet count (Oral presentation and Travel Award)

World Congress of Epidemiology, 2021, Virtual conference. Title: Effects of adiposity on the human proteome: Mendelian randomization study using individual-level data (Oral presentation)

International Genetic Epidemiology Society, 2021, Virtual conference. Title: Combining Mendelian randomization and randomized control trial study designs to determine effects of adiposity on the plasma proteome (Oral presentation for shortlisted posters)

Elizabeth Blackwell Institute Mechanisms to Populations, 2021, Virtual meeting. Title: Exploring the effect of BMI on platelet function: an interdisciplinary study (Oral presentation)

Bristol Heart Institute Meeting, 2021, Virtual meeting. Title: Combining Mendelian randomisation and randomised control trial study designs to determine effects of adiposity on the plasma proteome (Oral presentation).

1.6 Awards and prizes

Best talk in epidemiology category, 2021, Bristol Heart Institute Meeting

Poster prize - 2nd place, 2021, International Genetic Epidemiology Society.

Travel Award, 2021, International Society of Thrombosis and Haemostasis.

1.7 Supplementary material

This thesis reports all results where possible. Results which are too long to provide in full are located on GitHub, a platform which has been used for version control when writing this thesis in RMarkdown. The link to the thesis repository is <https://github.com/lucygoudswaard/mythesis>. Specific links to supplementary material will be provided where presented in the relevant results chapter.

Chapter 2

Introduction

2.1 The history of body mass index

Although body fatness (adiposity) has long been associated with adverse health, the use of the term body mass index (BMI), is relatively recent. BMI is thought to have been first defined in 1972 by Ancel Keys¹. This term was given to define the ratio of weight in kilograms over the square of the height in metres. This measure was found to reflect overall body fatness well, while being easy and cheap to measure. The World Health Organization first defined the BMI categories in 1995² used today: the National Health Service (NHS) defines a body mass index (BMI) of 25 kg/m^2 as having overweight and 30 kg/m^2 or higher as having obesity. These bounds were chosen based on associations with mortality. The average BMI of the UK adult population is $\sim 27 \text{ kg/m}^2$ ³, which is in the ‘overweight’ category. Overweight is also more common than ‘normal-weight’ in many other higher-income countries⁴, and prevalence of overweight is increasing in lower-income countries⁵. It is currently estimated that 40% of adults in the United States and 26% of adults in the United Kingdom have obesity⁶. As of 2016, the number of individuals globally with obesity surpassed 650 million⁷.

2.2 Cardiovascular diseases are the leading cause of deaths worldwide

Diseases affecting the heart and blood vessels are the major cause of mortality in many countries⁸. In 2019, global deaths from coronary heart disease (CHD) and stroke amounted to ~ 9 million and ~ 6.5

million deaths, respectively⁸. Current known risk factors for such diseases include smoking, physical inactivity, poor diet and high cholesterol⁹. Overweight and obesity are also well established risk factors for such diseases and are now considered the leading years lived with disability and injury risk factors in the United States⁹. Various studies have found a link between adiposity and cardiovascular risk through various study designs. For example, large observational studies have found that adults with overweight or obesity have a higher risk of cardiovascular events and death from cardiovascular disease¹⁰. Another study found that an average increase in BMI of 4 kg/m² across a population increased the odds of ischaemic heart disease by 20-30%¹¹.

2.3 Mendelian randomisation can be used to explore causality

Risk factors have often been identified through observational associations. Observational studies are often limited by bias, confounding and reverse causation¹². Mendelian randomisation (MR) is a technique which exploits the random assortment of alleles to estimate the causal association between a modifiable risk factor and disease outcomes. It can be likened to a natural randomised control trial (RCT)¹³. This technique is widely implemented due to the availability of genetic data in large cohorts, such as in ~500,000 UK Biobank participants¹⁴. An example of how MR and RCTs can be used to determine causality is shown in Figure 2.1, using BMI and cardiovascular events as an example¹⁵. Here, genotypes that are associated with BMI can be used. These genotypes should only be associated with BMI and not other confounding factors. If people with genotypes which are associated with higher BMI also have higher rates of cardiovascular events, it is likely that the relationship between BMI and cardiovascular events is causal. Although a useful technique in exploring causality, MR has its own biases and limitations, therefore other lines of evidence and study designs such as RCTs are required. In an RCT, if a weight loss intervention reduces BMI compared to the control group, and also reduces the rate of cardiovascular events, this also supports a causal role of BMI in the onset of cardiovascular events.

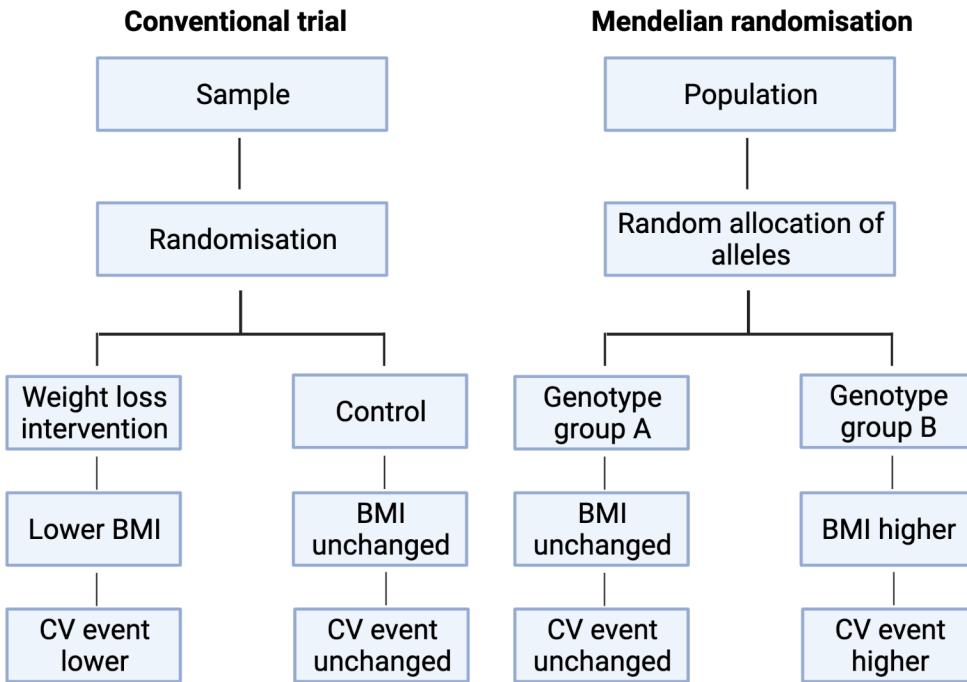


Figure 2.1: **Example of how Mendelian randomisation and a randomised control trial can be used to determine whether higher BMI increases the risk of cardiovascular events.** Figure made using BioRender.com.

2.4 The genetics of obesity

As indicated above, Mendelian randomization can be used to estimate the causal relationship of BMI on various outcomes. There are strong correlations between BMI and more direct measures of fat mass measured by dual-energy X-ray absorptiometry (DXA)¹⁶, such as total fat mass index and regional fat mass indices. Due to the specialised equipment needed for fat mass measures, BMI is often much more widely available within cohort studies, trials and in the clinic. Environmental factors are well known to explain a large amount of variance in BMI, but genetic variants also influence BMI. Monogenic obesity was first identified where mutations in the ob (leptin) gene¹⁷ lead to mice having obesity. This was later discovered to be because leptin was secreted by adipose tissue and was found to influence food consumption¹⁸. The first genome-wide association study (GWAS) for obesity traits was performed in 2007 for around 5,000 individuals¹⁹, which found evidence that 10 SNPs were associated with BMI. Since then, GWAS have been performed in increasingly larger cohort sizes²⁰, with the largest and most recent GWAS performed in 2018 by Yengo et al.²¹. This GWAS found 941 near-independent genetic variants which associate with BMI. These have been shown to explain around 6% of the variance in

BMI. Of these SNPs, 656 are considered as primary associations²¹.

2.5 Types of cardiovascular disease

As indicated in section 2.2, CHD and stroke affect millions of people worldwide every year. Cardiovascular disease is used as a term to describe the group of diseases which affect the heart or blood vessels. Within cardiovascular disease there are various diseases which range in their clinical presentation and pathology. CHD (also known as coronary artery disease (CAD) or ischaemic heart disease (IHD)) is caused by a build up of plaques in the arteries (atherosclerosis). These plaques are caused by low density lipoproteins (LDLs) accumulating in the intima of the arteries, where they undergo oxidation²². This then activates immune response and macrophages and lymphocytes also accumulate within the intima²³. The plaques build in size and may eventually rupture the endothelium, initiating platelet activation and thrombus formation (arterial thrombosis, Figure 2.2A), thereby causing a heart attack. Other types of cardiovascular disease include stroke, which can be either ischaemic (another form of arterial thrombosis) or haemorrhagic. Arterial thrombi are estimated to be composed of 31% platelets²⁴.

Thrombi can also form in veins (venous thromboembolism/VTE), thereby causing deep venous thrombosis (DVT) when the thrombus is in the legs or pulmonary embolism when an embolus becomes lodged in the lungs. Unlike arterial thrombi, venous thrombi form low shear rates²⁵. DVT is thought to start in the legs close to the valves as these are areas of stasis and hypoxia^{26,27}. This slowing of the blood reduces oxygen tension and increases haematocrit²⁷. The stasis and hypoxia also lead to downregulation of anticoagulant proteins such as thrombomodulin and endothelial protein C receptor (EPCR)²⁷ and an upregulation of procoagulant proteins such as the adhesion molecule P-selectin. Venous thrombi mainly consist of red cell dense fibrous clot (Figure 2.2B). Despite not as highly present in venous thrombi as arterial thrombi, platelets still play a role and form an inner layer of the thrombus²⁷.

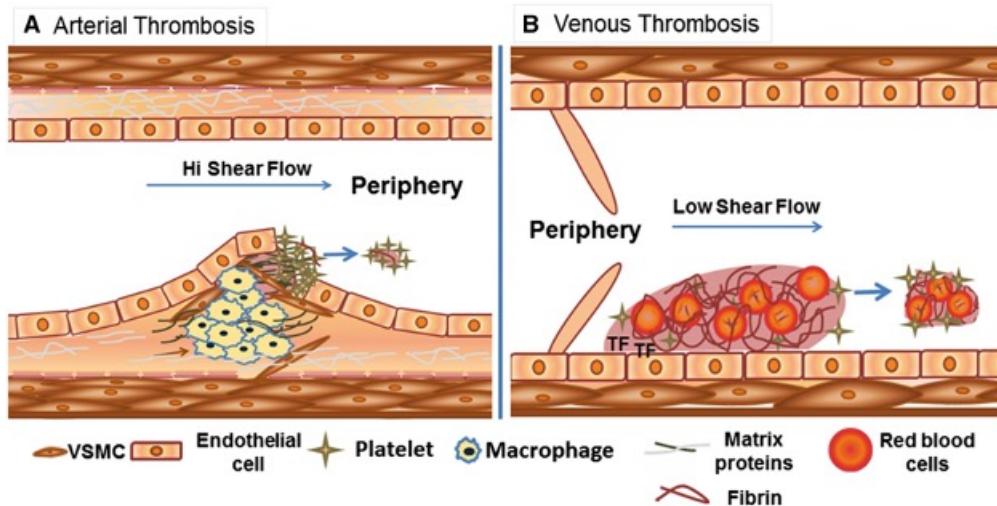


Figure 2.2: Cartoon of arterial and venous thrombosis. A) Arterial thrombi form when atherosclerotic plaques rupture and are rich in platelets. B) Venous thrombi mostly occur with an intact endothelial wall and are rich in fibrin, with red blood cells and a smaller proportion of platelets. Figure from Koupenova et al.²⁵.

2.6 Higher BMI has a causal effect on ischaemic heart disease

Studies which have used MR to estimate a causal effect have indicated that BMI may have an even greater effect on IHD than observational studies suggested. Nordestgaard et al. utilised single nucleotide polymorphisms (SNPs) which are associated with BMI to estimate that $4\text{kg}/\text{m}^2$ higher BMI increases the odds of IHD by as much as 52%¹¹. Other studies which have used more specific measures of adiposity, such as waist hip ratio adjusted for BMI (WHRadjBMI), have also found evidence for a causal effect of adiposity on coronary heart disease risk²⁸. Further, higher BMI has been shown to have a causal relationship with more specific measures of cardiovascular events such as ischaemic stroke²⁹. A separate line of evidence for a causal effect of BMI on cardiovascular outcomes is that more weight loss after bariatric surgery reduces the occurrence of major adverse cardiovascular events (MACE)³⁰. Despite evidence for causal relationships, changes which occur as a result of BMI which lead to these cardiovascular events are unclear.

2.7 How does higher BMI cause increased risk of CHD?

There is extensive evidence that higher BMI causes hypertension and T2D, which contribute to the increased risk of a heart attack or a stroke. As well as this, studies which have explored the molecular

footprint of adiposity have tended to focus on the lipidome, such as cholesterol and triglycerides in lipoprotein subtypes, including low-density lipoproteins (LDL), very-low-density lipoproteins (VLDL) and high-density lipoproteins (HDL)^{31,32}, where a higher BMI is associated with an increase in LDLs and a decrease in large HDLs. This has been explored in a causal framework. LDLs are therefore an important example of circulating molecules which are causally related to both BMI and disease risk. Although LDLs are important in mediating the increased risk of CHD³³, this does not account for all the risk. Therefore, it is likely that there are other factors which circulate that are mediating this association. There are thousands of detectable proteins which circulate in the blood³⁴, which have a range of roles from inflammation to coagulation. It is likely that adiposity causes an alteration in such proteins, which may have a downstream effect on platelet function and cardiovascular risk. Assessing the overall protein profile of adiposity may provide more of a mechanistic insight into how adiposity causes disease and identify druggable targets.

2.8 Platelets are key cells in thrombosis

Platelets are anucleate cells which are generated from megakaryocytes in the bone marrow. Platelets are one of the key cells involved in haemostasis³⁵. Under normal conditions, the endothelium releases nitric oxide (NO) and prostaglandin I2 (PGI2) which suppress platelet activation³⁶. When the endothelium is damaged, platelets adhere to the injured vessel wall through the glycoprotein Ib-IX-V (GPIb-IX-V) receptor and the glycoprotein VI (GPVI) collagen receptor. As a result, platelets secrete alpha and dense granules and undergo shape change²³. Alpha granules release growth factors (e.g. IGF-1), clotting factors and chemokines³⁷, whereas dense granules release molecules such as adenosine diphosphate (ADP), which further activate platelets by interacting with the P2Y₁ and P2Y₁₂ platelet receptors. Integrins are heterodimeric transmembrane glycoprotein complexes, which are important in platelet activation³⁸. One of the main integrins for platelet activation is integrin $\alpha_{IIb}\beta_3$ ³⁹, of which there are 50,000-100,000 integrins per resting platelet. Under resting conditions, integrin $\alpha_{IIb}\beta_3$ adopts a low affinity state. Binding of agonists such as thrombin (or the synthetic peptide agonist PAR1-AP/TRAP-6) to the protease-activated receptor 1 (PAR1) results in activation of integrin $\alpha_{IIb}\beta_3$. This is known as inside-out signalling. Here, inositol trisphosphate (IP3) and diacylglycerol are generated, which leads to an increase in cytosolic calcium (Ca^{2+}). This in turn activates CALDAG-GEF1 as well as protein kinase C (PKC)⁴⁰. The small GTPase, Rap1 is then activated which recruits proteins such as talin and kindlin⁴¹. This leads to a change in conformation to increase the affinity of integrin $\alpha_{IIb}\beta_3$ for its ligand,

fibrinogen³⁹. Neighbouring platelets then become activated due to the release of local mediators such as ADP and thromboxane A₂ (Tx_A₂, which acts at the TP receptor)⁴². Subsequent fibrinogen binding results in cross-linking of activated platelets (platelet aggregation) via integrin $\alpha_{IIb}\beta_3$, resulting in the cessation of bleeding³⁵. These key platelet agonists and receptors are shown in Figure 2.3. Despite these processes being essential for haemostasis, when platelets become hyperactive, the balance is tipped in favour of thrombosis following plaque rupture. This can cause a myocardial infarction and stroke.

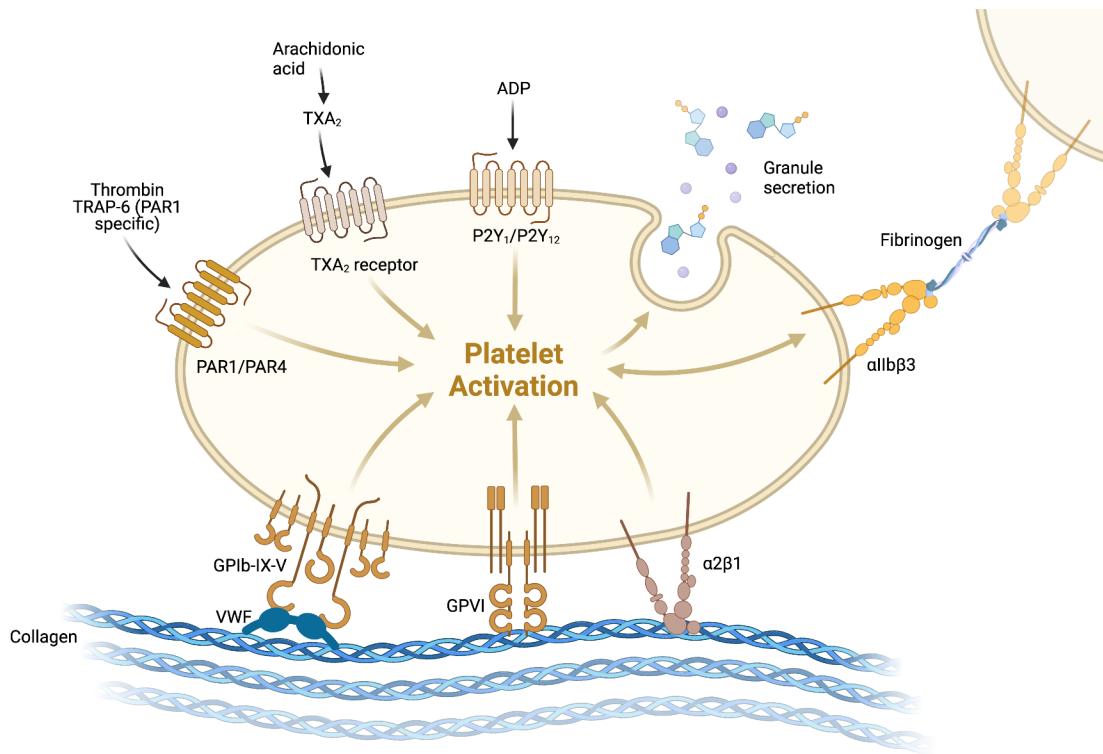


Figure 2.3: Key platelet receptors for platelet activation. Figure made using BioRender.com.

2.9 Platelets are therapeutic targets for thrombosis

When a patient has a clot in one of the coronary arteries, they often undergo percutaneous coronary intervention (PCI), which consists of inserting a catheter in order to place a stent to open up the occluded space. Following this, patients are commonly prescribed dual antiplatelet therapy (DAPT)^{43,44} and anticoagulants, which have been shown to reduce occurrence of future myocardial infarction⁴⁵. DAPT is generally consists of aspirin which inhibits the enzyme cyclooxygenase-1 (COX-1) in platelets, resulting in a blockade of production of Tx_A₂⁴⁶, and a P2Y₁₂ antagonist such as clopidogrel or ticagrelor⁴⁷.

Together, these therapies dampen platelet activation. Anticoagulants are also prescribed as the coagulation system results in conversion of fibrinogen to an insoluble fibrin rich platelet plug and is therefore a key component of thrombosis⁴⁸. Although these treatments are effective in many patients, myocardial infarction recurs in some patients.

As BMI is associated with increased rates of thrombotic events and platelets are one of the key cells involved in thrombosis, it is possible that high BMI causes platelet hyperactivity. Various studies have attempted to address this question. There is mixed evidence for an effect of BMI on platelet hyperactivity. For example, in patients who were treated with clopidogrel there was a positive association between BMI and platelet activation in response to the agonist ADP⁴⁴. Studies looking at BMI and platelet function are often limited by small sample sizes and are often restricted to patients being on antiplatelet therapy^{49–51}. Less is known about the association between BMI and platelet function in the absence of antiplatelet therapies. To address this, there needs to be a cohort which has information about BMI, potential circulating intermediates and platelet function. Genetic data would also help address the directionality of the effect. As this is currently not all available within a large cohort, separate cohorts are required to explore different associations.

2.10 Experimental techniques to measure platelet function

Platelet function can be measured by various laboratory techniques. Light transmission aggregometry (LTA) quantifies platelet aggregation by how much light passes through the sample. The more aggregation, the clearer the solution and the more light passes through and is detected. Other techniques routinely used include using flow cytometry. Measuring resting levels of receptors using fluorescently labelled antibodies provides information about the function of the platelet⁵². Some receptors change conformation and become exposed in response to platelet agonists (such as the integrin $\alpha_{IIb}\beta_3$). The adhesion receptor P-selectin is stored in α -granules and becomes rapidly expressed on the platelet plasma membrane in response to agonist stimulation. Such platelet agonists include as protease-activating receptor activating peptide 1 (PAR1-AP), adenosine diphosphate (ADP) and collagen-related peptide (CRP). Another technique which can provide useful information about platelets is measuring calcium mobilisation by using a fluorescent calcium indicator. Activation of various receptors causes downstream activation of phospholipase C (PLC). In turn, PLC produces inositol-1,4,5-trisphosphate (IP₃), thereby releasing calcium from the intracellular stores via IP₃ receptors (IP₃-R) in the endoplasmic reticulum⁵³. Another marker of platelet activation and coagulation is phosphatidylserine (PS)

exposure. Under basal conditions, PS is mainly present in the inner leaflet of the phospholipid bilayer. Upon activation (e.g. by dual stimulation using collagen and thrombin), the PS becomes exposed on the outer surface of the membrane, thereby providing a binding site for factor Xa (FXa) and thus fibrin formation⁵⁴. Therefore PS reflects the changes in PS exposure can be measured using a fluorescently labelled antibody.

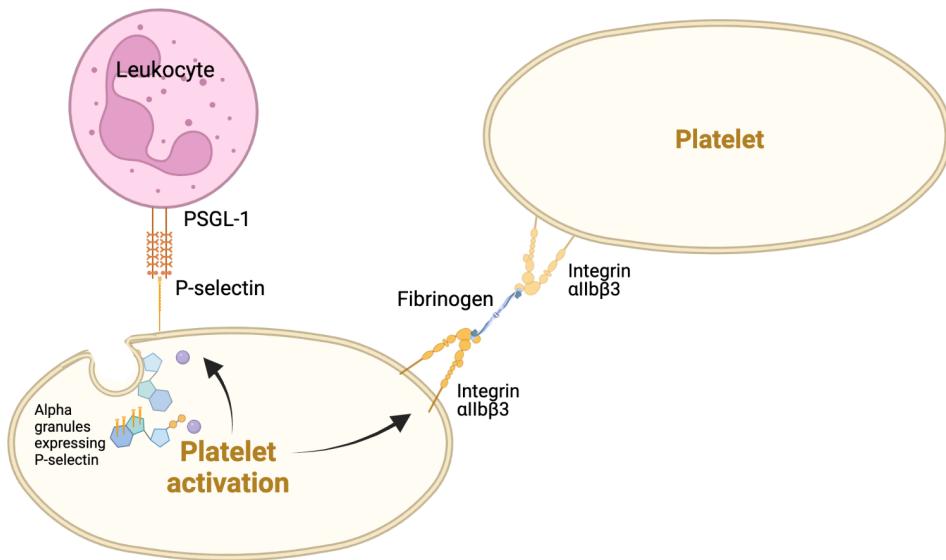


Figure 2.4: Cartoon of integrin $\alpha IIb\beta 3$ activation, P-selectin expression and platelet-leukocyte interactions. Upon platelet activation by a strong agonist such as CRP, the integrin $\alpha IIb\beta 3$ exposes its binding site for fibrinogen and cross-links platelets. P-selectin is expressed in the membrane of alpha granules within platelets. Upon platelet activation, alpha granules fuse with the membrane and P-selectin is then expressed on the outer membrane. P-selectin on platelets can bind to P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes. Figure made using BioRender.com.

As well as platelets aggregating to one another, platelets are also able to bind to leukocytes such as neutrophils. These interactions are important for neutrophil localization for inflammatory responses as well as in leukocyte phagocytosis and generation of neutrophil extracellular traps (NETs) as well as activation of coagulation⁵⁵. When activated, platelets express P-selectin. Neutrophils express P-selectin glycoprotein ligand-1 (PSGL-1)⁵⁶ (Figure 2.4), therefore these cells can form aggregates through these adhesion receptors. The extent of these interactions can also be used to measure platelet activation. This can be done under basal conditions or after stimulation with an agonist such as CRP. Firstly, neutrophils are gated based on their forward scatter (FSC) and using an antibody for the leukocyte common antigen (CD45). An antibody for the platelet receptor CD41 (integrin αIIb) is then used to detect the pro-

portion of neutrophils bound to platelets.

Techniques such as Western Blotting can be used to quantify the levels of total protein or phosphoprotein. This can therefore be used to explore changes in signalling in platelets under basal conditions, or after stimulation with agonists or antagonists. PGE1 acts at a G_s-coupled prostaglandin G protein coupled receptor (GPCR), which activates adenylyl cyclase and leads to subsequent production of cAMP. Protein kinase A (PKA) subsequently phosphorylates the vasodilator-stimulator phosphoprotein (VASP) at sites including Ser¹⁵⁷ and Ser²³⁹⁵⁷. Phosphorylation of VASP has been shown to inhibit integrin $\alpha_{IIb}\beta_3$ activation, thereby suppressing platelet activity⁵⁸. The P2Y₁₂ receptor is coupled to G_{i/o}, therefore the P2Y₁₂ agonist ADP opposes the effects of PGE1. Measuring phosphorylation of VASP at these sites is used as an indicator of how well P2Y₁₂ antagonists are inhibiting platelets. The inability to reduce PGE1-mediated VASP phosphorylation by ADP indicates more inhibition by drugs such as clopidogrel and ticagrelor⁵⁷.

Western blotting requires a lot of material and is limited by the number of proteins/phosphoproteins that can be detected at once. A solution to this issue is using Mass Spectrometry (MS). Using this method, platelets that have been isolated from blood are washed in a buffer and are lysed before MS analysis. Proteins are fragmented into peptides which are then ionised before being captured by the mass spectrometer. Computational methods are required to interpret the peaks of the spectra and to determine proteins present⁵⁹. This method enables detection of thousands of platelet proteins⁶⁰, therefore providing extensive detail of the platelet environment.

2.11 Platelet traits as proxies for platelet function

Although there are not many large studies directly exploring platelet function, other measures of platelet characteristics are widely used in the clinic and among cohort studies. These characteristics are often reported as part of a routine full blood count. These include platelet count (PLT, absolute number of platelets), plateletcrit (PCT, the volume occupied by platelets), mean platelet volume (MPV, a measurement of the average size of platelets) and platelet distribution width (PDW, a measure of how similar platelets are in size). These are often used as diagnostic tools, for example a low platelet count is demonstrative of thrombocytopenia and a high platelet count is known as thrombocytosis/thrombocythaemia. Platelet traits have also been implicated in cardiovascular disease: there is MR evidence that higher PLT contributes to the risk of ischaemic stroke⁶¹, thereby suggesting that these

platelet parameters have functional and clinical relevance. There is also evidence that alterations in these parameters associate with platelet function. For example, higher platelet count has been shown to associate with increased platelet aggregation^{62,63}. Previous studies have explored the association between BMI and platelet parameters. Some studies have found evidence for an association between BMI and both platelet count and mean platelet volume, however other studies have lacked evidence for an effect^{64–67}. These observational studies may be subject to confounding, bias and reverse causation, therefore it is unclear whether there is a causal effect of BMI on these platelet traits, or whether effects are due to other factors.

More recent haematology analysers such as the Sysmex XN series analysers can provide additional information about platelet structure. They use a combination of fluorescence and impedance flow cytometry. Additional readouts provided by these analyzers include immature platelet count (IPC, the number of immature platelets) as well as immature platelet fraction (IPF, the fraction of platelets which are immature), based on a gating of their forward scatter (FSC, size) and side fluorescence (SFL, an indicator of mRNA content). Detection of PLT and IPF by flow cytometry is shown in Figure 2.5⁶⁸. Immature platelets, or reticulated platelets, are the youngest platelets in circulation. They persist for between 24 and 36 hours⁶⁸. Immature platelets are larger in size and have more mRNA than older circulating platelets. Over time the mRNA degrades and they gradually decrease in size. There are various studies which have suggested that a larger number of immature platelets may increase the risk of suffering from major adverse cardiovascular events (MACE)⁶⁹. This suggests that immature platelets may be more reactive.

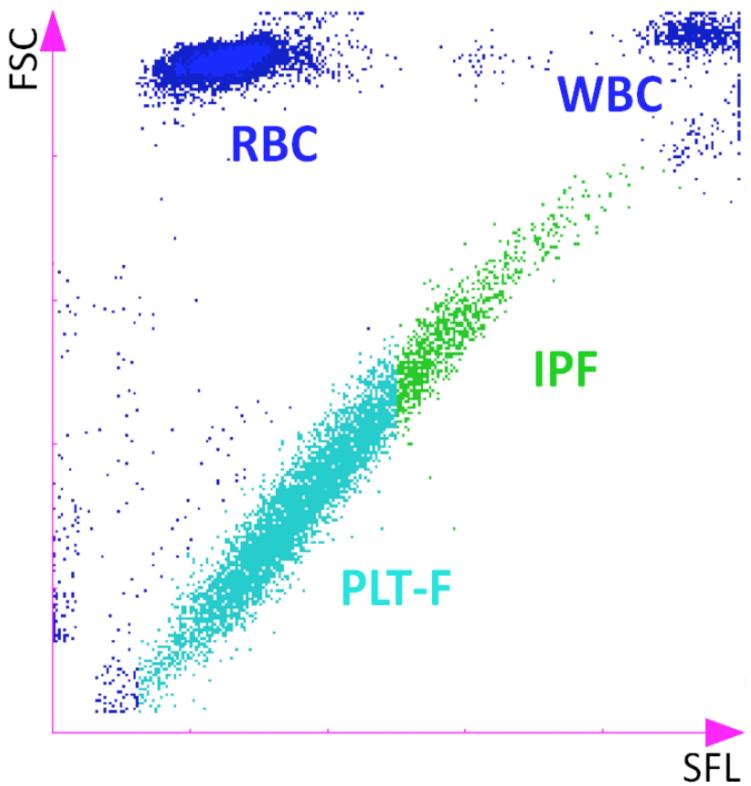


Figure 2.5: Scatter plot indicating how platelets and immature platelets are detected using a Sysmex haematology analyser. The forward scatter (FSC, a measure of cell size) and side fluorescence (SFL, a measure of cell mRNA content) are used to detect cell types. Immature platelets have a larger FSC and SFL. PLT-F = fluorescent platelet count, IPF = immature platelet fraction, RBC = red blood cells, WBC = white blood cells. Figure from Corpataux et al.⁶⁸.

2.12 Platelet primers

Platelets express receptors for various circulating molecules. Previous studies have reported that a range of soluble ligands, growth factors and cytokines which can enhance platelet function are elevated in obesity^{@ 70}. These ‘platelet primers’ include insulin-like growth factor-1 (IGF-1)^{71,72} and thrombopoietin (TPO)^{73,74}. There is evidence other chemokines may be able to activate platelets. The C-C motif chemokine receptor 4 (CCR4) receptor is activated by the chemokines macrophage derived chemokine (MDC/CCL22) and thymus and activation-regulated chemokine (TARC/CCL17)^{75,76}. There is observational evidence that higher BMI increases the circulating levels of MDC⁷⁷. These chemokines are important for chemotaxis of leukocytes. It has also been observed that these chemokines can activate platelets as measured in platelet aggregation and calcium mobilisation experiments⁷⁶. It is unclear how these chemokines are able to exert effects. Known platelet primers (e.g. TPO) potentiate platelet

activation through activation of the lipid kinase phosphoinositide-3-kinase (PI3K) pathway^{73,74}. This activation leads to recruitment of PH-domain proteins including protein kinase B (PKB/Akt) to the plasma membrane among other downstream regulators. PKB is subsequently phosphorylated on Threonine³⁰⁸ and Serine⁴⁷³ residues leading to its activation⁷⁸. Another pathway that has been implicated in platelet priming effects is the mitogen-activated protein kinase (MAPK) pathway. TPO can enhance thromboxane A₂ (TxA₂) synthesis through increasing the activation of extracellular signal regulated kinase (ERK2)^{79,80}. It is possible that these pathways are also important in signalling via CCR4.

2.13 Mechanisms of obesity-related disease

It is not fully understood how a high BMI might lead to changes in platelet function. Inflammatory mediators could be one such mechanism due to likely interactions between inflammatory mediators and platelets in the blood. Obesity commonly presents with a state of low-grade chronic inflammation. There are two types of fat: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT secretes proteins and peptides which have pro-inflammatory effects^{81,82}. There is evidence that obesity is associated with increases in interleukin-6 (IL-6), tumour necrosis factor- α (TNF- α), therefore supporting a pro-inflammatory state⁸³. Previous studies have also identified a causal link between higher BMI and higher circulating levels of the inflammatory molecule C-reactive protein (CRP)⁸⁴ and leptin⁸⁵, further suggesting these effects are BMI driven. More recent studies have used MR and have found an effect of BMI on a range of cytokines⁸⁶. As platelet activation can be modified by inflammatory mediators and there is evidence for a causal relationship between BMI and CRP/cytokines, it is likely that inflammation could mediate this effect.

2.14 The role of circulating platelet primers and disease

Recent studies have used large cohorts of people to identify SNPs which are associated with changes in levels of certain proteins. These are known as protein Quantitative Trait Loci (pQTL)³⁴. Sun et al. identified 1,927 genetic associations with 1,478 proteins. As a result of these pQTLs, there have been numerous studies that have been able to estimate causal effects of proteins in disease by using two sample MR. For a two sample MR, the exposure are measured in a separate cohort from the cohort of the outcome. An example of this is linking the interleukin-6 receptor (IL6R) to diseases such as rheumatoid arthritis and coronary artery disease⁸⁷. The chemokines MDC and TARC both have SNPs

which are associated with their levels³⁴. MDC and TARC have been implicated in platelet hyperactivity, however it is unknown whether raised levels of MDC and TARC would have clinical implications such as a role in ischaemic heart disease. As a polymorphism in the receptor for MDC and TARC (CCR4 receptor) has been implicated in myocardial infarction⁸⁸, it is important to see how raised levels of MDC and TARC would relate to cardiovascular disease outcomes.

2.15 Evaluating the circulating proteome may reveal mechanistic insight into obesity-related diseases

Through literature searches, there is evidence for an association between BMI and chemokines such as MDC and TARC⁷⁷. Based on these observations, there may be many more circulating proteins which may be raised in obesity and have downstream effects on platelet function. Many studies so far have had hypothesis driven approaches when looking at the effect of BMI on circulating protein levels⁸⁴. This is due to the often limited range of the number of proteins detected in laboratory assays, such as enzyme-linked immunosorbent assays (ELISAs). Recent technologies have now been developed which allow protein detection in unprecedented scope and detail, for example the SOMAScan by SomaLogic⁸⁹. This array can detect around 4000 unique proteins using SOMAmers (Slow Off-rate Modified Aptamers), that are specific for individual proteins and allow quantification using a DNA microarray. Studies that have attempted to characterise the plasma proteome of higher BMI have often only been in an observational framework⁹⁰. As the majority of druggable targets are proteins, it is valuable to explore the effects of BMI on circulating plasma proteins. It is estimated that 25% of proteins in the human proteome circulate in the blood⁹¹, including enzymes, cytokines and transport proteins. Therefore, focusing on effects of adiposity on the circulating proteome may allow for the development of or repurposing of therapeutic interventions to reduce obesity-related disease. To explore the link between BMI, circulating proteins and platelet function, the blood is the tissue of interest. Many studies have blood/plasma/serum samples analysed from participants, therefore enabling exploration of a range of blood measures.

2.16 Importance of interdisciplinary methods

To understand mechanisms of disease, various study designs are required. Each experimental approach used when testing hypotheses has biases and limitations. For example, using basic science

it is possible to explore mechanistic effects *in vitro*, *in vivo* or *ex vivo*, which is crucial. However, it is often difficult to fully understand how findings might translate in a clinical setting. Similarly, observational studies can provide useful clinical information, but these can generally only be used to identify correlation, not causation. Randomised control trials (RCTs) are considered the gold standard way to determine efficacy and causality, especially in drug trials, however these trials are often time consuming and expensive⁹². To overcome this issue, MR utilises genetic information to be able to provide causal estimates. MR is widely utilised since the cost of sequencing genomes has become more affordable. However, this technique requires large sample sizes and can also suffer from issues such as horizontal pleiotropy. Therefore, a combination of laboratory experiments, observational clinical studies, RCTs and MR can therefore help to provide separate sources of evidence in investigating mechanisms of disease and can account for their separate limitations.

2.17 Thesis aims

It is well established that patients with obesity have an increased risk of CVD, however, the underlying mechanism is still largely unknown. In this study I hypothesise that an increase in BMI alters the plasma proteome leading to enhanced platelet activity and CVD. To test this hypothesis, I will explore the relationship between BMI, the plasma proteome, platelets and CVD, using a multidisciplinary approach (Figure 2.6).

The specific sub-aims of the thesis are:

- 1) Explore the effect of BMI on platelet traits, platelet function and signalling.
- 2) Characterise the effects of the chemokines macrophage-derived chemokine (MDC) and thymus and activation regulated chemokine (TARC) on platelet function.
- 3) Determine the effects of adiposity on the plasma proteome and the potential contribution to platelet function.

Overview of thesis hypotheses

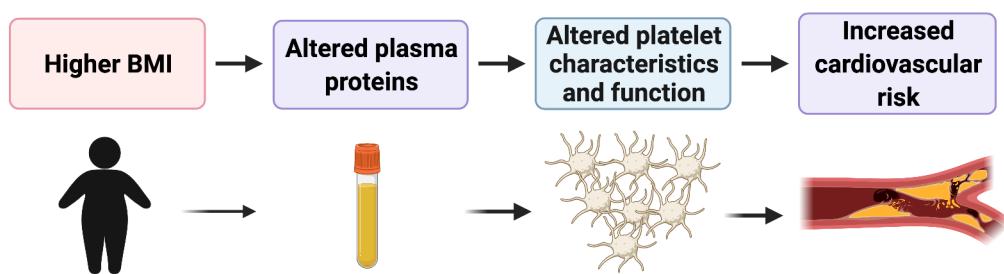


Figure 2.6: Schematic of the multi-step pathway addressed in the thesis

2.18 Thesis structure

To address the thesis aims, there will be six results chapters in this thesis:

- 1) Using observational and MR frameworks to determine the effect of body mass index on platelet characteristics (Chapter 3)
- 2) The effect of obesity on platelet function: a clinical pilot study (Chapter 4)
- 3) Pathways linking plasma proteins and platelet function: do the chemokines MDC and TARC play a role? (Chapter 5)
- 4) Exploring the plasma proteome as a link between adiposity and platelet function: Observational and Mendelian randomization estimates (Chapter 6)
- 5) Further elucidating the effect of adiposity on the plasma proteome using a weight loss intervention (Chapter 7)
- 6) Combining MR and an RCT to determine effects of adiposity on the plasma proteome. (Chapter 8)

Chapter 3

Using observational and MR frameworks to determine the effect of body mass index on platelet characteristics

This work is published in the journal Platelets⁹³. I performed all analyses, created all figures and tables and wrote the manuscript.

3.1 Background

As indicated in the background chapter (Chapter 2), clinical studies and genetic association studies have identified BMI as an independent risk factor for thrombotic disorders such as CHD and stroke^{11,28,94}. Platelet hyperactivity can be an indicator of those who may be at an increased risk of thrombosis⁹⁵. There is evidence that people with a higher BMI have hyperactive platelets^{44,96}, which could explain why a higher BMI is linked to an increase in thrombosis. Platelet function can be directly assessed by various methods (section 2.10, but these techniques are not widely available or readily standardized. Haematology analyzers such as the Sysmex XN series are more commonly used to provide full blood counts, providing detailed readouts of platelet characteristics. Mechanisms of platelet hyperactivity are unclear, but it is possible that alterations in properties of platelets such as PLT, MPV and IPF/IPC may play a role.

Immature platelets (also known as reticulated platelets) are the youngest platelets in circulation and

are detected based on having a greater forward scatter (FSC, an indicator of their size) and a greater side fluorescence (SFL, an indicator of their mRNA content)⁶⁹. More immature platelets are indicative of enhanced platelet production⁹⁷ and these platelets are believed to be more prothrombotic than older circulating platelets, with more dense granule release and increased P-selectin expression^{69,97,98}. Higher immature platelet count is associated with adverse cardiovascular outcomes in patients with coronary artery disease⁶⁹ and reduced effectiveness of antiplatelet therapies^{98,99}, suggesting that hyperactive immature platelets may contribute to adverse vascular events. Immature platelets have also been suggested to be less responsive to antiplatelet therapies such as prasugrel in acute coronary syndrome patients⁹⁸.

As these platelet characteristics provide potential information about platelet hyperactivity and given the increased thrombotic risk seen with adiposity, it is important to assess how adiposity affects platelet properties. Previous studies with modest samples sizes have implemented observational epidemiological methods to explore the effect of BMI on PLT, plateletcrit (PCT) and MPV. There is conflicting observational evidence regarding an association between BMI and PLT^{64,65}, with some studies suggesting a positive association between BMI and MPV⁶⁶ and other studies reporting no such association⁶⁷. Furthermore, as there is evidence that immature platelet production is increased in patients with metabolic syndrome and type II diabetes^{100,101}, it is therefore important to explore whether BMI may be an independent predictor of this trait. It is currently unknown whether the influence of BMI on platelet properties and function are causal and independent of confounding effects.

In this chapter, data from the INTERVAL prospective cohort (N=33388) was used to explore the association between BMI and platelet traits. Figure 3.1 shows the associations of the overall thesis hypothesis addressed in the chapter. Here, observational and Mendelian randomization (MR) approaches were employed to test the hypothesis that higher BMI leads to changes in platelet characteristics. Although observational studies can demonstrate associations between BMI and platelet characteristics, they cannot determine direct causality. To address the latter, we therefore employed MR, using a genetic risk score derived from single nucleotide polymorphisms (SNPs) associated with higher BMI (Figure 3.2). This allowed estimation of the causal effect of BMI on platelet traits, reducing the effect of confounding factors that are inherent to observational studies. To assess functional implications of BMI-platelet associations, a follow-up analysis was designed to explore the associations between platelet characteristics and whole blood aggregation in a cohort of cardiac surgery patients.

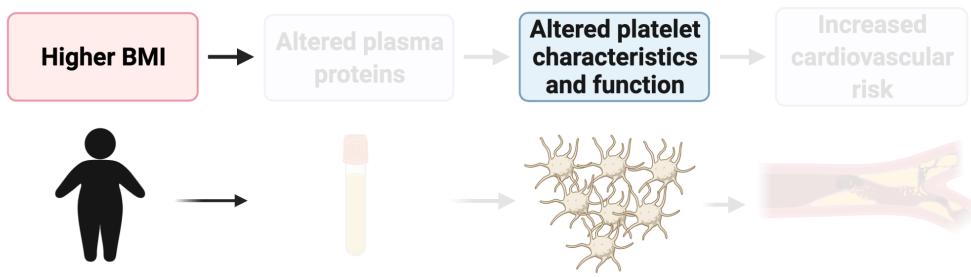
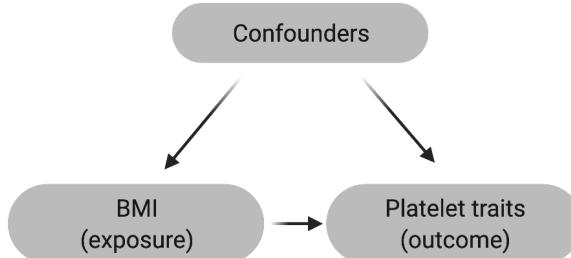


Figure 3.1: Schematic of the associations explored in the current chapter: the relationship between body mass index and platelet traits. Figure made using BioRender.com.

Linear regression



Mendelian randomization (two stage least squares)

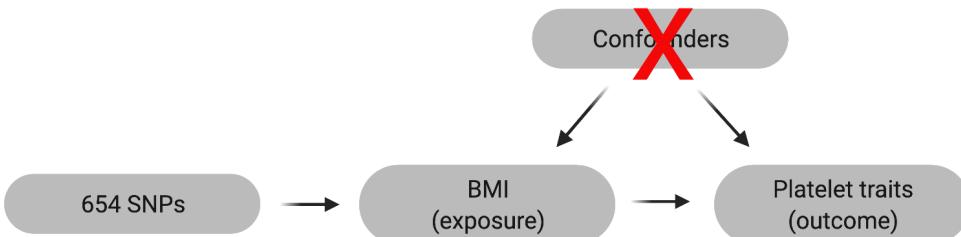


Figure 3.2: Schematic of linear regression and Mendelian randomization analyses. Linear regression assesses the association between **BMI (exposure)** and **platelet traits (outcome)**, with adjustment for potential confounders. Under the assumptions of Mendelian randomization (MR), the genetic risk score (GRS) for body mass index (BMI) does not associate with confounding variables, and if there is a causal association, the GRS should only associate with platelet outcomes through its association with body mass index.

3.2 Methods

3.3 The INTERVAL study

3.3.1 Study overview

INTERVAL is a prospective cohort study that initially aimed to test the safety of reducing the time interval between donation of whole blood in 50,000 participants¹⁰². Participants were over the age of 18 years, able to provide informed consent and free from a history of major disease. Participants were recruited between June 11th, 2012 and June 15th, 2014 from 25 National Health Service Blood and Transplant (NHSBT) centres across England. They filled out online questionnaires including self-reported height and weight, smoking status and alcohol consumption. Blood samples were also taken at baseline (before randomization within the study) where full blood counts were obtained. The study was approved by Cambridge East Research Ethics Committee. Permission for data access was provided by the Data Access Committee. Data contains sensitive content and requires permission to use therefore cannot be made publicly available. Access to data needs to be approved by the INTERVAL team www.intervalstudy.org.uk/more-information.

3.3.2 Assessment of BMI and covariates

Participants completed online questionnaires wherein they reported their height and weight. BMI was calculated as weight in kilograms divided by the square of their height in metres (kg/m^2). Available covariates were age, sex, previous or current smoking frequency (in three categories of: never, occasional, most days or every day) and alcohol intake frequency (in four categories of: rarely, less than once a week, 1-2 times a week, 3-5 times a week or most days for the proteomics analysis, or three categories of never, previous and current in the platelet trait analysis). These covariates were chosen as they were measured in the INTERVAL collection and are measures which are thought to influence adiposity and cardiometabolic health³¹.

3.3.3 Genetic data and instrument for BMI

INTERVAL participant genotyping was performed on the Affymetrix GeneTitan Multi-Channel (MC) Instrument using the UK Biobank Axiom Array (ThermoFisher Scientific, Loughborough, UK) and the QC of genotype data was implemented as described by Astle et al..¹⁰³ The imputation panel used was

the 1000 genomes phase-3-UK-10K¹⁰³. A genetic instrument for BMI was constructed using 654 genetic variants that were associated with BMI at $P < 5 \times 10^{-8}$ in the inverse variance weighted fixed-effect meta-analysis of GWAS of 700000 individuals of European ancestry²¹. This meta-analysis consisted of around 250000 adults from the Genetic Investigation of ANthromopetric Traits (GIANT) consortium²⁰ and 450000 adults from the UK Biobank study. Only 0.05 % of UK Biobank participants were included in the current INTERVAL study (of N=2737). These participants were not excluded to increase power. The weighted GRS was made using PLINK 2.0 software¹⁰⁴ using the effect alleles and beta coefficients from the source GWAS. The score was calculated by multiplying the number of effect alleles at each SNP by its effect estimate (beta), summing these, and dividing by the total number of SNPs included. The GRS therefore can be interpreted as the average per-SNP effect on BMI for each individual.

3.3.4 Study population

The present study was conducted on up to 33388 European ancestry INTERVAL participants living in the United Kingdom. These were the participants who had genetic data, had basic phenotype data and full blood count measures (Figure 3.3).

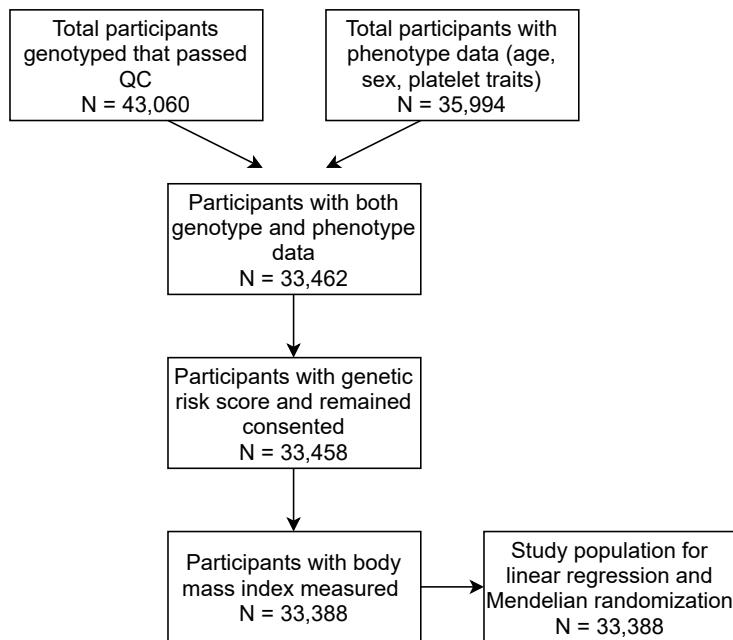


Figure 3.3: A STROBE diagram outlining participants included in the INTERVAL study for BMI and platelet trait analyses.

