



University
of Glasgow

McNeill, Susan Heather (2026) *An investigation of the pathophysiology underlying the prothrombotic effect of obesity during pregnancy*. MD thesis.

<https://theses.gla.ac.uk/86270/>

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses

<https://theses.gla.ac.uk/>
research-enlighten@glasgow.ac.uk

An Investigation of the Pathophysiology Underlying the Prothrombotic Effect of Obesity During Pregnancy

Dr Susan Heather McNeill

MBChB, MRCP, FRCPath

Submitted in fulfilment of the requirements for Doctor of Medicine

Institute of Cardiovascular Medicine

College of MVLS

University of Glasgow

June 2025

Abstract

Obesity has been over-represented in the UK-MBRRACE maternal mortality reports, with 73% of VTE-related deaths in between 2019-2021 occurring in women who were either overweight or obese. The pathophysiology underlying the increased thrombotic risk in overweight and obese pregnant women is poorly understood. In the non-pregnant obese population, a chronic inflammatory state, dysfunctional adipocytes and adipose tissue distribution are thought to contribute to the prothrombotic state. Whether these elements also contribute to increased thrombotic risk in the obese pregnant population is yet to be determined

This thesis aimed to investigate, in a prospective observational study, the pathophysiology underlying the prothrombotic state in obese pregnant women. Blood samples and anthropomorphic measurements (BMI, waist and hip circumference, SAT and VAT thickness) were obtained from 262 pregnant women (lean n=109, overweight/obese class I n=115, obese class II n=24, obese class III n=14) at the booking appointment (means 10.2 weeks' gestation). Adipokines (leptin, adiponectin, IL-6, TNF- α and IL-1 β) and adipose tissue distribution were correlated with thrombin generation and other laboratory measures of the prothrombotic state (FVIII, fibrinogen Clauss, D-dimer, PAI-1, protein S and TFPI).

BMI at the booking appointment had a significant positive association with thrombin generation, as assessed by ETP ($p=0.0058$). BMI was also positively associated with fibrinogen ($p<0.0001$), TFPI ($p=0.002$) and PAI-1 ($p=0.039$). There a significant interaction between time x BMI with ETP ($p=0.0498$), TFPI ($p=0.047$) and PAI-1 ($p<0.0001$) which, together with the data from the booking appointment, reflected that obese pregnant women have a more prolonged prothrombotic state compared to their lean counterparts. Waist circumference, SAT and IL-6 were able to explain 32.01% of variance in fibrinogen at booking ($p<0.0001$) whilst BMI, SAT and leptin were able to predict 13.62% of variance in ETP at booking ($p<0.0001$).

The increased risk of VTE for obese pregnant women may relate to the more prolonged prothrombotic state, with greater loss of naturally occurring

anticoagulants. Central obesity, as measured by waist circumference, and SAT, may be driving the procoagulant state via leptin and IL-6.

Table of Contents

Abstract	2
List of Tables	8
List of Figures	10
Acknowledgements	15
Author's Declaration	16
Definitions/Abbreviations	17
Chapter 1 Introduction	18
1.1 <i>Epidemiology of VTE in pregnancy</i>	18
1.2 <i>Pathophysiology of VTE</i>	19
1.3 <i>Risk Factors for VTE</i>	20
1.4 <i>Assessing and managing the risk of VTE during pregnancy</i>	22
1.5 <i>Pharmacological prevention of VTE during pregnancy</i>	23
1.6 <i>Normal haemostasis in the non-pregnant population</i>	25
1.6.1 Procoagulant factors	26
1.6.2 Naturally occurring anticoagulants	27
1.6.3 Fibrinolysis	28
1.7 <i>Laboratory assessment of the prothrombotic state</i>	28
1.8 <i>Haemostatic changes during normal pregnancy</i>	31
1.8.1 Procoagulant factors during pregnancy	31
1.8.2 Naturally occurring anticoagulants during pregnancy	32
1.8.3 Fibrinolysis during pregnancy	33
1.9 <i>Haemostatic changes associated with obesity in the non-pregnant population</i>	34
1.10 <i>Obesity</i>	35
1.10.1 Assessment of obesity	35
1.10.2 Adipose tissue depots	36
1.10.3 Adipocytes and normal expansion/function	37
1.10.4 Adipose tissue dysfunction in obesity	38
1.11 <i>Adipose tissue distribution changes in healthy and obese pregnancy</i>	41
1.11.1 Adipose tissue depots in healthy and obese pregnancy	41
1.11.2 Adipocyte dysfunction in obese pregnancy	42
1.11.3 Cardiometabolic changes in healthy and obese pregnancy	43
1.12 <i>Obesity as a prothrombotic state</i>	44
1.12.1 Role of leptin in the pathophysiology of VTE	45
1.12.2 Role of adiponectin in the pathophysiology of VTE	47
1.12.3 Role of chronic inflammation in the pathophysiology of VTE	47
1.12.4 Tissue factor-bearing microparticles and the prothrombotic state	48
1.12.5 Adipose tissue depot and thrombotic risk	49
1.13 <i>The prothrombotic effect of obesity during pregnancy</i>	49
1.13.1 Thrombin generation data in obese pregnancy	50
1.14 <i>Hypothesis</i>	51
1.14.1 Aims	52
Chapter 2 Materials and Methods	55
2.1 <i>Introduction</i>	55

2.2	<i>Study Design</i>	55
2.2.1	Ethical considerations	55
2.2.2	Study outline and recruitment considerations	56
2.2.3	Inclusion/Exclusion criteria	59
2.2.4	Power Calculation	60
2.2.5	Loss to Follow-up	63
2.2.6	Study recruitment.	65
2.2.7	Data and sample collection	66
2.3	<i>Laboratory Methodologies</i>	69
2.3.1	Haemostasis analyses of plasma samples	69
2.4	<i>Thrombin Generation</i>	73
2.4.1	Thrombin Generation Reagents	75
2.4.2	Thrombin Generation Method.....	77
2.4.3	Thrombin Generation Reference Plasma and Coefficient of Variation	79
2.5	<i>ELISA</i>	80
2.5.1	ELISA Principle	80
2.5.2	Adiponectin.....	81
2.5.3	Leptin	83
2.5.4	PAI-1.....	85
2.5.5	TFPI.....	86
2.5.6	IL-6	87
2.5.7	IL-1 β	89
2.5.8	TNF- α	90
2.5.9	ELISA Coefficients of Variation (CV%).....	93
2.6	<i>Statistical Analysis</i>	94
Chapter 3	The prothrombotic effect of obesity during pregnancy	95
3.1	<i>Introduction</i>	95
3.1.1	Routine assessment of the thrombotic state in clinical practice	96
3.1.2	Thrombin generation as a biomarker for thrombotic risk in pregnancy	97
3.1.3	Hypothesis and Aims	98
3.1.4	Specific research questions	99
3.2	<i>Methods</i>	100
3.2.1	Study Recruitment.....	100
3.2.2	Laboratory Methods	100
3.2.3	Statistical Methods	101
3.3	<i>Results</i>	102
3.3.1	Study population demographics	102
3.3.2	Procoagulant Tests	108
3.3.3	Inhibitors of Coagulation	112
3.3.4	Fibrinolysis	114
3.3.5	Thrombin Generation	116
3.4	<i>Discussion</i>	120
3.4.1	Main findings	121
3.4.2	Secondary findings: Pregnancy outcomes and relationship with obesity	124
3.4.3	Limitations	125
3.5	<i>Summary</i>	126
Chapter 4	Adipokine changes during pregnancy in women of different BMI and their relationship to markers of haemostasis	128
4.1	<i>Introduction</i>	128
4.1.1	Dysfunctional, pro-inflammatory adipose tissue in obese pregnancy	128
4.1.2	Endothelial dysfunction in obese pregnancy.....	129
4.1.3	Relating markers of the procoagulant state to dysfunctional adipose tissue and chronic low-grade inflammation in obese pregnancy	130
4.2	<i>Hypothesis, Aims and Objectives</i>	132

4.2.1	Aims	132
4.2.2	Specific Research Questions	132
4.3	<i>Methods</i>	133
4.3.1	Study Recruitment.....	133
4.3.2	Laboratory Methods	133
4.3.3	Statistical Methods	133
4.4	<i>Results</i>	135
4.4.1	Leptin	135
4.4.2	Adiponectin.....	138
4.4.3	IL-6	141
4.4.4	TNF- α	145
4.4.5	IL-1B.....	146
4.5	<i>Discussion</i>	148
4.5.1	Main findings	148
4.5.2	Evidence of dysfunctional adipose tissue in obese pregnancy	149
4.5.3	Relationship between adipokines and haemostasis.....	149
4.5.4	Summary	152
4.5.5	Limitations	153
Chapter 5	Adipose tissue distribution and the prothrombotic state in pregnancy	155
5.1	<i>Background</i>	155
5.1.1	Influence of body shape on thrombotic risk outwith pregnancy	155
5.1.2	Influence of adipose tissue depots on cardiometabolic and thromboembolic risk	156
5.2	<i>Aims and Objectives</i>	157
5.2.1	Hypothesis	157
5.2.2	Aims	157
5.2.3	Specific Research Questions	157
5.3	<i>Methods</i>	158
5.3.1	Study recruitment	158
5.3.2	Anthropometric methods	159
5.3.3	Statistical methods.....	160
5.4	<i>Results</i>	163
5.4.1	Comparison of anthropomorphic measurements of adipose tissue distribution and body mass index.....	163
5.5	<i>Relationship between adipokines and measurements of adipose tissue distribution</i>	168
5.5.1	Leptin and adipose tissue distribution.....	168
5.5.2	Adiponectin and adipose tissue distribution	171
5.5.3	TNF- α and adipose tissue distribution	174
5.5.4	IL-6 and adipose tissue distribution.....	176
5.5.5	IL-1B and adipose tissue distribution	179
5.6	<i>Relationship between haemostasis parameters and measurements of adipose tissue distribution</i>	180
5.6.1	FVIII and adipose tissue distribution	180
5.6.2	Fibrinogen and adipose tissue distribution.....	181
5.6.3	D-dimer and adipose tissue distribution.....	185
5.6.4	TFPI and adipose tissue distribution	186
5.6.5	Protein S and adipose tissue distribution	189
5.6.6	PAI-1 and adipose tissue distribution	191
5.7	<i>Relationship between thrombin generation and measurements of adipose tissue distribution</i>	194
5.7.1	Lagtime and adipose tissue distribution.....	194
5.7.2	Peak thrombin and adipose tissue distribution.....	196
5.7.3	ETP and adipose tissue distribution.....	199
5.7.4	Time to peak (ttP) and adipose tissue distribution	201
5.7.5	Velocity Index and adipose tissue distribution	202
5.8	<i>Discussion</i>	205

5.8.1	Main findings	205
5.8.2	Relationship between measures of adipose tissue distribution and BMI	207
5.8.3	Relationship between measures of adipose tissue distribution and adipokines	209
5.8.4	Relationship between measures of adipose tissue distribution and markers of haemostasis	210
5.8.5	Relationship between measures of adipose tissue distribution and thrombin generation.....	211
5.8.6	Limitations	212
5.8.7	Summary	214
Chapter 6	The pathophysiology underlying the prothrombotic state in obese pregnant women	219
6.1	<i>Background</i>	219
6.2	<i>Aims and Objectives</i>	220
6.2.1	Aims	220
6.2.2	Hypothesis	220
6.2.3	Specific Research Questions	220
6.3	<i>Methods</i>	221
6.3.1	Study population.....	221
6.3.2	Statistical analyses	221
6.4	<i>Results</i>	222
6.4.1	Predictive models for fibrinogen, TFPI and PAI-1.....	222
6.4.2	Predictive model for ETP using significant adipokine and anthropometric parameters	225
6.5	<i>Proposed pathophysiological pathways for the development of the prothrombotic state in obese pregnant women</i>	225
6.5.1	Analysis of the SAT-ETP pathway.....	228
6.5.2	Analysis of the SAT-fibrinogen pathway.....	228
6.5.3	Analysis of the VAT-fibrinogen pathway.....	229
6.6	<i>Discussion</i>	230
6.6.1	Proposed pro-coagulant pathways in obese pregnancy	230
6.6.2	Limitations	230
6.6.3	Summary	231
Chapter 7	Discussion	233
7.1	<i>Main findings</i>	234
7.2	<i>Conclusions</i>	239
7.3	<i>Limitations</i>	240
7.3.1	Study design limitations	240
7.3.2	Limitations related to loss to follow-up and COVID-19 pandemic	241
7.4	<i>Implications on clinical practice and future research</i>	241
Appendix 1	Participant Information Leaflet	244
Appendix 2	Consent form	250
Appendix 3	Demographics form	251
Appendix 4	USS data collection form	252
References	253	

List of Tables

Table 1 Royal College of Obstetricians and Gynaecologists Green Top Guideline 37a VTE risk assessment tool	22
Table 2 IPOP study BMI groups and WHO BMI classification system.	56
Table 3 Study inclusion and exclusion criteria	60
Table 4 Loss to follow-up.....	64
Table 5 Example thrombin generation plate plan.	78
Table 6 Thrombin generation inter-assay coefficient of variation (%).	80
Table 7 Example ELISA plate plan.....	81
Table 8 ELISA control coefficient of variation (CV%)	93
Table 9 Study population characteristics by BMI group	103
Table 10 Pregnancy outcomes by BMI group.....	105
Table 11 Leptin associations with haemostasis parameters and thrombin generation.	136
Table 12 Multiple regression modelling to ascertain the contribution of IL-6 in mediating the prothrombotic effects of leptin.	138
Table 13 Adiponectin associations with haemostasis parameter and thrombin generation.	140
Table 14 IL-6 associations with haemostasis parameter and thrombin generation.	143
Table 15 TNF- α associations with haemostasis parameter and thrombin generation.	146
Table 16 IL-1 β associations with haemostasis parameter and thrombin generation.	148
Table 17 SAT and VAT quartile divisions	161
Table 18 Adipose tissue distribution multiple regression model definitions.....	162
Table 19 Leptin and adipose tissue distribution multiple regression model construction.	170
Table 20 Adiponectin and adipose tissue distribution multiple regression model construction.	173
Table 21 TNF- α and adipose tissue distribution multiple regression model construction.	175
Table 22 IL-6 and adipose tissue distribution multiple regression model construction.	178

Table 23 Fibrinogen and adipose tissue distribution multiple regression model construction.	184
Table 24 TFPI and adipose tissue distribution multiple regression model construction.	188
Table 25 PAI-1 and adipose tissue distribution multiple regression model construction.	193
Table 26 Lagtime and adipose tissue distribution multiple regression model construction.	196
Table 27 Peak thrombin and adipose tissue distribution multiple regression model construction.....	198
Table 28 ETP and adipose tissue distribution multiple regression model construction.	201
Table 29 Velocity index and adipose tissue distribution multiple regression model construction.	205
Table 30 Multiple regression model for prediction of variability in fibrinogen at booking.....	222
Table 31 Multiple regression model for prediction of variability in TFPI at booking.....	223
Table 32 Multiple regression model for prediction of variability in PAI-1 at booking.....	224
Table 33 Multiple regression model for prediction of variability in ETP at booking.....	225
Table 34 Multiple regression analysis of SAT-ETP pathway.....	228
Table 35 Multiple regression analysis of SAT-fibrinogen pathway.....	229
Table 36 Multiple regression analysis of VAT-fibrinogen pathway	229

List of Figures

Figure 2-1 Virchow's triad.	20
Figure 2-2 Coagulation cascade	26
Figure 2-3 Thrombogram with measurement parameters.	30
Figure 2-1 Study design flowchart.	58
Figure 2-2 Consort diagram of study design and recruitment, including loss to follow-up	63
Figure 2-3 Blood sample requirements for each study visit	67
Figure 2-4 Anatomical landmarks on cross-sectional view for measurement of abdominal visceral and subcutaneous adipose tissue.	69
Figure 2-5 Coagulation cascade	71
Figure 2-6 Thrombogram with measurement parameters.	75
Figure 2-7 Adiponectin ELISA standard curve	83
Figure 2-8 Leptin ELISA standard curve	84
Figure 2-9 PAI-1 ELISA standard curve.....	86
Figure 2-10 TFPI ELISA standard curve	87
Figure 2-11 IL-6 ELISA standard curve	89
Figure 2-12 IL-1 β ELISA standard curve	90
Figure 2-13 TNF- α ELISA standard curve	92
Figure 3-1 PT at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).....	108
Figure 3-2 APTT at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).....	109
Figure 3-3 Fibrinogen Clauss at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).....	110
Figure 3-4 Factor VIII at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).....	111
Figure 3-5 Protein S at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).....	112
Figure 3-6 TFPI at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).....	113
Figure 3-7 PAI-1 (ng/mL) raw data, median and IQR, at booking and 28 weeks by BMI group.	114

Figure 3-8 D-dimer at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).....	115
Figure 3-9 Lagtime (1pM tissue factor + thrombomodulin) at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).....	116
Figure 3-10 Peak thrombin (1pM tissue factor + thrombomodulin) at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).	117
Figure 3-11 ETP (1pM tissue factor + thrombomodulin) at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed)..	118
Figure 3-12 ttP (1pM tissue factor + thrombomodulin) at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).	119
Figure 3-13 Velocity index (1pM tissue factor + thrombomodulin) at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).	120
Figure 3-14 Approximation of ETP across pregnancy and the puerperium in lean and obese.....	124
Figure 4-1 Leptin at booking and 28 weeks' gestation (raw data, median and IQR), subdivided by BMI group.....	135
Figure 4-2 Correlations between log-leptin and a) log-IL-6 b) log-fibrinogen c) log-ETP.	137
Figure 4-3 Adiponectin at booking and 28 weeks' gestation (raw data, median and IQR), subdivided by BMI group.....	139
Figure 4-4 Correlations between log-adiponectin and a) log-IL-6 b) log-PAI-1 c) log-lagtime.	141
Figure 4-5 IL-6 at booking and 28 weeks' gestation (raw data, median and IQR), subdivided by BMI group.....	142
Figure 4-6 Correlations between log-IL-6 and a) log-TNF- α b) log-fibrinogen c) log-TFPI d) log-lagtime.	144
Figure 4-7 TNF- α at booking and 28 weeks' gestation (raw data, median and IQR), subdivided by BMI group.....	145
Figure 4-8 IL-1 β at booking and 28 weeks' gestation, subdivided by BMI group.	147
Figure 4-9 Adipokine involvement in the prothrombotic state in obese pregnancy.	152
Figure 5-1 Consort diagram of IPOP study recruitment.	159

Figure 5-2 Anatomical landmarks on cross-sectional view for measurement of abdominal visceral and subcutaneous adipose tissue.	160
Figure 5-3 Correlation between BMI and waist circumference; correlation between BMI and hip circumference.	163
Figure 5-4 Correlation between waist:hip ratio (WHR) and BMI.	164
Figure 5-5 Correlation between BMI and (a) SAT and (b) VAT	165
Figure 5-6 Stacked bar chart depicting percentage frequency of each SAT and VAT quartile within each BMI group.	166
Figure 5-7 Frequency of VAT quartile associated with SAT quartiles	167
Figure 5-8 Non-linear regression log SAT vs log VAT.	167
Figure 5-9 Correlation between log-leptin and (a) log-SAT and (b) log-VAT.....	168
Figure 5-10 Correlation between log-leptin and (a) waist circumference and (b) hip circumference.	169
Figure 5-11 Correlation between adiponectin and (a) SAT, and (b) VAT.....	171
Figure 5-12 Correlation between log-adiponectin and waist:hip ratio	172
Figure 5-13 Correlation between log-TNF- α and (a) log-SAT, and (b) log-VAT..	174
Figure 5-14 Correlation between TNF- α and (a) waist circumference and (b) hip circumference.	175
Figure 5-15 Correlation between log IL-6 and (a) log-SAT, and (b) log-VAT.	176
Figure 5-16 Correlation between IL-6 and (a) waist circumference and (b) hip circumference.	177
Figure 5-17 Correlation between waist:hip ratio and IL-6.....	177
Figure 5-18 Correlation between IL-1 β and (a) SAT and (b) VAT.	179
Figure 5-19 Correlation between IL-1 β and (a) waist circumference and (b) hip circumference.	180
Figure 5-20 Correlation between log-FVIII and (a) log-SAT and (b) log-VAT.	180
Figure 5-21 Correlation between log-FVIII and (a) log-waist circumference and (b) log-hip circumference.	181
Figure 5-22 Correlation between log-fibrinogen Clauss and (a) log-SAT and (b) log-VAT.....	181
Figure 5-23 Correlation between log-fibrinogen and (a) log-waist circumference and (b) log-hip circumference.	182
Figure 5-24 log-fibrinogen subdivided by waist:hip ratio (healthy vs unhealthy).	183

Figure 5-25 Correlation between log-D dimer and (a) log-SAT and (b) log-VAT.	185
Figure 5-27 Correlation between log-D-dimer and (a) waist circumference and (b) hip circumference.	185
Figure 5-28 Correlation between log-TFPI and (a) log-SAT and (b) log-VAT.	186
Figure 5-29 Correlation between log-TFPI and (a) log-waist circumference and (b) log-hip circumference.	187
Figure 5-30 Correlation between log-protein S and (a) log-SAT and (b) log-VAT.	189
Figure 5-31 Correlation between log-protein S and (a) log-waist circumference and (b) log-hip circumference.	190
Figure 5-32 Correlation between log-PAI-1 and (a) log-SAT and (b) log-VAT. ...	191
Figure 5-33 Correlation between log-PAI-1 and (a) log-waist circumference and (b) log-hip circumference.	192
Figure 5-34 Pearson correlation between log-lagtime and (a) log-SAT and (b) log-VAT.	194
Figure 5-35 Correlation between log-lagtime and (a) log-waist circumference and (b) log-hip-circumference.	195
Figure 5-36 Correlation between log-peak thrombin and (a) log-SAT and (b) log-VAT.	197
Figure 5-37 Correlation between log-peak thrombin and (a) log-waist circumference and (b) log-hip circumference.	197
Figure 5-38 Correlation between log-ETP and (a) log-SAT and (b) log-VAT.....	199
Figure 5-39 Correlation between log-ETP and (a) log-waist circumference and (b) log-hip circumference.	200
Figure 5-40 Correlation between log-time to peak (ttP) and (a) log-SAT and (b) log-VAT.....	202
Figure 5-41 Correlation between log-time to peak (ttP) and (a) log-waist circumference and (b) log-hip circumference.	202
Figure 5-42 Correlation between log-velocity index and (a) log-SAT and (b) log-VAT.	203
Figure 5-43 Correlation between log-velocity index and (a) log-waist circumference and (b) log-hip circumference.	204
Figure 5-44 Associations between anthropomorphic measurements and markers of haemostasis and adipokines. Each variable is associated independently of BMI.	

Variables which showed a threshold effect also included and denoted by asterisk.....	207
Figure 5-45 Adipose tissue distribution in obese pregnancy and association with the prothrombotic state.....	217
Figure 6-1 Pathophysiology underlying the increased risk of thrombosis in obese pregnant women.....	227
Figure 7-1 Schematic representing proposed changes in procoagulant and anticoagulant substances in lean and obese pregnant women.	235
Figure 7-2 Schematic representation of changes in procoagulant state in lean and obese women during pregnancy.....	236

Acknowledgements

I would like to thank my supervisors, Dr Dilys Freeman and Dr Catherine Bagot, for their support and guidance throughout the entirety of my research period. Particular acknowledgement to Dilys for her endless patience explaining statistics to me; and to Catherine who inspired me with her enthusiasm for thrombosis research to undertake this study and gained the funding to make it possible. Thank you to the British Heart Foundation for funding this research and supporting me with a Clinical Research Training Fellowship.

I would like to thank Caroline Lawrence and Dr Vivienne Gibson from the Haemostasis Laboratory at Glasgow Royal Infirmary for training me in the haemostasis laboratory tests, thrombin generation and ELISA methodology and for their invaluable assistance in setting up the study. Thank you also to the Haemostasis lab MLAs, in particular, Ronnie Bailey who helped to process and store the study samples, allowing me more time to run the clinical side of the study.

Thank you to Mrs Fiona Jordan who carried out the adipokine ELISAs at the University of Glasgow, allowing the research to progress whilst I was on maternity leave. And also, to Dr Angela Kanny who assisted in outcome data collection.

I would also like to thank the midwives of the antenatal clinic at the Princess Royal Maternity Hospital, along with the CRF midwives without whom I could not have recruited study participants so efficiently.

Finally, I would like to thank my children, Charlotte and Jacob, for their patience whilst I have written up my thesis, and special thank you to my husband Terry for his invaluable support in keeping everyone entertained at home during my oncall weekends and research days, and occasional useful thesis-writing tips!

Author's Declaration

"I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution."

Printed Name: _____Susan McNeill_____

Signature: _____June 2025_____

Definitions/Abbreviations

Abbreviation	Definition
APTT	activated prothrombin time
ATM	adipose tissue macrophage
BMI	body mass index
DSAT	deep subcutaneous adipose tissue
ETP	endogenous thrombin potential
GDM	gestational diabetes mellitus
IL-1 β	interleukin 1-beta
IL-6	interleukin-6
PT	prothrombin time
SAT	subcutaneous adipose tissue
SSAT	superficial subcutaneous adipose tissue
TNF- α	tissue necrosis factor alpha
ttP	time to peak
VAT	visceral adipose tissue
WHR	waist:hip ratio

Chapter 1 Introduction

1.1 Epidemiology of VTE in pregnancy

Venous thromboembolism (VTE) affects approximately 1 in 1000 pregnancies in the United Kingdom, and has remained the leading direct cause of maternal mortality for more than a decade. (Knight M *et al.*, 2021; Sultan *et al.*, 2012; Heit *et al.*, 2005; Pomp *et al.*, 2008) The most recent MBRRACE-UK report, the triennial confidential enquiry into maternal deaths during the period 2021-23, identified the rate of thromboembolic deaths in pregnancy to be more than twice that of any other direct cause of maternal death (2.1 per 100,000 pregnancies). Black women were at significantly greater risk of death (OR 6.51 vs 2.33) from VTE compared to white women, and overall obese women (BMI ≥ 30) constituted more than 30% of women who died from causes directly related to pregnancy. (Felker A, 2025)

Pregnancy is recognised as a prothrombotic state, with the risk of antenatal VTE 4-6 times higher than that of the age and sex-matched general population. The risk in the postnatal period is even further increased at 60 times that of age and sex-matched non-pregnant controls. (Pomp *et al.*, 2008) Obesity, defined as a body mass index (BMI) ≥ 30 , is an independently recognised risk factor for VTE in both pregnant and non-pregnant populations, with studies in the non-pregnant population identifying the odds ratios of VTE in obesity as between 2.50 and 3.04. (Pomp *et al.*, 2007; Stein, Beemath and Olson, 2005) Several population-based studies have identified BMI ≥ 30 as a risk factor for VTE during pregnancy and the puerperium, with adjusted odds ratios between 1.7 and 5.3. (Liu *et al.*, 2009; Simpson *et al.*, 2001; Knight and UKOSS, 2008) A large population-based cohort study by Butwick *et al* found a statistically significant increase in both antepartum and postpartum VTE with increasing pre-pregnancy BMI ($p < 0.0001$). In the antepartum period VTE prevalence in underweight women was 0.02%, compared to 0.11% in obese class III women. (Butwick *et al.*, 2019) Furthermore, both the 2014-2016 and 2017-2019 triennial confidential enquiry reports into maternal mortality identified that 73% of VTE-related deaths during pregnancy and the puerperium in the UK and the Republic of Ireland occurred in overweight or obese women, suggesting that obesity is likely to be a significant contributory factor to the thrombotic risk already associated with pregnancy. (Knight M *et al.*,

2021; M *et al.*, 2018) The incidence of obesity is increasing worldwide, and this is also apparent within the maternity population. The National Maternity and Perinatal Audit in 2016/17 found, for the first time, that more than half of women were overweight (BMI >25) or obese (BMI >30) at the antenatal booking appointment, and it is expected that this will have further increased given the increase in national average BMI since then. (NMPA, 2018) Whilst older mothers are recognised to be at greatest risk for the majority of pregnancy-related complications, VTE-related deaths appear to affect pregnant women of all ages, with 25% of the women who died in the 2021 UK-MBRRACE survey aged 25 years or less. However, 63% of these women were morbidly obese with BMI >40 which alludes to the significant risk obesity poses, even in young mothers. (Knight M *et al.*, 2021)

Obesity in pregnancy was identified as an independent risk factor for all-cause maternal death in a 2020 study by Saucedo *et al.*, with OR 3.40 for women with class II-III obesity (BMI ≥ 35). (Saucedo *et al.*, 2021) This study is one of many which also illustrated that obese mothers are at increased risk of other cardio-metabolic complications of pregnancy compared to mothers with a normal BMI (18-25 kg/m²), which may suggest an overlap in pathophysiology with VTE. (Saucedo *et al.*, 2021) Risk factors for pre-eclampsia were examined in a case-control study by Bohiltea *et al.*, with obesity identified as a significant risk factor for pregnancy-induced hypertension and mild pre-eclampsia. (Bohiltea *et al.*, 2020) Whilst an earlier systematic review found that for each 5-7kg/m² increase in pre-pregnancy BMI, the risk of pre-eclampsia was doubled. (O'Brien, Ray and Chan, 2003)

1.2 Pathophysiology of VTE

Venous thromboembolism (VTE), encompassing both deep vein thromboses (DVT) and pulmonary embolism (PE), is a disease with annual incidence of 1-2 per 1000 within the general population. The pathophysiology of VTE was first proposed in Virchow's triad as an interaction between venous stasis, hypercoagulability and endothelial injury, and whilst understanding of the disease has advanced since this theory was devised, many risk factors can be traced back to these three elements of Virchow's triad, as depicted in Figure 1-1. (Bagot and Arya, 2008)

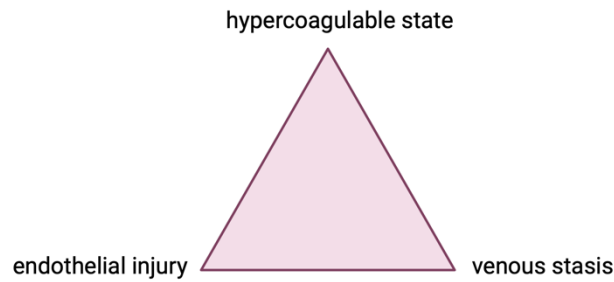


Figure 1-1 Virchow's triad.

Virchow proposed that the development of venous thromboembolism was the effect of the interaction between three variables: a hypercoagulable state, endothelial injury and venous stasis.

Endothelial injury, as caused by surgery, trauma or simply indwelling vascular access devices, results in the exposure of tissue factor which initiates the coagulation cascade, whilst exposed collagen and release of von Willebrand Factor activate and aggregate platelets. Disruption to blood flow within the vessel, due to immobility or mechanical obstruction, results in venous stasis which makes blood more prone to clotting. Finally, increased levels of pro-coagulant factors or reduced levels of naturally-occurring anticoagulants also predispose to the development of thrombosis. There can be many inherited or acquired changes in coagulation factors, including genetic deficiencies of anticoagulants protein C, S or antithrombin, or the increase in procoagulant factors observed in response to surgery, systemic infection or pregnancy. (Schulman *et al.*, 2024)

One single abnormality within the Virchow's triad may not lead to thrombosis, but the cumulative effect of multiple risk factors may tip the balance towards a prothrombotic state.

1.3 Risk Factors for VTE

A study by Anderson *et al* found, in a series of more than 1200 VTE cases, >96% had one identifiable risk factor present. (Anderson and Wheeler, 1992) There are many recognised risk factors for VTE, which can be related to the elements described by Virchow. For example, surgery and trauma, in simplest terms as mediators of endothelial injury, have an odds ratio for VTE quoted as high as 21.7 (95% CI 9.4-49.9). (Anderson and Wheeler, 1992; Meknas *et al.*, 2023; Heit *et al.*, 2000) Whilst malignancy, a recognised hypercoagulable state, increases the risk

Chapter 1

of VTE four-fold compared to those without cancer.(Heit *et al.*, 2000) Venous stasis may be induced by immobility, due to prolonged bed rest or hospital inpatient stay, which has an odds ratio for VTE risk of 8.0 (95% CI 4.5-14.2).(Heit *et al.*, 2000; Anderson and Wheeler, 1992)

Increased age is known to be a significant risk factor, with risk of VTE increased 60-fold in the over 60 year old population. However, this same level of risk is reached during pregnancy and the postpartum period for younger women.(Pomp *et al.*, 2008)

Healthy pregnancy therefore is a significant risk factor in itself for VTE occurrence and there are many features of Virchow's triad identifiable in the pathogenesis: reduced mobility and the pressure of the gravid uterus on pelvic veins results in venous stasis; there is the development of a hypercoagulable state with an increase in procoagulant factors and decrease in naturally-occurring anticoagulants which is widely considered to be an evolutionary adaptation aimed at preventing excessive blood loss at delivery when separation of the placenta from the uterine wall can expose the blood flow from the uterine spiral arteries; and, delivery, particularly with operative delivery, results in endothelial injury. But aside from these inherent risks of pregnancy, further inherited and acquired risk factors can compound the risk from pregnancy alone. Obesity is a significant risk factor for VTE in pregnancy (OR 2.4) and is a known risk factor for VTE in the non-pregnant population also.

A prospective study of 87,226 non-pregnant women identified a positive correlation between BMI and the incidence of pulmonary embolism, with a relative risk of 2.32-5.53 for those with BMI 25-35.(Kabrhel *et al.*, 2009) The thrombotic risk associated with obesity is most marked in younger women. Stein *et al* calculated the relative risk of VTE to be 6.10 for women under 40, compared to that of age-matched men which was 3.71, however this study did not account for pregnancy or the oral contraceptive pill which could be potential confounders.(Stein, Beemath and Olson, 2005) A further observational study by Hotoleanu described an increase in the risk of VTE with increasing BMI class: for Class I obesity OR 2.86, Class II OR 5.29, Class III OR 6.65, demonstrating that VTE risk is positively correlated with BMI.(Hotoleanu, 2020)

Taking this data together with what is already known about the risk of thrombosis in pregnancy, with the increase in maternal age and population levels of obesity, women are entering pregnancy with additional non-modifiable risk factors which have a cumulative effect on the VTE risk.

1.4 Assessing and managing the risk of VTE during pregnancy

As part of the current strategy to mitigate VTE risk during pregnancy, the Royal College of Obstetricians and Gynaecologists have devised a risk stratification tool for health professionals to help identify those women at greatest risk of VTE. This weighted scoring system, which is depicted in Table 1 adapted from the RCOG Green Top Guideline: Reducing the Risk of Venous Thromboembolism during Pregnancy and the Puerperium (37a), is based upon the adjusted odds ratios for VTE from a large body of literature on the risk factors for VTE during pregnancy. (RCOG, 2015)

Table 1 Royal College of Obstetricians and Gynaecologists Green Top Guideline 37a VTE risk assessment tool

Score ≥ 4 consider thromboprophylaxis from the first trimester; score 3 consider thromboprophylaxis from 28 weeks; score ≥ 2 postnatally consider thromboprophylaxis for at least 10 days. Range of adjusted odds-ratio from evidence within guideline. nr=not recorded. (adapted from RCOG guideline 37a)

Risk Factors for VTE		Score	Adj OR
Pre-existing risk	Previous VTE	4	24.8
	Previous VTE provoked by major surgery	3	nr
	Known high risk thrombophilia	3	4.1-50
	Medical comorbidities	3	1.7-43.4
	Family history unprovoked/oestrogen-related VTE in 1 st degree relative	1	nr
	Known low risk thrombophilia	1	2.1-2.3
	Age > 35years	1(2)	1.2-1.4
	Obesity: BMI ≥ 30 / (BMI ≥ 40)	1	1.7-5.3
	Parity ≥ 3	1	2.4
	Smoker	1	1.4-2.7
	Gross varicose veins	1	2.4

Obstetric risk	Pre-eclampsia	1	2.9-5.8
	Assisted reproductive technology	1	1.8-4.3
	Multiple pregnancy	1	1.7-4.2
	Caesaerean section in labour	2	2.7
	Elective caesarean section	1	1.8-3.6
	Mid-cavity/rotational forceps delivery	1	nr
	Prolonged labour (>24hours)	1	nr
	Postpartum haemorrhage (>1 litre or transfusion)	1	4.1-12
	Preterm birth <37 weeks	1	2.4-2.7
	Stillbirth	1	6.24
Transient risk	Surgical procedure during pregnancy	3	nr
	Hyperemesis	3	2.5-4.4
	Ovarian hyperstimulation syndrome	4	nr
	Systemic infection	1	4.1-6.1
	Immobility, dehydration	1	7.7-10.8

Despite the over-representation of obesity in the maternal death statistic, in the absence of other risk factors, thromboprophylaxis with low molecular weight heparin (LMWH) would only be recommended by the RCOG guidelines for mothers with BMI ≥ 40 in the postpartum period.(RCOG, 2015; Larsen TB, 2007)

1.5 Pharmacological prevention of VTE during pregnancy

Low molecular weight heparin (LMWH) has been demonstrated as a safe and effective measure to prevent venous thrombosis in the non-pregnant medical inpatient population for over two decades.(Samama MM and Janbon C, 1999; Leizorovicz *et al.*, 2004) As LMWH is known not to cross the placenta and has a lower risk of complications compared with alternative anticoagulants, the data from medical populations has been extrapolated to pregnancy, and prophylactic LMWH is used in those pregnant women considered to be at high thrombotic risk.(Bates *et al.*, 2018) Safety data has accumulated over the years of LMWH usage, and studies have shown no significant increase in blood loss at delivery in women using LMWH.(Roeters van Lennep *et al.*, 2011) However, as there have been no randomised control trials of prophylactic LMWH in the pregnant

Chapter 1

population, data for efficacy and dose-finding is limited and as such some observational studies have found the low dose LMWH to be ineffective in the highest risk women, for example those with thrombophilia or prior VTE.(Roeters van Lennep *et al.*, 2011; Rodger *et al.*, 2014) The Highlow study, published in 2022, was one of the first multi-centre randomised, controlled trials to compare the efficacy of low dose LMWH with weight-adjusted intermediate dose LMWH in pregnant women with a prior history of VTE. This study found no significant difference in either the incidence of VTE or risk of major bleeding, although there was a non-significant trend toward incidence of PE in the low dose group. (Bistervels *et al.*, 2022) A further area of contention is dose-finding of LMWH prophylaxis for overweight and obese women during pregnancy and the puerperium.

Due to the over-representation of obese women in maternal VTE-related deaths, and appreciative of the lack of high-quality evidence, in 2009 the RCOG took a pragmatic decision to increase the dose of prophylactic LMWH for women weighing >90kg based upon the findings of a study by Rottenstreich *et al* in which weight-adjusted LMWH dosing in post-caesarean section women weighing >90kg resulted in a reduction in thrombin generation.(RCOG, 2015; Rottenstreich *et al.*, 2018) Subsequently, a small study of obese pregnant women during the third trimester found a weight-adjusted dose of tinzaparin prophylaxis (75 iu/kg) could achieve target anti-Xa levels of 0.1-0.5iu/mL, which is within the range recognised as safe and effective for thromboprophylaxis in non-pregnant populations. There was also a corresponding reduction in thrombin generation and an absence of any major bleeding.(Ismail *et al.*, 2014)

Although not routinely requiring monitoring, given the changes in pharmacokinetics which are known to occur during pregnancy, a method of ensuring adequate anticoagulation with LMWH is desirable and anti-Xa levels are the current method of choice to assess LMWH drug levels. During pregnancy, studies have shown glomerular filtration rate and clearance of enoxaparin to have increased by up to 50% and due to the pregnancy-associated plasma volume expansion, the volume of distribution of enoxaparin increased by almost 50%.(Anderson, 2005; Lebaudy *et al.*, 2008) Patel *et al* found that as pregnancy progressed, anti Xa peak levels reduced, and trough levels increased which indicated a prolongation of the half-life of LMWH.(Patel *et al.*, 2013) But other

studies have cast doubt upon the reliability of anti-Xa levels as an assessment of the anticoagulation effect: although anti-Xa levels were within therapeutic range, the ability of LMWH to reduce thrombin generation declined with gestation and showed significant inter-individual variability. (Chowdary *et al.*, 2015; Selmeczi *et al.*, 2015)

Previous studies to assess the feasibility of a randomised control trial in pregnant women to assess the efficacy of prophylactic LMWH were met with significant recruitment difficulties, with many potential participants not wishing to take the placebo drug, or citing difficulty in attending study visits in the post-partum period. (Rodger *et al.*, 2015) Given the difficulties in undertaking studies in the pregnant population, much of the data obtained to assess efficacy of LMWH in pregnancy have been accrued from studies of laboratory haemostasis.

1.6 Normal haemostasis in the non-pregnant population

Haemostasis is a finely balanced process of blood clotting, ultimately resulting in the production of the substance thrombin which is converted to the fibrin clot. The sequential activation of procoagulant clotting factors are balanced through the action of naturally occurring anticoagulants and fibrinolytic substances which aim to halt the coagulation process once haemostasis is achieved to prevent excessive clot formation

Haemostasis can be considered in two phases: primary and secondary, both of which are critical to the formation of the clot and regulation of the process. Primary haemostasis involves the activation of platelets which migrate to the site of injury to form the platelet plug, thereby sealing the injury site. Simultaneously, there is release of tissue factor from the endothelium which stimulates the secondary haemostasis process in which procoagulant factors are sequentially activated in the coagulation cascade (see Figure 1-2).

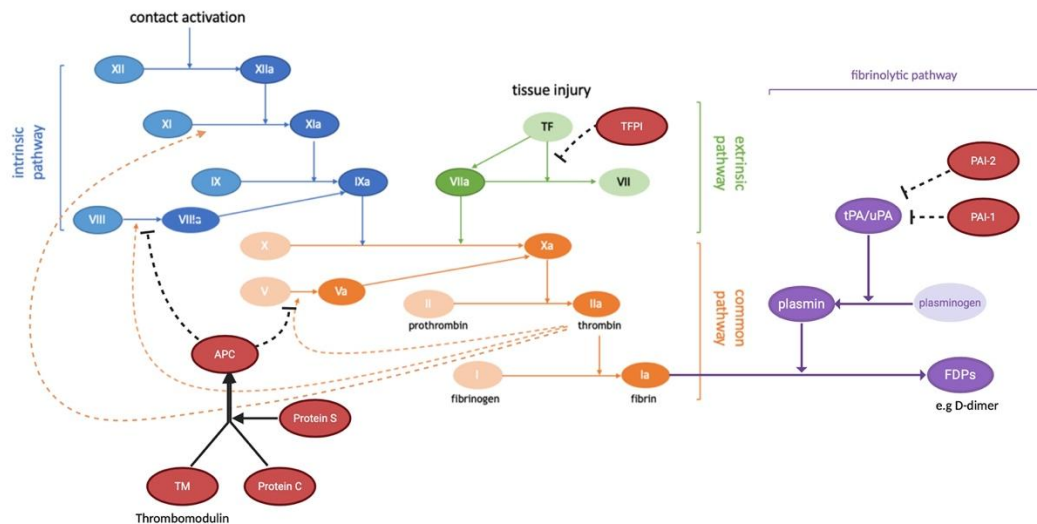


Figure 1-2 Coagulation cascade

The extrinsic pathway is activated by tissue factor following tissue damage. Tissue factor forms a complex with Factor VIIa, which leads to the activation of Factor Xa and the initiation of the common pathway when Factor Xa forms the prothrombinase complex with Factor Va (activated by thrombin). This in turn converts prothrombin to thrombin which converts fibrinogen to the fibrin clot. Thrombin formation provides positive feedback and amplifies the coagulation process through activation of the intrinsic pathway. The intrinsic pathway can also be initiated by contact activation which results in the activation of Factor XII. There is sequential activation of factors XI and IX, and then Factor VIII (which is activated by thrombin) forms the tenase complex with activated Factor IX. The tenase complex initiates the common pathway as previously outlined. The system is regulated by naturally-occurring anticoagulants including protein C, protein S, and TFPI. Fibrinolysis is the process of breaking down the clot once formed to prevent excessive thrombosis.

The coagulation cascade can broadly be considered as three pathways: the extrinsic pathway (initiated by activated tissue factor), the intrinsic pathway (initiated by the activation of contact factor and high molecular weight kininogens) and the common pathway (which can be initiated by either the intrinsic or extrinsic pathway to result in generation of thrombin and subsequently the fibrin clot).

1.6.1 Procoagulant factors

The procoagulant factors (fibrinogen, Factors II, V, VII, VIII, IX, and XI) exist in the circulation as inactive zymogens, which once activated trigger the next part of the coagulation cascade. Following vessel wall injury, the extrinsic pathway is initiated by the activation of tissue factor (TF) which binds to Factor VIIa, forming the TF-FVIIa complex which subsequently activates Factors IX and X. Activated Factor X (Xa) in combination with activated factor V (Va), is known as the

Chapter 1

prothrombinase complex which converts prothrombin (Factor II) to thrombin. A small amount of thrombin is produced by this method, however the production of thrombin itself results in a positive feedback loop by activating Factors V, VIII, IX and XI. Activated Factors VIII and IX combine to form the tenase complex which activates Factor X allowing further formation of the prothrombinase complex with Factor Va. This amplification of haemostasis results in the rapid generation of thrombin, known as the 'thrombin burst'. In the final step, thrombin converts fibrinogen (Factor I) to fibrin which is then crosslinked by Factor XIII to form the fibrin clot. (DAVIE and RATNOFF, 1964; MACFARLANE, 1964)

1.6.2 Naturally occurring anticoagulants

Whilst the haemostatic system is essential to prevent blood loss, the system must remain in careful balance to prevent excessive clotting which would result in obstruction of blood flow within vessels, and any clot formed must in time be dissolved to restore normal flow within the injured vessel once it has been repaired.

Whilst haemostasis is achieved through the activation of the procoagulant factors, natural inhibitors of coagulation, including thrombomodulin, antithrombin, protein C, protein S and tissue factor pathway inhibitor (TFPI) are simultaneously activated to prevent excessive clot formation.

Antithrombin is a serine protease inhibitor which primarily inhibits Factors IXa, Xa and thrombin. (Rezaie and Giri, 2020) Protein C and Protein S are Vitamin-K dependent naturally-occurring anticoagulants which regulate the coagulation cascade via negative feedback loops. Thrombomodulin is a transmembrane receptor located on endothelial cells which forms a complex with thrombin to prevent the binding of FV, FVIII and fibrinogen. Thrombin-bound thrombomodulin activates protein C which, with the co-factor protein S, inactivates FVa and FVIIIa. (Clark *et al.*, 1998) Protein S is also a co-factor for tissue factor pathway inhibitor (TFPI), a 3-kunitz domain glycoprotein released from endothelial cells, or platelets activated by thrombin, and directly inhibits the TF-FVIIIa complex and FXa. (Hackeng and Rosing, 2009) 80% of TFPI is bound to the endothelium and 20% is free circulating, yet it is not clear whether free or bound TFPI, is most responsible for the anti-coagulant effect. (Sandset, Bendz and Hansen, 2000)

1.6.3 Fibrinolysis

Once the fibrin clot has been formed and successfully secured haemostasis at the site of vessel injury, fibrinolytic mechanisms are in place to dissolve the fibrin clot to restore normal calibre and flow to the vessel. This process is triggered simultaneously by the procoagulant process, with the binding of fibrin to plasminogen triggering the conversion of plasminogen to plasmin by tissue plasminogen activator (tPA). Plasmin targets and cleaves fibrin and fibrinogen into numerous fibrin degradation products (FDPs), including D-dimers, which are a breakdown product of cross-linked fibrin polymers. The fibrinolytic process is controlled by the inhibitors, plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2). PAI-1 is a serpin which inhibits tPA and uPA and is produced and secreted by endothelial cells, but is also present in platelet α -granules and more recently found to be produced by other cells, including adipocytes. (Longstaff and Kolev, 2015) PAI-2 is produced predominantly during pregnancy by placental trophoblasts, but can also be upregulated in periods of acute inflammation. (Astedt, Lindoff and Lecander, 1998)

When in balance, these complex anticoagulant and fibrinolytic systems are able to tightly regulate the haemostatic process and limit clot formation to that which is essential. However, the disruption of this balance results in the development of a prothrombotic state which can lead to venous thromboembolic disease.

1.7 Laboratory assessment of the prothrombotic state

Unlike with bleeding disorders, or deficiencies of coagulation factors, characterising the prothrombotic state is more challenging. Standard laboratory tests of haemostasis assess time taken for a clot to form, and measurement ceases upon the formation of the fibrin clot. It is known that by this point, only a small part of the total amount of thrombin generated has been formed, with 95% of the thrombin-generating capacity of the plasma unmeasured. (Brummel *et al.*, 2002) Thus, standard laboratory haemostasis tests have limited utility in conditions which result in hypercoagulability.

The current approach in clinical practice is to measure each element of the haemostasis pathway individually to ascertain where deficiencies or functional

Chapter 1

abnormalities in naturally-occurring anticoagulants may lie. In some situations, this may identify the underlying cause for VTE, however the majority of unprovoked VTEs remain unexplained by laboratory tests.

Global assays of coagulation, such as thrombin generation, thromboelastography (TEG) and rotational thromboelastography (ROTEM), have been used in haemostasis research for several decades. Such assays are considered to be more representative of *in vivo* haemostasis as they provide an overall assessment of the balance of pro-coagulant and anticoagulant factors and are unique in being able to identify a prothrombotic state.

Ex vivo thrombin generation, a global coagulation assays, has been shown to accurately reflect a thrombotic phenotype and has been characterised in several prothrombotic conditions, including normal pregnancy. (Hron *et al.*, 2006; Tripodi *et al.*, 2008; Bagot *et al.*, 2010; Haidl *et al.*, 2006; Rosenkranz *et al.*, 2008; Selmeczi *et al.*, 2015; Bagot *et al.*, 2017; McLean, Bernstein and Brummel-Ziedins, 2012)

The current and most widely accepted methodology was devised by Hemker *et al* and utilises a fluorochrome which emits fluorescence in the presence of thrombin. (Hemker *et al.*, 2003) Thrombin generation is measured by the Calibrated Automated Thrombogram (CAT) analyser, an analyser capable of dispensing reagents and measuring fluorescence within a 96-well microplate. Experimental plasma samples are assessed in duplicate alongside a calibrator well to overcome the effect plasma colour may have on the fluorescence measured. Coagulation is triggered by the addition of a reagent containing tissue factor and phospholipids to platelet poor plasma. The concentration of tissue factor used determines which components of the coagulation pathways are assessed by the assay. In a high concentration of tissue factor (5pM), the thrombogram reflects the activity of the extrinsic pathway within the sample. In a lower concentration of tissue factor (1pM), coagulation requires the activation of the intrinsic pathway. Therefore, by altering the experimental conditions, different aspects of the coagulation system be assessed. The activity of the naturally-occurring anticoagulants can also be assessed by this assay through the addition of thrombomodulin (TM). (Hemker *et al.*, 2003) Thrombomodulin binds to protein C and increases its activation 20,000-fold, which in turn is inhibitory to thrombin

generation. Given that acquired protein C resistance is known to occur during pregnancy, an overview of the protein C system is desirable when assessing the plasma of pregnant women.(Clark, Walker and Greer, 1999) Finally, the coagulation process is initiated once calcium, mixed in solution with a fluorochrome (amino-methlycoumarin) is added by the dispenser to the wells. As thrombin is produced by the active coagulation process, it binds to and splits the fluorochrome emitting a fluorescent signal which can be measured by the CAT analyser. Fluorescent intensity is proportional to the thrombin concentration when compared to the calibrator of known thrombin concentration. The thrombogram for each well, such as the example provided in Figure 1-3, is generated by CAT analyser software and a series of 5 parameters are calculated by the Thrombinoscope® software to provide an objective assessment of Thrombin generation:

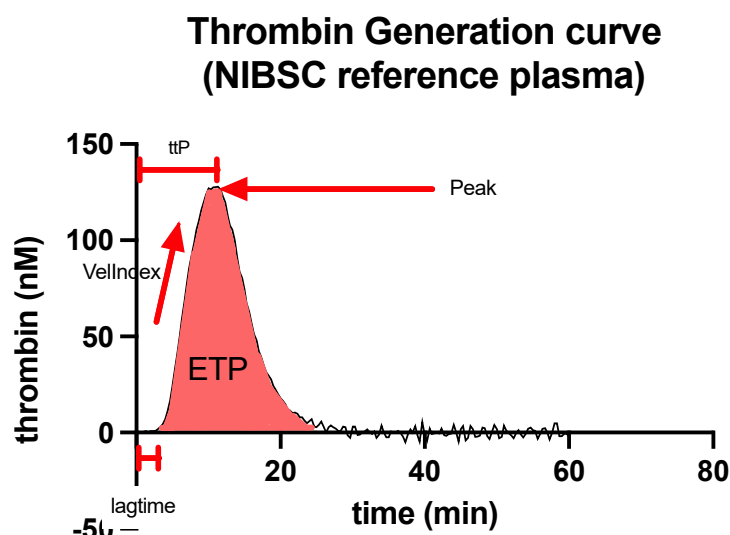


Figure 1-3 Thrombogram with measurement parameters. Raw data from NIBSC reference plasma used to demonstrate the thrombogram produced by the CAT analyser and the measurement parameters obtained.

Lagtime: time (minutes) taken from initiation of clotting to the start of the thrombin burst during the propagation phase of thrombin generation

Peak: maximum amount of thrombin (nM) generated during the assay

Time to peak (ttP): time (minutes) from initiation of the assay to Peak thrombin.

Endogenous thrombin potential (ETP): total amount of thrombin formed, quantified by calculating the area under the curve (nM*min)

Velocity index (VelIndex): rate of thrombin production during the thrombin burst (nM/min)

In addition to providing a global assessment of the haemostatic system, thrombin generation has been shown to correlate with thrombotic risk and therefore can be a useful biomarker when the desired primary endpoint of VTE may be of low incidence, for example in pregnancy. (Hron *et al.*, 2006)

1.8 Haemostatic changes during normal pregnancy

It is widely accepted that pregnancy is associated with the development of a prothrombotic state. This prothrombotic state has been well-characterised through standard laboratory measures of haemostasis with changes in all aspects of the regulation of haemostasis.

1.8.1 Procoagulant factors during pregnancy

During pregnancy and the puerperium, there is an incremental rise in levels of pro-coagulant factors. In a similar manner to that observed in studies of the combined oral contraceptive pill, the increased levels of oestrogen in pregnancy are thought to induce hepatic protein synthesis and thereby upregulate production of coagulation factors from hepatocytes. (Tchaikovski and Rosing, 2010)

Several longitudinal studies have evaluated the plasma levels of the coagulation factors at time points throughout pregnancy and the puerperium. All agree that levels of factors VII, VIII and von Willebrand factor antigen and activity increase progressively with gestation, and some identified increases in factors V, IX and X. (Stirling *et al.*, 1984; Choi and Pai, 2002; Clark *et al.*, 1998; Sánchez-Luceros *et al.*, 2003; Szecsi *et al.*, 2010) Peak levels of factors VII, VIII, and IX are reached by the third trimester, with mean FVIII levels more than 3 times that of non-pregnant controls. (Choi and Pai, 2002) Fibrinogen is also markedly increased in pregnancy and exhibits a positive correlation with gestational age, with levels at term nearly twice those pre-pregnancy. (Stirling *et al.*, 1984; Szecsi *et al.*, 2010; Tang *et al.*, 2019)

It is thought that the increase in procoagulant factors during pregnancy is part of a physiological effort to protect against the risk of major haemorrhage at delivery, and as such, all factor levels return to non-pregnant values by 8 weeks postpartum. (Choi and Pai, 2002)

1.8.2 Naturally occurring anticoagulants during pregnancy

Although there are increases in pro-coagulant factors during pregnancy, alterations in the levels and activity of naturally occurring inhibitors of coagulation, antithrombin, protein C, protein S and TFPI, also contribute to the prothrombotic state.

Antithrombin levels are known to fall during pregnancy, with a negative correlation with gestational age identified between second trimester and term and an overall 20% reduction in antithrombin. The most significant drop in antithrombin is in the immediate postpartum period, thought to reflect consumption at delivery. (James *et al.*, 2014)

During pregnancy there is a progressive fall in Protein S, from as early as the 5th gestational week and there is development of acquired protein C resistance. (Bagot *et al.*, 2017; Kovac *et al.*, 2015; Kjellberg *et al.*, 1999) Tchaikovski *et al* have suggested the fall in Protein S may result from haemodilution due to the increase in maternal plasma volume during pregnancy as there is a positive correlation between haematocrit and Protein S levels. (Tchaikovski *et al.*, 2011)

The decreasing Protein S levels associated with pregnancy may result in the progressively impaired function of its co-factor, activated Protein C (APC), as the prevalence of APC resistance has been seen to correlate positively with gestation and negatively with Protein S levels. (Walker *et al.*, 1997; Tchaikovski *et al.*, 2011) It has previously been recognised that APC resistance is an independent risk factor for VTE in both the pregnant and non-pregnant population (Bergrem *et al.*, 2011). When APC resistance occurs in the non-pregnant, 90% of cases are due to the inheritance of Factor V Leiden, a single nucleotide polymorphism in the Factor V gene which confers resistance to inactivation by Protein C. (Sedano-Balbás *et al.*, 2011) In contrast, there is evidence of an acquired and progressive resistance to APC during pregnancy, in the absence of the Factor V Leiden mutation, which has

been shown to correlate with increased generation of thrombin-antithrombin complexes, indicative of increased thrombin generation.(Clark *et al.*, 1998; Bergrem *et al.*, 2011; Walker *et al.*, 1997)

There is conflicting data on the changes in TFPI during pregnancy: Some studies have demonstrated that TFPI levels are positively correlated with gestational age,(Erez *et al.*, 2010; Rosenkranz *et al.*, 2008) and others have found either a significant fall or no difference in TFPI antigen or activity levels between pregnant and non-pregnant controls.(Uszyński *et al.*, 2001; Tchaikovsky *et al.*, 2011; Egan *et al.*, 2017) A low TFPI level is recognised to be a weak risk factor for VTE and has been found in association with oestrogen-containing contraceptive preparations.(Raps *et al.*, 2013; Dahm *et al.*, 2003) TFPI resistance has also been reported to be associated with increased risk of VTE, and has been found during pregnancy which is thought to relate to the reduction in Protein S levels associated with pregnancy, as Protein S is a co-factor to TFPI.(Tardy-Poncet *et al.*, 2012; Hackeng *et al.*, 2009) Taken together, these data may suggest a role for TFPI in the pathophysiology underlying the prothrombotic state induced by pregnancy.

1.8.3 Fibrinolysis during pregnancy

In addition to imbalances between pro-coagulant and anticoagulant factors, fibrinolysis is impaired in pregnancy which further contributes to the prothrombotic state. Pro-fibrinolytic substances, plasminogen and its activators, tissue plasminogen activator, (tPA) and urokinase-type plasminogen activator (uPA), are increased in pregnancy, mirroring gestational increases in fibrinogen.(Hellgren, 2003; Choi and Pai, 2002) The fibrin degradation product and biomarker of fibrinolytic activity, D-dimer, progressively increases from the 5th gestational week and peaks at delivery, reflective of the increased thrombin generation and subsequent clot lysis associated with pregnancy. (Bagot *et al.*, 2019; Szecsi *et al.*, 2010; Stirling *et al.*, 1984; Kovac *et al.*, 2015) However, the total fibrinolytic capacity of the system is impaired due to increased concentrations of potent inhibitors of fibrinolysis: plasminogen activator inhibitor-1 (PAI-1), which is released from the endothelium, and to a lesser extent, plasminogen activator inhibitor-2 (PAI-2), which is predominantly produced by the placenta, but also in smaller quantities by macrophages.(Hellgren, 2003; Kruithof *et al.*, 1987) These serine protease inhibitors are the principle inhibitors of tPA

and uPA which prevent the conversion of plasminogen to plasmin and reduce the activity of plasmin on cross-linked fibrin. (Cesarman-Maus G and KA, 2005; Kruithof *et al.*, 1987)

PAI-1 is the predominant tPA inhibitor during pregnancy and begins to increase in the second trimester with peak levels attained during the third trimester when they are approximately 3-5x that of the non-pregnant population. (Kruithof *et al.*, 1987; Choi and Pai, 2002; Jørgensen *et al.*, 1987) PAI-2 levels also increase throughout pregnancy, and have been inversely associated with placental complications during pregnancy, including intrauterine growth restriction, pre-eclampsia and low birthweight. (Coolman *et al.*, 2012; Astedt, Lindoff and Lecander, 1998; Yatsenko *et al.*, 2025) The consequence is comparatively diminished fibrinolysis, despite the elevated D-dimer, within a significantly activated coagulation system, contributing still further to the overall prothrombotic state.

Overall, this hypercoagulable state in pregnancy has been extensively investigated, but the further increased thrombotic risk in overweight and obese pregnant women remains poorly understood.

1.9 Haemostatic changes associated with obesity in the non-pregnant population

Laboratory haemostasis parameters in the non-pregnant obese population show changes reflective of a prothrombotic state. Several studies have associated obesity with an increase in tissue factor (TF) and coagulation factors VII, VIII, von Willebrand's factor, prothrombin and fibrinogen. (Chitongo *et al.*, 2017; Kopp *et al.*, 2003; Mertens and Van Gaal, 2002) Fibrinolysis is known to be impaired in obesity with increased plasma PAI-1 and D-dimer levels, yet coagulation inhibitors protein C, protein S and TFPI are also elevated, possibly as a mechanism to rebalance the coagulation system. (Chitongo *et al.*, 2017)

The overall effect of the coagulation factor changes associated with obesity are reflected in thrombin generation data from several studies which have found endogenous thrombin potential (ETP) to be positively correlated with BMI,

confirming a prothrombotic phenotype. (Chitongo *et al.*, 2017; Campello *et al.*, 2015; Sonnevi *et al.*, 2013)

Ay *et al* strengthened the evidence for a relationship between obesity and a hypercoagulable state, by observing that the prothrombotic changes observed in obesity could be reversed following weight loss surgery, demonstrating a significant reduction in tissue factor, PAI-1, prothrombin fragment 1+2 and thrombin generation in patients who had lost weight as a consequence of bariatric surgery.(Ay *et al.*, 2010)

Ex vivo thrombin generation data confirms a relationship between increased waist:hip ratio and thrombotic risk with a study by Beijers *et al* which identified a positive association between BMI, central adiposity and thrombin generation parameters (lag time, peak and endogenous thrombin potential) independent of recognised cardiovascular risk factors.(Beijers *et al.*, 2010)

The pathophysiology underlying the increased risk of venous thromboembolism observed in obesity is complex but an increasing body of work is recognising the importance of adipose tissue distribution and dysfunctional adipose tissue function resulting in a chronic pro-inflammatory state, abnormal cytokine release and the development of the prothrombotic state.

1.10 Obesity

1.10.1 Assessment of obesity

Obesity is defined by the World Health organisation (WHO) as a body mass index (BMI) $\geq 30\text{kg/m}^2$. Body Mass Index (BMI) is a clinical tool which can quickly identify individuals who are overweight or obese by assessing body weight relative to height. BMI is calculated by dividing body weight (kilograms) by height (metres) squared. (WHO, 2010)

However, evidence has shown that BMI underestimates total body adiposity, with the difference more marked in women compared with men.(Gallagher *et al.*, 1996) Accumulating evidence has shown that the location and type of adipose tissue can influence its function, with different phenotypes of fat distribution and function associated with varying cardio-metabolic risk.(Brook *et al.*, 2001; Borch

et al., 2009; Després, 2001) Given that BMI provides no insight into adipose tissue distribution, this may account for its indiscriminate prediction of those who are at increased risk of developing the metabolic complications of obesity such as type 2 diabetes mellitus, hyperlipidaemia and ischaemic heart disease, again a phenomenon particularly pronounced in women.

A 'pear' shape, where the majority of adipose tissue is carried around the hips, has been recognised as more protective against cardiovascular disease than the 'apple' shape, which signifies the presence of increased abdominal adipose tissue. (Peters, Bots and Woodward, 2018) Indeed, a waist:hip ratio of >0.85 has been defined as 'unhealthy' by the World Health Organisation (WHO) which is backed up by data that increased waist:hip ratio and central obesity are associated with vascular endothelial dysfunction, cardiometabolic disease and venous thromboembolism. (WHO, 2008; Wang *et al.*, 2023; Brook *et al.*, 2001; Phillips *et al.*, 2018) In addition to location of adipose tissue, the depot in which it is stored also appears to be important.

1.10.2 Adipose tissue depots

Over the last few decades, research has provided a greater understanding of the functions of adipose tissue beyond that of an energy store, and it is well recognised that adipose tissue is metabolically active with the ability to interact with the surrounding environment via production of cytokines. (Vilahur, Ben-Aicha and Badimon, 2017) The functionality of the adipose tissue is influenced by the depot in which it is stored, with some adipose tissue depots more metabolically active than others.

Adipose tissue is comprised of thermogenic brown adipose tissue, and white adipose tissue. White adipose tissue can be categorised as subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT), with each depot differing in metabolic and endocrine function. (Suárez-Cuenca *et al.*, 2021; Smith *et al.*, 2001) Visceral adipose tissue (VAT), which is predominantly located around the abdominal organs, is known to be the more metabolically active depot, which when in excess has been more closely associated with cardiovascular risk. Whilst subcutaneous adipose tissue (SAT), has traditionally been considered as a more stable store of excess energy, with the storage of fat in the gluteofemoral region

associated with insulin sensitivity.(Karpe and Pinnick, 2015) SAT can be further subdivided into superficial SAT (SSAT) and deep SAT (DSAT), anatomically separated by the fascia superficialis, with the latter possessing inflammatory and metabolic properties more akin to VAT. (Smith *et al.*, 2001)

There are sex-specific differences in the location of, and risk associated with, each of the adipose tissue depots. Women tend to carry more subcutaneous adipose tissue (SAT), particularly superficial SAT (SSAT), whereas men had more VAT than SAT.(Kammerlander *et al.*, 2021; Smith *et al.*, 2001; Kelley *et al.*, 2000) The Framingham heart study data was analysed to assess sex-based differences in cardiovascular risk and found that visceral adipose tissue (VAT) was more closely related to cardiovascular risk factors in women than men. For men, BMI is an accurate surrogate measure of VAT, but the same was not seen for women in whom the measurement of VAT provided additional predictive value for cardiovascular risk.(Kammerlander *et al.*, 2021) the underlying reason for the different properties of the adipose tissue depots appears to relate to differences in the adipocytes contained within them.

1.10.3 Adipocytes and normal expansion/function

White adipose tissue adipocytes are the predominant store of excess energy and differ in function and structure to brown adipocytes. White adipocytes contain a large central lipid droplet and eccentrically positioned nucleus and organelles. Healthy adipocytes expand hyperplastically and, in health, excess adipose tissue accumulates within subcutaneous depots. In times of energy excess, adipocytes secrete lipoprotein lipase which releases free fatty acids from circulating dietary-derived triglycerides. Free fatty acids and glucose (converted to glycerol) are stored as triglycerides within the adipocytes, which can be broken down via lipolysis to be released at time of energy requirement. (Ziegler and Scheele, 2024; Feingold *et al.*, 2000)

The sensitivity of adipocytes to insulin is crucial in the homeostasis of adipose tissue. Insulin suppresses lipolysis but when there is resistance to insulin this process is unopposed and results in high levels of circulating free fatty acids which can be deposited in ectopic fat stores, for example in the liver.

Chapter 1

In addition to providing an accessible energy store, adipocytes have important endocrine and paracrine functions. Adipokines, such as leptin and adiponectin, are cytokines produced by adipose tissue which, along with inflammatory cytokines such as IL-6 and TNF- α , are involved in numerous metabolic pathways to regulate glucose and insulin metabolism, energy intake and expenditure, in addition to some inflammatory processes. Yet in obesity, normal adipose tissue function is dysfunctional and the loss of these normal functions is known to be associated with a chronic pro-inflammatory state and the development of the cardiometabolic complications of obesity. (Mertens and Van Gaal, 2002; Samad and Ruf, 2013)

1.10.4 Adipose tissue dysfunction in obesity.

In obese adipose tissue, hypertrophy of adipocytes leads to increased apoptosis and a resultant inflammatory response. (Samad and Ruf, 2013; Lumeng *et al.*, 2008) Adipocyte size has been demonstrated to relate to function, with small adipocytes protective against metabolic dysfunction and hypertrophic adipocytes more susceptible to lipolysis. (Suárez-Cuenca *et al.*, 2021) The dysfunction of adipose tissue in obesity is not only evident at cellular level, but also in the pattern of distribution across the adipose tissue depots.

The “adipose tissue overflow” hypothesis, has been described by Sniderman *et al* and several others, initially as an ethnic variation in fat distribution, but is also considered as a potential explanation for differential adipose tissue distribution associated with cardiometabolic risk. In obesity, the superficial subcutaneous (SSAT) depot reaches capacity whilst there is concurrent hypertrophic expansion of adipocytes resulting in overspill of free fatty acids. The circulating free fatty acids are deposited in ectopic adipose tissue depots contributing to the expansion of visceral (VAT) and deep subcutaneous (DSAT) compartments. (Sniderman *et al.*, 2007) In the obese population, increasing VAT has been associated with insulin resistance and cardiovascular disease, with subsequent resolution of these pathologies when there is a reduction in visceral adiposity. (Björntorp, 1991; Borch *et al.*, 2009; Després, 2001; Seven *et al.*, 2016)

The pathophysiology underlying the cardiometabolic complications of obesity are thought to relate to the effect of dysfunctional adipose tissue which can be

demonstrated by alterations in the release of adipokines, such as leptin and adiponectin, and inflammatory cytokines, as outlined below:

1.10.4.1 **Pro-inflammatory cytokines**

Evidence of the association between adipose tissue distribution and a chronic inflammatory state has been demonstrated in several studies. Park *et al* found a positive correlation between both BMI and waist:hip ratio (WHR) and CRP, IL-6 and TNF- α . (Park, Park and Yu, 2005) Whilst both SAT and VAT have been found to be similarly positively associated with markers of inflammation (CRP, IL-6 and fibrinogen). Schlecht *et al* found the relationship between VAT and IL-6 to be stronger in the overweight/obese group whereas SAT was more strongly associated with IL-6 in the lean group. (Park, Park and Yu, 2005; Schlecht *et al.*, 2016) In another study, VAT was more strongly associated with further inflammatory markers, including monocyte chemoattractant protein-1 (MCP-1) and isoprostanes, independently of BMI and waist circumference.

Inflammatory cytokines in obesity are produced by adipose tissue macrophages (ATMs) and are pathognomic of adipose tissue dysfunction. The increased apoptotic rate, along with high levels of the satiety hormone leptin, and increased production of monocyte chemotactic protein-1 (MCP-1) results in recruitment of greater numbers of adipose tissue macrophages (ATMs). (Weisberg *et al.*, 2003) Weisberg *et al* were the first to discover ATMs to be positively correlated with both BMI and adipocyte size, and recognise the importance of these inflammatory cells in the pathophysiology of obesity-associated disease. (Weisberg *et al.*, 2003) In obesity, ATMs comprise up to 40% of adipose tissue cells, compared to <10% in lean adipose tissue. (Weisberg *et al.*, 2003) However in addition to the absolute increase in number of macrophages within adipose tissue, there is also a change in their function, with a phenotypic switch from the anti-inflammatory M2 macrophage to the pro-inflammatory M1 macrophage. (Samad and Ruf, 2013; Weisberg *et al.*, 2003; Acedo *et al.*, 2013; Suárez-Cuenca *et al.*, 2021) Acedo *et al* incubated human CD14⁺ cells with leptin and discovered the macrophages bore surface markers of the M2 macrophage but produced the pro-inflammatory cytokines, TNF- α , IL-6, IL-1 β , IL-1ra, monocyte chemotactic protein-1 (MCP-1) and IL-10, which are associated with M1-polarised macrophages. (Acedo *et al.*, 2013)

1.10.4.2 **Leptin**

Leptin, the satiety hormone, is responsible for maintenance of body weight through its effect on the hypothalamus to suppress hunger and reduce energy intake.(Vilahur, Ben-Aicha and Badimon, 2017) Obesity is associated with hyperleptinaemia due to an apparent 'leptin resistance' which results in dysregulation of hypothalamic-mediated body weight homeostasis.

