



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 in the Faculty of Life Sciences

Physiology, Pharmacology and Neuroscience / Population Health Sciences, March
2022, Word Count:

Abstract

This is copied from my TF application and will be edited.

Variation in adiposity is associated with thrombotic outcomes, but mechanisms leading from this exposure to disease are unclear. The overall aims were to assess the effect of adiposity on platelet function (key cells involved in thrombosis) and identify potential mechanisms.

Data from INTERVAL (a UK study of blood donors), COPTIC (Bristol-based cardiac surgery cohort) and DiRECT (Diabetes REmission Clinical Trial in Glasgow/Newcastle) were used. The effect of body mass index (BMI) on platelet traits measured by Sysmex XN-1000 was assessed in INTERVAL (N=33,388) using Mendelian randomization (MR). The association between platelet traits and platelet function was explored using linear regression in COPTIC (N=655). Bariatric patient (BMI >40kg/m²) platelet function was also compared with controls (BMI 18.5-25kg/m²). MR evidence in INTERVAL suggested that higher BMI raises immature platelet count (IPC - newly produced platelets). Results from COPTIC indicated that greater IPC is associated with greater platelet aggregation. Validation work in a specifically designed study of bariatric patients and healthy weight controls is ongoing.

To identify the involvement of the circulating proteome in adiposity-related platelet changes, the causal effect of BMI on >3600 proteins measured by SomaLogic was estimated using MR (N=2,737). To complement this, the effect of weight loss on the serum proteome was examined using linear regression in the DiRECT cohort (N=265). Adiposity had a substantial effect on the plasma proteome: higher BMI raised levels of leptin and fumarylacetoacetate while reducing sex hormone binding-globulin levels.

These analyses suggest that higher BMI raises immature platelet count (newly produced platelets), which are pro-thrombotic. The proteomic analyses from different study designs point towards potential proteins which may influence platelet production and function which require further investigation. Future analyses would include developing approaches to compare protein profiles across cohorts. Overall, these analyses provide novel mechanistic insight into how higher BMI causes disease.

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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Preface

Chapter 1

Introduction

1.1 Format of the introduction

This section will provide some information about the PhD such as the approaches that have been integrated throughout the thesis. Details of publications from the PhD will be provided, along with details of presentations and various meetings and conferences as well as details of awards and prizes. The following chapter (Chapter 2) will discuss the current literature surrounding the work in the thesis and introduce the hypotheses and aims of the thesis.

1.2 Collaborations from the PhD

This PhD project is part of the 4 year British Heart Foundation Integrative Cardiovascular Science Programme. 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 doctors on the COVID-19 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 on 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 All publications from the duration of the PhD

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. (2021) Platelets. doi:.

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>

Sledz, K. M., Moore, S. F., Vijayaragavan, V., Mallah, S., **Goudswaard, L. J.**, Williams, C.

M., Hunter, R. W. & Hers, I. Redundant role of ASK1-mediated p38MAPK activation in human platelet function. (2020) Cellular Signalling. 68, 11 p., 109528. [doi:10.1016/j.cellsig.2020.109528](https://doi.org/10.1016/j.cellsig.2020.109528)

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 from the PhD

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.

Chapter 2

Background

This chapter will give a more extensive introduction to the themes covered within the results chapters. It will set out where the current gaps are in the literature and provide an overview of the aims and hypotheses of the thesis.

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 Keys.¹ 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 countries,⁴ and prevalence of overweight is increasing in lower-income countries.⁵ It is currently estimated that 40% of adults in the United States and 26% of adults in the United Kingdom have obesity.⁶ As of 2016, the number of indi-

viduals globally with obesity surpassed 650 million.⁷

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 countries.⁸ In 2019, global deaths from coronary heart disease (CHD) and stroke amounted to ~9 million and ~6.5 million deaths, respectively.⁸ Current known risk factors for such diseases include smoking, physical inactivity, poor diet and high cholesterol.⁹ 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 States.⁹ 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 disease.¹⁰ Another study found that a 4gk/m² 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 causation. 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).¹² This technique is widely implemented due to the availability of genetic data in large cohorts, such as in ~500,000 UK Biobank participants.¹³ An example of how MR and RCTs can be used to determine causality is shown in Figure 2.1, using low density lipoproteins-cholesterol (LDL-C) and cardiovascular events as an example.¹⁴ Here, single nucleotide polymorphisms (SNPs) that are associated with levels of LDL-C can be used. These SNPs should also not be associated with other confounding factors such as smoking. If people with SNPs which lower LDL-C also have lower rates of cardiovascular events, it is likely that the relationship between LDL-C and cardiovascular events is causal. Although a useful technique

in exploring causality, MR has its own biases and therefore other lines of evidence and study designs such as RCTs are required.

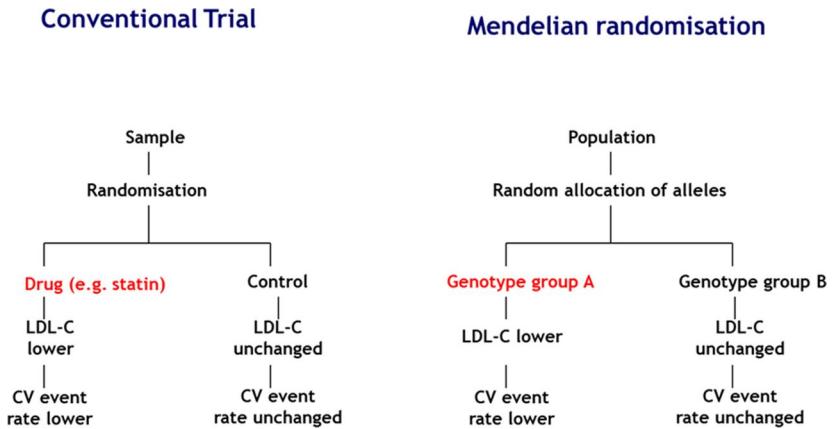


Figure 2.1: Example of how Mendelian randomisation and a randomised control trial can be used to determine whether lowering levels of low density lipoproteins can decrease risk of cardiovascular events. Figure from Bennett et al.¹⁴

2.4 The genetics of obesity

Mendelian randomization can be used to estimate the causal relationship between a modifiable exposure, such as BMI, and outcomes. There are strong correlations between BMI and more direct measures of fat mass measured by dual-energy X-ray absorptiometry (DXA),¹⁵ 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) gene¹⁶ lead to mice having obesity. The first genome-wide association study (GWAS) for obesity traits was performed in 2007 for around 5,000 individuals,¹⁷ which found evidence that 10 SNPs were associated with BMI. Since then, GWAS have been performed in increasingly larger cohort sizes,¹⁸ with the largest and most recent GWAS performed in 2018 by Yengo et al.¹⁹ 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 associations.¹⁹

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 or ischaemic heart disease) is caused by a build up of plaques in the arteries (atherosclerosis). These build ups can lead to blockages in the arteries (arterial thrombosis), thereby causing a heart attack. Other types of cardiovascular disease include stroke, which can be either ischaemic (another form of arterial thrombosis) or haemorrhagic. 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 it is in the lungs. Arterial and venous thrombi both are composed of platelets, however arterial thrombosis has a much higher proportion (31%).²⁰ Although not one of the main components of venous thrombi, platelets are still involved in the pathology.²¹

2.6 Higher BMI has a causal effect on ischaemic heart disease

Studies which have used MR and therefore estimate the causal effect have indicated that BMI may have an even greater effect on IHD. Nordestgaard et al. utilised single nucleotide polymorphisms (SNPs) which are associated with BMI to estimate that 4kg/m² 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 risk.²² Further, higher BMI has been shown to have a causal relationship with more specific measures of cardiovascular events such as ischaemic stroke.²³ 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).²⁴ 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 thrombosis?

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),^{25,26} 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 CHD,²⁷ this does not account for all the risk. Therefore, it is likely that there are other factors mediating this association. There are thousands of detectable proteins which circulate in the blood [reference], 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 haemostasis.²⁸ Under normal conditions, the endothelium releases nitric oxide (NO) and prostaglandin I2 (PGI2) which suppress platelet activation.²⁹ 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 change.³⁰ Alpha granules release growth factors (e.g. IGF-1), clotting factors and chemokines,³¹ whereas dense granules release molecules such as 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 activation.³² Binding of agonists such as

thrombin (or the synthetic peptide agonist PAR1-AP/TRAP-6) to the protease-activated receptor 1 (PAR-1AP) (??) results in activation of the 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).³³ The small GTPase, Rap1 is then activated which recruits proteins such as talin and kindlin.³⁴ Subsequent fibrinogen binding results in platelet aggregation, thrombus formation and cessation of bleeding.²⁸ These key platelet receptors are shown in figure ???. Despite these processes being essential for haemostasis, when platelets become hyperactive, the balance is tipped in favour of thrombosis. This can cause a myocardial infarction and stroke.

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),^{35,36} which has shown to reduce occurrence of future myocardial infarction.³⁷ DAPT generally consists of aspirin and a P2Y₁₂ antagonist such as clopidogrel or ticagrelor.

As BMI is associated with increased rates of thrombotic events and platelets are one of the key cells involved in thrombosis, it is likely that platelets are hyperactive compared to people who have a BMI in the healthy range. 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 ADP.³⁶ Studies looking at BMI and platelet function are often limited by small sample sizes and are often restricted to patients being on antiplatelet therapy.³⁸⁻⁴⁰ 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 therefore help address causality. 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 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 commonly used include using fluorescently labelled antibodies to detect surface receptors. Measuring basal levels of receptors may give information about the function of the platelet. Some receptors increase their expression in response to platelet agonists (such as the integrin $\alpha_{IIb}\beta_3$ and the adhesion receptor P-selectin) such 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, this causes intracellular calcium release.⁴¹ Another marker of platelet activation is phosphatidylserine (PS) exposure. Under basal conditions, PS is mainly present in the inner leaflet of the phospholipid bilayer. Upon activation (e.g. dual stimulation by collagen and thrombin), the PS becomes exposed on the outer surface of the membrane, thereby activating factors in the coagulation pathway.⁴² Changes in PS exposure can be measured using a fluorescently labelled antibody. Techniques such as Western Blotting can be used to quantify the levels of total protein of phosphoprotein. This can therefore be used to explore changes in signalling in platelets under basal conditions, or after stimulation with an agonist. These methods are very well established, however they are often time consuming and expensive.

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) and platelet distribution width (PDW). 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 stroke,⁴³ 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.⁴⁴ Previous studies have explored the association between BMI and platelet paramters. Some studies have foudn evidence for an association between BMI and both platelet count and mean platelet volume, however other studies have lacked evidence for an effect,^{45–48} suggesting that these effects are null. 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 cyotemtry. 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 2.2.⁴⁹ Immature platelets, or reticulated platelets, are the youngest platelets in circulation. They persist for between 24 and 36 hours.⁴⁹ 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).⁵⁰ This suggests that immature platelets may be more reactive.

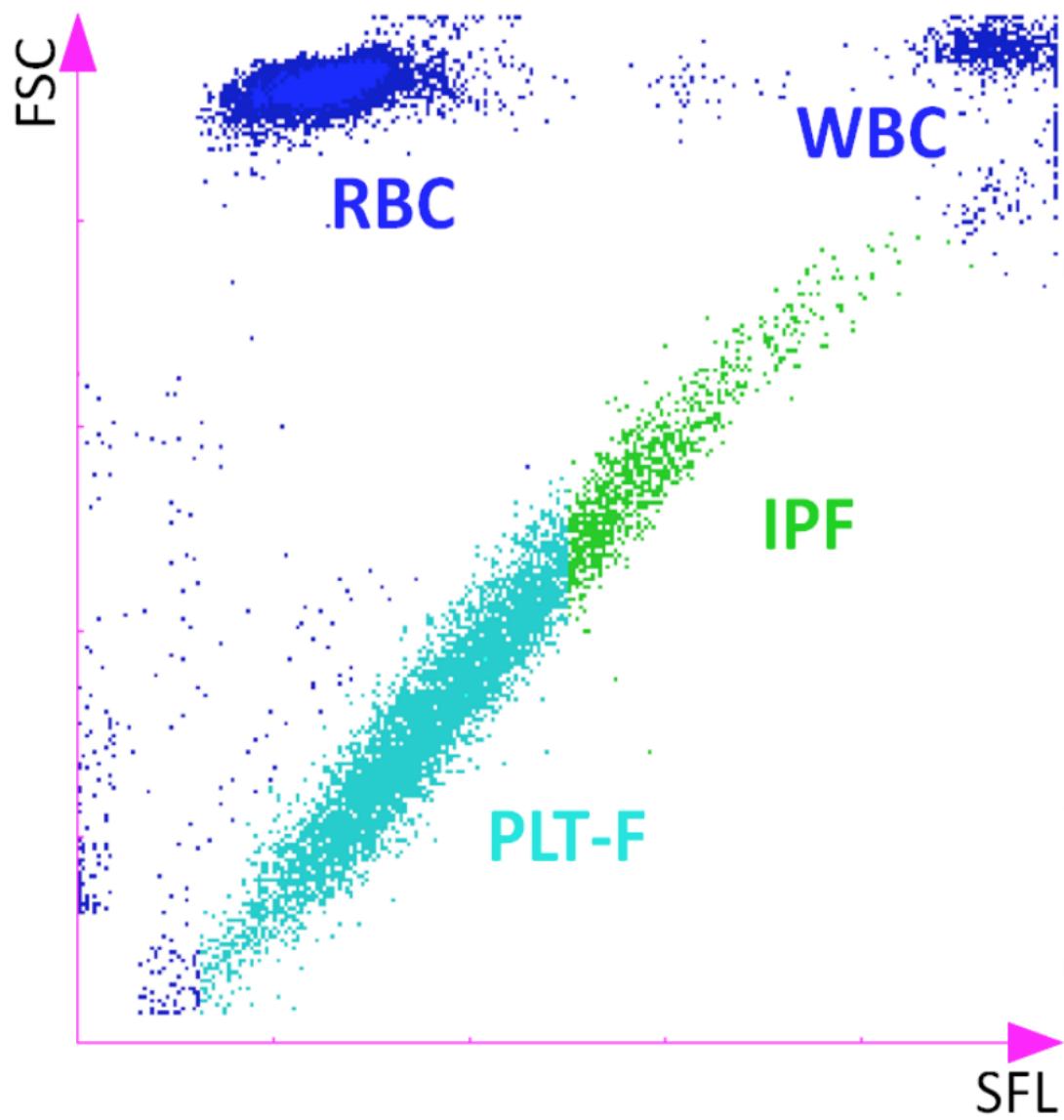


Figure 2.2: **Scatter plot indicating how platelets and immature platelets are detected using a Sysmex haematology analyser.** The forward scatter (FSC, a measure of cell size) and side fluorescence (SFL, a measure of cell mRNA content) are used to detect cell types. Immature platelets have a larger 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.⁴⁹

2.12 Platelet primers

Platelets express receptors for various circulating proteins. Previous studies have reported that a range of soluble ligands, growth factors and cytokines which can enhance platelet function are elevated in obesity. These ‘platelet primers’ include insulin-like growth factor-1 (IGF-1)⁵¹ and thrombopoietin (TPO).^{52,53} There is evidence that other chemokines may be able to activate platelets. The CCR4 receptor is activated by the chemokines macrophage derived chemokine (MDC/CCL22) and thymus and activation-regulated chemokine (TARC/CCL17).^{54,55} There is observational evidence that higher BMI increases the circulating levels of MDC.⁵⁶ These chemokines are important for chemotaxis of leukocytes. It has also been observed that these chemokines can activate platelets, inducing aggregation and calcium mobilisation.⁵⁵ 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.^{53,57} In turn, this leads to phosphorylation and activation of protein kinase B (PKB/Akt) and an increase in aggregation.⁵⁸ Another pathway which is important for platelet priming is the mitogen activated protein kinase (MAPK) pathway, in particular, the extracellular related kinases (ERKs).⁵⁹ 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. Platelets also have a role in the immune response and can be activated by various inflammatory mediators (ref). 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.^{60,61} There is evidence that obesity is associated with increases in interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α).⁶² Previous studies have identified a causal link between higher BMI and higher circulating levels of the inflammatory molecule C-reactive protein (CRP).⁶³ More recent studies have used MR and have found an effect of BMI

on a range of cytokines.⁶⁴ As platelets can be activated 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).⁶⁵ 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 disease.⁶⁶ The chemokines MDC and TARC both have SNPs which are associated with their levels. MDC and TARC have been implicated in platelet hyperactivity, however it is important to explore whether raised levels of MDC and TARC would have clinical implications such as a role in ischaemic heart disease. As a polymorphism in the CCR4 receptor has been implicated in myocardial infarction,⁶⁷ 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 TARC.⁵⁶ Based on these observations, there may be many more circulating proteins which may also 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 levels.⁶³ This is due to the often limited range of protein detection 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 SomaLogic,⁶⁸ which can detect around 4000 unique proteins. Studies that have attempted to characterise the plasma proteome of higher BMI have often only been in an observational framework.⁶⁹ 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 blood,⁷⁰ including enzymes, protein kinases 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.

2.16 Associations can be explored using blood

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. This is in contrast to other studies, where other tissues are being sampled that are not as easy to get hold of due to issues with invasiveness and ethics.

2.17 Importance of interdisciplinary methods

Each experimental approach used when trying to answer hypotheses has biases and limitations. For example, using basic science it is possible to determine effects *in vitro* or *ex vivo*, however it is 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 expensive.¹⁴ 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. 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.

2.18 Cohorts utilised within the thesis

This thesis approaches the hypotheses with a combination of approaches mentioned in the previous paragraph. These include the INTERVAL study, DiRECT, lab work, patient study. The studies will be introduced more thoroughly in the respective results chapter.

2.19 Thesis aims

The aims of the thesis are indicated below, with a schematic of the multi-step pathway which this thesis aims to explore (Figure 2.3). The 3 overall aims 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.

2.20 Thesis hypotheses

- 1) Higher BMI leads to platelet hyperactivity
- 2) Proteins such as the chemokines in MDC and TARC which circulate in the blood contribute to platelet hyperactivity
- 3)

Overview of thesis hypotheses

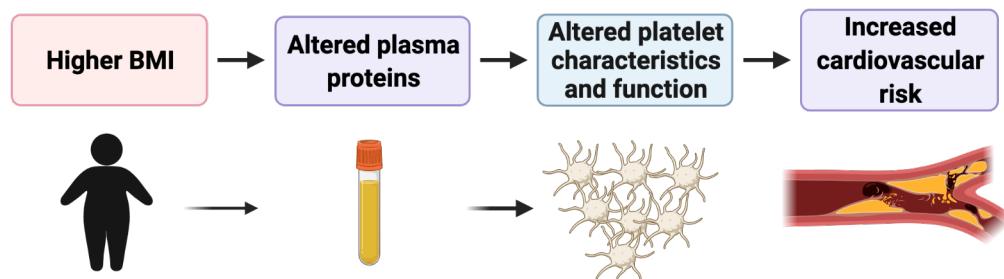


Figure 2.3: Schematic of the multi-step pathway addressed in the thesis

2.21 Thesis structure

There will be 5 results chapters in this thesis:

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

Chapter 3

Common Resources and Methods

This chapter will provide an overview of methods which are utilised in more than one results chapter. Each chapter will refer back to these methods where relevant and include further methodological details specific to the chapter.

3.1 Supplementary material

This paper 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. GitHub has also been used to store supplementary results. 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.

3.2 Platelet function experiments

3.2.1 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.

3.2.2 Patient study experiments

Details of recruitment and ethical approval are provided in Chapter 5.

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.

Platelet lysates

Acid citrate dextrose (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.

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).

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 to a final volume of 50 uL. Agonists used were protease-activated receptor 1 activating protein (PAR1-AP), adenosine diphosphate (ADP) and collagen related peptide (CRP), which were freeze-dried onto the plates.⁷¹ 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.

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.

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+.

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 Ultimate 3000 nano-LC system in line with an Orbitrap Fusion Tribrid Mass Spectrometer (ThermoFisher Scientific).

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 \log_2 transformed, where the logFC was calculated by subtracting the \log_2 normalised protein abundance of the control subjects from the bariatric surgery group. The \log_2 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, however P values of all comparisons are provided in the supplementary results in Chapter 5. False discovery rate (FDR) adjusted p-values are also provided in the full results . A positive logFC indicates an increase in protein level in the bariatric samples.

Ingenuity pathway analysis enrichment

Enrichment analyses were performed using the Ingenuity Pathway Analysis (IPA) bioinformatics tool (QIAGEN Inc., <https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis>) to determine whether proteins altered in bariatric patients with obesity showed enrichment in canonical pathways or diseases compared to the full protein list. IPA utilises the directionality of the change in protein, allowing interpretation of the likely acti-

vation or inhibition of the associated pathways. This is expressed as a Z-score. Proteins LogFCs which match the expected pattern of LogFCs contribute to a positive Z-score, and those which disagree contribute to a negative z-score.

3.2.3 Platelet priming experiments

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 μ M 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 μ M 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.

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

Washed platelets were diluted to a concentration of 2×10^7 /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.

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.

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.

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.

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.

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) (PVDF) 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.

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 x 10⁸/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.

3.2.4 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 plot-

ted 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'.⁷²

Chapter 4

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

This work is published in the journal Platelets (add reference). I performed all analyses, created all figures and tables and wrote the manuscript.