3.3.5 Measurement of platelet traits

Blood was taken by venipuncture into 3mL ethylenediamine tetraacetic acid (EDTA) tubes. Platelet parameters were measured using the Sysmex XN-1000 instrument¹⁰³. This analyzer provides information on cell counts by using a combination of fluorescence (PLT-F) and impedance (I) flow cytometry. The PLT-F channel used a Fluorocell fluorescent dye (oxazine), whereas the impedance method uses electrical resistance to detect platelets. Platelet indices included in the current study, along with the raw units, are provided in Table 3.1. These measurements were pre-adjusted for technical covariates such as time between venipuncture and blood count, as well as instrument drift, seasonal and weekly variation¹⁰³. Adjustment was performed with cyclic, thin-plate and P-splines in a generalized additive model (GAM)¹⁰⁵. Resulting platelet trait values along with their units of measurement are reported in Table 3.1. These data were rank normal transformed to normalize the distribution of each trait. Therefore, each platelet index is measured in normalized standard deviation (SD) units.

Table 3.1: Platelet traits measured by Sysmex XN-1000.

Short name	Full name	Derivation of platelet trait	Units	Mean (SD)
P-LCR	Platelet large cell ratio	(platelet number with volume>12 fL)/(platelet number of ordinary volume) x 100	%	36.9 (7.7)
H-IPF	High fluorescence immature platelet fraction	(high fluorescence immature platelet number)/(platelet count) x 100	%	1.3 (1.0)
PLT (F)	Platelet count (PLT-F channel)	Total number of platelets per litre	10E9/L	255 (57)
PLT (I)	Platelet count (impedance channel)	Total number of platelets per litre	10E9/L	250 (55)
PCT	Plateletcrit	Volume percentage of blood occupied by platelets	%	0.28 (0.06)
PDW	Platelet distribution width	Standard deviation of platelet volume distribution, estimated as width at 20% modal height of platelet volume histogram	fL	14.0 (1.8)
IPC	Immature platelet count	Immature platelet count	10E9/L	10.1 (5.0)
IPF	Immature platelet fraction	(Immature platelet number)/(platelet count) x 100	%	4.2 (2.5)
MPV	Mean platelet volume	Mean platelet volume	fL	11.1 (1.0)
SFL	Side fluorescence	Side fluorescence measures the nucleic acid (mRNA) content of the platelet	RFUs	80.3 (5.0)
FSC	Forward scatter	Forward scatter is a measure of the size of the platelet	RFUs	53.0 (6.4)
SSC	Side scatter	Side scatter is a measure of the granularity of the platelet	RFUs	37.9 (2.9)

3.3.6 Statistical analysis

Analyses were performed using R version 3.4.2¹⁰⁶. To visualize correlation between outcome platelet variables, a correlation matrix was made using the pairwise Pearson correlation coefficients of the rank normal transformed data. A dendrogram was also created to show the hierarchical relationship between platelet traits (<https://github.com/hughesvoanth/iPVs>), where the height at which variables are joined is set at (1 – Pearson correlation coefficient (r)). To explore observational associations between BMI and platelet traits, linear regression models were used (`lm()` function from “stats” package). Two regression models were used: firstly, adjusting for age and sex and secondly, additionally adjusting for smoking and alcohol consumption as ordinal variables. The results of the regression reflect the change in platelet traits (in normalized SD units) per normalized SD higher BMI (4.7 kg/m²). Only participants with all covariables were included in the linear regression models. The associations between potential covariables with both BMI and platelet traits were also explored using linear regression.

To understand properties of the GRS for BMI, the association between the GRS with both BMI and covariables were explored. The MR analysis was performed by using a two-stage least squares (2SLS) regression model (using `systemfit()` function from “systemfit” package¹⁰⁷). The MR causal estimates reflect the change in platelet traits (in SD units) per SD increase in BMI. A Wu-Hausman test was performed to test for endogeneity between observational and MR estimates. Exact beta coefficients, confidence intervals and P values are provided throughout and guide strength of association.

Where there was consistent observational and MR evidence for a BMI effect on platelet traits with potential clinical relevance, additional sensitivity analyses were carried out. We explored whether there were different magnitudes of associations across NHS BMI categories indicative of a non-linear relationship. Specifically, participants were stratified by BMI and the association between BMI and IPC estimated within BMI categories.

3.3.7 Follow-up analysis to explore the association between immature platelets and aggregation

Motivated by findings from the primary analyses described above, additional observational analyses were conducted using data from the COagulation and Platelet laboratory Testing in Cardiac Surgery (COPTIC) study. The COPTIC study was an observational, single centre cohort study of adults undergoing cardiac surgery at the Bristol Heart Institute with primary objective of examining the relationship between coagulation laboratory parameters and bleeding outcomes after surgery

in 2,541 participants¹⁰⁸. This study was approved by the UK NHS Research Ethics Committee (09/H0104/53).

3.3.8 COPTIC study variables

Age and sex were reported at baseline. Height and weight were obtained from medical notes. BMI was derived from weight and height (kg/m^2). Smoking was reported as a categorical variable (0=never smoker, 1=ex-smoker for > 5 years, 2=ex-smoker for 1-5 years, 3=ex-smoker for 30 days-1 year, 4=current smoker). Platelet variables (PLT, MPV, IPF, IPC) were measured using the Sysmex XE-2100 Automated Haematology System (Oxford, UK). Blood samples were taken pre-operatively into 3.2% sodium citrate vacutainers (BD Biosciences, Milton Keynes, UK). Platelet aggregation was measured using Multiplate multiple electrode aggregometry (MEA) (Roche, Rotkreuz, Switzerland), which detects change in electrical impedance when platelets aggregate on metal electrodes. Aggregation was determined by the area under the curve (AUC) in response to platelet agonists including adrenaline (100 mg/mL), thrombin receptor activator peptide 6 test (TRAP-test), ADP-test and arachidonic acid (ASPI-test). Use of a combination of agonists therefore reflects activation of platelets via different pathways: adrenaline acts at the α_2 adrenergic receptor, TRAP-6 acts at the protease-activated receptor-1 (PAR-1), ADP acts at the P2Y12 receptor and arachidonic acid at the Thromboxane A2 (TxA2) receptor.

3.3.9 COPTIC statistical analysis

The COPTIC dataset in the current analysis included 2,518 participants (23 out of 2,541 participants did not consent for future research). For the current study, only those who were not on antiplatelet therapies (prasugrel, clopidogrel or aspirin) were included (N=655). Extreme outliers that were ± 5 SDs from the mean were removed, and exposures and outcomes were rank normal transformed. Linear regression was used to generate estimates of the association between BMI and platelet parameters within this cohort. Linear regression was used to explore the association between immature platelet count and aggregation, adjusting for age, sex and smoking status.

Table 3.2: Characteristics of included INTERVAL participants for BMI-platelet analyses

Variable	Mean (SD) or %	N
Age	45.3 (14.2)	33388
Sex		33388
Male	49.8%	
Female	50.2%	
Weight (kg)	78.2 (16.0)	33455
Height (cm)	171.8 (9.6)	33388
Body mass index (kg/m ²)	26.4 (4.7)	33388
Smoking frequency		32867
Never	58.9%	
Previous	33.3%	
Current	7.8%	
Alcohol intake frequency		29538
Rarely	12.5%	
Less than once a week	17.1%	
One or two times a week	37.7%	
Three to five times a week / most days	32.7%	

3.4 Results

3.4.1 INTERVAL participant characteristics

Of INTERVAL participants included in the current study (N=33,388), 50.2 % were female. The mean age was 45.3 years (SD of 14.2 years, Table 3.2). The mean BMI was 26.4 kg/m² (SD of 4.7 kg/m²). The majority of participants were never smokers (58.9 %), with 33.3% and 7.8 % reported as previous and current smokers, respectively. Nearly a third (32.7 %) of participants reported drinking alcohol at least three times a week.

3.4.2 Correlation between platelet traits in INTERVAL

The Sysmex XN-1000 haematology analyzer measures multiple platelet traits, however many of these traits are closely related measurements and therefore may not be completely independent. Indeed, platelet traits showed a high degree of correlation with each other (Figure 3.4), in particular among similar measures. For example, measures of PLT (PLT I/F) and PCT, the latter a measurement of platelet mass, were highly positively correlated with each other but were weakly inversely correlated with other platelet measures. Measures of platelet maturity (IPF, IPC, H-IPF) were highly correlated with each other. In addition, platelet size variables (MPV, P-LCR and PDW) showed strong positive

correlations with each other as well as with measures of immature platelets.

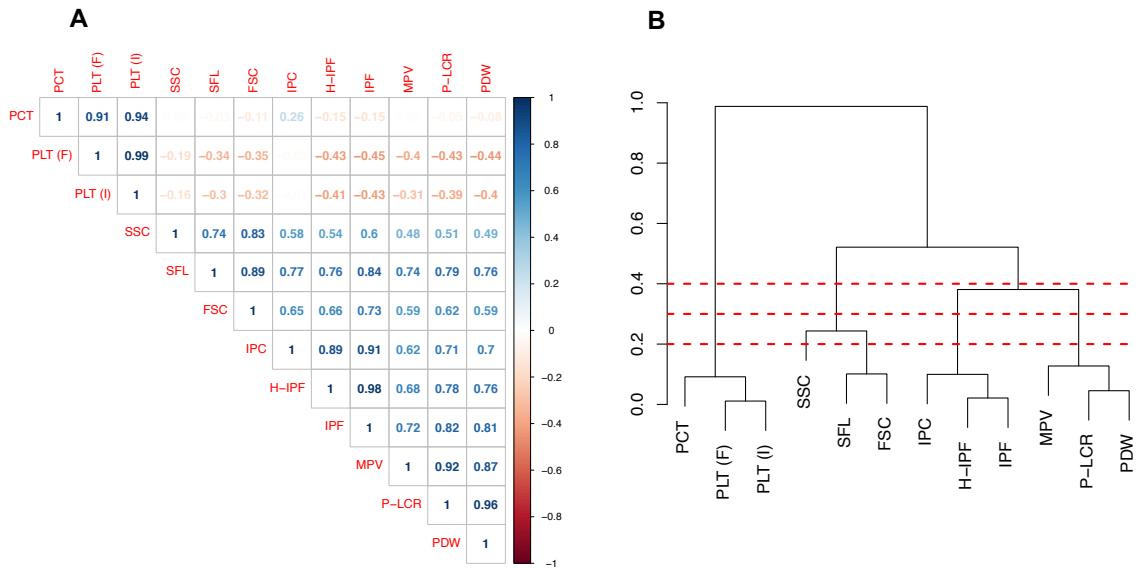


Figure 3.4: Correlation matrix and dendrogram of the relationship between platelet traits. A) Correlation coefficients are provided within the matrix. Dark blue indicates a correlation coefficient (r) of 1, with dark red indicating a correlation coefficient of -1. B) A dendrogram showing the hierarchical relationship between platelet traits, where the height at which two platelet traits join is $(1 - \text{Pearson correlation coefficient } r)$. PCT = plateletcrit, PLT (F) = platelet count (PLT-F channel), PLT (I) = platelet count (impedance channel), SSC = side scatter, SFL = side fluorescence, FSC = side scatter, IPC = immature platelet count, H-IPF = high fluorescence immature platelet fraction, IPF = immature platelet fraction, MPV = mean platelet volume, P-LCR = platelet large cell ratio, PDW = platelet distribution width. Dashed red lines indicate a height of 0.2, 0.3 and 0.4.

3.4.3 Observational associations between BMI and platelet traits in INTERVAL

Linear regression was performed to determine whether a higher BMI was associated with a change in platelet characteristics. In the confounder adjusted linear regression (Table 3.3), BMI was positively associated with PCT (0.12 SD higher per SD higher BMI, 95% CI 0.11 to 0.13, $P = 9.2 \times 10^{-88}$) and platelet count (PLT (I) 0.11 SD higher per SD higher BMI, 95% CI 0.09 to 0.12, $P = 1.0 \times 10^{-67}$). The next strongest association with BMI was the association with SFL (0.06 SD higher per SD increase in BMI, 95% CI 0.05 to 0.07, $P = 4.7 \times 10^{-23}$). BMI also showed a positive association with IPC (0.06 SD higher per SD increase in BMI, 95% CI 0.05 to 0.08, $P = 4.8 \times 10^{-22}$). These results demonstrate that BMI is positively associated with PCT, PLT (I), SFL and IPC in this population. These estimates were very similar to the age and sex only adjusted estimates (Table 3.4).

Table 3.3: Observational associations between BMI and platelet measures adjusted for age, sex, smoking status and alcohol consumption. β eta coefficient is the change in platelet measure in SDs per normalized SD increase in BMI. PCT = plateletcrit, PLT (I) = platelet count (impedance channel), PLT (F) = platelet count (PLT-F channel), SFL = side fluorescence, IPC = immature platelet count, PDW = platelet distribution width, FSC = forward scatter, H-IPF = high fluorescence immature platelet fraction, IPF = immature platelet fraction, P-LCR = platelet large cell ration, SSC = side scatter, MPV = mean platelet volume. Eta squared is the proportion of variance explained by the platelet trait in an ANOVA, whereas adjusted R squared is the variance explained by all the predictor variables in the regression model.

Platelet trait	N	β eta coefficient	SE	P value	R2	Eta2	95% CI	Lower 95% CI	Upper 95% CI
PCT	24562	0.12	0.006	9.17e-88	0.133	0.107	0.012	0.11	0.13
PLT (I)	26044	0.11	0.006	1.03e-67	0.096	0.081	0.012	0.09	0.12
PLT (F)	23921	0.1	0.006	5.62e-57	0.094	0.082	0.012	0.09	0.11
SFL	26082	0.06	0.006	4.73e-23	0.02	0.000	0.012	0.05	0.07
IPC	23909	0.06	0.007	4.84e-22	0.008	0.001	0.013	0.05	0.08
PDW	24563	0.03	0.007	5.21e-05	0.006	0.004	0.013	0.01	0.04
FSC	26082	0.02	0.006	0.01	0.026	0.001	0.012	0.01	0.03
H-IPF	26068	-0.02	0.006	0.01	0.006	0.005	0.012	-0.03	-0.01
IPF	23909	0.01	0.007	0.04	0.008	0.007	0.013	0.00	0.03
P-LCR	27015	0.01	0.006	0.19	0.003	0.000	0.012	0.00	0.02
SSC	26082	0.01	0.006	0.28	0.04	0.000	0.012	-0.01	0.02
MPV	24559	0.01	0.007	0.37	0.007	0.000	0.013	-0.01	0.02

Table 3.4: Age and sex adjusted estimates for the association between BMI and platelet traits.

β Beta coefficient is the change in platelet measure in SDs per SD higher BMI. Eta squared is the proportion of variance explained by the platelet trait in an ANOVA, whereas adjusted R squared is the variance explained by all the predictor variables in the regression model.

Full name	Short Name	N	Beta co-efficient	SE	P value	R2	Eta2	95% CI	Lower 95% CI	Upper 95% CI
Plateletcrit	PCT	27997	0.126	0.006	1.46e-108	0.132	0.107	0.011	0.115	0.137
Platelet count (impedance channel)	PLT (I)	29682	0.11	0.006	4.5e-84	0.097	0.082	0.011	0.099	0.121
Platelet count (PLT-F channel)	PLT (F)	27268	0.104	0.006	6.5e-70	0.094	0.083	0.012	0.093	0.116
Side fluorescence	SFL	29721	0.064	0.006	4.08e-28	0.021	0.000	0.011	0.053	0.076
Immature platelet count	IPC	27255	0.067	0.006	1.27e-27	0.007	0.001	0.012	0.055	0.079
Platelet distribution width	PDW	27998	0.029	0.006	2.09e-06	0.006	0.004	0.012	0.017	0.041
Forward scatter	FSC	29721	0.022	0.006	0.00014	0.026	0.001	0.011	0.011	0.034
High fluorescence immature platelet fraction	H-IPF	29708	-0.017	0.006	0.00443	0.005	0.005	0.012	-0.028	-0.005
Immature platelet fraction	IPF	27255	0.015	0.006	0.0129	0.007	0.007	0.012	0.003	0.027
Side scatter	P-LCR	29721	0.01	0.006	0.0781	0.041	0.000158	0.011	-0.001	0.022
Platelet large cell ratio	SSC	30788	0.01	0.006	0.0909	0.002	4.37e-05	0.011	-0.002	0.021
Mean platelet volume	MPV	27994	0.009	0.006	0.155	0.006	4.57e-06	0.012	-0.003	0.021

3.4.4 Associations of covariables with BMI and platelet traits in INTERVAL

The association of covariables (age, sex, smoking and alcohol) with BMI were evaluated (Table 3.5). Included covariables in the analysis showed associations with BMI. Males had a higher BMI than females (0.18 SD, 95% CI 0.16 to 0.21, $P = 9.64 \times 10^{-64}$). Age was positively associated with BMI (0.011 SD higher per year older, 95% CI 0.010 to 0.012, $P = 1.11 \times 10^{-182}$). Alcohol showed an inverse association with BMI and smoking showed a weak positive association with BMI. Associations between covariables (age, sex, smoking, alcohol) and platelet traits were also explored (Figure 3.5). Higher age was generally inversely associated with platelet measures. Males had a lower platelet count and PCT compared to females (PLT-F was 0.57 lower 95% CI -0.60 to -0.55, $P = 9.9 \times 10^{-324}$). Weak associations were detected between smoking and platelet traits such as a positive association between smoking status and immature platelets. Higher alcohol consumption also showed inverse associations with measures of PCT and PLT.

Table 3.5: Associations between age, sex, smoking and alcohol consumption (exposures) with standardised BMI (outcome). Beta coefficient is the change in BMI (normalized SDs) per unit increase in exposure.

Variable	N	Beta coefficient per 1-unit increase in confounder (SDs)	Standard error	P value	Adjusted R2	F statistic	95% CI	Lower 95% CI	Upper 95% CI
Sex (1=female, 2=male)	33388	0.184	0.011	9.64e-64	0.008	285	0.021	0.162	0.205
Age (years)	33388	0.011	3.81E-04	1.11e-182	0.025	841	0.001	0.01	0.012
Alcohol intake frequency (1=rarely, 2=<1 a week, 3=1-2 times a week, 4=most days/every day)	29538	-0.096	0.006	1.57e-61	0.009	275	0.011	-0.108	-0.085
Smoking frequency (1=never, 2=occasional, 3=most days/ every day)	32867	0.073	0.009	1.53e-17	0.002	72	0.017	0.057	0.09

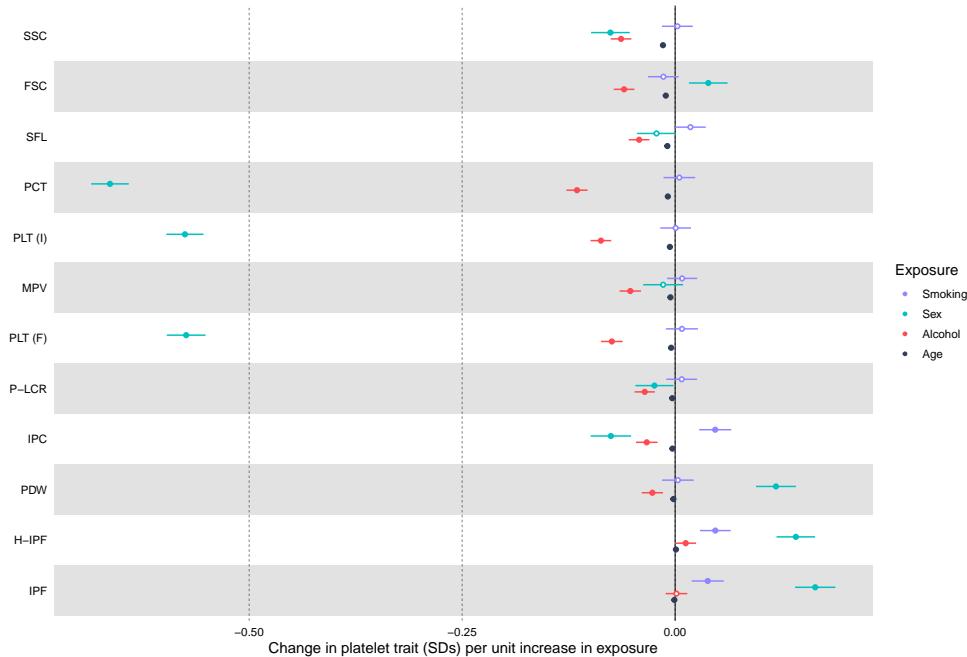


Figure 3.5: Linear regression results for the association between age, sex, smoking and alcohol with platelet traits. PCT = plateletcrit, PLT (F) = platelet count (PLT-F channel), PLT (I) = platelet count (impedance channel), SSC = side scatter, SFL = side fluorescence, FSC = side scatter, IPC = immature platelet count, H-IPF = high fluorescence immature platelet fraction, IPF = immature platelet fraction, MPV = mean platelet volume, P-LCR = platelet large cell ratio, PDW = platelet distribution width. For age: beta coefficient is the difference in platelet characteristics per 1 year increase in age. For sex: Beta coefficient is the difference in platelet measure in SDs in men compared with women (for sex, 1=female and 2=male). For smoking: beta coefficient is the change in platelet traits (SDs) per unit change in smoking category (1 = never, 2 = previous, 3 = current). For alcohol: beta coefficient is the change in platelet traits in SDs per unit increase in alcohol consumption (1=Rarely , 2= Less than weekly, 3=One or two weekly, 4= 3-5 weekly or every day)

3.4.5 GRS for BMI associations with BMI and covariates

To estimate the causal effect of BMI on platelet characteristics, MR was performed. The GRS for BMI (the average per SNP effect on BMI) showed a normal distribution (mean 0.08, SD 0.30, range -1.15 to 1.34) and was positively associated with BMI to the degree expected ($R^2 = 0.042$, $P = 2.3 \times 10^{-312}$, $F=1458$, Table 3.6). The GRS did not associate with sex, however weak associations were detected with alcohol consumption, age, and smoking status. The amount of variance explained by the GRS for BMI on any covariate included did not exceed $R^2 = 0.002$ (Table 3.6). As the GRS associates with BMI but does not strongly associate with the measured covariates, it is thus likely a valid instrument to perform MR.

Table 3.6: Association between genetic risk score for BMI with both BMI and covariates. Beta coefficient is the change in outcome variable per unit increase in the genetic risk score for BMI.

Variable	N	Beta coefficient (per 1-unit increase in GRS)	Standard error	P value	Adjusted R2	F statistic
BMI (SDs)	33388	0.69	0.018	2.3E-312	0.042	1458.4
Age (years)	33388	-1.46	0.26	2.2E-08	0.001	31.3
Sex (1=female, 2=male)	33388	-0.01	0.009	4.7E-01	-1.4E-05	0.5
Smoking frequency (1=never, 2=previous, 3=current)	32867	0.05	0.012	9.2E-05	4e-04	15.3
Alcohol intake frequency (1=rarely, 2=<1 a week, 3=1-2 times a week, 4=most days/every day)	29538	-0.15	0.02	2.9E-15	0.002	62.4

3.4.6 Mendelian randomization estimates for the association between BMI and platelet traits

In the MR analyses, BMI was associated with fewer traits than in the observational analysis (Table 3.7, 3.6). The causal estimate for the effect of BMI on SFL was 0.08 SDs per SD increase in BMI (95% CI 0.03 to 0.14, $P = 0.003$). This estimate was of larger magnitude than the observational estimate. The causal estimate for BMI and IPC was 0.06 SDs per SD increase in BMI (95% CI 0.006 to 0.12, $P = 0.03$), a similar magnitude of effect to the observational estimate. In the MR analysis, unlike in the observational analysis, the causal estimate did not suggest an effect of BMI on either PCT or PLT. MR estimates did not provide evidence for associations between BMI and other platelet variables. The point estimates were consistently positive, aligning with the positive observational associations seen across the platelet traits. Across all but one (H-IPF) of the platelet phenotypes we did not observe differences in directionality of effect comparing observational effects and causal effects predicted by Mendelian randomization (Figure 3.6). The Wu-Hausman test suggested that observational and MR estimates were similar for the majority of platelet traits ($P > 0.05$), except for measures of platelet count and plateletcrit ($P < 0.001$) (Table 3.7).

Table 3.7: Mendelian randomization estimates for the effect of BMI on platelet traits. Beta coefficient is the change in platelet measure in SDs per SD higher BMI.

Full name	Short Name	N	βeta co-efficient	SE	P value	R2	Eta2	95% CI	Lower 95% CI	Upper 95% CI	Wu-Hausman P value	F-statistic (GRS and BMI association)
Side fluorescence	SFL	29721	0.083	0.028	3.44E-03	0.132	0.107	0.056	0.027	0.139	0.973	676
Immature platelet count	IPC	27255	0.064	0.03	3.15E-02	0.097	0.082	0.058	0.006	0.122	0.672	641
Forward scatter	FSC	29721	0.052	0.028	6.62E-02	0.094	0.083	0.056	-0.003	0.108	0.774	652
Plateletcrit	PCT	27997	0.052	0.029	7.22E-02	0.021	0	0.057	-0.005	0.11	3e-04	706
Immature platelet fraction	IPF	27255	0.044	0.03	1.34E-01	0.007	0.001	0.058	-0.014	0.103	0.334	603
Platelet count (impedance channel)	PLT (I)	29682	0.035	0.028	2.19E-01	0.006	0.004	0.056	-0.021	0.091	0.001	729
Platelet large cell ratio	P-LCR	30788	0.034	0.028	2.25E-01	0.026	0.001	0.055	-0.021	0.088	0.527	678
Platelet distribution width	PDW	27998	0.035	0.029	2.29E-01	0.005	0.005	0.057	-0.022	0.093	0.936	625
Mean platelet volume	MPV	27994	0.029	0.029	3.23E-01	0.007	0.007	0.057	-0.028	0.086	0.746	614
Platelet count (PLT-F channel)	PLT (F)	27268	0.026	0.03	3.81E-01	0.041	0.000158	0.058	-0.032	0.084	0.001	671
Side scatter	SSC	29721	0.018	0.028	5.37E-01	0.002	4.37e-05	0.056	-0.038	0.073	0.433	661
High fluorescence immature platelet fraction	H-IPF	29708	0.015	0.028	6.10E-01	0.006	4.57e-06	0.056	-0.041	0.07	0.223	650

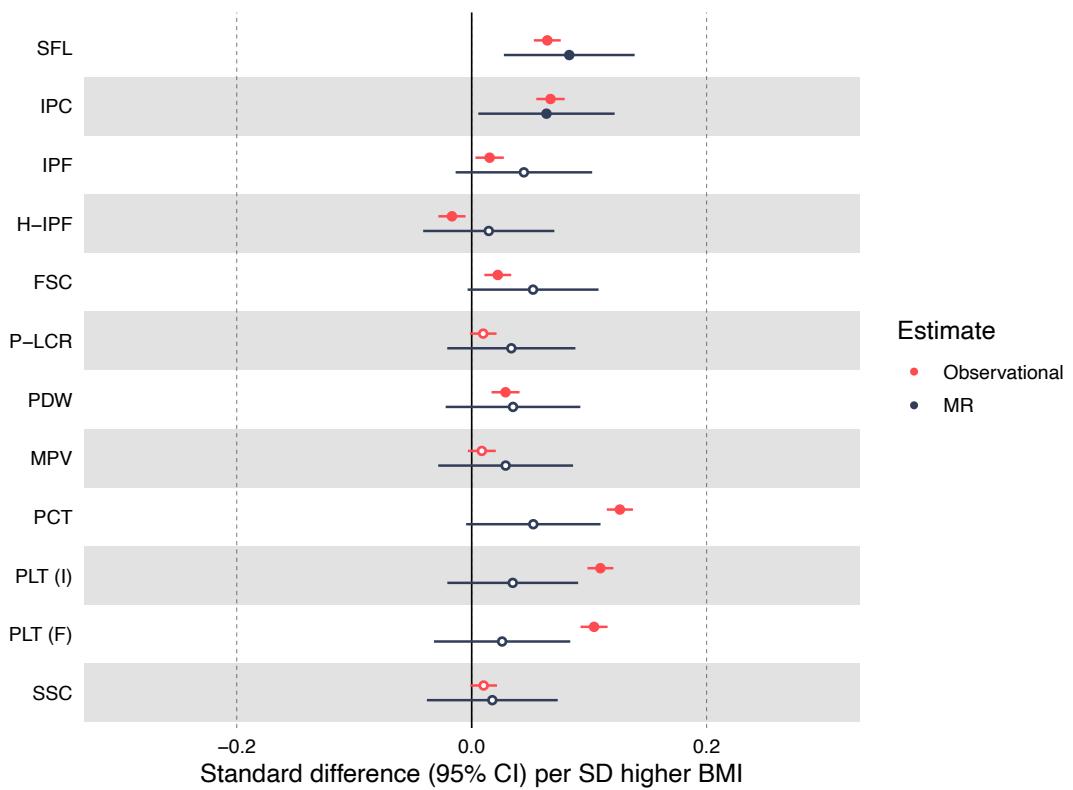


Figure 3.6: Forest plot of the Mendelian randomization (MR) and unadjusted observational estimates for the effect of BMI on platelet traits. Estimate points are filled where $P < 0.05$. SFL = side fluorescence, IPC = immature platelet count, H-IPF = high fluorescence immature platelet function, FSC = forward scatter, P-LCR = platelet large cell ratio, PDW = platelet distribution width, MPV = mean platelet volume, PCT = plateletcrit, PLT (I) = platelet count (impedance channel), PLT (F) = platelet count (PLT-F channel), SSC = side scatter.

3.4.7 Stratifying BMI and IPC associations by BMI group

We explored whether associations between BMI and IPC varied across NHS BMI groups (underweight $<18.5 \text{ kg/m}^2$, healthy weight $18.5\text{-}24.9\text{kg/m}^2$, overweight $25\text{-}29.9 \text{ kg/m}^2$, obesity $30+\text{ kg/m}^2$). IPC had consistent BMI effects across observational and MR analyses. Stratifying by BMI category suggested that the strongest association between BMI and IPC was the overweight category (Table 3.8).

Table 3.8: Associations between BMI and immature platelet count (IPC) stratified by NHS BMI category. Beta coefficient is the change in IPC (SDs) per SD increase in BMI

Adjustment	Group	BMI range	N	Beta coefficient	SE	P value	R^2	95%	Lower 95%	Upper 95%
								CI	CI	CI
Age and sex	Underweight	<18.5	169	0.202	0.834	0.461	-0.009	1.635	-1.434	1.837
Age and sex	Healthy	18.5-24.9	11889	0.039	0.018	0.026	0.003	0.035	0.005	0.074
Age and sex	Overweight	25-29.9	10189	0.092	0.034	0.006	0.006	0.066	0.027	0.158
Age and sex	Obese	30+	5008	0.032	0.032	0.315	0.004	0.063	-0.031	0.096
Fully adjusted	Underweight	<18.5	149	0.269	0.296	0.366	0.033	0.58	-0.311	0.849
Fully adjusted	Healthy	18.5-24.9	10500	0.033	0.019	0.079	0.004	0.037	-0.004	0.069
Fully adjusted	Overweight	25-29.9	8918	0.07	0.036	0.052	0.006	0.07	0	0.14
Fully adjusted	Obese	30+	4342	0.018	0.035	0.614	0.005	0.069	-0.051	0.086

3.4.8 Follow-up associations between IPC and whole blood aggregation in COP-TIC

Given evidence for a causal effect of BMI on IPC in the MR analysis, we sought to evaluate the relationship between IPC and platelet activity as a biological parameter of clinical relevance. Whilst this analysis could not be conducted in INTERVAL due to a lack of suitable data, we were able to utilize data from the COPTIC study to address this question. The COPTIC study is a cohort of cardiac surgery patients, with samples taken pre-surgery. These participants have whole blood aggregation measured, therefore making it possible to determine associations between IPC and aggregation in a clinical setting.

3.4.9 COPTIC participant characteristics

The total number of COPTIC participants was 2,541. Of these, 2,518 participants gave consent for future research (Table 3.9). Participants included in the analysis were those not on antiplatelet therapy (N=655). The majority of participants were male (61.8%), with a mean age of 63.9 years (SD of 16.1). Similar to the INTERVAL cohort, the mean BMI was in the overweight category (27.2 kg/m^2 with a SD of 4.9 kg/m^2). The majority of participants were either never smokers or ex-smokers for more than 5 years (89.2 %). The effect estimate for the effect of BMI on IPC was 0.06 (95% CI -0.01 to 0.14, P=0.09) and therefore consistent with that estimated in the INTERVAL cohort (Table 3.10).

Table 3.9: Characteristics of COPTIC study participants

Variable	Mean (SD) or % (all)	N (all)	Mean (SD) or % (included)	N (included)
Age	66.7 (11.9)	2502	63.9 (16.1)	655
Sex		2518		655
Female	24.9 %		38.2 %	
Male	75.1 %		61.8 %	
Body mass index (kg/m ²)	27.9 (4.6)	2422	27.2 (4.9)	649
Smoking		2469		654
Never	38.5 %		50.8 %	
Ex-smoker (>5 years)	47.4 %		38.4 %	
Ex-smoker (1-5 years)	8.3 %		6.3 %	
Ex-smoker (30 days to 1 year)	1.9 %		2.1 %	
Current smoker	3.9 %		2.4 %	
Antiplatelet medications		2467		655
0	26.6 %		100 %	
1	50.7 %			
2	22.5 %			
3	0.2 %			
ADP aggregation (AU)	138.2 (50.1)	2377	141 (46.5)	625
Adrenaline aggregation (AU)	53.0 (28.2)	2363	56.8 (32.3)	621
Arachidonic acid aggregation (AU)	86.9 (62.1)	2375	148.9 (52.4)	623
TRAP-6 aggregation (AU)	200.0 (50.1)	2373	198.7 (50.0)	623
Platelet count (x 10E9 /L)	209.8 (60.5)	2409	200.9 (57.6)	637
Mean platelet volume (fL)	10.6 (1.0)	2400	10.6 (0.98)	634
Immature platelet fraction (%)	3.2 (1.9)	2379	3.3 (2.0)	628
Immature platelet count (x 10E9/L)	6.3 (3.4)	2383	6.2 (3.3)	629

Table 3.10: Association between BMI and platelet parameters in COPTIC (adjusted for age, sex, smoking). Beta coefficient is the change in platelet measure ins SDs per SD higher BMI.

Outcome	N	Beta coefficient	SE	P value	R ²	95% CI	Lower 95% CI	Upper 95% CI
MPV	627	0.08	0.04	0.04	0.003	0.07	0.00	0.15
IPC	622	0.06	0.04	0.09	0.013	0.07	-0.01	0.14
PLT	630	0.04	0.04	0.32	0.071	0.07	-0.03	0.1
IPF	621	0.03	0.04	0.36	0.014	0.07	-0.04	0.11

3.4.10 Association between IPC and aggregation in COPTIC cohort

To determine the potential functional effects of variation in IPC, the COPTIC cohort was used to assess the observational association between IPC and whole blood platelet aggregation in response to a range of platelet agonists (Figure 3.7A). In participants who were not on antiplatelet therapy (up to N=655), there was evidence for a positive association between IPC and aggregation induced by adrenaline (0.13 SD increase per SD increase in IPC, 95% CI 0.04 to 0.21, $p=3.7\times10^{-3}$), TRAP-6 (0.11 SD increase per SD increase in IPC, 95% 0.03 to 0.18, $p=7.8\times10^{-3}$) and ADP (0.08 SD per SD increase in IPC, 95% 0.01 to 0.015, $p=0.04$). As there was a high correlation among platelet traits (Figure 3.7A, Figure 3.7B), the effect of MPV, PLT and IPF were also assessed. PLT was associated with all four measures of aggregation, with a larger effect estimate than IPC (Figure 3.7A). The finding that IPC does not correlate with PLT however suggests that the effect of IPC on platelet aggregation is independent of the effect of PLT on platelet aggregation (Figure 3.7B). Fitting PLT alongside IPC as an additional predictor of aggregation had little effect on the IPC effect estimate providing further evidence of independent contributions from the two traits (Figure 3.7C). Adjustment for PLT in the regression model for the association between IPF and aggregation provided estimates of a similar magnitude to that of IPC and aggregation.

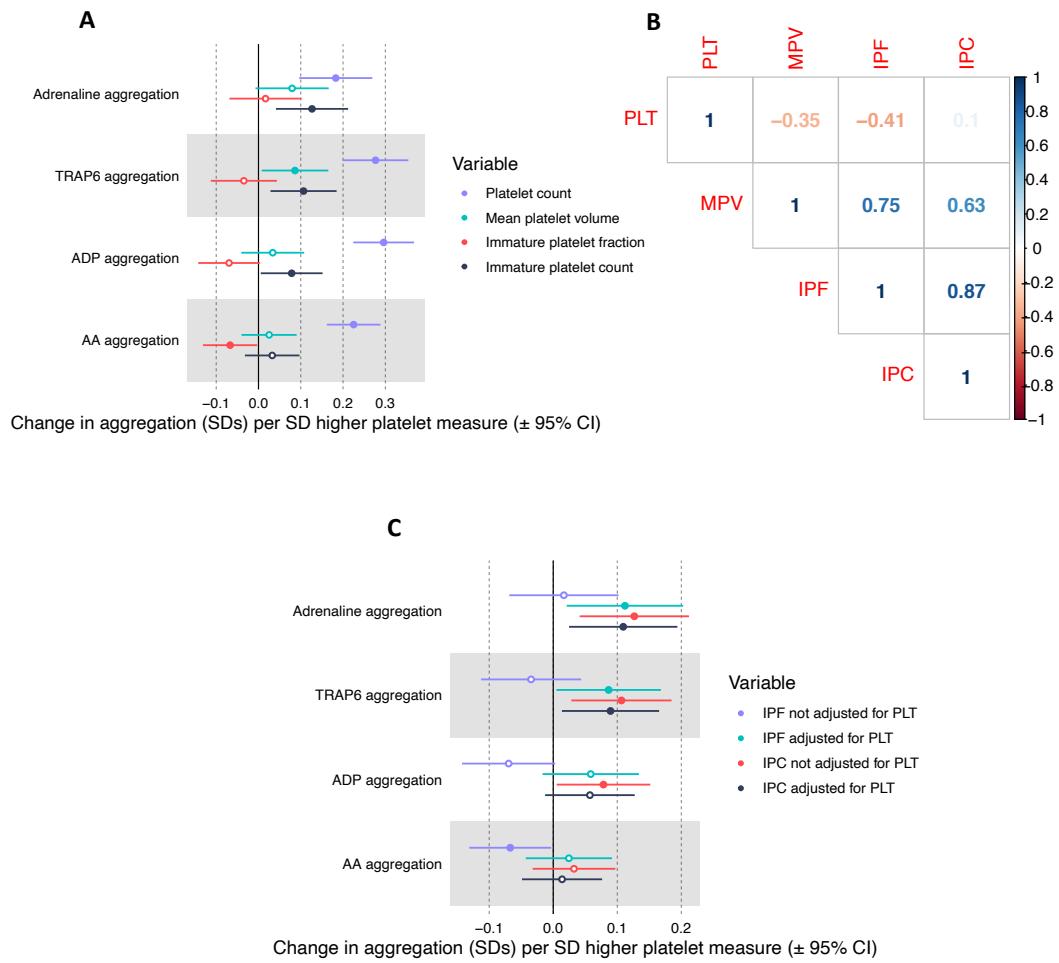


Figure 3.7: Association between platelet measures and whole blood platelet aggregation in COP-TIC trial participants. A) Forest plot displaying the association between platelet measures (exposure) and aggregation (outcomes). B) Correlation matrix displaying the Pearson's correlation coefficient (r) between platelet traits. C) Forest plot displaying the effect estimate for the association between immature platelet count (IPC) or immature platelet fraction (IPF) and measures of aggregation, with or without adjustment for platelet count (PLT). ADP = adenosine diphosphate, AA = arachidonic acid, MPV = mean platelet volume.

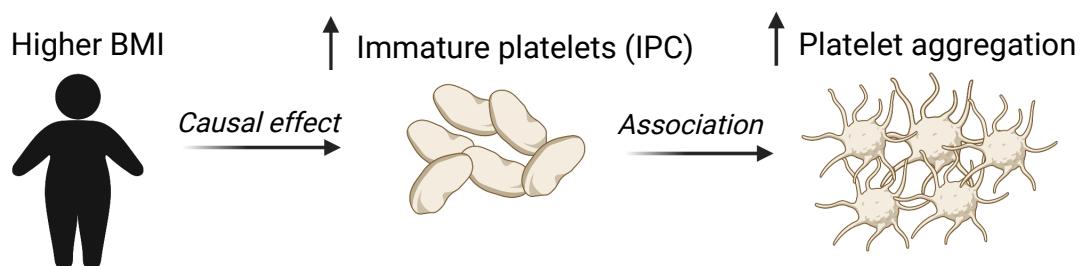


Figure 3.8: Graphical summary of findings

3.5 Discussion

In this study we used data from 33,388 healthy blood donors to study the effect of BMI on platelet phenotypes including measures of count, size and maturity. The combination of observational and MR estimates suggested a positive causal effect of BMI on SFL and IPC. The observational analysis revealed a strong association between BMI and both PLT and PCT, however the MR estimates did not provide evidence to support this association as causal. Observational analysis using data from a cardiac surgery cohort provided evidence for a positive association between IPC and aggregation induced by adrenaline, TRAP6 and ADP.

The observational and MR analyses in the current study provide evidence for a positive association between BMI and both IPC and SFL. Stratifying by NHS BMI category suggested that this estimate is strongest in the overweight category. As IPC is derived using SFL, the measure of mRNA content, these measures are highly correlated. Whilst few studies have explored the direct association between BMI and immature platelet measures, there is some evidence for increased immature platelets in subjects with metabolic syndrome (MS) and subjects with type II diabetes compared with control subjects^{100,101}. In these studies, participants with MS or type II diabetes also had a higher BMI, which could be a possible cause of this increase in immature platelets. Indeed, evidence from our analysis suggests that BMI, independent of metabolic syndrome or type II diabetes, may causally influence IPC and SFL.

As IPC is an indirect measure of platelet production⁹⁷, these results suggest that an increased BMI may lead to an increased production of platelets. It has been reported that immature platelets have increased thrombotic potential^{97,109} and may be predictive of cardiovascular events^{43,69}, suggesting that an increase in the number of immature platelets may be of functional and clinical relevance. A follow-up analysis confirmed that higher IPC was associated with greater platelet aggregation independent of absolute platelet count within a cardiac surgery cohort. Positive associations were found between IPC and aggregation induced by adrenaline, TRAP-6 and ADP, suggesting general platelet hyperactivity with more immature platelets. A previous study in a coronary artery disease cohort also found a positive correlation between IPC and aggregation induced by arachidonic acid, collagen and ADP¹⁰⁹. These findings suggest that IPC can be used as a proxy for platelet hyperactivity within a cohort with a history of cardiovascular disease. Measures of immature platelets are currently not considered in the clinic when prescribing antiplatelet therapy, however if a higher immature platelet count is demonstrative of newly produced, hyperactive platelets then this could be important in guiding dosage regimes to ensure

sufficient platelet inhibition⁹⁸. Observational studies have also found that patients with COVID-19 have elevated immature platelet counts and fraction, which could partly explain high rates COVID-19 induced thrombosis¹¹¹. Despite the association between BMI and IPC, there was less evidence for an effect of BMI on IPF. This lack of association may be because IPF is the proportion of immature platelets, therefore if someone had a higher number of immature platelets, but also a higher number of platelets overall (for example due to increased platelet lifespan), there would be no increase in the immature platelet fraction.

In general, previous studies have reported positive observational associations between BMI and both PLT and PCT⁶⁴. More direct measures of body fat such as total fat mass, waist-hip ratio and waist circumference have also been reported to be positively associated with both PLT and PCT⁶⁵; the observational evidence from the current study is in agreement with this. The MR analysis did not detect causal effects of BMI on measures of PLT/PCT, however the estimates were consistently positive. It is possible that IPC could be raised in absence of raised PLT as platelets may be produced at an increased rate, but could also be being destroyed at an increased rate. The small P values in the Wu-Hausman test for endogeneity for plateletcrit and platelet count variables support that the observational and MR estimates are different. Estimates could be biased due to confounding factors, reverse causation or other sources of bias. Possible routes of confounding could include stress, inflammation and nutrition, which could exert independent effects on both BMI and platelet count.

Both observational and MR estimates suggested that BMI is not associated with measures of platelet size, such as MPV and P-LCR. There are conflicting findings in the literature, with some studies suggesting that there is a positive association between BMI and MPV⁶⁶ and others showing no association^{64,65,67}. The results of the current study suggest that associations seen previously between BMI and MPV are likely due to confounding of observational estimates. The lack of association between BMI and MPV may be surprising given a correlation coefficient of 0.62 between IPC and MPV. As BMI was positively associated with immature platelet count, and immature platelets tend to be larger, it may be surprising that BMI was not associated with measures of platelet size. However, immature platelets make up a small percentage of overall platelets (~3-6 %)^{69,98}, therefore the increase in immature platelets may not affect the overall median size of the whole platelet population.

Although this study suggests potential effects of BMI on platelet traits such as SFL and IPC, it does not provide mechanistic insight into how BMI exerts these effects. Previous studies have suggested that inflammation driven by adiposity can stimulate megakaryocyte proliferation, thereby increasing platelet

numbers⁶⁵. There is evidence that inflammatory mediators such as interleukin-6 (IL-6) could be one such factor¹¹². Further study would be warranted to explore these mechanisms, as well as replicate the current findings, such as through independent population or clinical studies.

3.5.1 Limitations

There are a few limitations to the study that should be recognised. Firstly, BMI was derived from self-reported height and weight. Although there is potential for this to bias observations, previous studies have found that self-reported BMI and BMI measured in the clinic are strongly associated¹¹³. The GRS also associates with BMI to the extent expected. Secondly, there may be other confounders which were not recorded within INTERVAL and therefore could not be accounted for in our models, such as, socio-economic position, which may affect both BMI and risk of thrombosis. Therefore, residual confounding of observational estimates cannot be ruled out. Whilst the INTERVAL study provided relatively precise estimates of effect overall, larger sample sizes within each BMI category would be required to further investigate potential nonlinearity in the relationship between BMI and IPC. With respect to the observational analysis conducted in COPTIC, the sample size is modest, which may limit power to detect associations. Furthermore, this cohort required cardiac surgery and therefore it is possible that associations found may not be generalizable to the wider population, as participants also displayed greater aggregatory responses than the reference ranges¹¹⁴. However, these findings do indicate that immature platelets may be a biomarker of platelet hyperactivity in patients with a history of cardiovascular disease. It is important to note that the anticoagulant used (EDTA) has been reported to affect platelet parameters, such as increasing MPV and decreasing PLT¹¹⁵. Despite this, EDTA is the standard anticoagulant used for full blood counts in the NHS. The Sysmex analyzers used in INTERVAL and COPTIC were different models, however, it has been shown that there is strong association between platelet measures between the XE and newer XN analyzers¹¹⁶.

Altogether, we show observational and MR evidence that an increased BMI is associated with an increase in number of immature platelets. Observational evidence indicates that higher immature platelet count is associated with enhanced aggregation in a cardiac surgery cohort. Together, these results indicate that higher BMI may enhance platelet function and thrombosis by increasing platelet production and immature platelet count.

Chapter 4

The effect of obesity on platelet function: a clinical pilot study

The following chapter reports a specifically designed patient study. Recruitment into this study has been severely affected by the pandemic as approvals were set in place to start recruitment in March 2020. Further details of the initial plans for this study are provided in the discussion chapter (Chapter 9). The platelet-neutrophil assay data was performed by Dr Chris Williams and the Sysmex haematology analyzer data was collected by Dr Kate Burley. Help was required here due to time constraints when receiving patient samples. Proteomic data were generated by Dr Marieangela Wilson and Dr Kate Heesom at the Proteomics facility at the University of Bristol. Analytical support was provided by Dr Phil Lewis from the Proteomics facility. Optimised methods have been used within a study to explore the effect of COVID-19 on platelet function given the increased risk of thrombosis. The COVID-19 research has been written up as a manuscript but it has not been included in the thesis due to not aligning with the current aims.