Several studies would suggest that leptin may have a role in the phenotypic switch of adipose tissue macrophages.(Acedo *et al.*, 2013; Zarkesh-Esfahani *et al.*, 2001) In addition to inducing the pro-inflammatory phenotypic switch of ATMs, increased levels of leptin also contribute to a pro-inflammatory state by reducing proliferation of T regulatory cells, promoting generation of pro-inflammatory T helper (Th1) cells and inducing endothelial dysfunction.(Abella *et al.*, 2017; Korda *et al.*, 2008)

1.10.4.3 **Adiponectin**

Adiponectin, the other prominent adipokine released from adipose tissue, has anti-inflammatory properties and is associated with increased insulin sensitivity.

Alongside leptin, adipose tissue also secretes adiponectin, an anti-inflammatory adipokine which has vasculoprotective functions relating to insulin sensitivity, atheroma formation and the innate immune system.(Ouchi *et al.*, 1999; Yokota *et al.*, 2000) Adiponectin is reduced in obese individuals, and increases following weight loss surgery.(Askarpour *et al.*, 2020) Demonstration of the anti-inflammatory properties of adiponectin were observed in an atherosclerotic *ex vivo* model by Okamoto *et al.*, where adiponectin was observed to inhibit macrophage tissue factor.(Okamoto *et al.*, 2013) The low levels of adiponectin in obesity could potentially result in a loss of the aforementioned vascular protective functions and anti-inflammatory properties.(Ouchi *et al.*, 1999)

1.11 Adipose tissue distribution changes in healthy and obese pregnancy

1.11.1 Adipose tissue depots in healthy and obese pregnancy

Pregnancy is associated with gestational increase in body weight and adjustments to adipose tissue distribution to help the body support the growing fetus. In healthy pregnancy, SAT declines with gestational age whilst VAT increases and is thought to contribute to the necessary metabolic changes which occur during pregnancy.(Dutra *et al.*, 2014; Selovic, Sarac and Missoni, 2016; Straughen, Trudeau and Misra, 2013) Gene expression analysis in lean term pregnancies showed greater gene transcription within VAT compared to SAT, indicating VAT as the more dominant depot for metabolic regulation during pregnancy. However, in obese pregnancy, both VAT and SAT demonstrate equivalent level of gene transcripts, thus altering the ratio of VAT:SAT gene expression and indicating the dominance of VAT for metabolic regulation may be lost in obese pregnancy.(Bashiri *et al.*, 2014)

In healthy pregnancy, VAT is preferentially deposited (Kinoshita and Itoh, 2006), but obese women may be entering pregnancy with excess VAT deposited due to adipocyte dysfunction and as such this may affect the ability of the obese VAT depot to respond to pregnancy in the same way that the lean VAT depot would. This pattern was observed in a study of gestational weight gain by Straughen *et al.*: Weight gain was at a slower rate in overweight compared to lean pregnant women and the distribution of adipose tissue between SAT and VAT compartments was subtly different. Total fat thickness increased in lean and decreased in overweight/obese women, but the proportion of VAT increased more in lean compared to overweight/obese.(Straughen, Trudeau and Misra, 2013)

SAT has been identified in some studies to be a predictor for gestational diabetes and adverse pregnancy outcomes(Kansu-Celik *et al.*, 2018; Kennedy *et al.*, 2016), however these studies did not quantify VAT alongside SAT therefore it is unknown if SAT or VAT would have been the better predictor when directly compared. Work by several groups have shown that VAT, rather than SAT, is more closely associated with pro-inflammatory states in pregnancy, such as pre-eclampsia and gestational diabetes (Chitongo *et al.*, 2017; Huda *et al.*, 2017; Martin *et al.*, 2009; Trivett,

Lees and Freeman, 2021) When SAT and VAT were directly compared, VAT measurements in the first trimester were found to correlate better with measurement of insulin resistance (HOMA-IR), blood pressure, triglycerides and cholesterol than both SAT and BMI.(Bartha *et al.*, 2007; Martin *et al.*, 2009)

1.11.2 Adipocyte dysfunction in obese pregnancy

1.11.2.1 Adipokine profile in healthy and obese pregnancy

In healthy pregnancy, adipose tissue accumulation leads to increased leptin production by adipocytes which, combined with placental leptin production, results in a gestational increase in leptin.(Savard *et al.*, 2022) The function of hyperleptinaemia in pregnancy is to increase maternal appetite to provide nutrition for the growing fetus, and there is the development of leptin-insensitivity, similar to that which occurs in obesity.(Tessier, Ferraro and Gruslin, 2013). In obese pregnancy, much like in non-pregnant individuals, leptin is positively correlated with BMI.(Hendler *et al.*, 2005) Andersson-Hall *et al* characterised gestational changes in adipokine concentrations during pregnancy between healthy weight and obese women. Leptin concentration was higher in obese throughout pregnancy and post-partum and, in contrast to lean pregnancy, did not increase with gestation.(Andersson-Hall *et al.*, 2020)

Adiponectin decreases during pregnancy, and low adiponectin:leptin ratio has been associated with both pre-eclampsia and serum amyloid A, a biomarker of dysfunctional adipose tissue which is associated with insulin resistance.(Frühbeck *et al.*, 2019; Hendler *et al.*, 2005) Andersson-Hall *et al* found adiponectin concentrations were significantly lower in obese women at the start of pregnancy and fall similarly to those of lean women until the third trimester when lean women had a sharp drop in adiponectin levels.(Andersson-Hall *et al.*, 2020; Savard *et al.*, 2022) The loss of the anti-inflammatory properties of adiponectin in obesity means the pro-inflammatory state goes unopposed which may contribute to the pathologies associated with the low adiponectin:leptin ratio.

1.11.2.2 Pro-inflammatory cytokines in healthy and obese pregnancy

Pregnancy itself is an inflammatory state, with evidence of an increase in inflammatory markers that are counterbalanced in healthy pregnancy by anti-

inflammatory factors, including adiponectin and IL-10.(Fialová *et al.*, 2006) The reasons for this increased inflammation is not well understood but may relate to adaptations to the immune system necessary to allow successful implantation and placental development.(Zhou *et al.*, 2021) Successful implantation is associated with a shift from the Th1 response of cellular immunity, characterised by release of IL-2, interferon gamma, TGF- β and TNF- α , to the humoral response via Th2 cytokines IL-4, IL-5, IL-6. Imbalance in these responses has been postulated as a potential mechanism for complications such as pre-eclampsia which is more prevalent in the obese. Our group has previously identified that evidence of an excessive Th2 response in obese pregnant women, as assessed by low TNF- α :IL-6 ratio, was associated with endothelial dysfunction.(Stewart *et al.*, 2007)

IL-6 has been demonstrated to be significantly higher in obese women in the first trimester, whilst lean women had a gestational increase in IL-6 which reached that of obese women by the third trimester.(Stewart *et al.*, 2007; Zembala-Szczerba *et al.*, 2017) In a study by Savard *et al.*, IL-6 was compared across all three trimesters of pregnancy in women of different BMI groups (lean, BMI 25-30, BMI >30) and there was a significant effect of both gestation and BMI by two-way ANOVA.(Savard *et al.*, 2022) In addition, increased IL-6, TNF- α and CRP have been linked to both pre-eclampsia and PAI-1, a marker of endothelial dysfunction.(Udenze, Arikawe and Makwe, 2017) Taken together, these data might indicate that the pro-inflammatory phenotype obese women have in the first trimester, which leads to endothelial dysfunction and pre-eclampsia, stems from a pre-pregnancy adipose-derived inflammation.

1.11.3 Cardiometabolic changes in healthy and obese pregnancy

Pregnancy is a period of increased metabolic demands, with a progressive increase in insulin resistance, increased lipid mobilisation and improved microvascular function.(Stewart *et al.*, 2007) Data from Sonagra *et al.* showed a progressive increase in fasting serum insulin levels and HOMA-IR with advancing gestation, demonstrating the development of insulin resistance that characterises the catabolic state which ensues in late pregnancy.(Sonagra *et al.*, 2014; Lain and Catalano, 2007) This insulin resistance of healthy pregnancy results in elevated maternal glucose and free fatty acids which is a physiological mechanism to ensure

adequate supply of carbohydrate-derived energy and nutrients to the fetus. (Sivan *et al.*, 1998) In addition, HDL in pregnancy is known to have antithrombotic, antioxidant and vasodilatory functions. (Sulaiman *et al.*, 2016)

However, in obesity these mechanisms are disrupted, possibly as a result of adipocyte dysfunction. In obese pregnancy there is more pronounced insulin resistance with increased incidence of gestational diabetes. (Trivett, Lees and Freeman, 2021) In contrast to the improvement in microvascular function, there can be impairment of endothelial function which contributes to the increased risk of pre-eclampsia in overweight and obese pregnant women. (Udenze, Arikawe and Makwe, 2017) The underlying pathophysiology in obesity-induced endothelial dysfunction has some similarities between pregnant and non-pregnant populations with inflammatory markers, including CRP, TNF- α and IL-6, considered as mediators. (Esteve *et al.*, 2007; Ramsay *et al.*, 2002; Stewart *et al.*, 2007; Fialová *et al.*, 2006)

And whilst increased mobilisation of lipids is intended to provide nutrition to the fetus, obese pregnancy has been associated with higher first trimester triglyceride levels, reduced HDL, an atherogenic LDL subtype and increased neonatal birthweight, suggesting that the fetus may be exposed to elevated levels of circulating fatty acids. (Jarvie *et al.*, 2020; Meyer *et al.*, 2013; Jarvie *et al.*, 2010; Sulaiman *et al.*, 2016)

Saucedo *et al* identified an overlap in the risk factors responsible for the cardiometabolic complications of obese pregnancy, with those attributable to VTE in pregnancy, which may suggest a shared pathophysiology in the development of the prothrombotic state in obese pregnancy.

1.12 Obesity as a prothrombotic state

The increased risk of VTE in obesity in the non-pregnant is thought to be linked to dysfunctional adipose tissue and abnormal adipose tissue distribution. Several studies have observed BMI and adipokines to be associated with elevated procoagulant factors, thrombin generation and the generation of tissue factor-bearing microparticles.

1.12.1 Role of leptin in the pathophysiology of VTE

Leptin is a recognised risk factor for arterial thrombotic events, and leptin levels positively correlate with coronary heart disease risk. (Wallace *et al.*, 2001) The evidence base in venous thrombosis is much smaller, however hyperleptinaemia has been shown to be an independent risk factor for VTE in a cohort of patients post total knee arthroplasty where elevated pre-operative leptin was found to be significantly associated with post-operative deep vein thrombosis (DVT) in multivariate analysis. (Lu *et al.*, 2018) In addition, high leptin:low adiponectin ratio has been associated with the development of the post-thrombotic syndrome. (Mrozinska *et al.*, 2018) Laboratory evidence of an activated coagulation system exists, which supports a role for leptin in the pathophysiology of obesity-related VTE: Leptin has been positively correlated with levels of fibrinogen, PAI-1, mean platelet volume, FVIII and platelet microparticles (Csongrádi *et al.*, 2017; Pieterse, Schutte and Schutte, 2015; Singh *et al.*, 2010; De Mitrio *et al.*, 1999) Leptin has also been significantly correlated with clot strength and formation as measured by ROTEM in a cohort of bariatric patients. but leptin has never been directly related to thrombin generation. (Taura *et al.*, 2014)

Leptin receptors are present not only in hypothalamic tissue where they are involved in satiety signalling, but also in peripheral blood mononuclear cells. (Rafail *et al.*, 2008; Zarkesh-Esfahani *et al.*, 2001) Zarkesh-Esfahani *et al.* demonstrated the presence of the leptin receptor on PBMCs and that stimulating these cells with leptin resulted in the release of pro-inflammatory cytokines: There was a 5-fold increase in TNF- α , whilst IL-6 production was increased 19-fold, suggesting that leptin contributes to the chronic pro-inflammatory state in obesity. (Zarkesh-Esfahani *et al.*, 2001) There is also evidence that leptin induces monocyte production of tissue-factor bearing microparticles with high levels of such microparticles being associated with a previous history of VTE. (Petrini *et al.*, 2016; Bucciarelli *et al.*, 2012) In vitro stimulation of PBMCs by leptin increased tissue-factor mRNA expression and activity in a dose-dependent manner, and was abolished by JAK-2 inhibition and anti-TNF α inhibition, suggesting that both TNF- α and JAK-STAT signalling is implicated in the process. (Rafail *et al.*, 2008)

Chapter 1

Enhanced platelet activity, thought to be mediated through activation of the JAK-STAT pathway and the GPIIb/IIIa receptor, has also been demonstrated with leptin. Obese patients, with high levels of leptin, demonstrate accentuated *in vitro* ADP-dependent platelet aggregation compared with normal weight subjects.(Dellas *et al.*, 2008) Corsonello *et al* identified a dose-dependent relationship between leptin and *in vitro* ADP-dependent platelet aggregation in lean individuals, however they found this effect to be blunted in the obese group, which the investigators suggested may reflect a degree of leptin-resistance within the platelet leptin-receptor.(Corsonello *et al.*, 2003) However, a further study identified that leptin at higher concentration enhances ADP-dependent platelet aggregation, which may mean that supra-therapeutic doses of leptin may overcome the apparent leptin-resistance *in vitro*.(Nakata *et al.*, 1999)

Leptin has been shown to promote endothelial dependent vasodilation via nitric oxide in healthy individuals, a function which is impaired in hyperleptinaemia resulting in endothelial dysfunction.(Wang *et al.*, 2013; Vecchione *et al.*, 2002; Korda *et al.*, 2008) In most of the studies, endothelial function was assessed by real-time non-invasive methods, which are able to directly assess vasodilation under different conditions, to identify differences in endothelial-dependent and -independent vasodilation, but plasma biomarkers for endothelial function have also been investigated.

PAI-1 and von-willebrand factor are coagulation factors located in endothelial cells and are non-specific markers of endothelial damage. In a study by Pieterse *et al*, leptin was assessed alongside PAI-1, von Willebrand factor (vWF) and urinary albumin:creatinine ratio (ACR), the latter being a marker of renal vascular damage. Leptin was found to be independently associated with PAI-1, fibrinogen and clot lysis time, rather than with vWF and ACR.(Pieterse, Schutte and Schutte, 2015)

There seem to be several possible mechanisms by which leptin contributes to thrombotic risk: by inducing a pro-inflammatory state which is known to be a driver of coagulation, by inducing PBMC production of tissue-factor bearing microparticles, by increasing platelet aggregation and by inducing endothelial dysfunction.

1.12.2 Role of adiponectin in the pathophysiology of VTE

In contrast to leptin, adiponectin has been shown to have both anti-inflammatory and directly antithrombotic properties. Studies *in vitro* have demonstrated dose-dependent inhibition of macrophage proliferation, reduced macrophage TNF- α production and inhibition of TNF α -induced monocyte adhesion. Production of adhesion molecules, V-CAM-1, E-selectin and I-CAM-1, which are biomarkers for endothelial dysfunction and involved in the early development of atherosclerosis were also inhibited by adiponectin. (Ouchi *et al.*, 1999; Yokota *et al.*, 2000) Okamoto *et al* stimulated adiponectin pre-treated macrophages with lipopolysaccharide (LPS) and quantified both tissue factor and tissue factor mRNA, both of which were reduced. Furthermore, an adiponectin knock-out mice study found accelerated thrombus formation following laser arterial injury in the knock-out model which was attenuated by adiponectin supplementation. These results were strengthened by *in vitro* evidence of increased platelet aggregation and enhanced thrombus formation in blood-collagen studies in the knock-out mice. (Okamoto *et al.*, 2013; Kato *et al.*, 2006)

Taken together, these data suggest that the loss of the protective antithrombotic effects, when adiponectin is reduced in obesity, combined with the procoagulant effects of leptin may in part explain the increased thrombotic potential of obesity.

1.12.3 Role of chronic inflammation in the pathophysiology of VTE

The link between the pro-inflammatory state and thrombosis is well-recognised, particularly with further recognition of acute thromboinflammation in the setting of COVID-19. An association exists also between chronic low-grade inflammation, such as that seen in obesity, and thrombotic risk. As previously discussed, inflammatory cytokines including TNF- α , IL-6, CRP and MCP-1 are increased in obesity due to increased production by adipocytes or release from adipose tissue macrophages. TNF- α is known to induce IL-6 production which in turn stimulates the hepatic acute phase response resulting in increased synthesis of CRP, fibrinogen and serum amyloid A by the liver. (Cottam *et al.*, 2004)

Analysis of the inflammatory marker C-reactive protein (CRP) in a large prospective longitudinal study investigating risk factors for VTE, found a positive

correlation between obesity and CRP. CRP was found to be independently associated with thromboembolic events, once adjusted for BMI, and the addition of CRP to hazards model resulted in an attenuation of the effect of BMI on VTE risk which the researchers proposed indicated the importance of inflammation in the pathophysiology of VTE in the obese. (Olson *et al.*, 2014; Kunutsor *et al.*, 2017)

The increased expression of monocyte chemotactic protein-1 (MCP-1) may provide a link to the increased risk of thrombosis in obesity, as this protein has been shown to generate tissue factor and is linked to thrombus formation.(Charo and Taubman, 2004)

1.12.4 Tissue factor-bearing microparticles and the prothrombotic state

Several studies have identified obesity to be associated with upregulation of the tissue factor pathway.(Okamoto *et al.*, 2013; Rafail *et al.*, 2008; Egorina *et al.*, 2005) Increased lipolysis associated with the expansion of adipose tissue results in the release of inflammatory cytokines, which in turn induce the production of tissue factor mRNA by monocytes. This results in an increased in monocytes expressing tissue factor and tissue-factor bearing microparticles.(Petrini *et al.*, 2016; Samad and Ruf, 2013)

Tissue factor-bearing microparticles, derived from the membranes of activated or apoptotic cells, have been shown to have a prothrombotic role and have been identified in several thrombotic conditions including cardiovascular disease and venous thromboembolism in addition to obesity.(Bucciarelli *et al.*, 2012; Campello *et al.*, 2015) By binding tissue factor and expressing phosphatidylserine, the microparticles present an optimum environment for initiation and propagation of thrombosis. Microparticles are known to be increased in obesity and corresponding thrombin generation data shows an increase in ETP, peak thrombin and a reduction in lag-time when compared to normal weight individuals.(Campello *et al.*, 2015)

Tissue factor is present throughout the body, with the large majority associated with solid organs, however there is a small dormant pool in endothelial cells and circulating myelomonocytic cells.(Fleck *et al.*, 1990; Egorina *et al.*, 2005) In the

setting of inflammation, endothelial cells and monocytes are activated by cytokines and lipopolysaccharides which results in the induction of tissue factor mRNA production, a significantly enhanced expression of tissue factor and a reduction in thrombomodulin expressed on the endothelium, causing a shift towards a procoagulant environment.(Samad, Pandey and Loskutoff, 1998; Szotowski *et al.*, 2005; Archipoff *et al.*, 1991)

1.12.5 Adipose tissue depot and thrombotic risk

As VAT is known to be the adipose depot most closely correlated with risk of cardiovascular disease, it might be assumed that VAT would also predict for risk of VTE. However, in one study, SAT rather than VAT was found to be independently predictive of PE. A large autopsy-based case-control study by Ogren *et al* examined the autopsy data from nearly 24,000 in-hospital deaths and found that 50% had PE at time of death, with 50% of these deemed to be the cause of death. The investigators identified that BMI and SAT were both positive predictors of progression from DVT to PE, independently of age, gender and presence of cancer. (Ogren *et al.*, 2005)

The association between the adipose tissue compartment and thrombin generation is conflicting, with Chitongo *et al* finding VAT to be more closely associated with an increase in thrombin generation parameters than SAT, and Prüller *et al* finding SAT to be the adipose tissue depot most predictive of the thrombin generation parameter, endogenous thrombin potential (ETP).(Chitongo *et al.*, 2017; Prüller *et al.*, 2012)

1.13 The prothrombotic effect of obesity during pregnancy

Considering the evidence outlined thus far, both pregnancy and obesity are recognised as independent risk factors for venous thrombosis, but when occurring simultaneously the effect appears to be multiplicative. Jacobsen *et al* showed in a case-control study that BMI ≥ 25 was associated with an increased risk of thrombosis during pregnancy, with adjusted odds ratio of 2.4. With the addition of antepartum immobilisation this risk increased to an adjusted odds ratio of 40.1 for women with BMI ≥ 25 , compared to 10.8 for women with BMI <25 .(Jacobsen,

Skjeldestad and Sandset, 2008) This suggests that obesity likely has a significant multiplicative effect on pre-existing thrombotic risk factors.

Oestrogen is known to be the significant driver of the prothrombotic state in healthy pregnancy, with oestrogen inducing hepatic synthesis of many coagulation factors linked to the increase in thrombin generation in early pregnancy. (Bagot *et al.*, 2019) However, in obese pregnancy there is likely the added influence of those factors which increase the thrombotic risk in obesity: adipose tissue distribution, adipose tissue dysfunction and the chronic inflammatory state.

A small study by Smrtka *et al* may provide some laboratory evidence of this: Obese (BMI >30kg/m²) and non-obese (BMI <30kg/m²) pregnant women had a range of coagulation parameters measured in late third trimester. Plasminogen, fibrinogen, factors II, VII and VIII were measured, along with naturally-occurring anticoagulants protein C (and activated protein C resistance), and protein S (total and free). Only plasminogen (p=0.04) and fibrinogen (p<0.001) were found to be significantly higher in the obese group, with a positive correlation identified between BMI and fibrinogen. However, a further study by Morgan *et al* found no significant change in fibrinogen, Protein S, PAI-1 or platelet count when lean and obese pregnant women were compared in the third trimester of pregnancy. Thromboelastography was also assessed and no BMI-related effect was noted. (Morgan *et al.*, 2017)

Although VTE is a significant and potentially life-threatening complication of pregnancy, the actual population incidence is low, at 1 in 1000 pregnancies, making studying this condition challenging. In addition, given the dynamic changes in balance of pro-coagulant and anti-coagulant factors during pregnancy, measuring individual factor assays does not give an accurate representation of the prothrombotic state. Several assays of global coagulation have been studied, with the largest body of research undertaken using the Calibrated Automated thrombogram (CAT) method of thrombin generation.

1.13.1 Thrombin generation data in obese pregnancy

The calibrated automated thrombogram (CAT) method of thrombin generation is the global coagulation assay most investigated in obese pregnancy, yet there is

Chapter 1

not a large body of evidence in this area and data has been conflicting. Ismail *et al* observed that thrombin generation parameters, endogenous thrombin potential (ETP) and peak thrombin, were increased more in morbidly obese pregnant women compared to normal weight pregnant women when measured during the third trimester.(Ismail *et al.*, 2014) The MaTOPs study compared thrombin generation in obese women (BMI ≥ 30) with lean women (BMI 18.5-25). In contrast to the earlier study by Ismail *et al*, no difference in ETP was found between lean and obese at 26-28 weeks' gestation, whilst interestingly obese women were found to have lower ETP than lean at term. ETP reduced in both groups in the postpartum period.(Mandlebe *et al.*, 2021)

O'Keefe *et al* compared three global coagulation assays in pregnant women at term with non-pregnant controls. 41.7% of the pregnant group were obese (BMI ≥ 30). Thromboelastography (TEG), CAT thrombin generation and overall haemostatic potential assay (OHP), the latter measuring fibrin generation, all showed hypercoagulability in the pregnant group compared to non-pregnant controls. There was no difference in any of the CAT thrombin generation or OHP parameters at term when lean (BMI 18.5-24.9kg/m², n=13) were compared with obese (BMI ≥ 30 kg/m², n=25), however maximum amplitude as assessed by TEG was significantly higher in obese pregnant women at term.(O'Keefe *et al.*, 2021) However, a further study of rotational thromboelastography (ROTEM) at term found no difference in coagulation between lean and obese pregnant women at term.(Lee *et al.*, 2020)

The limitations of each of these studies is that measurements were only taken during the third trimester or term. It is known that there is evidence of a prothrombotic state from the 5th gestational week, and that 50% of maternal deaths from VTE occur in the antenatal period, therefore there may be unmeasured changes in the prothrombotic state in earlier pregnancy which these studies have not accounted for.

1.14 Hypothesis

The incidence of VTE-related maternal death has remained unchanged for over a decade, despite the use of prophylactic low molecular weight heparin in those deemed, by risk-stratification, to be at greatest risk. Overweight and obese

Chapter 1

women are over-represented within the maternal mortality data which suggests that the current strategy to identify women at greatest risk of VTE is insufficient for this population. Common aspects of the pathophysiology between thrombosis and the metabolic syndrome, including a low-grade inflammatory state and central adiposity, have been identified in non-pregnant individuals.

A more thorough understanding of the pathophysiology underlying the prothrombotic state in pregnancy complicated by obesity would allow better identification of risk factors pertinent to this population, and in turn allow development of further strategies to mitigate the increased thrombotic risk posed to this population. Due to the relatively low incidence of VTE in pregnancy, conducting a study with an outcome measure of VTE incidence would require a large study population and long duration of study before any meaningful conclusions can be drawn. The use of a reliable biomarker can circumvent this difficulty, and *ex vivo* thrombin generation has been previously associated with risk of VTE and has been measured in healthy pregnancy.

This thesis aims to test the hypothesis that the increased risk of venous thromboembolism in obese pregnant women, as measured by thrombin generation, is related to low-grade chronic inflammation due to adipocyte dysfunction and adipose tissue distribution.

1.14.1 Aims

A longitudinal study of pregnant women within 4 different BMI classes (lean, overweight/obese class I, obese class II and obese class III) was devised to allow the characterisation of the prothrombotic phenotype of obese pregnancy and investigate the underlying pathophysiology.

As the hypothesis was that adipose tissue distribution and adipocyte dysfunction contributed to the prothrombotic state, an assessment of the prothrombotic state (using individual coagulation factor assays alongside *ex vivo* thrombin generation) was made and correlated with measurements of adipose tissue distribution (Waist and hip circumference, abdominal SAT and VAT depths) and adipokines known to be associated with dysfunctional adipose tissue (leptin, adiponectin, IL-6, TNF- α and IL-1 β).

Chapter 1

Blood samples were collected at the antenatal booking visit (8-12 weeks) and at 28 weeks' gestation to assess for gestational changes in haemostasis and adipokine parameters.

Objective 1 (Chapter 3)

To characterise the prothrombotic effect of obesity during pregnancy by examining the changes in laboratory markers of haemostasis in women within each of the 4 BMI classes (lean, overweight/obese class I, obese class II and obese class III) between early pregnancy (8-12 weeks' gestation) and the third trimester (28 weeks' gestation). The prothrombotic state will be assessed by quantification of procoagulant factors (Factor VIII and fibrinogen), naturally-occurring anticoagulants (Protein S and TFPI), and markers of fibrinolysis (D-dimer and PAI-1), in addition to assessment of the global coagulation assay and biomarker for thrombotic risk, thrombin generation.

Objective 2 (Chapter 4)

To characterise the difference in the pattern of adipokines and inflammatory cytokines in women within each of the 4 BMI classes (lean, overweight/obese class I, obese class II and obese class III) between early pregnancy (8-12 weeks' gestation) and the third trimester (28 weeks' gestation). Adipokines known to be altered in obesity (leptin and adiponectin) and markers of the low-grade inflammatory state (TNF- α , IL-6 and IL-1 β) will be correlated with the thrombin generation and other haemostatic parameters to identify drivers of the prothrombotic state in obese pregnancy.

Objective 3 (Chapter 5)

To compare abdominal adipose tissue distribution, measured by waist and hip circumference at booking and ultrasound scan at 12-17 weeks' gestation, in women within each of the 4 BMI classes (lean, overweight/obese class I, obese class II and obese class III) and investigate the relationship between adipose tissue distribution, and the adipokine and haemostasis profile measured at booking.

Objective 4 (Chapter 6)

Chapter 1

To determine, based on the findings of Chapters 3-5, which of the adipokines and which measurements of adipose tissue distribution are most closely related to thrombin generation and the other measured haemostasis parameters known to be affected by BMI in obese pregnant women.

Chapter 2 Materials and Methods

2.1 Introduction

This chapter provides an outline of the design and methodology of the study used to address the research questions in this thesis, “An Investigation of the Pathophysiology underlying the increased risk of venous thrombosis in overweight and Obese Pregnant women (The IPOP study)”. The IPOP study was carried out between April 2019 and March 2020. Funding for the study and a Clinical Research Fellowship was the result of a successful application to the British Heart Foundation which I wrote along with my supervisors, Dr Catherine Bagot and Dr Dilys Freeman. I was personally successful in my application for an NHS Greater Glasgow and Clyde endowment fund award which was used to fund some of the laboratory analyses. I devised the study design alongside both of my supervisors, and performed all of the haemostasis laboratory analyses, thrombin generation and ELISAs for TFPI and PAI-1. The remaining ELISAs were conducted by Mrs Fiona Jordan at the University of Glasgow due to time constraints. I performed all of the statistical analyses with support from Dr Dilys Freeman.

2.2 Study Design

2.2.1 Ethical considerations

Ethical approval for the study, and subsequent amendments, was granted by the West of Scotland Research Ethics Committee 4 (Reference number 19/WS/0022) on the 6th of February 2019 and Board approval was issued by Greater Glasgow and Clyde Research and Development (R&D reference GN18HA013).

Written informed consent was obtained from study participants after they had read the participant information leaflet (PIL) (see Appendix 1) and been given 30 minutes to consider whether they wished to take part.

2.2.2 Study outline and recruitment considerations

This was a prospective observational study with four different body mass index (BMI)-based cohorts. The study was designed to allow the assessment of changes in coagulation parameters and adipokines with advancing pregnancy, and how these differ dependent upon BMI.

Body mass index (BMI) was calculated as follows:

$$\text{BMI (kg/m}^2\text{)} = \text{Weight (kg)} \div (\text{height(m)})^2$$

BMI groups were assigned according to the World Health Organisation (WHO) classification (see Table 2).(WHO, 2010)

Table 2 IPOP study BMI groups and WHO BMI classification system.

***Adapted from World Health Organisation “A healthy lifestyle” factsheet, 2010.(WHO, 2010)**

BMI	WHO Nutritional Status*	IPOP Study group
<18.5	Underweight	Lean
18.5-24.9	Normal weight	
25.0-29.9	Pre-obesity / Overweight	Overweight/Obese Class I
30-34.9	Obesity class I	
35.0-39.9	Obesity class II	Obese class II
≥40	Obesity class III	Obese class III

2.2.2.1 Study population

The study population was recruited from women attending for the antenatal booking appointment at the Princess Royal Maternity Hospital, Glasgow Royal Infirmary. The booking appointment is a routine part of antenatal care and ideally takes place between 8-15 weeks’ gestation. The appointment is conducted by a midwife in either a hospital or community clinic setting with the purpose of identifying any issues pertaining to the health of the woman or the pregnancy which may need further monitoring during her antenatal period.

Chapter 2

It has previously been demonstrated that changes in coagulation occur very early in pregnancy, from the 5th gestational week, however identifying women eligible to participate in antenatal studies during these early stages of pregnancy is challenging as the majority of women are not yet aware of the pregnancy, nor will they have made contact with antenatal services. (Bagot *et al.*, 2017) Therefore, the booking appointment was selected as the point for study recruitment as it is usually the first contact that most women will have with obstetric services. Measurements and blood samples taken at the booking appointment can provide a surrogate baseline with which to compare samples obtained later in pregnancy to show the further changes which occur as pregnancy progresses.

2.2.2.2 Study design flowchart

The study consisted of three study visits, as outlined in Figure 2-1. The first and third visit were chosen to coincide with routine antenatal care to reduce the number of attendances to hospital for participants and in turn encourage attendance to study follow-up appointments. The first visit, at which women were recruited to the study, coincided with the booking appointment. At this visit blood samples and anthropometric measurements were obtained, along with demographic data collection.

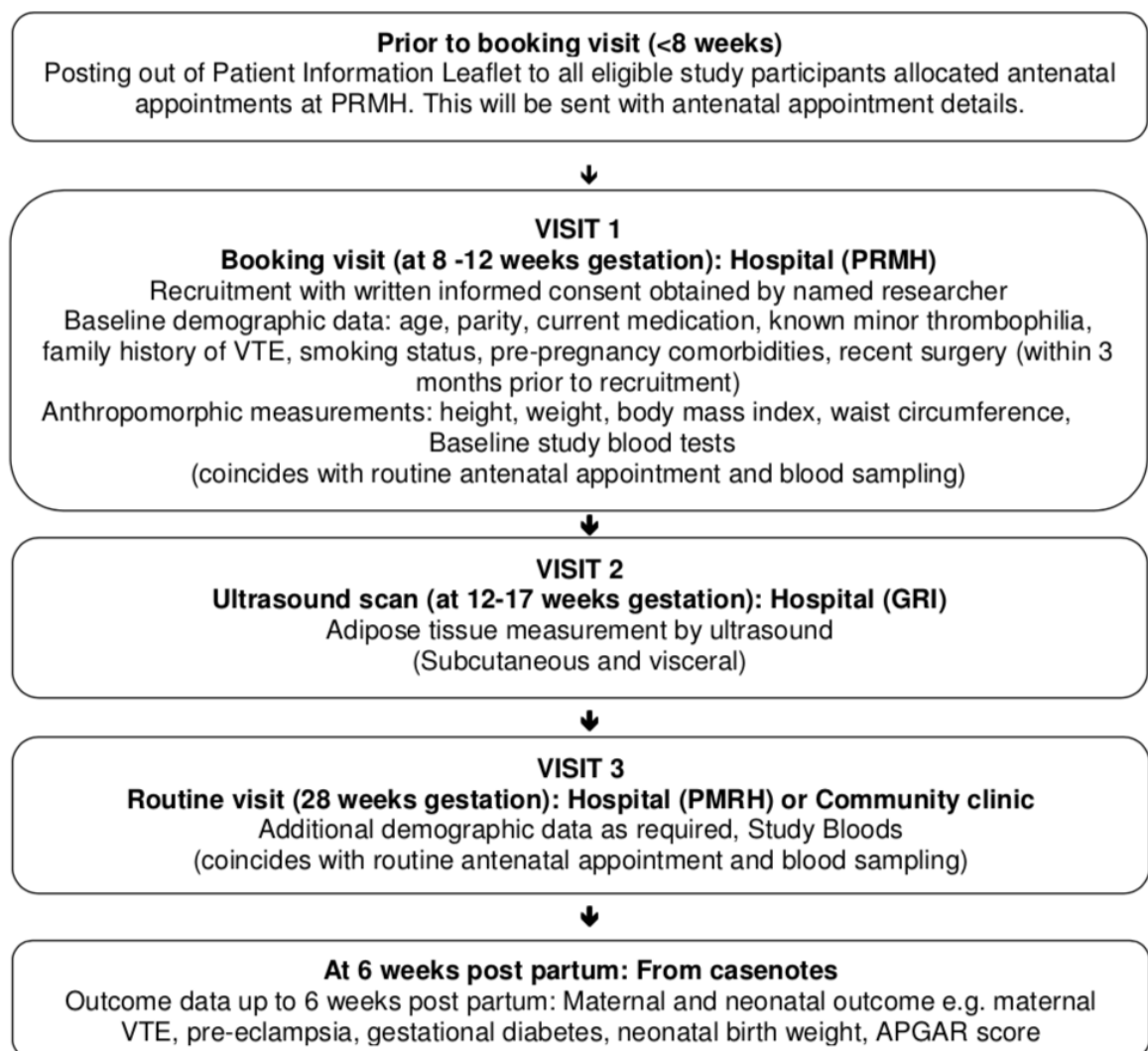


Figure 2-1 Study design flowchart.

The study consisted of 3 visits, with the data collected at each visit outlined above.

The second study visit was an additional attendance for an ultrasound scan to measure subcutaneous and visceral abdominal fat. This was arranged between 12-17 weeks' gestation. The third study visit coincided with the 28 week (± 2 weeks)

routine antenatal clinic attendance. Repeat study bloods were taken alongside routine antenatal clinic blood samples. Outcome data was collected from case notes once 6 weeks postpartum had elapsed.

2.2.3 Inclusion/Exclusion criteria

Inclusion and exclusion criteria were as summarised in Table 3. All women attending for a booking appointment at the Princess Royal Maternity hospital antenatal clinic who were ≥ 18 years age and were pregnant at ≤ 15 weeks' gestation were invited to take part in the study. Women were ineligible to participate in the study if they were taking low molecular weight heparin or had a previous history of venous thromboembolism; if they had a known major thrombophilia (protein C, protein S or antithrombin deficiencies); were unable to provide informed consent without the need for an interpreter; or were known to be having a twin pregnancy at time of booking appointment. Women with medical comorbidities were not excluded.

Gestation at time of recruitment to the study was estimated from the last menstrual period (LMP), and only women with an estimated gestation of 8-15 weeks were recruited to the study. Due to variability in menstrual cycle length, the gestation by LMP is an estimate, and the actual gestational age at time of booking was calculated from the dating scan performed around 12 weeks' gestation.

Chapter 2

Table 3 Study inclusion and exclusion criteria

Inclusion/Exclusion Criteria	Rationale
Inclusion	
≥ 18 years of age	Women <18 years usually attend different hospital clinic for antenatal care with added social support
≤ 15 weeks' gestation	First blood samples to reflect first/early second trimester
Exclusion	
Currently prescribed low molecular weight heparin (LMWH)	Anticoagulation affects coagulation studies and may impact development of normal prothrombotic state during pregnancy
Previous VTE/major thrombophilia	Women would require prophylactic anticoagulation during pregnancy.
Unable to provide informed consent without the need for an interpreter	Attendance of in-person interpreter not always possible, therefore true informed consent could not be guaranteed. Interpreter costs not available for second study visit which did not coincide with usual antenatal care
Twin pregnancy	Investigating only singleton pregnancies to minimise additional confounding variables.

2.2.4 Power Calculation

Scottish statistics on the prevalence of obesity in the pregnant population, around the time of study development in 2017, indicated approximately 30% of pregnant women were overweight, 16% obese and 2% morbidly obese (BMI ≥ 40)(NSS, November 2016). This prevalence data was taken into consideration when sample size was calculated so that the sampling of the population was representative of the population as a whole.

Sample size calculations were undertaken using GraphPad StatMate version 2.00 by The Robertson Institute, University of Glasgow. Calculations were performed using sample size for unpaired t-test, and were based upon standard deviation (SD) data from previous thrombin generation studies. All calculations assumed 80% power with a Type 1 error rate of 0.05 (two-tailed).

Chapter 2

Ismail *et al* investigated the effect of obesity in pregnancy on thrombin generation by conducting a study in late third trimester with 20 normal weight women and 20 morbidly obese women (BMI >40). The difference in mean endogenous thrombin potential (ETP) between lean and obese participants was 402nM*min (21% increase) with maximum standard deviation (SD) of 234.(Ismail *et al.*, 2014) Thrombin generation is known to correlate with BMI in non-pregnant populations, with a smaller difference in thrombin generation parameters reported between normal weight and overweight/obese women than in the study by Ismail *et al.* One study compared normal weight to overweight and obese non-pregnant women. This study reported a 6.3% and 8.4% increase in ETP in overweight and obese women respectively (maximum S.D. = 236).(Beijers *et al.*, 2010) In our laboratory, a study performed on pregnant women compared a mean ETP of 1610 +/- 273 nM*min in the first trimester compared to a mean ETP of 1201 +/- 222 nM*min in the same women in a non-pregnant state (34% increase in ETP).(Bagot *et al.*, 2017)

Given the above data, using the mean ETP obtained in our laboratory in pregnant women during the first trimester (1610 nM*min) and the highest standard deviation obtained in these studies (273 nM*min), it was proposed to recruit 100 lean women with BMI 18-24.9 and 100 women with a BMI 25-35. Assuming a mean increase in ETP of 240 and 330 nM*min in pregnant women with a BMI ≥ 35 and BMI >40 respectively, it was proposed to recruit 20 women with a BMI ≥ 35 and 12 women with a BMI >40.

Previous observational studies undertaken at the Princess Royal Maternity Hospital reported <1% loss to follow-up. It was initially anticipated that there would be a combined lost to follow up and study withdrawal of < 5%. This included an estimate of approximately 2% of women who may suffer a second trimester miscarriage after recruitment into the study.(Ammon Avalos, Galindo and Li, 2012) During recruitment however, it became apparent that the loss to follow-up was greater than anticipated, primarily due to first trimester miscarriage as most women had not undergone a viability scan prior to attending for their booking appointment and agreeing to participate in the study. As such, loss to follow-up due to first trimester miscarriage was 6%. Loss to follow-up due to other causes (e.g. participant choice to withdraw, participant moved out of healthboard area) was observed to be 5%. In order to maintain 80% power of the study, the Research Ethics Committee was consulted during the course of recruitment and a

Chapter 2

substantial amendment was submitted to alter the recruitment target, reflecting the greater loss to follow-up of 10-15%. In accordance with the informed consent form signed by participants at study entry, all data and blood samples collected up until the point of loss to follow up were included in the final analysis.

Based upon loss to follow-up of up to 15%, final target recruitment numbers were: 115 women with normal BMI (18-24.9), 115 women with a BMI 25-34.9, 23 women with a BMI 35-39.9 and 14 women with a BMI ≥ 40 .

2.2.5 Loss to Follow-up

A consort diagram of study recruitment and loss-to-follow-up is provided in Figure 2-2. In total 263 women were recruited to the study. Once ineligible women were excluded, there were 109 lean (BMI 17-24.9), 115 overweight/obese class I (BMI 25-34.9), 24 obese class II (BMI 35-39.9) and 14 obese class III (BMI ≥ 40).

The study ultrasound scan, to measure subcutaneous and visceral adipose tissue depth, was attended by 154 women. As this was separate to the blood sampling, women not attending this appointment were not excluded from further study follow-up. Results from the study ultrasound will be discussed in Chapter 5.

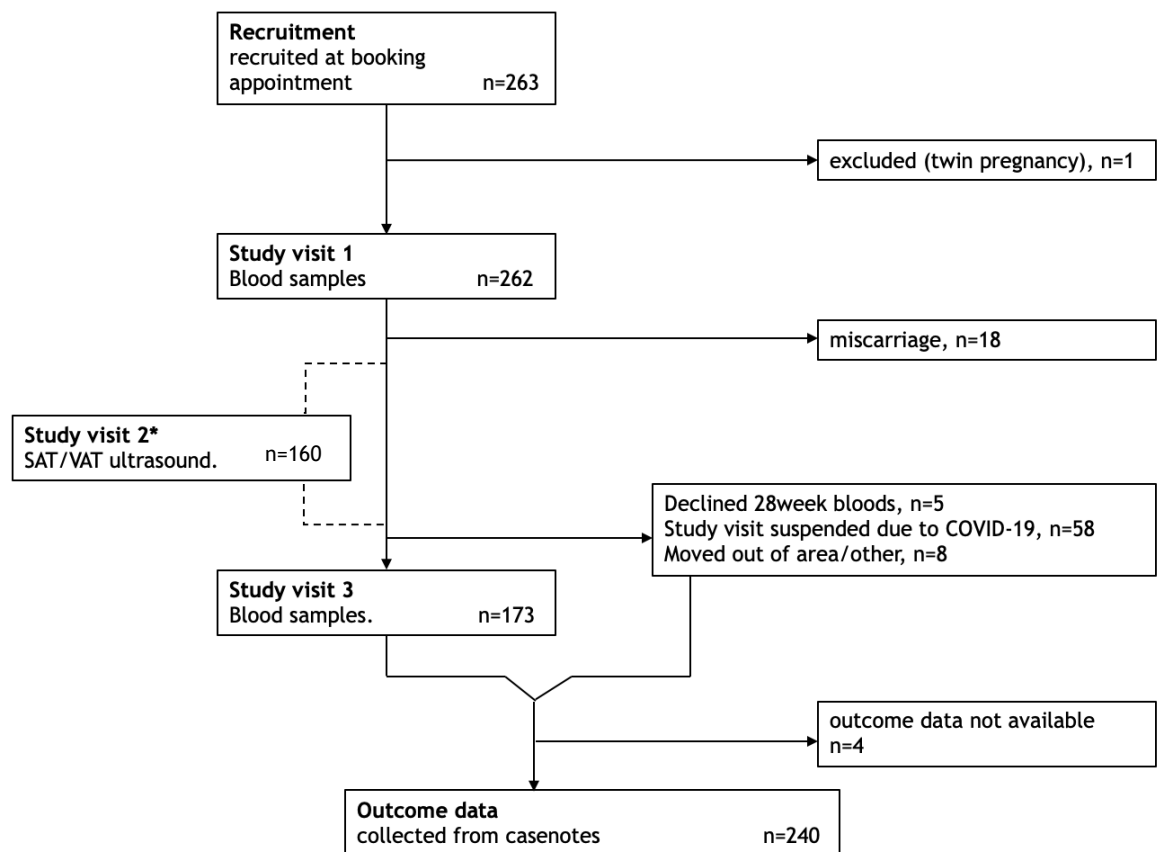


Figure 2-2 Consort diagram of study design and recruitment, including loss to follow-up

*study visit 2 was an additional attendance out with routine antenatal care. Many women were unable to attend this appointment but remained happy to continue within the study to provide research blood samples at study visit 3. For this reason, we did not exclude these women from continuing in the study and have not counted missing this appointment as lost to follow-up (this is further discussed in section 2.2.7.4).

Unfortunately, all study recruitment and follow-up visits were suspended with immediate effect in March 2020 due to the developing Coronavirus pandemic. This had a significant impact upon the rate of loss to follow-up, with the 28-week study

Chapter 2

visit and blood sampling being missed by 58 (22%) of the recruited women. All-cause loss to follow-up for each BMI group was: 40 (36%) of lean, 34 (29.6%) overweight/obese class I, 13 (54%) of obese class II and 2 (14%) of obese class III. Analysis of reasons for loss to follow-up are as per Table 4.

Table 4 Loss to follow-up

Loss to follow-up for 262 eligible women recruited to IPOP study. *other causes for loss to follow-up: participant moved out of area, research team unable to attend appointment. ow=overweight, ob=obese

Reason for loss to follow-up	Lean n=109	ow/ob class I n=115	ob class II n=244	ob class III n=14	Total (%)
COVID-19 study suspension	27	25	6	0	58 (22%)
First trimester miscarriage	6	4	5	2	17 (6.5%)
Second trimester miscarriage	1	0	0	0	1 (0.4%)
Participant choice	2	2	1	0	5 (1.9%)
Other*	4	3	1	0	8 (3.1%)
Subtotals, n (%)	40 (36%)	34 (29.6%)	13 (54.2%)	2 (14.3%)	89 (34%)

After loss to follow-up from miscarriage, COVID-19 and participant withdrawal, 173 participants completed both booking and 28 week blood sampling (lean=69, overweight/obese class I= 81, obese class II = 11 and obese class III = 12). Outcome data was collected from all women recruited to the study who had a viable pregnancy, providing consent for data collection had not been withdrawn. Outcomes were collected on 240 women, with 4 outcomes missing as the women had moved out of area and casenotes were not accessible.

In addition to the increased loss to follow-up, due to the COVID-19 pandemic and pause of all research work, a further cross-sectional study to investigate *ex vivo* cytokine release from adipocytes was unable to proceed. This small study aimed to collect samples of subcutaneous and visceral adipocytes from the abdominal adipose tissue of 20 pregnant women of normal BMI and 20 obese pregnant women undergoing planned caesarean section at term. Concurrent blood samples were to be obtained for thrombin generation analyses. Cultured adipocytes were to be assessed under basal and LPS-stimulated conditions with measurement of released cytokines in the supernatant measured by ELISA and results correlated with the

plasma thrombin generation results. It was hoped that findings of this cross-sectional study may have provided data to explain the pathophysiology of the associations identified between adipose tissue distribution and the prothrombotic state in the main observational study.

2.2.6 Study recruitment.

2.2.6.1 Participant recruitment process

Participant information leaflets (PIL) were initially sent out in the post by the antenatal booking office staff along with the appointment letter for all women attending PRMH for booking appointment. Booking appointments were allocated at random, therefore there was no bias towards more complex or high-risk pregnancies attending booking appointment in the hospital compared to community clinics. Once the woman had completed her booking appointment, the midwife then determined from the clinical history if the woman was eligible to have ongoing follow-up in the community (green pathway) or whether she had more complex antenatal care needs which necessitated hospital antenatal clinic follow-up (red pathway). (RCOG, 2015)

A member of the research team attended the antenatal clinic at PRMH and handed out the participant information leaflet to women who were deemed eligible upon case note review. Women were provided with a participant information leaflet upon arrival at the antenatal clinic and given a minimum of 30 minutes to consider if they wished to take part. Although PILs had initially been sent out in the post to give women awareness of the study before attending their appointment, we did not notice any change to recruitment when this was stopped, so we moved to a recruitment model whereby leaflets were only handed out to women on the day they attended for booking appointment when a researcher was present.

The antenatal clinic midwife undertook the booking antenatal assessment and if the patient was willing to participate in the study a member of the research team attended to confirm eligibility and obtain informed consent. Written informed consent was obtained and stored in the study site file, and a copy given to the study participant. Participants were allocated a unique study number and all data collected was labelled with this study number to allow for pseudo-anonymisation

Chapter 2

of data. A password-protected file kept participant details (name and community health index number) alongside study ID for the purpose of collecting outcome data from participant notes at a later date.

2.2.7 Data and sample collection

2.2.7.1 Demographics

Once informed consent was obtained, baseline demographic data was collected by a member of the research team. A demographics form (see Appendix 2), labelled only with the unique study ID, was completed for each participant. Information was collected on age, parity, past medical history, current medications, smoking status, and ethnicity.

Further demographic data was obtained at the 3rd study visit (28±2 weeks). Data was collected on any new comorbidity which may have developed since the booking appointment, current medications, smoking status, and pregnancy complications e.g. pregnancy-induced hypertension.

Outcome data was collected at 6 weeks post-partum from electronic case note review.

2.2.7.2 Anthropomorphic Measurements

Height (metres) and weight (kilograms) were measured by the antenatal clinic midwife as part of standard antenatal care. BMI (weight (kg)/height (m)²) was calculated by the antenatal care system, Badgernet, once height and weight were entered. Based upon the BMI calculated at the booking appointment, women were recruited into the appropriate study group: lean (BMI 18-24.9), overweight/obese class I (BMI 25-34.9), obese class II (BMI 35-39.9) or obese class III BMI ≥40 as previously indicated in Table 2.

Hip and waist circumference were measured, according to the CDC National Health and Nutrition Examination Survey guidelines, using a plastic measuring tape. (CDC, 1988) Hip measurement was taken at the widest point across the hips and encompassing the buttocks at their point of greatest protuberance. Waist circumference was obtained at the narrowest point between the lower ribs and

Chapter 2

anterior superior iliac spines. Measurements were taken in singlicate, correct to the nearest 0.5cm.

2.2.7.3 Venepuncture and blood sample preparation

Blood samples were collected from study participants at study visit 1 and study visit 3, to coincide with the timing of routine antenatal blood sampling, as outlined in Figure 2-3.

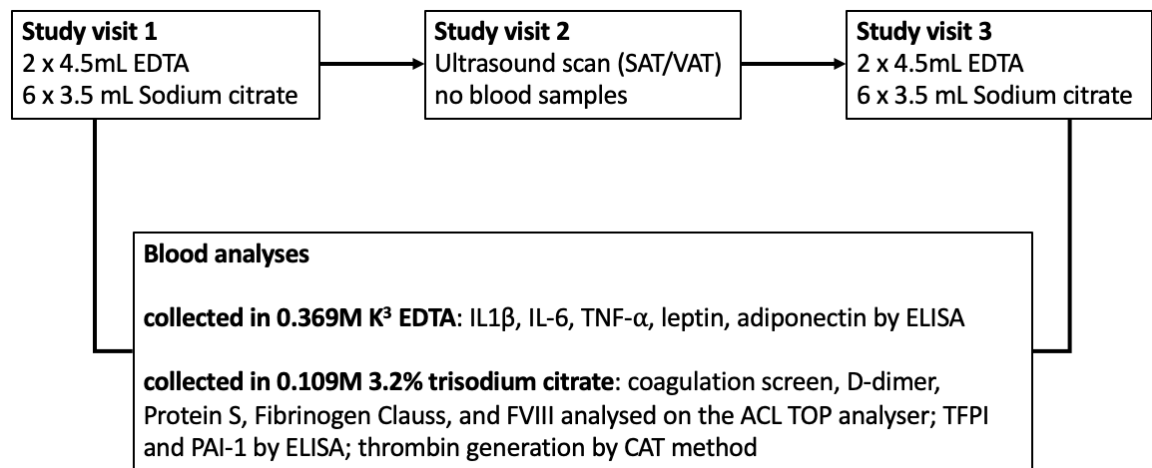


Figure 2-3 Blood sample requirements for each study visit

A total of 30mL blood samples were collected at study visit 1 and 3. Adipokine ELISA samples were collected in EDTA and all coagulation samples were collected in trisodium citrate and subsequently prepared in the laboratory as platelet poor plasma.

Blood samples were obtained by a member of the research team or antenatal clinic midwife from an antecubital or dorsal hand vein using a tourniquet and a butterfly needle (Greiner bio-one Vacuette®, minimum 23 gauge). Routine antenatal blood samples were collected first followed by a total of 30mL of blood for research purposes. Study blood samples were collected in 2 x 4.5mL 0.369M K³ EDTA and 6 x 3.5mL 0.109M 3.2% trisodium citrate vacutainers from study participants at the antenatal booking visit (8-15 weeks' gestation) and follow-up samples obtained at the 3rd study visit (26-30 weeks' gestation).

Samples for coagulation studies (PT, APTT, TCT, Ddimer, Protein S, Fibrinogen, FVIII, TFPI, PAI-1 and thrombin generation) were collected in 0.109M 3.2% trisodium citrate, with a total of 6 x 3.5mL vacutainers collected per participant per visit. Samples for adipokine ELISA (leptin, adiponectin, IL-1B, IL-6, TNF-α) were collected in 2 x 4.5mL 0.369M K³-EDTA vacutainers. Samples were

Chapter 2

transported to the Haemostasis Laboratory at Glasgow Royal Infirmary within 1 hour of venepuncture, and preparation completed within 2 hours from sample collection.

Samples underwent centrifugation at 3500g for 10 minutes, following which plasma was collected into a 10mL secondary tube. The secondary tube underwent a further centrifugation step at 3500g for 10minutes to separate platelets from plasma, then plasma was aspirated from the tube without disturbing the platelet-rich layer in the bottom 0.5mL. The resultant platelet poor plasma (PPP) was frozen at -80°C in 1 mL aliquots to allow future batch analysis.

2.2.7.4 **Ultrasound scan**

Participants were provided with an appointment for the study ultrasound scan to measure subcutaneous and visceral adipose tissue depth. Travel expenses up to a maximum value of £20 or a £20 shopping voucher were provided as compensation for attending this additional appointment.

Ultrasound scan was undertaken between 12-17 weeks' gestation, after pregnancy was confirmed viable by a routine 12 week antenatal scan. The same Consultant Radiologist performed all of the study ultrasound scans, except on one occasion where the scan was performed by a senior sonographer.

The scan was performed according to the protocol devised by Martin *et al*, as illustrated in Figure 2-4.(Martin *et al.*, 2009) Subcutaneous adipose tissue (SAT) depth in millimetres was measured from the outer border of the rectus abdominus muscle to the skin surface, and visceral adipose tissue (VAT) depth was measured from the inner border of the rectus abdominus muscle to the anterior wall of the abdominal aorta at the level of the linea alba. Three measurements were taken, and an average calculated and the data documented on a data collection form (see Appendix 4).

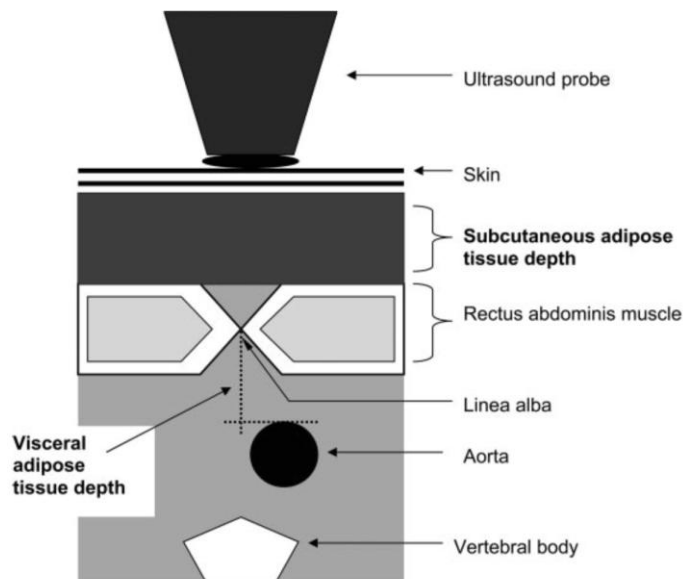


Figure 2-4 Anatomical landmarks on cross-sectional view for measurement of abdominal visceral and subcutaneous adipose tissue.

Original diagram and measurement technique by Martin *et al* (Martin *et al.*, 2009)

Due to the study scan being an additional appointment outwith routine antenatal care, there was some additional loss to follow-up for this appointment alone (see Figure 2-2). It was decided that missing this appointment did not mean exclusion from further follow-up, as the data from the blood analysis could stand alone from the ultrasound data. SAT and VAT measurements by ultrasound were collected on 154 women (58.8% of the total cohort), comprising 66 (60.1%) lean, 71 (61.7%) overweight/obese class I, 9 (37.5%) obese class II and 8 (57%) obese class III.

2.3 Laboratory Methodologies

2.3.1 Haemostasis analyses of plasma samples

All coagulation studies were performed on platelet-poor plasma, prepared as per section 2.2.7.3, in the Haemostasis Laboratory of Glasgow Royal Infirmary. This is a clinical laboratory serving Glasgow Royal Infirmary and the surrounding area's community healthcare settings. At time of analysis, the laboratory held a managed service contract with Instrumentation Laboratory, Werfen. As such, all analyses were undertaken on the ACL TOP 750 analyser using reagents purchased from Werfen. The laboratory Standard Operating Procedure (SOP), based upon manufacturer's instructions, was followed for each assay. The same lot numbers

Chapter 2

were used for the entire study sample analyses, and calibration for each lot was undertaken prior to commencement of study analyses. Internal quality control, using Werfen control material, was performed on each day of analyses (30 samples per day) and only accepted if within 1SD of the mean. Control values and coefficient of variation were not documented for the coagulation studies performed in the clinical laboratory. The laboratory participates in the NEQAS external quality control scheme, with all results from recent exercises 'within consensus'. Booking and 28 weeks' samples for the same participant were analysed simultaneously to reduce the impact of inter-assay variability.

2.3.1.1 Coagulation Screen (PT, APTT, TCT)

Prothrombin Time (PT), Activated Partial Thromboplastin Time (APTT) and Thrombin Clotting Time (TCT) were measured according to manufacturer's guidance for the ACL TOP 750 analyser (Instrumentation Laboratory, Werfen, Bedford, MA 01730, USA) at Glasgow Royal Infirmary Haematology Laboratory. The analyser used a turbidimetric clot detection method which measured the change in optical density over time to determine the time taken for clot to form in the presence of specific reagents to give information on different aspects of the coagulation cascade.

The PT assesses the extrinsic and common pathways (Factors II, V, VII, X and Fibrinogen as illustrated in Figure 2-5). RecombiPlastin 2G (Lot number N1081865, Werfen, Warrington, UK) and calcium were added to thawed participant platelet poor plasma at 37°C, and the time taken for clot to form was measured by change in optical density.

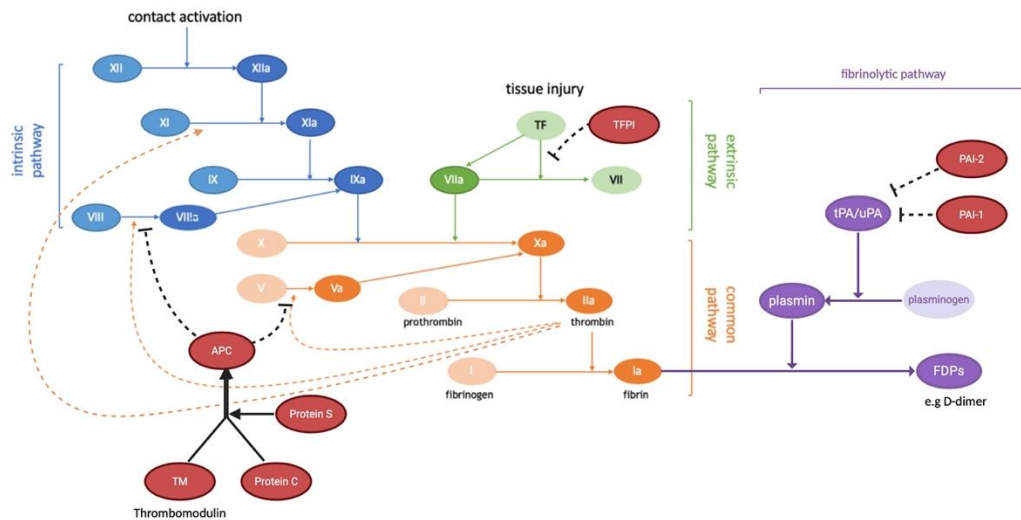


Figure 2-5 Coagulation cascade

The intrinsic pathway is initiated by contact activation which results in the activation of Factor XII. There is sequential activation of factors XI and IX, and then Factor VIII (which is activated by thrombin) forms the tenase complex with activated Factor XI. The tenase complex initiates the common pathway by activating Factor Xa which forms the prothrombinase complex with Factor Va (activated by thrombin). This in turn convert prothrombin to thrombin which converts fibrinogen to the fibrin clot. The extrinsic pathway is activated by tissue factor following tissue damage. Tissue factor forms a complex with Factor VIIa, which leads to the activation of Factor Xa and the initiation of the common pathway as previously outlined. Thrombin formation provides positive feedback and amplifies the coagulation process through activation of the intrinsic pathway. Fibrinogen is converted to fibrin to form the clot, which is subsequently broken down by fibrinolysis to fibrin degradation products (FDPs) including D-dimer. PAI-1 and PAI-2 regulate the fibrinolytic pathway. Natural anticoagulants activated protein C (APC) and it's co-factor protein S inhibit coagulation Factor V and VIII, whilst TFPI inhibits tissue factor.

The APTT assesses the intrinsic (or contact) pathway (Factors VIII, IX, XI, XII, pre-kallikrein and high molecular weight kininogen as illustrated in Figure 2-5). Coagulation was activated using silica and phospholipid in the SynthaSil reagent (Lot number N1181982), Werfen, Warrington, UK) which was added to thawed participant platelet poor plasma at 37°C. Calcium chloride was added after a 3-minute incubation period and time taken for clot to form measured using changes in optical density.

The TCT is a screening test to assess Fibrinogen activity. Bovine thrombin solution (3UNIH/mL; Lot number N1081482, Werfen, Warrington, UK) was added to thawed participant platelet poor plasma at 37°C and time taken for clot to form, following conversion of fibrinogen to fibrin, measured using changes in optical density.

Chapter 2

2.3.1.2 **Fibrinogen Clauss**

The Fibrinogen Clauss method was used to assess functional fibrinogen levels. Bovine thrombin solution at high concentration (100UNIH/mL; lot number N0797528, Werfen, Warrington, UK) was added to thawed participant platelet poor plasma which had been pre-diluted to 1:10 and warmed to 37°C. Time taken for conversion of fibrinogen to fibrin was measured by the ACL TOP analyser using changes in optical density.

2.3.1.3 **D-dimer**

An automated latex enhanced immunoassay (lot number B31673, Werfen, Warrington, UK) was used to give a quantitative assessment of D-dimer in thawed participant platelet poor plasma. Latex particles, coated with a monoclonal antibody specific to D-dimer, bind and agglutinate in the presence of the reaction buffer. The degree of agglutination was proportional to the concentration of D-dimer in the sample and measured by the ACL TOP analyser using changes in optical density.

2.3.1.4 **Free Protein S**

An automated latex ligand immunoassay (lot number B31644, Werfen, Warrington, UK) was used to determine concentration of free protein S. *In vivo* 40% of Protein S is free-circulating, and the remainder is bound to complement C4b-binding protein (C4BP). Latex particles adsorbed with C4BP were added to thawed participant citrated platelet poor plasma and bound with high affinity to free protein S. An anti-Protein S monoclonal antibody adsorbed onto latex particles was added and in the presence of Protein S agglutination of the latex particles was measurable by the ACL TOP analyser with changes in optical density. The degree of agglutination was proportional to the concentration of free Protein S in the plasma.

2.3.1.5 **Factor VIII**

Concentration of factor VIII was assessed by an APTT-based turbidimetric method. FVIII deficient plasma (lot number N0290477, Werfen, Warrington, UK) was added to thawed participant citrated platelet poor plasma which had been diluted to 1/10 and 1/20 concentrations. The APTT was measured as described above (see

Chapter 2

section 2.3.1.1) and the concentration of FVIII determined based upon the degree of correction of the APTT following addition of the test sample, in comparison to a reference line derived from a reference plasma of known FVIII concentration.

The average of the 1/10 and 1/20 dilution was taken as the FVIII result. If the 1/10 dilution provided a result $>140\text{ iU/dL}$, reflex testing rules set up on the ACL-TOP analyser mandated a 1/40 dilution be performed as values $>140\text{ iU/dL}$ were outwith the linear range of the 1/10 calibration curve. In the event of results $>140\text{ iU/dL}$, the value of the 1/40 dilution was taken as the FVIII result.

If the values of the 1/10 and 1/20 dilutions showed inter-assay variability $>10\%$, the assays were repeated. In a small number of cases there was evidence of inter-assay variability $>10\%$ which persisted upon repeat. These results showed a 'reverse non-parallelism', with decreasing FVIII concentrations as dilutions increase, which can be observed in the clinical laboratory and is often indicative of an activated coagulation sample due to pre-analytical factors, for example due to difficult venepuncture. In this situation, to obtain the FVIII result, the dilution which resulted in a plot with a value within the linear range of the calibration curve was used.

2.4 Thrombin Generation

Thrombin generation is an *ex-vivo* global coagulation assay which provides information on the balance of pro-coagulant factors and naturally-occurring anticoagulants in platelet-poor plasma. This provides a more complete assessment of the coagulation system than standard laboratory tests, which are based upon measuring the time for the initial clot to form. By the time the initial clot forms, only 5% of the total amount of thrombin generated by the coagulation process will have been formed, therefore much of the coagulation process is not captured by standard laboratory tests (Brummel *et al.*, 2002). The Thrombin Generation Assay is also unique in providing laboratory evidence of a procoagulant state which cannot be achieved by routine clinical laboratory coagulation tests.

The current and most widely accepted methodology was devised by Hemker *et al* and utilises a fluorochrome which emits fluorescence in the presence of thrombin. (Hemker *et al.*, 2003) Thrombin Generation is measured by the

Chapter 2

Calibrated Automated Thrombogram (CAT) analyser, an analyser capable of dispensing reagents and measuring fluorescence within a 96-well microplate. Experimental plasma samples are assessed in duplicate alongside a calibrator well to overcome the effect plasma colour may have on the fluorescence measured. Coagulation is triggered by the addition of a reagent containing tissue factor and phospholipids to platelet poor plasma. The concentration of tissue factor used determines which components of the coagulation pathways are assessed by the assay. In a high concentration of tissue factor (5pM), the thrombogram reflects the activity of the extrinsic pathway within the sample. In a lower concentration of tissue factor (1pM), coagulation requires the activation of the intrinsic pathway. Therefore, by altering the experimental conditions, different aspects of the coagulation system be assessed. The activity of the naturally-occurring anticoagulants can also be assessed by this assay through the addition of thrombomodulin (TM). (Hemker *et al.*, 2003) Thrombomodulin binds to protein C and increases its activation 20,000-fold, which in turn is inhibitory to thrombin generation. Given that acquired protein C resistance is known to occur during pregnancy, an overview of the protein C system is desirable when assessing the plasma of pregnant women. (Clark, Walker and Greer, 1999) Finally, the coagulation process is initiated once calcium, mixed in solution with a fluorochrome (amino-methylcoumarin) is added by the dispenser to the wells. As thrombin is produced by the active coagulation process, it binds to and splits the fluorochrome emitting a fluorescent signal which can be measured by the CAT analyser. Fluorescent intensity is proportional to the thrombin concentration when compared to the calibrator of known thrombin concentration. The thrombogram for each well, such as the example provided in Figure 2-6, is generated by CAT analyser software and a series of 5 parameters are calculated by the Thrombinoscope® software to provide an objective assessment of Thrombin Generation.

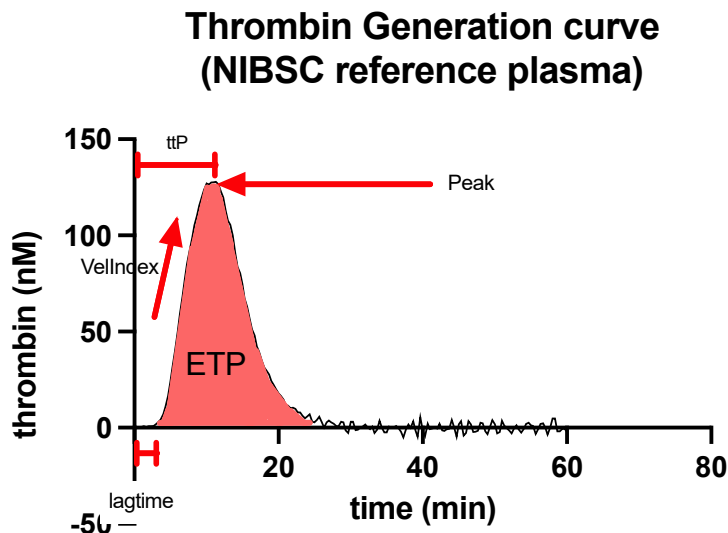


Figure 2-6 Thrombogram with measurement parameters. Raw data from NIBSC reference plasma used to demonstrate the thrombogram produced by the CAT analyser and the measurement parameters obtained.

Lagtime: time (minutes) taken from initiation of clotting to the start of the thrombin burst during the propagation phase of thrombin generation

Peak: maximum amount of thrombin (nM) generated during the assay

Time to peak (ttP): time (minutes) from initiation of the assay to Peak thrombin.

Endogenous thrombin potential (ETP): total amount of thrombin formed, quantified by calculating the area under the curve (nM*min)

Velocity index (VelIndex): rate of thrombin production during the thrombin burst (nM/min)

2.4.1 Thrombin Generation Reagents

2.4.1.1 PPP low

PPP low reagent (Ref TS31.0, Lot number PPL1910/01) was purchased from Diagnostica Stago, Theale, UK. It consists of Tissue factor (1pM) and phospholipid for use with platelet poor plasma (PPP). PPP low was reconstituted with 1mL distilled water prior to use.

Chapter 2

2.4.1.2 **Calibrator**

Thrombin Calibrator (Ref TS20.0, Lot number TC2001/01, activity 730nM) was purchased from Diagnostica Stago, Theale, UK. The calibrator contains citrate buffered saline, bovine proteins and saccharides. The composition of the calibrator is based upon the formulation of a thrombin calibrator devised by Hemker (Hemker *et al.*, 2003) in which α -2-macroglobulin is bound to a bovine thrombin-like enzyme. This α -2-macroglobulin-thrombin complex is mixed with antithrombin and heparin to prevent the calibrator from clotting. As the calibrator contains a known concentration of thrombin and is non-clotting, it provides a continuous reaction with the fluorogenic substrate from which test sample thrombin concentrations can be interpolated.(Hemker *et al.*, 2003). Thrombin Calibrator was reconstituted with 1mL distilled water prior to use.

2.4.1.3 **Thrombomodulin**

Thrombomodulin (ST09-009, batch 00510), 43 μ M, was kindly gifted by the Cardiovascular Research Institute Maastricht (CARIM). A 1 μ M stock solution of thrombomodulin was required for Thrombin Generation, therefore this was prepared by the Haemostasis laboratory clinical scientist by diluting Thrombomodulin in a Hepes-NaCl 0.5% BSA buffer.

The Hepes-NaCl buffer was made by adding 25mM HEPES to 175mM NaCl, creating a solution of pH 7.7 to which 0.5% BSA (w/v) was added.