4.1 Background

Obesity (body mass index $\geq 30 \text{ kg/m}^2$) has nearly tripled worldwide in the last 45 years.⁶ This is a major health concern as a higher body mass index (BMI) is an important risk factor for various noncommunicable disorders, including cardiovascular disease.⁷³ Clinical studies and genetic association studies have identified BMI as an independent risk factor for thrombotic disorders, including coronary heart disease and stroke.^{11,22,74}

Platelets are blood cells which are essential for the cessation of bleeding upon injury to a blood

vessel and are involved in thrombosis and progression of cardiovascular disease.²¹ When pathologically activated, platelets can aggregate to form thrombi thereby occluding arteries and triggering a myocardial infarction or stroke.²¹ Platelet hyperactivity can be an indicator of those who may be at an increased risk of thrombosis.⁷⁵ There is evidence that people with a higher BMI have hyperactive platelets,^{36,76} which could explain why a higher BMI is linked to an increase in thrombosis. Mechanisms of platelet hyperactivity are unclear, but it is possible that changes in platelet numbers, size and immaturity may play a role. Furthermore, an alteration in circulating metabolites or proteins induced by obesity could modulate the activation of platelets.⁷⁷

Platelet function can be directly assessed by various methods; these methods commonly include platelet aggregation experiments and/or using antibodies to detect platelet receptors that are expressed upon activation. One of the limitations of measuring platelet function directly is that these techniques are not widely available or readily standardized. Haematology analyzers are more commonly used to provide full blood counts, providing detailed readouts of platelet characteristics. Some of these characteristics have been reported to be indirect measures of platelet function.⁴⁴ For example, an increase in mean platelet volume (MPV) has been reported to be predictive of vascular mortality and ischaemic heart disease.⁷⁸ Another commonly measured platelet characteristic is the number of platelets in circulation (platelet count, PLT). An increase in platelet count has been reported to be associated with platelet hyperactivity⁴⁴ and there is evidence that higher platelet count is associated with ischaemic stroke.⁴³

Sysmex haematology analyzers are able to provide additional platelet characteristics, such as immature platelet fraction (IPF) and immature platelet count (IPC).⁷⁹ 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).⁵⁰ Higher levels of immature platelets are indicative of enhanced platelet production⁸⁰ and these platelets are believed to be more pro-thrombotic than older circulating platelets, with more dense granule release and increased P-selectin expression.^{50,80,81} Higher immature platelet count is associated with adverse cardiovascular outcomes in patients with coronary artery disease⁵⁰ and reduced effectiveness of antiplatelet therapies,^{81,82} 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 patients.⁸¹

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, plateletrit (PCT) and MPV. There is conflicting observational evidence regarding an association between BMI and PLT,^{45,46} with some studies suggesting a positive association between BMI and MPV⁴⁷ and other studies reporting no such association.⁴⁸ Furthermore, as there is evidence that immature platelet production is increased in patients with metabolic syndrome and type II diabetes,^{83,84} 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 study, we used data from the INTERVAL prospective cohort (N=33388) to explore the association between BMI and platelet traits. Figure 4.1 shows the associations of the overall thesis hypotheses that are addressed in the chapter. We combined observational and Mendelian randomization (MR) approaches 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 4.2). 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.

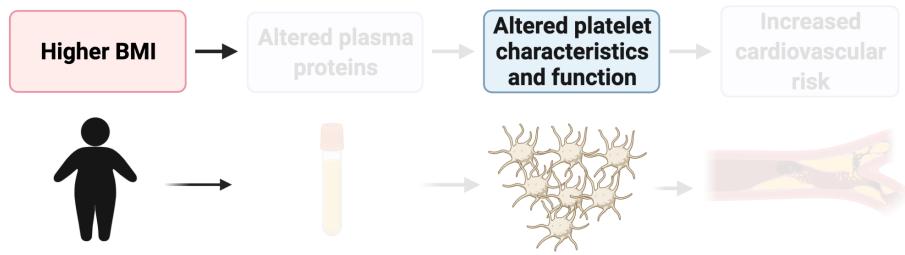
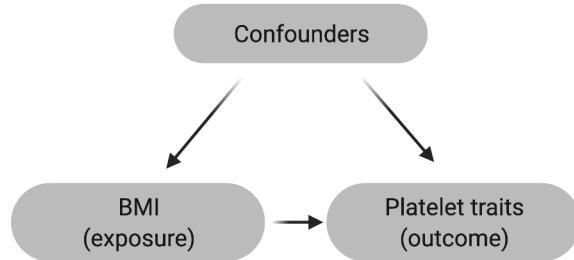


Figure 4.1: Schematic of the associations explored in the current chapter: the relationship between body mass index and platelet traits. Figure made using BioRender.com.

Linear regression



Mendelian randomization (two stage least squares)

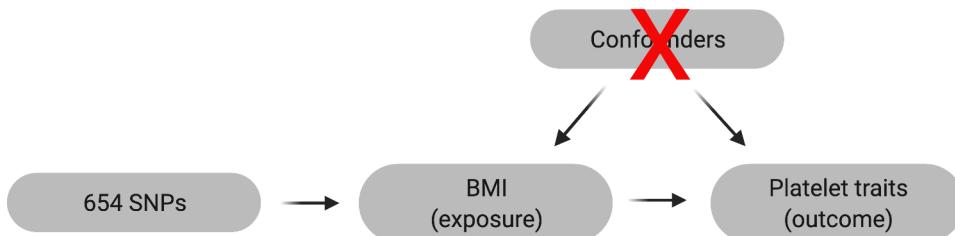


Figure 4.2: **Schematic of linear regression and Mendelian randomization analyses.** **Linear regression assesses the association between BMI (exposure) and platelet traits (outcome), with adjustment for potential confounders.** Under the assumptions of Mendelian randomization (MR), the genetic risk 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.

4.2 Methods

4.3 The INTERVAL study

4.3.1 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 participants.⁸⁵ 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.

4.3.2 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 health.²⁵

4.3.3 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..⁸⁶ The imputation panel used was the 1000 genomes phase-3-UK-10K.⁸⁶ 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 ancestry.¹⁹ This meta-analysis consisted of around 250000 adults from the Genetic Investigation of ANthromopetric Traits (GIANT) consortium¹⁸ 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 software⁸⁷ 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.

4.3.4 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 4.3). Participants were mostly of European descent. More details of this study can be found in Chapter 3.

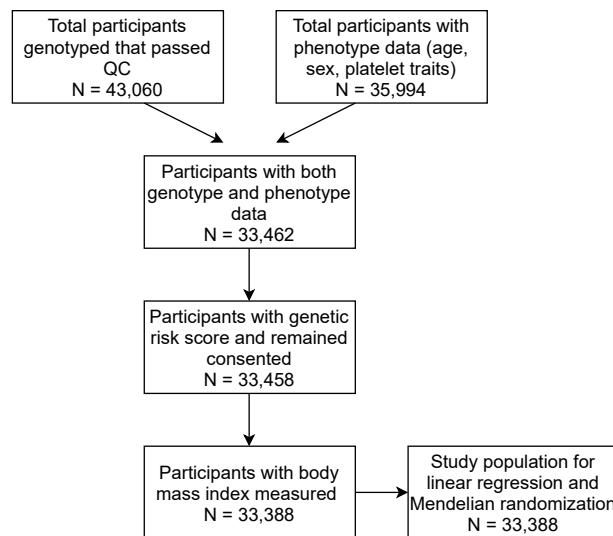


Figure 4.3: A STROBE diagram outlining participants included in the INTERVAL study for BMI and platelet trait analyses.

4.3.5 Measurement of platelet traits

Blood was taken by venipuncture into 3mL EDTA tubes. Platelet parameters were measured using the Sysmex XN-1000 instrument.⁸⁶ 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 4.1. These measurements were pre-adjusted for technical covariates such as time between venipuncture and blood count, as well as instrument drift, seasonal and weekly variation.⁸⁶ Adjustment was performed with cyclic, thin-plate and P-splines in a generalized additive model (GAM).⁸⁸ Resulting platelet trait values along with their units of measurement are reported in Table 4.1. 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 4.1: 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	109/L	255 (57)
PLT (I)	Platelet count (impedance channel)	Total number of platelets per litre	109/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	109/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)

4.3.6 Statistical analysis

Analyses were performed using R version 3.4.2.⁸⁹ 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/m^2). Only participants with all covariates were included in the linear regression models. The associations between potential covariates 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 covariates were explored. The MR analysis was performed by using a two-stage least squares (2SLS) regression model (using `systemfit()` function from “systemfit” package).⁹⁰ 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.

4.3.7 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 participants.⁹¹ This study was approved by the UK NHS Research Ethics Committee (09/H0104/53).

4.3.8 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 $\alpha 2$ 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.

4.3.9 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.

4.4 Results

4.4.1 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 4.2). 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.

4.4.2 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 4.4), 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.

Table 4.2: 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%	

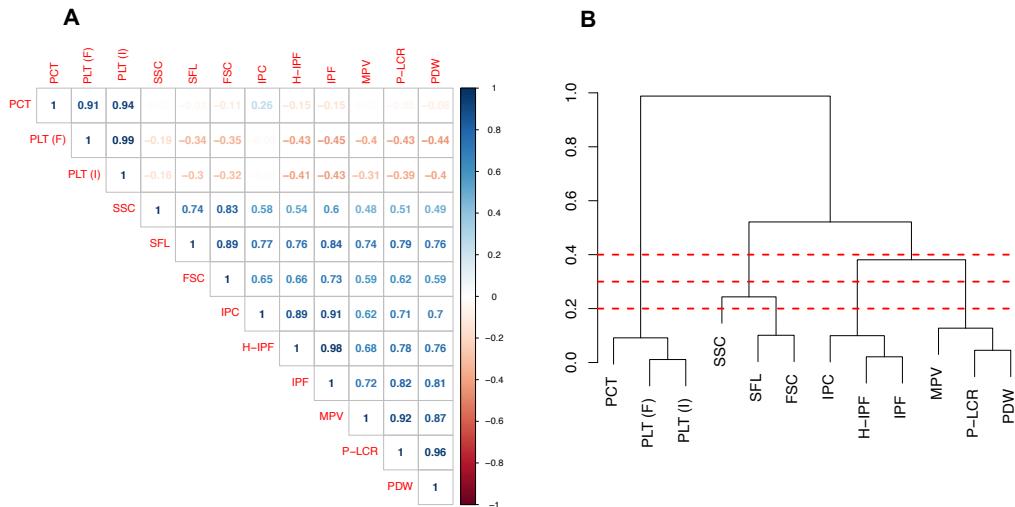


Figure 4.4: Correlation matrix and dendrogram of the relationship between platelet traits. A) Correlation coefficients are provided within the matrix. Dark blue indicates a correlation coefficient (r) of 1, with dark red indicating a correlation coefficient of -1. B) A dendrogram showing the hierarchical relationship between platelet traits, where the height at which two platelet traits join is $(1 - \text{Pearson correlation coefficient } r)$. PCT = plateletrcrit, 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.

4.4.3 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 4.3), 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 4.4).

Table 4.3: 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	Eta squared	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 4.4: Age and sex adjusted estimates for the association between BMI and platelet traits. 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	βeta 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

4.4.4 Associations of covariates with BMI and platelet traits in INTERVAL

The association of covariates (age, sex, smoking and alcohol) with BMI were evaluated (Table 4.5). Included covariates 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 covariates (age, sex, smoking, alcohol) and platelet traits were also explored (Figure @ref(fig(confounder-platelets))). 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 plateletcrit and platelet count.

Table 4.5: 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=occasinal, 3=most days/ every day)	32867	0.073	0.009	1.53e-17	0.002	72	0.017	0.057	0.09

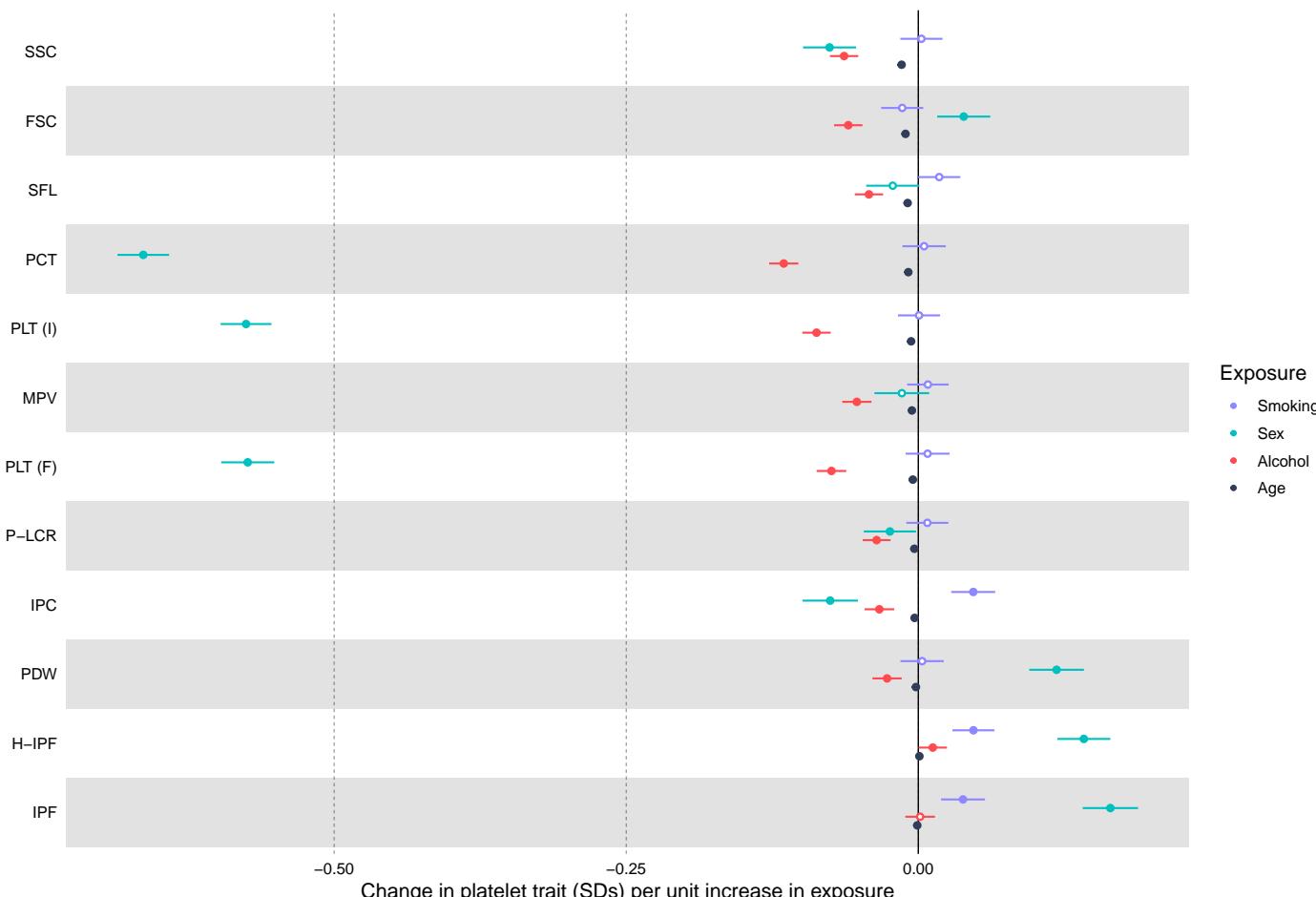


Figure 4.5: Linear regression results for the association between age, sex, smoking and alcohol with platelet traits. PCT = plateletcrit, PLT (F) = platelet count (PLT-F channel), PLT (I) = platelet count (impedance channel), SSC = side scatter, SFL = side fluorescence, FSC = side scatter, IPC = immature 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=

4.4.5 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 4.6). 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 4.6). 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 4.6: 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

4.4.6 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 4.7, 4.6). 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 4.6). 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 4.7).

Table 4.7: 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	beta coefficient	SE	P value	R2	Eta2	95% CI	Lower 95% CI	Upper 95% CI	Wu-Hausman P value	F-Hausman 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	0.0001580.058	-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

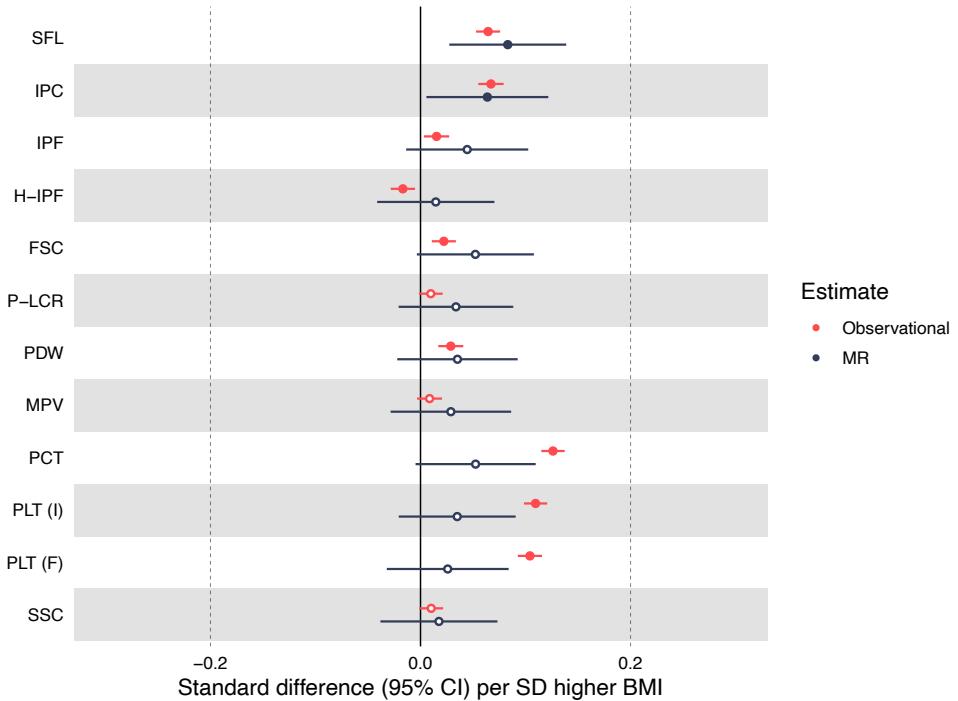


Figure 4.6: Forest plot of the Mendelian randomization (MR) and unadjusted observational estimates for the effect of BMI on platelet traits. Estimate points are filled where $P < 0.05$. SFL = side fluorescence, IPC = immature platelet count, H-IPF = high fluorescence immature platelet function, FSC = 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.

4.4.7 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 4.8).

Table 4.8: 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	R2	95% CI	Lower 95% CI	Upper 95% 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

4.4.8 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.

4.4.9 COPTIC participant characteristics

The total number of COPTIC participants was 2,541. Of these, 2,518 participants gave consent for future research (Table 4.9). 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 4.10).

Table 4.9: 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 10 ⁹ /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 10 ⁹ /L)	6.3 (3.4)	2383	6.2 (3.3)	629

Table 4.10: 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	β eta coefficient	SE	P value	R2	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

4.4.10 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 4.7A). 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=3.7x10-3), TRAP-6 (0.11 SD increase per SD increase in IPC, 95% 0.03 to 0.18, p=7.8x10-3) 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 4.7A, Figure 4.7B), 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 4.7A). 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 4.7B). 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 4.7C). 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.

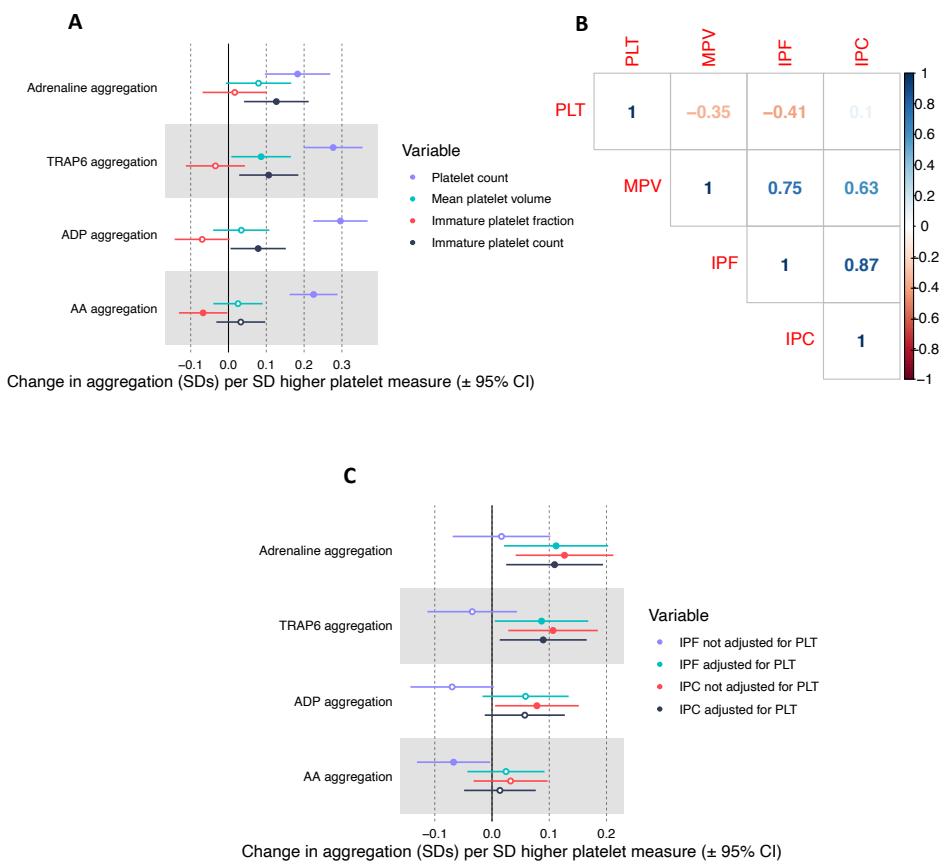


Figure 4.7: Association between platelet measures and whole blood platelet aggregation in COPTIC trial participants. A) Forest plot displaying the association between platelet measures (exposure) and aggregation (outcomes). B) Correlation matrix displaying the Pearson's correlation coefficient (r) between 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.

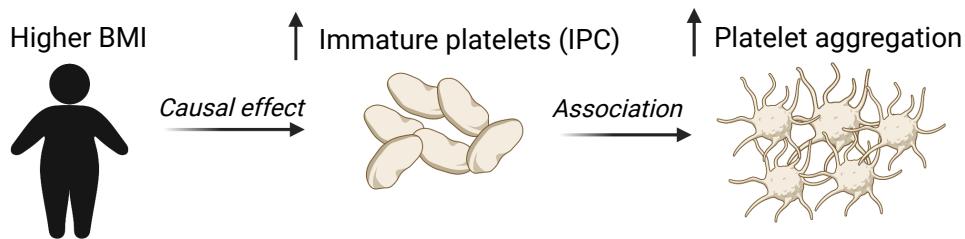


Figure 4.8: Graphical summary of findings

4.5 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.^{83,84} 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 production,⁸⁰ 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^{80,92} and may be predictive of cardiovascular events,^{35,50} 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 ADP.⁹² 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 inhibition.⁸¹ 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 thrombosis.⁹⁴ 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 PCT.⁴⁵ 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 PCT;⁴⁶ 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. Possi-

ble 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 MPV⁴⁷ and others showing no association.^{45,46,48} 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 %),^{50,81} 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 numbers.⁴⁶ There is evidence that inflammatory mediators such as interleukin-6 (IL-6) could be one such factor.⁹⁵ Further study would be warranted to explore these mechanisms, as well as replicate the current findings, such as through independent population or clinical studies.

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 associated.⁹⁶ 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 ranges.⁹⁷ 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 PLT.⁹⁸ 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 analyzers.⁹⁹

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 5

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. All data in this chapter was collected by myself, apart from the platelet-neutrophil assay data, which was performed by Dr Chris Williams and the Sysmex haematology analyzer data, which was collected by Dr Kate Burley. Help was required here due to time constraints when receiving patient samples. Proteomic data were analysed by Dr Marieangela Wilson and Dr Kate Heesom at the Proteomics facility at the University of Bristol. 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.