4.1 Background

As detailed in Chapter 3, there is evidence that people with a higher BMI have an increased risk of cardiovascular events such as coronary heart disease (CHD) or stroke^{11,28,94}. As platelets are one of the key cells involved in haemostasis and thrombosis²⁵, it is likely that a higher BMI has a direct effect on platelet function. The previous chapter provided evidence that higher BMI causes an increase

in the production of immature platelets, evidenced by an increase in immature platelet count (IPC). IPC was also positively associated with whole blood platelet aggregation in response to various platelet agonists (adrenaline, protease activating receptor 1 peptide (PAR1-AP/TRAP-6) and adenosine diphosphate (ADP)) in a cardiac surgery cohort. Although these analyses suggest that higher BMI leads to platelet hyperactivity, this association was not directly assessed due to the limited platelet function data available. In COPTIC, the only platelet function data was whole blood platelet aggregation; there are other platelet function assays which may provide important information about the platelets activation. COPTIC was also a cohort which had a history of cardiovascular disease, therefore associations could be confounded by the presence of cardiovascular disease.

Upon activation, platelets adhere to the vessel endothelium, where they undergo shape change, release their granules and aggregate. There are numerous laboratory techniques that can be used to measure platelet function¹¹⁷. Experiments include flow cytometry which use fluorescently tagged antibodies that bind to platelet receptors. Some receptors increasingly expose their ligand binding sites upon stimulation such as the integrin $\alpha_{IIb}\beta_3$, which is important for aggregation of platelets (Figure 4.1). The adhesion receptor P-selectin is expressed on α -granules and is therefore only expressed when platelet secrete their α -granules. Platelet agonists which are able to further expose the integrin $\alpha_{IIb}\beta_3$ include PAR1-AP and collagen-related peptide (CRP). Other experiments commonly used to test platelet function include light transmission aggregometry (LTA), which measures the degree of platelet aggregation in response to platelet agonists by how much light passes through the sample. Techniques such as LTA are difficult to implement with patient samples due the requirement of a substantial amount of sample and due to experiments being time consuming. As platelet function declines over a few hours after removal from circulation¹¹⁸, it is preferable to opt for experiments which can be performed quickly. It is also possible to explore the intracellular signalling which occurs within the platelets by using techniques such as Western blotting and tandem mass tag mass spectrometry (TMT-MS), which use targeted and untargeted methods, respectively, to measure protein or phosphoprotein levels.

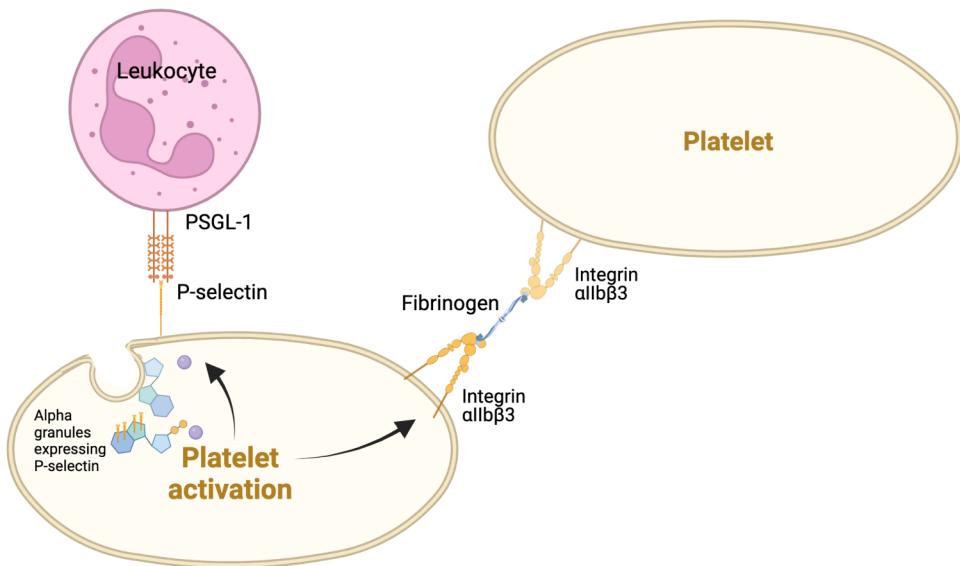


Figure 4.1: Cartoon of integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression on platelets. Upon platelet activation by a strong agonist such as CRP, the integrin $\alpha_{IIb}\beta_3$ exposes its binding site for fibrinogen and cross-links platelets. P-selectin is expressed in the membrane of alpha granules within platelets. Upon platelet activation, platelets secrete these granules and P-selectin is then expressed on the outer membrane. P-selectin on platelets can bind to PSGL-1 leukocytes. Figure made using BioRender.com.

Previous studies have attempted to determine the direct effects of BMI on platelet function and signalling, where there is conflicting evidence regarding how BMI affects platelet function^{44,49,50,96}. Such studies have focused on the relationship between BMI and platelet function in patients receiving dual antiplatelet therapy (DAPT). In these studies there is evidence that in people with higher BMI, antiplatelet drugs such as prasugrel, which acts at the P2Y₁₂ receptor, may not be as effective at suppressing platelet activity⁴⁹. These findings have come from measuring phospho-VASP as an indicator of platelet function at the P2Y₁₂ receptor. PGE1 suppresses platelet function by acting at a G_s coupled receptor, causing a rise in cAMP and phospho-VASP. Activation of the P2Y₁₂ receptor, which is G_{i/o} coupled, by the platelet agonist ADP reduces this VASP phosphorylation. Lower phospho-VASP levels are therefore indicative of less inhibition by the antiplatelet drug and more activation of the platelet by ADP. It is less clear what the relationship is between BMI and platelet function in those who are not on any antiplatelet therapy and across other platelet function assays.

The aims of this chapter were to:

- 1) Optimise laboratory techniques to assess platelet function in bariatric patients pre-surgery (BMI)

$>40\text{kg}/\text{m}^2$) and healthy controls (BMI 18-25 kg/m^2).

- 2) Determine whether high BMI is associated with an alteration in platelet function and signalling (Figure 4.2).

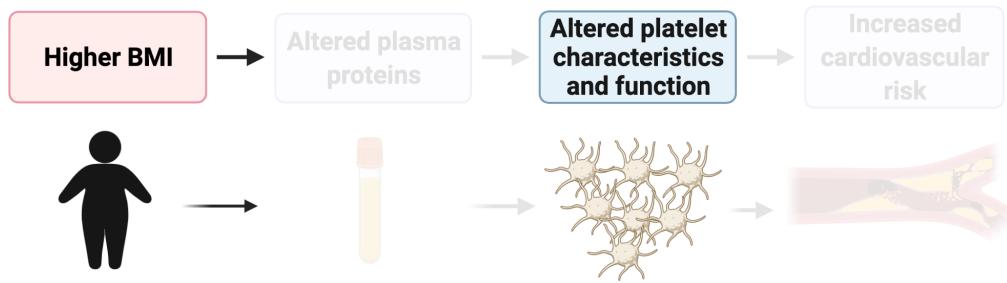


Figure 4.2: Schematic of the associations explored: this chapter explores the relationship between body mass index and platelet function. Figure made using BioRender.com.

4.2 Materials and Methods

4.2.1 Materials

Protease-activated receptor 1 (PAR-1)-activating peptide (TRAP-6/SFLLRN-NH₂) was from Bachem (Bubendorf, Switzerland), crosslinked collagen-related peptide (CRP-XL) from Prof. Richard Farndale (Department of Biochemistry, University of Cambridge, UK). Adenosine diphosphate (ADP) was from Sigma-Aldrich (Poole, UK). Complete mini protease inhibitor tablets and phosSTOP phosphatase inhibitors were from Roche Life Sciences (Welwyn Garden City, UK). The Pierce bicinchoninic acid (BCA) assay was from ThermoFisher Scientific (Altrincham, UK). Sodium Citrate Vacutainer® tubes, FixLyse, PE-Cy5-conjugated mouse anti-human CD42b antibody, FITC mouse anti-human CD61 antibody, Fc block, PE mouse anti-human platelet GPVI antibody, FITC mouse anti-human PAC-1 and PE mouse anti-human CD62P antibodies were from BD (Wokingham, UK). FITC mouse anti-human CD41 and

CD42b antibodies were from BioLegend (London, UK). Human MDC (69 amino acids) and TARC were from PeproTech (London, UK). Indomethacin, PGE1, Triton X-100, apyrase, HEPES Tyrode's (10 mM HEPES, 1 mM magnesium, 0.5 mM monosodium phosphate, 145 mM sodium chloride, 3 mM potassium chloride, pH 7.2), 2-butanol, ethanol, methanol and bovine serum albumin (BSA) were purchased from Sigma-Aldridge (Poole, UK). Phosphate buffered saline (PBS), trisodium citrate, acid citrate dextrose (ACD), D-glucose, ammonium persulfate (APS), Tris buffered saline (TBS), polysorbate 20 (Tween 20), Donkey anti-Rabbit IgG (H+L) Highly Cross Adsorbed Secondary Antibody Alexa Fluor 647 and Fura-2 were purchased from Thermo Fisher Scientific (Loughborough, UK). 30 % acrylamide mix (37.5:1), 1.0 M Tris pH 6.8 solution and 1.5 M Tris pH 8.8 solution purchased from National Diagnostics (Nottingham, UK). Immobilon-FL PVDF membranes (0.45-micron filter) were purchased from Merck Millipore (Hertfordshire, UK). Sodium dodecyl sulphate (SDS) was purchased from GE Healthcare worldwide (Hatfield, UK). SDS solution (10 %), tetramethylethylenediamine (TEMED), filter paper and Precision Plus protein pre-stained standards were purchased from Bio-Rad laboratories (Bredbury, UK). Blocking buffer was purchased from LI-COR Biosciences (Cambridge, UK). Rabbit pSer239 VASP and pSer157 VASP antibodies and pSer425 Talin were purchased from Cell Signaling Technology (Hertfordshire, UK). Alexa Fluor 680 AffiniPure Donkey Anti-Goat IgG and Alexa Fluor 680 AffiniPure Donkey Anti-Rabbit IgG were obtained from Jackson Immunoresearch (Cambridgeshire, UK). Paraformaldehyde was purchased from Acros Organics (Geel, Belgium). Annexin V Alexa Fluor 568 conjugate was from ThermoFisher. The inhibitor AZD-2908 was from Bio-Techne, YM-284890 was from Tebu-Bio Ltd and Y27632 was from Generon Ltd. Tween-20 (Sigma G02062) and hydrogenated gelatine (Sigma P1379) were from Sigma-Aldrich (Poole, UK).

4.2.2 Ethical approval process

This study was designed in July 2019. To be able to carry out a patient study, there were various documents that needed to be prepared. These were submitted using the Integrated Research Application System (IRAS). Documents included were a research protocol, statistical review, IRAS form, consent forms and participant information sheets for bariatric patients and control subjects, GP letters and a copy of the email invite sent out to control participants. Upon ethical approval from the London Riverside Research Ethics Committee (REC reference 19/LO/1781) and approval from Southmead Hospital, this study was first approved to start recruitment in March 2020. Recruitment was put on hold until May 2021 due to the pandemic. During this time, two amendments were made to be able to account for: 1) whether the participant had previously had a COVID-19 infection and 2) whether the participant had

been vaccinated for COVID-19 due to reports that COVID-19 infection and the AstraZeneca vaccine lead to an increased risk of thrombotic outcomes^{119,120}. The second amendment was not approved by Southmead Hospital in time and therefore the vaccination status could not be recorded for bariatric patients.

4.2.3 Study population

All participants provided written informed consent. Patients, who were due to undergo bariatric surgery, with a body mass index of $>40\text{kg}/\text{m}^2$, were recruited from the Department of Bariatric and Metabolic Surgery at Southmead Hospital, Bristol. Control participants with a BMI between 18.5 and 25 kg/m^2 were recruited from the University of Bristol. Inclusion criteria for participants were: aged 18 years and over, able to give written informed consent and sufficient level of English to understand the study and ask any questions. Participants were excluded if they were pregnant or lactating, had any known clotting or bleeding disorders (e.g. von Willebrand disease or drug-induced thrombocytopenia), or if they had taken any antiplatelet medication within the previous 14 days such as clopidogrel, ticagrelor or aspirin or any other non-steroidal anti-inflammatory drug (NSAID). Recruitment occurred between May 2021 and June 2021. As a pilot study, a total of four bariatric patients with obesity and four age and sex-matched control participants were recruited. Bariatric patients donated blood before bariatric surgery.

4.2.4 Isolation of platelet rich plasma (PRP)

Blood was taken by venipuncture into vacutainers containing sodium citrate (3.2%). Blood was centrifuged (1000 RPM, 17 mins). PRP was diluted 1:40 in HEPES Tyrode's (145 mM NaCl, 3 mM KCl, 0.5 mM Na₂HPO₄, 1 mM MgSO₄.7H₂O, 10 mM HEPES, pH 7.4, 0.1% [w/v] D-glucose) for experiments where indicated.

4.2.5 Platelet lysates

ACD (1:7) and apyrase (0.02 U/mL) were added to PRP. PRP was centrifuged (1700 RPM, 10 mins) to pellet platelets. Platelets were resuspended in CGS buffer (120 mM NaCl, 25.8 mM sodium citrate dihydrate, 0.1% [w/v] D-glucose, 0.02 U/mL apyrase, pH 6.5) Platelets were centrifuged again (1700 RPM, 10 mins) to pellet platelets and were resuspended in CGS buffer. Platelets were pelleted and resuspended in radioimmunoprecipitation assay buffer (RIPA: 25 mM HEPES, 200 mM NaCL, 1mM

EDTA, 1 % (v/v) NP40, 0.5 % (w/v) sodium deoxychelate, 0.1 % (w/v) SDS) supplemented with a protease and phosphatase inhibitor for 10 minutes on ice. Lysates were centrifuged (10000 RPM, 4°C for 5 minutes). Supernatant was removed and a bicinchoninic acid (BCA) assay was performed to calculate sample protein concentrations. Samples were stored in a -80°C freezer until proteomics analysis.

4.2.6 Platelet parameters measured by Sysmex

Citrated whole blood (200 uL) was analysed using the Sysmex XN-20 haematology analyser. Platelet readouts were platelet count (PLT) ($\times 10^9/L$), immature platelet fraction (IPF) (%), immature platelet count (IPC) ($\times 10^9/L$), side fluorescence (SFL, a measure of mRNA content in relative fluorescent units/RFUs), forward scatter (FSC, a measure of platelet size measured in RFUs) and side scatter (SSC, a measure of granularity in RFUs).

4.2.7 Freeze-dried agonist plates

Agonist was freeze dried onto plates to reduce day-to-day variation in agonist concentrations, as described previously.¹²¹ 96 well plates were coated with 10 mM PBS with 0.75% w/v hydrogenated gelatine and 0.05% v/v Tween-20 with a pH of 0.74 and left for 4 hours at room temperature. Plates were washed with dH₂O with 0.01% v/v, followed by two washes with dH₂O and left to dry in a fume hood. Agonist was added to the plates (5uL per well at 10x final concentration): agonists used were protease-activated receptor 1 activating protein (PAR1-AP), adenosine diphosphate (ADP) and collagen related peptide (CRP). Plates were then stored in a -80°C freezer for 1 hour, then placed into the freeze dryer (-50°C) and left overnight. Once freeze-dried, plates were vacuum packed.

4.2.8 Integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression measured by flow cytometry

Isolated PRP was diluted 1:40 in HEPES Tyrode's. FITC-conjugated PAC1 and PE-conjugated CD62P antibodies were added in a 2:1 ratio (5uL PAC1 and 2.5uL CD62P per well). This master mix was added to pre-prepared vacuum packed 96 well plates with freeze-dried agonist to a final volume of 50 uL. HEPES Tyrode's was also freeze-dried onto plates, which was used for a measurement of unstimulated integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression. Platelets were stimulated with agonists for 10 minutes. Platelets were fixed with 1% paraformaldehyde (PFA) for 10 minutes. Platelets were gated

and the median fluorescence intensity (MFI) in arbitrary units (AUs) was reported from a total of 10,000 events.

4.2.9 Surface receptor levels measured by flow cytometry

Diluted PRP (1:40) was used for unstimulated surface receptor detection. Antibodies were added (1:10 v/v) to a final volume of 50 uL. Antibodies used include anti-human CD41 (α_{IIb}), CD42b (GP1b α), CD61 (β_3) (FITC-conjugated) and GPVI and CD110 (thrombopoietin/TPO receptor) (PE-conjugated). Platelets were fixed with 1% PFA for 10 minutes. Platelets were gated and the MFI was reported in AUs from a total of 10,000 events.

4.2.10 Platelet-neutrophil assay measured by flow cytometry

Citrated whole blood was diluted 1:10 in HEPES-Tyrode's buffer and incubated with FITC-conjugated anti-CD41 and PE-conjugated anti-C45 antibodies at room temperature for 15 mins (antibodies used 1:10 v/v). Samples were either stimulated (+ 5 ug/mL CRP) or unstimulated (+ vehicle) for the same 15 minute duration. Samples were fixed with FixLyse for 10 minutes, followed by a final concentration of 4 % PFA for 10 minutes. Fluorescence was quantified using the flow cytometer, where 1000 neutrophil events were analysed based on a gating of CD45 and SSC parameters. Aggregates of platelets and neutrophils were defined by being both CD41+ and CD45+.

4.2.11 Tandem Mass Tag Mass Spectrometry (TMT-MS) quantification of platelet proteins

Platelet lysate samples (50 ug total protein) were digested with trypsin and labelled with TMT 15 plex reagents (ThermoFisher Scientific, Loughborough, UK). Labelled samples were pooled. The pooled sample was evaporated to dryness then resuspended in formic acid (5%). The pooled sample was then desalted using a SepPak cartridge (Waters, Milford, Massachusetts, USA). Eluate from the SepPak cartridge was evaporated to dryness and resuspended prior to fractionation by high pH reversed-phase chromatography using an Ultimate 3000 liquid chromatography system (Thermo Fisher Scientific). Further fractionation occurred using high pH RP fractions using the Ultamte 3000 nano-LC system in line with an Orbitrap Fusion Tribrid Mass Spectrometer (ThermoFisher Scientific). Further details of the methods used for mass spectrometry within the proteomics facility have been previously published¹²².

4.2.12 Statistical analysis for platelet function assays

Data was analysed using GraphPad Prism 8. All data points are presented where possible to be fully transparent. If data displayed a normal distribution (based on Shapiro-Wilk p-value and W statistic), a parametric test was used (e.g. unpaired t-test or one way ANOVA), otherwise a nonparametric test was used. Concentration-response curves were plotted using a four parameter variable slope. To compare concentration-response curve parameters, pEC50s or curve maxes were compared. A Fisher's exact test was used to compare proportions of categorical variables. Statistical tests used for each experiment are provided in figure legends. P-values for results are presented throughout, however these were not dichotomised as either 'significant' or 'not significant'¹²³ as the current study has low power and the groups are unlikely to be representative of the respective populations. A P-value threshold of 0.05 may therefore not be appropriate in this case.

4.2.13 Analysis of proteomic data

A principal component analysis (PCA) was performed to reduce the data and to identify major axes (principal components, PCs). This method helps to determine whether the major axes of the data can likely be explained by the group (bariatric patient with obesity or control participant). Normalised protein abundances were log₂ transformed, where the logFC was calculated by subtracting the log₂ normalised protein abundance of the control subjects from the bariatric surgery group. The log₂ normalised protein abundances of bariatric patients were compared to controls using a two-tailed unpaired t-test. P-values were not adjusted for multiple testing, due to the observation that false discovery rate (FDR) adjustment may be too blunt in low power proteomics experiments¹²⁴. P-values of all comparisons are provided in the supplementary results in Chapter 4. (FDR) adjusted p-values are also provided in the full results as a reference. A positive logFC indicates an increase in protein level in the bariatric samples.

4.3 Results

4.3.1 Participant characteristics

The characteristics of participants included in the study are shown in Table 4.1. The mean BMI in the control group was 21.8 kg/m² (SD 2.1 kg/m²) and 49.3 kg/m² (SD 8.8 kg/m²). Participants in the control group and bariatric patient group were age and sex matched. All bariatric patients and control participants were non-smokers. Half of the bariatric patients presented with type 2 diabetes (T2D) and

Table 4.1: Characteristics of included participants in the body mass index and platelet function study

Variable	Control mean (SD) or %	Bariatric patient mean (SD) or %	P value for difference
Age	42.2 (14.1)	46 (18.0)	0.77
Sex	75 %	75 %	1
Body mass index (kg/m2)	21.8 (2.1)	49.3 (8.8)	9e-04
Smoker	0 %	0 %	1
Hypertensive (140/90 mmHg)	0 %	25 %	1
Type 2 Diabetes	0 %	50 %	0.43
Type 2 Diabetes medication	NA		NA
Metformin		25 %	
Dapagliflozin		25 %	
Dulaglutide		25 %	
Previous COVID-19 infection	0 %	75 %	0.14

were receiving T2D medication, whereas none of the control participants had T2D. Similarly, 75% of the bariatric patients had previously had a positive test for COVID-19 but none of the control participants had ever tested positive for COVID-19.

4.3.2 Platelet parameters

Platelet traits measured by Sysmex were compared across bariatric and control groups (Table 4.2). There was no statistical evidence for an effect of obesity on platelet parameters. However, IPC and IPF did display larger mean values compared to controls, but they also had relatively large SDs in the bariatric patient group (IPC 9.3 (SD 4.9) $\times 10^9/L$ in control vs 16.3 (SD 16.3) $\times 10^9/L$ in bariatric group, p=0.45).

Table 4.2: Comparison of platelet traits measured by Sysmex XE-2100 across bariatric and control groups

Variable	Control mean (SD)	Bariatric patient mean (SD)	P value for difference
Platelet count (PLT) ($10^9/L$)	257.8 (29.1)	235.8 (61.5)	0.54
Immature platelet fraction (IPF) (%)	3.8 (2.3)	7.3 (8.3)	0.44
Immature platelet count (IPC) ($10^9/L$)	9.3 (4.9)	16.3 (16.3)	0.45
Side fluorescence (SFL)	82.0 (5.8)	85.1 (9.8)	0.61
Forward scatter (FSC)	53.8 (5.5)	60.5 (8.8)	0.24
Side scatter (SSC)	40.3 (1.1)	41.8 (3.0)	0.37

4.3.3 Basal receptors

Activation of the integrin $\alpha_{IIb}\beta_3$ and expression of P-selectin were compared across bariatric and control groups to see whether there was a difference in basal platelet activation. Basal integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression were similar across groups (Figures 4.3A and B). Expression of other platelet surface receptors in unstimulated platelets were also compared using specific antibodies. Levels of GPVI, CD42b (GP1b α), CD41 (integrin α_{IIb}), CD61 (Integrin β_3) and CD110 (thrombopoietin/TPO receptor) were not different across groups (Figure 4.3C).

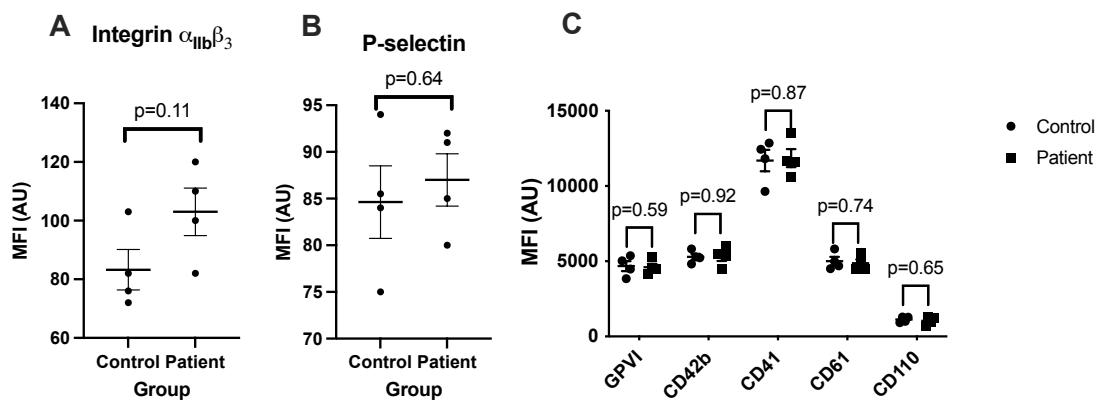


Figure 4.3: Comparison of basal integrin $\alpha_{IIb}\beta_3$ activation, P-selectin expression and surface receptors levels across control and bariatric patient groups. Diluted PRP was used for experiments, where platelets were incubated with antibodies for 10 minutes before fixing. Bar graphs comparing A) Basal integrin $\alpha_{IIb}\beta_3$ activation B) Basal P-selectin expression and C) Basal surface receptor expression across bariatric patient and control groups. Antibodies were used to detect surface expression of GPVI, CD42b (GP1b α), CD41 (integrin α_{IIb}), CD61 (Integrin β_3) and CD110 (thrombopoietin/TPO receptor). N=4. P-values indicated for unpaired t-test results. Mean + SEM displayed.

4.3.4 Agonist-induced integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression

Markers of platelet activation were compared in response to different platelet agonists. There was weak evidence that the integrin $\alpha_{IIb}\beta_3$ maximum response was reduced in the bariatric patient group in response to PAR1-AP (2479 AU (SEM 126) in control vs 2105 AU (SEM 101) in bariatric group, $p=0.06$) and ADP (2526 AU (SEM 121) in control group vs 2274 AU (SEM 478), $p=0.05$), but there was no change in the pEC50s across groups. There was insufficient data to compare the pEC50s and max of the curves for CRP, however comparing the highest concentration of CRP, there was no difference in response across the bariatric and control group for activation of the integrin $\alpha_{IIb}\beta_3$ (Figures 4.4A-C). There was no difference in the levels of P-selectin across bariatric patients and control groups in response to PAR1-AP or ADP (Figures 4.4D and E) as determined by the pEC50 or the curve maximum

values. There was no evidence for a difference in expression of P-selectin across bariatric and control groups in response to the highest concentration of CRP ((Figure 4.4F)).

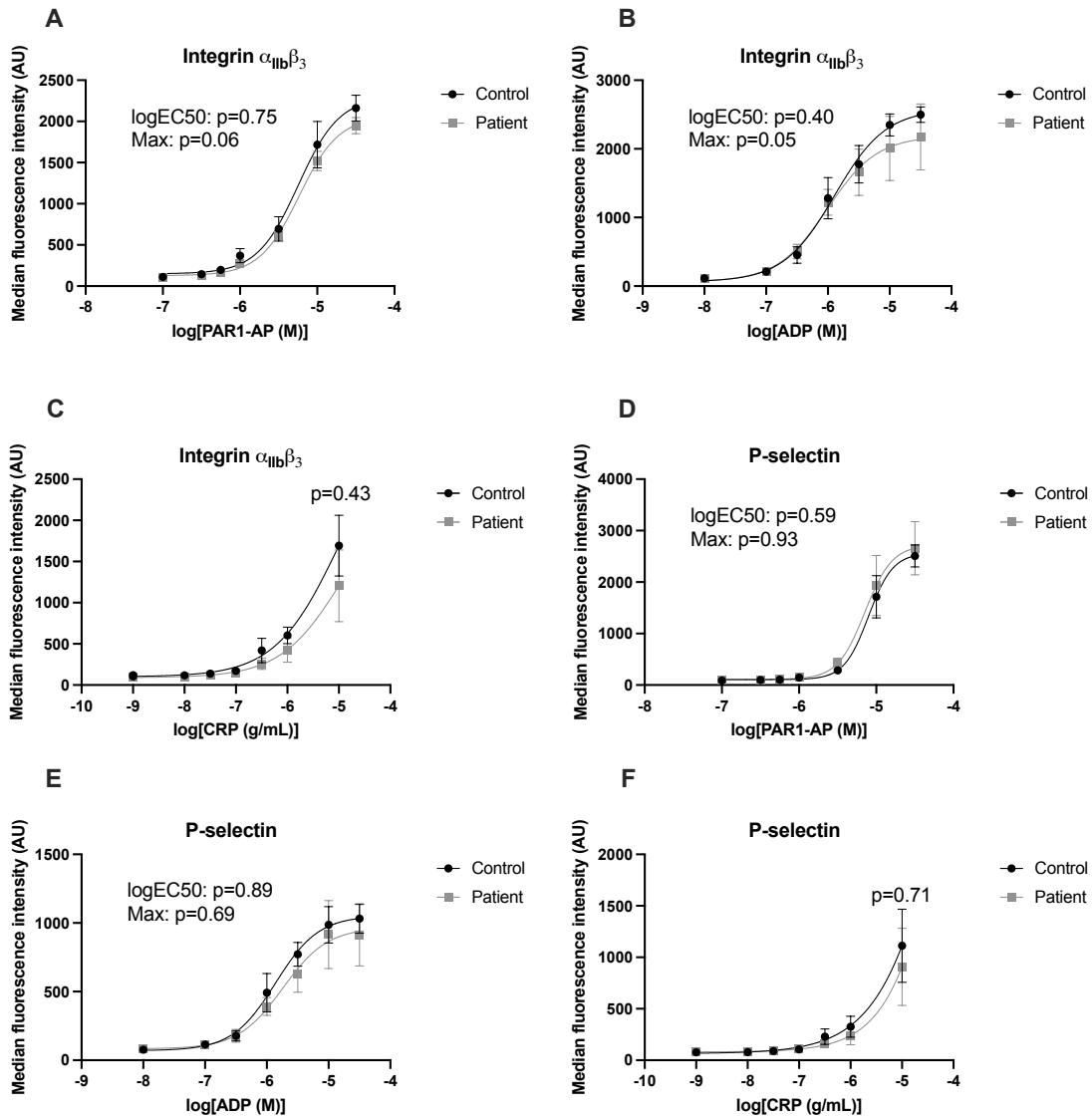


Figure 4.4: Concentration-response curves of integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression for bariatric patient and control groups in platelet rich plasma diluted in HEPES-Tyrode's. Integrin $\alpha_{IIb}\beta_3$ activation in response to A) PAR1-AP, B) ADP and C) CRP. P-selectin expression in response to D) PAR1-AP, E) ADP, F) CRP. N=4. Mean \pm SEM displayed. P values are the comparison of either the pEC50 or the maximum of the curve for the individual concentration-response curves across the bariatric patient and control groups. For CRP, the p value for an unpaired two-tailed t-test comparing the response to 1×10^{-5} g/mL CRP across the bariatric patient and control groups is displayed.

4.3.5 Platelet-neutrophil aggregates

Next, platelet-neutrophil interactions were explored to determine whether there was a difference in the proportion of neutrophils that are bound to platelets, therefore demonstrative of a difference in hyperactive platelets. This was explored by gating neutrophils using an antibody for the specific neutrophil marker, CD45, and their side scatter (granularity). Within this gate, the percentage of neutrophils interacting with platelets was determined by using an antibody for the platelet specific marker CD41 (integrin α_{IIb} subunit) and determining the percentage of events that were both CD45+ and CD41+. There was no difference in the percentage of neutrophils bound to platelets under basal conditions (7.0 % (SEM 1.5 %) in control v 11.2 % (SEM 3.2%) in bariatric patient group, $p=0.30$). Similarly, after stimulation with CRP, there was no difference in the percentage of neutrophil-platelet aggregates (38.6 % (SEM 2.6 %) in control group v 35.5 % (SEM 2.7 %), $p=0.48$).

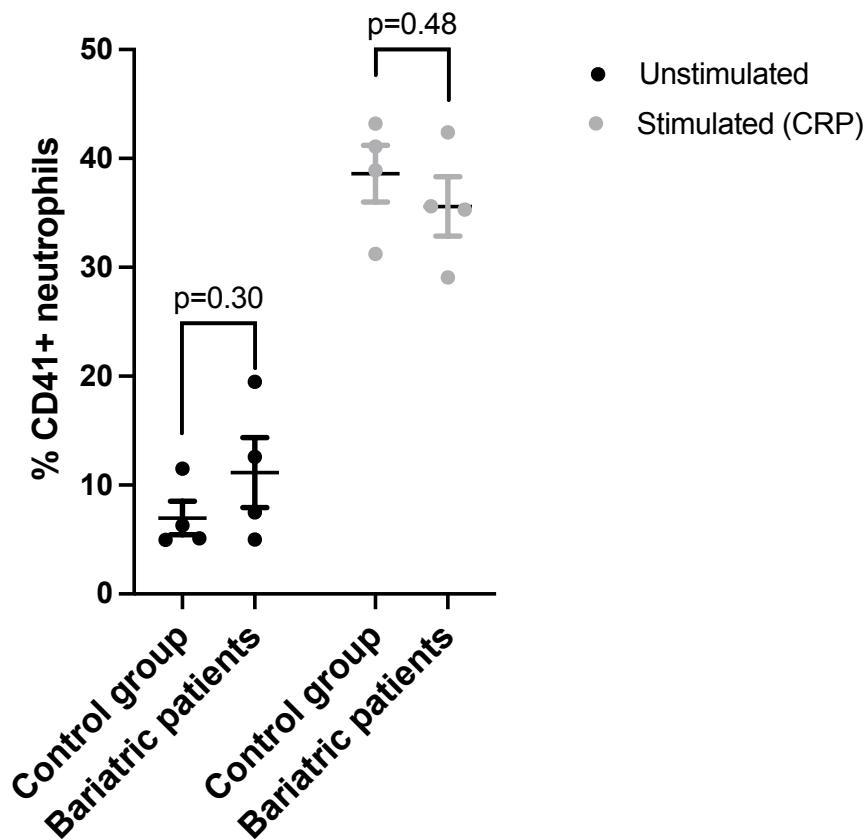


Figure 4.5: **Comparison of neutrophil-platelet aggregates across bariatric and control groups.** Neutrophil-platelet aggregates were determined using CD45 and CD41 antibodies. Aggregates were compared across bariatric patient and control participant groups in unstimulated and stimulated (5 ug/mL CRP) conditions. N=4. P-values provided for the results of a two-way ANOVA with Sidak's multiple comparisons tests. Mean + SEM displayed.

4.3.6 Platelet proteomics

A total of 5318 proteins were detected from platelets of both groups of participants by tandem mass tag mass spectrometry (TMT-MS). A principal component analysis (PCA) was performed on the protein data. PC1 and PC2 explained 27.1 % and 14.4 % of the variance in the data, respectively. A PC plot of PC1 against PC2 is shown in Figure 4.6. PC1 plotted against PC2 did not separate the bariatric patient samples from the control participant samples, despite grouping of three patients (Figure 4.6). PC3 and PC4 explained 13.1% and 12.6% of the data respectively. When plotting PC3 against PC4, there is separation among the groups (Figure 4.7). This suggests that the group was not the main source of variation between the data.

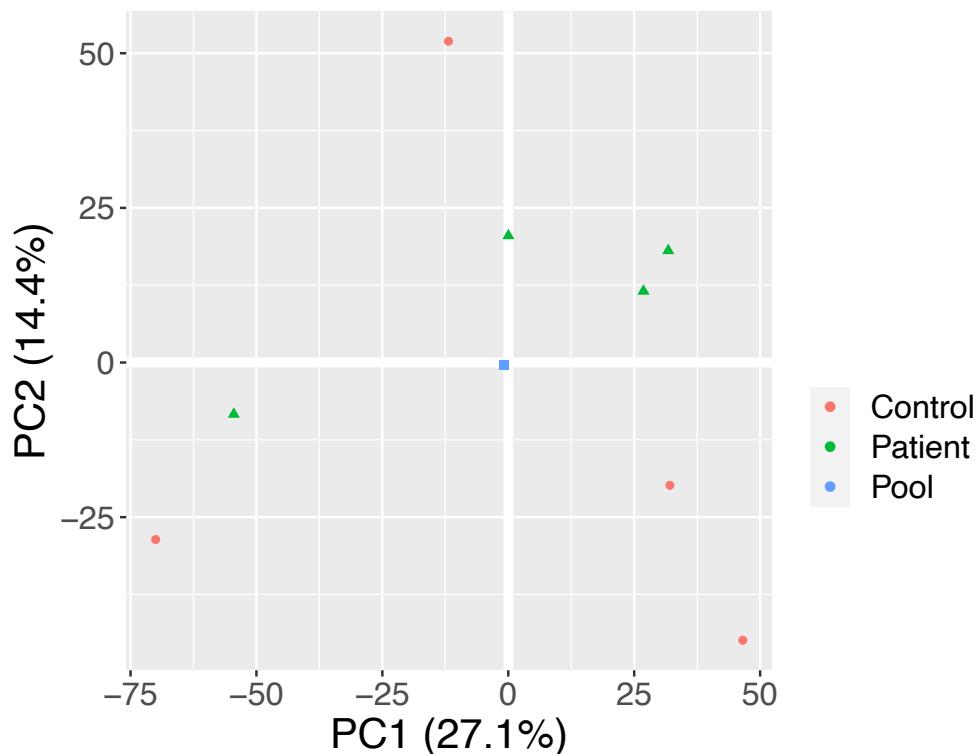


Figure 4.6: **Principal component analysis (PCA): scatter plot of PC1 and PC2.** Each point represents a participant. Control samples are coloured red and bariatric samples coloured blue, with the pooled reference sample of both control and bariatric samples coloured in blue.

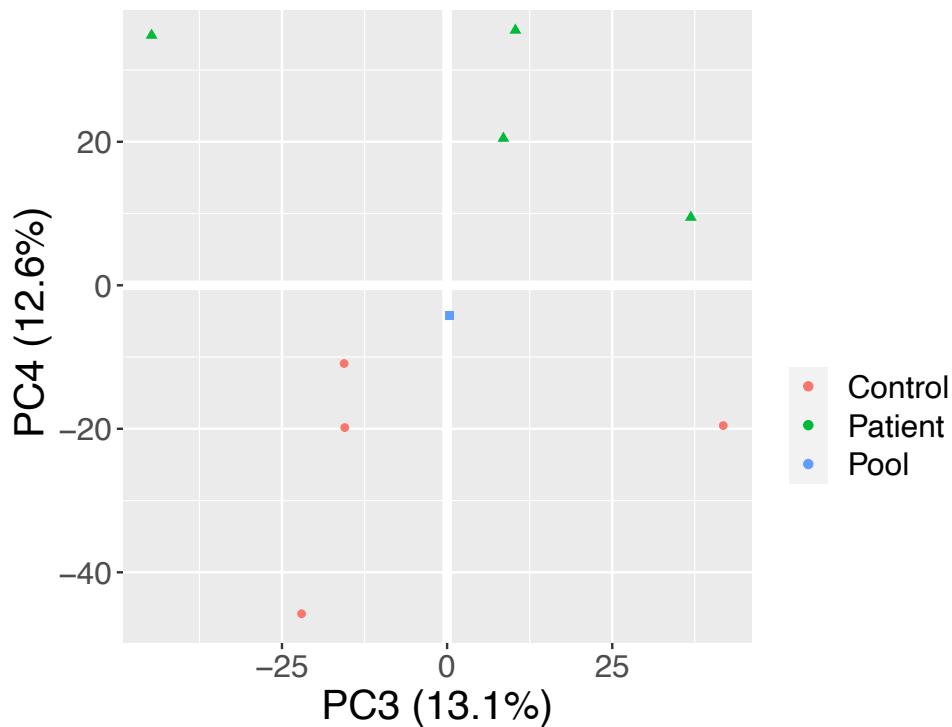


Figure 4.7: Principal component analysis (PCA): scatter plot of PC3 and PC4. Each point represents a participant. Control samples are coloured pink and bariatric samples coloured blue, with the pooled reference sample of both control and bariatric samples coloured in blue.

There was evidence ($p<0.05$) that 188 out of a total of 5318 detected platelet proteins (3.5%) were altered in bariatric patients with obesity compared to controls (Figure 4.8). Positive logFC values indicate the protein having higher levels in the bariatric group. The protein which had the strongest evidence for a BMI effect was phosphatidylinositol 4-kinase beta (PI4K-beta) ($\log FC\ 0.45, p=6.3\times 10^{-5}$). The signalling protein G protein-coupled receptor kinase (GRK6) was lower in bariatric patients ($\log FC\ -0.15, p=0.002$), whereas the adipocyte plasma membrane-associated protein (APMAP) was increased in bariatric patients ($\log FC\ 0.23, p=0.006$). Platelet proteins previously reported⁹⁶ to be associated with BMI replicated here, including gelsolin ($\log FC\ -0.14, p=0.018$), actin (cytoplasmic 1, $\log FC\ 0.51, p=0.03$) and septin-2 ($\log FC\ 0.14, p=0.03$). Platelet transcripts have been reported to be associated with BMI, including transcripts for myeloperoxidase and protein S100-A9¹²⁵. The current study provides evidence that these alterations in transcripts directly relate to protein levels measured in the proteomics analysis as BMI group was associated with both myeloperoxidase ($\log FC\ 1.15, p=0.04$) and protein S100-A9 ($\log FC\ 1.48, p=0.049$). Similar to the flow cytometry results, no alteration in protein receptors such as the α_{IIb} integrin and GPVI receptor was detected. The full platelet proteomic results can be found here <https://github.com/lucygoudswaard/mythesis/blob/f17f0250ad50bad74d7e38>

Volcano plot

EnhancedVolcano

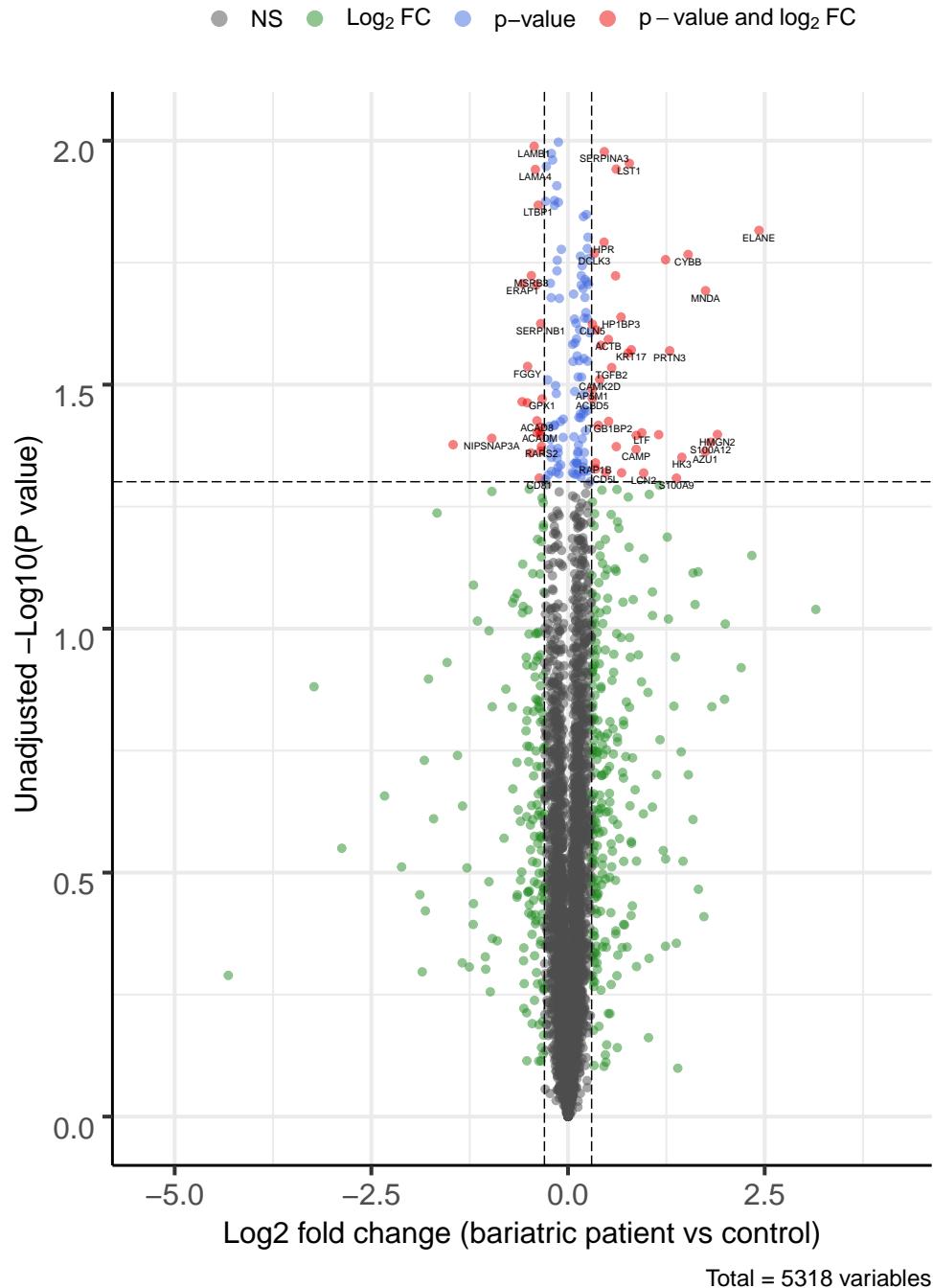


Figure 4.8: A volcano plot comparing the \log_2 fold change in protein levels in bariatric patients compared to controls. Dashed lines indicate a \log_2 fold change of 0.3 and a pvalue cut off of 0.05 ($-\text{Log}_{10}$ pvalue of 1.3)

4.4 Discussion

This chapter explored the effect of obesity on platelet parameters and platelet function by collecting pre-surgery samples from bariatric patients and age and sex-matched controls. Here, I have optimised common experimental laboratory techniques for use within a patient study. In addition, these techniques have also been used to study platelet function within another patient group (COVID-19) and can be implemented in future patient studies. The present study did not detect functional differences in platelet parameters and between bariatric patients compared to healthy controls, but proteomics analysis by TMT-MS detected differences in platelet protein abundance, which may have implications for platelet signalling.

This study did not detect differences in platelet characteristics such as IPC when comparing the high BMI bariatric surgery group with the control group. The small sample size and large standard deviation in the high BMI group would explain why the BMI and IPC association detected in Chapter 3 was not detected in the current study. There was also no evidence for a difference in levels of platelet receptors under basal conditions. A difference in P-selectin expression in response to agonists across the bariatric patient and control group was not detected, however, there was weak evidence that activation of the integrin $\alpha_{IIb}\beta_3$ was impaired in the bariatric patient group. This reduction is modest so it is unclear whether this would impact the ability of platelets to aggregate. A previous study (unpublished) from the Hers group and published data from other groups¹²⁶ indicated that in severe COVID-19 there is a large reduction (~60% reduction) in platelet integrin $\alpha_{IIb}\beta_3$ activation, but an increase in the percentage of platelets bound to neutrophils therefore suggesting there is hyperactivity. It is therefore possible that impaired integrin activation may be a marker of platelet hyperactivity. A similar reduction in integrin $\alpha_{IIb}\beta_3$ activation was reported in platelets from patients with myeloproliferative disorders who also have a prothrombotic phenotype¹²⁷. Despite this, in the current study there was no difference in the percentage of neutrophils bound to platelets in the bariatric surgery group. This may be because the reduction in integrin $\alpha_{IIb}\beta_3$ activation was not large enough (it was only ~10% reduction in the bariatric surgery group in response to ADP).

One previous study detected an increase in GPVI receptor levels and GPVI mediated platelet aggregation⁹⁶. Despite this previous finding, Barrachina et al. did not directly measure activation of the integrin $\alpha_{IIb}\beta_3$, so it is unclear how this increase in expression would translate to the activation of this integrin. A larger sample size would be required to be able to draw conclusions here. As well as this, aggregation experiments would have also been useful to compare platelet function across

groups, however this technique was not chosen due to time restraints and large amount of sample required.

The principal component analysis of the proteomic data suggested that PC4 was able to separate the bariatric and control groups. Although PC4 was not the main source of variance, even with a small N number, this provided evidence that the group (bariatric/control) could explain variation in proteins. Therefore, some protein signatures may be due to the difference in BMI. However, it could also be due to other factors which differed across groups such as previous COVID-19 infection or T2D diagnosis. COVID-19 infection can cause venous thromboembolism and is more likely to occur in people with obesity^{111,128,129}. Therefore, it is possible that the COVID-19 infection in the bariatric patients may cause longer-term changes in platelet activity and confound results. Despite this, concordance with previous studies in terms of proteins reportedly altered with BMI also suggests that the current study is likely capturing platelet protein signatures of obesity. Table 4.3 shows the results that have replicated in the current study when comparing to three previous studies that have studied the platelet proteome⁹⁶, platelet microvesicle proteome¹³⁰ and platelet transcriptome in relation to obesity¹²⁵.

Table 4.3: Summary of current literature on the effect of body mass index on the platelet proteome

Paper	Author, year	Overlapping findings
GPVI surface expression and signalling pathway activation are increased in platelets from obese patients: Elucidating potential anti-atherothrombotic targets in obesity	Barrachina et al., 2019	Proteins altered: Actin, cytoplasmic 1 (Fragment), Septin-2, Gelsolin, Glycerol-3-phosphate dehydrogenase
Platelet-Derived Microparticles From Obese Individuals: Characterization of Number, Size, Proteomics, and Crosstalk With Cancer and Endothelial Cells	Grande et al., 2019	Platelet microvesicle proteins altered: LIM and senescent cell antigen-like containing domain protein 1
Relation of platelet and leukocyte inflammatory transcripts to body mass index in the Framingham heart study	Freedman et al., 2010	Platelet transcripts altered: myeloperoxidase (MPO), S100-A9

Evidence from the current study and results from Barrachina et al⁹⁶. point towards platelet proteins which may be altered with obesity such as actin, gelsolin and septin-2. Actin is important in platelet shape change and activation^{96,131}. Higher actin levels in platelets from patients with obesity could contribute to platelet hyperactivity. Mutations in the gene which encodes actin (ACTB) can cause Baraitser-Winter cerebrofrontofacial syndrome (BWCFF). Patients with this syndrome have been reported to have a low platelet count (thrombocytopenia) with enlarged platelets and a higher immature platelet fraction¹³². This actinopathy is known as ACTB-associated syndromic thrombocytopenia. It is therefore possible that increased levels of actin may be indicative of more newly produced platelets. Gelsolin is another platelet cytoskeletal protein, which was lower in the bariatric patient group. Gelsolin levels were shown to be lower in PRP from patients with stable angina pectoralis compared with controls, however they were higher in patients with unstable angina pectoralis and acute myocardial infarction patients¹³³. Recombinant human gelsolin has been shown to be protective against thromboembolism in mice, thereby suggesting that reduced levels seen in the current study could increase thrombosis risk¹³⁴. Septins are cytosolic proteins which are also reported to be important in integrin $\alpha_{IIb}\beta_3$ activation, platelet spreading and clot contraction¹³⁵. Septin-2 levels were higher in platelets from bariatric patients, suggesting this may lead to increased platelet activation. Although no difference was found in $\alpha_{IIb}\beta_3$ activation, it would be useful to explore whether there are differences in platelet aggregation and platelet spreading.