The 1 μ M Thrombomodulin stock solution was then made by adding 50 μ L of the 43 μ M thrombomodulin to 2100 μ L of the HEPES-NaCl 0.5% BSA buffer.

The optimal concentration for this batch of thrombomodulin to reduce ETP by 50% was previously determined to be a working solution of 0.4nM, therefore the 1 μ M stock required further dilution prior to thrombin generation analyses.

A 30nM stock solution of thrombomodulin with 1pM tissue factor was made by diluting 3 μ L of 1 μ M thrombomodulin in 97 μ L of reconstituted PPP low (see section 2.4.1.1). A 0.4nM working solution of thrombomodulin + 1pM tissue factor was prepared by adding 54 μ L of the stock solution to 621 μ L of reconstituted PPP low.

Chapter 2

2.4.1.4 **FluCa**

Fluo substrate (TS50-10, lot number FLS2003/01), which contains the fluorochrome, and Fluo buffer (TS50-20, lot number FLB2002/01), containing calcium, were purchased from Diagnostica Stago, Theale, UK. Prior to use in the Thrombin generation assay, 3200µL Fluo buffer was warmed to 37°C in the waterbath. Fluo substrate, 80µL, was diluted in the Fluo buffer to make the FluCa solution which was protected from light and used within one hour of dilution.

2.4.1.5 **Thrombin Generation Test Reference Plasma**

A lyophilised Thrombin Generation test reference plasma (lot number 06/232) was kindly gifted by NIBSC (Potters Bar, Hertfordshire, UK). This was reconstituted with 1mL of distilled water and remained stable at 4°C for 3 hours. A new vial was reconstituted for each plate run. An acceptance range (mean $\pm$ 2SD) was devised for the TG reference plasma (see section 2.4.3) as previously described by our laboratory. (Bagot and Leishman, 2015)

2.4.2 Thrombin Generation Method

Thrombin generation was performed according to the method developed by Hemker *et al.* using a Fluroskan Ascent fluorometer (Thermo Labsystems OY, Helsinki, Finland). (Hemker *et al.*, 2003)

Reagents and thawed, vortexed participant samples were added to an Immulon 2HB clear U-bottom 96 well plate as per the plate plan in Table 5

Chapter 2

Table 5 Example thrombin generation plate plan. Samples assayed in duplicate under each condition (with and without thrombomodulin), and with calibrator. Corresponding booking and 28 week samples were assayed on the same 96-well plate. NIBSC = reference plasma, IPOPOP001= example study number.

	1pM TF		1pM TF + TM		Calibrator		1pM TF		1pM TF + TM		Calibrator	
	1	2	3	4	5	6	7	8	9	10	11	12
A	NIBSC		NIBSC		NIBSC		IPOP005 booking		IPOP005 booking		IPOP005 booking	
B	NIBSC		NIBSC		NIBSC		IPOP005 28 weeks		IPOP005 28 weeks		IPOP005 28 weeks	
C	IPOP001 booking		IPOP001 booking		IPOP001 booking		IPOP006 booking		IPOP006 booking		IPOP006 booking	
D	IPOP001 28 weeks		IPOP001 28 weeks		IPOP001 28 weeks		IPOP006 28 weeks		IPOP006 28 weeks		IPOP006 28 weeks	
E	IPOP003 booking		IPOP003 booking		IPOP003 booking		IPOP007 booking		IPOP007 booking		IPOP007 booking	
F	IPOP003 28 weeks		IPOP003 28 weeks		IPOP003 28 weeks		IPOP007 28 weeks		IPOP007 28 weeks		IPOP007 28 weeks	
G	IPOP004 booking		IPOP004 booking		IPOP004 booking		IPOP008 booking		IPOP008 booking		IPOP008 booking	
H	IPOP004 28 weeks		IPOP004 28 weeks		IPOP004 28 weeks		IPOP008 28 weeks		IPOP008; 8 weeks		IPOP008 28 weeks	

The plate was pre-incubated in the fluorometer for 10 minutes, following which the dispenser was washed with distilled water heated to 37°C before being filled with warmed FluCa solution. FluCa (20µL) was automatically dispensed to each well of the 96-well plate to initiate the assay. Fluorescence was measured at an excitation wavelength of 390nm and an emission wavelength of 460nm.

Thrombinoscope® software automatically calculated the individual values for lag time, ETP, peak thrombin, time to peak, velocity index and start tail. This data was exported to Excel for analysis.

2.4.3 Thrombin Generation Reference Plasma and Coefficient of Variation

At present, the Thrombin Generation assay remains primarily a research tool whilst its translation to clinical practice is under development. Thrombin Generation, as measured by the CAT analyser, is subject to significant intra-assay and inter-assay variability. Moreover, variability between individuals is greater than with standard laboratory assessment of coagulation. Historically, to allow comparison of results between individuals and reduce the impact of inter-assay variability, it has been the practice to normalise results to a control sample run on each plate. However, work by Bagot and Leishman showed that by introducing a standard reference plasma with an acceptance range for individual thrombin generation parameters, it was possible to reduce inter-assay variability, exclude experiments with erroneous results and give confidence in the production of accurate data. (Bagot and Leishman, 2015)

The practice of using a reference plasma was adopted for all thrombin generation analyses in this study. NIBSC reference plasma was analysed in duplicate alongside participant samples on every plate. The values with intra-assay CV of <5% were taken and compared with the values of NIBSC reference plasma for all plates once analyses of all samples was completed. The inter-assay CVs for all NIBSC reference plasma are depicted in Table 6 and an acceptance range of mean $\pm$ 2SD was derived, using the same methodology as Bagot and Leishman. (Bagot and Leishman, 2015) If the NIBSC plasma values for lag time, ETP, peak or time to peak were outwith the acceptance range, the analyses of all samples on that plate were discarded and the experiment repeated to ensure that the values obtained across different plates could be directly comparable. Any participant samples which had lag-time, ETP, peak, or time to peak values with an intra-assay CV >10% were repeated. Velocity index has been repeatedly shown by our lab to be highly variable and as such samples with CV >10% in Velocity index alone were not repeated.

Table 6 Thrombin generation inter-assay coefficient of variation (%) NIBSC plasma values $\pm$ 2SD; CV(%)

	Lagtime	ETP	Peak	Time to Peak	Velocity Index
1pM TF	4.86	1279.35	127.52	11.43	19.69
Mean ($\pm$ 2SD)	(4.56, 5.16)	(1044.70, 1514.01)	(90.08, 164.95)	(10.47, 12.38)	(11.21, 28.18)
CV (%)	3%	9%	15%	4%	22%
1pM TF + TM	4.59	652.12	93.55	8.95	21.59
Mean ($\pm$ 2SD)	(4.16, 5.02)	(398.93, 905.30)	(54.52, 132.58)	(8.41, 9.50)	(11.38, 31.80)
CV (%)	5%	19%	21%	3%	24%

2.5 ELISA

2.5.1 ELISA Principle

The Enzyme-Linked Immunosorbent Assay (ELISA) utilises the immunological action of a specific antibody to detect and quantify the analyte of interest. In the quantitative sandwich ELISA kits utilised in this study, 96-well plates were pre-coated with an antibody specific to the analyte being measured. As per the example plate plan in Table 7, standards, test samples and controls were added to the pre-coated plates. Following an incubation phase, any excess unbound antigen was washed off with buffered surfactant. An enzyme-linked antibody specific for the analyte of interest was then added to the plate. Following a further incubation and wash step, any unbound enzyme-linked antibody was removed and a chromogenic substrate (tetramethylbenzidine) added. The enzyme-substrate reaction was stopped after 30minutes by the addition of 2 N sulphuric acid and the colour change observed was proportional to the concentration of bound analyte of interest. Colour intensity was measured by microplate-reader at an absorbance of 450nm and the optical density values measured were compared to the standard curve to interpolate the analyte concentration of each sample.

Chapter 2

Table 7 Example ELISA plate plan

TFPI plate 3. S= standard, P =participant sample (booking/28weeks), C= control, X=empty

	1	2	3	4	5	6	7	8	9	10	11	12
A	S 2000pg/mL	S 2000pg/mL	C	P91 (28)	P95 (28)	P100 (b)	P104 (b)	P108 (b)	P112 (b)	P116 (28)	P121 (b)	P125 (b)
B	S 1000pg/mL	S 1000pg/mL	P88 (b)	P92 (b)	P96 (b)	P100 (28)	P104 (28)	P108 (28)	P112 (28)	P117 (b)	P121 (28)	P125 (28)
C	S 500pg/mL	S 500pg/mL	P88 (28)	P92 (28)	P96 (28)	P101 (b)	P105 (b)	P109 (b)	P113 (b)	P117 (28)	P122 (b)	P126 (b)
D	S 250pg/mL	S 250pg/mL	P89 (b)	P93 (b)	P97 (b)	P101 (28)	P105 (28)	P109 (28)	P113 (28)	P118 (b)	P122 (28)	P126 (28)
E	S 125pg/mL	S 125pg/mL	P89 (28)	P93 (28)	P98 (b)	P102 (b)	P106 (b)	P110 (b)	P114 (b)	P119 (b)	P123 (b)	P127 (b)
F	S 62.5pg/mL	S 62.5pg/mL	P90 (b)	P94 (b)	P98 (28)	P102 (28)	P106 (28)	P110 (28)	P115 (b)	P119 (28)	P123 (28)	P127 (28)
G	S 31.3pg/mL	S 31.3pg/mL	P90 (28)	P94 (28)	P99 (b)	P103 (b)	P107 (b)	P111 (b)	P115 (28)	P120 (b)	P124 (b)	X
H	blank	blank	P91 (b)	P95 (b)	P99 (28)	P103 (28)	P107 (28)	P111 (28)	P116 (b)	P120 (28)	P124 (28)	C

Platelet-poor plasma samples collected in 0.369M K³-EDTA were used for adiponectin, leptin, TNF- α , IL-1 β , and IL-6 assays, as recommended in the product literature for the ELISA kits. Platelet-poor plasma samples collected in 0.109M 3.2% trisodium citrate were used for TFPI and PAI-1 ELISAs as recommended in the package insert. Samples which were paired, booking and 28 weeks' gestation for the same study participant, were analysed together to reduce the impact of inter-assay variability upon the ability to directly compare analyte values. Samples which were above the linear range of detection were further diluted by a factor of 50% and analysis repeated. All samples were analysed in singlicate due to cost restrictions. Sample analyses for adipokines (leptin, adiponectin, TNF- α , IL-1 β and IL-6) were undertaken at the University of Glasgow by Mrs F. Jordan, Laboratory Research Technician for the School of Cardiovascular and Metabolic Health. TFPI and PAI-1 ELISAs were undertaken at the Glasgow Royal Infirmary Haemostasis laboratory.

2.5.2 Adiponectin

Adiponectin was assessed using R&D systems Human Total Adiponectin/Acrp30 solid-phase Quantikine[®] ELISA (catalogue number DRP300, lot number P247897)

Chapter 2

which supplied a 96-well plate pre-coated with a monoclonal antibody specific for the globular domain of adiponectin.

A lyophilised recombinant adiponectin was provided as a standard to produce a curve from which test sample concentrations could be determined. The standard was reconstituted and serial dilutions produced by diluting with Calibrator Diluent RD6S (buffered protein base with preservatives). The undiluted adiponectin standard served as the top concentration (250ng/mL) with subsequent doubling dilutions of 125ng/mL, 62.5ng/mL, 313.3ng/mL, 15.6ng/mL, 7.8ng/mL and 3.9ng/mL making up the rest of the standard curve. Calibrator diluent RD6S served as the zero standard, labelled 'blank' on the plate plan.

Participant samples were diluted 100-fold prior to analysis. Following the addition of 100 μ L of assay diluent RD1W, 50 μ L each of participant samples, Adiponectin Standard dilutions and in-house control samples were added to the 96-well plate in accordance with the plate plan (see Table 7). The sealed plate was incubated at room temperature for 2 hours before undergoing 4 washes using 300 μ L of Wash Buffer (buffered surfactant) per well. Human Adiponectin Conjugate (200 μ L), an enzyme-linked monoclonal antibody specific for the globular domain of adiponectin, was added to each well prior to a further 2-hour incubation at room temperature. The Conjugate was removed by a further 4 washes using 300 μ L of Wash Buffer per well, and 200 μ L of chromogenic Substrate Solution (tetramethylbenzidine mixed with hydrogen peroxide) was added to each well. The sealed plate was incubated, protected from light, for 30 minutes to allow the action of the chromogen, before 50 μ L of Stop solution (2 N sulphuric acid) was added to each well to halt chromogenic activity. Optical density was measured within 30 minutes using the microplate reader (MULTISKAN EX 230V microplate Reader, Thermofisher Scientific) set to 450nm. Adiponectin standards were plotted on log-log graph using GraphPad PRISM software and study and control

sample values were interpolated from the standard curve (

Adiponectin ELISA standard curve (plate 1)

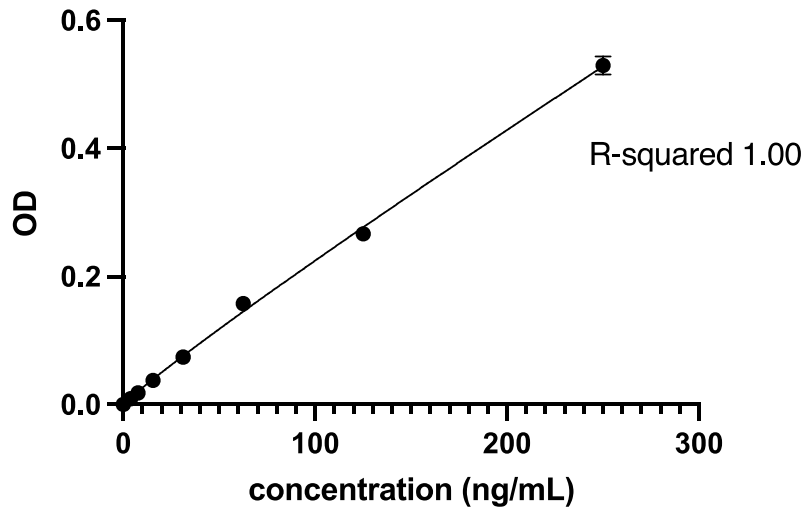


Figure 2-7). Values were multiplied by 100 to account for the original dilution.

Adiponectin ELISA standard curve (plate 1)

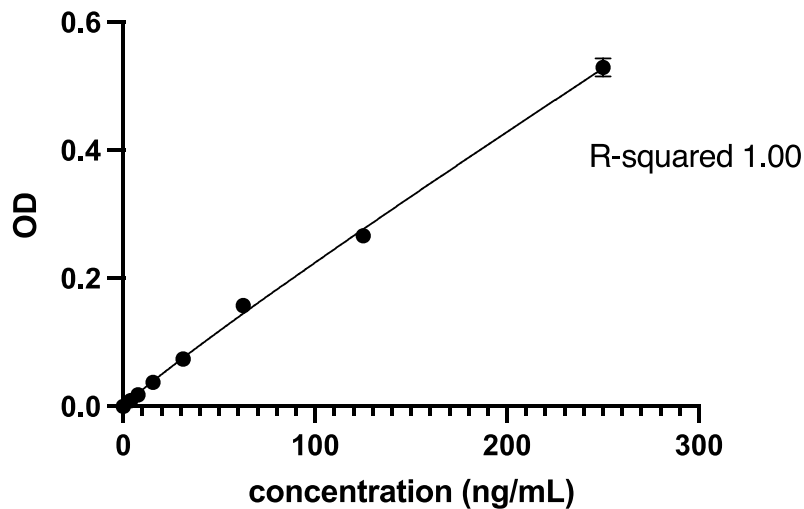


Figure 2-7 Adiponectin ELISA standard curve

Log-log graph plotting mean and SD of the adiponectin standard (2 replicates), with nonlinear regression line. The optical density of each participant sample was read from this graph to obtain the concentration of adiponectin in the sample.

2.5.3 Leptin

Leptin was assessed using R&D systems Human Leptin solid-phase Quantikine® ELISA kit (DLP00, lot number P243227) which included a 96-well plate pre-coated with a monoclonal antibody specific for *E.coli*-expressed recombinant Human Leptin. Participant samples were diluted 100-fold with Calibrator Diluent in

Chapter 2

preparation for analysis. Participant samples were assayed alongside serial dilution of a reconstituted Leptin Standard and an in-house control.

The Leptin Standard was reconstituted with distilled water to produce a 10,000pg/mL solution. 100µL of the standard was added to 900µL of Calibrator Diluent RD5P (diluted 1:5) which served as the top standard, 1000pg/mL. Calibrator diluent was used to create the serial dilutions at concentrations of 500pg/mL, 250pg/mL, 125pg/mL, 62.5pg/mL, 31.3pg/mL, and 15.6pg/mL. The Calibrator Diluent served as the zero standard.

Assay Diluent RD1-19 (100µL) was added to the 96-well plate prior to 100µL of Leptin Standard, diluted participant samples and controls. The sealed plate was incubated for 2 hours at room temperature following which the plate was washed 4 times with 300µL of Wash Buffer (buffered surfactant) per well to remove any unbound leptin in the sample wells. Human Leptin Conjugate (200µL), an enzyme-linked monoclonal antibody specific for leptin, was added to each well prior to a 1-hour incubation. Any unbound monoclonal antibody was removed during a further 4 washes prior to 200µL of a chromogenic Substrate Solution (equal parts tetramethylbenzidine and hydrogen peroxide) being added to each of the wells. Stop Solution (2 N sulphuric acid) was used to stop the chromogenic reaction following a 30-minute incubation in the dark, and the optical density was read by the plate-reader (MULTISKAN EX 230V microplate Reader, Thermofisher Scientific) set to 450nm within 30 minutes. GraphPad PRISM software was used to generate a log-log curve of the leptin Standard which participant and control values were interpolated from (Figure 2-8). Interpolated values were multiplied by 100 to correct for the dilution factor applied.

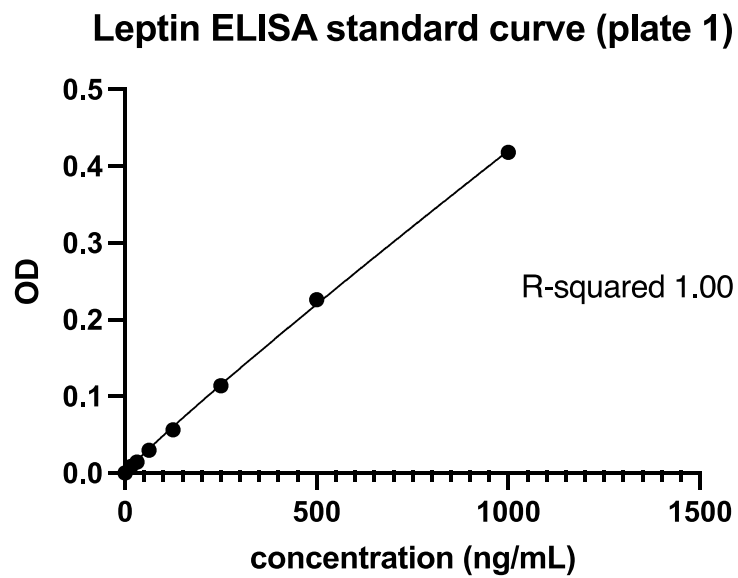


Figure 2-8 Leptin ELISA standard curve

Log-log graph plotting mean and SD of the leptin standard (2 replicates), with nonlinear regression line. The optical density of each participant sample was read from this graph to obtain the concentration of leptin in the sample.

2.5.4 PAI-1

R&D Systems solid-phase Quantikine® ELISA plates were used to quantify PAI-1 (DTSE100, lot number P277485) as per the general procedure in section 2.5.1 and according to manufacturer's instructions. Participant samples were diluted to 1 in 10 concentration with Calibrator Diluent and assayed alongside serial dilutions of a PAI-1 Standard, and PAI-1 low positive control (R&D systems, control set 822, lot number 1338374).

The PAI-1 Top Standard (20ng/mL) was produced by diluting 50µL of the reconstituted 200ng/mL Standard with 450µL of Calibrator Diluent RD5-26. Serial doubling dilutions of 10ng/mL, 5ng/mL, 2.5ng/mL, 1.25ng/mL, 0.625ng/mL and 0.313ng/mL were made by diluting 200µL of the previous dilution with 200µL of Calibrator Diluent. Calibrator Diluent served as the zero standard.

The 96-well plate supplied in the ELISA kit was pre-coated with a PAI-1-specific polyclonal antibody. Following application of 50µL of assay diluent, diluted participant samples, controls and PAI-1 Standard dilutions were added to the plate and then incubated at room temperature for 2 hours. Wash buffer, 300µL per well, was used to remove any unbound PAI-1 in the samples, and the wash step was repeated for a total of 4 washes. An enzyme-linked polyclonal antibody to PAI-1 (200µL) was added to the wells prior to a further 2-hour incubation. Following another series of 4 plate washes, 200µL of a chromogenic Substrate Solution (equal parts hydrogen peroxide and tetramethylbenzidine) was added to the wells. The reaction was stopped with 2 N sulphuric acid after 30 minutes incubation protected from the light and optical density was read by the microplate reader (Sunrise, Tecan, Austria GmbH) at 450nm absorbance. A four-parameter logistic (4-PL) standard curve was generated using GraphPad PRISM software (Figure 2-9), and PAI-1 concentrations were read from this curve and then multiplied by the dilution factor of 10.

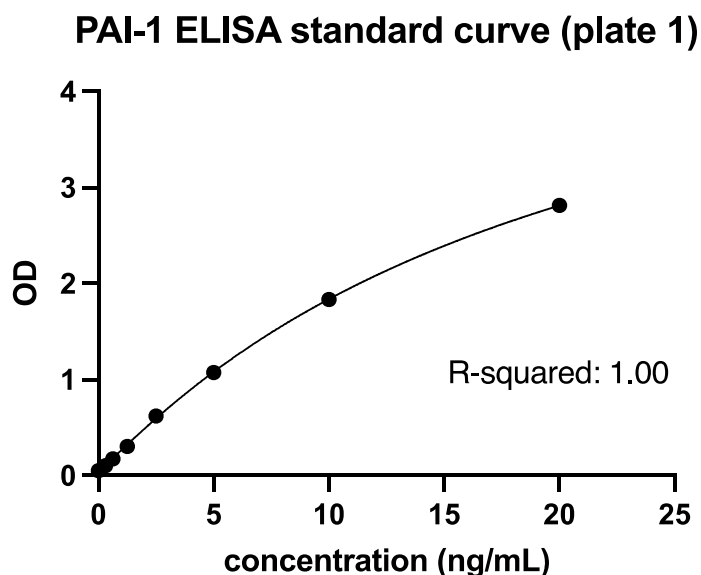


Figure 2-9 PAI-1 ELISA standard curve

4-PL curve plotting mean and SD of the PAI-1 standard (2 replicates). The optical density of each participant sample was interpolated from this curve to obtain the concentration of leptin in the sample.

2.5.5 TFPI

R&D Systems' Human TFPI Quantikine® ELISA kit (DTFP10, lot P274140) was used to measure plasma TFPI.

Serial dilutions of a TFPI Standard were produced by diluting a 20,000pg/mL of the Standard with Calibrator Diluent RD5-20. The final concentrations of the standards were 2000pg/mL ('top standard'), 100pg/mL, 500pg/mL, 250pg/mL, 125pg/mL, 62.5pg/mL, and 31.3pg/mL, with the Calibrator Diluent serving as the 'zero standard'.

Assay Diluent RD1-89 (100µL) was first added to the 96-well plate pre-coated with a TFPI-specific monoclonal antibody. Participant samples were diluted 100-fold prior to being added to the plate alongside the serial dilutions of the TFPI standard and R&D Systems' medium positive control (Control set 860, lot number 1363476). The plate underwent 4 washes with 300µL of Wash Buffer (buffered surfactant) per well after a 2-hour room-temperature incubation on a microplate-shaker. Following this 200µL of TFPI Conjugate, an enzyme-linked TFPI-specific monoclonal antibody, was added to the wells. The wash-step was repeated following a further 1-hour incubation at room temperature on the microplate-

shaker. Chromogenic substrate (200 μ L) was added to the plate and stop solution (2 N sulphuric acid) halted the reaction after a 30-minute incubation in the dark. Optical density was read from each well by the microplate reader (Sunrise, Tecan, Austria GmbH) at an absorbance of 450nm. GraphPad PRISM software was used to generate a four-parameter logistic (4-PL) curve of the TFPI standard and participant samples were interpolated from this curve (Figure 2-10). Final concentration of TFPI for each sample was calculated following correction for the 100-fold dilution.

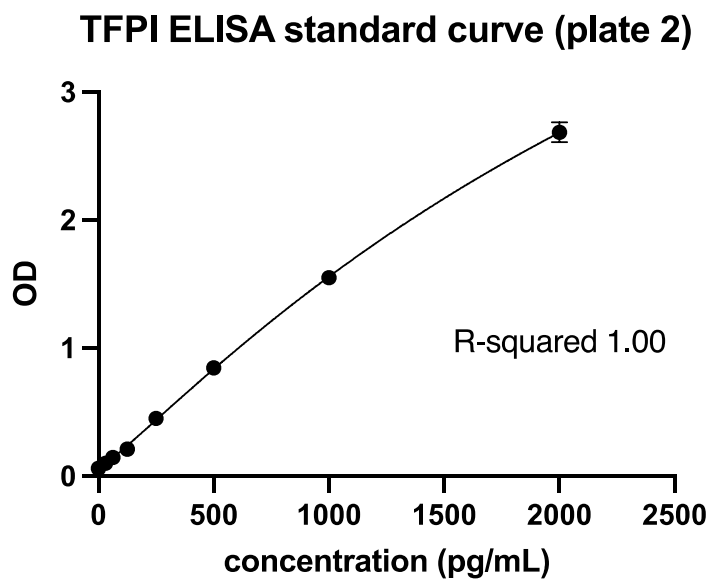


Figure 2-10 TFPI ELISA standard curve

4-PL curve plotting mean and SD of the TFPI standard (2 replicates). The optical density of each participant sample was interpolated from this curve to obtain the concentration of TFPI in the sample.

2.5.6 IL-6

Plasma IL-6 was quantified with R&D Systems' human IL-6 Quantikine High Sensitivity ELISA kit (HS600C, lot number P249514) using a 96-well plate pre-coated with monoclonal antibody to IL-6. Participant samples were assayed alongside IL-6 Standard dilutions and in-house control material of known concentration.

The IL-6 Standard (100pg/mL) was diluted with Calibrator Diluent RD5-4 to a concentration of 10pg/mL, which served as the 'top standard'. Doubling dilutions, produced by diluting 500 μ L of the previous concentration with 500 μ L Calibrator Diluent, resulted in the final series of dilutions: 5pg/mL, 2.5pg/mL, 1.25pg/mL,

Chapter 2

0.625pg/mL, 0.313pg/mL and 0.156pg/mL. Calibrator Diluent served as the 'zero standard'.

Assay diluent (100 μ L) was added to the wells, followed by 100 μ L of standard, sample or control as appropriate. The plate was sealed and incubated for 2 hours at room temperature on a microplate shaker. The plate underwent 4 washes with Wash Buffer (buffered surfactant) then 200 μ L of human IL-6 HS Conjugate, a biotinylated polyclonal antibody specific for IL-6, was added. Following a further 1-hour incubation on the plate-shaker, the wash step was repeated and 200 μ L of streptavidin Polymer-HRP was added to the wells. Streptavidin binds with strong affinity to biotin which was bound to the anti-IL-6 antibody, therefore the wash step repeated after a 30-minute incubation removed any unbound Streptavidin and left only that which was bound to the anti-IL-6 antibody. Substrate solution (equal parts Tetramethylbenzidine and Hydrogen Peroxide (200 μ L) was added to the plate and protected from light for 30mins to allow the chromogenic reaction to occur in proportion to the concentration of bound IL-6. A stop solution (2 N sulphuric acid) was added and optical density measured using the plate reader (MULTISKAN EX 230V microplate Reader, Thermofisher Scientific) set to 450nm absorbance. A four-parameter logistic (4-PL) standard curve was generated using GraphPad PRISM software and participant sample concentrations interpolated from this curve (Figure 2-11).

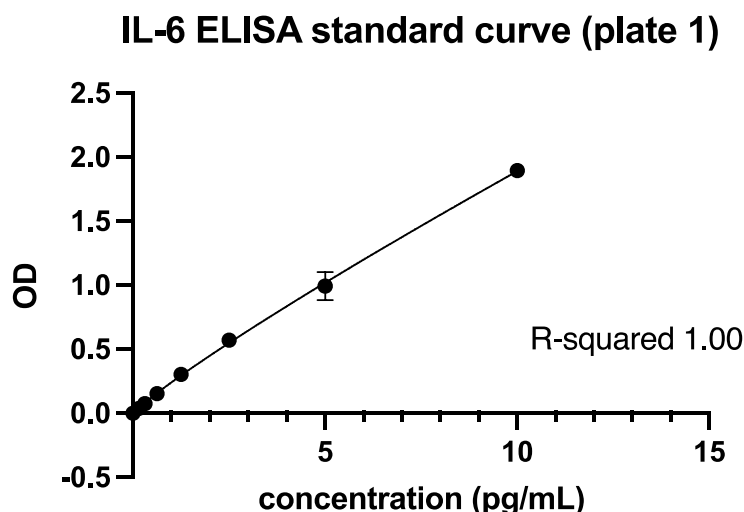


Figure 2-11 IL-6 ELISA standard curve

4-PL curve plotting mean and SD of the TFPI standard (2 replicates). The optical density of each participant sample was interpolated from this curve to obtain the concentration of TFPI in the sample.

2.5.7 IL-1 β

R&D System's Quantikine® High Sensitivity IL-1 β ELISA kit (catalogue number HSLB00D, lot number P248971) was used to quantify IL-1 β in participant samples. The 96-well plate provided was pre-coated with a monoclonal antibody specific to human IL-1 β .

Serial Dilutions of IL-1 β standard provided a standard curve from which test sample concentrations were read. The IL-1 β Top Standard (8pg/mL) was produced by diluting 100 μ L of the reconstituted 80pg/mL Standard with 900 μ L of Calibrator Diluent RD5T. Serial doubling dilutions of 4pg/mL, 2pg/mL, 1pg/mL, 0.5pg/mL, 0.25pg/mL and 0.125pg/mL were made by diluting 500 μ L of the previous dilution with 500 μ L of Calibrator Diluent. Calibrator Diluent served as the zero standard.

Assay Diluent (50 μ L) was added to the wells, followed by 100 μ L of IL-1 β Standard, participant samples and controls as per the plate plan. The plate was incubated on the horizontal orbital microplate shaker for 2 hours at room temperature and then any unbound IL-1 β was washed off using Wash buffer (300 μ L per well, 4 washes). Human IL-1 β HS Conjugate (biotinylated polyclonal antibody specific to IL-1 β) (200 μ L) was added to the wells and the wash step was repeated following a 1-hour incubation on the plate shaker. Streptavidin polymer-HRP, which has a

Chapter 2

high affinity for biotin, was added and a further 30-minute incubation was undertaken. The wash step was repeated to remove any unbound Streptavidin and the chromogenic Substrate (tetramethylbenzidine and hydrogen peroxide) was added to the plate. Following 30 minutes of incubation at room temperature, protected from light, the stop solution (2 N Sulphuric Acid) was added to stop the reaction. Optical density was measured at absorbance of 450nm on the microplate reader. (MULTISKAN EX 230V microplate Reader, Thermofisher Scientific) A four-parameter logistic curve of the IL-1 β Standard dilutions was generated using GraphPad PRISM and participant sample and control values interpolated from this (Figure 2-12).

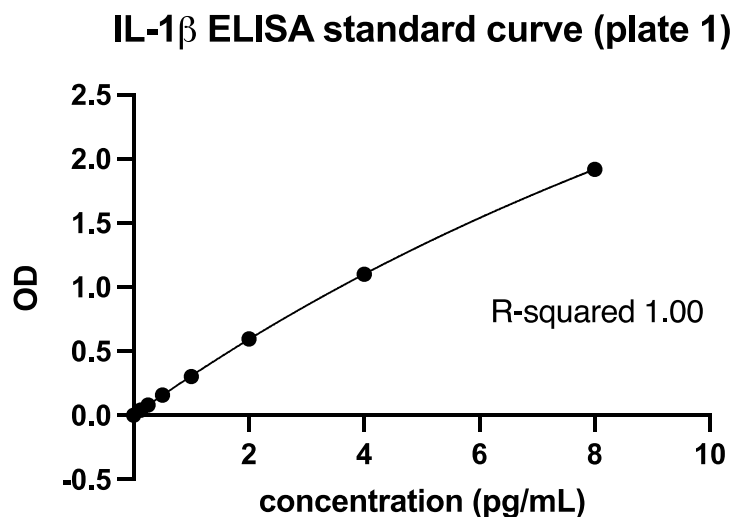


Figure 2-12 IL-1 β ELISA standard curve

4-PL curve plotting mean and SD of the IL-1 β standard (2 replicates). The optical density of each participant sample was interpolated from this curve to obtain the concentration of IL-1 β in the sample.

Concentrations for IL-1 β in this assay were undetectable at booking in 24% and in 32% of samples at 28 weeks, despite a high sensitivity assay being used. Unfortunately, data for plate 6 was lost in a fire suffered by our laboratory at the University of Glasgow. As the yield of detectable results was so low over the rest of the data series, we opted not to repeat this plate due to cost and timing constraints.

2.5.8 TNF- α

TNF- α was quantified by R&D Systems Quantikine® high Sensitivity ELISA kit (catalogue number HSTA00E, lot number P245804). A 96-well plate pre-coated

Chapter 2

with E.coli-derived recombinant human TNF- α antibodies was provided in the kit purchased from R&D Systems.

Serial dilutions of the TNF- α standard were produced. The Top Standard (10ng/mL) was produced by diluting 50 μ L of the reconstituted 100pg/mL Standard with 450 μ L of Calibrator Diluent RD6S. Serial doubling dilutions of, 5pg/mL, 2.5pg/mL, 1.25pg/mL, 0.625pg/mL, 0.313pg/mL and 0.156pg/mL were made by diluting 200 μ L of the previous dilution with 200 μ L of Calibrator Diluent. Calibrator Diluent served as the zero standard.

Assay diluent was added to each well of the plate (50 μ L), followed by 50 μ L of the serial dilutions of TNF- α Standard, in-house controls and participant samples. The plate was incubated at room temperature for 2 hours on an orbital microplate shaker, before the wells were washed with 300 μ L of Wash Buffer (x4) to remove any unbound samples. Biotinylated polyclonal antibody to TNF- α (200 μ L) was added to each of the wells and the plate was incubated again for an hour at room temperature on the microplate shaker. Unbound TNF- α antibody was removed with a further wash step following which 200 μ L of Streptavidin-Polymer-HRP was added to the wells. This was washed off after a 30-minute incubation under agitation on the plate shaker and the chromogenic substrate was added. After 30 minutes incubation in the dark, the chromogenic reaction was stopped with 2 N sulphuric acid and the optical density of the wells read by the plate reader set to 450nm. The optical density and concentrations of the TNF- α standard dilutions were input into GraphPad PRISM and a four-parameter logistic curve generated from which the concentrations of TNF- α in participant samples and controls could be interpolated (Figure 2-13).

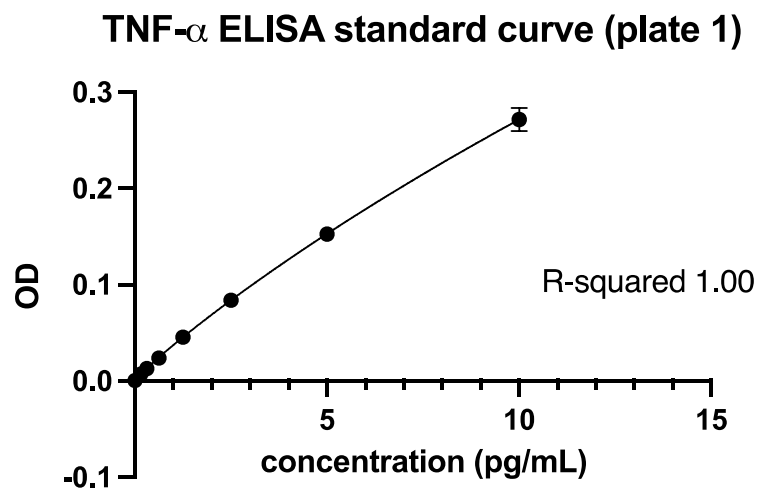


Figure 2-13 TNF- α ELISA standard curve

4-PL curve plotting mean and SD of the TNF- α standard (2 replicates). The optical density of each participant sample was interpolated from this curve to obtain the concentration of TNF- α in the sample.

2.5.9 ELISA Coefficients of Variation (CV%)

Control material was purchased from R&D systems for PAI-1 and TFPI ELISAs. Material was reconstituted on the first day of analyses then aliquots were frozen for use on subsequent plates. For PAI-1 controls, material reconstituted fresh for plate 1 provided values slightly below the acceptance range, whereas the same material which went through one freeze-thaw cycle provided values within the acceptance range which were consistent across the remaining 5 plates (CV for PAI-1 plates 2-6 =7%). For all other ELISAs, an in-house control of known value was used. Controls were performed in duplicate, except on occasion when a control well was required to repeat a participant sample. Intra- and inter-assay CVs are depicted in Table 8, controls not performed in duplicate are annotated as 'single'. Singlicate control samples were necessary when repeat study samples were required to be analysed in the control well due to a shortage of capacity for repeat analyses.

Table 8 ELISA control coefficient of variation (CV%)

Controls performed in singlicate marked as 'single'. *IL-1 β plate 6 results lost.

Control material	Intra-assay CV (%)						Inter-assay CV (%)
	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	
Adiponectin (in-house control)	11%	13%	4%	14%	1%	1%	12%
Leptin (in-house control)	8%	1%	single	1%	single	single	13%
PAI-1 (R&D low positive control)	0%	4%	6%	6%	0%	single	21%
TFPI (R&D medium positive control)	0%	1%	6%	8%	3%	2%	9%
IL-6 (in-house control)	1%	1%	1%	4%	1%	4%	4%
IL-1β (in-house control)	6%	17%	7%	6%	10%	n/a*	17%
TNF-α (in-house control)	4%	6%	7%	3%	4%	single	10%

2.6 Statistical Analysis

Data were assessed for normality using D'Agostino-Pearson normality test by GraphPad PRISM software. Where data were normally distributed, mean and SD values are provided, and in the case of non-parametric data median and interquartile ranges are supplied. Data which was non-parametric was transformed to log10 or square root to allow the use of more powerful parametric statistical tests.

Within the subsequent results chapters, coagulation and cytokine values are displayed graphically as median and IQR to demonstrate change between the two study timepoints, booking and 28 weeks' gestation. Two-way ANOVA with mixed effects modelling on log-transformed data was used to determine the effect of both time and BMI on each parameter, and whether there was a different effect on time for different BMI groups. *Post hoc* analysis by Tukey's multiple comparison test identified any differences between the BMI groups at each time-point.

Correlations between BMI and both coagulation and cytokine parameters were performed using Pearson's correlation, on log-transformed data where appropriate. Non-linear associations have goodness-of-fit statistics, adjusted R^2 displayed.

Further detail on multivariate statistical analysis is provided in the relevant results chapters.

Chapter 3 The prothrombotic effect of obesity during pregnancy

3.1 Introduction

Pregnancy is known to induce a prothrombotic state, with extensive evidence demonstrating an increase in procoagulant factors such as Factor VIII, von Willebrand Factor and fibrinogen occurring from early in the antenatal period.(Choi and Pai, 2002; Sánchez-Luceros *et al.*, 2003) This increase in procoagulant factors is accompanied by a fall in the naturally-occurring anticoagulant protein S, the development of activated protein C resistance and an impairment of the clot breakdown process, fibrinolysis.(Clark, Walker and Greer, 1999; Walker *et al.*, 1997; Choi and Pai, 2002) All of these changes contribute to the prothrombotic state necessary to prevent excessive bleeding at delivery, but also result in the increased risk of venous thromboembolism (VTE) during pregnancy and the puerperium.

With obesity also known to be a risk factor for VTE in the non-pregnant population, it could be assumed that the combination of obesity and pregnancy would produce a greater risk of thrombosis.(Hotoleanu, 2020; Stein, Beemath and Olson, 2005; Pomp *et al.*, 2007) Data on maternal morbidity and mortality confirms this theory, with an increased incidence of thrombosis in obese pregnant women, compared to their lean counterparts.(Liu *et al.*, 2009; Knight and UKOSS, 2008; Simpson *et al.*, 2001)

Having the ability to identify women at risk from venous thromboembolic disease during pregnancy may provide the opportunity for earlier intervention to prevent a disease which can prove fatal to new mothers. In current clinical practice, no single test exists which can predict the development of VTE, and as such identification of mothers at risk of thrombosis is through the application of a clinical risk scoring system.(RCOG, 2015) However, given that VTE remains the leading direct cause of maternal death, and with the increasing prevalence of obesity within the maternity population, a more comprehensive strategy is required, and the introduction of a biomarker for thrombotic risk could provide a valuable adjunct to current risk stratification methods.

3.1.1 Routine assessment of the thrombotic state in clinical practice

Assessment of haemostasis can be performed by measurement of clotting times (including the PT and APTT) which act as screening tests and may indicate deficiencies in clotting factors, or by measurement of individual clotting factors (including fibrinogen, FVIII and von Willebrand factor). However, assessment in the laboratory of the prothrombotic state is more challenging.

The prothrombin time (PT) predominantly assesses the extrinsic pathway of coagulation, which encompasses factors VII and the inhibitor TFPI. The activated partial thromboplastin time (APTT) is used to assess the intrinsic pathway which consists of factors VIII, IX, XI and XII, and is inhibited by activated protein C and protein S. Both tests can also be affected by changes within the common pathway (factors X, V, prothrombin and fibrinogen). A shorter clotting time, measured in seconds, may indicate a more prothrombotic sample.

It can be valuable to measure concentrations of individual factors, as these are known to correlate in some studies with risk of thrombosis, particularly Factor VIII and Fibrinogen. Fibrinogen, or Factor I, is a zymogen converted by thrombin to fibrin which then is cross-linked to form the fibrin clot. Fibrinogen is a critical substance within haemostasis, with the fibrin clot the main end product of the coagulation cascade, and the formed fibrin network provides a structure to which coagulation factors and cellular components of the coagulation process (including red blood cells, leucocytes, platelets and endothelial cells) can adhere. (Aleman *et al.*, 2014) Recent research has indicated that alterations within fibrin clot structure, including reduced permeability and impaired susceptibility to lysis, are associated with both first and recurrent VTE. (Baker *et al.*, 2019) Hyperfibrinogenaemia is a recognised risk factor for VTE. (Ariëns, 2013) There is also laboratory evidence of increased thrombus formation following the administration of fibrinogen concentrate to mice, and data showing the use of siRNA molecules to reduce fibrinogen synthesis in murine models of trauma resulted in reduction in thrombus formation, confirming the importance of fibrinogen concentration in VTE risk. (Machlus *et al.*, 2011; Seadler *et al.*, 2024)

Chapter 3

Factor VIII is a coagulation factor produced predominantly by the liver. Factor VIII circulates in the blood bound to von Willebrand Factor which prolongs the half-life of FVIII by preventing its degradation as a free molecule. Activated Factor VIII acts as a cofactor to activated Factor IX in converting Factor X to its active form which subsequently can convert prothrombin to thrombin. Factor VIII also acts as an acute phase protein, which increases in response to inflammation, hence its relevance in obesity which is associated with a chronic low-grade inflammatory state.

It is recognised that deficiencies of the naturally-occurring anticoagulants have been associated with thrombotic risk, particularly in inherited deficiencies known as thrombophilias. Protein S is a naturally-occurring anticoagulant which exerts its function via a number of direct and indirect mechanisms. It enhances the anticoagulant activity of its cofactor TFPI, inhibiting the Tissue Factor-Factor VIIa complex and acts as a cofactor to activated protein C which in turn inhibits Factor Va and Factor VIIIa. In addition, Protein S directly binds to activated Factor IXa preventing the formation of the tenase complex (FVIIIa-FIXa), and also binds to Factor Va and Factor Xa preventing formation of the prothrombinase complex which cleaves prothrombin. (Pilli, Plautz and Majumder, 2016)

Accurately characterising the dynamic change in balance between procoagulant and anticoagulant factors which occurs during pregnancy can be challenging, but this difficulty may be overcome by the use of a global coagulation assay, such as thrombin generation. Thrombin generation provides an assessment of the amount of thrombin produced over time, thus encapsulating the activity of the entirety of the coagulation cascade. This assay can give a unique perspective on global haemostasis which is preferable given that this is closer to coagulation *in vivo* compared to standard haemostasis laboratory tests which quantify individual factor levels.

3.1.2 Thrombin generation as a biomarker for thrombotic risk in pregnancy

Thrombin generation has been studied in pregnancy before, with increases in peak, ETP and velocity index, indicative of the development of the prothrombotic state, starting from as early as the 5th gestational week. (Bagot *et al.*, 2017) The

association between obesity and thrombin generation has also been investigated, with one study finding a reduction in ETP following obesity surgery, suggesting BMI and thrombin generation may be correlated.(Tripodi *et al.*, 2019) Only a few studies exist which have examined the combined impact of obesity and pregnancy. Mandelebe *et al* measured thrombin generation in late pregnancy and at 12 weeks postpartum. They showed that ETP was not significantly different between obese and non-obese at 26-28 weeks' gestation, but by 36-40 weeks' gestation non-obese pregnant women had statistically higher ETP than the obese group. By 12 weeks' postpartum, they found that non-obese women had a significant decrease in ETP but the decrease in obese women was non-significant.(Mandlebe *et al.*, 2021) Two further studies have compared thrombin generation in the third trimester between lean and obese pregnant women, finding significantly greater ETP in the obese groups.(Ismail *et al.*, 2014; O'Connor H 2014) Indication of an enhanced prothrombotic state in obese pregnancy was also observed in a study of the global, point of care coagulation assay thromboelastography (TEG).(O'Keefe *et al.*, 2021) These studies add to the accumulating evidence that obese women have greater thrombin generation during pregnancy than lean women. Yet by looking only at the later part of pregnancy and postpartum, where the majority of work has been done to-date, it remains unknown how long these women have greater thrombotic risk.

Given the over-representation of obese women in VTE-related maternal mortality, an accurate characterisation of the prothrombotic state in obese pregnancy is necessary to allow future studies of strategies to mitigate the apparent increased risk.

3.1.3 Hypothesis and Aims

It was hypothesised that obese pregnancy would be characterised by a greater increase in thrombin generation between the first and third trimesters, and the measurement of standard laboratory measures of haemostasis would strengthen the thrombin generation data by ensuring the haemostasis findings of the cohort were comparable to those of previous studies.

The main aim of this chapter is to characterise the influence of obesity on the prothrombotic state during pregnancy by examining the changes in laboratory

Chapter 3

markers of haemostasis in women within each of the 4 BMI classes (lean, overweight/obese class I, obese class II and obese class III) between early pregnancy (8-12 weeks' gestation) and the third trimester (28 weeks' gestation). The prothrombotic state will be assessed by quantification of procoagulant factors (Factor VIII and fibrinogen), naturally-occurring anticoagulants (Protein S and TFPI), and markers of fibrinolysis (D-dimer and PAI-1), in addition to assessment of the global coagulation assay and biomarker for thrombotic risk, thrombin generation.

The coagulation screen provides no significant insight into the assessment of the prothrombotic state, but was utilised to ensure the validity of the samples (i.e to ensure samples not activated) and exclude any significant coagulation factor deficiencies which may affect the results of thrombin generation. Thrombin generation is the key biomarker for this study, with correlations to thrombotic risk already well-established in the non-pregnant population. The inclusion of the other coagulation factor analyses aimed to confirm the findings in existing studies of pregnant women which would ensure the thrombin generation findings were representative, and also to investigate the underlying pathophysiology of the prothrombotic state given fibrinogen and PAI-1, for example, are known to increase in obesity and fibrinogen has been associated with thrombotic risk.

3.1.4 Specific research questions

What changes are observed in the following measures and components of the coagulation system between the booking appointment (8-15 weeks' gestation) and 28 weeks' gestation, and how do they differ between women of different BMIs during pregnancy:

- Coagulation screen (prothrombin time, activated partial thromboplastin time)
- Procoagulants (fibrinogen Clauss, Factor VIII)
- Naturally-occurring anticoagulants (Protein S, tissue factor pathway inhibitor)

- Markers of fibrinolysis (plasminogen activator inhibitor-1, D-dimer)
- Global coagulation assay, thrombin generation (as measured by the CAT method)

3.2 Methods

3.2.1 Study Recruitment

This was a prospective longitudinal study with blood samples collected at two timepoints during pregnancy (booking appointment and 28 weeks' gestation). The study population consisted of pregnant women, recruited within four different BMI groups (lean, overweight/obese class I, obese class II and obese class III). Study design and recruitment methods were as previously described in the Materials and Methods chapter (section 2.2).

3.2.2 Laboratory Methods

Laboratory methods are as previously described in detail in the materials and methods chapter (section 2.3) In brief, participant blood samples were collected in 3.2% sodium citrate in the antenatal clinic by a member of the study team and transported to the haemostasis laboratory at Glasgow Royal Infirmary within 1 hour. Platelet poor plasma was prepared within 2 hours of sample collection, and frozen in 1mL aliquots to allow batch analysis of samples at a later date.

Paired samples, collected at booking and 28 weeks' gestation, from the same participant were analysed simultaneously to minimise differences due to inter-assay variability. The ACL TOP automated analyser was used to perform a routine coagulation screen, Fibrinogen Clauss, D-dimer, Factor VIII and Protein S. TFPI and PAI-1 were quantified by ELISA, and thrombin generation was measured by the calibrated automated thrombogram (CAT) method.

A routine coagulation screen, consisting of prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin clotting time (TCT) was performed on all study samples to ensure samples were not activated (initiation of coagulation in the collection tube due to pre-analytical variables) and exclude any major coagulation abnormalities which may affect further tests.

Chapter 3

TFPI and PAI-1 were both quantified by ELISA, as previously described in detail in the materials and methods chapter (section 2.5).

3.2.2.1 Thrombin generation

Thrombin generation using platelet poor plasma was performed by the calibrated automated thrombogram (CAT) method, as described in detail in the materials and methods chapter (section 2.4). Paired samples (booking and 28 weeks) from the same study participant, where available, were analysed concurrently to minimise the impact of inter-assay variability.

Samples were analysed under two conditions: 1pM tissue factor (TF) with and without thrombomodulin (TM). Low (1pM) concentration tissue factor allows increased sensitivity to the intrinsic pathway of coagulation, and the addition of thrombomodulin provides sensitivity to disorders of the activated protein C system, such as acquired protein C resistance which can be observed during pregnancy. For this reason, the data presented is that analysed with thrombomodulin (TM).

3.2.3 Statistical Methods

3.2.3.1 Demographic and outcome data

Demographic data (Table 9) and outcome data (Table 10), where normally distributed, are expressed as mean $\pm$ SD. Where data was not normally distributed, data is expressed as median with interquartile ranges, and comparisons were made on log-transformed data. Comparison between the four BMI groups was by one-way ANOVA, with *post hoc* analysis by Tukey's multiple comparisons test to identify where any significant differences lay between the groups.

Categorical data is expressed as number and percentages, with paired comparisons made by Fisher's exact test and comparisons of 3 or more groups by Chi-squared test. Where a significant p-value was found through Chi-squared analyses, Z-scores with Bonferroni correction were calculated to ascertain where the significant difference lay.

3.2.3.2 Haemostasis data

All coagulation laboratory data was non-parametric, and therefore prior to statistical analyses this data was log-transformed to fit a normal distribution. However, given the familiarity of clinicians with the laboratory values and normal ranges, raw data (expressed as median and interquartile range) has been plotted alongside the statistical analyses.

Data was analysed by two-way ANOVA with mixed effects model as this statistical method was able to overcome the interindividual variability between thrombin generation measurements and allowed for missing values, given the loss to follow-up between the two study visits. Data was grouped by time and BMI class. P-values <0.05 were accepted as significant where a single variable was analysed. In the case of mixed effects model output with interaction of variables (time x BMI), p values <0.05 were accepted as significant. Tukey's *post hoc* test was used to identify differences between each of the groups.

3.3 Results

3.3.1 Study population demographics

3.3.1.1 Baseline demographic data

The characteristics of the study population are shown in Table 9. Participants were well-matched for age and smoking status in each of the four BMI groups studied, and the population was predominantly Caucasian. As would be anticipated, weight, hip and waist circumference were statistically different between each of the four groups, all increasing with BMI. Median waist:hip ratio in all groups was considered within the 'healthy' range by WHO standards.(WHO, 2008) An unhealthy waist:hip ratio (>0.85) was present in 2.75% of lean participants, 23.48% of overweight/obese class I, 29.17% of obese class II and 28.57% of obese class III.

Table 9 Study population characteristics by BMI group

Continuous normally distributed data expressed as mean (SD) with comparison by one-way ANOVA, * denotes non-parametric data expressed as median (interquartile range) with comparison by one-way ANOVA performed on log-transformed data. One-way ANOVA *post hoc* analysis with Tukey's test: different superscript letters denote significant difference. # Chi-squared test of association used to compare categorical data (ethnicity comparing caucasian vs black/s.asian/other to achieve statistical power), §significant difference contributing to p-value (significant Z-score following Bonferroni correction). Previous smokers stopped at least 6 months ago.

		Lean	Overweight/ obese class I	Obese class II	Obese class III	<i>p-value</i>
Age (mean, SD)		31.0 (5.7)	30.9 (5.3)	32.1 (5.3)	33.4 (5.0)	0.37
Ethnicity #, n (%)	<i>Caucasian</i>	95 (87)	107 (93)	23 (96)	14 (100)	0.19
	<i>Black</i>	6 (6)	4 (3)	0 (0)	0 (0)	
	<i>S.Asian</i>	5 (5)	4 (3)	1 (4)	0 (0)	
	<i>Other</i>	3 (3)	(0)	0 (0)	0 (0)	
Weight*, kg (IQR)		61.0 (55.8,65.9) ^a	77.0 (72.4,83.7) ^b	99.4 (94.1, 107.8) ^c	126.0 (114.6, 145.4) ^d	<0.0001
Waist circumference*, cm (IQR)		76.0 (73.5, 80.0) ^a	90.0 (85.5, 95.0) ^b	101.5 (98.0, 104.8) ^c	120.5 (114.5, 131.5) ^d	<0.0001
Hip circumference*, cm (IQR)		100.0 (96.0, 104.0) ^a	110 (107.0, 115.0) ^b	126 (122.0, 131.0) ^c	145.5 (135.0, 163.5) ^d	<0.0001
Waist:hip ratio* (IQR)		0.76 (0.74, 0.80) ^a	0.82 (0.78, 0.85) ^b	0.81 (0.76, 0.86) ^b	0.80 (0.79, 0.89) ^b	<0.0001
Parity #, n (%)	<i>primiparous</i>	49 (45)	48 (42)	7 (29)	3 (21)	0.23
	<i>multiparous</i>	60 (55)	67 (58)	17 (71)	11 (79)	
Pre-pregnancy	<i>Yes</i>	27 (25) [§]	42 (37)	16 (67) [§]	7 (50)	0.001
comorbidity, n (%)	<i>No</i>	82 (75) [§]	73 (63)	8 (33) [§]	7 (50)	
Smoker, n (%)	<i>Current</i>	11 (10)	11 (9.6)	2 (8.3)	3 (21)	0.58
	<i>Previous</i>	25 (23)	28 (24)	8 (33.3)	1 (7)	
	<i>Never</i>	73 (67)	76 (66)	14 (58.3)	10 (71)	
Gestation at booking (weeks)		10.1 (1.8)	9.9 (2.0)	9.5 (1.9)	9.8 (1.3)	0.98

Chapter 3

Parity was subdivided into primiparous (no previous pregnancies) and multiparous (≥ 1 previous pregnancy) and no significant difference was found in parity between the 4 BMI groups ($p=0.23$) when compared by Chi-squared test. Pre-pregnancy comorbidity was more prevalent in the obese groups. There was a significant difference in co-morbidity between different BMI groups, with statistically more comorbidity in obese class II group compared to the lean group, as demonstrated by Chi-squared test of association *post hoc* analysis using Bonferroni correction of Z-scores.

The mean gestational age at time of study recruitment (booking appointment), based upon the dating ultrasound scan, was 9.9 weeks (range 4-17 weeks), and there was no significant difference in gestational age at booking between the different BMI groups ($p=0.98$) as documented in Table 9.

3.3.1.2 Outcome Data

Pregnancy outcomes are depicted in Table 10. The secondary outcome measure of VTE only occurred in one participant, consisting of a superficial thrombophlebitis extending 6cm and to within 3cm of the saphenofemoral junction. Given that the incidence of VTE during pregnancy is 1 in 1000, it would not have been anticipated to have more than one episode of VTE in a study population of this size. (Pomp *et al.*, 2008)

Table 10 Pregnancy outcomes by BMI group.

Outcome data below on 240 women (loss to follow-up due to miscarriage or moved outwith healthboard). Categorical data displayed as number (%) and analysed by Fisher's exact test (comparing each group to lean). Continuous, normally distributed data expressed as mean (SD) and analysed for significance using one-way ANOVA (p value in brackets indicates significant value on Tukey's multiple comparison *post hoc test*). Non-parametric data expressed as median (IQR) and log-transformed prior to analysis by one-way ANOVA. Shared superscript indicates no significant difference, SVD = spontaneous vaginal delivery, LUSCS = lower uterine segment caesarean section.

	Lean n=101	Overweight/Obese Class I n=106	Obese Class II n=21	Obese Class III n=12	p value
Pregnancy-induced hypertension/pre-eclampsia, n (%)	2 (2.0%) ^a	6 (5.7%) ^a	0 ^a	1 (8.3%) ^a	p=0.28 (lean v ow/ob1) p>0.99 (ow/ob1 vs ob2) p=0.28 (ob2 vs ob3)
Venous thromboembolism, n (%)	0	1 (0.9%)	0	0	n/a
Gestational diabetes mellitus, n (%)	1 (0.99%) ^a	5 (4.7%) ^a	3 (14%) ^b	4 (33.3%) ^c	p=0.21 (lean v ow/ob1) p=0.016 (ow/ob1 vs ob2) p=0.0004 (ob2 vs ob3)
Gestation at delivery, weeks (SD)	39.4 (1.5)	39.1 (2.3)	38.5 (1.3) ^a	37.7 (2.1) ^a	p=0.015

Table 2 Pregnancy outcomes by BMI group (continued)

	Lean n=101	Overweight/Obese Class I n=106	Obese Class II n=21	Obese Class III n=12	p value
Mode of delivery; n (%)					
SVD	62 (61.4%)	52 (49%)	11 (52.4%)	4 (33.3%)	p=0.09 (lean v ow/ob1) p=0.47 (ow/ob1 vs ob2) p=0.07 (ob2 vs ob3)
Elective LUSCS	21 (20.8%)	37 (34.9%)	7 (33.3%)	5 (41.7%)	p=0.03 ^a p=0.26 (ow/ob1 vs ob2) p=0.46 (ob2 vs ob3)
Emergency LUSCS	18 (17.8%)	17 (16%)	3 (14.3%)	3 (25%)	p=0.85 (lean v ow/ob1) p=1.0 (ow/ob1 vs ob2) p=0.24 (ob2 vs ob3)
EBL (median, IQR)	500 (300, 600)	500 (337.5, 952.5)	500 (375,825)	700 (425, 1075)	p=0.074
Neonatal birth weight, grams (SD)	3398 (487)	3390 (665)	3570 (422)	3472 (780)	p=0.60
Duration hospital inpatient from 28weeks, hours (IQR)	48 (34,78.5) ^a	57 (36,96) ^a	72 (50,127)	93.5 (57.5,133.75)	p=0.005 (lean v ow/ob1) (p=0.035) (ow/ob1 vs ob2) (p=0.04) (ob2 vs ob3)

Chapter 3

Metabolic pregnancy-related complications were more likely in the obese groups. The incidence of gestational diabetes was greater in obese class II ($p=0.016$) and obese class III ($p=0.0004$) when compared to lean. There was no difference in the incidence of pregnancy-induced hypertension/pre-eclampsia when each of the overweight/obese groups were compared to lean.

Higher BMI was associated with earlier delivery, with mean gestation at delivery significantly lower in obese class III mothers compared to lean mothers (37.7 vs 39.4 weeks, $p=0.022$). Mode of delivery was not significantly different between the BMI groups, although the percentage of caesarean section deliveries (both elective and emergency) increased with BMI. Overweight/obese class I women were more likely to have an elective caesarean section compared to lean ($p=0.03$), but this difference was not found in either of the other obese groups. Emergency caesarean section occurred in 25% of obese class III mothers, compared to an incidence of 14-17% in all other groups, however this was not found to be statistically significant when compared to lean ($p=0.24$).

Cumulative duration of hospital inpatient stay from 28 weeks' gestation was significantly greater in obese class II and obese class III compared to lean mothers ($p=0.035$ and $p=0.04$ respectively).

No significant difference was found by ANOVA between BMI groups and neonatal birthweight in this cohort ($p=0.60$).

3.3.2 Procoagulant Tests

3.3.2.1 Screening tests (PT and APTT)

Figure 3-1 illustrates the changes in prothrombin time between booking and 28 weeks' gestation, with data sub-divided by BMI class.

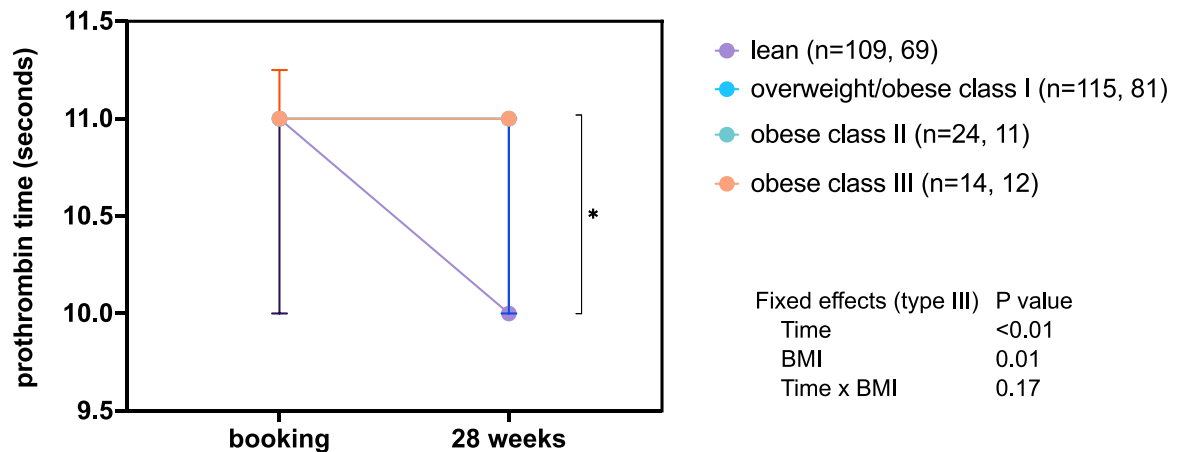


Figure 3-1 PT at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed). The data was non-parametric, therefore two-way ANOVA with mixed effects model was performed on log-transformed data. Tukey's *post hoc* test was used to identify differences between the four BMI groups at each time point. * $p < 0.05$. Data points for overweight/obese class I, obese class II and obese class III overlap in this figure.

Two-way ANOVA with mixed effects modelling showed a significant negative association with both time ($p < 0.01$) and BMI ($p = 0.01$) on PT between booking and 28 weeks' gestation. The interaction between time and BMI was not significant ($p = 0.17$) Tukey's *post hoc* analysis found no significant difference in PT between each of the four BMI groups at booking. The only significant difference at 28 weeks was shorter PT in lean compared to overweight/obese class I groups ($p = 0.01$).

Chapter 3

Activated partial thromboplastin time (APTT) at each of the two study timepoints is depicted in Figure 3-2.

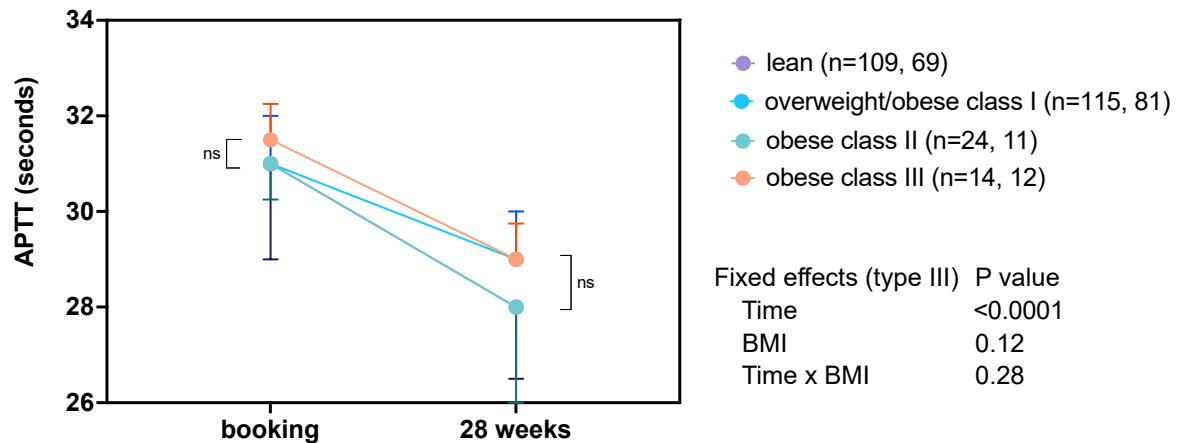


Figure 3-2 APTT at booking and 28 weeks, by BMI group (raw data, median ± IQR displayed). The data was non-parametric so log-transformed data was used for two-way ANOVA with mixed effects model. Tukey's *post hoc* test was used to identify significant differences between the BMI groups at each time point. APTT normal range 27-38 seconds. ns=non-significant

Mixed effect modelling showed a significant correlation with time ($p < 0.0001$), with significant shortening of APTT with advancing gestation, but no association with BMI on APTT and no interaction between time and BMI was found.

3.3.2.2 Fibrinogen

Fibrinogen Clauss raw data is depicted in Figure 3-3, and illustrates the increase in Fibrinogen in each of the 4 BMI groups as pregnancy advances.

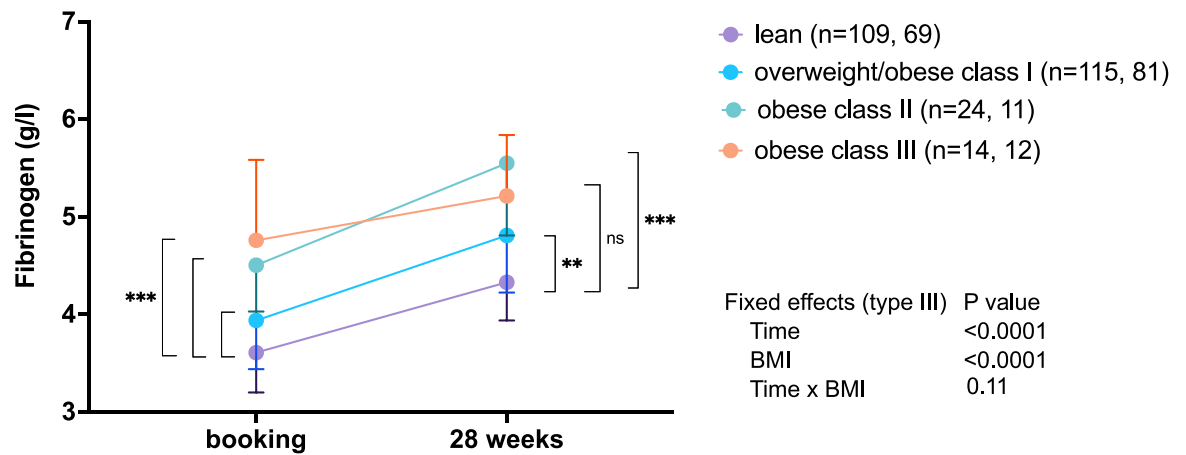


Figure 3-3 Fibrinogen Clauss at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).

Data was non-parametric so was log-transformed prior to statistical analysis with two-way ANOVA and mixed effects model. Normal range fibrinogen Clauss 1.7-4.0g/l. *** $p < 0.001$, ** $p < 0.01$, ns=non-significant

Median fibrinogen levels were increased above the normal range (1.7-4.0 g/L) in obese class II and obese class III from the booking appointment. By 28 weeks' gestation, median fibrinogen levels were elevated outwith the normal reference range for all BMI groups.

Two-way ANOVA with a mixed effects model confirmed a significant increase in Fibrinogen Clauss over time ($p < 0.0001$) and an increase with each BMI group ($p < 0.0001$). The interaction between time and BMI was not statistically significant.

Post hoc analysis by Tukey's multiple comparison test identified significantly higher Fibrinogen at booking in each of the overweight and obese groups when compared to lean (all $p < 0.001$). By 28 weeks this difference was reduced and the only significant differences were between lean and overweight/obese class I ($p = 0.007$), lean and obese class II ($p = 0.001$); and overweight/obese class I and obese class II ($p = 0.044$). The attenuated difference between the BMI groups by 28 weeks' gestation reflects the interaction between BMI and time, with less of an increase observed in Fibrinogen Clauss for the obese Class III group.

3.3.2.3 Factor VIII

Figure 3-4 shows the concentrations of Factor VIII between booking and 28 weeks' gestation, subdivided by BMI group. Two-way ANOVA with mixed effects modelling found a significant increase in Factor VIII with time ($p < 0.0001$), but no difference in Factor VIII levels with BMI ($p = 0.53$) and no interaction between time and BMI ($p = 0.30$) as illustrated in Figure 3-4. At the time of the booking appointment, 35% of women had Factor VIII levels above the normal range for our laboratory (58-152iu/dL). Between booking and 28 weeks' gestation, there was an increase in Factor VIII in all BMI groups which reached mean values of >200 iu/dL.

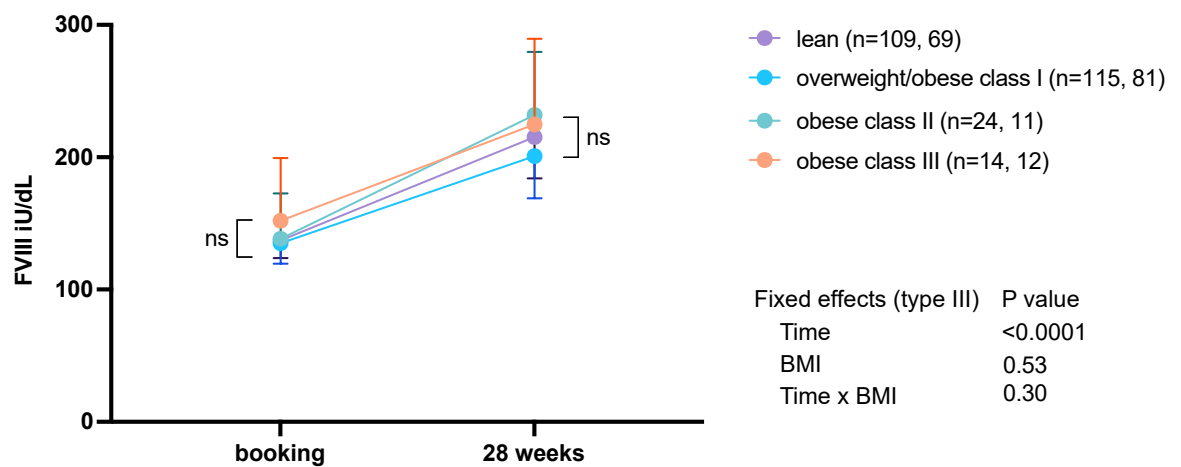


Figure 3-4 Factor VIII at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).

Data was non-parametric so was log-transformed prior to statistical analysis with two-way ANOVA and mixed effects model. ns=non-significant. Normal range 58-152iu/dL.

3.3.3 Inhibitors of Coagulation

3.3.3.1 Protein S

Two-way ANOVA with mixed effects modelling found a significant decrease in Protein S with time ($p<0.0001$), but no correlation with BMI ($p=0.13$). The interaction between time and BMI did not reach statistical significance ($p=0.28$).

