CORA Cork Open Research Archive
Cartlann Taighde Oscailte Chorcaí

Title	Characterisation of the role of mitochondrial dysfunction and meta-inflammation as a shared pathogenic network in gestational diabetes mellitus
Authors	McElwain, Colm
Publication date	2023-05-02
Original Citation	McElwain, C. 2023. Characterisation of the role of mitochondrial dysfunction and meta-inflammation as a shared pathogenic network in gestational diabetes mellitus. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
Rights	© 2023, Colm McElwain. - https://creativecommons.org/licenses/by/4.0/
Download date	2026-11-06 12:59:25
Item downloaded from	https://hdl.handle.net/10468/14583



UCC
University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

Ollscoil na hÉireann, Corcaigh

National University of Ireland, Cork



Characterisation of the Role of Mitochondrial Dysfunction and Meta-inflammation as a Shared Pathogenic Network in Gestational Diabetes Mellitus

Thesis presented by

Colm McElwain, MPharm, MPSI

for the degree of

Doctor of Philosophy

Department of Pharmacology and Therapeutics

Head of Department: Prof. Christian Waeber

Supervisors: Dr. Cathal McCarthy and Dr. Fergus McCarthy

2023

Table of Contents

Declaration x

Presentations and Publications xviii

List of Tables xx

List of Figures xxi

List of Abbreviations x

Acknowledgements xxvi

Thesis Abstract 1

Chapter 1 *Introduction* 4

1.1 Gestational Diabetes Mellitus 5

1.2 Clinical Diagnosis of Gestational Diabetes Mellitus 5

1.3 Risk Factors for Gestational Diabetes Mellitus 6

1.3.1 Genetic Predisposition to Gestational Diabetes Mellitus 7

1.3.2 Maternal Obesity in Gestational Diabetes Mellitus 8

1.4 Gestational Diabetes Mellitus and Future Cardiometabolic Risk 9

1.4.1 Maternal Outcomes 9

1.4.2 Fetal Outcomes 11

1.5 Pathophysiology of Gestational Diabetes Mellitus 12

1.5.1 Pancreatic Beta Cell Dysfunction 13

1.5.2 Insulin Resistance 15

1.5.3 Insulin Signalling Cascade 15

1.5.4 Adipose Tissue....18

1.5.5. Insulin Growth Factor (IGF) and IGF-Binding Proteins in Adipocyte Expansion....20

1.5.6 Adipokines in Gestational Diabetes Mellitus....21

1.5.7 Role of the Placenta in Gestational Diabetes Mellitus....25

1.5.8 Mitochondrial Dysfunction and Oxidative Stress in Gestational Diabetes Mellitus....30

1.5.9 Role of Inflammation in Gestational Diabetes Mellitus....33

1.5.10 Role of Immune Cells in Gestational Diabetes Mellitus....36

1.6 Pharmacological Treatment Strategies for Gestational Diabetes Mellitus....45

1.7 Thesis Hypothesis, Aims, & Objectives....50

1.7.1 Hypothesis....50

1.7.2 Aims....50

1.7.3 Objectives....51

Chapter 2 *Circulating Markers of Insulin Resistance and Meta-inflammation in Gestational Diabetes Mellitus*....52

Abstract....53

2.1 Introduction....56

2.2 Methods....59

2.2.1 Study design and participant recruitment....59

2.2.2 Sample collection....60

2.2.3 Immunoassays....60

2.2.4 Peripheral blood mononuclear cell (PBMC) isolation.....61

2.2.5 Flow cytometry analysis.....62

2.2.6 Cf-mtDNA quantification.....63

2.2.7 TLR9 activation assay in HEK-blue hTLR9 cells.....63

2.2.8 Statistics.....64

2.3 Results.....65

2.3.1 Baseline characteristics of study participants.....65

2.3.2 Elevated systemic insulin resistance in GDM.....66

2.3.3 Phenotype heterogeneity in GDM classification.....68

2.3.4 Serum triglyceride and FFA lipid profile is not altered in GDM.....70

2.3.5 Circulating levels of IGF and PAPPA are not significantly different in GDM.....72

2.3.6 Adiponectin is significantly reduced in maternal circulation in GDM.....73

2.3.7 Circulating cf-mtDNA, a marker of mitochondrial dysfunction, is significantly increased in GDM.....74

2.3.8 No change in the phenotype of circulating monocyte/macrophage populations in GDM.....77

2.3.9 Mitochondrial superoxide production by circulating $CD14^+$ cells is not significantly altered in GDM.....79

2.3.10 Significant increase in circulating inflammatory mediators in GDM.....81

2.4 Discussion.....85

2.5 Conclusion.....94

Chapter 3 ***Adipocyte Hypoplasia and Impaired Insulin Signalling in Gestational Diabetes Mellitus*** **.....95**

Abstract 96

3.1 Introduction 98

3.2 Methods 101

3.2.1 Study design and participant recruitment 101

3.2.2 Sample collection 102

3.2.3 Immunohistochemical analysis 102

3.2.4 Protein isolation and immunoblotting 104

3.2.5 Omental VAT and placental explant culture 105

3.2.6 Immunoassays 105

3.2.7 Isolation of leukocytes from omental tissue 106

3.2.8 Flow cytometry analysis 107

3.2.9 Statistics 108

3.3 Results 110

3.3.1 Reduced adipocyte count in omental VAT in GDM 110

3.3.2 Elevated insulin resistance correlates with adipocyte hypoplasia in GDM 113

3.3.3 Expression of IGFBP-4 is significantly reduced in VAT in GDM 114

3.3.4 Omental or placental secretion of PAPPA is not significantly altered in GDM 116

3.3.5 Disruption in insulin signalling in omental VAT in GDM 117

3.3.6 Omental production of adipokines is not significantly altered in GDM .120

3.3.7 Macrophage phenotype is not significantly altered in omental VAT in GDM 121

3.3.8 Macrophage production of mitochondrial superoxide is not altered in GDM omental VAT 124

3.3.9 No evidence of localised inflammation in omental VAT in GDM.....125

3.4 Discussion 128

3.5 Conclusion.....134

Supplementary material related to Chapter 3 135

Chapter 4 ***Mitochondrial Dysfunction Promotes a Dysregulated Placental Inflammatory Response in Gestational Diabetes Mellitus*****.....139**

Abstract 140

4.1 Introduction 143

4.2 Methods.....148

4.2.1 Study design and patient recruitment.....148

4.2.2 Sample collection.....149

4.2.3 Placental and omental explant culture and treatments.....149

4.2.4 Immunohistochemical Staining 150

4.2.5 Image analysis.....150

4.2.6 Immunoassays.....151

4.2.7 Isolation of leukocytes from placental tissue.....152

4.2.8 Flow cytometry analysis 153

4.2.9 TLR9 activation assay 154

4.2.10 Protein isolation and immunoblotting 155

4.2.11 Statistics 156

4.3. Results 157

4.3.1 Placental villous immaturity in GDM 157

4.3.2 Macrophage phenotype is not significantly altered in placental tissue in GDM 159

4.3.3 Generation of mitochondrial superoxide by placental macrophages is significantly increased in GDM 161

4.3.4. Pervasive oxidative damage is not evident in placental tissue in GDM . 162

4.3.5 Placental-derived mediators do not trigger TLR-9 activation 163

4.3.6 Placental expression of NLRP3 inflammasome cascade members are not altered in GDM 165

4.3.7 Increased placental production of IL-18, a NLRP3-related cytokine, in GDM 167

4.3.8 ERG significantly attenuates NLRP3-related cytokine release from placental tissue in GDM 169

4.3.9 Omental expression of NLRP3 inflammasome cascade members are not altered in GDM 170

4.3.10 Circulating and omental production of NLRP3-related cytokines IL-1β and IL-18 are not significantly increased in GDM 172

4.3.11 Placental inflammation is significantly increased in GDM 173

4.3.12 Dysregulation in placental adipokine production in GDM 176

4.4 Discussion 178

4.5 Conclusion 186

Supplementary material related to Chapter 4 187

Chapter 5 ***Investigating adipose-placental cross-talk in mediating the pathophysiology of GDM*** **..... 190**

Abstract 191

5.1 Introduction 194

5.2 Methods 197

5.2.1 Study design and participant recruitment 197

5.2.2 Sample collection 198

5.2.3 Placental and omental explant culture 198

5.2.4 Cell culture 199

5.2.5 Adipogenesis assay 200

5.2.6 Immunoblotting 201

5.2.7 Lipid utilisation assay 202

5.2.8 Glucose uptake assay 202

5.2.9 Mitochondrial superoxide assay 203

5.2.10 Omental-stimulated HTR8 cell cytokine release 204

5.2.11 Statistics 205

5.3 Results 206

5.3.1 Lack of evidence of placental-mediated increase in adipogenesis in GDM 206

5.3.2 Placental-derived mediators in GDM do not affect adipocyte IGFBP expression 207

5.3.3 Adipocyte IC lipid content is not altered by placental mediators in GDM 209

5.3.4 Placental-derived mediators in GDM do not affect adipocyte glucose uptake.....210

5.3.5 Insulin signalling in adipocytes is not significantly altered by placental-derived mediators in GDM 211

5.3.6 Placental-derived mediators in GDM have no significant impact on adipocyte mROS production.....214

5.3.7 Effect of omental-derived mediators in GDM on the placental production of mROS 215

5.3.8 Omental-derived mediators in GDM do not alter placental cytokine release 216

5.4 Discussion 219

5.5 Conclusion.....226

Chapter 6 *General discussion*.....229

6.1 Overview and summary of key findings 230

6.2 Interpretation of key findings.....234

6.3 Strengths and limitations.....242

6.4 Implications for Future Research 243

6.5 Conclusion.....247

References 248

List of Abbreviations

2DG6P - 2-deoxyglucose-6-phosphate

ACOG - American College of Obstetricians and Gynecologists

ADHD - Attention-deficit/hyperactivity disorder

ADIPOR1 - Adiponectin receptor type 1

ADIPOR2 - Adiponectin receptor type 2

AGE - Advanced glycation end product

AKT/PKB - Protein kinase B

AMP - Adenosine monophosphate

AMPK - AMP-activated protein kinase

APC - Adipocyte progenitor cell

APC - Antigen presenting cell

APPL1 - Adaptor protein containing a pleckstrin homology domain 1

ASC - Apoptosis-associated speck-like protein containing a CARD

ASD - Autism spectrum disorders

ASP - Acylation stimulating protein

AT - Adipose tissue

ATP - Adenosine triphosphate

BMI - Body mass index

CDKAL1 - CDK5 regulatory subunit associated protein 1 like 1

CDKN2A/2B - Cyclin dependent kinase inhibitor 2A/B

CfDNA - Cell-free DNA

Cf-mtDNA - Cell free mtDNA

CMPF - 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid

CRP - C-reactive protein

CUMH - Cork University Maternity Hospital

CVD - Cardiovascular disease

DAMP - Damage-associated molecular pattern

DC - Dendritic cell

DNA - Deoxyribonucleic acid

DNK - Decidual NK

DVI - Distal villous immaturity

EDC - Endocrine disrupting chemical

EDTA - Ethylenediaminetetraacetic acid

ENOS - Endothelial nitric oxide synthase

ERG - L-ergothioneine

ERK1/2 - Extracellular signal-regulated kinase 1/2

ETC – Electron transport chain

FA - Fatty acid

FAO - Fatty acid β-oxidation

FFA - Free fatty acid

FGR - Fetal growth restriction

FMD - Flow-mediated dilation

FTO - Fat-mass and obesity-associated gene

GAP - GTPase-activating protein

GDM - Gestational diabetes mellitus

GLUT4 - Glucose transporter type 4

GM-CSF - Granulocyte macrophage colony-stimulating factor

GSV - GLUT4 storage vesicle

GWG - Gestational weight gain

HBC - Hofbauer cell

HDL - High-density lipoprotein

HHEX - Hematopoietically expressed homeobox

HLA - Human leukocyte antigen

HOMA-IR - Homeostasis model assessment for insulin resistance

HPGH - Human placental growth hormone

HPL - Human placental lactogen

HSE - Health Service Executive

IADPSG - International Association of Diabetes and Pregnancy Study Groups

IGF - Insulin growth factor

IGF2BP2 - Insulin-like growth factor 2 mRNA-binding protein 2

IGFBP - IGF-binding protein

IGT - Impaired glucose tolerance

IKK - IκB kinase

IL - Interleukin

IRS - Insulin receptor substrate

IUGR - Intrauterine growth restriction

JNK - C-Jun N-terminal kinase

KCNQ1 - Potassium voltage-gated channel subfamily Q member 1

LGA - Large for gestational age

LPS - Lipopolysaccharide

MAPK - Mitogen-activated protein kinase

MC4R - Melanocortin 4 receptor

MCP-1 - Monocyte chemoattractant protein-1

MDA - Malondialdehyde

MIF - Migration-inhibitory factor

MHC - Major histocompatibility complex

MLR - Monocyte-lymphocyte ratio

MRNA - Messenger RNA

MROS - Mitochondrial ROS

MtDNA - Mitochondrial DNA

MTNR1B - Melatonin receptor 1B

MTORC - Mammalian target of rapamycin complex

NAFLD - Non-alcoholic fatty liver disease

NF-κB - Nuclear factor kappa B

NGT - Normal glucose tolerance

NK – Natural killer

NLRP3 - NOD-, LRR- and pyrin domain-containing protein 3

NO - Nitric oxide

Nrf2 - Nuclear factor erythroid 2–related factor 2

OCTN1 - Organic cation transporter novel type 1

OGTT - Oral glucose tolerance test

OxLDL - Oxidised low-density lipoproteins

PA - Palmitic acid

PAMP - Pathogen-associated molecular pattern

PAPPA - Pregnancy-associated plasma protein A

PBMC - Peripheral blood mononuclear cell

PCOS - Polycystic ovary syndrome

PdE - Placenta-derived exosome

PDK1 - Phosphoinositide-dependent kinase 1

PE - Pre-eclampsia

PGC-1α - Peroxisome proliferator-activated receptor-γ coactivator 1α

PI3K - Phosphatidylinositol 3-kinase

PIP2 - Phosphatidylinositol-4,5-diphosphate

PIP3 - Phosphatidylinositol-3,4,5-trisphosphate

PLL - Poly-L-lysine

PPAR - Peroxisome proliferator-activated receptor

PROX1 - Prospero homeobox 1

PSR - Picro sirius red

RGC - RAL–GAP complex

RNA - Ribonucleic acid

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

SAT - Subcutaneous adipose tissue

SCD14 - Soluble CD14

SCD163 - Soluble CD163

SdLDL - Small dense low-density lipoprotein

SFlt-1 - Soluble fms-like tyrosine kinase-1

SLC30A8 - Solute carrier family 30 member 8

SOD - Superoxide dismutase

T1DM - Type 1 diabetes mellitus

T2DM - Type 2 diabetes mellitus

TCF7L2 - Transcription factor 7 like 2

Th1 - T-helper 1

Th17 - T-helper 17

Th2 - T-helper 2

TLR - Toll-like receptor

TNF - Tumour necrosis factor

Treg - Regulatory T cell

TXNIP - Thioredoxin-interacting protein

UCP-1 - Uncoupling protein 1

VAT - Visceral adipose tissue

VEGF - Vascular endothelial growth factor

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

Author contribution

All work was personally and independently performed by the author with the following exceptions:

Chapter 2

Dr. Andrea Musumeci assisted with leukocyte isolation, cell staining and flow cytometry analysis.

Chapter 3

Chloe Rouchon, Anne Marie O'Callaghan and Mustafa Bakhit Ebad assisted with the blinded histological analysis of adipocyte morphology and fibrosis. Isaac Sylvester assisted with the immunoblotting analysis of insulin signalling components. Dr. Andrea Musumeci assisted with leukocyte isolation, cell staining and flow cytometry analysis.

Chapter 4

Diego Alberto Mendez Ceja assisted with the blinded histological analysis of placental morphology. Dr. Andrea Musumeci assisted with leukocyte isolation, cell staining and flow cytometry analysis.

Presentations and Publications

- Irish Association of Pharmacologists (IAP) 2019 – Poster presentation: *"Investigating the role of the NLRP3 inflammasome in the pathophysiology of pre-eclampsia and gestational diabetes mellitus"*
- New Horizons University College Cork 2019 – Oral presentation: *"Investigating the role of mitochondrial dysfunction in gestational diabetes mellitus and elucidating if BMI is a causative mediator"*
- New Horizons University College Cork 2020 – Poster presentation: *"Investigating a Role for the NLRP3 Inflammasome in the Pathophysiology of Gestational Diabetes Mellitus"*
- Society for Reproductive Investigation (SRI) 2021 – Poster presentation: *"Investigating a Role for the NLRP3 Inflammasome in the Pathophysiology of Gestational Diabetes Mellitus"*
- International Federation of Placenta Associations (IFPA) 2021 – Oral presentation: *"Elucidating a Role for the NLRP3 Inflammasome in Gestational Diabetes Mellitus"*
- Irish Association of Pharmacologists (IAP) 2021 - Poster presentation: *"Investigating a Role for the NLRP3 Inflammasome in the Pathophysiology of Gestational Diabetes Mellitus"* – best poster presentation award
- EMBO workshop 2021 "The inflammasomes: The next frontier" – Poster presentation: *"Investigating a Role for the NLRP3 Inflammasome in the Pathophysiology of Gestational Diabetes Mellitus"*
- Biochemical Society "The adipocyte across biological scales" 2021 – Poster presentation: *"Investigating Omental Visceral Adipose Tissue Dysfunction in the Pathophysiology of Gestational Diabetes Mellitus"*

- The American Society for Reproductive Immunology (ASRI) 2022 – Oral presentation: *"Mitochondrial Dysfunction Drives a Dysregulated Inflammatory Response in Gestational Diabetes Mellitus"*
- 9th ESE Young Endocrinologists and Scientists' annual meeting 2022 – Oral presentation: "*Placental Signalling Contributes to Adipokine Dysregulation and Systemic Insulin Resistance in Gestational Diabetes Mellitus*" – best oral presentation award
- College of Medicine and Health (UCC) Research Conference "from Molecules to People" 2022 – Poster presentation: "*Mitochondrial Dysfunction Drives a Dysregulated Inflammatory Response in Gestational Diabetes Mellitus*"
- Keystone Symposium 2023: Adipose Tissue: Energizing Good Fat – Poster presentation: *"Adipocyte hypoplasia and metabolic dysfunction promotes insulin resistance in gestational diabetes mellitus"*

Publications relating to this thesis

– **McElwain CJ**, Tuboly E, McCarthy FP, McCarthy CM. Mechanisms of Endothelial Dysfunction in Pre-eclampsia and Gestational Diabetes Mellitus: Windows Into Future Cardiometabolic Health? Front Endocrinol (Lausanne). 2020 Sep 11;11:655.

– **McElwain CJ**, McCarthy FP, McCarthy CM. Gestational Diabetes Mellitus and Maternal Immune Dysregulation: What We Know So Far. International Journal of Molecular Sciences. 2021; 22(8):4261.

List of Tables

- Table 1.1. A summary of the studies describing immune cell populations in maternal circulation, placental tissue and adipose tissue in GDM pregnancies and how their cellular function is altered in GDM relative to uncomplicated pregnancies.
- Table 2.1. Clinical characteristics of study participants.
- Table 2.2. Circulating levels of pro-inflammatory cytokines in NGT and GDM participants.
- Table 3.1. Omental VAT levels of pro-inflammatory cytokines in NGT and GDM participants.
- Table 4.1. Placental tissue levels of pro-inflammatory cytokines in NGT and GDM participants.
- Table 5.1. HTR8 production of pro-inflammatory cytokines following treatment with NGT and GDM omental supernatant.
- Table 6.1. Summary of macrophage phenotype, cytokine and adipokine findings in maternal serum, omental VAT and placental tissue.

List of Figures

- Figure 1.1. Insulin signalling regulates GLUT4 exocytosis by engaging intracellular trafficking machinery.
- Figure 1.2. An overview characterising immune cell phenotypes in maternal circulation, adipose tissue and placental tissue in healthy uncomplicated pregnancy compared to a pregnancy complicated by GDM.
- Figure 1.3. Glycaemic and immune-mediated effects of clinically approved therapeutic options for GDM.
- Figure 2.1. GDM participants show significant systemic insulin resistance.
- Figure 2.2. Lean GDM participants have significantly lower circulating insulin levels and HOMA-IR scores relative to obese GDM participants.
- Figure 2.3. Circulating levels of triglycerides and FFAs are not significantly different in GDM.
- Figure 2.4. Circulating levels of IGF-1 and PAPPA are not altered in GDM.
- Figure 2.5. GDM participants have significantly lower circulating levels of adiponectin.
- Figure 2.6. Increased circulating cf-mtDNA in GDM.
- Figure 2.7. Phenotype of circulating monocyte/macrophage populations in GDM.
- Figure 2.8. Mitochondrial-specific superoxide production in circulating monocyte/macrophage populations in GDM.
- Figure 2.9. Increase in pro-inflammatory cytokines in the maternal circulation in GDM.
- Figure 3.1. Adipocyte hypoplasia in visceral adipose tissue in GDM.

- Figure 3.2. Insulin resistance correlates with adipocyte hypoplasia in GDM participants.
- Figure 3.3. IGFBP-4 expression is significantly decreased in omental VAT in GDM.
- Figure 3.4. Release of PAPPA from placental tissue or VAT is not altered in GDM.
- Figure 3.5. Altered insulin signalling in omental VAT in GDM.
- Figure 3.6. Adipokine secretion from omental VAT in GDM.
- Figure 3.7. Macrophage phenotype is unchanged in omental VAT depot in GDM.
- Figure 3.8. Mitochondrial-specific superoxide production in omental VAT macrophages is not altered in GDM.
- Figure 3.9. No change in inflammatory cytokines in omental VAT explants in GDM.
- Figure S3.1. Omental VAT protein expression of IGFBP-3 and IGFBP-4 was measured in NGT (n=8) and GDM (n=8) samples, matched by maternal age and BMI. Matched pairs were loaded adjacent to each other over two separate blots (A + B).
- Figure S3.2. Omental VAT protein expression of IRS-1, IRS-2, phospho-IRS-1(ser312), phospho-IRS-1(ser318), phospho-IRS-1(ser612), PDK1 and PI3K was measured in NGT (n=8) and GDM (n=8) samples, matched by maternal age and BMI. Matched pairs were loaded adjacent to each other over two separate blots (A + B).
- Figure 4.1. Activation of the NLRP3 inflammasome.
- Figure 4.2. Reduction in placental villi maturation in GDM.

- Figure 4.3. Macrophage phenotype in placental tissue in GDM.
- Figure 4.4. Increased mitochondrial superoxide production in $CD14^+$ and $CD14^+CD86^+CD206^-$ placental macrophages in GDM.
- Figure 4.5. Oxidative stress-induced damage is not increased in placental tissue in GDM.
- Figure 4.5. Placental derived mediators do not alter TLR9-induced inflammatory signalling in GDM.
- Figure 4.6. Placental expression of the NLRP3 inflammasome cascade in GDM.
- Figure 4.7. Placental secretion of NLRP3-related cytokines IL-1β and IL-18.
- Figure 4.8. ERG dampens placental production of pro-inflammatory cytokines IL-18 and IL-1β in GDM.
- Figure 4.9. Expression of the NLRP3 inflammasome cascade in adipose tissue in GDM.
- Figure 4.10. Circulating and omental production of NLRP3-related cytokines IL-18 and IL-1β.
- Figure 4.11. Increase in pro-inflammatory cytokines IL-6 and TNF-α in placental tissue in GDM.
- Figure 4.12. Placental adipokine dysregulation in GDM.
- Figure S4.1. Placental tissue protein expression of NLRP3, ASC and pro-caspase-1 was compared in NGT placenta (n=8) relative to GDM placenta (n=8) matched by maternal age and BMI. Matched pairs were loaded adjacent to each other over two separate blots (A + B).
- Figure S4.2. Omental tissue protein expression of NLRP3, ASC and pro-caspase-1 was compared in NGT adipose tissue (n=8) relative to GDM

adipose tissue (n=8), matched by maternal age and BMI. Matched pairs were loaded adjacent to each other over two separate blots (A + B).

- Figure 5.1. Placental-derived mediators in GDM do not increase adipogenesis relative to NGT.
- Figure 5.2. Primary mature adipocyte expression of IGFBP-3 and IGFBP-4 following treatment with placental explant supernatant.
- Figure 5.3. Placental-derived mediators in GDM do not significantly alter adipocyte lipolysis.
- Figure 5.4. Placental-derived mediators in GDM do not affect adipocyte glucose uptake.
- Figure 5.5. Primary adipocyte expression of IRS-1 and phospho-IRS-1(ser312) following treatment with placental explant supernatant.
- Figure 5.6. Placental-derived mediators in GDM do not affect adipocyte mROS production.
- Figure 5.7. Omental-derived mediators in GDM do not affect trophoblast mROS production.
- Figure 5.8. No change in omental-mediated cytokine release from HTR8 cells.
- Figure S5.1. Primary adipocyte protein expression of IGFBP-3 and IGFBP-4 was compared after 24-hour treatment with NGT (n=4) and GDM (n=4) placental explant culture supernatant. NGT and GDM-treated protein lysates were loaded in alternating order.
- Figure S5.2. Primary adipocyte protein expression of IRS-1 and phospho-IRS-1(ser312) was compared after 24-hour treatment with NGT (n=4) and

GDM (n=4) placental explant culture supernatant. NGT and GDM-treated protein lysates were loaded in alternating order.

Acknowledgements

First and foremost, I would like to express my sincere gratitude to all the participants in this study who gave up their time and energy to partake in this research. The findings in this thesis would be impossible without their contribution. I would like to thank my PhD supervisors, Dr. Cathal McCarthy and Dr. Fergus McCarthy, for their guidance throughout my PhD journey. I would particularly like to thank Cathal for his constant and unwavering support, guidance and encouragement. His expertise and invaluable feedback has been instrumental in shaping my research and helping me to reach my full potential during my studies. I would also like to extend my appreciation to Sam Manna for showing me the ropes when I joined the team and for her continued support throughout and to my postdoctoral colleague, Dr. Andrea Musumeci, for sharing his vast knowledge and experience in the field of immunology.

I am deeply grateful to HRB Ireland for providing me with the financial support that made this research possible. Their contribution has allowed me to pursue my academic goals and has provided me with the resources I needed to complete my PhD and present my research internationally over the past four years.

I would also like to extend my heartfelt thanks to the members of the Department of Pharmacology and Therapeutics, UCC. Their assistance and friendship has been invaluable to me and has made my time as a PhD student both productive and enjoyable.

Finally, I would like to acknowledge my parents and family, who have been supportive and encouraging throughout my studies and have been a constant source of inspiration and motivation. The sacrifices and efforts of my parents to provide me

with the best opportunities in life have made me who I am today. I am forever grateful and this achievement would not have been possible without them.

Thesis Abstract

Background

Gestational diabetes mellitus (GDM) is one of the most prevalent obstetric complications, with a growing incidence resulting from upward trends in global obesity and metabolic syndrome. GDM pathology is considered a state of exaggerated insulin resistance in pregnancy, mediated by pancreatic β-cell insufficiency, adipose dysregulation and systemic meta-inflammation. Although the pathophysiology of GDM is complex, adipose tissue dysfunction is an established cause of metabolic dysfunction and systemic insulin resistance. Furthermore, the placental microenvironment is highly sensitive to GDM physiology and there is significant evidence that the hyperglycaemic state of GDM drives placental dysfunction, in part mediated by exaggerated oxidative stress and inflammation. This thesis aims to characterise the role of systemic and local tissue mitochondrial dysfunction and meta-inflammation in promoting GDM pathology.

Methods

In this study, the inflammatory and metabolic profiles of omental visceral adipose tissue (VAT), placental tissue and fasting maternal blood were extensively studied to identifiy pathways which may drive insulin resistance in GDM and contribute to both acute and chronic adverse maternal and fetal outcomes. Samples were collected at term pregnancy from nulliparous women with normal glucose tolerant pregnancies and women diagnosed with GDM. Various immune-endocrine mediators were investigated in VAT, placental tissue and the maternal circulation including markers of mitochondrial dysfunction, monocyte/macrophage population frequency, hormones, inflammatory cytokines and insulin signalling transduction.

Results

Firstly, distinct phenotypes of GDM were confirmed, relating to both insulin-deficient and insulin-resistant pathologies. Insulin-deficient GDM participants had lower body mass indexes (BMIs) and were significantly hypoinsulinaemic compared to insulin-resistant GDM participants, who had elevated BMIs with substantial VAT dysfunction, including adipocyte hypoplasia and dysregulated insulin signalling capacity. The placental microenvironment was also negatively affected in GDM, with a potent pro-inflammatory secretome including increased release of IL-6, TNF-α and IL-18. This inflammatory phenotype was mirrored in the maternal circulation, confirming that systemic low-grade inflammation is present in term GDM physiology and is, in part, facilitated by placental signalling. Mitochondrial dysfunction was also confirmed in GDM participants, characterised by exaggerated mitochondrial superoxide production in placental macrophages and elevated circulating levels of cell-free mitochondrial DNA. *Ex vivo* mitochondrial antioxidant therapy attenuated excessive placental inflammation in GDM, proposing a promising therapeutic avenue for alleviating systemic inflammation in GDM patients.

Conclusion

In this thesis, we have provided substantial evidence of metabolic dysfunction and inflammation in the maternal circulation, omental VAT and placental tissue of women with GDM. This includes evidence of defects in adipocyte expansion and impaired insulin signal transduction, in addition to placental and systemic mitochondrial dysfunction and inflammation, which may ultimately orchestrate GDM pathophysiology. These findings identify novel pathological pathways in

GDM, which may be further delineated to establish their potential as therapeutic targets to alleviate adverse maternal outcomes.

Chapter 1

Introduction

In this introductory chapter, an overview of the available literature on the clinical considerations and pathophysiology of gestational diabetes mellitus (GDM) is provided. Firstly, an outline of diagnostic guidelines, risk factors and adverse clinical outcomes of GDM are given to highlight the relevance of this condition in the context of our research. Finally, a background of the current findings on various aspects of GDM patholophysiology, with a particular focus on meta-inflammatory pathways, highlights the areas which require further investigation.

Excerpts from this chapter appear in the following publications:

- **McElwain CJ**, Tuboly E, McCarthy FP, McCarthy CM. Mechanisms of Endothelial Dysfunction in Pre-eclampsia and Gestational Diabetes Mellitus: Windows Into Future Cardiometabolic Health? Front Endocrinol (Lausanne). 2020 Sep 11;11:655.

- **McElwain CJ**, McCarthy FP, McCarthy CM. Gestational Diabetes Mellitus and Maternal Immune Dysregulation: What We Know So Far. International Journal of Molecular Sciences. 2021; 22(8):4261.

1.1 Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. GDM is a serious obstetric complication that affects approximately 5-10% of pregnancies worldwide and is characterised by an insufficient insulin response to compensate for the insulin resistant state of pregnancy (1, 2). The global prevalence of GDM has risen in the last decade, largely due to increased maternal age and maternal BMI at conception (3). A recent systematic review has estimated that the European prevalence of GDM in pregnancy is 10.9%. There is a reported 2.14-fold increased risk of GDM complications in pregnancies with maternal age ≥30 years and a 1.47-fold increased risk if the diagnosis was made in the third trimester (versus second trimester). Maternal BMI is also a contributory risk factor for GDM diagnosis as there is a 6.79-fold increased risk in obese women and 2.29-fold increased risk in overweight women (4).

1.2 Clinical Diagnosis of Gestational Diabetes Mellitus

The International Association of Diabetes and Pregnancy Study Groups (IADPSG) recently updated the diagnostic criteria for GDM where an oral glucose tolerance test (OGTT) is performed in a fasting state using 75g of glucose at 24-28 weeks of pregnancy. GDM is diagnosed if any one of the following cut-off's is met i.e. fasting plasma glucose ≥ 92 mg/dl (≥ 5.2 mmol/l) or ≥ 180 mg/dl (≥ 10 mmol/l) at 1 hour post-OGTT or ≥ 153mg/dl (≥ 8.5 mmol/l) at 2 hours post-OGTT (5). A recent study found that in Europe, 82.1% (23 out of 28 surveyed) of clinical guidelines recommend screening for GDM at the first prenatal visit and 67.9% (19 out of 28) recommend screening before 24 weeks of pregnancy. All guidelines recommend

screening for GDM ≥24 weeks. However, universal screening with a 75g OGTT was only recommended in 36.8% of European guidelines included in this survey study, with other screening methods largely based on risk factor assessment (6). In contrast, national clinical guidelines in Australia recommend GDM screening with a 75g OGTT for all pregnant women, regardless of existing risk factors (7). In cases of diagnosed GDM, lifestyle modifications, dietary control and pharmacological intervention have reduced the development of maternal type 2 diabetes mellitus (T2DM) by up to 40%, emphasising the importance of accurate diagnosis, early intervention and monitoring practices (8).

1.3 Risk Factors for Gestational Diabetes Mellitus

Currently, risk prediction for GDM in Europe is largely based on the mother's medical history and other clinical risk factors. In Ireland, the Health Service Executive (HSE) recommends a 75g OGTT at 24-28 weeks if any of the following risk factors are identified at the initial antenatal visit:

- Family history of diabetes in a first degree relative
- Body mass index ≥ 30kg/m^2
- Maternal age ≥ 40 years
- Previous unexplained perinatal death
- Current glycosuria
- Long term use of steroids
- Previous delivery of a baby weighing ≥ 4.5kg
- Polycystic Ovary Syndrome (PCOS)
- Polyhydramnios and/or macrosomia in existing pregnancy

- Ethnicity associated with a high prevalence of diabetes: (India/ Pakistan/ Bangladesh/ Black Caribbean/ Saudi Arabia/ United Arab Emirates/ Iraq/ Jordan/ Syria/ Oman/ Qatar/ Kuwait/ Lebanon/Egypt) (9)

Obesity and PCOS are particularly common risk factors for GDM. PCOS drives metabolic dysfunction, including dysregulation in hormone release and insulin resistance. Although not fully elucidated, these metabolic alterations may be the cause for increased incidence of GDM in women with PCOS (10). Recently, other non-traditional risk factors have been identified which may increase GDM incidence. Elevated exposure to environmental toxins, including endocrine disrupting chemicals (EDCs) such as arsenic, polychlorinated biphenyls and polybrominated diphenyl ethers, are associated with impaired glucose tolerance and GDM in pregnancy (11, 12). Furthermore, maternal depression in the first and second trimester has been prospectively related to increased GDM risk (13).

Like most European countries, pregnant women in the Irish healthcare system are selectively screened for GDM based on the presence of maternal or fetal risk factors, with no universal screening procedures. However, compliance with implementation of selective GDM screening based on appropriate risk factor evaluation is not monitored on a national level (14).

1.3.1 Genetic Predisposition to Gestational Diabetes Mellitus

Development of GDM during pregnancy is considered an interplay between genetic risk factors and environmental influence. GDM often occurs in family clusters suggesting a significant role for genetic predisposition (15). A recent Chinese population study found that genetic variants associated with susceptibility to obesity and metabolic diseases were also associated with GDM risk, including *FTO (fat-*

mass and obesity-associated gene) rs1121980, *KCNQ1 (potassium voltage-gated channel subfamily Q member 1)* rs163182, *MC4R (melanocortin 4 receptor)* rs12970134, and *PROX1 (prospero homeobox 1)* rs340841 (16). Genetic variants that are significantly associated with T2DM are also genetic risk factors for GDM, including genetic variants in *CDKAL1 (CDK5 regulatory subunit associated protein 1 like 1), CDKN2A/2B (cyclin dependent kinase inhibitor 2A/B), HHEX (hematopoietically expressed homeobox), IGF2BP2 (insulin-like growth factor 2 mRNA-binding protein 2), SLC30A8 (solute carrier family 30 member 8),* and *TCF7L2 (transcription factor 7 like 2)* (17-19). A recent review of 83 GDM candidate genes found that polymorphisms in *TCF7L2, MTNR1B (melatonin receptor 1B), CDKAL1, IRS1 (insulin receptor substrate 1)* and *KCNQ1* are the most prevalent genetic risk factors for GDM (20). Polymorphisms in these genes were found to modulate pancreatic β-cell dysfunction, adiposity, obesity and insulin resistance through several mechanisms. Environmental stressors, such as poor diet, cause epigenetic changes in these genes, resulting in a loss of insulin sensitivity. Genome-wide association studies represent an exciting new avenue in diabetic research to further understand the genetic determinants of exaggerated insulin resistance in GDM pregnancy.

1.3.2 Maternal Obesity in Gestational Diabetes Mellitus

Maternal obesity is the most significant modifiable risk factor for GDM diagnosis (4). Due to the increase in the global prevalence of obesity, GDM incidence has continued to increase in the last decade (1). Pre-pregnancy maternal obesity, rather than excessive gestational weight gain (GWG), is the predominant risk factor, with women who are obese pre-pregnancy being 4-8 times more likely to develop GDM compared with normal weight women (21). GWG should also be monitored, as

women who gain excessive weight during pregnancy are more likely to retain weight in the postpartum period, leading to other obesity-related metabolic complications including pre-pregnancy obesity for subsequent pregnancies (22). High pre-pregnancy BMI and excessive GWG are risk factors for pregnancy complications other than GDM, including a higher incidence of large for gestational age (LGA) babies, requirement for caesarean section and gestational hypertension (23). The risk of childhood obesity for the offspring of GDM pregnancies is also significant, however this risk drops significantly after adjustment for pre-pregnancy BMI (24). The nature of this relationship between obesity and GDM may result in a vicious cycle of intergenerational obesity and subsequent diabetes diagnosis.

1.4 Gestational Diabetes Mellitus and Future Cardiometabolic Risk

1.4.1 Maternal Outcomes

Unmanaged GDM can cause significant maternal morbidity. GDM is an independent risk factor for future maternal adverse outcomes such as T2DM, recurrent GDM, cardiovascular disease, metabolic syndrome, as well as ophthalmic, psychiatric, and renal disease (25). The cause of GDM-associated T2DM risk has been linked to long-term alteration of maternal endothelial function due to insulin resistance and hyperglycaemia (26). Women diagnosed with GDM have a significantly lower flow-mediated dilation (FMD) compared with healthy pregnancies, suggestive of increased endothelial damage (27). Persistent endothelial dysfunction in these women may lead to maternal cardiovascular disease (CVD) and poor cardiometabolic health. A recent US study established a 63% increased risk of CVD amongst women with a history of GDM, which was only partly explained by correlation with BMI (28), suggesting other key players in GDM pathophysiology

are driving poor maternal outcomes. In women with no history of diabetes or hypertension, those diagnosed with GDM develop impaired myocardial contractility and higher left ventricular mass (29). Similarly, the shift in glucose metabolism, during which maternal hyperinsulinemia compensates to support fetal growth, serves as a cardiometabolic stressor that may culminate in long-term cardiovascular complications (30). The compensatory systemic hyperinsulinaemia associated with insulin resistance has been linked to vascular plaque formation in atherosclerosis, induced by changes in gene expression of the estrogen receptor (31, 32).

Insulin resistance-induced dyslipidaemia is also postulated to be a significant risk factor for CVD, as it is characterised by increased circulating triglycerides, decreased high-density lipoproteins (HDLs) as well as the formation of small dense low-density lipoproteins (sdLDLs) (31). The detrimental effects of both insulin resistance and altered lipid signalling on metabolic function are believed to stem from endothelial-derived nitric oxide (NO) deficiency and reactive oxygen species (ROS) production. This relationship exists as a negative feedback loop where insulin resistance and cellular metabolic disturbances increase the production of ROS and reactive nitrogen species (RNS). These species deleteriously affect the function of endothelial nitric oxide synthase (eNOS) and NO, rendering them incapable of their homeostatic vascular function. In turn, induced endothelial dysfunction impairs insulin action due to damage of microcirculatory blood flow and the capillary network, reducing insulin fluency in circulation (33).

GDM is also strongly associated with the long term development of non-alcoholic fatty liver disease (NAFLD), a recognised hepatic manifestation of the metabolic syndrome (34). GDM during pregnancy increases the risk of future maternal neurological complications, including development of psychological disorders and

increased incidence of antenatal depression (35). GDM can also cause short-term implications for the mother during pregnancy, including increased risk of gestational hypertension and pre-eclampsia (PE) (36).

Improvement in risk prediction, early intervention and prophylaxis of adverse GDM symptoms could negate the short and long-term complications of GDM diagnosis.

1.4.2 Fetal Outcomes

GDM is associated with an elevated risk of neonatal morbidity and mortality, with an increased risk of adverse perinatal complications such as macrosomia, respiratory distress syndrome and fetal hyperinsulinaemia (37). Maternal hyperglycaemia, whether from pre-existing diabetes or from gestational diabetes, can lead to fetal hyperglycaemia as glucose is easily distributed across the placenta (38), therefore maintaining optimal blood glucose levels is crucial to reducing these risks for mother and fetus. Population-based studies have shown that neurodevelopmental disorders, including autism spectrum disorders (ASD) and attention-deficit/hyperactivity disorder (ADHD), and type 1 diabetes mellitus (T1DM) are more prevalent in children from GDM pregnancies (39, 40). The significant implications of GDM-related obstetric complications on the fetus, such as an increased risk of endocrine, nutritional, and metabolic disturbances, including T2DM, are often evident in adolescence and early-adulthood (41, 42).

A randomized clinical trial to assess the therapeutic benefit of aggressive multipartite treatment of women with GDM (including comprehensive dietary advice, blood glucose monitoring, and insulin therapy) reported a reduction from a 4% to 1% incidence of severe perinatal and postnatal fetal outcomes (defined as death, shoulder dystocia, bone fracture, and nerve palsy) (43). Furthermore, a systematic review of

ten studies consisting of 3317 pregnant women who were treated for mild GDM reported a reduced the risk of adverse maternal outcomes including preeclampsia, macrosomia, LGA babies, caesarean section and shoulder dystocia without increasing the risk of small for gestational age (44).

1.5 Pathophysiology of Gestational Diabetes Mellitus

During healthy pregnancy, the maternal physiological response adapts to compensate for many changes in energy demands, including glucose metabolism. As the fetus develops, a surge in maternal hormone secretion, including placental lactogen and estrogen, promotes a state of insulin resistance, which facilitates a rise in blood glucose levels to support fetal growth (45). Appropriate blood glucose levels are then maintained through homeostatic mechanisms such as hypertrophy and hyperplasia of pancreatic β-cells (46). However, in GDM, normal metabolic adaptions to pregnancy fail to adequately control glycaemic levels, resulting in hyperglycaemia and secondary complications such as vasculopathy and complications at delivery (47).

The pathophysiological origins of GDM are not fully understood and there are conflicting opinions on how, and where, the condition originates. The primary hypothesis proposes that pancreatic β-cell insufficiency and an inability to produce sufficient insulin to counteract pregnancy-induced insulin resistance instigates GDM pathology (45). There is a secondary hypothesis which suggests that aberrant placental hormone expression may stimulate maternal metabolic dysfunction and diminished insulin functionality (48). This concept originates from our understanding that, as pregnancy develops, the placenta plays a key role in the regulation of peripheral insulin resistance through the release of hormones such as estrogen, progesterone, prolactin, leptin, cortisol, placental lactogen, and placental

growth hormone. Alteration in the levels of these hormone mediates an increase in glycaemic levels by reducing insulin sensitivity and consequently increasing glucose levels to fuel fetal growth. This theory is supported by the fact that maternal insulin sensitivity returns to pre-pregnancy levels within days of delivery (49). A further hypothesis proposes that adipocyte dysfunction and the resultant disruption in the maternal insulin signalling pathway, which may subclinically precede pregnancy, can also contribute to GDM pathophysiology (50).