There is also overlap between platelet proteins altered with BMI in the current study and platelet transcripts that are reported to be altered with higher BMI¹²⁵. For example, S100A9 (Myeloid-related protein-14 or MRP-14) transcripts were altered with BMI and the levels of the protein were increased in the bariatric group in the current study. Studies in mice have found evidence that S100A9 is involved in thrombus formation¹³⁶, and there is evidence for its involvement in myocardial infarction¹³⁷. Likewise, myeloperoxidase (MPO) transcripts were associated with BMI and there were higher protein levels in bariatric patients with obesity. A previous study has shown evidence that MPO can potentiate ADP-induced platelet aggregation in PRP¹³⁸.

These platelet proteins have previously been reported to be associated with BMI and are therefore more likely to be robust associations. This study has also provided some novel platelet proteins which have different levels in bariatric patients with obesity compared with healthy weight controls. For example, there was an increase in levels of APMAP in platelets from the high BMI group. A SNP which is associated with higher plasma levels of APMAP (rs8125909) is associated with higher BMI and greater

weight¹³⁹, therefore it is possible that the protein is involved in weight regulation, rather than higher BMI altering levels of the protein. Following up this protein after bariatric surgery could help determine the directionality of the effect. There was also a decrease in levels of G protein-coupled receptor kinase (GRK6) in the high BMI group. GRKs phosphorylate G protein coupled receptors (GPCRs) that have been activated by agonists, thereby leading to receptor desensitisation¹⁴⁰. GRK6 knockout mice have been shown to have larger aggregatory responses to agonists and more dense and α -granule secretion, along with increased thrombus formation¹⁴⁰ as a result of less receptor desensitisation occurring. Together, these results therefore reflect a platelet proteomic footprint of higher BMI that implicates a hyperactive platelet environment. Investigating the role of these proteins would be informative. It would also be important to explore additional measures of platelet activation such as platelet aggregation, calcium mobilisation and in vitro thrombus formation, as well as replicating experiments in a larger cohort.

In the proteomics analysis, a post hoc power calculation was performed to determine the range of power for the effect sizes where $p < 0.05$. This study had at least 67% power to detect effect sizes, but for some proteins which had smaller p values, there was 100% power. A larger sample size would be required to be able to detect smaller effect sizes.

Overall, this study optimised experimental techniques to test platelet function from patients. Sample sizes were limited and only small differences were detected in platelet function experiments and Sysmex platelet parameters. Platelet proteomic analysis replicated previous findings in relation to proteins which may be altered by BMI and implicated some novel proteins which are altered by BMI and may lead to changes in platelet function. Larger sample sizes of participants which display more similar characteristics would be required to confirm findings.

Chapter 5

Pathways linking plasma proteins and platelet function: do the chemokines MDC and TARC play a role?

5.1 Background

Platelets play an important role in haemostasis³⁵. In healthy people, prostacyclin (PGI2) and nitric oxide (NO) are released by the endothelium to suppress platelet activity³⁶. When the endothelium is damaged, platelets adhere to the injured vessel wall through the glycoprotein Ib-IX-V receptor and the GPVI collagen receptor. As a result, platelets secrete α - and dense-granules and undergo shape change²³. Alpha granules release growth factors (e.g. IGF-1), clotting factors and chemokines³⁷, whereas dense granules release molecules such as ADP, which further activate platelets by interacting with the P2Y₁ and P2Y₁₂ platelet receptors. Platelet activation also results in activation of integrins such as $\alpha_{IIb}\beta_3$ and subsequent fibrinogen binding results in platelet aggregation, thrombus formation and cessation of bleeding³⁵. Despite these processes being essential for haemostasis, when platelets become hyperactive, the balance is tipped in favour of thrombosis.

Platelets likely contribute to the increased CVD risk related to obesity and type II diabetes. Platelets from patients with obesity are reported to be hyperactive and have a lower sensitivity to antiplatelet therapies such as clopidogrel⁴⁴. These therapies are critical in the prevention of ischaemic events in certain circumstances. The underlying mechanism that causes platelet hyperactivity is not completely

understood. It is likely to involve a combination of factors. One such factor is a decrease in the production of endothelial PGI₂ and nitrous oxide¹⁴¹. Another contributing factor could be an alteration in the circulating levels of soluble ligands, growth factors and cytokines as a result of low grade chronic inflammation⁸³. These proteins may subsequently enhance platelet function (known as platelet priming). Platelet primers are signaling molecules which enhance agonist-induced platelet function but in isolation are unable to activate platelets. Molecules that have been reported to be able to prime platelets include insulin-like growth factor-1 (IGF-1)^{44,73} and thrombopoietin (TPO)⁷⁰.

There are other biomarkers which are also suggested to be raised with obesity as a result of low-grade chronic inflammation⁸³, including the chemokines macrophage-derived chemokine (MDC/CCL22) and thymus and activation regulated chemokine (TARC/CCL17)¹⁴². These inflammatory chemokines act at the receptor CCR4, which is expressed on platelets¹⁴³. MDC and TARC have been shown to potentiate platelet aggregation¹⁴⁴. For example, the presence of TARC and MDC have been demonstrated to greatly enhance platelet aggregation in the presence of low levels of the platelet agonist ADP¹⁴⁴. Furthermore, gene polymorphisms of MDC and CCR4 are associated with myocardial infarction (MI), therefore implicating MDC in thrombosis pathophysiology⁸⁸. Previous studies have shown that multiple platelet primers increase platelet function through activation of the lipid kinase phosphoinositide-3-kinase (PI3K)^{73,74,145}. Another pathway that has been implicated in platelet priming effects is the mitogen-activated protein kinase (MAPK) pathway. TPO can enhance thromboxane A₂ (TxA₂) synthesis through increasing the activation of extracellular signal regulated kinase (ERK2)^{79,80}. The common involvement of both PI3K and MAPK pathways in platelet priming mediated by IGF-1, TPO and matrix metalloproteinase-2 (MMP-2) suggests that MDC and TARC could also exert priming effects through these common pathways.

As blood/plasma levels of MDC have been reported to be elevated in obese people¹⁴² and polymorphisms of its receptor are associated with MI, understanding the mechanisms by which MDC can enhance platelet function may provide important insight into how platelets are hyperactive in obesity and potentially provide a novel target for new antiplatelet/thrombotic or preventative therapies for those with an increased risk of cardiovascular events.

The aims of this chapter are therefore to:

- 1) Determine the effects and mechanisms of MDC and TARC on platelet function and signalling.
- 2) Explore the effect of increasing levels of MDC and TARC on disease using two sample Mendelian randomization.

Figure 5.1 indicates the parts of the overall thesis hypotheses which are addressed in the current chapter.

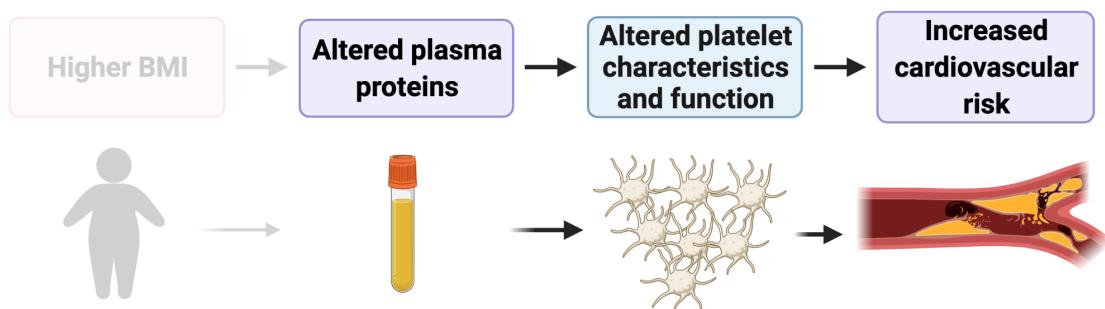


Figure 5.1: **Schematic of the associations explored in Chapter 5.** This chapter explores the effect of the chemokines MDC and TARC on platelet function and aims to explore their involvement in disease. Figure made using BioRender.com.

5.2 Methods

5.2.1 Materials

Materials used for the current chapter are listed in section 4.2.1.

5.2.2 Isolation of human platelets

Fresh human venous blood was obtained from healthy volunteers by venipuncture. Blood was taken into the syringe with a 1 in 10 volume of 4 % trisodium citrate, then mixed with a 1 in 7 volume of acid citrate dextrose (ACD). Blood was placed into 5 mL LP4 tubes then centrifuged at 1000 revolutions per minute (RPM) for 17 minutes. Platelet rich plasma (PRP) was removed. PRP was supplemented with inhibitors such as 0.02 U/mL apyrase, 140 nM PGE1 or 10 µM indomethacin. Supplemented PRP was either used for the experiment or centrifuged for a further 10 minutes at 1700 RPM. Platelet poor plasma (PPP) was removed leaving a platelet pellet. This pellet was resuspended in HEPES Tyrode's (supplemented with 0.1 % glucose and 0.02 U/mL apyrase, and 10 µM indomethacin if already applied to PRP). Platelets were counted using a Z1 coulter particle counter by diluting 1 in 2000 in 10 mL MQ water. Platelets were diluted further using the supplemented HEPES Tyrode's to a platelet concentration of 4×10^8 /mL and left to rest for 30 mins in a 30°C water bath.

5.2.3 Integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression measured by flow cytometry

Washed platelets were diluted to a concentration of $2 \times 10^7/\text{mL}$ using supplemented HEPES Tyrode's buffer with FITC-conjugated PAC1 and PE-conjugated CD62P antibodies in a 2:1 ratio, where PAC1 was present at a 1:10 volume and CD62P at 1:20. Platelets were incubated with 200 ng/ml MDC, TARC or vehicle for 5 mins followed by stimulation with increasing concentrations of PAR1-AP for 10 minutes. Platelets were fixed with a final concentration of 1 % paraformaldehyde (PFA) for 10 minutes. Plates were analysed using the BD Accuri C6 Plus flow cytometer (BD Biosciences, Wokingham, UK), where the median fluorescence intensity was derived from 10,000 gated platelets.

5.2.4 Platelet aggregation

Platelets were diluted to a concentration of $2 \times 10^8/\text{mL}$ using supplemented HEPES Tyrode's buffer. A volume of 250 μL platelets was added to aggregometer cuvettes containing a Teflon stir bar. HEPES Tyrode's (500 μL) was added to the reference PPP (blank) channel. Platelets were left to rest in the cuvettes for 2 mins. For priming experiments, platelets were either preincubated with vehicle (MQ) or 1 $\mu\text{g}/\text{ml}$ MDC or TARC for 5 mins. A Chrono-log model 490 aggregometer was used. For experiments which were not exploring platelet priming, platelets were added to the cuvettes for 2 minutes and agonist was added for 5 minutes. Aggregation traces were derived from the amount of light transmitted through the sample. Recordings were taken for 5 minutes with a stirring speed of 1200 RPM and at 37 °C. Platelets were used within 3 hours from resting.

5.2.5 Plate platelet aggregation

PRP supplemented with apyrase was used. For each donor, the platelet count was recorded. PPP was used as a reference. PRP was added to the wells of a 96 well plate and left to rest for 2 minutes. 200 ng/ml MDC, 200 ng/ml TARC or vehicle were preincubated with platelets for 5 minutes. After 5 minutes, PAR1-AP was added to the wells and the plate was shaken with an Eppendorf ThermoMixer® for 5 minutes at 1200 RPM and at 37 °C. The plate was immediately read using a Labtech LT-4500 automatic microplate absorbance reader. Responses were normalized so that basal PRP = 0 % aggregation and PPP = 100 % aggregation.

5.2.6 Phosphatidylserine (PS) exposure

Washed platelets were used at a concentration of $2 \times 10^7/\text{mL}$. Platelets were preincubated with vehicle, MDC or TARC. Platelets were stimulated 5 $\mu\text{g}/\text{mL}$ CRP as well as increasing concentrations of thrombin. PE-conjugated annexin V (1:10 v/v) was used to bind to PS. Plates were read using the BD Accuri flow cytometer, where the % positive (annexin V) were recorded.

5.2.7 Flow cytometry: phospho-VASP

Washed platelets were diluted to a concentration of $1 \times 10^8/\text{mL}$. Platelets were left to rest in a 96 well plate for 2 minutes to warm to 37°C . 200 ng/ml MDC, 200 ng/mL TARC, 10 μM ADP or vehicle were preincubated with platelets for 5 mins at 37°C . PGE1 (concentration-response curve) was added for a further 5 minutes. Platelets were fixed with 1 % PFA for 10 minutes. Platelets were centrifuged in a 96 well plate at 2651 rpm for 10 minutes (Heraeus Megafuge with M-20 Microplate Swinging Bucket Rotor, ThermoFisher Scientific). Supernatant was then discarded, and platelets were permeabilized with 0.1% TritonTM X-100 for 10 minutes. The plate was centrifuged again at 2651 rpm for 10 minutes. Supernatant was discarded and platelets were washed and resuspended in 1 x PBS. Platelets were centrifuged at 2651 rpm for 10 minutes. Supernatant was discarded and platelets were resuspended with rabbit pSer157 VASP primary antibody (1 in 1000) for 30 minutes on ice. Platelets were centrifuged at 2651 rpm for 10 minutes. Supernatant was discarded and platelets were washed and resuspended with 1 x PBS. Platelets were centrifuged at 2651 RPM for 10 minutes. Supernatant was discarded and platelets were resuspended with secondary antibody (Alexa Fluor™ 647 anti-rabbit IgG, 1 in 1000) for 45 minutes on ice. Platelets were centrifuged at 2651 RPM for 10 minutes. Supernatant was discarded and platelets were washed with 1 x PBS. Platelets were centrifuged once more at 2651 rpm for 10 minutes, resuspended once more in 1 x PBS and the plate was read using a BD Accuri C6 Plus flow cytometer and using the APC-A channel.

5.2.8 Western blotting

Platelets were used at a concentration of $4 \times 10^8/\text{mL}$. Platelets were preincubated with 200ng/mL MDC, 200ng/mL TARC or vehicle for 5 minutes followed by 5 minutes application of PAR1-AP (all at 30°C). For phospho-VASP assay, MDC, TARC or ADP were preincubated with platelets for 5 minutes, with PGE1 added for a further 5 minutes. Platelets were lysed with 4 X LDS NuPAGE sample buffer supplemented with 50 mM dithiothreitol (DTT). Samples were stored at -20°C . Samples were heated to 70°C for

10 minutes prior to SDS-PAGE. A 10 % Resolving gel was cast for Tris-glycine SDS-polyacrylamide gel electrophoresis, and a 5 % Stacking gel was cast for loading. As a standard, 4 µl Precision Plus protein pre-stained standard was loaded, along with 20 µl of the lysate sample. The samples were placed in a tank containing 4-morpholinepropanesulfonic acid (MOPS) running buffer (50 mm MOPS, 50 mm Tris, 1 mm EDTA, 0.1% SDS, 5 mm sodium bisulfite) and protein electrophoresis was carried out for 90 minutes at 100 V. Following this, proteins were transferred to a poly(vinylidene fluoride) (PDVF) membrane in the presence of transfer buffer (25 mM Tris-HCl (pH 7.6), 192 mM glycine, 20% methanol) for 60 minutes and at 100 V. The membrane was then incubated in LI-COR blocking buffer with 0.1 % TBS-Tween in a 1:1 ratio for 1 hour. Membranes were incubated with the primary antibody (1 in 1000 dilution) at room temperature overnight. Talin was used as a loading control. The membrane was washed 3 times with 0.1 % TBS-Tween for 5 minutes. The membrane was incubated with Fluor680 labelled secondary antibody (1 in 5000 dilution) for 1 hour. The membrane was washed 6 times for 5 minutes, then membranes were scanned using the LI-COR Odyssey® CLx Imaging System. Bands were quantified using Image Studio Lite.

5.2.9 Calcium mobilisation

Fura-2 (4 µM) was added to PRP after the addition of indomethacin and apyrase and incubated for 1 hour wrapped in foil at 37 °C. PRP was centrifuged and platelets were resuspended in supplemented HEPES Tyrode's as described above, with washed platelets (at $4 \times 10^8/\text{mL}$) protected from the light with foil. Platelets were recalcified with 1 mM calcium chloride using the Tecan Infinite M200 multimode plate reader (Mannedorf, Switzerland). Fluorescence of fura-2 was measured as the ratio at wavelengths 340:380 nm and provided a measurement of free calcium. 10 basal recordings were taken over 1 minute before addition of MDC, TARC or ADP. Upon agonist stimulation, 20 readings were taken over 2 minutes. A final concentration of 1 % Triton X-100 was added to permeabilise platelets and further readings were taken for calibration.

5.2.10 Statistical analysis for platelet function assays

Data was analysed using GraphPad Prism 8. If data displayed a normal distribution (based on Shapiro-Wilk p-value and W statistic), a parametric test was used (e.g. unpaired t-test or one way ANOVA), otherwise a nonparametric test was used. Concentration-response curves were plotted using a four parameter variable slope. To compare concentration-response curve parameters, pEC50s or curve

maxes were compared. A Fisher's exact test was used to compare proportions of categorical variables. Statistical tests used for each experiment are in figure legends. Strength of evidence is determined by p-values, however results will not be dichotomised as either 'significant' or 'not significant'¹²³.

5.2.11 Two sample MR look-up using EpigraphDB

Mendelian randomization is a method which uses genetic variants as instrumental variables to provide a causal estimate of the relationship between a modifiable exposure and health outcome¹². Two sample MR uses summary statistics and allows for causal effects to be estimated where the exposure and outcome are measured in different populations¹². EpigraphDB is a tool which performs two sample MR to determine how the human proteome contributes to complex diseases¹⁴⁶. Protein quantitative trait loci (pQTLs) were available for MDC and TARC from the INTERVAL study (N=3301), which were rank normal transformed³⁴. Therefore a systematic exploration of the possible roles of MDC and TARC in disease was performed. These SNPs were associated with these proteins at $p < 5 \times 10^{-8}$. GWAS summary statistics that were publically available for disease outcomes were included. A list of the available outcomes are presented on EpigraphDB <https://www.epigraphdb.org/pqtl/list/outcomes>. As an example, the venous thromboembolism variables were from Neale lab: https://github.com/Nealelab/UK_Biobank_GWAS). The venous thromboembolism (VTE) units are derived from a case/control linear mixed model. For MDC, there was one instrument available, therefore a Wald ratio was used to estimate the causal effect¹⁴⁷. There were two instruments available for TARC, therefore the inverse variance weighted (IVW) method was used, which assumes all genetic variants are valid instrumental variables¹⁴⁸. The pQTLs used were trans pQTLs(located >500 kb from the leading pQTL). The instruments used were categorised as Tier 1 instruments, which passed both pleiotropy and consistency tests¹⁴⁶. As a total of 225 outcomes were searched, a Bonferroni-adjusted p-value of $0.05/225 = 2.2 \times 10^{-4}$ was utilised to account for multiple testing.

5.3 Results

5.3.1 Priming effects of MDC and TARC on platelet aggregation in washed platelets using light transmission aggregometry

To investigate the effect of MDC and TARC on agonist-induced platelet aggregation, MDC and TARC were preincubated before addition of PAR1-AP. Figure 5.2A shows an example aggregation trace from

one donor where PAR1-AP and vehicle induced only shape change, but the presence of MDC and TARC induced a maximal aggregation response. A submaximal concentration of PAR1-AP (ranging from 0.7 to 1 μ M) and vehicle induced a mean aggregation of 7.0 % (SEM 2.9 %) aggregation (Figure 5.2B). In the presence of 1 μ g/ml MDC, this aggregation increased to 33 % (SEM 13 %) (MDC v vehicle, $p = 0.15$). In the presence of 1 μ g/ml TARC, there was 46 % (SEM 11 %) aggregation (TARC v vehicle, $p < 0.05$). Similar effects were observed with MDC and TARC on the area under the aggregation curve (Figure 5.2C).

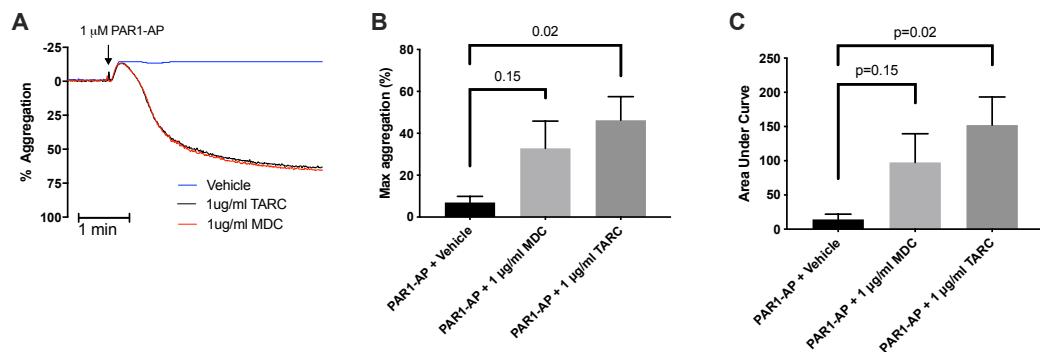


Figure 5.2: The priming effect of the chemokines MDC and TARC on PAR1-AP induced platelet aggregation in washed platelets. Washed platelets at 2×10^8 /mL supplemented with PGE1 and apyrase were preincubated with MDC or TARC (5 mins) before application of PAR1-AP-induced aggregation (5 mins). A) Raw aggregation trace comparing the effect of vehicle, 200 ng/mL MDC or 200 ng/mL TARC on PAR1-AP induced aggregation. B) Bar chart comparing the maximum aggregation reached when adding vehicle, MDC or TARC. C) Bar chart comparing the area under the curve of the aggregation trace in response to vehicle, MDC or TARC. Results analysed with a repeated measures one-way ANOVA with Dunnett's multiple comparisons. N=7. Mean+SEM displayed.

5.3.2 Priming effects of MDC and TARC on platelet aggregation in PRP

Next, the priming effect of MDC and TARC was explored on platelet aggregation in PRP using plate aggregation. Plate aggregation allows exploration of more conditions than LTA, therefore full concentration-response curves can be constructed. MDC and TARC were not able to potentiate the aggregation induced by increasing concentrations of PAR1-AP (Figure 5.3A). There was no difference in the pEC50 for comparing vehicle with MDC and TARC (Figure 5.3B).

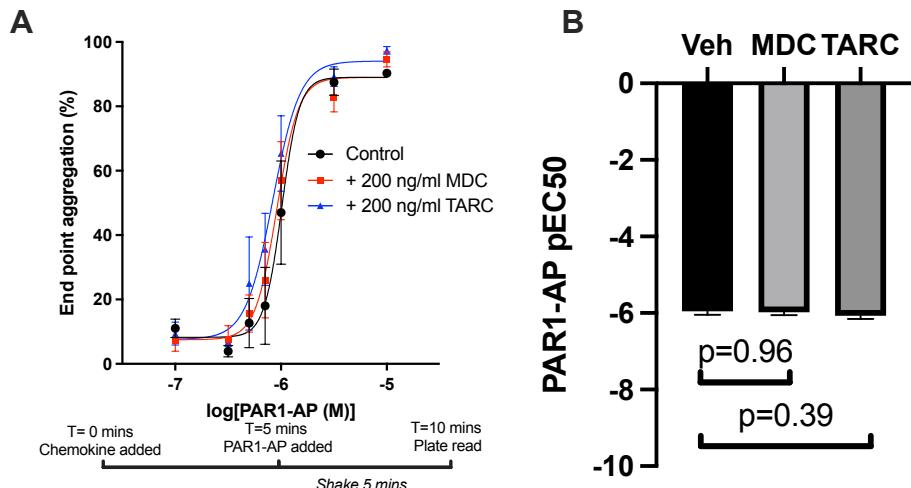


Figure 5.3: The priming effect of the chemokines MDC and TARC on PAR1-AP induced platelet aggregation in PRP using plate aggregation. PRP supplemented with apyrase was preincubated with MDC or TARC (5 mins) before application of PAR1-AP and aggregation induced by a plate shaker (5 mins). A) Concentration-response curves of the effect of vehicle, 200 ng/mL MDC or 200 ng/mL TARC on PAR1-AP induced aggregation. N=7. B) Bar chart of the pEC50s of the PAR1-AP concentration response curve in the presence of vehicle, MDC or TARC. Results analysed using a repeated measures one-way ANOVA with Dunnett's multiple comparisons. N=7.

5.3.3 Priming effects of MDC and TARC on PAR1-AP induced integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression

When a platelet becomes activated by an agonist, the integrin $\alpha_{IIb}\beta_3$ changes conformation and is exposed on the membrane of the platelet. This allows fibrinogen to bind and aggregation to occur. P-selectin is also expressed on the membrane as a result of stimulation and is a marker of alpha granule secretion. To assess whether MDC and TARC affect integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression, washed platelets were incubated with vehicle, 200 ng/ml MDC or TARC prior to PAR1-AP stimulation. MDC enhanced integrin $\alpha_{IIb}\beta_3$ activation, with a leftward shift in the PAR1-AP concentration response curve ($pEC50 = -5.54$ M (SEM 0.10 M) to -5.66 M (SEM 0.08 M), $p=0.02$). Preincubation with TARC had the same effect as MDC, left shifting the concentration-response curve ($pEC50 = -5.54$ M (SEM 0.11 M) to -5.64 M (SEM 0.11 M), $p=0.01$, Figure 5.4A). MDC and TARC did not cause a significant increase in the maximal integrin activation. As the concentration of PAR1-AP applied to the platelets increased, the amount of P-selectin exposed increased and therefore there was an increase in the MFI. The $pEC50$ for the PAR1-AP effect on P-selectin expression was -5.36 (SEM 0.07) (Figure 5.4B). Preincubation with 200 ng/ml MDC caused a small leftward shift in $pEC50$ to -5.44 (SEM 0.07) (MDC v vehicle, $p=0.006$),

Table 5.1: **Comparison of the effect of MDC and TARC on the pEC50 for PAR1-AP induced integrin activation and P-selectin expression.** Estimate compared with a one-way ANOVA (N=4).

Condition	Integrin activation pEC50 (M)	Integrin pEC50 SEM	Integrin P value	P-selectin pEC50 (M)	P-selectin pEC50 SEM	P-selectin p value
Vehicle	-5.54	0.1		-5.36	0.07	
MDC	-5.66	0.08	0.019	-5.44	0.07	0.006
TARC	-5.64	0.11	0.014	-5.46	0.11	0.21

but preincubation with 200 ng/ml TARC did not alter the sensitivity to PAR1-AP. Together these results show that MDC and TARC are able to potentiate platelet activation mainly through integrin $\alpha_{IIb}\beta_3$. In the absence of PAR1-AP, both MDC and TARC had a weak effect on integrin $\alpha_{IIb}\beta_3$ activation: increasing basal levels from a median fluorescence intensity (MFI) of 161 (SEM 20) to 222 (SEM 27) (vehicle v MDC, p=0.006) and to 285 ± 36 (vehicle v TARC, p=0.007) respectively (Figure 5.4C). This effect was specific to integrin $\alpha_{IIb}\beta_3$ as it was not seen with P-selectin.

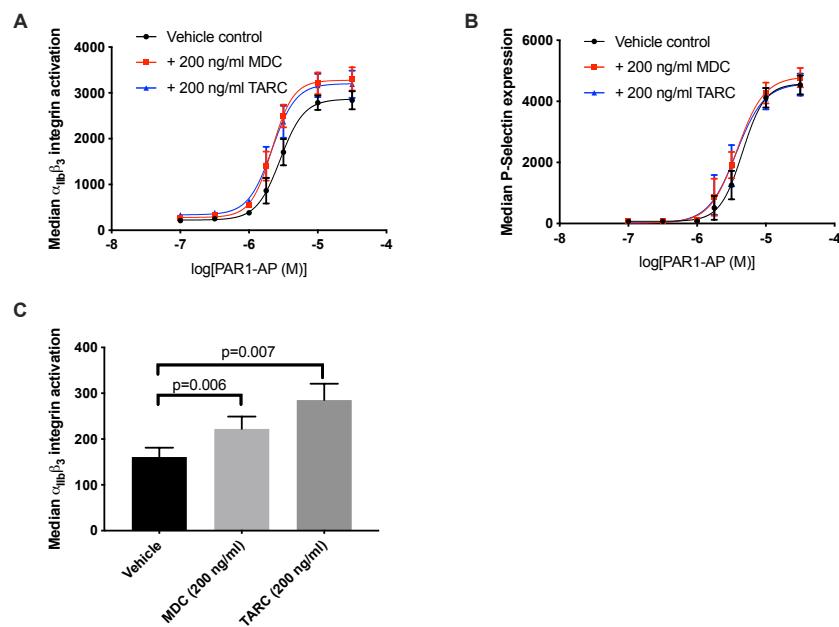


Figure 5.4: **The priming effect of the chemokines MDC and TARC on PAR1-AP induced $\alpha_{IIb}\beta_3$ activation and P-selectin expression.** Washed platelets at $2 \times 10^7/\text{mL}$ supplemented with PGE1 and apyrase were preincubated with MDC or TARC (5 mins) before application of PAR1-AP (5 mins). Integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression were measured using flow cytometry. A) The effect of vehicle, 200 ng/mL MDC or 200 ng/mL TARC on PAR1-AP induced $\alpha_{IIb}\beta_3$ activation measured as the median fluorescence intensity (AU). B) The effect of vehicle, MDC or TARC on PAR1-AP induced P-selectin expression measured using median fluorescence intensity (AU). pEC50s compared using a one-way ANOVA with Dunnett's multiple comparisons in Table 5.1 C) MDC and TARC alone increase integrin $\alpha_{IIb}\beta_3$ activation. Mean + SEM displayed. N=4.

5.3.4 Effects of MDC and TARC on PS exposure

When a platelet is activated by strong agonists, such as dual stimulation by collagen-related peptide (CRP) and thrombin, PS is translocated from the inner platelet membrane to the outer, thereby facilitating coagulation by providing binding sites for prothrombinase complex and supporting thrombin generation¹⁴⁹. MDC and TARC did not affect the overall percentage of annexin V positive platelets induced by CRP and thrombin (Figure 5.5).

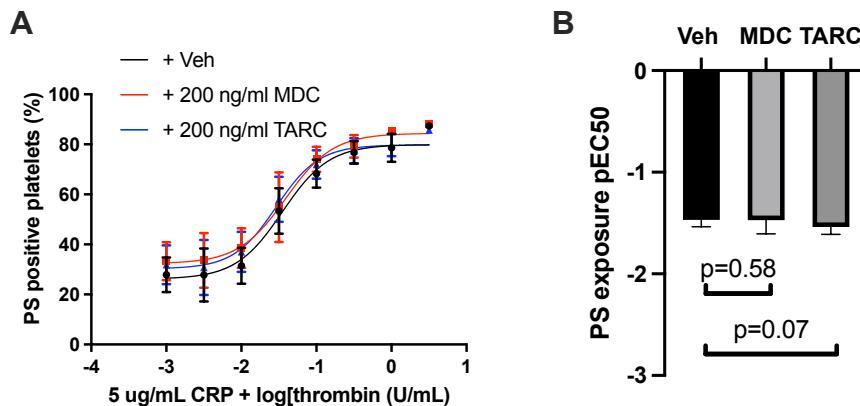


Figure 5.5: The effect of the chemokines MDC and TARC on PAR1-AP induced PS exposure. A) Washed platelets at $2 \times 10^7/\text{mL}$ supplemented with indomethacin and apyrase were preincubated with vehicle, 200ng/mL MDC or 200 ng/mL TARC (5 mins) before application of CRP and increasing concentrations of thrombin (10 mins). PS exposure was measured using flow cytometry (N=4). B) Bar chart comparing the effect of vehicle, MDC or TARC on the pEC50s (M) of thrombin induced PS exposure. pEC50s were compared by a Friedman's test.

5.3.5 Effects of MDC and TARC alone on aggregation in washed platelets

MDC and TARC were applied to washed platelets to determine whether they exert effects on platelet aggregation in the absence of an agonist. MDC and TARC did not cause platelet aggregation, however they did cause an upward deflection on the aggregation trace (Figure 5.6). This upward deflection is a reduction in light passing through the sample, indicating that the platelets may be undergoing shape change.

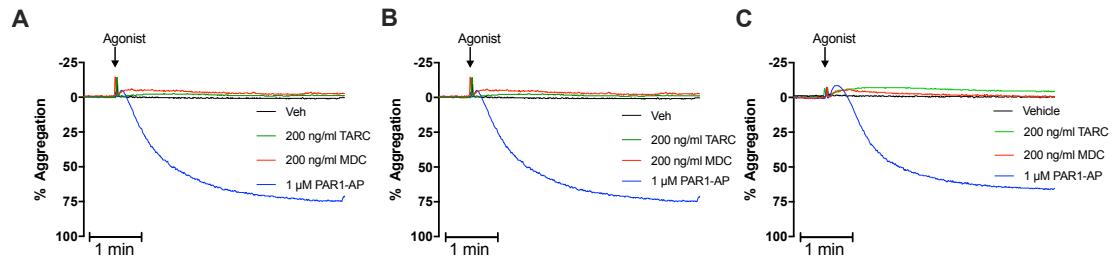


Figure 5.6: The effect of the chemokines MDC and TARC alone on aggregation in washed platelets. A-C) Washed platelets at $2 \times 10^8/\text{mL}$ supplemented with PGE₁ and apyrase were stimulated with vehicle, 200ng/mL MDC, 200 ng/mL TARC or 1 μM PAR1-AP (5 mins). Aggregation was measured using light transmission aggregometry. Each graph is a representative trace from a separate donor

5.3.6 Effects of MDC and TARC alone on calcium mobilisation

Calcium mobilisation was measured by using a fluorescent dye, Fura-2, which binds to intracellular calcium. Increasing concentrations of ADP were added to washed platelets as a positive control, where increasing calcium mobilisation was observed (5.7A). MDC and TARC alone were also able to increase calcium mobilisation in a concentration-dependent manner (5.7B), however caused a much smaller calcium response than ADP.

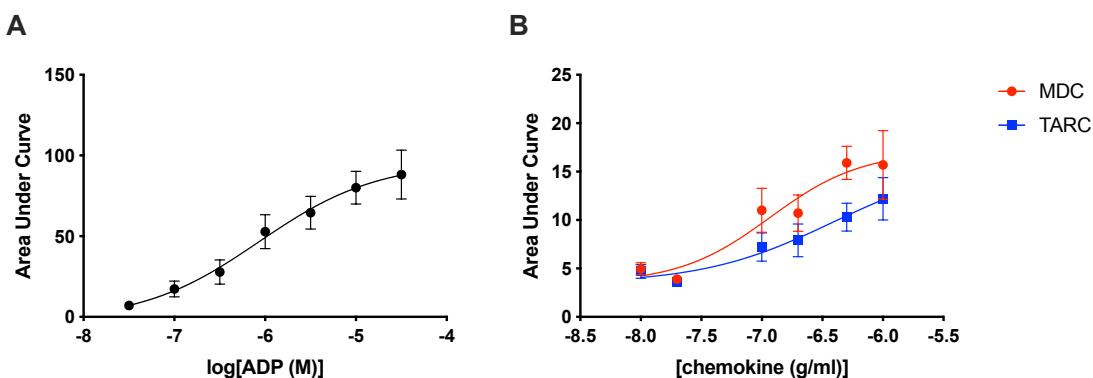


Figure 5.7: The effect of ADP and the chemokines MDC and TARC alone on calcium mobilisation in washed platelets. Washed platelets at $2 \times 10^8/\text{mL}$ supplemented with indomethacin and apyrase were loaded with Fura-2. Platelets were stimulated with increasing concentrations of A) ADP or B) 200ng/mL MDC or 200 ng/mL TARC. Area under curve represents the free calcium and is ratio of fluorescence at wavelengths 340:380 nm.

5.3.7 Mechanism of MDC and TARC priming in washed platelets

MDC and TARC activate the platelet receptor CCR4, which can reportedly couple to G_{i/o}. PGE₁ inhibits platelets by acting at a G_s coupled receptor, thereby increasing levels of cAMP and activating protein

kinase A (PKA). PKA subsequently phosphorylates cAMP. One mechanism by which MDC and TARC may therefore increase platelet function is by activating the G_i pathway, which would oppose cAMP inhibition of platelet function. ADP acts at the P2Y₁₂ receptor which is G_{i/o} coupled, therefore it reduces PGE₁ induced VASP phosphorylation. To explore whether MDC and TARC activate G_i signaling, the effect of MDC and TARC on VASP phosphorylation in the presence and absence of PGE₁ was explored. PGE₁ increased the phosphorylation of VASP at both Ser¹⁵⁷ and Ser²³⁹. Figure 5.8 shows a representative blot from one donor. There was little evidence for an effect of MDC, TARC and ADP reducing basal VASP levels (Figure 5.9A, C). As a positive control, ADP (10 μM) reduced the phosphorylation of VASP induced by 100 nM PGE₁ at both Ser²³⁹ by 62 % SEM (18 %) (ADP v vehicle, p=0.048, Figure 5.9B) and at Ser¹⁵⁷ by 44 % (SEM 21 %) (ADP v vehicle, p=0.048, Figure 5.9D). In contrast, there was not strong evidence that MDC and TARC reduced the phosphorylation of VASP induced by 100 nM PGE₁ at Ser²³⁹ or at Ser¹⁵⁷ (Figures 5.9B, D). This was also true for 10 nM and 30 nM PGE₁ (data not shown). Phospho-VASP at Ser¹⁵⁷ was also measured using flow cytometry (Figure 5.10). PGE₁ increased phosphorylation of VASP in a concentration-dependent manner, with a pEC50 of -8.36 M (SEM 0.16 M). Pre-incubation with ADP shifted this response to the right to give a pEC50 of -6.95 M (SEM 0.17 M) (ADP v vehicle, p=0.002). MDC and TARC did not alter the PGE₁ phosphorylation of VASP. Together, these results indicate that MDC and TARC do not signal through G_i-coupled receptors in human platelets, or too weakly to detect using Western blotting or flow cytometry.

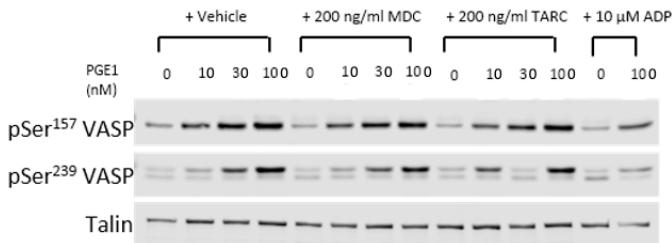


Figure 5.8: A representative blot of the effect of 200 ng/mL MDC, 200 ng/mL TARC and 10 uM ADP on phospho-VASP at Serine¹⁵⁷ and Serine²³⁹ in the presence of increasing concentrations of PGE₁. Quantification is shown in Figure 5.9.

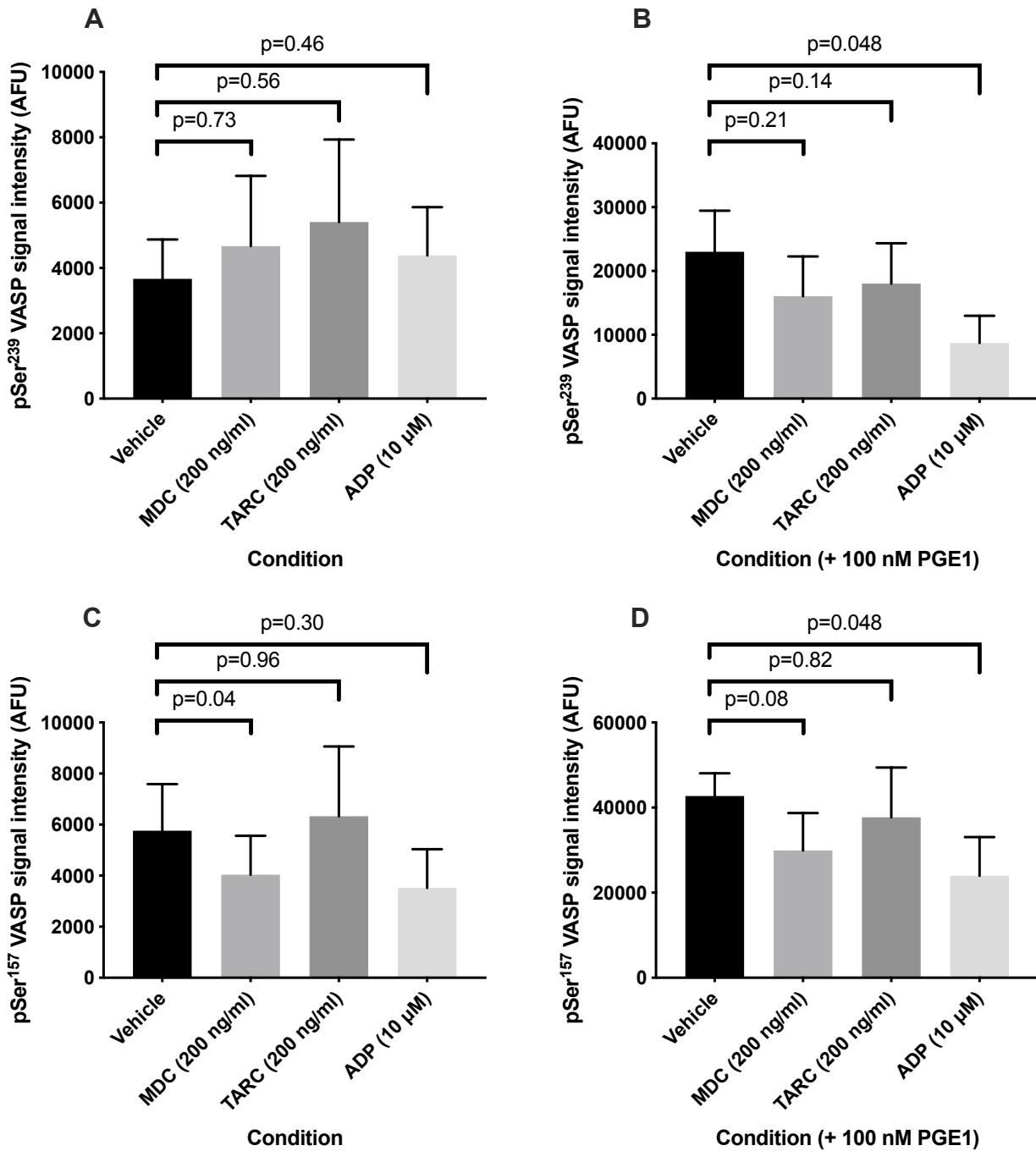


Figure 5.9: Quantification of the effect of MDC, TARC and ADP on PGE₁ stimulated phospho-VASP. Bar charts of the effect of 200 ng/mL MDC, 200 ng/mL TARC and 10 uM ADP on phospho-VASP at A) Serine 239 in the absence of PGE₁, B) Serine 239 in the presence of 100 nM PGE₁, C) Serine 157 in the absence of PGE₁, D) Serine 157 in the presence of 100 nM PGE₁. N=4. Results analysed with a repeated measures one-way ANOVA with Dunnett's Multiple comparisons.

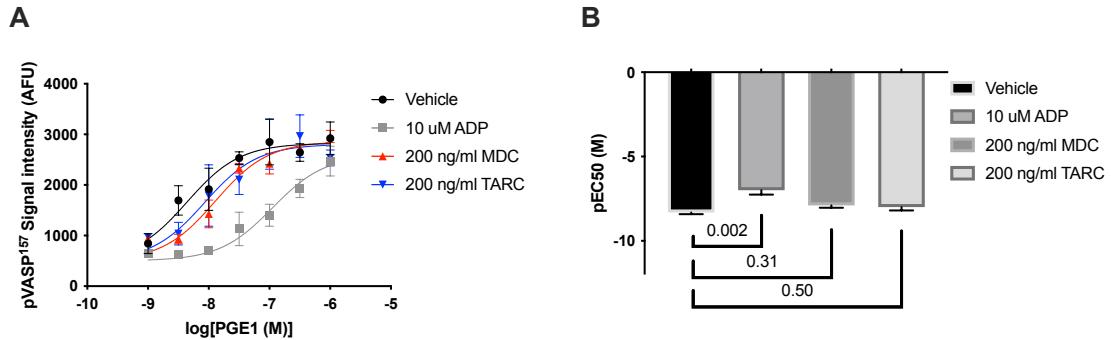


Figure 5.10: The effect of the chemokines MDC and TARC on phospho-VASP levels using flow cytometry. A) Washed platelets at $1 \times 10^8/\text{mL}$ were supplemented with indomethacin and apyrase. Platelets were preincubated with vehicle, 200ng/mL MDC, 200 ng/mL TARC or 10 μM ADP (5 mins) before application of increasing concentrations of PGE₁ (5 mins). Phospho-VASP at Ser¹⁵⁷ was measured using flow cytometry ($N=3$). B) Bar chart of the pEC50s of each concentration response curve from A. pEC50s were compared using a one-way ANOVA with Dunnett's multiple comparisons.

5.3.8 Mechanisms of the effect of MDC on platelet aggregation in PRP

The mechanism by which these chemokines cause platelet aggregation in PRP was further characterised just using MDC. MDC-induced platelet aggregation was explored using various inhibitors. To test that the antagonists used were specific, they were first applied to PAR1-AP. The G_q inhibitor YM-254890 inhibited PAR1-AP induced platelet aggregation, but the CCR4 antagonist AZD-2098 did not affect PAR1-AP platelet aggregation (Figures 5.11A, B). Y27632 inhibited the G_{12/13} mediated shape change induced by low concentrations of PAR1-AP (0.75 μM) due inhibiting rho-associated protein kinase (ROCK) (data not shown).

The effects of these inhibitors on MDC-induced aggregation were then explored. MDC by itself was able to consistently cause over 60% aggregation in PRP. The CCR4 inhibitor AZD-2098 was able to reduce this aggregation, but only at 50 μM (Figures 5.11C, 5.12A, B). The G_q inhibitor YM-254890 was able to reduce the maximum aggregation induced by MDC (Figure 5.11C, Figure 5.12C), suggesting CCR4 couples to G_q. Y27632 did not affect aggregation induced by MDC, suggesting that this aggregation is not dependent on G_{12/13} and ROCK (Figure 5.11D, 5.12D). Application of MDC to PRP did not cause shape change.