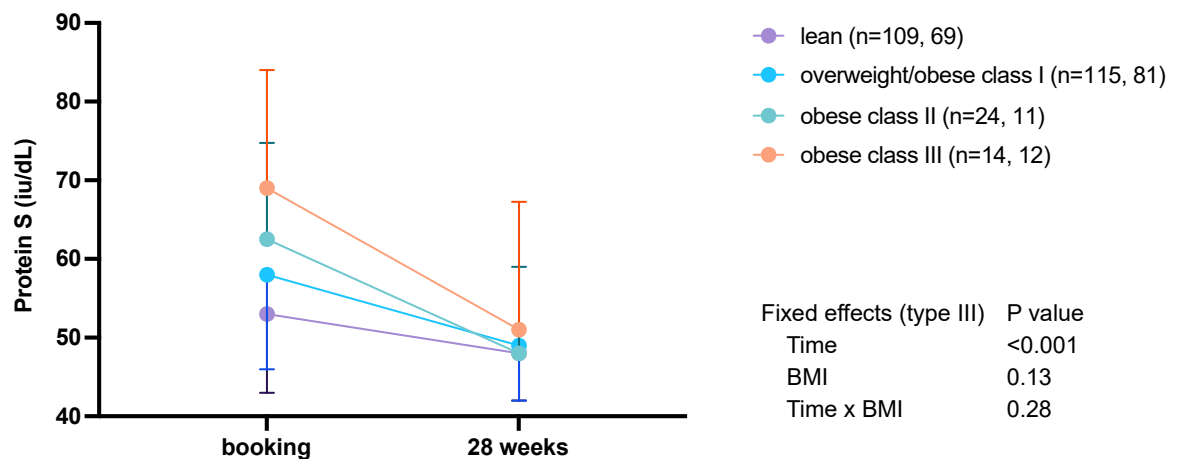


Figure 3-5 Protein S at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).

Data was non-parametric so was log-transformed prior to statistical analysis with two-way ANOVA and mixed effects model. Normal range 65-137iU/dL.

Protein S at booking was below the reference range set for our clinical laboratory (65-137iU/dL) in 69% of lean and overweight/obese class I women and 50% of obese class II and class III women. Protein S fell in all women between booking and 28 weeks' gestation and was below the reference range in 90.1% of all women by 28 weeks' gestation. This lack of statistical significance may be due to the small sample size in the obese groups as the graph depiction suggests a greater decrease in obese class II and class III which has not been borne out in the statistical analyses.

3.3.3.2 Tissue Factor Pathway Inhibitor (TFPI)

TFPI median $\pm$ IQR at booking and 28 weeks' gestation for each of the four BMI groups are illustrated in Figure 3-6. Two-way ANOVA with mixed effects model identified a significant reduction in TFPI with time ($p < 0.0001$) and that BMI was positively correlated with TFPI ($p=0.0002$). In addition, an interaction between time and BMI ($p=0.047$) illustrates gestational change in TFPI observed during

Chapter 3

pregnancy differs according to BMI group. As illustrated in Figure 3-6, at the time of the booking appointment, TFPI was significantly higher in obese class II and class III when compared to lean ($p=0.0004$ and $p<0.0001$ respectively). There was a reduction in TFPI in all BMI groups between the two study timepoints, then by 28 weeks there was no longer a significant difference between lean and obese groups. This would suggest the interaction between time and BMI signifies a greater reduction in TFPI in more obese women between booking and 28 weeks' gestation.

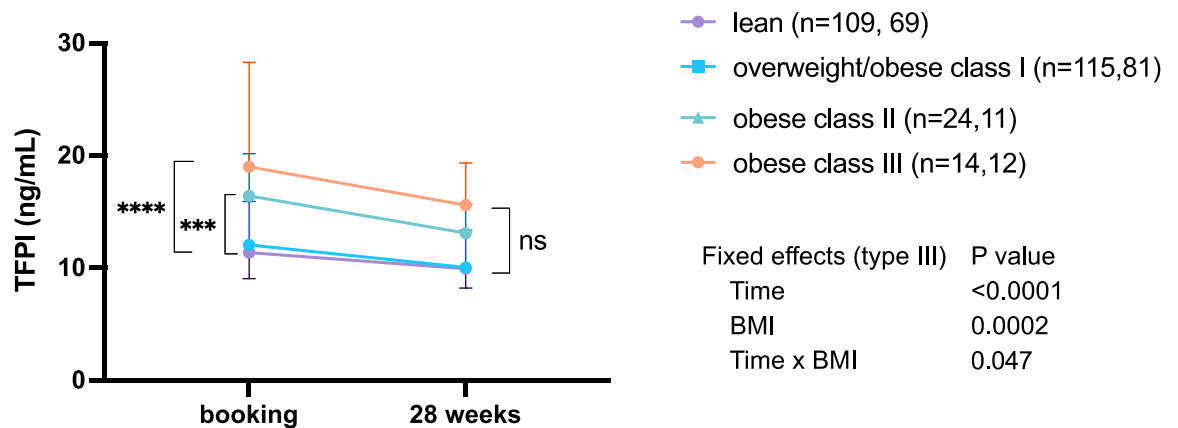


Figure 3-6 TFPI at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).

Data was non-parametric so log-transformed data was used for statistical analysis by two-way ANOVA with mixed effect modelling. **** $p<0.0001$, *** $p<0.001$, ns=non-significant.

3.3.4 Fibrinolysis

3.3.4.1 Plasminogen Activator Inhibitor 1 (PAI-1)

Figure 3-7 shows the change in PAI-1 between booking and 28 weeks' gestation in each of the four BMI groups. PAI-1 data at booking and 28 weeks' gestation was subdivided by BMI class and analysed by two-way ANOVA with mixed effects modelling which found a significant increase in PAI-1 with time ($p<0.0001$), a positive association with BMI ($p=0.039$) and interaction between time and BMI ($p<0.0001$).

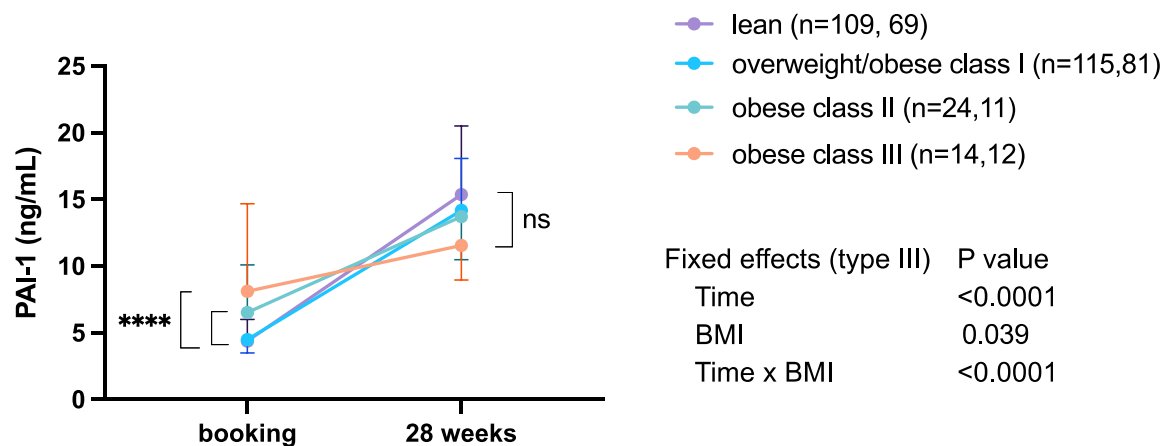


Figure 3-7 PAI-1 (ng/mL) raw data, median and IQR, at booking and 28 weeks by BMI group. Two-way ANOVA with mixed effects model performed on log-transformed data as raw data was non-parametric. Tukey's *post hoc* test confirmed obese class II and obese class III groups had significantly higher PAI-1 at booking compared to lean, but there was no significant difference between any of the groups at 28 weeks. ** $p<0.0001$, ns=non-significant.**

PAI-1 at booking was significantly greater in obese class II and obese class III groups when compared to lean (both $p<0.0001$). Between booking and 28 weeks' gestation, PAI-1 increased in all BMI groups with the increase statistically significant for all groups except obese class III (lean $p<0.0001$, overweight/obese class I $p<0.0001$, obese class II $p=0.006$, obese class III $p=0.83$). By 28 weeks' gestation PAI-1 values between the four BMI groups were not statistically different. The increase in PAI-1 was greatest in lean and least in obese class III which likely accounts for the noted interaction in the two-way ANOVA between BMI and time and results in a reversal of the positive correlation between BMI and PAI-1 at 28 week's gestation.

Chapter 3

3.3.4.2 D-dimer

Two way ANOVA with mixed effects modelling showed a positive correlation with time ($p < 0.0001$), with D-dimer increasing between booking and 28 weeks' gestation in all BMI groups, but there was no association with BMI ($p = 0.12$) and no interaction between time and BMI ($p = 0.54$).

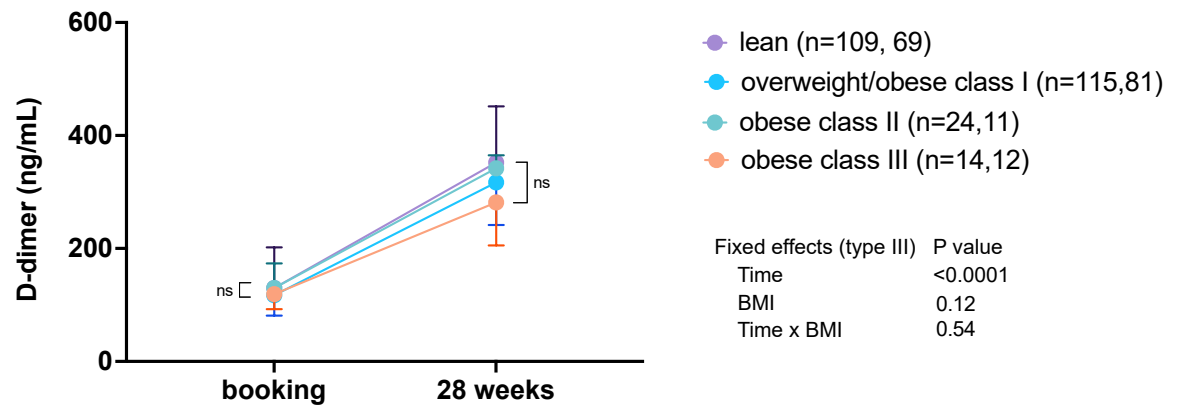


Figure 3-8 D-dimer at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed). Data was non-parametric so log-transformed prior to analysis by two-way ANOVA with mixed effects modelling. Ns=non-significant

3.3.5 Thrombin Generation

3.3.5.1 Lagtime

Two-way ANOVA with mixed effects modelling found there was no correlation with gestation ($p=0.72$) but there was positive association with BMI ($p=0.0006$) with prolongation of lagtime in more obese groups. There was no interaction identified between time and BMI ($p=0.20$).

Post hoc analysis by Tukey's multiple comparison test found a significantly longer lagtime at booking in Obese Class II ($p=0.0027$) and Obese Class III ($p=0.002$) when compared to the Lean group. No such difference existed at 28 weeks gestation.

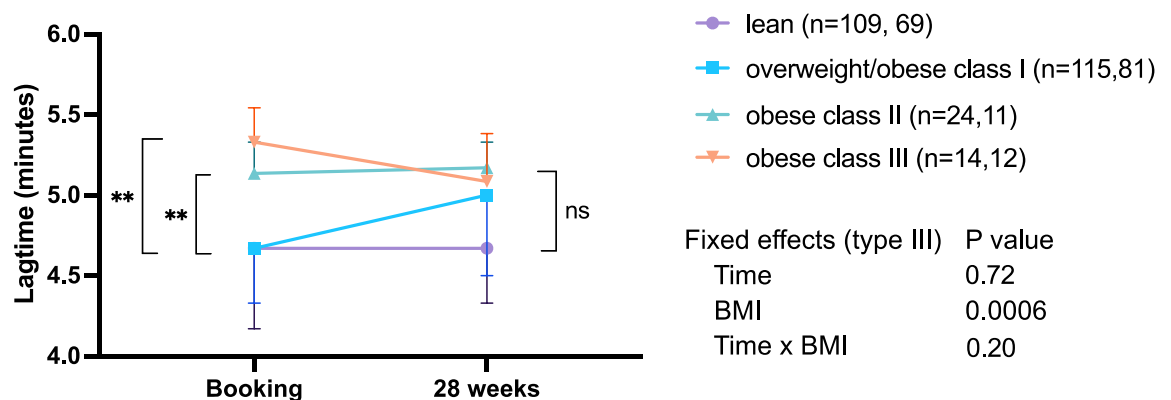


Figure 3-9 Lagtime (1pM tissue factor + thrombomodulin) at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed). Data was non-parametric so log-transformed prior to analysis by two-way ANOVA with mixed effects model. Tukey's *post hoc* analysis found a significant difference in lagtime at booking between lean and obese class II and class III but no difference at 28 week's gestation. ** $p<0.01$, ns=non-significant.

3.3.5.2 Peak

Figure 3-10 shows peak thrombin at booking and 28 weeks' gestation, subdivided by BMI group. When the data was considered overall, two-way ANOVA with mixed effects model found a significant positive correlation with time ($p < 0.0001$) and BMI ($p = 0.0058$) on peak thrombin values, but there was no significant interaction present between time and BMI ($p = 0.10$).

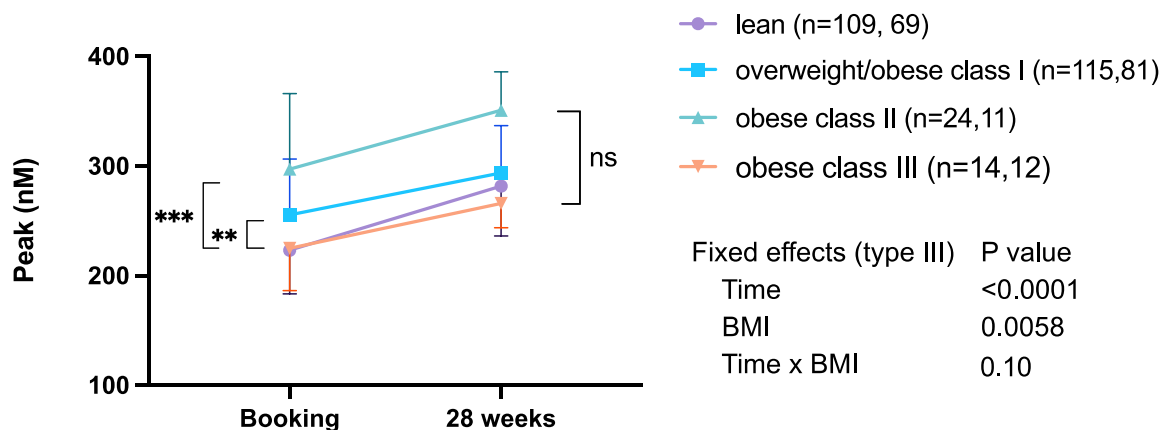


Figure 3-10 Peak thrombin (1pM tissue factor + thrombomodulin) at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).

Data was non-parametric so log-transformed prior to analysis by two-way ANOVA with mixed effects modelling. *** $p < 0.001$, ** $p < 0.01$, ns=non-significant

Post hoc analysis was by Tukey's multiple comparison test. At booking, peak thrombin was significantly greater in overweight/obese class I ($p = 0.0027$) and obese class II ($p = 0.0004$) compared to lean, however no difference was found between lean and obese class III ($p = 0.80$). Whilst peak thrombin at booking was seen to increase with each increase in BMI class, Obese Class III women did not follow the trend and had lower than anticipated peak thrombin when compared to the rest of the study population. By 28 weeks' gestation, no differences were found between any of the 4 groups which may reflect the interaction between time and BMI, as the differences evident at booking between the BMI groups were no longer apparent by 28 weeks suggesting the rate of change must be different dependent upon BMI group.

These data demonstrate that whilst peak thrombin is greatest in obese class II at booking and remains so by 28 weeks' gestation, the amplification of thrombin

Chapter 3

generation is greatest in lean participants between booking and 28 weeks' gestation, such that any differences initially evident at booking between the BMI groups were no longer apparent by 28 weeks' gestation as the lean group had increased peak thrombin to reach that of obese class II women. Obese Class III participants did not fit into the trend as expected, i.e lower than lean when expected to be higher than Obese Class II, which may be due to small sample size in this cohort.

3.3.5.3 Endogenous Thrombin Potential (ETP)

Raw ETP data is depicted in Figure 3-11. Two way ANOVA with mixed effects model on log-transformed data found a positive correlation between both time ($p=0.0069$) and BMI ($p=0.0057$) and ETP, and also identified an interaction between time and BMI ($p=0.0498$) suggesting that the BMI group is associated with how much ETP increases as pregnancy progresses.

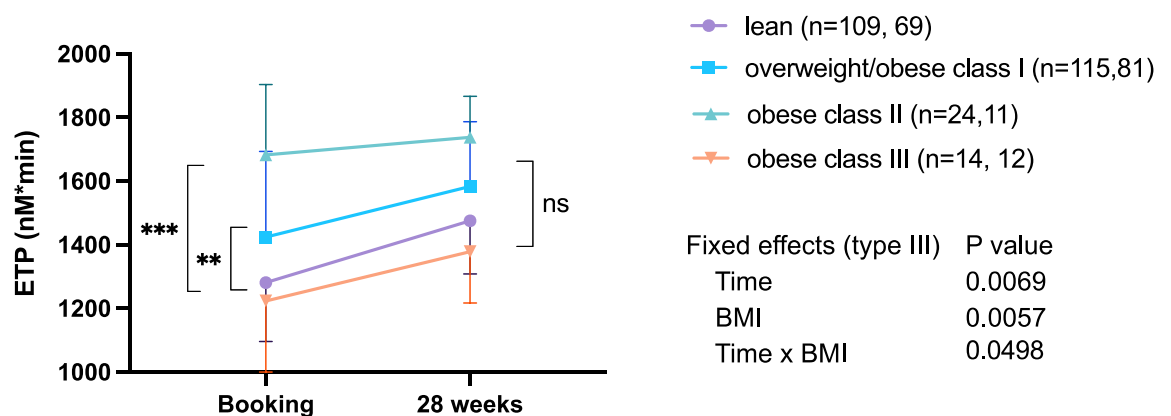


Figure 3-11 ETP (1pM tissue factor + thrombomodulin) at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed)..

Log-transformed data was analysed by two-way ANOVA with mixed effects modelling and *post hoc* analysis by Tukey's multiple comparison. *** $p<0.001$, ** $p<0.01$, ns=non-significant.

There was an increase in booking ETP with each subsequent increase in BMI group. ETP at booking was observed to be significantly greater in Obese Class II and Overweight/Obese Class I compared to Lean ($p=0.0003$ and $p=0.0019$, respectively). However Obese Class III did not follow this trend, with no statistical difference between Obese Class III and Lean ETP at booking ($p=0.99$). These findings were similar to the pattern observed with peak thrombin data, as Obese Class III had lower than expected peak thrombin within the context of the other BMI groups.

Chapter 3

ETP increased in all BMI groups, as reflected in the mixed effects model by the correlation between time and ETP, but by 28 weeks' gestation the differences between the BMI groups were no longer apparent and no statistical difference was identified between any of the BMI groups. This, similar to the peak thrombin data, signifies the interaction between time and BMI and that the rate of increase in ETP between booking and 28 weeks' gestation differs between the BMI groups

3.3.5.4 Time to Peak (ttP)

Figure 3-12 shows raw data for ttP in each of the 4 BMI groups at booking and 28 weeks' gestation. When all log-transformed data was considered in a two-way ANOVA mixed effects model, a significant decrease in ttP with time was identified ($p=0.0056$) but there was no relationship with BMI ($p=0.25$) and no interaction between time and BMI ($p=0.76$).

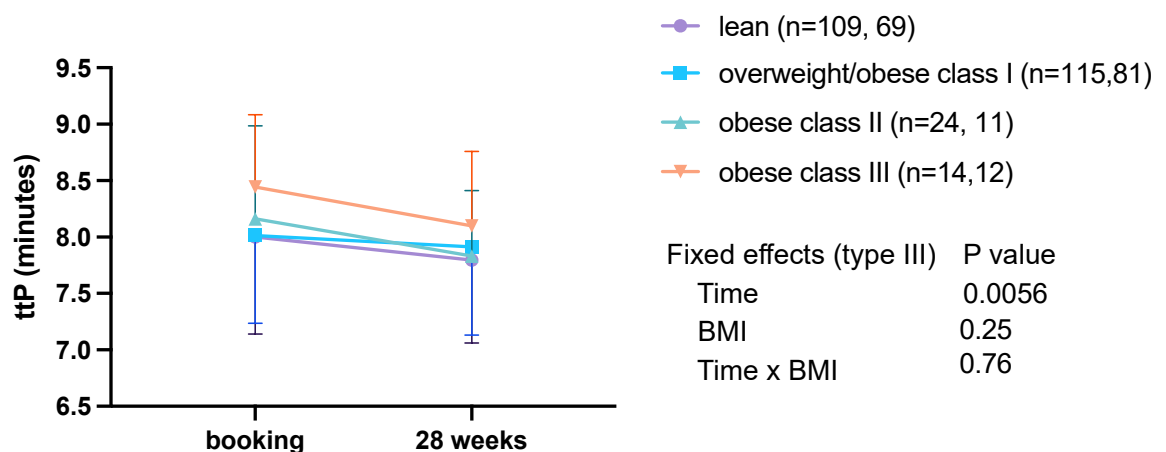


Figure 3-12 ttP (1pM tissue factor + thrombomodulin) at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).

Log-transformed data was analysed by two-way ANOVA with mixed effects modelling.

3.3.5.5 Velocity Index

Raw data for velocity index at booking and 28 weeks' gestation is depicted in Figure 3-13. Two-way ANOVA on log-transformed data found a significant positive association with time ($p<0.0001$) and BMI ($p=0.0021$) on Velocity Index, but the interaction between time and BMI did not reach statistical significance ($p=0.16$).

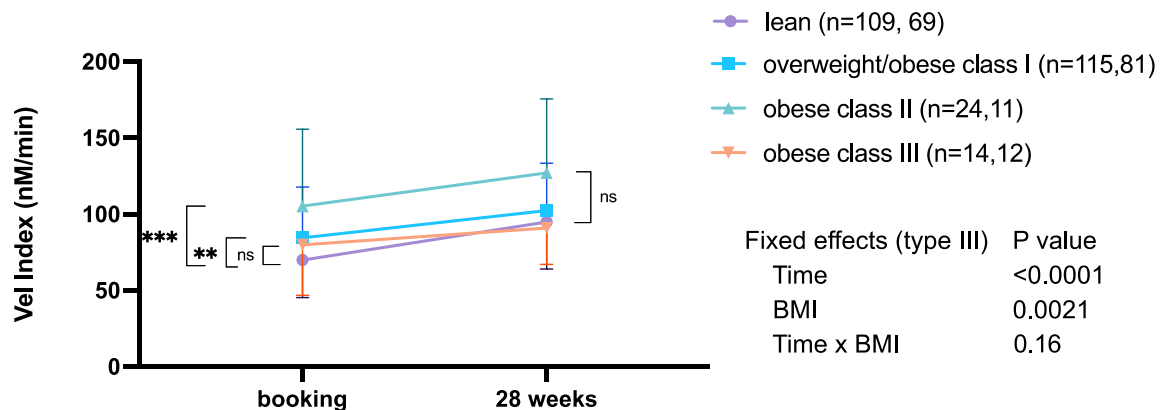


Figure 3-13 Velocity index (1pM tissue factor + thrombomodulin) at booking and 28 weeks, by BMI group (raw data, median $\pm$ IQR displayed).

Two-way ANOVA with mixed effects model on log-transformed data and Tukey's *post hoc* analysis. *** $p<0.001$, ** $p<0.01$, ns=non-significant.

At booking, velocity index was seen to increase with each increase in BMI group, but as has been seen with the previously discussed thrombin generation parameters, the Obese Class III group did not fit into this pattern. There was a significant increase in Velocity index between lean and overweight/obese class I ($p=0.0024$), and lean and obese class II ($p=0.0001$) at booking, but no difference between lean and obese class III ($p=0.6048$). Velocity index increased in all groups between booking and 28 weeks but there were no longer any significant differences between the BMI groups by 28 weeks' gestation.

3.4 Discussion

This study provides a unique assessment of the changes to haemostasis which occur during pregnancy and how these differ between lean and obese women. Although previous studies have described the changes in individual factors which occur antenatally, there are few which have investigated the influence obesity has on these factors, and fewer which have simultaneously measured thrombin generation. Following a recent literature search, and to the best of our

knowledge, this study is the first to compare thrombin generation between different BMI groups prospectively and from early pregnancy through to the third trimester.

3.4.1 Main findings

The original hypothesis assumed that thrombin generation, and related prothrombotic substances would show a greater increase in obese women compared to lean, where in fact the opposite has been demonstrated. Whilst it has been demonstrated that thrombin generation (peak, ETP and velocity index), was positively affected by BMI group at booking ($p=0.0057$, $p=0.0058$ and $p=0.0021$ respectively), thrombin generation was not found to increase more in obese compared to lean pregnancies by 28 weeks' gestation (time $\times$ BMI $p=0.10$, $p=0.0498$, $p=0.76$ respectively), and instead there was no significant difference in peak, ETP and velocity index between the four different BMI groups at 28 weeks' gestation.

Although the initial hypothesis was not proven, these data shown the prothrombotic state in obese pregnancy onsets earlier than in lean women. Obese women had higher thrombin generation at booking appointment, and the prothrombotic state developed between booking and 28 weeks' gestation in lean women, so that thrombin generation was similar in all BMI groups by 28 weeks' gestation

These findings are strengthened by the following haemostasis parameters.

3.4.1.1 Procoagulant substances in obese pregnancy

Fibrinogen was positively correlated with BMI at both study timepoints, and increased with advancing gestation, which is concordant with data from Smrcka et al and others. (Smrcka *et al.*, 2012) Fibrinogen was greater than the upper threshold of the normal reference range at booking in obese class II and class III women. However the gestational increase in fibrinogen was attenuated in the obese groups compared to lean, which mirrors the attenuated increase in thrombin generation observed in obese women.

Chapter 3

Factor VIII did not show any relationship with BMI, but did increase significantly between booking and 28 weeks' gestation, in concordance with the previously summarised findings in the literature.

Screening tests **PT** and **APTT** showed a significant negative correlation with time ($p < 0.01$ and $p < 0.0001$, respectively), but no association with BMI. Given that **APTT** is reflective of the intrinsic pathway, particularly **Factor VIII**, the shortening of the **APTT** likely reflects the increase in **Factor VIII**.

3.4.1.2 Inhibitors of coagulation in obese pregnancy

Plasma concentrations of **TFPI** correlated positively with BMI at booking but fell with advancing gestation. There was an interaction between time x BMI ($p = 0.0047$) with the data plot indicating the loss of **TFPI** in pregnancy is greatest in the most obese. By 28 weeks' gestation there was no significant difference in **TFPI** between the four BMI groups. Therefore, it may stand to reason that having such a proportionally greater loss of coagulation inhibition could be pushing the balance towards a more prothrombotic state, based on the knowledge that a reduction in **TFPI** in the non-pregnant population has been associated with risk of VTE (Dahm *et al.*, 2003)

Protein S significantly reduced by 28 week's gestation in all groups, similar to the findings of the previously summarised literature. The difference between the BMI groups at booking did not reach statistical significance.

3.4.1.3 Fibrinolysis in obese pregnancy

PAI-1 was significantly greater in obese compared to lean women at booking and increased significantly between booking and 28 weeks in all except obese class III women. There was an interaction between time x BMI ($p < 0.0001$) noted which manifest as an increase in **PAI-1** which was greatest in lean women and most attenuated in obese class III, such that by 28 weeks' gestation there was no significant difference in **PAI-1** levels between the BMI groups.

As outlined in previous literature, the data from this study also found an increase in **D-dimer** between booking and 28 weeks' gestation, but there was no relationship identified between BMI and **D-dimer**.

3.4.1.4 Thrombin generation in obese pregnancy

ETP was the only thrombin generation parameter which showed a significant interaction between time and BMI ($p=0.498$), but a similar pattern was observed between booking and 28 weeks' gestation for each of **peak**, **ETP** and **velocity index**, with thrombin generation positively associated with each parameter at booking, increasing between booking and 28 weeks' gestation but no longer a significant difference between the BMI groups by 28 weeks. The most obese women entered their booking appointment with higher baseline thrombin generation and had minimal change by 28 weeks' gestation, whereas lean women start started with lower parameters and matched the levels of thrombin generation by 28 weeks that obese women had been exposed to from early pregnancy.

Obese class III women did not follow the trends set by the other BMI classes, with thrombin generation similar to that of lean women, which was suspected to relate to the small sample size in this group ($n=12$).

3.4.1.5 Interpretation of main findings

In this study, all measured substances within each of the domains of the haemostasis system (procoagulant, anticoagulant and fibrinolysis) were altered during pregnancy. Factors from each of the domains also showed a relationship with BMI: fibrinogen (procoagulant), TFPI (anticoagulant) and PAI-1 (fibrinolysis). Yet only TFPI, PAI-1 and thrombin generation (ETP) showed an interaction between time x BMI.

Examining the pattern of change in thrombin generation, TFPI and PAI-1 indicate that obese women show less 'flexibility' in their response to pregnancy compared to the response of lean women.

In concordance with our data, a recent study by Mandelebe et al compared thrombin generation in lean and obese women during late pregnancy and the puerperium found no significant difference in ETP at 26-30 weeks' gestation. However, by term, lean women had higher ETP than obese women. At the final study time point 6-12 weeks postpartum, ETP in the lean group had decreased more sharply and was lower than obese group.(Mandlebe *et al.*, 2021) If we

consider the data from our study and this study together as a complete picture of thrombin generation during pregnancy and the puerperium (see illustration in Figure 3-14), this may fit with the theory that lean women have a more flexible haemostatic system which responds more acutely to physiological changes such as pregnancy with a rapid return to normal postpartum. Obese women, on the other hand, are prothrombotic to begin with and have less ability to respond to changes, resulting in a more prolonged prothrombotic period with greater area under the curve. This difference in response to pregnancy between lean and obese subjects has been recognised before in metabolic parameters, and obese women are recognised to be less “metabolically flexible” in response to physiological stressors such as pregnancy. (Meyer *et al.*, 2013)

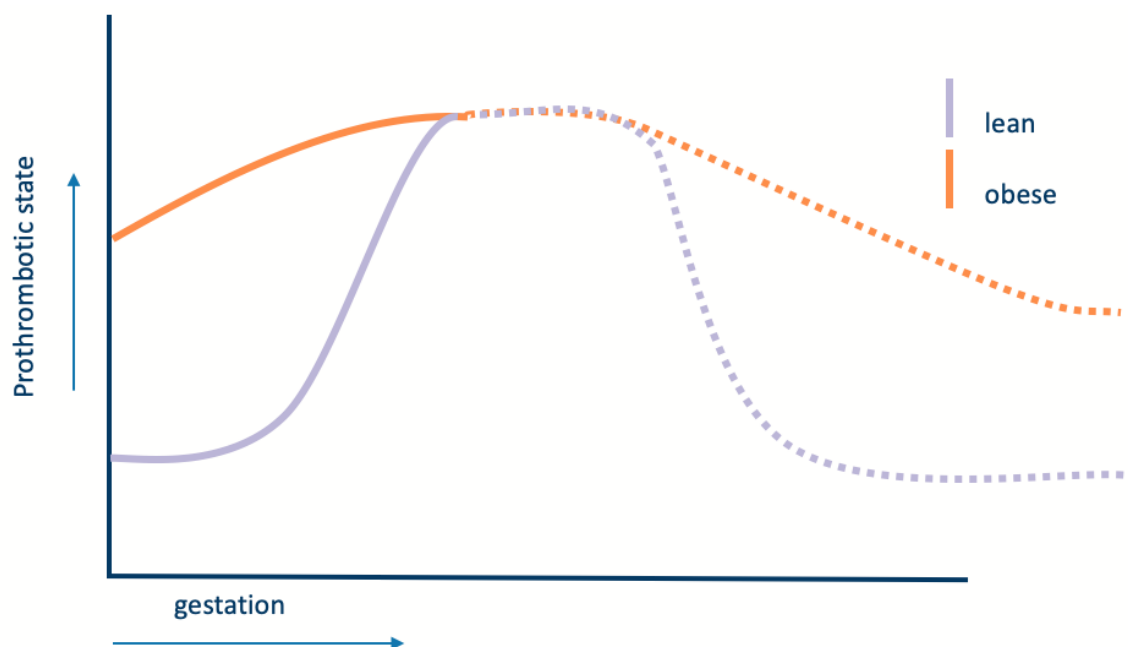


Figure 3-14 Approximation of ETP across pregnancy and the puerperium in lean and obese.

Hypothesis of changes in ETP across pregnancy combining trends observed in our data (solid line) and data from Mandlebe et al 2021 (dotted line). Lean women respond more rapidly to physiological change such as pregnancy, obese women have higher levels of ETP outwith pregnancy and lower percentage change across pregnancy.

3.4.2 Secondary findings: Pregnancy outcomes and relationship with obesity

VTE occurred in one study participant during the study and follow-up period. Given the incidence of VTE during pregnancy is around 1 in 1000, this single occurrence in our study population would be in keeping with the known incidence. However, as may have been anticipated given the link between obesity and metabolic

Chapter 3

diseases, the incidence of gestational diabetes was significantly greater in obese class II (14%, $p=0.016$) and obese class III (33.3%, $p=0.0004$) groups when compared to lean. There was no significant difference in incidence of pregnancy-induced hypertension or pre-eclampsia between the four BMI groups, despite this being found to be more common within obese women during pregnancy in many previous studies, which could relate to the small sample size for obese class II and class III women.(Bohiltea *et al.*, 2020; Ehrental *et al.*, 2011)

As has been recognised in previous studies, obesity was also associated with earlier delivery, with gestational age at delivery significantly earlier in obese class III compared to lean (37.7 vs 39.4 weeks, $p=0.022$). (Bone *et al.*, 2022) Obesity is known to be associated with both pre-term and post-dates labour, with an increased risk of induction failure and incidence of operative delivery. (Bone *et al.*, 2022; Slack *et al.*, 2019; Ellis *et al.*, 2019) The earlier delivery rate in this study population could relate to an increased incidence of elective caesarean section deliveries in obese class III compared to lean (41.7% vs 20.8%), as planned deliveries tend to be arranged around 39 weeks' gestation, however the difference in delivery modes was not found to be statistically significant between the BMI groups.

Estimated blood loss at delivery and neonatal birth weight showed no difference between the four BMI groups. However, duration of hospital inpatient stay was significantly different when the four BMI groups were compared by one-way ANOVA ($p=0.005$), with the difference between lean and obese class II ($p=0.035$) and lean and obese class III ($p=0.04$) contributing to the statistically significant results. It was suspected that this relationship may relate to the greater incidence of operative delivery for obese women within the study.

3.4.3 Limitations

The original hypothesis, that ETP would increase more in obese pregnant women, has been proven to be false. Thrombin generation in obese class III women did not correlate with BMI as it was seen to in the other obese groups, or in the other haemostasis data. Following review of the power calculations, it is most likely that the small sample size for Class II and Class III obesity groups have resulted in these anomalous results. In addition, a greater loss to follow-up than originally

Chapter 3

anticipated (consequent on first trimester miscarriage and cancellation of 28 weeks' gestation follow-up visits due to COVID-19 pandemic) has contributed to a reduced sample size at the 28 week's gestation time point.

For the purposes of power calculations, it was hypothesised that ETP would be greatest in the most obese women, Obese Class III, with an estimated 20% increase in ETP when compared to lean women. Mean ETP for each group was compared to lean ETP. ETP was 14% higher at booking in overweight/obese class I women, 30% higher in obese class II women and only 3.5% higher in obese class III women.

Sample size was considered as the most likely factor which could be influencing the results, and the power for detecting a difference between the means compared to the lean group was recalculated. The power for overweight/obese class I and obese class II were 93% and 69% respectively but given the small difference in the mean between lean and obese class III the power was only 5%, with a required sample size of 408 women necessary to detect a significant difference. (Ismail *et al.*, 2014)

Conducting a study to include this many women with BMI ≥ 40 would be challenging and potentially biased, given the frequency in the general population. The current study has a population which is an accurate reflection of the BMI distribution of the pregnant population in the West of Scotland. Given the other haemostatic parameters for this group appeared to following the trends of the other BMI groups, it may be that the small sample size for an analyses such as thrombin generation which has significant inter-individual variability has resulted in a significant skewing of the results. Future study could allow repetition on a scaled-up sample size to determine if the results were reproducible.

3.5 Summary

Taking the factor assays and thrombin generation together, these data reflect the development of a pro-coagulant state and impaired fibrinolytic capacity known to be associated with advancing gestational age. These findings are comparable to those from previous studies which provides confidence that the novel findings relating to obesity are from a representative sample of the pregnant

population. (Choi and Pai, 2002; Stirling *et al.*, 1984; Sánchez-Luceros *et al.*, 2003; Kovac *et al.*, 2015; Bagot *et al.*, 2017)

What is new, is that we have observed that obese pregnant women are subjected to a **more prolonged prothrombotic state** compared to lean women, and have proportionally greater loss of the usual compensatory mechanisms, the naturally occurring inhibitors of coagulation. This more prolonged exposure to the prothrombotic state may explain why obese pregnant women are at greater risk of VTE during pregnancy.

A thorough understanding of the underlying pathophysiology of the prothrombotic state in obese pregnancy is necessary and has not previously been fully elucidated. Taking what is known of the development of thrombosis in the obese, non-pregnant population, it may relate to dysfunctional adipose tissue and a chronic pro-inflammatory state. In the next chapter, changes in inflammatory markers and adipokines known to be dysregulated in obesity will be compared between the different BMI groups and across pregnancy and related to the haemostatic measurements of this chapter.

Chapter 4 Adipokine changes during pregnancy in women of different BMI and their relationship to markers of haemostasis

4.1 Introduction

There are several maternal metabolic adaptations observed in healthy pregnancy to support the growing fetus and prepare the mother for delivery and lactation. These include development of insulin resistance, increased lipid mobilisation, improved vascular endothelial function and the development of a prothrombotic state. In obese pregnancy, dysfunctional adipocytes and the associated low-grade inflammatory state interfere with the mechanisms involved in these adaptations, resulting in reduced metabolic capacity to respond to the pregnant state and as such puts obese women at higher risk of complications including pre-eclampsia, gestational diabetes, and thrombosis.

4.1.1 Dysfunctional, pro-inflammatory adipose tissue in obese pregnancy

In the non-pregnant obese population, dysfunctional adipose tissue has been associated with an altered adipokine profile, characterised by high leptin, low adiponectin and elevated levels of inflammatory cytokines. The adipocyte dysfunction relates to the hypertrophic expansion of adipose tissue in obesity, resulting in an increased lipolytic rate, increased recruitment of adipose tissue macrophages and their polarisation towards the pro-inflammatory M1 phenotype which is evidenced by increased levels of proinflammatory cytokines, including IL-6, TNF- α , IL-1 β , CRP and MCP-1.(Cottam *et al.*, 2004; Vilahur, Ben-Aicha and Badimon, 2017)

Gestational changes in adipokines differ between lean and obese pregnant women. Leptin was found to be persistently higher in obese during pregnancy and the postpartum period without any significant increase, in contrast to the marked gestational increase observed in lean pregnant women.(Andersson-Hall *et al.*, 2020) Similarly, adiponectin was observed to remain lower in obese women with little gestational change during pregnancy, in contrast to the steep drop-off observed in the lean pregnant group.(Andersson-Hall *et al.*, 2020)

This ‘metabolic inflexibility’ observed in the adipokines of obese women has also been demonstrated in the gestational changes of IL-6 in obese pregnancy. IL-6 was higher in obese pregnant women in the first trimester, but the increase in the lean group was greater resulting in IL-6 concentration comparable to those of the obese group by the third trimester. (Stewart *et al.*, 2007; Savard *et al.*, 2022)

Several groups have investigated gestational changes in inflammatory cytokines, comparing lean and obese pregnant women. CRP has been observed to correlate with BMI in pregnancy, but showed no significant gestational change. (Savard *et al.*, 2022; Ramsay *et al.*, 2002; Stewart *et al.*, 2007) Whereas, TNF- α showed a significant gestational increase in the same study by Stewart *et al.*, and no difference between lean and obese was observed. (Stewart *et al.*, 2007)

The apparent ‘inflexibility’ of adipokine response to the changes required by pregnancy, which may result in a greater exposure to the pro-inflammatory cytokines throughout the course of pregnancy, is one factor which may contribute to the increased risk of metabolic complications and potentially of VTE in obese pregnant women.

4.1.2 Endothelial dysfunction in obese pregnancy

The vascular endothelium is recognised to be integral to several physiological processes including vascular tone, immune cell recruitment, angiogenesis and haemostasis. (Aird, 2008; Palomo *et al.*, 2023) Endothelial cell activation is a normal physiological function, triggered by cytokines including IL-1, TNF- α and endotoxin, as an adaptation to, for example infection or stress. This process allows greater vascular permeability, expression of leucocyte adhesion molecules and increase in cytokine production to support the immune system. There is also the development of a locally prothrombotic state with increase in tissue factor and PAI-1 expression and reduction in the antithrombotic thrombomodulin. (Hunt and Jurd, 1998) However, this process can become maladaptive and considered dysfunctional in some disease states if, for example, there is prolonged stimulation. (Palomo *et al.*, 2023) Impaired vasomotor function was first demonstrated to be involved in the pathophysiology underlying atherosclerotic disease but has subsequently been identified in other disease states. (Aird, 2008)

Microvascular endothelial function is also known to be impaired in obese pregnancy. Previous work by our group has identified lower endothelium-dependent and -independent vasodilation in obese pregnant women compared to lean. (Stewart *et al.*, 2007) This impairment of endothelial function is considered to be mediated by the inflammatory cytokines, CRP, IL-6 and TNF- α , which act unopposed in obesity due to reduced plasma levels of protective factors, including HDL and adiponectin. Endothelial dysfunction is implicated in the underlying pathophysiology of metabolic complications of pregnancy, for example preeclampsia. (Esteve *et al.*, 2007; Ramsay *et al.*, 2002; Stewart *et al.*, 2007; Fialová *et al.*, 2006; Sulaiman *et al.*, 2016)

Endothelial dysfunction can be indicated by elevated concentrations of nitric oxide, but also by increased levels of the coagulation factors, PAI-1 and von Willebrand factor, which are released by endothelial cells. Leptin has been considered as a potential mediator of endothelial dysfunction in obesity in the non-pregnant population given the independent association with PAI-1. (Wang *et al.*, 2013; Vecchione *et al.*, 2002; Korda *et al.*, 2008) (Pieterse, Schutte and Schutte, 2015)

Evidence has shown that metabolic adaptations are altered in obese pregnancy, with endothelial dysfunction contributing to placental insufficiency and preeclampsia, greater insulin resistance leading to gestational diabetes mellitus and a more active inflammatory response which may contribute to many of these vascular complications of pregnancy, including VTE. Given the role leptin appears to play in endothelial dysfunction, and the association between markers of the prothrombotic state, this may suggest that one mechanism for the development of the prothrombotic state in obesity is through endothelial dysfunction, induced by hyperleptinaemia.

4.1.3 Relating markers of the procoagulant state to dysfunctional adipose tissue and chronic low-grade inflammation in obese pregnancy

In the non-pregnant population, leptin has been linked both with procoagulant factors, fibrinogen, FVIII and FIX, and with the development of VTE. (Mrozinska *et al.*, 2018; Buis *et al.*, 2020; Lu *et al.*, 2018; Pieterse, Schutte and Schutte, 2015)

Chapter 4

There seem to be several possible mechanisms by which leptin contributes to thrombotic risk: by inducing a pro-inflammatory state which is known to be a driver of coagulation, through endothelial dysfunction, by inducing peripheral blood mononuclear cell (PBMC) production of tissue-factor bearing microparticles, and through impairment of fibrinolysis.

PBMCs, when stimulated by leptin, produce increased significantly elevated levels of pro-inflammatory cytokines TNF- α and IL-6, in addition to microparticles of the procoagulant substance, tissue factor (Zarkesh-Esfahani *et al.*, 2001; Petrini *et al.*, 2016; Bucciarelli *et al.*, 2012; Rafail *et al.*, 2008)

In the non-pregnant, leptin has been associated with endothelial dysfunction, a known mediator of the procoagulant state. Due to low levels of the anti-inflammatory cytokine adiponectin in obesity, the actions of leptin on the endothelium go unopposed, and as such the high leptin:low adiponectin phenotype associated with obesity may contribute to the prothrombotic state.

In the non-pregnant obese population, studies have shown increased release of inflammatory cytokines including TNF- α and IL-6 from adipocytes and adipose tissue macrophages. (Park, Park and Yu, 2005; Weisberg *et al.*, 2003) An *in vitro* study by Szotowski *et al* found a significant increase in tissue factor production by endothelial cells in response to stimulation by TNF- α and IL-6, with demonstrable procoagulant activity with a significant increase in generation of activated Factor X in the cell supernatant only 5 hours after the cytokines were introduced. (Szotowski *et al.*, 2005) IL-6 in turn induces hepatic production of CRP, fibrinogen and serum amyloid A. CRP has been associated both with laboratory evidence of the prothrombotic state, and clinically associated with increased risk of VTE. (Olson *et al.*, 2014; Kunutsor *et al.*, 2017) (Prüller *et al.*, 2012).

How the cytokine changes in obesity interact with those which develop in pregnancy relate to the development of a prothrombotic state in obese pregnancy is not well-studied and understanding this is likely to be valuable in developing clinical strategies to reduce the risk of VTE in this population.

In order to address this gap in knowledge, this study planned to assess laboratory measures of haemostasis, and the global coagulation assay thrombin generation

which is known to act as a biomarker for VTE, alongside adipokines and inflammatory markers known to be dysregulated in obesity to ascertain the relationship between the dysfunctional adipose tissue in obese pregnancy and the prothrombotic state.

4.2 Hypothesis, Aims and Objectives

It was hypothesised that dysfunctional adipose tissue (as identified by elevated leptin and low adiponectin), alongside a chronic inflammatory state (evidenced by elevated IL-6, TNF- α and IL-1 β) would be associated with markers of the prothrombotic state: increased thrombin generation (peak, ETP and velocity index), increased procoagulant factors (FVIII and fibrinogen), reduced natural anticoagulants (protein S and TFPI), and impaired fibrinolysis (elevated PAI-1, elevated D-dimer).

4.2.1 Aims

To characterise the difference in the pattern of adipokines and inflammatory cytokines in women within each of the 4 BMI classes (lean, overweight/obese class I, obese class II and obese class III) between early pregnancy (8-12 weeks' gestation) and the third trimester (28 weeks' gestation). Adipokines known to be altered in obesity (leptin and adiponectin) and markers of the low-grade inflammatory state (TNF- α , IL-6 and IL-1 β) will be correlated with the thrombin generation and other haemostatic parameters to identify drivers of the prothrombotic state in obese pregnancy.

4.2.2 Specific Research Questions

1. What changes are observed in the adipokines leptin, adiponectin, IL-6, TNF- α and IL-1 β between the booking appointment (mean 9.9 weeks' gestation) and 28 weeks' gestation, and how do they differ between women of different BMIs during pregnancy?
2. How do the adipokines leptin, adiponectin, IL-6, TNF- α and IL-1 β relate to measures of haemostasis (fibrinogen, Factor VIII, protein S, TFPI, PAI-1 and D-dimer) and thrombin generation (lagtime, peak, ETP, time to peak, velocity index) at booking and 28 weeks' gestation.

4.3 Methods

4.3.1 Study Recruitment

This was part of the prospective longitudinal study as previously described in section 2.2.2. The study population is the same as described in the previous chapter and consisted of pregnant women, recruited within four different BMI groups (lean, overweight/obese class I, obese class II and obese class III). Blood samples were collected at two timepoints during pregnancy (booking appointment and 28 weeks' gestation). Study design and recruitment methods were as previously described in the Materials and Methods chapter (section 2.2.2). The study population characteristics were as detailed in section 3.3.1

4.3.2 Laboratory Methods

The adipokines investigated were quantified by ELISA as outlined in detail in the Materials and Methods chapter section 2.5.

In brief, blood samples were collected in 0.369M K³-EDTA and platelet-poor plasma was collected for analysis of adiponectin, leptin, TNF- α IL-1 β and IL-6 assays as dictated in the product literature. Booking and 28 week samples for each participant were assayed on the same plate alongside a standard. Due to cost restriction on the study, participant samples were assayed in singlicate.

4.3.3 Statistical Methods

All adipokine data was non-parametric and was log-transformed to fit a normal distribution prior to analysis, with the exception of IL-1 β which was unable to transform, so analysed non-parametrically. Data for booking samples (n=262) and the paired 28 weeks' gestation samples (n=173) were analysed together by two-way ANOVA with mixed effect modelling which allowed for missing values. The two-way ANOVA was able to assess for associations with BMI group, gestation and also to look for an interaction between BMI and gestational changes (BMI x time). P-values of <0.05 were accepted as statistically significant and *post hoc* testing by Tukey's test was used to identify differences between each of the subgroups. Pearson's correlation was used to determine the strength of the relationships between the cytokines and BMI at booking. As BMI was not remeasured at 28

Chapter 4

weeks' gestation, correlations between BMI and cytokine levels at 28 weeks' gestation were not undertaken as the BMI value was not valid for the time of blood sampling. IL-1 β was only detectable in 24% of booking samples and 32% of 28 week samples therefore could not be analysed in the same method as the other adipokines. Paired booking and 28 week samples were compared by Friedman rank test to assess for evidence of a significant difference. Spearman's rank was used for correlations with markers of haemostasis and other adipokines.

Simple linear regression, on log-transformed data, was used to characterise the relationship between adipokines and thrombin generation and haemostasis parameters. Multiple regression analysis was used to test if the relationship at booking was independent of BMI. BMI was not adjusted for in the 28 week regressions as it was not representative of adipose tissue mass at the time of blood sampling. The relationships which were independent of BMI were plotted with linear regression line and Pearson's correlation r value. A significance level of $p < 0.01$ was set for this chapter to allow focus on the parameters which showed the strongest relationships with measures of haemostasis.

4.4 Results

4.4.1 Leptin

4.4.1.1 Associations between leptin, BMI and gestation during pregnancy

Two-way ANOVA on log-transformed leptin data (Figure 4-1) found a significant positive correlation with BMI ($p < 0.0001$), although no individual effect of time was identified ($p = 0.13$) and the interaction between time and BMI did not reach statistical significance ($p = 0.12$).

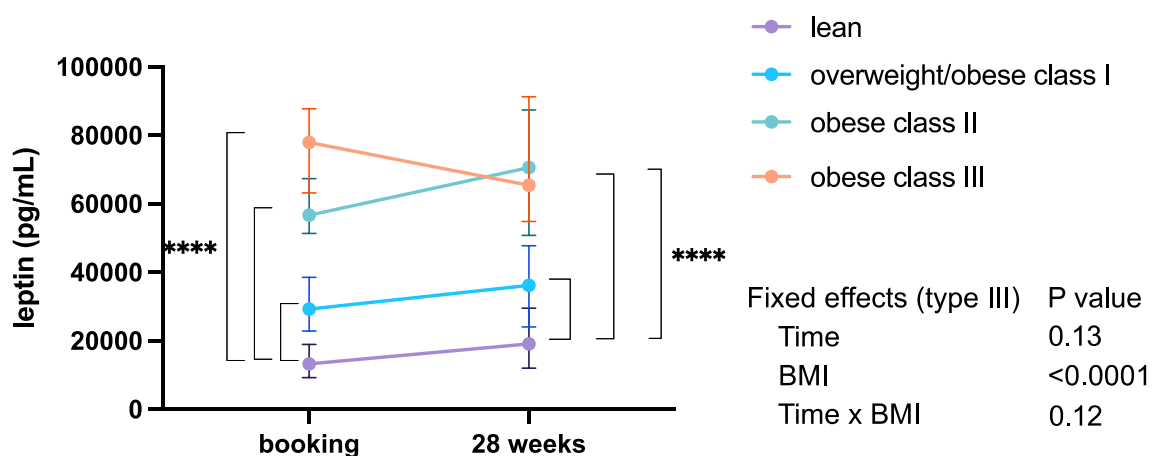


Figure 4-1 Leptin at booking and 28 weeks' gestation (raw data, median and IQR), subdivided by BMI group.

Statistical analysis by two-way ANOVA on log-transformed data, with *post hoc* analysis by Tukey's test. **** $p < 0.0001$.

Leptin correlated positively with BMI group at booking ($r = 0.77$, $p < 0.0001$, data not shown) and *post hoc* analysis with Tukey's multiple comparison test on the two-way ANOVA found a significant difference in booking leptin level existed between each of the BMI groups when compared to lean (all $p < 0.0001$). Between booking and 28 weeks' gestation, leptin increased in all groups, except for obese class III in which leptin decreased.

4.4.1.2 Relationships between leptin, other adipokines and measures of haemostasis

Leptin was positively associated with IL-6, TNF- α , fibrinogen, FVIII, protein S, TFPI, PAI-1, lagtime, peak, ETP and velocity index at booking. However following adjustment for BMI only the relationships between leptin and IL-6, fibrinogen, peak thrombin, ETP and velocity index remained robust. At 28 weeks' gestation

Chapter 4

there were significant associations with IL-6, TNF- α , fibrinogen and most thrombin generation parameters however BMI could not be accounted for as it was not recalculated at that time. The strongest association was between leptin and IL-6, which further increased by 28 weeks (adj-R²=15.65% to 21.18%).

Table 11 Leptin associations with haemostasis parameters and thrombin generation.

Simple linear and multiple regression analyses. Multiple regression analysis adds BMI to the model and overall adjusted-R² displayed. 28 week samples were not adjusted for BMI as BMI was not remeasured and as such associations would be with booking BMI and not representative of 28 week adipose mass.

	Association with leptin at booking (n=262)		Association with leptin at booking (adjusted for BMI, overall adj-R ²)		Association with leptin at 28 weeks (n=173)	
	adj-R ²	p-value	adj-R ²	p-value	adj-R ²	p-value
IL-6	15.65%	<0.001	18.54%	0.042	21.18%	<0.0001
TNF- α	4.06%	0.001	5.78%	0.721	3.75%	0.006
Fibrinogen	18.18%	<0.0001	20.08%	0.005	11.05%	<0.0001
FVIII	1.16%	0.045	0.84%	0.34	0%	0.44
Protein S	2.13%	0.010	4.04%	0.78	0.03%	0.31
TFPI	10.76%	<0.0001	13.68%	0.219	0.24%	0.24
PAI-1	6.39%	<0.0001	11.11%	0.867	0.59%	0.16
D-dimer	0%	0.828	0%	0.938	1.16%	0.085
Lagtime	6.07%	<0.0001	8.37%	0.564	2.68%	0.018
Peak	12.88%	<0.0001	13.71%	<0.0001	2.93%	0.014
ETP	13.03%	<0.0001	15.04%	<0.0001	3.03%	0.013
ttP	0.11%	0.256	1.06%	0.472	0%	0.61
Velocity index	12.54%	<0.0001	12.66%	<0.0001	3.32%	0.010

Chapter 4

Correlations between the adipokines and haemostasis measures which were independent of BMI in their relationship with leptin are shown in Figure 4-2

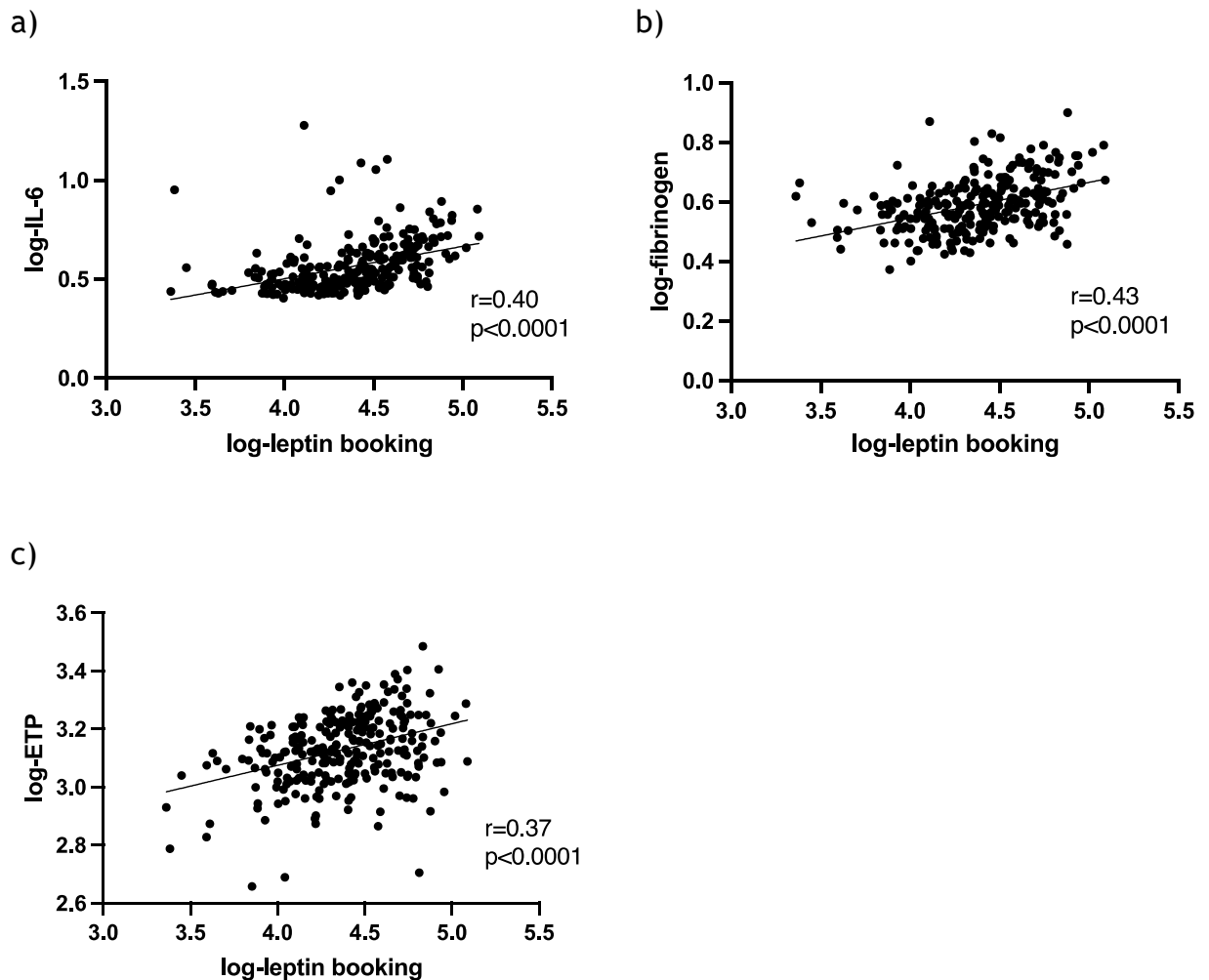


Figure 4-2 Correlations between log-leptin and a) log-IL-6 b) log-fibrinogen c) log-ETP.

Relationships between leptin and adipokines/markers of haemostasis which were independent of BMI on multiple regression analysis, as in Table 11. Pearson's correlation used to demonstrate relationship.

Considering the potential mechanisms by which leptin may be associated with the pro-coagulant state, and the BMI-independent association between IL-6 and leptin, a further regression model was tested to ascertain if the prothrombotic state associated with leptin was being mediated via a pro-inflammatory pathway.

A multiple regression model including leptin, IL-6 and BMI was tested against fibrinogen, peak, ETP and velocity index as in Table 12. These models identify that the relationship between leptin and fibrinogen is not independent of BMI or

Chapter 4

IL-6, and that IL-6 is more closely associated with fibrinogen ($\beta = 0.21$, $p < 0.0001$) than leptin ($\beta 0.053$, $p = 0.024$). Together, leptin, IL-6 and BMI were able to predict 27.81% of variance in fibrinogen levels. In contrast, the relationship between leptin and the thrombin generation parameters were independent of IL-6, suggesting the prothrombotic state is mediated via a different mechanism, unrelated to the inflammatory markers assessed in this study.

Table 12 Multiple regression modelling to ascertain the contribution of IL-6 in mediating the prothrombotic effects of leptin.

	β co-efficient	p-values	model adjusted- R^2
Fibrinogen	log-leptin 0.053	0.024	27.81%
	log-IL-6 0.21	<0.0001	
	BMI 0.0018	0.086	
Peak	log-leptin 0.20	<0.0001	13.37%
	log-IL-6 0.0016	0.98	
	BMI-0.03	0.068	
ETP	log-leptin 0.22	<0.0001	14.71%
	log-IL-6 -0.0027	0.97	
	BMI -0.0042	0.0098	
Velocity Index	log-leptin 0.24	<0.0001	12.36%
	log-IL-6 0.027	0.76	
	BMI-0.0028	0.23	

4.4.2 Adiponectin

4.4.2.1 Associations between adiponectin, BMI and gestation during pregnancy

Figure 4-3 shows there was a significant negative correlations for both time and BMI with adiponectin (both $p < 0.0001$) but no interaction between time and BMI ($p = 0.73$) suggesting the fall in adiponectin with advancing gestation was similar in each of the BMI groups.

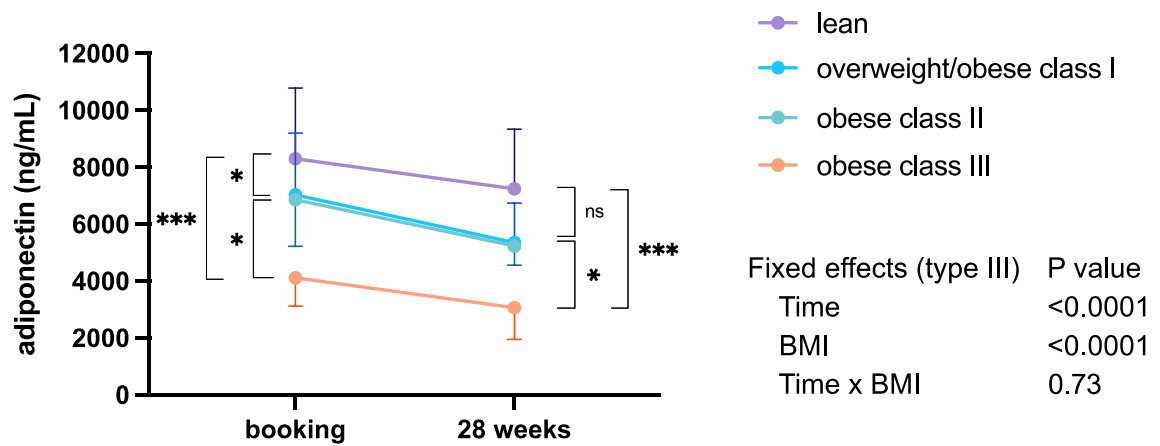


Figure 4-3 Adiponectin at booking and 28 weeks' gestation (raw data, median and IQR), subdivided by BMI group. Data was non-parametric so log-transformed prior to analysis by two-way ANOVA with mixed effects model. *Post hoc* analysis by Tukey's multiple comparison test. *** $p < 0.001$, * $p < 0.05$, ns = non-significant

Adiponectin correlated weakly with BMI at booking ($r = -0.24$, $p < 0.0001$, data not shown). Tukey's *post hoc* test found, when compared to lean, adiponectin was significantly lower in overweight/obese class I ($p = 0.015$) and obese class III ($p = 0.0003$) groups. Obese class III was also significantly lower than both overweight/obese class I ($p = 0.035$) and obese class II ($p = 0.045$) at booking. Adiponectin decreased similarly in all groups between booking and 28 weeks' gestation, and remained significantly lower in obese class III when compared to lean ($p = 0.0001$) and overweight/obese class I ($p = 0.011$) at 28 weeks' gestation.

4.4.2.2 Relationships between adiponectin, other adipokines and measures of haemostasis

Considering the relationship between adiponectin and the other adipokines (Table 13), adiponectin was negatively associated with IL-6 ($\beta -0.075$, $p = 0.019$) which remained robust once BMI was added to the regression model. The addition of BMI improved the predictive value and was able to explain 18.98% of variance in IL-6 levels but showed continued contribution of adiponectin.

Table 13 Adiponectin associations with haemostasis parameter and thrombin generation.

Simple linear and multiple regression analyses. Multiple regression analysis adds BMI to the model and overall adjusted-R² displayed. 28 week samples were not adjusted for BMI as BMI was not remeasured and as such associations would be with booking BMI and not representative of 28 week adipose mass.

	Association with adiponectin at booking (n=262)		Association with adiponectin at booking (adjusted for BMI, overall adj-R ²)		Association with adiponectin at 28 weeks (n=173)	
	adj-R ²	p-value	adj-R ²	p-value	adj-R ²	p-value
IL-6	4.99%	0.00016	18.98%	0.019	11.53%	<0.0001
TNF- α	2.58%	0.005	7.03%	0.060	2.64%	0.019
Fibrinogen	3.33%	0.002	18.44%	0.11	1.64%	0.052
FVIII	0%	0.80	0.51%	0.85	0%	0.46
Protein S	0.02%	0.30	5.48%	0.046	0%	0.77
TFPI	1.73%	0.019	13.48%	0.33	0%	0.98
PAI-1	4.03%	0.001	12.80%	0.026	1.74%	0.046
D-dimer	0%	0.69	0%	0.64	0.37%	0.20
Lagtime	6.97%	<0.0001	12.44%	0.001	2.99%	0.013
Peak	0%	0.40	3.82%	0.99	0.78%	0.13
ETP	0%	0.53	2.75	0.91	0.91%	0.11
ttP	3.88%	0.001	4.14%	0.003	0.38%	0.20
Velocity index	0%	0.38	4.81%	0.96	0.90%	0.11

Adiponectin was also negatively associated with fibrinogen (β -0.073), TFPI (β -0.10), PAI-1 (β -0.20), lagtime (β -0.068) and ttP (β -0.037) at booking but only the relationships with PAI-1, lagtime and ttP remained robust once BMI was accounted for. ttP is consequent on the duration of lagtime therefore further analyses were concentrated on lagtime. These most significant relationship between adiponectin and IL-6, PAI-1 and lagtime are depicted in Figure 4-4.

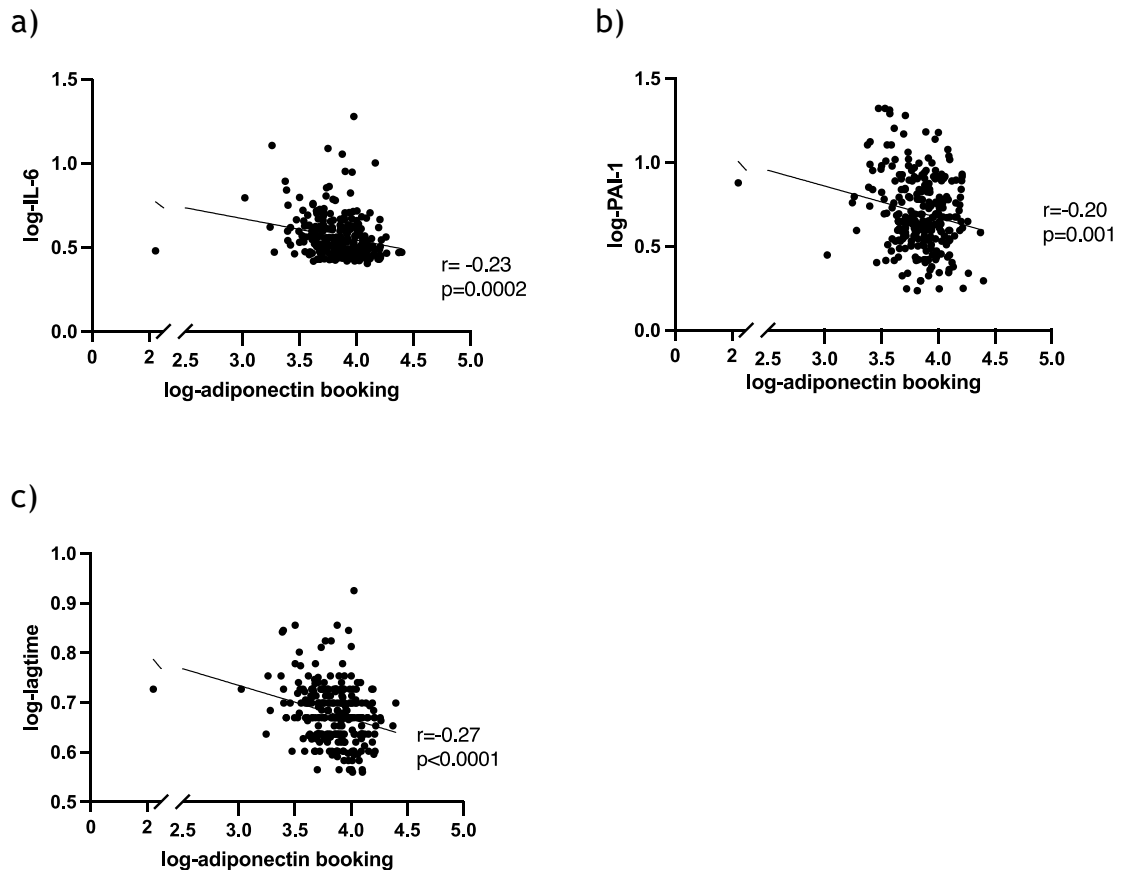


Figure 4-4 Correlations between log-adiponectin and a) log-IL-6 b) log-PAI-1 c) log-lagtime.

Relationships between adiponectin and adipokines/markers of haemostasis which were independent of BMI on multiple regression analysis, as in Table 13. Pearson's correlation used to demonstrate relationship.

Lagtime is known to be affected by high levels of TFPI, but the weak association between adiponectin and TFPI suggests this may not explain the relationship between adiponectin and lagtime here. To confirm, multiple regression analysis was undertaken which found adiponectin (β -0.047, $p=0.001$), TFPI (β 0.12, $p<0.001$) and BMI (β 0.0011, $p=0.03$) to all be independently associated with lagtime and able to explain 21.61% of variance in lagtime.

4.4.3 IL-6

4.4.3.1 Associations between IL-6, BMI and gestation during pregnancy

As seen in Figure 4-5, there was a significant positive relationship between BMI and IL-6 concentration ($p<0.0001$), but no association with time ($p=0.42$) as IL-6 levels remained stable between booking and 28 week's gestation. No interaction between time and BMI was observed ($p=0.73$). IL-6 was significantly higher at both

Chapter 4

timepoints for all overweight and obese groups when compared to lean women. (lean vs obese class III $p < 0.0001$ at both timepoints)

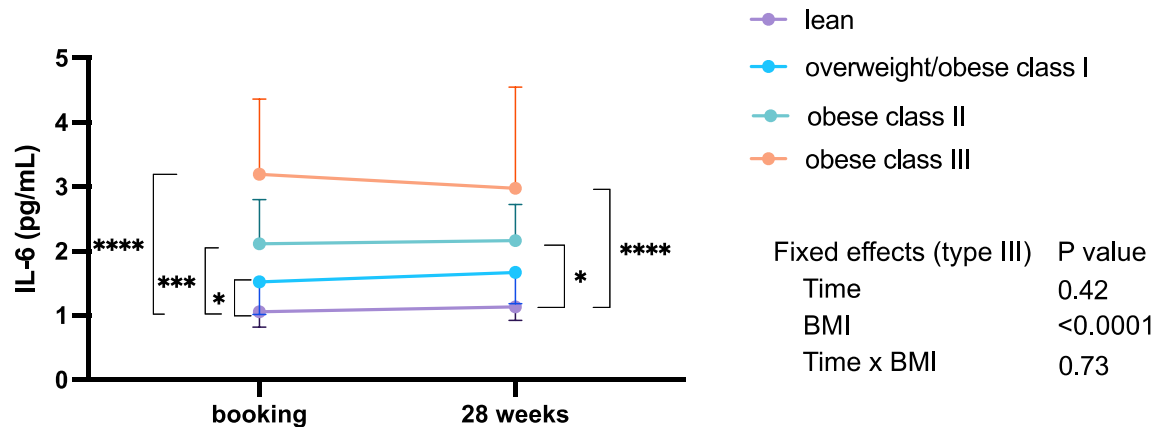


Figure 4-5 IL-6 at booking and 28 weeks' gestation (raw data, median and IQR), subdivided by BMI group.