5.1 Background

As detailed in Chapter 4, there is evidence that people with a higher BMI have an increased risk of cardiovascular events such as coronary heart disease (CHD) or stroke.^{11,22,74} As platelets are one of the key cells involved in haemostasis and thrombosis,²¹ 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 function.¹⁰⁰ 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 5.1). 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 circulation,¹⁰¹ 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.

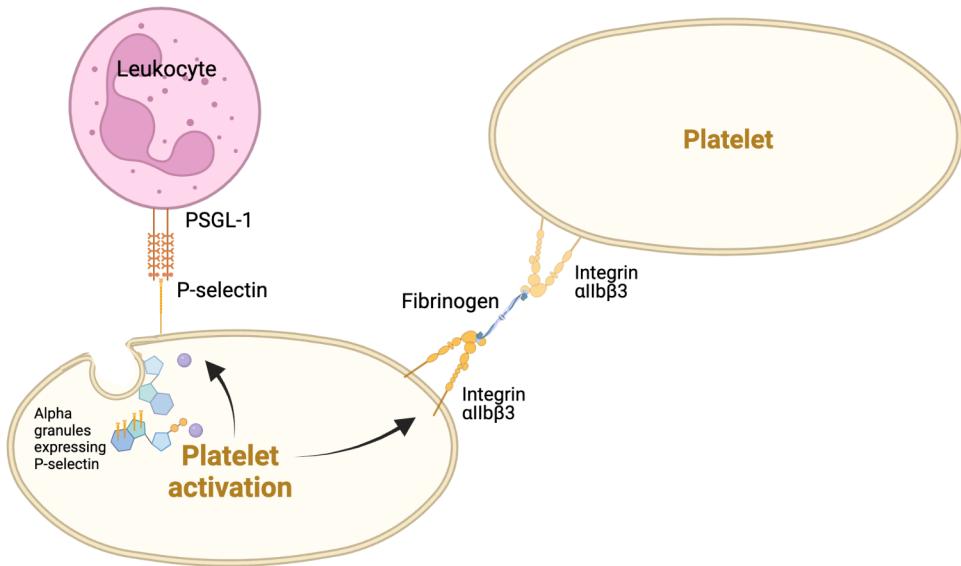


Figure 5.1: Cartoon of integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression on platelets. Upon platelet activation by a strong agonist such as CRP, the integrin $\alpha_{IIb}\beta_3$ exposes its binding site for fibrinogen and cross-links platelets. P-selectin is expressed in the membrane 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.^{36,38,39,76} 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 activity.³⁸ 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 ($BMI > 40 \text{ kg/m}^2$) and healthy controls ($BMI 18-25 \text{ kg/m}^2$).
- 2) Determine whether high BMI is associated with: an increase in immature platelet count, an increase in platelet function and an alteration in platelet protein expression by comparing platelets (Figure 5.2).

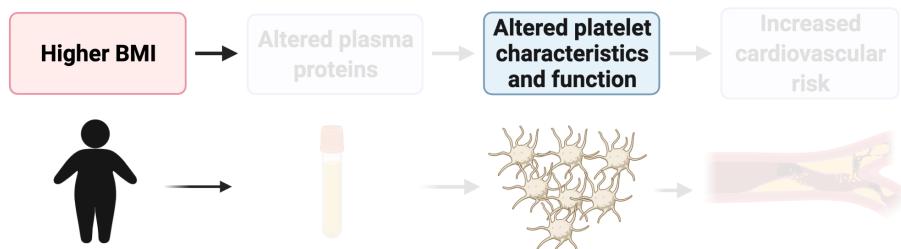


Figure 5.2: Schematic of the associations explored: this chapter explores the relationship between body mass index and platelet function. Figure made using BioRender.com.

5.2 Methods

The platelet function methods and statistical analyses utilised within this study are provided in the Methods chapter (Chapter 3).

5.3 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, participant information sheets, 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 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. The second amendment was not approved by Southmead Hospital in time and therefore the vaccination status could not be recorded for bariatric patients.

5.3.1 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 $25\text{ kg}/\text{m}^2$ 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.

Table 5.1: 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/m ²)	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

5.4 Results

5.4.1 Participant characteristics

The characteristics of participants included in the study are shown in Table 5.1. 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 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.

5.4.2 Platelet parameters

Platelet traits measured by Sysmex were compared across bariatric and control groups (Table 5.2). 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 5.2: 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

5.4.3 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 5.3A 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 5.3C).

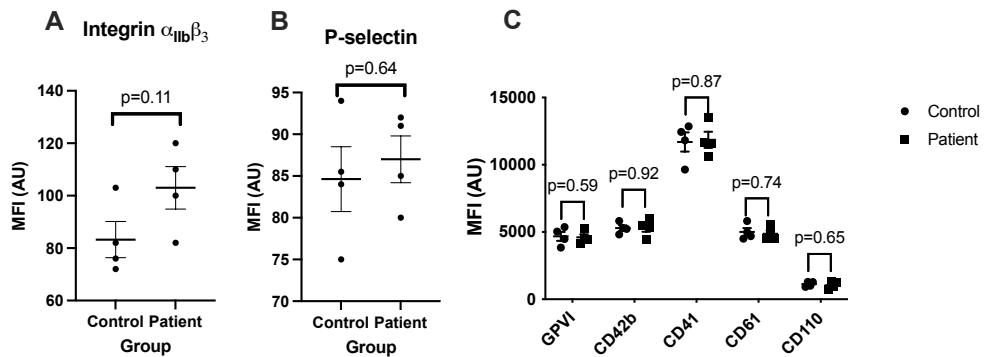


Figure 5.3: Comparison of basal integrin $\alpha_{IIb}\beta_3$ activation, P-selectin expression and surface receptors levels across control and bariatric patient groups.
 Diluted PRP was used for experiments, where platelets were incubated with antibodies for 10 minutes before fixing. Bar graphs comparing 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.

5.4.4 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 5.4A-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 5.4D 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 5.4F).

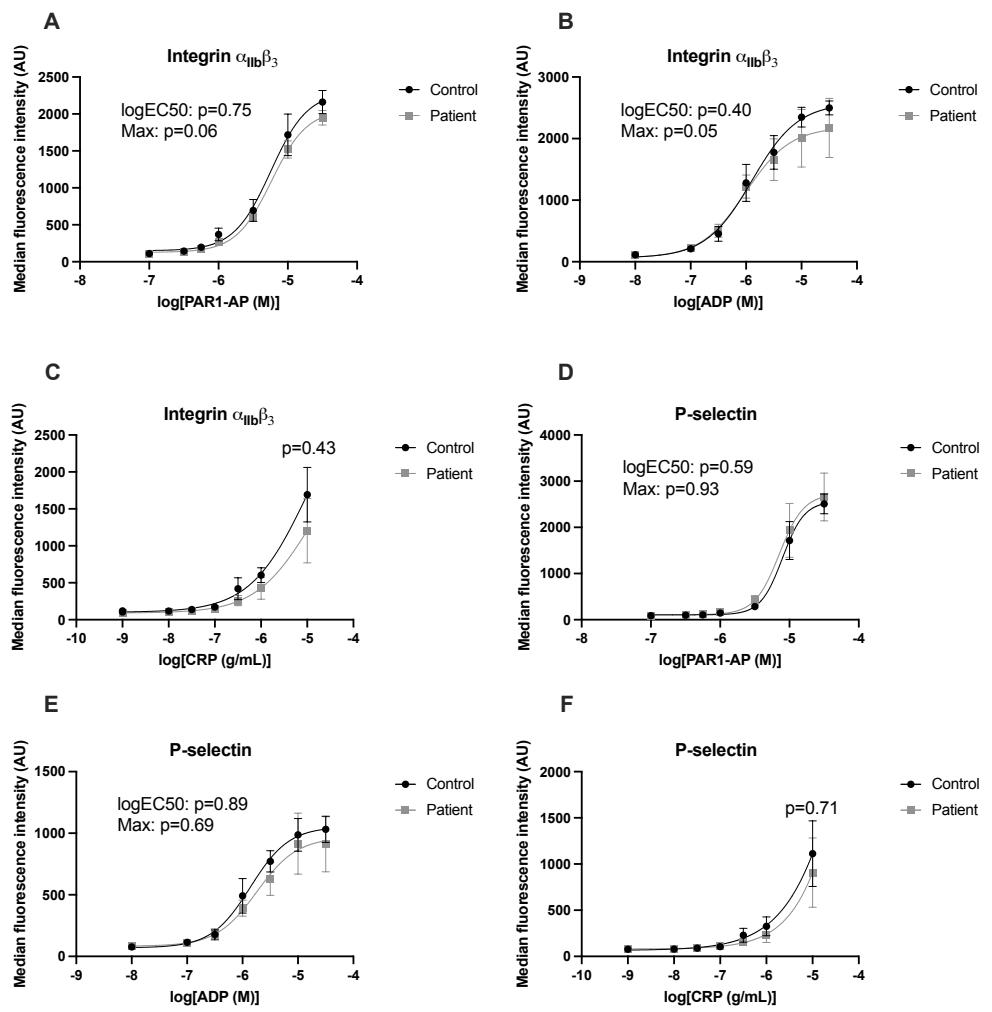


Figure 5.4: Concentration-response curves of integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression for bariatric patient and control groups in platelet rich plasma diluted in HEPES-Tyrode's. Integrin $\alpha_{IIb}\beta_3$ activation in response to A) PAR1-AP, B) ADP and C) CRP. P-selectin 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.

5.4.5 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 inflammation and 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).

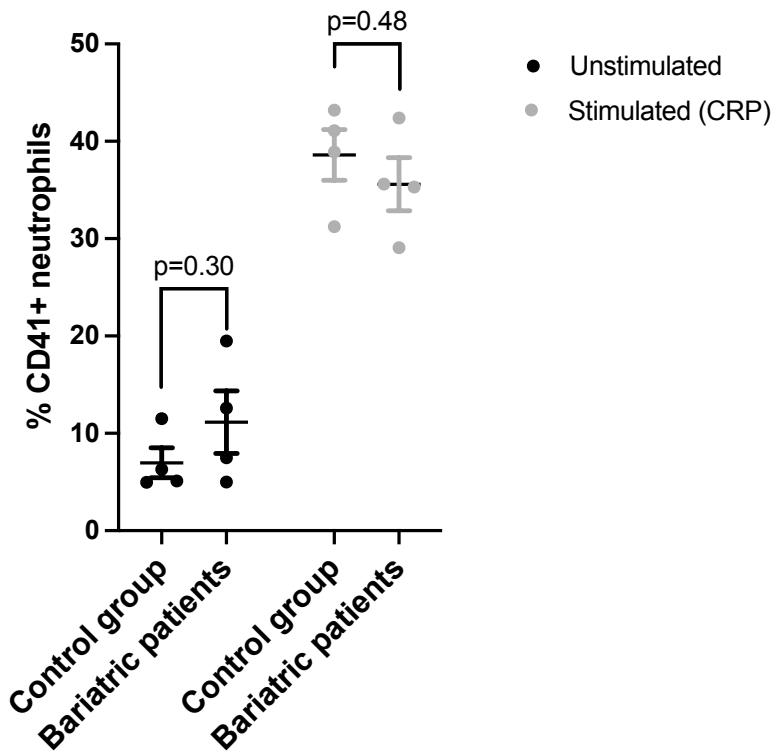


Figure 5.5: Comparison of neutrophil-platelet aggregates across bariatric and control groups. Neutrophil-platelet aggregates were determined using CD45 and CD41 antibodies. Aggregates were compared across bariatric patient and control participant groups in unstimulated and stimulated (5 ug/mL CRP) conditions. N=4. P-values provided for the results of a two-way ANOVA with Sidak's multiple comparisons tests. Mean + SEM displayed.

5.4.6 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 5.6. PC1 plotted against PC2 did not separate the bariatric patient samples from the control participant samples, despite grouping of three patients (Figure 5.6). 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 5.7). This suggests that the group was not the main source of variation between the data.

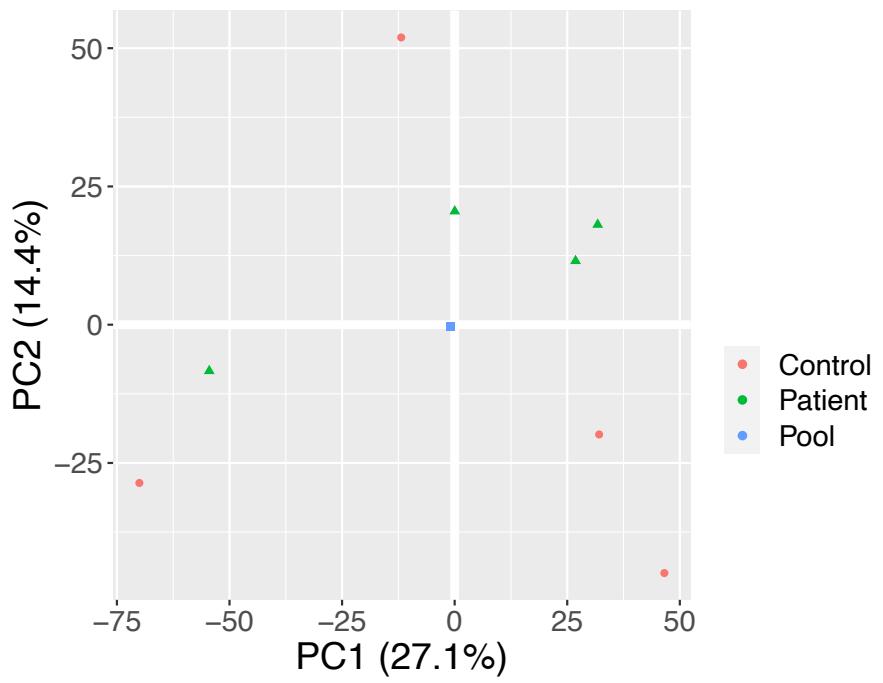


Figure 5.6: **Principal component analysis (PCA): scatter plot of PC1 and PC2.** Each point represents a participant. Control samples are coloured red and bariatric samples coloured blue, with the pooled reference sample of both control and bariatric samples coloured in blue.

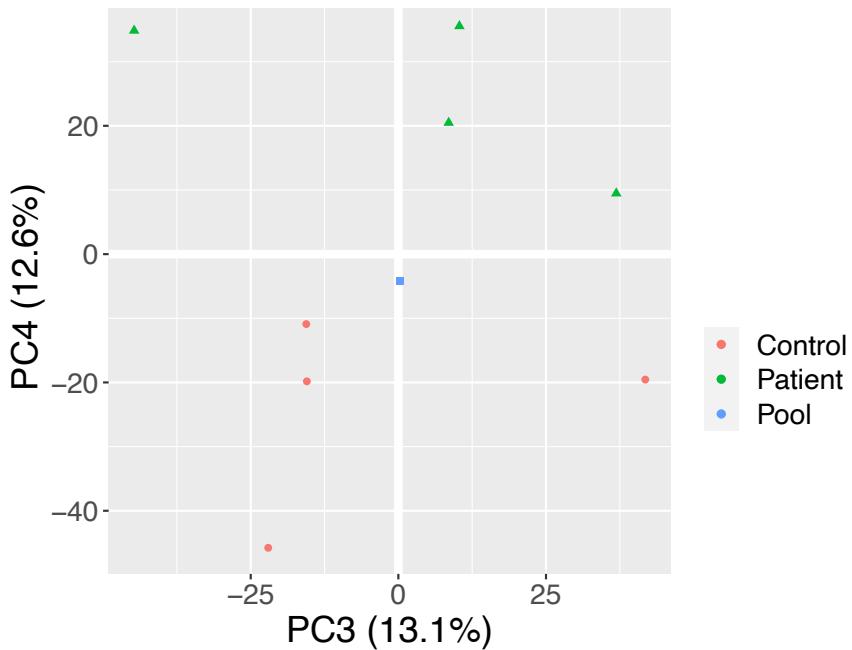


Figure 5.7: Principal component analysis (PCA): scatter plot of PC3 and PC4.
Each point represents a participant. Control samples are coloured pink and bariatric samples coloured blue, with the pooled reference sample of both control and bariatric samples coloured in blue.

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 5.8). 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) (logFC 0.45, $p=6.3\times 10^{-5}$). The signalling protein G protein-coupled receptor kinase (GRK6) was lower in bariatric patients (logFC -0.15, $p=0.002$), whereas the adipocyte plasma membrane-associated protein (APMAP) was increased in bariatric patients (logFC 0.23, $p=0.006$). Platelet proteins previously reported⁷⁶ to be associated with BMI replicated here, including gelsolin (logFC -0.14, $p=0.018$), actin (cytoplasmic 1, logFC 0.51, $p=0.03$) and septin-2 (logFC 0.14, $p=0.03$). Platelet transcripts have been reported to be associated with BMI, including transcripts for myeloperoxidase and protein S100-A9.¹⁰² 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 (LogFC 1.15, $p=0.04$) and protein

S100-A9 (logFC 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/f17f0250ad50bad74d7e38225d060aa6cd83321b/index/figure/Bariatric_study/Proteomics_results.xlsx

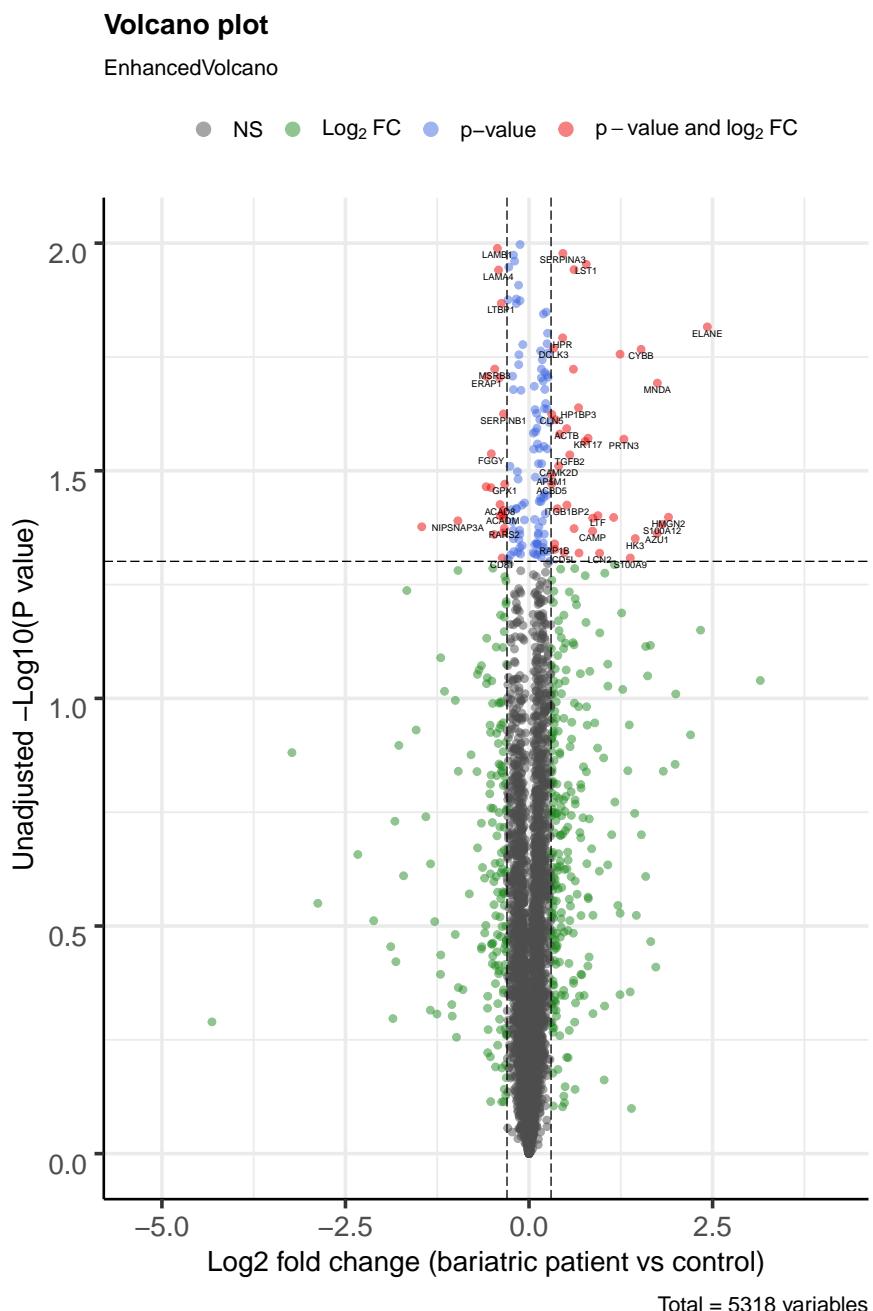


Figure 5.8: A volcano plot comparing the Log₂ fold change in protein levels in bariatric patients compared to controls. Dashed lines indicate a log₂ fold change of 0.3 and a pvalue cut off of 0.05 (- Log_{10} pvalue of 1.3)

5.5 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 4 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 groups¹⁰³ 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 phenotype.¹⁰⁴ 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

aggregation.⁷⁶ 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. 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 5.3 shows the results that have replicated in the current study when comparing to three previous studies that have studied the platelet proteome,⁷⁶ platelet microvesicle proteome¹⁰⁵ and platelet transcriptome in relation to obesity.¹⁰²

Table 5.3: 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 al.⁷⁶ 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.^{76,106} 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 fraction.¹⁰⁷ 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 patients.¹⁰⁸ 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 risk.¹⁰⁹ Septins are cytosolic proteins which are also reported to be important in integrin $\alpha_{IIb}\beta_3$ activation, platelet spreading and clot contraction.¹¹⁰ 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 BMI.¹⁰² 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 formation,¹¹¹ and there is evidence for its involvement in myocardial infarction.¹¹² 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 PRP.¹¹³

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 weight,¹¹⁴ 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 desensitisation.¹¹⁵ GRK6 knockout mice have been shown to have larger aggregatory responses to agonists and more dense and α -granule secretion, along with increased thrombus formation¹¹⁵ 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 6

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

6.1 Background

Platelets play an important role in haemostasis.²⁸ In healthy people, prostacyclin (PGI2) and nitric oxide (NO) are released by the endothelium to suppress platelet activity.²⁹ 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 change.³⁰ Alpha granules release growth factors (e.g. IGF-1), clotting factors and chemokines,³¹ 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 bleeding.²⁸ 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 clopidogrel.³⁶ These therapies are critical in the prevention of ischaemic events. 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 oxide.¹¹⁶ Another contributing factor could be an alteration in the circulating levels of soluble ligands, growth factors and cytokines which can enhance platelet function (known as platelet primers). Platelet primers are signaling molecules which enhance agonist-induced platelet function but in isolation are unable to stimulate platelet aggregation or integrin activation. Molecules that have been reported to be able to prime platelets include insulin-like growth factor-1 (IGF-1)^{36,53} and thrombopoietin (TPO).⁵²

Previous studies have shown that multiple platelet primers increase platelet function through activation of the lipid kinase phosphoinositide-3-kinase (PI3K).^{53,57,117} This activation leads to recruitment of PH-domain proteins including protein kinase B (PKB/AKT) to the plasma membrane. PKB is subsequently phosphorylated on Threonine³⁰⁸ and Serine⁴⁷³ residues leading to its activation.⁵⁸ 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).^{59,118} 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 platelets may also be activated by other platelet primers. There are other biomarkers which are also suggested to be raised with obesity as a result of low-grade chronic inflammation,⁶² including the chemokines macrophage-derived chemokine (MDC/CCL22) and thymus and activation regulated chemokine (TARC/CCL17).¹¹⁹ The inflammatory chemokines MDC and TARC act at the receptor CCR4, which is expressed on platelets.¹²⁰ These chemokines have been shown to potentiate platelet aggregation.¹²¹ For example, the presence of TARC and MDC have been demonstrated to greatly enhance platelet aggregation in the presence of low levels of ADP or thrombin.¹²¹ Furthermore, gene polymorphisms of MDC and CCR4 are associated with myocardial infarction (MI).⁶⁷ As blood/plasma levels of MDC have been reported to be elevated in obese people¹¹⁹ and polymorphisms of its

receptor are associated with MI, understanding the mechanisms by which MDC can enhance platelet function may give us an 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:

- 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 6.1 indicates the parts of the overall thesis hypotheses which are addressed in the current chapter.