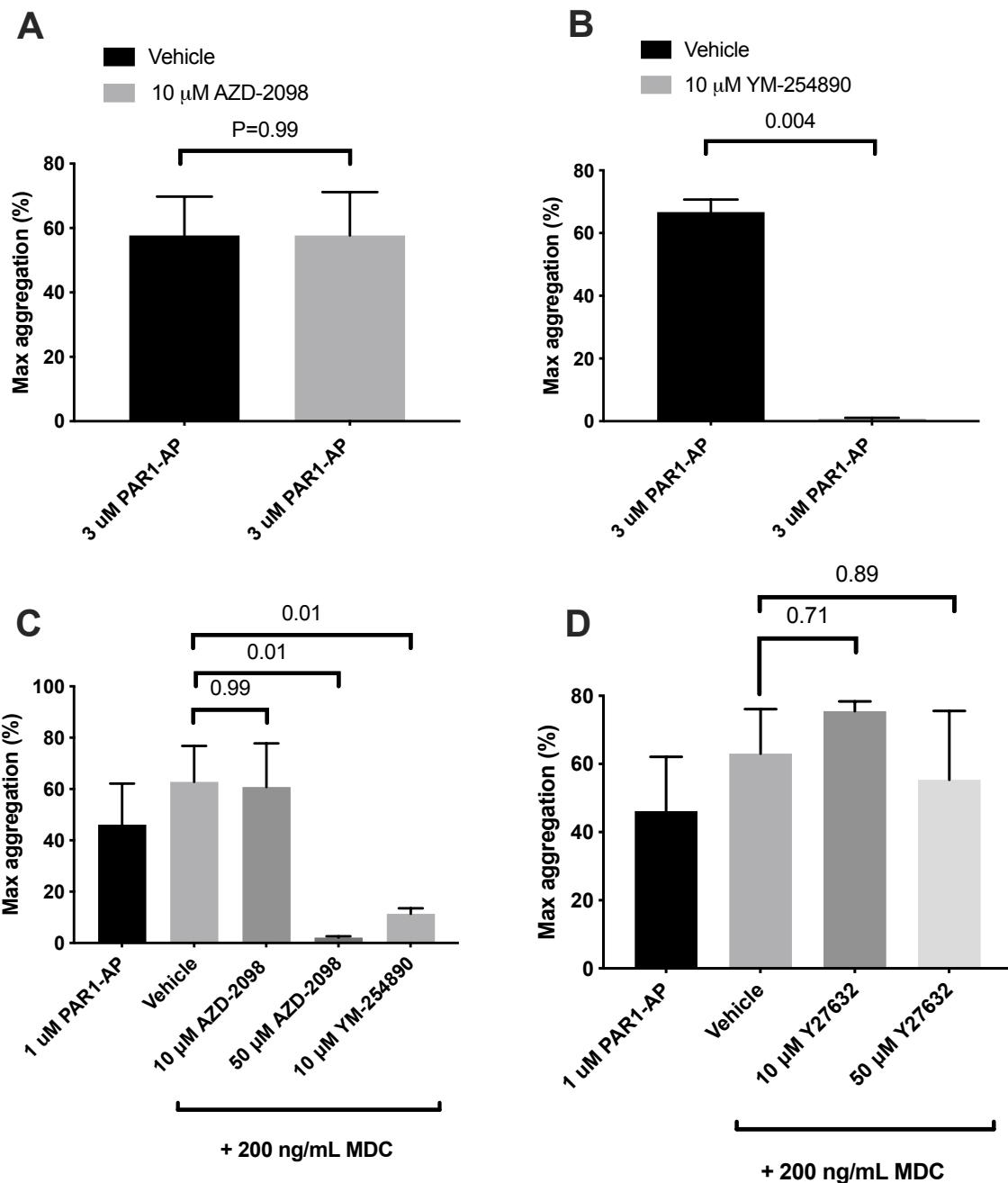


Figure 5.11: The effect of inhibitors on PAR1-AP and MDC induced aggregation in PRP. Platelet rich plasma (PRP) supplemented with apyrase was incubated for 5 minutes with the antagonist indicated, followed by stimulation by PAR1-AP or MDC. A) Effect of 10 μ M AZD-2098 (CCR4 antagonist) on maximal aggregation induced by PAR1-AP B) Effect of 10 μ M YM-254890 (G_q inhibitor) on maximal aggregation induced by PAR1-AP C) Effect of AZD-2098 and YM-254890 on aggregation induced by 200 ng/mL MDC D) Effect of the ROCK inhibitor Y27632 on aggregation induced by 200 ng/mL MDC Results are analysed with a paired two-tailed t-test or repeated measures one-way ANOVA with Dunnett's Multiple comparisons. N=3-5.

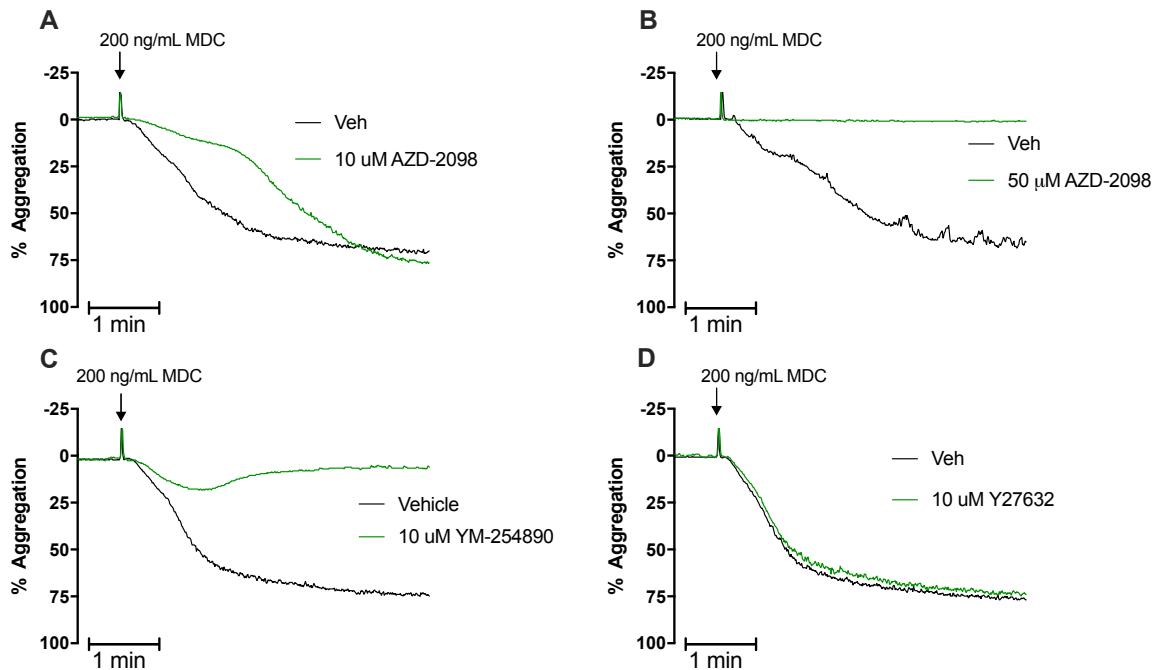


Figure 5.12: Aggregation traces of the effect of inhibitors on MDC induced platelet aggregation in PRP. Representative aggregation traces of platelet rich plasma (PRP) supplemented with apyrase, incubated with A) 10 uM CCR4 antagonist AZD-2098 B) 50 uM AZD-2098 C) 10 uM G_q inhibitor YM-254890 D) 10 uM ROCK inhibitor Y27632, followed by addition of 200 ng/mL MDC.

5.3.9 Two sample Mendelian randomisation to assess the role of MDC (CCL22) and TARC (CCL17) in disease

To estimate potential causal relationships between circulating levels of MDC and TARC and disease, a two sample MR was performed using pQTL data for these chemokines and available disease outcome data. Results are displayed for estimates where $p < 0.05$, however due to multiple testing, the p -value threshold was adjusted to 2.2×10^{-4} . Results are presented as the change in outcome per SD higher protein. There was evidence from the Wald ratio MR that higher MDC had a causal effect on three outcomes: high cholesterol (Effect size = 0.22 SD, SE = 0.02, $p = 2.2 \times 10^{-26}$), DVT (Effect size = 0.22, SE 0.021, $p = 2.2 \times 10^{-26}$) and pulmonary embolism (Effect size = 0.33, SE 0.08, $p = 2.0 \times 10^{-5}$). There was not strong evidence for an effect of higher levels of TARC on any of the included outcomes. Results of the two sample MR are shown for MDC and TARC in tables 5.2 and 5.3.

Table 5.2: Estimates for the effect of MDC on disease outcomes using two sample Mendelian randomization

Protein	Outcome trait	N SNP (rsID)	Method	Effect size	Standard error	P value
MDC/CCL22	Non-cancer illness code self-reported: high cholesterol	1 (rs77542162)	Wald ratio	0.2224	0.0209	2.2e-26
MDC/CCL22	Non-cancer illness code self-reported: deep venous thrombosis (dvt)	1 (rs77542162)	Wald ratio	0.2936	0.0512	9.81e-09
MDC/CCL22	Non-cancer illness code self-reported: pulmonary embolism (with or without) dvt	1 (rs77542162)	Wald ratio	0.3308	0.0775	1.969e-05
MDC/CCL22	Forced expiratory volume in 1-second (FEV1)	1 (rs77542162)	Wald ratio	0.0312	0.0084	0.0001961
MDC/CCL22	Forced vital capacity (FVC)	1 (rs77542162)	Wald ratio	0.0285	0.0079	0.0003201
MDC/CCL22	Eye problems or disorders: Glaucoma	1 (rs77542162)	Wald ratio	0.2292	0.0654	0.000457
MDC/CCL22	Diagnoses - main ICD10: M16	1 (rs77542162)	Wald ratio	0.2217	0.0648	0.0006186
MDC/CCL22	Coxarthrosis [arthrosis of hip]					
MDC/CCL22	Non-cancer illness code self-reported: gout	1 (rs77542162)	Wald ratio	-0.4045	0.1237	0.001075
MDC/CCL22	Non-cancer illness code self-reported: hypothyroidism or myxoedema	1 (rs77542162)	Wald ratio	0.1203	0.0381	0.001607
MDC/CCL22	Schizophrenia	1 (rs77542162)	Wald ratio	-0.1477	0.048	0.002086
MDC/CCL22	Systolic blood pressure automated reading	1 (rs77542162)	Wald ratio	-0.0283	0.0099	0.004246
MDC/CCL22	Alcohol intake frequency	1 (rs77542162)	Wald ratio	-0.04	0.0143	0.005124
MDC/CCL22	Non-cancer illness code self-reported: osteoporosis	1 (rs77542162)	Wald ratio	0.1593	0.0665	0.01655
MDC/CCL22	Diagnoses - main ICD10: K80	1 (rs77542162)	Wald ratio	-0.1965	0.0826	0.01731
MDC/CCL22	Cholelithiasis					
MDC/CCL22	Fracture resulting from simple fall	1 (rs77542162)	Wald ratio	0.0528	0.0236	0.02527
MDC/CCL22	Cancer code self-reported: small intestine or small bowel cancer	1 (rs77542162)	Wald ratio	0.5966	0.2745	0.02976
MDC/CCL22	Eye problems or disorders: Cataract	1 (rs77542162)	Wald ratio	0.0991	0.0487	0.04163
MDC/CCL22	Diagnoses - main ICD10: R04	1 (rs77542162)	Wald ratio	0.2189	0.1094	0.04552
MDC/CCL22	Haemorrhage from respiratory passages					
MDC/CCL22	Diagnoses - main ICD10: I80	1 (rs77542162)	Wald ratio	0.2269	0.1145	0.04755
MDC/CCL22	Phlebitis and thrombophlebitis					

Table 5.3: Estimates for the effect of TARC on disease outcomes using two sample Mendelian randomization

Protein	Outcome trait	N SNP (rsID)	Method	Effect size	Standard error	P value
TARC/CCL17	Rheumatoid arthritis	2 (rs983545, rs222846)	Inverse variance weighted	0.1015	0.0386	0.00851
TARC/CCL17	Anorexia nervosa	2 (rs983545, rs222846)	Inverse variance weighted	0.2182	0.0845	0.009826
TARC/CCL17	Non-cancer illness code self-reported: gastro-oesophageal reflux (gord) or gastric reflux	2 (rs983545, rs222846)	Inverse variance weighted	0.003	0.0012	0.01172
TARC/CCL17	Systemic lupus erythematosus	2 (rs983545, rs222846)	Inverse variance weighted	0.2716	0.114	0.01717
TARC/CCL17	Ischemic stroke	2 (rs983545, rs222846)	Inverse variance weighted	-0.081	0.0373	0.02999
TARC/CCL17	Red blood cell count	2 (rs983545, rs222846)	Inverse variance weighted	-0.0116	0.0058	0.04445

5.4 Discussion

This study explored the effects of the chemokines MDC and TARC on platelet function and signalling. Evidence suggested that MDC and TARC were able to potentiate platelet function induced by the agonist PAR1-AP in washed platelets. There was not strong evidence that this potentiation was mediated by PKB (unlike other platelet primers) or by a $G_{i/o}$ -coupled receptor. Functional experiments indicated that MDC and TARC are not only able to prime platelets, but that they are also able to cause a small calcium response and platelet shape change when applied alone in washed platelets. Experiments in PRP indicated that MDC is able to cause full aggregation and that this is due to the CCR4 receptor, and that this is G_q coupled. Two sample MR was performed to estimate the causal effect of higher levels of MDC and TARC on disease. These results suggested that higher levels of MDC increased the risk of DVT and PE. These effects could be due to the activation of platelets.

Previous studies have attempted to explore the effects of MDC and TARC on platelet function. There is evidence that MDC and TARC potentiate ADP-induced aggregation¹⁴⁴. The current study provides evidence that this is also the case with PAR1-AP, therefore this effect is not agonist specific. MDC and TARC alone have been shown to elicit a calcium response, but only in PRP can aggregation occur⁷⁵. This is likely due to components in PRP such as arachidonic acid. The current chapter confirmed these findings, where only shape change was achieved in washed platelets, but in PRP MDC could induce aggregation. As well as confirming previous findings, this chapter provided some novel observations. For example, this chapter explored whether MDC and TARC can potentiate PS exposure. There was no evidence that MDC and TARC could potentiate the procoagulant effects induced by CRP and thrombin. This may be because the signalling elicited by MDC and TARC may not be strong enough to affect PS exposure.

Platelets express chemokine receptors as they are also key cells in inflammation and host defense¹⁴³. MDC and TARC can also cause activation of platelets, thereby demonstrating the close link between inflammation and haemostasis³⁷.

As well as functional effects, this chapter aimed to assess whether effects are due to the CCR4 receptor and the downstream signalling involved. It has been reported that MDC inhibits calcium currents in HEK293 cells and barium currents dorsal root ganglia neurones and is therefore $G_{i/o}$ coupled¹⁵¹. MDC and TARC were not able to reduce VASP phosphorylation induced by PGE1, suggesting that the effects are not via $G_{i/o}$ in platelets. It is possible that this experiment was not sensitive enough to detect the

reduction in VASP phosphorylation, as ADP did not reduce VASP levels as much as previous reports⁵⁷. Another study suggested that CCR4 is coupled to G_q due to the calcium response observed by MDC⁷⁵. Inhibiting G_q coupling in the current study inhibited the aggregation induced by MDC. It is therefore likely that CCR4 couples to G_q.

MDC and TARC have been implicated in cardiovascular disease such as atherosclerosis¹⁵², but experiments focusing on the effect of MDC and TARC on platelet function have not used genetic epidemiology to explore whether genetic variants which lead to alterations in the levels of circulating MDC and TARC in the plasma are associated with an increased risk of disease. As MDC and TARC can activate platelets, the effect of MDC and TARC on disease was explored using two sample MR. The SNP that was associated with levels of MDC, rs77542162, was also associated with increased risk of DVT and pulmonary embolism. This effect may occur due to increased platelet priming by higher levels of MDC. Despite the evidence for a causal effect, it is possible that this SNP could affect the outcome through routes other than through the exposure (horizontal pleiotropy). This is also reflected by other outcomes being related to increased levels of MDC such as forced vital capacity (FVC). As there is only one SNP, sensitivity analyses cannot be performed to test this. Further analyses such as a colocalisation analysis could help determine whether the relationship between MDC and DVT/PE is causal as this method estimates whether an exposure and an outcome share a causal top signal in a particular genomic region¹⁵³.

This chapter has provided evidence for a an effect of MDC and TARC on platelet function and indicated possible mechanisms of effect. As mentioned in the introduction of the current chapter, there is evidence that patients with obesity have higher circulating levels of MDC¹⁴², however the effect of BMI and levels of these chemokines needs to assessed in a larger cohort and using a causal framework. The SOMAScan proteomics panel detects these circulating chemokines, therefore further exploration of the effect of BMI on these chemokines will be presented in Chapter 6 by using one sample MR and Chapter 7 which utilises a weight loss RCT.

5.4.1 Conclusions

Overall, this chapter provided evidence that the chemokines MDC and TARC potentiate platelet aggregation induced by PAR1-AP. On their own in washed platelets, a small calcium response can be observed as well as shape change, but PRP is required to observe full aggregation. Signalling experiments suggest MDC and TARC act at the CCR4 receptor on platelets, and that G_q coupling is respon-

sible for the functional effects observed. There was evidence that higher MDC may have a causal effect on DVT and PE. MDC may therefore be a biomarker for venous thromboembolism. Further work is required to determine signalling, and further cohort studies would be useful to confirm effects of MDC on DVT and PE.

Chapter 6

Exploring the plasma proteome as a link between adiposity and platelet function: Observational and Mendelian randomization estimates

The work within this chapter has been published in the International Journal of Obesity¹⁵⁴. I am the first author of the publication; I performed all analyses, created all figures and tables and wrote the manuscript.

Supplementary Tables 1-10 can be found here https://github.com/lucygoudswaard/mythesis/blob/master/index/figure/BMI_protein_INTERVAL/BMI_protein_supplementary_Tables.xlsx

6.1 Introduction

The previous chapters (Chapter 3 and Chapter 4) have provided evidence that higher body mass index raises immature platelet count and alters levels of platelet proteins which are important in platelet activation. As well as this, chapter 5 indicated that application of MDC and TARC on platelets *ex vivo* leads to platelet activation. Despite this evidence, it is unclear how a higher BMI leads to an increased risk of thrombosis, in particular an increase in platelet production and changes in platelet signalling proteins. It

is possible that molecules which circulate in the blood may be mediating this effect as platelets also circulate in the blood and blood vessels are the site of thrombi. Previous studies have largely focused on the impact of higher BMI on the lipidome including traits such as cholesterol and triglycerides in lipoprotein subtypes (e.g. low-density lipoprotein (LDL) and high-density lipoprotein (HDL) particles)^{31,32} and on inflammatory molecules such as C-reactive protein (CRP)^{32,84}. Fewer studies have explored the effect of adiposity on the circulating proteome profile⁹⁰.

A benefit of studying the systematic effects of BMI on the circulating proteome is that proteins are often more suitable pharmacological targets than metabolites. Efforts to study the effect of BMI on the proteome have generally been in an observational framework⁹⁰. It is estimated that 25% of proteins in the human proteome circulate in blood⁸⁹, which is important as the majority of druggable targets are such proteins⁹¹. Studying the effect of BMI on a large set of proteins has only recently become possible with newly developed proteomic technologies such as the SomaLogic platform, with the ability to quantify enzymes, protein kinases and transport proteins with unprecedented sensitivity¹⁵⁵. Utilisation of SomaLogic within a trial or cohort setting has recently become more widespread, such as within the INTERVAL study, a UK cohort of blood donors³⁴. There is evidence that proteins which change as a result of a higher BMI may contribute to thrombosis (ref Andrei's paper): identification of such proteins is important in understanding how higher BMI causes disease and to identify targets which may benefit from pharmacological intervention. It is possible that proteins that are altered by BMI may contribute to thrombosis by activating platelets (Chapter 5).

In this chapter, the aim is to measure associations between adiposity and the human proteome and to also estimate the underlying effects in a causal framework (Figure 6.1). Using data on 2737 participants from INTERVAL, the effects of BMI on 4034 (3622 unique) plasma protein traits will be estimated in both observational and MR frameworks. The specific effect of BMI on platelet proteins will be explored, as well as a disease enrichment analysis.

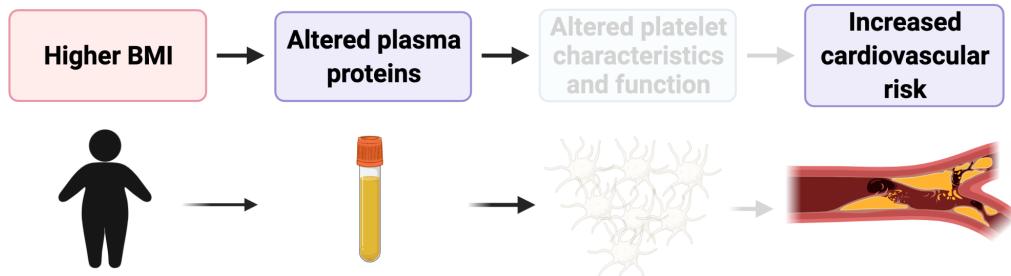


Figure 6.1: **Schematic of the associations explored in Chapter 6.** This chapter explores the causal effect of BMI on the plasma proteome and explores whether these changes might contribute to cardiovascular diseases. Figure made using BioRender.com.

6.2 Methods

6.2.1 Study population

The present study was conducted on a random subset of participants from INTERVAL who had basic phenotype data and plasma proteins measured by SomaLogic. This included up to 2737 participants mostly of European descent across analyses described below. More details of the INTERVAL study can be found in Chapter 3 in section 3.3.

6.2.2 Measurement of circulating proteins

Plasma proteins were measured in INTERVAL participants at baseline (before randomisation of assignment to the time interval between blood donation) using the SomaScan® by SomaLogic³⁴. This platform uses 4034 modified nucleotides known as Slow Off-rate Modified Aptamers (SOMAmers) which make direct contact with proteins, enabling detection of 3622 unique proteins or protein complexes and quantifies them using a DNA microarray¹⁵⁵. Separate SOMAmers can bind to isoforms of the same protein,

but can also bind to the same protein at different sites (which can be impacted by post-translational modifications or complexes formed with other proteins). All 4034 SOMAmers were therefore included. The extensive number of proteins measured, with no missingness and in a cohort of 2737 participants, provides a rich proteomic dataset. The proteins were measured in relative fluorescence units (RFUs) and quality control (QC) was performed as described by Sun et al.³⁴. There was no missingness across protein variables. The proteomic data used had been pre-adjusted (using linear regression) for age, sex, duration between blood draw and sample processing (1 day or less vs >1 day), and the first three genetic principal components, with the residuals inverse normal rank transformed. All following analyses use this ‘pre-adjusted’ data as input.

6.2.3 Statistical analyses

The population characteristics of INTERVAL participants with SomaLogic data who were included in this study (N range: 2422 to 2737 due to missing data for covariables) were compared to those INTERVAL participants who were not included (N range: 27174 to 30721) to assess generalisability of any BMI-protein associations to the wider INTERVAL sample. Population characteristics evaluated were age, sex, weight, height, BMI, smoking frequency, and alcohol intake. Differences in population characteristics among the two INTERVAL sub-sets were tested by a two-sided t-test for continuous traits and a two-sided Chi-square test for categorical variables. Observational analyses were conducted using linear regression to examine associations between BMI (in normalized standard deviation (SD) units based on a rank normal transformation (`rntransform()` from “moosefun” package <https://github.com/hughesvoanth/moosefun>) and each standardised protein trait as a dependent (outcome) variable. Two linear models were used (using `lm()` function from R “stats” package): 1) adjusted for age and sex and 2) additionally adjusted for smoking and alcohol consumption (each as an ordered categorical variable). Given that the procedure which generates the “pre-adjusted” data (adjustment for covariables before rank normal transformation of proteins) can reintroduce correlations¹⁵⁶, age and sex are again used as covariables here. The estimates derived from models 1) and 2) therefore reflect the normalized SD-unit difference in each protein trait per normalized SD-unit (4.8 kg/m²) higher BMI. Associations of covariables with BMI and protein traits were also examined using linear regression.

A Shapiro-Wilk test was used to confirm whether the GRS showed a normal distribution. MR analyses were conducted using two-stage least squares (2SLS) regression models with robust standard errors,

using the systemfit function as part of the systemfit package¹⁰⁷, with measured BMI in SD units and the GRS for BMI as the instrumental variable. These MR estimates reflect the normalized SD-unit difference in each protein trait per normalized SD-unit (4.8 kg/m^2) higher BMI. Estimates are reported from the direct linear associations between BMI and proteins as “observational” estimates and those from the 2SLS causal effect estimates as “MR estimates”. Agreement between observational and MR estimates was examined using a separate linear regression. This was performed: 1) for all proteins, and 2) excluding the proteins that fell below our P-value reference point for strong evidence (defined below) to examine whether agreement is limited to “top hits” or applies throughout the effect distribution. Agreement between observational estimates and MR estimates would suggest that there are causal effects of BMI across the general proteome, with differences in estimates suggesting confounding of observational estimates.

To account for multiple testing, a Bonferroni correction was used to adjust results. This was informed by the correlation between proteins, adjusting only for the estimated number of independent traits (Figure 6.2). Correlation was assessed by a Spearman’s correlation matrix. From a starting number of 4034, the number of independent proteins was 3655 (using a correlation cut-off of $r = 0.8$ or tree cut height = 0.2 between proteins, Figure 6.3), dendrogram made using “iPVs” package <https://github.com/hughesevanth/iPVs>). A Bonferroni adjusted P-value of $0.05/3655 = 1.4 \times 10^{-5}$ was utilised to indicate strong evidence in this sample. Full results are presented in the supplementary material.

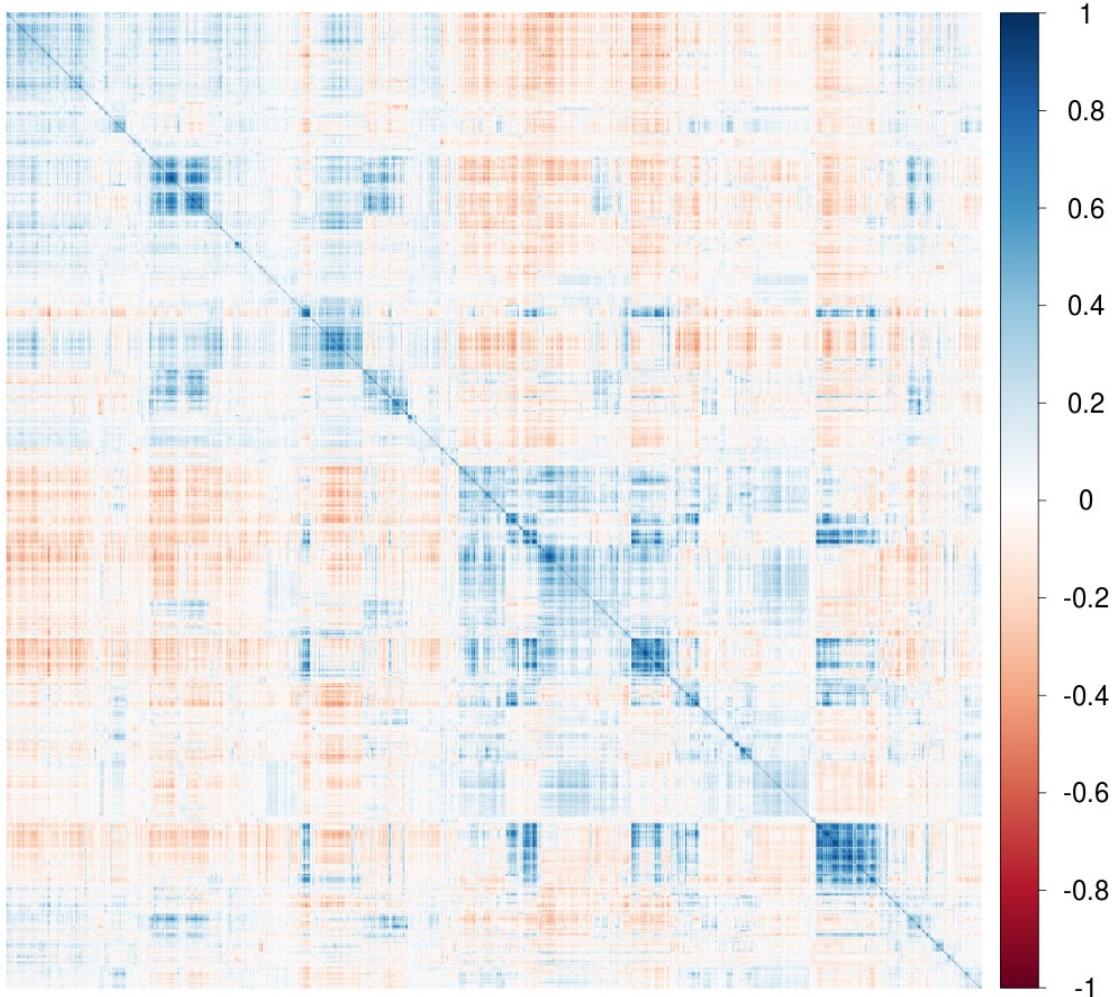


Figure 6.2: **Correlation matrix of all protein traits analysed by SomaLogic (4,034).** The colour corresponds to the correlation coefficient ranging from 1 (dark blue) to -1 (dark red).

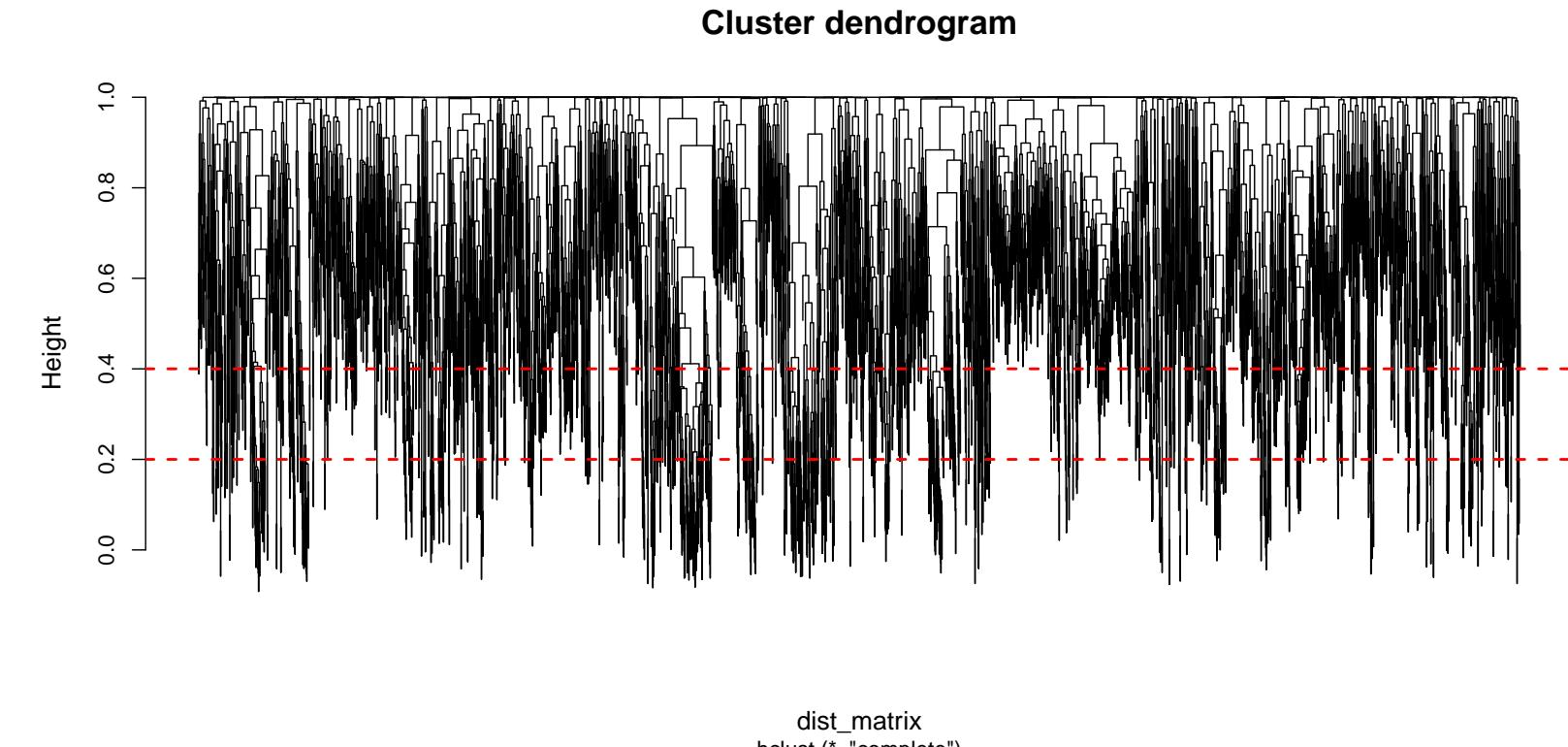


Figure 6.3: Cluster dendrogram showing the hierarchical relationship between protein levels.

Height is calculated as (1-correlation coefficient). At a height of 0.2 and 0.4 (red dashed lines), the number of independent proteins was 3,655 and 3,016 respectively

The association between BMI and specific platelet proteins were also explored. Here, GO pathways which include the term “platelet” were selected in AmiGO2 <http://amigo.geneontology.org/> (Supplementary Table 1). These pathways were then searched in BioMart <https://www.ensembl.org/biomart/martview/afd335dff2c91c86501d3f1f77b8b805>, to provide a list of all the proteins involved in these platelet pathways (Supplementary Table 2). Unique proteins in Supplementary Table 2 were searched within the results to extract the estimate for BMI on platelet proteins. As the chemokines MDC and TARC were explored in the previous chapter, the specific effects of BMI on MDC and TARC were looked up.

6.2.4 Enrichment analysis

To investigate whether any protein groups showed a particularly strong relationship with BMI and disease (signal detection), protein features were clustered for further analysis. First, a principal component analysis (PCA; `prcomp()` function from the R “stats” package) on the proteins, not the individuals, was performed on the “pre-adjusted” (see above) dataset (Figure 6.4A-D). The top “n” PC eigenvectors, as identified by a scree plot of the PCA eigenvalues (Figure 6.4E), were carried forward into an unsupervised k-means analysis (`kmeans()` function from the R “stats” package). Nineteen k-means analyses were run altering the value of k (number of clusters) from two to 20. To identify an appropriate number of protein clusters (k) a scree like plot was generated (Figure 6.4F). Here the variance explained by clusters was plotted, for each k, as estimated as the sum of squares explained by clusters (betweenness) over the total sums of squares, and looked for the smallest k with the maximum variance explained (a plateau). In summary, a data reduction method (PCA) was used to identify major axes (PCs) of the protein data that were then utilised in a machine learning clustering algorithm (k-means) to identify clusters of proteins that share abundance similarities across individuals.

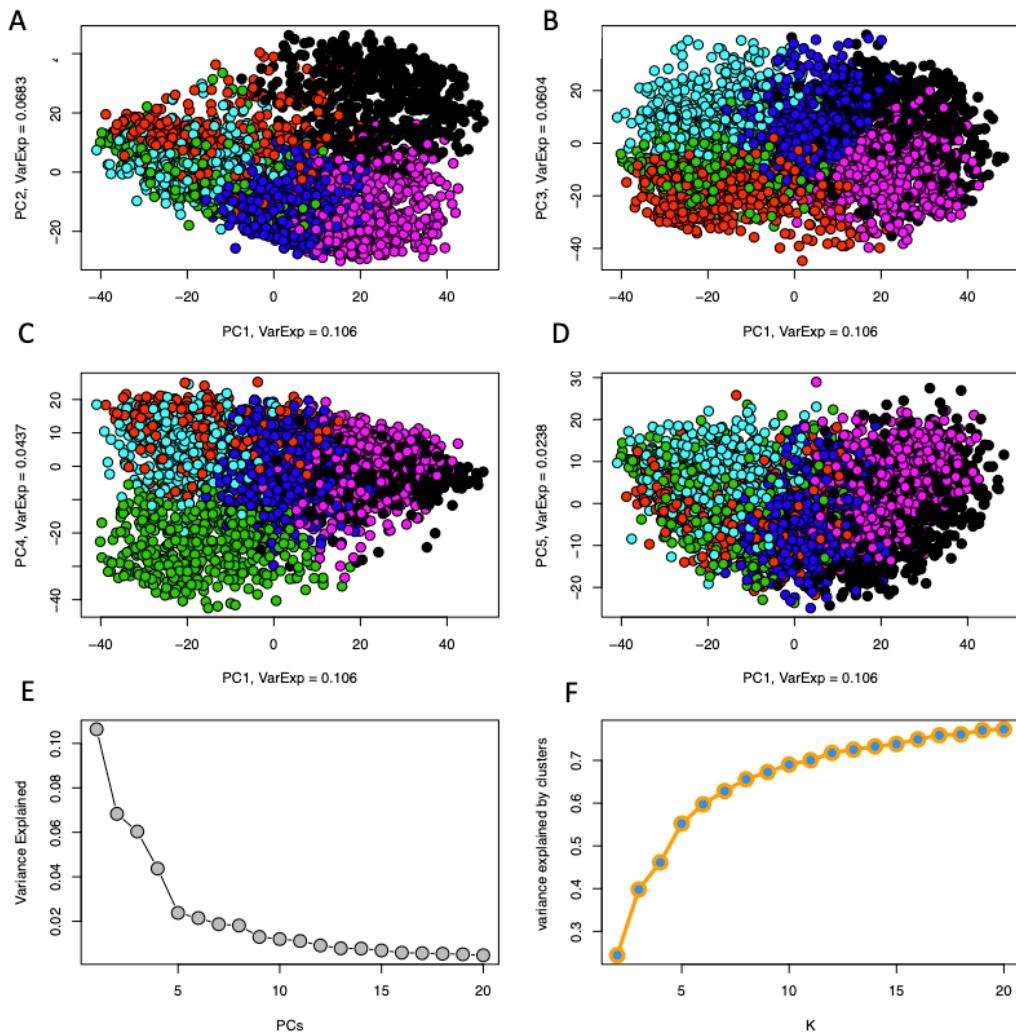


Figure 6.4: Principal component analysis (PCA) and k-means clustering of proteins. Principal component analysis (PCA) and k-means clustering of proteins provide evidence for five clusters. A-D) Principal component (PC) 1 vs PC2-PC5 for the study protein data. Each dot represents a protein, and the colors represent the clusters identified by the k-means analysis (1=black, 2=red, 3=green, 4=dark blue, 5=light blue, 6=pink). E) PC scree plot displaying the proportion of variance explained by each of the first 20 PCs. F) K-means scree like plot displaying the variance explained by clusters for each of the 19 k-means analysis, where in each analysis k (the number of clusters) was set from two to 20.

To explore whether there was a systematic difference in the association of proteins within these clusters and BMI, the beta coefficients from the observational linear regressions or MR models were transformed into their absolute values and divided by their standard error (SE). The absolute betas divided by their SEs in each cluster was compared using a one-tailed pairwise Wilcox test to identify which clusters showed a stronger association with BMI. For the cluster(s) showing evidence for larger absolute beta coefficients, an enrichment analysis was performed using DAVID bioinformatics resources

6.8¹⁵⁷. Enrichment was assessed by using the uniprot IDs for the proteins in the cluster and comparing these proteins with the uniprot IDs of the full SomaLogic protein list. Enrichment for protein involvement in disease (using the genetic association database (GAD) disease classes¹⁵⁸) of the protein cluster was assessed by fold enrichment and a Bonferroni-corrected P-value to account for multiple testing. Disease classes are broadly grouped, therefore the main disease class of interest is cardiovascular disease as this class would incorporate CHD/stroke. Proteins that were associated with BMI in confounder-adjusted observational analyses at $P < 1.4 \times 10^{-5}$ were also entered into the disease enrichment tool and compared with the total proteins (as described for cluster enrichment). Analyses were performed using R version 3.4.2¹⁰⁶.

6.3 Results

6.3.1 Participant characteristics

INTERVAL participants included in this study (those with proteomic data), had a mean age of 45.0 years (SD of 14.1 years) and 48.3% were female (Table 6.1). Mean BMI was 25.9 kg/m² (SD of 4.8 kg/m²) and the majority of participants were non-smokers (59.1%). Nearly a quarter (23.5%) reported currently or previously smoking daily and 71.5% reported drinking alcohol at least once a week. Participants with proteomic data were representative of the full INTERVAL cohort (Table 6.1).

Table 6.1: Comparison of included vs excluded INTERVAL participants for the BMI-proteome analysis

Variable	Included Mean (SD) or %	Included N	Excluded Mean (SD) or %	Excluded N	P value for difference (Two tailed t-test or Chi^2^ test)
Age	45.0 (14.1)	2737	44.9 (14.2)	30721	0.76
Sex		2737		30721	0.04
Male	51.70%		49.70%		
Female	48.30%		50.30%		
Weight (kg)	78.5 (16.1)	2736	78.6 (16.0)	30719	0.26
Height (cm)	172.7 (9.7)	2730	172.2 (9.6)	30661	0.005
Body mass index (kg/m ²)	25.9 (4.8)	2729	26.4 (4.7)	30659	0.66
Smoking frequency		2675		29958	0.88
Never	59.10%		59.40%		
Occasional	11.90%		12.00%		
Most days or every day	29.00%		28.50%		
Alcohol intake frequency		2422		27124	0.34
Rarely	11.70%		12.60%		
Less than once a week	16.90%		17.10%		
One or two times a week	37.40%		37.70%		
Three to five times a week / most days	34.10%		32.50%		

6.3.2 Observational estimates of associations of BMI with protein traits

In a linear regression model adjusted for age and sex among 2729 adults, BMI (per SD higher) was associated with 1576 proteins (39%) at the level $P < 1.4 \times 10^{-5}$ (multiple testing reference point, Supplementary Table 3). In a second model additionally adjusting for frequencies of smoking and alcohol intake among 2380 adults, there were 1447 associations at the same reference point (Supplementary Table 4). The strongest positive associations were with leptin (0.74 SD, 95% CI 0.71-0.77, $P=9.9 \times 10^{-324}$) and adipocyte fatty acid binding protein (FABP4) (0.58 SD, 95% CI 0.55-0.62, $P=6.4 \times 10^{-211}$). BMI (per SD) was also strongly positively associated with inflammatory proteins such as Complement Factor I (0.46 SD, 95% CI 0.43-0.50, $P=5.6 \times 10^{-122}$) and CRP (0.44 SD, 95% CI 0.41-0.48, $P=8.2 \times 10^{-112}$). BMI (per SD) also showed strong negative associations with proteins such as insulin-like growth factor-binding protein (IGFBP) 2 (-0.48 SD, 95% CI -0.51 to -0.44, $P=2.7 \times 10^{-133}$) and sex hormone-binding globulin (SHBG) (-0.43 SD, 95% CI -0.47 to -0.39, $P=2.4 \times 10^{-106}$).

There were 262 unique platelet proteins that were analysed in INTERVAL, 121 were associated with BMI in the confounder adjusted model (46%). There was evidence that proteins involved in platelet pathways were associated with BMI. Higher BMI was negatively associated with antithrombin-III (-0.26 SD, 95% CI -0.30 to -0.22, $p=9.6 \times 10^{-42}$). BMI was also negatively associated with platelet-activating factor acetylhydrolase (PAFAH) (-0.26 SD, 95% CI -0.30 to -0.23, $p=4.9 \times 10^{-39}$), as was platelet endothelial aggregation receptor 1 (PEAR1) (-0.20 SD, 95% CI -0.24 to -0.16, $p=1.24 \times 10^{-23}$). Other proteins that were negatively associated with BMI include platelet endothelial cell adhesion molecule (PECAM-1), platelet glycoprotein Ib alpha chain (GPIb α) and platelet-derived growth factor receptor beta (PDGF Rb) (Supplementary Table 4 & Supplementary Table 5). BMI was positively associated with the chemokine MDC (0.18 SD, 95% CI 0.14-0.22, $p=3.6 \times 10^{-21}$), but was not associated with TARC (Supplementary Table 3).

6.3.3 Observational associations of covariates with BMI and protein traits

Age, sex, and frequencies of smoking and alcohol intake were each associated with BMI (Table 6.2). Males had a higher BMI than females (0.17 SD, 95% CI 0.10-0.25, $P=5.8 \times 10^{-6}$). Age was positively associated with BMI (0.01 SD higher per year older, 95% CI 0.009-0.015, $P=1.2 \times 10^{-18}$). Smoking frequency was positively associated with BMI, but alcohol intake frequency was negatively associated with BMI. Covariates (age, sex, smoking and alcohol) showed associations with protein traits (Supplementary Tables 6-9 and Figures 6.5 A-D). There was evidence for 18 associations between age

and proteins, 26 associations between sex and proteins, 38 proteins associated with smoking and 137 proteins associated with alcohol at the Bonferroni-adjusted level of $p<1.4\times10^{-5}$.

Table 6.2: **Associations between covariables (exposure) and standardised BMI (outcome)**

Variable	N	Beta coefficient per 1-unit increase in confounder (SDs)	Standard error	P value	Adjusted R2	F statistic	95% CI	Lower 95% CI	Upper 95% CI
Age (years)	2729	0.012	0.001	1.23e-18	0.028	78.8	0.003	0.009	0.015
Sex (1=female, 2=male)	2729	0.17	0.038	5.8e-06	0.007	20.6	0.075	0.1	0.25
Smoking frequency (1=never, 2=occasinal, 3=most days/ every day)	2667	0.022	0.022	3.16e-05	0.006	17.4	0.043	0.05	0.13
Alcohol intake frequency (1=rarely, 2=<1 a week, 3=1-2 times a week, 4=most days/every day)	2415	0.021	0.021	7.08e-05	0.006	15.8	0.041	-0.12	-0.04

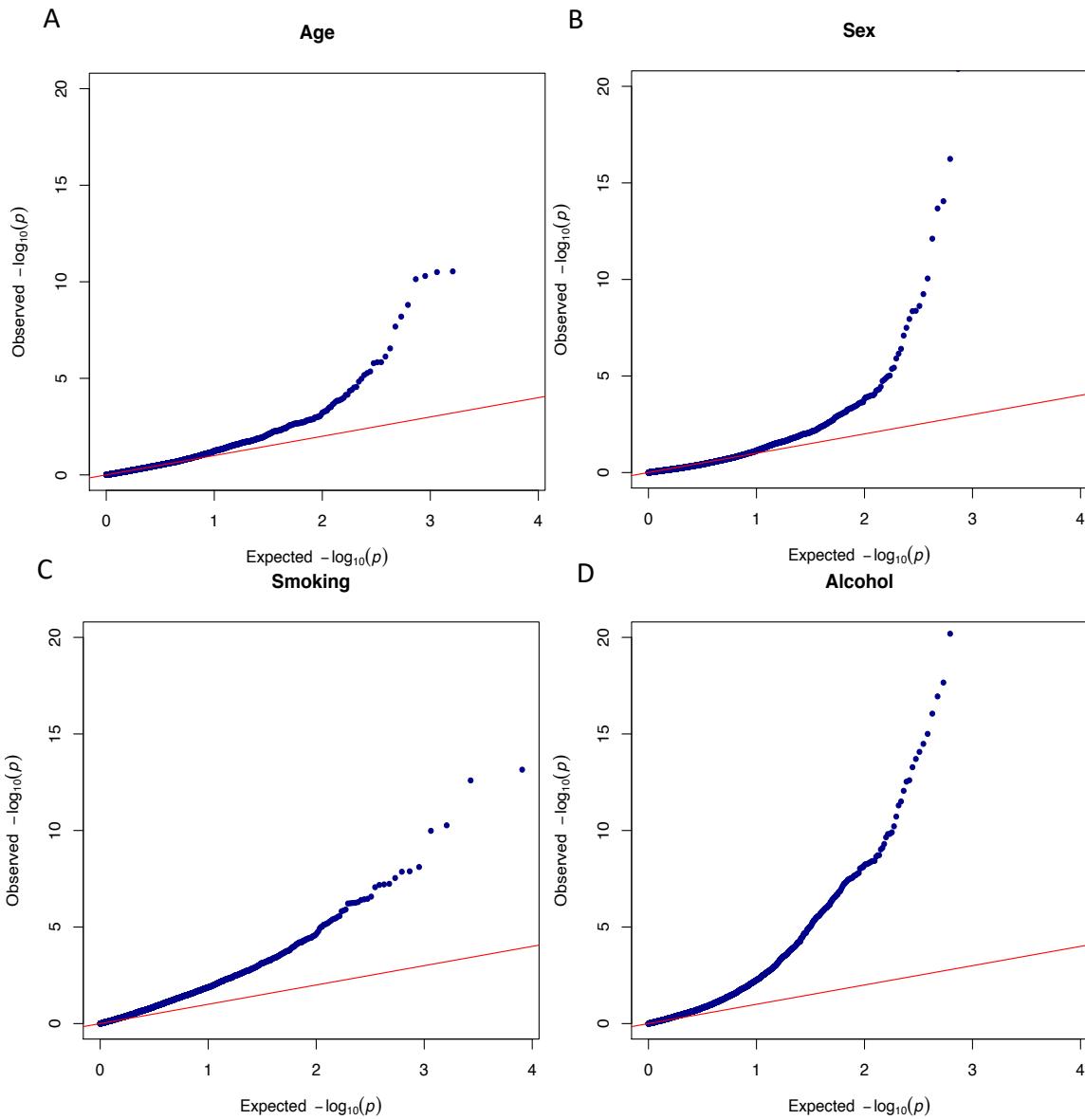


Figure 6.5: **Q-Q plots for the expected against observed $-\log_{10}(p)$ values for associations of age (A), sex (B), smoking (C) and alcohol (D) with protein traits**

6.3.4 Associations of the GRS for BMI with measured BMI and covariates

The distribution of the GRS among participants was normal (mean=0.08, SD=0.29, W=0.99, P=0.73, N=2729). The GRS was associated with BMI, explaining 2.8% of its variance ($R^2=0.028$, $P=1.6\times 10^{-18}$, Table 6.3). There was no strong evidence of association between GRS and age ($R^2=0.001$, $P=0.11$), sex ($R^2=6\times 10^{-5}$, $P=0.28$), smoking frequency ($R^2=< 0.0001$, $P=0.91$), or alcohol intake ($R^2<0.0001$, $P=0.44$).

Table 6.3: Associations of the genetic risk score for BMI with reported BMI and covariates

Variable	N	Beta coefficient (per 1-unit increase in GRS)	Standard error	P value	Adjusted R2	F statistic
BMI (SDs)	2729	0.57	0.06	1.64e-18	0.028	78.2
BMI (kg/m ²)	2729	2.54	0.31	4.82e-16	0.024	66.68
Age	2737	-1.47	0.92	0.11	0.001	2.55
Sex (1=female, 2=male)	2737	-0.04	0.03	0.28	5.96e-05	1.16
Smoking frequency (1=never, 2=occasional, 3=most days/every day)	2675	-0.02	0.14	0.91	-4e-04	0.01
Alcohol intake frequency (1=rarely, 2=<1 a week, 3=1-2 times a week, 4=most days/every day)	2422	-0.06	0.07	0.44	-2e-04	0.6

6.3.5 MR estimates of associations between BMI and protein traits

In MR analyses, eight unique BMI-protein associations were detected at the level $P<1.4\times10^{-5}$ (multiple testing reference point, Figure 6.6). MR estimates provide an estimate of the causal association between protein (in SDs) per SD higher BMI. The strongest association of BMI was again with leptin (0.63 SD, 95% CI = 0.48-0.79; $P=1.6\times10^{-15}$); this was followed by the association with FABP4 (0.65 SD, 95% CI = 0.46-0.83; $P=6.7\times10^{-12}$). A strong negative association was also seen between BMI (per SD) and SHBG (-0.45 SD, 95% CI -0.65 to -0.25, $P=1.4\times10^{-5}$). Other BMI-protein associations ($P<1.4\times10^{-5}$) included positive associations with fumarylacetoacetate (FAAA), inhibin beta C chain and complement C5, and negative associations with receptor-type tyrosine-protein phosphatase delta and PILR alpha-associated neural protein.