Data was non-parametric so log-transformed prior to analysis by two-way ANOVA with mixed effects modelling. *Post hoc* testing by Tukey's multiple comparison test. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns=non-significant.

4.4.3.2 Relationships between IL-6, other adipokines and measures of haemostasis

IL-6 was positively associated with TNF- α , fibrinogen, FVIII, Protein S, PAI-1, lagtime, peak, ttP and velocity index. The associations with PAI-1 and protein S were no longer robust after adjustment for BMI. Together, IL-6 and BMI were able to predict 28.03% of variance in TNF- α , 26.44% of variance in fibrinogen, 17.22% of variance in TFPI levels and 18.23% of variance in lagtime duration.

Table 14 IL-6 associations with haemostasis parameter and thrombin generation.

Simple linear and multiple regression analyses. Multiple regression analysis adds BMI to the model and overall adjusted-R² displayed. 28 week samples were not adjusted for BMI as BMI was not remeasured and as such associations would be with booking BMI and not representative of 28 week adipose mass.

	Association with IL-6 at booking (n=262)		Association with IL-6 at booking (adjusted for BMI, overall adj-R ²)		Association with IL-6 at 28 weeks (n=173)	
	adj-R ²	p-value	adj-R ²	p-value	adj-R ²	p-value
TNF-α	17.81%	<0.0001	28.03%	<0.0001	16.35%	<0.0001
Fibrinogen	19.84%	<0.0001	26.44%	<0.0001	17.46%	<0.0001
FVIII	3.25%	0.002	3.00%	0.01	5.98%	0.001
Protein S	2.67%	0.005	4.85%	0.13	3.53%	0.008
TFPI	11.11%	<0.0001	17.22%	0.0005	4.57%	0.003
PAI-1	4.61%	0.0003	11.87%	0.14	0%	0.62
D-dimer	0%	0.71	0%	0.97	0%	0.82
Lagtime	16.59%	<0.0001	18.23%	<0.0001	11.45%	<0.0001
Peak	1.23%	0.04	3.99%	0.51	2.32%	0.026
ETP	0.96%	0.062	2.91%	0.51	2.40%	0.024
ttP	6.31%	<0.0001	6.0%	0.0002	3.27%	0.01
Velocity index	1.80%	0.017	5.10%	0.38	2.99%	0.013

Chapter 4

The most significant relationships with IL-6 were with TNF- α , fibrinogen, TFPI and lagtime as correlated in Figure 4-6. The association between IL-6 and prolonged lagtime may again be mediated by TFPI as discussed in 4.4.2.2. However, multiple regression analysis found that IL-6 (β 0.14, $p < 0.0001$) and TFPI (β 0.17, $p < 0.0001$) were both independently associated with lagtime and able to predict 24.41% of variance in lagtime.

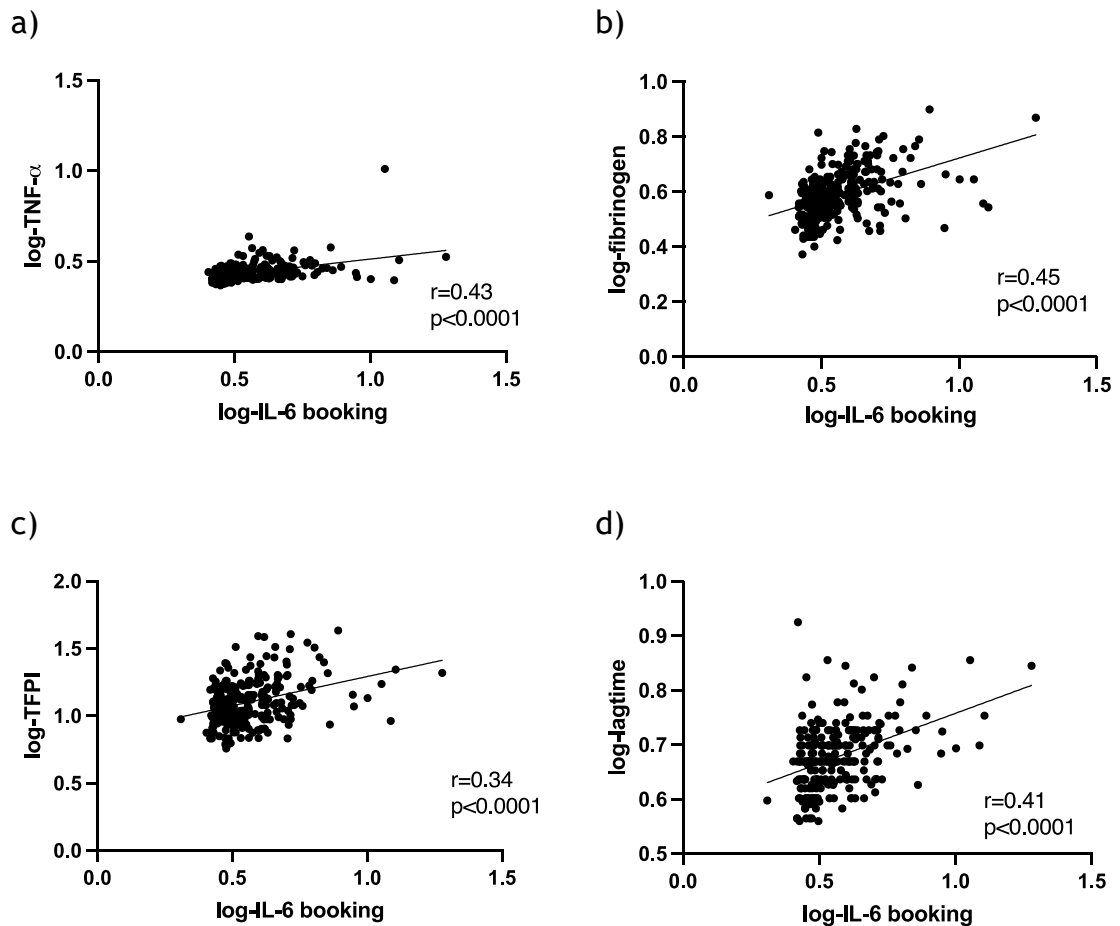


Figure 4-6 Correlations between log-IL-6 and a) log-TNF- α b) log-fibrinogen c) log-TFPI d) log-lagtime.

Relationships between adiponectin and adipokines/markers of haemostasis which were independent of BMI on multiple regression analysis, as in Table 14. Pearson's correlation used to demonstrate relationship.

4.4.4 TNF- α

4.4.4.1 Associations between TNF- α , BMI and gestation during pregnancy

Figure 4-7 shows significant positive associations for both time and BMI with TNF- α ($p < 0.0001$ for both), but no interaction between time x BMI ($p = 0.89$). On *post-hoc* analysis, Obese Class III women had significantly higher TNF- α levels than each of the other groups at both study timepoints ($P < 0.0001$ at booking, $p < 0.001$ at 28 weeks' gestation) which was likely responsible for the apparent significant relationship with BMI in this mixed effects model.

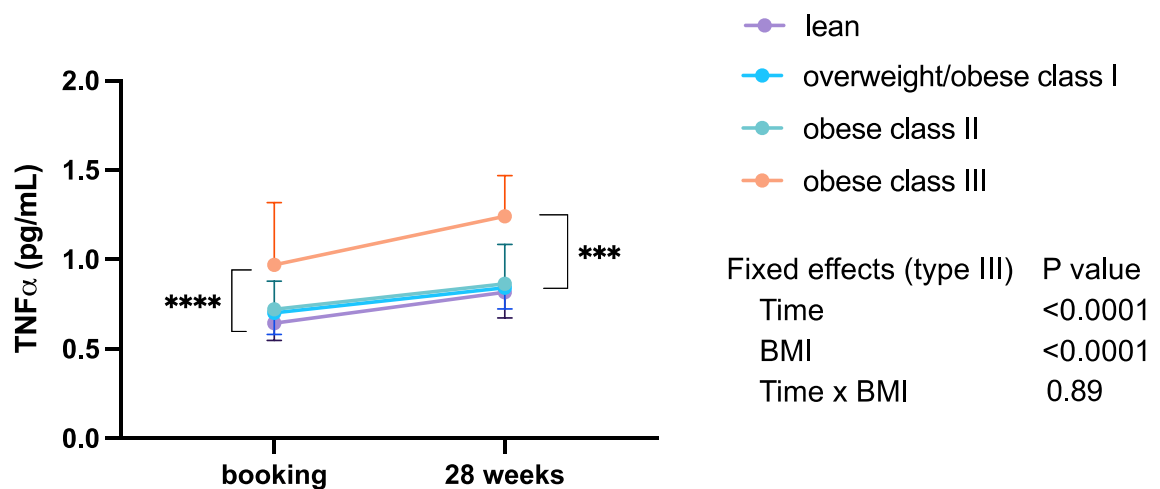


Figure 4-7 TNF- α at booking and 28 weeks' gestation (raw data, median and IQR), subdivided by BMI group.

Data was non-parametric so log-transformed prior to analysis by two-way ANOVA with mixed effects model with *post hoc* Tukey's multiple comparisons testing. *** $p < 0.001$, **** $p < 0.0001$.

4.4.4.2 Relationships between TNF- α , other adipokines and measures of haemostasis

At booking, TNF- α was weakly associated with an increase in fibrinogen, TFPI, PAI-1, lagtime and ttP. The relationships with fibrinogen, TFPI and PAI-1 were no longer significant once BMI was taken into account. Significant associations with peak and ETP were only present once BMI was added to the model suggesting these relationships were due to BMI rather than TNF- α . Only lagtime and ttP were weakly associated at 28 weeks' gestation.

Table 15 TNF- α associations with haemostasis parameter and thrombin generation.

Simple linear and multiple regression analyses. Multiple regression analysis adds BMI to the model and overall adjusted-R² displayed. 28 week samples were not adjusted for BMI as BMI was not remeasured and as such associations would be with booking BMI and not representative of 28 week adipose mass.

	Association with TNF- α at booking (n=262)		Association with TNF- α booking (adjusted for BMI, overall adj-R ²)		Association with TNF- α at 28 weeks (n=173)	
	adj-R ²	p-value	adj-R ²	p-value	adj-R ²	p-value
Fibrinogen	3.47%	0.001	18.19%	0.11	1.03%	0.099
FVIII	0.65%	0.10	1.06%	0.22	0.36%	0.21
Protein S	1.13%	0.047	4.23%	0.24	0%	0.42
TFPI	3.17%	0.002	14.17%	0.096	0%	0.35
PAI-1	2.57%	0.006	12.06%	0.14	0%	0.36
D-dimer	0.42%	0.15	0.15	0.13	0%	0.98
Lagtime	7.33%	<0.0001	12.44%	0.0004	4.83%	0.002
Peak	0%	0.74	4.62%	0.020	0%	0.54
ETP	0%	0.40	3.99%	0.088	0.41%	0.19
ttP	3.92%	0.001	4.08%	0.003	2.29%	0.027
Velocity index	0%	0.95	5.34%	0.33	0%	0.84

4.4.5 IL-1 β

4.4.5.1 Associations between IL-1 β , BMI and gestation during pregnancy

Due to the very low concentrations of IL-1 β detectable, raw data with median and interquartile ranges were plotted. As the median values for most of the BMI group were zero, analysis by two-way ANOVA was not undertaken. Raw data is plotted in Figure 4-8 and was compared using Friedman rank test. No statistically significant difference existed between paired booking and 28 week IL-1 β concentrations.

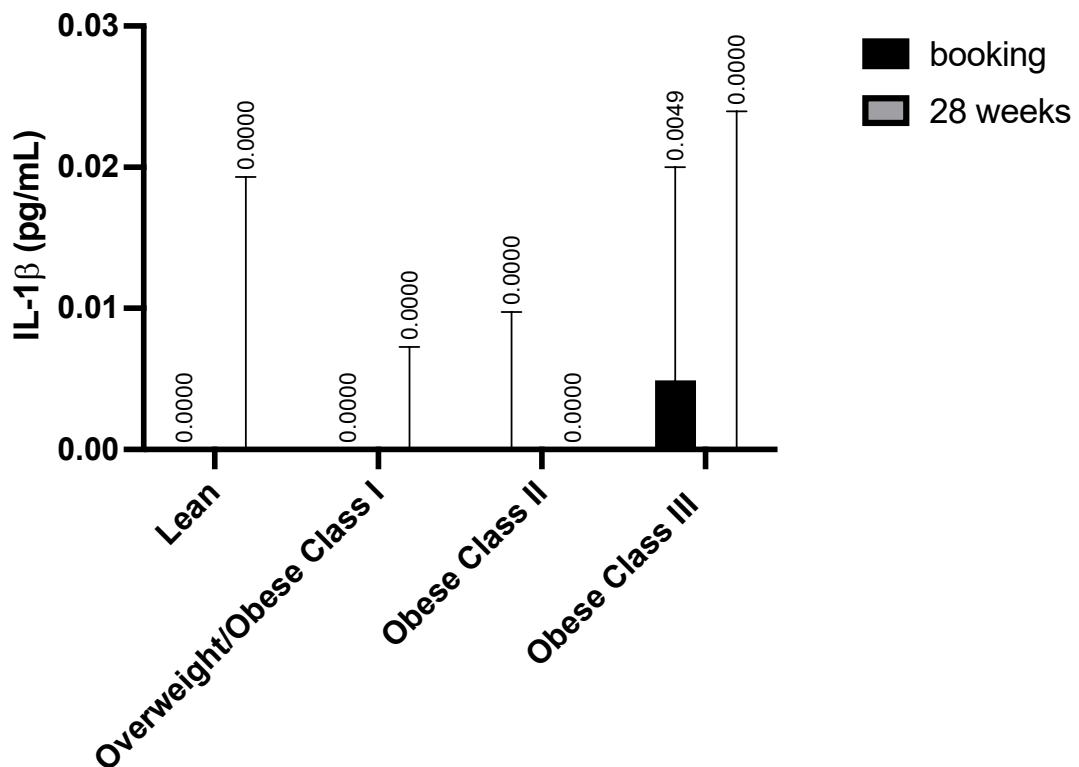


Figure 4-8 IL-1 β at booking and 28 weeks' gestation, subdivided by BMI group.

Data plotted as median (annotated value) and interquartile range (depicted by error bars)

4.4.5.2 Relationships between IL-1 β , other adipokines and measures of haemostasis

IL-1 β was correlated with other adipokines and haemostasis parameters at booking and 28 weeks' gestation using Spearman's rank test. Correlation co-efficients and p-values are displayed in Table 16. The most significant positive correlations at booking were with other inflammatory cytokines, TNF- α ($r=0.27$, $p<0.0001$) and IL-6 ($r=0.15$, $p=0.025$). IL-1 β was only significantly associated with 2 haemostasis parameters at booking: positively with TFPI ($r=0.18$, $p=0.0081$) and negatively with ETP ($r=-0.15$, $p=0.027$). By 28 weeks' gestation, the moderately positive correlations between IL-1 β and TNF- α and IL-6 persisted, the correlations with TFPI only just reached statistical significance ($r=0.15$, $p=0.047$) and ETP was no longer associated.

Table 16 IL-1 β associations with haemostasis parameter and thrombin generation. Spearman rank correlations and p-values at booking and 28 weeks' gestation.

	Association with IL-1 β at booking (n=216)		Association with IL-1 β at 28 weeks (n=173)	
	correlation	p-value	correlation	p-value
leptin	0.062	0.36	-0.02	0.80
adiponectin	-0.13	0.053	-0.11	0.17
IL-6	0.15	0.025	0.25	0.001
TNF- α	0.27	<0.0001	0.23	0.003
Fibrinogen	0.015	0.83	-0.05	0.51
FVIII	0.008	0.91	0.12	0.11
Protein S	0.023	0.74	0.098	0.20
TFPI	0.18	0.0081	0.15	0.047
PAI-1	0.005	0.95	0.03	0.69
D-dimer	0.086	0.21	0.023	0.77
Lagtime	0.053	0.44	0.094	0.22
Peak	-0.12	0.073	0.018	0.82
ETP	-0.15	0.027	-0.004	0.96
ttP	0.053	0.44	0.075	0.33
Velocity index	-0.11	0.11	0.018	0.81

4.5 Discussion

4.5.1 Main findings

The main hypothesis for this chapter was that adipokines, known to be characteristic of dysfunctional adipose tissue, and inflammatory cytokines, would be associated with evidence of the prothrombotic state. This chapter has proven the hypothesis correct with increased BMI associated with elevated leptin, low adiponectin and increased IL-6 and TNF- α , all characteristic of dysfunctional adipose tissue and a pro-inflammatory state. In turn, each of the measured adipokines and inflammatory cytokines (excluding IL-1 β) has been associated with at least one haemostatic parameter, with leptin and IL-6 most closely related to measures of the prothrombotic state - ETP (leptin), fibrinogen (leptin and IL-6) and TFPI (IL-6).

4.5.2 Evidence of dysfunctional adipose tissue in obese pregnancy

BMI group was positively correlated with leptin and negatively with adiponectin at booking, displaying the high leptin:adiponectin ratio characteristic of dysfunctional adipose tissue.(Frühbeck *et al.*, 2019). There was also evidence of a pro-inflammatory state, with positive associations between BMI group and both IL-6 and TNF- α at booking, although the increase in TNF- α was only evident in the most obese women (obese class III). Previous work by our group found no effect of BMI on TNF- α , however in the study by Stewart *et al* the mean BMI in the obese group was 34.2 ($\pm$ 4.5) so potentially the study population in that cohort did not allow for identification of the relationship with obese class III women.(Stewart *et al.*, 2007)

In the current study, there were gestational changes in the adipokines as previously observed in other studies. This study identified that pregnancy resulted in a fall in adiponectin as has been seen previously. However, although leptin did increase with gestation in lean, overweight/obese class I and obese class II groups, in keeping with findings of previous studies ,this was not found to be statistically significant likely due to the slight decrease in leptin in the most obese group.(Andersson-Hall *et al.*, 2020) Leptin was noted to show no gestational increase in obese pregnant women in the study by Andersson-Hall *et al* , but a significant increase in the healthy weight group, which would fit with our findings and provides further evidence of the ‘metabolic inflexibility’ observed in previous studies of obese pregnant women.(Stewart *et al.*, 2007) TNF- α increased with gestation, but unlike in previous studies we did not see a gestational rise in IL-6.(Stewart *et al.*, 2007; Savard *et al.*, 2022)

4.5.3 Relationship between adipokines and haemostasis

Due to BMI not being recalculated at 28 weeks’ gestation, and the relationship with obesity being most prominent at booking, only relationships between adipokines and markers of haemostasis at booking will be discussed.

Booking leptin and IL-6 were the cytokines most closely related to coagulation. Independently of BMI, leptin was strongly and positively associated with fibrinogen (adj-R²=20.08%, p=0.005), and thrombin generation (Peak adj-R²=13.71, ETP adj-

Chapter 4

$R^2=15.04\%$, velocity index adj- $R^2=12.66\%$, $p<0.0001$ for all). *Ex vivo* administration of leptin in rat studies has been identified to be associated with a concentration-dependent increase in ETP (Lagrange 2017) suggesting that leptin has a direct role in increasing thrombin generation. There are several potential mechanisms by which leptin may increase thrombin generation: by inducing PBMC production of tissue-factor bearing microparticles, by increasing platelet aggregation. by inducing endothelial dysfunction and by inducing a pro-inflammatory state which is known to be a driver of the prothrombotic state.(Vecchione *et al.*, 2002; Zarkesh-Esfahani *et al.*, 2001; Rafail *et al.*, 2008) The association between IL-6, leptin, fibrinogen and ETP demonstrated by this study supports the hypothesis that the pro-inflammatory state in obese pregnant women is contributing to the prothrombotic state,

To investigate whether the associations of leptin with fibrinogen and thrombin generation were driven via IL-6 pro-inflammatory pathways, a further multiple regression model was built to add IL-6 alongside leptin and BMI. These models identified that 27.81% of variance in fibrinogen could be explained by leptin and IL-6, with leptin more strongly associated which suggests that increases in fibrinogen due to leptin may be mediated via IL-6. This would fit with previous studies which have shown IL-6 induces hepatic synthesis of fibrinogen. However, interestingly the same pattern was not observed in the regression analysis for markers of thrombin generation. Peak, ETP and velocity index all remained positively associated with leptin independently of both BMI and IL-6, suggesting the procoagulant effect of leptin as measured by thrombin generation is IL-6 independent. Whilst the increased fibrinogen will undoubtedly be contributing to increased thrombin generation, these data suggest that there may be further mechanisms by which thrombin generation is increased, for example by leptin's direct actions on stimulating tissue factor production from monocytes. There was no association between leptin and markers of endothelial dysfunction, TFPI and PAI-1 observed in this study, however more specific assessment of endothelial function would be required to exclude this as a contributing factor in thrombin generation.

The loss of the anti-inflammatory functions of adiponectin in obesity may result in increased inflammation, as demonstrated in the negative associations between adiponectin, IL-6 and TNF- α , or endothelial dysfunction, as indicated by weak

Chapter 4

negative associations with TFPI and PAI-1. Lagtime and ttP both correlated negatively with adiponectin. Lagtime, and by inference ttP, is known to be sensitive to elevated TFPI therefore it was considered whether the relationship between adiponectin and lagtime could be mediated by TFPI.(de Laat-Kremers *et al.*, 2022) However, the relationship between lagtime and adiponectin remained robust once TFPI was adjusted for, and the predictive value of the regression model improved suggesting that the relationship between adiponectin and lagtime was independent of TFPI. Our data does show a weak association between low adiponectin and the pro-inflammatory state and evidence of endothelial dysfunction, but does not clearly show that these are the mechanisms by which coagulation is affected.

In addition to the association with fibrinogen, IL-6 was also positively associated with TNF- α , FVIII, TFPI, lagtime and ttP and PAI-1, although the PAI-1 relationship was not independent of BMI. Many of these observations have been well-recognised in the literature: TNF- α is known to induce production of IL-6, which may explain the close correlation observed, whilst IL-6 is known to induce hepatic synthesis of FVIII as an acute phase protein.(Stirling, Hannant and Ludlam, 1998; Cottam *et al.*, 2004) As discussed previously, elevated TFPI can cause a prolongation of lagtime *in vitro* therefore the prolongation of lagtime and ttP could relate to the effects of IL-6 on TFPI.(de Laat-Kremers *et al.*, 2022) To the best of our knowledge, IL-6 is not known to directly influence the release of TFPI as it has been observed to with the acute phase proteins, but it could represent endothelial dysfunction. The microvascular endothelium is thought to be the predominant source of TFPI, with production also occurring in megakaryocytes and storage of TFPI in platelets. TFPI release from the endothelium is triggered by thrombin and it has been considered as a marker of endothelial dysfunction due to its association with subclinical atherosclerosis.(Morange *et al.*, 2001; Mitchell *et al.*, 2009; Lupu *et al.*, 1995) Elevated TFPI has been demonstrated in obesity before and potentially could reflect IL-6-induced endothelial dysfunction as IL-6 has been identified to increase endothelial permeability.(Desai *et al.*, 2002; Vambergue *et al.*, 2001)

4.5.4 Summary

Taking together the findings of this chapter with existing knowledge in the literature, the proposed involvement of adipokines in the development of the prothrombotic state in obese pregnancy are illustrated in Figure 4-9.

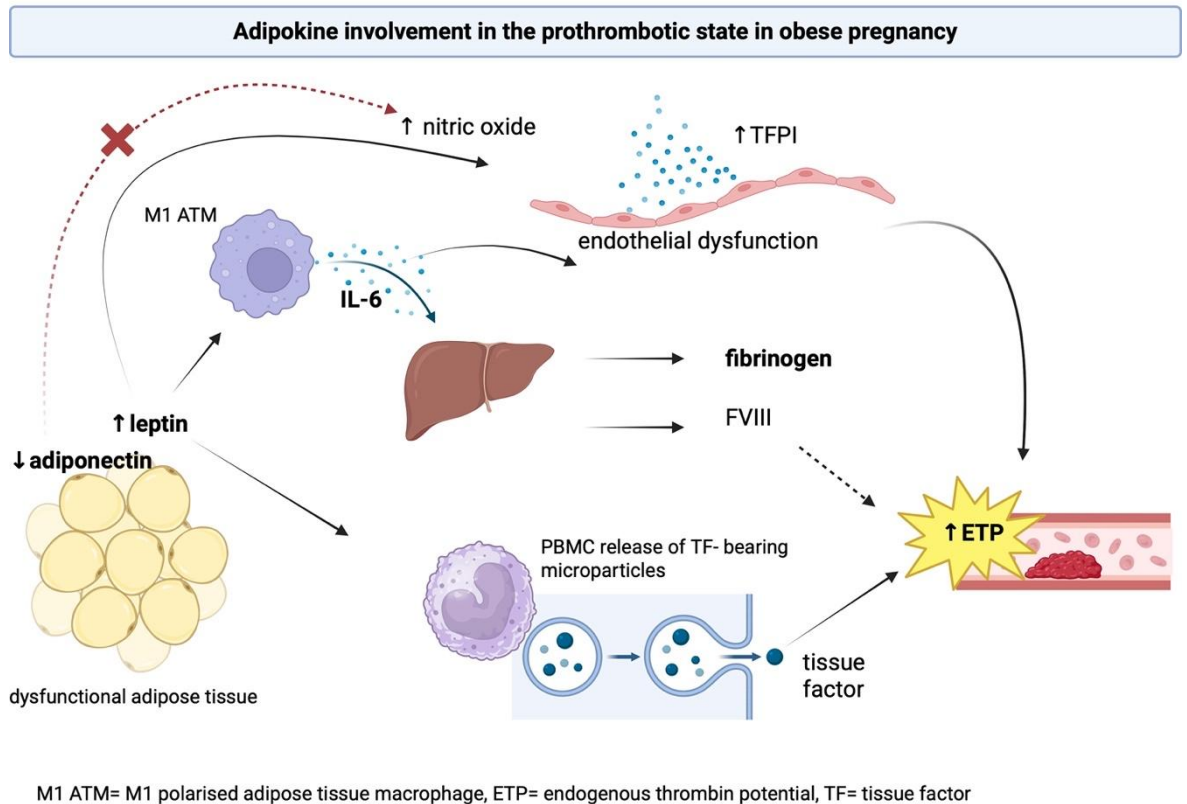


Figure 4-9 Adipokine involvement in the prothrombotic state in obese pregnancy.

Dysfunctional adipocytes produce increased leptin and reduced adiponectin whilst recruiting and polarising adipose tissue macrophages towards the pro-inflammatory M1 phenotype. Leptin induces endothelial dysfunction (as recognised by increased TFPI) via high levels of nitric oxide, the usual inhibition of which is impaired due to reduced adiponectin. Leptin also stimulates release of TF-bearing microparticles by monocytes. Pro-inflammatory ATMs release IL-6 which contributes to endothelial dysfunction and also induces hepatic production of FVIII and fibrinogen. The procoagulant contributions of FVIII, tissue factor and potentially the effects of endothelial dysfunction are measurable by increased ETP, whilst fibrinogen also contributes to the thrombotic state through mechanisms not assessable by thrombin generation. Created in <https://BioRender.com>

Dysfunctional adipose tissue produces increased leptin and reduced adiponectin. Leptin is known to promote endothelial-dependent vasodilation via nitric oxide, causing endothelial dysfunction, which can be indicated by elevated TFPI. (Wang *et al.*, 2013; Vecchione *et al.*, 2002; Korda *et al.*, 2008; Mitchell *et al.*, 2009; Morange *et al.*, 2001) The reduced adiponectin levels are therefore unable to counteract the inflammatory and oxidative state and the resultant endothelial dysfunction predisposes to thrombosis. The effects of leptin on primary

haemostasis were not assessed in this study, but existing literature indicates that leptin induces platelet aggregation. (Corsonello *et al.*, 2003; Dellas *et al.*, 2008)

The infiltration of dysfunctional adipose tissue by increased numbers of adipose tissue macrophages which are then polarised toward the pro-inflammatory M1 phenotype results in increased IL-6. IL-6 contributes to endothelial dysfunction by increasing endothelial permeability but also induces hepatic synthesis of FVIII and fibrinogen. (Desai *et al.*, 2002) The increase in these factors can be demonstrated by increased thrombin generation, however FVIII and fibrinogen do not fully explain the increase in ETP seen in obese pregnant women at booking. It is proposed that a contribution from the tissue factor pathway, via leptin stimulation of PBMC release of tissue factor-bearing microparticles may be contributing to increased ETP.

4.5.5 Limitations

Due to budgetary limitations, only a small selection of adipokines were able to be analysed in this study. IL-1 β has been observed to be poorly predictive of the prothrombotic state, and was only detectable in a minority of study participants. IL-1 β is a cytokine elevated in inflammation but given the chronic low-grade inflammation involved in obesity this may not be sufficient to cause a significant measurable increase. If this study were to be repeated, then measurement of a highly sensitive CRP would be better value for money as a general marker of inflammation which is known to be associated with thrombotic risk. (Prüller *et al.*, 2012)

As BMI was not recalculated based on current weight at 28 weeks' gestation, BMI could not be used in the analysis of 28 weeks' gestation adipokines as the booking BMI would not have been representative of the current adipose tissue mass at time of blood sampling. Therefore, it is unknown whether the changes in adipokines by 28 weeks' gestation relate specifically to pregnancy or to gestational weight gain.

It is known that BMI provides a poor assessment of adipose tissue mass and does not provide insight to adipose tissue function which is likely of greater consequence when considering the adipokine profile during pregnancy. The next chapter will investigate the application of alternative measurements of adipose

Chapter 4

tissue distribution and their relationship to adipokines and markers of the prothrombotic state.

Chapter 5 Adipose tissue distribution and the prothrombotic state in pregnancy

5.1 Background

Adipose tissue function is closely linked to adipose tissue distribution, with differing adipokine profiles between subcutaneous and visceral adipose tissue. Obesity is known to be an important cardiometabolic risk factor, however BMI has been shown to be an indiscriminate predictor for the metabolic complications of obesity, particularly in women (Gallagher *et al.*, 1996). Instead, adipose tissue distribution and depot, as measured by waist:hip ratio and by quantifying SAT and VAT, have been proven to be more predictive of cardiometabolic disease. (Brook *et al.*, 2001; Borch *et al.*, 2009; Després, 2001) VAT has been identified in epidemiological studies to be associated with cardiovascular mortality, and has been associated with ectopic fat deposition. Both VAT and ectopic fat, for example epicardial fat or liver steatosis, are associated with the release of pro-inflammatory cytokines, insulin resistance, oxidative stress and increased vascular resistance. (Lim and Meigs, 2014; van den Munckhof *et al.*, 2024) Extrapolating what is known in the non-pregnant female population would lead us to expect that similar risk factors in the pregnant population are contributing to a greater thrombotic potential.

5.1.1 Influence of body shape on thrombotic risk outwith pregnancy

Using *ex vivo* thrombin generation as a biomarker of the prothrombotic state, the link between adipose distribution and thrombotic risk has been characterised by several groups, including Beijers *et al* who identified a positive association between BMI, central adiposity (i.e waist circumference) and thrombin generation parameters (lag time, peak and endogenous thrombin potential). (Wang *et al.*, 2023; Beijers *et al.*, 2010; Prüller *et al.*, 2012) Furthermore, these correlations identified by Beijers *et al* and similar by Peverill *et al* were only present in women, providing additional evidence of the differing consequence of adipose tissue distribution between the sexes. In women in particular, central adiposity is associated with a greater risk of cardiometabolic and thrombotic disease. (Beijers *et al.*, 2010) (Peverill *et al.*, 2007) Central adiposity reflects greater waist

circumference, and although waist circumference encompasses both subcutaneous (SAT) and visceral adipose tissue (VAT), higher waist circumference implies greater VAT thickness. (Pouliot *et al.*, 1994) What is not known, is whether subcutaneous adipose tissue (SAT) or visceral adipose tissue (VAT) carries the greater risk of thrombosis. (Prüller *et al.*, 2012)

5.1.2 Influence of adipose tissue depots on cardiometabolic and thromboembolic risk

In the obese population, VAT was associated with increased thrombin generation, independently of BMI. (Beijers *et al.*, 2010; Chitongo *et al.*, 2017) Similarly, when Pou *et al* compared VAT with SAT, VAT was also found to be more robustly associated with inflammatory markers, known mediators of a prothrombotic state (Charo and Taubman, 2004; Pou *et al.*, 2007) In this same study, SAT was only found to be associated independently of BMI with fibrinogen, but in an autopsy-based study by Ogren *et al*, presence of thromboembolic disease was more closely associated with SAT rather than VAT. (Pou *et al.*, 2007; Ogren *et al.*, 2005)

In pregnancy, it is known that VAT increases with advancing gestation while SAT falls. (Selovic, Sarac and Missoni, 2016; Straughen, Trudeau and Misra, 2013; Dutra *et al.*, 2014). VAT has been more closely associated with the development of pre-eclampsia, gestational diabetes and correlated with blood pressure, triglycerides, HOMA-IR and cholesterol. (Kansu-Celik *et al.*, 2018; Kennedy *et al.*, 2016) (Chitongo *et al.*, 2017; Huda *et al.*, 2017; Martin *et al.*, 2009; Trivett, Lees and Freeman, 2021) (Bartha *et al.*, 2007; Martin *et al.*, 2009)

Taking these data together, in the non-pregnant population, central adiposity appears to be more closely associated with thrombotic risk, and whilst it may be assumed this indicates a greater proportion of VAT, there is no clear consensus on whether SAT or VAT is the better predictor of thrombotic risk. However in both the pregnant and non-pregnant populations, there were more robust association between VAT and the pro-inflammatory and cardiometabolic complications.

The investigation in this chapter will aim to identify the adipose tissue distribution, assessed both by waist and hip circumference and abdominal SAT and

VAT thickness, which is most closely associated with inflammatory adipokines and markers of the prothrombotic state.

5.2 Aims and Objectives

5.2.1 Hypothesis

With the evidence of the importance of adipose tissue distribution in the underlying pathophysiology of thrombosis in non-pregnant individuals, and with the evidence that VAT is associated with dysfunctional metabolic and pro-inflammatory states in pregnancy, it was hypothesised that VAT is similarly associated with risk of thrombosis, as assessed by the biomarker thrombin generation, in obese pregnant women.

5.2.2 Aims

To compare abdominal adipose tissue distribution, measured by waist and hip circumference at booking and ultrasound scan at 12-17 weeks' gestation, in women within each of the 4 BMI classes (lean, overweight/obese class I, obese class II and obese class III) and investigate the relationship between adipose tissue distribution, and the adipokine and haemostasis profile measured at booking.

5.2.3 Specific Research Questions

1. What are the relationships between waist circumference (cm), hip circumference (cm), waist:hip ratio (WHR) and each of the following:

- BMI/WHO obesity classification (lean, overweight/obese class I, obese class II and obese class III)
- adipokines (leptin, adiponectin, IL-6, TNF- α and IL-1 β)
- measures of haemostasis (PT, APTT, FVIII, fibrinogen, TFPI, protein S, D-dimer, PAI-1)
- biomarker of venous thromboembolism, thrombin generation

2. What are the relationships between subcutaneous adipose tissue (SAT) thickness and visceral adipose tissue (VAT) thickness and each of the following:

- BMI/WHO obesity classification (lean, overweight/obese class I, obese class II and obese class III)
- adipokines (leptin, adiponectin, IL-6, TNF- α and IL-1 β)
- measures of haemostasis (PT, APTT, FVIII, fibrinogen, TFPI, protein S, D-dimer, PAI-1)
- biomarker of venous thromboembolism, thrombin generation

5.3 Methods

5.3.1 Study recruitment

Study participants were recruited as previously described in the Materials and Methods chapter as part of the IPOP study. All study participants had basic anthropometric measurements obtained at the booking appointment, and all were provided with an appointment to attend for an ultrasound scan between 12-17 weeks' gestation for the measurement of abdominal subcutaneous and visceral fat thickness (as previously described). As the attendance for the study ultrasound scan was outwith routine antenatal care, a £20 shopping voucher, or travel expenses up to the value of £20, were offered as compensation for the study participant's time.

Due to loss to follow-up of 39%, the study population for this anthropometry 'sub-study' consisted of 160 women in total, subdivided into the same four BMI groups. Lean (n=70), overweight/obese class I (n=72), obese class II (n=10), obese class III (n=8). The women included in this part of the study were recruited within the main IPOP study recruitment process, as detailed in the Materials and Methods chapter and as outlined in the consort diagram in Figure 5-1. All women included in the anthropometry 'sub-study' had booking blood samples obtained at study visit 1, and had measurement of height, weight, waist and hip circumference collected at the booking visit in the same way as all other study participants. The mean gestational age at the ultrasound study visit was 14.6 weeks.

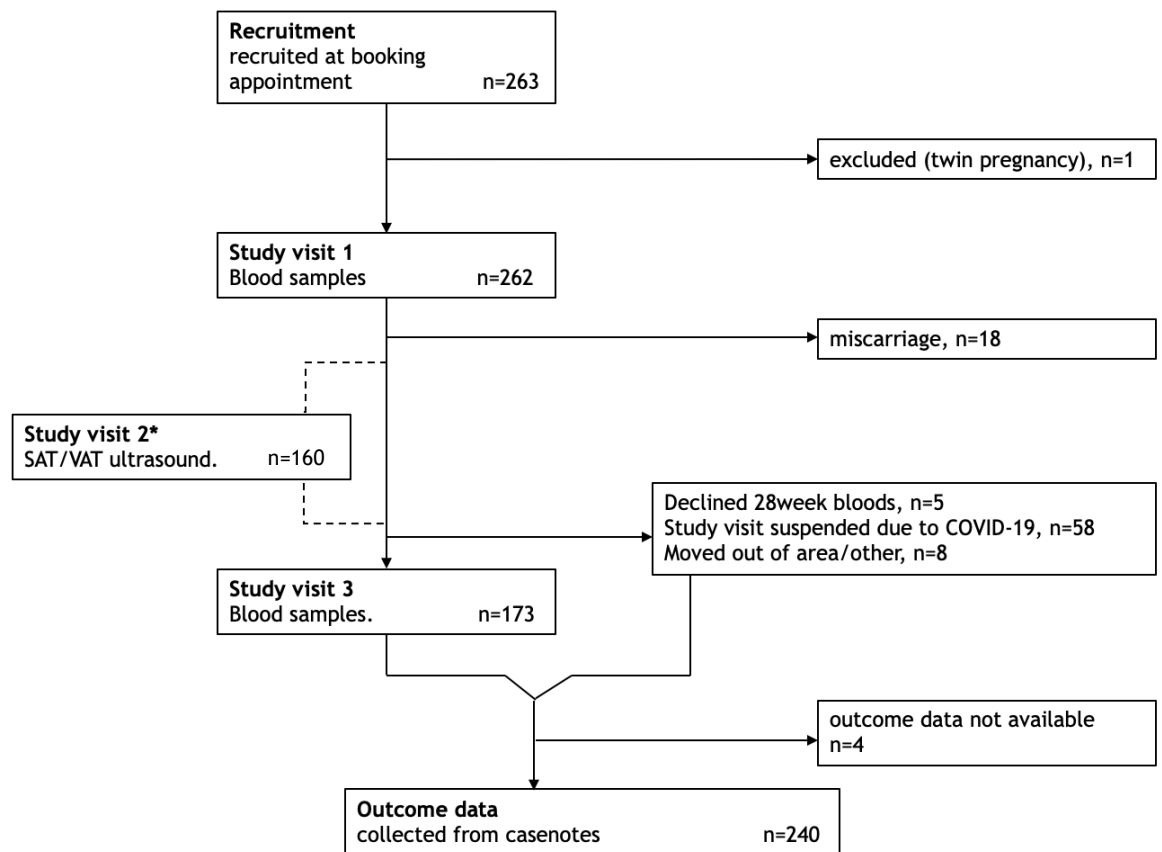


Figure 5-1 Consort diagram of IPOP study recruitment.

*denotes anthropometry sub-study population. Scan undertaken at mean gestation of 14.6 weeks.

5.3.2 Anthropometric methods

5.3.2.1 Body mass index (BMI)

Body mass index was calculated as weight (kg) divided by squared-height (m), and participants were grouped by BMI according to the WHO BMI classification system, as outlined previously in the Materials and Methods chapter.

5.3.2.2 Waist and hip circumference

Waist circumference was measured, in centimetres, at the narrowest point between the lower ribs and the hips. Hip circumference was measured, in centimetres, at the widest point around the buttocks and hips.

Chapter 5

Waist:hip ratio (WHR) was calculated by dividing waist circumference by hip circumference. The World Health organisation (WHO) have previously defined WHR <0.85 as 'healthy' and WHR >0.85 as 'unhealthy'. (WHO, 2008)

5.3.2.3 Ultrasound scan of abdominal SAT and VAT

Study participants attended ultrasound scan between 12-17 weeks' gestation (mean 14.6 weeks' gestation). Subcutaneous adipose tissue (SAT) measurement in millimetres was obtained, in triplicate, from the surface of the skin to the outer border of the rectus abdominis muscle (as in Figure 5-2). Visceral adipose tissue (VAT) thickness was measured in millimetres, and in triplicate, from the inner border of the rectus abdominis muscle to the anterior wall of the abdominal aorta and the level of the linea alba. An average of each of the measurements was taken to minimise the impact of intra-operator variability.

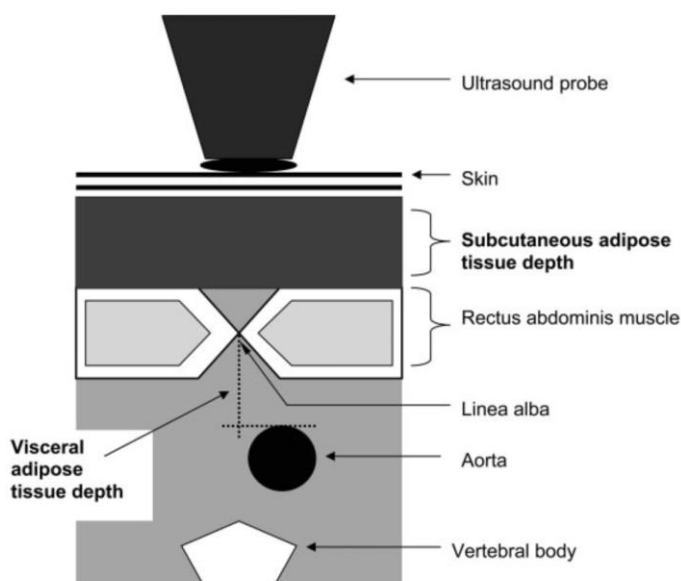


Figure 5-2 Anatomical landmarks on cross-sectional view for measurement of abdominal visceral and subcutaneous adipose tissue.

Original diagram and measurement technique by Martin et al. (Martin et al., 2009)

5.3.3 Statistical methods

To account for multiple comparisons and reduce type I error, for the conclusions of this chapter a significance level of $p < 0.001$ was used to allow focus on only the most significant relationships and therefore those which may have the most clinically significant value.

Chapter 5

Anthropometric measurements, waist and hip circumference, waist:hip ratio, SAT and VAT thickness were first related to BMI as a continuous variable to assess for correlation and then to BMI groups to ascertain the pattern of adipose tissue distribution within each of the different BMI groups.

SAT and VAT measurements were next related to the adipokine and haemostasis data to identify whether adipose tissue distribution was associated with any of these parameters. SAT and VAT measurements were not normally distributed and therefore were log-transformed prior to analyses. To identify the nature of the relationship between these anthropometric measurements and the markers of haemostasis and adipokines, the data were analysed both as continuous variables using Pearson's correlation, and as quartiles (SAT and VAT quartiles divisions as in Table 17) using one-way ANOVA to identify any threshold patterns associated with fat distribution. Where a significant threshold effect was identified, the quartile-based analyses have been displayed alongside the correlation analyses and statistics.

Table 17 SAT and VAT quartile divisions

Quartile	SAT thickness (mm), n	VAT thickness (mm), n
Q1	5.0-11.9, n=44	2.0-7.9, n=52
Q2	12.0-16.9, n=42	8.0-10.9, n=35
Q3	17.0-24.79, n=34	11.0-19.79, n=33
Q4	24.8-47, n=40	19.8-65, n=40

Waist and hip circumference were non-parametric and were log-transformed prior to analysis. Waist and hip circumference were correlated with adipokines and measures of haemostasis, including thrombin generation. Any significant correlations were further analysed by multiple regression analysis as described above, with SAT and VAT measurements included in the model to identify whether the relationship with the adipokines were independent of the other measurements of body fat distribution.

When a significant correlation or significant difference between the groups was identified (defined as $p < 0.001$), multiple regression analysis was used to identify if the variance was attributable to the anthropometric measurement or if there was collinearity with BMI group, waist or hip circumference, SAT or VAT. Four

Chapter 5

different models were used as outlined in Table 18, with combinations of these models being used to identify which factors were most predictive of the parameter assessed. For the final regression model, stepwise multiple regression analysis using all anthropometric parameters was used to avoid interpretation bias. For this stepwise model an α value of 0.15 was used to enter or remove a term.

Table 18 Adipose tissue distribution multiple regression model definitions

Multiple regression model	Variables assessed within model
Model 1	BMI
Model 2	SAT and VAT
Model 3	waist circumference and hip circumference
Model 4	waist:hip ratio
Final model	Stepwise using combination of above variables ($\alpha=0.15$ to enter/remove)

5.4 Results

5.4.1 Comparison of anthropomorphic measurements of adipose tissue distribution and body mass index

5.4.1.1 Waist and hip circumference

Figure 5-3 illustrates that BMI strongly correlated with both waist circumference (adj $R^2=0.88$, $p<0.0001$) and hip circumference (adj $R^2=0.88$, $p<0.0001$) at booking. The relationship between BMI and hip circumference was linear, however the relationship between BMI and waist circumference appeared to reach a plateau when BMI reached >40 suggesting that once BMI is >40 additional adipose tissue deposition was predominantly in the hip region.

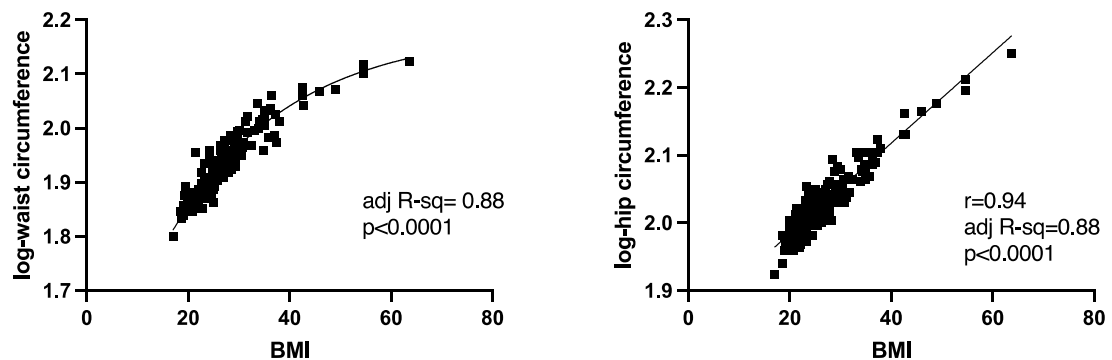


Figure 5-3 Correlation between BMI and waist circumference; correlation between BMI and hip circumference.

Waist and hip measurements were non-parametric so log-transformed prior to correlation with BMI. Pearson's r value and p value displayed for waist circumference, non-linear regression line of best fit and adjusted R^2 displayed for hip circumference.

Waist:hip ratio (WHR) was correlated with BMI in Figure 5-4. The relationship was non-linear with an exponential plateau line providing the best fit line (although adjusted R^2 only 0.21, $p < 0.0001$). This fits with what we observed in the individual waist and hip circumference plots, that although total body mass is increasing, there is a plateau in waist circumference and an ongoing increase in hip circumference so no increase in WHR.

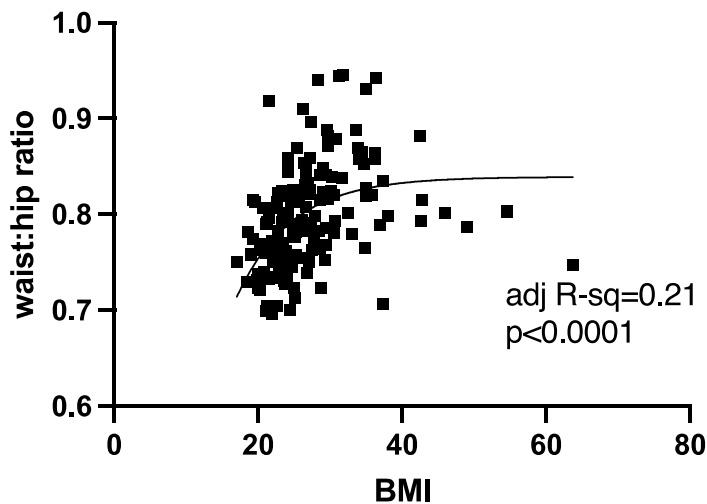


Figure 5-4 Correlation between waist:hip ratio (WHR) and BMI.

WHR data was normally distributed. Non-linear regression line displayed along with adjusted R^2 value.

When subdivided into the WHO 'healthy' (< 0.85) and 'unhealthy' (> 0.85) WHR, the majority of the study group (84.4%) had WHR within the WHO 'healthy' range, < 0.85 , despite 56.3% of the study group having a BMI > 25 which would be deemed unhealthy (data not shown).

5.4.1.2 Subcutaneous (SAT) and Visceral Adipose Tissue (VAT)

There was a strong relationship between BMI and VAT (adj $R^2=0.46$, $p<0.001$) and an even stronger relationship between BMI and SAT (adj $R^2=0.70$, $p<0.0001$). A plateau in both the SAT and VAT curves was observed in those with BMI >40 making an exponential non-linear regression line the line of best-fit for both variables (Figure 5-5). The plateau was more marked with the SAT measurements, whereas VAT did continue to increase with BMI but at an attenuated rate, in contrast to the linear relationship observed between hip circumference and BMI. This may suggest that excess adipose tissue is deposited in the hip region in the most obese.

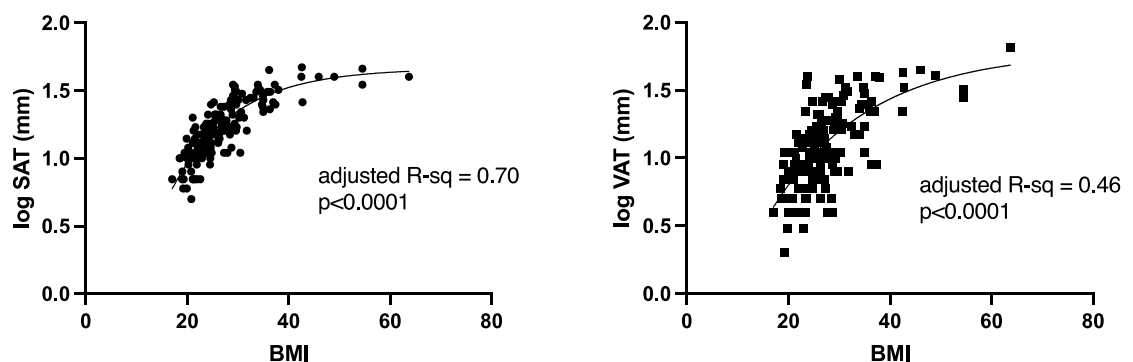


Figure 5-5 Correlation between BMI and (a) SAT and (b) VAT

Non-linear regression used to plot line of best fit (goodness of fit denoted by adjusted R^2 value).

Chapter 5

To further investigate the distribution of SAT and VAT within BMI groups, SAT and VAT quartiles were plotted with BMI groups to determine the frequency of each SAT and VAT quartile within different BMI groups (Figure 5-6).

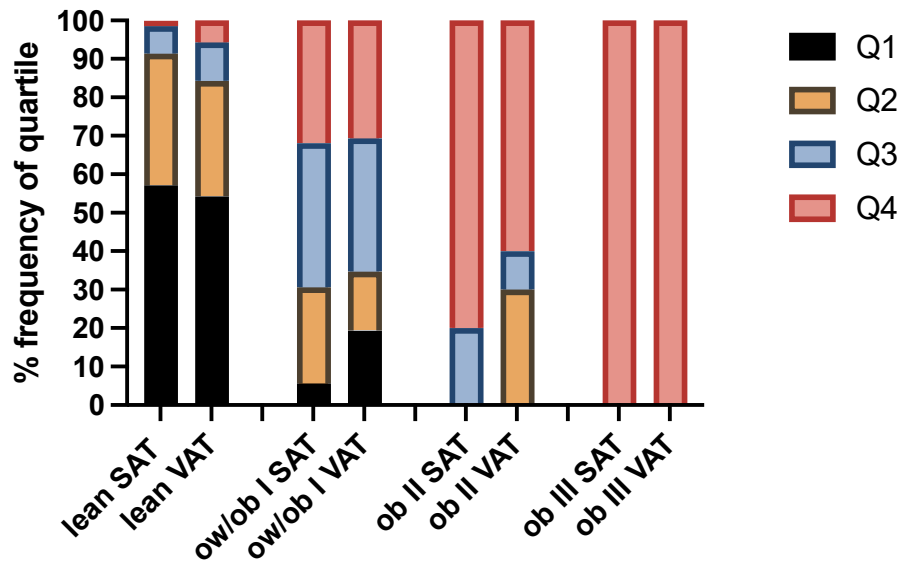


Figure 5-6 Stacked bar chart depicting percentage frequency of each SAT and VAT quartile within each BMI group.

Lean participants had no discrepancy between SAT and VAT quartiles, with similar frequencies within each quartile and the majority of participants had SAT and VAT thickness within the lowest quartile. Within the Overweight/Obese Class I group, the majority of participants had SAT and VAT thickness in Q3 or Q4. Obese Class II and Class III groups both had the majority of participants within Q4 thickness of SAT and VAT.

Chapter 5

To assess the relationship between SAT and VAT, the frequency of VAT quartile within each SAT quartile were plotted in Figure 5-7 and log-transformed SAT and VAT data were plotted against each other (Figure 5-8). These charts show that as SAT thickness increases, VAT thickness also increases and displays an exponential relationship when higher thickness of SAT are reached.

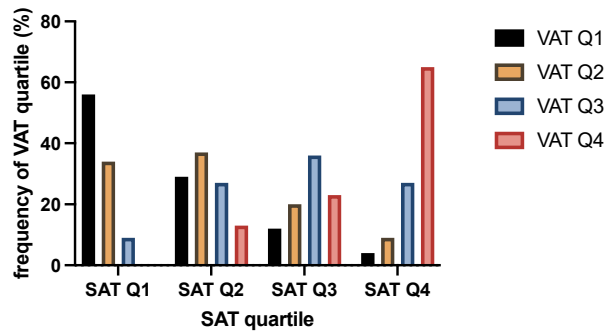


Figure 5-7 Frequency of VAT quartile associated with SAT quartiles

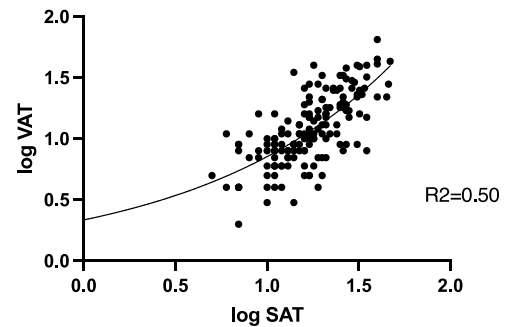


Figure 5-8 Non-linear regression log SAT vs log VAT.

To ascertain if there was a difference in fat distribution with increasing obesity, SAT:VAT ratio within each of the four BMI groups was compared by one-way ANOVA, with no significant difference identified, $p=0.32$ (data not shown).

5.5 Relationship between adipokines and measurements of adipose tissue distribution

5.5.1 Leptin and adipose tissue distribution

5.5.1.1 SAT and VAT

Leptin correlated strongly with both SAT ($r=0.84$, $p<0.0001$) and VAT ($r=0.66$, $p<0.0001$) thickness (Figure 5-9). When analysed within quartiles (data not shown), there was no evidence of a threshold effect but a continuous increase in leptin with each quartile.

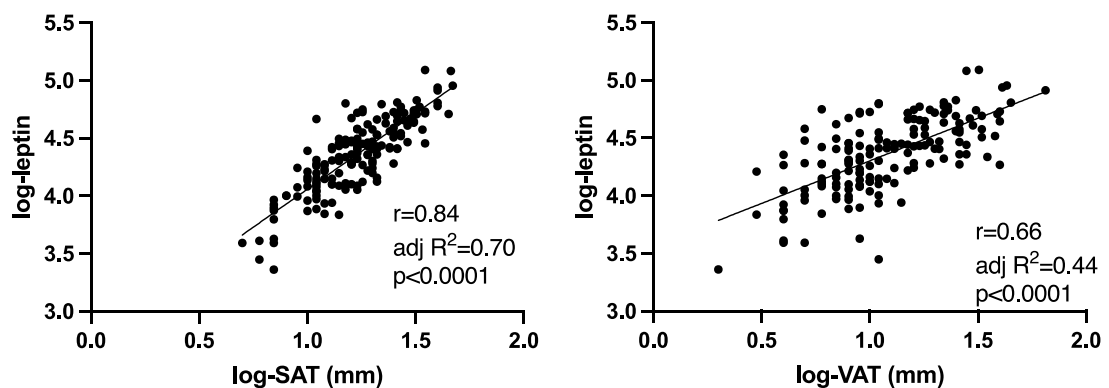


Figure 5-9 Correlation between log-leptin and (a) log-SAT and (b) log-VAT.

Pearson r value and p -values displayed. Linear regression line plotted and goodness of fit denoted by adjusted R^2 value.

5.5.1.2 Waist and hip circumference

Figure 5-10 shows a positive, curvilinear relationship between leptin and both waist circumference ($\text{adj}R^2=0.68$, $p<0.0001$) and hip circumference ($\text{adj}R^2=0.68$, $p<0.0001$), reflecting that despite further increases in both waist and hip circumference there was a plateau in leptin concentration.

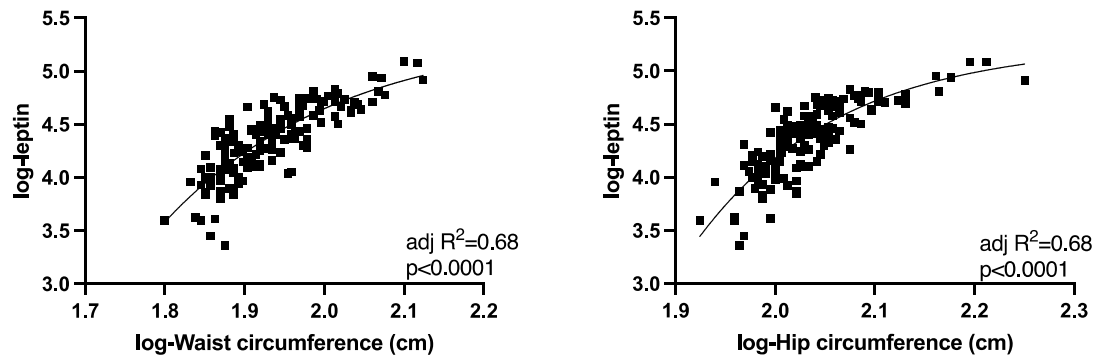


Figure 5-10 Correlation between log-leptin and (a) waist circumference and (b) hip circumference.

Non-linear regression on log-transformed data. The line of best fit was a curvilinear line, with goodness of fit denoted by adjusted R^2 value.

Waist:hip ratio was also moderately correlated with leptin ($r=0.36$, $p<0.0001$, data not shown). When participants were divided by healthy waist:hip ratio (<0.85) and unhealthy waist:hip ratio (>0.85), mean leptin was higher in the unhealthy group ($p=0.006$), but did not reach the statistical threshold set for this study.

5.5.1.3 Multiple regression analysis leptin and anthropometry

As leptin was previously demonstrated in Chapter 4 to be closely correlated with BMI, multiple regression analysis was used to ascertain if SAT, hip and waist circumference and waist:hip ratio were predictive of leptin, independently of BMI. The multiple regression models were built up as illustrated in Table 19.

Table 19 Leptin and adipose tissue distribution multiple regression model construction.

Leptin was analysed within four individual models (numbered 1-4) as previously defined in statistical methods section (page 160). Each model was combined with model 1 (BMI) to ascertain if the anthropometric measurements were correlated with leptin independently of BMI. A final stepwise model ($\alpha = 0.15$ to keep/remove) was used to find the variables which were the strongest predictors of leptin concentration.

Regression Model	β co-efficient	p-value	adjusted R^2
model 1 (BMI)	0.035	<0.0001	58.86%
model 2 (SAT and VAT)	log-SAT 1.16 log-VAT 0.16	<0.0001 0.019	71.18%
model 3 (WC, HC)	log-wc 2.50 log-hc 2.34	<0.0001 0.0003	67.22%
model 4 (WHR)	2.09	<0.0001	11.75%
model 1 + model 2	BMI 0.013 log-SAT 0.89 log-VAT 0.085	<0.0001 <0.0001 0.193	74.18%
model 1 + model 3	BMI -0.005 log-wc 2.71 log-hc 2.78	0.46 <0.0001 0.0017	67.12%
model 1 + model 4	BMI 0.034 WHR 0.69	<0.0001 0.03	59.83%
final model (stepwise)	log-hc 2.28 log-SAT 1.23 log-VAT -0.32 SAT:VAT -0.11	<0.0001 <0.0001 0.092 0.025	77.00%

The relationship between leptin and SAT was independent of BMI, whereas the relationship with VAT was no longer significant when BMI was added to the model (model 1+2) suggesting collinearity between BMI and VAT thickness. The positive

correlation between leptin and both waist circumference and hip circumference were independent of BMI (model 1+3).

The best fitting regression model (devised via stepwise method) identified that the combination of SAT, VAT, hip circumference and SAT:VAT ratio were able to explain 77% of variance (adjusted $R^2=77.0\%$, $p<0.0001$). Log-SAT and log-hip circumference both positively predicted log-leptin, whilst log-VAT and SAT:VAT ratio negatively predicted log-leptin. These relationships were independent of BMI which was removed from the model due to p value >0.15 .

5.5.2 Adiponectin and adipose tissue distribution

5.5.2.1 SAT and VAT

Figure 5-11 shows correlation between log-adiponectin, log-SAT and log-VAT. Adiponectin was moderately negatively correlated with both SAT ($r=-0.33$, $p<0.0001$) and VAT ($r=-0.30$, $p<0.0001$).

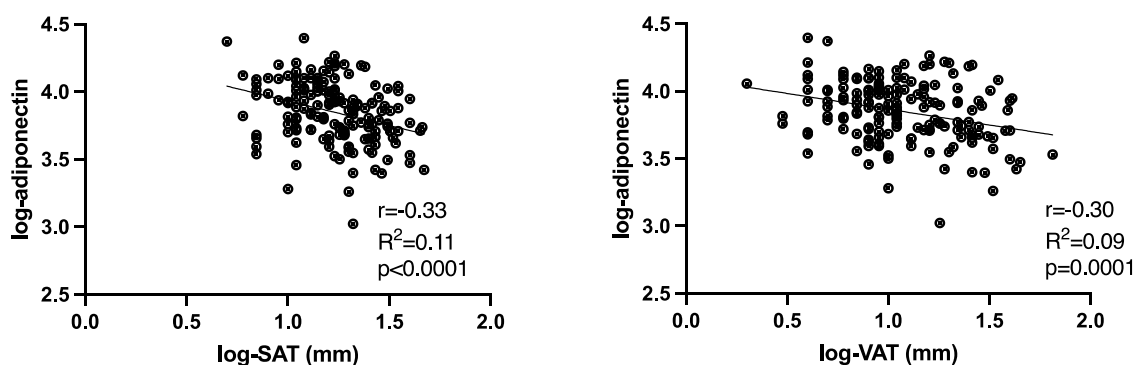


Figure 5-11 Correlation between adiponectin and (a) SAT, and (b) VAT.

Linear regression and correlation analysis performed. Pearson r value and p values displayed. Linear regression line and goodness of fit denoted by R^2 value.

When adiponectin was analysed within SAT and VAT quartiles (data not shown), adiponectin concentration was observed to follow a linear reduction with each increase in VAT quartile. However, there appeared to be a threshold effect within SAT quartiles, with a fall in adiponectin when SAT thickness reached the third quartile (SAT thickness $>17\text{mm}$, Q2 vs Q3 $p=0.0013$), although this was just outwith the threshold set for statistical significance for this study.

5.5.2.2 Waist and hip circumference

Log-waist circumference and log-adiponectin were significantly inversely correlated ($r=0.29$, $p=0.0003$, data not shown). A weaker association was seen between log-hip circumference and log adiponectin ($r=0.18$, $p=0.026$, data not shown). The strongest relationship was between waist:hip ratio and log-adiponectin ($r=-0.31$, $p<0.0001$).

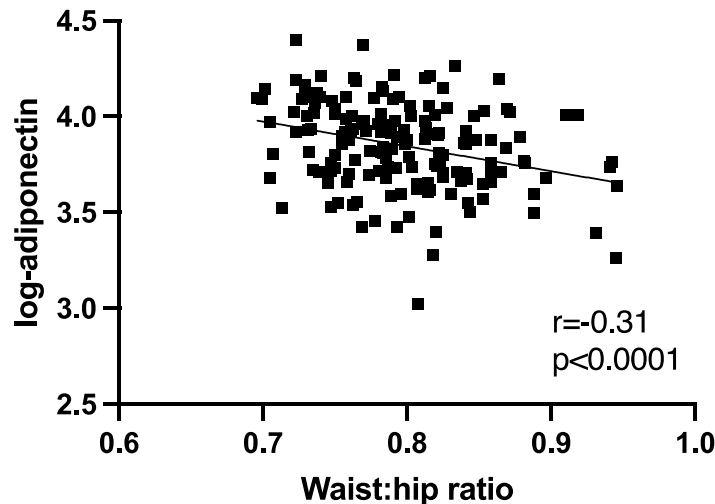


Figure 5-12 Correlation between log-adiponectin and waist:hip ratio

Linear regression and correlation analyses performed on log-transformed data. Pearson r value and p values displayed.

When waist:hip ratio was subdivided into healthy and unhealthy groups (data not shown), mean adiponectin was significantly lower in the unhealthy group ($p=0.041$), however didn't meet statistical significance level set for this study.

5.5.2.3 Multiple regression analysis adiponectin and anthropometry

To ascertain if the moderate correlations between adiponectin, SAT and VAT were due to collinearity with BMI, a multiple regression analysis was performed.

Chapter 5

Table 20 Adiponectin and adipose tissue distribution multiple regression model construction.

Adiponectin was analysed within four individual models (numbered 1-4) as previously defined in statistical methods section (page 160). Each model was combined with model 1 (BMI) to ascertain if the anthropometric measurements were correlated with adiponectin independently of BMI. A final stepwise model ($\alpha=0.15$ to keep/remove) was used to find the variables which were the strongest predictors of adiponectin.

Regression Model	β co-efficient	p-value	adjusted R^2
model 1 (BMI)	-0.0078	0.002	5.07%
model 2 (SAT and VAT)	log-SAT -0.26 log-VAT -0.10	0.028 0.22	10.39%
model 3 (WC, HC)	log-WC -2.25 log-HC 1.67	0.0003 0.027	9.81%
model 4 (WHR)	-1.29	<0.0001	8.75%
model 1 + model 2	BMI 0.0022 log-SAT -0.31 log-VAT -0.12	0.57 0.033 0.183	10.0%
model 1 + model 3	BMI -0.01 log-WC -1.86 log-HC 2.50	0.24 0.007 0.016	10.04%
model 1 +model 4	BMI -0.005 WHR -1.083	0.05 0.002	10.39%
final model (stepwise)	log-SAT -0.26 WHR -0.842	0.005 0.018	12.69%

Waist:hip ratio was one of the parameters most closely associated with adiponectin ($\beta = -1.29$, $p < 0.0001$). This negative relationship fits with the individual findings of waist and hip circumference that the ‘pear shape’ of lower WHR is associated with higher concentrations of the anti-inflammatory and vasculoprotective adipokine, adiponectin.

The negative relationship between log-SAT and adiponectin was found to be independent of BMI ($p = 0.033$). This relationship was weak and was only able to explain 10% of variance in adiponectin at booking. A stepwise model looking at all of the anthropometric variables only found log-SAT and waist:hip ratio, and not BMI, to be negatively predictive of adiponectin concentrations, yet this model only explained 12.69% of variance and neither of the predictors achieved the level

of significance set for the study, suggesting anthropometric measurements are poor predictors of plasma adiponectin levels.

5.5.3 TNF- α and adipose tissue distribution

5.5.3.1 SAT and VAT

Figure 5-13 depicts the correlations between TNF- α and both SAT and VAT which whilst correlated did not reach the level of statistical significance set for this study. SAT correlated moderately positively with TNF- α ($r=0.24$, $p=0.0026$) and VAT also correlated positively but the relationship was weaker ($r=0.17$, $p=0.029$). No significant difference in TNF- α was found when analysed within SAT and VAT quartiles.

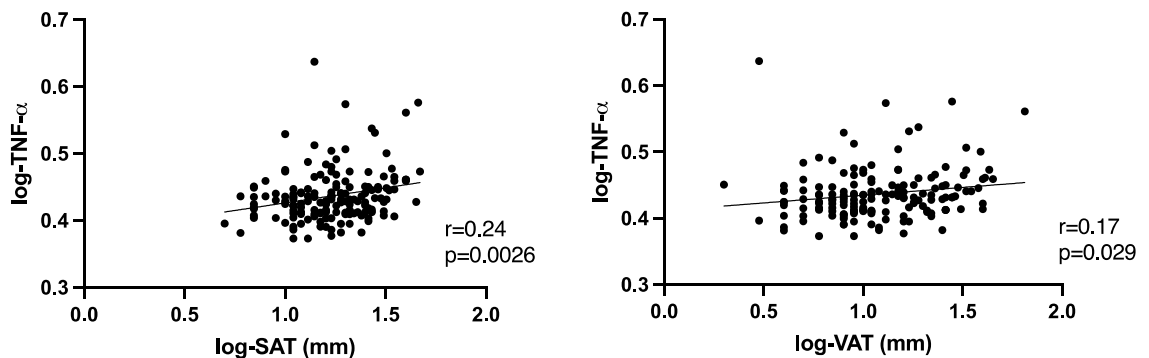


Figure 5-13 Correlation between log-TNF- α and (a) log-SAT, and (b) log-VAT.

Pearson r value and p -values displayed.

5.5.3.2 Waist and Hip Circumference

Figure 5-14 shows there was a moderately positive correlation between TNF- α and waist circumference ($r=0.26$, $p=0.0012$), and a slightly stronger positive correlation between TNF- α and hip circumference ($r=0.32$, $p<0.0001$). Hip circumference was the only parameter which reached the statistical significance threshold set for this study. Waist:hip ratio did not significantly correlate with TNF- α (data not shown).

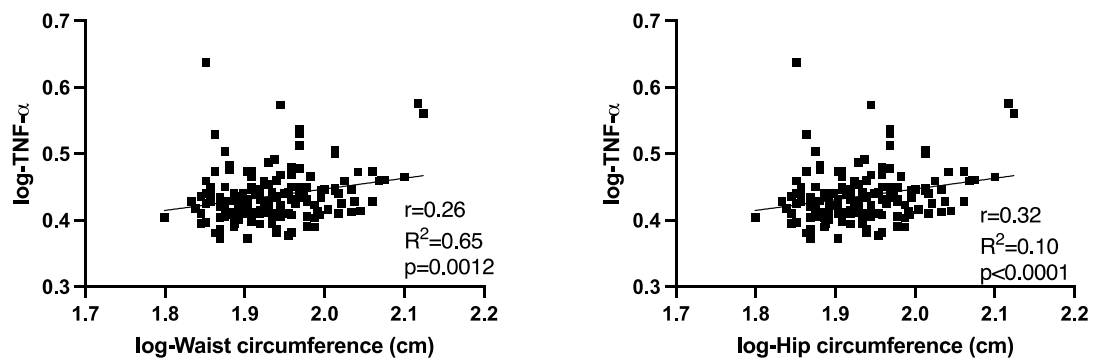


Figure 5-14 Correlation between TNF- α and (a) waist circumference and (b) hip circumference.