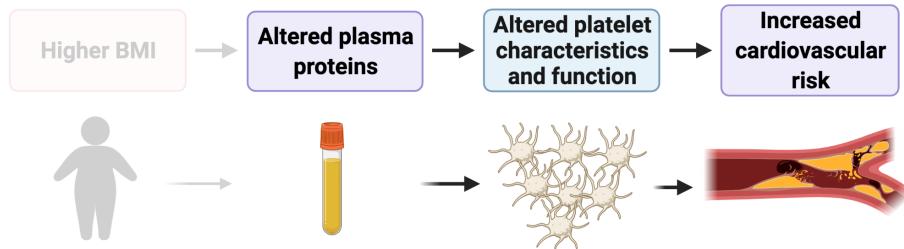


Figure 6.1: Schematic of the associations explored in Chapter 6. This chapter explores the effect of the chemokines MDC and TARC on platelet function and aims to explore their involvement in disease. Figure made using BioRender.com.

6.2 Methods

The platelet function methods and details of statistical analyses used within this chapter are provided in the Methods chapter (Chapter 3).

6.2.1 Two sample MR lookup using EpigraphDB

Two sample MR uses summary statistics and allows for causal effects to be estimated where the exposure and outcome are measured in different populations. EpigraphDB is a tool which performs two sample MR to determine how the human proteome contributes to complex diseases.¹²² Using protein quantitative trait loci (pQTLs) for MDC and TARC from the INTERVAL study⁶⁵ (N=3301) and GWAS summary statistics for disease outcomes (mainly from Neale lab: https://github.com/Nealelab/UK_Biobank_GWAS), it is possible to explore whether plasma levels of these chemokines contribute to disease. For MDC, a Wald ratio was used to estimate the causal effect and for TARC the inverse variance weighted (IVW) method was used.

6.3 Results

6.3.1 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 6.2A 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 µM) and vehicle induced a mean aggregation of 7.0 % (SEM 2.9 %) aggregation (Figure 6.2B). In the presence of 1 µg/ml MDC, this aggregation increased to 33 % (SEM 13 %) (MDC v vehicle, p = 0.15). In the presence of 1 µg/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 6.2C).

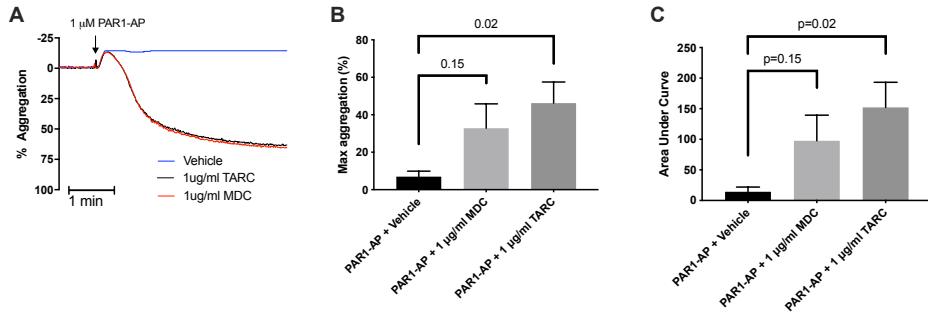


Figure 6.2: The priming effect of the chemokines MDC and TARC on PAR1-AP induced platelet aggregation in washed platelets. Washed platelets at $2 \times 10^8/\text{mL}$ supplemented with PGE1 and apyrase were preincubated with MDC or TARC (5 mins) before application of PAR1-AP-induced aggregation (5 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.

6.3.2 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 6.3A). There was no difference in the pEC50 for comparing vehicle with MDC and TARC (Figure 6.3B).

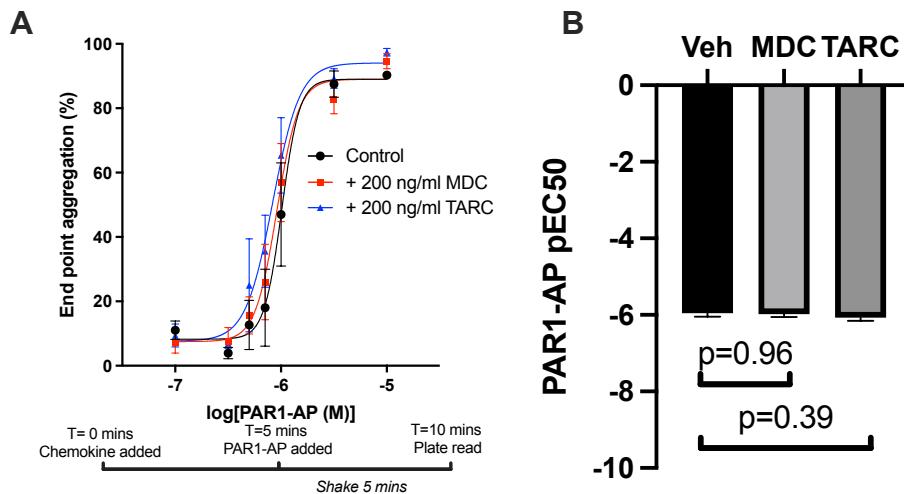


Figure 6.3: The priming effect of the chemokines MDC and TARC on PAR1-AP induced platelet aggregation in PRP using plate aggregation. PRP supplemented with apyrase was preincubated with MDC or TARC (5 mins) before application of PAR1-AP and aggregation induced by a plate shaker (5 mins). A) Concentration-response 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.

6.3.3 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 confirmation 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 PAR-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 6.4A). 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 6.4B). Preincubation with 200 ng/ml MDC caused a small leftward shift in pEC50 to -5.44 (SEM 0.07) (MDC v vehicle, $p=0.006$), 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 6.4C). This effect was specific to integrin $\alpha_{IIb}\beta_3$ as it was not seen with P-selectin.

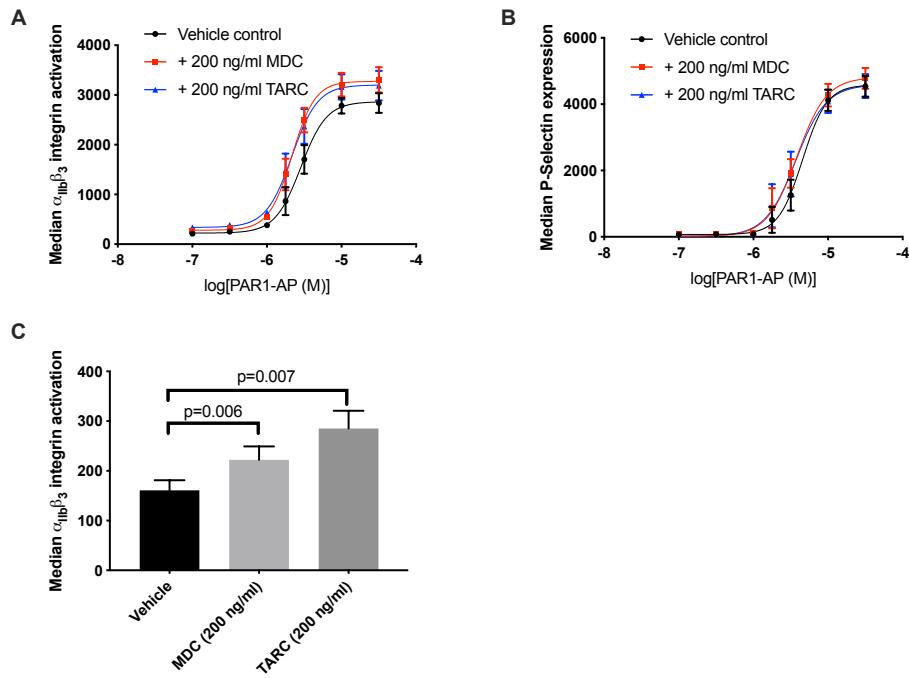


Figure 6.4: The priming effect of the chemokines MDC and TARC on PAR1-AP induced $\alpha_{IIb}\beta_3$ activation and P-selectin expression. Washed platelets at $2 \times 10^7/\text{mL}$ supplemented with PGE1 and apyrase were preincubated with MDC or TARC (5 mins) before application of PAR1-AP (5 mins). 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 6.1 C) MDC and TARC alone increase integrin $\alpha_{IIb}\beta_3$ activation. Mean + SEM displayed. N=4.

Table 6.1: 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

6.3.4 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 generation.¹²³ MDC and TARC did not affect the overall percentage of annexin V positive platelets induced by CRP and thrombin (Figure 6.5).

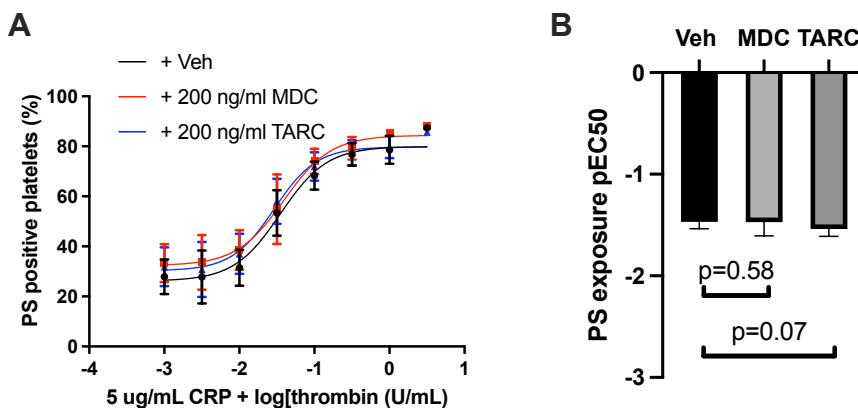


Figure 6.5: **The effect of the chemokines MDC and TARC on PAR1-AP induced PS exposure.** A) Washed platelets at $2 \times 10^7/\text{mL}$ supplemented with indomethacin and apyrase were preincubated with vehicle, 200ng/mL MDC or 200 ng/mL TARC (5 mins) before application of CRP and increasing concentrations 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.

6.3.5 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 6.6). This upward deflection is a reduction in light passing through the sample, indicating that the platelets may be undergoing shape change.

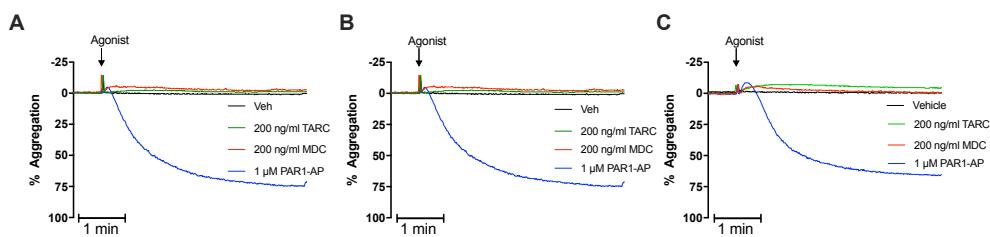


Figure 6.6: The effect of the chemokines MDC and TARC alone on aggregation in washed platelets. A-C) Washed platelets at $2 \times 10^8/\text{mL}$ supplemented with PGE1 and apyrase were stimulated with vehicle, 200ng/mL MDC, 200 ng/mL TARC or 1 μM PAR1-AP (5 mins). Aggregation was measured using light transmission aggregometry. Each graph is a representative trace from a separate donor

6.3.6 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 (6.7A). MDC and TARC alone were also able to increase calcium mobilisation in a concentration-dependent manner (6.7B), however caused a much smaller calcium response than ADP.

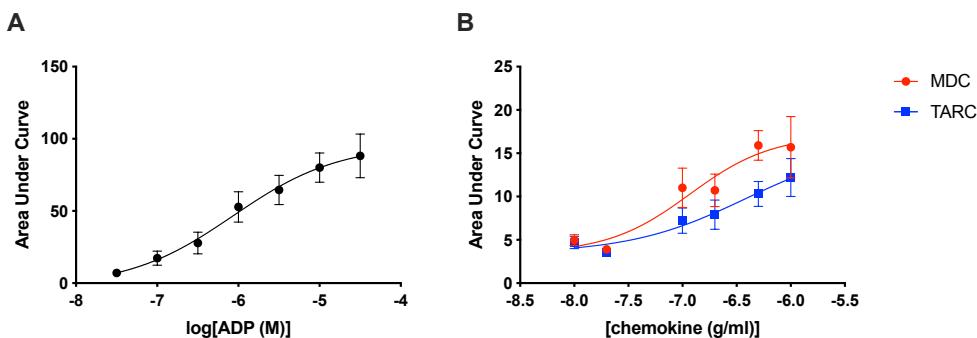


Figure 6.7: The effect of ADP and the chemokines MDC and TARC alone on calcium mobilisation in washed platelets. Washed platelets at $2 \times 10^8/\text{mL}$ supplemented with indomethacin and apyrase were loaded with Fura-2. Platelets were stimulated with increasing concentrations of A) ADP or B) 200ng/mL MDC or 200 ng/mL TARC. Area under curve represents the free calcium and is ratio of fluorescence at wavelengths 340:380 nm.

6.3.7 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 6.8 shows a representative blot from one donor. There was little evidence for an effect of MDC, TARC and ADP reducing basal VASP levels (Figure 6.9A, 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 6.9B) and at Ser¹⁵⁷ by 44 % (SEM 21 %) (ADP v vehicle, p=0.048, Figure 6.9D). 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 6.9B, 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 6.10). 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.

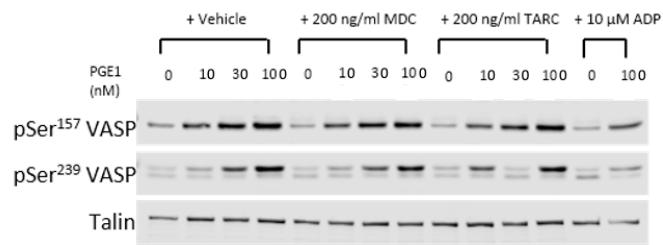


Figure 6.8: A representative blot of the effect of 200 ng/mL MDC, 200 ng/mL TARC and 10 μ M ADP on phospho-VASP at Serine¹⁵⁷ and Serine²³⁹ in the presence of increasing concentrations of PGE₁. Quantification is shown in Figure 6.9.

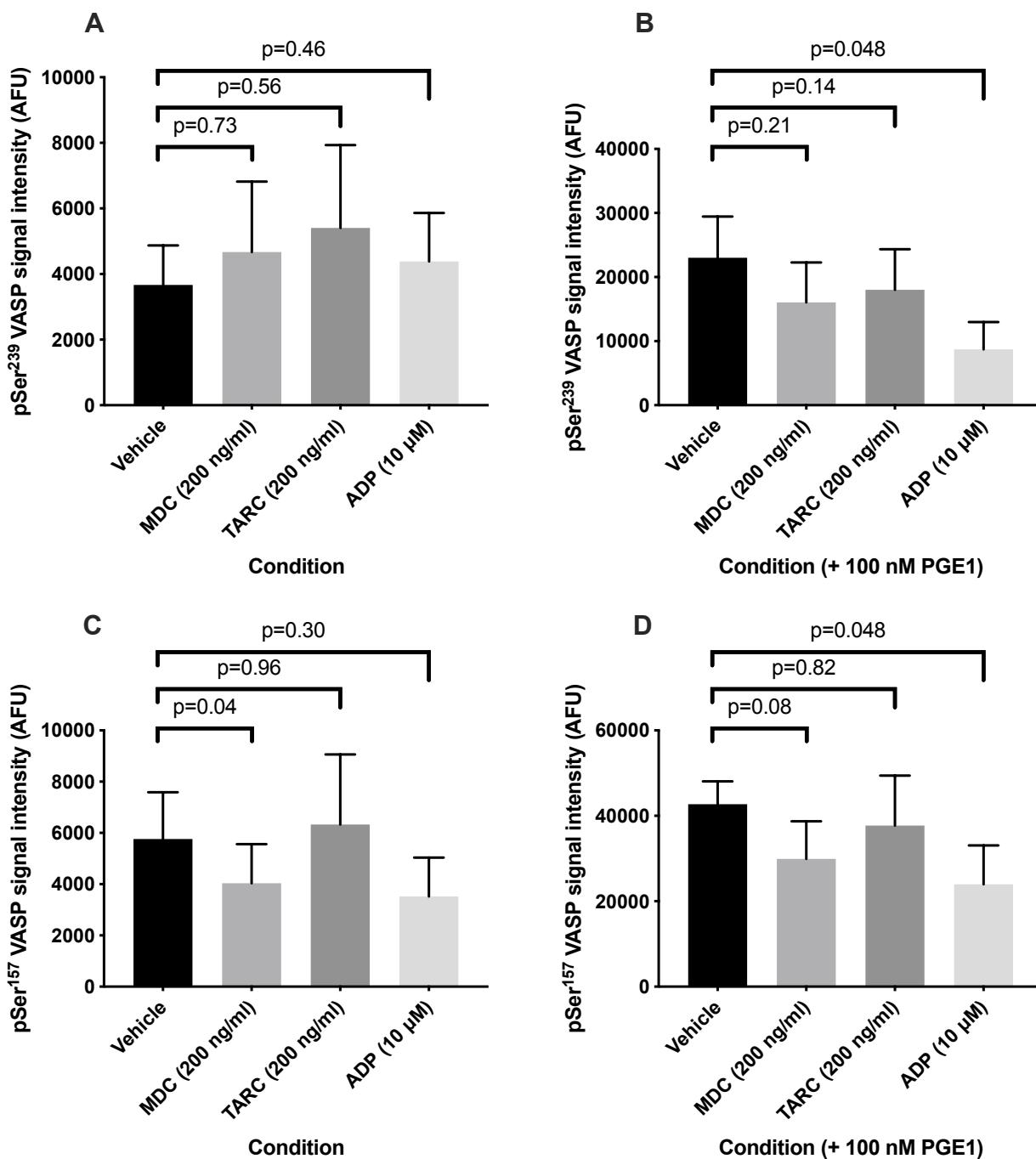


Figure 6.9: Quantification of the effect of MDC, TARC and ADP on PGE₁ stimulated phospho-VASP. Bar charts of the effect of 200 ng/mL MDC, 200 ng/mL TARC and 10 μ M ADP on phospho-VASP at A) Serine 239 in the absence of PGE₁, B) Serine 239 in the presence of 100 nM PGE₁, C) Serine 157 in the absence of 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.

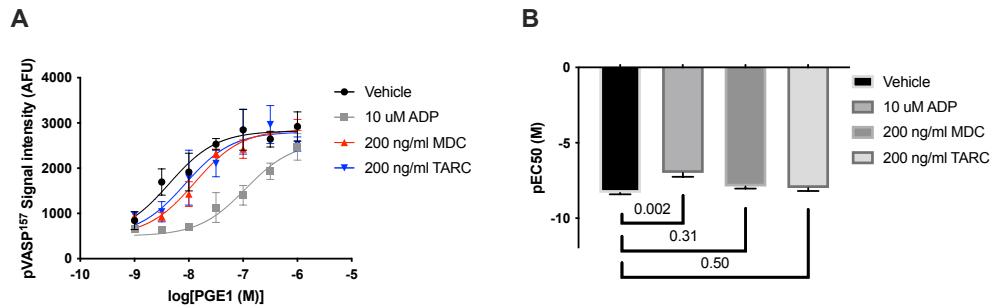


Figure 6.10: The effect of the chemokines MDC and TARC on phospho-VASP levels using flow cytometry. A) Washed platelets at $1 \times 10^8/\text{mL}$ were supplemented with indomethacin and apyrase. Platelets were preincubated with vehicle, 200ng/mL MDC, 200 ng/mL TARC or 10 uM ADP (5 mins) before application 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.

6.3.8 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 6.11A, B). Y27632 inhibited the G_{12/13} mediated shape change induced by low concentrations of PAR1-AP (0.75 uM) 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 uM (Figures 6.11C, 6.12A, B). The G_q inhibitor YM-254890 was able to reduce the maximum aggregation induced by MDC (Figure 6.11C, Figure 6.12C), 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 6.11D, 6.12D). Application of MDC to PRP did not cause shape change.