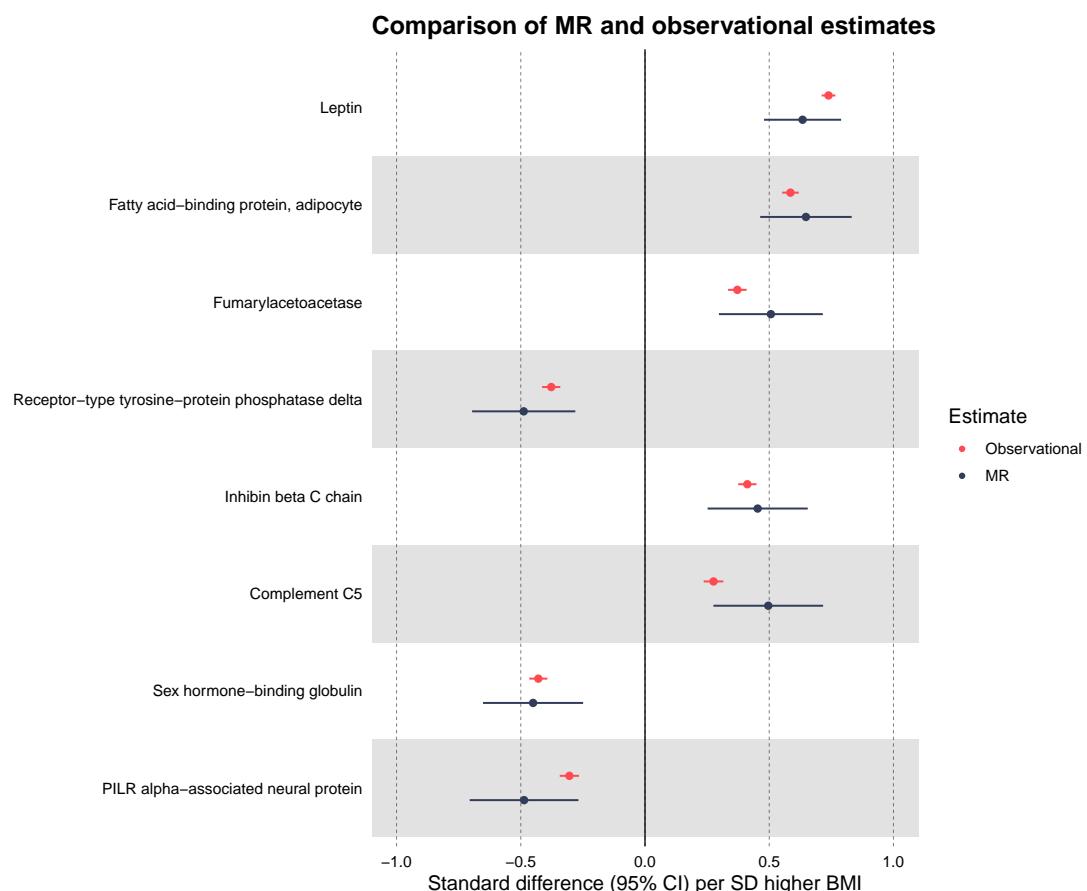


Figure 6.6: Forest plot of the strongest BMI-protein Mendelian randomisation estimates and the corresponding observational estimates

The estimates for the effect of BMI on platelet specific proteins did not pass multiple testing adjustment. Despite this, the MR estimates for the effect of BMI on platelet proteins generally were of a similar

magnitude to the fully adjusted observational analysis, however none of the platelet proteins passed the multiple testing adjusted p-value. The causal estimate for the effects of BMI on PAFAH was -0.24 SD (95% CI -0.46 to -0.03, $p=0.028$). Similarly, the causal estimate for the effect of BMI on PEAR1 was similar to the observational estimate (-0.21 SD, 95% CI -0.43 to 0.005, $p=0.06$). Despite a similar magnitude of estimate, these associations were not detected as these estimates were less precise. Supplementary Table 10 provides the full MR results and Supplementary Table 5 has the observational and MR estimate for each platelet protein combined. The MR estimate for the effect of BMI on MDC was similar in magnitude (0.20 SDs, 95% CI -0.02 to 0.42, $p=0.069$), but was less precise than the observational estimate, whereas there was no evidence for an effect on TARC (Figure 6.7).

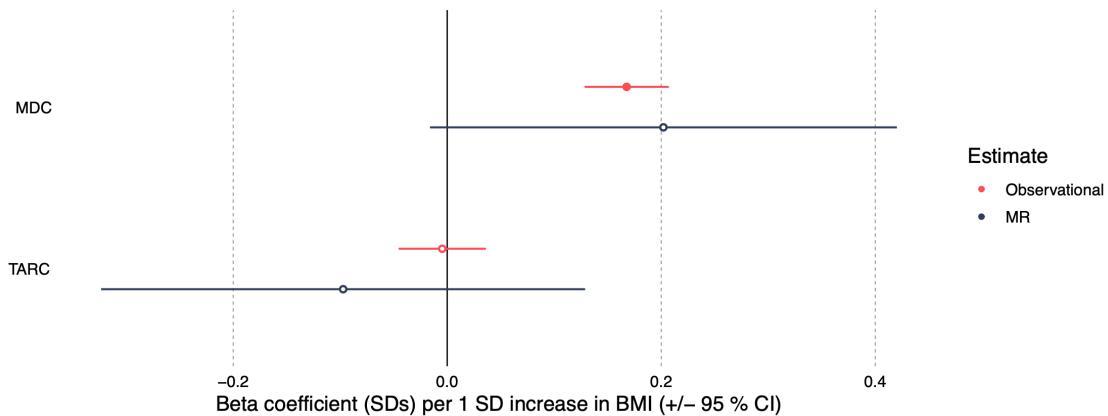


Figure 6.7: Forest plot of the Mendelian randomisation estimates and the corresponding observational estimates for MDC and TARC. Filled estimates indicate $p<1.4\times10^{-5}$.

6.3.6 Comparison of observational and MR estimates

The distribution of P-values for associations between BMI and protein traits suggested an overrepresentation of signal for the observational estimates of BMI and protein traits; far more than expected from chance alone (Figure 6.8A). In contrast to this, the extent of this overrepresentation was reduced considerably in the MR (Figure 6.8B).

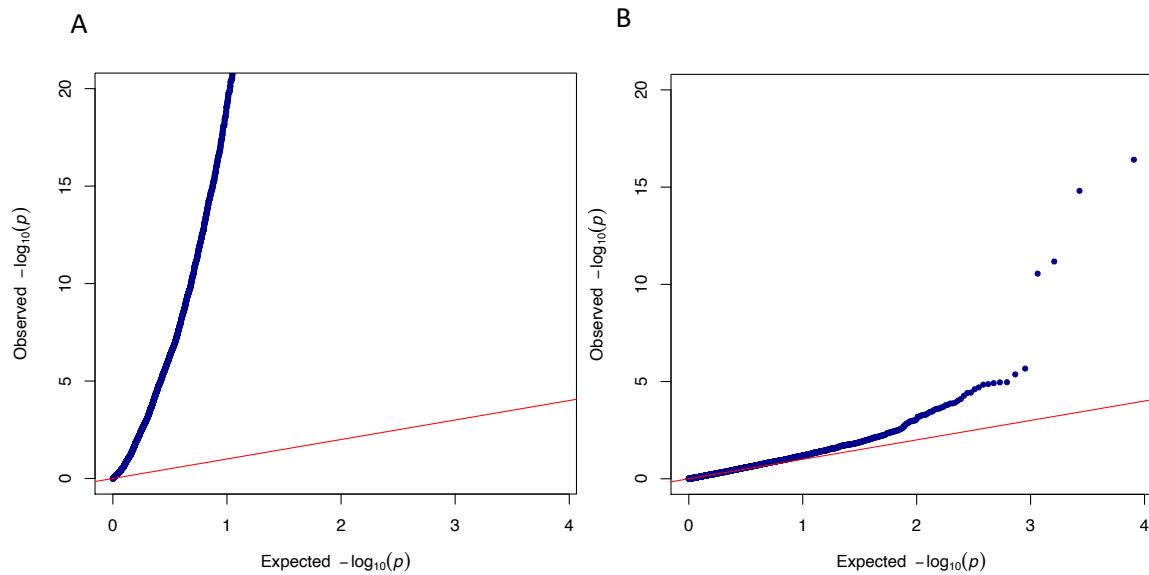


Figure 6.8: Q-Q plot of the expected against observed $-\log_{10}(\text{pvalues})$ for BMI-protein estimates.
A) Q-Q plot of expected against observed $-\log_{10}(\text{pvalues})$ for the unadjusted observational BMI-protein trait estimates
B) Q-Q plot of expected against observed $-\log_{10}(\text{p})$ values for the Mendelian Randomization BMI-protein trait estimates.

The unadjusted and confounder-adjusted regression coefficients for BMI and protein traits were strongly associated ($\text{Beta}=0.99$ SDs, $R^2=0.99$, $P=9.9 \times 10^{-324}$, Figure 6.9). Compared with the observational estimates, the MR estimates were less precise, but there was a strong positive association between the beta coefficients from observational and MR estimates ($\text{Beta}=0.68$ SDs, $R^2=0.33$, $P=9.9 \times 10^{-324}$) (Figure 6.10). After removing the proteins where $P < 1.4 \times 10^{-5}$, the strength of association between unadjusted and adjusted observational estimates remained, but the association between observational and MR estimates attenuated slightly (Figures 6.11 A/B). These results suggest causal effects of BMI across the general proteome.

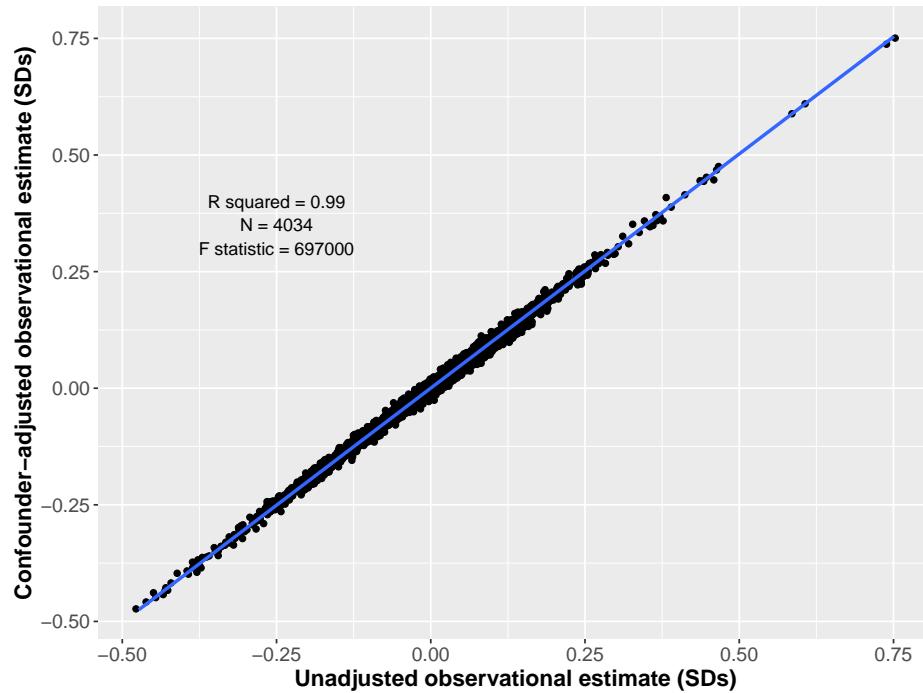


Figure 6.9: Scatter plot of the unadjusted (age and sex adjusted) observational estimates and the confounder-adjusted observational estimates for BMI and protein traits. Regression line shown in blue.

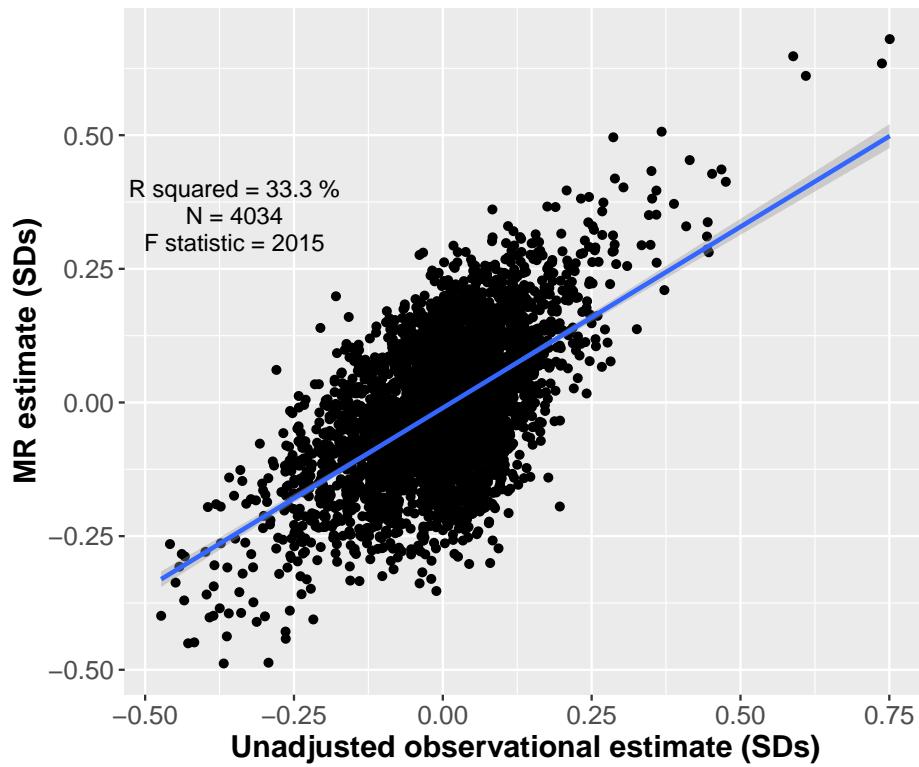


Figure 6.10: **Scatter plot of the unadjusted (age and sex adjusted) observational estimates and the MR estimates for BMI and protein traits.** Regression line shown in blue.

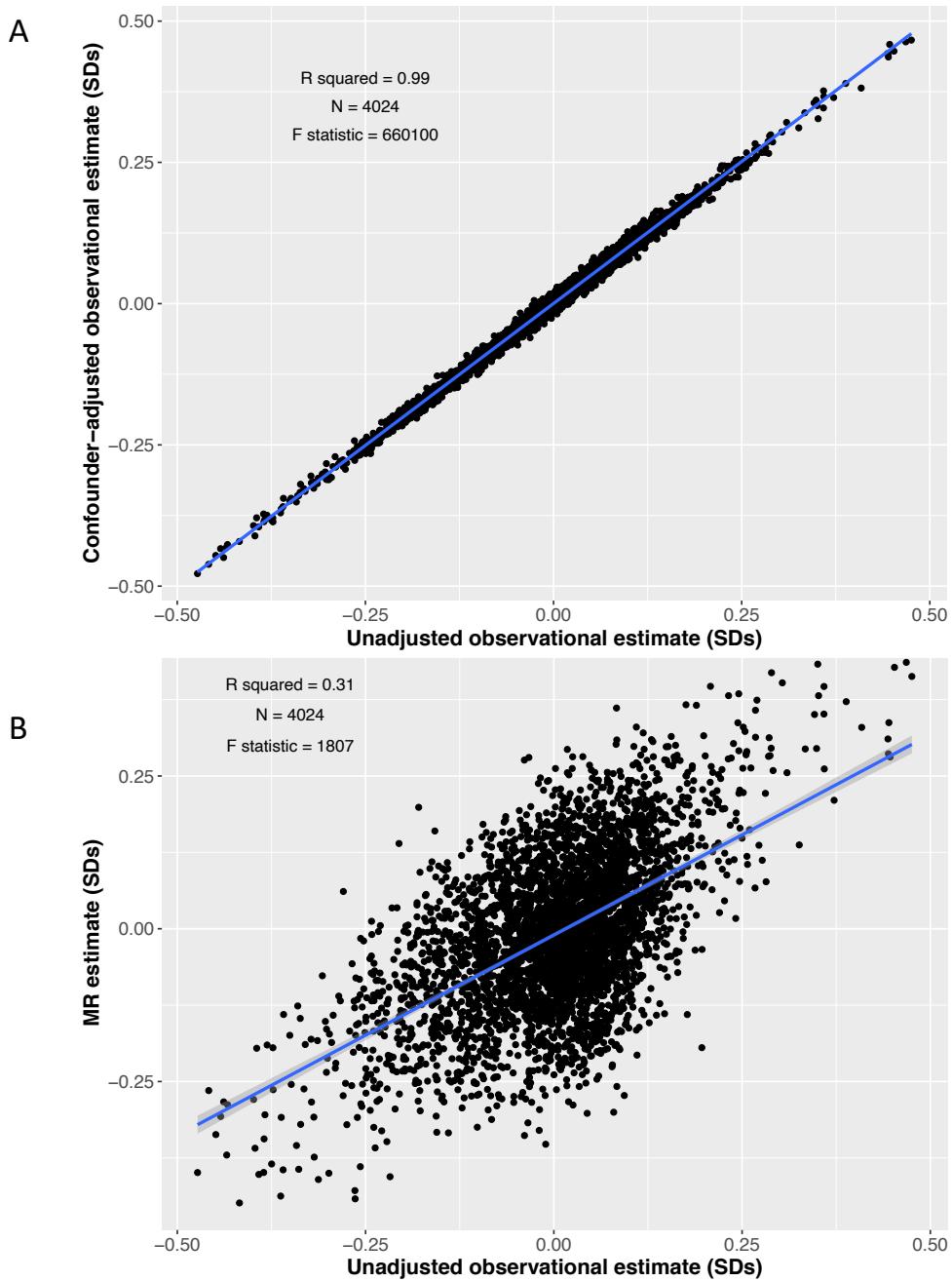


Figure 6.11: Scatter plot comparing BMI-protein estimates across models with top eight BMI-associated proteins excluded. A) Scatter plot of the unadjusted (age and sex adjusted) observational estimates and the confounder-adjusted observational estimates for BMI and protein traits with a regression line (blue). B) Scatter plot of the unadjusted (age and sex adjusted) observational estimates and the MR estimates for BMI and protein traits with a regression line (blue).

6.3.7 Enrichment analysis of strongest BMI-protein associations

In examining the clustering of proteins, visual representation using a scree plot suggested there were five PCs that explained 30.3% of the variance (Figure 6.4). After PC5 there was clear drop in variance explained, therefore other PCs were excluded. These five PCs were entered into a k-means analysis, which provided evidence for five clusters (grouping of individual proteins is included in Supplementary Table 10). To identify which cluster was most strongly affected by BMI, the median absolute beta coefficient divided by the SE for each cluster was compared with the overall estimate. Six of the proteins out of the eight strongest BMI-protein MR estimates were in cluster 2 (Supplementary Table 10). There was consistent evidence that cluster 2 showed a stronger association with BMI than the overall average BMI-protein effect both observationally (3.79 (IQR 1.62-7.06) vs 3.35 (IQR 1.57-5.83) respectively, $P=3.7\times 10^{-4}$) and in MR (0.85 (IQR 0.41-1.46) vs 0.74 (IQR 0.32-1.14), $P=5.3\times 10^{-6}$, Table 6.4). Cluster 2 showed consistent evidence of having the largest BMI effect. Compared with the full protein list in SomaLogic, the proteins in cluster 2 were enriched for disease (Table 6.5), including cardiovascular disease (1.14 fold enrichment, $P=1.3\times 10^{-4}$), renal disease (1.22 fold enrichment, $P=1.0\times 10^{-3}$) cancer (1.1 fold enrichment, $P=9.5\times 10^{-3}$) and metabolic disease (1.08 fold enrichment, $P=4.2\times 10^{-2}$). No other individual cluster showed enrichment for disease. Enrichment for disease was also explored by comparing the proteins which had an association with BMI ($P<1.4\times 10^{-5}$) in the confounder-adjusted regression model with the total protein list. Compared with the full protein list, the proteins which showed a stronger observational association with BMI were enriched for renal disease (1.21 fold enrichment, $P=0.001$) and metabolic disease (1.9 fold enrichment, $P=0.015$, Table 6.6).

Table 6.4: Comparison of cluster median(absolute beta coefficient / SE) with the overall median for observational and MR analyses. *One-tailed pairwise Wilcox test

Cluster	N	Median absolute observational beta coefficient divided by SE (IQR)	P value* for cluster v all proteins (Observational)	Median MR beta coefficient divided by SE (IQR)	P value* for cluster v all proteins (MR)
1	1061	3.28 (1.56-5.26)	1	0.69 (0.31-1.22)	1
2	1406	3.79 (1.62-7.06)	3.70E-4	0.85 (0.41-1.46)	5.3E-6
3	581	2.77 (1.39-4.30)	1	0.74 (0.37-1.25)	1
4	502	3.01 (1.25-5.6)	1	0.68 (0.34-1.19)	1
5	484	4.44 (2.2-7.05)	1.80E-5	0.63 (0.32-1.14)	1
All	4034	3.35 (1.57-5.83)		0.74 (0.35-1.28)	

Table 6.5: Cluster 2 vs full SomaLogic protein enrichment results for disease class using DAVID bioinformatics 6.8¹⁵⁷

Term (Genetic Association Database disease class)	Count	%	List Total	Population Hits	Population Total	Fold Enrichment	P Value	Bonferroni-adjusted P value
Cardiovascular	439	34.1	1024	1023	2723	1.14	7.3E-06	1.3E-04
Renal	216	16.8	1024	472	2723	1.22	5.8E-05	1.0E-03
Cancer	401	31.2	1024	958	2723	1.11	5.3E-04	9.5E-03
Pharmacogenomic	357	27.8	1024	856	2723	1.11	1.9E-03	3.4E-02
Metabolic	504	39.2	1024	1243	2723	1.08	2.4E-03	4.2E-02
Vision	100	7.8	1024	216	2723	1.23	5.9E-03	1.0E-01
Haematological	168	13.1	1024	388	2723	1.15	9.8E-03	1.6E-01
Reproduction	152	11.8	1024	351	2723	1.15	1.4E-02	2.3E-01
Immune	360	28	1024	886	2723	1.08	1.5E-02	2.4E-01
Neurological	311	24.2	1024	759	2723	1.09	1.6E-02	2.5E-01
Aging	117	9.1	1024	278	2723	1.12	7.5E-02	7.5E-01

Table 6.6: Disease enrichment of proteins associated with BMI in confounder adjusted model compared with all proteins in SomaLogic (DAVID Bioinformatics 6.8)¹⁵⁷

Term (Genetic Association Database disease class)	Count	%	List Total	Population Hits	Population Total	Fold Enrichment	P Value	Bonferroni-adjusted P value
Renal	218	16.4	1039	472	2723	1.21	7.7E-05	0.001
Pharmacogenomic	372	28	1039	856	2723	1.14	8.8E-05	0.002
Metabolic	515	38.8	1039	1243	2723	1.09	8.3E-04	0.015
Aging	129	9.7	1039	278	2723	1.22	2.7E-03	0.047
Cardiovascular	424	32	1039	1023	2723	1.09	4.1E-03	0.072
Unknown	233	17.6	1039	541	2723	1.13	6.4E-03	0.109
Psych	228	17.2	1039	534	2723	1.12	1.2E-02	0.189
Reproduction	154	11.6	1039	351	2723	1.15	1.4E-02	0.228
Normal variation	86	6.5	1039	185	2723	1.22	1.5E-02	0.236
Other	211	15.9	1039	497	2723	1.11	2.1E-02	0.313
Cancer	391	29.5	1039	958	2723	1.07	2.2E-02	0.332
Haematological	164	12.4	1039	388	2723	1.11	5.0E-02	0.605
Neurological	309	23.3	1039	759	2723	1.07	5.5E-02	0.638
Developmental	160	12.1	1039	381	2723	1.1	6.6E-02	0.708

6.4 Discussion

This chapter sought to estimate the effects of adiposity on a comprehensive set of protein traits only recently measurable by untargeted proteomics using observational and MR methods. This analysis was performed as alterations in the plasma proteins may have implications for cardiovascular disease, and therefore relating to platelet function. Observational results provided evidence for associations between BMI and 1576 proteins and MR was performed to reduce confounding. MR results suggest that BMI alters protein traits involved in regulating appetite, sex hormones, inflammation, and other systems; specific proteins most altered by BMI include leptin, FABP4, and SHBG. Results of follow-up analyses suggest that the cluster of proteins most altered by BMI is enriched for genes associated with cardiovascular and metabolic disease.

This chapter explored the effect of BMI on a large set of circulating proteins in an MR framework. Previous studies have used observational epidemiology to explore the effect of obesity on the plasma proteome: one study used mass spectrometry and found an increase in Complement Factors I, B and H and an increase in CRP⁹⁰. These findings were replicated in our current observational analysis using the SomaLogic platform, indicating that associations are detectable across different proteomic platforms. The only association that did not replicate in the current analysis was the positive association with protein S100-A9. Although the MR analysis did not support some of these BMI-protein associations as being causal based on a P-value reference point, the strong association between the observational and MR estimates throughout the entire effect distribution suggests that disagreements between methods are likely an issue of power given current sample sizes.

Previous work implementing MR to examine the relationship between BMI and ~1000 proteins (measured using the same SomaLogic array) provided corroborative evidence to that shown here⁸⁵. Both studies suggested a positive association between BMI and leptin, as well as a negative association with SHBG. Other proteins, such as IGFBP1/2 and growth hormone receptor, did not pass our multiple testing threshold, but the direction and magnitude of estimates were in agreement, suggesting a possible causal effect that was not detectable in the current analysis. Building on previous work, the current chapter provides MR estimates for >3600 proteins, offering a wider proteomic profile and detecting additional associations such as that between BMI and fumarylacetoacetate and inhibin beta C chain. Furthermore, the inclusion of over threefold more proteins allowed a more comprehensive enrichment analysis to be performed.

For proteins with stronger MR derived association evidence, it is important to explore whether they have a potential role in disease. Identification of individual proteins could help to guide future intervention if changes in proteins can be mapped to disease outcomes. Our results suggest a strong positive effect of BMI on levels of leptin, a hormone released by white adipose tissue which suppresses appetite¹⁵⁹. The direction of effect agrees with estimates from previous cross-sectional and MR studies^{32,160}, indicating leptin receptor resistance¹⁶¹. There is observational evidence in humans that higher leptin can induce greater aggregation of platelets (cells involved in haemostasis)¹⁶². In a larger observational study, leptin was found to be associated with higher risk of coronary events independent of BMI¹⁶³.

Our results help to provide contextualisation for proteins which have already been implicated in disease. For example, results suggest a strong positive effect of BMI on FABP4, an adipokine found primarily in adipocytes and macrophages¹⁶⁴. This MR estimate supports the association which has been suggested in previous observational studies¹⁶⁵. FABP4 has been implicated in cardiometabolic disease: a SNP which increases FABP4 was found to raise the odds of type II diabetes among adults¹⁶⁶, potentially through its contribution to higher insulin resistance¹⁶⁷. FABP4 has also been associated with higher risk of atherosclerosis among adults¹⁶⁸. A strong SHBG-lowering effect of higher BMI was also suggested here. The SHBG molecule is a glycoprotein which binds androgens and oestrogens and suppresses their activity¹⁶⁹; a reduction in SHBG is therefore expected to lead to higher levels of circulating sex hormones. The negative effect of BMI on SHBG seen here supports observational findings^{170–172}. When evaluating the role of SHBG in disease, MR analysis suggests that an increase in SHBG contributes to a decrease in risk of cardioembolic stroke¹⁴⁶. Other studies have also implicated lower SHBG levels in increasing type II diabetes risk.¹⁷³ The exact mechanisms leading from decreased SBHG to ill-health is unclear, but may arise as a result of the increased bioavailability of testosterone and oestrogen¹⁶⁹.

There were also a range of platelet proteins which were detected in the plasma (262 proteins). These are proteins which have been linked to platelet GO pathways. The observational analysis detected an inverse association between BMI and platelet proteins, including PAFAH and PEAR1. These estimates were similar in the MR analysis, however the estimates did not pass multiple testing adjustment. Interestingly, higher BMI led to a decrease in levels of these proteins circulating in the plasma. PAFAH is a phospholipase A₂ enzyme.¹⁷⁴ PAFAH binds to lipoproteins and can degrade platelet activating factor (PAF). PAFAH has been shown to bind to lipoproteins in mice¹⁷⁵ and is thought to therefore reduce lipoprotein oxidation, thereby being antiatherogenic. The reduction in PAFAH that was associated with

higher BMI could therefore be atherogenic. It is unclear why higher BMI would lead to a reduction in other proteins such as PEAR1. PEAR1 is a transmembrane protein involved in megakaryopoiesis and platelet activation, where it plays a key role in stabilising the integrin $\alpha_{IIb}\beta_3$ ¹⁷⁶. Lower circulating levels of PEAR1 in participants with higher BMI could be explained by PEAR1 levels being retained more within the platelet (and therefore could lead to platelet hyperactivity). Although alterations in plasma proteins may help understanding of the environment platelets are in, it would be informative to explore the platelet proteome in a cohort as large as INTERVAL, however the material is not available for this analysis.

The involvement of the chemokines MDC and TARC in platelet function was presented in Chapter 5, where there was evidence that they can modulate platelet activation. There was also MR evidence that higher levels of MDC could contribute towards VTE pathophysiology. These chemokines were also included in the SomaLogic panel in the current chapter, therefore the effect of BMI on the circulating levels of these chemokines was explored. MDC was associated with BMI observationally, and the magnitude of effect was similar in the MR, however this estimate did not withstand multiple testing adjustment. It is possible that MDC could be an intermediate in between BMI and platelet function, however this analysis needs to be replicated in a larger cohort. There was no evidence to support TARC as an intermediate. Differences in regulation of MDC and TARC need to be further elucidated.

Despite these possible protein involvements in cardiometabolic disease and platelet function, it remains difficult to assess the contribution of individual proteins as they are not entirely independent and any pathological effects would likely be due to a global change in protein composition. There are not distinct groupings in the SomaLogic data as there often are with, for example, metabolomics data. Proteins grouped into clusters of similar features were examined, where the BMI-protein estimates of each cluster with overall estimates were compared and enrichment for genes related to disease were explored. The cluster most altered by BMI (cluster 2) included most of the eight proteins with the strongest BMI effects from MR analyses, as well as various complement factors, chemokines and coagulation factors. Therefore, it is very likely that this global change in proteins would activate platelets. This cluster was also found to be enriched for genes related to cardiovascular disease, renal and metabolic diseases, and cancer. Enrichment was similar when comparing the proteins that had an observational association with BMI with all proteins included, with enrichment appearing greatest for renal and metabolic disease. Together, this suggests that changes in proteins may mediate effects of obesity on cardiometabolic diseases; more focused investigations of these proteins are now needed, especially to assess their impact

on platelet function.

This analysis has some limitations. The limitations that have arisen through use of the INTERVAL study have been discussed in section 3.5.1. One of the limitations specific to the proteomic data in INTERVAL is that although it is one of largest existing cohorts to have untargeted proteomic data based on the SomaLogic platform, the sample size is still relatively modest and may have low power to detect some associations when using MR. Based on the detectable ($P < 1.4 \times 10^{-5}$) median absolute observational effect size (0.13 SDs), our analyses had 80% power to detect MR effect sizes ≥ 0.33 SDs ($\alpha = 0.05$) for our sample size ($N = 2737$)¹⁷⁷. With greater statistical power, there would likely be more proteins detected with MR. This was reinforced by the strong agreement in the magnitude of effect estimates seen in observational and MR analyses which applied throughout the effect distribution. Additionally, the proteins examined are highly correlated and therefore changes in individual proteins may not fully be described. Evidence from case-control cohorts as well as functional and animal studies would help isolate individual proteins that are altered and contribute to disease. Finally, although analyses provide insight into the proteomic effects of BMI, it does not distinguish between the type of adiposity. It would be useful to distinguish between the effects of subcutaneous and visceral fat using dual-energy X-ray absorptiometry (DXA) derived measurements, but these were not available in the INTERVAL data set.

This chapter utilised SomaLogic to explore the relationship between BMI and plasma proteins in unprecedented scope and detail, in both an observational and MR framework. There is evidence for a broad impact of higher adiposity on the circulating proteome. Causal evidence was strongest for BMI in relation to proteins involved in regulating appetite, sex hormones, and inflammation. Protein alterations were found to be enriched for genes related to cardiovascular and metabolic disease, therefore implicating an environment which may lead to platelet hyperactivity. Altogether, these results help to focus attention onto new potential proteomic signatures of obesity-related disease. Further characterisation of the role of such proteomic profiles in cardiovascular disease using MR is warranted, as well as characterising the role of such proteins on platelet function.

Chapter 7

Further elucidating the effect of adiposity on the plasma proteome using a weight loss intervention

7.1 Background

Obesity is associated with an increased risk of type II diabetes (T2D), cardiovascular diseases, musculoskeletal diseases, and types of cancer^{10,178,179}. These associations are well established, however mechanisms of disease are less clear. It is likely that a change in the composition of proteins circulating in the blood plays a role in obesity-related cardiovascular risk¹⁵⁴. The previous results chapter (Chapter 6) examined the effect of BMI on the plasma proteome and provided estimates for the average change in proteins per average difference in BMI in a general population, using observational estimates and one sample Mendelian randomization (MR). There was evidence of a BMI effect on proteins such as leptin, sex hormone binding globulin (SHBG), fatty acid binding protein 4 (FABP4) and fumarylacetoacetate. Within an enrichment analysis, this change in circulating levels of these proteins was implicated in cardiovascular diseases. There is also evidence that obesity affects platelet function, thereby elevating the risk of thrombotic events. There is evidence from the literature and from Chapter 5 that circulating proteins could mediate this risk.

Although MR helps to overcome issues inherent to observational studies such as confounding and reverse causation, MR has its own limitations. For example, it is possible that genetic variants used for

the genetic risk score could affect protein levels through routes other than changes in BMI. This is known as horizontal pleiotropy¹². As well as this, although there were over 2700 participants within the MR, this is a relatively small sample size for the study design. Additionally, it is important to not only look at which proteins are altered within the population with higher BMI; identifying which proteins can be modulated with weight loss is important as this may help reveal mechanistic insight into mechanisms of obesity-related cardiovascular disease. Triangulation can therefore be used in efforts to further determine the BMI effects on the plasma proteome. Triangulation is defined as strengthening causal inference through the combination of study designs which each have separate sources of bias¹⁸⁰. Therefore, to add an independent source of evidence to the MR in the previous chapter, the Diabetes REmission Clinical Trial (DiRECT) can be utilised. The DiRECT trial consisted of a group of patients with overweight/obesity and T2D who were either given guideline T2D care or an acute intervention, consisting of a low calorie diet, to help with weight loss and T2D remission¹⁸¹. This chapter therefore aims to: examine the effect of reducing BMI with caloric restriction on the plasma proteome.

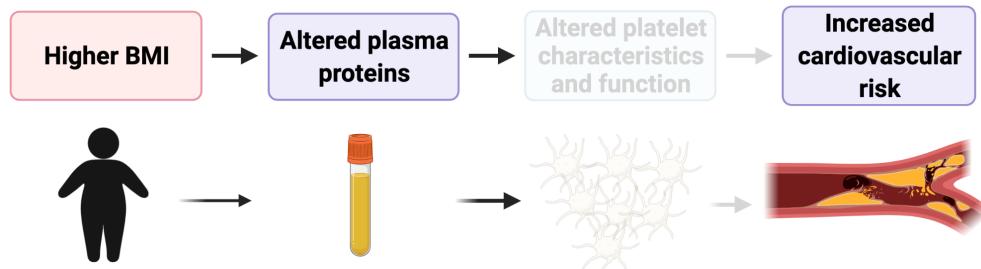


Figure 7.1: **Schematic of the associations explored in Chapter 7. This chapter explores the effect of caloric restriction-induced weight loss on the plasma proteome.** Figure made using BioRender.com.

7.2 Methods

7.2.1 Study design and participants

Samples analysed within this study were collected from participants enrolled in the Diabetes Remission clinical trial (DiRECT). DiRECT was a cluster-randomised trial which took place at 49 primary care practices in Scotland and Tyneside¹⁸¹. Ethical approval was granted from West 3 Ethics Committee (January, 2014) and the National Health Service (NHS). Participants were recruited between 25th July 2014 and 5th August 2016. Details of the protocol have previously been published¹⁸². Participants enrolled were between 20-65 years, diagnosed with T2D within previous 6 years, had a BMI of between

$27\text{-}45\text{kg/m}^2$. Participants were excluded if they were: using insulin, had a HbA_{1c} concentration of $\geq 12\%$ ($\geq 108\text{ mmol/mol}$), weight loss of $>5\text{ kg}$ in the preceding 6 months, an estimated glomerular filtration rate of $<30\text{ mL/min per }1.732\text{ m}^2$. Other exclusion criteria include heart failure, participation in other clinical trials, substance abuse, cancer, recent MI (<6 months), learning difficulties, current use of anti-obesity drugs, eating disorders, pregnancy or admission to hospital for depression or use of antipsychotic medication¹⁸¹. Participants in the control group received best-practice care by guidelines. The intervention group were asked to follow the Counterweight-Plus weight management programme¹⁸³. This programme consisted of a total diet replacement (TDR) phase using a low energy diet (825-853 kcal/day) for 3-5 months, followed by structured food reintroduction of 2-8 weeks, with ongoing monthly long-term weight loss maintenance visits. Those in the intervention group had their antidiabetic and antihypertensive drugs discontinued. In total, there were 146 patients in the control group and 119 in the intervention group who remained enrolled in the study at 1 year, as detailed in Figure 7.2.

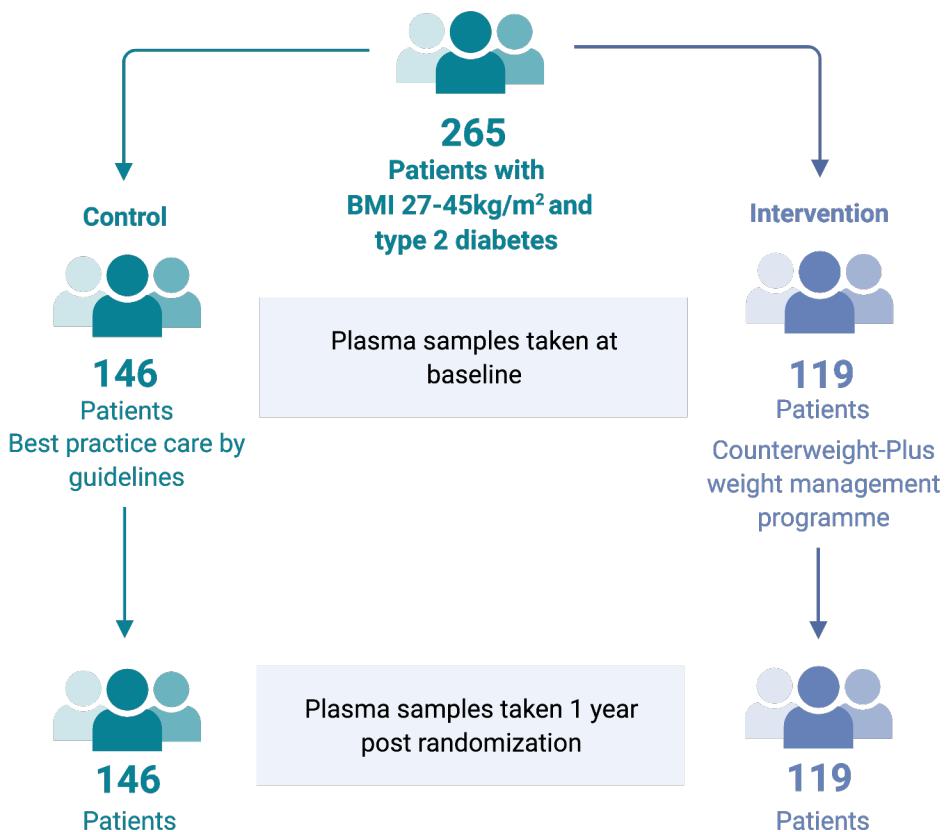


Figure 7.2: Overview of participants in the DiRECT trial. Figure made using BioRender.com.

7.2.2 Variables included in analysis

Age and sex were self-reported. Height was measured with the Frankfort plane horizontal, with a portable stadiometer (Chasmors Ltd, London). Weight was measured using Class 111 approved calibrated scales (Marsden Group UK)¹⁸². Blood was donated at various timepoints including at baseline (week 0) and at 1 year (~week 52), where HDL cholesterol, triglycerides, HbA1c and plasma glucose were measured. Systolic blood pressure was measured with the patient seated, rested and with legs uncrossed for ≥ 5 mins. BMI was calculated by dividing the weight (kg) by the square of the height (m). BMI change was calculated by subtracting BMI at baseline from BMI at 1 year. This BMI change was rank normal transformed (function rntransform() from “moosefun” R package <https://github.com/hughesvoanth/moosefun/>). The centre attended and list size of the practice (>5700 or ≤ 5700) determined whether participants were allocated to control or intervention (minimisation variables) and therefore were recorded.

7.2.3 Proteomics

Blood was taken from participants into 9 mL vacutainers with EDTA (an anticoagulant) at baseline and 1-year post-randomisation. Blood samples were centrifuged to derive plasma samples and plasma was stored at -80 °C. Protein detection was performed by the SomaScan assay by SomaLogic. As mentioned before, this technique uses Slow Off-rate Modified Aptamers (SOMAmers) which make direct contact with proteins and quantifies protein levels by using a DNA microarray¹⁵⁵. There were 5284 proteins included in the array, of which 4601 proteins passed internal quality control checks. The proteomic data was further cleaned using the “metaboprep” R package¹⁸⁴, with protein levels from both timepoints all QC’d together. This R package identified no extreme missingness (defined at 0.2 for sample and protein missingness), removed outliers (based on more than 5 SDs based on principal components (PCs) 1 and 2). The package also calculated the number of independent proteins by using pairwise correlation coefficients between proteins (869 representative proteins based on correlation coefficient of 0.5). The Shapiro-Wilk test was implemented to identify proteins which have a normal distribution ($W \geq 0.95$). Only 399 out of 4601 proteins had W statistics ≥ 0.95 . As a result of this, all data was transformed to meet normality assumptions for analyses. This data log₂ transformed to match protein transformation by Sun et al.³⁴ then adjusted for age and sex (data had already undergone technical adjustment). Protein change was calculated for each individual by subtracting the baseline protein levels from protein levels at one-year. These protein change values were rank normal transformed.

7.2.4 Statistical analysis

Analyses were performed using R version 3.6.1. A total of 265 participants were included in the current analysis. Participant baseline characteristics were summarised as the mean and SD. Baseline characteristics were calculated for both control group (N=146) and intervention (N=119) and were compared across groups using either a two-tailed unpaired t-test or a Chi² test.

The associations between BMI change and protein changes were explored using linear regression adjusting for centre, list size, age and sex (function lm() from base R “stats” package). Due to the nature of linear regression, outputs of regression provide the mean individual change in protein (in SDs) per SD increase in BMI. Therefore if more weight loss reduces the levels of protein, the beta coefficient is positive¹⁸⁵. The association between potential confounders (age and sex) and both exposure (BMI change) and outcome (protein change) were explored using linear regression. The difference in BMI change in intervention group compared to control was performed using linear regression, adjusting for centre, list size, age and sex. Covariables (age, sex, centre and list size) were compared across treatment groups. Treatment group was therefore used as an instrument for BMI change in a two stage least squares analysis 7.3 to estimate the effects of BMI change on protein change using the ivreg() function from the “AER” R package (<https://github.com/cran/AER/blob/master/R/ivreg.R>). BMI change-protein change point estimates for 4601 proteins from the multiple linear regression analyses and two stage least squares analyses were compared using linear regression.

The association between BMI change and change in platelet proteins were also examined by searching for the UniProt IDs of the same platelet proteins as the previous chapter (Chapter 6). This approach was used due to the lack of distinct groupings in the SomaLogic protein list. As a sensitivity analysis, for proteins which associated with BMI change, baseline protein levels were compared across control and intervention groups. Similar to the previous chapter, the estimates for the effect of BMI change on a change in levels of the chemokines MDC and TARC were also explored. P-values were adjusted for multiple testing based on the number of representative proteins ($0.05/869 = 5.8 \times 10^{-5}$). As a separate sensitivity analysis, a linear mixed model was used to account for baseline and endpoint protein levels (as the change in protein variable derived does not account for this). For the linear mixed model, the lmer() function from the “lme4” package, protein levels were used as the dependent variable, with treatment group and visit entered as fixed effects (and exploration of an interaction) and subject as a random effect. Estimates from the interaction of treatment group and visit were derived for each protein. These estimates were compared with the two stage least squares estimates.

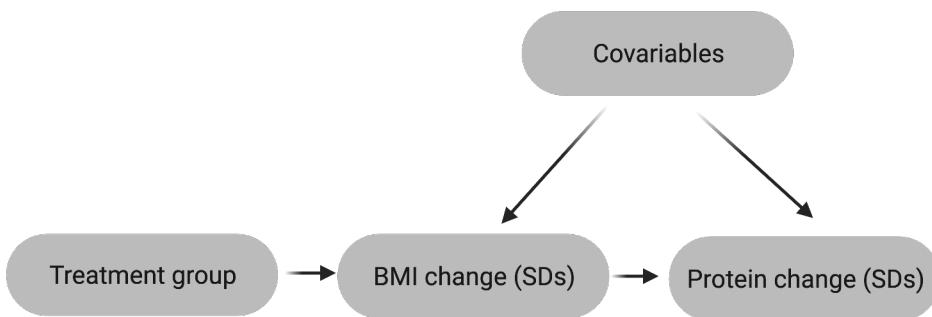


Figure 7.3: **Schematic of two stage least squares analysis performed in the current chapter.**
Figure made using BioRender.com.

7.2.5 Disease class enrichment

Similarly to the previous chapters, proteins which had a BMI effect were taken forward into an enrichment analysis using DAVID Bioinformatics 6.8.¹⁵⁷ In total, 144 proteins were compared with all proteins detected (4601). Enrichment was explored by using the genetic association database (GAD). Enrichment was assessed by fold enrichment and by Bonferroni-corrected p-values. StringDB¹⁸⁶ was also used for a pathway enrichment analysis as DAVID Bioinformatics does not allow assigning of a rank order (such as by P value) to the input. Here the UniProt IDs of the proteins along with their $-\log_{10}$ p value (smallest first) for both the observational analysis and the instrumental variable analysis. Pathway analysis was explored using gene ontology (GO) terms and KEGG pathways¹⁸⁷.

7.3 Results

7.3.1 Participant characteristics

Participants displayed similar characteristics across control and intervention groups (Table 7.1). Baseline BMI was similar across treatment groups (34.2 kg/m^2 with SD of 4.3 kg/m^2 in the control group vs 35.0 kg/m^2 with SD of 4.6 kg/m^2 in the intervention group, $p=0.19$). Other variables relating to cardiometabolic health were also similar across groups, including systolic blood pressure, HbA1c, glucose and insulin levels. The participants in the intervention group were younger and included fewer participants from Scotland but more from Tyneside.