A likely scenario is that several biological factors contribute to the diabetic syndrome observed in GDM. Two endocrine organs which undoubtedly play a prominent pathophysiological role in GDM are the placenta and adipose tissue (AT). Both metabolic organs release a myriad of signals, which can induce deleterious effects on the maternal vasculature, with probable long-term consequences. Potential mediators directing this deleterious communication between both metabolic organs and the maternal endothelium include ROS, mitochondrial DNA (mtDNA), pro-inflammatory cytokines, adipokines and lipid-derived signals. In this thesis, we explore the intertwined signalling relationships of placenta and AT on the maternal syndrome and characterise how these mediators play a pathophysiological role in GDM pregnancies.

1.5.1 Pancreatic Beta Cell Dysfunction

Healthy uncomplicated pregnancy is characterised by a significant level of insulin resistance, where fasting insulin levels are 29% higher in the second trimester of pregnancy and 61% higher in the third trimester, compared with non-pregnant controls (51). The development of the insulin resistant state is required to increase circulating glucose levels, facilitating the additional maternal energy needs of pregnancy (approximately 300 kcal/day over routine energy intake) in addition to

providing the necessary nutritional requirements of the growing fetus (approximately 33μmol/kg/min by the 3rd trimester). β-cell adaptions compensate for peripheral insulin resistance and maintain glucose homeostasis through increased insulin secretion. These compensatory adaptions are in part stimulated by pregnancy-related hormones prolactin and human placental lactogen (hPL), which promote β-cell mass proliferation and reduce β-cell apoptosis (52, 53).

GDM may be viewed as an inability to compensate for the insulin resistance of pregnancy and pathological outcomes have been linked to impaired insulin sensitivity caused by insufficient β-cell compensation (54). β-cell dysfunction has also been implicated in T2DM pathology as well as T1DM, where autoimmunity drives β-cell loss and insulin depletion (55). However, the mechanism by which β-cell insufficiency drives GDM pathology remains somewhat unclear. 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), a furan fatty acid metabolite, has emerged as a potential negative regulator of β-cell function. A clinical study found that CMPF was an independent predictor of reduced insulin secretion in GDM and was not sufficiently compensated for by increased prolactin or hPL release, suggesting it as a circulating determinant of β-cell dysfunction and hyperglycemia in women with GDM. (56). *In vitro* analysis of differentially methylated cell-free DNA (cfDNA), as a potential biomarker of β-cell loss, reported that β-cell death is reduced in GDM, suggesting that deficiency in pancreatic β-cell number is not a factor for GDM-related insulin resistance (57). Although there is established impairment of β-cell function in the majority of women who develop GDM, the origin and cause of this, in the context of GDM-related lipotoxicity, inflammation, oxidative stress and impairments in adipokine and placental signalling, remain unclear (53, 58).

1.5.2 Insulin Resistance

As previously mentioned, healthy pregnancy is characterised by systemic insulin resistance, which facilitates a rise in blood glucose levels to fuel fetal growth and maternal energy needs. At 12-14 weeks' gestation, there is a subtle increase in insulin sensitivity which gradually declines for the remainder of the pregnancy (59). By the third trimester, insulin sensitivity may gradually drop to 50% of normal function (60). GDM emerges when the maternal system fails to compensate for this maternal insulin resistance. Obesity-related adipose dysfunction, a significant risk factor for both GDM and non-pregnancy related insulin resistance, is likely to be a significant culprit. In the last decade there has been a significant increase in our understanding of the pathogenic role of AT inflammation in mediating the disruption of insulin signalling with resulting insulin resistance, particularly in T2DM (61). Immune cell infiltration, and an increase in meta-inflammation and lipid signalling, disrupts triglyceride storage resulting in increased free fatty acid (FFA) release. Increased FFAs further amplify insulin resistance in both the muscle and liver (62). There is significant heterogeneity in insulin resistance levels in GDM pregnancies (63), hence a more detailed description of the mechanisms contributing to maternal insulin resistance will be dicussed in the remaining sections.

1.5.3 Insulin Signalling Cascade

Insulin stimulates glucose uptake into fat and muscle cells through activation of insulin receptors. Receptor mediated activation initiates the trafficking of various intracellular cascades leading to the increased membrane translocation of glucose transporter type 4 (GLUT4), an insulin-sensitive glucose transporter that acts to promote insulin-stimulated glucose uptake by adipose, skeletal and cardiac tissues (64). Two main pathways involved in insulin signalling are activated upon insulin

binding to the insulin receptor, namely the phosphatidylinositol 3-kinase (PI3K, a lipid kinase)/AKT (also known as PKB or protein kinase B) pathway and the Raf/Ras/MEK/MAPK pathway (65). While the mitogen-activated protein kinase (MAPK) pathway predominantly controls cellular growth and differentiation, the PI3K pathway is responsible for the metabolic activity of insulin and impairment of this pathway is linked to many insulin-resistant disease states, including diabetes (66).

Insulin receptor substrate (IRS) proteins are a family of cytoplasmic adaptor proteins that transmit signals from the insulin receptor to mediate a cellular response. Two of these substrates, IRS-1 and IRS-2 are ubiquitously expressed and are the primary mediators of insulin-dependent regulation of glucose metabolism in most cell types (67). Insulin receptor activation stimulates PI3K-dependent signalling cascade by phosphorylating IRS proteins, thereby providing docking sites for the recruitment and activation of PI3K. This kinase converts phosphatidylinositol-4,5-diphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3), which functions as a platform for the recruitment of phosphoinositide-dependent kinase 1 (PDK1) and AKT. At the plasma membrane, AKT is phosphorylated by PDK1 and mammalian target of rapamycin complex 2 (mTORC2). AKT promotes GLUT4 storage vesicle (GSV) exocytosis by phosphorylating and inactivating two GTPase-activating proteins (GAPs), AS160 and the RAL–GAP complex (RGC), which regulate small GTPases that are involved in GSV retention and targeting, respectively (Figure 1.1) (64).

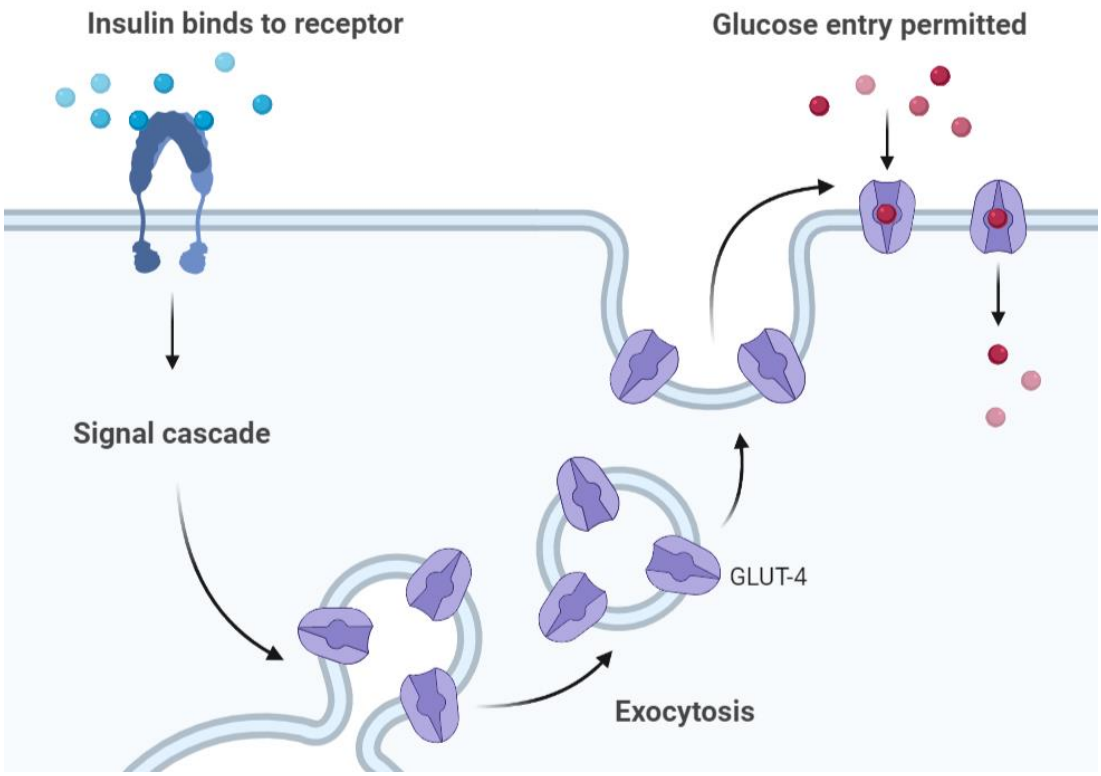


Figure 1.1. Insulin signalling regulates GLUT4 exocytosis by engaging intracellular trafficking machinery. Created with Biorender.com. Insulin binding to the insulin receptor stimulates an intracellular signalling cascade which leads to GSV exocytosis resulting in GLUT4 mediated glucose uptake (64).

Various modifications of proteins in the insulin signalling cascade are evident in GDM. The rate of insulin-stimulated glucose uptake is reduced by 54% in GDM relative to healthy uncomplicated pregnancy (68). While it appears that the expression of the insulin receptor in skeletal muscle is unaffected in GDM, the capacity for insulin-stimulated tyrosine phosphorylation of the insulin receptor β-subunit is hindered in GDM patients (69, 70). GDM-related defects of IRS-1 include decreased IRS-1 expression, reduced IRS-1 tyrosine phosphorylation and increased basal serine phosphorylation (68, 71, 72). Phosphorylation of IRS-1 at the serine

residue impairs its ability to bind to PI3K and stimulate glucose metabolism, resulting in significant insulin resistance (73).

However, these findings are largely evident in skeletal muscle with little known about the regulation of the insulin signalling cascade in VAT in GDM. Interestingly, abnormal glucose metabolism in AT, caused by adipocyte dysregulation and insufficient insulin signalling, can negatively affect systemic glucose homeostasis at other tissue sites. For example, disruption of stimulated adipocyte GLUT4 translocation has been shown to limit the suppressive action of insulin on hepatic glucose production (74). Insulin signalling is also altered in the placental tissue of insulin-controlled GDM patients, including a significant increase in IRS-1 expression, reduction in IRS-2 expression and a decrease in expression of PI3K p85α and GLUT4, relative to healthy control pregnancies (75).

1.5.4 Adipose Tissue

Adipose tissue is comprised of stromal cells such as preadipocytes, mature adipocytes and immune cells, which support the proliferation and differentiation of preadipocytes to adipocytes through the secretion of various growth factors and cytokines. Adipogenesis is the process by which precursor cells differentiate into mature adipocytes. Mature adipocytes are believed to have lost the ability to divide following the completion of terminal differentiation. Adipose tissue is composed of various types of adipocytes, including white adipocytes, brown adipocytes and beige adipocytes. Brown adipogenesis is distinct from that of white adipose tissue (WAT) and occurs mainly during embryonic development and in response to cold exposure. While WAT stores excess energy as triglycerides, brown and beige adipose tissue are specialized in dissipating energy as heat, a process known as thermogenesis (76). Subcutaneous AT (SAT) exists directly beneath the skin whereas VAT

predominantly surrounds the abdominal organs. VAT has been characterized as a predominantly metabolic pro-inflammatory organ, which produces hormones and adipokines in addition to possessing a substantial immune cell population and VAT adipocyte hypertrophy is associated with a higher risk for T2DM, hypertension and dyslipidemia (77). Increased energy intake can often result in insufficient number of mature adipocytes and a deficiency in their storage capacity in both SAT and VAT depots (78). Adipose tissue physiologically responds to this deficiency via two mechanisms, initiating hyperplasia and/or hypertrophy. Adipocyte hyperplasia results from the activation of adipogenesis. Secondly, adipocyte hypertrophy responds to increased energy intake, by increasing the size of mature adipocytes which facilitates extra lipid content in these depots. During healthy pregnancy, AT expands to support the nutritional needs of the growing fetus, reaching a peak towards the end of the second trimester, and deficient AT expansion is associated with insulin resistance, attributed to ectopic lipid deposition (79). Adipocytes play a key regulatory role in dyslipidaemia. Impaired adipocyte function leads to a reduction in the suppression of FFA release in response to insulin, as well as inducing hypertriglyceridaemia and decreasing HDL concentrations (80), which can account for an increased CVD risk.

Maternal AT morphology and adipocyte function are dysregulated in GDM, with reduced hyperplasia and excessive hypertrophy contributing to a reduced ability to retain fatty acids, resultant ectopic fat accumulation and impaired glucose metabolism (78, 79). Due to this altered adipocyte phenotype, women with GDM are prone to insufficient insulin-mediated suppression of lipolysis, resulting in exaggerated FFA and glucose production, and excessive insulin resistance (81). Maternal VAT mass is a key component in the regulation of FFA and glucose

uptake. Ultrasound measurement of increased maternal VAT depth during the first half of pregnancy reported a positive association with GDM diagnosis (82, 83).

1.5.5. Insulin-like Growth Factor (IGF) and IGF-Binding Proteins in Adipocyte Expansion

An important mediator of preadipocyte differentiation and AT mass is insulin-like growth factor-1 (IGF-1), which is primarily produced by the liver. Most circulating and tissue-localised IGF molecules are bound by one of the IGF-binding protein (IGFBP) family, produced in many peripheral tissues including AT, which function to regulate IGF signalling, potentiating the effects of IGF-1 by increasing local retention. Another important function of IGFBPs, particularly those in circulation, is to prevent IGFs from binding to insulin receptors and activating the insulin signalling pathway, which could potentially induce hypoglycaemia (84). Circulating levels of IGF-1, believed to be orchestrated by placental hormone release, are significantly higher in the third trimester of healthy pregnancy (85).

Various mediators of the IGF pathway are dysregulated in GDM. Firstly, circulating IGF-1 levels are increased in GDM. Findings in 24-28 weeks gestation have found that circulating IGF-1 concentrations significantly correlate with BMI, insulin levels and HOMA-IR scores in GDM (86). Zhao et al. found that IGF-1 levels correlated with fasting blood glucose levels, suggesting a potential homeostatic role in IGF-1 in these women (87). Local functional dysregulation is also evident, as Rojas-Rodriguez et al. found significantly decreased gene expression of IGFBP-5 and IGF-1 in SAT from GDM pregnancies versus normal glucose tolerance (NGT) pregnancies (79). IGFBP-5 expression levels did not correlate with BMI in these women, suggesting that the observed differences were independent of maternal BMI. However, a recent systematic review has highlighted inconsistencies as to the exact

role of the IGF axis in GDM. Wang et al. found that, in two studies, GDM was associated with lower IGFBP-2 concentrations in early, mid-, or late gestation, while five other studies reported increased IGFBP-3 concentrations in third trimester GDM patients (88). These studies only recorded circulating levels, with no supportive information on local tissue expression. These aforementioned findings suggest a pathogenic link between adipocyte dysregulation and concurrent maternal metabolic dysfunction in GDM pathology but emphasize the need for further characterisation of the role of IGFBPs in regulating IGF.

1.5.6 Adipokines in Gestational Diabetes Mellitus

Adipokines are hormones secreted predominantly by adipose tissue but also by other organs such as the placenta. They play a pivotal role in regulating crucial biological processes including metabolic adaptation to pregnancy. Various studies have established significant changes in the secretion of adipokines during pregnancy and altered adipokine production is evident in obstetric complications such as GDM, PE and fetal growth abnormalities (89).

Adiponectin

Adiponectin possesses both anti-inflammatory and insulin-sensitising properties. Adiponectin acts on two main receptors, adiponectin receptor type 1 (ADIPOR1), predominantly expressed in skeletal muscle, and adiponectin receptor type 2 (ADIPOR2), largely expressed in the liver (90). Once adiponectin binds to its receptors, the adaptor protein containing a pleckstrin homology domain 1 (APPL1) binds intracellularly to ADIPOR1 or ADIPOR2, triggering a complex signal transduction cascade resulting in the activation of peroxisome proliferator-activated receptor-alpha (PPAR-α) and phosphorylation of adenosine monophosphate (AMP)-activated protein kinase (AMPK) in peripheral tissues. This cascade regulates fatty

acid oxidation and glucose uptake in skeletal muscle, in addition to suppression of glucose production, lipogenesis and inflammation in the liver, positively modifying systemic insulin sensitivity (91).

Adiponectin also has potent anti-inflammatory properties as it can promote cellular differentiation of monocytes to M2-like macrophages and suppress the release of leptin and pro-inflammatory cytokines such as interleukin (IL)-6 and tumour necrosis factor (TNF)-α (92). It has also been shown *in vitro* to attenuate NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome activation, a protein complex responsible for the release of pro-inflammatory cytokines IL-1β and IL-18, by modulating the AMPK-ROS signalling pathway, via up-regulation of AMPK phosphorylation and the suppression of ROS generation (93-95).
Adiponectin plays a critical role in regulating the metabolic adaptions of pregnancy and systemic levels of adiponectin gradually decrease over the gestational period, with the lowest levels evident in the third trimester, when insulin resistance peaks (96). Adiponectin levels are systemically lower and are inversely correlated with fetal growth in GDM pregnancies compared to healthy NGT pregnancies (96-98). A reduction in adiponectin secretion during pregnancy is thought to be a significant risk factor for decreased insulin sensitivity and the development of GDM (99).

Leptin

Leptin is a hormone which predominantly regulates satiety and energy homeostasis (100). It is now understood that leptin is produced by a variety of tissues, including the placenta, in addition to AT (101). Leptin levels are elevated in obesity, hyperglycaemia, hyperinsulaemia and in response to inflammatory cytokine signalling (102). In AT, leptin expression is increased in response to pro-inflammatory markers such as TNF-α and IL-1β (103). Equally, leptin has pro-

inflammatory properties, increasing the secretion of various pro-inflammatory cytokines including IL-6, IL-12 and TNF-α, which may instigate a deleterious inflammatory feedback loop that promotes chronic low-grade inflammation (104). During pregnancy, leptin plays a crucial role in regulating trophoblast proliferation, protein synthesis, invasion and apoptosis, particularly in the first trimester. Disruption in leptin secretion is evident in PCOS, recurrent miscarriage, PE and intrauterine growth restriction (IUGR) (105). GDM is characterized by increased leptin and leptin receptor expression, which increases pro-inflammatory cytokine release and positively correlates with insulin resistance (106, 107). Although placental leptin production appears to play a prominent role in this phenotype (108, 109), it is likely that AT also contributes to the leptin-related pathology in GDM as circulating leptin levels generally correlate with AT size (110). Although findings to date are conflicting, hyperinsulinaemia also appears to stimulate leptin release suggesting an important relationship between these hormones in regulating metabolic homeostasis (111).

Adipsin

Adipsin is a protease with close homology to human complement D and is secreted by muscle, lung, peripheral nerves, placenta and AT. Adipsin is the rate-limiting enzyme in the formation of acylation stimulating protein (ASP), a factor contributing to lipid storage in AT. ASP enhances both glucose and fatty acid uptake, an effect independent and additive to the lipogenic effect of insulin, promoting the accumulation of fat in AT through the inhibition of lipolysis and the stimulation of glucose transport (112). Adipsin has also been shown to have a beneficial role in maintaining β-cell function (113). While there is little known about the biological role of adipsin in healthy pregnancy, levels are lower in pregnant women compared

to non-pregnant women and increase postpartum (114). In non-pregnant individuals, serum adipsin levels are significantly increased in obesity and positively relate to BMI (115). However, Zhou et al. found that levels were lower in T2DM and impaired glucose tolerance (IGT) patients (116). Elevated circulating levels of adipsin have been reported in GDM pregnancies (117, 118) but no studies to date have investigated tissue-specific regulation of adipsin release and the contribution that his may have to GDM pathology.

Resistin

Resistin is another secretory hormone that was originally thought to be adipocyte-specific but has since been localised in various tissues. It is named due to its resistant effect on insulin through the inhibition of insulin signalling in adipocytes. Resistin is also considered a pro-inflammatory mediator which is expressed by immune cells and contributes to chronic inflammatory diseases, metabolic diseases, infectious diseases and cancers (119, 120). Resistin additionally acts as a host defence peptide through anti-microbial activity and suppression of microbial product-induced inflammation (121, 122). Resistin is thought to play a particularly important role in the pathology of insulin resistance and diabetes (123). A recent systematic review found that in T2DM and obesity, resistin levels positively correlated with insulin resistance in those with elevated circulating levels (124). Increased mRNA expression of resistin in SAT was reported in a cohort of obese women who had significant insulin resistance, relative to non-obese women (125), suggesting AT as a probable source of pathological resistin production.

In healthy pregnancy, it appears that elevated systemic resistin levels contribute to insulin resistance, as increased plasma levels were reported in pregnant compared to non-pregnant women. Plasma levels also increase with gestational age, in line with

the gradual increase in insulin resistance that develops during pregnancy (126). A recent meta-analysis reported that elevated circulating levels of resistin are associated with an increased risk of developing GDM and that this increase may be related to pathological severity (127). However, Megia et al. found significantly lower serum resistin levels in their GDM cohort (128) while other studies found no significant difference in circulating resistin levels in healthy and GDM pregnancies (129, 130). In omental VAT, significantly increased protein expression of resistin has been identified in GDM (118, 131). Although the exact role that resistin plays in GDM pathophysiology is unclear, there is evidence that a pathological link may exist.

1.5.7 Role of the Placenta in Gestational Diabetes Mellitus

Role of the placenta in Normal Pregnancy

During normal placentation, cytotrophoblast cells facilitate remodelling of the maternal uterine spiral arteries, allowing for sufficient blood flow and nutrient supply to the growing fetus (132). The fusion of cytotrophoblasts to terminally differentiated syncytiotrophoblasts allows for the formation of gap junctions and the exchange of small metabolites and secondary messengers (133). Beneath the trophoblast layer is the extraembryonic mesoderm, fetal macrophages or Hofbauer cells (HBCs), and capillaries originating from endothelial cells. These layers form the maternal-fetal barrier, which regulates material transfer between the maternal and fetal circulation (134). An essential structure in the regulation of placental function are placental villi, or villous “trees”, which can be stratified based on their proximity to either the fetal or maternal side of the placenta. Structural stem villi are connected to the chorionic plate and are positioned closest to the umbilical cord. During the first and second trimester, intermediate villi are formed and are the principal sites of

exchange during the early stages of pregnancy. During the third trimester, intermediate villi differentiate to smaller terminal villi, which are in contact with the maternal surface and support efficient diffusive exchange between maternal and fetal compartments and also serves as a physical barrier to protect the fetus from harmful substances (135, 136).

Immunological Role of the Placenta in Healthy Pregnancy

One of the important functions of the placenta is to protect the semi-allogeneic fetus from maternal immune recognition. During pregnancy, the mother's immune system must tolerate the presence of the developing fetus, which has a unique set of genetic material inherited from both parents. Therefore, the placenta acts as a barrier that limits the transfer of immune cells and molecules between the maternal and fetal circulation. This immunological barrier function is achieved through a combination of different mechanisms, including the expression of specific proteins such as human leukocyte antigen (HLA)-G. HLA-G is a non-classical major histocompatibility complex (MHC) class I molecule that is expressed specifically by trophoblasts and plays a critical role in regulating immune tolerance during pregnancy. HLA-G inhibits the activation and proliferation of maternal immune cells, such as T cells and natural killer cells, which would otherwise recognize and attack fetal cells as foreign (137). In healthy pregnancy, trophoblasts do not express other HLA class I molecules, such as HLA-A and HLA-B and their absence likely serves as a protective measure, preventing the presentation of antigens to maternal immune cells which would trigger an immune response (138).

Morphological Changes in GDM Placenta

Numerous studies have reported macroscopic changes in placental tissue in GDM, in particular increased placental weight and surface area (139, 140). Placental

histological findings from GDM and hyperglycaemic pregnancies provide evidence of villous immaturity, villous fibrinoid necrosis, chorangiosis (capillary hyperplasia in terminal villi due to chronic placental hypoperfusion or low grade tissue hypoxia), and increased angiogenesis with increased placental size. (141, 142). Distal villous immaturity (DVI) is a placental phenotype characterized by enlarged villi with excessive stroma and reduced villi growth. DVI results from excessive maternal glucose levels leading to release of fetal insulin and other growth factors that promote excessive placental growth at the expense of villous maturation, and is thought to promote a hypoxic intrauterine environment in the third trimester which may make the fetus more susceptible to adverse pregnancy outcomes (143, 144). Therefore, while a hyperglycaemic environment drives villous immaturity and reduced placental efficiency, modifications such as chornangiosis and angiogenesis are believed to be a homeostatic response to maintain tissue efficiency in GDM. El-Tarhouny et al. found that DVI pathology, which is largely observed in term or near-term placenta and in maternal conditions such as IGT, obesity and excessive GWG, was significantly increased in GDM placenta (145).

Placental Hormones

The placenta is a highly active endocrine organ and the release of placental hormones plays a vital role in mediating the maternal adaption to pregnancy. Certain physiological changes are at least partly coordinated by hormones and other mediators secreted by the placenta including estradiol, progesterone, prolactin, cortisol, hPL, and human placental growth hormone (hPGH), which are proposed to facilitate pregnancy-induced peripheral insulin resistance (146, 147). Placental lactogen and prolactin increase maternal food intake by the induction of central leptin resistance, and promote maternal β-cell expansion and insulin production to

counteract hyperglycaemia (148). Inappropriate secretion of placental hormones during pregnancy has been linked to obstetric complications including GDM, PE, fetal growth restriction (FGR), macrosomia and pre-term birth (149).

Significant increases in prolactin levels during the first trimester positively correlate with GDM risk, suggesting that impaired regulation of prolactin release contributes to GDM pathology (150). While there is strong evidence for the potential pathophysiological role of prolactin in GDM, a definitive role for hPL in GDM is somewhat ambiguous. Elevated levels of first trimester hPL were found in pregnant women with T1DM but not in women who developed GDM (151). Other studies also found no GDM-related effect on circulating maternal hPL levels, however amniotic fluid concentrations of hPL were shown to be significantly higher in GDM pregnancies (56, 152).

Adiponectin receptors ADIPOR1 and ADIPOR2 are expressed in placental tissue and while adiponectin is predominantly produced by AT the placenta also contributes to adiponectin secretion during pregnancy (153, 154). Placental mRNA expression of adiponectin is downregulated in GDM (97) and concentrations are reduced in umbilical artery blood in GDM. Adiponectin levels in umbilical cord blood negatively correlated with GDM development and risk of abnormal pregnancy outcomes, although the source of adiponectin is believed to be of fetal, rather than placental or maternal origin (155). A recent *in vivo* study found that placental deficiency of adiponectin induces placental dysfunction, including diminished IGF-1 signalling and reduced nutrient transport (156).

Leptin is equally produced by syncytiotrophoblasts and endothelial cells of the placenta and regulates placental function and the expression of tolerogenic maternal

response molecules such as HLA-G (105, 157, 158). Given its previously mentioned pleotrophic roles, increased placental expression of leptin and leptin receptor expression is a significant culprit in GDM-related inflammation. It is also suggested that the effect of altered placental leptin secretion in GDM contributes to endocrine disruption, resulting in excessive maternal AT expansion and subsequent exaggerated insulin resistance (159). Multiple studies have confirmed a significant correlation between elevated systemic leptin concentrations and obesity in pregnancy (160, 161).

Adipsin and ASP are equally produced by the placenta, with evidence that placental production exceeds AT secretion, potentially explaining the elevated circulating ASP levels at term (162). Although GDM has been associated with elevated adipsin serum levels at term (118), there are no studies which have specifically measured placental adipsin release in GDM.

Although predominantly produced by AT, the expression of resistin has been established in human placental tissue, with an increased placental levels at term compared to first trimester (163). Only two recent studies have quantified placental resistin protein expression in GDM, where levels were significantly higher than in healthy pregnancies (164, 165).

Pregnancy-associated plasma protein A (PAPPA) is a protease which is produced by uterine tissue in non-pregnant women and is predominantly produced by the placenta in pregnancy (166). PAPPA cleaves IGFBP-4 and IGFBP-5, thereby increasing IGF bioavailability (167). First trimester circulating levels of PAPPA are significantly reduced in GDM, possibly affecting placental and fetal development (168, 169). Additionally, lower systemic PAPPA levels during pregnancy have been postulated

to disrupt AT function and potentiate GDM pathology. Rojas-Rodriguez et al. recently identified that PAPPA stimulates AT expansion *ex-vivo* and that PAPPA$^{-/-}$ mice have impaired AT remodelling and exaggerated pregnancy-induced insulin resistance (170). These findings highlight the potential impact of impaired placental signalling on adipocyte function and glucose metabolism in GDM.

1.5.8 Mitochondrial Dysfunction and Oxidative Stress in Gestational Diabetes Mellitus

Pregnancy is associated with oxidative stress, largely orchestrated by placental mitochondrial activity and the production of ROS, by-products of normal cellular activity (171). Mitochondria serve as the primary source of endogenous ROS, however the endoplasmic reticulum and peroxisomes also produce ROS (172). During pregnancy, placental development requires an increased mitochondrial capacity to facilitate metabolic requirements, including adenosine triphosphate (ATP) production, hormone synthesis and trophoblast oxygen sensing in addition to supporting the fetal growth (173).

However, insufficient placentation and the resulting ischaemic placental microenvironment result in excessive ROS generation leading to the release of deleterious mediators into the maternal circulation (174). A recent *in vitro* study investigating the role of placental redox protein thioredoxin-interacting protein (TXNIP) and GDM pathology found that a high glucose environment increased TXNIP expression, mitochondrial dysfunction and ROS generation (175). Furthermore, Fisher et al. provided evidence of mitochondrial dysfunction in isolated mitochondria from both the cytotrophoblast and syncytiotrophoblast cell lineages in GDM compared to healthy placenta (176). In particular, their findings indicated an increased requirement for superoxide dismutase (SOD) in mitochondria

from the syncytial layer in GDM due to an increase in ROS production. These results reflect previous findings by Coughlan et al., which showed that SOD activity and protein carbonyl content (a marker of oxidative damage) were elevated in GDM placenta (177).

Although no research to-date has explicitly examined adipocyte ROS production in GDM pathology, related findings suggest that a pathogenic link may exist between GDM pregnancies and oxidative stress responses. In non-pregnant individuals, increasing VAT mass increases adipocyte dysfunction, with a resulting elevation in adipocyte ROS production. This elevated ROS generation has been correlated with an increase in insulin resistance in both adipose and other peripheral tissues (178). Systemically, hyperglycaemic diabetic patients have significantly lower levels of SOD activity, lower total antioxidant capacity and higher levels of malondialdehyde (MDA), a marker of increased free radical production (179).

Exaggerated ROS generation thereby represents an important cause of metabolic dysfunction in GDM, resulting in potentially permanent vascular damage which may have significant long term consequences (180).

Mitochondrial DNA

An additional mechanism by which mitochondrial dysfunction can disrupt systemic metabolic function is by releasing mitochondrial DNA (mtDNA) which can provoke an inflammatory response by driving the expression of pro-inflammatory cytokines. Previous studies have found that cell free mtDNA (cf-mtDNA) copy numbers increase with mtDNA damage or mitochondrial dysfunction (181) and during oxidative stress (182).

Mitochondria have their own genome with multiple copies of small circular mtDNA molecules, whose number is regulated through the processes of biogenesis and mitophagy (183). MtDNA is particularly susceptible to oxidative damage because of its proximity to the electron transport chain (ETC) and its deficiency of protective histones (184). A change in membrane potential, as a result of increased oxidative stress, induces mitochondrial membrane depolarisation and increases permeability (185). Damage to mtDNA can induce malfunction of the ETC and ATP production, resulting in overproduction of ROS by complex I and III (186). The above pathway describes a pernicious feedback loop, where mtDNA can be damaged by ROS-mediated oxidation leading to diminished oxidative phosphorylation capacity, mtDNA fragment release and further ROS production (187). These biochemical disruptions facilitate the release of mitochondrial components such as ROS and mtDNA into the cytosol, triggering various inflammatory and apoptotic pathways (188). A recent cohort study found significant correlations between women carrying mtDNA oxidative phosphorylation mutations and an increased incidence of pregnancy loss, GDM, IUGR and postpartum haemorrhage (189).

Secreted mtDNA fragments act as damage-associated molecular patterns (DAMPs), endogenous signalling molecules that are released from damaged cells and activate an innate immune response (190). Although the majority of circulating cf-mtDNA in the maternal circulation during pregnancy is of maternal origin, approximately 5-20% is of fetal/placental origin (191). MtDNA motifs can instigate a pro-inflammatory response in the maternal circulation by binding to toll-like receptor (TLR)-9 expressed on immune cells. Recent work in our group established that pathogenic plasma mediators (including mtDNA) in PE increased TLR-9 activation with ensuing neutrophil activation (192, 193). Our group has also provided evidence

of increased circulating levels of mtDNA in GDM pregnancies, which was associated with higher maternal BMI (194).

An additional source of cf-mtDNA in the maternal circulation could be from AT, as mitochondrial dysfunction in the omental AT has been linked to a significant increase in mtDNA content (195). Adipocyte mitochondria have been shown to play an integral signalling role through their ability to influence key biochemical processes central to the adipocyte, such as fatty acid esterification and lipogenesis, as well as their impact on the production and release of key adipokines (196).

The importance of mitochondria in influencing metabolic function cannot be underestimated, whether mediated directly via ROS-induced oxidative stress or indirectly via the instigation of pro-inflammatory signalling pathways.

1.5.9 Role of Inflammation in Gestational Diabetes Mellitus

Inflammation is a key process in reproductive success as it is necessary for implantation, pregnancy maintenance, and parturition (197). The maternal physiological immune response to pregnancy is a complex process which is delicately balanced between immune activation and suppression during healthy pregnancy (198-200). Cytokines regulate the functions of the immune system and facilitate metabolic and inflammatory communication between cells (201). The placenta is a key instigator of inflammatory signalling during pregnancy. In healthy NGT pregnancy, cytokine signalling in the placenta mediates local vascular growth and development. Tissue-specific expression of cytokines facilitates additional functions during gestation, for example, IL-8 expression in the myometrium has been shown to be specifically upregulated during both term and preterm labour (202).

It has been postulated that GDM may develop from aberrant adaption of the maternal immune system to pregnancy and an increase in circulating inflammatory factors linked to innate immunity, with ensuing endothelial dysfunction (203, 204). Although the significance of immune dysregulation as a causative mediator in GDM pathophysiology is currently unclear, aberrant maternal immune responses contribute to secondary complications of GDM such as maternal, and fetal, cardiovascular and metabolic disorders. This stems from evidence in individuals with T2DM, where immune cell infiltration of VAT results in the pathological disruption of insulin signalling contributing to insulin resistance (205). Hyperglycaemia can equally cause immune dysfunction, resulting in altered neutrophil chemotaxis, macrophage function and phagocytic responses (206). Hyperglycaemia in GDM is associated with increased placental inflammation (207), and dysregulation of placental cytokine release has been demonstrated to correlate with GDM pathology, leading to inflammation, endothelial dysfunction and insulin resistance (208, 209). Individual studies have found elevated circulating levels of pro-inflammatory cytokines such as IL-8, IL-18, IL-6 and TNF-α in GDM (98, 210, 211). However, a review by Perez Gomes et al. found that significant heterogeneity exists in the current evidence investigating cytokine production in GDM with no significant collective conclusions. This review included 22 studies reporting on 9 cytokines (IL-1β, IL-2, IL-6, IL-10, IL-13, IL-18, IFN-ɣ, TGF-β, TNF-α) measured in a total of 1982 participants, including 1027 participants diagnosed with GDM. Meta-analysis was hindered in this review due to methodological differences in study design, including participant selection criteria, gestational age at sampling, sample type, assay method and data reporting. Furthermore, a lack of detailed information on sample collection, handling, storage and assessment methods was evident in most studies. This review

highlighted that additional studies are required to remove potential confounding factors and provide clear, detailed evidence of alterations in circulating cytokine levels (212).

Placental macrophages or Hofbauer cells (HBCs) have been implicated in regulating placental inflammatory signalling in GDM. Sisino et al. demonstrated a shift in the phenotype of HBCs from the anti-inflammatory M2 to the pro-inflammatory M1 phenotype in a rat model of gestational diabetes (213). However, analysis of primary HBCs in GDM placenta showed an increase in M2 markers such as CD206, compared to healthy placenta (214). Maternal adiposity can also disrupt placental-derived cytokine signalling. Challier at al. reported that obese pregnant women had a two-threefold increase in the number of placental macrophages, characterized by an increased expression of IL-1, TNF-α and IL-6 mRNA (215). Similar studies have found an increased placental expression of IL-8 and leptin in GDM (216).

The metabolically active VAT secretes adipokines and cytokines which regulate both pro- and anti-inflammatory responses. Some of the key AT-derived mediators involved in these responses are leptin, adiponectin, resistin, TNF-α, IL-6 and IL-1β (217). While adiponectin can act in both an anti- and pro-inflammatory capacity (218), these mediators predominantly exert a pro-inflammatory response. Adipokines can also regulate vascular function, in a deleterious or protective manner (219). For example, adiponectin exerts cardioprotective effects in vascular disease, via adiponectin receptor 1 and 2 activation, resulting in an increase in differentiation and survival of both endothelial and vascular smooth muscle cells while reducing the pro-inflammatory macrophage response (220). However, these inflammatory motifs generally propagate a negative effect on metabolic health. TNF-α downregulates insulin-stimulated cellular glucose uptake by interfering with GLUT4 translocation

(221). Furthermore, obese mice deficient in either TNF-α or its receptors (both p55 and p75 subtypes) were protected against insulin resistance (222). TLR-4 activation in adipocytes initiates AT inflammation and may contribute to insulin resistance through ceramide biosynthesis (223). Although the exact mechanism of this TLR-4 receptor activation is still unclear, one potential mechanism is activation by FFAs, which are increased in obesity.

1.5.10 Role of Immune Cells in Gestational Diabetes Mellitus

Pregnancy and fetal growth pose a substantial challenge for the maternal immune system. From the early stages of implantation and decidual formation, to successful fetal delivery, key maternal immunological mediators such as macrophages, natural killer (NK) cells and regulatory T cells (Tregs) must be meticulously balanced by the maternal immune system to avoid adverse pathology and/or disruption of pregnancy (224). How various immune mediators adapt to the state of pregnancy will thereby impact both fetal and maternal health outcomes. Both frequency and function of various immune cell populations are altered in GDM pathophysiology (Table 1.1 and Figure 1.2). These alterations are evident in maternal circulation and locally in the placenta and AT.

Table 1.1. A summary of the studies describing immune cell populations in maternal circulation, placental tissue and adipose tissue in GDM pregnancies and how their cellular function is altered in GDM relative to uncomplicated pregnancies (225).

Immune Cell Populations		Functional Role	Studies	Alterations in GDM
In Circulation				
Neutrophils		Phagocytosis; cytokine secretion	(226-232)	Blood count ↑ Activation ↑
NK cells		Regulates innate immune response; cytokine secretion	(233-235)	NKCD56dim cell count ↑ NKCD56bright cell count ↓ Pro-inflammatory cytokine release ↑
NKT cells		Cytokine production	(236, 237)	↔
Monocytes		Phagocytosis; antigen-presenting cells	(238, 239)	$CD14^+$ cell count ↑ Activation ↑ Intermediate monocyte count ↓
Dendritic cells		Antigen-presenting cells	(240)	↔
T cells	T helper cells	Inflammation; defence against IC bacterial pathogens	(204)	Th1 cell count ↑ Th17 cell count ↑
	Regulatory T cells	Regulates immune response	(204, 233, 241-245)	Mixed findings on Treg count and functionality
	γδ T cells	Inflammation and cytotoxicity	(240, 246, 247)	γδ T cell count ↑
	Cytotoxic T cells		(237, 247)	CD8+ T cell count ↑
	Naïve/memory phenotype		(236, 245)	Naïve T cell count ↓ Memory T cell count ↑
B cells		Antibody secretion	(248)	Blood count ↑ Activation ↑

Platelets		Coagulation; vasoconstriction	(228, 230, 232, 249-259)	Blood count ↑ Activation ↑
Placental tissue				
Neutrophils		Phagocytosis; cytokine secretion	(226)	Infiltration ↑ Activation ↑
Decidual NK cells		Promoting decidual vascularization	(235)	$DNKCD56^{dim}$ cell count ↑
Macrophages		Homeostasis of placenta environment and host defense against infections	(214, 260-263)	Activation ↑ Mixed findings on M1/M2 polarity
Adipose tissue (visceral and subcutaneous)				
Macrophages		Lipid and energy metabolism; adipocyte mitochondrial function	(261, 264)	Infiltration ↑ Activation ↑

Immune Cells and Meta-inflammation

In the last decade there has been a significant increase in our understanding of the pathogenic role of AT pro-inflammatory responses in mediating vascular damage and disrupting insulin signalling with resulting insulin resistance (61). Meta-inflammation refers to a chronic, low-grade inflammatory state that occurs in AT, liver, and other organs in response to factors such as obesity, insulin resistance and metabolic dysfunction. Meta-inflammation differs from classical inflammation that occurs in response to an injury or infection, involving transient and acute inflammatory responses of the innate immune system, and is mediated by immune cell populations such as monocytes, macrophages, dendritic cells, NK cells, mast cells and granulocytes infiltrating VAT and disrupting energy homeostasis (265). Meta-inflammation has been implicated in the development of a variety of chronic

diseases, including T2DM, cardiovascular disease and NAFLD. In GDM, meta-inflammation plays a role in the development of insulin resistance, a key feature of the condition. During pregnancy, the placenta produces hormones that can impair insulin action in the mother, leading to insulin resistance. In women predisposed to insulin resistance due to obesity or a family history of diabetes, this can result in the development of GDM (216, 266). Meta-inflammation exacerbates this process by further impairing insulin signalling, and can lead to β-cell dysfunction and impaired glucose tolerance. This deleterious level of insulin resistance impairs triglyceride storage, resulting in increased FFA release. Increased FFAs secretion into circulation futher induces insulin resistance and inflammation in both the muscle and liver (62). VAT-infiltrating neutrophils are strongly linked to initiating a state of meta-inflammation, partly by increased elastase production, an important mediator of acute and chronic inflammatory responses (267). A recent preclinical study demonstrated that naringenin, a citrus flavonoid, attenuated tissue inflammation and adipokine dysregulation by suppressing immune cell infiltration of VAT, particularly neutrophils, by inhibiting monocyte chemoattractant protein-3 (268).

There is substantial *in vivo* evidence from individuals with T2DM that both monocyte and macrophage subpopulations are the dominant cellular mediators involved in the development of meta-inflammation, by disrupting insulin signalling cascades which leads to the development of insulin resistance. Infiltration of VAT by monocyte/macrophage populations, and subsequent meta-inflammation, are particularly relevant to GDM due to the contribution of shared pathophysiology including insulin resistance, inflammation, oxidative stress and endothelial dysfunction. The potency of these pathogenic mediators are further amplified in obesity, a disease which itself increases the maternal risk of developing GDM.

Monocytes

Monocytes are critical cells in the innate immune response and are characterised by the expression of the CD14 receptor antigen which stratifies them into three subtypes: the main 'classical monocytes' ($CD14^{++}CD16^{-}$), which have significant phagocytic activity, and two additional subtypes, the 'non-classical' ($CD14^{+}$ $CD16^{++}$) and the 'intermediate' ($CD14^{+}$ $CD16^{+}$) (269). Monocytes promote an innate immune response by recognising pathogenic molecules via pattern recognition receptors and respond through phagocytosis, antigen presentation, chemokine secretion and proliferation. These cells also exhibit high levels of plasticity enabling them to alter their phenotype according to their local microenvironment, facilitating differentiation into monocyte-derived macrophages or monocyte-derived dendritic cells (DCs) (270, 271).