Pearson correlation on log-transformed data as both sets of data were non-parametric.

5.5.3.3 Multiple regression analysis TNF- α and anthropometry

Table 21 TNF- α and adipose tissue distribution multiple regression model construction.

TNF- α was analysed within four individual models (numbered 1-4) as previously defined in statistical methods section (page 160). Each model was combined with model 1 (BMI) to ascertain if the anthropometric measurements were correlated with TNF- α independently of BMI. A final stepwise model ($\alpha = 0.15$ to keep/remove) was used to find the variables which were the strongest predictors of TNF- α . Waist:hip ratio (WHR) was not analysed alongside BMI as when analysed alone the regression model was not found to have a statistically significant p-value.

Regression Model	β co-efficient	p-value	adjusted R^2
model 1 (BMI)	0.0018	<0.0001	9.59%
model 2 (SAT and VAT)	log-SAT 0.045 log-VAT 0.0016	0.038 0.92	4.41%
model 3 (WC, HC)	log-WC -0.089 log-HC 0.35	0.39 0.007	9.61%
model 4 (WHR)	regression model p=0.93		
model 1 + model 2	BMI 0.002 log-SAT 0.0035 log-VAT -0.0094	0.005 0.89 0.53	8.67%
model 1 + model 3	BMI 0.0017 log-WC -0.16 log-HC 0.20	0.23 0.19 0.25	9.88%
model 1 +model 4	not performed		
final model (stepwise)	log-HC 0.25	<0.0001	9.77%

Chapter 5

Multiple regression modelling identified BMI and hip circumference as significant positive predictors of TNF- α , although each was only able to account for <10% of variance in TNF- α concentrations and they were not independent of each other. Stepwise modelling identified log-hip circumference as the best predictor of TNF- α , although the predictive value of this relationship was poor, explaining only 9.77% of variance.

5.5.4 IL-6 and adipose tissue distribution

5.5.4.1 SAT and VAT

Figure 5-15 shows a strong positive correlation between IL-6 and both SAT ($r=0.45$, $p<0.0001$) and VAT ($r=0.42$, $p<0.0001$). When IL-6 was analysed within SAT and VAT quartiles, there was a continuous increase in IL-6 with increasing quartiles with no threshold effect demonstrated (data not shown).

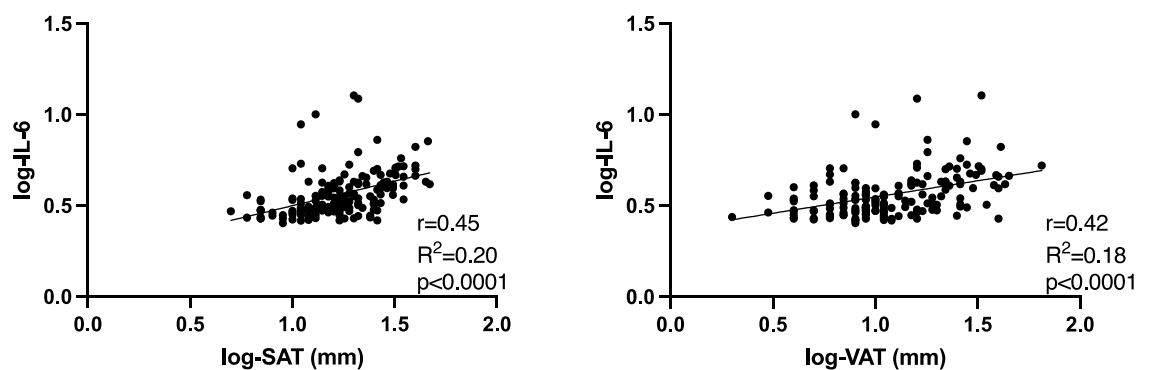


Figure 5-15 Correlation between log IL-6 and (a) log-SAT, and (b) log-VAT.

Analysis by Pearson correlation and simple linear regression. Pearson r -value and p -values displayed. Linear regression line and goodness of fit denoted by R^2 value.

5.5.4.2 Waist and hip circumference

Figure 5-16 demonstrates the strong positive correlation between IL-6 and both waist circumference ($r=0.49$, $p<0.0001$) and hip circumference ($r=0.44$, $p<0.0001$)

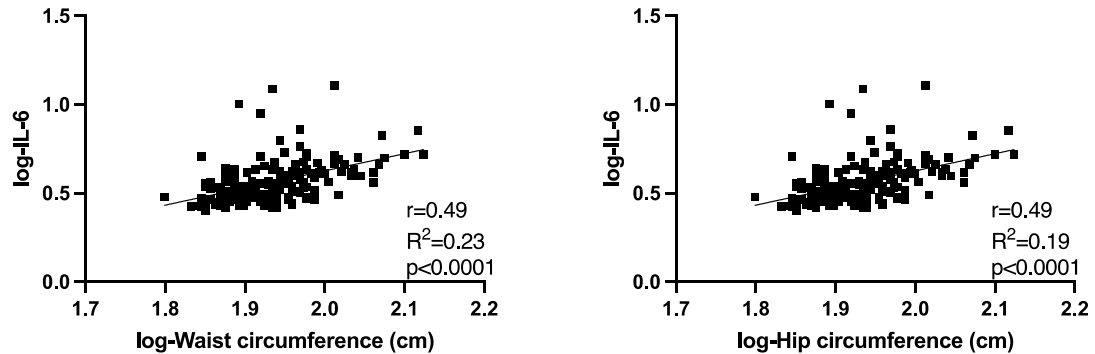


Figure 5-16 Correlation between IL-6 and (a) waist circumference and (b) hip circumference.

All data non-parametric and log-transformed for analysis. Pearson r -value and p -values displayed. Linear regression line and goodness of fit denoted by R^2 value.

Waist:hip ratio was found to be significantly and moderately positively correlated with IL-6 ($r=0.28$, $p=0.0004$), as in Figure 5-17. When IL-6 was analysed according to waist:hip ratio category, healthy (<0.85) and unhealthy (>0.85), IL-6 was found to be higher in those with an unhealthy waist:hip ratio, but did not reach the level of statistical significance set for this study ($p=0.027$, data not shown).

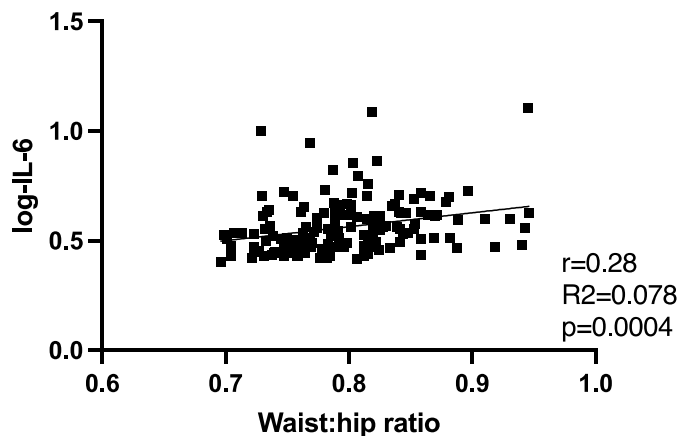


Figure 5-17 Correlation between waist:hip ratio and IL-6.

All data non-parametric and log-transformed for analysis. Pearson r -value and p -values displayed. Linear regression line and goodness of fit denoted by R^2 value.

5.5.4.3 Multiple regression analysis IL-6 and anthropometry

Multiple regression analysis was used to determine the predictive value of the anthropomorphic measurements on IL-6 levels at booking and assess whether there was any collinearity from BMI group. A model (Table 22) was drawn up using the pre-defined models 1-4, including BMI, SAT, VAT, waist circumference, hip circumference and waist: hip ratio included as predictors. A stepwise method was used to refine the final model to reduce interpretation bias.

Table 22 IL-6 and adipose tissue distribution multiple regression model construction.

IL-6 was analysed within four individual models (numbered 1-4) as previously defined in statistical methods section (page 160). Each model was combined with model 1 (BMI) to ascertain if the anthropometric measurements were correlated with IL-6 independently of BMI. A final stepwise model ($\alpha = 0.15$ to keep/remove) was used to find the variables which were the strongest predictors of IL-6 concentration.

Regression Model	β co-efficient	p-value	adjusted R^2
model 1 (BMI)	0.0081	<0.0001	20.45%
model 2 (SAT and VAT)	log-SAT 0.18 log-VAT 0.086	0.002 0.043	21.27%
model 3 (WC, HC)	log-WC 0.88 log-HC 0.12	0.004 0.76	22.7%
model 4 (WHR)	0.64	0.0004	7.15%
model 1 + model 2	BMI 0.004 log-SAT 0.99 log-VAT 0.064	0.038 0.16 0.14	22.92%
model 1 + model 3	BMI 0.0019 log-WC 0.81 log-HC -0.051	0.64 0.02 0.92	22.32%
model 1 + model 4	BMI 0.0072 WHR 0.33	<0.0001 0.049	21.89%
final model (stepwise)	log-WC 0.74 log-VAT 0.072	<0.0001 0.068	24.28%

SAT and VAT did not remain independently associated with IL-6 once BMI was included in the model (model 1+2). However, waist circumference did remain an independent predictor of IL-6 when adjusted for BMI (model 1+3)

Chapter 5

The best fitting model was only able to explain 24.28% of variance (adjusted $R^2=24.28\%$, $p<0.0001$) and included waist circumference and VAT, but not BMI which was removed as $p>0.15$. Only waist circumference was independently and positively associated with IL-6 levels at booking, as VAT was not found to be independently associated with IL-6 once waist circumference was included.

5.5.5 IL-1 β and adipose tissue distribution

5.5.5.1 SAT and VAT

Figure 5-18 illustrates that there was no correlation observed between IL-1 β and either SAT or VAT.

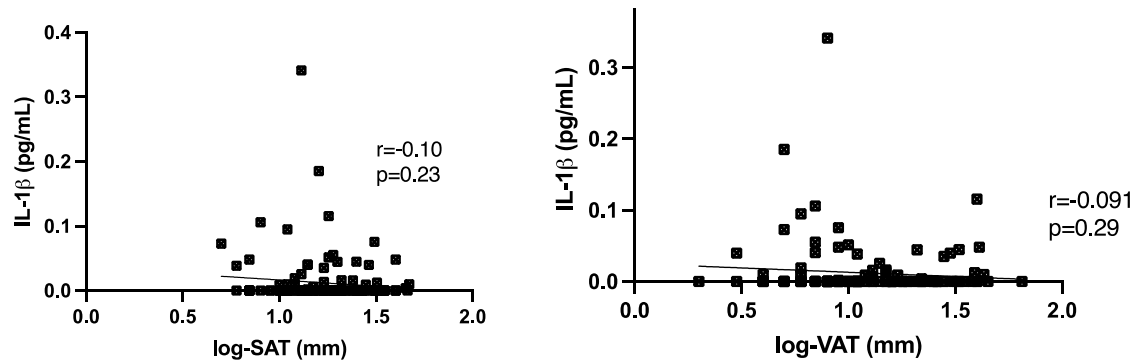


Figure 5-18 Correlation between IL-1 β and (a) SAT and (b) VAT.

Pearson r-value and p-values displayed.

5.5.5.2 Waist circumference and hip circumference

There was no correlation between IL-1 β and either waist or hip circumference, as depicted in Figure 5-19

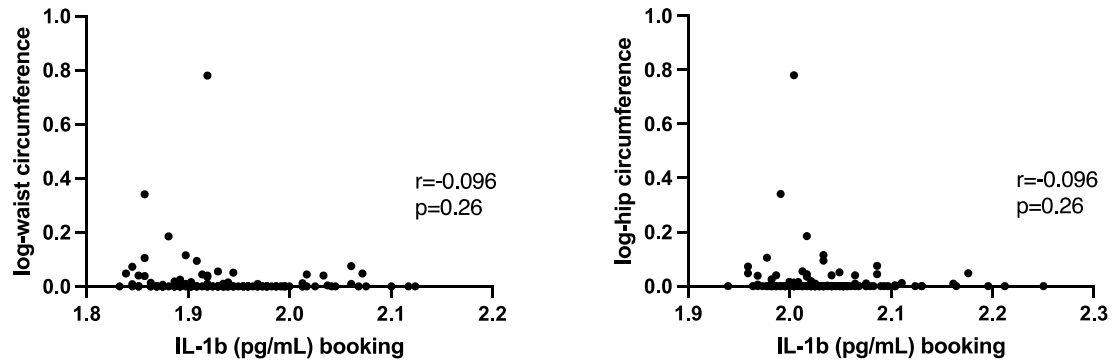


Figure 5-19 Correlation between IL-1 β and (a) waist circumference and (b) hip circumference.

Pearson r value and p -values displayed.

5.6 Relationship between haemostasis parameters and measurements of adipose tissue distribution

5.6.1 FVIII and adipose tissue distribution

5.6.1.1 SAT and VAT

There were no significant correlations between FVIII levels at booking and SAT or VAT thickness (Figure 5-20).

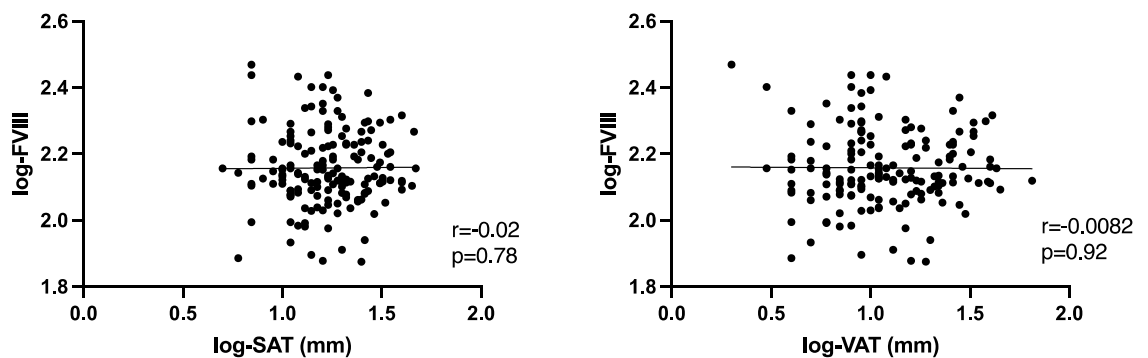


Figure 5-20 Correlation between log-FVIII and (a) log-SAT and (b) log-VAT.

Chapter 5

Pearson r-value and p-values displayed

5.6.1.2 Waist and hip circumference

As with SAT and VAT, FVIII did not correlate with either waist or hip circumference (Figure 5-21).

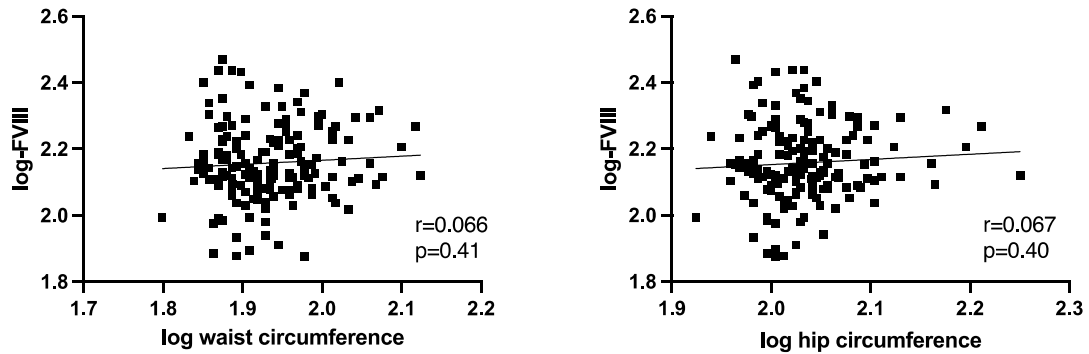


Figure 5-21 Correlation between log-FVIII and (a) log-waist circumference and (b) log-hip circumference.

Pearson r-value and p-values displayed.

5.6.2 Fibrinogen and adipose tissue distribution

5.6.2.1 SAT and VAT

As illustrated in Figure 5-22, there was a significant positive linear correlation between fibrinogen Clauss and SAT ($r=0.52$, $p<0.0001$) and also with VAT ($r=0.41$, $p<0.0001$).

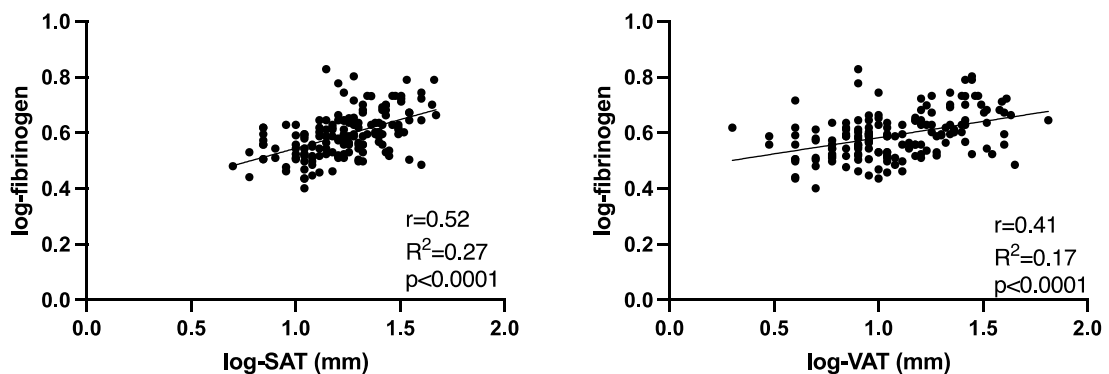


Figure 5-22 Correlation between log-fibrinogen Clauss and (a) log-SAT and (b) log-VAT.

Pearson r-value and p-values displayed. Linear regression line plotted with goodness-of-fit denoted by R^2 value.

When fibrinogen was analysed with SAT and VAT quartiles (data not shown), there was a threshold effect on fibrinogen when VAT reached the 4th quartile (VAT thickness > 19.8mm, $p=0.0086$) however this didn't quite reach the statistical threshold set for this study. The increase in fibrinogen with increasing SAT quartiles appeared linear.

5.6.2.2 Waist and hip circumference

Both waist and hip circumference were strongly positively and linearly correlated with fibrinogen at booking (Figure 5-23). Pearson's correlation found the relationship between fibrinogen and waist circumference ($r=0.53$, $p<0.0001$) to be slightly stronger than that with hip circumference ($r=0.41$, $p<0.0001$) with linear regression showing a better predictive value for waist circumference also ($R^2=0.28$ vs 0.18).

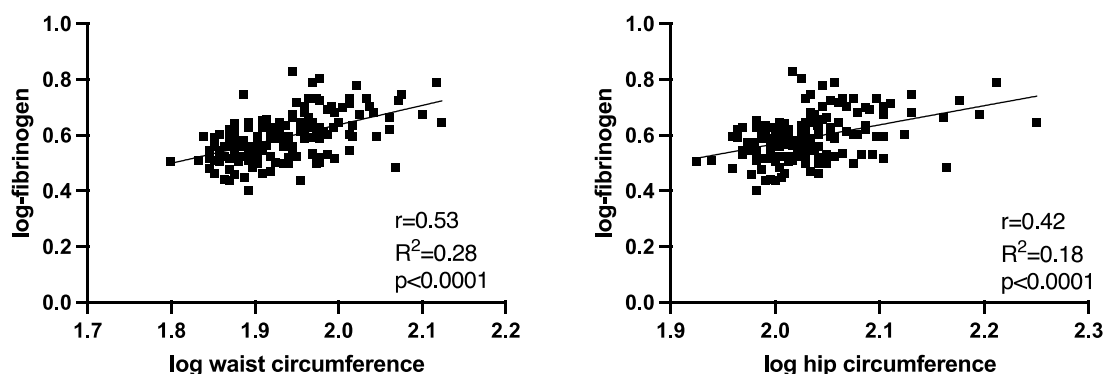


Figure 5-23 Correlation between log-fibrinogen and (a) log-waist circumference and (b) log-hip circumference.

Pearson r value and p-values displayed with linear regression line and goodness of fit denoted by R^2 ,

When fibrinogen was analysed within healthy and unhealthy waist:hip ratio groups (Figure 5-24), participants with an unhealthy waist:hip ratio at booking were found to have a significantly higher fibrinogen ($p=0.0007$).

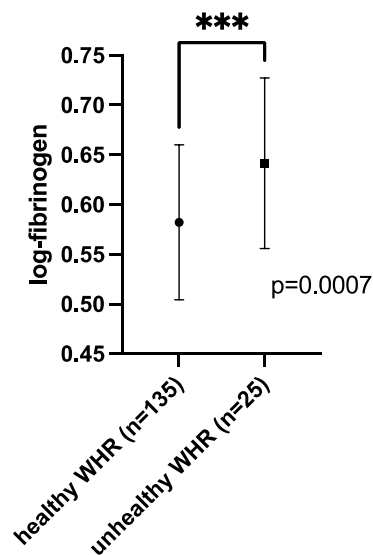


Figure 5-24 log-fibrinogen subdivided by waist:hip ratio (healthy vs unhealthy).

Healthy WHR <0.85, unhealthy WHR >0.85. Analysed by unpaired t-test, ***p<0.001

5.6.2.3 Multiple regression analysis fibrinogen and anthropometry

Multiple regression analysis was used to identify which of the anthropomorphic measures were able to best predict fibrinogen levels at booking. Whilst individually, each of BMI, SAT, VAT, waist circumference, hip circumference and WHR were significantly positively correlated with fibrinogen, when the four regression models (1-4 as previously described) were used it was evident some of these relationships were not independently associated.

Table 23 Fibrinogen and adipose tissue distribution multiple regression model construction.

Fibrinogen was analysed within four individual models (numbered 1-4) as previously defined in statistical methods section (page 160). Each model was combined with model 1 (BMI) to ascertain if the anthropometric measurements were correlated with fibrinogen independently of BMI. A final stepwise model ($\alpha = 0.15$ to keep/remove) was used to find the variables which were the strongest predictors of fibrinogen concentration.

Regression Model	β co-efficient	p-value	adjusted R^2
model 1 (BMI)	0.0055	<0.0001	21.72%
model 2 (SAT and VAT)	log-SAT 0.18 log-VAT 0.026	<0.0001 0.34	26.51%
model 3 (WC, HC)	log-WC 0.95 log-HC -0.36	<0.0001 0.14	27.7%
model 4 (WHR)	0.6	<0.0001	15.23%
model 1 + model 2	BMI 0.0019 log-SAT 0.14 log-VAT 0.016	0.136 0.002 0.578	27.09%
model 1 + model 3	BMI 0.0029 log-WC 0.83 log-HC -0.61	0.26 0.0002 0.065	27.82%
model 1 + model 4	BMI 0.0045 WHR 0.41	<0.0001 0.0002	
final model (stepwise)	log-SAT 0.12 log-WC 0.67 log-HC -0.41	0.013 0.003 0.086	30.04%

When SAT and VAT were analysed together, the relationship between VAT and fibrinogen was not independent of SAT, but the relationship between SAT and fibrinogen was independent of both VAT and BMI (models 1+2). The relationship between waist circumference and fibrinogen remained statistically significant once BMI was taken into consideration (models 1+3), as did the relationship between WHR and fibrinogen (models 1+4).

Overall, using stepwise multiple regression analysis to identify the best predictors of fibrinogen ($\alpha = 0.15$ to keep/remove), SAT, waist circumference and hip circumference were the variables identified which were able to predict 30.04% of variance in fibrinogen levels at booking. BMI was not included in the final model.

5.6.3 D-dimer and adipose tissue distribution

5.6.3.1 SAT and VAT

There was no correlation between either SAT or VAT with D-dimer at booking

Figure 5-25 Correlation between log-D dimer and (a) log-SAT and (b) log-VAT.

.

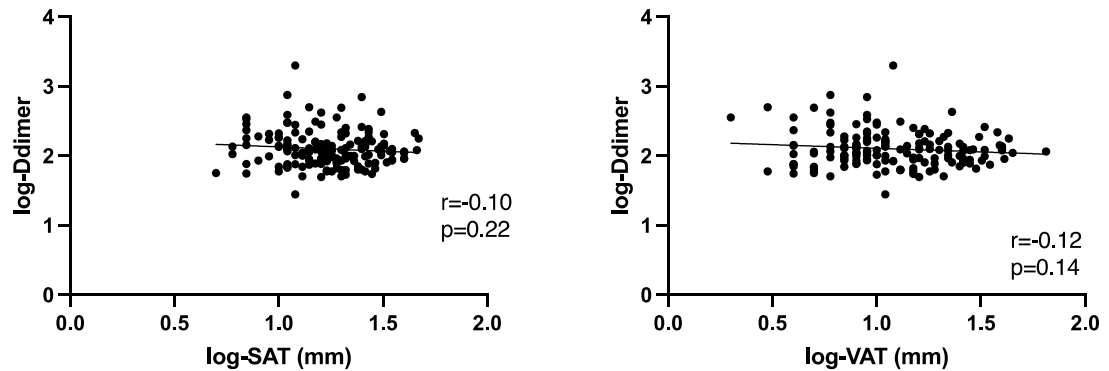


Figure 5-25 Correlation between log-D dimer and (a) log-SAT and (b) log-VAT.

Pearson r-value and p values displayed.

5.6.3.2 Waist and hip circumference

No correlation was present between D-dimer and either waist or hip circumference (Figure 5-26).

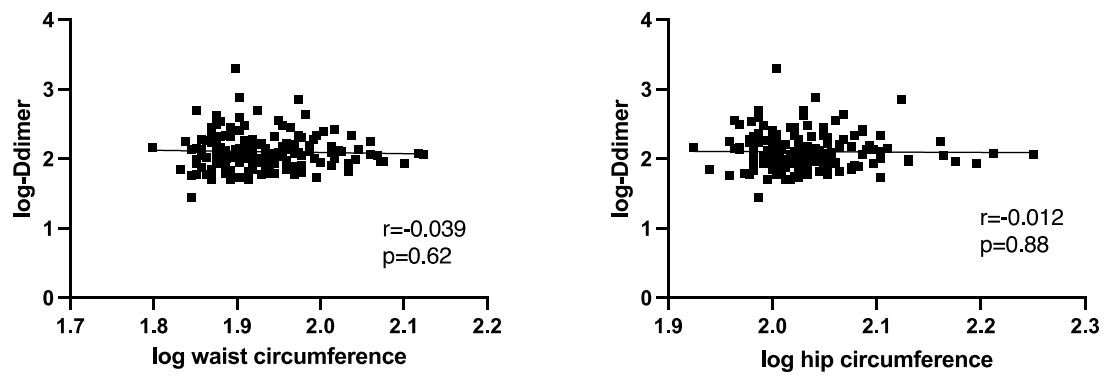


Figure 5-26 Correlation between log-D-dimer and (a) waist circumference and (b) hip circumference.

Pearson r-value and p-values displayed.

5.6.4 TFPI and adipose tissue distribution

5.6.4.1 SAT and VAT

Figure 5-27 shows the moderately positive correlation between booking TFPI level and both SAT ($r=0.27$, $p=0.0005$) and VAT ($r=0.33$, $p<0.0001$). Linear regression found the ability of SAT and VAT to predict TFPI levels weak with $R^2=0.11$ for each. When TFPI was analysed with SAT and VAT quartiles, there was a continuous increase in mean SAT with each quartile increase and no evidence of a threshold effect (data not shown).

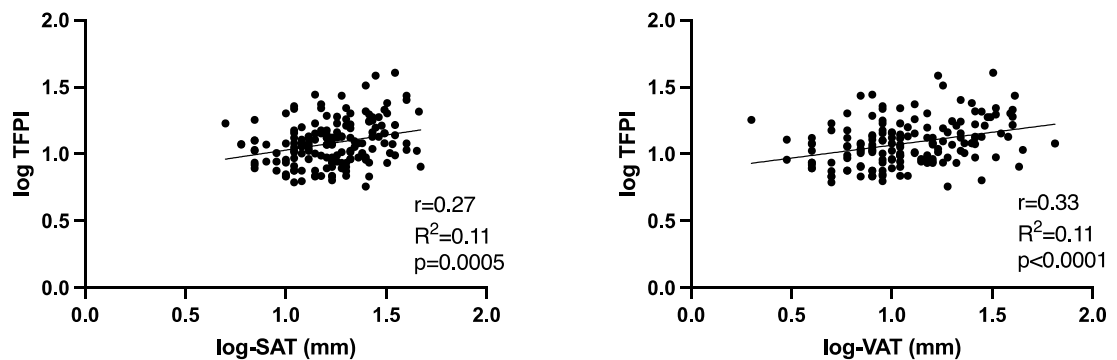


Figure 5-27 Correlation between log-TFPI and (a) log-SAT and (b) log-VAT.

Pearson r -value and p -values displayed. Linear regression line plotted with goodness-of-fit denoted by R^2 value.

5.6.4.2 Waist and hip circumference

Figure 5-28 depicts the moderately positive correlation between TFPI and both waist circumference ($r=0.31$, $p<0.0001$) and hip circumference ($r=0.28$, $p=0.0003$). However, linear regression analysis identified the predictive value of waist circumference and hip circumference on TFPI levels to be very low ($R^2=0.098$ and 0.079 , respectively)

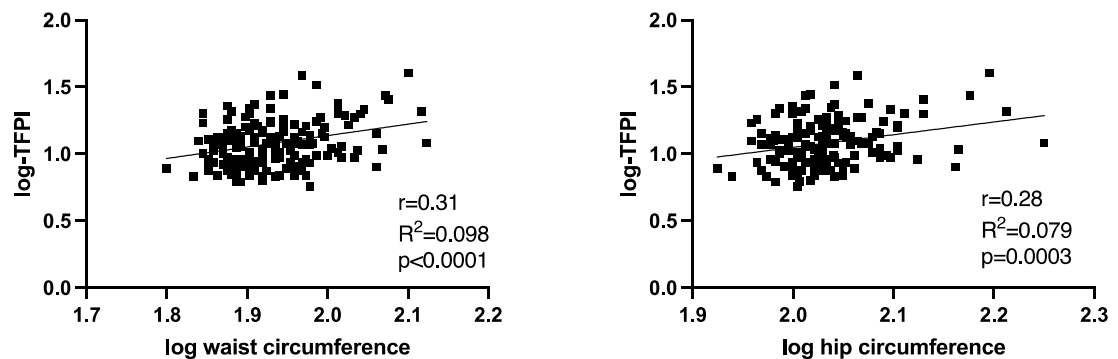


Figure 5-28 Correlation between log-TFPI and (a) log-waist circumference and (b) log-hip circumference.

Pearson r -value and p -values displayed. Linear regression line plotted with goodness-of-fit denoted by R^2 value.

When the population was subdivided by healthy and unhealthy WHR (data not shown), there was no significant difference in TFPI concentrations between the two groups ($p=0.43$).

5.6.4.3 Multiple regression analysis TFPI and anthropometry

Multiple regression analysis identified that the positive correlation between TFPI and SAT was not independent of VAT or BMI, however the positive correlation between VAT and TFPI remained robust once BMI was included in the model. Neither relationship with waist or hip circumference and TFPI was independent of BMI. The final stepwise regression model identified BMI ($p=0.047$) and log-VAT ($p=0.042$) to be the best independent predictors of TFPI, but this model was only able to explain 11.99% of variance.

Table 24 TFPI and adipose tissue distribution multiple regression model construction.

TFPI was analysed within four individual models (numbered 1-4) as previously defined in statistical methods section (page 160). Each model was combined with model 1 (BMI) to ascertain if the anthropometric measurements were correlated with TFPI independently of BMI. A final stepwise model ($\alpha = 0.15$ to keep/remove) was used to find the variables which were the strongest predictors of TFPI concentration.

Regression Model	β co-efficient	p-value	adjusted R^2
model 1 (BMI)	0.0079	<0.0001	10.22%
model 2 (SAT and VAT)	log-SAT 0.068 log-VAT 0.16	0.44 0.011	10.09%
model 3 (WC, HC)	log-WC 0.82 log-HC 0.053	0.071 0.92	8.65%
model 4 (WHR)	0.572	0.021	2.73%
model 1 + model 2	BMI 0.0054 log-SAT -0.044 log-VAT 0.13	0.061 0.67 0.044	11.53%
model 1 + model 3	BMI 0.011 log-WC 0.38 log-HC -0.89	0.068 0.46 0.24	10.00%
model 1 + model 4	BMI 0.0072 WHR 0.27	0.0002 0.28	10.32%
final model (stepwise)	BMI 0.0047 log-VAT 0.12	0.047 0.042	11.99%

5.6.5 Protein S and adipose tissue distribution

5.6.5.1 SAT AND VAT

There was no correlation between protein S and either SAT or VAT at booking (Figure 5-29).

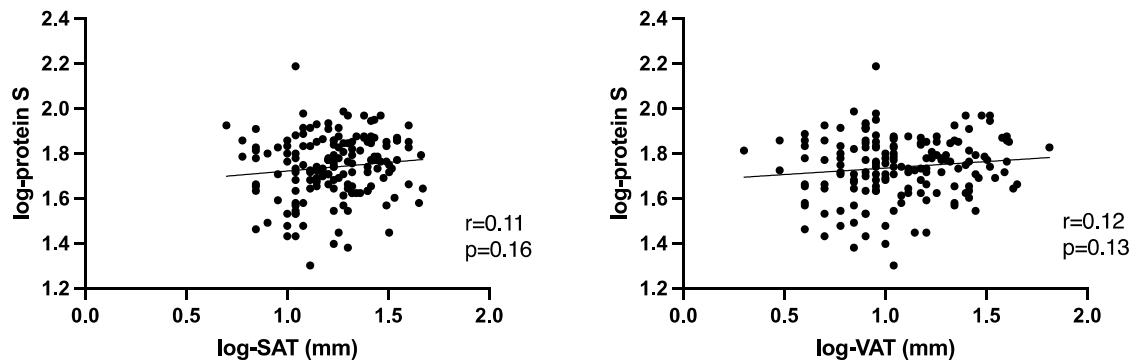


Figure 5-29 Correlation between log-protein S and (a) log-SAT and (b) log-VAT.

Pearson r-value and p values displayed.

5.6.5.2 Waist and hip circumference

There was a weak positive correlation between protein S and waist circumference ($r=0.19$, $p=0.015$), and a weak positive correlation between protein S and hip circumference ($r=0.16$, $p=0.049$) as seen in Figure 5-30. The linear regression analyses identified waist circumference and hip circumference to be poor predictors of protein S ($R^2=0.037$ and 0.024 , respectively) and the threshold for statistical significance for this study was not reached.

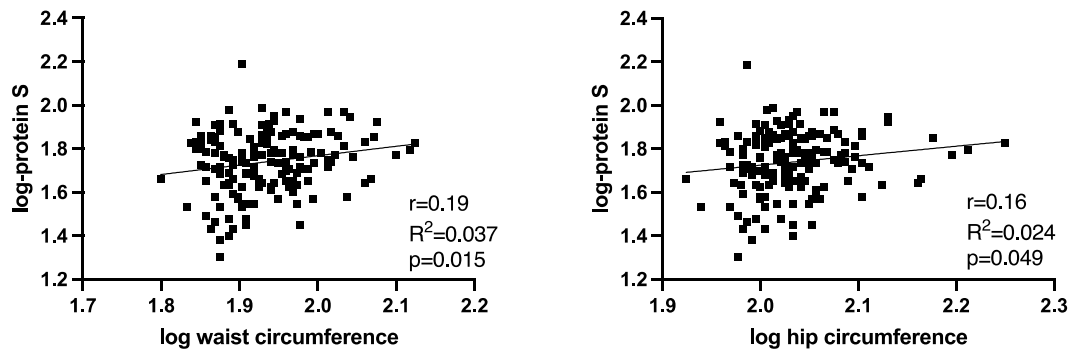


Figure 5-30 Correlation between log-protein S and (a) log-waist circumference and (b) log-hip circumference.

Pearson r value and p values displayed. Linear regression line plotted with goodness-of-fit denoted by R^2 value.

The study population was divided according to healthy (<0.85) and unhealthy (>0.85) waist: hip ratio and protein S levels compared between the two groups. There was no significant difference between the healthy WHR and unhealthy WHR groups ($p=0.11$, data not shown).

5.6.6 PAI-1 and adipose tissue distribution

5.6.6.1 SAT and VAT

There was a weakly positive non-linear association between PAI-1 and SAT ($R^2=0.12$ $p<0.0001$) and between PAI-1 and VAT ($R^2=0.09$, $p=0.0002$) as depicted in Figure 5-31.

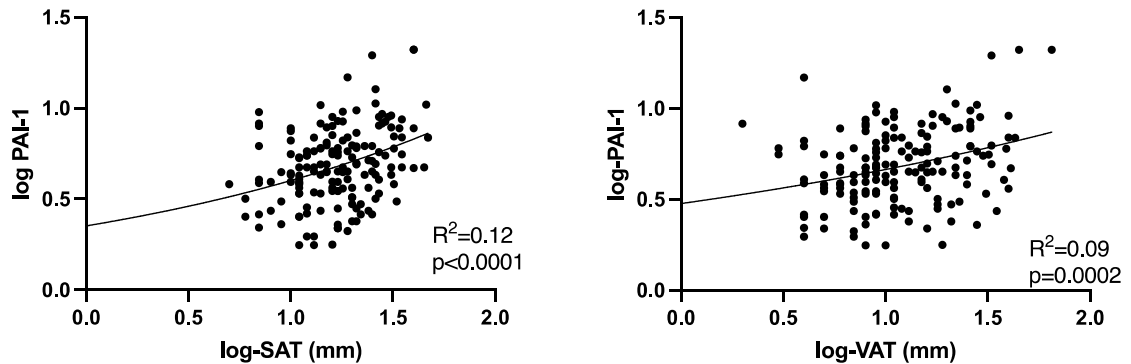


Figure 5-31 Correlation between log-PAI-1 and (a) log-SAT and (b) log-VAT.

Non-linear regression lines plotted and goodness-of-fit denoted by R^2 values.

The line of best fit for both SAT and VAT was a non-linear exponential line and predictive value of SAT for PAI-1 was stronger than that of VAT ($R^2=0.12$ vs 0.09 , respectively). This suggests that at greater thickness of VAT, and more so SAT, there is a greater increase in PAI-1. This relationship was also identified when TFPI was analysed within SAT and VAT quartiles (data not shown) however due to the small sample size in these groups did not reach the level of statistical significance set for this study ($p<0.001$).

When PAI-1 was analysed within SAT and VAT quartiles (data not shown), one-way ANOVA confirmed a significant difference in PAI-1 between the SAT quartiles ($p<0.0001$) and VAT quartiles ($p=0.0007$). On *post hoc* analysis it was evident that there was a threshold effect, with the only significant increase between the groups existing when SAT or VAT thickness reached the 4th quartile (comparing Q3 to Q4: SAT $>24.8\text{mm}$ $p=0.0053$, VAT $>19.8\text{mm}$ $p=0.048$), however this was below the level of significance set for the study.

5.6.6.2 Waist and hip circumference

Figure 5-32 shows the moderately positive correlation between PAI-1 and waist circumference ($r=0.31$, $p<0.0001$) and hip circumference ($r=0.33$, $p<0.0001$). when the study population was divided by waist:hip ratio into healthy (<0.85) and unhealthy (>0.85) groups, there was no significant difference in level of PAI-1 between the two groups (data not shown).

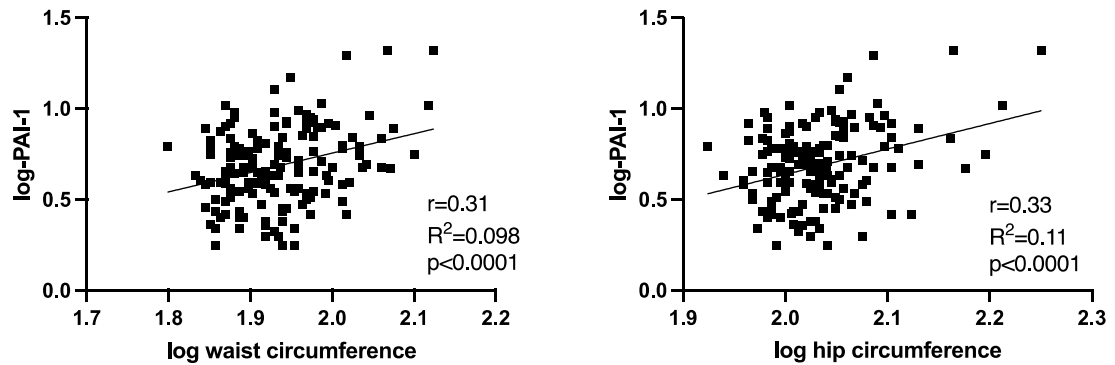


Figure 5-32 Correlation between log-PAI-1 and (a) log-waist circumference and (b) log-hip circumference.

Pearson r value and p values displayed. Goodness of fit denoted by R^2 .

5.6.6.3 Multiple regression analysis PAI-1 and anthropometric measures

Multiple regression models were built up as described in Table 25. The previously demonstrated correlations with SAT and VAT were not found to be independent from BMI. Similarly, the correlations between waist circumference and hip circumference were explained by collinearity with BMI. Overall, the best fitting model, as identified through stepwise modelling, used only BMI as a predictor for PAI-1 which was able to predict only 11.93% of variance.

Table 25 PAI-1 and adipose tissue distribution multiple regression model construction.

PAI-1 was analysed within four individual models (numbered 1-4) as previously defined in statistical methods section (page 160). Each model was combined with model 1 (BMI) to ascertain if the anthropometric measurements were correlated with PAI-1 independently of BMI. A final stepwise model ($\alpha = 0.15$ to keep/remove) was used to find the variables which were the strongest predictors of PAI-1.

Regression Model	β co-efficient	p-value	adjusted R^2
model 1 (BMI)	0.011	<0.0001	11.93%
model 2 (SAT and VAT)	log-SAT 0.27 log-VAT 0.08	0.015 0.30	10.71%
model 3 (WC, HC)	log-WC 0.30 log-HC 1.07	0.59 0.12	10.04%
model 4 (WHR)	regression model p value 0.25		
model 1 + model 2	BMI 0.0065 log-SAT 0.13 log-VAT 0.043	0.071 0.32 0.59	12.00%
model 1 + model 3	BMI 0.011 log-WC -0.16 log-HC 0.072	0.12 0.80 0.94	10.84%
model 1 + model 4	BMI 0.011 WHR -0.092	<0.0001 0.77	11.42%
final model (stepwise)	BMI 0.011	<0.0001	11.93%

5.7 Relationship between thrombin generation and measurements of adipose tissue distribution

5.7.1 Lagtime and adipose tissue distribution

5.7.1.1 SAT and VAT

There was a moderate positive correlation between lagtime and both SAT ($r=0.31$, $p<0.0001$) and VAT ($r=0.31$, $p<0.0001$) as displayed in Figure 5-33. However linear regression R^2 values were low for both SAT ($R^2=0.095$) and VAT ($R^2=0.094$) suggesting the predictive value of these measurements on lagtime was poor.

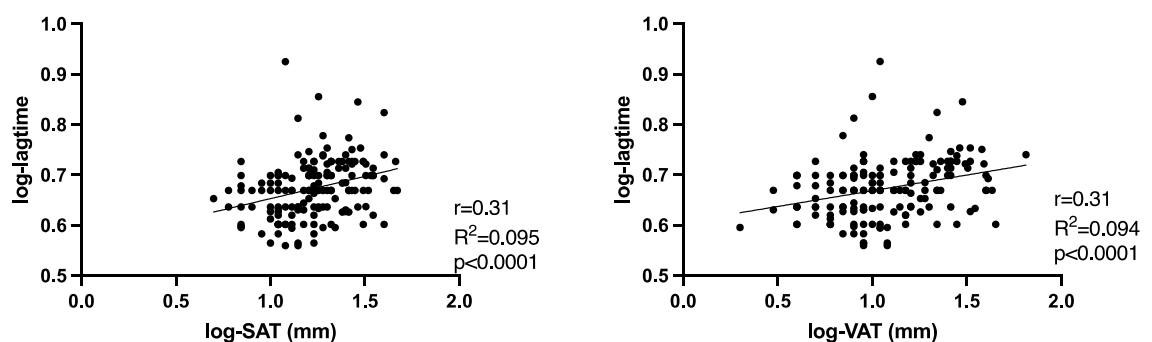


Figure 5-33 Pearson correlation between log-lagtime and (a) log-SAT and (b) log-VAT. Pearson r-value and p-values displayed.

When lagtime was analysed with SAT and VAT quartiles (data not shown) the increase with each SAT quartile appeared to be continuous, whereas there was a threshold effect with VAT quartile. There was an increase between Q3 and Q4 VAT ($VAT>19.8\text{mm}$), however this didn't reach the level of statistical significance set for this study.

5.7.1.2 Waist and hip circumference

Figure 5-34 shows a moderate positive correlation between lagtime and waist circumference ($r=0.26$, $p=0.0007$) but the correlation between lagtime and hip circumference was not statistically significant ($r=0.16$, $p=0.050$). When subdivided by healthy (<0.85) and unhealthy (>0.85) waist:hip ratio, there was no significant difference in lagtime between the two groups ($p=0.054$, data not shown).

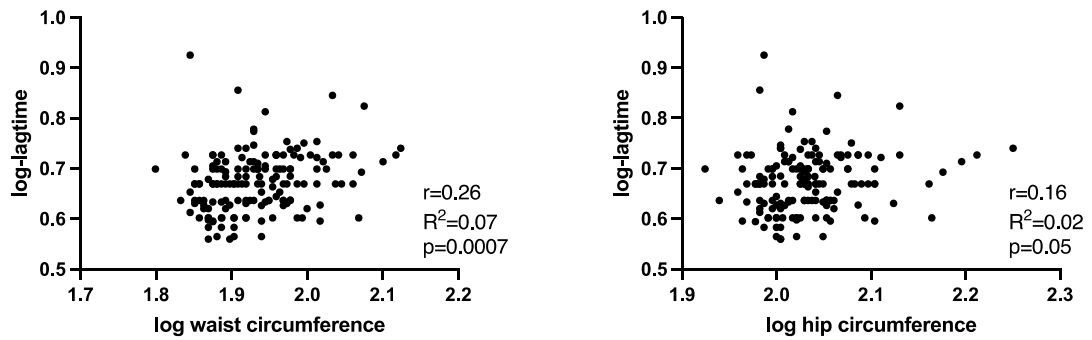


Figure 5-34 Correlation between log-lagtime and (a) log-waist circumference and (b) log-hip-circumference.

Pearson r value and p values displayed.

5.7.1.3 Multiple regression analysis lagtime and anthropometric measures

Multiple regression analysis was used to ascertain the variables independently predictive of lagtime at booking. Hip circumference remained negatively predictive of lagtime once BMI was adjusted for and WHR was the only other parameter to remain independently associated with lagtime following the addition of BMI to the model. However, the stepwise multiple regression identified WHR and VAT, rather than BMI, as the best independent predictors of lagtime, but this equation was only able to predict 12.56% of variance.

Table 26 Lagtime and adipose tissue distribution multiple regression model construction.

Lagtime was analysed within four individual models (numbered 1-4) as previously defined in statistical methods section (page 160). Each model was combined with model 1 (BMI) to ascertain if the anthropometric measurements were correlated with lagtime independently of BMI. A final stepwise model ($\alpha = 0.15$ to keep/remove) was used to find the variables which were the strongest predictors of lagtime.

Regression Model	B co-efficient	p-value	adjusted R ²
model 1 (BMI)	0.0022	0.001	6.23%
model 2 (SAT and VAT)	SAT 0.053 VAT 0.026	0.082 0.098	9.98%
model 3 (WC, HC)	WC 0.57 HC -0.44	0.00038 0.024	8.84%
model 4 (WHR)	0.33	0.00011	8.45%
model 1 + model 2	BMI 0.0001 log-SAT 0.051 log-VAT 0.035	0.92 0.17 0.12	9.40%
model 1 + model 3	BMI 0.0063 WC 0.31 HC -0.99	0.002 0.072 0.00018	13.63%
model 1 + model 4	BMI 0.0015 WHR 0.26	0.021 0.003	10.93%
final model (stepwise)	VAT 0.047 WHR 0.24	0.004 0.006	12.56%

5.7.2 Peak thrombin and adipose tissue distribution

5.7.2.1 SAT and VAT

Figure 5-35 shows the moderate positive correlation between peak thrombin and SAT ($r=0.28$, $p=0.00027$). There was also a very weak positive correlation between peak thrombin and VAT but it did not reach statistical significance thresholds set for this study ($r=0.19$, $p=0.018$). When analysed within SAT and VAT quartiles (data not shown), there was a continuous increase in peak thrombin with each increase in SAT or VAT quartile, with no evidence of a threshold effect.

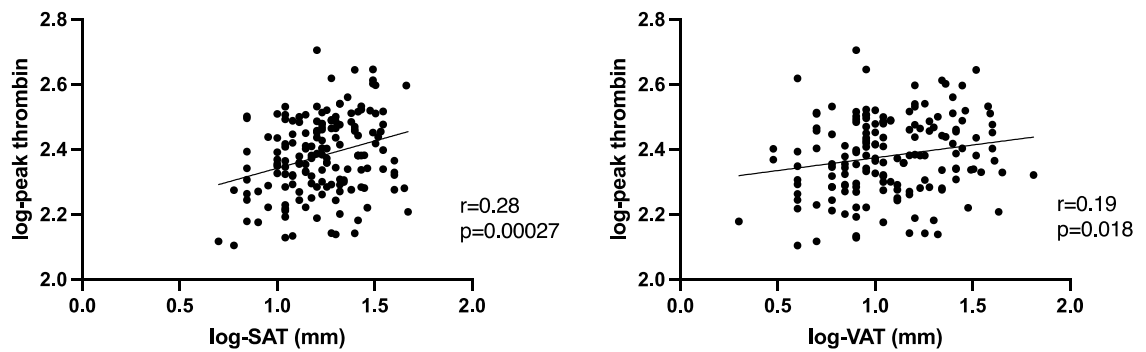


Figure 5-35 Correlation between log-peak thrombin and (a) log-SAT and (b) log-VAT.

Pearson r value and p values displayed.

5.7.2.2 Waist and hip circumference

There was a moderately positive correlation between peak thrombin and waist circumference, however it did not reach level of statistical significance set for this study ($r=0.24$, $p=0.0026$). The correlation between peak thrombin and hip circumference did not reach statistical significance ($r=0.16$, $p=0.05$). There was no significant difference in peak thrombin when it was compared between participants with a healthy waist:hip ratio and those with an unhealthy waist:hip ratio ($p=0.081$, data not shown).

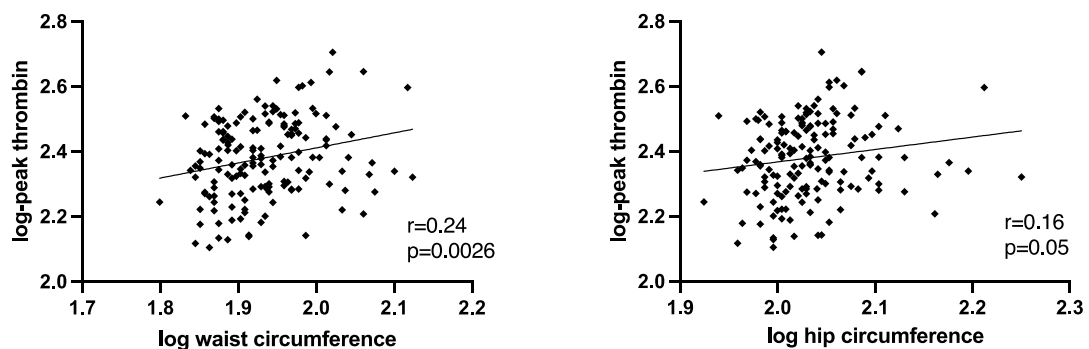


Figure 5-36 Correlation between log-peak thrombin and (a) log-waist circumference and (b) log-hip circumference.

Pearson r value and p values displayed

5.7.2.3 Multiple regression analysis peak thrombin and anthropometry

Multiple regression modelling identified that both SAT and waist circumference remained independently correlated with peak thrombin once BMI was adjusted for, however waist circumference was not independent of SAT. The final

Chapter 5

regression model was able to account for 7.5% of variance and used only SAT as the predictor.

Table 27 Peak thrombin and adipose tissue distribution multiple regression model construction.

Peak thrombin was analysed within four individual models (numbered 1-4) as previously defined in statistical methods section (page 160). Each model was combined with model 1 (BMI) to ascertain if the anthropometric measurements were correlated with peak thrombin independently of BMI. A final stepwise model ($\alpha = 0.15$ to keep/remove) was used to find the variables which were the strongest predictors of peak thrombin. Waist:hip ratio (WHR) was not included in the multiple regression as there was no significant difference between peak thrombin when subdivided by healthy and unhealthy WHR in the *a priori* analysis.

Regression Model	β co-efficient	p-value	adjusted R^2
model 1 (BMI)	0.0029	0.032	2.27%
model 2 (SAT and VAT)	SAT 0.18 VAT -0.012	0.006 0.82	6.94%
model 3 (WC, HC)	WC 0.89 HC -0.0560	0.007 0.14	5.72%
model 4 (WHR)	0.53	0.003	5.00%
model 1 + model 2	BMI -0.002 SAT 0.22 VAT 0.0004	0.35 0.005 0.99	6.86%
model 1 + model 3	BMI -0.0023 WC 0.98 HC -0.40	0.60 0.009 0.48	5.29%
model 1 + model 4	BMI 0.0018 WHR 0.46	0.20 0.014	5.40%
final model (stepwise)	SAT 0.17	0.00027	7.50%

5.7.3 ETP and adipose tissue distribution

5.7.3.1 SAT and VAT

Figure 5-37 shows the moderate positive correlation between ETP and SAT ($r=0.31$, $p<0.0001$). There was a weak positive correlation between ETP and VAT ($r=0.21$, $p=0.009$) however this did not reach the level of statistical significance set for this study.

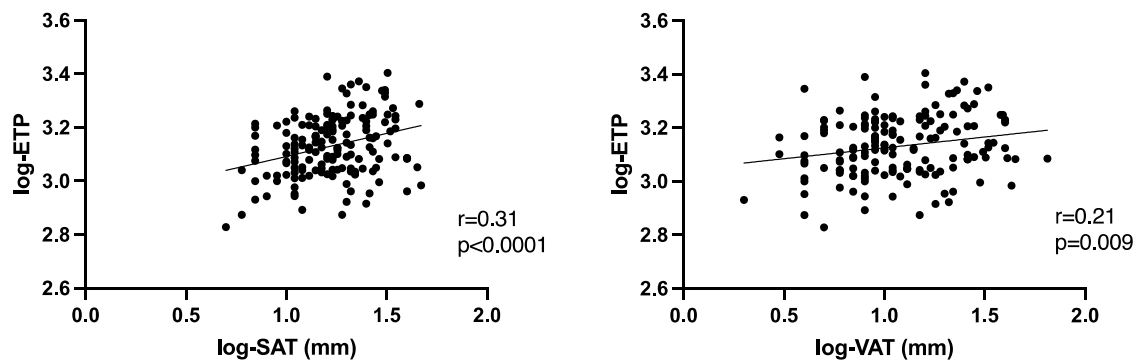


Figure 5-37 Correlation between log-ETP and (a) log-SAT and (b) log-VAT.

Pearson r value and p values displayed.

When ETP was analysed within SAT quartiles (data not shown), there was evidence of a threshold effect with a significant increase in ETP when SAT increased from Q1 to Q2 ($p=0.0086$) but this was below the study level of significance. There was no further significant difference between the subsequent quartiles. There was no significant difference in ETP when compared between VAT quartiles, although overall there was a small increase in ETP as VAT increased ($p=0.025$) which was below the statistical threshold for significance set for this study.

5.7.3.2 Waist and hip circumference

There was a weak positive correlation between ETP and waist circumference ($r=0.22$, $p=0.0045$) as shown in Figure 5-38, however this did not reach the level of significance set for this study ($p<0.001$). The correlation between ETP and hip circumference did not reach statistical significance ($r=0.16$, $p=0.05$). When the population was subdivided by waist:hip ratio, into healthy (<0.85) and unhealthy (>0.85) groups, there was no difference observed between the two groups ($p=0.19$).

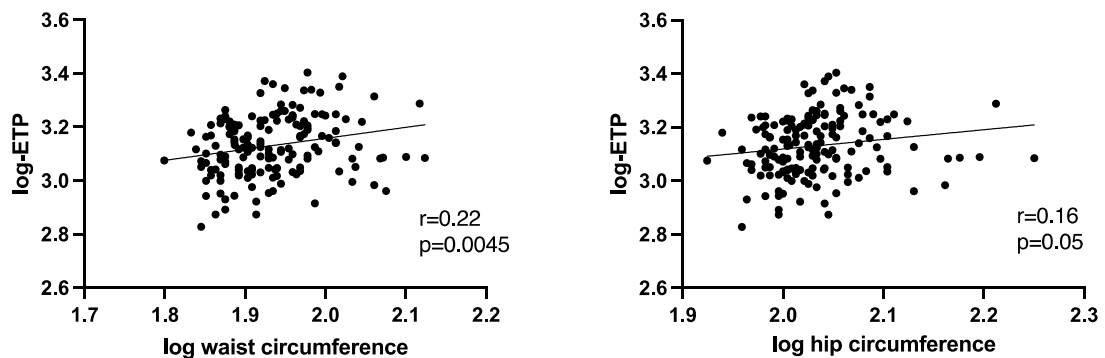


Figure 5-38 Correlation between log-ETP and (a) log-waist circumference and (b) log-hip circumference.

Pearson r values and p values displayed.

5.7.3.3 Multiple regression analysis ETP and anthropometry

Multiple regression analysis found the relationship between SAT and ETP was independent of BMI, as was the relationship between waist circumference and BMI. Waist circumference was no longer significantly correlated with ETP once SAT was added to the model. However, the final stepwise multiple regression model identified BMI and SAT as the best predictors of BMI, able to account for 10.06% of variance. BMI was not independent from SAT.

Table 28 ETP and adipose tissue distribution multiple regression model construction.

ETP was analysed within four individual models (numbered 1-4) as previously defined in statistical methods section (page 160). Each model was combined with model 1 (BMI) to ascertain if the anthropometric measurements were correlated with ETP independently of BMI. A final stepwise model ($\alpha = 0.15$ to keep/remove) was used to find the variables which were the strongest predictors of ETP.

Regression Model	β co-efficient	p-value	adjusted R^2
model 1 (BMI)	0.0026	0.044	1.93%
model 2 (SAT and VAT)	SAT 0.18 VAT -0.01	0.002 0.81	8.58%
model 3 (WC, HC)	WC 0.72 HC -0.43	0.021 0.26	4.57%
model 4 (WHR)	0.33	0.0001	8.45%
model 1 + model 2	BMI -0.0032 SAT 0.25 VAT 0.007	0.11 0.001 0.86	9.50%
model 1 + model 3	BMI -0.0032 WC 0.85 HC -0.16	0.44 0.017 0.77	4.32%
model 1 + model 4	BMI 0.0015 WHR 0.26	0.021 0.003	10.93%
final model (stepwise)	BMI -0.0031 SAT 0.25	0.11 0.00014	10.06%

5.7.4 Time to peak (ttP) and adipose tissue distribution

5.7.4.1 SAT and VAT

There were only very weak positive correlations (Figure 5-39) between time to peak thrombin (ttP) and SAT ($r=0.17$, $p=0.036$) and VAT ($r=0.19$, $p=0.016$) which did not reach the significance threshold for this study. When ttP was analysed within SAT and VAT quartiles (data not shown), there was no significant difference between any of the groups.

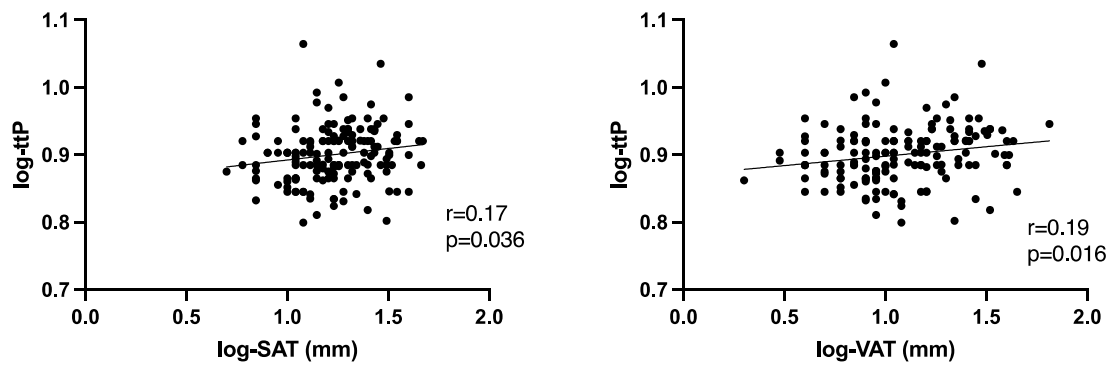


Figure 5-39 Correlation between log-time to peak (ttP) and (a) log-SAT and (b) log-VAT.

Pearson r value and p values displayed.

5.7.4.2 Waist and hip circumference

There was no correlation between ttP and either waist or hip circumference. There was no significant difference in ttP when participants with a healthy waist:hip ratio were compared to participants with an unhealthy waist:hip ratio ($p=0.64$, data not shown).

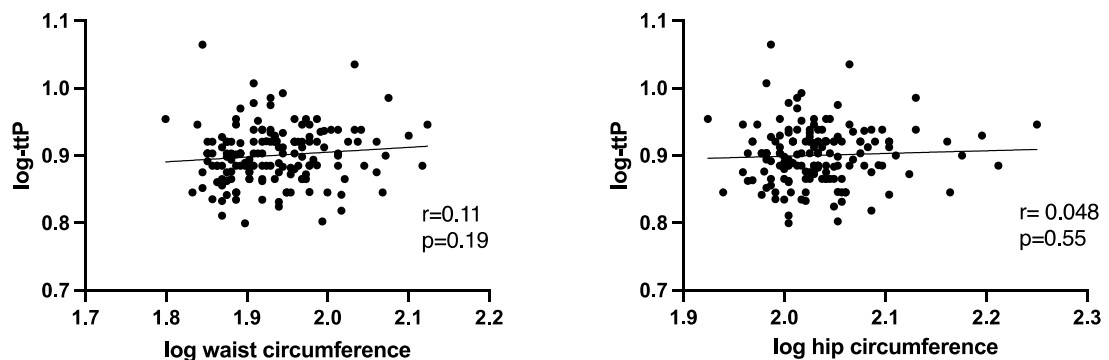


Figure 5-40 Correlation between log-time to peak (ttP) and (a) log-waist circumference and (b) log-hip circumference.

Pearson r value and p values displayed.

5.7.5 Velocity Index and adipose tissue distribution

5.7.5.1 SAT and VAT

Figure 5-41 shows the moderate positive correlation which was present between velocity index and SAT ($r=0.27$, $p=0.0006$). A weaker correlation existed between velocity index and VAT ($r=0.18$, $p=0.026$), however this didn't reach statistical

Chapter 5

significance for this study. When data was analysed within SAT and VAT quartiles (data not shown), the increase in velocity index with each SAT quartile was observed to be continuous with no evidence of a threshold effect (data not shown).

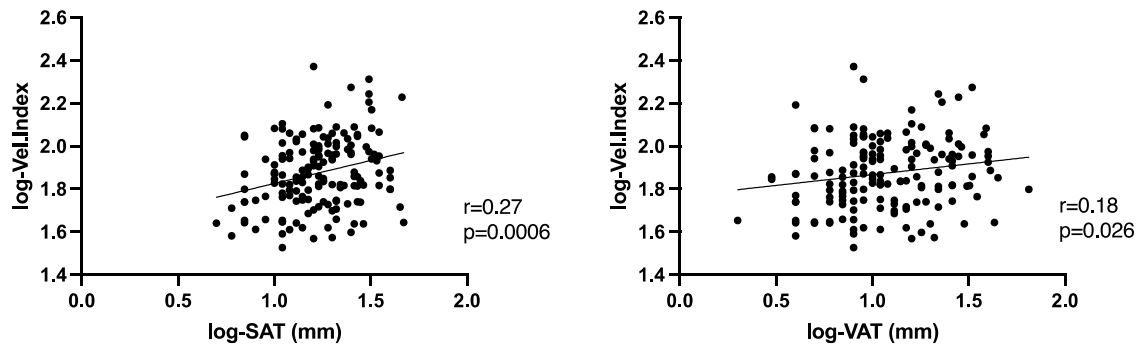


Figure 5-41 Correlation between log-velocity index and (a) log-SAT and (b) log-VAT. Pearson r value and p values displayed.

5.7.5.2 Waist and hip circumference

In Figure 5-42 the moderate positive correlation between velocity index and waist circumference is displayed ($r=0.25$, $p=0.0014$). There was a weaker positive correlation between velocity index and hip circumference ($r=0.17$, $p=0.033$). However, neither of these correlations reached statistical significance threshold set for this study. There was no significant difference in velocity index when the study group was subdivided into healthy and unhealthy waist:hip ratio groups ($p=0.11$, data not shown).

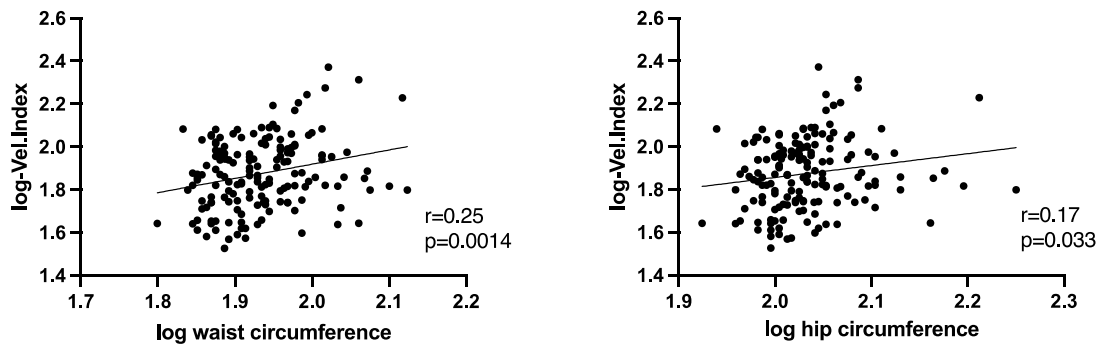


Figure 5-42 Correlation between log-velocity index and (a) log-waist circumference and (b) log-hip circumference.

Pearson r value and p values displayed.

5.7.5.3 Multiple regression analysis velocity index and anthropometry

Multiple regression analysis identified that the relationship between SAT and velocity index was independent from BMI, and that the correlation between waist circumference and velocity index remained robust once BMI was accounted for. Stepwise multiple regression analysis identified WHR and SAT as the variables able to predict 8.08% of variance in velocity index, however only SAT was independently predictive and neither relationship reached the statistical threshold set.

Table 29 Velocity index and adipose tissue distribution multiple regression model construction.

Velocity index was analysed within four individual models (numbered 1-4) as previously defined in statistical methods section (page 160). Each model was combined with model 1 (BMI) to ascertain if the anthropometric measurements were correlated with velocity index independently of BMI. A final stepwise model ($\alpha = 0.15$ to keep/remove) was used to find the variables which were the strongest predictors of velocity index.

Regression Model	B co-efficient	p-value	adjusted R ²
model 1 (BMI)	0.0044	0.018	2.89%
model 2 (SAT and VAT)	SAT 0.23 VAT -0.014	0.009 0.82	6.05%
model 3 (WC, HC)	WC 1.26 HC -0.83	0.005 0.13	6.45%
model 4 (WHR)	0.76	0.002	5.58%
model 1 + model 2	BMI -0.00093 SAT 0.25 VAT -0.0085	0.75 0.02 0.89	5.51%
model 1 + model 3	BMI -0.0018 WC 1.33 HC -0.67	0.78 0.009 0.38	5.91%
model 1 + model 4	BMI 0.0028 WHR 0.64	0.14 0.01	6.28%
final model (stepwise)	SAT 0.16 WHR 0.49	0.023 0.062	8.08%

5.8 Discussion

5.8.1 Main findings

The main aim of the analysis in this Chapter was to assess the influence of adipose tissue distribution on the prothrombotic state via the biomarker, *ex vivo* thrombin generation. The hypothesis that VAT would be predictive of thrombin generation was incorrect as it was identified that SAT was the parameter with the most robust relationship to ETP. However, together, SAT and BMI were only able to predict 10.06% of the variance in ETP. This finding is in contrast with the only other previous study in this area by Chitongo *et al* who identified, in a non-pregnant population, that VAT was associated with thrombin generation parameters.

Chapter 5

The other main findings of this study were consistent with the pre-existing body of literature, that BMI incompletely reflects adiposity, and that in pregnancy, measurements of adipose tissue distribution are better able to predict leptin, IL-6 and fibrinogen, mediators of the procoagulant state than BMI alone. The most significant associations are depicted in Figure 5-43 and outlined below:

- In this study, identification of central adiposity in early pregnancy, with increased waist circumference, was found to be a strong predictor for plasma IL-6 at booking (adjusted $R^2=24.28\%$). To the best of our knowledge, this is a novel finding in the pregnant population, and does correlate with the evidence base in the non-pregnant population(Charo and Taubman, 2004; Pou *et al.*, 2007).
- Upper and lower body SAT, as measured by abdominal SAT thickness, waist and hip circumference, were strong predictors of plasma fibrinogen concentrations (adj $R^2=30.04\%$) independently of BMI, similar to findings observed in non-pregnant individuals by Pou *et al.*(Pou *et al.*, 2007)
- In keeping with the data in non-pregnant individuals, leptin was reflective of total fat mass, with waist circumference, hip circumference, SAT and VAT taken together all strongly predictive of plasma leptin concentrations, and providing a greater predictive value than BMI alone (adj $R^2=77.00\%$ vs 58.86%)

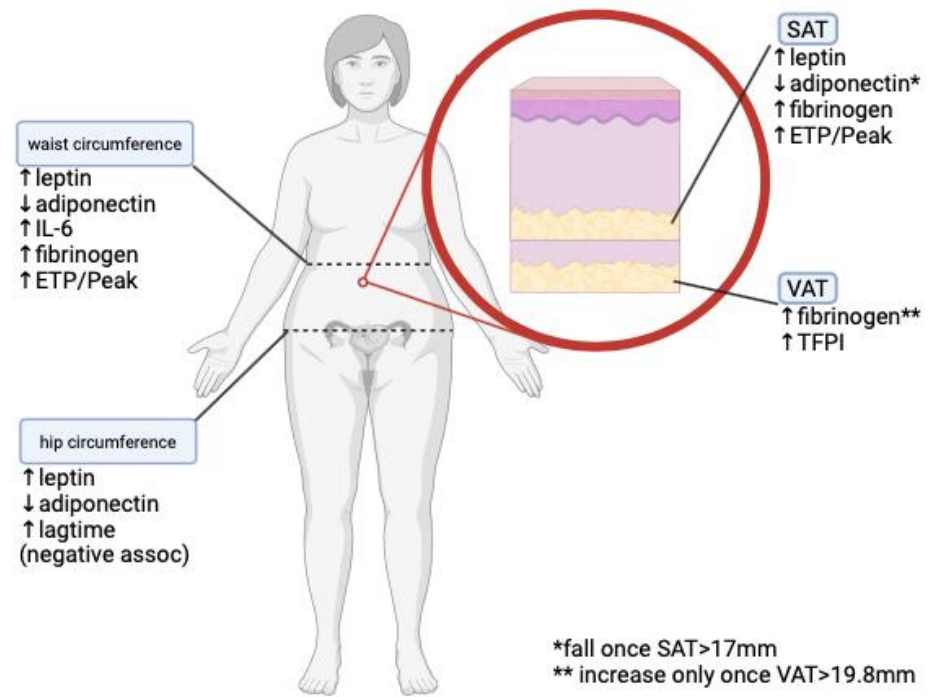


Figure 5-43 Associations between anthropomorphic measurements and markers of haemostasis and adipokines. Each variable is associated independently of BMI. Variables which showed a threshold effect also included and denoted by asterisk.