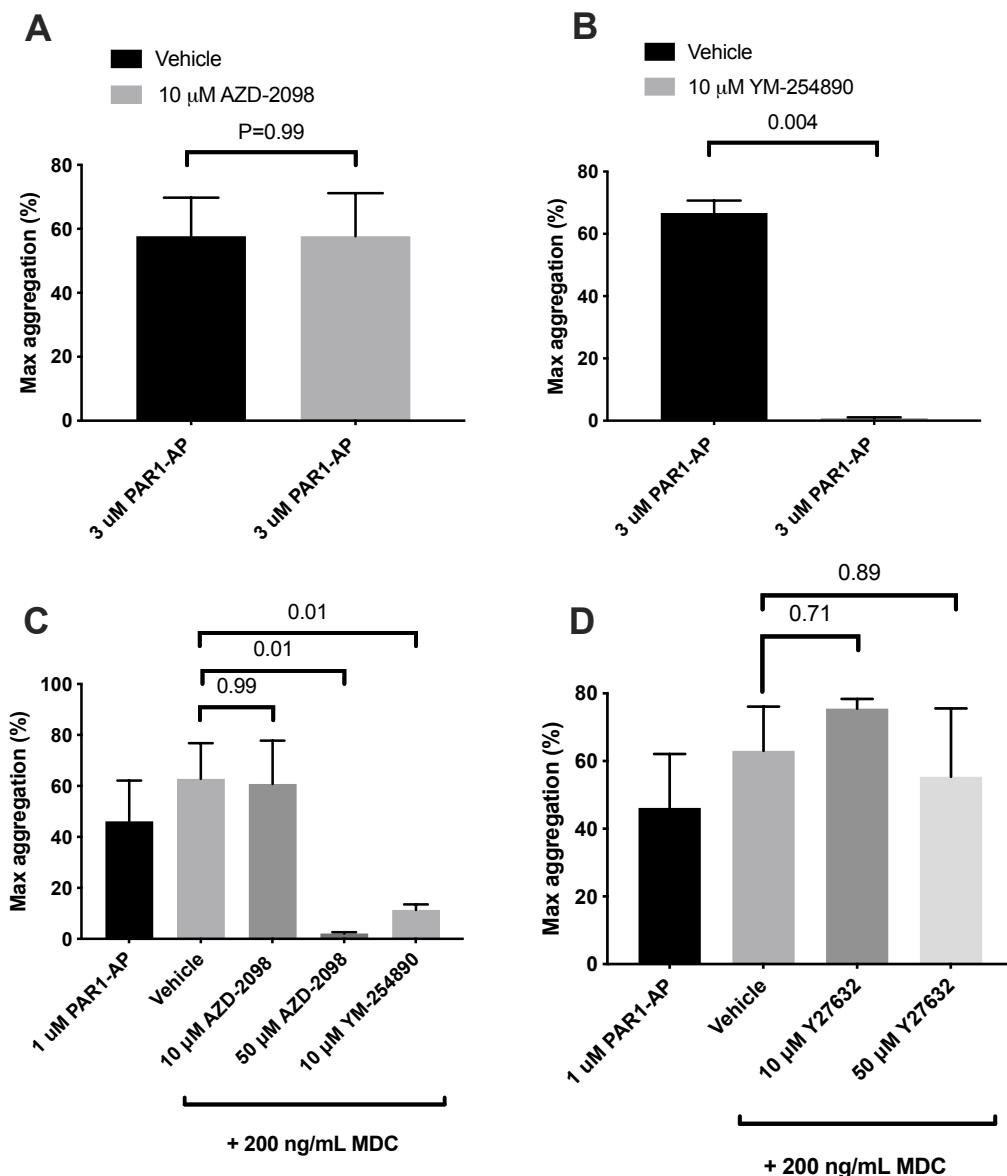


Figure 6.11: The effect of inhibitors on PAR1-AP and MDC induced aggregation in PRP. Platelet rich plasma (PRP) supplemented with apyrase was incubated for 5 minutes with the antagonist indicated, followed by stimulation by PAR1-AP or MDC. A) Effect of 10 μ M AZD-2098 (CCR4 antagonist) on maximal aggregation 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.

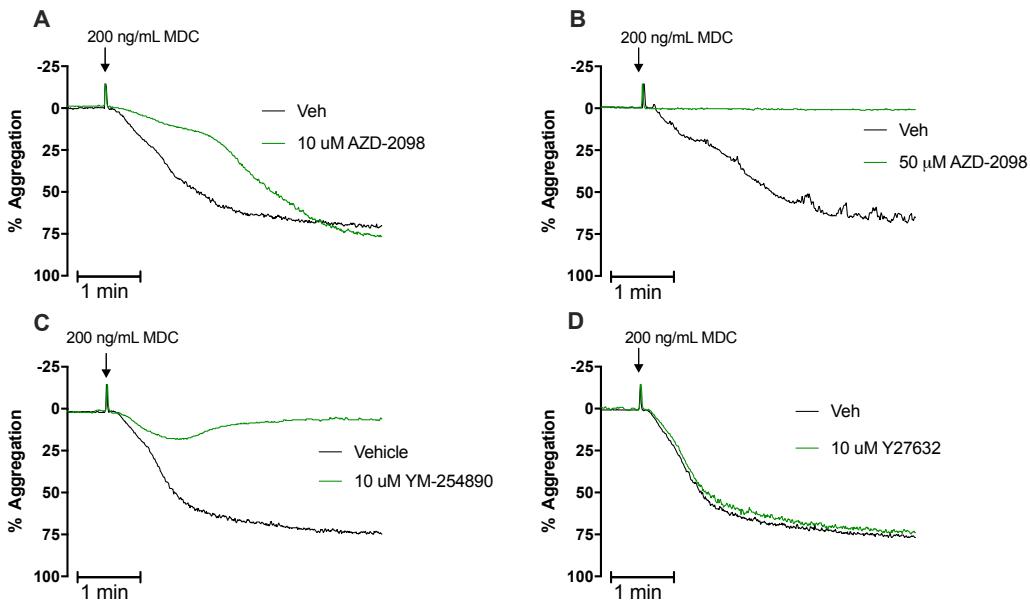


Figure 6.12: Aggregation traces of the effect of inhibitors on MDC induced platelet aggregation in PRP. Representative aggregation traces of platelet rich plasma (PRP) supplemented with apyrase, incubated with A) 10 uM CCR4 antagonist AZD-2098 B) 50 uM AZD-2098 C) 10 uM G_q inhibitor YM-254890 D) 10 uM ROCK inhibitor Y27632, followed by addition of 200 ng/mL MDC.

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

To determine whether levels of MDC or TARC contribute to disease, a two sample MR was performed using pQTL data for these chemokines and available disease outcome data. Results of the two sample MR are shown for MDC and TARC in tables 6.2 and 6.3. There was evidence from the Wald ratio MR that higher MDC has a causal effect on 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 weaker evidence for causal effects of TARC on disease, with evidence that higher levels of TARC have an effect on rheumatoid arthritis (Effect size 0.10, SE 0.04, $p=8.51\times 10^{-3}$). There was weak evidence for higher TARC decreasing the risk of ischaemic stroke (Effect size -0.08, SE 0.04, $p=0.03$).

Table 6.2: 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 Phlebitis and thrombophlebitis	1 (rs77542162)	Wald ratio	0.2269	0.1145	0.04755

ref(caption-TARC-disease-MR) Estimates for the effect of TARC on disease outcomes using two sample Mendelian randomization

Table 6.3: ref(caption-TARC-disease-MR)

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

6.4 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 aggregation.¹²¹ 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 occur.⁵⁴ 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 defense.¹²⁰ MDC and TARC can also cause activation of platelets, thereby demonstrating the close link between inflammation and haemostasis.³¹

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} coupled.¹²⁵ 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 reports.¹²⁶ Another study suggested that CCR4 is coupled to G_q due to the calcium response observed by MDC.⁵⁴ 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 atherosclerosis,¹²⁷ 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 region.¹²⁸

In efforts to determine whether levels of MDC or TARC are affected by BMI, a one sample MR was performed in Chapter 7. Here, the methods and full results can be seen, where there is weak evidence that higher BMI raises levels of MDC.

6.4.1 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 responsible 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 7

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 Obesity.⁷⁷ I am the first author of the publication; I performed all analyses, created all figures and tables and wrote the manuscript.

Supplementary Tables 1-7 can be found here https://github.com/lucygoudswaard/mythesis/blob/master/index/figure/BMI_protein_INTERVAL/BMI_protein_supplementary_Tables.xlsx

7.1 Introduction

Obesity has tripled worldwide since 1975, now affecting around 40% of adults in the United States and 26% of adults in the UK.⁶ The average body mass index (BMI) of the UK adult pop-

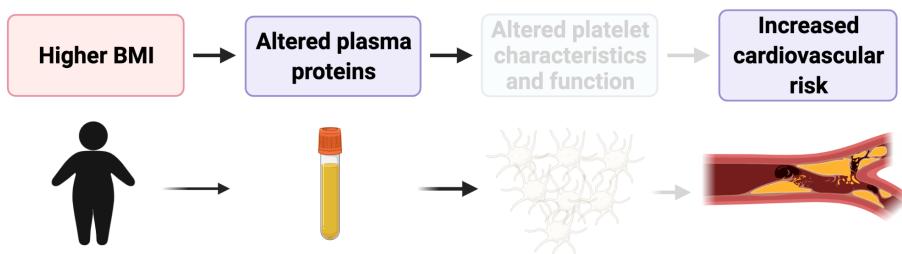
ulation is now in the conventional ‘overweight’ category (BMI between 25 and 30 kg/m²)³ and ‘overweight’ is now more common than ‘normal-weight’ in middle age in many high-income countries.⁴ BMI is often used as a proxy for adiposity given high correlations between BMI and more objectively measured fat mass indices.²⁵ Higher adiposity is a major risk factor for various noncommunicable diseases including cardiovascular diseases such as coronary heart disease (CHD) and stroke.^{11,22,74} These BMI-disease associations are supported by prospective observational studies and, more recently, by Mendelian randomization (MR) studies^{11,22} which use genetic variation reliably associated with BMI to re-estimate effects of BMI on disease outcomes. Given the properties of genetic variation, this method helps to overcome issues such as confounding and reverse causation which commonly occur with observational studies.¹²⁹

Despite MR studies supporting a causal role of adiposity for cardiometabolic diseases, and randomised trials supporting the effectiveness of weight loss in reducing disease risk,¹³⁰ the molecular footprint of adiposity is not well understood. 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)^{25,26} and on inflammatory molecules such as C-reactive protein (CRP).^{26,63}

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 framework.⁶⁹ It is estimated that 25% of proteins in the human proteome circulate in blood,⁶⁸ which is important as the majority of druggable targets are such proteins.⁷⁰ 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 sensitivity.¹³¹ 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 donors.⁶⁵ There is evidence that proteins which change as a result of a higher BMI may contribute to cardiometabolic disease: 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 cardiometabolic

disease by activating platelets (Chapter 6).

In this chapter, the aim was to measure associations between adiposity and the human proteome and to also estimate the underlying effects in a causal framework (Figure ??). Using data on 2737 participants from INTERVAL, the effects of BMI on 4034 (3622 unique) plasma protein traits were estimated, in both observational and MR frameworks. The agreement between effect estimates from different methods were examined and enrichment analyses of the most strongly altered proteins were performed to map their potential relevance to cardiovascular disease.



7.2 Methods

7.2.1 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 this study can be found in Chapter 4 in section 4.3.

7.2.2 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 SomaLogic.⁶⁵ 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 microarray.¹³¹ 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). We therefore have included all 4034 SOMAmers. 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..⁶⁵ 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.

7.2.3 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/hughesveoanth/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 covariates before rank normal transformation of proteins) can reintroduce correlations,¹³² 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/m^2) 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 package,⁹⁰ 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. We report estimates 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 7.1). 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 7.2), dendrogram made using “iPVs” package <https://github.com/hughesvoanth/iPVs>). We utilised a Bonferroni adjusted P-value of $0.05/3655 = 1.4 \times 10^{-5}$ to indicate strong evidence in this sample. Full

results are presented in the supplementary material.

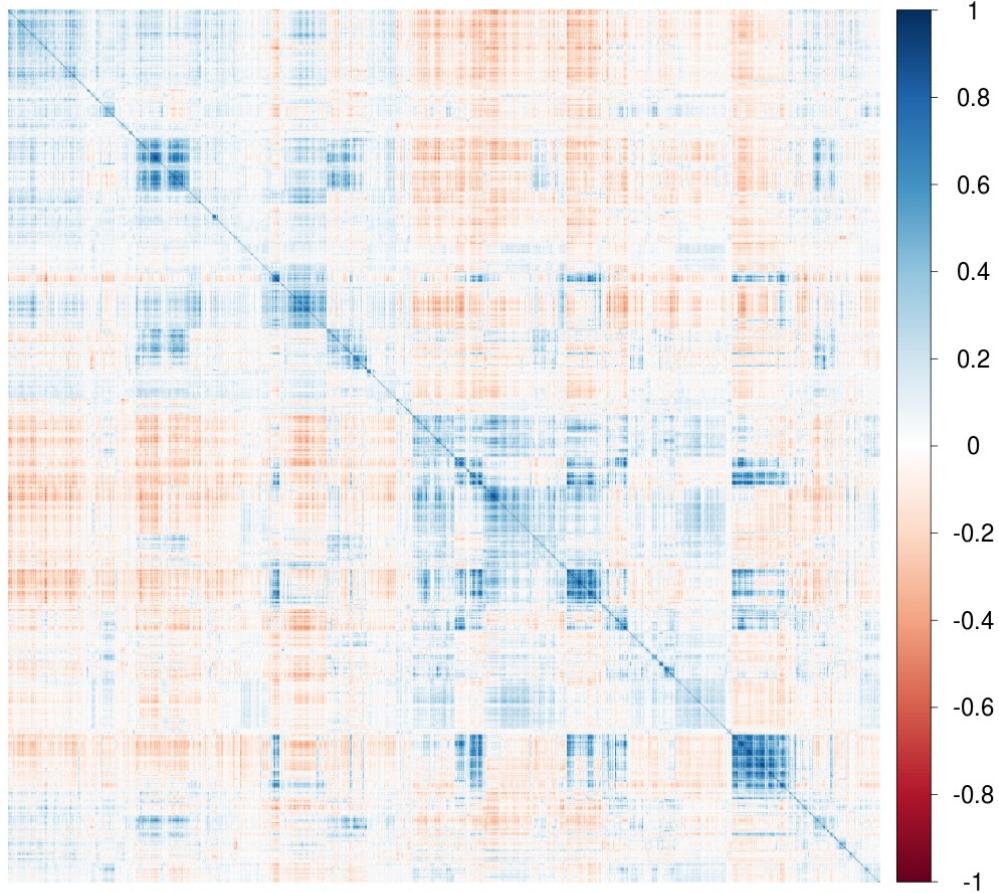


Figure 7.1: Correlation matrix of all protein traits analysed by SomaLogic (4,034). The colour corresponds to the correlation coefficient ranging from 1 (dark blue) to -1 (dark red).

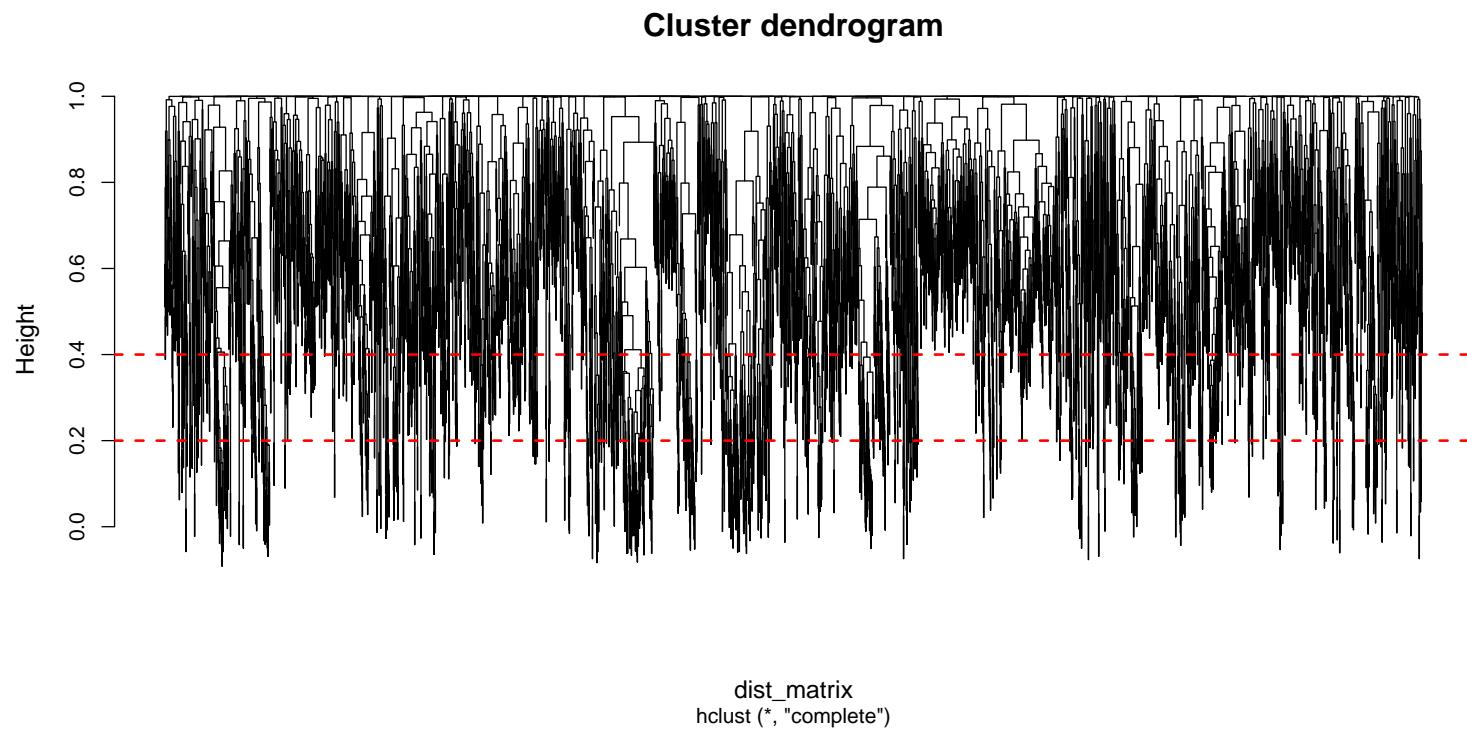


Figure 7.2: Cluster dendrogram showing the hierarchical relationship between protein levels. Height is calculated as (1-correlation coefficient). At a height of 0.2 and 0.4 (red dashed lines), the number of independent proteins was 3,655 and 3,016 respectively

7.2.4 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 7.3A-D). The top “n” PC eigenvectors, as identified by a scree plot of the PCA eigenvalues (Figure 7.3E), 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) we generated a scree like plot (Figure 7.3F). Here we plotted the variance explained by clusters, 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, we used a data reduction method (PCA) 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.

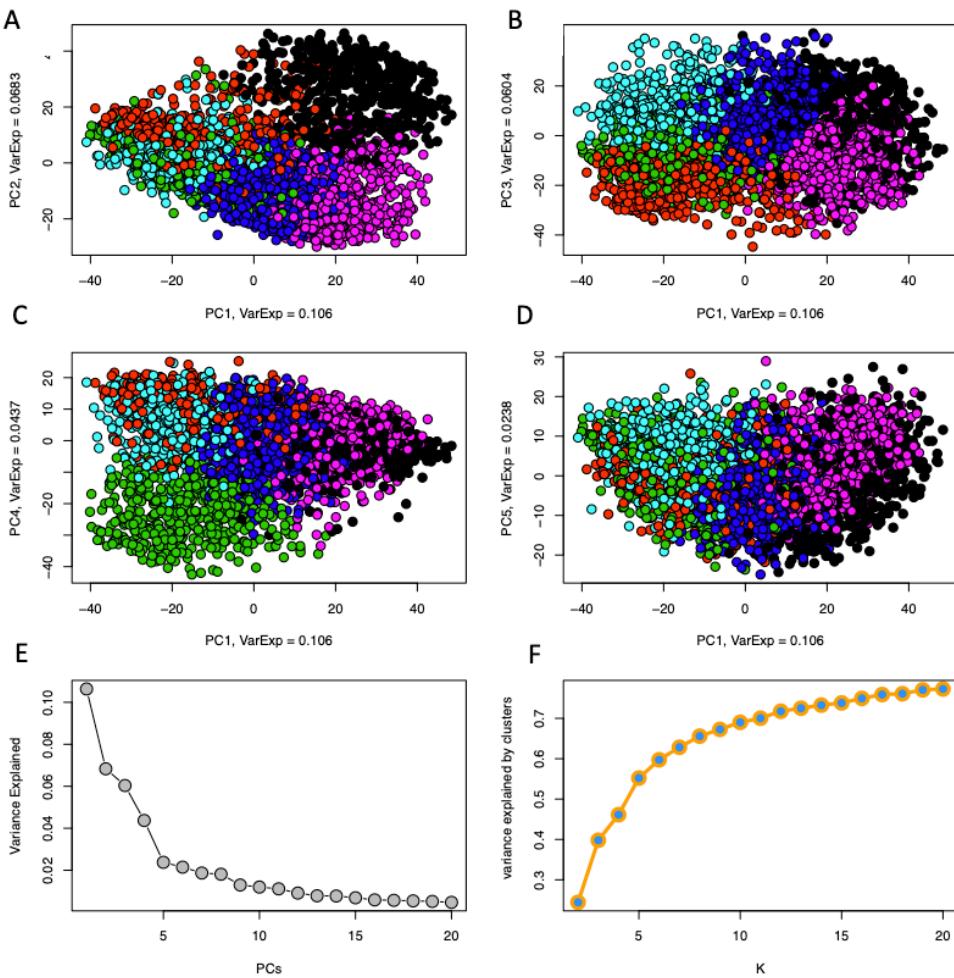


Figure 7.3: Principal component analysis (PCA) and k-means clustering of proteins. Principal component analysis (PCA) and k-means clustering of proteins provide evidence for five clusters. A-D) Principal component (PC) 1 vs PC2-PC5 for the study protein data. Each dot represents a protein, and the colors 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 abso-

lute 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 6.8.¹³³ 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 classes)¹³⁴ of the protein cluster was assessed by fold enrichment and a Bonferroni-corrected P-value to account for multiple testing. 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 3.4.2.⁸⁹ R code used for analyses available upon request.

7.3 Results

7.3.1 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 7.1). 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 7.1).

Table 7.1: 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 Chi2 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%		

7.3.2 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 1). In a second model additionally adjusting for frequencies of smoking and alcohol intake among 2 380 adults, there were 1447 associations at the same reference point (Supplementary Table 2). 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, $P=2.4 \times 10^{-106}$).

7.3.3 Observational associations of covariates with BMI and protein traits

Age, sex, and frequencies of smoking and alcohol intake were each associated with BMI (Table 7.2). 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 3-6 and Figures 7.4 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 $p < 1.4 \times 10^{-5}$.