As maternal innate immunity adapts to the initial stages of pregnancy, the number of circulating monocytes increases significantly, particularly the nonclassical and intermediate subtypes (272). Placental cellular products are believed to act systemically in activating monocytes, with increased expression of surface activation markers such as CD11b, CD54 and CD64. Circulating monocytes in pregnancy also have increased expression of IL-12 and IL-1β cytokines relative to non-pregnant women. Monocyte activation proportionally increases from first trimester through to late gestation, confirming the critical role of innate immunity in maintaining a healthy pregnancy (273).

A recent case-control study found that the percentage of circulating $CD14^{+}$ cells were increased in GDM pregnancies. However, lower frequencies of intermediate monocytes ($CD14^{+}CD16^{+}$) and circulating monocytes (classical, intermediate and non-classical) expressing TLR-4 were observed in GDM, while soluble $CD14^{+}$

levels ($sCD14^+$) were higher in GDM pregnancies compared to healthy pregnancies (238). $SCD14^+$ is a complex moiety that can stimulate either pro-inflammatory or anti-inflammatory responses and has been defined as a nonspecific marker of monocyte activation (274). Further clinical assessment has confirmed that women with pregnancy-associated hyperglycaemia exhibits higher absolute monocyte counts with increased monocyte-lymphocyte ratios (MLR) (239). The described increase in circulating monocyte numbers and activation levels in GDM may be linked to elevated levels of circulating monocyte chemoattractant protein-1 (MCP-1), a key factor in the recruitment and activation of both peripheral blood and adipose-resident leukocytes. Telejko et al. reported that MCP-1 significantly correlated with fasting glucose levels in GDM pregnancies, where MCP-1 levels increased with higher plasma glucose levels in these women (275).

Macrophages

Macrophages are antigen presenting cells (APCs), derived from monocyte differentiation, with distinct phenotypic classification into either M1-like, pro-inflammatory and microbicidal, or M2-like, anti-inflammatory and immunoregulatory subtypes (276). These subtypes are characterised by the expression of specific markers including human leukocyte antigen–DR isotype (HLA-DR), CD11c and CD86 on M1-like macrophages and CD163, CD204, CD206 and vascular endothelial growth factor (VEGF) on M2-like macrophages (277). Specifically, CD86 (M1) and CD206 (M2) have been used as reliable characterization markers of macrophage subtypes in recent studies on diabetes and adipose tissue inflammation (278, 279). During pregnancy, macrophages play an important role in mediating tolerance at the maternal-fetal interface, comprising 20-30% of decidual leukocytes (280).

Specific placental tissue-resident macrophages, namely HBCs, are located in the chorionic villi and are largely associated with regulatory and anti-inflammatory functions. These cells are of fetal origin and while their role in pregnancy is not yet fully understood, their function is believed to be altered in GDM (281). The function of HBCs resembles activated M2-like macrophage subtypes, playing an important role in placental vasculogenesis and angiogenesis. High-dimensional flow cytometry analysis identified ten functionally diverse HBC subsets present throughout pregnancy, with varying expression of activation markers such as CD14, CD68 and HLA-DR. However, upon pathogenic stimulation, HBCs can undergo pro-inflammatory reprogramming to produce pro-inflammatory cytokines such as IL-6 and TNF-α (282, 283). Nonetheless, the described characteristics of HBCs suggest a regulatory, tissue remodelling role in the placenta rather than that of an inflammatory mediator (284).

Although, to date, no studies have explicitly measured specific peripheral macrophage populations in GDM pregnancies, there has been some evidence assessing the molecular communication between macrophage activation and GDM pathophysiology. Upon activation, macrophages shed their haemoglobin-haptoglobin scavenger receptor CD163, or soluble CD163 (sCD163), rendering it useful as an *in vivo* marker of macrophage activation (285). Ueland et al. reported that GDM pregnancies had increased circulating levels of sCD163 as early as 14-16 weeks gestation and 5 years postpartum, compared with healthy uncomplicated pregnancies. This increase in sCD163 was independent of BMI and other risk factors, proposing monocyte/macrophage activation as an important early mediator in GDM pathology (286). These women also had reduced circulating adiponectin levels. Furthermore, transgenic murine models expressing human adiponectin have

shown that macrophages expressing adiponectin can physiologically modulate metabolic activities such as insulin sensitivity *in vivo* by improving metabolism in distal tissues (287). In addition to its insulin-sensitising properties, adiponectin also regulates macrophage proliferation and function, suggesting a possible link between AT dysfunction and concurrent imbalance in peripheral immune regulation. Similar results found that the significant increase in circulating sCD163 levels in GDM women correlated with increased sCD163 release from both placenta and AT, suggesting that these endocrine organs are sources of the circulating risk predictor (261).

Elevated circulating levels of macrophage migration-inhibitory factor (MIF) have also been associated with GDM (288). MIF is a ubiquitously expressed pro-inflammatory cytokine that can stimulate T-helper 1 (Th1) cell type responses and IL-17 release and increases TLR-4 expression on macrophages, priming them for inflammatory stimulation (289). Furthermore, MIF-associated genotypes are susceptibility factors in the pathogenesis of GDM (290, 291). These results further indicate a potential interplay between maternal monocytes/macrophage activation and metabolic cytokine production as a culprit in the early development of GDM.

In GDM pregnancies, HBCs were shown to maintain their M2-like anti-inflammatory phenotype, characterised by CD206 and CD209 expression, with no significant difference in cytokine production relative to healthy uncomplicated pregnancies (214). This study was, however, limited by a small sample size. Further research found increased expression of CD163 on macrophage-like cells in GDM placental tissue samples with no difference in CD68 expression, suggesting a decrease in the M1/M2 ratio and a dominant anti-inflammatory phenotype (260). Bari et al. found similar results, where $CD163^{+}$ cells were significantly increased in

GDM placenta compared with controls. GDM placental explants also released increased concentrations of sCD163 compared to control explants (261).

Other studies have observed a pro-inflammatory shift towards an M1-like phenotype, with an increased number of $CD68^+$ cells and increased expression of IL-6, IL-8, TNF-α and TLR-2 in GDM placental tissue compared to healthy placental tissue (262, 263). Similar to the increase in maternal circulating levels of MIF reported in GDM pregnancies, placental MIF expression is increased in GDM and this correlated with maternal insulin resistance (292). These limited clinical findings show contrasting results in the role that macrophages and HBCs may play in placental-related immune dysregulation in GDM.

Similarly, current evidence exploring the role of AT inflammation in GDM is limited. One study reported that macrophage infiltration is significantly increased in the omental VAT in GDM pregnancies compared to uncomplicated pregnancies, which significantly correlates with maternal insulin resistance (264). Furthermore, Bari et al. found significantly increased $CD163^+$ infiltrates and increased concentrations of sCD163 in SAT explants in GDM pregnancies relative to uncomplicated pregnancies (261). In this thesis, the role of monocyte/macrophage populations in maternal circulation, VAT and placental tissue in GDM pregnancies will be characterised, particularly their function in inducing meta-inflammation.

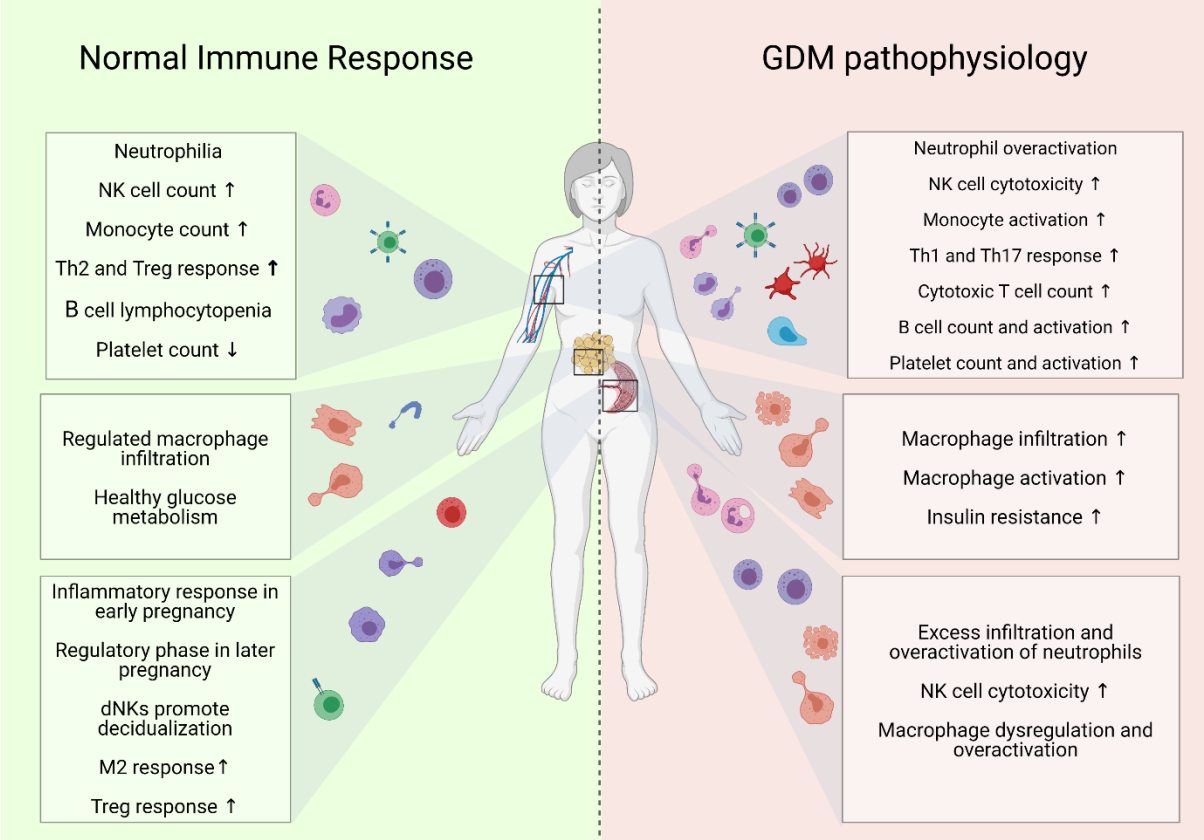


Figure 1.2. An overview characterising immune cell phenotypes in maternal circulation, adipose tissue and placental tissue in healthy uncomplicated pregnancy compared to a pregnancy complicated by GDM. Created with Biorender.com. GDM, gestational diabetes mellitus; NK, natural killer; Th2, T-helper 2 cell; Th1, T-helper 1 cell; Th17, T-helper 17 cell; Treg, regulatory T cell; dNK, decidual NK cell (225).

1.6 Pharmacological Treatment Strategies for Gestational Diabetes Mellitus

The American College of Obstetricians and Gynecologists (ACOG) categorises GDM into two subgroups, dependent on their therapeutic management. GDM Class 1 (A1GDM) women are those who respond to dietary intervention (low glycemic

index meals with low simple sugar and high fibre content) and exercise. GDM Class 2 (A2GDM) patients require pharmacologic intervention to achieve target glycaemic levels (Figure 1.3) (293).

The current first line pharmacological intervention for the treatment of GDM, when diet and lifestyle modification fail to adequately control glycaemic levels, focuses on insulin therapy. Insulin is administered subcutaneously and maintains glucose homeostasis by acting on skeletal muscle, liver and white adipocytes to stimulate glycogen synthesis, suppress lipolysis and increase glucose transport (294). Insulin is available in a range of formulations that can be tailored for individual patient needs and does not cross the placental barrier into the fetal circulation. However, insulin administration is invasive and inconvenient for the patient, with risk of injection site infection and hypoglycaemic episodes. Recent evidence has suggested that insulin may have additional effects on the immune system, as a range of cells not typically involved in glucose homeostasis, including immune cells, express insulin receptors. Both *in vitro* and *in vivo* clinical evidence from T2DM patients have shown that insulin can mediate anti-inflammatory responses in circulating mononuclear cells, through downregulation of TLR transcription (295, 296). In patients with T1DM, insulin administration can suppress inflammatory markers such as C-reactive protein (CRP), IL-1β and TNF-α, reduce CD14 transcription and lipopolysaccharide (LPS)-stimulated apoptosis in macrophages, and attenuate neutrophil ROS generation (297). Insulin also has indirect anti-inflammatory implications via the management of hyperglycaemia, thereby reducing 'glucose toxicity', preventing cell stress and reducing the generation of inflammatory mediators such as advanced glycation end products (AGEs) (298).

Oral anti-diabetic pharmacological therapies such as metformin or sulfonylureas may also be considered for the treatment of GDM. The exact pharmacodynamic profile of metformin is complex and not yet fully understood, however, it is an effective insulin sensitising agent and reduces hepatic glucose production. Metformin has minimal risk of inducing hypoglycaemic episodes and has a reduced impact on weight gain, thereby improving patient outcomes (299). Although metformin crosses the placenta, several studies assessing fetal outcomes in metformin-treated pregnancies have indicated its safe use in pregnancy (300, 301). Evidence suggests that metformin equally suppresses immune responses through induction of cellular AMPK and subsequent inhibition of mTORC1 (302). Inhibition of mTORC results in increased IL-12 production, altered antigen presentation in DCs, and increased expression and proliferation of Tregs (303). In murine models fed a high fat diet, metformin improved obesity-related inflammation by inducing polarisation of M2-like macrophages via AMPK dependent mechanisms, resulting in decreased circulating concentrations of IL-6 and TNF-α and an overall anti-inflammatory phenotype (304). *In vitro* findings have shown that metformin also attenuates mitochondrial ROS production, reducing mitochondrial-mediated apoptosis and oxidative stress responses (305).

Sulfonylurea drugs, such as glyburide, appear to be more effective than metformin in managing GDM-induced hyperglycaemia, however some studies have suggested a correlation with PE, macrosomia and neonatal hypoglycaemia (306). The predominant clinical effect of glyburide is through the stimulation of pancreatic insulin release (307). Glyburide also stimulates an anti-inflammatory response, particularly through M2-like polarisation and inflammasome inhibition in

macrophages, which reduces the release of pro-inflammatory cytokines such as IL-1β and IL-18 (308, 309).

Although a significant proportion of women with GDM will require pharmacological intervention during pregnancy, lifestyle modification is the first line of intervention for managing the symptoms of GDM. Reduction of dietary glucose intake and careful glycaemic monitoring can successfully manage insulin resistance, without the need for pharmacological intervention, in less severe GDM pregnancies.

Unfortunately, the availability of therapeutic options for obstetric complications are hindered by the ethical and clinical reservations associated with the participation of pregnant women in clinical trials for novel drug treatments. Nonetheless, the recent EMERGE clinical trial (ClinicalTrials.gov Identifier: NCT02980276), is currently investigating the most effective way to implement lifestyle modifications in addition to metformin, to reduce the need for insulin and the occurrence of hypoglycaemic episodes and maternal weight gain in GDM pregnancies. Extensive pre-clinical investigations into safe and effective therapeutics, with appropriate pre-clinical models targeting the diverse pathogenic mediators of GDM pathophysiology, is key for clinical progression.

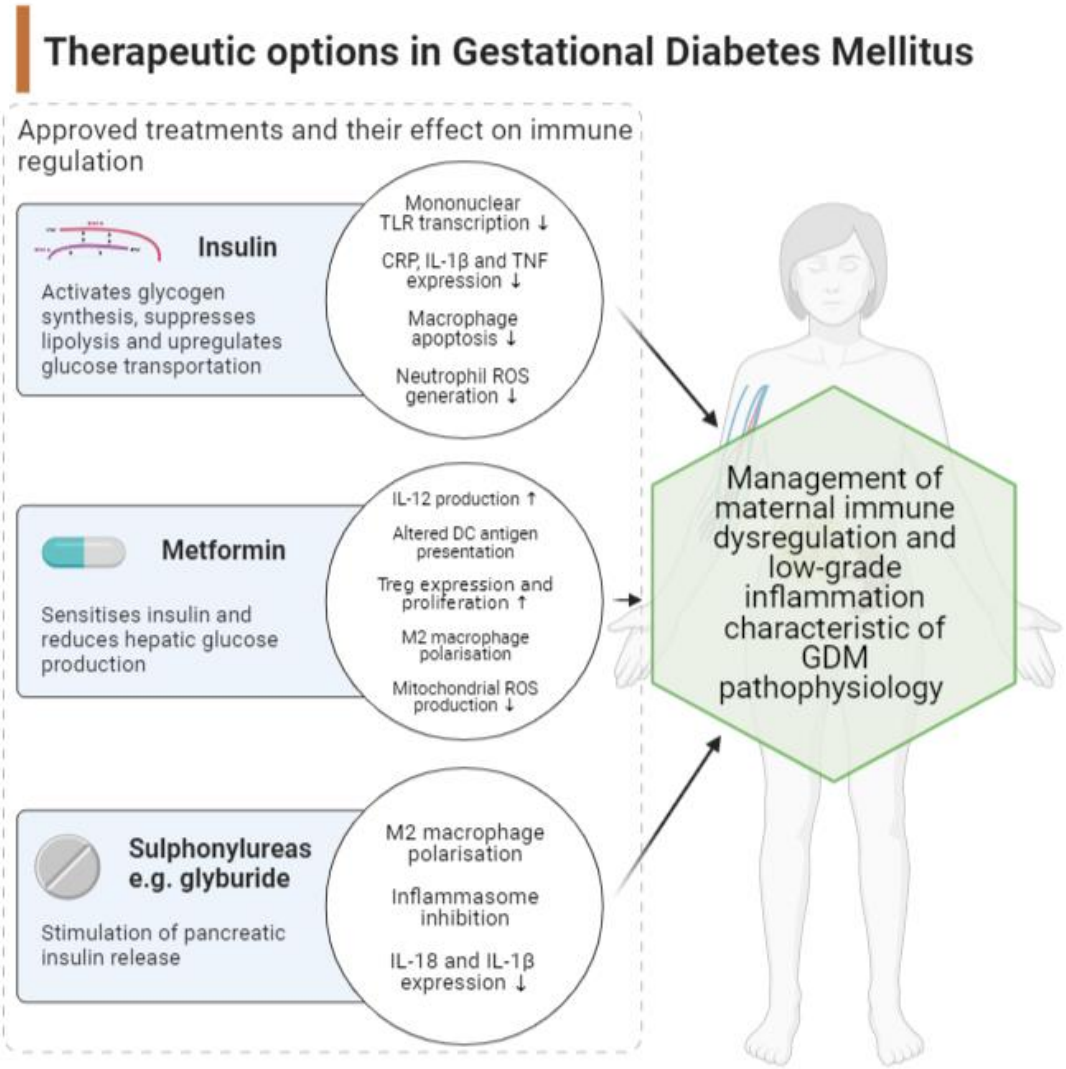


Figure 1.3. Glycaemic and immune-mediated effects of clinically approved therapeutic options for GDM. Created with Biorender.com. TLR, toll-like receptor; CRP, c-reactive protein; IL, interleukin; TNF, tumour necrosis factor; ROS, reactive oxygen species; DC, dendritic cell; Treg, regulatory T cell (225).

1.7 Thesis Hypothesis, Aims, & Objectives

1.7.1 Hypothesis

A review of the current literature on GDM suggests that a link exists between hyperglycaemia, metabolic dysfunction, and systemic low-grade inflammation, which instigates diabetic symptoms and adverse maternal outcomes in GDM. This relationship may be driven by local tissue meta-inflammation and the release of mitochondrial-derived DAMPs, which propagate inflammatory pathways both locally and systemically. Identifying these molecular signalling networks between the placenta-adipose tissue- axis will allow us to identify clinical interventions to effectively manage GDM pathology. To date no clinical cohort study has comprehensively characterised these pathways by adopting a multi-organ approach.

1.7.2 Aims

The overaching aim of this thesis is to characterise maternal GDM-mediated meta-inflammation and mitochondrial dysfunction locally in both the adipose and placenta tissue and systemically in the maternal circulation. This will be the first translational study to investigate global metabolic dysfunction in fasting blood, omental VAT and placental tissue from the same cohort of GDM participants. Furthermore, many of the pathways which we aim to characterise in this study have not previously been investigated in GDM pathology, such as omental VAT dysfunction, which has been largely implicated in T2DM etiology. This may allow the delineation of the mechanistic pathways involved in promoting insulin resistance in GDM. Additionally, in this thesis, the interactions between placental mediators and AT dysfunction which may contribute to impaired insulin sensitivity will be investigated.

1.7.3 Objectives

In order to achieve these aims, the following objectives were identified:

- **Objective 1:** To characterise the metabolic, mitochondrial and inflammatory mediators which adversely affect insulin sensitivity in the maternal circulation in GDM (**Chapter 2**).

- **Objective 2**: To define whether omental VAT adipocyte dysfunction and meta-inflammation drive insulin resistance in GDM (**Chapter 3**).

- **Objective 3:** To examine whether mitochondrial dysfunction activates the NLRP3 inflammasome and induces immune dysregulation in the placenta in GDM (**Chapter 4**).

- **Objective 4:** To identify if a bidirectional relationship exists between placental tissue and AT in GDM, in particular how placental-derived mediators may impact adipocyte function and impair insulin sensitivity (**Chapter 5**).

Chapter 2

Circulating Markers of Insulin Resistance and Meta-inflammation in Gestational Diabetes Mellitus

After the overview of available literature pertaining to the various pathogenic mediators contributing to the pathophysiology of GDM and their clinical implication, this chapter will explore circulating markers of metabolic dysfunction and inflammation in GDM. More specifically, pathological insulin resistance in GDM is characterised, followed by an investigation into the extent of systemic mitochondrial dysfunction and meta-inflammation which adversely affects maternal insulin sensitivity in GDM.

Abstract

Background

GDM pathology is considered a state of exaggerated insulin resistance, mediated by pancreatic β-cell insufficiency, adipose dysfunction and systemic meta-inflammation. In mild cases, GDM symptoms can be managed by lifestyle modification, promoting normal glucose metabolism. In more severe cases, pharmacological intervention with either insulin or metformin is required. The pathophysiology of GDM is complex with many potential contributors including oxidative stress and immune imbalance. This chapter aims to characterise the metabolic, mitochondrial and inflammatory mediators in the maternal circulation which adversely affect insulin sensitivity in GDM.

Methods

GDM-diagnosed (n=30) and healthy NGT control (n=30) participants were recruited on the day of elective caesarean section. Fasting whole blood was obtained pre-surgery and plasma and serum samples were isolated. Plasma concentrations of insulin, glucose, IGF-1 and serum concentrations of PAPP-A, triglycerides and FFAs were quantified by ELISA. Serum cytokine and plasma adipokine profiles were quantified using a multiplex immunoassay. Cell-free mitochondrial DNA (cf-mtDNA) copy number was quantified by Real-time PCR. Peripheral blood mononuclear cells were collected for phenotyping of circulating M1-like ($CD14^+CD86^+CD206^-$) and M2-like ($CD14^+CD86^+CD206^+$) monocyte/macrophage populations by flow cytometry. Mitochondrial superoxide production of circulating monocyte/macrophage populations was measured by flow cytometry using $MitoTracker^+MitoSOX^+$ staining.

Results

GDM participants had significantly higher fasting circulating insulin (p=0.006) and glucose (p=0.04) levels and a significantly higher maternal BMI (p=0.004), which correlated with HOMA-IR scores (p=0.03). HOMA-IR scores were significantly higher in metformin and insulin treated GDM groups, but not in the diet-controlled group, when compared to the NGT group. Additionally, a subgroup of lean (BMI <29 kg/m^2) GDM participants had significantly lower insulin levels (p=0.007) and HOMA-IR scores (p=0.01) relative to obese GDM participants, suggestive of insulin deficiency. Serum triglyceride and FFA levels were not significantly altered in GDM compared to NGT participants (p=0.19 and p=0.71, respectively). IGF-1 and PAPPA plasma levels were not significantly different in GDM compared to NGT participants (p=0.17 and p=0.61, respectively). Circulating cf-mtDNA copy numbers were significantly higher in GDM when compared to NGT participants (109233 copies/µl ± 111578 copies/µl vs. 16657 copies/µl ± 18740 copies/µl, p=0.002). There were no significant changes between study groups in the abundance of monocyte/macrophage M1-like/M2-like populations or their production of mitochondrial superoxide. However, GDM participants had significantly increased circulating concentrations of pro-inflammatory cytokines IL-6 (p=0.003), IL-17A (p=0.04), IL-12p70 (p=0.05) and MCP-1 (p=0.03) and lower circulating levels of adiponectin (p=0.03).

Conclusion

Insulin resistance is significantly increased in GDM participants, which may be mediated by higher maternal BMI. In the maternal circulation, GDM participants had significantly higher cf-mtDNA levels, increased production of pro-inflammatory cytokines and lower levels of the insulin-sensitizing adipokine, adiponectin. This

study suggests that inflammation and mitochondrial dysfunction could contribute to the pathophysiology of GDM by altering metabolic function.

2.1 Introduction

Insulin resistance is a well-defined physiological response to healthy pregnancy, providing sufficient glucose for maternal and fetal requirements (59). GDM pathology results from a failure of the maternal system to regulate this systemic insulin resistance response, resulting in uncontrolled hyperglycaemia. In this state, physiological levels of circulating insulin cannot maintain normoglycaemia through insulin receptor binding and subsequent glucose uptake and metabolism, which promotes excessive insulin secretion. When this hyperinsulinemic state fails to counteract insulin resistance, GDM develops. Aberrant glucose homeostasis during pregnancy has significant health implications, including increased maternal risk of hypertensive disorders of pregnancy, T2DM, metabolic syndrome and cardiovascular disease (25) and an increased risk of LGA or macrosomia, respiratory distress syndrome and hyperinsulinaemia in the neonate (37, 63). Furthermore, insulin resistance in GDM can cause changes to maternal fat metabolism, promoting an altered lipid profile with elevated circulating triglyceride and FFA levels. These pro-inflammatory mediators can further impair insulin sensitivity and promote adverse pregnancy outcomes (310, 311).

Hyperglycaemia-induced ROS and oxidative stress equally have the potential to dysregulate maternal metabolism in GDM. Hyperglycaemia can disrupt the mitochondrial ETC and increase the production of AGEs, which increase superoxide production (312). Exaggerated production of ROS without an appropriate antioxidant response results in deleterious oxidative stress, which orchestrates maternal endothelial dysfunction evident in GDM. This can subsequently result in permanent vascular damage with increased maternal cardiometabolic risk (180). Mitochondria are the primary source of cellular ROS generation and mitochondrial dysfunction is

associated with aberrant ROS production which can directly stimulate the production of pro-inflammatory cytokines (313). In response to cellular injury and ROS-induced oxidative stress, mitochondria release mtDNA into the extracellular space which can induce inflammation, predominantly through TLR9 activation and subsequent nuclear factor kappa B (NF-κB) activation, driving the expression of pro-IL-1β and -18 (314). The pathogenic implications of ROS generation and concurrent inflammation is established in many inflammatory diseases but has not been fully investigated in GDM.

The role of circulating monocytes and macrophages as inflammatory mediators is well established. The recruitment of monocytes to sites of inflammation is critical for host defense, allowing monocytes to infiltrate sites of injury and differentiate into macrophages which suppress inflammation and initiate wound repair. Macrophages can also drive local low-grade inflammation, impairing localized insulin activity (315). However, recent evidence from obesity and T2DM studies has established that these immune cell populations can also directly influence metabolic signalling leading to the development of insulin resistance. In obese individuals with insulin resistance, an increase in scavenger receptor CD36 on monocytes increases oxidized LDL (oxLDL) uptake, promoting the development of atherogenesis-induced insulin resistance (316). Macrophage-specific expression of adiponectin can mediate metabolic activities *in vivo* by improving metabolism in distal tissues (287). This evidence shows that the physiological role of monocytes and macrophages extends beyond direct inflammatory signalling, and systemic dysregulation of these leukocyte populations may have substantial metabolic impacts on maternal health.

Furthermore, elevated levels of circulating inflammatory cytokines promote inflammation, endothelial dysfunction and insulin resistance (209), however the role

of circulating cytokines in GDM is poorly characterised. Perez Gomes et al. extensively reviewed current GDM studies reporting circulating cytokine levels but found significant variation in study methods and results, with no significant collective conclusions (212). However, individual studies have found elevated levels of pro-inflammatory cytokines such as IL-8, IL-18, IL-6 and TNF-α in GDM (98, 210). This study aims to quantify 13 inflammatory cytokines in the maternal circulations at term pregnancy using explicitly defined recruitment, sample collection and analysis methods to address previous issues of heterogeneity in circulating cytokine level reporting in GDM. Research reporting on circulating adipokine levels in GDM is also lacking, however some consensus is available. The expression of leptin, an adipokine implicated in energy intake and expenditure, has been shown to be increased in GDM, which may increase pro-inflammatory cytokine release and insulin resistance (106). Increased leptin production has been also linked to decreased NO bioavailability through an increase in oxidative stress (317). On the contrary, adiponectin levels are systemically lower in GDM and are inversely correlated with fetal growth (96-98). Other secreted adipokines such as resistin, which is elevated in GDM and significantly relates to GDM risk after 28 weeks gestation, may also have significant influence on glucose homeostasis. Resistin has been shown to impair insulin function through the regulation of IRS-1 and AKT, thereby reducing GLUT4 translocation and glucose uptake (127, 318).

The principal aim of this study was to characterise the metabolic, mitochondrial and inflammatory mediators in the maternal circulation which adversely affect maternal insulin sensitivity in GDM. We wanted to test the hypothesis that GDM pathology may be driven by systemic mitochondrial dysfunction, with subsequent activation of a pro-inflammatory response which may disrupt metabolic homeostasis.

2.2 Methods

2.2.1 Study design and participant recruitment

GDM-diagnosed (n=30) and healthy NGT control (n=30) participants were recruited from Cork University Maternity Hospital (CUMH), Ireland between 2019 and 2022 as part of the COMRADES study, a non-interventional cohort study of nulliparous singleton pregnancies with the aim of characterizing mitochondrial dysfunction and meta-inflammation in GDM. The COMRADES study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all the procedures were approved by the Clinical Research Ethics Committee of the Cork Teaching (ECM4 (ff) 04/12/18). All participants provided informed written consent. Inclusion criteria for all study participants included; women aged between 18-50 years, nulliparous pregnancy, singleton pregnancy as determined by ultrasound scan, opted for an elective caesarean section and had provided written informed consent. As per national HSE guidelines, selective screening for GDM was carried out on identified at-risk pregnant women between 24-28 weeks gestation using the 75g oral glucose tolerance test (OGTT). A diagnosis of GDM was confirmed if one of the following values were met or exceeded: fasting blood glucose levels of 5.1mmol/L, blood glucose levels of 10.0mmol/L 1 hour post-OGTT or blood glucose levels of 8.5mmol/L 2 hours post-OGTT.

Healthy NGT control participants were defined as pregnant women who had not been diagnosed with GDM, as per the above guidelines, and did not present with any other diagnosed comorbidities. Exclusion criteria included women who: had a multiple pregnancy; were multiparous; had already started to labour, who were outside the age range of 18-50 years of age or had an emergency caesarean section. Women with known pregnancy or other health related conditions namely: pre

pregnancy essential hypertension; known liver or renal diseases; known Human Immunodeficiency Viruses (HIV), Hepatitis B or Hepatitis C positive; known Systemic Lupus Erythematous; known antiphospholipid syndrome and known or suspected fetal anomalies were excluded from the study.

Although our study cohort size for each group was n=30, smaller sample sizes were used for individual assays. Where possible, samples were chosen from the pool of 30 samples to allow for matching by maternal age and/or BMI (e.g. histology and immunoblotting analysis). Where this occured, it is explicitly stated. Where this was not possible, samples were chosen to exclude participants with either extreme age and/or BMI parameters and to include participants for which we had a complete biological sample profile (fasting blood, placental tissue and omental tissue).

2.2.2 Sample collection

Fasting blood samples were collected from study participants with a gestational age ≥37 weeks pre-surgery on the morning of elective caesarean section. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes for plasma collection and vacutainer serum tubes for serum collection. Samples were centrifuged at 2400 g for 10 minutes at 4°C before plasma and serum was collected. This method was performed according to a standardised lab protocol. Samples were then stored at -80°C until analysis was performed.

2.2.3 Immunoassays

A variety of immunoassays were used in the quantification of circulating markers in either plasma or serum samples from GDM (n=20) and NGT (n=20) participants. Plasma insulin, glucose and IGF-1 were measured using the following kits: Insulin Quantikine ELISA kit (Bio-Techne DINS00), Glucose assay (Abcam ab65333),

IGF-1 Quantikine ELISA kit (Bio-Techne DG100B). Serum triglyceride and free fatty acid (FFA) levels were quantified using a triglyceride assay (Abcam 65336) and a FFA assay (Abcam 65341). Serum PAPPA was measured by PAPPA Quantikine ELISA kit (Bio-Techne DPPA00). To determine the extent of systemic insulin resistance in our study cohort, circulating glucose and insulin levels were used to calculate homeostatic model assessment for insulin resistance (HOMA-IR) values according to the formula: fasting insulin (μU/L) x fasting glucose (nmol/L)/22.5.

Cytokine panel including IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33 were measured in participant serum samples using the LEGENDPlex Human Inflammation Panel 1 kit (BioLegend 740809). Plasma adiponectin, adipsin, leptin, resistin were measured using the LEGENDplex Human Metabolic Panel 1 (BioLegend 740212). All assays were performed as per the manufacturer instructions.

2.2.4 Peripheral blood mononuclear cell (PBMC) isolation

Following sample collection, 1-3mls of whole blood collected in EDTA vacutainer was overlaid onto 5mL Histopaque (3.1ml Histopaque 1119 overlaid by 1.9ml Histopaque 1077) in a 15ml falcon tube followed by centrifugation at 700g for 30 minutes, with reduced acceleration and deceleration. Cells at the interface were collected with a pasteur pipette and washed in 5ml PBS. This suspension was passed through a 70μm cell strainer (Sigma CLS431751) to remove cells aggregates. The resultant cell suspension was pelleted by centrifugation at 300g for 5 minutes. Supernatant was discarded and the cell pellet was stained for flow cytometry analysis.

2.2.5 Flow cytometry analysis

Following maternal blood PBMC isolation, single cell suspensions were kept on ice. Approximately $1x10^6$ cells of the single cell suspensions were placed in 5ml round-bottomed tubes (Falcon 352008) and centrifuged at 300g for 5 minutes and supernatants were discarded. Cell pellets were incubated with 50µl FC blocking buffer (10% human serum (heat-inactivated)(Sigma S1-M) in 1x PBS (without Ca^{2+}/Mg^{2+})(Sigma P4417)) containing Fixable Viability Dye eFluor™ 455UV (eBioscience 65-0868-14) (1:1000) on ice for 10 minutes. 50µl of the antibody mix containing CD14 (eBioscience 25-0149-42)(61D3, 1:20), CD86 (BioLegend 374214)(BU63, 1:20), CD206 (eBioscience 48-2069-42)(19.2, 1:20), MitoTracker DeepRed (Invitrogen M22426)(50nM) and MitoSOX Red (Invitrogen M36008)(5µM) was added and cells were incubated for a further 20 minutes on ice. Cell suspensions were then washed with 2ml FACS buffer (EDTA 2mM (Sigma E6758), FBS 2% (heat-inactivated)(Gibco 16140071) in 1x PBS (without Ca^{2+}/Mg^{2+})) and centrifuged at 300g for 5 minutes. The supernatant was discarded and 100µl of fixation buffer (PFA 4% (Sigma 158127) in 1x PBS (without Ca^{2+}/Mg^{2+})) was added. Cell suspensions were incubated for 30 minutes at room temperature in the dark. Cell suspensions were then washed in 2ml PBS and centrifuged at 300g for 5 minutes and supernatants were discarded. Finally, stained cells were resuspended in 150µl PBS and stored in the dark at 4°C until acquisition.

Samples were analysed within 24 hours on a BD LSR II flow cytometer. Rainbow Calibration particles (BioLegend 422903) were used to standardise instrument settings for longitudinal analysis. Data was analysed using FlowJo (v10.8.1) according to the following initial gating strategy: singlet cells (as determined by FSC-H/FSC-A), leukocytes (as determined by SSC-A/FSC-A), live cells (as

determined by live/dead stain), monocytes/macrophages (defined as $CD14^+$), M1-like monocytes/macrophages (defined as $CD14^+CD86^+CD206^-$), M2-like monocytes/macrophages (defined as $CD14^+CD86^+CD206^+$), cells containing superoxide-producing mitochondria (defined as $MitoTracker^+MitoSOX^+$). Gates were set according to unstained control samples. Cell proportions were quantified as a percentage of their parent gate e.g. $CD14^+$ as a percentage of live cells, $CD14^+CD86^+CD206^-$ and $CD14^+CD86^+CD206^-$ cells as a percentage of $CD14^+$ cells and $MitoSOX^+$ cells as a percentage of $MitoTracker^+$ cells.

2.2.6 Cf-mtDNA quantification

DNA was isolated from plasma samples using the QIAamp DNA Mini Kit (Qiagen 51304), following manufacturer protocols. Isolated DNA was sonicated for 10 minutes to optimise DNA yield followed by quantification on a NanoDrop (Thermo Scientific). Mitochondrial DNA copy number was determined by real-time PCR determined using predesigned Taqman assays (hMitoF5, hMitoR5) (319) and Taqman Mastermix (Bio-sciences 4369016) on the StepOne Plus Detection platform (Thermo Scientific). Absolute quantification of cf-mtDNA concentration was determined by standard curve analysis and presented as mtDNA copies/µl.

2.2.7 TLR9 activation assay in HEK-blue hTLR9 cells

TLR9 ligand activity was monitored using HEK-blue TLR9 Reporter cells (InvivoGen) which are designed for studying the stimulation of human TLR9 (hTLR9) by monitoring the activation of NF-κB. HEK-Blue hTLR9 cells were seeded at $4x10^5$ cells/well in a 96-well plate and cultured overnight in growth medium containing DMEM, 4.5 g/l glucose, 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml Normocin™, 2 mM L-glutamine. Following a media change, adherent cells were treated for 24-hours with 5% plasma

from NGT (n=20) and GDM (n=20) participants. 5% plasma treatment was selected following preliminary experiments with 1%, 3% and 5% plasma treatments, where only 5% treatment was sufficient to consistently stimulate activation above untreated control. ODN 2006 (InvivoGen #tlrl-2006) was used as a positive control and sterile distilled water was used as a negative control. HEK-Blue hTLR9 supernatants were subsequently incubated with Quanti-Blue detection medium (InvivoGen) and the NF-κB activity was read on a Tecan Spark spectrophotometer at a wavelength of 620 nm.

2.2.8 Statistics

Statistical analysis was performed using GraphPad Prism 5.03. Where applicable, data is represented as mean ± standard deviation for parametric analysis and median [95% confidence interval] for non-parametric analysis. Where specified, GDM cases were matched with controls by maternal age (± 3 years) and BMI (± 3 kg/m^2 units). P values < 0.05 were considered as statistically significant. The Kolmogorov–Smirnov test was used to test for data normality. For statistical comparisons, either the Student's t-test or the Mann-Whitney U test was used depending on data normality and test suitability. Wilcoxon matched-pairs signed rank test was used to compare matched samples. Fisher's exact test was used to compare categorical outcomes. Spearman's correlation analysis was used to examine the correlation between clinical parameters. The Benjamini–Hochberg method with a 20% false discovery rate was used for multiple hypothesis testing in cytokine (n=13) panels to decrease the false discovery rate. Grubbs's test was used to detect outliers in data sets.

2.3 Results

2.3.1 Baseline characteristics of study participants

Sixty nulliparous pregnant women were included in this study, including NGT pregnancies (n=30) and GDM-complicated pregnancies (n=30). Of these 30 GDM participants, 13 were diet-controlled, 9 were insulin-treated and 8 were metformin-treated. Clinical characteristics were recorded from electronic clinical charts in CUMH for each individual and any differences between participant groups were assessed (**Table 2.1**). There were no significant differences observed between study groups for maternal age, gestational age at caesarian delivery, fetal weight, placenta weight or fetal gender. GDM participants had a significantly higher BMI relative to NGT participants (p=0.004).

Table 2.1. Clinical characteristics of study participants.

	Participant study group		
	NGT	*GDM*	***p-value***
Maternal age (years)	33.12 ± 3.77	35.04 ± 5.52	0.16
Maternal BMI	27.11 ± 6.31	32.41 ± 5.94	0.004 **
Gestational age (weeks)	38.73 ± 0.67	38.62 ± 0.5	0.55
Fetal weight (g)	3408.95 ± 545.24	3595.65 ± 342.66	0.18
Placenta weight (g)	682.32 ± 171.65	714.75 ± 126.69	0.49
Fetal gender	Male – 36.6% Female – 63.3%	Male – 53.3% Female – 46.6%	0.3
GDM intervention	N/A	Diet – 43% Metformin – 27% Insulin – 30%	N/A

Clinical information for NGT (n=30) and GDM (n=30) participants. Student's t-test was used to compare means between continuous variables. Fisher's exact test was used to compare categorical outcomes. Where applicable, data is represented as mean ± standard deviation. Significance levels are shown as: ** $p<0.01$.

2.3.2 Elevated systemic insulin resistance in GDM

It has been well established that GDM pathology is associated with systemic insulin resistance (68). GDM participants had significantly higher circulating levels of insulin (39.24pmol/L ± 14.36pmol/L vs. 135.3pmol/L ± 151.5 pmol/L, p=0.006) (**Figure 2.1A**), significantly higher circulating levels of glucose (6.03mmol/L ± 0.49mmol/L vs. 6.3mmol/L ± 0.26mmol/L, p=0.04) (**Figure 2.1C**) and significantly higher HOMA-IR scores (1.73 ± 0.71 vs. 5.45 ± 6.11, p=0.009) (**Figure 2.1D**) relative to NGT participants. However, due to the artificial effect that insulin therapy has on plasma insulin levels and subsequently, HOMA-IR scores, the analysis of circulating insulin levels was repeated excluding participants on insulin therapy (39.24pmol/L ± 14.36pmol/L vs. 74.16pmol/L ± 90.17pmol/L, p=0.09) (**Figure 2.1B**).

Similarly, GDM subgroups were stratified and HOMA-IR scores were compared by intervention (**Figure 2.1E**). There was no significant difference in HOMA-IR scores between NGT and diet-controlled GDM participants (1.73 ± 0.71 vs. 1.69 ± 0.88). GDM participants treated with metformin (5.77 ± 5.71) and insulin (11.09 ± 7.6) had significantly higher HOMA-IR scores compared to NGT participants. Insulin-treated participants were expected to have significantly higher HOMA-IR scores due to the confounding effect of exogenous insulin administration. Finally, the potential effect of maternal BMI on elevated HOMA-IR scores was determined in the GDM cohort (excluding insulin-treated participants to reduce confounding effects). There was a

significant correlation between HOMA-IR and maternal BMI in GDM (r_S=0.60, n=13, p=0.03) (**Figure 2.1F**), suggesting that elevated maternal BMI contributes to exaggerated insulin resistance in GDM.