These findings and how they may relate to the underlying pathophysiology of the prothrombotic state in obese pregnancy will be discussed in further detail.

5.8.2 Relationship between measures of adipose tissue distribution and BMI

Whilst BMI is a useful tool to quickly identify overweight and obese subjects, it is known to provide a poor assessment of body fat distribution, which has been shown to be an important indicator of cardiovascular risk.

As might have been anticipated, waist and hip circumference at booking both strongly correlated with BMI (adjusted $R^2=0.88$, $p<0.0001$). Waist:hip ratio, which provides an assessment of body shape, did not correlate as strongly with BMI as the individual measurements (adjusted $R^2=0.21$), indicating that BMI does not reflect body shape. Both SAT and VAT thickness at booking correlated positively with BMI (adjusted $R^2=0.70$ and 0.46 , respectively) but one-way ANOVA comparing

the four BMI groups found no significant difference in SAT:VAT ratio ($p=0.32$) implying that BMI provides no insight into the composition of adipose tissue.

It was observed that waist circumference reached a plateau when BMI reached 40, whereas hip circumference continued to have a linear relationship with BMI suggesting excess adipose tissue is deposited more in the lower body SAT depots than centrally. The analyses of the relationship between BMI and abdominal SAT and VAT thickness found that central SAT thickness plateaued once BMI reached 40 whilst the relationship with VAT remained linear. Taken together, these data might suggest that in obese women, excess adipose is preferentially deposited centrally as VAT rather than SAT, or it is being deposited outwith the area measured by ultrasound in this study e.g into hip region. It is not possible to distinguish at this study point whether the adipose distribution relates to pregnancy or to pre-gestational obesity but given the early gestation at which the scan was undertaken (mean 14.6 weeks) it seems most likely that pre-pregnancy body composition is having the greatest influence.

The “adipose tissue overflow” hypothesis, described by Sniderman *et al* and several others initially as an ethnic variation in adipose tissue distribution, may offer an explanation for the differences observed in adipose tissue distribution in those with BMI >40 in this study. They suggested that the expansion of adipose tissue into the deep SAT (DSAT) and VAT compartments occurs once the more stable subcutaneous SAT (SSAT) depot reaches capacity. (Sniderman *et al.*, 2007) There is evidence to support this theory when the current study population was analysed within SAT quartiles, the VAT thickness increased exponentially as SAT reached the 3rd quartile.

The adipose tissue overflow hypothesis has been recognised previously within the pregnant population in conditions such as gestational diabetes mellitus and pre-eclampsia, where VAT thickness has been positively correlated with metabolic parameters. (Trivett, Lees and Freeman, 2021; Bartha *et al.*, 2007; Gur *et al.*, 2014). Martin *et al* and others found a significant positive association between VAT thickness and the development of gestational diabetes. (Martin *et al.*, 2009; Li *et al.*, 2024) Whilst Huda *et al* demonstrated *ex vivo* stimulation of visceral adipocytes from mothers with pre-eclampsia released greater concentrations of inflammatory markers including IL6, TNF- α and CCL-2, compared to visceral

adipocytes from healthy pregnant women. (Huda *et al.*, 2017) In healthy pregnancy, VAT is preferentially deposited (Kinoshita and Itoh, 2006), but obese women may be entering pregnancy with excess VAT deposited due to adipocyte dysfunction and as such this may affect the ability of the obese VAT depot to respond to pregnancy in the same way that the lean VAT depot would. This pattern was observed in a study of gestational weight gain by Straughen *et al.*: Weight gain was at a slower rate in overweight compared to lean pregnant women and the distribution of adipose tissue between SAT and VAT compartments was subtly different. Total fat thickness increased in lean and decreased in overweight/obese women, but the proportion of VAT increased more in lean compared to overweight/obese. (Straughen, Trudeau and Misra, 2013)

These data have identified in this pregnant population, as has been shown previously in the general population, that whilst BMI is a useful tool in assessing total mass of body fat, it does not accurately reflect the distribution of body fat and the inherent risks with which different phenotypes of adipose tissue distribution may be associated.

5.8.3 Relationship between measures of adipose tissue distribution and adipokines

Leptin was the adipokine which most strongly correlated with measures of adipose tissue distribution, showing strong positive correlations with both upper and lower body SAT. Given that leptin is produced by adipocytes, our findings may reflect that leptin is associated with total fat mass rather than location or differing properties of adipose tissue.

IL-6, in contrast, was associated with evidence of central obesity, with waist circumference independently positively correlated with IL-6. VAT appeared to be colinear with BMI but was included in the final regression model alongside waist circumference which was able to predict 24% of variance in IL-6. This finding may reflect the already well-recognised pro-inflammatory effects of central obesity within the pregnant and non-pregnant populations. However, the lack of independent association between VAT and IL-6 was surprising, given the evidence in pregnant and non-pregnant individuals that VAT is the more pro-inflammatory depot. (Stewart *et al.*, 2007; Kahn *et al.*, 2022; Pou *et al.*, 2007; Schlecht *et al.*,

2016) Given the relationship with waist circumference, it could be that the production of IL-6 relates to the inflammatory properties of unmeasured deep SAT, which has been associated with inflammatory markers and the metabolic syndrome similarly to VAT. (Smith *et al.*, 2001; Kim *et al.*, 2016)

In summary, it appears that plasma leptin concentrations reflected total fat mass, whereas the anti-inflammatory adipokine, adiponectin, was negatively associated with central adiposity and SAT. IL-6, was associated with waist circumference but not VAT. TNF- α and IL-1 β showed no significant associations with adipose tissue distribution.

Taken together these data suggest that in pregnant women at booking, central adiposity, but not exclusively VAT, results in a pro-inflammatory state. This could reflect lesser-recognised inflammatory properties of SAT, changes which develop in pregnancy, or may suggest that what we are detecting is the influence of deep SAT, which was not quantified in this study.

5.8.4 Relationship between measures of adipose tissue distribution and markers of haemostasis

Anthropometric measures correlated with fibrinogen Clauss, TFPI and PAI-1, but the relationship with PAI-1 was colinear with BMI and therefore not independent. There was no correlation between any of the measures of adipose tissue distribution and Factor VIII, D-dimer or protein S.

Fibrinogen was strongly positively correlated with waist circumference and SAT. The devised multiple regression model comprised of SAT, waist circumference and a negative relationship with hip circumference was able to explain 30.04% of variance in fibrinogen concentrations, independently of BMI. This suggests that higher fibrinogen concentrations are associated with the deposition of additional SAT in the waist region in preference to the hip region, i.e. a central obesity distribution.

VAT was not found to be independently correlated with fibrinogen, due to collinearity with SAT. However, when fibrinogen was analysed within VAT quartiles, a threshold effect was evident showing a significant ($p=0.008$) increase in fibrinogen once VAT reached a thickness of 19.8mm (4th quartile) a finding

which may have been too small to have identified within multiple regression modelling. This may reflect the inflammatory potential of VAT, as fibrinogen is known to be an acute phase protein released as part of the inflammatory response, however it does also suggest that having very high levels of VAT (>19.8mm) may contribute exponentially to the prothrombotic state in obese pregnancy. A previous study by Gur *et al* found a VAT thickness of 19.5mm was the optimal cut-off point in prediction of gestational diabetes mellitus, therefore although these findings weren't below the significance level in this study, they may suggest a shared pathophysiology between thrombotic risk and the metabolic syndrome in pregnancy. (Gur *et al.*, 2014)

Central obesity, measured by VAT, in combination with BMI were found to be positively and independently predictive of TFPI, able to account for 11.99% of variance suggesting that obesity and adipose tissue distribution have only a minor influence on TFPI levels. TFPI is a recognised naturally occurring anticoagulant, therefore its positive association with markers of obesity seems contrary to the hypothesis that obesity is associated with a prothrombotic state. TFPI has also been recognised as a marker of endothelial dysfunction and could also reflect a regulatory mechanism developed in obesity to counter the increased thrombotic risk.

In summary, central adipose tissue distribution, measured by waist circumference and SAT rather than VAT, has a strong positive association with fibrinogen levels. Although there was a significant increase in fibrinogen when VAT thickness was >19.8m, SAT showed the closer association with fibrinogen. This again suggests that the central adiposity link with the pro-inflammatory and prothrombotic state may be mediated via inflammatory SAT or deep SAT rather than VAT as was hypothesised. The positive correlation between VAT and TFPI would not contribute to the prothrombotic state but may reflect evidence of endothelial dysfunction in obesity.

5.8.5 Relationship between measures of adipose tissue distribution and thrombin generation

SAT thickness was the anthropomorphic measure most strongly associated with the thrombin generation parameters, with independent and positive association

Chapter 5

with each of lagtime, ETP, peak thrombin and velocity index. The adjusted R^2 values of the multiple regression models were low for all of these parameters suggesting that anthropomorphic measures do not have a strong influence on thrombin generation.

Interestingly, a prolongation of lagtime was associated with both upper body SAT and VAT (both $r=0.31$, $p<0.0001$), with evidence that once VAT reaches 19.8mm there was a significant increase in lagtime ($p=0.039$). The multiple regression analysis final stepwise model found that SAT, VAT, BMI and hip circumference were the strongest predictors for lagtime, but this model was only able to predict 16.93% of the variance in lagtime. In a prothrombotic state, a shortening of lagtime would be expected, however a similar phenomenon has been found in association with high levels of TFPI, fibrinogen and protein S which can prolong lagtime *in vitro* without affecting ETP and peak thrombin. (Chitongo *et al.*, 2017; Brodin *et al.*, 2009) TFPI, fibrinogen and protein S were all positively correlated with BMI and VAT so this could be the explanation for this apparent discrepancy in lagtime results.

In summary anthropometry is a poor predictor of thrombin generation, however SAT correlates most frequently, and does contribute independently of BMI to the prothrombotic state.

5.8.6 Limitations

There were several limitations of this study in determining the influence of adipose tissue distribution on thrombin generation.

Sample size

The main IPOP study group consisted of 262 participants, yet complete adipose distribution data to include SAT and VAT thickness were only available on 160 women. Unfortunately, due to time pressures during the routine antenatal dating scan which all women receiving at around 12 weeks' gestation, the obstetric sonographers felt they would not be able to undertake study measurements of SAT and VAT, and as such a research-specific scan was arranged to obtain these measurements. As this was an additional voluntary attendance to the hospital,

and with no direct benefit to the study participant's pregnancy, the attendance rate was only 61%. A substudy group of the 160 women who did attend for analysis purposes, was therefore analysed which did have a proportionate spread of BMIs, comparable to the main study. However, ideally the study scan measurements would have been obtained at the routine antenatal scan to maximise collection of SAT and VAT data, providing a larger sample size for more robust conclusions.

Method for measurement of SAT and VAT

The 'gold standard' for accurate quantification of visceral adipose tissue is by CT or MRI scan as these give accurate, reproducible cross-sectional images in multiple planes by which accurate volumetric assessment of VAT can be ascertained. However, these methodologies are generally confined to research-use due to availability in clinical practice. In addition, CT uses ionising radiation which is harmful to the unborn foetus and to developing maternal breast tissue and reproductive organs, and as such would not be suitable for use in the pregnant population. The rationale for using ultrasound scan in this study was to utilise a readily-available technique which pregnant women would already be undergoing during pregnancy, so that any identified associations with thrombotic risk could be easily extrapolated into clinical risk scoring for VTE assessment during pregnancy. By using ultrasound, we recognise the measurements are only 2-dimensional and can be prone to inter- and intra-operator variability.

Timing of SAT/VAT scan

As the SAT/VAT scan required women to find time to attend for an additional appointment which only took place weekly, some women may not have been able to attend until close to the 17 week gestation cut off for completing the scan. The average gestation at time of the SAT/VAT scan was 14.6 weeks, with most women already into the second trimester of pregnancy before SAT and VAT thickness were measured. As it is known that VAT expands and SAT declines during the second trimester, SAT and VAT thickness may have been influenced by these pregnancy-related changes rather than reflecting early pregnancy adipose tissue depots, which have been identified predictors of metabolic disease in pregnancy. (Li *et al.*, 2024)

One potential explanation for why SAT was more closely related to pro-inflammatory cytokines and the prothrombotic state than VAT is that there may be an unmeasured proportion of the more metabolically active deep SAT which is influencing the prothrombotic state. In this study subdivisions in the measurement of SAT were not assessed so it is impossible to draw any conclusions on this hypothesis, but this could be something to consider in future studies.

5.8.7 Summary

Considering all of the anthropometric data together, SAT and waist circumference appeared to be the most frequently associated anthropometric measures, independent of BMI, in predicting adipokines and markers of haemostasis. With regard to the primary study endpoint in predicting thrombotic risk, SAT was the strongest anthropometric predictor of thrombin generation (ETP) and added to the predictive value of BMI, however the overall predictive value of the multiple regression model was low at 10.66%, suggesting that in isolation anthropometric measures are not strong predictors of thrombotic risk. These findings are in contrast to the work of Chitongo et al, whose study in class III obese non-pregnant individuals found VAT to have the strongest association with thrombin generation parameters.(Chitongo *et al.*, 2017)

There are a number of differences in the study design between this study and that of Chitongo et al which could be contributing to our opposing findings. Firstly, the Chitongo study was in older (mean age 44), non-pregnant individuals so the differences could reflect phenotypic alterations in SAT and VAT attributable to both age and pregnancy. Secondly, the method of quantifying adipose tissue differed as our study used abdominal ultrasound compared to software-assisted volume quantification by CT scanning which likely provides a more accurate and reproducible measurement. And thirdly, the Chitongo study focused on class III obese individuals, therefore, as the current study has demonstrated, there are differences in properties of SAT and VAT in women of different BMI groups. Our study had only a small proportion of participants with BMI ≥ 40 therefore associations specific to this group may have been missed. Furthermore, both studies only measured SAT and VAT, meaning that any functional differences in

superficial SAT and deep SAT will not have been identified and correlations between the more metabolically active DSAT and proinflammatory cytokines and thrombin generation potentially overlooked.

SAT has been associated with VTE previously in an autopsy-based study by Ogren *et al.* (Ogren *et al.*, 2005) It may be that in both Ogren's study and the current study that it is the more metabolically active subdivision of SAT, DSAT, that is exerting the pro-inflammatory effect. DSAT is predominantly located in the abdomen and separated from the superficial subcutaneous adipose tissue (SSAT) by a fascial plane. Several studies have explored the role of deep subcutaneous adipose tissue (DSAT) in cardiovascular risk, with the metabolic properties of DSAT similar to those of VAT. (Kelley *et al.*, 2000) When compared to SSAT, DSAT is more vascular, has less stable triglyceride storage, and is strongly associated with features of the metabolic syndrome including insulin resistance, dyslipidaemia and potentially with pro-inflammatory cytokines. (Sniderman *et al.*, 2007)

Considering data within the pregnant population, Straughen *et al.*'s study investigating gestational weight gain, the rate of change of the VAT:SAT ratio was greater in lean compared to overweight/obese women, a phenomenon seen in some haemostasis parameters in our study and may explain the reduced flexibility observed by our group with some metabolic parameters in pregnancy. (Stewart *et al.*, 2007)

The impaired response to pregnancy observed in metabolic parameters in obese pregnancy, and in the current study with attenuated gestational changes in thrombin generation, PAI-1 and TFPI, may relate to adipocyte dysfunction as outlined previously in the adipocyte expandability hypothesis by Suárez-Cuenca *et al.* (Suárez-Cuenca *et al.*, 2021) Adipocyte size has been demonstrated to relate to function, with small adipocytes protective against metabolic dysfunction. In the expansion of adipose tissue, adipocytes can expand in a 'healthy' hyperplastic manner or, in the case of adipocyte dysfunction, in a hypertrophic fashion. The hypertrophic adipocytes are associated with increased leptin and reduced adiponectin (as observed in our cohort) then more prone to lipolysis, with increased cell death attracting more adipose tissue macrophages. The macrophages become polarised towards the pro-inflammatory M1 phenotype and there is increased release of cytokines including TNF- α , IL-1 β and IL-6, which in

turn stimulate the increase in fibrinogen. Our cohort demonstrated increased IL-6 and fibrinogen associated with increasing adiposity, but Q3 VAT (>17mm) was associated with a significant fall in adiponectin and Q4 VAT (>19.8mm) associated with increases in PAI-1 and fibrinogen suggesting at this critical VAT mass there is an alteration in adipocyte function.

Our initial hypothesis that pro-inflammatory cytokines produced by excess VAT contribute to increased thrombin generation was not correct, as SAT was seen to be more closely associated with thrombin generation than VAT. However, if the SAT and waist circumference measurements were known to reflect accumulation of DSAT, then it remains possible that the correlations with leptin, adiponectin, IL-6, fibrinogen relate to the more metabolically active DSAT portion and that the theory of the chronic proinflammatory state being the driver for thrombotic risk in obesity remains true. The proposed influence of adipose tissue distribution on the prothrombotic state in obese pregnancy is illustrated in Figure 5-44.

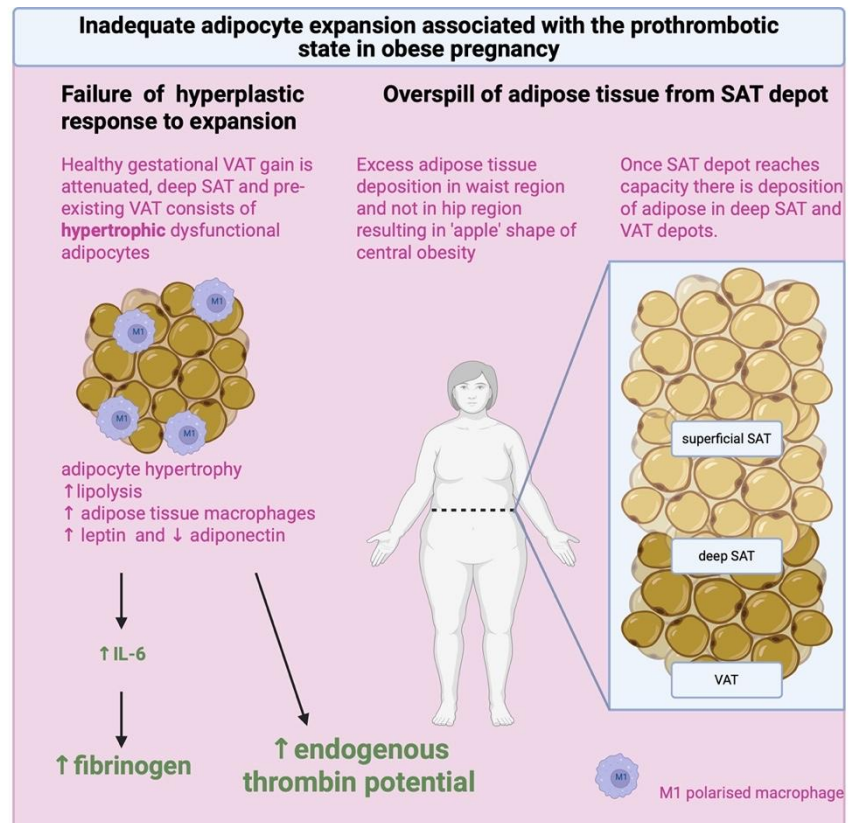


Figure 5-44 Adipose tissue distribution in obese pregnancy and association with the prothrombotic state.

Obese women enter pregnancy with an excess of adipose tissue which, for women at greatest risk, is deposited in the waist region rather than the hips resulting in the 'apple' shape of central obesity. SAT was more closely related to thrombotic risk in this study and we propose that excess adipose tissue is deposited in VAT and the similarly active deep SAT. Normal healthy gestational VAT gain is attenuated and the adipose tissue in the deep SAT and VAT depots consists of dysfunctional, hypertrophic adipocytes. These adipocytes are prone to increased lipolysis, increased recruitment of adipose tissue macrophages and polarisation of these macrophages to the pro-inflammatory M1 phenotype which results in release of pro-inflammatory cytokines, particularly IL-6. Adipokines are dysregulated resulting in increased leptin and a fall in the anti-inflammatory adiponectin. IL-6 and fibrinogen are increased and there is also increased thrombin generation (as measured by ETP)

The data in this chapter has shown that the assessment of waist circumference and SAT thickness provides additional information over and above that of BMI, but *ex vivo* adipocyte studies looking at the differing properties of SAT (superficial and deep) and VAT adipocytes in healthy women of different BMI during pregnancy, alongside thrombin generation data, may help to elucidate how SAT and VAT properties differ in these BMI groups and how this might be affecting thrombin generation.

In the final chapter, using the most significant findings of the preceding chapters, the adipokine and anthropomorphic parameters will be analysed alongside the

Chapter 5

haemostasis markers to determine the pathways involved in the pathophysiology underlying the prothrombotic state in obese pregnancy.

Chapter 6 The pathophysiology underlying the prothrombotic state in obese pregnant women

6.1 Background

The rationale for undertaking this study was to characterise the prothrombotic phenotype of obese pregnancy, which would allow identification of risk factors for thrombotic risk in this population and ultimately targets for developing strategies to allow mitigation of this risk. In order to do this, an accurate understanding of the pathways involved in the development of the prothrombotic state is necessary. The previous chapters have examined the individual relationships between measures of the prothrombotic state, adipokines and adipose tissue distribution but in order to appreciate the underlying pathophysiology leading to the prothrombotic state in obese pregnancy the interactions between all of these factors need to be understood. The strongest relationships from each of the previous chapters will be taken together in this chapter to determine the characteristics which best predict the prothrombotic state, as measured by thrombin generation, during pregnancy.

In the current study, measures of the prothrombotic state; ETP, Fibrinogen, TFPI and PAI-1; were observed to show a significant association with time and BMI during pregnancy. Considering the adipokine and adipose tissue distribution parameters which may be driving these prothrombotic measures, the adipokine leptin, in addition to anthropometric assessment by BMI, waist circumference and SAT, was found to be positively correlated with ETP. Fibrinogen Clauss was found to be strongly associated with leptin, waist circumference and SAT, and studies have previously identified fibrinogen to be associated with thrombotic risk.(Aleman *et al.*, 2014; Klovaite *et al.*, 2013) TFPI was independently associated with BMI and the measure of central obesity, VAT, but had no significant associations with any adipokines. PAI-1, whilst showing a significant correlation with both time and BMI during pregnancy, was only significantly associated with BMI and none of the other anthropomorphic measures at booking.

In support of the original hypothesis for this study, that pro-inflammatory dysfunctional adipocytes and adipose tissue distribution in obese pregnancy were driving thrombotic risk, the relationship between leptin and fibrinogen appeared

to be IL-6 dependent. In turn, IL-6 was associated with SAT and waist circumference, suggesting that the central obesity phenotype, although not VAT, contributes to the prothrombotic state.

In this short chapter, statistical analyses will identify the variables identified so far which best predict endogenous thrombin potential (ETP), the biomarker of thrombosis used in this study. In addition, surrogate measures of haemostasis which each showed an association BMI at booking; fibrinogen, PAI-1 and TFPI, will be analysed to identify which adipokine and adipose tissue distribution variables are most closely associated. The analysis of fibrinogen and TFPI will aim to strengthen the thrombin generation findings, whilst PAI-1 will show the impact of obesity during pregnancy on fibrinolysis, an important determinant of thrombotic risk.

6.2 Aims and Objectives

6.2.1 Aims

To determine, based on the findings of Chapters 3-5, which of the adipokines and which measurements of adipose tissue distribution are most closely related to thrombin generation and the other measured haemostasis parameters known to be affected by BMI in obese pregnant women.

6.2.2 Hypothesis

The increased risk of VTE in obese pregnant women, as measured by thrombin generation, is positively correlated with leptin and IL-6, BMI, waist circumference and SAT.

6.2.3 Specific Research Questions

1. What is the best statistical model to predict fibrinogen, incorporating the parameters known to be associated with fibrinogen: leptin, IL-6, SAT, waist circumference and BMI
2. What is the best statistical model to predict TFPI, incorporating the parameters known to be associated with TFPI: VAT, IL-6 and BMI

3. What is the best statistical model to predict PAI-1, incorporating the parameters known to be associated with PAI-1: adiponectin and BMI

4. What is the statistical model best able to predict ETP, incorporating parameters known to be associated with ETP: leptin, SAT, waist circumference, BMI

6.3 Methods

6.3.1 Study population

The study population was the ‘sub-study’ group (n=160) as previously described in detail in Chapter 5 as this population allowed analysis of the anthropometric data alongside the haemostasis and adipokine data obtained at booking.

6.3.2 Statistical analyses

Analyses were focussed on ETP, fibrinogen, TFPI and PAI-1 at booking as these were the haemostatic parameters which showed a significant relationship with BMI. In addition, haemostasis parameters at the booking appointment were able to be related to both plasma concentrations of adipokines and measurements of adipose tissue distribution taken at the same time point. BMI and adipose tissue distribution were not re-measured at 28 weeks’ gestation therefore relating adipokine and haemostasis data accurately to the adipose tissue distribution was not possible.

Multiple regression analysis for each of the haemostatic parameters (ETP, fibrinogen, TFPI and PAI-1) were undertaken. In each model, the adipokine and anthropometric parameters found to be significantly associated in the relevant previous chapters were nominated as predictors, alongside BMI. Stepwise multiple regression analysis was used, with α -value set at 0.15 to remain/remove.

Taking together all of the conclusions from earlier chapters, a theory for how each of the variables measured interact to explain the increased risk of thrombosis in obese pregnancy has been proposed. A curated multiple regression analysis model was used to test these theories. To prove a dependent relationship or pathway, a reduction in adjusted R^2 and non-significance of the p-values were observed with the addition of a parameter.

6.4 Results

6.4.1 Predictive models for fibrinogen, TFPI and PAI-1

6.4.1.1 Prediction of fibrinogen

Plasma fibrinogen concentration was seen to be positively correlated with BMI and increase with advancing gestation in this study. Fibrinogen concentration is one component of the procoagulant state which has been extensively associated in previous studies to be associated with VTE risk, and understanding which adipokine and anthropomorphic parameters have influenced fibrinogen can aid in determining the pathophysiology of the prothrombotic state. The parameters identified as significantly associated with fibrinogen in this study were BMI, waist circumference (adj R^2 =27.82% p =0.0002 independent of BMI and hip circumference), SAT (adj- R^2 =27.09%, p =0.002 independent of BMI and VAT), leptin (adj R^2 =20.08%, p <0.0001 independent of BMI) and IL-6 (adj- R^2 =26.44%, p <0.0001 independent of BMI). The stepwise multiple regression model in Table 30 was able to predict 32.01% of variance in plasma fibrinogen concentrations (p <0.0001):

Table 30 Multiple regression model for prediction of variability in fibrinogen at booking.

Stepwise multiple regression, α =0.15 to keep/remove

$$\text{Log-fibrinogen} = 0.2736 + 0.101 \log\text{-SAT} + 0.0013 \log\text{-waist circ} + 0.14 \log\text{-IL-6}$$

adj- R^2 = 32.01%, p <0.0001

Variable	β co-efficient	p-value
log-SAT	0.101	0.023
log-waist circumference	0.0013	0.06
log-IL-6	0.14	0.006

This regression model suggests the central obesity body shape, pro-inflammatory state and increased SAT are positively associated with fibrinogen, mirroring the findings seen with ETP. The association between leptin and fibrinogen which was demonstrated in Chapter 4 was no longer evident in this model, but this may be due to collinearity with SAT.

6.4.1.2 Prediction of TFPI

TFPI was weakly but independently associated with VAT (adj $R^2=11.99\%$, $p=0.042$) and BMI (adj $R^2=11.99\%$, $p=0.047$) in Chapter 5, and with IL-6 (adj $R^2=17.22\%$, $p=0.0005$ independent of BMI) in Chapter 4. A stepwise multiple regression model was used to identify the parameters most predictive of TFPI levels (Table 31). The final model ($p<0.0001$) was able to predict 12.91% of variance in TFPI levels:

Table 31 Multiple regression model for prediction of variability in TFPI at booking.

Stepwise multiple regression, $\alpha=0.15$ to keep/remove

$$\text{Log-TFPI} = 0.7659 + 0.189 \log\text{-IL-6} + 0.1008 \log\text{-VAT} \text{ and } 0.0367 \text{ BMI}$$

adj- $R^2=12.91\%$, $p<0.0001$

Variable	β co-efficient	p-value
log-IL-6	0.189	0.11
log-VAT	0.1008	0.085
BMI	0.0367	0.14

TFPI was positively associated with BMI, IL-6 and VAT, but p-values were all non-significant, implying collinearity. This suggests TFPI at booking is associated with the pro-inflammatory central obesity phenotype and it may be increased in obesity in an effort to counteract the procoagulant substances increased in obesity. Alternatively, TFPI is considered a marker of endothelial dysfunction which is known to occur in obesity, therefore elevated TFPI may reflect the endothelial dysfunction in obesity with a 'double effect' of some anticoagulant activity which is not at a sufficient level to mitigate the procoagulant substances in obesity. (Morange *et al.*, 2001) Increased TFPI in the obese groups explains the contradictory thrombin generation profiles, which showed prolongation of lagtime (indicative of reduced coagulation) yet elevated peak, ETP and velocity index (parameters associated with increased thrombotic potential). Previous studies have identified TFPI prolongs lagtime in the thrombin generation assay but is known to not have significant effect on ETP, therefore the increase in TFPI must not be sufficient to counterbalance the prothrombotic substances contributing to thrombin generation. (Brodin *et al.*, 2009)

6.4.1.3 Prediction of PAI-1

PAI-1 was found to be significantly positively associated with BMI ($p < 0.0001$) and very weakly negatively associated with adiponectin (adj $R^2 = 12.80\%$, $p = 0.026$, independent of BMI). Linear regression found these variables to predict 13.22% of variance in PAI-1.

Table 32 Multiple regression model for prediction of variability in PAI-1 at booking.

Stepwise multiple regression, $\alpha = 0.15$ to keep/remove

$$\text{Log-PAI-1} = 0.914 + 0.0096 \text{ BMI} - 0.13 \text{ log-adiponectin}$$

adj- $R^2 = 13.22\%$, $p < 0.0001$

Variable	B co-efficient	p-value
log-adiponectin	-0.13	0.069
BMI	0.0096	<0.0001

PAI-1 reflects fibrinolysis which is known to be dysregulated in obesity and pregnancy. It would not contribute to ETP, as the CAT methodology does not assess fibrinolysis but does contribute to thrombotic risk. BMI is the most important predictor of PAI-1, but low adiponectin is indicative of dysfunctional adipose tissue and may have a role.

6.4.2 Predictive model for ETP using significant adipokine and anthropometric parameters

A stepwise multiple regression model was used to identify, using the significant parameters identified in previous chapters, exclusive of other haemostasis parameters, which were greatest at predicting variance in ETP. Included in this model were BMI, waist circumference (adj $R^2=4.32\%$, $p=0.017$ independent of BMI), SAT (adj- $R^2=9.50\%$, $p=0.001$ independent of BMI), and leptin (adj- $R^2=15.04\%$, $p<0.0001$ independent of BMI). The stepwise mode used an α value of 0.15 to keep/remove. The final regression equation ($p<0.0001$) was able to predict 13.62% of variance in ETP:

Table 33 Multiple regression model for prediction of variability in ETP at booking.

Stepwise multiple regression, $\alpha=15$ to keep/remove

$$\text{Log-ETP} = 2.51 - 0.00504 \text{ BMI} + 0.1187 \log \text{ SAT} + 0.1408 \log \text{-leptin}$$

adj- $R^2=13.62\%$, $p<0.0001$

Variable	β co-efficient	p-value
BMI	-0.00504	0.012
log-SAT	0.1187	0.14
log-leptin	0.1408	0.007

Leptin ($p=0.007$) and BMI ($p=0.012$) were independently associated with ETP, whilst waist circumference and SAT did not remain associated despite SAT being the anthropomorphic measure most associated with ETP in Chapter 5. This may reflect the strong positive relationship between SAT and leptin, which may be indicative of collinearity in this statistical model.

6.5 Proposed pathophysiological pathways for the development of the prothrombotic state in obese pregnant women

Taking together all of the most significant results from this and the previous chapters, a proposed mechanism for the development of the prothrombotic state in obese pregnant women at booking is outlined in Figure 6-1.

Chapter 6

The current study identified that obese pregnant women started pregnancy with higher fibrinogen and ETP, but also higher concentrations of naturally occurring anticoagulants, particularly TFPI, when compared to lean counterparts. PAI-1 was also positively correlated with BMI.

Based upon the findings of Chapter 5, central obesity and accumulation of SAT, and to a lesser extent VAT, is associated with hyperleptinaemia which may lead to the recruitment and phenotypic switch of adipose tissue macrophages to the pro-inflammatory M1 phenotype, as described by Acedo *et al.* M1 ATMs release inflammatory cytokines, in particular IL-6 which induces hepatic productions of fibrinogen which likely contributes to the prothrombotic state. The procoagulant state, as assessed by ETP is associated with BMI, SAT and leptin, but the mechanism appears to be IL-6 independent, suggesting alternative methods through which hyperleptinaemia induces a prothrombotic state (see Figure 6-1). Release of IL-6 from VAT contributes to the increase in TFPI, which in addition to having anticoagulant properties is also a measure of endothelial dysfunction.

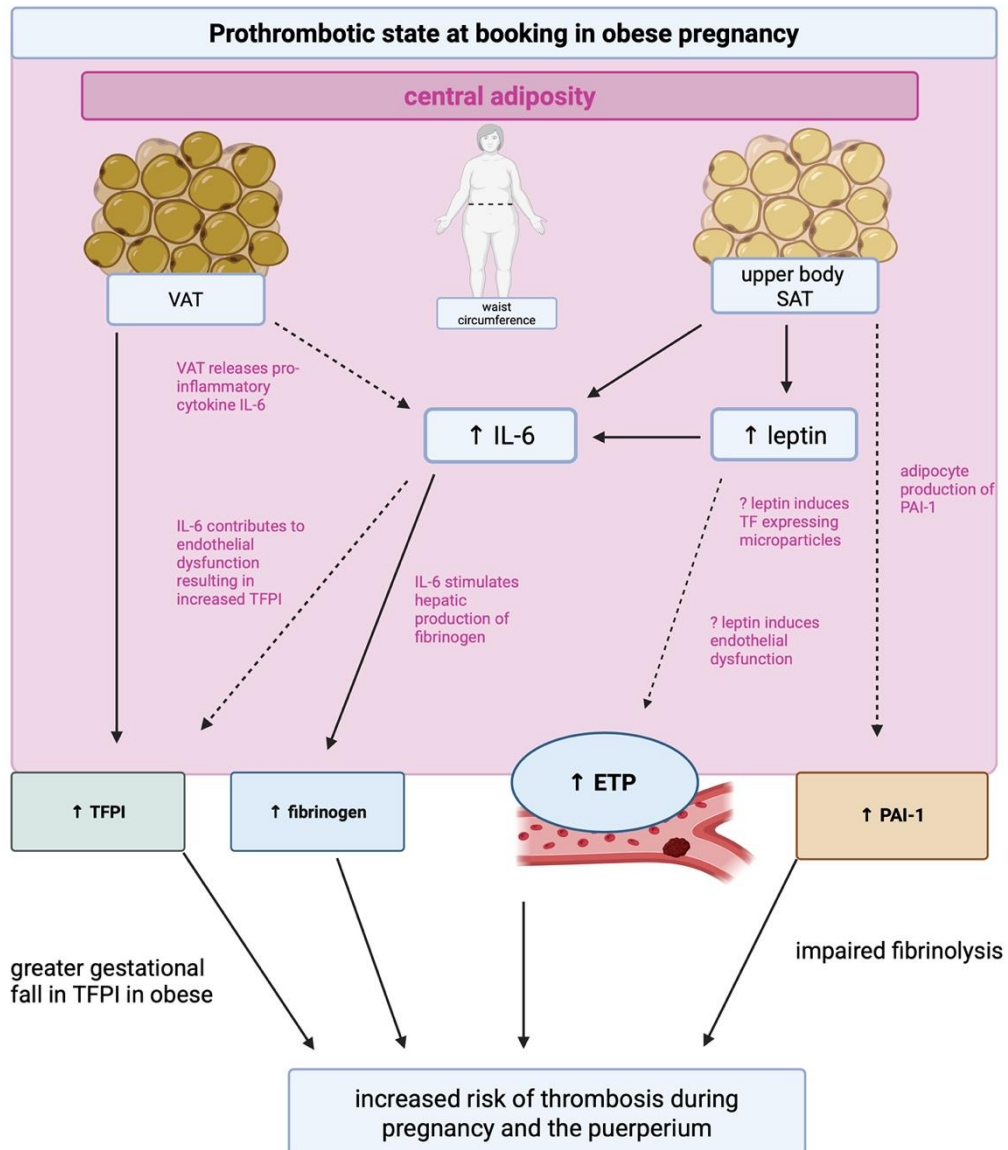


Figure 6-1 Pathophysiology underlying the increased risk of thrombosis in obese pregnant women.

The central obesity phenotype, with increased waist circumference and increased SAT, was seen in the study to be associated with hyperleptinaemia and elevated IL-6. IL-6 stimulates hepatic production of fibrinogen resulting in hyperfibrinogenaemia which may contribute to the prothrombotic state. Leptin may cause increased thrombin generation via induction of tissue factor-expressing microparticles or endothelial dysfunction. VAT was positively associated with TFPI, which may be a marker of endothelial dysfunction, whilst SAT was associated with PAI-1 which indicates impaired fibrinolysis. The prothrombotic state in obese pregnancy is present from early pregnancy and therefore more prolonged than in lean pregnancy, and may be further exacerbated by the greater gestational fall in TFPI with advancing gestation.

Solid arrows indicate associations demonstrated in the current study, dashed arrows indicate supporting theories from literature. Created in <https://BioRender.com>.

For the proposed pathway towards increased ETP, as suggested in Figure 6-1, multiple regression analysis was used to confirm if the variables within the pathway were truly dependent upon each other.

6.5.1 Analysis of the SAT-ETP pathway

Table 34 shows the sequential multiple regression analysis of the parameters from the SAT-leptin-ETP pathway (Figure 6-1).

Table 34 Multiple regression analysis of SAT-ETP pathway

SAT-ETP pathway			
Variables in model	β co-efficient	p-value	model adj-R ²
log-SAT	0.17	<0.0001	9.13%
log-SAT	0.049	0.52	10.64%
log-leptin	0.094	0.057	

The analysis in Table 34 demonstrates loss of significance of SAT when leptin is added to the model which suggests the proposed pathway from SAT to ETP may have merit. However, given that this model is only able to explain 10.64% of variance in ETP, it indicates there are other pathways which contribute to the ETP increase in obese pregnant women.

6.5.2 Analysis of the SAT-fibrinogen pathway

To strengthen the thrombin generation findings with further evidence of the prothrombotic state and confirm the hypothesised pathways in Figure 6-1, the pathways thought to contribute to increased fibrinogen were analysed by multiple regression modelling.

The SAT-fibrinogen pathway is outlined in Table 35.

Table 35 Multiple regression analysis of SAT-fibrinogen pathway*Fibrinogen pathway (SAT)*

Variables in model	B co-efficient	p-value	model adj-R ²
log-SAT	0.21	<0.0001	26.54%
log-SAT	0.16	0.002	26.67%
log-leptin	0.036	0.26	
log-SAT	0.14	0.005	30.62%
log-leptin	0.019	0.54	
log-IL-6	0.16	0.002	

Table 35 shows the significant positive relationship between SAT and fibrinogen, which remained independent following the addition of leptin to the model with minimal change in the adjusted-R² suggesting that the SAT and leptin relationship was dependent. IL-6 was identified to have a significant correlation with fibrinogen, independently of SAT.

6.5.3 Analysis of the VAT-fibrinogen pathway

The parameters from the VAT-fibrinogen pathway, as indicated in Figure 6-1, were analysed within a multiple regression analysis model as built up in Table 36.

Table 36 Multiple regression analysis of VAT-fibrinogen pathway*Fibrinogen pathway (VAT)*

Variables in model	B co-efficient	p-value	model adj-R ²
log-VAT	0.12	<0.0001	16.46%
log-VAT	0.080	0.00029	23.97%
log-IL-6	0.21	<0.0001	

The VAT-fibrinogen pathway was also assessed (Table 36) and both VAT and IL-6 found to be independent predictors of fibrinogen. This model was able to explain 23.97% of variance in fibrinogen, yet VAT and IL-6 have been identified as independent of each other, suggesting an alternate influence on IL-6 production.

Given the findings in Table 34, in which SAT and IL-6 were independent predictors of fibrinogen, it may stand to reason that the persistent independence of IL-6 across both models suggest the dual influence of both SAT and VAT on IL-6 production.

6.6 Discussion

6.6.1 Proposed pro-coagulant pathways in obese pregnancy

At booking, this study has shown obese women to have higher fibrinogen, ETP, TFPI and PAI-1 compared to lean counterparts. As depicted in Figure 6-1 and analysed in detail above, central obesity and SAT appear to have the greatest influence upon the development of the prothrombotic state. The SAT-ETP pathway model was able to predict 10.64% of variance in ETP suggesting there are additional mechanisms, which have not been investigated in the current study, through which ETP is increased in obese pregnant women.

However, the relationships between fibrinogen and the measurements of adipose tissue distribution were considerably stronger than those seen with ETP. SAT and VAT were both implicated in increasing fibrinogen in pregnancy complicated by obesity, via IL-6. The SAT-fibrinogen pathway was superior at predicting the procoagulant state compared to the VAT pathway (30.62% vs 23.97%), and both pathways demonstrated closer associations with fibrinogen than those with ETP. These analyses also identified independent associations between SAT, IL-6 and fibrinogen, and suggested the dual influence of SAT and VAT on IL-6 production.

These findings in pregnancy are unique to this study, as the influence of adipokines and adipose tissue distribution on thrombin generation have not, in the literature published to date, been elucidated.

6.6.2 Limitations

The analyses for this chapter were limited to the ultrasound substudy group (n=160) due to the availability of complete data sets of both laboratory data and anthropometric data, therefore the sample size is relatively small and so weaker associations may not have been powered enough to become evident.

Chapter 6

In addition, the data was only analysed at one time point, the booking appointment. Therefore, haemostasis and adipokine observations at 28 weeks' gestation, which are likely to have been influenced by the unmeasured body composition changes at this time point, may have been overlooked and their ability to strengthen any findings between adipose tissue distribution and the prothrombotic state missed.

The curated multiple regression analyses performed to confirm the pathophysiological pathways are unable to accurately appreciate multi-directional pathways or dual influence on parameters. Statistical pathway analysis software exists but requires specialist software add-ons and statistician input, therefore was not within the scope of this thesis.

Finally, the proposed pathophysiological pathways, as indicated in Figure 6-1, have been derived based on multiple regression analysis, therefore conclusions are hypothesised and based on statistical analyses and interpretation of previous literature rather than scientifically proven. Analyses of adipokine secretion from SAT and VAT of obese pregnant women would provide direct laboratory evidence to confirm or refute these proposed pathways and give greater strength to the hypothesised pathophysiology.

6.6.3 Summary

This is the first study which has aimed to establish the relationships between adipokines, adipose tissue distribution and thrombin generation in pregnancy. This study has identified that central obesity, with SAT rather than VAT, is part of a pathway involving leptin which is able to predict 10.64% of variance in ETP.

Two pathways, starting with VAT and abdominal SAT respectively, which culminate in increased levels of fibrinogen via leptin and IL-6, were also proposed. Leptin and IL-6 appear to be associated more closely with the SAT-fibrinogen pathway which had a greater ability to predict fibrinogen than the VAT-fibrinogen pathway.

Although associations between adipose tissue distribution, adipokines and thrombin generation were identified, these only explain a small percentage of

Chapter 6

variance in ETP, suggesting there are alternative pathophysiology underlying the increased thrombin generation in obese pregnancy which have yet to be elucidated. However, the relationships observed between fibrinogen and measurements of obesity were considerably stronger than those observed with ETP, suggesting that fibrinogen may be a valuable biomarker in identifying women at risk from VTE during pregnancy.

Chapter 7 Discussion

The triennial MBRRACE maternal mortality reports have identified VTE as the leading direct cause of maternal death in the UK and Ireland for several years.(Knight M *et al.*, 2021) Despite risk-stratification guidelines for the use of prophylactic low molecular weight heparin in selected women during pregnancy and the puerperium, the incidence of VTE-related mortality has remained unchanged in over a decade. Overweight and obese women are over-represented in the mortality data, and with the worldwide prevalence of obesity increasing, understanding the factors which contribute to the prothrombotic effect of obesity during pregnancy is essential in developing strategies to both identify those at greatest risk and to help mitigate this risk to prevent future maternal deaths.

Reflecting the pathophysiology underlying thrombosis in non-pregnant obese individuals, it was hypothesised that a chronic low-grade inflammatory state driven by dysfunctional adipose tissue, consequent on adipose tissue distribution, would be associated with increased thrombin generation in obese pregnant women.

In order to investigate this hypothesis, 262 pregnant women, comprising 109 lean, 115 overweight/obese class I, 24 obese class II and 14 obese class III, were recruited into the current prospective observational study. Assessment of adipose tissue distribution was undertaken by BMI, waist and hip circumference and abdominal SAT and VAT measurements. Blood samples were obtained at the antenatal booking appointment (mean 10.2 weeks' gestation) and repeated at 28 weeks' gestation. Blood analysis included haemostatic parameters to assess the prothrombotic state (Factor VIII, fibrinogen, protein S, TFPI, D-dimer, PAI-1 and *ex vivo* thrombin generation) and adipokines which were known to be dysregulated in obesity (leptin, adiponectin, TNF- α , IL-6, IL-1 β). Two-way ANOVA was used to identify the relationships between BMI and gestation on each of the haemostasis parameters and adipokines, whilst multiple regression analysis was used to determine the associations between the measures of adipose tissue distribution, adipokines and haemostatic markers.

The aims of the current study were to characterise the prothrombotic state in obese pregnancy, characterise the adipokine changes in obese pregnancy,

Chapter 7

characterise the differences in adipose tissue distribution in obese pregnant women at booking, and finally relate the adipokine and adipose tissue distribution data to the haemostatic parameters to determine the underlying pathophysiology for the prothrombotic state in obese pregnancy.

7.1 Main findings

Chapter 3 has provided a characterisation of the development of the prothrombotic state in pregnancy in women of the four BMI groups. This chapter showed that for all women, Factor VIII, fibrinogen, D-dimer and PAI-1 increased whilst the naturally occurring anticoagulants, protein S and TFPI, decreased with advancing gestation as has previously been extensively demonstrated in the literature. As these haemostatic parameters are comparable with the literature, it provides strength to the novel thrombin generation findings in this cohort.

Fibrinogen, TFPI, PAI-1 and ETP were the only haemostatic parameters to show an association with both time and BMI, and were all positively correlated with BMI at the booking appointment. However, by 28 weeks' gestation, whilst fibrinogen remained significantly higher in obese, there was no longer a difference in TFPI, PAI-1 or ETP between the four BMI groups. The attenuated increase in ETP and PAI-1 between booking and 28 weeks' gestation in obese women (Figure 7-1) is similar to the pattern of 'metabolic inflexibility' previously observed in the changes in lipids in obese pregnant women by Meyer et al, which may suggest that obesity during pregnancy impairs the ability to respond to adaptations necessary during pregnancy.

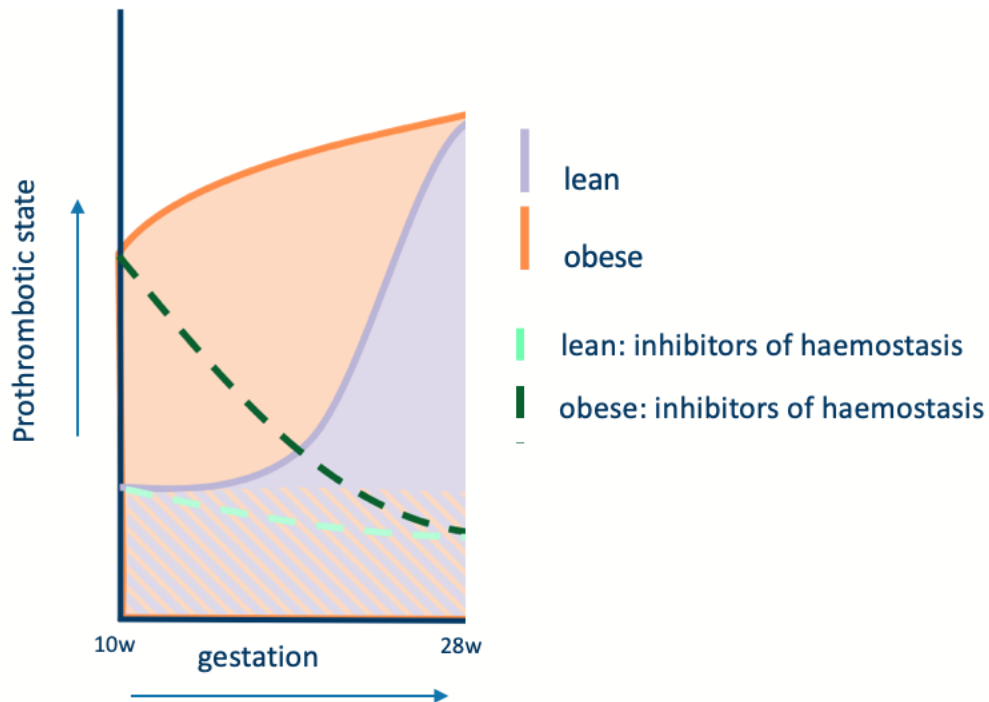


Figure 7-1 Schematic representing proposed changes in procoagulant and anticoagulant substances in lean and obese pregnant women.

The converse was observed with TFPI, which showed a proportionally greater loss of TFPI for the obese women (Figure 7-1). Low TFPI has previously been associated with occurrence of VTE, therefore it may be that the more prolonged prothrombotic state which obese women have during pregnancy is then compounded by the gestational loss of TFPI, resulting in the greater incidence of VTE during pregnancy in this group.

Mirroring the findings in the current study, Mandelebe et al found no significant difference in ETP between lean and obese pregnant women at the 26-28 weeks' gestation time point, but then ETP was significantly higher in lean women by term. When this data is taken along with that of the current study, it may suggest that although obese women have greater thrombin generation in early pregnancy, it is matched by that of lean women by 28 weeks' gestation, yet obese women do not recover post-partum to the same level as lean women and remain prothrombotic. This may suggest, as illustrated in Figure 7-2, that the prothrombotic state which develops in pregnancy is more flexible in its onset and ability to recover in lean women, whereas obese women have a greater area under the curve representing a more prolonged prothrombotic state.

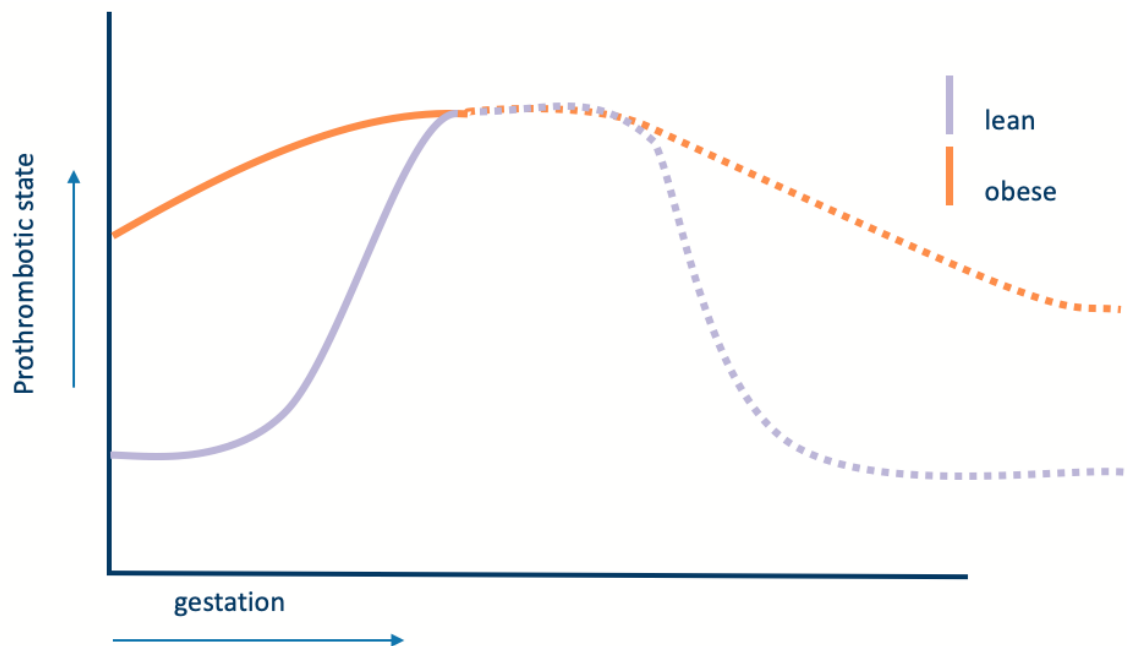


Figure 7-2 Schematic representation of changes in procoagulant state in lean and obese women during pregnancy.

Based upon data of the current study and the work of Mandlebe *et al.* Solid line indicates findings from current study, dotted line indicates findings of Mandlebe *et al.*

In the existing literature, studies investigating *ex vivo* thrombin generation in obese pregnancy have predominantly examined the assay in the third trimester, often at a single timepoint. Epidemiological data has shown that the risk of VTE occurs from early in pregnancy, with 50% of antenatal thrombotic episodes occurring in the first trimester. Whilst it has previously been demonstrated by Bagot *et al* that thrombin generation is known to increase from the 5th gestational week, the current study offers novel thrombin generation data for overweight and obese women from the first trimester through to the early third trimester. Based upon the data in the current study it could be proposed that, against our initial hypothesis, obese women are not at greater risk of thrombosis during pregnancy due to a greater increase in thrombin generation but that it is the longer duration of the prothrombotic state, coupled with greater loss of compensatory mechanisms, which contribute to the occurrence of thrombotic events.

It was hypothesised that dysfunctional adipose tissue and a chronic low grade inflammatory state were the drivers behind the prothrombotic state in obese pregnant women. In Chapter 4, I aimed to characterise the changes in adipokines and inflammatory cytokines between the booking appointment and 28 weeks'

Chapter 7

gestation in women of the four different BMI groups, and then assess for associations between the adipokines and measures of the prothrombotic state.

Leptin was positively correlated with both BMI and gestation, whilst adiponectin negatively correlated with BMI and gestation, as has previously been described in the literature. Pro-inflammatory cytokines, TNF- α and IL-6, were both positively correlated with BMI, although TNF- α was only significantly increased in the most obese group and neither showed significant relationship with gestation. IL-1 β was only detectable in a minority of participants and showed no correlation with BMI or gestation, possibly reflecting that the inflammatory state in obesity is too low-grade for this to be detectable.

Considering the relationships between the adipokines and prothrombotic state, leptin was strongly positively associated with fibrinogen and ETP, independently of BMI. Acedo *et al* have previously identified hyperleptinaemia to contribute to the recruitment of adipose tissue macrophages and their switch to the pro-inflammatory M1 phenotype which release higher concentrations of IL-6. Based upon the existing literature, and multiple regression analysis in this cohort, the relationship between leptin and fibrinogen is hypothesised to be mediated by increased circulating IL-6 which induces hepatic production of fibrinogen. The relationship between leptin and ETP was independent of IL-6, but previous work by Petrini *et al* has shown leptin stimulates release of tissue factor-bearing microparticles from PBMCs which may have a direct impact on thrombin generation. Leptin is also known to increase thrombotic risk via inducing endothelial dysfunction, which increases thrombotic risk which may not necessarily be captured in the measurement of *ex vivo* thrombin generation.

It was initially hypothesised that adipocyte dysfunction and the chronic inflammatory state would be identified as the driver of the prothrombotic state. The findings of Chapter 4 partially prove my hypothesis for this chapter, with an association between leptin, as an indicator of dysfunctional adipocytes, and both fibrinogen and ETP. However, there is less of an association between the measured inflammatory markers and the prothrombotic state. IL-6 was the only inflammatory cytokine to be significantly associated with haemostasis, via a previously well-described association with fibrinogen. Fibrinogen has been associated with risk of thrombosis in several studies, but isn't considered to be as

Chapter 7

robust a biomarker for thrombotic risk as thrombin generation. This study has not demonstrated a link between the pro-inflammatory state in obesity and increased thrombin generation, and it may be that thrombin generation in obese pregnant women is related to leptin-induced endothelial dysfunction and increased release of tissue factor-bearing microparticles.

Adipokine and inflammatory cytokine release in obesity is consequent on the amount and location of adipose tissue. Adipose tissue distribution has been found to be an important determinant of cardiometabolic and thrombotic risk in non-pregnant individuals, and associated with metabolic complications of pregnancy. Therefore, in Chapter 5, I aimed to identify associations between anthropomorphic measurements of adipose tissue distribution and the measurements of the pro-inflammatory and prothrombotic states in women of the four BMI groups at the antenatal booking appointment.

As has been recognised in previous studies, the current study found BMI to be an inaccurate measure of adipose tissue distribution. Whilst waist and hip circumferences and SAT and VAT thickness did positively correlate with BMI, these relationships were less robust in those with BMI >40. In the most obese, excess adipose tissue was preferentially deposited centrally as VAT, which may be supported by the previously described ‘adipose tissue overflow’ hypothesis in which less stable depots of adipose tissue, DSAT and VAT, expand once the SAT depot reaches capacity. This then contributes to the chronic low grade inflammatory state in obesity as SAT and VAT have increased adipose tissue macrophage recruitment and polarisation towards the pro-inflammatory M1 ATM phenotype. There was not strong evidence of the pro-inflammatory state in the current study, as inflammatory cytokines IL-6, TNF- α and IL-1 β were not associated, independently of BMI, with VAT as expected based on the existing literature. However, there was a significant BMI-independent relationship between IL-6 and waist circumference which led to the consideration that the central obesity association with IL-6 may be via unmeasured DSAT.

Based upon the existing data in cardiovascular disease and previous data in metabolic complications of pregnancy, it was hypothesised that central obesity and specifically VAT would be positively associated with thrombin generation due to the pro-inflammatory phenotype associated with this adipose tissue depot. This

Chapter 7

hypothesis was incorrect, as in the current study SAT was the adipose tissue depot most closely associated with ETP, independently of BMI. SAT and waist circumference were also associated with fibrinogen concentration.

The current study has found that ETP was positively correlated with the adipokine leptin and measures of adiposity: BMI, waist circumference and SAT. This data is strengthened by similar positive relationships between fibrinogen and leptin, SAT, and waist circumference. Taken together, these data would suggest that leptin, BMI, waist circumference and SAT may be relevant to the underlying pathophysiology of the prothrombotic state in obese pregnancy.

To try to further refine these relationships, Chapter 6 aimed to take the adipokine and anthropomorphic measures which had the closest relationships to ETP and fibrinogen to identify the pathophysiological pathways leading to the prothrombotic state in obese pregnant women at the antenatal booking appointment. Multiple regression analysis confirmed dependent relationships between SAT-leptin-ETP, and SAT-leptin-IL-6-fibrinogen. SAT and leptin were only able to predict 10.64% of variance in ETP, which suggests there are other pathways contributing to the increase in ETP observed in obese pregnant women, but 32.01% of variance in fibrinogen was explained by SAT, leptin and IL-6.

7.2 Conclusions

In conclusion, this study is the first to compare thrombin generation in pregnant women of different BMI from the first trimester, and the first to demonstrate that the increased thrombotic risk in obese pregnant women may be due to a more prolonged prothrombotic state, as demonstrated by increased ETP and fibrinogen, which is present from the antenatal booking appointment and is compounded by the gestational loss of TFPI.

The prothrombotic state in obese pregnant women in this study, as measured by ETP and fibrinogen, was associated with central adiposity, as measured by waist circumference, and SAT rather than VAT. IL-6 was positively correlated with SAT, leptin and fibrinogen. The same was not true for thrombin generation, as ETP was associated with leptin, but not IL-6.

7.3 Limitations

There were a number of limitations to this study, as previously mentioned in the relevant results chapters.

7.3.1 Study design limitations

The main limitation relating to study design is the sample size for the most obese groups, obese class II and class III, which were initially planned to be $n=25$ and $n=14$. These were based upon the expectation that ETP would show a greater percentage increase between booking and 28 weeks' gestation in these groups, whereas this study has shown that although greater at booking, the increase in ETP between booking and 28 weeks' is inversely related to BMI. Due to the attenuated increase in ETP in the most obese, the small group size has resulted in thrombin generation results for obese class III women which are much lower than may have been anticipated, based upon the trends observed with the other three BMI classes.

Another significant flaw in the study design was the omission of a repeat BMI and anthropomorphic measurements at 28 weeks' gestation. It may have been difficult to determine if there were gestational-related weight gain, and measuring waist circumference would not have been reflective of adipose tissue mass and rather the growing uterus, but the adipokine concentrations at 28 weeks' gestation were unable to be reliably linked to any measurements of adipose tissue as the study measurements were obtained at booking and would not reflect the gestational changes in adipose tissue deposition between booking and 28 weeks' gestation.

Whilst thrombin generation has been the assay most-studied in relation to thrombotic risk, it is not a readily available test in clinical practice, meaning that it limits the immediate clinical applicability of this work. Point-of-care global coagulation assays (e.g TEG and ROTEM) are used in obstetrics frequently, predominantly for assessment of coagulation in situations of major obstetric haemorrhage. TEG is predominantly used for assessment of deficiencies of clotting factors and haemostasis, however the study by O'Keefe et al describes a profile suggestive of a prothrombotic state which was observed in obese pregnant women. Whilst TEG/ROTEM are clinically available, further work would need to be

undertaken to determine if their use was able to identify those women at increased risk of VTE in pregnancy. (O'Keefe *et al.*, 2021)

7.3.2 Limitations related to loss to follow-up and COVID-19 pandemic

The rate of loss to follow-up was greater than initial estimates as these were based upon rates of second trimester miscarriage and expected drop out for studies in which all study visits coincide with routine antenatal care. As women were recruited prior to the booking ultrasound scan, which confirms pregnancy viability, several were recruited who had suffered undetected first trimester miscarriage. In addition, due to the study ultrasound scan involving an additional hospital attendance, many women dropped out of this portion of the study.

The most significant reason for loss to follow-up was the development of the COVID-19 pandemic, due to which non-COVID-related studies were suspended and as such 60 women could not complete the second set of study bloods. Despite this, and due to the rapid achievement of recruitment targets earlier in the study period, a large number (n=173) of women attended both study visits which provided a significant study population which was representative of the local maternity population.

In addition to the increased rate of loss to follow-up, a pause on recruitment for all non-COVID-19 studies meant that the planned *ex vivo* adipocytes work could not take place. Samples of SAT and VAT adipocytes collected at caesarean section from pregnant women of different BMI were to be collected and measurement of released adipokines compared under basal and LPS-stimulated conditions, and compared to contemporary thrombin generation. If this work were to be undertaken, isolating adipocytes from deep SAT would potentially further the theories of this thesis that pro-inflammatory cytokines from deep SAT are driving the prothrombotic state in obese pregnancy.

7.4 Implications on clinical practice and future research

The clinical knowledge gaps which exist for obese pregnant women are: accurately identifying those who are at risk of VTE during pregnancy and the puerperium,

identifying at which point thromboprophylaxis should be started, and what the most effective dose of low molecular weight heparin should be used in this group.

The current study has demonstrated that obese women have a more prolonged prothrombotic state, yet current risk stratification guidelines would only recommend prophylactic LMWH in the post-partum period if BMI ≥ 40 is a sole risk factor. It may be that measurement of leptin, IL-6, waist circumference and SAT in addition to thrombin generation could identify those women who may benefit from prophylactic LMWH from earlier in pregnancy.

The findings of the current study should first be validated by repeating the work in a larger cohort. Sample size calculations for each BMI group should reflect that ETP starts higher in obese but does not increase more in this group with advancing gestation, and a larger cohort of obese class II and III would be required. Analyses should be focussed on thrombin generation, fibrinogen, TFPI and PAI-1, alongside leptin and IL-6. The contribution of deep SAT to the pathophysiology needs further exploration, and as such in a larger cohort, ultrasound should be used to quantify thickness of VAT, deep SAT and superficial SAT. The planned *ex vivo* adipocytes study would also help to contextualise the findings of this prospective cohort and further the understanding of the pathophysiology of the prothrombotic state in obese pregnant women.

Should a follow-up study confirm the current findings, then leptin, IL-6, SAT ($\pm$ deep SAT) and waist circumference could be incorporated into the pre-existing antenatal risk stratification system to identify those women at greatest risk of VTE. Future studies would also be required to determine the optimal gestation at which to start thromboprophylaxis and the correct dosing regimen.

Consideration should also be taken to the role of glucagon-like peptide-1 receptor agonists (GLP1-Ras) in the weight reduction and subsequent thrombotic risk in pregnancy. GLP1-RA use for weight loss has grown exponentially, yet our current understanding on whether this is an effective strategy pre-pregnancy to reduce obstetric risks is lacking. Given what this thesis and the observational data has demonstrated on the additional risks of obesity during pregnancy on thrombotic risk, reducing weight pre-pregnancy by using GLP1-RAs may appear to be a viable solution to the maternal mortality related to VTE. However, a recent meta-

Chapter 7

analysis by Liu et al found a significant increase in the risk of DVT (25 more events per 10,000 person-years). This association was most marked in those using GLP1-RAs for >78 weeks duration (OR 2.32), but no increased risk of PE was identified. The median age of study participants was older (60 years) and predominantly male, so the findings may not be truly extrapolated to the pregnant population, however further work is needed in this area given the increasing prevalence of GLP1-RA use in women of child-bearing age. (Liu *et al.*, 2025)

Further work is warranted to make the findings of this study clinically translatable, however it has identified several potential risk factors which may help to move forward the risk stratification of obese pregnant women to prevent future VTE-related maternal deaths.

Appendix 1 Participant Information Leaflet



Department of Haematology
Macewan Building
Glasgow Royal Infirmary
84 Castle Street
G4 0SF

Patient Information Sheet Version 2.0

Title: A study to determine how body weight affects the risk of developing a blood clot in pregnancy

We wish to invite you to participate in this clinical study. Before deciding if you wish to take part, please read the information below carefully to ensure you understand the purpose of the study and what it will involve for you if you choose to take part. If you have any questions relating to any part of the study, please don't hesitate to ask your midwife to direct you to a member of the research team.

Please read Part 1. If the information given there interests you and you are considering taking part in the study, please read the additional information in Part 2 before making any decision. Part 3 provides information on the General Data Protection Regulations (GDPR)

Part 1

Purpose of the study

Blood clots in veins are the commonest cause of death in pregnant women. Pregnant women who are overweight have a greater chance of developing and dying from blood clots than normal weight pregnant women. This study will try to understand why overweight pregnant women have a higher risk of blood clots. It might be due to increased inflammation in the body caused by an increased quantity of fat both under the skin and deeper in the body.

Why have I been invited to take part?

You are eligible to take part because you are pregnant. You can be either of normal weight or overweight to take part in the study.

Do I have to take part?

You do not have to take part in the study. There will be no effect on your routine pregnancy care should you choose not to take part. If you do take part, you are free to decide to withdraw from the study at any time.

What will happen if I decide to take part in the study?

If you decide to take part in the study after reading this information sheet, you will meet with a member of the research team who will be able to answer any questions you may have. We will then ask you to sign a consent form to confirm you understand what the study involves for you and that you are happy to take part. The researcher will then ask you some

Appendix 1

questions about your medical history, family history and obtain some basic body measurements (height, weight and waist circumference).

You will then have a blood sample taken from one of the veins in your arm. We will ask you if you prefer to have the blood taken from a particular arm. If you have ever had a blood test taken previously at your GP or another clinic this will be very similar. We expect that the blood taken for the research will be taken as a single sample at the same time as the blood which is taken as part of your routine pregnancy care. It will either be the midwife allocated to you at your antenatal appointment who will take your blood, or one of the research team members who have all been trained and are experienced in taking blood. The additional quantity of blood taken for the research will be approximately 30ml, the equivalent of 2 tablespoons of blood. It is expected that the amount of time you spend with the research team will increase the length of your first routine antenatal appointment by about 30 minutes.

The blood tests for the study will be repeated again when you have routine antenatal blood tests taken at around 28 weeks of pregnancy. It is expected that the amount of time you spend with the research team will increase the length of this antenatal appointment by about 15 minutes.

On each occasion, your blood will be tested to determine how well it clots and also measure proteins that are associated with increased inflammation in the body.

We will also arrange for you to attend for an additional ultrasound scan between 12-17 weeks of pregnancy. This scan will measure the amount of fat tissue under your skin and in your abdomen. This test is safe for you and your baby. It will be very similar to the ultrasound scans you will also have as part of routine pregnancy care. This scan will not be assessing the development of your baby and it is possible, depending on your stage of pregnancy, that your baby may not be seen at all during this scan. There is a small possibility of identifying an unexpected abnormality when the ultrasound scan is being performed. If this were to occur then suitable further follow-up would be arranged as necessary.

After 28 weeks there will be no further tests or additional clinic visits required for the study. The research team will review your case notes after you have had your baby to obtain information about how your health has been for the remainder of your pregnancy, the delivery of your baby and the health of your baby when it was first born.

Expenses

Travel expenses, up to a limit of £20, will be provided for the additional ultrasound scan between 12 and 17 weeks of pregnancy as this is an additional visit to your routine antenatal care. Travel expenses will not be provided for travel to routine antenatal appointments which may involve study blood tests being taken.

What are the risks of taking part in the study?

There may be discomfort or bruising after blood is taken, similar to any time you have a blood sample taken.

There will be no effect on your routine care during pregnancy by taking part in this study.

What are the benefits of taking part in the study?

The study will not be of direct benefit to you.

What happens when the research stops?

You will continue to receive normal pregnancy care before, during and following completion of the study.

What if there is a problem?

Any complaint about the way you are being dealt with during the study or any possible harm you might suffer will be dealt with. The detailed information on this is given in Part 2.

Will my taking part in the study be kept confidential?

Yes. We will follow standard ethical and legal practice and all information about you will be kept strictly confidential. The details are included in Part 2.

Part 2**What will happen if I do not want to continue with the study?**

We would like you to contact a member of the research team if you decide to withdraw from the study. You are free to do this at any time, without giving a reason, and this will not affect the standard of medical care you receive. Any stored blood that can still be identified as yours will be destroyed if you wish. We will use the data collected up to the time of your withdrawal from the study.

If you lose the ability to give consent after entering the study, you will be withdrawn from the study at the time you lose this ability. We will use any blood or data collected up to the time of your withdrawal from the study.

What if there is a problem?

If you have worries about any aspect of the study, you should ask to speak to a member of the research team (contact names and telephone numbers at end of document).

If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints procedure. Details can be obtained from the hospital.

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against Greater Glasgow and Clyde Health Board but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

What will happen to any blood samples I give?

All blood samples collected will be securely stored in a freezer in the Specialist Haemostasis laboratory at Glasgow Royal Infirmary. Members of the research team will have access to the samples. This freezer will also be accessible to other laboratory staff, but your research samples will be identified with a unique number that will only be traceable back to you by members of the research team.

Appendix 1

Samples will not be transferred out of the UK. Some of your blood will be sent to the University of Leeds, to assess how your body forms and breaks down blood clots. These tests are unable to be performed in Glasgow but researchers at the University of Leeds are experts in performing these blood tests. The samples sent to the University of Leeds will not be able to be identified as being yours.

Other samples will only be sent to a different laboratory in the UK if appropriate equipment in the laboratory at Glasgow Royal Infirmary is unavailable for an unpredicted reason e.g. equipment failure.

Once the research outlined in this document is completed, the samples will be stored. These samples may be used again in future tests that are relevant to the investigation of the effect of pregnancy on the risk of developing blood clots.

Will genetic tests be done?

No

What will happen to my data?

All information that is collected about you during the course of the study will be kept strictly confidential. The data will not be recognized as being yours but will be identified with a unique number. Only members of the research team will have access to data that can be identified as yours.

The data collected from you will be used only in the research outlined in this document. It will be stored securely on a computer in the Haematology laboratory at Glasgow Royal Infirmary. The data will be password protected and only members of the research team will have this password.

Any data that can be recognised as yours will be destroyed two years after the study is completed. Any data that cannot be identified as yours will be retained for 15 years and then destroyed unless it is used in further studies. If this occurs, further ethical approval and consent from you will have to be obtained before it can be used.