Table 7.1: DiRECT participant characteristics

Variable	Control mean (SD) or %	Control N	Intervention mean (SD) or %	Intervention N	P value for difference (Two tailed t-test or Chi2 test)
Age	56.2 (6.9)	146	53.9 (7.1)	119	0.01
Sex		146		119	0.54
Male	62 %		57 %		
Female	38 %		43 %		
Body mass index (kg/m ²)	34.2 (4.3)	146	35.0 (4.6)	119	0.19
Systolic Blood Pressure (mmHg)	137 (15)	146	135 (17)	119	0.19
HbA1c (mmol/mol)	58 (11)	146	60 (13)	119	0.15
Glucose (mmol/l)	8.8 (2.5)	146	9.2 (3.2)	118	0.24
Insulin (uu/ml)	22 (14)	146	24 (15)	119	0.27
Cholesterol (mmol/l)	4.3 (1.1)	146	4.3 (1.1)	118	0.9
HDL (mmol/l)	1.2 (0.3)	146	1.1 (0.3)	118	0.04
Triglycerides (mmol/l)	1.9 (0.9)	146	2.0 (1.4)	118	0.48
Diabetes duration (years)	3.0 (1.7)	146	3.2 (1.6)	119	0.37
Number of antidiabetic medications		146		119	0.5
0	23 %		24 %		
1	53 %		44 %		
2	18 %		23 %		
3	5 %		7 %		
4	1 %		3 %		
Centre		146		119	0.01
Scotland	77 %		62 %		
Tyneside	23 %		38 %		
List size		146		119	0.2
>5700	49 %		58 %		
=<5700	51 %		42 %		

7.3.2 Observational association between BMI change and protein change

Linear regression was used to estimate the effect of BMI change on protein change, where BMI change (endpoint-baseline) was used as the exposure and protein change (endpoint-baseline) was used as the outcome and adjusting for confounders. After adjusting for multiple testing ($p<5.8\times10^{-5}$), 254 proteins out of 4601 proteins were associated with BMI change (5.5 % of all protein tested) (Supplementary Table 1). Although overall there was weight loss and a reduction in BMI, beta coefficients can be interpreted as the change in protein level (SDs) per SD increase in BMI. Negative associations were observed with BMI change and scavenger receptor class A member 5 (SCAR5, -0.62 SDs per SD increase in BMI, 95% CI -0.72 to -0.53 $p=3.7\times10^{-29}$) and apolipoprotein F (-0.58 SDs per SD increase in BMI, 95% -0.68 to -0.48, $p=1.8\times10^{-24}$). BMI change was positively associated with changes in levels of Proto-oncogene tyrosine-protein kinase receptor Ret (RET) (0.59 SDs increase per SD increase in BMI, 95% CI 0.49 to 0.69 $p=1.6\times10^{-25}$), growth hormone receptor (GHR) (0.59 SDs increase per SD increase in BMI, 95% CI 0.49 to 0.69 $p=5.8\times10^{-25}$).

Proteins involved in GO platelet pathways were explored within the results. There were 248 platelet proteins identified in DiRECT. In the linear regression analysis, there was evidence for an effect of BMI change on 31 platelet proteins (12.5%) (Supplementary Table 2). Associations include a positive association between BMI change and collagen type III (0.37 SDs increase per SD increase in BMI, 95% CI 0.26-0.48, $p=5.1\times10^{-10}$). There was also a positive association between BMI change and plasminogen activator inhibitor 1 (PAI-1, 0.29 SDs increase per SD increase in BMI, 0.17-0.40, $p=1.9\times10^{-6}$). The platelet primer insulin-like growth factor 1 (IGF-1) was negatively associated with BMI (-0.28 SDs per SD increase in BMI, 95% CI -0.40 to -0.16, $p=4.8\times10^{-6}$). The estimate for the association between BMI change and PAFAH was (-0.16 SDs per SD increase in BMI, 95% CI -0.28 to -0.04, $p=9.7\times10^{-3}$) and therefore did not pass multiple testing correction. The estimate for the effect of BMI change on the change in levels was 0.10 SD (95% CI -0.02 to 0.22, $p=0.099$) and was 0.07 SD for TARC (95% CI -0.04 to 0.19, $p=0.22$).

7.3.3 Association between confounders and exposure/outcomes

There was weak evidence for an association between age and BMI change (0.02 SDs increase in BMI change per 1 year, 95 % CIs -0.0003 to 0.034, $p=0.054$), where an increase in BMI change means that the participant is less likely to reduce their BMI. There was no evidence that sex was associated with BMI change (BMI change 0.073 SDs increase in BMI change in females compared with males, 95 % CI

-0.17 to 0.32, $p=0.56$) (Supplementary Table 2). Age and sex displayed weak associations with protein changes, but these did not pass multiple testing adjustment (Supplementary Tables 3 and 4).

7.3.4 Association between treatment group and BMI change

Mean BMI change in all participants was -1.8kg/m^2 ($\text{SD } 2.5 \text{ kg/m}^2$), with a mean change in the control group of -0.4 kg/m^2 ($\text{SD } 1.3$) vs -3.6kg/m^2 ($\text{SD } 2.5$) in the intervention. The difference in BMI change comparing intervention to control was -3.2 kg/m^2 ($\text{SE } 0.25$, $p=1.52\times10^{-29}$). These analyses suggest that treatment group is a valid instrument for BMI change.

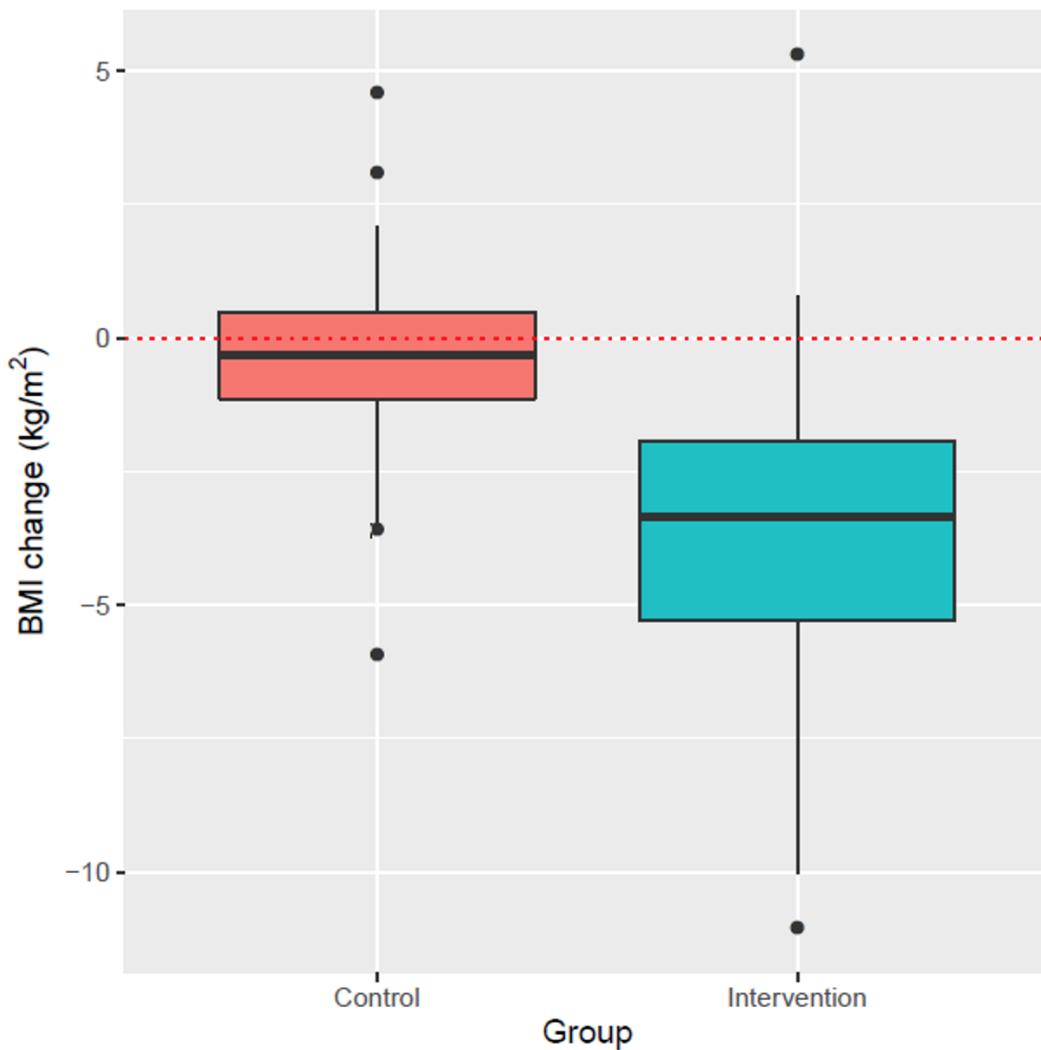


Figure 7.4: **Box plot of the distribution of BMI change (kg/m²) by treatment group.** Median and interquartile ranges displayed by the box. Dotted red line indicates where there is no change in BMI.

7.3.5 Two-stage least squares analysis

Treatment group was used as an instrument for BMI change in a two-stage least squares analysis. After adjustment for multiple testing, BMI change was associated with changes in levels of 171 proteins (3.7 %) (Supplementary Table 5). The protein which showed the strongest association with BMI change was SCAR5 (-0.91 SDs per SD increase in BMI, 95% CI -1.07 to -0.75, $p=1.54\times10^{-23}$). Other proteins displayed a positive association with BMI change, including fatty acid-binding protein, heart (FABP1, 0.86 SDs increase per SD increase in BMI, 95% CI 0.69-1.04, $p=6.3\times10^{-19}$). Aminoacyclase-1 was also positively associated with BMI change (0.80 SDs increase in protein per SD increase in BMI, 95% CI 0.64 to 0.97, $p=4.9\times10^{-18}$). Figure 7.5 shows the qq-plot for the observational associations (A) and instrumental variable two stage least squares analysis (B). The protein with the smallest observed P value in both models is SCAR5, however the other “top hits” are not the same proteins in Figure 7.5.

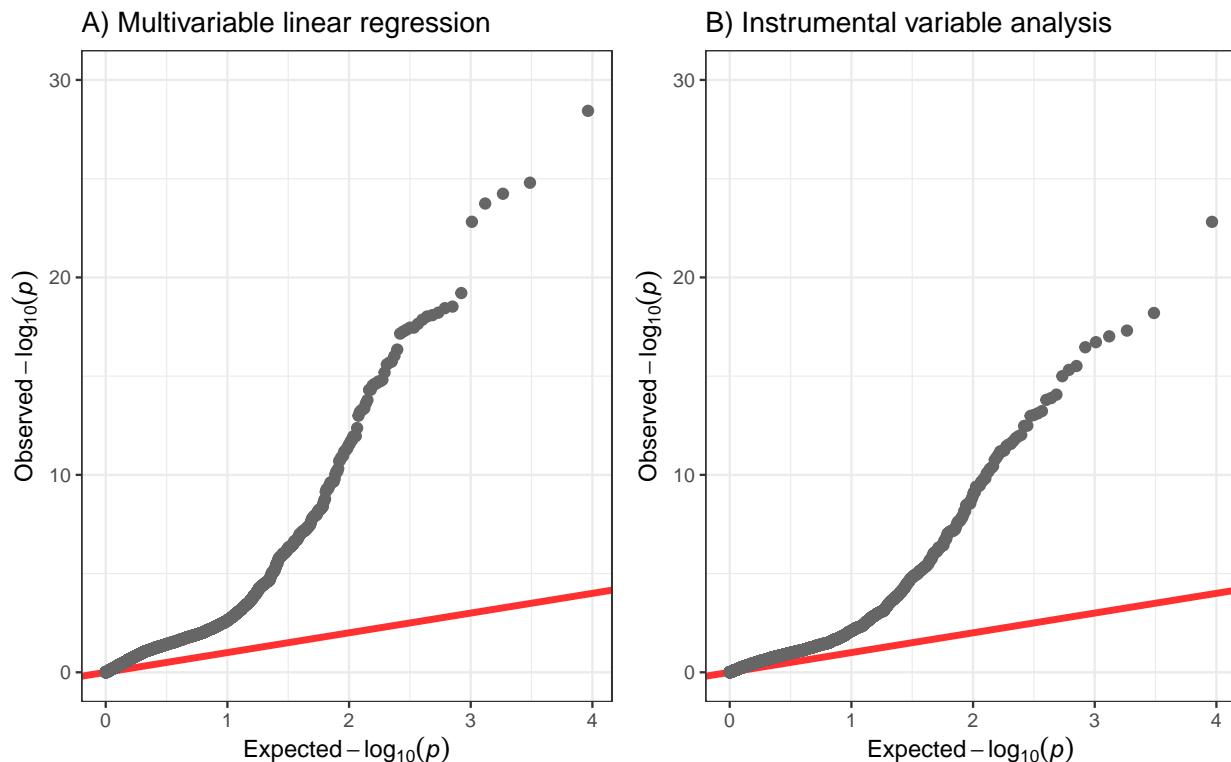


Figure 7.5: QQ-plots of the expected vs the observed $-\log_{10}(p\text{-values})$ for the BMI change-protein change effects. A) Linear regression B) Instrumental variable two stage least squares analysis.

In the two stage least squares analysis, there was evidence for an effect of BMI change on 13 platelet proteins. The estimate for the effect of BMI change on collagen type III was 0.53 SDs per SD increase

in BMI (95% CI 0.35 to 0.71, $p=2.0\times10^{-8}$), larger in magnitude than in the observational analysis (Supplementary Table 2). The estimate for the association between BMI change and the platelet protein, PAFAH, strengthened in the instrumental variable model, where the estimate was -0.42 (95% CI -0.61 to -0.22, $p=5.3\times10^{-5}$), thereby passing the multiple testing adjustment. The two stage least squares analysis did not provide evidence for an effect of BMI change on a change in levels of MDC or TARC.

7.3.6 Comparison of observational linear regression and TSLS results for the effect of BMI change on protein change

A total of 110 proteins were only associated within the observational linear regression model, whereas 27 proteins were only associated in the TSLS analysis using treatment group as an instrument for BMI change. 144 proteins were associated with BMI in both analyses (7.6), with largest effect estimates across methods for SCAR5, Adiponectin, Apolipoprotein F, growth hormone receptor (GHR), receptor-type tyrosine-protein phosphatase U (PTPRU) and sex hormone binding globulin. These can be seen in Figure 7.7. Of these 144 BMI change-protein change associations that were consistent across both models, 121 were positive, where weight loss led to a reduction in levels of the protein.

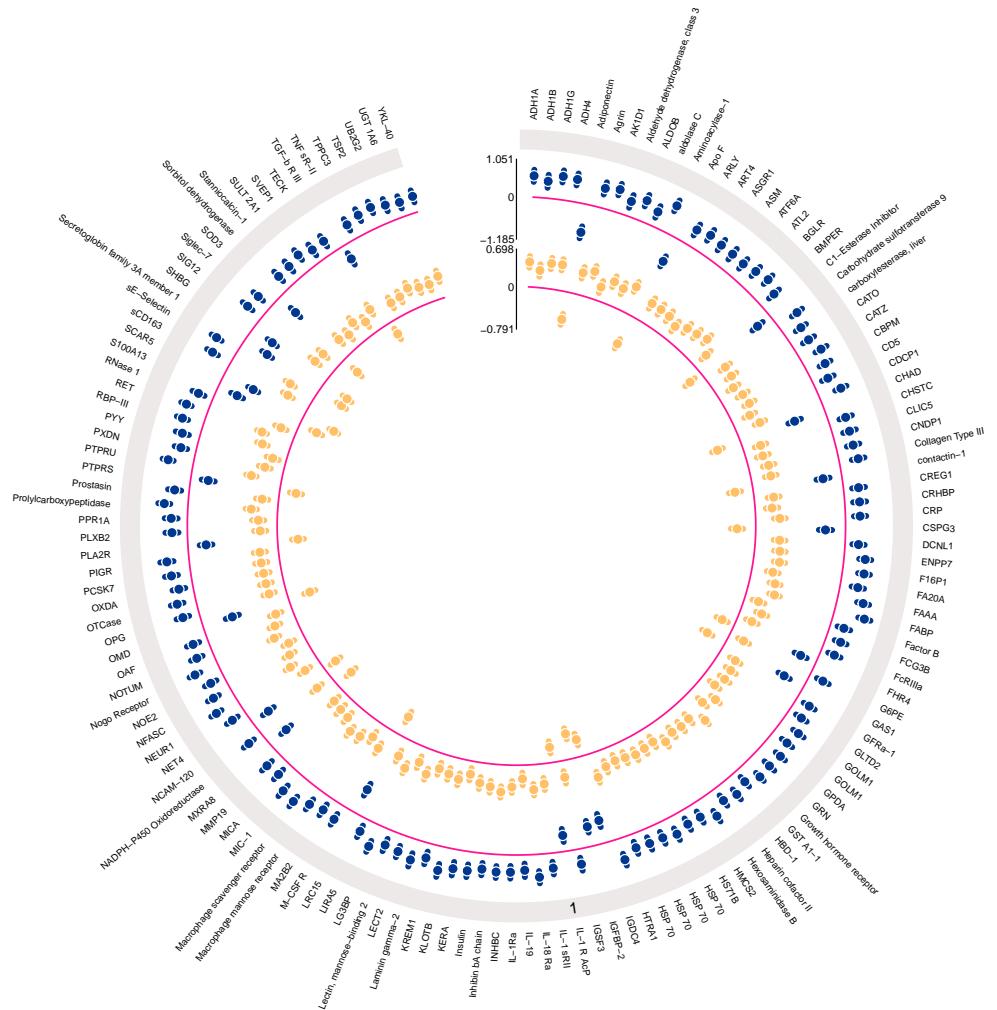


Figure 7.6: Circos plot displaying the protein change (SDs) per SD increase in BMI in DiRECT. Blue estimates are the two stage least squares estimates and the yellow estimates are from the linear regression. Proteins presented were associated in both models at $p=5.8\times10^{-5}$. Figure made using the EpiViz R package <https://github.com/mattlee821/EpiViz>.

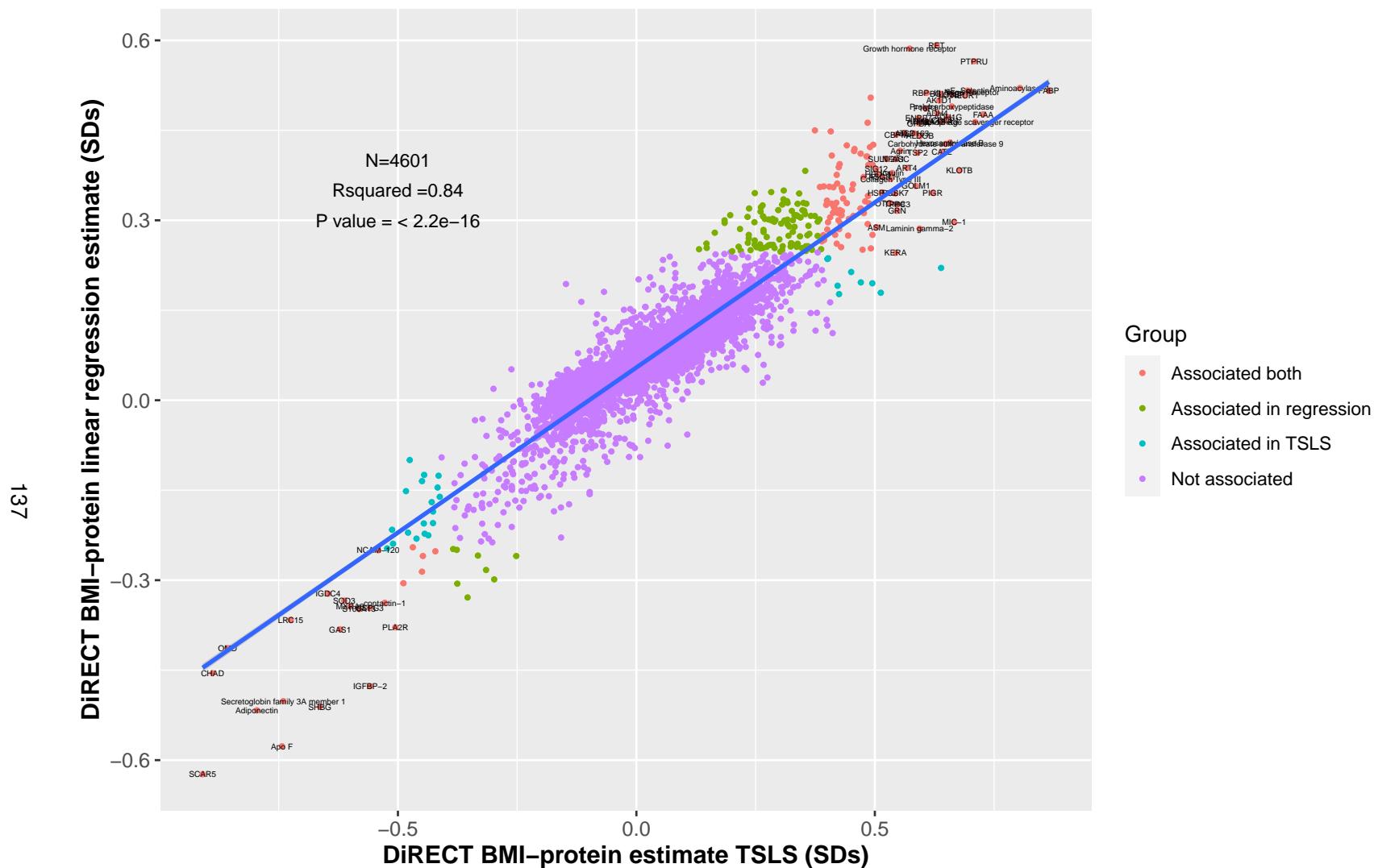


Figure 7.7: Scatter plot displaying the estimate for the change in protein (SDs) per SD increase in BMI in the two stage least squares and linear regression models. Points are coloured by which models the proteins were associated with BMI change ($p<5.8\times 10^{-5}$).

7.3.7 Sensitivity analysis

Comparison of protein levels at baseline by treatment group

Proteins which were associated with BMI change were compared across control and intervention groups at baseline. This was done as the delta protein variable used in the regression model does not account for absolute levels of protein at baseline, therefore it is possible that the change in levels of protein may appear to be associating with BMI change due to differing basal protein levels across groups. There was not strong evidence for any differences in baseline levels of proteins ($p < 5.8 \times 10^{-5}$). However, there was weak evidence that levels of certain proteins, for example apolipoprotein F (beta=-0.20, SE=0.07, p=0.005) and carbohydrate sulfotransferase (beta=0.11, SE=0.04, p=0.006) were different in treatment group compared to control. Figure 7.8 displays the boxplots for the distribution of levels of these proteins at both baseline and endpoint for the intervention and control group.

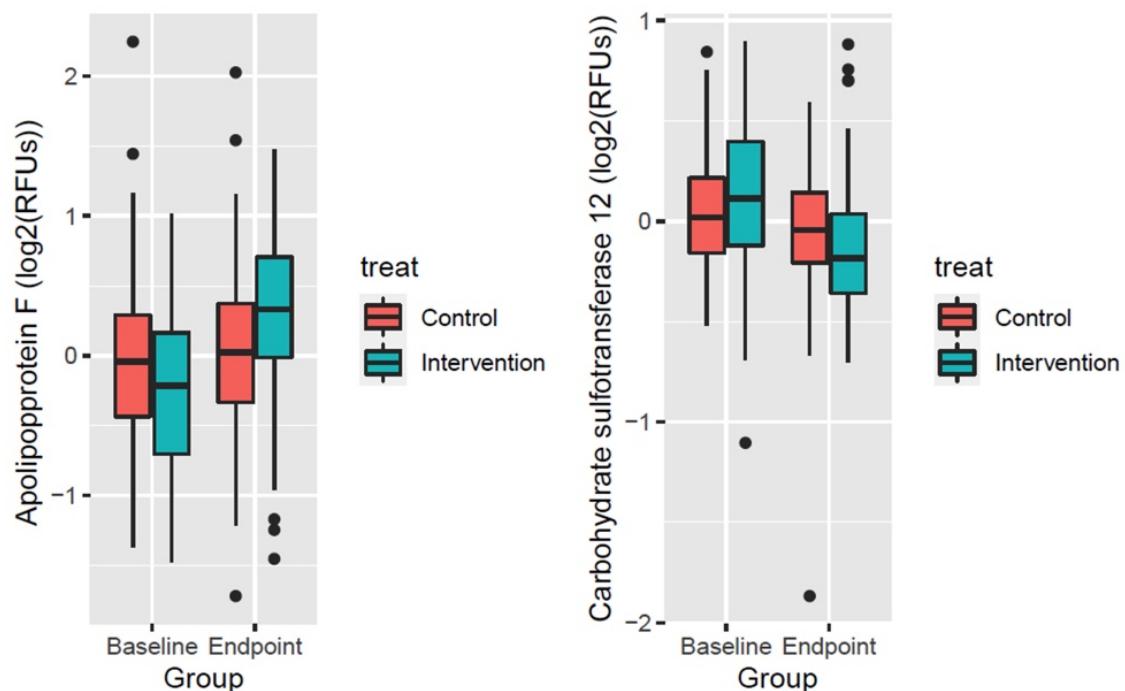


Figure 7.8: **Sensitivity analysis: boxplot of the levels of Apolipoprotein F and Carbohydrate Sulfotransferase at baseline and endpoint, grouped by treatment group.** Median and IQR is indicated by the box. Units are not rank normal transformed.

Using a linear mixed model to estimate effects of treatment group and timepoint on protein levels

To account for individual protein levels at each timepoint, a linear mixed model was used to determine the effect of the intervention on protein levels. The estimate for the interaction between treatment group and timepoint were derived for each protein, which can be interpreted as difference in gradient of protein change comparing the intervention to control. Estimates were compared with the two stage least squares analysis results which provided an estimate for the effect of BMI change on protein change, using the treatment group as an instrument. Units across models are different as the TSLS estimates protein change per SD higher BMI, while the linear mixed model estimates the gradient of the protein comparing endpoint to baseline, therefore the gradient with a reduction in BMI. The estimates from the two models were strongly associated ($R^2 = 0.91$, $p < 2.0 \times 10^{-16}$, $F = 49280$, intercept=0.017) (Figure 7.9). As a specific example using SCAR5, the estimate from the TSLS was that per SD increase in BMI, SCAR5 reduces by -0.91 SDs in BMI, 95% CI -1.07 to -0.75, $p = 1.54 \times 10^{-23}$. The linear mixed model suggests that the estimated difference in gradient of protein change (comparing endpoint to baseline) comparing intervention to control is 0.92 SDs (95% CI 0.75 to 1.09, $t = 10.8$).

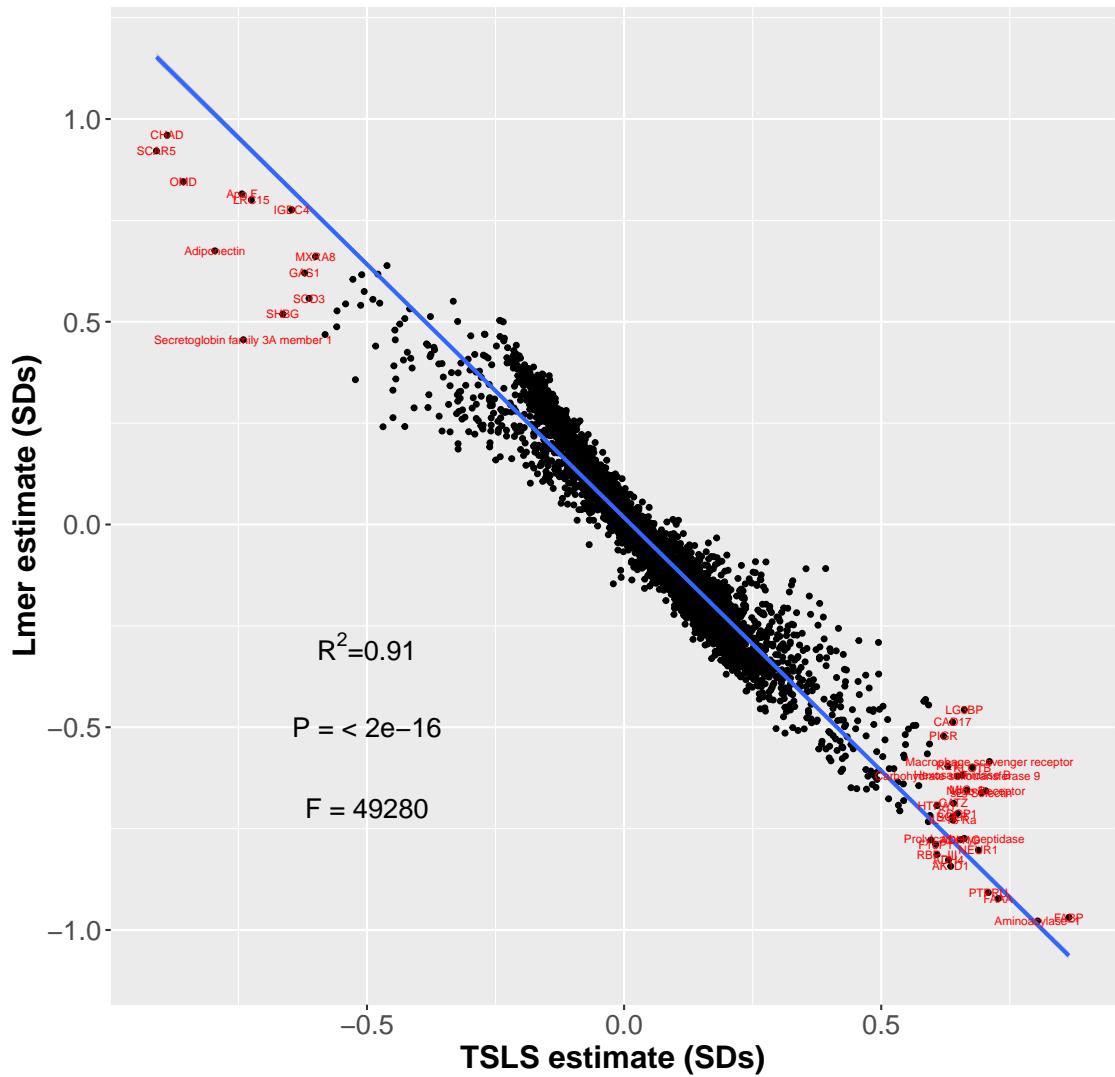


Figure 7.9: Scatter plot of the estimates from the TSLS and linear mixed model analyses.

7.3.8 Enrichment analysis

To explore disease class enrichment, DAVID Bioinformatics 6.8 was used¹⁵⁷. As there was consistent evidence for a BMI effect on 144 proteins across both models in DiRECT, these were compared to the full 4601 protein panel. There was strongest evidence for enrichment in proteins involved in metabolic disease (1.4 fold enrichment, $p=4.21\times 10^{-3}$), with evidence for enrichment of renal disease (1.8 fold enrichment, $p=1.02\times 10^{-2}$). Cardiovascular disease had weak evidence for enrichment, but this association did not pass multiple testing correction.

Table 7.2: DiRECT protein enrichment results for disease class using DAVID bioinformatics 6.8¹⁵⁷

Term (Genetic Association Database disease class)	Count	%	List Total	Population Hits	Population Total	Fold Enrichment	P Value	Bonferroni-adjusted P value
Metabolic	73	53	118	1451	3204	1.4	2.34E-04	4.21E-03
Renal	34	24	118	518	3204	1.8	5.72E-04	1.02E-02
Neurological	49	35	118	884	3204	1.5	9.61E-04	1.72E-02
Psych	36	26	118	589	3204	1.7	1.38E-03	2.45E-02
Pharmacogenomic	51	37	118	964	3204	1.4	2.11E-03	3.74E-02
Cancer	56	40	118	1093	3204	1.4	2.23E-03	3.94E-02
Reproduction	25	18	118	381	3204	1.8	4.49E-03	7.79E-02
Normal variation	16	12	118	209	3204	2.1	8.09E-03	1.36E-01
Aging	21	15	118	324	3204	1.8	1.21E-02	1.96E-01
Cardiovascular	56	40	118	1214	3204	1.2	2.51E-02	3.68E-01

Using StringDB and inputting p values for the association between BMI change and each protein change, there was evidence for enrichment of KEGG pathways. Using the UniProt IDs and p-values from the linear regression analysis for BMI change and protein change, there was evidence that proteins altered by protein change in the linear regression model were involved in cytokine-cytokine receptor interaction (enrichment score = 0.14, pathway size = 282, $p = 2.4 \times 10^{-4}$) and complement and coagulation cascades (enrichment score = 0.36, pathway size = 82, $p = 9.8 \times 10^{-4}$). These were the only pathways associated with the “top of the input”, therefore indicating enrichment of proteins most strongly associated with BMI change. There was also enrichment for platelet activation, however the analysis suggested that this enrichment was at the bottom of the protein list (enrichment score 4.9, pathway size 122, $p=6.5 \times 10^{-5}$), therefore more proteins involved in platelet activation were not associated with BMI change. The network of proteins involved in platelet activation is shown in Figure 7.10. KEGG enrichment results were not available by inputting the ranked p values for the proteins from the instrumental variable analysis.

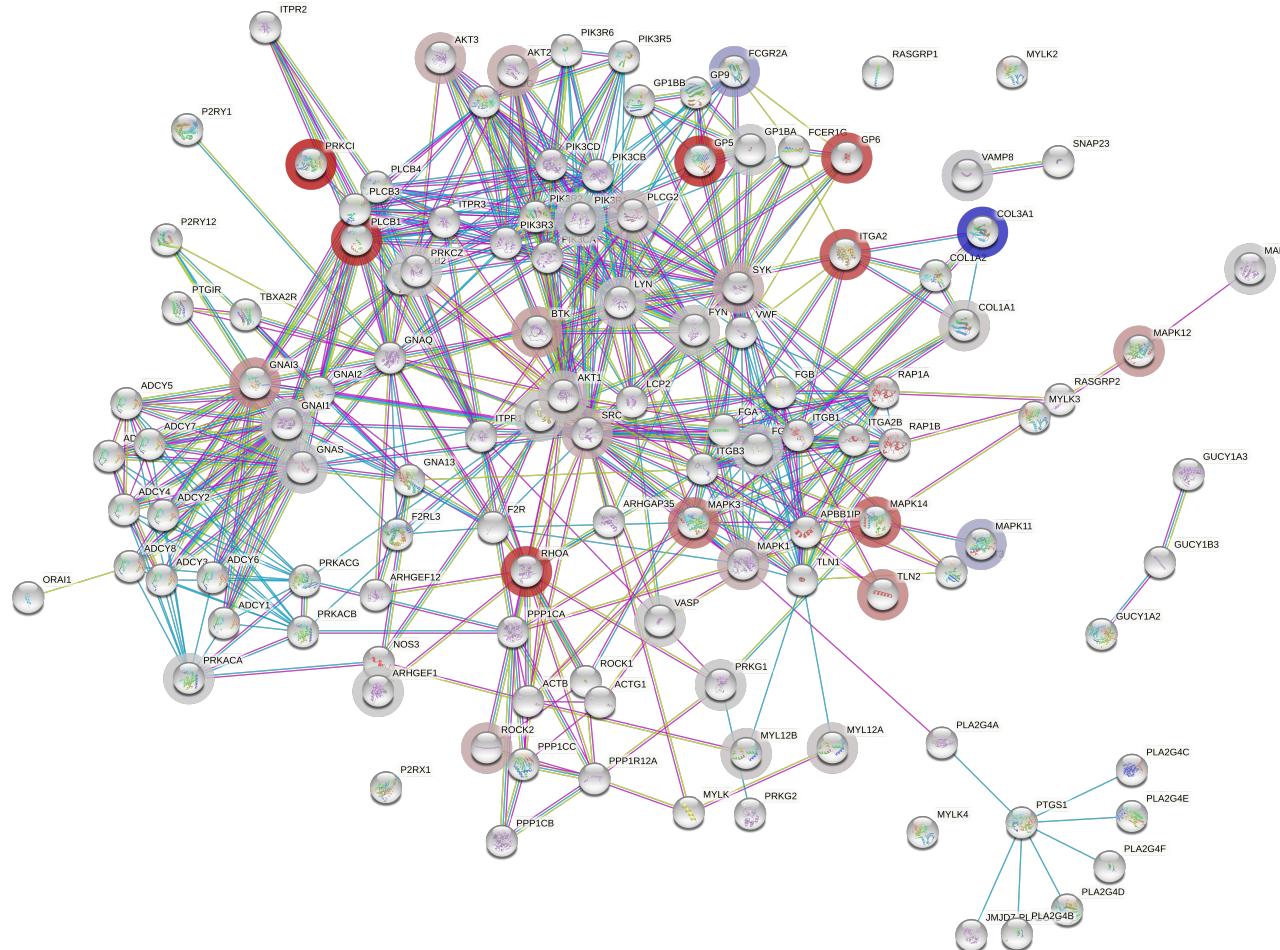


Figure 7.10: Network of proteins in DiRECT which are involved in platelet activation. Nodes represent individual proteins. The halo colour represents the rank of the protein where blue indicates the top of the list (most strongly associated with BMI change) and red indicates the bottom of the list. The edges indicate known interactions between proteins. Figure from made using string-db.org

7.4 Discussion

In this chapter, a clinical trial implementing caloric restriction-induced weight loss was utilised as a second line of evidence to assess the effect of BMI on the plasma proteome. This analysis provided evidence for a broad effect of weight loss on the plasma proteome, providing results the effect of caloric restriction on the most extensive set of proteins to date.

The current study identified 144 BMI-related protein changes that were consistent across both models. This includes a reduction in BMI reducing levels of apolipoprotein F (ApoF) and GHR, while increasing levels of SHBG and IGFBP1/2. Table 7.3 provides a summary of findings from the literature from previous studies which have explored the effect of caloric restriction on plasma proteins. As detailed in the table, previous studies have mainly used mass spectrometry. One study used SomaLogic¹⁸⁸, however this panel only included 1129 proteins. The current study utilised over fourfold more proteins, therefore providing more in depth readouts of biological processes. Findings are in concordance with previous studies^{185,188–192}. These protein changes replicate across cohorts and proteomic platforms and are more likely to be robust associations. Proteins altered with caloric restriction have been implicated in disease including coronary artery disease and T2D within the same cohort¹⁷³. A mediation analysis suggested that proteins mediate the risk between a T2D genetic risk score (GRS) and incidence of T2D, such as IGFBP1/2, SHBG and GHR. Looking at individual proteins, a SNP which increases levels of ApoF also increases the risk of myocardial infarction and coronary heart disease¹³⁹, therefore this apolipoprotein could mediate the increased risk of thrombotic events seen with a higher BMI.

Table 7.3: Summary of current literature on the effect of weight loss on the plasma proteome

Paper	Author, year	Proteomic platform	Study summary
Proteomics reveals the effects of sustained weight loss on the human plasma proteome	Geyer et al., 2016	Mass spectrometry	Participants: 43 individuals with obesity before and after 8-week weight loss. Total proteins associated: 93/1294. Overlapping findings: Weight loss leads to increase in ApoF, ITIH3, SHBG, corticosteroid-binding globulin.
Proteomic profiles before and during weight loss: Results from randomized trial of dietary intervention	Figarska et al., 2020	Olink CVD II, CVD III and inflammation	Participants: 609 from the diet intervention examining the factors interacting with treatment success (DIETFITS) study. Total proteins associated: 130/26 associated with change in BMI. Overlapping findings: Changes in IGFBP1/IGFBP2, E-selectin, CD163.
The differential plasma proteome of obese and overweight individuals undergoing a nutritional weight loss and maintenance intervention	Oller-Moreno et al., 2018	Mass spectrometry	Participants: 473 in the nutritional programme DiOGenes. Total proteins associated: 39/183. Overlapping findings: Changes in SHBG, Adiponectin, IGFBP2, galectin-3-binding protein.
Integrative Personal Omics Profiles during Periods of Weight Gain and Loss	Piening et al., 2018	Luminex and ProSeek by Olink	Participants: 23 with overweight or obesity undergoing weight loss. Total proteins associated: 27/276. Overlapping findings: changes in leptin.
Protein quantitative trait locus study in obesity during weight-loss identifies a leptin regulator	Carayol et al., 2017	SomaLogic	Participants: 494 participants on low DiOGenes on low calorie diet. Total proteins associated: 104/1129. Overlapping findings: Changes in leptin, GHR, TIG2, IGFBP2, RET proto-oncogene (granulysin).
Analysis of 1508 plasma samples by capillary-flow data-independent acquisition profiles proteomics of weight loss and maintenance	Bruderer et al., 2019	Mass spectrometry	Participants: 477 in DiOGenes. Total proteins associated: 271/465. Overlapping findings: Changes in Apolipoprotein F, INHBC, SHBG.

This study also provides some novel proteins associated with a reduction in BMI including SCAR5, chondroadherin (CHAD), fumarylacetoacetate (FAAA) and aminoacylase-1. A reduction in BMI led to an increase in plasma levels of SCAR5. This protein is involved in mediating commitment of mesenchymal stem cells into adipocytes and is highly expressed in white adipose tissue (WAT¹⁹³). A reduction in BMI also increased levels of chondroadherin (CHAD). Chondroadherin is a protein which is important in regulating extracellular matrix organisation in cartilage and bones^{194,195}. CHAD knockout mice show decreased bone strength compared with wildtype¹⁹⁴. This suggests that weight reduction may be beneficial for bone strength. The function of these proteins and relationship with adiposity needs to be further characterised.

The effect of BMI on specific platelet-related proteins was explored by extracting the estimates for proteins involved in platelet GO pathways. Both analyses indicated that a reduction in BMI reduces the levels of collagen type III. Collagen activates platelets by binding to receptors such as the GPVI receptor, therefore this reduction may reduce platelet activation¹⁹⁶. The instrumental variable analysis detected an effect of BMI change on PAFAH. Here, a reduction in BMI led to higher levels of PAFAH. This platelet protein was detected in Chapter 6. PAFAH is protein which has been shown to bind to lipoproteins, thereby reducing their oxidation¹⁷⁵. The comparison across studies will be discussed further in Chapter 8. The chemokines utilised in Chapter 5, MDC and TARC, were included in the SomaLogic panel, therefore were also specifically looked up within the results. The evidence suggested that a reduction in BMI did not alter levels of MDC and TARC, however it is also possible that the sample size was limited to detect smaller magnitudes of effects.

The enrichment results comparing the 144 proteins which were altered with BMI change to the full 4601 proteins tested confirmed this as there was strongest evidence for enrichment of proteins involved in metabolic disease and renal disease, however the effect on cardiovascular disease did not pass multiple testing adjustment. Pathway enrichment using StringDB also suggested that proteins most altered with BMI change were involved in cytokine-cytokine receptor interactions and complement and coagulation cascades, rather than platelet activation. It could be that the effect on inflammation and coagulation may be more immediate effects, and that changes in platelet activation may either require a more sustained weight loss, or a further reduction in BMI to see effects.

Limitations from the current analysis therefore include possible protein effects that are not caused by a change in BMI. Despite the use of the treatment group as an instrument in a two stage least squares analysis, the treatment group was also associated with an alteration in other factors: those who were

allocated TDR also were taken off any T2D medication and were more likely to achieve remission of T2D. It is possible that the analysis is therefore picking up protein effects that are caused by a change in medication or T2D remission, rather than as a result in loss of fat. Participants would have also lost muscle mass throughout the intervention¹⁹⁷, therefore it is not certain that effects are attributable to loss of fat. Despite this, there is evidence that in a group of participants with obesity, the majority of weight lost is fat mass¹⁹⁸. Thirdly, BMI decreased by an average of 0.4kg/m² in the control group and by 3.6 kg/m². As participants started at a mean BMI of 34-35 kg/m², the majority of participants will still have obesity even after the intervention. This may limit protein effects observed, as a certain BMI may need to be reached to see an alteration in protein levels (i.e. the relationship may not be linear). Finally, it is unclear how generalisable results would be to the wider population. It is often the case with diets and caloric restriction that they only result in weight loss short-term and this is likely because the body adapts to the lower intake of calories. Therefore the changes which are seen in levels of proteins may not be so dynamic. The use of bariatric surgery may help to ensure weight loss and the associated changes are sustained.

Overall, this study provided evidence that weight loss leads to a substantial change in the composition of plasma proteins, replicating effects seen previously and providing some novel associations. Proteins altered with higher BMI in a Mendelian randomisation framework can be reversed with weight loss. Proteins altered with weight loss were enriched for renal disease and metabolic disease.

Chapter 8

Combining MR and an RCT to determine effects of adiposity on the plasma proteome

8.1 Background

The two previous results chapters (Chapter 6 and Chapter 7) aimed to determine the effect of adiposity on circulating proteins by using two different study designs: an MR study which evaluated the effect that prolonged high BMI has on protein levels and an RCT which explored the effect of weight loss through caloric restriction on the plasma proteome. These two studies provided two separate line of evidence for a causal role of adiposity in circulating protein composition. Both studies also provided evidence that the change in circulating proteins may be contributing to the increased risk of disease such as metabolic disease or cardiovascular disease. There was also separate lines of evidence for an effect of BMI on certain proteins which may be important in platelet pathways.

This overall shift in proteins provides important information. One of the benefits of having the same proteomics platform in both studies is that there is a large overlap in the number of proteins measured. Each approach has its own biases and limitations and both the concordance and divergence in effects across studies provides insight into possible mechanisms. The overall aim of this chapter is to compare BMI-protein effects across MR and RCT study designs and evaluate implications for platelet function and cardiovascular disease.

8.2 Methods

In efforts to further elucidate which proteins are altered by adiposity, a comparison between the BMI-protein associations in INTERVAL (Chapter 6) and the BMI change-protein change associations in Chapter 7 was performed. There were 2803 unique proteins (by UniProt ID) which were detected in both INTERVAL and DiRECT. Estimates across the two cohorts were compared using linear regression. To be able to compare whether estimates were similar across study designs, estimates from each analysis were scaled using the scale() function from the “base” R package. Estimates were categorised as: similar if the scaled estimates were within 1 SD of each other; different if estimates were more than 1 SD apart and extreme if within 1 SD of each other and with an absolute scaled estimate of more than 1 SD.

Estimates for the specific platelet proteins highlighted in Chapters 6 and 7 were also extracted to evaluate the effect of BMI on platelet proteins. Estimates were extracted from the MR interval and for the two stage least squares analysis in DiRECT as these were the more robust models.

8.3 Results

8.3.1 Comparison of BMI effects across DiRECT and INTERVAL

BMI-protein estimates from this DiRECT were compared with effect estimates from INTERVAL (Chapter 6), where 2803 proteins were detected across both studies. Both cohorts, through use of different study designs, provided evidence for an effect of BMI on the plasma proteome. Figure 8.1 shows the BMI-protein estimates for the proteins which were included in both DiRECT and INTERVAL ($R^2=0.11$, $P=1.0 \times 10^{-77}$). There was consistent evidence across both studies that higher BMI leads to higher levels of various proteins including fumarylacetoacetate (FAAA), alcohol dehydrogenase 4 (ADH4), growth hormone receptor (GHR) and carboxypeptidase M (CBPM). Furthermore, higher BMI had a lowering effect on levels of sex hormone binding globulin (SHBG), as well as insulin-like growth factor binding protein 1/2 (IGFBP1/2). Although some of these estimates did not pass multiple testing in INTERVAL, the concordance in estimates in terms of effect size and direction of effect suggests that this is an issue of power.

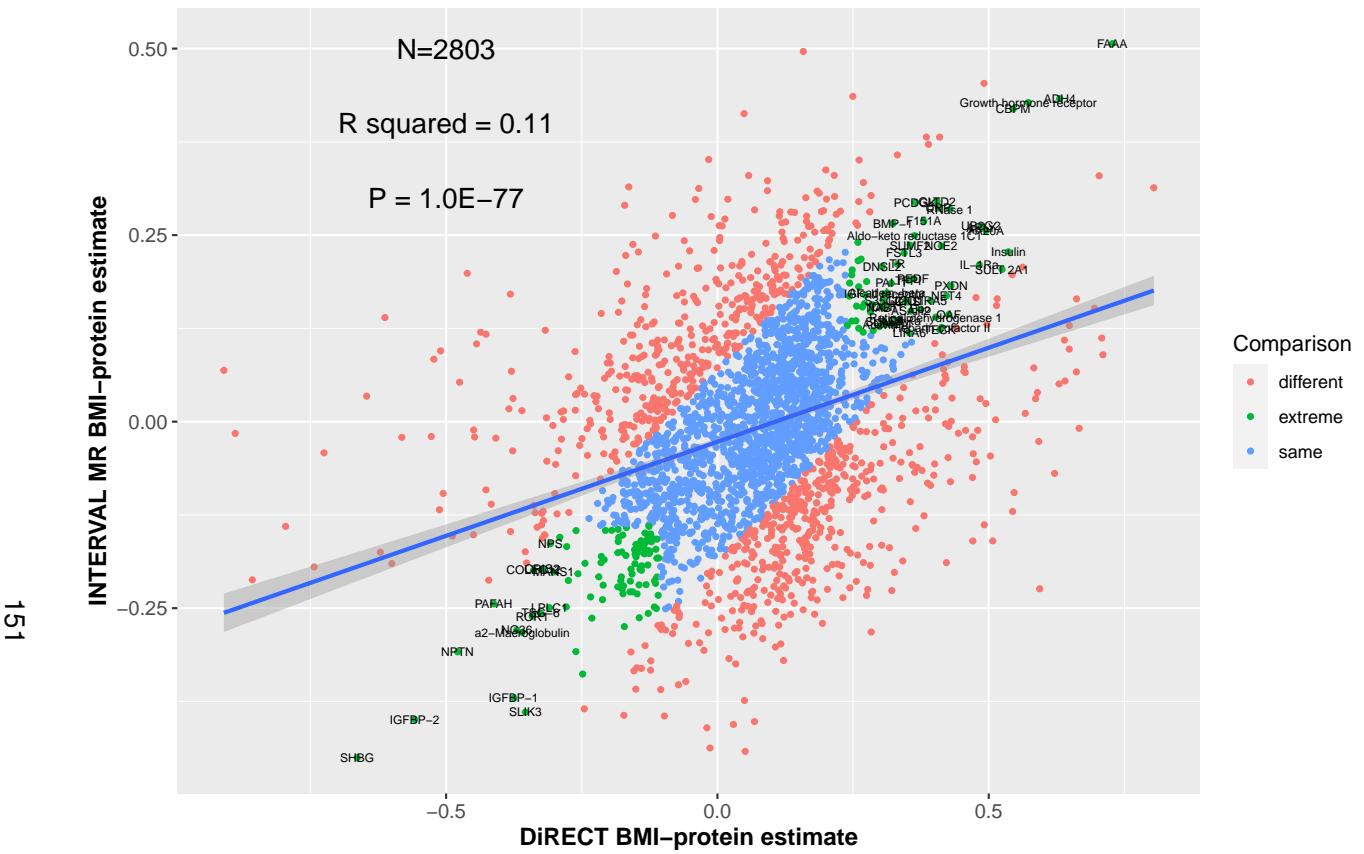


Figure 8.1: Scatter plot comparing the two stage least squares estimate for the effect of BMI change on protein change in DiRECT with the Mendelian randomisation estimate for the effect of BMI on protein levels in INTERVAL. Proteins that are similar across analyses with a large effect size in both studies are coloured in green, whereas those which display similar estimates across studies with small effect size are shown in blue. Proteins do not show similar effects across studies are shown in pink. A linear regression line is indicated in blue.