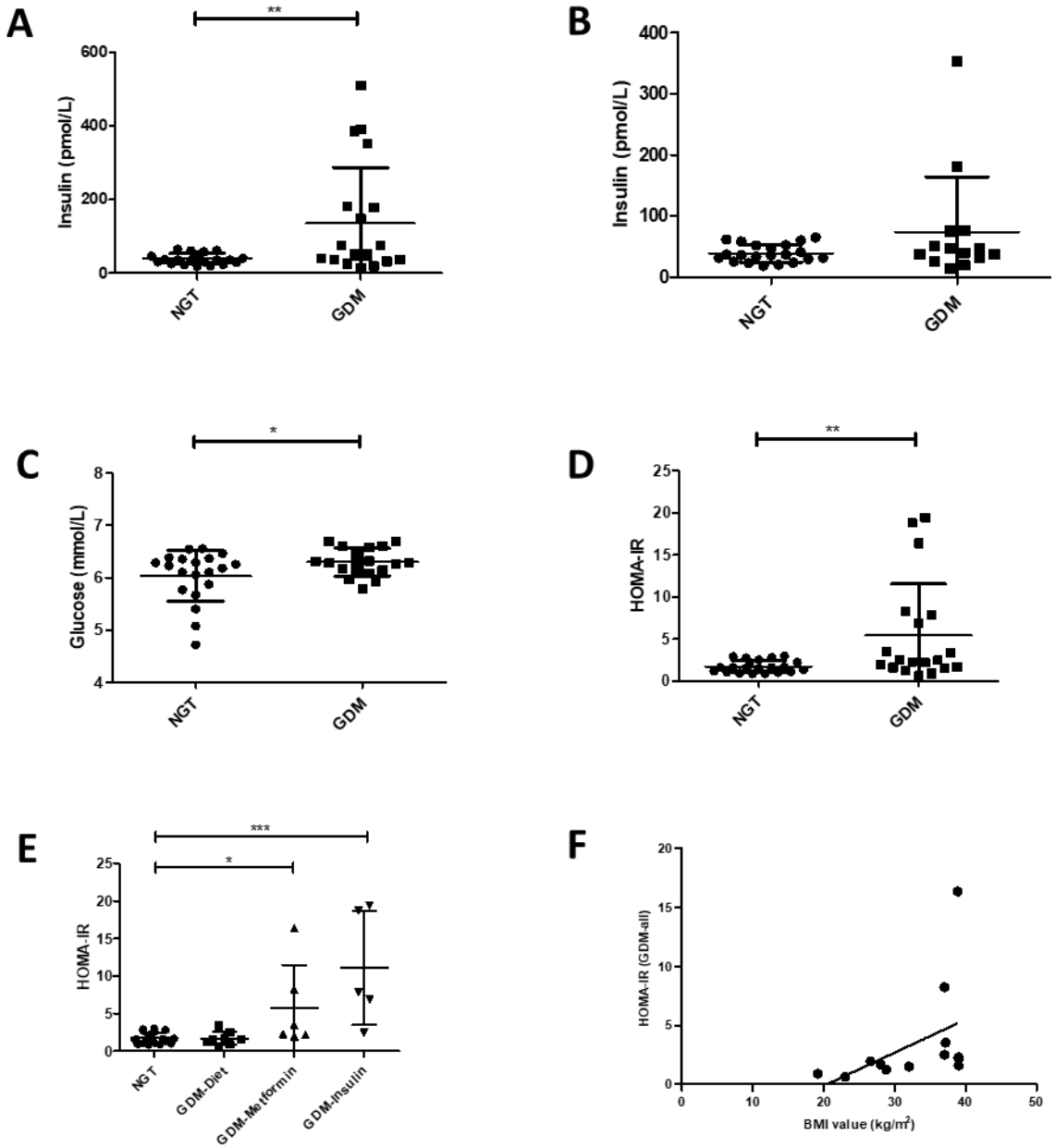


Figure 2.1. GDM participants show significant systemic insulin resistance. All values were measured in NGT (n=20) versus GDM (n=20) participants. **(A)** Plasma insulin levels (pmol/L) in NGT and GDM participants and **(B)** plasma insulin levels

(pmol/L) excluding GDM participants who were receiving insulin therapy. **(C)** Glucose levels (mmol/L) in NGT and GDM participants. **(D)** HOMA-IR scores in NGT and GDM participants. **(E)** Comparison of HOMA-IR scores between NGT and GDM participants stratified by therapeutic intervention. **(F)** Correlation of HOMA-IR score to maternal BMI in women with GDM excluding participants who were receiving insulin therapy. Student's t-test was used to compare the means of variables between dichotomous groups. One-way ANOVA and Dunnett's Multiple Comparison Test was used to compare HOMA-IR scores between intervention groups. Spearman's correlation was used to assess the relationship between HOMA-IR score and BMI. Grubb's test was used to remove statistical outliers from figure C and D. Significance levels are shown as: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

2.3.3 Phenotype heterogeneity in GDM classification

Recent work by Powe et al. identified heterogeneity in the classification of GDM and characterised 3 pathological subtypes. Women with GDM may be (1) insulin-deficient, (2) insulin-resistant or (3) a mix of these subtypes depending on the relative contribution of β-cell insufficiency or insulin resistance to their pathology. Lean GDM patients often present with a hyperglycemic but hypoinsulinemic phenotype, whereas insulin resistance, with concurrent hyperinsulinemia, is more evident in obese GDM patients (320). To identify if similar heterogeneity was evident in GDM participants in this study, circulating insulin and glucose levels, as well as HOMA-IR scores, were compared between GDM participants with a BMI <29 and GDM participants with a BMI >29. To exclude the confounding effect of insulin administration, GDM participants receiving insulin therapy were excluded from this analysis. Relative to obese GDM participants (BMI >29), lean GDM participants (BMI <29) had significantly lower plasma insulin levels (25.97pmol/L

[13.54, 41.26] vs. 50.82pmol/L [19.43, 180.9], p=0.007) (**Figure 2.2A**) and HOMA-IR scores (1.25 [0.61, 1.96] vs. 2.52 [0.90, 8.37], p=0.01) (**Figure 2.2C**). There was no significant difference in plasma glucose levels (6.17mmol/L [6.0, 6.6] vs. 6.28mmol/L [6.05, 6.48], p=1.00) (**Figure 2.2B**) between groups. This suggests that, in this study, lean GDM participants have a deficiency in insulin secretion which drives GDM pathology, whereas insulin resistance drives GDM pathology in obese GDM participants.

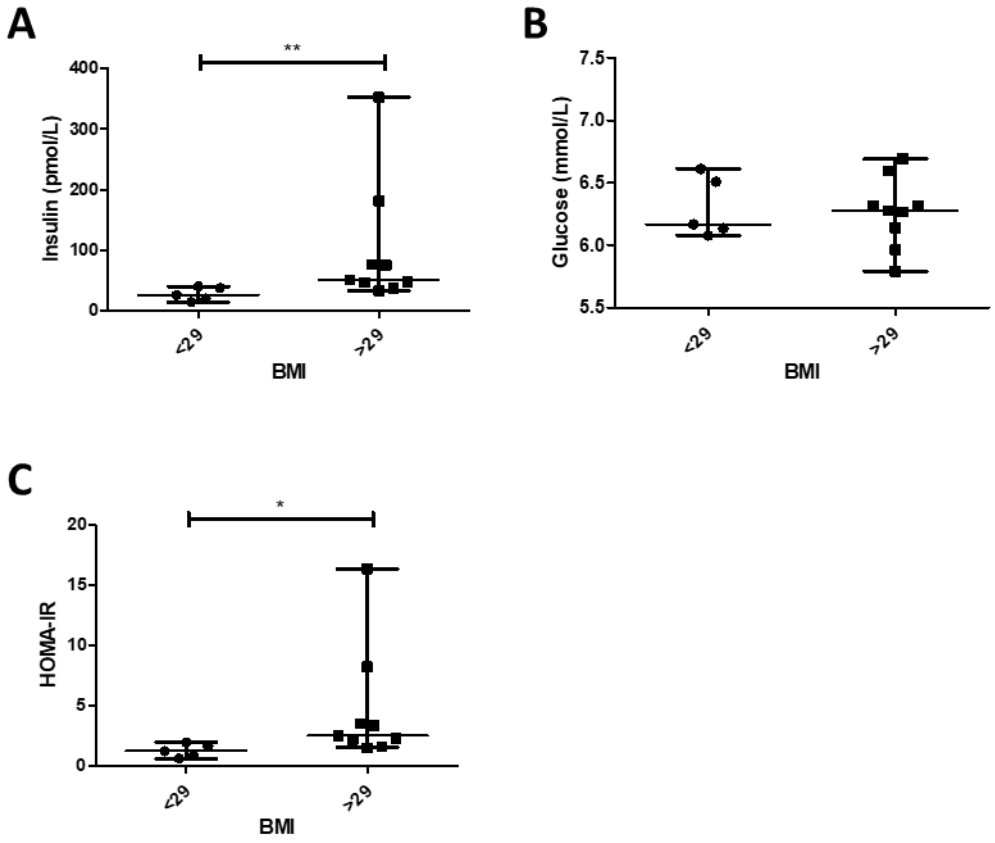


Figure 2.2. Lean GDM participants have significantly lower circulating insulin levels and HOMA-IR scores relative to obese GDM participants. **(A)** Plasma insulin, **(B)** plasma glucose and **(C)** HOMA-IR scores were measured in GDM participants with a BMI <29 (n=5) and GDM participants with a BMI >29 (n=9), excluding GDM participants who were receiving insulin therapy (n=6). The Mann-

Whitney U test was used to compare differences between groups. Significance levels are shown as: * $p<0.05$, ** $p<0.01$.

2.3.4 Serum triglyceride and FFA lipid profile is not altered in GDM

In conditions of insulin resistance and inadequate glucose utilization, adipose tissue lipolysis is enhanced to provide an alternative source of fuel, resulting in dyslipidaemia. Elevated circulating levels of triglycerides and FFAs are frequently reported in women with GDM (311, 321, 322). To identify if dyslipidaemia was evident in GDM in this study, triglyceride and FFA serum levels were measured using biochemical assay kits. There were no significant differences in triglyceride (0.76mM ± 0.67mM vs. 1.12mM ± 1.02mM, p=0.19) (**Figure 2.3A**) or FFA (0.10mM ± 0.06mM vs. 0.11mM ± 0.09mM, p=0.71) (**Figure 2.3C**) levels between NGT and GDM participants. The GDM cohort was then subgrouped by therapeutic intervention. Triglyceride levels were, again, not significantly different between NGT participants when compared to diet-controlled (1.05mM ± 0.99mM), metformin-treated (1.05mM ± 1.18mM) or insulin-treated (1.28mM ± 1.07mM) GDM participants (**Figure 2.3B**). Similarly, FFA levels were not significantly different between NGT participants and diet-controlled (0.16mM ± 0.09mM), metformin-treated (0.09mM ± 0.07mM) or insulin-treated (0.07mM ± 0.09mM) GDM participants (**Figure 2.3D**).

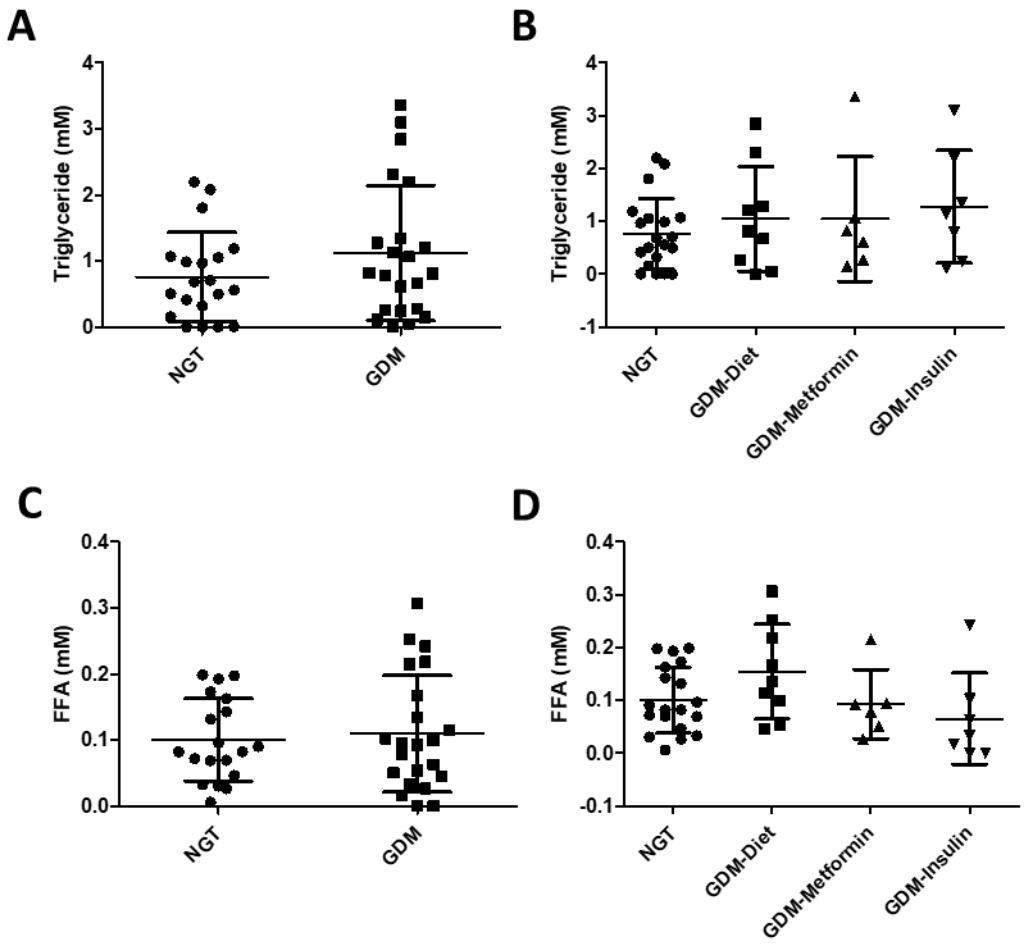


Figure 2.3. Circulating levels of triglycerides and FFAs are not significantly different in GDM. Triglyceride and FFA levels were measured in fasting serum samples from NGT (n=20) and GDM (n=20) participants. Circulating levels of **(A)** triglycerides (mM) and **(B)** subgrouped by therapeutic intervention in GDM. Circulating levels of **(C)** FFA levels (mM) and **(D)** subgrouped by therapeutic intervention in GDM. Student's t-test was used to compare the means of variables between dichotomous groups. One-way ANOVA was used to compare levels between intervention groups. Grubb's test was used to remove statistical outliers from figure C and D.

2.3.5 Circulating levels of IGF and PAPPA are not significantly different in GDM

IGF-1 is a hormone which promotes tissue development and is also an important signalling molecule for promoting insulin sensitivity. IGF-1 can bind to hybrid insulin-IGF-1 receptors as well as insulin receptors to promote glucose metabolism (323). The bioavailability of IGF-1 is modulated by PAPPA, a highly specific metalloproteinase, through proteolysis of IGFBPs. In this study, there were no significant differences in circulating plasma levels of IGF-1 (51.27ng/ml ± 20.62ng/ml vs. 58.4ng/ml ± 9.55ng/ml, p=0.17) (**Figure 2.4A**) or serum PAPPA (148.0ng/ml ± 48.25ng/ml vs. 156.5ng/ml ± 55.31ng/ml, p=0.61) (**Figure 2.4B**) in NGT compared to GDM participants respectively.

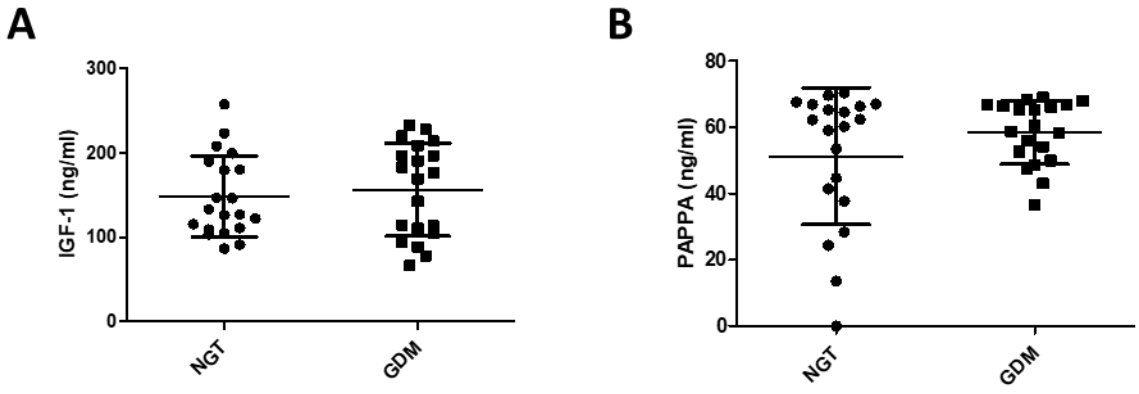


Figure 2.4. Circulating levels of IGF-1 and PAPPA are not altered in GDM. **(A)** Plasma IGF-1 **(**ng/ml) and **(B)** serum PAPPA levels (ng/ml) in NGT and GDM participants. Student's t-test was used to compare the means of variables between groups.

2.3.6 Adiponectin is significantly reduced in maternal circulation in GDM

Dysregulation of circulating adipokine levels have been associated with insulin sensitivity in T2DM patients (324, 325). Four adipokines (leptin, adiponectin, resistin and adipsin) were measured in fasting plasma samples from both study groups. Circulating plasma levels of adiponectin were significantly lower in GDM versus NGT participants (37.85µg/ml ± 20.22µg/ml vs. 49.7µg/ml ± 10.85µg/ml, p=0.033) (**Figure 2.5A**). There were no significant differences in the circulating levels of leptin (22.07ng/ml ± 14.12ng/ml vs. 28.93ng/ml ± 17.31ng/ml, p=0.187) (**Figure 2.5B**), adipsin (424.92ng/ml ± 53.78ng/ml vs. 463.39ng/ml ± 76.4ng/ml, p=0.073) (**Figure 2.5C**) or resistin (2.77ng/ml ± 1.33ng/ml vs. 2.62ng/ml ± 0.98ng/ml, p=0.700) (**Figure 2.5D**) in NGT participants compared to GDM participants.

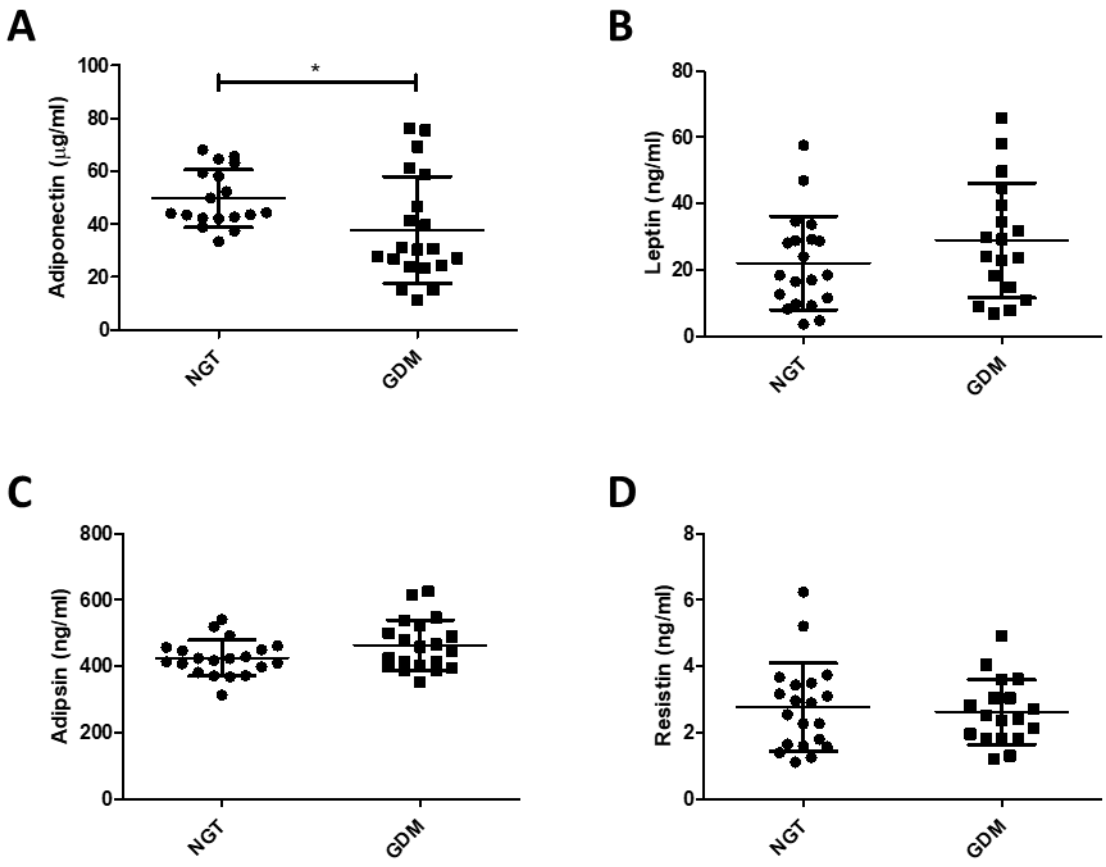


Figure 2.5. GDM participants have significantly lower circulating levels of adiponectin. Adipokine levels were measured in fasting plasma samples from NGT (n=20) and GDM (n=20) participants. Circulating levels of **(A)** adiponectin, **(B)** leptin, **(C)** adipsin and **(D)** resistin. Student's t-test was used to compare the means of variables between groups. Grubb's test was used to remove statistical outliers from figure A, B and D. Significance levels are shown as: * $p<0.05$.

2.3.7 Circulating cf-mtDNA, a marker of mitochondrial dysfunction, is significantly increased in GDM

Cf-mtDNA, a marker of mitochondrial dysfunction, has been associated with impaired insulin secretion and glucose intolerance through decreased β-oxidation and ATP production and increased ROS production (326, 327). As such, cf-mtDNA was explored in both study groups. Circulating copy numbers of cf-mtDNA were

quantified in participant plasma and there was a significant increase in cf-mtDNA in GDM compared to NGT (109233 copies/µl ± 111578 copies/µl vs. 16657 copies/µl ± 18740 copies/µl, p=0.002) (**Figure 2.6A**).

Cf-mtDNA copy number did not significantly correlate with maternal age in either GDM (r_s=-0.23, n=19, p=0.33) (**Figure 2.6B**) or NGT participants (r_s=0.22, n=17, p=0.39) (**Figure 2.6C**). Additionally, cf-mtDNA copy number did not significantly correlate with maternal BMI in GDM (r_s=-0.27, n=17, p=0.30) (**Figure 2.6D**) or NGT participants (r_s=-0.21, n=16, p=0.44) (**Figure 2.6E**). Next, we examined the relationship between insulin resistance (HOMA-IR) and increased circulating cf-mtDNA copy numbers in GDM participants. There was also no significant correlation between GDM participant HOMA-IR and plasma mtDNA copies/µl (r_s=0.24, n=16, p=0.37) (**Figure 2.6F**).

Cell free mtDNA has been shown to induce inflammation in part by binding and activating the endosomal TLR9 receptor, leading to an up-regulation of pro-inflammatory transcription factors and increased release of pro-inflammatory cytokines (328). In the next experiment, HEK-TLR9 cells were incubated with 5% plasma from both study groups. There was no evidence of increased activation of the TLR9 receptor between NGT and GDM participants following treatment with 5% plasma (0.188 ± 0.069 vs. 0.178 ± 0.056, p=0.63) (**Figure 2.6G**).

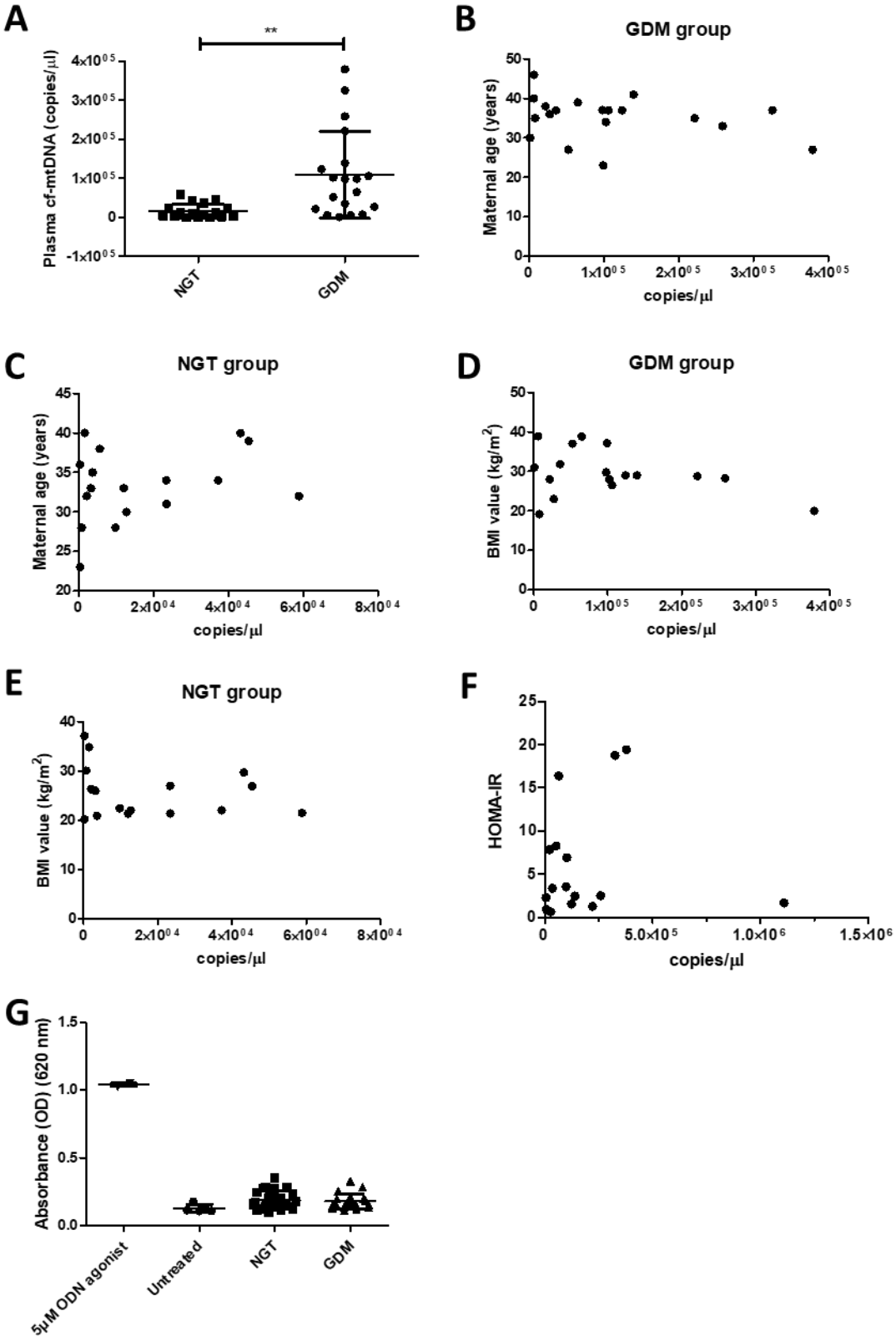


Figure 2.6. Increased circulating cf-mtDNA in GDM. Cf-mtDNA copy number was quantified by real-time PCR in plasma of NGT (n=20) and GDM (n=20)

participants. **(A)** Relative circulating levels of cf-mtDNA were measured as copies/µl. Cf-mtDNA levels did not significantly correlate with **(B)** maternal age in GDM participants, **(C)** maternal age in NGT participants, **(D)** BMI in GDM participants or **(E)** BMI in NGT participants. **(F)** Correlation of HOMA-IR score to circulating levels of cf-mtDNA in GDM participants. **(G)** 5% plasma-induced TLR9 activation was measured in NGT (n=20) versus GDM (n=20) samples. Student's t-test was used to compare the mean of cf-mtDNA copy numbers and TLR9 activation between groups. Spearman's rank correlation coefficient was used to examine correlation between cf-mtDNA copy numbers and participant characteristics. Grubb's test was used to remove statistical outliers from figure A and G. Significance levels are shown as: ** $p<0.01$.

2.3.8 No change in the phenotype of circulating monocyte/macrophage populations in GDM

As monocyte/macrophages express insulin receptors, hyperinsulinaemia can instigate metabolic dysregulation by modifying both pro-inflammatory and anti-inflammatory responses in these cells, which can further affect insulin resistance (329). To examine the potential contribution of circulating monocyte/macrophage populations to systemic insulin resistance, circulating levels of $CD14^{+}CD86^{+}CD206^{-}$ cells (M1-like) and $CD14^{+}CD86^{+}CD206^{+}$ cells (M2-like) were investigated by flow cytometry in NGT and GDM participants. Cell population gating was performed as described in section 2.2.5. (**Figure 2.7A**).

There were no significant differences in cell proportions of $CD14^{+}CD86^{+}CD206^{-}$ cells (97.35% [94.47, 98.39] vs. 98.05% [93.05, 99.5], p=0.45) (**Figure 2.7B**) or $CD14^{+}CD86^{+}CD206^{+}$ cells (2.17% [1.3, 2.87] vs. 1.26% [0.48, 3.49], p=0.89) (**Figure 2.7C**) between NGT and GDM participants. The M1/M2 ratio was equally

not significantly different between NGT and GDM participants (42.03 [26.09, 60.65] vs. 52.18 [18.92, 90.22], p=0.68) (**Figure 2.7D**).

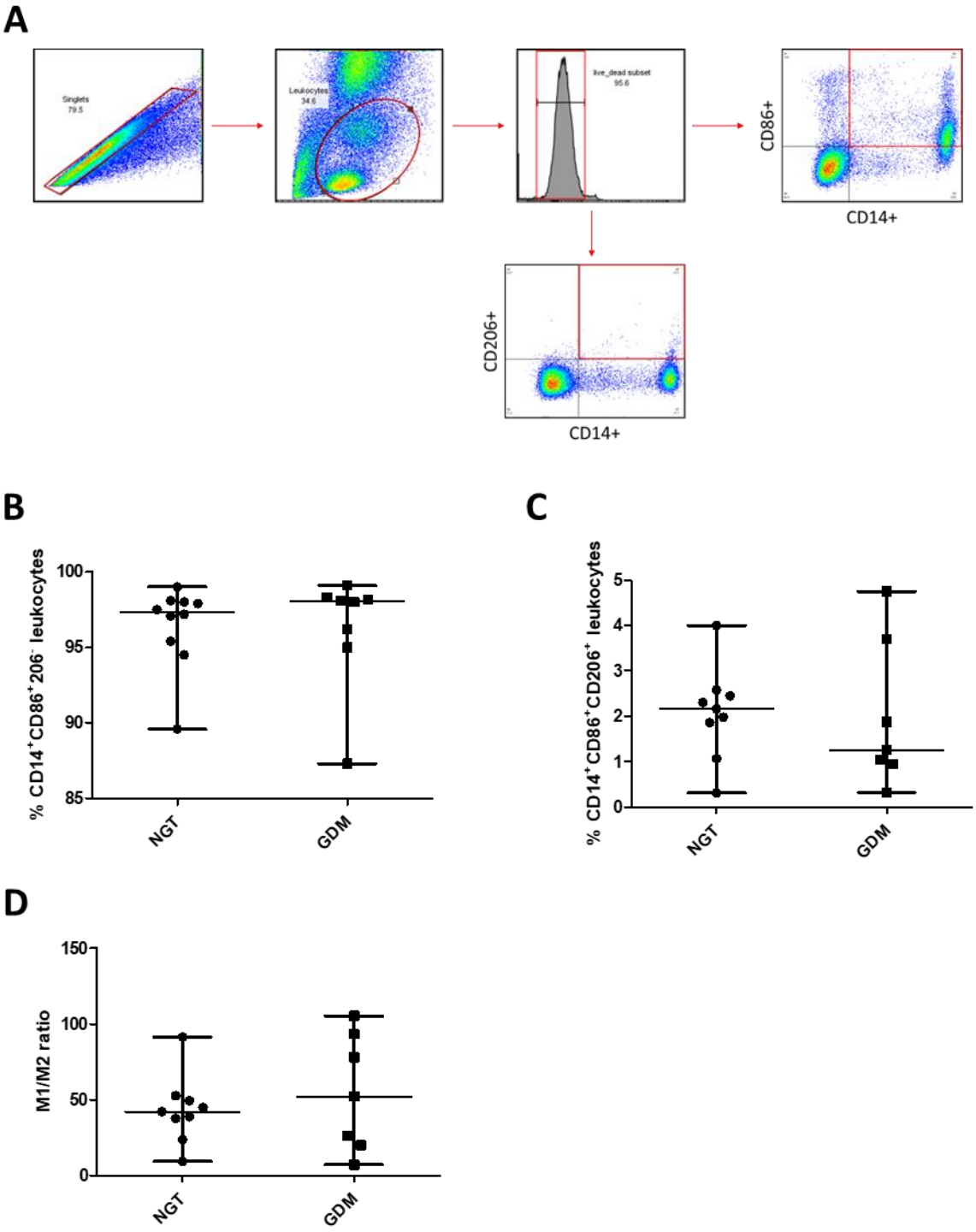


Figure 2.7. Phenotype of circulating monocyte/macrophage populations in GDM. Monocyte/macrophage cell phenotype was characterised in fasting blood

samples from NGT (n=10) and GDM (n=8) participants. **(A)** Representative graphs are shown for $CD14^+CD86^+CD206^-$/$CD14^+CD86^+CD206^+$ flow cytometry gating strategies. Cell proportions, as a percentage of total $CD14^+$ cell count, are shown for **(B)** $CD14^+CD86^+CD206^-$ cells (M1-like) and **(C)** $CD14^+CD86^+CD206^+$ cells (M2-like). **(D)** M1/M2 ratio of cell populations for NGT and GDM participants. The Mann-Whitney U test was used to compare cell proportion differences between groups. Grubb's test was used to remove statistical outliers from figure C and D.

2.3.9 Mitochondrial superoxide production by circulating $CD14^+$ cells is not significantly altered in GDM

Given there was an increase in circulating cf-mtDNA in GDM participants, we wanted to explore if circulating immune cells contributed to this phenotype of mitochondrial dysfunction in GDM. Monocytes and macrophages are the predominant instigators of meta-inflammatory signalling pathways, mediated in part by the NLRP3 inflammasome cascade, which itself may be triggered by mitochondrial superoxide (330). To investigate if circulating M1-like and M2-like monocyte/macrophage populations identified in Figure 2.7 increase mitochondrial ROS in GDM, mitochondrial-specific superoxide production was measured. The total monocyte/macrophage ($CD14^+$ cells) population and stratified into M1-like monocyte/macrophage ($CD14^+CD86^+CD206^-$) and M2-like monocyte/macrophage ($CD14^+CD86^+CD206^+$) populations, as identified in Figure 2.7A, were analysed for their production of mitochondria-specific superoxide (defined as $MitoTracker^+MitoSOX^+$) (**Figure 2.8A**).

There was no significant difference in total monocyte/macrophage production of mitochondrial ROS ($CD14^+MitoTracker^+MitoSOX^+$) (19.55% [11.36, 31.92] vs. 26.95% [18.35, 39.47], p=0.33) (**Figure 2.8B**) between NGT and GDM participants.

Additionally, when stratifying monocyte/macrophage populations into M1-like $CD14^{+}CD86^{+}CD206^{-}MitoTracker^{+}MitoSOX^{+}$ cells (19.35% [11.82, 31.85] vs. 24.95% [17.57, 37.98], p=0.4) (**Figure 2.8C**) and M2-like $CD14^{+}CD86^{+}CD206^{+}MitoTracker^{+}MitoSOX^{+}$ cells (33.85% [16.08, 43.24] vs. 36.55% [21.62, 66.48], p=0.46) (**Figure 2.8D**), there was no significant difference in mitochondrial ROS production between NGT and GDM participants.

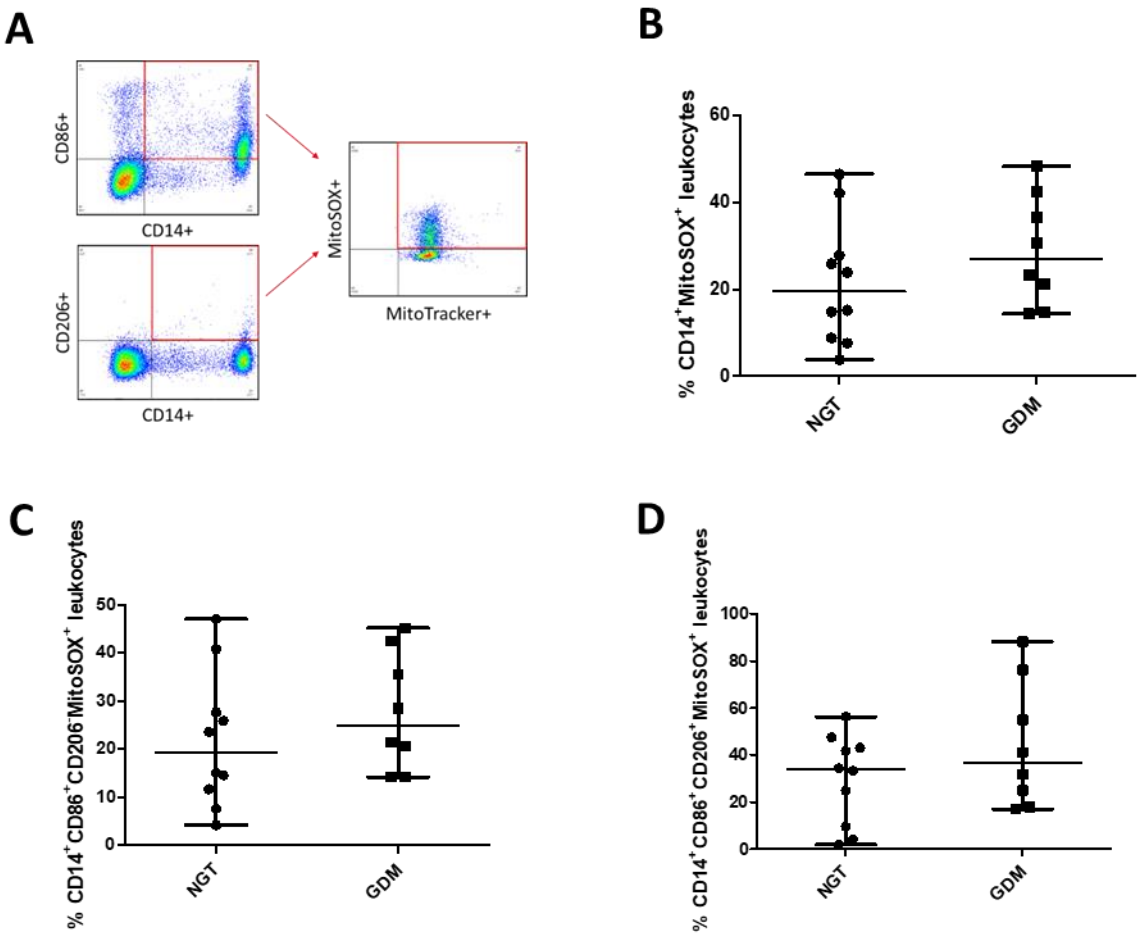


Figure 2.8. Mitochondrial-specific superoxide production in circulating monocyte/macrophage populations in GDM. Mitochondrial superoxide production was measured in circulating $CD14^{+}$ cell populations in NGT (n=10) and GDM (n=8). **(A)** Representative graphs are shown for $MitoTracker^{+}MitoSOX^{+}$ flow cytometry gating strategies of M1-like and M2-like monocyte/macrophage populations.

Population frequencies are shown for **(B)** $CD14^+MitoSOX^+$ cells, **(C)** M1-like $CD14^+CD86^+CD206^-MitoSOX^+$ cells and **(D)** M2-like $CD14^+CD86^+CD206^+MitoSOX^+$ cells. The Mann-Whitney U test was used to compare cell proportion differences between groups.

2.3.10 Significant increase in circulating inflammatory mediators in GDM

Elevated levels of circulating inflammatory cytokines promote inflammation, endothelial dysfunction and insulin resistance (209). A recent systematic review of cytokine production in GDM by Perez Gomes et al. found significant variation in findings, with no significant conclusions (212). To quantifycytokine dysregulation in term GDM physiopathology, inclusive of strict participant exclusion criteria and explicitly defined sample collection and analysis methods, the production of 13 key cytokines were measured in the fasting serum samples from NGT (n=20) and GDM (n=20) participants (**Table 2.2**). Circulating levels of four pro-inflammatory cytokines were significantly increased in GDM participants, including IL-6, IL-17A, IL-12p70 and MCP-1.

Table 2.2. Circulating levels of pro-inflammatory cytokines in NGT and GDM participants

Cytokine	**NGT (Mean ± SD)(pg/ml)**	**GDM (Mean ± SD)(pg/ml)**	**P value**
IL-6	7.03 ± 3.09	13.46 ± 7.47	0.003
IL-17A	1.32 ± 1.13	1.98 ± 1.36	0.04
IL-12p70	3.3 ± 1.83	6.29 ± 5.21	0.05
MCP-1	277.1 ± 162.4	441 ± 253.9	0.03
TNF-α	9.49 ± 8.13	5.12 ± 1.92	0.18

IFN-α2	4.92 ± 2.67	5.06 ± 2.44	0.79
IL-23	14.35 ± 12.63	12.68 ± 9.6	0.87
IL-10	10.33 ± 6.73	19.65 ± 23.41	0.07
IFN-ɣ	5.74 ± 5.12	4.94 ± 4.53	0.56
IL-8	85.68 ± 81.54	76.65 ± 77.28	0.93
IL-33	67.13 ± 33.4	99.11 ± 66.89	0.25
IL-1β	29.0 ± 25.7	21.8 ± 16.0	0.33
IL-18	978.7 ± 514.5	847.6 ± 407.4	0.39

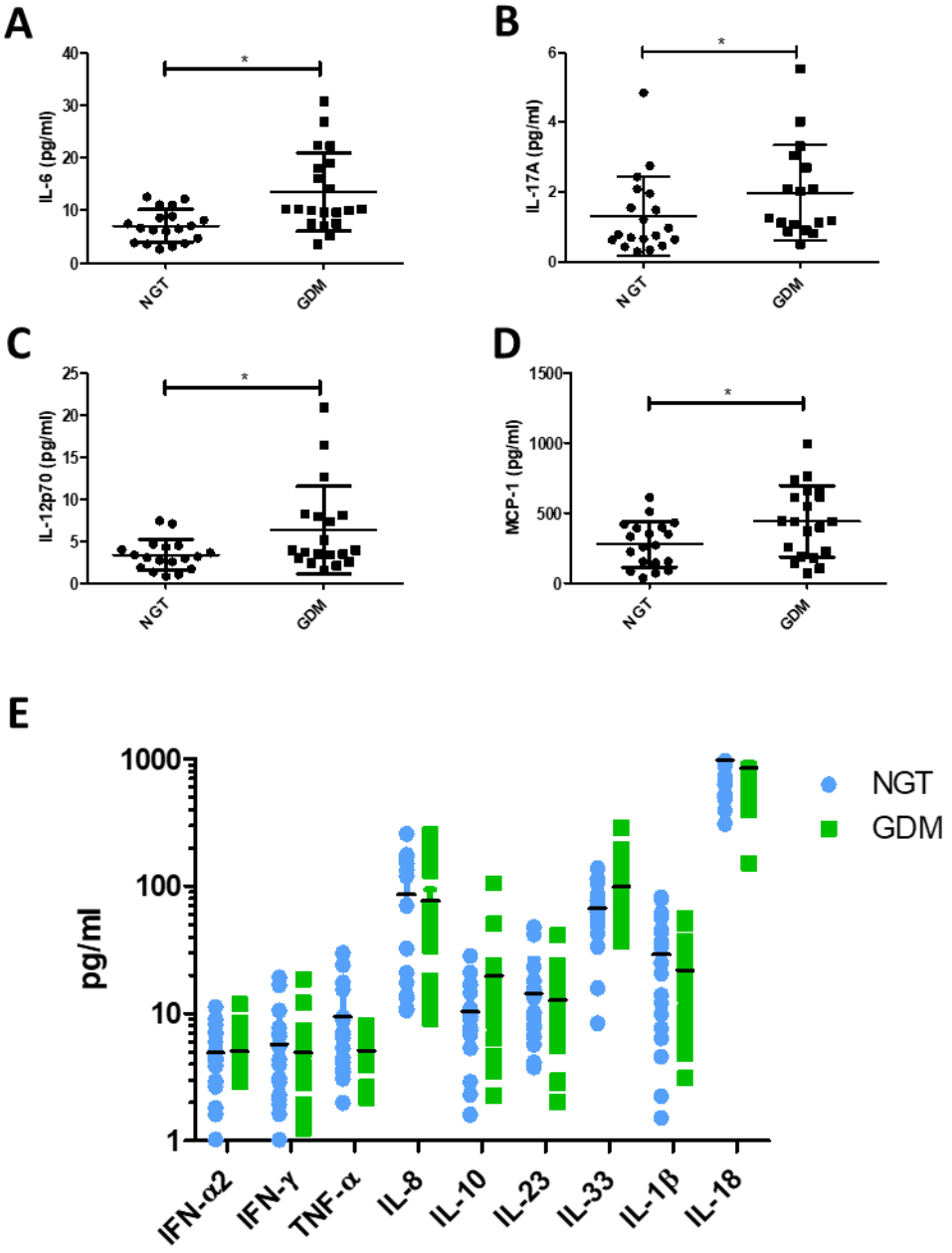


Figure 2.9. Increase in pro-inflammatory cytokines in the maternal circulation in GDM. Serum concentration of 13 key cytokines were quantified in NGT (n=20) and GDM (n=20) participants. Circulating levels of **(A)** IL-6, **(B)** IL-17A, **(C)** IL-12p70, **(D)** MCP-1, **(E)** IFN-α2, IFN-ɣ, TNF-α, IL-8, IL-10, IL-23, IL-33, IL-1β and IL-18. Student's t-test was used to compare the means of variables between groups. Grubb's test was used to remove statistical outliers from figure B, C, D and E. The

Benjamini–Hochberg method with a false discovery rate of 20% was used for multiple hypothesis testing.Corrected significance levels are shown as: * $p<0.05$.