Involvement of the General Practitioner (GP)

We would like to inform your GP that you are participating in this study. We will seek your consent before your GP is informed of your participation in the study.

What will happen to the results of the study?

When you first consent to join the study we will ask you if you wish to be informed of any results arising from the research. When results are obtained we will send a letter to you providing an outline of the broad scientific results of the trial, with references for any work that has been published in medical journals.

Will my involvement in the study be confidential?

Yes. As with all medical information, any details about you in the study will be confidential. You will be allocated a trial number and all data will be stored under the trial number rather than your name.

Who is organising and funding the research?

The study is organised and sponsored by NHS Greater Glasgow and Clyde, with funding provided through a grant from the British Heart Foundation.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed by the West of Scotland Ethics Committee. In addition, this study has also been reviewed by the British Heart Foundation and the University of Glasgow Institute of Cardiovascular Medicine and Science (ICAMS)

Further information and contact details:

1) General information about research

The following website addresses provide general information about research

National Electronic Library for Health <http://www.library.nhs.uk/trials>

The National Research Register (UK database of research projects) <http://www.nrr.nhs.uk>

INVOLVE (Promoting public involvement in the NHS) <http://www.invo.org.uk>

2) Specific information about this research project

Dr. Susan McNeill 0141 211 5125

Dr. Catherine Bagot 0141 211 4671

Dr. Vicki Brace 0141 211 5286

Part 3 General Data Protection Regulation (GDPR) Information

NHS Greater Glasgow & Clyde is the sponsor for this study based in Scotland. We will be using information from your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. NHS Greater Glasgow and Clyde will keep identifiable information about you for 10 years after the study has finished.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information by contacting Dr Catherine Bagot.

NHS Greater Glasgow and Clyde will collect information about you for the study 'An investigation of the pathophysiology underlying the increased risk of venous thrombosis in overweight and obese pregnant women' from your maternity medical notes. This information will include your name, NHS number, postcode and date of birth and health information, which is regarded as a special category of information. We will use this

Appendix 1

information to relate our study findings to maternal characteristics (e.g. how does maternal weight affect the research findings?) and to delivery characteristics (e.g. how are the research findings related to baby birth weight?).

When you agree to take part in a research study, the information about your health and care may be provided to researchers running other research studies in this organisation and in other organisations. These organisations may be universities, NHS organisations or companies involved in health and care research in this country or abroad. Your information will only be used by organisations and researchers to conduct research in accordance with the [UK Policy Framework for Health and Social Care Research](#).

This information will not identify you and will not be combined with other information in a way that could identify you. The information will only be used for the purpose of health and care research, and cannot be used to contact you or to affect your care. It will not be used to make decisions about future services available to you, such as insurance.

Appendix 2 Consent form

Department of Haematology
3rd Floor, Macewen Building
Glasgow Royal Infirmary
84 Castle Street
Glasgow G4 0SF

Tel. No. 0141 211 5125
Fax No. 0141 211 4931



CONSENT FORM v2

Title of Project: **A study to determine how body weight affects the risk of developing a blood clot in pregnancy.**

Name of Researcher: Dr Susan McNeill

Please initial each box

Initials

1. I confirm that I have read and understand the information sheet dated 11/02/19 v2.0 for the above study. I have had the opportunity to consider the information and ask questions.
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.
3. I agree to stored samples being used for future testing where it relates to the investigation of blood clotting during pregnancy.
4. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the research team and also by regulatory research and ethical authorities in the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.
5. I agree to my GP being informed of my participation in the study
6. I agree to take part in the above study

Name of Patient

Date

Signature

I confirm that I have explained the study to the participant and have answered their questions honestly and fully.

Name of Person
taking consent

Date

Signature

When completed, 1 for patient; 1 for researcher site file (original); 1 to be filed in patient's notes.

11th February 2019

v2.0

Appendix 3 Demographics form

APPENDIX A – CASE REPORT FORM

Subject Name:

Subject Study Number:

Demographic data

Age (years)	
Gravity (no. of previous pregnancies)	
Parity (no. of previous viable pregnancies)	
Pre-pregnancy comorbidities	Yes/No If yes, please specify:
Known minor thrombophilia (i.e FVLeiden or PT G20210A heterozygosity)	Yes/No If yes, confirm type:
Smoking status	Never/Current/Previous
Recent surgery (within 3 months)	Yes/No If yes, specify type of surgery:
Family history of VTE	Yes/No If yes: state affected family members
Current medications	Yes/No If yes, please specify:

Anthropomorphic data

Height (cm)	
Weight (kg)	
BMI (kg/m ²)	
Waist circumference (cm)	

Appendix 4 USS data collection form

The prothrombotic effect of obesity during pregnancy
R&D number: GN18HA013



Imaging Proforma

Participant Name

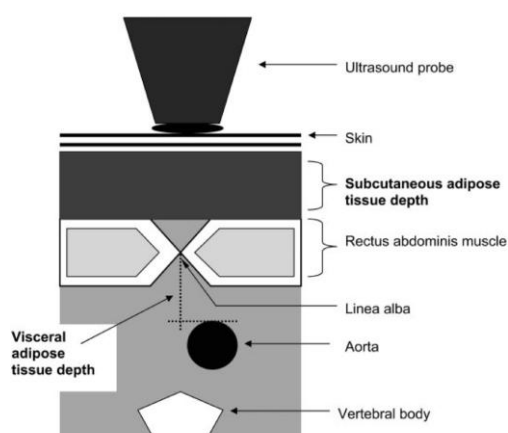
Study Number

Sonographer Name

Date

SAT depth (mm)	
VAT depth (mm)	

Measurement of SAT & VAT by transabdominal ultrasound



Subcutaneous adipose tissue (SAT) to be measured from the outer border of the rectus abdominis muscle to the skin surface, at the intersection of the linea alba and umbilicus.

Visceral adipose tissue (VAT) to be measured from the inner border of the rectus abdominis muscle to the anterior wall of the abdominal aorta, at the level of the linea alba.

Figure 1—Cross-sectional representation of the abdominal wall and subcutaneous and visceral fat compartments measured by ultrasound.

Modified from: Martin AM et al. Abdominal visceral adiposity in the first trimester predicts glucose intolerance in later pregnancy. *Diabetes Care*. 2009;32:1308-1310.

Once completed, please scan form into CRIS and submit one copy to the research file.

Dr Susan McNeill – Clinical Research Fellow in Haematology.
Susanmcneill1@nhs.net

11th December 2018

v1.0

References

- Abella, V., Scotece, M., Conde, J., Pino, J., Gonzalez-Gay, M. A., Gómez-Reino, J. J., Mera, A., Lago, F., Gómez, R. and Gualillo, O. (2017) 'Leptin in the interplay of inflammation, metabolism and immune system disorders', *Nat Rev Rheumatol*, 13(2), pp. 100-109.
- Acedo, S. C., Gambero, S., Cunha, F. G., Lorand-Metze, I. and Gambero, A. (2013) 'Participation of leptin in the determination of the macrophage phenotype: an additional role in adipocyte and macrophage crosstalk', *In Vitro Cell Dev Biol Anim*, 49(6), pp. 473-8.
- Aird, W. C. (2008) 'Endothelium in health and disease', *Pharmacol Rep*, 60(1), pp. 139-43.
- Aleman, M. M., Walton, B. L., Byrnes, J. R. and Wolberg, A. S. (2014) 'Fibrinogen and red blood cells in venous thrombosis', *Thromb Res*, 133 Suppl 1(0 1), pp. S38-40.
- Ammon Avalos, L., Galindo, C. and Li, D. K. (2012) 'A systematic review to calculate background miscarriage rates using life table analysis', *Birth Defects Res A Clin Mol Teratol*, 94(6), pp. 417-23.
- Anderson, F. A. and Wheeler, H. B. (1992) 'Physician practices in the management of venous thromboembolism: a community-wide survey', *J Vasc Surg*, 16(5), pp. 707-14.
- Anderson, G. D. (2005) 'Pregnancy-induced changes in pharmacokinetics: a mechanistic-based approach', *Clin Pharmacokinet*, 44(10), pp. 989-1008.
- Andersson-Hall, U., Svedin, P., Svensson, H., Lönn, M., Mallard, C. and Holmäng, A. (2020) 'Longitudinal changes in adipokines and free leptin index during and after pregnancy in women with obesity', *Int J Obes (Lond)*, 44(3), pp. 675-683.
- Archipoff, G., Beretz, A., Freyssinet, J. M., Klein-Soyer, C., Brisson, C. and Cazenave, J. P. (1991) 'Heterogeneous regulation of constitutive thrombomodulin or inducible tissue-factor activities on the surface of human saphenous-vein endothelial cells in culture following stimulation by interleukin-1, tumour necrosis factor, thrombin or phorbol ester', *Biochem J*, 273 (Pt 3)(Pt 3), pp. 679-84.
- Ariëns, R. A. (2013) 'Fibrin(ogen) and thrombotic disease', *J Thromb Haemost*, 11 Suppl 1, pp. 294-305.
- Askarpour, M., Alizadeh, S., Hadi, A., Symonds, M. E., Miraghajani, M., Sheikhi, A. and Ghaedi, E. (2020) 'Effect of Bariatric Surgery on the Circulating Level of Adiponectin, Chemerin, Plasminogen Activator Inhibitor-1, Leptin, Resistin, and Visfatin: A Systematic Review and Meta-Analysis', *Horm Metab Res*, 52(4), pp. 207-215.
- Astedt, B., Lindoff, C. and Lecander, I. (1998) 'Significance of the plasminogen activator inhibitor of placental type (PAI-2) in pregnancy', *Semin Thromb Hemost*, 24(5), pp. 431-5.

Ay, L., Kopp, H. P., Brix, J. M., Ay, C., Quehenberger, P., Schernthaner, G. H., Pabinger, I. and Schernthaner, G. (2010) 'Thrombin generation in morbid obesity: significant reduction after weight loss', *J Thromb Haemost*, 8(4), pp. 759-65.

Bagot, C. N. and Arya, R. (2008) 'Virchow and his triad: a question of attribution', *Br J Haematol*, 143(2), pp. 180-90.

Bagot, C. N. and Leishman, E. (2015) 'Establishing a reference range for thrombin generation using a standard plasma significantly improves assay precision', *Thromb Res*, 136(1), pp. 139-43.

Bagot, C. N., Leishman, E., Onyiaodike, C. C., Jordan, F. and Freeman, D. J. (2017) 'Normal pregnancy is associated with an increase in thrombin generation from the very early stages of the first trimester', *Thromb Res*, 157, pp. 49-54.

Bagot, C. N., Leishman, E., Onyiaodike, C. C., Jordan, F., Gibson, V. B. and Freeman, D. J. (2019) 'Changes in laboratory markers of thrombotic risk early in the first trimester of pregnancy may be linked to an increase in estradiol and progesterone', *Thromb Res*, 178, pp. 47-53.

Bagot, C. N., Marsh, M. S., Whitehead, M., Sherwood, R., Roberts, L., Patel, R. K. and Arya, R. (2010) 'The effect of estrone on thrombin generation may explain the different thrombotic risk between oral and transdermal hormone replacement therapy', *J Thromb Haemost*, 8(8), pp. 1736-44.

Baker, S. R., Zabczyk, M., Macrae, F. L., Duval, C., Undas, A. and Ariëns, R. A. S. (2019) 'Recurrent venous thromboembolism patients form clots with lower elastic modulus than those formed by patients with non-recurrent disease', *J Thromb Haemost*, 17(4), pp. 618-626.

Bartha, J. L., Marín-Segura, P., González-González, N. L., Wagner, F., Aguilar-Diosdado, M. and Hervias-Vivancos, B. (2007) 'Ultrasound evaluation of visceral fat and metabolic risk factors during early pregnancy', *Obesity (Silver Spring)*, 15(9), pp. 2233-9.

Bashiri, A., Heo, H. J., Ben-Avraham, D., Mazor, M., Budagov, T., Einstein, F. H. and Atzmon, G. (2014) 'Pregnancy complicated by obesity induces global transcript expression alterations in visceral and subcutaneous fat', *Mol Genet Genomics*, 289(4), pp. 695-705.

Bates, S. M., Rajasekhar, A., Middeldorp, S., McLintock, C., Rodger, M. A., James, A. H., Vazquez, S. R., Greer, I. A., Riva, J. J., Bhatt, M., Schwab, N., Barrett, D., LaHaye, A. and Rochwerg, B. (2018) 'American Society of Hematology 2018 guidelines for management of venous thromboembolism: venous thromboembolism in the context of pregnancy', *Blood Adv*, 2(22), pp. 3317-3359.

Beijers, H. J., Ferreira, I., Spronk, H. M., Bravenboer, B., Dekker, J. M., Nijpels, G., ten Cate, H. and Stehouwer, C. D. (2010) 'Body composition as determinant of thrombin generation in plasma: the Hoorn study', *Arterioscler Thromb Vasc Biol*, 30(12), pp. 2639-47.

Bergrem, A., Dahm, A. E., Jacobsen, A. F., Mowinckel, M. C., Sandvik, L. and Sandset, P. M. (2011) 'Resistance to activated protein C is a risk factor for

pregnancy-related venous thrombosis in the absence of the F5 rs6025 (factor V Leiden) polymorphism', *Br J Haematol*, 154(2), pp. 241-7.

Bistervels, I. M., Buchmüller, A., Wiegers, H. M. G., Ní Áinle, F., Tardy, B., Donnelly, J., Verhamme, P., Jacobsen, A. F., Hansen, A. T., Rodger, M. A., DeSancho, M. T., Shmakov, R. G., van Es, N., Prins, M. H., Chauleur, C., Middeldorp, S., committee, H. B. w. and Investigators, H. (2022) 'Intermediate-dose versus low-dose low-molecular-weight heparin in pregnant and post-partum women with a history of venous thromboembolism (Highlow study): an open-label, multicentre, randomised, controlled trial', *Lancet*, 400(10365), pp. 1777-1787.

Björntorp, P. (1991) 'Adipose tissue distribution and function', *Int J Obes*, 15 Suppl 2, pp. 67-81.

Bohiltea, R. E., Zugravu, C. A., Nemescu, D., Turcan, N., Paulet, F. P., Gherghiceanu, F., Ducu, I. and Cirstoiu, M. M. (2020) 'Impact of obesity on the prognosis of hypertensive disorders in pregnancy', *Exp Ther Med*, 20(3), pp. 2423-2428.

Bone, J. N., Joseph, K. S., Mayer, C., Platt, R. and Lisonkova, S. (2022) 'The association between pre-pregnancy body mass index and perinatal death and the role of gestational age at delivery', *PLoS One*, 17(3), pp. e0264565.

Borch, K. H., Braekkan, S. K., Mathiesen, E. B., Njølstad, I., Wilsgaard, T., Størmer, J. and Hansen, J. B. (2009) 'Abdominal obesity is essential for the risk of venous thromboembolism in the metabolic syndrome: the Tromsø study', *J Thromb Haemost*, 7(5), pp. 739-45.

Brodin, E., Appelbom, H., Osterud, B., Hilden, I., Petersen, L. C. and Hansen, J. B. (2009) 'Regulation of thrombin generation by TFPI in plasma without and with heparin', *Transl Res*, 153(3), pp. 124-31.

Brook, R. D., Bard, R. L., Rubenfire, M., Ridker, P. M. and Rajagopalan, S. (2001) 'Usefulness of visceral obesity (waist/hip ratio) in predicting vascular endothelial function in healthy overweight adults', *Am J Cardiol*, 88(11), pp. 1264-9.

Brummel, K. E., Paradis, S. G., Butenas, S. and Mann, K. G. (2002) 'Thrombin functions during tissue factor-induced blood coagulation', *Blood*, 100(1), pp. 148-52.

Bucciarelli, P., Martinelli, I., Artoni, A., Passamonti, S. M., Previtali, E., Merati, G., Tripodi, A. and Mannucci, P. M. (2012) 'Circulating microparticles and risk of venous thromboembolism', *Thromb Res*, 129(5), pp. 591-7.

Buis, D. T. P., Christen, T., Smit, R. A. J., de Mutsert, R., Jukema, J. W., Cannegieter, S. C., Lijfering, W. M. and Rosendaal, F. R. (2020) 'The association between leptin concentration and blood coagulation: Results from the NEO study', *Thromb Res*, 188, pp. 44-48.

Butwick, A. J., Bentley, J., Leonard, S. A., Carmichael, S. L., El-Sayed, Y. Y., Stephansson, O. and Guo, N. (2019) 'Prepregnancy maternal body mass index and venous thromboembolism: a population-based cohort study', *BJOG*, 126(5), pp. 581-588.

- Campello, E., Zabeo, E., Radu, C. M., Spiezia, L., Gavasso, S., Fadin, M., Woodhams, B., Vettor, R. and Simioni, P. (2015) 'Hypercoagulability in overweight and obese subjects who are asymptomatic for thrombotic events', *Thromb Haemost*, 113(1), pp. 85-96.
- CDC, C. f. D. C. 1988. National Health and Nutrition Examination Survey III: Body Measurements (Anthropometry). Rockville, MD: CDC.
- Cesarman-Maus G and KA, H. (2005) 'Molecular mechanisms of fibrinolysis', *British Journal of Haematology*, 129, pp. 307-21.
- Charo, I. F. and Taubman, M. B. (2004) 'Chemokines in the pathogenesis of vascular disease', *Circ Res*, 95(9), pp. 858-66.
- Chitongo, P. B., Roberts, L. N., Yang, L., Patel, R. K., Lyall, R., Luxton, R., Aylwin, S. J. B. and Arya, R. (2017) 'Visceral Adiposity Is an Independent Determinant of Hypercoagulability as Measured by Thrombin Generation in Morbid Obesity', *TH Open*, 1(2), pp. e146-e154.
- Choi, J. W. and Pai, S. H. (2002) 'Tissue plasminogen activator levels change with plasma fibrinogen concentrations during pregnancy', *Ann Hematol*, 81(11), pp. 611-5.
- Chowdary, P., Adamidou, D., Riddell, A., Aghighi, S., Griffioen, A., Priest, P., Moghadam, L., Kelaher, N., Huq, F. Y., Kadir, R. A., Tuddenham, E. G. and Gatt, A. (2015) 'Thrombin generation assay identifies individual variability in responses to low molecular weight heparin in pregnancy: implications for anticoagulant monitoring', *Br J Haematol*, 168(5), pp. 719-27.
- Clark, P., Brennand, J., Conkie, J. A., McCall, F., Greer, I. A. and Walker, I. D. (1998) 'Activated protein C sensitivity, protein C, protein S and coagulation in normal pregnancy', *Thromb Haemost*, 79(6), pp. 1166-70.
- Clark, P., Walker, I. D. and Greer, I. (1999) 'Acquired activated protein-C resistance in pregnancy and association with increased thrombin generation and fetal weight', *Lancet*, 353(9149), pp. 292-3.
- Coolman, M., Timmermans, S., de Groot, C. J., Russcher, H., Lindemans, J., Hofman, A., Geurts-Moespot, A. J., Sweep, F. C., Jaddoe, V. V. and Steegers, E. A. (2012) 'Angiogenic and fibrinolytic factors in blood during the first half of pregnancy and adverse pregnancy outcomes', *Obstet Gynecol*, 119(6), pp. 1190-200.
- Corsonello, A., Perticone, F., Malara, A., De Domenico, D., Loddo, S., Buemi, M., Ientile, R. and Corica, F. (2003) 'Leptin-dependent platelet aggregation in healthy, overweight and obese subjects', *Int J Obes Relat Metab Disord*, 27(5), pp. 566-73.
- Cottam, D. R., Mattar, S. G., Barinas-Mitchell, E., Eid, G., Kuller, L., Kelley, D. E. and Schauer, P. R. (2004) 'The chronic inflammatory hypothesis for the morbidity associated with morbid obesity: implications and effects of weight loss', *Obes Surg*, 14(5), pp. 589-600.

- Csongrádi, É., Káplár, M., Nagy, B., Koch, C. A., Juhász, A., Bajnok, L., Varga, Z., Seres, I., Karányi, Z., Magyar, M. T., Oláh, L., Facskó, A., Kappelmayer, J. and Paragh, G. (2017) 'Adipokines as atherothrombotic risk factors in obese subjects: Associations with haemostatic markers and common carotid wall thickness', *Nutr Metab Cardiovasc Dis*, 27(6), pp. 571-580.
- Dahm, A., Van Hylckama Vlieg, A., Bendz, B., Rosendaal, F., Bertina, R. M. and Sandset, P. M. (2003) 'Low levels of tissue factor pathway inhibitor (TFPI) increase the risk of venous thrombosis', *Blood*, 101(11), pp. 4387-92.
- DAVIE, E. W. and RATNOFF, O. D. (1964) 'WATERFALL SEQUENCE FOR INTRINSIC BLOOD CLOTTING', *Science*, 145(3638), pp. 1310-2.
- de Laat-Kremers, R., Di Castelnuovo, A., van der Vorm, L., Costanzo, S., Ninivaggi, M., Cerletti, C., Huskens, D., De Curtis, A., Gialluisi, A., Bai, C., de Gaetano, G., Yin, D., Donati, M. B., de Laat, B., Iacoviello, L. and Investigators, M.-s. (2022) 'Increased BMI and Blood Lipids Are Associated With a Hypercoagulable State in the Moli-sani Cohort', *Front Cardiovasc Med*, 9, pp. 897733.
- De Mitrio, V., De Pergola, G., Vettor, R., Marino, R., Sciaraffia, M., Pagano, C., Scaraggi, F. A., Di Lorenzo, L. and Giorgino, R. (1999) 'Plasma plasminogen activator inhibitor-I is associated with plasma leptin irrespective of body mass index, body fat mass, and plasma insulin and metabolic parameters in premenopausal women', *Metabolism*, 48(8), pp. 960-4.
- Dellas, C., Schäfer, K., Rohm, I., Lankeit, M., Ellrott, T., Faustin, V., Riggert, J., Hasenfuss, G. and Konstantinides, S. (2008) 'Absence of leptin resistance in platelets from morbidly obese individuals may contribute to the increased thrombosis risk in obesity', *Thromb Haemost*, 100(6), pp. 1123-9.
- Desai, T. R., Leeper, N. J., Hynes, K. L. and Gewertz, B. L. (2002) 'Interleukin-6 causes endothelial barrier dysfunction via the protein kinase C pathway', *J Surg Res*, 104(2), pp. 118-23.
- Després, J. P. (2001) 'Health consequences of visceral obesity', *Ann Med*, 33(8), pp. 534-41.
- Dutra, L. P., Cisneiros, R. M., Souza, A. S., Diniz, C. P., Moura, L. A., Figueiroa, J. N. and Alves, J. G. (2014) 'Longitudinal variance of visceral fat thickness in pregnant adolescents', *Aust N Z J Obstet Gynaecol*, 54(1), pp. 91-3.
- Egan, K., O'Connor, H., Kevane, B., Malone, F., Lennon, A., Al Zadjali, A., Cooley, S., Monteith, C., Maguire, P., Szklanna, P. B., Allen, S., McCallion, N. and Ní Áinle, F. (2017) 'Elevated plasma TFPI activity causes attenuated TF-dependent thrombin generation in early onset preeclampsia', *Thromb Haemost*, 117(8), pp. 1549-1557.
- Egorina, E. M., Sovershaev, M. A., Bjørkøy, G., Gruber, F. X., Olsen, J. O., Parhami-Seren, B., Mann, K. G. and Østerud, B. (2005) 'Intracellular and surface distribution of monocyte tissue factor: application to intersubject variability', *Arterioscler Thromb Vasc Biol*, 25(7), pp. 1493-8.

Ehrenthal, D. B., Jurkovitz, C., Hoffman, M., Jiang, X. and Weintraub, W. S. (2011) 'Prepregnancy body mass index as an independent risk factor for pregnancy-induced hypertension', *J Womens Health (Larchmt)*, 20(1), pp. 67-72.

Ellis, J. A., Brown, C. M., Barger, B. and Carlson, N. S. (2019) 'Influence of Maternal Obesity on Labor Induction: A Systematic Review and Meta-Analysis', *J Midwifery Womens Health*, 64(1), pp. 55-67.

Erez, O., Romero, R., Vaisbuch, E., Kusanovic, J. P., Mazaki-Tovi, S., Chaiworapongsa, T., Gotsch, F., Fareed, J., Hoppensteadt, D., Than, N. G., Yoon, B. H., Edwin, S., Dong, Z., Espinoza, J., Mazor, M. and Hassan, S. S. (2010) 'High tissue factor activity and low tissue factor pathway inhibitor concentrations in patients with preterm labor', *J Matern Fetal Neonatal Med*, 23(1), pp. 23-33.

Esteve, E., Castro, A., López-Bermejo, A., Vendrell, J., Ricart, W. and Fernández-Real, J. M. (2007) 'Serum interleukin-6 correlates with endothelial dysfunction in healthy men independently of insulin sensitivity', *Diabetes Care*, 30(4), pp. 939-45.

Feingold, K. R., Ahmed, S. F., Anawalt, B., Blackman, M. R., Boyce, A., Chrousos, G., Corpas, E., de Herder, W. W., Dhatariya, K., Dungan, K., Hofland, J., Kalra, S., Kaltsas, G., Kapoor, N., Koch, C., Kopp, P., Korbonits, M., Kovacs, C. S., Kuohung, W., ... and Wilson, D. P. (2000) 'Endotext'.

Felker A, P. R., Kotnis R, Kenyon S, Knight M (Eds.) on behalf of MBRRACE-UK 2025. Saving Lives, Improving Mothers' Care Compiled Report - Lessons learned to inform maternity care from the UK and Ireland Confidential Enquiries into Maternal Deaths and Morbidity 2021-23. Oxford: National Perinatal Epidemiology Unit: University of Oxford.

Fialová, L., Malbohan, I., Kalousová, M., Soukupová, J., Krofta, L., Stípek, S. and Zima, T. (2006) 'Oxidative stress and inflammation in pregnancy', *Scand J Clin Lab Invest*, 66(2), pp. 121-7.

Fleck, R. A., Rao, L. V., Rapaport, S. I. and Varki, N. (1990) 'Localization of human tissue factor antigen by immunostaining with monospecific, polyclonal anti-human tissue factor antibody', *Thromb Res*, 59(2), pp. 421-37.

Frühbeck, G., Catalán, V., Rodríguez, A., Ramírez, B., Becerril, S., Salvador, J., Colina, I. and Gómez-Ambrosi, J. (2019) 'Adiponectin-leptin Ratio is a Functional Biomarker of Adipose Tissue Inflammation', *Nutrients*, 11(2).

Gallagher, D., Visser, M., Sepúlveda, D., Pierson, R. N., Harris, T. and Heymsfield, S. B. (1996) 'How useful is body mass index for comparison of body fatness across age, sex, and ethnic groups?', *Am J Epidemiol*, 143(3), pp. 228-39.

Gur, E. B., Ince, O., Turan, G. A., Karadeniz, M., Tatar, S., Celik, E., Yalcin, M. and Guclu, S. (2014) 'Ultrasonographic visceral fat thickness in the first trimester can predict metabolic syndrome and gestational diabetes mellitus', *Endocrine*, 47(2), pp. 478-84.

Hackeng, T. M., Maurissen, L. F., Castoldi, E. and Rosing, J. (2009) 'Regulation of TFPI function by protein S', *J Thromb Haemost*, 7 Suppl 1, pp. 165-8.

- Hackeng, T. M. and Rosing, J. (2009) 'Protein S as cofactor for TFPI', *Arterioscler Thromb Vasc Biol*, 29(12), pp. 2015-20.
- Haidl, H., Cimenti, C., Leschnik, B., Zach, D. and Muntean, W. (2006) 'Age-dependency of thrombin generation measured by means of calibrated automated thrombography (CAT)', *Thromb Haemost*, 95(5), pp. 772-5.
- Heit, J. A., Kobbervig, C. E., James, A. H., Petterson, T. M., Bailey, K. R. and Melton, L. J. (2005) 'Trends in the incidence of venous thromboembolism during pregnancy or postpartum: a 30-year population-based study', *Ann Intern Med*, 143(10), pp. 697-706.
- Heit, J. A., Silverstein, M. D., Mohr, D. N., Petterson, T. M., O'Fallon, W. M. and Melton, L. J. (2000) 'Risk factors for deep vein thrombosis and pulmonary embolism: a population-based case-control study', *Arch Intern Med*, 160(6), pp. 809-15.
- Hellgren, M. (2003) 'Hemostasis during normal pregnancy and puerperium', *Semin Thromb Hemost*, 29(2), pp. 125-30.
- Hemker, H. C., Giesen, P., Al Dieri, R., Regnault, V., de Smedt, E., Wagenvoord, R., Lecompte, T. and Béguin, S. (2003) 'Calibrated automated thrombin generation measurement in clotting plasma', *Pathophysiol Haemost Thromb*, 33(1), pp. 4-15.
- Hendler, I., Blackwell, S. C., Mehta, S. H., Whitty, J. E., Russell, E., Sorokin, Y. and Cotton, D. B. (2005) 'The levels of leptin, adiponectin, and resistin in normal weight, overweight, and obese pregnant women with and without preeclampsia', *Am J Obstet Gynecol*, 193(3 Pt 2), pp. 979-83.
- Hotoleanu, C. (2020) 'Association between obesity and venous thromboembolism', *Med Pharm Rep*, 93(2), pp. 162-168.
- Hron, G., Kollars, M., Binder, B. R., Eichinger, S. and Kyrle, P. A. (2006) 'Identification of patients at low risk for recurrent venous thromboembolism by measuring thrombin generation', *JAMA*, 296(4), pp. 397-402.
- Huda, S. S., Jordan, F., Bray, J., Love, G., Payne, R., Sattar, N. and Freeman, D. J. (2017) 'Visceral adipose tissue activated macrophage content and inflammatory adipokine secretion is higher in pre-eclampsia than in healthy pregnancies', *Clin Sci (Lond)*, 131(13), pp. 1529-1540.
- Hunt, B. J. and Jurd, K. M. (1998) 'Endothelial cell activation. A central pathophysiological process', *BMJ*, 316(7141), pp. 1328-9.
- Ismail, S. K., Norris, L., O'Shea, S. and Higgins, J. R. (2014) 'Weight-adjusted LMWH prophylaxis provides more effective thrombin inhibition in morbidly obese pregnant women', *Thromb Res*, 134(2), pp. 234-9.
- Jacobsen, A. F., Skjeldestad, F. E. and Sandset, P. M. (2008) 'Ante- and postnatal risk factors of venous thrombosis: a hospital-based case-control study', *J Thromb Haemost*, 6(6), pp. 905-12.

- James, A. H., Rhee, E., Thames, B. and Philipp, C. S. (2014) 'Characterization of antithrombin levels in pregnancy', *Thromb Res*, 134(3), pp. 648-51.
- Jarvie, E., Hauguel-de-Mouzon, S., Nelson, S. M., Sattar, N., Catalano, P. M. and Freeman, D. J. (2010) 'Lipotoxicity in obese pregnancy and its potential role in adverse pregnancy outcome and obesity in the offspring', *Clin Sci (Lond)*, 119(3), pp. 123-9.
- Jarvie, E. M., Stewart, F. M., Ramsay, J. E., Brown, E. A., Meyer, B. J., Olivecrona, G., Griffin, B. A. and Freeman, D. J. (2020) 'Maternal Adipose Tissue Expansion, A Missing Link in the Prediction of Birth Weight Centile', *J Clin Endocrinol Metab*, 105(3).
- Jørgensen, M., Philips, M., Thorsen, S., Selmer, J. and Zeuthen, J. (1987) 'Plasminogen activator inhibitor-1 is the primary inhibitor of tissue-type plasminogen activator in pregnancy plasma', *Thromb Haemost*, 58(3), pp. 872-8.
- Kabrhel, C., Varraso, R., Goldhaber, S. Z., Rimm, E. B. and Camargo, C. A. (2009) 'Prospective study of BMI and the risk of pulmonary embolism in women', *Obesity (Silver Spring)*, 17(11), pp. 2040-6.
- Kahn, D., Macias, E., Zarini, S., Garfield, A., Zemski Berry, K., MacLean, P., Gerszten, R. E., Libby, A., Solt, C., Schoen, J. and Bergman, B. C. (2022) 'Exploring Visceral and Subcutaneous Adipose Tissue Secretomes in Human Obesity: Implications for Metabolic Disease', *Endocrinology*, 163(11).
- Kammerlander, A. A., Lyass, A., Mahoney, T. F., Massaro, J. M., Long, M. T., Vasan, R. S. and Hoffmann, U. (2021) 'Sex Differences in the Associations of Visceral Adipose Tissue and Cardiometabolic and Cardiovascular Disease Risk: The Framingham Heart Study', *J Am Heart Assoc*, 10(11), pp. e019968.
- Kansu-Celik, H., Karakaya, B. K., Tasci, Y., Hancerliogullari, N., Yaman, S., Ozel, S. and Erkaya, S. (2018) 'Relationship maternal subcutaneous adipose tissue thickness and development of gestational diabetes mellitus', *Interv Med Appl Sci*, 10(1), pp. 13-18.
- Karpe, F. and Pinnick, K. E. (2015) 'Biology of upper-body and lower-body adipose tissue--link to whole-body phenotypes', *Nat Rev Endocrinol*, 11(2), pp. 90-100.
- Kato, H., Kashiwagi, H., Shiraga, M., Tadokoro, S., Kamae, T., Ujiie, H., Honda, S., Miyata, S., Ijiri, Y., Yamamoto, J., Maeda, N., Funahashi, T., Kurata, Y., Shimomura, I., Tomiyama, Y. and Kanakura, Y. (2006) 'Adiponectin acts as an endogenous antithrombotic factor', *Arterioscler Thromb Vasc Biol*, 26(1), pp. 224-30.
- Kelley, D. E., Thaete, F. L., Troost, F., Huwe, T. and Goodpaster, B. H. (2000) 'Subdivisions of subcutaneous abdominal adipose tissue and insulin resistance', *Am J Physiol Endocrinol Metab*, 278(5), pp. E941-8.
- Kennedy, N. J., Peek, M. J., Quinton, A. E., Lanzarone, V., Martin, A., Benzie, R. and Nanan, R. (2016) 'Maternal abdominal subcutaneous fat thickness as a predictor for adverse pregnancy outcome: a longitudinal cohort study', *BJOG*, 123(2), pp. 225-32.

- Kim, S. H., Chung, J. H., Song, S. W., Jung, W. S., Lee, Y. A. and Kim, H. N. (2016) 'Relationship between deep subcutaneous abdominal adipose tissue and metabolic syndrome: a case control study', *Diabetol Metab Syndr*, 8, pp. 10.
- Kinoshita, T. and Itoh, M. (2006) 'Longitudinal variance of fat mass deposition during pregnancy evaluated by ultrasonography: the ratio of visceral fat to subcutaneous fat in the abdomen', *Gynecol Obstet Invest*, 61(2), pp. 115-8.
- Kjellberg, U., Andersson, N. E., Rosén, S., Tengborn, L. and Hellgren, M. (1999) 'APC resistance and other haemostatic variables during pregnancy and puerperium', *Thromb Haemost*, 81(4), pp. 527-31.
- Klovaite, J., Nordestgaard, B. G., Tybjaerg-Hansen, A. and Benn, M. (2013) 'Elevated fibrinogen levels are associated with risk of pulmonary embolism, but not with deep venous thrombosis', *Am J Respir Crit Care Med*, 187(3), pp. 286-93.
- Knight M, Bunch K, Tuffnell D, Patel R, Shakespeare J, Kotnis R, Kenyon S and MBRRACE-UK., K. J. E. o. b. o. 2021. Saving Lives, Improving Mothers' Care - Lessons learned to inform maternity care from the UK and Ireland Confidential Enquiries into Maternal Deaths and Morbidity 2017-19. Oxford: National Perinatal Epidemiology Unit, University of Oxford 2021.
- Knight, M. and UKOSS (2008) 'Antenatal pulmonary embolism: risk factors, management and outcomes', *BJOG*, 115(4), pp. 453-61.
- Kopp, C. W., Kopp, H. P., Steiner, S., Kriwanek, S., Krzyzanowska, K., Bartok, A., Roka, R., Minar, E. and Schernthaner, G. (2003) 'Weight loss reduces tissue factor in morbidly obese patients', *Obes Res*, 11(8), pp. 950-6.
- Korda, M., Kubant, R., Patton, S. and Malinski, T. (2008) 'Leptin-induced endothelial dysfunction in obesity', *Am J Physiol Heart Circ Physiol*, 295(4), pp. H1514-21.
- Kovac, M. K., Lalic-Cosic, S. Z., Dmitrovic, J. M., Djordjevic, V. J. and Radojkovic, D. P. (2015) 'Thrombin generation, D-dimer and protein S in uncomplicated pregnancy', *Clin Chem Lab Med*, 53(12), pp. 1975-9.
- Kruithof, E. K., Tran-Thang, C., Gudinchet, A., Hauert, J., Nicoloso, G., Genton, C., Welti, H. and Bachmann, F. (1987) 'Fibrinolysis in pregnancy: a study of plasminogen activator inhibitors', *Blood*, 69(2), pp. 460-6.
- Kunutsor, S. K., Seidu, S., Blom, A. W., Khunti, K. and Laukkanen, J. A. (2017) 'Serum C-reactive protein increases the risk of venous thromboembolism: a prospective study and meta-analysis of published prospective evidence', *Eur J Epidemiol*, 32(8), pp. 657-667.
- Lain, K. Y. and Catalano, P. M. (2007) 'Metabolic changes in pregnancy', *Clin Obstet Gynecol*, 50(4), pp. 938-48.
- Larsen TB, S. H., Gislum M, Johnsen SP 2007. Maternal smoking, obesity and risk of venous thromboembolism during pregnancy and the puerperium: a population-based nested

case-control study. *Thromb Res*.

Lebaudy, C., Hulot, J. S., Amoura, Z., Costedoat-Chalumeau, N., Serreau, R., Ankri, A., Conard, J., Cornet, A., Dommergues, M., Piette, J. C. and Lechat, P. (2008) 'Changes in enoxaparin pharmacokinetics during pregnancy and implications for antithrombotic therapeutic strategy', *Clin Pharmacol Ther*, 84(3), pp. 370-7.

Lee, J., Eley, V. A., Wyssusek, K. H., Kimble, R. M. N., Way, M., Cohen, J. and van Zundert, A. A. (2020) 'The influence of obesity on coagulation in healthy term pregnancy as assessed by rotational thromboelastometry', *Aust N Z J Obstet Gynaecol*.

Leizorovicz, A., Cohen, A. T., Turpie, A. G., Olsson, C. G., Vaitkus, P. T., Goldhaber, S. Z. and Group, P. M. T. S. (2004) 'Randomized, placebo-controlled trial of dalteparin for the prevention of venous thromboembolism in acutely ill medical patients', *Circulation*, 110(7), pp. 874-9.

Li, W., Jiang, Y., Feng, L. and Yu, J. (2024) 'Visceral Adipose Tissue Depth as a Novel Predictor for Gestational Diabetes Mellitus: A Comprehensive Meta-Analysis and Systematic Review', *Medicina (Kaunas)*, 60(4).

Lim, S. and Meigs, J. B. (2014) 'Links between ectopic fat and vascular disease in humans', *Arterioscler Thromb Vasc Biol*, 34(9), pp. 1820-6.

Liu, S., Rouleau, J., Joseph, K. S., Sauve, R., Liston, R. M., Young, D. and Kramer, M. S. (2009) 'Epidemiology of pregnancy-associated venous thromboembolism: a population-based study in Canada', *J Obstet Gynaecol Can*, 31(7), pp. 611-620.

Liu, Y., He, L., Han, S., Ping, F., Li, W., Xu, L., Zhang, H. and Li, Y. (2025) 'Glucagon-Like Peptide-1 Receptor Agonists and Risk of Venous Thromboembolism: A Systematic Review and Meta-Analysis of Randomized Controlled Trials', *J Am Heart Assoc*, 14(9), pp. e039446.

Longstaff, C. and Kolev, K. (2015) 'Basic mechanisms and regulation of fibrinolysis', *J Thromb Haemost*, 13 Suppl 1, pp. S98-105.

Lu, W., Zhou, S., Xue, L., Dai, B., Qiao, L., Chen, D., Xu, Z., Yao, Y., Teng, H. and Jiang, Q. (2018) 'High preoperative serum leptin level is an independent risk factor for deep vein thrombosis after total knee arthroplasty in osteoarthritis patients: A prospective and cross-sectional study', *Medicine (Baltimore)*, 97(21), pp. e10884.

Lumeng, C. N., DelProposto, J. B., Westcott, D. J. and Saltiel, A. R. (2008) 'Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes', *Diabetes*, 57(12), pp. 3239-46.

Lupu, C., Lupu, F., Dennehy, U., Kakkar, V. V. and Scully, M. F. (1995) 'Thrombin induces the redistribution and acute release of tissue factor pathway inhibitor from specific granules within human endothelial cells in culture', *Arterioscler Thromb Vasc Biol*, 15(11), pp. 2055-62.

- M, K., K, B., D, T., H, J., J, S., R, K., S, K. and MBRRACE-UK., K. J. E. o. b. o. 2018. Saving Lives, Improving Mothers' Care - Lessons learned to inform maternity care from the UK and Ireland Confidential Enquiries into Maternal Deaths and Morbidity 2014-16. Oxford: National Perinatal Epidemiology Unit, University of Oxford.
- MACFARLANE, R. G. (1964) 'AN ENZYME CASCADE IN THE BLOOD CLOTTING MECHANISM, AND ITS FUNCTION AS A BIOCHEMICAL AMPLIFIER', *Nature*, 202, pp. 498-9.
- Machlus, K. R., Cardenas, J. C., Church, F. C. and Wolberg, A. S. (2011) 'Causal relationship between hyperfibrinogenemia, thrombosis, and resistance to thrombolysis in mice', *Blood*, 117(18), pp. 4953-63.
- Mandlebe, B., Orundami, O. I., Lynch, L. A., Teale, G., Said, J. M. and Cutts, B. A. (2021) 'Maternal thrombin generation and D-dimer levels in obesity and pregnancy: results from the maternal thrombin generation in obesity and pregnancy (MaTOPs) study', *Blood Coagul Fibrinolysis*, 32(6), pp. 394-400.
- Martin, A. M., Berger, H., Nisenbaum, R., Lausman, A. Y., MacGarvie, S., Crerar, C. and Ray, J. G. (2009) 'Abdominal visceral adiposity in the first trimester predicts glucose intolerance in later pregnancy', *Diabetes Care*, 32(7), pp. 1308-10.
- McLean, K. C., Bernstein, I. M. and Brummel-Ziedins, K. E. (2012) 'Tissue factor-dependent thrombin generation across pregnancy', *Am J Obstet Gynecol*, 207(2), pp. 135.e1-6.
- Meknas, D., Brækkan, S. K., Hansen, J. B. and Morelli, V. M. (2023) 'Surgery As a Trigger for Incident Venous Thromboembolism: Results from a Population-Based Case-Crossover Study', *TH Open*, 7(3), pp. e244-e250.
- Mertens, I. and Van Gaal, L. F. (2002) 'Obesity, haemostasis and the fibrinolytic system', *Obes Rev*, 3(2), pp. 85-101.
- Meyer, B. J., Stewart, F. M., Brown, E. A., Cooney, J., Nilsson, S., Olivecrona, G., Ramsay, J. E., Griffin, B. A., Caslake, M. J. and Freeman, D. J. (2013) 'Maternal obesity is associated with the formation of small dense LDL and hypoadiponectinemia in the third trimester', *J Clin Endocrinol Metab*, 98(2), pp. 643-52.
- Mitchell, C. T., Kamineni, A., Palmas, W. and Cushman, M. (2009) 'Tissue factor pathway inhibitor, vascular risk factors and subclinical atherosclerosis: the Multi-Ethnic Study of Atherosclerosis', *Atherosclerosis*, 207(1), pp. 277-83.
- Morange, P. E., Renucci, J. F., Charles, M. A., Aillaud, M. F., Giraud, F., Grimaux, M. and Juhan-Vague, I. (2001) 'Plasma levels of free and total TFPI, relationship with cardiovascular risk factors and endothelial cell markers', *Thromb Haemost*, 85(6), pp. 999-1003.
- Morgan, E. S., Wilson, E., Melody, T., Parmar, K., Zhang, Y., Gao, F. and Hunt, B. J. (2017) 'An observational study of haemostatic changes, leptin and soluble endoglin during pregnancy in women with different BMIs', *Blood Coagul Fibrinolysis*, 28(1), pp. 50-55.

Mrozinska, S., Cieslik, J., Broniatowska, E. and Undas, A. (2018) 'Elevated leptin and decreased adiponectin independently predict the post-thrombotic syndrome in obese and non-obese patients', *Sci Rep*, 8(1), pp. 6938.

Nakata, M., Yada, T., Soejima, N. and Maruyama, I. (1999) 'Leptin promotes aggregation of human platelets via the long form of its receptor', *Diabetes*, 48(2), pp. 426-9.

NMPA, N. M. a. P. A. T.-. 2018. National Maternity and Perinatal Audit: Clinical Report 2017. RCOG London.

NSS, N. N. S. S. (November 2016) *Births in Scottish Hospitals. Information Services Division Publication Report*.

O'Brien, T. E., Ray, J. G. and Chan, W. S. (2003) 'Maternal body mass index and the risk of preeclampsia: a systematic overview', *Epidemiology*, 14(3), pp. 368-74.

O'Keefe, D., Lim, H. Y., Tham, J., Ho, P. and Hui, L. (2021) 'Assessing maternal clotting function with novel global coagulation assays: A prospective pilot study', *Int J Lab Hematol*, 43(3), pp. 458-467.

Ogren, M., Eriksson, H., Bergqvist, D. and Sternby, N. H. (2005) 'Subcutaneous fat accumulation and BMI associated with risk for pulmonary embolism in patients with proximal deep vein thrombosis: a population study based on 23 796 consecutive autopsies', *J Intern Med*, 258(2), pp. 166-71.

Okamoto, Y., Ishii, S., Croce, K., Katsumata, H., Fukushima, M., Kihara, S., Libby, P. and Minami, S. (2013) 'Adiponectin inhibits macrophage tissue factor, a key trigger of thrombosis in disrupted atherosclerotic plaques', *Atherosclerosis*, 226(2), pp. 373-7.

Olson, N. C., Cushman, M., Lutsey, P. L., McClure, L. A., Judd, S., Tracy, R. P., Folsom, A. R. and Zakai, N. A. (2014) 'Inflammation markers and incident venous thromboembolism: the REasons for Geographic And Racial Differences in Stroke (REGARDS) cohort', *J Thromb Haemost*, 12(12), pp. 1993-2001.

Ouchi, N., Kihara, S., Arita, Y., Maeda, K., Kuriyama, H., Okamoto, Y., Hotta, K., Nishida, M., Takahashi, M., Nakamura, T., Yamashita, S., Funahashi, T. and Matsuzawa, Y. (1999) 'Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin', *Circulation*, 100(25), pp. 2473-6.

O'Connor H, K. B., Cooley S, et al 2014. The effect of morbid obesity on thrombin generation and sensitivity to the anticoagulant activated protein C in pregnant women. *Archives of Disease in Childhood - Fetal and Neonatal Edition*.

Palomo, M., Moreno-Castaño, A. B., Salas, M. Q., Escribano-Serrat, S., Rovira, M., Guillen-Olmos, E., Fernandez, S., Ventosa-Capell, H., Youssef, L., Crispi, F., Nomdedeu, M., Martinez-Sanchez, J., De Moner, B. and Diaz-Ricart, M. (2023) 'Endothelial activation and damage as a common pathological substrate in different pathologies and cell therapy complications', *Front Med (Lausanne)*, 10, pp. 1285898.

- Park, H. S., Park, J. Y. and Yu, R. (2005) 'Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF-alpha and IL-6', *Diabetes Res Clin Pract*, 69(1), pp. 29-35.
- Patel, J. P., Green, B., Patel, R. K., Marsh, M. S., Davies, J. G. and Arya, R. (2013) 'Population pharmacokinetics of enoxaparin during the antenatal period', *Circulation*, 128(13), pp. 1462-9.
- Peters, S. A. E., Bots, S. H. and Woodward, M. (2018) 'Sex Differences in the Association Between Measures of General and Central Adiposity and the Risk of Myocardial Infarction: Results From the UK Biobank', *J Am Heart Assoc*, 7(5).
- Petrini, S., Neri, T., Lombardi, S., Cordazzo, C., Balìa, C., Scalise, V., Paggiaro, P., Pedrinelli, R. and Celi, A. (2016) 'Leptin induces the generation of procoagulant, tissue factor bearing microparticles by human peripheral blood mononuclear cells', *Biochim Biophys Acta*, 1860(6), pp. 1354-61.
- Peverill, R. E., Teede, H. J., Malan, E., Kotsopoulos, D., Smolich, J. J. and McGrath, B. P. (2007) 'Relationship of waist and hip circumference with coagulation and fibrinolysis in postmenopausal women', *Clin Sci (Lond)*, 113(9), pp. 383-91.
- Phillips, J., McBride, C. A., Morris, E., Crocker, A. M. and Bernstein, I. (2018) 'Adiposity, but not Obesity, Is Associated With Arterial Stiffness in Young Nulliparous Women', *Reprod Sci*, 25(6), pp. 909-915.
- Pieterse, C., Schutte, R. and Schutte, A. E. (2015) 'Leptin links with plasminogen activator inhibitor-1 in human obesity: the SABPA study', *Hypertens Res*, 38(7), pp. 507-12.
- Pilli, V. S., Plautz, W. and Majumder, R. (2016) 'The Journey of Protein S from an Anticoagulant to a Signaling Molecule', *JSM Biochem Mol Biol*, 3(1).
- Pomp, E. R., le Cessie, S., Rosendaal, F. R. and Doggen, C. J. (2007) 'Risk of venous thrombosis: obesity and its joint effect with oral contraceptive use and prothrombotic mutations', *Br J Haematol*, 139(2), pp. 289-96.
- Pomp, E. R., Lenselink, A. M., Rosendaal, F. R. and Doggen, C. J. (2008) 'Pregnancy, the postpartum period and prothrombotic defects: risk of venous thrombosis in the MEGA study', *J Thromb Haemost*, 6(4), pp. 632-7.
- Pou, K. M., Massaro, J. M., Hoffmann, U., Vasan, R. S., Maurovich-Horvat, P., Larson, M. G., Keaney, J. F., Meigs, J. B., Lipinska, I., Kathiresan, S., Murabito, J. M., O'Donnell, C. J., Benjamin, E. J. and Fox, C. S. (2007) 'Visceral and subcutaneous adipose tissue volumes are cross-sectionally related to markers of inflammation and oxidative stress: the Framingham Heart Study', *Circulation*, 116(11), pp. 1234-41.
- Pouliot, M. C., Després, J. P., Lemieux, S., Moorjani, S., Bouchard, C., Tremblay, A., Nadeau, A. and Lupien, P. J. (1994) 'Waist circumference and abdominal sagittal diameter: best simple anthropometric indexes of abdominal visceral adipose tissue accumulation and related cardiovascular risk in men and women', *Am J Cardiol*, 73(7), pp. 460-8.

Prüller, F., Raggam, R. B., Posch, V., Almer, G., Truschnig-Wilders, M., Horejsi, R., Möller, R., Weghuber, D., Ille, R., Schnedl, W. and Mangge, H. (2012) 'Trunk weighted obesity, cholesterol levels and low grade inflammation are main determinants for enhanced thrombin generation', *Atherosclerosis*, 220(1), pp. 215-8.

Rafail, S., Ritis, K., Schaefer, K., Kourtzelis, I., Speletas, M., Doumas, M., Giaglis, S., Kambas, K., Konstantinides, S. and Kartalis, G. (2008) 'Leptin induces the expression of functional tissue factor in human neutrophils and peripheral blood mononuclear cells through JAK2-dependent mechanisms and TNFalpha involvement', *Thromb Res*, 122(3), pp. 366-75.

Ramsay, J. E., Ferrell, W. R., Crawford, L., Wallace, A. M., Greer, I. A. and Sattar, N. (2002) 'Maternal obesity is associated with dysregulation of metabolic, vascular, and inflammatory pathways', *J Clin Endocrinol Metab*, 87(9), pp. 4231-7.

Raps, M., Helmerhorst, F. M., Fleischer, K., Dahm, A. E., Rosendaal, F. R., Rosing, J., Reitsma, P., Sandset, P. M. and van Vliet, H. A. (2013) 'The effect of different hormonal contraceptives on plasma levels of free protein S and free TFPI', *Thromb Haemost*, 109(4), pp. 606-13.

RCOG 2015. Royal College of Obstetricians and Gynaecologists: Reducing the Risk of Thrombosis and Embolism during Pregnancy and the Puerperium (Green-top Guideline No. 37a).

Rezaie, A. R. and Giri, H. (2020) 'Anticoagulant and signaling functions of antithrombin', *J Thromb Haemost*, 18(12), pp. 3142-3153.

Rodger, M. A., Hague, W. M., Kingdom, J., Kahn, S. R., Karovitch, A., Sermer, M., Clement, A. M., Coat, S., Chan, W. S., Said, J., Rey, E., Robinson, S., Khurana, R., Demers, C., Kovacs, M. J., Solymoss, S., Hinshaw, K., Dwyer, J., Smith, G., ... and Investigators, T. (2014) 'Antepartum dalteparin versus no antepartum dalteparin for the prevention of pregnancy complications in pregnant women with thrombophilia (TIPPS): a multinational open-label randomised trial', *Lancet*, 384(9955), pp. 1673-83.

Rodger, M. A., Phillips, P., Kahn, S. R., James, A. H., Konkle, B. A. and Investigators, P. (2015) 'Low-molecular-weight heparin to prevent postpartum venous thromboembolism. A pilot randomised placebo-controlled trial', *Thromb Haemost*, 113(1), pp. 212-6.

Roeters van Lennep, J. E., Meijer, E., Klumper, F. J., Middeldorp, J. M., Bloemenkamp, K. W. and Middeldorp, S. (2011) 'Prophylaxis with low-dose low-molecular-weight heparin during pregnancy and postpartum: is it effective?', *J Thromb Haemost*, 9(3), pp. 473-80.

Rosenkranz, A., Hiden, M., Leschnik, B., Weiss, E. C., Schlembach, D., Lang, U., Gallistl, S. and Muntean, W. (2008) 'Calibrated automated thrombin generation in normal uncomplicated pregnancy', *Thromb Haemost*, 99(2), pp. 331-7.

Rottenstreich, A., Levin, G., Elchalal, U., Kleinstern, G., Spectre, G., Ziv, E., Yagel, S. and Kalish, Y. (2018) 'The effect on thrombin generation and anti-Xa

levels of thromboprophylaxis dose adjustment in post-cesarean obese patients - A prospective cohort study', *Thromb Res*, 170, pp. 69-74.

Samad, F., Pandey, M. and Loskutoff, D. J. (1998) 'Tissue factor gene expression in the adipose tissues of obese mice', *Proc Natl Acad Sci U S A*, 95(13), pp. 7591-6.

Samad, F. and Ruf, W. (2013) 'Inflammation, obesity, and thrombosis', *Blood*, 122(20), pp. 3415-22.

Samama MM, C. A., Darmon J, Desjardins L, Eldor A, and Janbon C, L. A., Nguyen H, Olsson C, Turpie AG, Weisslinger N 1999. A comparison of enoxaparin with placebo for the prevention of venous thrombo-embolism in acutely ill medical patients. *N Engl J Med*.

Sandset, P. M., Bendz, B. and Hansen, J. B. (2000) 'Physiological function of tissue factor pathway inhibitor and interaction with heparins', *Haemostasis*, 30 Suppl 2, pp. 48-56.

Saucedo, M., Esteves-Pereira, A. P., Pencolé, L., Rigouzzo, A., Proust, A., Bouvier-Colle, M. H., Deneux-Tharaux, C. and group, C. s. (2021) 'Understanding maternal mortality in women with obesity and the role of care they receive: a national case-control study', *Int J Obes (Lond)*, 45(1), pp. 258-265.

Savard, C., Lemieux, S., Plante, A. S., Gagnon, M., Leblanc, N., Veilleux, A., Tchernof, A. and Morisset, A. S. (2022) 'Longitudinal changes in circulating concentrations of inflammatory markers throughout pregnancy: are there associations with diet and weight status?', *Appl Physiol Nutr Metab*, 47(3), pp. 287-295.

Schlecht, I., Fischer, B., Behrens, G. and Leitzmann, M. F. (2016) 'Relations of Visceral and Abdominal Subcutaneous Adipose Tissue, Body Mass Index, and Waist Circumference to Serum Concentrations of Parameters of Chronic Inflammation', *Obes Facts*, 9(3), pp. 144-57.

Schulman, S., Makatsariya, A., Khizroeva, J., Bitsadze, V. and Kapanadze, D. (2024) 'The Basic Principles of Pathophysiology of Venous Thrombosis', *Int J Mol Sci*, 25(21).

Seadler, M. S., Ferraresso, F., Bansal, M., Haugen, A., Hayssen, W. G., Flick, M. J., de Moya, M., Dyer, M. R. and Kastrup, C. J. (2024) 'Suppressing upregulation of fibrinogen after polytrauma mitigates thrombosis in mice', *J Trauma Acute Care Surg*, 97(6), pp. 948-953.

Sedano-Balbás, S., Lyons, M., Cleary, B., Murray, M., Gaffney, G. and Maher, M. (2011) 'Acquired activated protein C resistance, thrombophilia and adverse pregnancy outcomes: a study performed in an Irish cohort of pregnant women', *J Pregnancy*, 2011, pp. 232840.

Selmeczi, A., Roach, R. E., Móri, C., Batta, Z., Hársfalvi, J., van der Bom, J. G., Boda, Z. and Oláh, Z. (2015) 'Thrombin generation and low-molecular-weight heparin prophylaxis in pregnant women with thrombophilia', *Thromb Haemost*, 113(2), pp. 283-9.

- Selovic, A., Sarac, J. and Missoni, S. (2016) 'Changes in adipose tissue distribution during pregnancy estimated by ultrasonography', *J Matern Fetal Neonatal Med*, 29(13), pp. 2131-7.
- Seven, E., Thuesen, B. H., Linneberg, A. and Jeppesen, J. L. (2016) 'Abdominal Adiposity Distribution Quantified by Ultrasound Imaging and Incident Hypertension in a General Population', *Hypertension*, 68(5), pp. 1115-1122.
- Simpson, E. L., Lawrenson, R. A., Nightingale, A. L. and Farmer, R. D. (2001) 'Venous thromboembolism in pregnancy and the puerperium: incidence and additional risk factors from a London perinatal database', *BJOG*, 108(1), pp. 56-60.
- Singh, P., Peterson, T. E., Barber, K. R., Kuniyoshi, F. S., Jensen, A., Hoffmann, M., Shamsuzzaman, A. S. and Somers, V. K. (2010) 'Leptin upregulates the expression of plasminogen activator inhibitor-1 in human vascular endothelial cells', *Biochem Biophys Res Commun*, 392(1), pp. 47-52.
- Sivan, E., Whittaker, P. G., Sinha, D., Homko, C. J., Lin, M., Reece, E. A. and Boden, G. (1998) 'Leptin in human pregnancy: the relationship with gestational hormones', *Am J Obstet Gynecol*, 179(5), pp. 1128-32.
- Slack, E., Best, K. E., Rankin, J. and Heslehurst, N. (2019) 'Maternal obesity classes, preterm and post-term birth: a retrospective analysis of 479,864 births in England', *BMC Pregnancy Childbirth*, 19(1), pp. 434.
- Smith, S. R., Lovejoy, J. C., Greenway, F., Ryan, D., deJonge, L., de la Bretonne, J., Volafova, J. and Bray, G. A. (2001) 'Contributions of total body fat, abdominal subcutaneous adipose tissue compartments, and visceral adipose tissue to the metabolic complications of obesity', *Metabolism*, 50(4), pp. 425-35.
- Smrcka, M. P., Thames, B., Beckman, M., Rajgor, D., Gandhi, M. and James, A. H. (2012) 'Obesity-related coagulation changes in pregnancy', *Thromb Res*, 129(2), pp. 204-6.
- Sniderman, A. D., Bhopal, R., Prabhakaran, D., Sarrafzadegan, N. and Tchernof, A. (2007) 'Why might South Asians be so susceptible to central obesity and its atherogenic consequences? The adipose tissue overflow hypothesis', *Int J Epidemiol*, 36(1), pp. 220-5.
- Sonagra, A. D., Biradar, S. M., K, D. and Murthy D S, J. (2014) 'Normal pregnancy- a state of insulin resistance', *J Clin Diagn Res*, 8(11), pp. CC01-3.
- Sonnevi, K., Tchaikovski, S. N., Holmström, M., Antovic, J. P., Bremme, K., Rosing, J. and Lärffars, G. (2013) 'Obesity and thrombin-generation profiles in women with venous thromboembolism', *Blood Coagul Fibrinolysis*, 24(5), pp. 547-53.
- Stein, P. D., Beemath, A. and Olson, R. E. (2005) 'Obesity as a risk factor in venous thromboembolism', *Am J Med*, 118(9), pp. 978-80.
- Stewart, F. M., Freeman, D. J., Ramsay, J. E., Greer, I. A., Caslake, M. and Ferrell, W. R. (2007) 'Longitudinal assessment of maternal endothelial function

and markers of inflammation and placental function throughout pregnancy in lean and obese mothers', *J Clin Endocrinol Metab*, 92(3), pp. 969-75.

Stirling, D., Hannant, W. A. and Ludlam, C. A. (1998) 'Transcriptional activation of the factor VIII gene in liver cell lines by interleukin-6', *Thromb Haemost*, 79(1), pp. 74-8.

Stirling, Y., Woolf, L., North, W. R., Seghatchian, M. J. and Meade, T. W. (1984) 'Haemostasis in normal pregnancy', *Thromb Haemost*, 52(2), pp. 176-82.

Straughen, J. K., Trudeau, S. and Misra, V. K. (2013) 'Changes in adipose tissue distribution during pregnancy in overweight and obese compared with normal weight women', *Nutr Diabetes*, 3(8), pp. e84.

Sulaiman, W. N., Caslake, M. J., Delles, C., Karlsson, H., Mulder, M. T., Graham, D. and Freeman, D. J. (2016) 'Does high-density lipoprotein protect vascular function in healthy pregnancy?', *Clin Sci (Lond)*, 130(7), pp. 491-7.

Sultan, A. A., West, J., Tata, L. J., Fleming, K. M., Nelson-Piercy, C. and Grainge, M. J. (2012) 'Risk of first venous thromboembolism in and around pregnancy: a population-based cohort study', *Br J Haematol*, 156(3), pp. 366-73.

Suárez-Cuenca, J. A., De La Peña-Sosa, G., De La Vega-Moreno, K., Banderas-Lares, D. Z., Salamanca-García, M., Martínez-Hernández, J. E., Vera-Gómez, E., Hernández-Patricio, A., Zamora-Alemán, C. R., Domínguez-Pérez, G. A., Ruíz-Hernández, A. S., Gutiérrez-Buendía, J. A., Melchor-López, A., Ortiz-Fernández, M., Montoya-Ramírez, J., Gaytán-Fuentes, O. F., Toríz-Ortiz, A., Osorio-Valero, M., Orozco-Vázquez, J., ... and Mondragón-Terán, P. (2021) 'Enlarged adipocytes from subcutaneous vs. visceral adipose tissue differentially contribute to metabolic dysfunction and atherogenic risk of patients with obesity', *Sci Rep*, 11(1), pp. 1831.

Szecsí, P. B., Jørgensen, M., Klajnbard, A., Andersen, M. R., Colov, N. P. and Stender, S. (2010) 'Haemostatic reference intervals in pregnancy', *Thromb Haemost*, 103(4), pp. 718-27.

Szotowski, B., Antoniuk, S., Poller, W., Schultheiss, H. P. and Rauch, U. (2005) 'Procoagulant soluble tissue factor is released from endothelial cells in response to inflammatory cytokines', *Circ Res*, 96(12), pp. 1233-9.

Sánchez-Luceros, A., Meschengieser, S. S., Marchese, C., Votta, R., Casais, P., Woods, A. I., Nadal, M. V., Salviú, M. J. and Lazzari, M. A. (2003) 'Factor VIII and von Willebrand factor changes during normal pregnancy and puerperium', *Blood Coagul Fibrinolysis*, 14(7), pp. 647-51.

Tang, J., Lin, Y., Mai, H., Luo, Y., Huang, R., Chen, Q. and Xiao, D. (2019) 'Meta-analysis of reference values of haemostatic markers during pregnancy and childbirth', *Taiwan J Obstet Gynecol*, 58(1), pp. 29-35.

Tardy-Poncet, B., Piot, M., Brunet, D., Chapelle, C., Bonardel, M., Mismetti, P., Morange, P. and Tardy, B. (2012) 'TFPI resistance related to inherited or acquired protein S deficiency', *Thromb Res*, 130(6), pp. 925-8.

Taura, P., Rivas, E., Martinez-Palli, G., Blasi, A., Holguera, J. C., Balust, J., Delgado, S. and Lacy, A. M. (2014) 'Clinical markers of the hypercoagulable state by rotational thrombelastometry in obese patients submitted to bariatric surgery', *Surg Endosc*, 28(2), pp. 543-51.

Tchaikovsky, S. N. and Rosing, J. (2010) 'Mechanisms of estrogen-induced venous thromboembolism', *Thromb Res*, 126(1), pp. 5-11.

Tchaikovsky, S. N., Thomassen, M. C., Costa, S. D., Peeters, L. L. and Rosing, J. (2011) 'Role of protein S and tissue factor pathway inhibitor in the development of activated protein C resistance early in pregnancy in women with a history of preeclampsia', *Thromb Haemost*, 106(5), pp. 914-21.

Tessier, D. R., Ferraro, Z. M. and Gruslin, A. (2013) 'Role of leptin in pregnancy: consequences of maternal obesity', *Placenta*, 34(3), pp. 205-11.

Tripodi, A., Legnani, C., Chantarangkul, V., Cosmi, B., Palareti, G. and Mannucci, P. M. (2008) 'High thrombin generation measured in the presence of thrombomodulin is associated with an increased risk of recurrent venous thromboembolism', *J Thromb Haemost*, 6(8), pp. 1327-33.

Tripodi, A., Primignani, M., Badiali, S., de Ruberto, F., Granelli, P., Tosetti, G., Clerici, M., Padovan, L., Chantarangkul, V., Scalabrino, E. and Peyvandi, F. (2019) 'Body mass index reduction improves the baseline procoagulant imbalance of obese subjects', *J Thromb Thrombolysis*, 48(1), pp. 52-60.

Trivett, C., Lees, Z. J. and Freeman, D. J. (2021) 'Adipose tissue function in healthy pregnancy, gestational diabetes mellitus and pre-eclampsia', *Eur J Clin Nutr*, 75(12), pp. 1745-1756.

Udenze, I. C., Arikawe, A. P. and Makwe, C. C. (2017) 'Early pregnancy plasminogen activator inhibitor-1 levels in Nigerian women and its relationship with preeclampsia', *Niger J Clin Pract*, 20(5), pp. 517-522.

Uszyński, M., Zekanowska, E., Uszyński, W. and Kuczyński, J. (2001) 'Tissue factor (TF) and tissue factor pathway inhibitor (TFPI) in amniotic fluid and blood plasma: implications for the mechanism of amniotic fluid embolism', *Eur J Obstet Gynecol Reprod Biol*, 95(2), pp. 163-6.

Vambergue, A., Rugeri, L., Gaveriaux, V., Devos, P., Martin, A., Fermon, C., Fontaine, P. and Jude, B. (2001) 'Factor VII, tissue factor pathway inhibitor, and monocyte tissue factor in diabetes mellitus: influence of type of diabetes, obesity index, and age', *Thromb Res*, 101(5), pp. 367-75.

van den Munckhof, I. C. L., Bahrar, H., Schraa, K., Brand, T., Ter Horst, R., van der Graaf, M., Dekker, H. M., Stienstra, R., de Graaf, J., Joosten, L. A. B., Netea, M. G., Riksen, N. P. and Rutten, J. H. W. (2024) 'Sex-specific association of visceral and subcutaneous adipose tissue volumes with systemic inflammation and innate immune cells in people living with obesity', *Int J Obes (Lond)*, 48(4), pp. 523-532.

Vecchione, C., Maffei, A., Colella, S., Aretini, A., Poulet, R., Frati, G., Gentile, M. T., Fratta, L., Trimarco, V., Trimarco, B. and Lembo, G. (2002) 'Leptin effect

on endothelial nitric oxide is mediated through Akt-endothelial nitric oxide synthase phosphorylation pathway', *Diabetes*, 51(1), pp. 168-73.

Vilahur, G., Ben-Aicha, S. and Badimon, L. (2017) 'New insights into the role of adipose tissue in thrombosis', *Cardiovasc Res*, 113(9), pp. 1046-1054.

Walker, M. C., Garner, P. R., Keely, E. J., Rock, G. A. and Reis, M. D. (1997) 'Changes in activated protein C resistance during normal pregnancy', *Am J Obstet Gynecol*, 177(1), pp. 162-9.

Wallace, A. M., McMahon, A. D., Packard, C. J., Kelly, A., Shepherd, J., Gaw, A. and Sattar, N. (2001) 'Plasma leptin and the risk of cardiovascular disease in the west of Scotland coronary prevention study (WOSCOPS)', *Circulation*, 104(25), pp. 3052-6.

Wang, J., Tan, J., Hua, L., Sheng, Q., Huang, X. and Liu, P. (2023) 'Genetic Predisposition of Both Waist Circumference and Hip Circumference Increased the Risk of Venous Thromboembolism', *Thromb Haemost*, 123(3), pp. 347-361.

Wang, J., Wang, H., Luo, W., Guo, C., Chen, Y. E., Chang, L. and Eitzman, D. T. (2013) 'Leptin-induced endothelial dysfunction is mediated by sympathetic nervous system activity', *J Am Heart Assoc*, 2(5), pp. e000299.

Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L. and Ferrante, A. W. (2003) 'Obesity is associated with macrophage accumulation in adipose tissue', *J Clin Invest*, 112(12), pp. 1796-808.

WHO, W. H. O. 2008. Waist circumference and waist-hip ratio: report of a WHO expert consultation, Geneva, 8-11 December 2008.

WHO, W. H. O. 2010. A Healthy Lifestyle: WHO recommendations (factsheet).

Yatsenko, T., Us, I., Korolova, D., Zhuk, S., Dziuba, H., Nalbat, A., Kharchenko, S., Vari, S. G. and Chernyshenko, V. (2025) 'Placental Dysfunction Is Associated with Dysregulated Fibrinolytic System Activation', *Int J Mol Sci*, 26(19).

Yokota, T., Oritani, K., Takahashi, I., Ishikawa, J., Matsuyama, A., Ouchi, N., Kihara, S., Funahashi, T., Tenner, A. J., Tomiyama, Y. and Matsuzawa, Y. (2000) 'Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages', *Blood*, 96(5), pp. 1723-32.

Zarkesh-Esfahani, H., Pockley, G., Metcalfe, R. A., Bidlingmaier, M., Wu, Z., Ajami, A., Weetman, A. P., Strasburger, C. J. and Ross, R. J. (2001) 'High-dose leptin activates human leukocytes via receptor expression on monocytes', *J Immunol*, 167(8), pp. 4593-9.

Zembala-Szczerba, M., Jaworowski, A., Huras, H., Babczyk, D. and Jach, R. (2017) 'Low-Grade Metabolically-Induced Inflammation Mediators Interleukin-6, Adiponectin, and TNF- α Serum Levels in Obese Pregnant Patients in the Perinatal Period', *Med Sci Monit Basic Res*, 23, pp. 1-7.

Zhou, M., Xu, H., Zhang, D., Si, C., Zhou, X., Zhao, H., Liu, Q., Xu, B. and Zhang, A. (2021) 'Decreased PIBF1/IL6/p-STAT3 during the mid-secretory phase

inhibits human endometrial stromal cell proliferation and decidualization', *J Adv Res*, 30, pp. 15-25.

Ziegler, A. K. and Scheele, C. (2024) 'Human adipose depots' diverse functions and dysregulations during cardiometabolic disease', *NPJ Metab Health Dis*, 2(1), pp. 34.