Table 7.2: 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

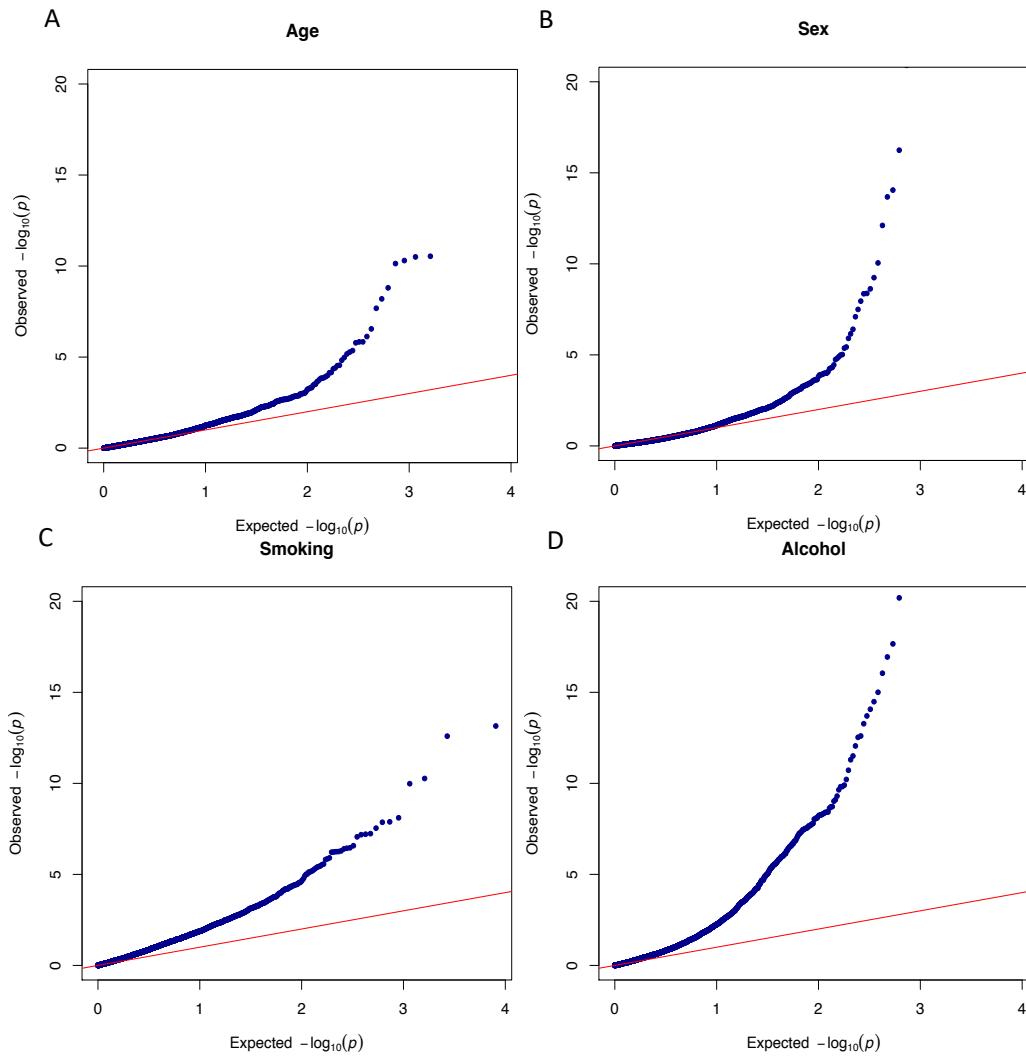


Figure 7.4: **Q-Q plots for the expected against observed $-\log_{10}(p)$ values for associations of age (A), sex (B), smoking (C) and alcohol (D) with protein traits**

7.3.4 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 7.3). 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 7.3: 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

7.3.5 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 7.5). 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. Supplementary Table 7 provides the full MR results.

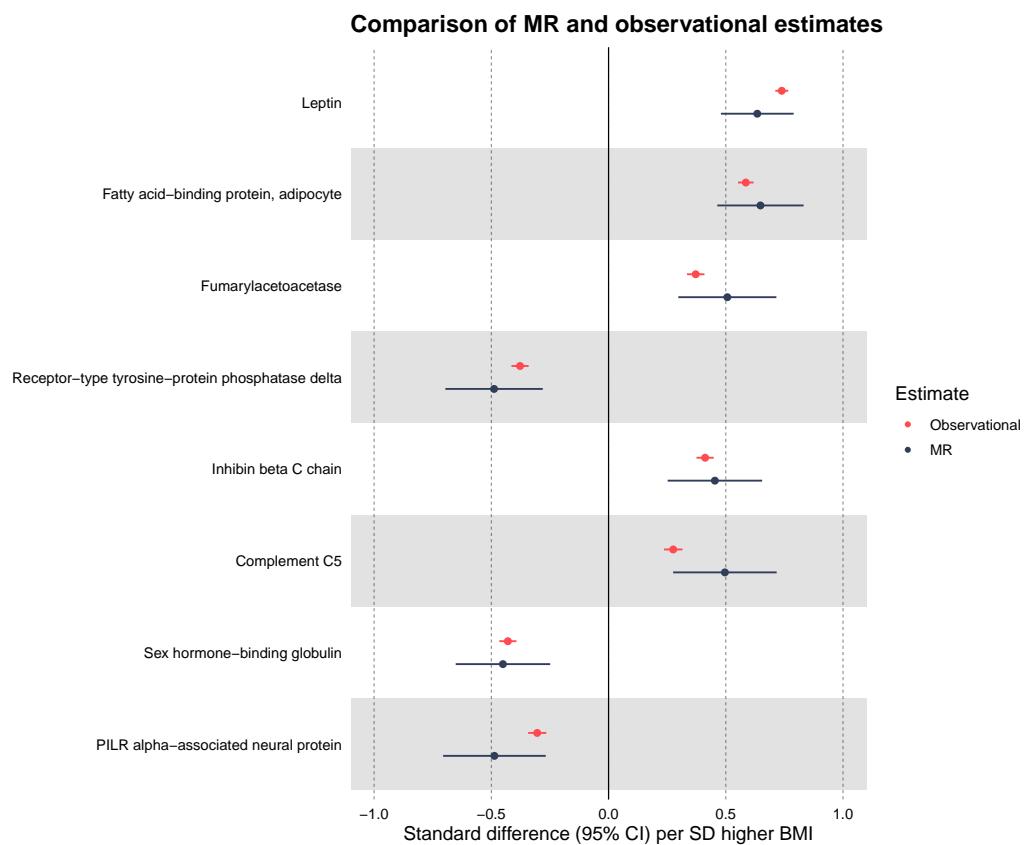


Figure 7.5: Forest plot of the strongest BMI-protein Mendelian randomisation estimates and the corresponding observational estimates

7.3.6 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 7.6A). In contrast to this, the extent of this overrepresentation was reduced considerably in the MR (Figure 7.6B).

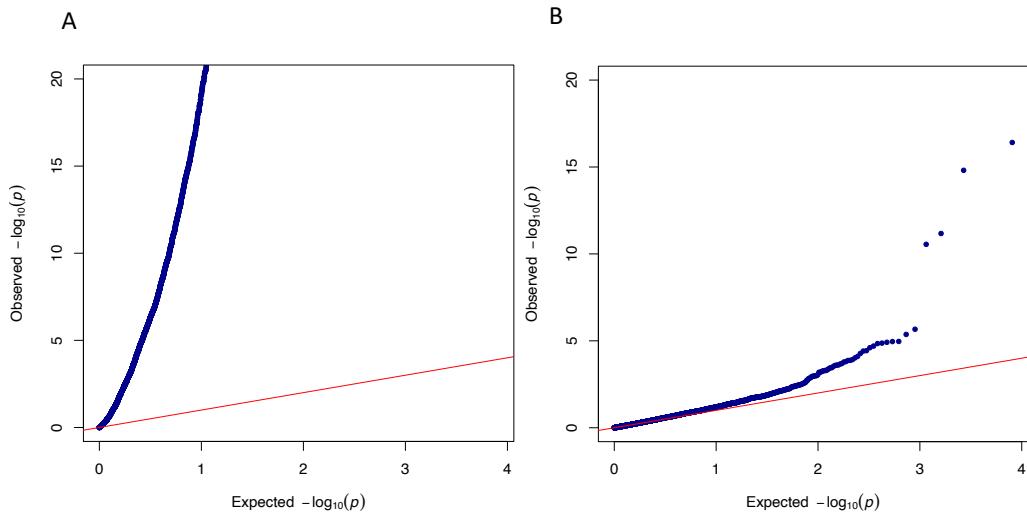


Figure 7.6: Q-Q plot of the expected against observed $-\log_{10}(p)$ values for BMI-protein estimates. A) Q-Q plot of expected against observed $-\log_{10}(p)$ values for the unadjusted observational BMI-protein trait estimates B) Q-Q plot of expected against observed $-\log_{10}(p)$ values for the 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 7.7). 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 7.8). 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 7.9 A/B). These results suggest causal effects of BMI across the general proteome.

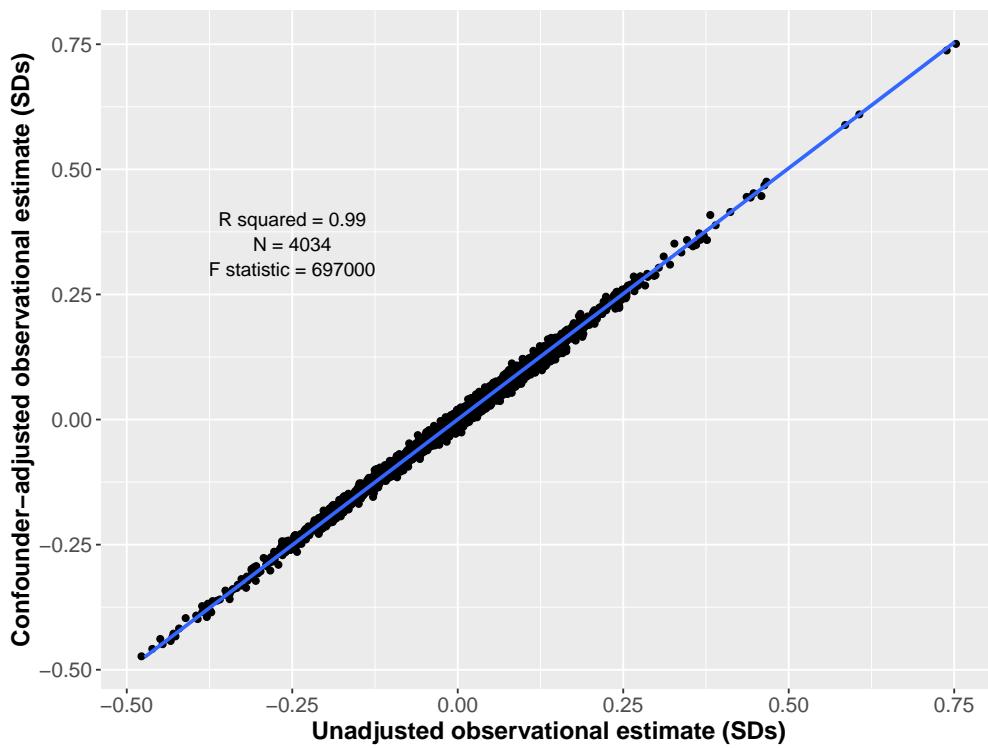


Figure 7.7: Scatter plot of the unadjusted (age and sex adjusted) observational estimates and the confounder-adjusted observational estimates for BMI and protein traits. Regression line shown in blue.

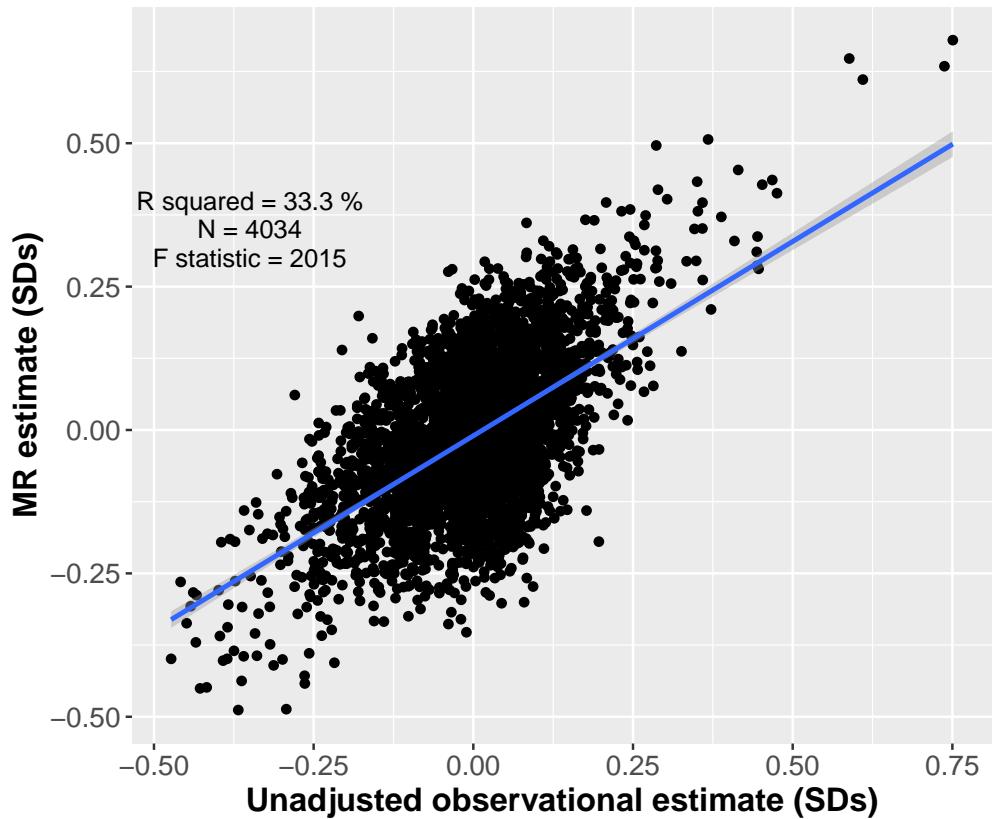


Figure 7.8: Scatter plot of the unadjusted (age and sex adjusted) observational estimates and the MR estimates for BMI and protein traits. Regression line shown in blue.

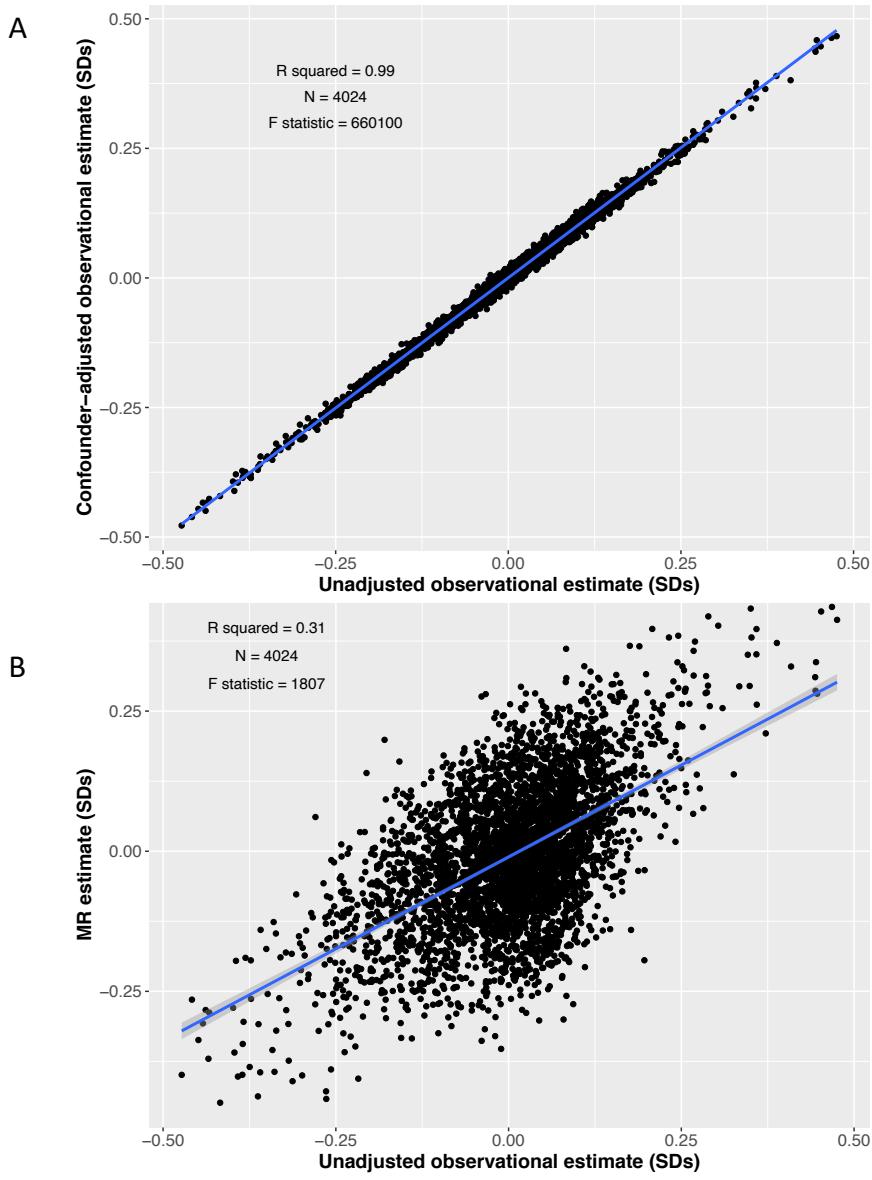


Figure 7.9: Scatter plot comparing BMI-protein estimates across models with top eight BMI-associated proteins excluded. A) Scatter plot of the unadjusted (age and sex adjusted) observational estimates and the confounder-adjusted observational estimates for BMI and protein traits with a regression line (blue) 6B) 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).

7.3.7 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 7.3). 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 7). 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 7). 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 7.4). 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 7.5), 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 7.6).

Table 7.4: 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 7.5: Cluster 2 vs full SomaLogic protein enrichment results for disease class using DAVID bioinformatics 6.8¹³³

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 7.6: Disease enrichment of proteins associated with BMI in confounder adjusted model compared with all proteins in SomaLogic (DAVID Bioinformatics 6.8)¹³³

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

7.4 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 CRP.⁶⁹ 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 here.¹³⁵ 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, of-

fering 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 appetite.¹³⁶ The direction of effect agrees with estimates from previous cross-sectional and MR studies,^{26,137} indicating leptin receptor resistance.¹³⁸ There is observational evidence in humans that higher leptin can induce greater aggregation of platelets (cells involved in haemostasis).¹³⁹ In a larger observational study, leptin was found to be associated with higher risk of coronary events independent of BMI.¹⁴⁰

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 macrophages.¹⁴¹ This MR estimate supports the association which has been suggested in previous observational studies.¹⁴² FABP4 has been implicated in cardiometabolic disease: a SNP which increases FABP4 was found to raise the odds of type II diabetes among adults,¹⁴³ potentially through its contribution to higher insulin resistance.¹⁴⁴ FABP4 has also been associated with higher risk of atherosclerosis among adults.¹⁴⁵ 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 activity;¹⁴⁶ 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.^{147–149} When evaluating the role of SHBG in disease, MR analysis suggests that an increase in SHBG contributes to a decrease in risk of cardioembolic stroke.¹²² Other studies have also implicated lower SHBG levels in increasing type II diabetes risk.¹⁵⁰ 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 oestrogen.¹⁴⁶

Despite these possible protein involvements in cardiometabolic disease, 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. We therefore examined proteins grouped into clusters of similar features, compared BMI-protein estimates of each cluster with overall estimates and explored enrichment for genes related to disease. 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. When IPC GWAS summary stats become available, it would be useful to perform a PheWAS of protein exposures to IPC to explore whether any proteins which are altered by BMI can affect IPC.

This analysis has some limitations. Firstly, although INTERVAL is one of the 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 ($N = 2737$).¹⁵¹ 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. Secondly, height and weight were self-reported which could bias results towards the null due to systematic error in BMI measurement. However, strong correlations are often reported between self-reported and measured BMI⁹⁶ and the validity of self-reported BMI is supported by the association between

the GRS for BMI and self-reported BMI in INTERVAL to the degree expected. Thirdly, the small degree of overlap between INTERVAL and UK Biobank (participants used for the source GWAS for BMI who were also in INTERVAL) may have a biasing effect on estimates, though this is likely to be towards the null. We anticipate that overall, this bias would make estimates more conservative.¹⁵² Fourth, we recognise a lack of availability of possible confounders such as socio-economic position (which likely affect both BMI and protein traits related to cardiovascular disease processes).^{153,154} Residual confounding may help account for the divergence between observed and expected P-values seen in observational versus MR models. Fifth, the proteins examined are highly correlated and we therefore may not fully be describing changes in individual proteins. 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 dataset.

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 human 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. 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 8

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

8.1 Background

Obesity is associated with an increased risk of type II diabetes (T2D), cardiovascular diseases, musculoskeletal diseases, and types of cancer.^{10,155,156} 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 risk.⁷⁷ The previous results chapter (Chapter 7) 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.

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 pleiotropy.¹⁵⁷ 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 bias.¹⁵⁸ Therefore, to complement 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 intervention, consisting of a low calorie diet, to help with weight loss and T2D remission.¹⁵⁹ This chapter therefore aimed to:

- 1) Examine the effect of reducing BMI on the plasma proteome.
- 2) Compare BMI-protein effects across MR and RCT study designs (Results from Chapter 7 (Figure 8.1)).

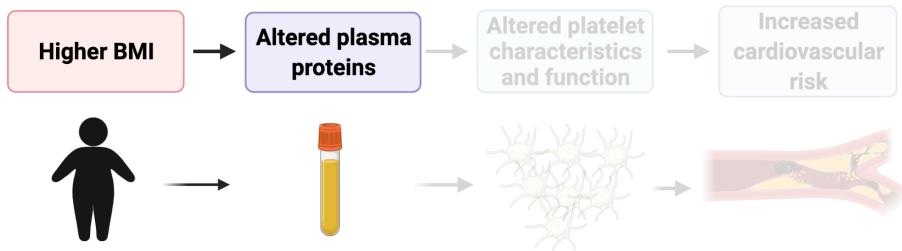


Figure 8.1: Schematic of the associations explored in Chapter 8. This chapter explores the effect of caloric restriction-induced weight loss on the plasma proteome. Figure made using BioRender.com.

8.2 Methods

8.2.1 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 Tyneside.¹⁵⁹ 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 published.¹⁶⁰ Participants enrolled were between 20-65 years, diagnosed with T2D within previous 6 years, had a BMI of between 27-45kg/m². Participants were excluded if they were: using insulin, had a HbA1c concentration of $\geq 12\%$ (≥ 108 mmol/mol), weight loss of > 5 kg in the preceding 6 months, an estimated glomerular filtration rate of < 30 mL/min per 1.732 m². More exclusion criteria are included in the main trial paper.¹⁵⁹ Participants in the control

group received best-practice care by guidelines. The intervention group were asked to follow the Counterweight-Plus weight management programme.¹⁶¹ 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 8.2.