2.4 Discussion

The main objective of this chapter was to characterise the metabolic, mitochondrial and inflammatory mediators in the maternal circulation which may adversely affect maternal insulin sensitivity in GDM. Nulliparous women at term pregnancy from two groups, those diagnosed with GDM during their pregnancy and those who had a healthy, NGT pregnancy, were recruited. In our GDM participants, we found significant evidence of systemic insulin resistance with lower levels of adiponectin, elevated quantities of cf-mtDNA and increased abundance of circulating pro-inflammatory cytokines, including IL-6, IL-17A, IL-12p70 and MCP-1.

Firstly, examination of a range of clinical characteristics of participants in this study established that women diagnosed with GDM had a significantly higher BMI relative to NGT participants. Given the global increase in obesity, which is equally evident in the pregnant population, this study confirms the importance of BMI as a risk factor for GDM development and reinforces the pathogenic role of obesity and concurrent metabolic dysfunction as potent instigators of hyperglycaemia in pregnancy. GDM participants in this study had an overall significant increase in systemic insulin resistance as determined using the HOMA-IR score, a useful indirect measure of insulin resistance that is calculated from fasting blood glucose and insulin levels. A HOMA-IR score of >3.63 is a strong indicator of pathological insulin resistance and a significant risk factor for future T2DM (331). As the mean HOMA-IR score of GDM participants was 5.45 vs 1.73 in NGT participants, we have shown evidence of deleterious insulin resistance in our term GDM cohort. This hyperinsulinsemic state has been reported in other studies of GDM pregnancies (332). However, stratifying GDM participants by maternal BMI identified that lean GDM participants (BMI <29) presented with a hypoinsulinaemic phenotype, relative to obese (BMI >29)

GDM participants. In this lean cohort, diabetic pathology appears to be driven by insulin deficiency rather than insulin resistance. Similar heterogeneity in GDM phenotype has been established previously, suggesting that a number of diverse pathways promote hyperglycaemia in GDM pathology (320). Stratification of GDM participants, based on clinical intervention, established that insulin resistance was increased in women who required pharmacological intervention but not in women whose hyperglycaemia could be managed through lifestyle modification alone. These findings likely portray an association between the severity of insulin resistance in GDM, as characterised by HOMA-IR score, and the need for pharmacological management. Those women who were diagnosed with GDM but presented with only mild impairment in glucose metabolism were sufficiently treated with lifestyle modification alone. Those with more severe impairment in glucose metabolism required either metformin or insulin therapy to control glycaemic levels.

In our GDM cohort, HOMA-IR scores significantly correlated with maternal BMI suggesting that increased maternal BMI may, at least partly, account for diminished insulin sensitivity in GDM. This is unsurprising as previous research has established that increased BMI is a risk factor for insulin resistance, largely mediated by adipocyte hypertrophy which impairs glucose metabolism and promotes inflammation (333-335). Dyslipidaemia, a hallmark of increased adiposity and insulin resistance, was assessed in study participants to investigate the role of alterations in maternal lipid profile in GDM. Elevated levels of triglycerides and FFAs have been previously established in patients with GDM and higher triglyceride levels are present in the first trimester, before GDM pathology is evident (311, 321, 322). However, neither triglyceride or FFA serum levels were significantly elevated in GDM versus NGT participants in this study. It is important to note that our

participants were recruited at term, on the day of their elective caesarean section. As such, GDM-diagnosed participants had been diagnosed and treated for approximately 15 weeks before our clinical samples were obtained. Any improvements in insulin resistance with therapeutic intervention may be equally effective in reducing dyslipidaemia and reversing the phenotype which may have been present earlier in pregnancy.

To interrogate potential molecular drivers of established GDM-related insulin resistance, circulating markers of IGF activity were investigated. IGF-1 and IGF-2 are peptide growth factors that are primarily produced by the liver and stimulate the growth and development of a wide range of cell types, including the regulation of adipogenesis in adipose tissue, an organ vital in regulating insulin resistance. IGF-1 and IGF-2 can also bind to insulin receptors, as well as IGF/insulin hybrid receptors, and IGF-1 selectively activates insulin signalling, via the PI3K/AKT pathway, to stimulate glucose metabolism (336). In this study, there was no evidence of differential IGF-1 secretion between NGT and GDM-complicated pregnancies. Circulating levels of PAPPA, a protease which is predominantly produced by the placenta in pregnancy (166) and increases IGF bioavailability via cleavage of IGFBP4 and IGFBP5, was also measured (167). Previous research reported that circulating PAPPA concentrations during pregnancy are inversely correlated with glycaemic control and the odds of developing GDM (170). However, there were no significant differences in plasma PAPPA levels at term in GDM participants.

IGF-1 levels gradually increase over pregnancy and often the first and second trimester concentrations of IGF-1 are used for the prediction of obstetric outcomes such as fetal birth weight and PE (337, 338). Similarly, maternal PAPPA increases dramatically over gestation, from 100 to 10,000 times non-pregnant levels in the first

and third trimester respectively (339). Lower first trimester serum levels of PAPPA have been observed in women who later develop GDM (168, 169). The levels of both IGF-1 and PAPPA in the third trimester may not be clinically relevant for the investigation of obstetric complications which develop earlier in pregnancy, such as GDM.

Due to the metabolic impact of adipose tissue on systemic insulin resistance, the role of adipokines in the pathophysiology of GDM is relatively well defined, with adiponectin and leptin being the major adipokines involved in regulating insulin sensitivity. Examples of established alterations in circulating adipokines in GDM include increased amounts of leptin and chemerin and lower levels of adiponectin, omentin-1 and visfatin (340, 341). Adiponectin acts as an endogenous insulin sensitizer, promoting glucose metabolism while also exhibiting anti-inflammatory and anti-atherogenic properties (342). From our study, there was no significant change in circulating leptin levels but adiponectin levels were significantly lower in GDM compared to NGT participants. This result is in agreement with multiple other studies of GDM (341, 343, 344). Equally, adiponectin levels recorded at 10-14 weeks gestation are inversely related to GDM risk (345). These findings suggest a pathogenic role of depleted systemic adiponectin early in pregnancy which subsequently leads to the development of GDM.

Mitochondrial dysfunction has also been postulated to play a pathogenic role in GDM by acting as a source of ROS and inducing oxidative stress (346). Mitochondrial superoxide production has previously been shown to impair insulin-stimulated glucose uptake and GLUT4 translocation to the plasma membrane of adipocytes and myotubes (347). Increased generation of mitochondrial-specific superoxide and mtDNA are recognised markers of mitochondrial dysfunction.

Circulating cell-free DNA is composed of both nuclear and mtDNA, yet mtDNA is particularly vulnerable to oxidative damage due to a proximity to the electron transport chain and lack of protective histones (348). In a study of plasma samples taken at 20 weeks' gestation, our group previously found that women with GDM have higher circulating cf-mtDNA copy numbers relative to NGT, with BMI presenting as a likely physiological mediator (194). In this study, mitochondrial dysfunction was initially determined by measuring cf-mtDNA in maternal plasma taken at term from GDM and NGT participants. Similar to previous work in our group, cf-mtDNA copy numbers were significantly higher in plasma from GDM compared to NGT participants. However, there was no correlation present in either study group between mtDNA copy numbers and either maternal BMI or maternal age, suggesting that BMI or age alone do not account for the exaggerated release of circulating mtDNA present in GDM participants. Additionally, using specific markers for measuring live mitochondria (MitoTracker) and superoxide-producing mitochondria (MitoSOX), we investigated if circulating monocyte/macrophage populations produced mitochondrial-specific ROS which may contribute to the mitochondrial dysfunction evident in GDM. However, there was no significant differences in mitochondrial superoxide production in these populations between our study groups.

While our results suggest a potential role for mitochondrial dysfunction in GDM, circulating macrophages do not appear to be major contributors, which suggests other potential maternal cellular sources. It is recognised that circulating cf-DNA in maternal plasma is mostly of maternal origin (haematopoietic and stromal derived), depending on the gestational week, while approximately 5-20% of circulating cf-DNA is derived from fetal/placental cells (191), which may equally account for our

results. In obese T2DM patients, circulating mtDNA levels positively correlated with individual insulin resistance (349). Although no such correlation was seen in our participants, there is strong evidence that mitochondrial function has an important role to play in maintaining insulin sensitivity, through the regulation of β-oxidation, ATP production and ROS generation (350, 351).

In addition to its established role as a marker of mitochondrial dysfunction, cf-mtDNA is equally classified as a DAMP which can induce inflammation. This mtDNA-induced inflammation is mediated in part by binding and activation of the endosomal TLR9 receptor (328). Given the increase in cf-mtDNA in GDM in our study, its potential to stimulate inflammation was explored *in vitro* using a HEK-TLR9 cell assay treated with maternal plasma. However, there was no significant difference in TLR9 activation between NGT and GDM plasma samples. One possible explanation for this result may be the acute exposure conditions, as cells were incubated for 24 hours, which may not be sufficient to replicate the chronic biological conditions in the maternal system.

Although circulating M1/M2 phenotypes have not been examined to date in GDM, there is evidence of increased frequency and activation of circulating $CD14^+$ monocyte populations in GDM, in addition to a decreased intermediate monocyte count (238, 239). To identify if these innate immune cells were involved in the pathophysiology in GDM, the phenotype of circulating monocyte/macrophage populations were characterised. The abundance of either circulating $CD14^+CD86^+CD206^-$ (M1-like) or $CD14^+CD86^+CD206^+$ (M2-like) monocyte/macrophage cells were not significantly different between study groups. Despite the lack of alterations in the circulating $CD14^+$ cell populations in GDM, these results only represent a snapshot of systemic inflammation. To further

interrogate a possible pathogenic role for inflammation in GDM, a multiplex panel of established cytokine mediators were measured. After statistical correction for multiple testing, the concentrations of IL-6, IL-17A, IL-12p70 and MCP-1 were all significantly elevated in GDM relative to NGT participants. Furthermore, the mean increase (%) of these cytokines in GDM fasting blood, relative to NGT, was substantial (IL-6 – 91%, IL-17A – 50%, IL-12p70 – 91% and MCP-1 – 59%). These findings demonstrate that there is increased production of monocyte/macrophage-specific pro-inflammatory cytokines (IL-12p70 and MCP-1) in GDM. This outcome may also indicate that alterations in other immune cell populations may be more relevant to GDM pathophysiology. For example, various other circulating populations are affected in GDM, such as increased T helper type 1 (Th1) and T helper type 17 (Th17) cell counts (204), increased CD8+ T cells (237, 247) and increased neutrophil count and activation (226-232). In support of this, we have shown in this study that T-cell related cytokines IL-6 and IL-17 are significantly increased in GDM compared to NGT participants.

IL-6 is produced by a variety of cell types, including dendritic cells, macrophages and T-cells, and is widely expressed in the gestational tissues. During pregnancy, IL-6 is involved in the regulation of placental development and immune tolerance to pregnancy (352). Elevated maternal serum IL-6 levels have previously been found at 24-28 weeks gestation in women with GDM, highlighting a chronic pro-inflammatory state (353), and have also been linked to the development of insulin resistance, potentially by inducing the expression of SOCS-3 which inhibits insulin signalling (354). This pathway represents a potential mechanistic link between IL-6 levels and insulin resistance in our term GDM participants. IL-6 equally promotes the differentiation of $CD4^+$ T cells into Th17 cells, the primary producer of IL-17A,

a key cytokine in promoting chronic inflammation which was elevated in our GDM participants (355). IL-17A can induce further production of TNF-α, IL-1β, and IL-6 in macrophages, contributing to a deleterious feedback cycle of chronic inflammatory stimulus (356). Unlike IL-6 however, IL-17A does not appear to impair insulin sensitivity or adipogenesis, suggesting it is predominantly responsible for the propagation of an inflammatory state (357). Elevated IL-17A levels have been associated with other obstetric complications, including prenatal distress and PE (358, 359). Our findings suggest that this exaggerated IL-6/IL17A feedback loop is driving inflammation in GDM, while also amplifying maternal insulin resistance.

IL-12p70 is a pro-inflammatory cytokine produced mainly by macrophages and DCs and is believed to play a substantial role in the induction of NK cells. IL-12p70 was previously found to be elevated in individuals with T2DM (360, 361) and elevated circulating levels have been linked to elevated HOMA-IR scores and reduced insulin sensitivity (209, 362). Findings in individuals with T2DM suggest that elevated IL-12 may be induced as a consequence of peripheral insulin resistance (361). Therefore, successful management or prevention of exaggerated maternal insulin resistance in GDM may prevent an increase of circulating mediators such as IL-12p70 which further propagate maternal cardiometabolic risk (363).

Increased circulating levels of MCP-1 in women with GDM in this study is in agreement with previous research (364, 365). In GDM, plasma MCP-1 concentrations are predicted by plasma glucose levels (364), which suggests that MCP-1 is an insulin-responsive gene. Both *in vitro* studies in 3T3-L1 adipocytes and *in vivo* studies of insulin-resistant obese mice have shown that insulin induces substantial MCP-1 expression and secretion, with WAT being the predominant source. Elevated MCP-1 may further aggravate insulin sensitivity by negatively

affecting insulin-stimulated glucose uptake and the expression of several adipogenic genes (366).

The strengths of our study include employing a strict exclusion criteria which included only nulliparous women, without any significant co-morbidities or physiological adaptive changes from previous pregnancies. Identical methods of sample collection and processing were used for each participant. However, some limitations do exist in this study. Firstly, our study is predominantly cross-sectional in nature, with recruitment on the day of elective caesarean section, eliminating the possibility to derive causation from our findings in the development of GDM. Our study was also a single site study with a relatively small sample size, limiting the scientific validity of the results required to influence widespread clinical practical. This research was carried out in Cork, Ireland, resulting in a largely white Irish participant group, thereby limiting the extrapolation of our findings to other ethnicities. This is of particular relevance to GDM as race-ethnicity and country of birth have a significant impact on the prevalence this condition (367). Overall, our findings provide evidence of systemic insulin resistance, maternal mitochondrial dysfunction and inflammation in women with GDM that warrant further examination in future longitudinal studies to fully decipher their pathogenic role.

2.5 Conclusion

GDM pathophysiology is a complex intersection of multi-organ communication which results in reduced insulin sensitivity, hyperglycaemia and concurrent systemic metabolic dysfunction. However, no causative pathological pathways, which precede the clinical diagnosis of GDM, have been conclusively characterised or therapeutically targeted. This study has identified multiple possible mechanisms of meta-inflammation and exaggerated insulin resistance in GDM. Firstly, two distinct phenotypes of GDM pathology were observed in this study; lean GDM participants with a hyperglycemic but hypoinsulinemic phenotype and obese GDM participants with significant insulin resistance and hyperinsulinemia. Cf-mtDNA levels were significantly increased in GDM, proposing a pathogenic role for mitochondrial dysfunction. Despite the lack of alteration in monocyte/macrophage populations between study groups, there was significant evidence that GDM is associated with a chronic inflammatory state, with increased pro-inflammatory cytokine levels of IL-6, IL-17A, IL-12p70 and MCP-1 and lower adiponectin levels, an anti-inflammatory, insulin-sensitizing adipokine. Prospective research, with sample collection at multiple timepoints throughout gestation and targeted phenotyping of the pathways highlighted in our findings, may uncover alternative therapeutic strategies.

Chapter 3

Adipocyte Hypoplasia and Impaired Insulin Signalling in Gestational Diabetes Mellitus

Following on from the described evidence of peripheral insulin resistance and systemic meta-inflammation in GDM pathology in Chapter 2, this chapter will investigate the pathophysiological role of omental VAT in regulating insulin sensitivity in GDM. Our hypothesis is that visceral adipocyte dysfunction impairs glucose metabolism, resulting in exaggerated insulin resistance and hyperinsulinaemia in GDM. Specifically, this chapter will examine mediators of adipocyte morphology and adipogenesis, in addition to assessing the insulin signalling pathway. Mediators of meta-inflammation will also be examined to elucidate their role in altering adipocyte signal transduction and glucose uptake in GDM.

Abstract

Background

Due it to the established pathogenic role of adipose tissue in the etiology of insulin resistance and T2DM, we propose that adipose tissue equally plays an integral role in the pathophysiology of GDM. AT dysfunction is an established cause of metabolic syndrome and systemic insulin resistance. Various mechanisms are responsible for this, including impaired fatty acid metabolism, inflammatory signalling, altered insulin signalling and diminished glucose uptake capacity. This chapter characterises the morphology of omental VAT, in order to elucidate if disruption of adipocyte function impairs insulin signalling and leads to systemic insulin resistance in GDM. Secondly, we investigated if mediators of immune-endocrine signalling in adipocytes also contribute to insulin resistance in GDM.

Methods

Visceral omental adipose tissue was collected post-elective caesarean section from nulliparous women with normal glucose tolerant pregnancies (n=20) and women diagnosed with GDM (n=20) in pregnancy. Immunohistochemical analysis was used to characterise adipocyte morphology and tissue fibrosis. Immunoblotting was used to quantify markers of adipocyte function including IGFBP-3, IGFBP-4, and members of the insulin signalling pathway, IRS-1, IRS-2, phospho-IRS-1(ser312), phospho-IRS-1(ser318), phospho-IRS-1(ser612), PDK1 and PI3K. Omental production of adipokines (adiponectin, adipsin, leptin and resistin) and inflammatory mediators (IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-23, IL-33) were measured in VAT explant cultures by multiplex ELISA. Characterisation of infiltrating M1-like ($CD14^{+}CD86^{+}CD206^{-}$) and M2-like

($CD14^+CD86^+CD206^+$) macrophage populations in VAT were determined by flow cytometry. Infiltrating macrophage production of mitochondrial-specific-ROS was measured by flow cytometry using $MitoTracker^+MitoSOX^+$ fluorescent staining.

Results

There was evidence of significant adipocyte hypoplasia (p=0.004) in VAT from GDM participants relative to NGT participants. Adipocyte hypoplasia significantly correlated with increased systemic insulin resistance, expressed as HOMA-IR score (p=0.03) in GDM. There was a significant reduction in the protein expression of IGFBP-4 (p=0.016) in VAT in GDM without a change in PAPPA secretion (p=0.39). There was also a significant impairment of insulin signalling in GDM, with increased phosphorylation of IRS-1 at the ser312 residue (p=0.04) and a reduction in total IRS-2 expression (p=0.008). There were no significant changes in the abundance of infiltrating macrophage M1-like/M2-like populations in VAT or in their production of mitochondrial superoxide between study groups. There was equally no significant differences in adipokine and cytokine profiles in VAT explants between NGT and GDM participants.

Conclusion

These findings suggest that AT dysfunction may contribute to systemic insulin resistance in GDM. Specifically, reduced adipogenesis may limit omental VAT metabolic capacity in women who develop GDM, resulting in hindered insulin activity and glucose metabolism.

3.1 Introduction

Adipose tissue performs a central role in regulating systemic glucose homeostasis via two primary mechanisms, firstly efficient storage of lipids and secondly modulating adipokine secretion. These mechanisms are pivotal in maintaining numerous metabolic signalling cascades which prevent glucotoxicity and lipotoxic-mediated effects (368).

Adipocyte dysfunction is a prominent driver of insulin resistance, with various mechanisms responsible for this pathological link. Insufficient fat sequestration in adipose tissue depots leads to elevated circulating levels of FFAs and triglycerides, which negatively affect insulin action on glucose metabolism (369). Increased adiposity can affect the ability of adipocytes to function as endocrine cells and secrete appropriate biological mediators. This can lead to imbalances including excessive release of MCP-1 with subsequent macrophage infiltration and meta-inflammation (366), or diminished secretion of adiponectin, resulting in reduced insulin sensitivity (370). Increased macrophage infiltration and activation in AT positively correlates with insulin resistance in adults at risk of T2DM. This association was established by quantification of soluble CD163 (sCD163), a marker of macrophage activation and inflammation (371).

Altered adipocyte morphology may directly regulate metabolic dysfunction. Adipocyte hypertrophy or increased adipocyte enlargement, in part due to excess caloric intake, can result in an insufficient ability to buffer excess dietary fat (372). Hypoplasia, a reduced adipocyte number in part caused by defective preadipocyte differentiation, has also been associated with dysregulated glucose, insulin and lipid profiles in obese female participants (373). An important mediator of normal preadipocyte differentiation is the action of IGF-1, which is regulated locally by

binding to IGFBPs. These binding proteins function to increase the half-life of IGF both locally and in circulation, but may also block potential binding to IGF-1 and insulin receptors (84).

Dysregulation of the intracellular adipocyte insulin signalling pathway can directly hinder glucose uptake. Previous studies in women with GDM have found reduced insulin-stimulated glucose uptake, with decreased IRS-1 expression, decreased tyrosine phosphorylation and increased serine phosphorylation of the IRS-1 protein (68, 71, 72). However, these findings were observed in serum and skeletal muscle samples, with a paucity of evidence available on how this pathway may be affected in VAT. However, a study by Ott et al. characterised a BMI-independent decrease in VAT insulin receptor mRNA and protein expression at term in women with GDM (374).

ROS are another important metabolic regulator of glucose-lipid balance. *In vitro* findings suggest that transient and acute adipocyte exposure to ROS may improve insulin signalling and glucose metabolism, however chronic ROS exposure leads to insulin resistance, through the inhibition of GLUT4 translocation and subsequent glucose uptake (375).

The AT secretome is vital to the maintenance of metabolic balance. VAT has been characterized as a more potent inflammatory tissue compared to SAT, with a larger number of infiltrating immune cells and hormone receptors (77). Adipocyte-derived factors such as MCP-1 and adiponectin directly influence insulin sensitivity and meta-inflammation. In addition, SAT and VAT explant cultures from GDM participants secrete higher levels of leptin, relative to NGT participants (153, 376). Various studies have reported increased IL-8 and chemerin mRNA expression in

VAT in women with GDM, but these have not been translated into elevated protein secretion (340, 377).

Dysregulation of the molecular cues that stimulate the physiological expansion of adipose tissue depots during pregnancy may have a pathogenic role in the development of insulin resistance, yet the mechanisms involved in GDM are yet to be fully elucidated. This will be the first study to characterise omental VAT metabolic function in GDM, by investigating adipocyte morphology, insulin signalling, markers of adipogenesis, adipokines and inflammatory mediators and will correlate these findings with clinical parameters that define GDM. The aim of this chapter was to define if VAT adipocyte dysfunction and meta-inflammation distort insulin signalling and promote the systemic insulin resistant state we have observed in GDM participants.

3.2 Methods

3.2.1 Study design and participant recruitment

GDM-diagnosed (n=30) and healthy, NGT control (n=30) participants were recruited from Cork University Maternity Hospital (CUMH), Ireland between 2019 and 2022 as part of the COMRADES study, a non-interventional cohort study of nulliparous singleton pregnancies with the aim of characterizing mitochondrial dysfunction and meta-inflammation in GDM. The COMRADES study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all the procedures were approved by the Clinical Research Ethics Committee of the Cork Teaching (ECM4 (ff) 04/12/18). Inclusion criteria for all study participants included; women aged between 18-50 years, nulliparous pregnancy, singleton pregnancy as determined by ultrasound scan, opted for an elective caesarean section and had provided written informed consent.

After receiving participant consent, samples were collected from nulliparous, singleton pregnancies undergoing elective pre-labour caesarean section. As per national HSE guidelines, selective screening for GDM was carried out on identified at-risk pregnant women between 24-28 weeks gestation using the 75g oral glucose tolerance test (OGTT). A diagnosis of GDM was confirmed if one of the following values were met or exceeded: fasting blood glucose levels of 5.1mmol/L, blood glucose levels of 10.0mmol/L 1 hour post-OGTT or blood glucose levels of 8.5mmol/L 2 hours post-OGTT. Healthy, NGT control participants were defined as pregnant women who had not been diagnosed with GDM, as per the above guidelines, and did not present with any other diagnosed comorbidities.

Exclusion criteria included women who: had a multiple pregnancy; were multiparous; had already started to labour, who were outside the age range of 18-50

years of age or were having an emergency caesarean section. Women with known pregnancy or other health related conditions namely: pre-pregnancy essential hypertension; known liver or renal diseases; known Human Immunodeficiency Viruses (HIV), Hepatitis B or Hepatitis C positive; known Systemic Lupus Erythematous; known antiphospholipid syndrome and known or suspected fetal anomalies were excluded from the study.

3.2.2 Sample collection

Omentum tissue was obtained during caesarean section and processed within 30 minutes post-surgery. Placental tissue samples were obtained immediately post caesarean section. Before cryopreservation, placental tissue was sectioned from 5 regions of each placenta within 30 minutes post-surgery. Biological tissue samples were stored in our biobank in the CUMH at -80 °C prior to analysis. Additionally, tissue samples were taken for explant culture experiments or fixed in 10% formalin for histological analysis.

3.2.3 Immunohistochemical analysis

Omentum tissue samples for histological investigation were fixed in 10% neutral buffered formalin (NBF) within 30 minutes of surgical biopsy. Fixed samples were dehydrated and embedded in paraffin as per standard laboratory protocols. 7μm tissue sections for each sample were sectioned using a Leica RM2125 RTS microtome and mounted on SuperFrost glass slides (VWR 631-0446). H&E staining was completed using the following protocol: sections were deparaffinized in xylene for 2 minutes (x2) and rehydrated by immersion in ethanol (2 minutes each) using a decreasing gradient concentration (96% - 70% - 50% - 30%), followed by immersion in tap water for 1 minute. Slides were then immersed in haematoxylin stain (3 minutes), washed in tap water (1 minute), differentiated in 1% acid alcohol (30

seconds), washed in tap water (1 minute), rinsed in Scott's tap water (1 minute), washed in tap water (1 minute) following by a 70% alcohol wash (1 min) and finally immersed in eosin stain (3 minutes). After a quick wash in 30% ethanol, sections were dehydrated in ethanol 70% and 100% (2 minutes each) and immersed in xylene for 2 minutes (x2). Finally, slides were coverslipped using CV mounting media (Leica 14046430011).

Brightfield microscopy was used to visualize sections at 20X magnification. All samples were blinded prior to image acquisition and analysis. Images were imported into Image J - Fiji software for analysis. Adipocyte cell counting was performed on five 20X images (one representative images from each of five sections per sample). Cell measurements were completed manually on ten 20X images images (two representative images from each of five sections per sample). Adipocyte area (μm^2) was calculated as the area of the ellipse from the perpendicular maximum (a) and minimum (b) axes using the following equation : Area = a*b*pi (378).

Picro Sirius Red (PSR) staining (Abcam ab150681) was used to quantify the extent of tissue fibrosis in omentum VAT. Sections were deparaffinized and hydrated prior to staining with PSR solution for 1 hour. Slides were then rinsed twice with acetic acid solution (0.5%). Dehydration and mounting steps were completed as per the H&E protocol described above. Finally, collagen fibres (red) in omental VAT were imaged using brightfield microscopy at 20X magnification. Pericellular fibrosis was quantified in ImageJ software by converting the image to grayscale and segmenting the red-stained collagen using thresholding as per an online ImageJ protocol titled "Quantifying Stained Liver Tissue" (https://imagej.nih.gov/ij/docs/examples/stained-sections/index.html).

3.2.4 Protein isolation and immunoblotting

Omental tissue samples (50mg ± 5mg) were incubated in T-PER™ Tissue Protein Extraction Reagent (Thermo Scientific 78510) supplemented with phosphatase inhibitor (Roche, PhosSTOP™) and protease inhibitor (Roche) and homogenized for 3x 20 seconds using a TissueLyser II (Qiagen). Isolated protein (30μg) was separated at 100V by SDS-PAGE on a 7.5% Tris-Glycine polyacrylamide gel and transferred to a methanol activated PVDF membrane (Amersham Hybond Sigma-Aldrich) at 400mA on ice for 2 hours. A protein ladder (Thermo Scientific 26634) was included in the run to confirm protein band kDa size. Membranes were blocked with 5% BSA in 0.1% TBS-T (tris-buffered saline with 0.1% Tween detergent) for 1 hour at room temperature and incubated overnight at 4°C with the following primary antibodies: IGFBP3 1:1000 (R&D Systems AF675), IGFBP4 1:3000 (R&D Systems AF804), IRS1 1:1000 (Cell Signalling #3407), IRS2 1:1000 (Cell Signalling #4502), phospho-IRS1(ser312) 1:1000 (Cell Signalling #2381), phospho-IRS1(ser318) 1:1000 (Cell Signalling #5610), phospho-IRS1(ser612) 1:1000 (Cell Signalling #3203), PDK1 1:1000 (Cell Signalling #3062), PI3K 1:1000 (Cell Signalling #4249) and GAPDH 1:1000 (Cell Signalling #97166).

After overnight incubation, membranes were washed and incubated at room temperature for 1 hour with secondary HRP-linked antibody, either anti-rabbit 1:2000 (Cell Signalling #7074), anti-mouse 1:2000 (Santa Cruz 516102) or anti-goat 1:1000 (R&D Systems HAF019). Membranes were washed in 0.1% TBS-T and developed using Pierce™ ECL Western Blotting Substrate (Thermo Scientific 32209) and LICOR Odyssey image analyser. Densitometry was performed using LICOR Image Studio Lite Version 5.2. All densitometry values were normalised by relative GAPDH expression.

3.2.5 Omental VAT and placental explant culture

Tissue explants were prepared from omental VAT samples collected from both NGT and GDM participants. 100mg ± 10mg of omental tissue were cultured in 12-well plates, containing 2ml of culture media containing Medium 199 1x (Gibco 31150022), FBS (heat-inactivated) 10% (Gibco 16140071), penicillin-streptomycin (10,000 U/mL) 1% (Gibco 15140122), amphotericin B 0.25µg/ml (Gibco 15290018). Tissue explants were also prepared from placental samples collected from both NGT and GDM participants. Following the removal of outer membrane and connective tissue, 200mg ± 20mg of placental tissue (combined from three regions of the placenta) was cultured in 12-well plates, containing 2ml of culture media. Placental explant media consisted of RPMI-1640 1x (Gibco 21875034), FBS (heat-inactivated) 10% (Gibco 16140071), penicillin-streptomycin (10,000 U/mL) 1% (Gibco 15140122), insulin 1µg/ml (SAFC 91077C), hydrocortisone 0.1µg/ml (Sigma H0888), retinol acetate 0.1µg/ml (Sigma R0300000), gentamicin 0.05µg/ml (Sigma G1397). Tissue weight was recorded and used for normalisation of all explant experimental analysis. Supernatants were subsequently stored at -80°C for further analysis.

3.2.6 Immunoassays

A variety of immunoassays were used in the quantification of omental-derived mediators in explant supernatants from GDM (n=10) and NGT (n=10) participants respectively. PAPPA was measured in omental and placental explant culture supernatant with the Pappalysin-1 ELISA kit (Bio-Techne DPPA00). Omental adiponectin, adipsin, leptin and resistin were measured in VAT explant culture supernatant using the LEGENDplex Human Metabolic Panel 1 (BioLegend 740212). A cytokine panel including IL-1β, IL-18, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8,

IL-10, IL-12p70, IL-17A, IL-23, IL-33 were measured in VAT explant culture supernatant using the LEGENDPlex Human Inflammation Panel 1 kit (BioLegend 740809). All assays were performed as per the manufacturer protocols.

3.2.7 Isolation of leukocytes from omental tissue

Omental tissue samples were initially stored in cold tissue preservative buffer which consisted of 154mM NaCl, 1.6mM $CaCl_2$, 10mM HEPES, 250mM sucrose, 5.4mM K_3PO_4, 1.2mM $MgCl_2$ in sterile, distilled water. Tissue samples were washed in PBS to remove excess blood. Omental tissue was then weighed in a clean petri dish and finely minced using a surgical scalpel, removing residual blood clots and connective tissue. Minced tissue was placed in a clean 50ml falcon tube containing 20mL DMEM/F-12 (Gibco 31330038), 10% FBS (heat-inactivated) (Gibco 16140071), 1% penicillin-streptomycin (10,000 U/mL) (Gibco 15140122), 0.2% collagenase type I (Sigma C0130), and 0.02% DNAse I (Sigma D5025). Tissue samples were incubated in a water bath at 37°C for 45 minutes, with agitation every 15 minutes to facilitate enzymatic digestion. Tissue suspensions were then filtered into a clean 50ml falcon tube through one layer of gauze (Steroswab 1905/SF). The tube used for enzymatic digestion was washed with 20ml PBS and the eluate was passed through the same layer of gauze. The filtrate was centrifuged at 300g for 5 minutes and the resultant cell pellet was re-suspended in 5mL PBS.

This mixture was overlaid onto 10mL Histopaque (6.2ml Histopaque 1119 overlaid by 3.8ml Histopaque 1077) in a 50ml falcon tube followed by centrifugation at 700g (room temperature) for 30 minutes, with reduced acceleration and deceleration. Cells at the interface (white cloudy band) were collected with a pasteur pipette and washed in 5ml PBS. This suspension was passed through a 70µm cell strainer (Sigma CLS431751) to remove cells aggregates. The resultant suspension was pelleted by

centrifugation at 300g for 5 minutes. The supernatant was discarded and the cell pellet was stained for flow cytometry analysis.

3.2.8 Flow cytometry analysis

Following isolation of immune cells from omental VAT tissue, single cell suspensions were kept on ice until ready for staining. Approximately $1x10^6$ cells from the single cell suspensions were placed in 5ml round-bottomed tubes (Falcon 352008), centrifuged at 300g for 5 minutes and supernatants were discarded. Cell pellets were incubated with 50µl FC block buffer (10% human serum (heat-inactivated) (Sigma S1-M) in 1x PBS (without Ca^{2+}/Mg^{2+})) (Sigma P4417) containing Fixable Viability Dye eFluor™ 455UV (eBioscience 65-0868-14) (1:1000) on ice for 10 minutes. 50µl of the antibody staining mix containing CD14 (eBioscience 25-0149-42) (61D3, 1:20), CD86 (BioLegend 374214) (BU63, 1:20), CD206 (eBioscience 48-2069-42) (19.2, 1:20), MitoTracker DeepRed (Invitrogen M22426) (50nM) and MitoSOX Red (Invitrogen M36008) (5µM) was added and cells were incubated for a further 20 minutes on ice. Cell suspensions were then washed with 2ml FACS buffer (EDTA 2mM (Sigma E6758), FBS 2% (heat-inactivated) (Gibco 16140071) in 1x PBS (without Ca^{2+}/Mg^{2+})) and centrifuged at 300g for 5 minutes. The supernatant was discarded and 100µl of fixation buffer (PFA 4% (Sigma 158127) in 1x PBS (without Ca^{2+}/Mg^{2+})) was added. Cell suspensions were incubated for 30 minutes at room temperature in the dark. Cell suspensions were then washed with 2ml PBS, centrifuged at 300g for 5 minutes and supernatants were discarded. Finally, stained cell suspensions were resuspended in 150µl PBS.

Samples were analysed within 24 hours on a BD LSR II flow cytometer. Rainbow Calibration particles (BioLegend 422903) were used to standardise instrument

settings for longitudinal analysis. Data was analysed using FlowJo (v10.8.1) according to the following initial gating strategy: singlet cells (as determined by FSC-H/FSC-A), leukocytes (as determined by SSC-A/FSC-A), live cells (as determined by live/dead stain), monocytes/macrophages (defined as $CD14^+$), M1-like monocytes/macrophages (defined as $CD14^+CD86^+CD206^-$), M2-like monocytes/macrophages (defined as $CD14^+CD86^+CD206^+$), cells containing superoxide-producing mitochondria (defined as $MitoTracker^+MitoSOX^+$). Gates were set according to unstained control samples. Cell proportions were quantified as a percentage of their parent gate e.g. $CD14^+$ as a percentage of live cells, $CD14^+CD86^+CD206^-$ and $CD14^+CD86^+CD206^-$ cells as a percentage of $CD14^+$ cells and $MitoSOX^+$ cells as a percentage of $MitoTracker^+$ cells.

3.2.9 Statistics

Analysis was performed using GraphPad Prism 5.03. Where applicable, data is represented as mean ± standard deviation for parametric analysis and median [95% confidence interval] for non-parametric analysis. Where specified, GDM cases were matched with NGT controls by maternal age (± 3 years) and BMI (± 3 kg/m^2 units). P values < 0.05 were considered as statistically significant. The Kolmogorov–Smirnov test was used to test for data normality. For statistical comparisons, either the Student's t-test or the Mann-Whitney U test was used depending on data normality and test suitability. Wilcoxon matched-pairs signed rank test was used to compare matched samples. Spearman's correlation analysis was used to examine the correlation between non-parametric parameters. Pearson's correlation analysis was used to examine the correlation between parametric parameters. The Benjamini–Hochberg method with a 20% false discovery rate was used for multiple hypothesis

testing in cytokine (n=13) panels to decrease the false discovery rate. Grubbs's test was used to detect outliers in data sets.

3.3 Results

3.3.1 Reduced adipocyte count in omental VAT in GDM

Adipose tissue responds to changes in energy requirement by expanding existing adipocytes (hypertrophy) or forming new adipocytes (hyperplasia). While hyperplasia is considered a protective function which contributes to systemic insulin sensitivity, hypertrophy is a recognized feature of AT dysfunction associated with cellular stress, metabolic imbalance and systemic diabetes (379). Additionally, obesity can cause AT fibrosis, which contributes to disturbances in glucose metabolism and insulin resistance (380). To evaluate omental adipocyte morphology and tissue fibrosis in our participants, biopsies of omental VAT were stained with haematoxylin and eosin (H&E) (**Figure 3.1A**).

To reduce the effect of confounding factors on our results, NGT and GDM participant samples were matched by maternal age (± 3 years). Although there was no difference in adipocyte size in omental VAT from NGT compared to GDM (7698μm^2 [5583, 9251] vs. 9141μm^2 [5663, 11338], p=0.5) (**Figure 3.1C**), GDM omental VAT had a significantly lower adipocyte count (per 20X image) relative to NGT (133.3 [124.8, 170.8] vs. 180.0 [158.8, 207.1], p=0.004) (**Figure 3.1B**). There were no significant differences in pericellular fibrosis between NGT and GDM omentum VAT (9.09% [6.64, 13.24] vs. 8.25% [5.48, 10.27], p=0.84) (**Figure 3.1D**). To further decipher the impact of hypoplasia in GDM, participants were subgrouped by therapeutic intervention. Only the insulin-treated GDM group (115.4 [106.5, 150.7], p=0.046) held a significantly lower adipocyte count (per 20X image) relative to NGT participants. No significant difference in adipocyte count (per 20X image) was seen in diet-controlled (127.3 [98.9, 201.1]) or metformin-treated (155.0 [42.87, 307.3]) GDM participants relative to NGT participants (**Figure 3.1E**). In

Chapter 2, we identified two phenotypes of GDM pathology where lean GDM participants (BMI <29) had significant hypoinsulinaemia and obese GDM participants (BMI >29) were hyperinsulinaemic and had significant insulin resistance. To investigate if omental adipocyte hypoplasia was specific to the insulin resistant GDM pathology, adipocyte counts were compared in these subgroups. Obese GDM participants had significantly lower adipocyte count (per 20X image) (113.2 [107.6, 132.7]) relative to both lean GDM (181.8 [130.5, 231.5]) and NGT (180.0 [158.8, 207.1]) participants, whereas no significant difference was seen in lean GDM participants relative to NGT (**Figure 3.1F**). This strengthens the theory that GDM pathology in insulin-resistant obese participants is orchestrated by omental VAT dysfunction, unlike in hypoinsulinaemic, lean participants.