The divergence in magnitude of estimates across study designs also suggests that some proteins are only associated with BMI in one study design. For example, the estimates in INTERVAL suggested that BMI has an effect on complement factors such as Complement C5 and Complement Factor I. As seen in Figure 8.2, these complement factors as well as others group together, where they appear to have a relationship with BMI in INTERVAL but are not affected by weight loss in DiRECT. There are other proteins which show the opposite: SCAR5 and CHAD displayed strong associations with BMI change in DiRECT, however the MR did not detect a causal effect of BMI on levels of these proteins 8.2.

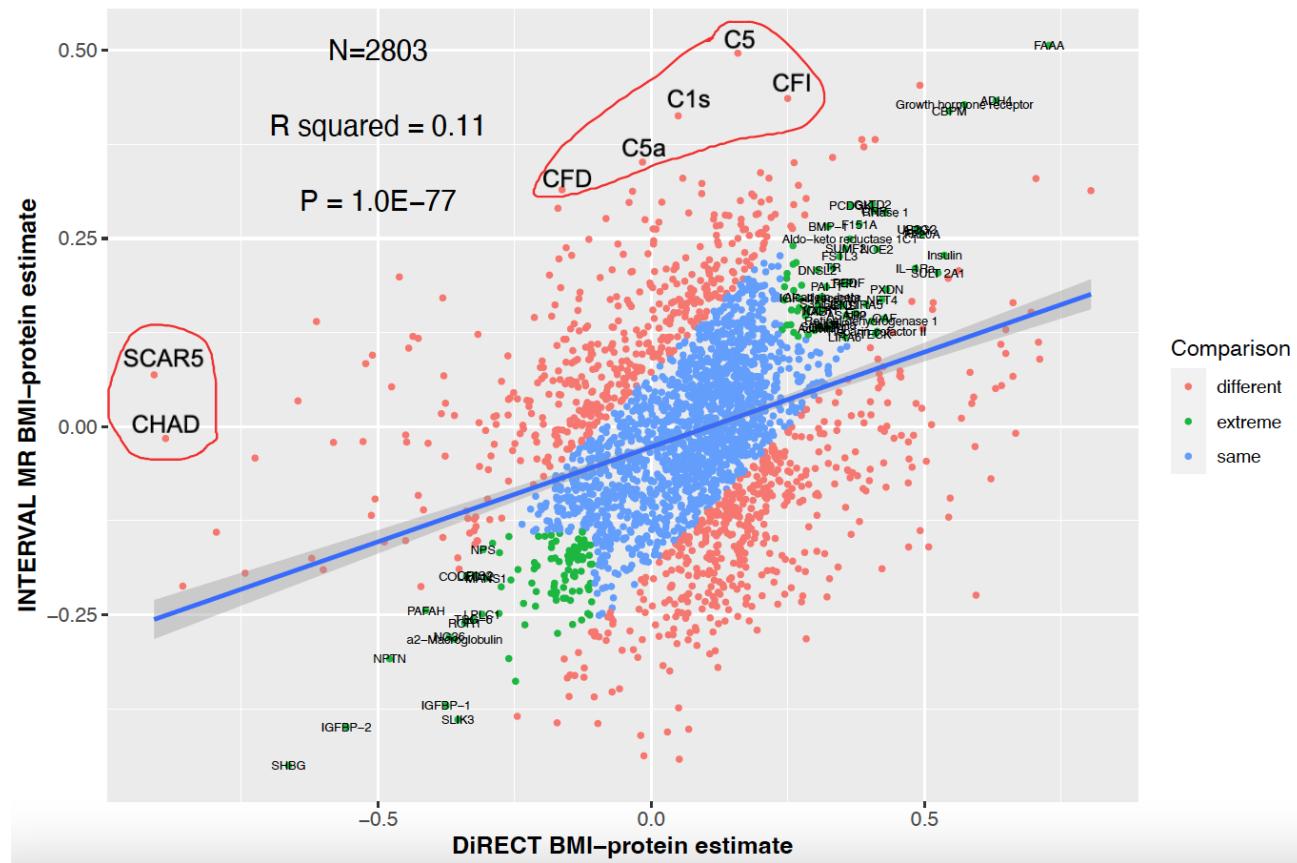


Figure 8.2: Scatter plot comparing the two stage least squares estimate for the effect of BMI change on protein change in DiRECT with the Mendelian randomisation estimate for the effect of BMI on protein levels in INTERVAL, with proteins that differ across models highlighted. Proteins that are similar across analyses with a large effect size in both studies are coloured in green, whereas those which display similar estimates across studies with small effect size are shown in blue. Proteins do not show similar effects across studies are shown in pink. A linear regression line is indicated in blue. Complement factors which group together are circled in red at the top. SCAR5 and CHAD are also indicated.

Across both INTERVAL and DiRECT, 170 unique platelet proteins were detected. The estimates for these proteins are shown in Figure 8.3. Overall, the estimates were associated with each other ($R^2=0.19$, $p=1.1\times 10^{-9}$), however there were divergences in estimates for some proteins. Despite this, there were consistencies for some proteins. PAFAH was associated with BMI in the observational analysis in INTERVAL. However, PAFAH did not pass multiple testing in the MR analysis. Despite this, the estimate was similar in magnitude to the observational estimate. PAFAH was also associated with BMI change in DiRECT in the instrumental variable analysis (Figure ??). The direction of the effect indicated that higher BMI is associated with reduction in circulating PAFAH. In agreement with this, a reduction in BMI lead to levels of PAFAH increasing. The estimates for PAFAH were similar and can be seen in Figure 8.3. Consistencies in the estimates across the two study designs for other proteins, such as for complement factor B, suggest other effects that may be mediated by BMI that could affect platelet function.

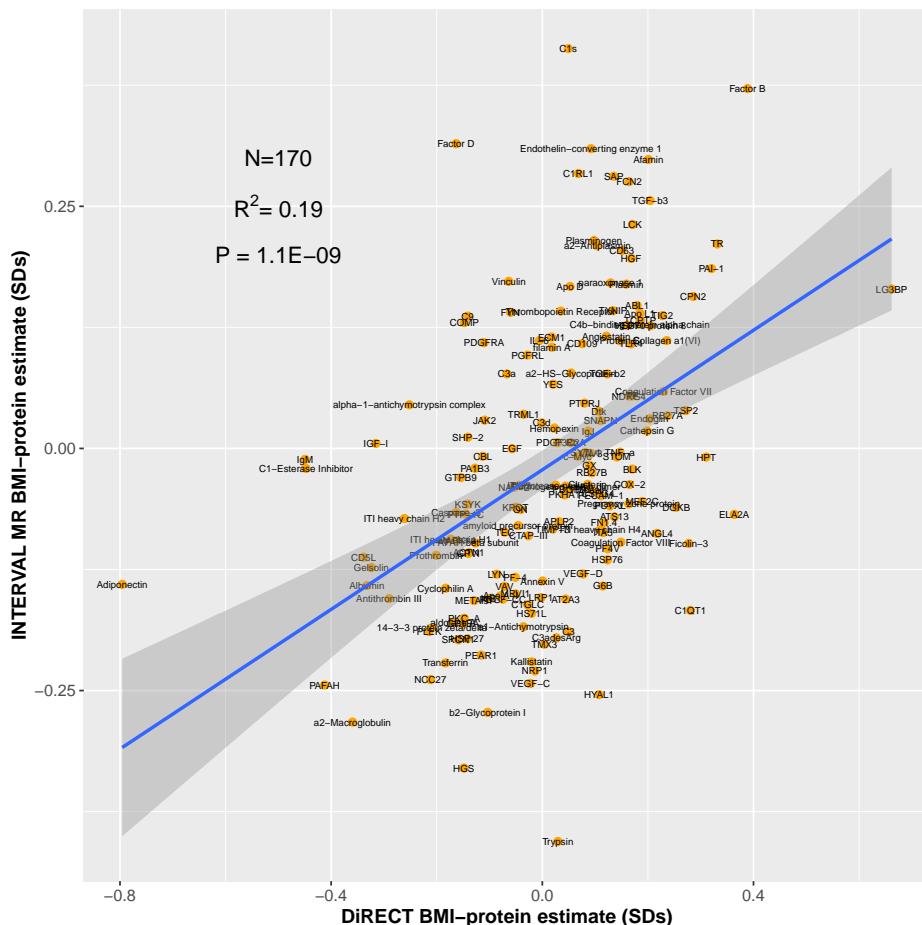


Figure 8.3: Scatter plot of the two stage least squares estimate in DiRECT and the Mendelian Randomisation estimates in INTERVAL and the effect of BMI on proteins involved in platelet pathways.

8.4 Discussion

This chapter brought together results from two different analyses to determine effects of adiposity on the plasma proteome (INTERVAL in Chapter 6 and DiRECT in Chapter 7). Comparisons of protein effects across the different study designs points towards proteins of interest which may be involved in the development or progression of obesity-associated cardiometabolic disease. This chapter also collated the results for the proteins which have been implicated in pathways relevant to platelets.

The comparison of results from Chapter 6, which used MR and observational associations to assess the effect of average higher BMI across a population on average protein levels, with results from an RCT in Chapter 7 provides two separate lines of evidence to suggest a causal role of BMI in levels of various proteins. The analysis in INTERVAL suggested that proteins most altered by BMI were enriched for diseases such as cardiovascular disease and metabolic disease. As the estimates for the effect of BMI on proteins were consistent across both DiRECT and INTERVAL, this suggests that weight loss is able to reverse the protein changes observed with higher BMI, thereby potentially reducing disease risk. Although previous studies have identified BMI effects on some of these proteins, this direct comparison of effect estimates across an MR study design and weight loss intervention for the general proteome has not yet been done in this manner. This is a key strength of the study. The RCT had fewer participants than INTERVAL (265 vs 2737), but had enough power to detect an effect of BMI change on over 100 proteins. Proteins identified which would benefit following up, based on consistencies across study designs, include SHBG, IGFBP1/2, and GHR. The comparison has also highlighted some more novel proteins such as FAAA and PAFAH, which require further study.

Overall, estimates for the effect of BMI on platelet proteins in DiRECT and INTERVAL displayed a positive association. The agreement in effect estimates across study designs for the platelet protein PAFAH suggests that higher BMI does affect levels of this protein and that it is likely the power issue which means it cannot be detected in the MR. The power analysis in the discussion of Chapter 6 supports this as it was calculated that an effect size of ± 0.33 SDs would be required to have a power of 80%. This protein has been suggested to be antiatherogenic, therefore lower levels would be proatherogenic¹⁷⁵. Validating this protein in an independent cohort and investigating this protein further in the lab could help reveal the functional implications of changes in PAFAH. This protein provides a good example of how triangulating can help tease apart the protein effects which are more likely to be driven by BMI.

Divergence in estimates across study designs may also suggest that there are spurious relationships

occurring. However, this divergence may also provide some insight into the biology due to the nature of each study design. In general, the MR study design provides insight into the effect on the proteome that a sustained high BMI has over the life course and is therefore indicative of chronic effects. In contrast, the estimates in DiRECT are the effect that acute weight loss (1 year) has on the proteome. Complement factors may be altered in the MR study, but not after a weight loss intervention because weight loss may not have been sustained long enough to induce changes at the proteome level. There was no evidence for a causal relationship between BMI and both SCAR5 and CHAD in the MR, but these proteins displayed strong associations with weight loss in DiRECT. This could be because these protein changes are signals of starvation or due to other changes that happen with caloric restriction, such as a change of medication or loss of muscle mass. This requires further exploration, and requires more formal analysis to disentangle effects.

One of the limitations of the analyses performed in Chapter 6 and Chapter 7 is that many proteins are not in the SOMAscan in both studies. Out of 4,034 SOMAmers in INTERVAL and 4601 SOMAmers in DiRECT, there were 2803 proteins in both. This means that there would be other proteins which would likely have reproducible effects across study (such as leptin). Therefore, this is limiting which proteins are discussed in the current chapter.

Overall, this chapter brought together two different study designs which aimed to determine the effect of adiposity on the plasma proteome. Through directly comparing the magnitude of estimates, this chapter identifies proteins which have the strongest evidence for a BMI effect (SHBG, IGFBP1/2, GHR, FAAA), as well as identifying a role of BMI in levels of circulating PAFAH, which may have implications for atherosclerosis. Divergence in estimates indicates effects which may be specific to one study design such as a causal effect of BMI on complement factors in the MR, but the inability to reverse these effects with weight loss. Replicating in other cohorts is now required to further understand these relationships.

Chapter 9

Discussion

9.1 Overall findings

The work presented in this thesis brought together an interdisciplinary approach to expand on previous literature to further explore the contribution of platelet function and the plasma proteome to obesity-related cardiovascular disease (Figure 9.1. The results and limitations from each individual results chapter have been discussed in the relevant chapters. This overall discussion chapter will recap the main findings from the thesis, address the overall strengths and limitations, discuss the wider clinical implications of findings as well as suggest future research to expand this area of research.

Overview of thesis hypotheses

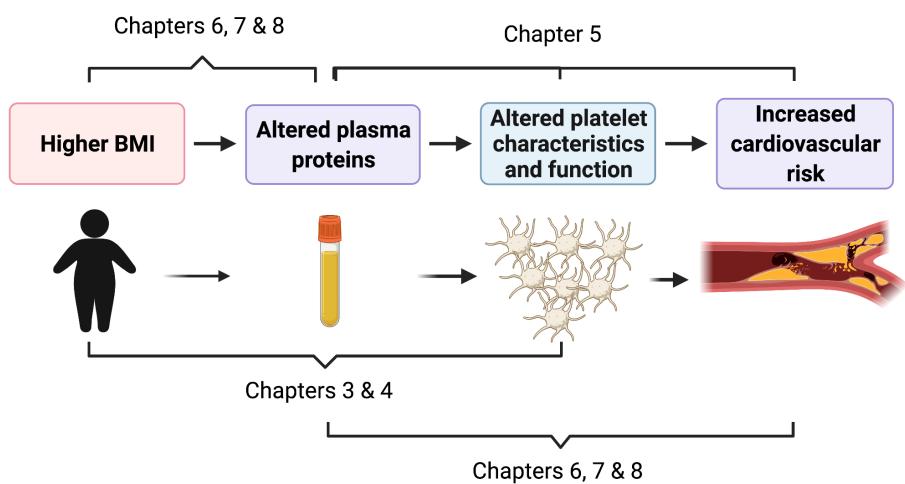


Figure 9.1: **Schematic of the multi-step pathway addressed in the thesis with chapter numbers where associations are explored.** Figure made using BioRender.com.

A summary of the main findings of the thesis is provided in Table 9.1. This thesis started with exploring the effect of BMI on platelet traits within a cohort of UK blood donors (INTERVAL) (Chapter 3). This chapter provided evidence, using both observational and MR analyses, that higher BMI increases IPC. As INTERVAL did not have measures of platelet function, associations between IPC and whole blood platelet aggregation were explored with a cardiac surgery cohort (COPTIC). Here IPC and platelet aggregation were positively associated, independent of the overall platelet count. The next chapter (Chapter 4) sought to determine the effect of BMI on more detailed readouts of platelet function by designing a patient study, recruiting patients who were due to undergo bariatric surgery. Here, alterations in the plasma proteome were found which may have implications for platelet function and signalling. Follow-up experiments using other platelet function techniques, with a larger sample size, are now required. Chapter 5 examined the effect of the chemokines MDC and TARC on platelet function, chemokines which may be raised a result of obesity-induced inflammation. MDC and TARC were found to potentiate PAR1-AP induced platelet function in washed platelets and MDC was able to cause platelet aggregation in PRP. This aggregation was found to be mediated by CCR4, with evidence that this receptor signals through G_q coupling. There was MR evidence that higher levels of MDC could contribute to DVT and PE. Chapters 6 and 7 both aimed to explore effects of adiposity on the plasma proteome. Chapter 6 used observational and MR frameworks and found causal evidence for an effect

of BMI on the plasma proteome whereas Chapter 7 used a weight loss intervention to explore effects of BMI on the plasma proteome. Chapter 8 brought together these study designs to identify consistent evidence for BMI effects on proteins.

Table 9.1: Summary of main findings from the thesis

Chapter	Main findings
Chapter 3	Higher body mass index (BMI) leads to higher immature platelet count (IPC) in a Mendelian randomization framework (INTERVAL). Within a cardiac surgery cohort (COPTIC), immature platelet count was positively associated with whole blood platelet aggregation.
Chapter 4	Laboratory experiments were optimised to assess platelet function from patients. In the clinical pilot study, the effect of high BMI on platelet function was inconclusive, but there was evidence for a difference in levels of platelet proteins across BMI groups.
Chapter 5	This study provided evidence that the chemokines MDC and TARC can potentiate PAR1-AP induced platelet function. The chemokines were also found to be able to activate platelets when applied alone. It is likely that these effects are through the CCR4 receptor which is likely to be G \sim q \sim coupled. A two sample Mendelian randomization analysis implicated higher levels of MDC in deep vein thrombosis and pulmonary embolism. Higher BMI has a causal effect on levels of proteins which circulate in the plasma. Proteins most altered by BMI were enriched for cardiovascular disease, metabolic disease and renal disease.
Chapter 6	Higher BMI has a causal effect on levels of proteins which circulate in the plasma. Proteins most altered by BMI were enriched for cardiovascular disease, metabolic disease and renal disease.
Chapter 7	Weight loss as a result of caloric restriction has a substantial effect on the plasma proteome, with changes enriched for metabolic disease and renal disease.
Chapter 8	Proteins altered by higher BMI in Chapter 6 can be reversed with weight loss in Chapter 7.

9.2 Strengths

As previously mentioned, this thesis has approached hypotheses using a range of study designs. Integrating clinical studies, basic science, cohort studies and trial data has been crucial to further understand the role of BMI in platelet function. This is therefore a strength of the current research. One of the major benefits of these techniques are that they are in line with the principles of the 3Rs (replacement, reduction and refinement). The thesis has made use of available human data from various cohorts to avoid use of animals in research (replacement).

9.3 Limitations

The associations within the pathway in Figure 9.1 were explored where there was available data or where it was possible to address hypotheses with specifically designed basic science experiments. Certain analyses were limited by the availability of data. For example, it would have been beneficial to have platelet function measured within the INTERVAL cohort as this would have made it possible to do a one sample MR for BMI to platelet function, and the plasma proteome findings could have been directly compared to platelet function. Although platelet function data is not available on this scale, cohorts such as the Framingham Heart Study (FHS) have platelet function data for ~3,000 participants¹⁹⁹. As they also have genetic data, it would be beneficial to perform a one sample MR for BMI to platelet function. The association between IPC and platelet function would benefit from being replicated in a larger cohort who are free from major disease such as the FHS, however this study does not have IPC measured.

As mentioned in Chapter 4, a major limitation is the lack of longitudinal data for the bariatric surgery and platelet function study. Before the COVID-19 pandemic, the plan was to have bariatric patients with $BMI > 40 \text{ kg/m}^2$ and controls followed up at four time points. These time points were: baseline (2-3 weeks before surgery), after the very low energy diet (VLED) that precedes bariatric surgery (day of surgery), 6 weeks post-surgery and 12 months post-surgery. This would have allowed exploration of the change in an individual's platelet function with weight loss. Previous studies have researched the effect of obesity and bariatric surgery on platelet function^{96,200}, but platelet function at four timepoints surrounding bariatric surgery has not yet been explored, more specifically before and after the VLED. This study aimed to add in a time point at 12 months, which is where there is evidence for maximal weight loss²⁰¹. Therefore this study would have been one of the most in depth platelet function studies

for bariatric surgery.

Another limitation of the thesis is that there was no stratification by sex in analyses. Analyses did adjust for sex and therefore should help to reduce the confounding effect from this variable, however there may be a need to explore whether there are any sex specific effects. It is possible that BMI has different effects on the plasma proteome in males and females. Previous studies have identified sex differences in inflammatory protein changes in individuals with and without metabolic syndrome, for example adiponectin²⁰². A difference in platelet reactivity between males and females has also been reported, where aggregation in response to a range of platelet agonists was greater in females²⁰³. Future analyses would benefit from stratifying analyses by sex to further investigate this, however larger sample sizes would be required.

9.4 Wider implications of findings

The research has contributed to the understanding of mechanisms of obesity-related cardiovascular disease. As well as providing evidence for a role of body mass index in the production of immature platelets, the research surrounding IPC has wider implications. As mentioned in Chapter 3, the standard full blood count used by the NHS does not include IPC. Similarly, some more detailed blood counts from newer haematology analyzers do not provide IPC within the summary, however this can be derived from IPF and PLT. The finding that IPC associates with whole blood platelet aggregation is important as it not only suggests that IPC should be considered in patients with obesity, but it could also be a useful biomarker of platelet hyperactivity across the population. Studies have indicated that there is a positive correlation between IPC and ADP-induced platelet aggregation in patients receiving either prasugrel or ticagrelor, therefore IPC could be used to predict which patients would be more responsive to antiplatelet treatment⁹⁸.

As well as IPC having the potential to be a useful biomarker, various plasma proteins could also be biomarkers for patients with obesity who may be at a greater risk of cardiovascular events and metabolic disease. Using MR, various proteins altered by BMI as shown in Chapter 6 have been implicated in disease. For example, in work by Constantinescu et al., their analysis suggested that certain proteins altered with BMI may have a causal role in DVT, including INHBC (Reference Andrei's paper). Proteins such as INHBC could therefore be therapeutic targets. These proteins could also be potential biomarkers.

9.5 Future studies

Based on the findings of the research, there have been more questions which have been raised and would require further research. These will be discussed in the following paragraphs.

The MR in Chapter 3 provided causal evidence for higher BMI raising IPC. There was also a positive association between BMI and IPC within the cardiac surgery cohort and although the magnitude of estimate was the same, this effect was weak, therefore this is potentially attributable to the modest sample size. It would be beneficial to validate this association within an independent cohort, such as UK Biobank. It would be useful to link IPC to medical records to be able to look at whether IPC is a predictor of cardiovascular events such as myocardial infarction or stroke. Mendelian randomization could also be implemented to look at the causal effect of IPC on cardiovascular outcomes. Once summary statistics become available for a GWAS of platelet function, with more participants, it would also be beneficial to do an MR analysis to explore the causal role of IPC in platelet function.

Following the findings of Chapter 4, although underpowered, it was observed that some bariatric patients with obesity had a high IPC, as high as 40%. Therefore, this needs to be evaluated in more bariatric patients. There are other bariatric cohorts such as the Stratification of Obese PHenotypes to optimize future obesity therapy (SOPHIA) study which would likely have the relevant full blood count data to perform these analyses. Further characterisation of the immature platelets in patients with obesity within the laboratory is also now required. Here it is possible to look at the expression of P-selectin within the gated immature platelets⁹⁸, to determine how this differs in obesity and people of normal weight. It is currently unclear why higher BMI would be linked to the production of more platelets, but the platelet proteomics experiment using mass spectrometry implicated that certain proteins which may be markers of platelet maturity may be altered in obesity. These proteins such as actin could be characterised further using laboratory experiments. Use of small-molecule proteolysis targeting chimeras (PROTACs)²⁰⁴, which can degrade proteins in platelets would be one such method to determine their role. GWAS for protein levels tend to be measured in the plasma or serum. This may be missing important changes which could be involved in disease progression. For example, levels of certain proteins could be unaltered in the plasma, but changes within certain cells such as neutrophils or platelets would provide additional information. If GWAS were performed for platelet proteins, Mendelian randomization could be performed to identify how this could affect cardiovascular outcomes. However, this would be labour intensive.

Analyses explored both the platelet proteome and the plasma proteome in relation to body mass index. The effect of obesity on microparticles released from platelets activated by thrombin has also been explored¹³⁰. A systematic comparison across these three proteomes could reveal how these proteomes relate to each other. For example, it could be possible to see whether specific proteins are affected in the same way in both platelets and plasma, or if there is a reciprocal relationship. Initial analyses on platelet proteomic data from patients with COVID-19 have identified that many plasma COVID-19 biomarkers are also altered in platelets (unpublished data from the Hers group). This may also be the case for other diseases such as MI/DVT and therefore requires further exploration.

The MR analysis in Chapter 6 provided evidence for a causal effect of BMI on a range of proteins. There was also MR evidence that higher BMI raises IPC. Exploring the possibility of a bidirectional effect for both of these analysis would be important as it cannot be ruled out that certain proteins could be involved in processes involved in weight regulation. It is unlikely that IPC would have a causal effect on BMI, however this would also need to be confirmed by use of a two sample MR. The possible effect of plasma proteins on IPC could also be explored using a two sample MR, using summary statistics for their respective GWAS. Another way to address the effects of proteins on IPC and platelet function could be to apply proteins such as fumarylacetoacetate, SHBG and leptin to megakaryocytes and to see whether there are changes in production of platelets compared to control. Proteins could also be applied in platelet aggregation experiments or *in vitro* thrombosis to see whether these cause platelet hyperactivity.

Exploring individual proteins is useful in understanding disease risk, however further work on protein profiles is required as it is more likely a change in various proteins mediates the risk. Techniques to explore profiles are not well developed, however there are a few approaches which could be implemented. One such technique would be network MR, to be able to identify the relationship between proteins altered by BMI. This could help reveal whether there are any cascade effects, where an alteration in one protein may lead to downstream changes in other proteins and as such be at the top of the cascade. Another technique to begin unpicking profile effects could be exploring the effect of BMI on ratios between proteins. This technique has been used for metabolites, for example a single measure of the ratio between monounsaturated fats to polyunsaturated fats circulating in the blood is used²⁰⁵. It is unclear whether this technique would be suitable for proteomics data, and if it is, which protein ratios would be biologically meaningful to express as a ratio.

Methods to assess the effect of BMI on proteins have used linear methods. Linear MR is commonly

used, however this may not always be the most appropriate method as there are often nonlinear relationships, for example between BMI and mortality, where there is a J-shaped relationship²⁰⁶. Nonlinearity has been observed for other associations, for example with vitamin D deficiency and CVD risk²⁰⁷. Most studies have implemented linear models when looking at the effect of BMI on proteins/metabolites, however these relationships should be re-examined for nonlinearity. Methods to do this are currently available, but they are not easily implementable on a large scale, such as when there are thousands of protein outcomes. Current methods such as the “nlmr” R package stratify estimates based on quantiles. As a result of this, the user cannot define their bounds of their strata and therefore makes it difficult to interpret results. The “glsmr” package was therefore developed to allow to do so and therefore provide estimates for causal effects in the predefined ranges of the exposure such as BMI. More detail and implementation of these packages is provided in Appendix A, with an example using the effect of BMI on levels of leptin. A limitation of this approach is that stratifying MR estimates reduces the power and therefore creates more imprecise estimates. Furthermore, when looking at the effects of obesity at the upper end of the distribution (e.g. BMI range of 40-60 kg/m²), there are fewer participants and therefore causing the same power issue. As a result, larger sample sizes would be needed with oversampling at the ends of the distributions to ensure sufficient power in each stratum. More recently, SomaLogic proteomic analysis has been performed on plasma samples from nearly 40,000 Icelanders²⁰⁸. Studies of this scale would help with stratifying into groups while maintaining power. Other studies such as the Qatar Biobank have higher rates of obesity than other cohorts, where 39% have obesity and the mean BMI is 29 kg/m²²⁰⁹. Studies such as this would be more suitable for nonlinear analyses.

The analyses in Chapter 7 utilise an RCT implementing caloric restriction to explore the effect of weight loss on the plasma proteome. As mentioned briefly within the limitations section of that chapter, the reduction in BMI was modest, so the majority of participants will still have obesity even 1 year after the intervention. It is also likely that this weight loss would not be sustainable long term. Therefore it may be difficult to determine the effects of reducing adiposity. Bariatric surgery is another intervention which typically causes a greater reduction in BMI. A previous study in adolescents indicated that a gastric bypass and sleeve gastrectomy resulted in a 3-year BMI reduction of 15 kg/m², around 30% reduction of baseline BMI²¹⁰. This drastic change in BMI would likely have a substantial effect on the plasma proteome. Previous studies have explored this and I plan to utilise some pilot data from the bariatric surgery trial, where serum samples have been analysed by the new Olink Explore 1536 panel. This will provide estimates for the effect of bariatric surgery-induced weight loss on the serum proteome. These estimate can be compared alongside the estimates presented in Chapter 8.

9.6 Overall conclusion

Overall

Appendix A

Exploring nonlinear associations between body mass index and plasma proteins

This appendix contains an example of nonlinearity occurring for the association between body mass index (BMI) and levels of certain plasma proteins within the INTERVAL dataset. This analysis was performed as a result of an observation within the group and from published data²¹¹ that the effect of BMI on certain circulating metabolites such as LDL-C may be nonlinear. Therefore it is likely that this is also the case with proteins: biologically, proteins might reach a threshold and therefore increasing BMI may lead to a plateau in the levels of the protein. Alternatively, certain technologies may reach a maximum in detection. The SomaLogic platform has a wide range of detection from femtomolar to micromolar²¹², therefore maximal detection could be reached. This possible nonlinear relationship has not been accounted for in the linear models and Mendelian randomisation analyses performed in Chapter 6. Nonlinearity may also explain why some proteins may be altered as a result of high BMI in the MR, but caloric restriction may not alter the levels of the same protein. There may be a certain BMI that needs to be reached before protein levels are altered therefore in DiRECT, as BMI remains in the obese category, effects may not be seen. Exploring nonlinearity is ongoing, but this appendix provides an example of the exploration of a possible nonlinear relationship between BMI (in kg/m²) and levels of leptin.

A.1 Observational association between BMI and leptin

Data from the INTERVAL study was used as was described in Chapter 6. BMI was not rank normal transformed to help with interpretation of nonlinearity. The association between BMI and leptin was explored observationally using both a linear model and a nonlinear generalized additive model (GAM) (Figure A.1). Here, using BMI as the exposure and leptin as the outcome (without covariates added in the model), there was evidence for a positive linear association between the two variables. However, the nonlinear GAM indicated that after a BMI of around 40 kg/m^2 the levels of leptin do not increase anymore. These two models were compared using an ANOVA (F test) with the hypothesis that the nonlinear GAM is a better fit than the linear model, where a small P value indicates that the GAM is a better fit. For leptin, R^2 for the linear model was 0.51 and it was 0.56 for the GAM, with a p value of (2.17×10^{-59}). This was the smallest p value comparing the two models for all 4,034 SOMAmers in INTERVAL, therefore indicating that the nonlinear association is the most appropriate for leptin.

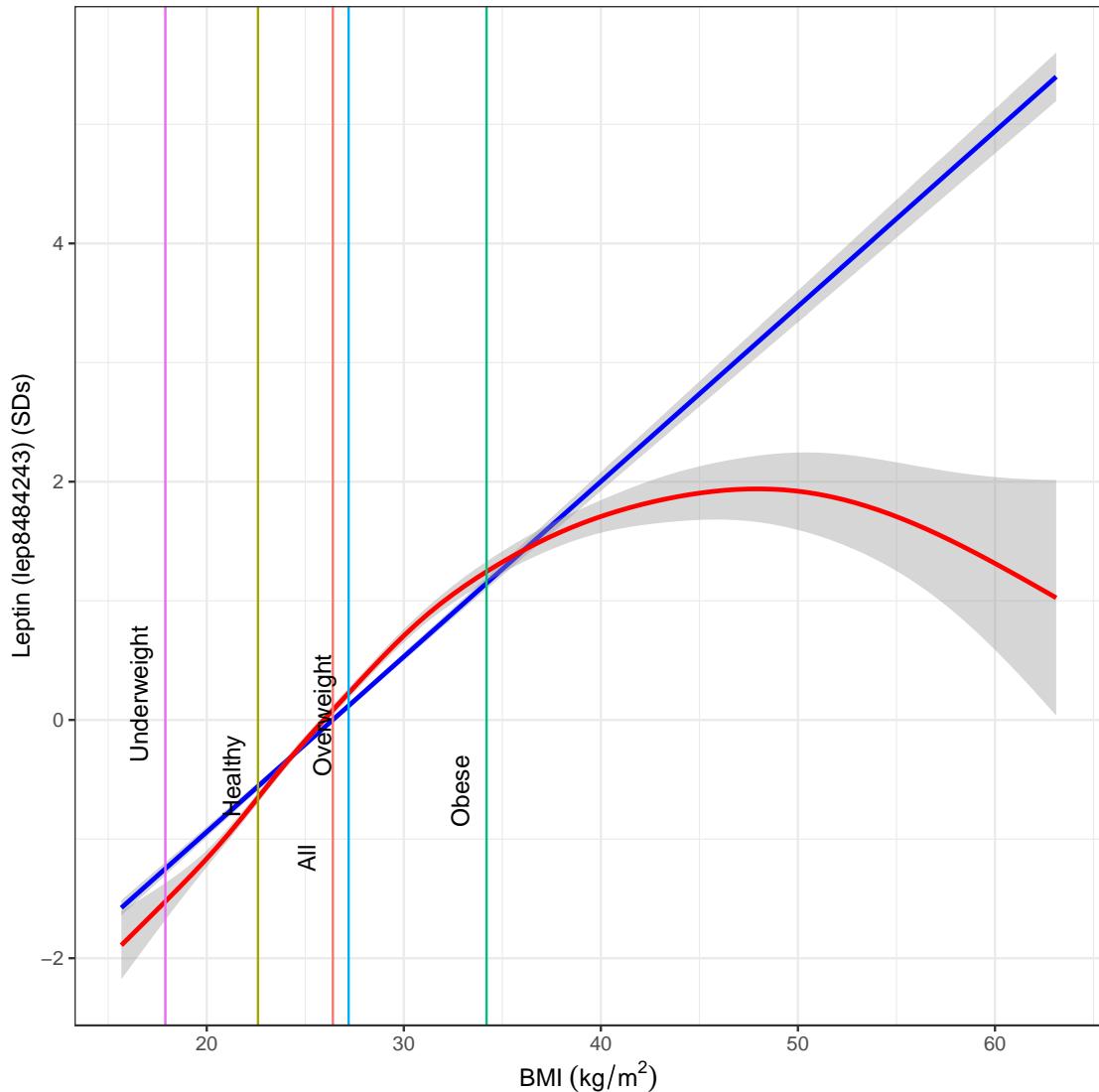


Figure A.1: Linear model (blue) and nonlinear generalized additive model (red) of the association between BMI and leptin with 95% confidence intervals. The vertical lines indicate the mean BMI for each BMI category (in order of underweight, healthy, all, overweight, obese).

A.2 Determining nonlinear effects using TSLS MR methods

Nonlinearity can be easily implemented using observational methods. However due to the nature of MR, regressing the genetic risk score on BMI provided estimates of genetically predicted BMI (the first stage of the TSLS analysis). The protein was then regressed on this genetically predicted BMI (second stage of TSLS) using a nonlinear function, however, this covered a limited range of BMI as seen in Figure A.2. Therefore it was more difficult to interpret a causal nonlinear effect over the full range of

BMI observed in the population.

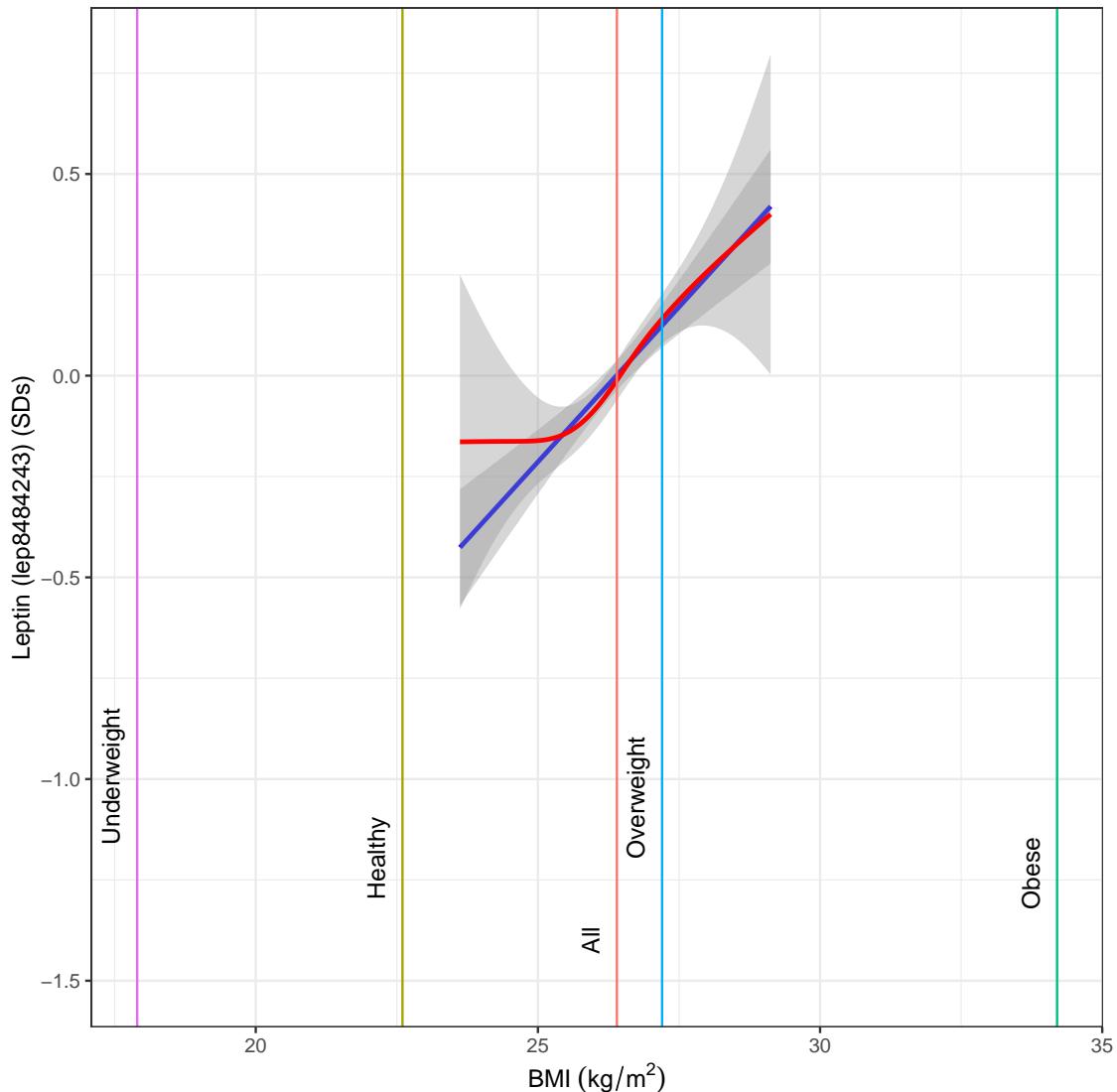


Figure A.2: Linear model and generalized additive model of the association between BMI and leptin using Mendelian randomization. Linear model (blue) and generalized additive model (red) of the association between BMI and leptin with 95% confidence intervals. The vertical lines indicate the mean BMI for each BMI category (underweight, healthy, all, overweight, obese)

A.3 Using nonlinear MR package

The nonlinear MR package (“nlmr”) was also utilised to overcome this issue. This package utilises two methods: fractional polynomials and piecewise MR.²¹³ Fractional polynomials consist of metaregression of the localised average causal effect (LACE) estimates against the mean of the exposure in each

stratum, whereas the piecewise MR estimates linear functions in a piecewise manner, where the gradient is the LACE estimate in each stratum. As indicated in Figure A.3A, the fractional polynomial method with five quantiles suggested that the levels of leptin start to plateau with higher BMI. This effect was less clear using the piecewise method in Figure A.3B, which also has been set to five quantiles. However, tests of nonlinearity such as the fractional polynomial nonlinearity p-value suggested that this fit is not better than a linear model ($p=0.13$). This was also the case using the Cochran Q test for both the fractional polynomial and piecewise linear model, where the p-values were both 0.62. LACE estimates suggest that the effect of BMI on leptin is strongest in the first quantile (Estimate = 0.20, 95% CI 0.10 to 0.30, $p=7.00\times10^{-5}$). For the fifth quantile, the estimate is 0.12 (95% CI 0.04 to 0.20, $p=0.0020$).

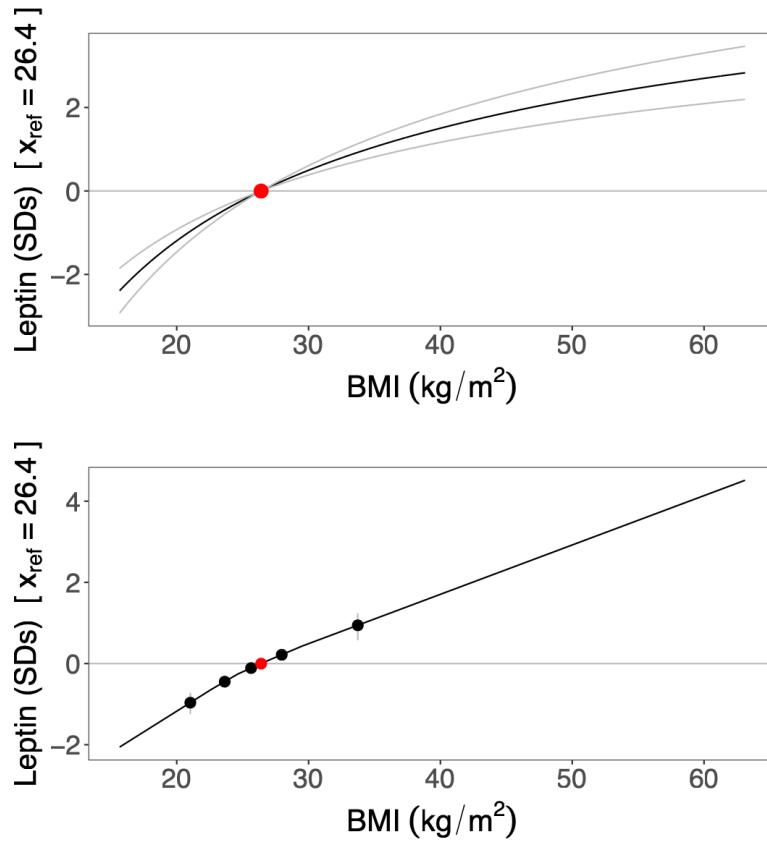


Figure A.3: Effects of BMI on levels of leptin using nonlinear MR from the **nlmr** R package.²¹³ A) fractional polynomial MR. B) Piecewise MR.

A.4 GAM and linear stratified MR (GLSMR)

The issue with these methods is that it isn't possible to estimate the effect of a certain range of BMI on protein levels. This is important to be able to understand the range of BMI where certain effects

on proteins are likely to occur. Therefore the package “*glsmr*” was created within the group by David Hughes. This package enables estimates to be derived for the effect of the exposure on the outcome for specified ranges in the exposure, using both observational associations and using Mendelian randomization. It also compares the linear and nonlinear models. More details can be found here <https://github.com/hughesveoanth/glsmr>. The effects of BMI on leptin levels were further explored using this package. For this analysis the strata were defined as 18.5-25 kg/m² (healthy), 25-30kg/m² (overweight) and 30-40kg/m² (obesity). Similar to Figures A.1 and A.2, Figures A.4A and B indicate the observational and MR linear and nonlinear GAMs with the comparison in model fit indicated by the p-values. Here, the nonlinear GAM is a better fit than the linear model ($p=5.4 \times 10^{-43}$), but this was not the case for the MR analysis ($p=0.07$). Figure A.4C indicates how as the BMI category increases, the magnitude of the estimate became smaller, where the estimate was halved when comparing the obesity group (Estimate =) with the healthy BMI group (Estimate =). The estimates also became smaller in magnitude in the MR (Figure A.4D)

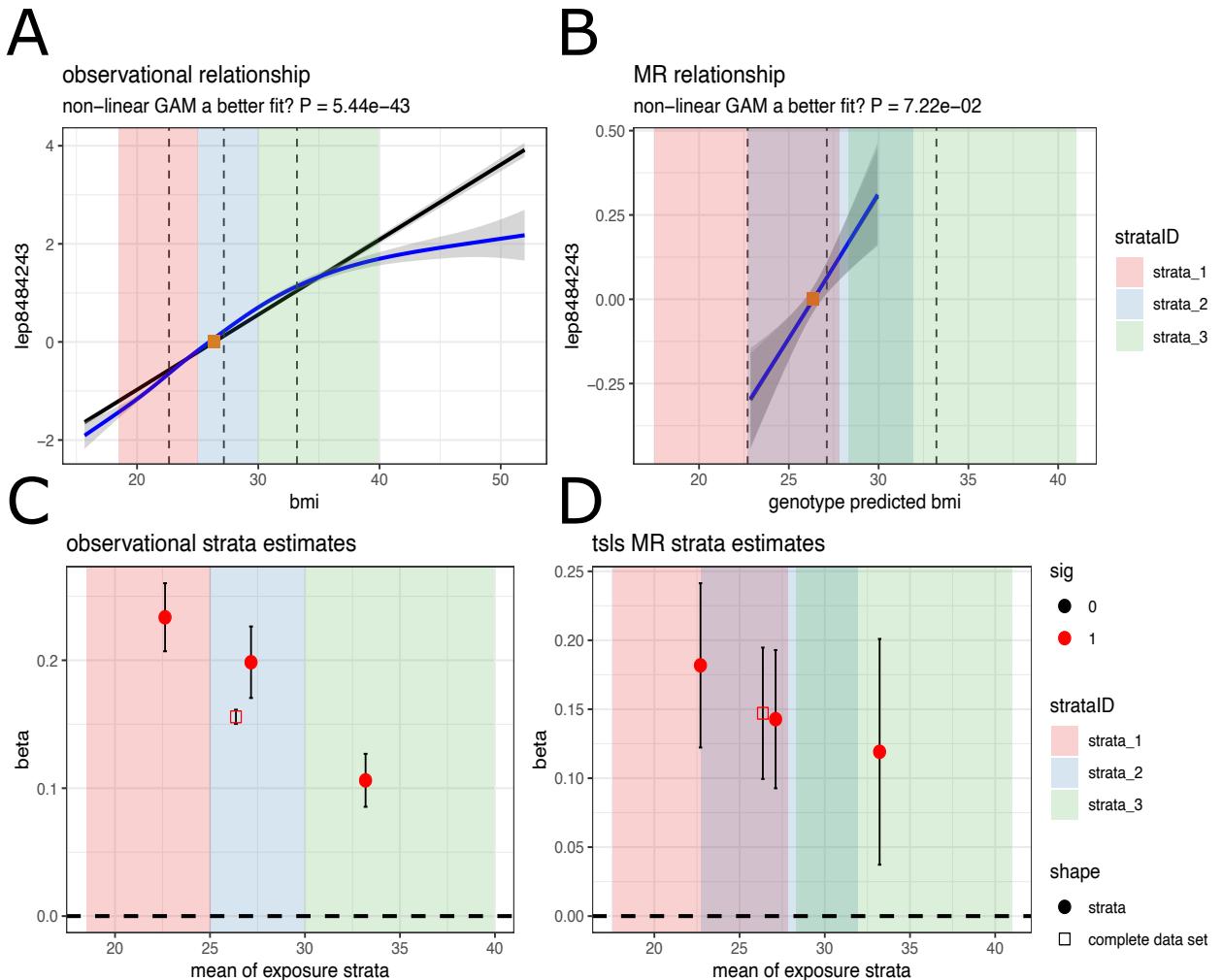


Figure A.4: Observational and Mendelian randomization estimates for the effect of BMI (kg/m^2) on leptin (SDs) stratified by BMI category. Shaded areas display the ranges of BMI of each stratum: 18.5-25 (red), 25-30 (blue), 30-40 (green) A) Comparison between the fit of the nonlinear and linear generalized additive models (GAMs) with age and sex as covariates. B) Comparison between the fit of the nonlinear and linear generalized additive models (GAMs) with age and sex as covariates for the two stage least squares Mendelian randomization (MR) analysis. C) Stratified estimates for the observational association between BMI and leptin (beta is SD change in leptin per 1 kg/m^2 increase in BMI). D) Stratified estimates for the MR estimates for the effect of BMI on leptin (beta is SD change in leptin per 1 kg/m^2 increase in BMI). Black indicates the estimate has a p value of >0.05 and red indicates $p<0.05$. Strata estimates are presented as circles with the estimate for the complete dataset represented by a square.

A.5 Conclusion

This appendix provided an example of how nonlinearity between BMI and protein outcomes can be explored using observational and MR methods. The development of the “glsmr” R package will help to

systematically characterise the nature of associations between BMI and levels of all circulating proteins in studies such as INTERVAL and DiRECT.

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