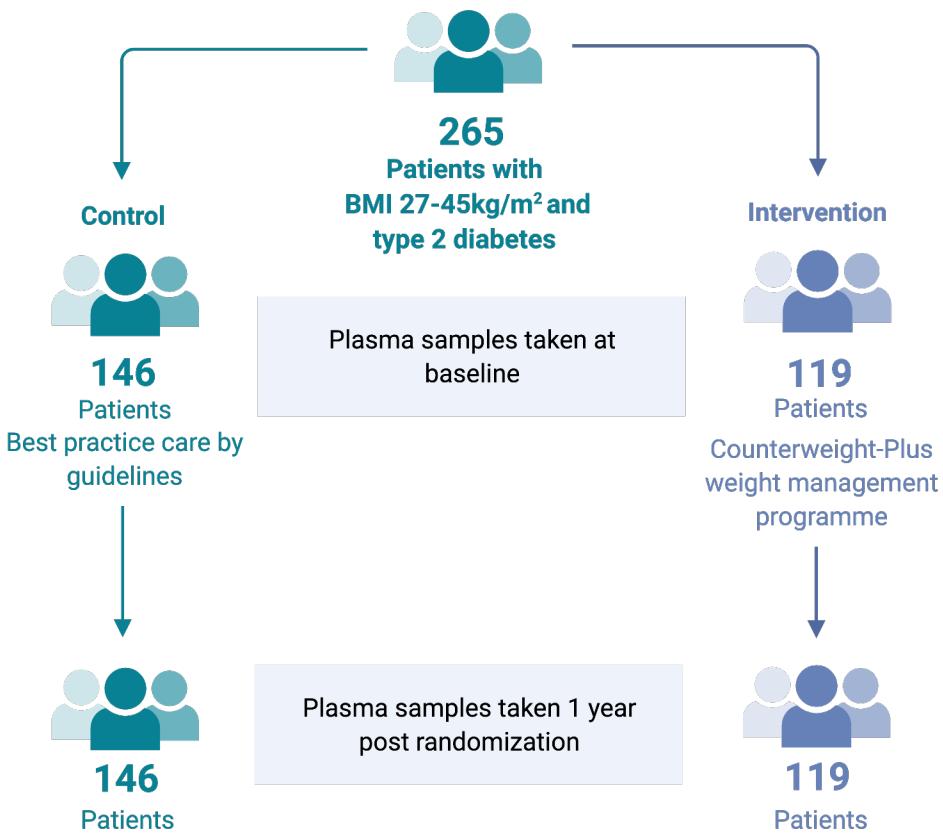


Figure 8.2: **Overview of participants in the DiRECT trial.** Figure made using BioRender.com.

8.2.2 Included variables

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 ap-

proved calibrated scales (Marsden Group UK).¹⁶⁰ 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 rtrans-form() from “moosefun” R package <https://github.com/hughesveoanth/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.

8.2.3 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 microarray.¹³¹ 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 package,¹⁶² 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 proteins had W statistics ≥ 0.95 . This data was then \log_2 transformed to match protein transformation by Sun et al.⁶⁵ 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.

8.2.4 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 multiple 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 positive.¹⁶³ 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. Covariates (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 8.3 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>). As a sensitivity analysis, for proteins which associate with BMI change, baseline protein levels were compared across control and intervention groups. P values were adjusted for multiple testing based on the number of representative proteins ($0.05/869 = 5.8 \times 10^{-5}$).

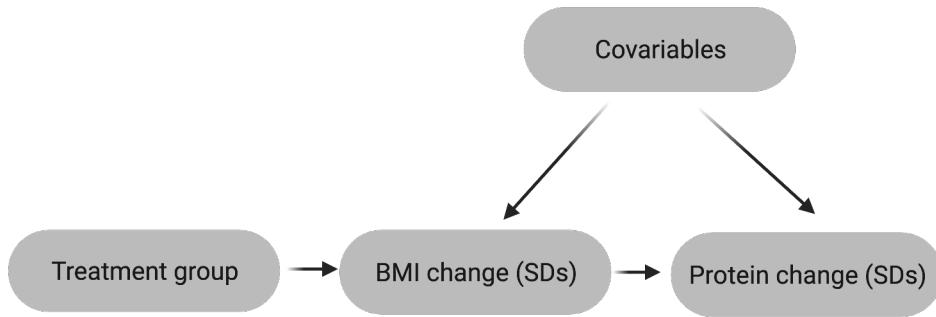


Figure 8.3: Schematic of two stage least squares analysis performed in the current chapter. Figure made using BioRender.com.

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. In efforts to further elucidate which proteins are altered by adiposity, a comparison between the BMI-protein associations in INTERVAL (Chapter 7) and the BMI change-protein change associations in the current analyses was also performed using a linear regression. There were 2803 unique proteins overlapping across the two cohorts. Estimates across the two cohorts were compared using linear regression.

8.2.5 Disease class enrichment

Similarly to the previous chapters, proteins which had a BMI effect were taken forward into an enrichment analysis using DAVID Bioinformatics 6.8.¹³³ 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. StringDB¹⁶⁴ 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 pathways.¹⁶⁵

8.3 Results

8.3.1 Participant characteristics

Participants displayed similar characteristics across control and intervention groups (Table 8.1). 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 8.1: 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 %		

8.3.2 Observational association between BMI change and protein change

Linear regression was used to estimate the effect of BMI change on protein change. 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 $p=5.8\times10^{-25}$).

8.3.3 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).

8.3.4 Association between treatment group and BMI change

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

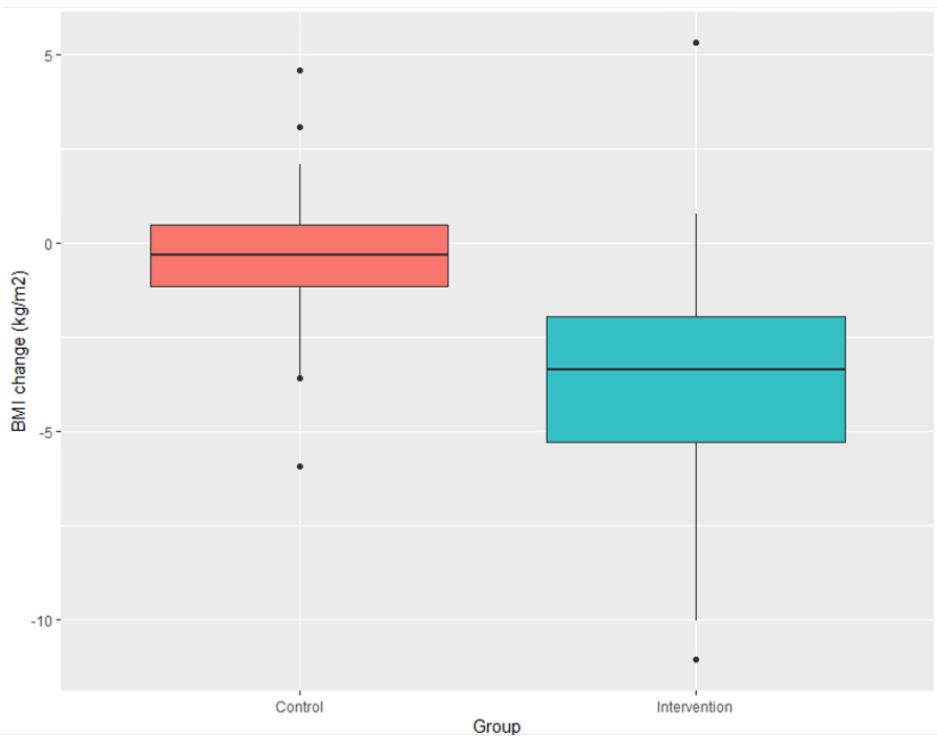


Figure 8.4: Box plot of the distribution of BMI change (kg/m²) by treatment group. Median and interquartile ranges displayed by the box.

8.3.5 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 8.5 shows the qq-plot for the observational associations (A) and instrumental variable two stage least squares analysis (B).

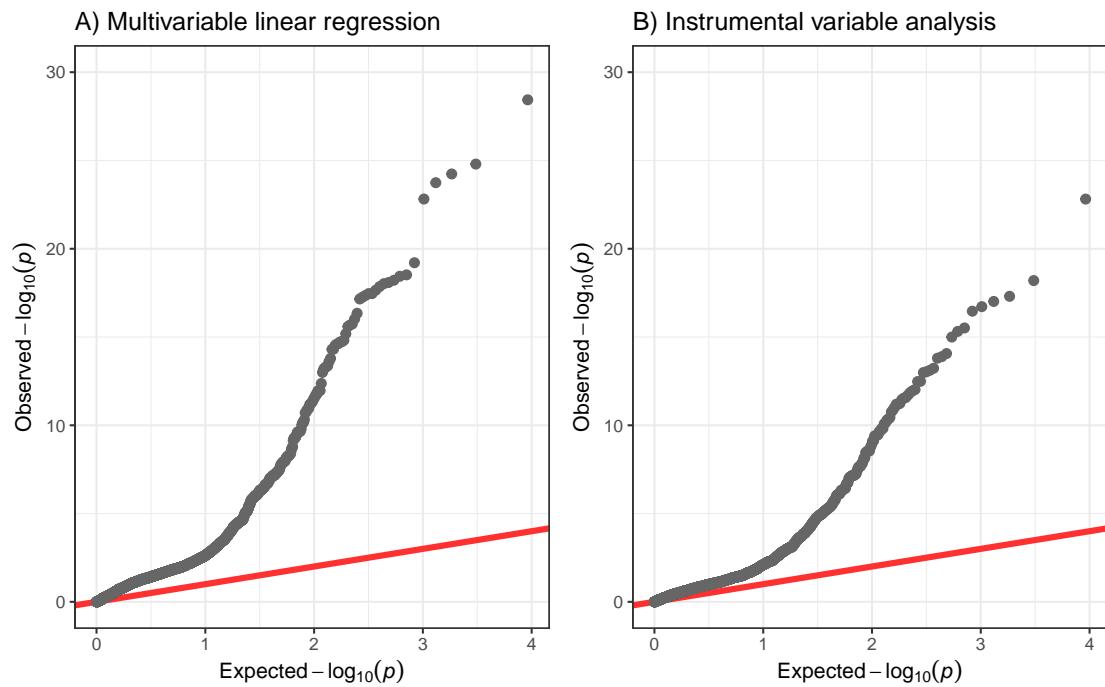


Figure 8.5: QQ-plots of the expected vs the observed $-\log_{10}(p\text{-values})$ for the BMI change-protein change effects. A) Multiple linear regression B) Instrumental variable two stage least squares analysis.

8.3.6 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 (8.6), 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 8.7. 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.

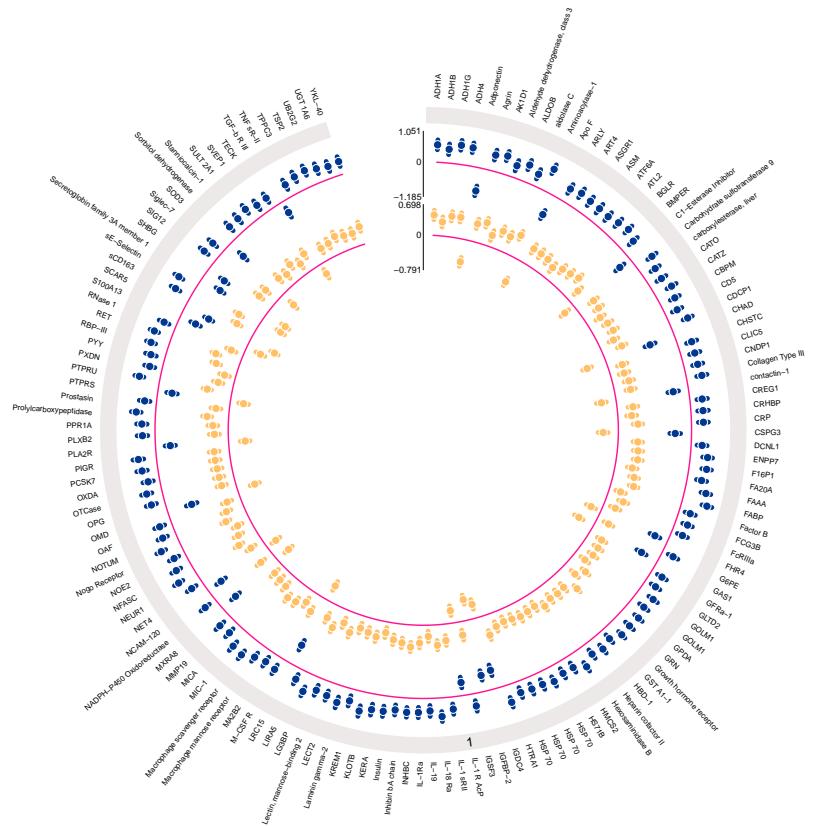


Figure 8.6: Circos plot displaying the protein change (SDs) per SD increase in BMI in DiRECT. Blue estimates are the two stage least squares estimates and the yellow estimates are from the multiple linear regression. Proteins presented were associated in both models at $p=5.8\times 10^{-5}$. Figure made using the EpiViz R package <https://github.com/mattlee821/EpiViz>.

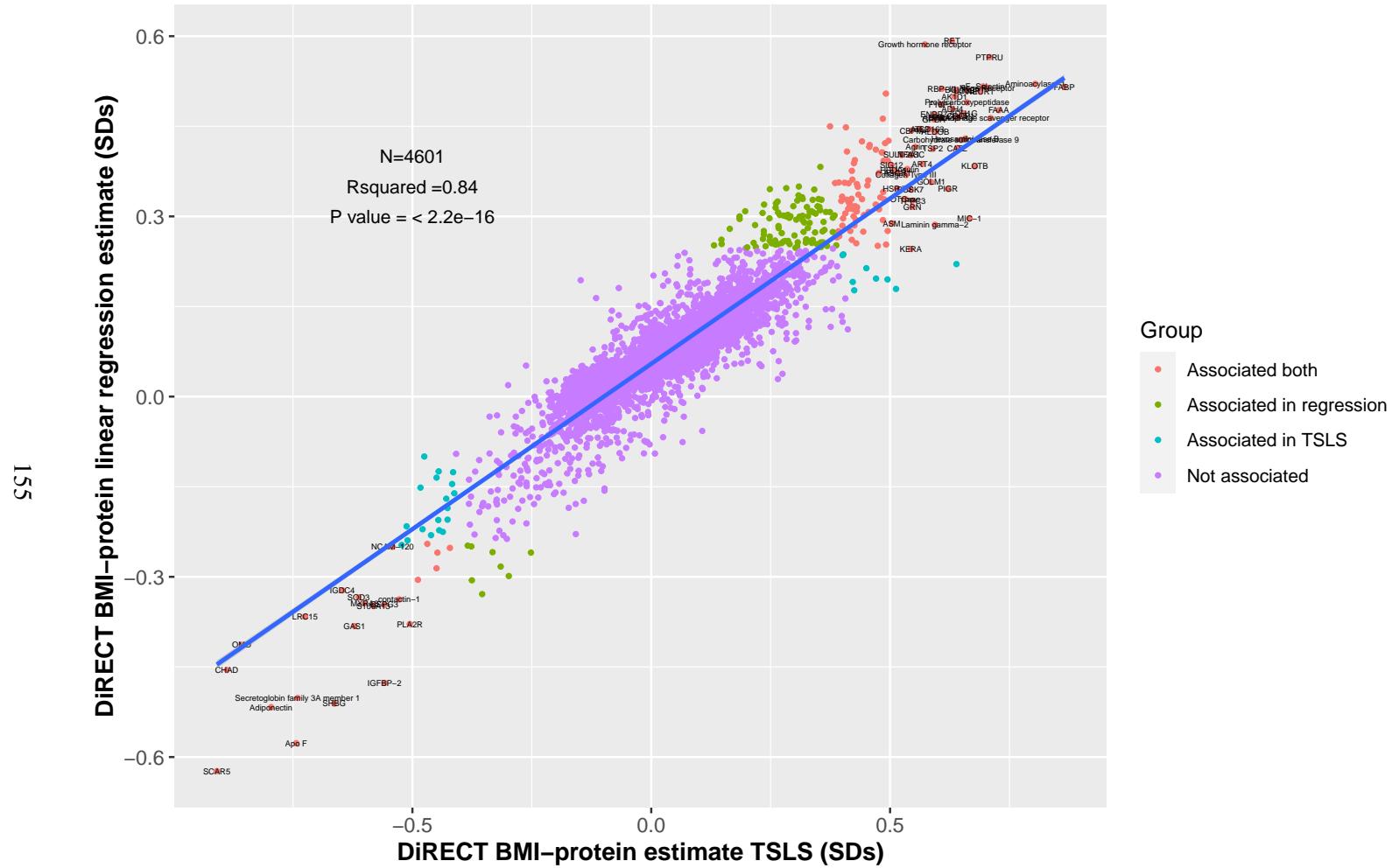
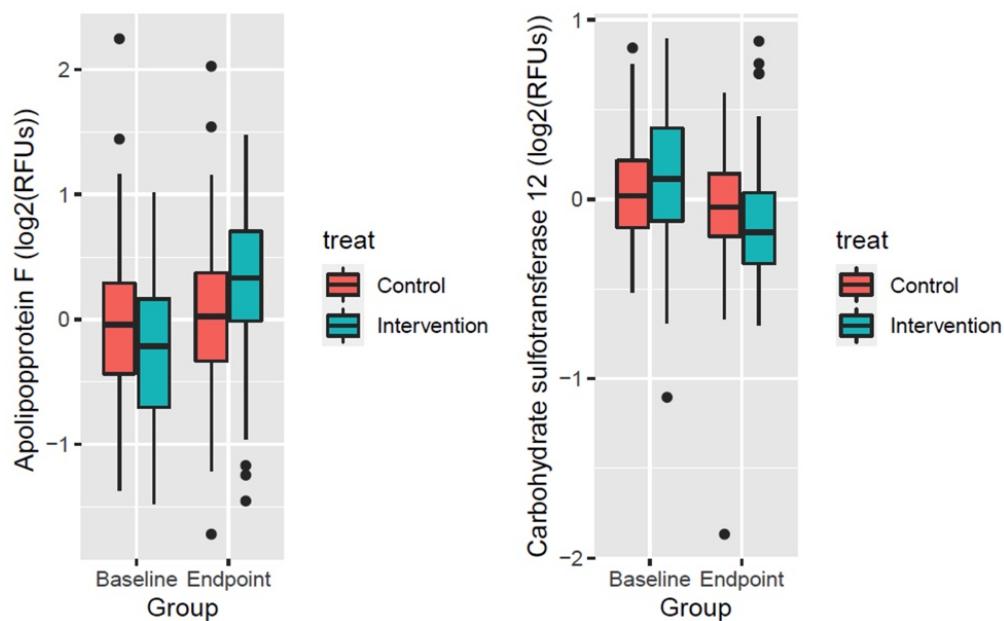


Figure 8.7: Scatter plot displaying the estimate for the change in protein (SDs) per SD increase in BMI in the two stage least squares and linear regression models. Points are coloured by which models the proteins were associated with BMI change ($p < 5.8 \times 10^{-5}$).

8.3.7 Sensitivity analysis

Proteins which were associated with BMI change were compared across control and intervention groups at baseline. 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 ?? displays the boxplots for the distribution of levels of these proteins at both baseline and endpoint for the intervention and control group.



8.3.8 Comparison of BMI effects across DiRECT and INTERVAL

BMI-protein estimates from this study were compared with effect estimates from INTERVAL (Chapter 7), 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 8.8 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.

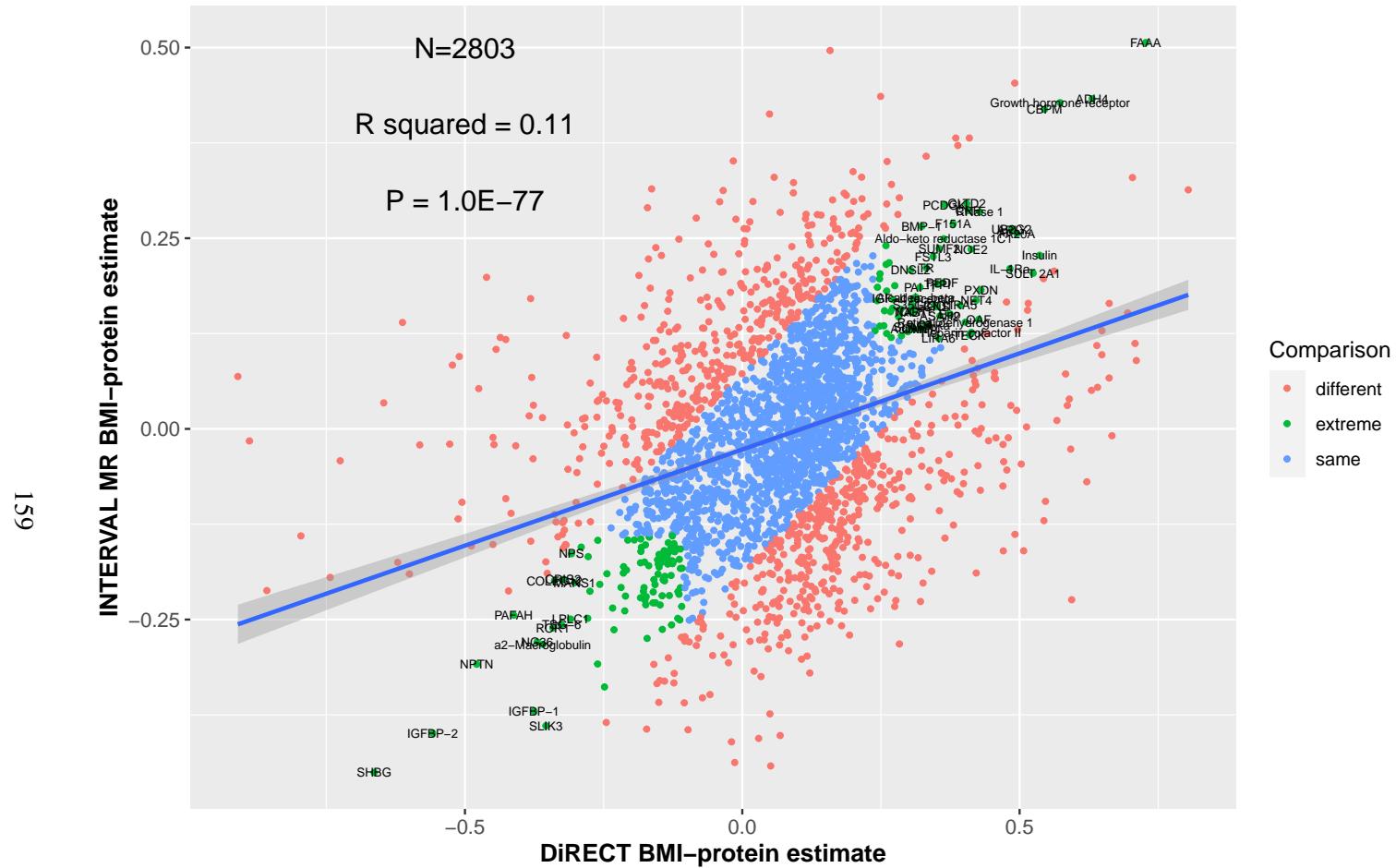


Figure 8.8: Scatter plot comparing the two stage least squares estimate for the effect of BMI change on protein change in DiRECT with the Mendelian randomisation estimate for the effect of BMI on protein levels in INTERVAL. Proteins that are similar across analyses with a large effect size in both studies 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.