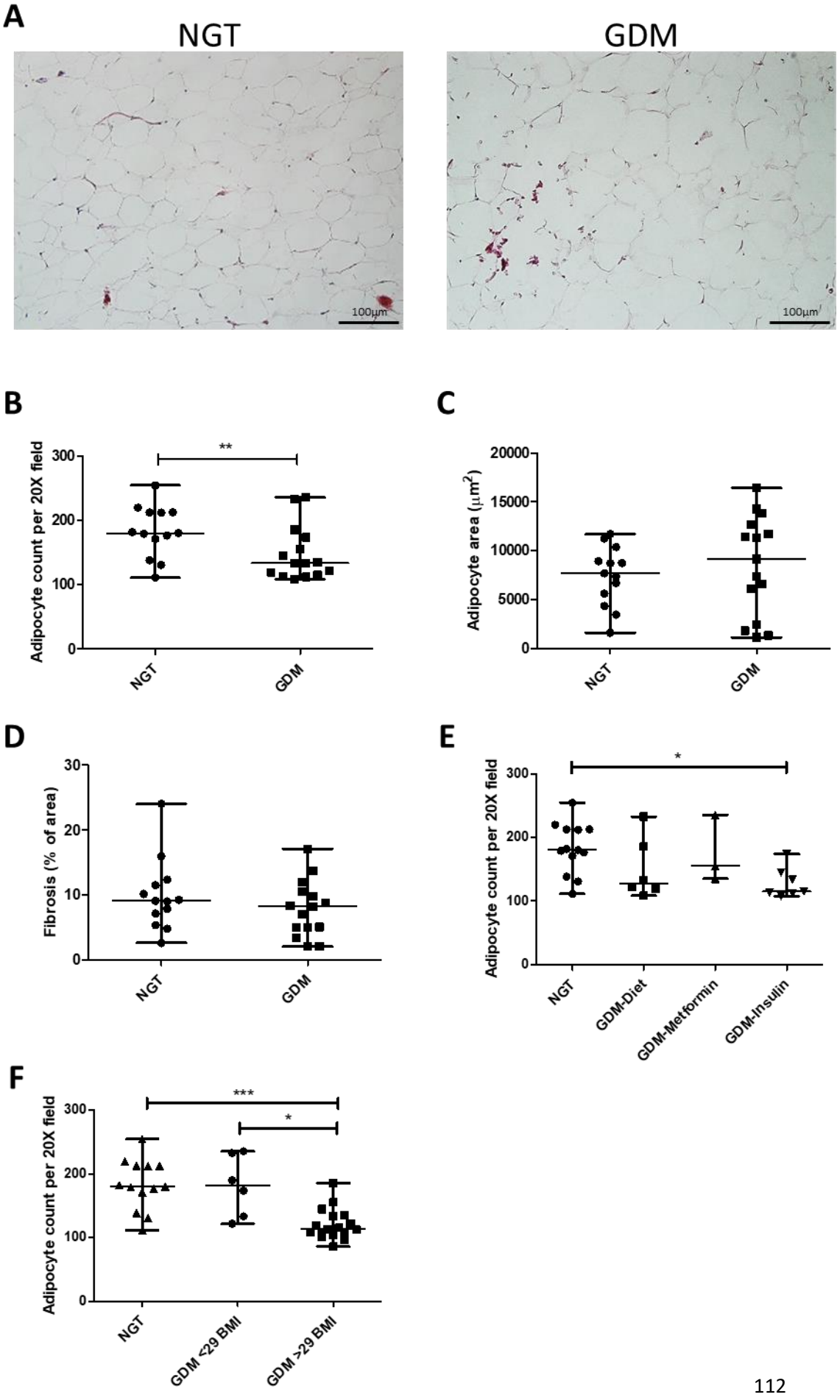

A
NGT
GDM
100µm
100µm
B
Adipocyte count per 20X field
300
200
100
0
**
NGT
GDM
C
Adipocyte area (µm²)
20000
15000
10000
5000
0
NGT
GDM
D
Fibrosis (% of area)
30
20
10
0
NGT
GDM
E
Adipocyte count per 20X field
300
200
100
0
*
NGT
GDM-Diet
GDM-Metformin
GDM-Insulin
F
Adipocyte count per 20X field
300
200
100
0
***
*
NGT
GDM <29 BMI
GDM >29 BMI

Figure 3.1. Adipocyte hypoplasia in visceral adipose tissue in GDM. Histological analysis of omental VAT samples from NGT (n=13) and GDM (n=15) pregnancies, matched by maternal age. **(A)** Representative images of H&E staining of NGT and GDM omental VAT samples. Comparison of **(B)** adipocyte count (per 20X field image) **(C)** adipocyte size (μm^2) and **(D)** fibrosis (% of image area) between NGT and GDM participants. Comparison of **(E)** adipocyte count (per 20X field image) between NGT and GDM participants subgrouped by therapeutic intervention. **(F)** Adipocyte count (per 20X image) was compared in NGT and GDM participants with a BMI <29 (n=6) and with a BMI >29 (n=17). Wilcoxon matched-pairs signed rank test was used to compare matched samples. The Kruskal-Wallis test and Dunnett's Multiple Comparison test was used to compare adipocyte count between subgroups. Significance levels are shown as: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.3.2 Elevated insulin resistance correlates with adipocyte hypoplasia in GDM

Adipocyte dysfunction has been linked to insulin resistance in obesity and T2DM. To assess the relationship between adipocyte hypoplasia and insulin resistance in GDM, HOMA-IR scores were compared with adipocyte count (per 20X image) in GDM participants. HOMA-IR scores in GDM participants significantly inversely correlated with omental adipocyte count (per 20X image), where a decrease in adipocyte count correlated with increased HOMA-IR scores (r_s=-0.53, n=17, p=0.03) (**Figure 3.2A**). To account for the effect of insulin therapy on HOMA-IR scores, the analysis was repeated excluding insulin-treated participants. In this case, HOMA-IR did not significantly correlate with adipocyte count (per 20X image) (r_s=-0.53, n=13, p=0.06) (**Figure 3.2B**).

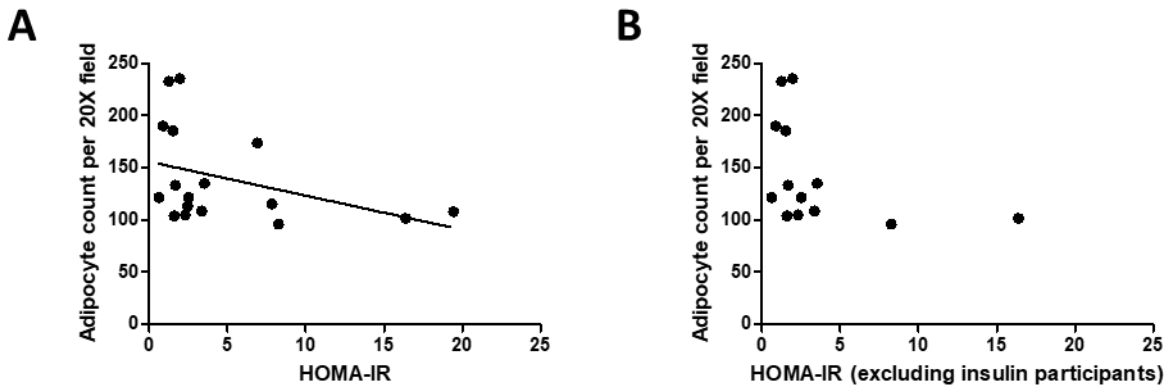


Figure 3.2. Insulin resistance correlates with adipocyte hypoplasia in GDM participants. Correlation of HOMA-IR and adipocyte count (per 20X image) in **(A)** GDM participants (n=11) and **(B)** GDM participants (n=7) not receiving insulin therapy. Spearman's rank correlation coefficient was used to examine correlation between adipocyte count and HOMA-IR value.

3.3.3 Expression of IGFBP-4 is significantly reduced in VAT in GDM

IGFBPs regulate IGF signalling, an important promoter of adipocyte proliferation and differentiation from adipocyte progenitor cells. In the previous chapter, we found that there was no significant difference in the circulating levels of plasma IGF-1 between study groups. To elucidate the role of the IGF/IGFBP axis in regulating adipocyte function in GDM, protein expression of IGFBP-3 and IGFBP-4 were measured in maternal BMI and age-matched NGT and GDM omental VAT (**Figure 3.3A**). While there was no significant difference in the expression of IGFBP-3 (0.49 [0.25, 0.69] vs. 0.2 [0.02, 0.59], p=0.20) (**Figure 3.3B**) in VAT from NGT and GDM, IGFBP-4 was significantly lower in omental VAT in GDM (0.21 [0.11, 0.29] vs. 0.09 [0.05, 0.2], p=0.016) (**Figure 3.3C**). To examine the relationship between IGFBP-4 expression and hypoplasia in GDM, correlation analysis was performed

with adipocyte count (per 20X image) from GDM participants, however no significant association was identified (r_p=0.16, n=8, p=0.69) (**Figure 3.3D**).

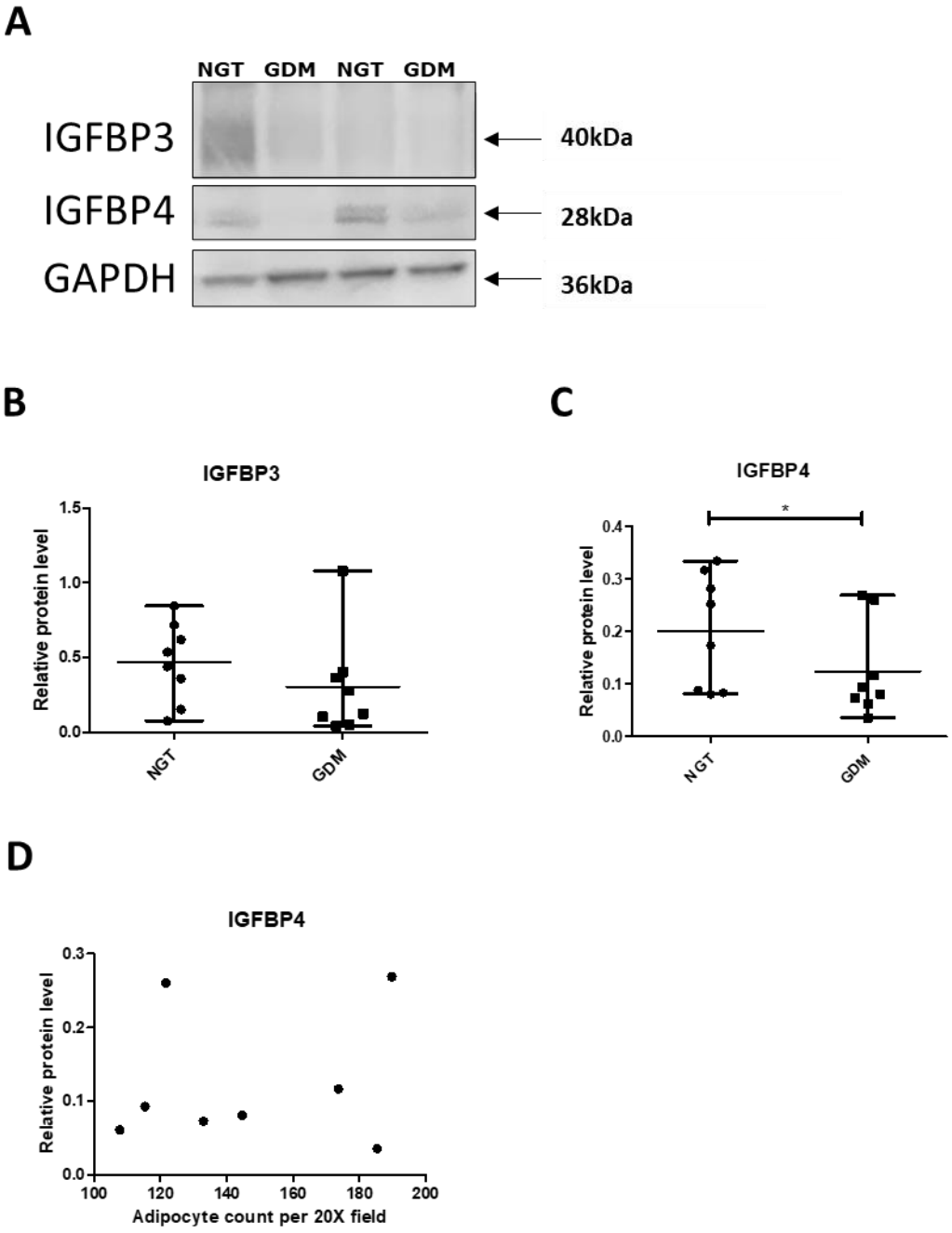


Figure 3.3. IGFBP-4 expression is significantly decreased in omental VAT in GDM. (A) Representative images of IGFBP-3 and IGFBP-4 protein expression as measured by Western blotting in omental VAT from NGT (n=8) and GDM (n=8), matched by maternal age and BMI. Densitometric analysis was used to quantify

expression of **(B)** IGFBP-3 and **(C)** IGFBP-4, with expression normalised to GAPDH. **(D)** Correlation of IGFBP-4 omental expression with adipocyte count (per 20X image). Wilcoxon matched-pairs signed rank test was used to compare matched samples. Pearson's correlation coefficient was used to examine correlation between adipocyte count and IGFBP4 expression. Significance levels are shown as: * $p<0.05$.

3.3.4 Omental or placental secretion of PAPPA is not significantly altered in GDM

PAPPA plays a role in increasing IGF bioavailability by cleaving IGFBP-4 and IGFBP-5 (167). During pregnancy, PAPPA is predominantly produced by the placenta, but is also expressed by other tissues such as AT. Despite the change in omental IGFBP-4 expression, we previously reported in Figure 2.4 that there were no changes in the circulating levels of either IGF-1 or PAPPA between study groups. To further extend our investigations, we measured the secretion of PAPPA in both omental and placental explant culture supernatants from NGT (n=10) and GDM (n=10) participants. Similar to Chapter 2, there were no significant differences in omental PAPPA in NGT compared to GDM (2.06ng/ml [1.3, 4.26] vs. 2.84ng/ml [2.11, 4.42], p=0.39) (**Figure 3.4A**) or placental PAPPA (34.97ng/ml [31.83, 39.77] vs. 38.75ng/ml [35.74, 41.89], p=0.21) (**Figure 3.4B**) between study groups.

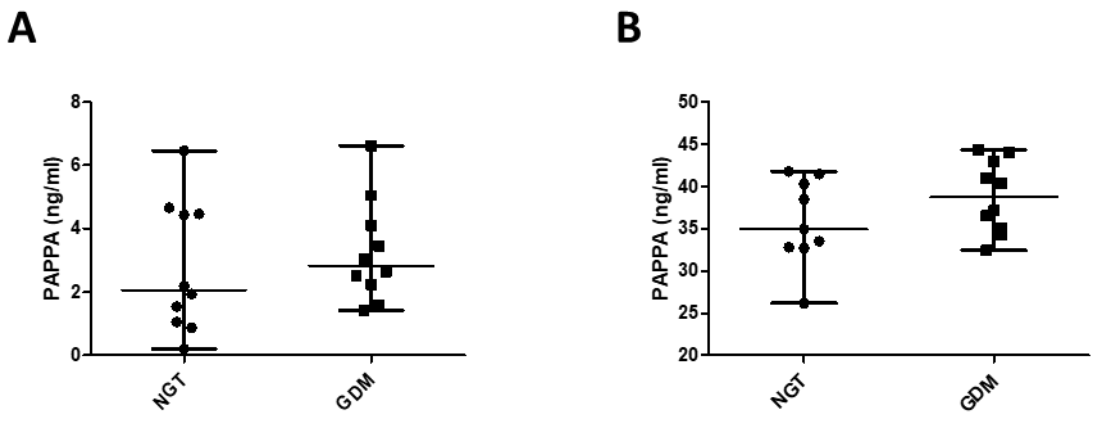


Figure 3.4. Release of PAPPA from placental tissue or VAT is not altered in GDM. (A) Omental PAPPA levels (ng/ml) and **(B)** Placental PAPPA levels (ng/ml) from NGT (n=10) and GDM (n=10) participants. The Mann-Whitney U test was used to compare differences between groups in explant culture supernatants. Grubb's test was used to remove statistical outliers from figure B.

3.3.5 Disruption in insulin signalling in omental VAT in GDM

Insulin resistance can result from insulin signalling disruption in AT. To eludicate if adipocyte dysfunction alters insulin signalling in GDM, protein expression of members of the insulin signalling cascade were measured in maternal BMI and age-matched NGT and GDM omental VAT **(Figure 3.5A**). Although there were no significant differences in protein expression of total IRS-1 in VAT from NGT compared to GDM (0.11 [0.003, 0.7] vs. 0.126 [0.03, 0.22], p=0.148) (**Figure 3.5B**), total IRS-2 was significantly decreased in VAT in GDM (0.6 [0.22, 0.92] vs. 0.06 [0.01, 0.21], p=0.008) (**Figure 3.5C**). There was also significantly increased phosphorylation of ser312 residue of IRS-1 relative to total IRS-1 in VAT in GDM (0.004 [-0.01, 0.05] vs. 0.03 [-0.15, 0.43], p=0.039) (**Figure 3.5D**). Ser312 residue is a key regulatory site that disrupts the IRS-1/IR interaction, inhibits insulin-mediated activation of the PI3K and MAPK pathways and has been implicated as a causative mechanism of insulin resistance (381, 382).

We found no significant difference in phosphorylation of other serine residues in VAT in GDM, namely ser318 (1.0 [0.22, 2.94] vs. 0.91 [-4.44, 20.94], p=0.46) (**Figure 3.5E**) or ser612 (10.06 [-5.63, 52.94] vs. 8.15 [-10.74, 83.87], p=0.74) (**Figure 3.5F**). Additionally, there was no significant difference in the protein expression of PDK1 (0.31 [0.26, 0.42] vs. 0.4 [0.25, 0.77] , p=0.55) (**Figure 3.5G**) or PI3K (0.45 [0.31, 0.69] vs. 0.35 [0.19, 0.65], p=0.195) (**Figure 3.5H**) in omental

VAT in NGT compared to GDM. These results suggest that the dysregulation of insulin signalling evident in VAT in GDM occurs upstream of these intracellular mediators. To investigate the relationship between insulin signalling disruption and hypoplasia in GDM, correlation analysis was performed on GDM participant adipocyte count (per 20X image) with both total IRS-2 and phospho-IRS-1(ser312)/IRS-1 expression. In GDM, IRS-2 expression significantly correlated with adipocyte count (per 20X image) (r_p=0.76, n=8, p=0.03) (**Figure 3.5I**), which was not evident for phospho-IRS-1(ser312)/IRS-1 expression (r_s=-0.38, n=8, p=0.36) (**Figure 3.5J**).

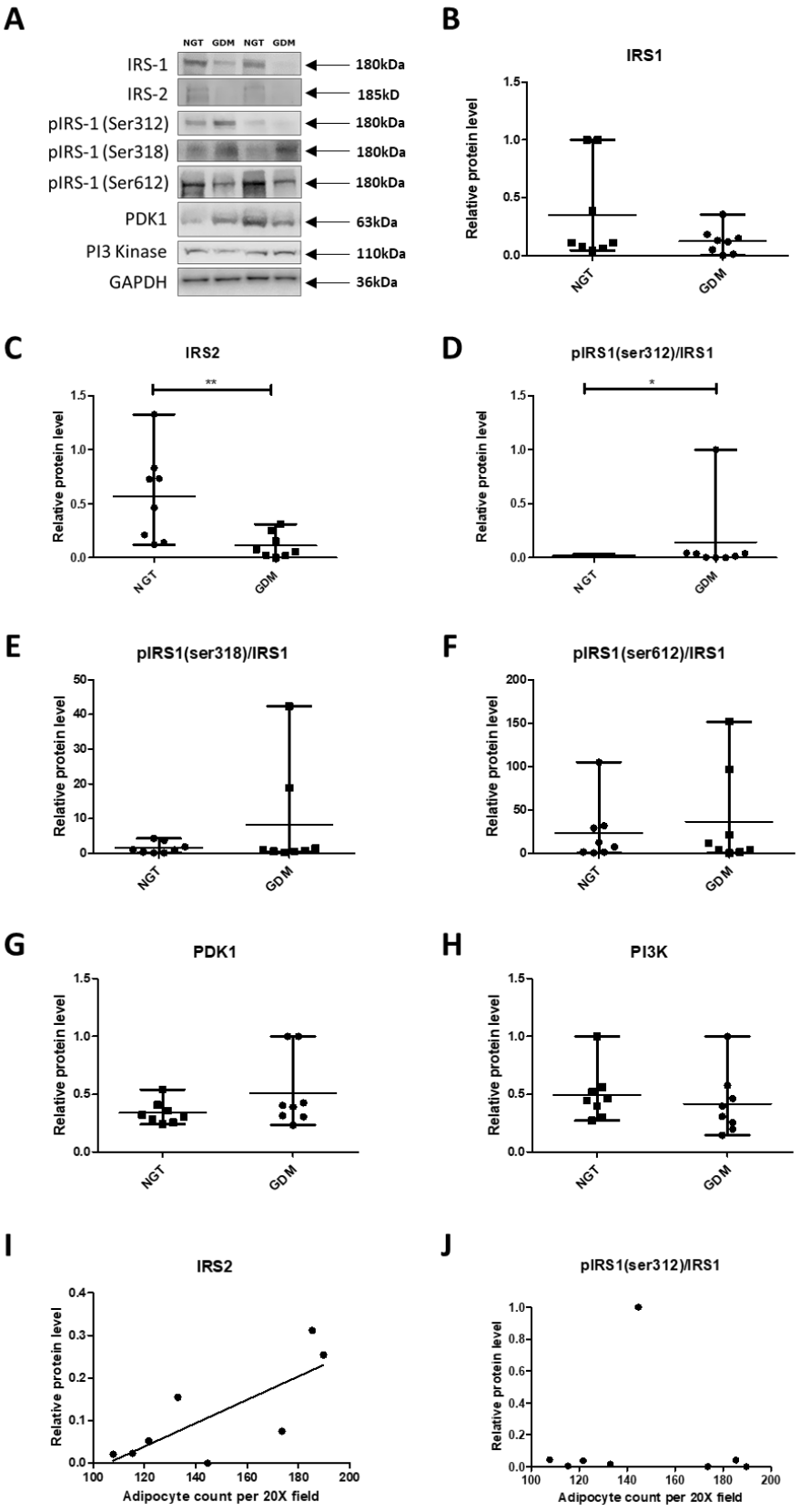


Figure 3.5. Altered insulin signalling in omental VAT in GDM.

(A) Representative images of IRS-1, IRS-2, phospho-IRS-1(ser312), phospho-IRS-

1(ser318), phospho-IRS-1(ser612), PDK1 and PI3K protein expression as measured by immunoblotting in omental VAT from NGT (n=8) and GDM (n=8), matched by maternal age and BMI **(B)** Densitometry analysis of total IRS-1, **(C)** total IRS-2, **(D)** phospho-IRS-1(ser312)/IRS-1, **(E)** phospho-IRS-1(ser318)/IRS-1, **(F)** phospho-IRS-1(ser612)/IRS-1, **(G)** PDK1 and **(H)** PI3K, with expression normalised to GAPDH. Correlation of adipocyte count (per 20X image) with **(I)** total IRS-2 and **(J)** phospho-IRS-1(ser312)/IRS-1 expression. Wilcoxon matched-pairs signed rank test was used to compare matched samples. Pearson's and Spearman's correlation coefficient was used to examine correlation of adipocyte count with IRS-2 and phospho-IRS-1(ser312)/IRS-1 expression, respectively. Significance levels are shown as: * $p<0.05$, ** $p<0.01$.

3.3.6 Omental production of adipokines is not significantly altered in GDM

The production of adipokines by either adipocytes or AT-infiltrating macrophages may lead to metabolic imbalance and meta-inflammation. Various adipokines have been implicated in the pathogenesis of GDM, particularly adiponectin and leptin, whose levels are altered as early as 10-18 weeks before GDM diagnosis (345). However, at term, there was no significant difference in omental VAT production of adiponectin (382.5ng/ml [290.2, 493.1] vs. 278.3ng/ml [226.4, 490.2], p=0.393) (**Figure 3.6A**), leptin (3.07ng/ml [1.49, 5.06] vs. 2.56ng/ml [1.31, 5.86], p=0.971) (**Figure 3.6B**), adipsin (67.71ng/ml [35.75, 192.6] vs. 70.32ng/ml [41.59, 90.92], p=0.813) (**Figure 3.6C**) or resistin (0.71ng/ml [0.32, 3.45] vs. 0.7ng/ml [0.27, 2.21], p=0.897) (**Figure 3.6D**) in NGT participants compared to GDM participants.

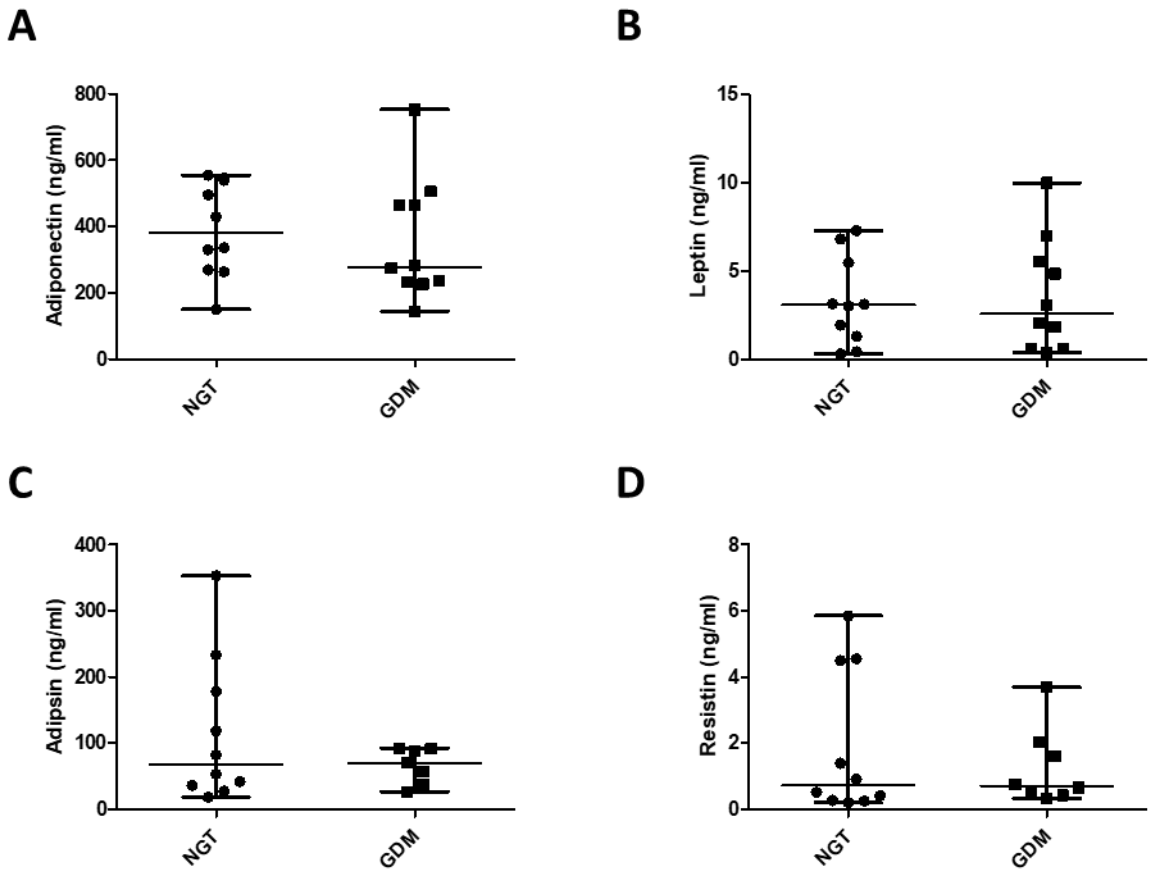


Figure 3.6. Adipokine secretion from omental VAT in GDM. Omental secretion of **(A)** adiponectin, **(B)** leptin, **(C)** adipsin and **(D)** resistin were measured in VAT explants from NGT (n=10) and GDM (n=10) participants. The Mann-Whitney U test was used to compare differences between groups. Grubb's test was used to remove statistical outliers from figure C and D.

3.3.7 Macrophage phenotype is not significantly altered in omental VAT in GDM

Previous research has proposed that macrophage infiltration and accumulation in VAT is associated with meta-inflammation and insulin resistance (383, 384). There is limited evidence of increased macrophage infiltration and activation in both visceral and subcutaneous adipose depots in GDM (261, 264). To assess the phenotype of endogenous and infiltrating macrophage populations in omental VAT

of study participants, $CD14^+CD86^+CD206^-$ (M1-like macrophages) and $CD14^+CD86^+CD206^+$ (M2-like macrophages) populations were quantified by flow cytometry. Gating of cell populations was performed as described in section 3.2.8. (**Figure 3.7A**). There were no significant differences in cell proportions, as a percentage of total $CD14^+$ cell count, of $CD14^+CD86^+CD206^-$ cells (56.5% [43.78, 65.8] vs. 52.75% [42.03, 67.32], p=0.88) (**Figure 3.7B**) or $CD14^+CD86^+CD206^+$ cells (43.35% [33.92, 55.73] vs. 46.4% [32.11, 57.66], p=0.89) (**Figure 3.7C**) between NGT and GDM omental tissues. The M1/M2 ratio was also not significantly different between NGT and GDM participants (1.31 [0.68, 2.2] vs. 1.14 [0.71, 2.26], p=0.88) (**Figure 3.7D**).

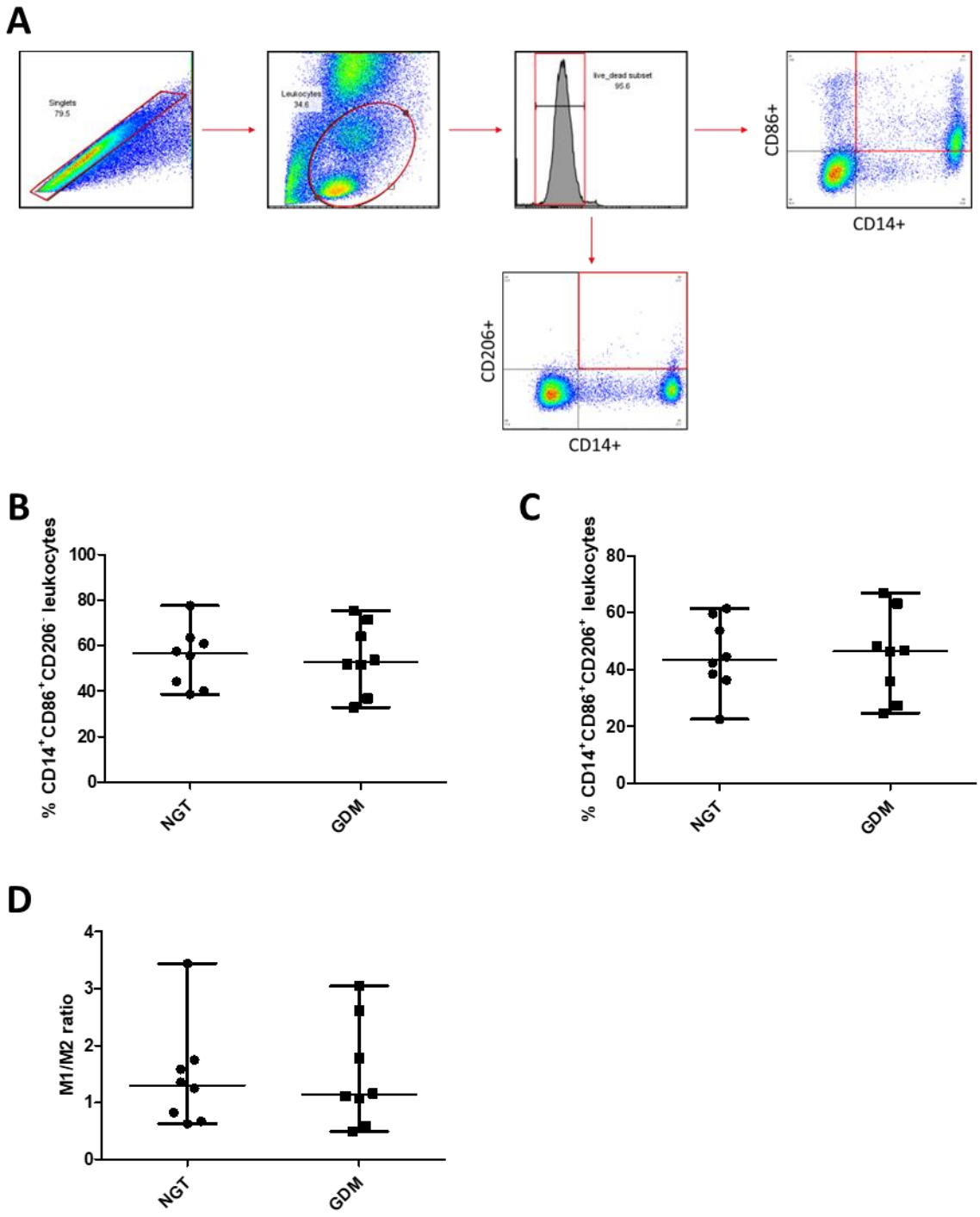


Figure 3.7. Macrophage phenotype is unchanged in omental VAT depot in GDM. Monocyte/macrophage cell phenotype was characterised in NGT (n=8) and GDM (n=8) omental VAT samples. **(A)** Representative graphs are shown for $CD14^+CD86^+CD206^-$/$CD14^+CD86^+CD206^+$ flow cytometry gating strategies. Cell proportions, as a percentage of total $CD14^+$ cell count, are shown for **(B)**

$CD14^+CD86^+CD206^-$ cells and **(C)** $CD14^+CD86^+CD206^+$ cells. **(D)** Comparison of the M1/M2 ratio as calculated for NGT and GDM participants. The Mann-Whitney U test was used to compare cell proportion differences between groups.

3.3.8 Macrophage production of mitochondrial superoxide is not altered in GDM omental VAT

Hyperglycaemia can induce cellular hypoxia and mitochondrial ROS generation, promoting diabetic symptoms (385). Mitochondrial ROS, and resulting oxidative damage can impact adipose tissue metabolism and contribute to insulin resistance (386, 387). To investigate the role of ROS production by omental macrophage populations in driving insulin resistance in our GDM participants, mitochondrial superoxide was measured in omental VAT $CD14^+$ cells in NGT and GDM tissues by flow cytometry. Gating of cell populations was performed as outlined in Figure 3.7A. Macrophages (defined as $CD14^+$), M1-like macrophages (defined as $CD14^+CD86^+CD206^-$) and M2-like macrophages (defined as $CD14^+CD86^+CD206^+$) were analysed for mitochondria-specific superoxide production (defined as $MitoTracker^+MitoSOX^+$) (**Figure 3.8A**).

There were no significant differences in total monocyte/macrophage production of $CD14^+MitoSOX^+$ cells (81.9% [67.9, 92.2] vs. 64.55% [54.78, 90.37], p=0.57) (**Figure 3.8B**) between NGT and GDM participants. Additionally, when stratifying monocyte/macrophage populations into M1-like $CD14^+CD86^+CD206^-MitoSOX^+$ cells (76.7% [56.76, 90.97] vs. 46.0% [34.73, 86.0], p=0.51) (**Figure 3.8C**) and M2-like $CD14^+CD86^+CD206^+MitoSOX^+$ cells (87.95% [83.06, 93.81] vs. 92.65% [83.35, 97.48], p=0.65) (**Figure 3.8D**), there was also no significant difference in mitochondrial ROS production between NGT and GDM participants.

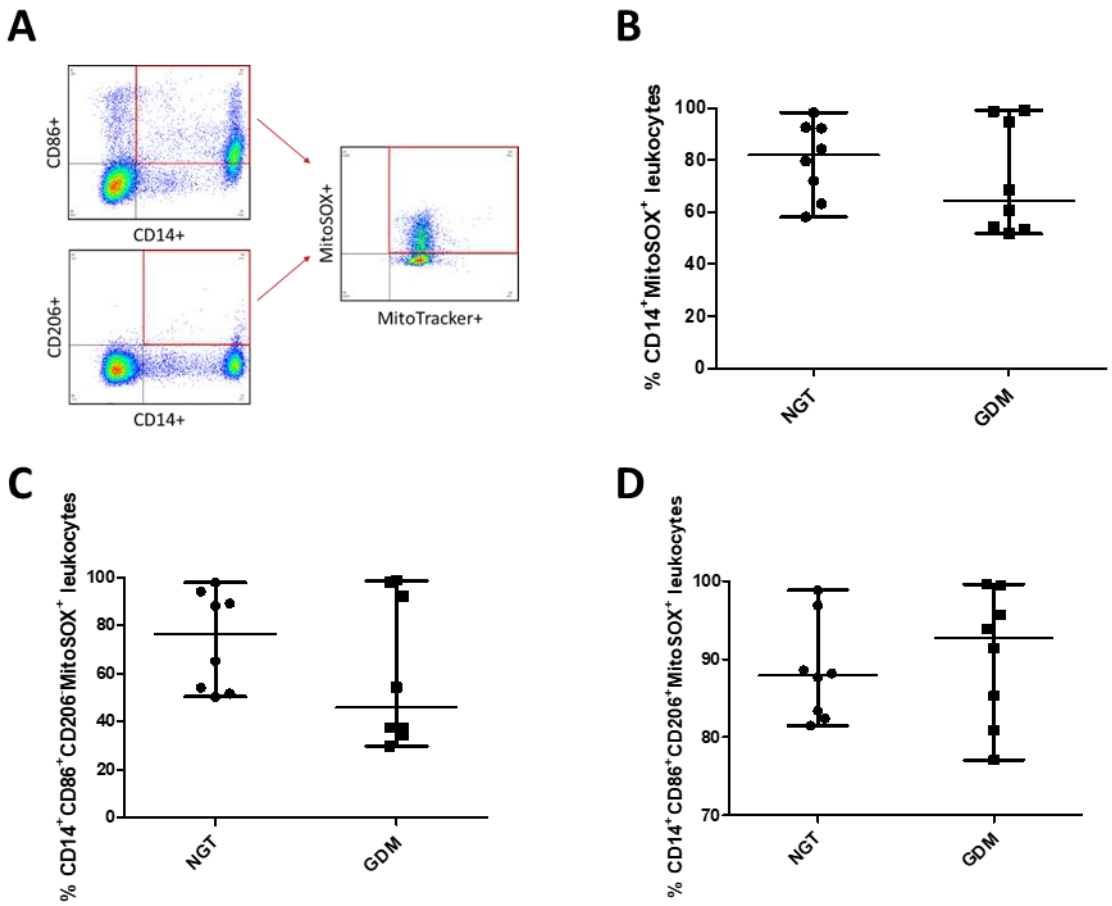


Figure 3.8. Mitochondrial-specific superoxide production in omental VAT macrophages is not altered in GDM. Mitochondrial superoxide production was measured in omental macrophage populations in NGT (n=8) and GDM (n=8). **(A)** Representative graphs are shown for MitoTracker^{+}MitoSOX^{+} flow cytometry gating strategies. Population frequencies are shown for **(B)** CD14^{+}MitoSOX^{+} cells, **(C)** CD14^{+}CD86^{+}CD206^{-}MitoSOX^{+} cells, **(D)** CD14^{+}CD86^{+}CD206^{+}MitoSOX^{+} cells. The Mann-Whitney U test was used to compare cell proportion differences between groups.

3.3.9 No evidence of localised inflammation in omental VAT in GDM

Increased inflammation in omental VAT has been shown to substantially contribute to the development of insulin resistance by disrupting insulin signalling in T2DM

patients (388). In order to determine if inflammatory mediators equally participate in altering insulin signalling in omental VAT in GDM, 13 key cytokines were measured in NGT and GDM explant culture supernatants (**Table 3.1**). These findings suggest that localised inflammation in the VAT depot is not orchestrating insulin resistance in GDM.

Table 3.1. Omental VAT levels of pro-inflammatory cytokines in NGT and GDM participants.

Cytokine	**NGT (Median [95% CI])(pg/ml)**	**GDM (Median [95% CI])(pg/ml)**	**P value**
IL-6	223340 [-4015530, 16242500]	1156580 [-8038650, 42111800]	0.28
IL-17A	6.42 [2.85, 8.23]	7.74 [5.06, 13.07]	0.13
IL-12p70	82.76 [42.41, 100.3]	90.20 [60.65, 146.9]	0.32
MCP-1	76413 [56655, 100765]	105179 [62778, 123308]	0.5
TNF-α	31.61 [13.63, 58.59]	31.43 [14.86, 77.91]	0.78
IFN-α2	49.31 [25.41, 65.04]	62.42 [37.57, 91.5]	0.22
IL-23	142.0 [71.15, 170.7]	154.3 [96.31, 245.1]	0.32
IL-10	365.5 [148.7, 494.1]	448.7 [218.4, 640.4]	0.3
IFN-ɣ	41.51 [12.61, 68.88]	43.91 [27.13, 113.0]	0.49
IL-8	467942 [173318, 506461]	506295 [278868, 706501]	0.19
IL-33	118.6 [17.5, 253.6]	161.9 [60.8, 490.4]	0.35
IL-1β	242.5 [134.8, 354.3]	293.9 [178.6, 507.3]	0.32
IL-18	244.7 [137.4, 306.7]	286.4 [195.9, 494.3]	0.35

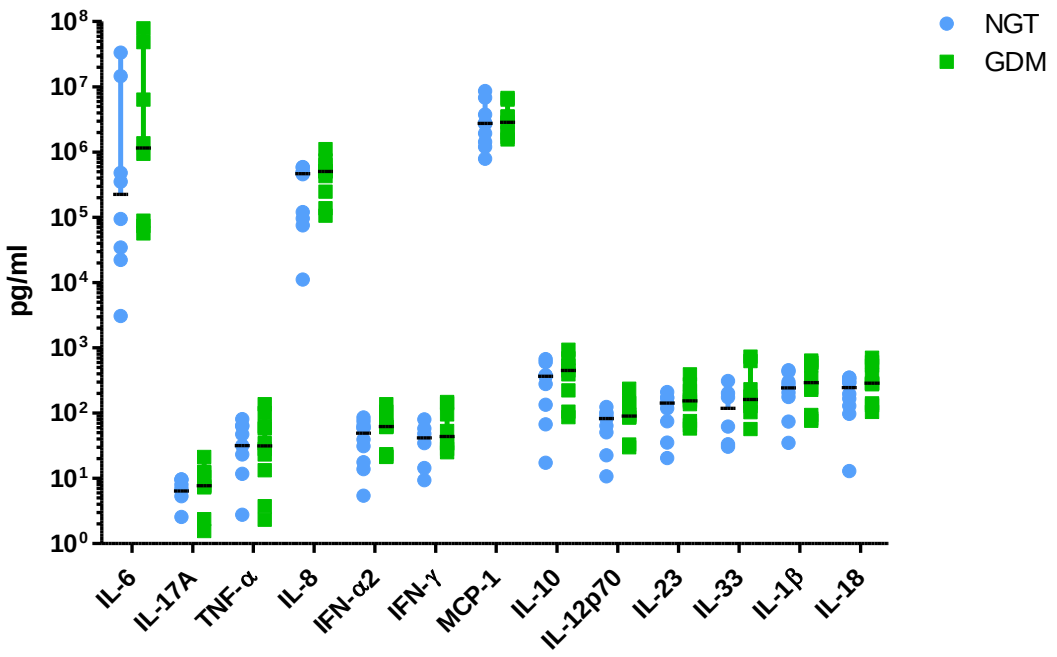


Figure 3.9. No change in inflammatory cytokines in omental VAT explants in GDM. Omental production of IL-6, IL-17A, TNF-α, IL-8, IFN-α2, IFN-ɣ, MCP-1, IL-10, IL-12p70, IL-23, IL-33, IL-1β and IL-18 were measured in VAT explant culture supernatants from NGT (n=10) and GDM (n=10) participants. The Mann-Whitney U statistical test was used to compare cytokine level differences between groups. Grubb's test was used to remove statistical outliers.

3.4 Discussion

In this chapter, we investigated the potential pathogenic contribution of adipocyte dysfunction and omental tissue meta-inflammation to systemic insulin resistance in GDM. We examined whether structural and functional changes in mature adipocytes in the metabolically active VAT depot modify insulin signalling in GDM. Additionally, we investigated if mediators of immune-endocrine signalling in the VAT depot also contribute to insulin resistance in GDM. Initially, we provide evidence of adipocyte hypoplasia in omental VAT which directly correlates with systemic insulin resistance. IGFBP-4, a key regulator of adipogenesis, was significantly reduced in omental VAT in GDM. Furthermore, we identified that insulin signalling is distorted in VAT in GDM participants, with reduced expression of IRS-2 and increased phosphorylation of IRS-1 at the ser312 residue, which ultimately inhibits insulin signal transduction. Finally, this study established a lack of localised meta-inflammation in VAT in GDM.

Firstly, we examined adipocyte morphology in omental VAT from NGT and GDM participants. It is widely recognised that hypertrophic, or enlarged adipocytes are insulin resistant and can develop due to lipid overload and defective differentiation capacity. This pathological state is independent of localised inflammation in these depots, with hypertrophy alone promoting insulin resistance in obese individuals (389). Adipocyte hypertrophy also inversely correlates with insulin sensitivity in non-obese individuals, suggesting that metabolic dysfunction, rather than obesity, promotes systemic insulin resistance (390). It is believed that hypertrophy occurs prior to hyperplasia in order to accommodate elevated requirements for fat storage. Hyperplasia subsequently acts to balance local tissue metabolism and promote AT homeostasis.

A deficiency in preadipocyte proliferation will expose individuals to exaggerated metabolic dysfunction and insulin resistance (391, 392). Although there were no significant differences observed in the size of adipocytes between our study groups, GDM participants had a significantly lower adipocyte count in their VAT depots. This reduction in adipocyte count significantly correlated with HOMA-IR score in women with GDM, suggesting that adipocyte hypoplasia may impair insulin sensitivity in omental VAT depots. The clinical relevance of this correlation is strengthened by the large range of HOMA-IR scores in this analysis (0.6 to 19.4), representing a potential link between reduced adipocyte count and severe insulin resistance. We repeated this analysis excluding insulin-treated participants, as exogenous insulin administration would have an artificial effect on calculated HOMA-IR scores. Although statistical significance was lost once this data was removed from the correlation analysis, it is important to note that this manipulation also removes the most severe GDM cases from our analysis.

IGF-1, produced predominantly by the liver, serves as an important promoter of adipocyte proliferation and differentiation (393). IGFBP family members bind available IGF-1 and thereby regulate IGF signalling by increasing its half-life and blocking receptor binding (84). Specifically, IGFBP-4 has been shown to potentiate the effects of IGF-1 by increasing local retention and promoting adipogenesis (394). The role of IGFBP-3 appears to be more complex. Certain *in vitro* findings suggest that IGFBP-3 can promote insulin resistance by reducing insulin-stimulated GLUT4 translocation (395). Other findings propose that IGFBP-3 inhibits NF-κB activation and has an anti-inflammatory affect while maintaining glucose uptake in human adipocytes (396). Additionally, De Silva et al. showed that endogenous IGFBP-3

may impair adipogenic differentiation by regulating adipocyte sensitivity towards the inhibitory effect of TGF-β (397).

We measured the expression of both IGFBP-3 and IGFBP-4 in omental VAT. While there was no difference in the omental expression of IGFBP-3 between study groups, omental expression of IGFBP-4 was significantly reduced in GDM. This finding suggests that although there were no difference in circulating plasma IGF-1 between study groups at term, a reduction in VAT IGFBP-4 expression in GDM may impair local retention of IGF-1. Equally, given the established role of IGBPP-4 in mediating adipogenesis, this reduction in omental expression could contribute to the adipocyte hypoplasia observed in VAT samples from GDM participants. Omental VAT samples from NGT and GDM groups were matched for maternal BMI and age, suggesting that reduction in omental IGFBP-4 expression in GDM is not age or obesity driven.

To further dissect the possible mechanism behind omental hypoplasia in VAT depots in our GDM cohort, we measured the localised secretion of PAPPA from omental and placental explant cultures. PAPPA is predominantly secreted by the placenta during pregnancy, but can also be produced by AT, and cleaves IGFBPs to increase the proportion of free, receptor-accessible IGF-1. IGFBP-4 is a principal substrate for PAPPA proteolysis activity (167). Rojas-Rodriguez et al. showed that PAPPA stimulates AT expansion *ex-vivo* and that $PAPPA^{-/-}$ mice had impaired AT remodelling and exaggerated pregnancy-induced insulin resistance (170). Furthermore, PAPPA expression is significantly higher in omental human preadipocytes, relative to mesenteric or SC preadipocytes, and is elevated by inflammatory cytokines such as TNF-α and IL-1β (398). However, we found no significant difference in the secretion of PAPPA from omental or placental tissue

explant cultures between NGT and GDM participants. This is in agreement with the outcome from Chapter 2, where we equally saw no difference in term circulating PAPPA levels between study groups. These findings suggest that reduced IGFBP-4 expression may be the rate-limiting step in the IGF-IGFBP-PAPPA axis, which hinders protective preadipocyte proliferation and differentiation in GDM by reducing the local retention of IGF-1 required for metabolic flexibility in VAT.

In order to elucidate if adipocyte dysfunction disrupts systemic glucose homeostasis, we interrogated the intracellular machinery that regulates glucose metabolism via GLUT4 translocation and glucose uptake. In this study, we found significantly lower expression of total IRS-2 in VAT of GDM participants relative to NGT participants. Catalano et al. quantified IRS-2 protein expression in subcutaneous AT and found GDM participants had significantly higher expression than non-pregnant participants, but did not observe a difference between NGT and GDM pregnancies (81). However, to date no studies have examined the omental expression of IRS-2 in GDM. In 3T3-L1 murine preadipocytes, knockdown of IRS-2 severely impaired insulin-stimulated AKT phosphorylation and glucose uptake, hindered preadipocyte differentiation into mature adipocytes and reduced the secretion of adiponectin, an insulin-sensitising adipokine (399). These results mirror our findings in GDM participants, where we have shown systemic insulin resistance and reduced omental adipocyte count. Although we did not see a significant reduction in the omental production of adiponectin, circulating plasma adiponectin levels were significantly reduced in GDM. These *in vitro* findings propose that depleted omental IRS-2 in VAT of our GDM participants may drive insulin resistance on three levels, by diminishing insulin signalling transduction, preventing homeostatic adipogenesis and reducing adiponectin production. The proposed impact of depleted IRS-2 expression

on adipogenesis is further strengthened by the significant positive correlation between omental VAT IRS-2 expression and adipocyte count in GDM participants in this study. We also found increased phosphorylation at the ser312 residue of IRS-1 in our GDM participants, impairing the ability of IRS-1 to activate downstream PI3K-dependent pathways.

Infiltrating macrophages and subsequent inflammation can distort insulin signalling in omental VAT depots leading to insulin resistance in obesity and T2DM (400). Serine phosphorylation of IRS-1 can be induced by the inhibitor κB kinase (IKK) complex, in response to inflammatory stimuli such as TNF-α. Adiponectin can inhibit this TNF-α induced IKK activation, thereby maintaining insulin sensitivity (401, 402). Furthermore, IL-6 also increases IRS-1 ser312 phosphorylation by activating the Jun N-terminal protein kinase (JNK) and extracellular signal-regulated kinase 1/2 (ERK1/2) pathways (403).

As such, we wanted to elucidate if localised VAT inflammation was involved in GDM and explore if these inflammatory mediators were responsible for impairing insulin signalling. However, the abundance of $CD14^{+}CD86^{+}CD206^{-}$ (M1-like) and $CD14^{+}CD86^{+}CD206^{+}$ (M2-like) macrophage cells were not significantly different in omental VAT between study groups. Excessive intracellular production of mitochondrial ROS (mROS) in macrophages can induce pro-inflammatory gene expression, hence we subsequently determined whether these macrophage populations produce excessive mROS. However, there were no significant differences in the production of mitochondrial-specific superoxide by these macrophage populations between study groups. Finally, we examined the local adipokine and cytokine profiles of the omental VAT secretome and found no significant difference in these mediators of meta-inflammaiton between study

groups. While we did not see any significant difference in the localised expression of adiponectin or inflammatory mediators in GDM, circulating plasma adiponectin levels were reduced while pro-inflammatory mediators, such as IL-6, were increased in our GDM participants. These systemic changes may leave IRS-1 susceptible to exaggerated serine phosphorylation and subsequent insulin resistance. As such, systemic meta-inflammation rather than a localised effect may be a greater contributor to exaggerated insulin resistance in GDM.

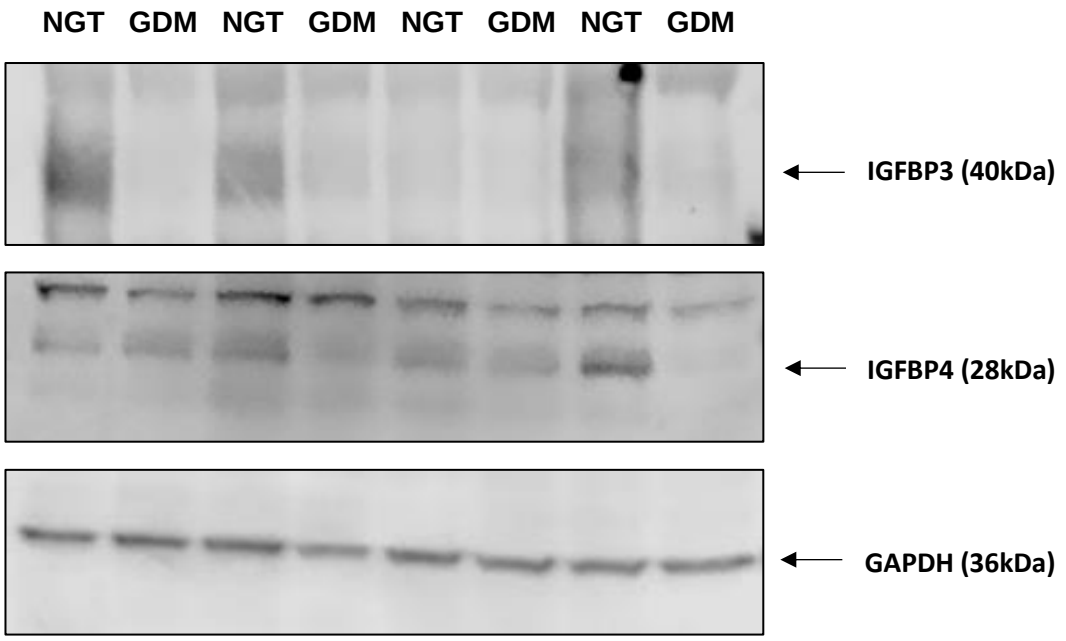
The strengths of this study include the use of omental VAT tissue from NGT and GDM participants that were matched maternal BMI and age, suggesting that effects were not mediated by age or obesity. Limitations include the cross-sectional nature of our study. Furthermore, this is a single site study that recruited a largely white Irish participant group, thereby limiting the extrapolation of our findings to other clinical settings or ethnicities. Overall, our findings provide substantial evidence of adipocyte dysregulation and impaired insulin signalling in GDM. Furthermore, adipocyte hypoplasia may be mediated by reduced IGFBP-4 and IRS-2 expression. These findings suggest a potential VAT-driven mechanism for the systemic insulin resistance in our GDM participants.

3.5 Conclusion

The physiological expansion of AT depots during pregnancy may have a direct mechanistic relationship with alterations in AT function, systemic glucose homeostasis and insulin resistance. In this study, we provide for the first time evidence of reduced adipocyte count and reduced protein expression of IGFBP-4 and IRS-2, key regulators of adipogenesis, which may be responsible for hypoplasia in the omental VAT of GDM participants. These findings suggest that impaired metabolic flexibility in omental VAT may contribute to systemic insulin resistance in GDM by limiting insulin signal transduction.

Supplementary material related to Chapter 3

A



B

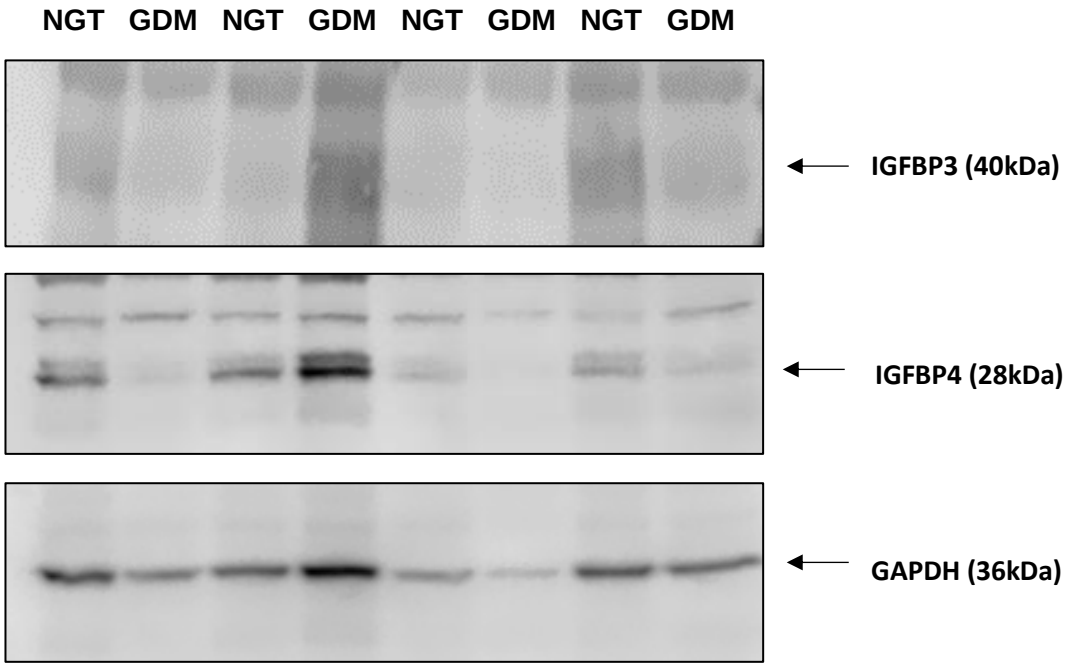
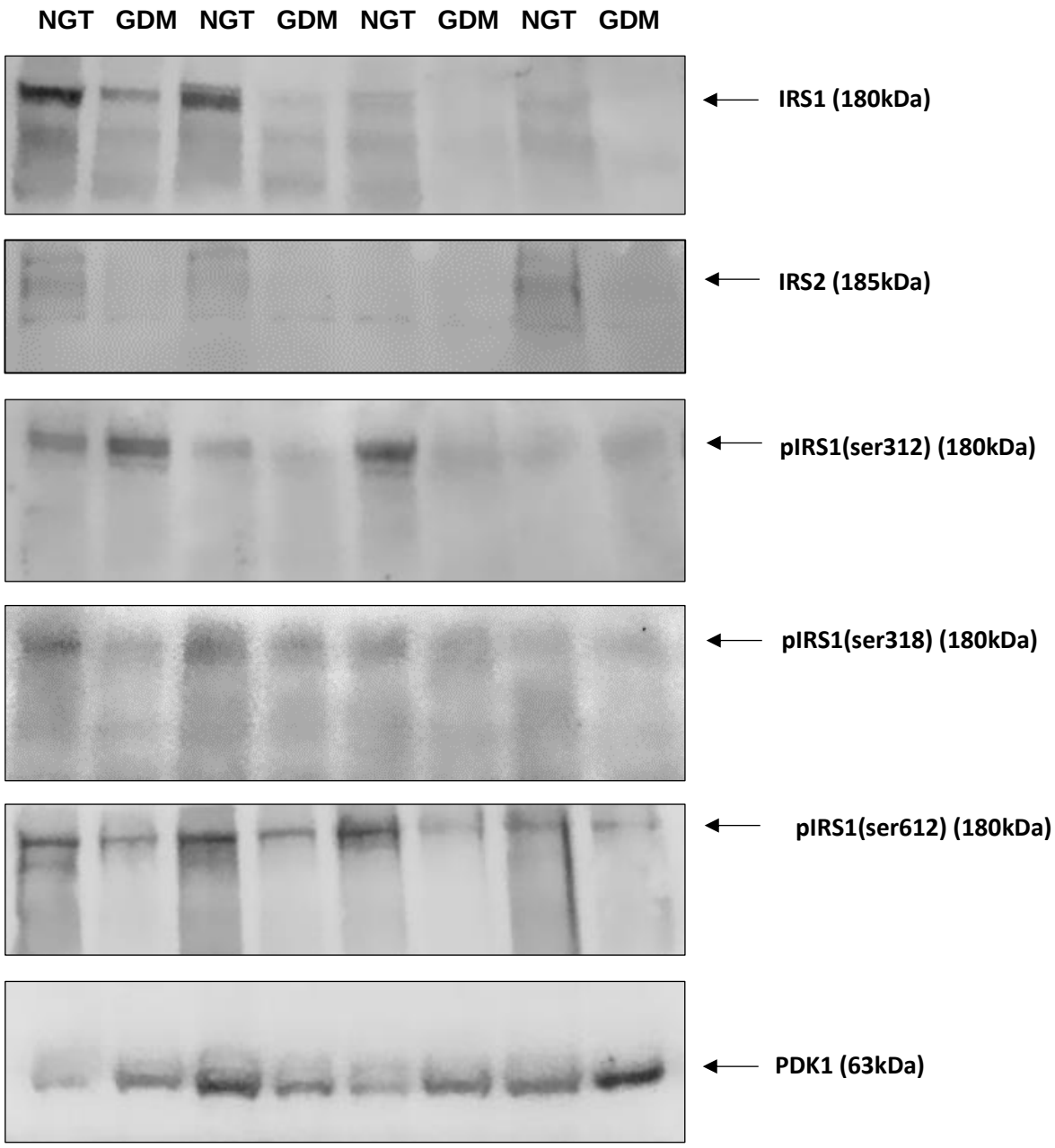
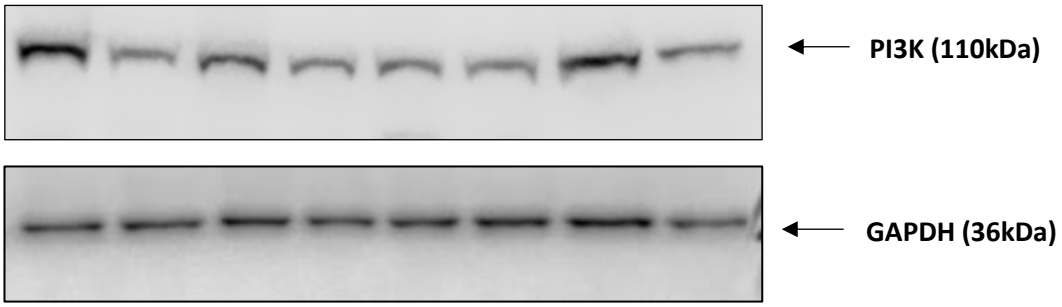


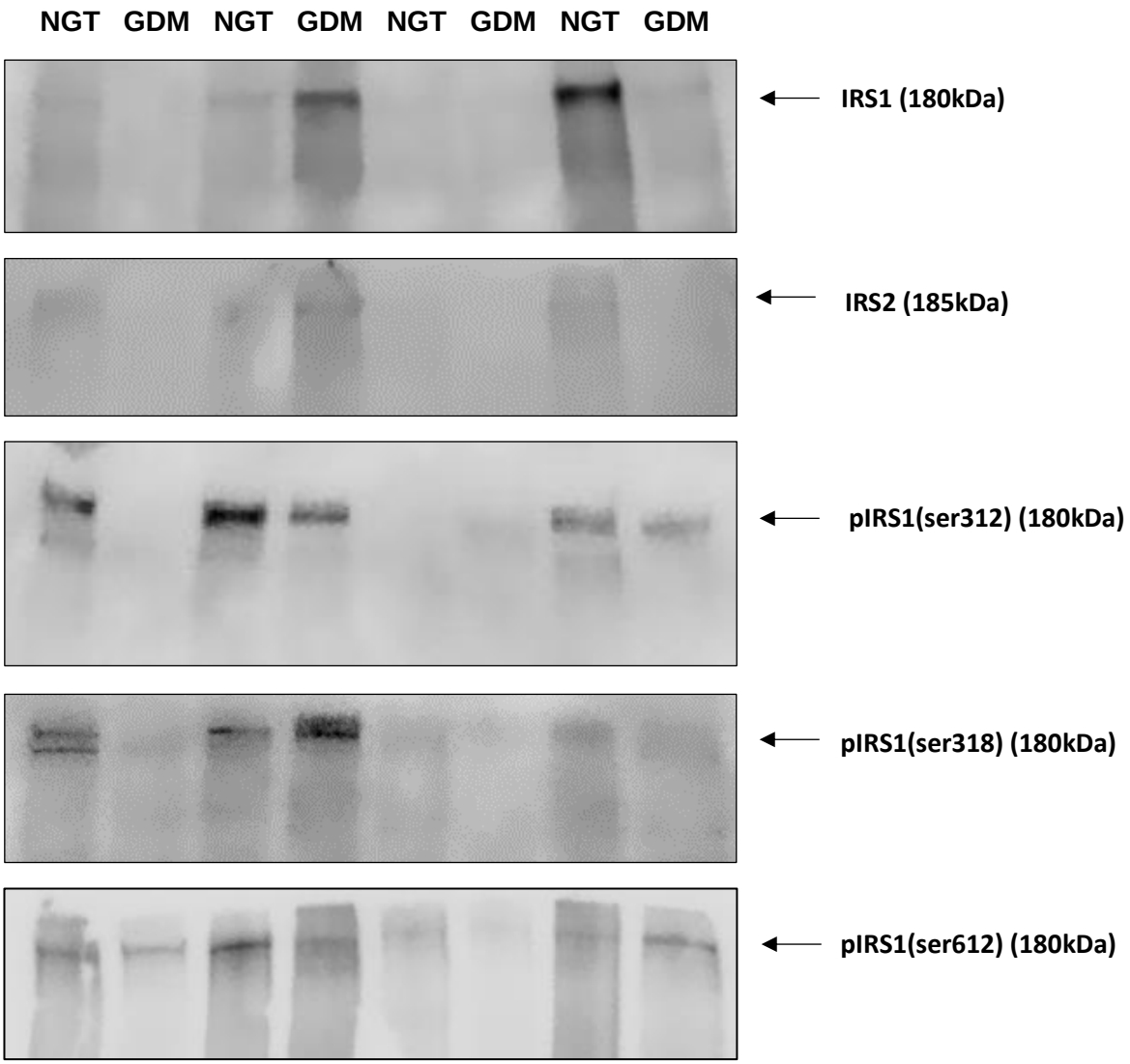
Figure S3.1. Omental VAT protein expression of IGFBP-3 and IGFBP-4 was measured in NGT (n=8) and GDM (n=8) samples, matched by maternal age and BMI. Matched pairs were loaded adjacent to each other over two separate blots **(A + B)**.

A




PI3K (110kDa)
GAPDH (36kDa)

B


NGT GDM NGT GDM NGT GDM NGT GDM
IRS1 (180kDa)
IRS2 (185kDa)
pIRS1(ser312) (180kDa)
pIRS1(ser318) (180kDa)
pIRS1(ser612) (180kDa)

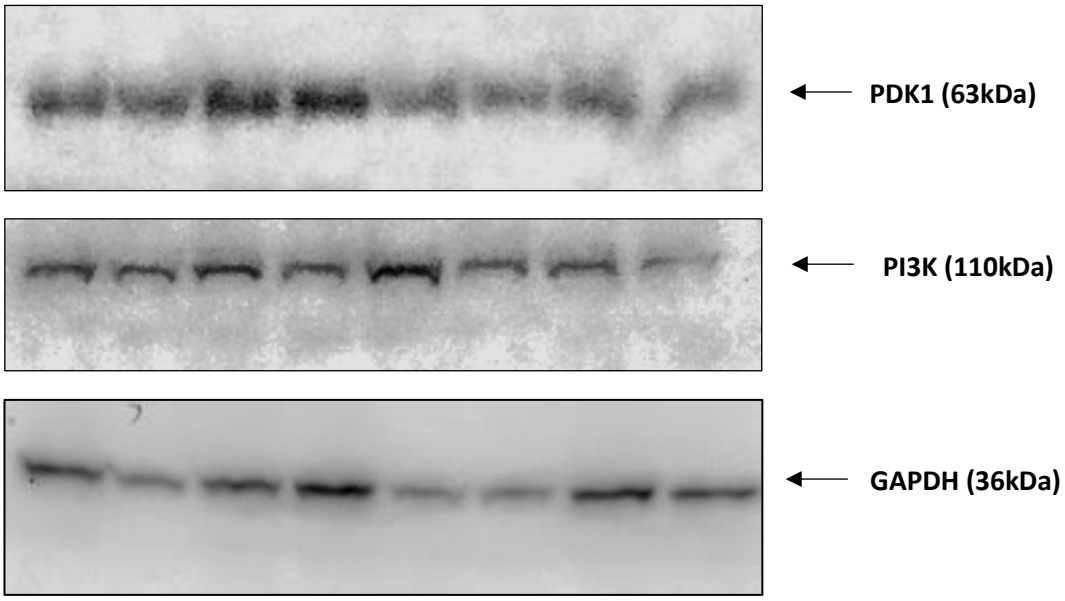


Figure S3.2. Omental VAT protein expression of IRS-1, IRS-2, phospho-IRS-1(ser312), phospho-IRS-1(ser318), phospho-IRS-1(ser612), PDK1 and PI3K was measured in NGT (n=8) and GDM (n=8) samples, matched by maternal age and BMI. Matched pairs were loaded adjacent to each other over two separate blots **(A + B)**.

Chapter 4

Mitochondrial Dysfunction Promotes a Dysregulated Placental Inflammatory Response in Gestational Diabetes Mellitus

Following on from the evidence provided in Chapter 2 and Chapter 3 of peripheral insulin resistance, systemic meta-inflammation and adipocyte dysfunction in GDM pathology, this chapter investigates the potential pathogenic interaction between mitochondrial dysfunction and inflammation at the local placental level. Our hypothesis is that placental mitochondrial dysfunction may instigate a local pro-inflammatory response which ultimately contributes to systemic inflammation and insulin resistance in GDM. In particular this chapter will focus on the NLRP3 inflammasome, a critical regulatory multiprotein complex that facilitates the intersection between mitochondrial dysfunction and inflammation.

Abstract

Background

The placental microenvironment *in utero* is highly sensitive to hyperglycaemia and hyperinsulinaemia which are evident in GDM. There is significant evidence that GDM pathology drives placental dysfunction, in part mediated by exaggerated oxidative stress. Equally there is evidence of increased placental inflammation in GDM. NLRP3 inflammasome activity is responsive to endogenous signals of mitochondrial dysfunction including mROS and mtDNA. Activation of the NLRP3 inflammasome results in the release of pro-inflammatory cytokines IL-1β and IL-18. Here we tested our hypothesis that local placental mitochondrial dysfunction orchestrates subsequent inflammatory signalling by activating the NLRP3 inflammasome, which may contribute to systemic inflammation in GDM. Secondly, we investigated if treatment with the antioxidants MitoQ and L-ergothioneine limit placental inflammation in GDM.

Methods

Placental tissue was collected post-elective caesarean section from nulliparous women with normal glucose tolerant pregnancies and women diagnosed with GDM. Characterisation of placental structure was performed by H&E staining. Placental explants from GDM (n=10) and NGT (n=10) participants were cultured for 24 hours with mitochondrial antioxidants (1mM L-ergothioneine, 1μM MitoQ) and the NLRP3 inhibitor (1μM MCC950). Placental production of adipokines (adiponectin, adipsin, leptin and resistin) and inflammatory mediators (IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33) were measured in explant cultures by multiplex ELISA. Characterisation of endogenous

and infiltrating M1-like ($CD14^+CD86^+CD206^-$) and M2-like ($CD14^+CD86^+CD206^+$) macrophage populations in the placenta were determined by flow cytometry. Placental macrophage production of mROS was measured by flow cytometry using $MitoTracker^+MitoSOX^+$ fluorescent staining. Immunoblotting for protein expression of members of the inflammasome cascade NLRP3, ASC and caspase-1 was performed in maternal BMI and age-matched GDM (n=8) and NGT (n=8) placental tissue. Statistical analysis was performed with GraphPad Prism 8.

Results

There was evidence of placental villous immaturity with a significantly higher intermediate/terminal villi ratio (p=0.016) in GDM compared to NGT placental tissues. There were no changes in the placental macrophage M1-like/M2-like populations between study groups. However, placental populations of both $CD14^+$ (p=0.02) and $CD14^+CD86^+CD206^-$ (p=0.03) macrophages produced significantly increased levels of mitochondrial superoxide in GDM compared to NGT participants. Placental production of leptin was significantly reduced (p=0.046) while adipsin was significantly increased (p=0.028) in GDM compared to NGT participants. There was significantly increased placental production of the pro-inflammatory cytokines of IL-6 (p=0.028), TNF-α (p=0.008) in GDM compared to NGT.

Although there was no significant difference in the expression of components of the NLRP3 inflammasome cascade between study groups, placental production of the NLRP3-mediated cytokine, IL-18 (p=0.04) was significantly increased in GDM. Additionally, the increase in placental IL-18 in GDM was attenuated by treatment

with either 1μM MCC950 ($p=0.0005$), an inhibitor of the NLRP3 inflammasome or 1mM L-ergothioneine ($p=0.007$), a mitochondrial antioxidant.

Conclusion

Placental inflammation is significantly increased in women with GDM. Furthermore, this increase may be initiated by elevated production of mitochondrial-specific ROS by macrophage subpopulations. The NLRP3 inflammasome appears to orchestrate this placental cross-talk between pathogenic mediators, a pathway which may be ameliorated by antioxidant therapy.

4.1 Introduction

GDM is associated with adverse fetal and neonatal outcomes, including macrosomia, neonatal hypoglycemia and respiratory distress syndrome. The placenta acts as a natural selective barrier between the maternal and fetal systems and is highly sensitive to metabolic stressors, such as hyperglycaemia, which promote changes in both the morphology and function of placental tissue (144). Upon gross examination, placenta from GDM pregnancies are generally larger, with reduced villous maturity, increased evidence of chorangiosis and have greater numbers of syncytial knots (134, 141, 404). Furthermore, changes in immune cell populations are also evident in GDM placental tissue, including increased infiltration and activation of neutrophils, elevated numbers of decidual NK cells and increased macrophage activation. The placental inflammatory secretome is also modified in GDM pathology, with evidence of increased placental release of IL-8, TNF-α and IL-6 pro-inflammatory cytokines (225).

Healthy pregnancy is characterized by placental ROS production, which facilitates implantation and tissue development. However, GDM is associated with an exaggerated oxidative stress response, resulting from excessive production of ROS, which can promote systemic insulin resistance by interfering with insulin receptor signal transduction and subsequent glucose metabolism. This may be as a consequence of disruption in the cellular redistribution of IRS-1 and PI3K from the cytosol to the internal membrane pool, resulting in reduced functional activity (405). Unfortunately, our current understanding of how oxidative stress contributes to metabolic dysfunction in GDM is limited.

Increased mitochondrial dysfunction and mROS production have been implicated in GDM pathophysiology (175, 176). Mitochondrial dysfunction, with an increase in

mtDNA release and mROS production, may also directly coordinate pro-inflammatory signalling. This may occur by mtDNA activation of the TLR-9 receptor on the endosomal surface of immune cells (192) or mROS instigating dissociation of the thioredoxin and thioredoxin-interacting protein complex with subsequent activation of NLRP3 (406). In this chapter, placental macrophage production of mROS and placental-meditated activation of the TLR-9 receptor will be measured to elucidate the intracellular communication between these pathophysiological pathways in GDM.

The NLRP3 inflammasome is a multiprotein complex that, upon activation, oligomerizes and activates caspase-1, initiating the cleavage and release of pro-inflammatory cytokines IL-1β and IL-18. Persistent and aberrant NLRP3 activation underlies many chronic inflammatory and degenerative diseases, such as T2DM and CVD (407-410). The NLRP3 inflammasome is activated by a wide variety of exogenous and endogenous signals, including pathogen-associated molecular patterns (PAMPs) and DAMPs, and is predominantly expressed in innate immune cells, particularly macrophages (197). NLRP3 inflammasome activation is a two-step process, namely priming (signal 1) and assembly (signal 2), with subsequent cytokine release (see Figure 4.1). The priming step involves PAMP and DAMP mediated signalling, leading to increased transcription of NLRP3 and pro-IL-1β. Apoptosis-associated speck-like protein containing a CARD (ASC) simultaneously becomes ubiquitinated and phosphorylated for inflammasome assembly. Assembly (signal 2), is triggered by stimuli including extracellular ATP, pore-forming toxins, RNA viruses, mROS and mtDNA. Pro-caspase-1 filaments are formed through caspase activation and recruitment domain (CARD) interaction, allowing auto-proteolytic activation of pro-caspase-1 (411). Multiple molecular or cellular events,

including ionic flux, mitochondrial dysfunction, mROS generation, mtDNA release and lysosomal damage, have been shown to activate the NLRP3 inflammasome cascade with the resulting release of pro-inflammatory cytokines IL-1β and IL-18, amplifying systemic inflammation (408, 412, 413).

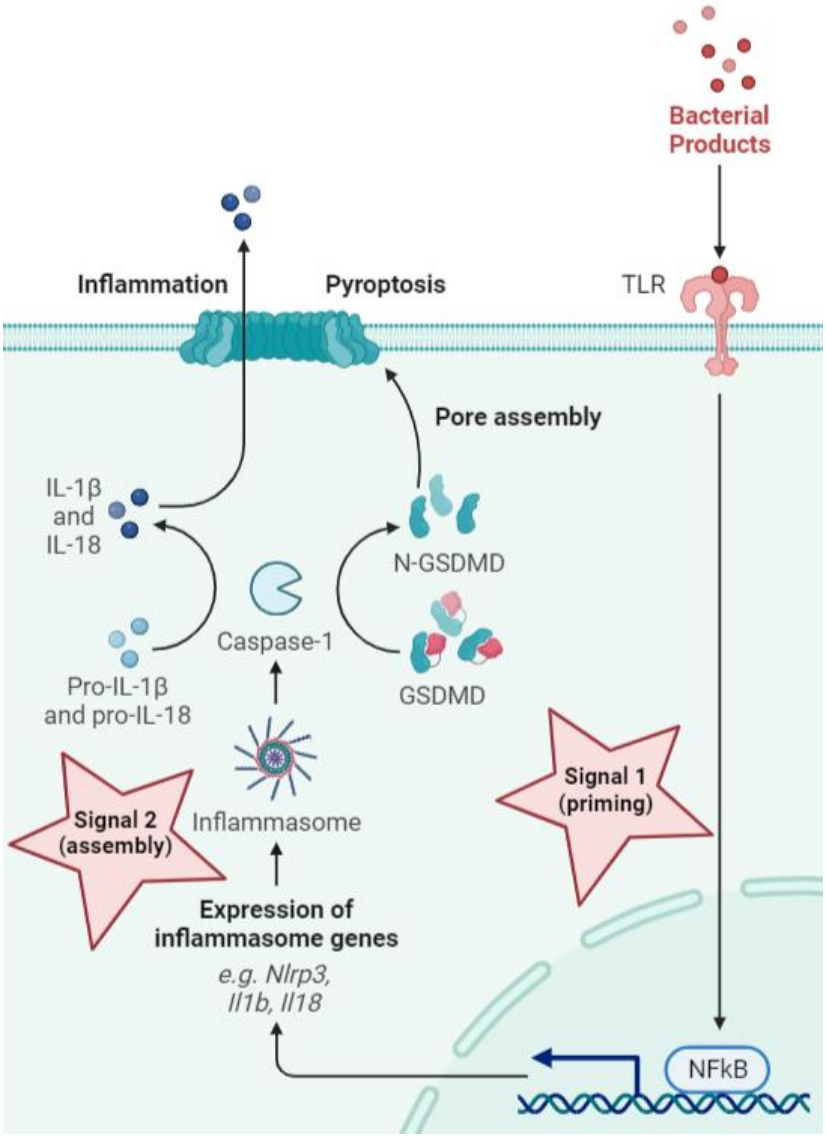


Figure 4.1. Activation of the NLRP3 inflammasome. Adapted from "Suppression of Inflammasome by IRF4 and IRF8 is Critical for T Cell Priming", by BioRender.com (2013). The activation of TLRs and the NF-κB signalling pathway induces the priming step of the NLRP3 cascade. Once primed, NLRP3 can be activated by a variety of signals, including mROS and mtDNA. These signals trigger

the assembly of the NLRP3 inflammasome, which involves the recruitment of ASC and the activation of caspase-1. Once activated, caspase-1 cleaves gasdermin D (GSDMD), which triggers its oligomerization and the formation of pores in the cell membrane. These pores allow for the release of pro-inflammatory cytokines and the initiation of pyroptosis.

In healthy pregnancy, components of the NLRP3 inflammasome are expressed in both the maternal and fetal compartments (414). Hyperglycaemia has been shown to stimulate placental NLRP3 inflammasome activation, inducing generation of IL-1β and IL-18 inflammatory cytokines (415). Furthermore, recent *in vitro* evidence has shown that palmitic acid, a surrogate of elevated saturated fat, induces NLRP3 inflammasome activation, increasing IL-1β expression and apoptosis in primary placental macrophages isolated from GDM term placental tissue (416). Another site of NLRP3 activity is in AT, where mtDNA and other recognised DAMPs can activate TLR-9 and trigger NLRP3 inflammasome recruitment (417). A recent *in vivo* study in a murine model of GDM demonstrated that Tranilast (NLRP3 inhibitor) attenuated NLRP3 expression in both placental and VAT tissue. Tranilast also significantly ameliorated maternal body weight, hyperglycaemia, insulin insufficiency, glucose intolerance and insulin resistance, enlarged litter size, and reduced litter body weight (418). These results demonstrate a potential therapeutic avenue for GDM by targeting exaggerated NLRP3 activation.

Given the pathogenic role of dysfunctional mitochondria in activating the NLRP3 inflammasome, and previous evidence of NLRP3 activation in T2DM and related metabolic complications, we hypothesise that directly targeting mitochondria may

dampen activation of the NLRP3 inflammasome and identify a clinical therapeutic pathway in GDM. MitoQ, a mitochondrial-targeted antioxidant, is a ubiquinone moiety linked to a lipophilic triphenylphosphonium (TPP) cation (419) which has shown promising results in ameliorating endothelial dysfunction in animal and human studies (420-422). Similarly, a nutraceutical mitochondria-targeted antioxidant, L-ergothioneine (ERG), has demonstrated potential therapeutic effects, particularly in a preclinical model of PE (423). ERG is a water-soluble amino acid derived from histidine, which accumulates preferentially in high oxidative stress organs through the action of a specific organic cation transporter novel type 1 (OCTN1) (424). This nutraceutical has a half-life of approximately 30 days and can cross both the blood brain barrier and the placenta (425, 426). These mitochondrial specific therapeutics show promising results in favour of a targeted antioxidant strategy, where other clinical interventions may be contraindicated.

The primary aim of this chapter was to elucidate if mitochondrial dysfunction increases placental inflammation in GDM by activating the NLRP3 inflammasome. A secondary aim was to determine if directly targeting mROS production with MitoQ or ERG ameliorates placental inflammation in GDM.

4.2 Methods

4.2.1 Study design and patient recruitment

Patients were recruited from Cork University Maternity Hospital (CUMH), Ireland between 2019 and 2022 as part of the COMRADES study, a non-interventional cohort study of nulliparous singleton pregnancies with the aim of characterizing mitochondrial dysfunction and meta-inflammation in GDM. The COMRADES study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all the procedures were approved by the Clinical Research Ethics Committee of the Cork Teaching (ECM4 (ff) 04/12/18). Inclusion criteria for all study participants included; women aged between 18-50 years, nulliparous pregnancy, singleton pregnancy as determined by ultrasound scan, opted for an elective caesarean section and had provided written informed consent. As per national HSE guidelines, selective screening for GDM was carried out on identified at-risk pregnant women between 24-28 weeks gestation using the 75g oral glucose tolerance test (OGTT). A diagnosis of GDM was confirmed if one of the following values were met or exceeded: fasting blood glucose levels of 5.1mmol/L, blood glucose levels of 10.0mmol/L 1 hour post-OGTT or blood glucose levels of 8.5mmol/L 2 hours post-OGTT.

Healthy, NGT control participants were defined as pregnant women who had not been diagnosed with GDM, as per the above guidelines, and did not present with any other diagnosed comorbidities. Exclusion criteria included women who: had a multiple pregnancy, were multiparous, had already started to labour, who were outside the age range of 18-50 years of age or were having an emergency caesarean section. Women with known pregnancy or other health related conditions were also excluded namely: pre pregnancy essential hypertension, known liver or renal

diseases, Human Immunodeficiency Viruses (HIV), Hepatitis B or Hepatitis C positive, Systemic Lupus Erythematous, antiphospholipid syndrome and known or suspected fetal anomalies.

4.2.2 Sample collection

Placental and omental adipose tissue samples were collected immediately post caesarean section. Placental tissue was taken from 5 defined regions of each placenta and tissue samples were stored at -80 °C until analysis was performed. Additionally, tissue samples were used for explant cultures or fixed in 10% formalin for histological analysis.

4.2.3 Placental and omental explant culture and treatments

Tissue explants were prepared from placental samples collected from both NGT and GDM participants. Following the removal of outer membrane and connective tissue, 200mg ± 20mg of placental tissue (combined from three regions of the placenta) were cultured in 12-well plates, containing 2ml of culture media. Placental explant media consisted of RPMI-1640 1x (Gibco 21875034), FBS (heat-inactivated) 10% (Gibco 16140071), penicillin-streptomycin (10,000 U/mL) 1% (Gibco 15140122), insulin 1µg/ml (SAFC 91077C), hydrocortisone 0.1µg/ml (Sigma H0888), retinol acetate 0.1µg/ml (Sigma R0300000), gentamicin 0.05µg/ml (Sigma G1397). Tissue explants were also prepared from omental AT samples collected from both NGT and GDM participants. 100mg ± 10mg of omental tissue were cultured in 12-well plates, containing 2ml of culture media containing Medium 199 1x (Gibco 31150022), FBS (heat-inactivated) 10% (Gibco 16140071), penicillin-streptomycin (10,000 U/mL) 1% (Gibco 15140122), amphotericin B 0.25µg/ml (Gibco 15290018). Tissue weight was recorded and used for normalisation of all explant experimental analysis. Explant cultures were treated, or cultured without treatment, for 24 hours as follows;

untreated, DMSO, 1µM MitoQ, 1µM MCC950 and 1mM ERG for 24 hours at 37°C/5% CO_2. Supernatants were subsequently stored at -80°C for further analysis.

4.2.4 Immunohistochemical Staining

Tissue samples for histological investigation were fixed in 10% neutral buffered formalin (NBF) within 30 minutes of surgical biopsy. Fixed samples were dehydrated and embedded in paraffin as per standard laboratory protocols. 7µm tissue sections were sectioned using a Leica RM2125 RTS microtome and mounted on SuperFrost glass slides (VWR 631-0446). H&E staining was completed using the following protocol: tissue sections were deparaffinized in xylene for 2 minutes (x2) and rehydrated by immersion in ethanol (2 minutes each) using a decreasing gradient concentration (96% - 70% - 50% - 30%), followed by immersion in tap water for 1 minute. Slides were then immersed in haematoxylin stain (3 minutes), washed in tap water (1 minute), differentiated in 1% acid alcohol (30 seconds), washed in tap water (1 minute), rinsed in Scott's tap water (1 minute), washed in tap water (1 minute) following by a 70% alcohol wash (1 minute) and finally immersed in eosin stain (3 minutes). After a quick wash in 30% ethanol, sections were dehydrated in ethanol 70% and 100% (2 minutes each) and immersed in xylene for 2 minutes (x2). Finally, slides were cover slipped using CV mounting media. Brightfield microscopy was used to visualize sections at 20X magnification.

4.2.5 Image analysis

For placental morphology analysis, all tissue samples were imaged at 20X magnification with an Olympus BX43 upright microscope. Three tissue sections per participant were imaged and three independent images per tissue section were acquired. All samples were blinded prior to image acquisition and analysis and images were imported into Image J - Fiji software. Villous maturity (characterised

by quantification of number of terminal villi and the number of intermediate villi), number of syncytial knots number and hypoplasia (measured by quantifying the intervillous space between five representative villi within each image and calculating the mean distance (μm)) were determined for each placental tissue sample. Characterisation of villi as either intermediate (diameter of 80-400μm characterized by a condensed fibrous stroma containing central arteries and veins with a thick smooth muscle wall) or terminal (diameter of 30-80μm with a high degree of capillarization and highly dilated sinusoids) villi was based on "Histology of Human Placenta" by Avagliano et al. (427).