8.3.9 Enrichment analysis

To explore disease class enrichment, DAVID Bioinformatics 6.8 was used.¹³³ 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. Using StringDB and inputting p values for the association between BMI change and each protein change, there was evidence that proteins altered by protein change in the linear regression model were involved in cytokine-cytokine receptor interaction and complement and coagulation cascades. This enrichment were associated with the “top of the input”, therefore indicating proteins with the smallest p values. There was enrichment for platelet activation, however the analysis indicated that this enrichment was at the bottom of the protein list, therefore more proteins involved in platelet activation were not associated with BMI change (data not shown). KEGG enrichment results were not available by inputting the ranked p values for the proteins from the instrumental variable analysis.

Table 8.2

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

8.4 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. Comparisons of protein effects across the different study designs (INTERVAL in Chapter 7) points towards proteins of interest which may be involved in the development or progression of obesity-associated cardiometabolic disease. Proteins which are only altered in one study design could also provide useful biological information as to how the individual protein may relate to adiposity.

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 8.3 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 SomaLogic,¹⁶⁶ 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.^{163,166–170} 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 cohort.¹⁵⁰ 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 disease,¹¹⁴ therefore this apolipoprotein could mediate the increased risk of thrombotic events seen with a higher BMI.

Table 8.3: 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, 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, 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, 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, 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, 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, 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 (WAT).¹⁷¹ 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.^{172,173} CHAD knockout mice show decreased bone strength compared with wildtype.¹⁷² 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 comparison of results from the previous chapter (Chapter 7), which used Mendelian randomization and observational associations to assess the effect of average higher BMI across a population on average protein levels, with results from a randomised control trial in the current chapter 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. 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.

Divergence in estimates across study designs may also provide some insight into the biology due to the nature of each study design. In general, the MR study design provides information on the effect on the proteome that a sustained high BMI has over the life course. In contrast,

the estimates in DiRECT are the effect that a relatively short-term weight loss intervention (1 year) has on the proteome. A protein 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. A protein may also not have evidence for a causal relationship in the MR but be altered in DiRECT. This could be because the protein is a signal of starvation or other changes that happen with caloric restriction.

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 remiss their diabetes. It is possible that the analysis is therefore picking up protein effects that are caused by a change in medication or diabetes remission, rather than as a result in loss of fat. Participants would have also lost muscle mass throughout the intervention,¹⁷⁴ 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 mass.¹⁷⁵ 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).

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 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 thrombosis. A summary of pathways explored in each results chapters is displayed in Figure 9.1, with a more detailed summary of findings in Table 9.1.

Overview of thesis hypotheses

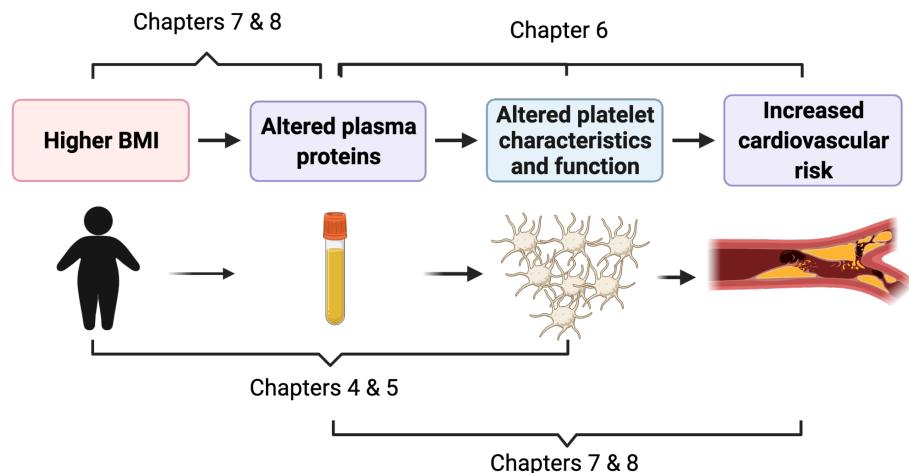


Figure 9.1: Schematic of the multi-step pathway addressed in the thesis with chapter numbers where associations are explored. Figure made using BioRender.com.

Table 9.1: Summary of main findings from the thesis

Chapters Main findings

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.

Laboratory experiments were optimised to assess platelet function from patients. In the clinical pilot study, there was no evidence for an effect of high BMI on platelet function, but there was evidence for a difference in levels of platelet proteins across groups. This study needs to be replicated with a larger N, controlling for confounders.

Main findings

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_q 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.

Weight loss as a result of caloric restriction has a substantial effect on the plasma proteome and protein levels altered as a result of higher BMI (Chapter 7) can be reversed with weight loss.

This thesis started with exploring the effect of BMI on platelet traits within a cohort of UK blood donors (INTERVAL) (Chapter 4). 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 5) 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 6 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 7 and 8 both aimed to explore

effects of adiposity on the plasma proteome. Chapter 7 used observational and MR frameworks and found causal evidence for an effect of BMI on the plasma proteome whereas Chapter 8 used a weight loss intervention to explore effects of BMI on the plasma proteome. There was consistent evidence for BMI effects on proteins across study designs and there was evidence that changes in such proteins were enriched for cardiovascular disease. Two of these chapters have been published in the International Journal of Obesity and Platelets journal, thereby highlighting the novelty of the contents of this thesis.

9.2 Strengths

As previously mentioned, 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.

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 participants.¹⁷⁶ 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 5, a major limitation is the sample size for the bariatric patient platelet study. The sample size (N=4) has limited power to detect associations, especially in experiments

which have more variability. Before the COVID-19 pandemic, the plan was to have bariatric patients with $\text{BMI} > 40 \text{ kg/m}^2$ 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 allow 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,^{76,177} 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 loss.¹⁷⁸ Therefore this study would have been one of the most in depth platelet function studies for bariatric surgery.

One of the limitations of the analyses performed in Chapter xx and Chapter xx 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.

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 4, 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 treatment.⁸¹

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 x and x 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. Further work on protein profiles is required, as it is more likely a change in various proteins mediates the risk.

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 4 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, 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 5, 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 would likely have the relevant variables 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 platelets,⁸¹ 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),¹⁷⁹ 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 both labour intensive to perform proteomics on thousands of people's platelets computationally intensive to perform GWAS for thousands of proteins.

The involvement of the chemokines MDC and TARC in platelet function was presented in Chapter xx, with Chapter 6 - Further signaling and functional work required. - These chemokines were measured within Chapter 7 - there was evidence for an association between BMI and MDC, and weak evidence for a causal effect ($p=0.07$). This did not pass multiple testing correction. It would be beneficial to look at these chemokines in other cohorts. In the DiRECT analysis, there was weak evidence that a reduction in weight reduces levels of MDC ($p=0.06$). Again, this did not pass multiple testing. The agreement in direction across the two studies (raised with higher BMI and reduced with weight loss), suggests that this effect could be real, however it may be a relatively small effect which may require more targeted proteomics. The effects were smaller for TARC, suggesting that if these effects are real, body mass index has a larger effect on MDC levels.

Chapter 7 The analyses in Chapter 7 provided evidence for a causal effect of BMI on a range of proteins. However, linear MR may not always be the most appropriate as there are often nonlinear relationships, for example between BMI and mortality, where there is a J-shaped relationship.¹⁸⁰ 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 do not allow the user to define their bounds of their strata and therefore makes it difficult to interpret results. The glsmr package was 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 has been provided in the Appendix A, with an example using the effect of BMI on levels of leptin. 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 Icelanders.¹⁸¹ Studies of this scale would help with stratifying into groups while maintaining power.

Chapter 8 The analyses in Chapter 8 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. 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 (from >40 gk/m²) to ..

- Utilise bariatric surgery data as a third line of evidence to explore effect of BMI/weight loss on the plasma proteome. Bring in that this will be performed as part of the transition fellowship.
- Work on proteomic networks to really determine key proteins in disease - this leads onto the transition fellowship.
- Instrument groups of proteins
- Two sample MR of proteins of interest to immature platelet count - .

9.6 Overall conclusion

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 data¹⁸² 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 micromolar, therefore this should be less of an issue. This possible nonlinear relationship has not been accounted for in the linear models and Mendelian randomisation analyses performed in Chapter 7. 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 possible

nonlinear relationship between BMI (in kg/m² so that it is biologically meaningful and effects can be related back to the NHS BMI categories) and levels of leptin.

A.1 Observational association between BMI and leptin

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/m² 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, R² for the linear model was 0.51 and it was 0.56 for the GAM, with a p value of (2.17x10⁻⁵⁹). This was the smallest p value comparing the two models for all 4,034 SOMAmers in INTERVAL.

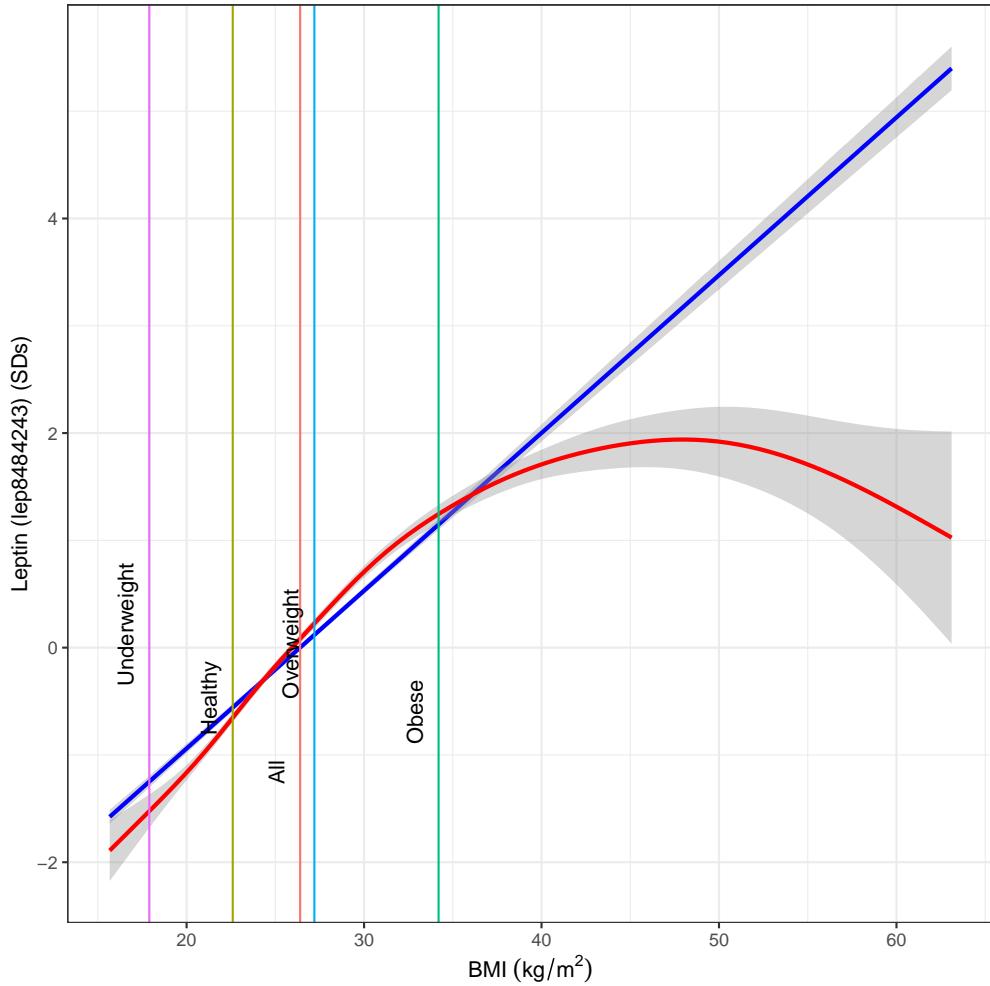


Figure A.1: Linear model (blue) and nonlinear generalized additive model (red) of the association between BMI and leptin with 95% confidence intervals. The vertical lines indicate the mean BMI for each BMI category (underweight, healthy, all, overweight, obese)

A.2 Determining nonlinear effects using MR

Nonlinear associations can be easily implemented using observational associations. However due to the nature of MR, regressing BMI on the genetic risk score provided estimates of genetically predicted BMI. The protein was then regressed on this genetically predicted BMI 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 this causal effect over the full range of BMI observed in the

population.

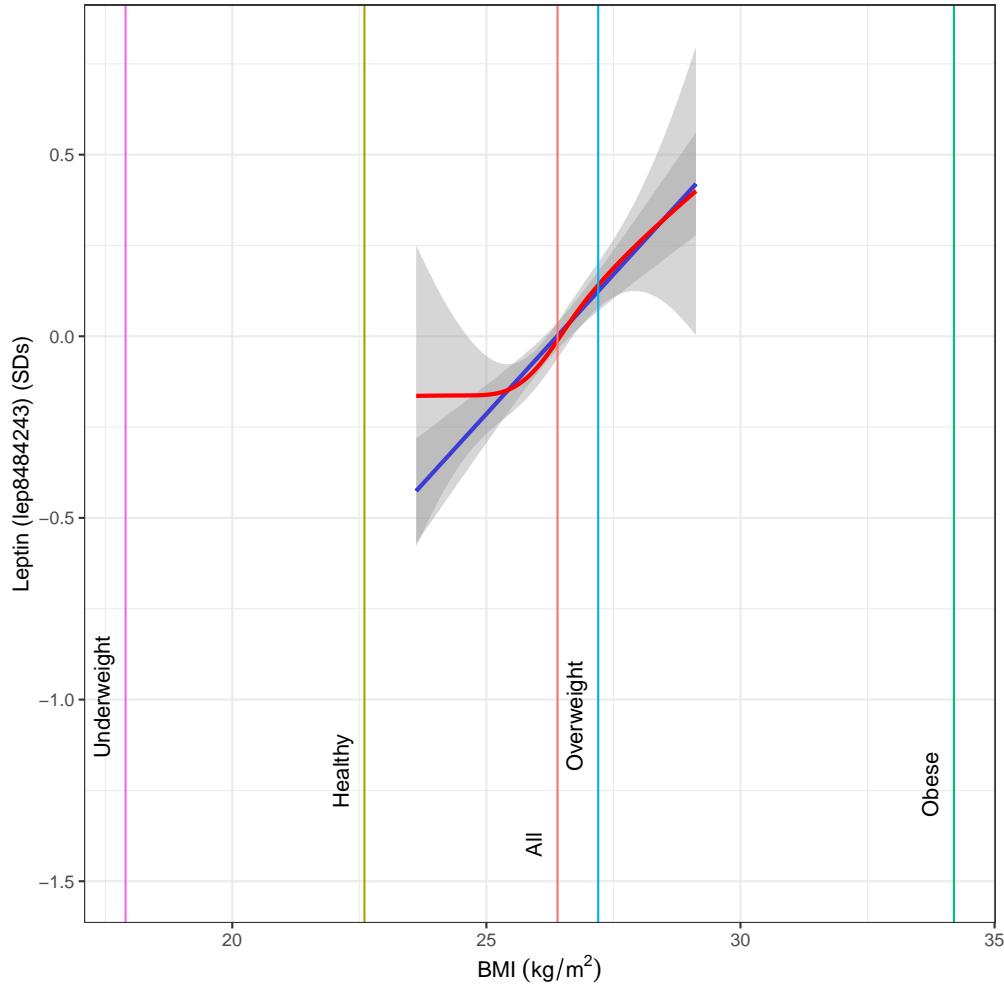


Figure A.2: Linear model and generalized additive model of the association between BMI and leptin using Mendelian randomization. Linear model (blue) and generalized additive model (red) of the association between BMI and leptin with 95% confidence intervals. The vertical lines indicate the mean BMI for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

A.3 Using nonlinear MR package

The nonlinear MR package (“nlmr”) was also be utilised. This package utilises two methods: fractional polynomials and piecewise MR.¹⁸³ 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, $p=7.00\times 10^{-5}$). For the fifth quantile, the estimate is 0.12 (95% CI 0.04 to 0.20, $p=0.0020$).

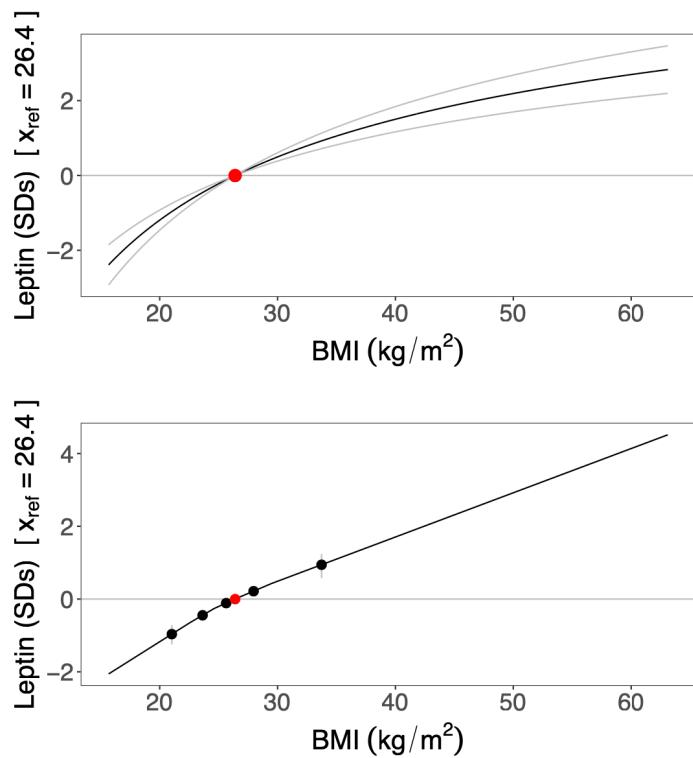


Figure A.3: Effects of BMI on levels of leptin using nonlinear MR from the nlmr R package.¹⁸³ A) fractional polynomial MR. B) Piecewise MR.

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 BMIs 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/hughesvoanth/glsmr>. The effects of BMI on leptin levels were further explored using this package. For this analysis the strata were defined as $18.5\text{-}25 \text{ kg/m}^2$ (healthy), $25\text{-}30\text{kg/m}^2$ (overweight) and $30\text{-}40\text{kg/m}^2$ (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. 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 with the healthy BMI group. The estimates also became smaller in magnitude in the MR (Figure A.4D)

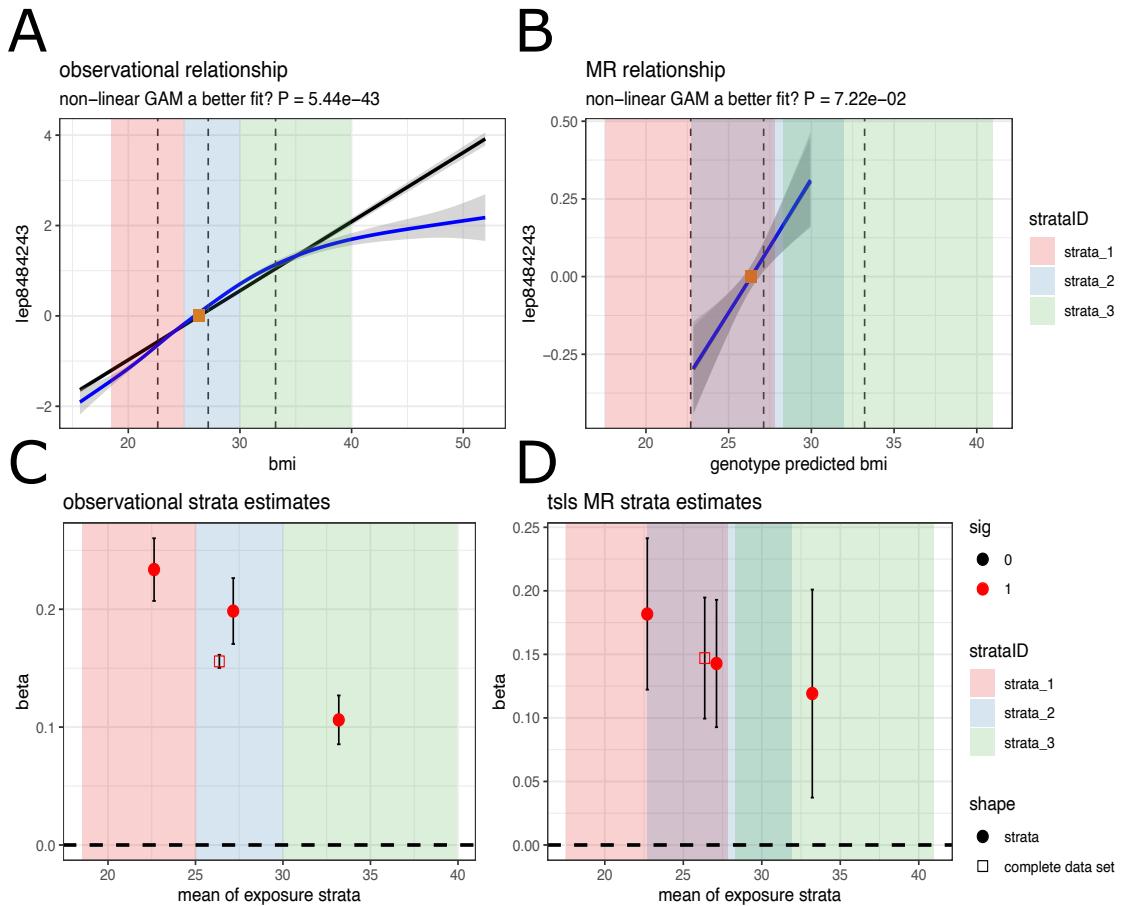


Figure A.4: Observational and Mendelian randomization estimates for the effect of BMI (kg/m^2) and leptin (SDs) stratified by BMI category. Shaded areas display the ranges of BMI of each stratum: 18.5-25 (red), 25-30 (blue), 30-40 (green).
 A) Comparison between the fit of the nonlinear and linear generalized 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.

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Abbreviations

```
```{r abbreviations, echo=FALSE} library(readxl) library(tidyr) library(kableExtra)
install.packages("knitr") library(knitr) abbreviations <- read_xlsx("figure/Abbreviations/Abbreviation")
abbreviations <- abbreviations[order(abbreviations$Abbreviation),]

knitr::kable(abbreviations, longtable = T, booktabs = T, format = "latex", escape = F,
caption = "Full list of abbreviations") %>% kable_classic_2(full_width = F) %>%
kable_styling(full_width = TRUE) %>% column_spec(1, width = "3cm") ``
```