4.2.6 Immunoassays

A variety of immunoassays were used in the quantification of placental and omental mediators in explant supernatants from GDM (n=10) and NGT (n=10) participants respectively. The oxidative stress marker 8-OHdG, was measured in placental explant culture supernatant using the 8-hydroxy 2 deoxyguanosine (8-OHdG) ELISA kit (Abcam ab201734). Placental adiponectin, adipsin, leptin and resistin were measured in explant culture supernatant using the LEGENDplex Human Metabolic Panel 1 (BioLegend 740212). Cytokine panel including IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33 were measured in placental explant culture supernatant using the LEGENDPlex Human Inflammation Panel 1 kit (BioLegend 740809). IL-1β and IL-18 in serum samples from NGT (n=20) and GDM (n=20) participants and omental explant culture supernatant were measured using the LEGENDPlex Human Inflammation Panel 1 kit (BioLegend 740809). For *ex vivo* drug treatments, placental IL-18 and IL-1β were measured in explant culture supernatant using the Human Total IL-18/IL-1F4 Quantikine ELISA kit (R&D/Bio-Techne Ltd DL180) and human IL-1β/IL-1F2

Quantikine ELISA kit (R&D/Bio-Techne Ltd DLB50). All assays were performed as per the manufacturer protocols.

4.2.7 Isolation of leukocytes from placental tissue

Placental tissue samples were initially stored in cold tissue preservative buffer until processing. Tissue preservative buffer consisted of 154mM NaCl, 1.6mM $CaCl_2$, 10mM HEPES, 250mM sucrose, 5.4mM K_3PO_4, 1.2mM $MgCl_2$ in sterile, distilled water. Tissue was washed with PBS to remove excess blood. Tissue samples were weighed in a clean petri dish and finely minced using a surgical scalpel ensuring removal of residual blood clots and connective tissue. Homogenised tissue was placed in a clean 50ml falcon tube containing 20mL DMEM/F-12 (Gibco 31330038), 10% FBS (heat-inactivated) (Gibco 16140071), 1% penicillin-streptomycin (10,000 U/mL) (Gibco 15140122), 0.2% collagenase type I (Sigma C0130), and 0.02% DNAse I (Sigma D5025). Tissue samples were incubated in a water bath at 37°C for 1 hour, with agitation every 15 minutes to facilitate enzymatic digestion. Tissue suspensions were then filtered into a clean 50ml falcon tube through one layer of gauze (Steroswab 1905/SF). The tube used for digestion was flushed with 20ml PBS and this eluate was also passed through the gauze layer. The filtrate was centrifuged at 300g for 5 minutes and the resultant cell pellet was re-suspended in 5mL PBS.

This mixture was then overlaid onto 10mL Histopaque (6.2ml Histopaque 1119 overlaid by 3.8ml Histopaque 1077) in a 50ml falcon tube followed by centrifugation at 700g (room temperature) for 30 minutes, with reduced acceleration and deceleration. Cells at the interface (white cloudy band) were collected with a pasteur pipette and washed in 5ml PBS. This suspension was passed through a 70µm cell strainer (Sigma CLS431751) to remove cells aggregates. The resultant suspension

was pelleted by centrifugation at 300g for 5 minutes. The supernatant was discarded and the cell pellet was stained for flow cytometry analysis.

4.2.8 Flow cytometry analysis

Following isolation of immune cells from placental tissue, single cell suspensions were kept on ice until ready for staining. Approximately $1x10^6$ cells of the single cell suspension were placed in 5ml round-bottomed tubes (Falcon 352008) and centrifuged at 300g for 5 minutes and supernatants were discarded. Cell pellets were incubated with 50µl FC blocking buffer (10% human serum (heat-inactivated) (Sigma S1-M) in 1x PBS (without Ca^{2+}/Mg^{2+})(Sigma P4417)) containing Fixable Viability Dye eFluor™ 455UV (eBioscience 65-0868-14) (1:1000) on ice for 10 minutes. 50µl of the antibody mix containing CD14 (eBioscience 25-0149-42) (61D3, 1:20), CD86 (BioLegend 374214) (BU63, 1:20), CD206 (eBioscience 48-2069-42) (19.2, 1:20), MitoTracker DeepRed (live cell mitochondrial stain) (Invitrogen M22426) (50nM) and MitoSOX Red (mitochondrial superoxide stain) (Invitrogen M36008) (5µM) was added and the cells were incubated for a further 20 minutes on ice. Cell suspensions were then washed with 2ml FACS buffer (EDTA 2mM (Sigma E6758), 2% FBS (heat-inactivated)(Gibco 16140071) in 1x PBS (without Ca^{2+}/Mg^{2+})) and centrifuged at 300g for 5 minutes. The supernatant was discarded and 100µl of fixation buffer (PFA 4% (Sigma 158127) in 1x PBS (without Ca^{2+}/Mg^{2+})) was added. Cell suspensions were incubated for 30 minutes at room temperature in the dark and then washed with 2ml PBS and centrifuged at 300g for 5 minutes. Supernatants were discarded. Finally, stained cell suspensions were resuspended in 150µl PBS and samples were stored at 4°C in the dark until acquisition.

Samples were analysed within 24 hours on a BD LSR II flow cytometer. Rainbow Calibration particles (BioLegend 422903) were used to standardise instrument settings for longitudinal analysis. Data was analysed using FlowJo (v10.8.1) according to the following initial gating strategy: singlet cells (as determined by FSC-H/FSC-A), leukocytes (as determined by SSC-A/FSC-A), live cells (as determined by live/dead stain), monocytes/macrophages (defined as $CD14^+$), M1-like monocytes/macrophages (defined as $CD14^+CD86^+CD206^-$), M2-like monocytes/macrophages (defined as $CD14^+CD86^+CD206^+$), cells containing superoxide-producing mitochondria (defined as $MitoTracker^+MitoSOX^+$). Gates were set according to unstained control samples. Cell proportions were quantified as a percentage of their parent gate e.g. $CD14^+$ as a percentage of live cells, $CD14^+CD86^+CD206^-$ and $CD14^+CD86^+CD206^-$ cells as a percentage of $CD14^+$ cells and $MitoSOX^+$ cells as a percentage of $MitoTracker^+$ cells.

4.2.9 TLR9 activation assay

HEK-Blue hTLR9 cells (InvivoGen #hkb-htlr9) were used to determine if placental secreted mediators activate TLR-9 signalling. HEK-Blue hTLR9 cells were seeded at $4x10^5$ cells/well in a 96-well plate and cultured overnight in growth medium containing DMEM, 4.5 g/l glucose, 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml Normocin™, 2 mM L-glutamine. Following a media change, adherent cells were incubated for 24 hours with 5% placental tissue explant culture supernatant from NGT (n=10) and GDM (n=10) participants. 5% supernatant treatment was selected following preliminary experiments with 1%, 3% and 5% supernatant treatments, where only 5% treatment was sufficient to consistently stimulate activation above untreated control. ODN 2006 (InvivoGen #tlrl-2006) was used as a positive control and sterile distilled water

was used as a negative control. HEK-Blue hTLR9 cell supernatants were subsequently incubated with Quanti-Blue detection medium (InvivoGen) and the NF-κB-induced SEAP activity was read on a Tecan Spark spectrophotometer at a wavelength of 620 nm.

4.2.10 Protein isolation and immunoblotting

Placental and omental tissue samples (50mg ± 5mg) were incubated in T-PER™ Tissue Protein Extraction Reagent (Thermo Scientific 78510) supplemented with phosphatase inhibitor (Roche, PhosSTOP™) and protease inhibitor (Roche) and homogenized for 3x 20 seconds using a TissueLyser II (Qiagen). Isolated protein (30µg) samples and protein ladder (Thermo Scientific 26634) were separated by SDS-PAGE on a 7.5% Tris-Glycine polyacrylamide gel and transferred to a methanol activated PVDF membrane (Amersham™ Hybond® Sigma-Aldrich). Membranes were blocked in 5% BSA for 1 hour at room temperature and incubated overnight at 4°C with the following primary antibodies: NLRP3 1:1000 (Novus/Bio-Techne 12446), caspase-1 1:500 (Novus/Bio-Techne 45433), ASC 1:500 (Novus/Bio-Techne 78977) and GAPDH 1:1000 (Cell Signalling #97166). After overnight incubation membranes were washed and incubated at room temperature for 1 hour with secondary HRP-linked antibody, either anti-rabbit 1:2000 (Cell Signalling #7074) or anti-mouse 1:2000 (Santa Cruz 516102). Membranes were washed in 0.1% TBS-T and developed using the Pierce™ ECL Western Blotting Substrate (Thermo Scientific 32209) and LICOR Odyssey image analyser. Densitometry was performed using LICOR Image Studio Lite Version 5.2. All densitometry values were normalised by relative GAPDH expression.

4.2.11 Statistics

Analysis was performed using GraphPad Prism 5.03. Where applicable, data is represented as mean ± standard deviation for parametric analysis and median [95% confidence interval] for non-parametric analysis. Where possible, GDM cases were matched with NGT controls by maternal age (± 3 years) and BMI (± 3 kg/m^2 units). P values < 0.05 were considered as statistically significant. For statistical comparisons, either the Student's t-test or the Mann-Whitney U test was used depending on data normality and test suitability. Wilcoxon matched-pairs signed rank test was used to compare matched samples. The Benjamini–Hochberg method with a 20% false discovery rate was used for multiple hypothesis testing in cytokine (n=13) panels to decrease the false discovery rate. Grubbs's test was used to detect outliers in data sets.

4.3. Results

4.3.1 Placental villous immaturity in GDM

It has been previously reported that placental morphology is altered in GDM, which may stem from glucose-induced excessive placental growth at the expense of villous maturation (134). Placental villous immaturity results in a decreased fetal reserve to withstand stressors such as transient hypoxia in the later stages of pregnancy, thereby increasing the risk of stillbirth (428). H&E staining was used to compare the placental morphology from NGT and GDM participants in our study cohort (**Figure 4.2A**). There were no significant differences in intervillous space between villi in NGT versus GDM placental tissues (22.52µm [10.89, 50.53] vs. 30.18µm [20.33, 37.34], p=0.84) (**Figure 4.2B**) or in the number of terminal villi (12.67 [9.74, 16.93] vs. 9.0 [3.78, 13.81], p=0.09) (**Figure 4.2C**). However, there was a significant increase in the number of intermediate villi (10.0 [7.24, 13.16] vs. 15.0 [11.72, 16.52], p=0.047) (**Figure 4.2D**) and the intermediate/terminal villi ratio (0.71 [0.48, 1.09] vs. 1.67 [0.65, 3.32], p=0.016) (**Figure 4.2E**) in GDM placental tissue. Finally, there were no significant differences in the number of syncytial knots between study groups (8.67 [3.73, 16.66] vs. 9.0 [5.06, 13.32], p=1.00) (**Figure 4.2F**).

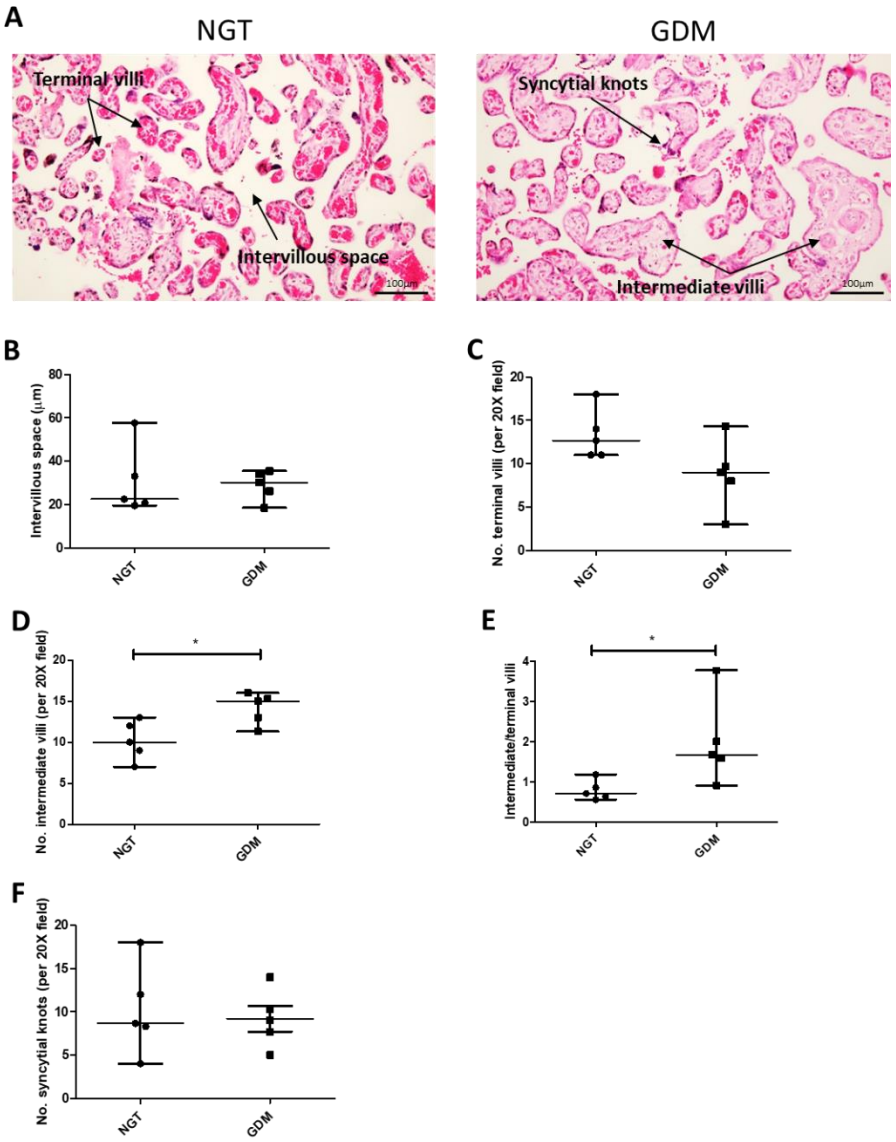


Figure 4.2. Reduction in placental villi maturation in GDM. (A) H&E staining was used to assess the placental morphology from maternal age and BMI-matched NGT (n=5) and GDM (n=5) participants. Placental features including **(B)** intervillous space, **(C)** terminal villi number, **(D)** intermediate villi number, **(E)** intermediate/terminal villi ratio and **(F)** number of syncytial knots were measured. Wilcoxon matched-pairs signed rank test was used to compare matched samples. Significance levels are shown as: * $p<0.05$.

4.3.2 Macrophage phenotype is not significantly altered in placental tissue in GDM

Placental macrophages are a heterogenous population of immune cells, present throughout gestation, that are essential for maintenance of a healthy placental environment and host defence against infection (429). Previous evidence has shown that the placental macrophage phenotype is altered in GDM, with an increase in macrophage activation but somewhat ambiguous findings regarding M1/M2 polarity (214, 260-263). Endogenous and infiltrating $CD14^{+}CD86^{+}CD206^{-}$ cells (M1-like macrophages) and $CD14^{+}CD86^{+}CD206^{+}$ cells (M2-like macrophages), were quantified by flow cytometry in the placental tissue of NGT and GDM participants. Gating of cell populations was performed as described in section 4.2.8 (**Figure 4.3A**). There were no significant differences in cell proportions of $CD14^{+}CD86^{+}CD206^{-}$ cells (88.9% [86.2, 92.2] vs. 89.65% [82.35, 94.12], p=0.96) (**Figure 4.3B**) or $CD14^{+}CD86^{+}CD206^{+}$ cells (10.95% [7.65, 13.74] vs. 10.36% [6.1, 16.83], p=0.59) (**Figure 4.3C**) between NGT and GDM placental tissues. The M1/M2 ratio was also not significantly different between NGT and GDM participants (8.15 [6.84, 12.54] vs. 8.69 [5.07, 15.35], p=0.83) (**Figure 4.3D**).

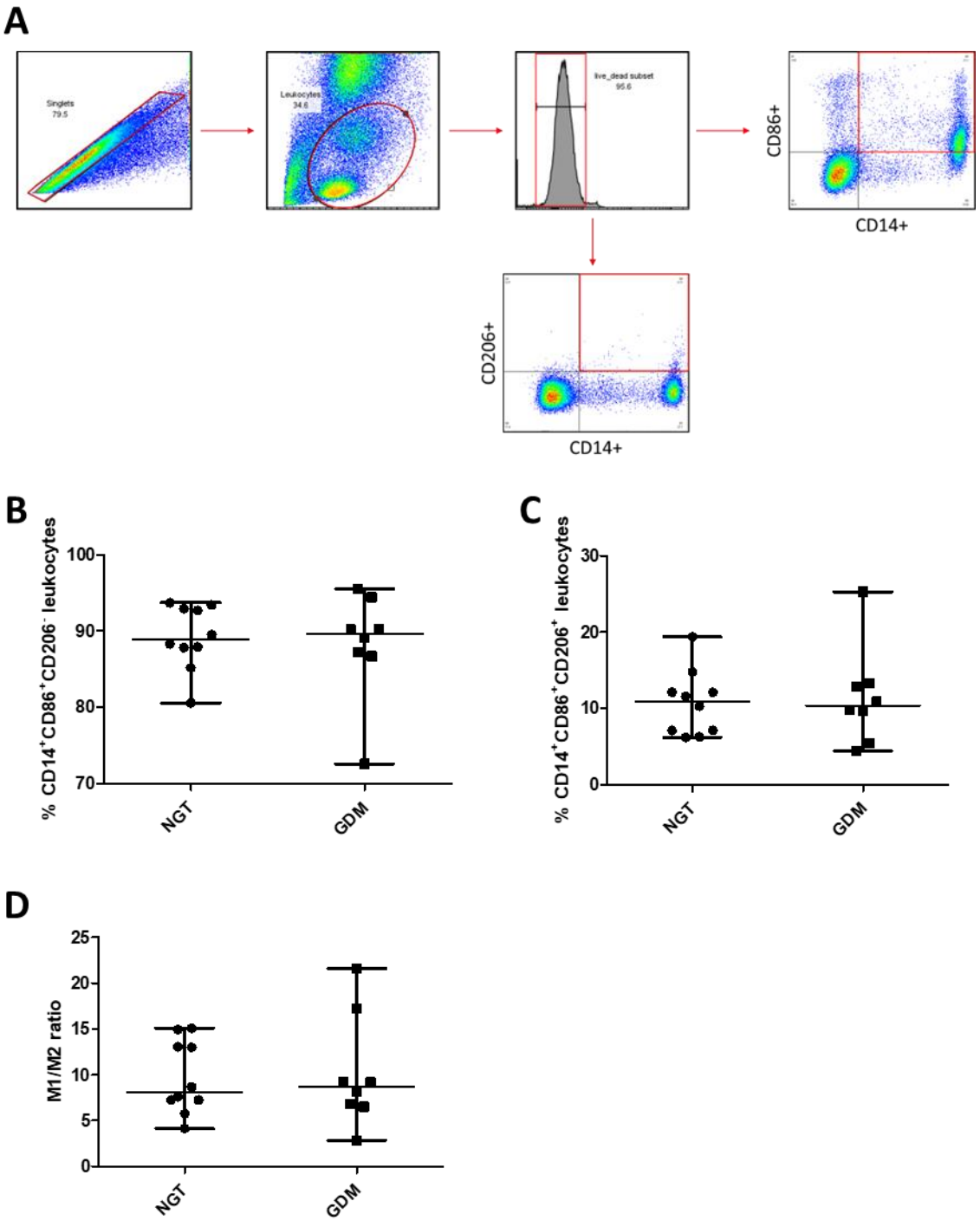


Figure 4.3. Macrophage phenotype in placental tissue in GDM.

Monocyte/macrophage $CD14^+$ cell phenotype was characterised in NGT (n=10) and GDM (n=8) placental tissue samples. **(A)** Representative graphs are shown for $CD14^+CD86^+CD206^-$/$CD14^+CD86^+CD206^+$ flow cytometry gating strategies. Cell proportions, as a percentage of total $CD14^+$ cell count, are shown for **(B)**

$CD14^+CD86^+CD206^-$ cells and **(C)** $CD14^+CD86^+CD206^+$ cells. **(D)** M1/M2 ratio of macrophage populations for NGT and GDM participants. The Mann-Whitney U test was used to compare cell proportion differences between groups.

4.3.3 Generation of mitochondrial superoxide by placental macrophages is significantly increased in GDM

Mitochondrial dysfunction has previously been reported in placental tissue in GDM, with reduced respiratory capacity, increased SOD activity and reduced citrate synthase activity (346). To investigate if the placental M1-like and M2-like macrophage populations identified in Figure 4.3 contribute to mitochondrial dysfunction, mitochondrial-specific superoxide production was measured in macrophage populations in NGT and GDM participants by flow cytometry. Macrophages (defined as $CD14^+$), M1-like macrophages ($CD14^+CD86^+CD206^-$) and M2-like macrophages ($CD14^+CD86^+CD206^+$) were analysed for mitochondria-specific superoxide production ($MitoTracker^+MitoSOX^+$) (**Figure 4.4A**).

Both $CD14^+MitoSOX^+$ cells (24.7% [15.77, 29.66] vs. 35.35% [23.74, 70.84], p=0.02) (**Figure 4.4B**) and M1-like macrophages $CD14^+CD86^+CD206^-MitoSOX^+$ cells (22.85% [14.6, 29.92] vs. 33.35% [20.83, 69.67], p=0.03) (**Figure 4.4C**) had increased production of mitochondrial superoxide in GDM placental tissue. There was no significant difference in mitochondrial superoxide production in M2-like macrophages $CD14^+CD86^+CD206^+MitoSOX^+$ cells (53.7% [29.7, 63.98] vs. 57.15% [46.53, 84.4], p=0.13) (**Figure 4.4D**) between study groups. This data demonstrates that the hyperglycaemic placental microenvironment in GDM increases macrophage mROS production, specifically in the pro-inflammatory M1-like phenotype, which may influence placental inflammation in GDM.

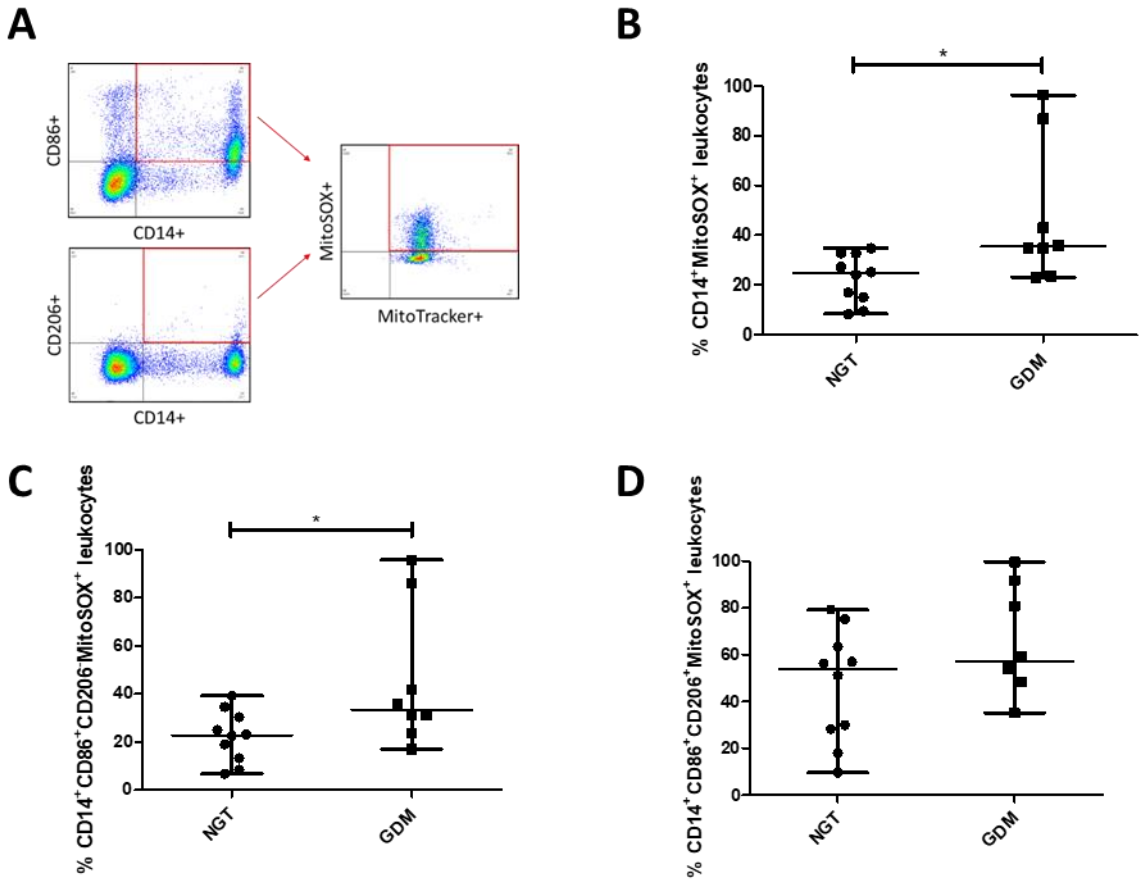


Figure 4.4. Increased mitochondrial superoxide production in $CD14^+$ and $CD14^+CD86^+CD206^-$ placental macrophages in GDM. Mitochondrial superoxide production was quantified in placental macrophage populations in NGT (n=10) and GDM (n=8). **(A)** Representative graphs are shown for $MitoTracker^+MitoSOX^+$ flow cytometry gating strategies. Population frequencies are shown for **(B)** $CD14^+MitoSOX^+$ cells, **(C)** M1-lke $CD14^+CD86^+CD206^-MitoSOX^+$ cells, **(D)** M2-like $CD14^+CD86^+CD206^+MitoSOX^+$ cells. The Mann-Whitney U test was used to compare cell proportion differences between groups. Significance levels are shown as: * $p<0.05$.

4.3.4. Pervasive oxidative damage is not evident in placental tissue in GDM

Given that placental macrophages produce increased mitochondrial superoxide, we then investigated if placental oxidative damage was prevalent throughout placental

tissue in GDM. 8-hydroxy-2-deoxy Guanosine (8-OHdG) is a product of oxidative stress-induced DNA damage resulting from increased reactive oxygen and nitrogen species (430). Increased placental production of 8-OHdG has been associated with pregnancy-induced hypertension (PIH) and lower gestational age at delivery (431). However, there were no significant differences in placental secretion of 8-OHdG in NGT compared to GDM (6.14ng/ml [3.29, 15.25] vs. 6.4ng/ml [4.2, 9.04], p=0.68) (**Figure 4.5**).

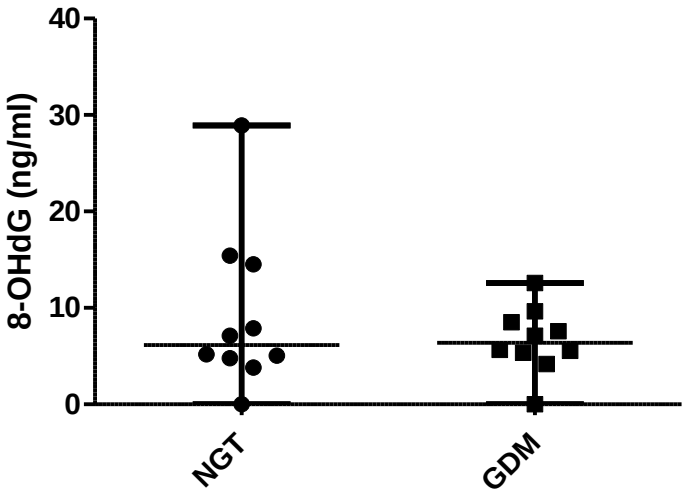


Figure 4.5. Oxidative stress-induced damage is not increased in placental tissue in GDM. 8-OHdG were measured in the supernatant of NGT (n=10) and GDM (n=10) placental explant cultures. Mann-Whitney U test was used to compare 8-OHdG levels differences between cohort groups.

4.3.5 Placental-derived mediators do not trigger TLR-9 activation

Mitochondrial dysfunction can result in the release of mtDNA which may induce inflammation by activating the TLR-9 pathway (432). Placental-derived cf-mtDNA has been implicated in systemic maternal TLR-9 activation and subsequent vascular dysfunction in PE (433). As we have established that cf-mtDNA is significantly

increased in maternal plasma in GDM in Chapter 2 and given that approximately 5-20% of circulating cf-DNA is derived from the placenta, we investigated if placental-derived mitochondrial mediators directly activate the TLR-9 receptor. HEK-TLR9 cells were incubated with 5% placental explant supernatant from NGT and GDM. Similar to Figure 2.6G, there was equally no significant difference in TLR-9 activation between study groups (1.11 [0.87, 1.59] vs. 1.31 [0.92, 1.58], p=0.97) (**Figure 4.6**).

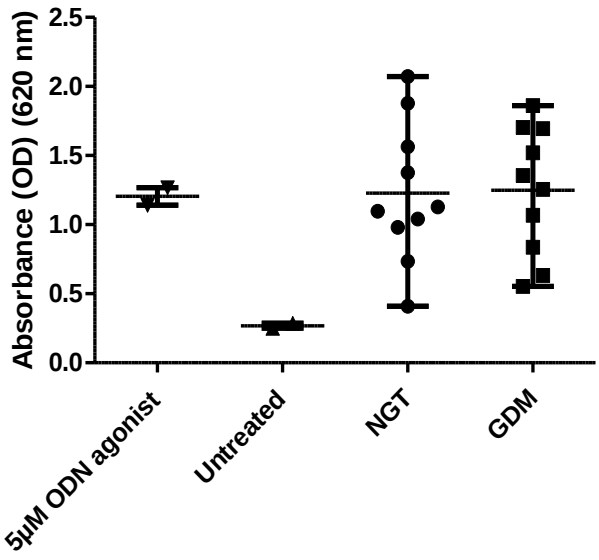


Figure 4.6. Placental derived mediators do not alter TLR9-induced inflammatory signalling in GDM. HEK-TLR9 activation was measured in response to incubation with 5% placental explant supernatant from NGT (n=10) and GDM (n=10). Mann-Whitney U test was used to compare the mean of TLR-9 activation between groups.

4.3.6 Placental expression of NLRP3 inflammasome cascade members are not altered in GDM

An alternative pathway by which mitochondrial dysfunction may incite inflammation is via mROS activation of the NLRP3 inflammasome. Activation of the NLRP3 inflammasome, particularly in macrophages, leads to exaggerated local and systemic inflammation, specifically the increased production of pro-inflammatory cytokines IL-1β and IL-18 (434). Recent *in vivo* murine studies have shown that obesity-induced NLRP3 activation, with subsequent increases in IL-1β and IL-18 cytokines, impairs insulin sensitivity and glucose homeostasis (435).

To investigate if the increased production of mROS by endogenous and infiltrating macrophages in placental tissue activates the NLRP3 inflammasome in GDM, protein expression of members of the inflammasome cascade, namely NLRP3, ASC and pro-caspase-1, were measured in maternal age and BMI-matched NGT and GDM placental tissue (**Figure 4.7A**). Densitometric analysis showed no significant difference in NLRP3 (0.079 [0.05, 0.11] vs. 0.072 [0.04, 0.12], p=0.74) (**Figure 4.7B**), ASC (0.24 [0.13, 0.74] vs. 0.36 [0.21, 0.63], p=0.64) (**Figure 4.7C**) or pro-capsase-1 (0.04 [0.02, 0.06] vs. 0.043 [0.03, 0.05], p=0.74) (**Figure 4.7D**) protein expression between NGT and GDM placental tissue.

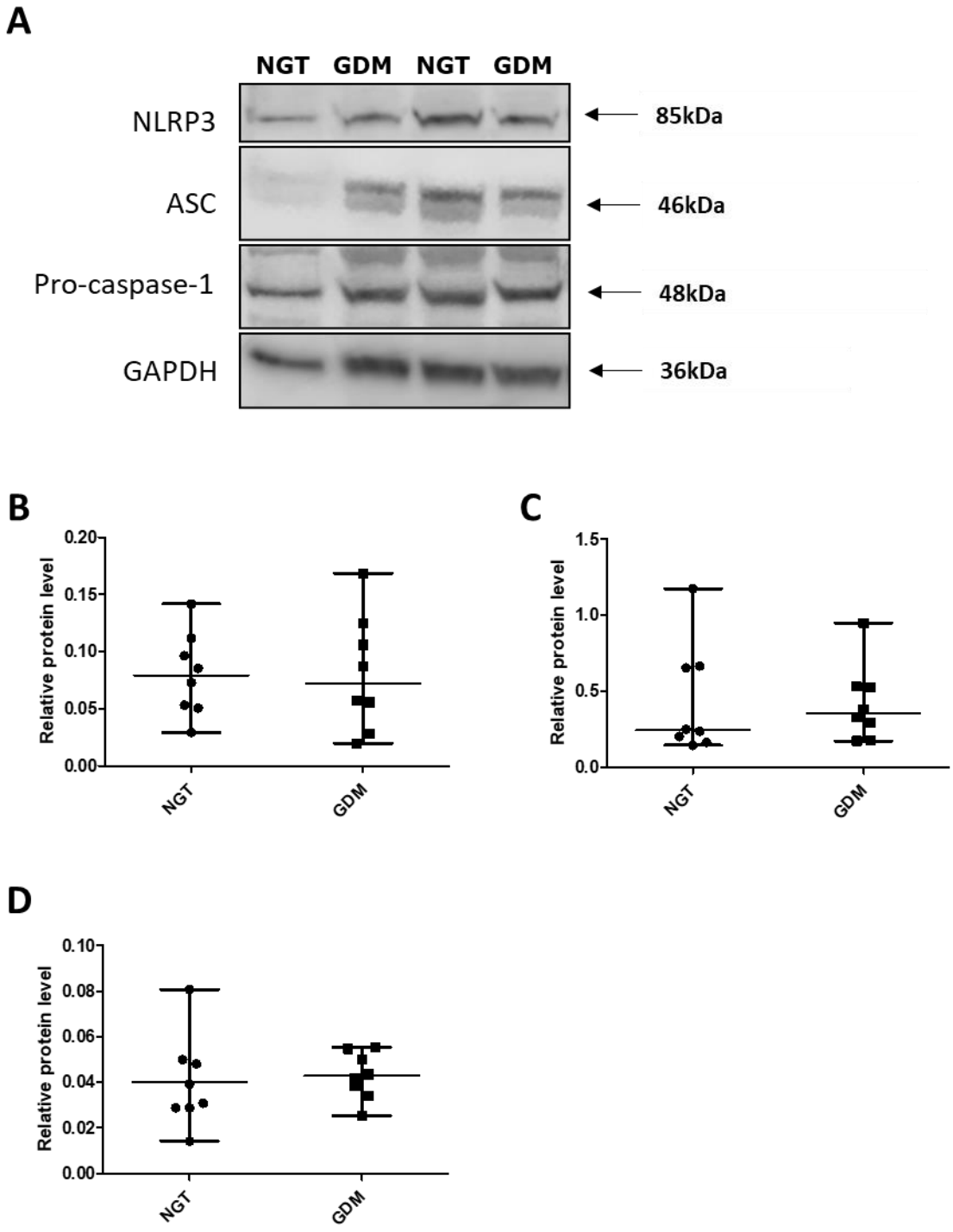


Figure 4.7. Placental expression of the NLRP3 inflammasome cascade in GDM. **(A)** Representative images of NLRP3, ASC and pro-caspase-1 protein expression as measured by Western blotting in placental tissue from NGT (n=8) and GDM (n=8), matched by maternal age and BMI. Densitometric analysis was used to quantify expression of **(B)** NLRP3, **(C)** ASC and **(D)** pro-capsase-1, with expression

normalised to GAPDH. Wilcoxon matched-pairs signed rank test was used to compare matched samples.

4.3.7 Increased placental production of IL-18, a NLRP3-related cytokine, in GDM

Activation of the NLRP3 inflammasome increases secretion of pro-inflammatory cytokines IL-1β and IL-18 (413). We next determined the placental production of IL-18 and IL-1β cytokines in explant culture supernatants from both study groups. While there was no significant increase in IL-1β (939.7pg/ml [443.8, 1221.0] vs. 336.2pg/ml [117.0, 1170], p=0.28) (**Figure 4.8A),** IL-18 (127.9pg/ml [89.75, 171.7] vs. 65.15pg/ml [39.84, 111.1], p=0.04) (**Figure 4.8B**) was significantly increased in placental supernatants in GDM relative to NGT. To determine if the placental increase in IL-18 in GDM was mediated via the NLRP3 inflammasome, GDM placental explants were treated with 1μM MCC950 (a direct inhibitor of the NLRP3 inflammasome). MCC950 significantly reduced the secretion of IL-1β (252.8pg/ml [144.1, 333.3] vs. 183.7pg/ml [81.25, 271.1], p=0.005) (**Figure 4.8C**) and IL-18 (13.95pg/ml [11.23, 20.76] vs. 8.12pg/ml [6.44, 10.13], p=0.0005) (**Figure 4.8D**) in GDM placental tissue explant cultures. Although placental expression of the NLRP3 inflammasome cascade is not altered in GDM (**Figure 4.6**), there is significantly higher levels of NLRP3-mediated IL-18 secretion from GDM placental tissue. This somewhat conflicting evidence may indicate that despite the lack of a significant induction of members of the NLRP3 inflammasome cascade, a threshold for activation appears to have been achieved which resulted in increased IL-18 release from GDM placental tissue.

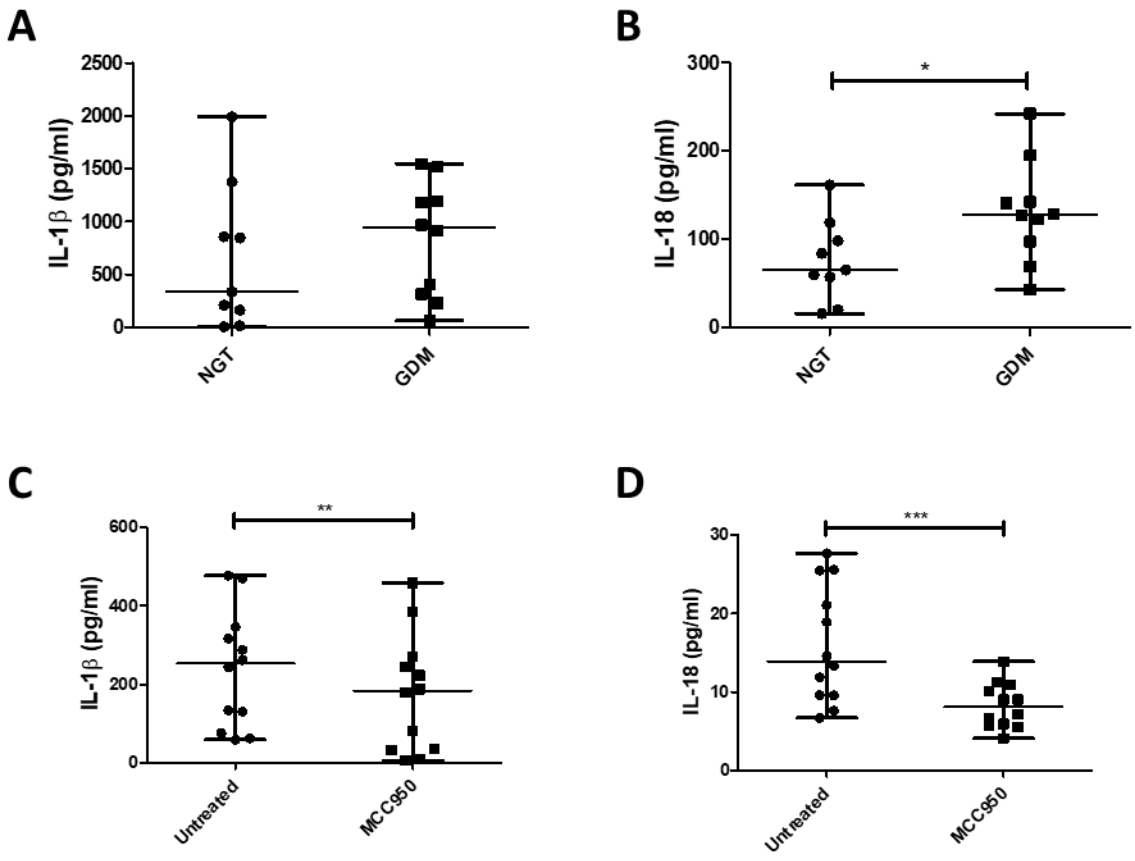


Figure 4.8. Placental secretion of NLRP3-related cytokines IL-1β and IL-18.

(A) IL-1β and **(B)** IL-18 levels were quantified in placental explant culture supernatant from NGT (n=10) and GDM (n=10) participants. GDM placental explants (n=12) were treated with 1μM MCC950 for 24 hours. Placental secretion of **(C)** IL-1β and **(D)** IL-18 was quantified. The Mann-Whitney U test was used to compare cytokine level differences between study groups. Wilcoxon matched-pairs signed rank test was used to compare treatment groups. Data was normalized to tissue weight. Grubb's test was used to remove statistical outliers from figure A and B. Significance levels are shown as: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.3.8 ERG significantly attenuates NLRP3-related cytokine release from placental tissue in GDM

It has previously been shown that antioxidant therapy can reduce NLRP3-mediated inflammation, through alleviating excessive mitochondrial dysfunction and subsequent ROS generation (436). Given the increase in placental mROS production and IL-18 release in GDM, we examined the effect of mitochondrial antioxidants on NLRP3-related cytokine production in GDM placental explants. ERG (1mM) significantly reduced both IL-18 (10.06pg/ml [7.75, 14.81] vs. 13.95pg/ml [11.23, 20.76], p=0.007) (**Figure 4.9A**) and IL-1β (215.9pg/ml [112.1, 297.7] vs. 252.8pg/ml [144.1, 333.3], p=0.04) (**Figure 4.9B**) in GDM placental tissue explants. However, MitoQ (1μM) did not significantly alter the production of either IL-18 (13.51pg/ml [10.81, 21.27] vs. 11.62pg/ml [9.49, 17.07], p=0.20) or IL-1β (241.7pg/ml [147.3, 366.1] vs. 232.0pg/ml [116.5, 285.6], p=0.09), relative to DMSO control in GDM placental tissue explants.

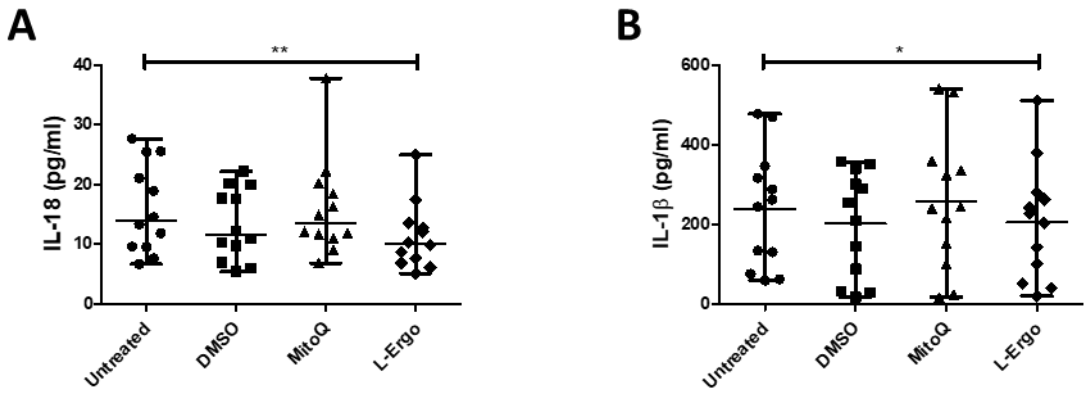


Figure 4.9. ERG dampens placental production of pro-inflammatory cytokines IL-18 and IL-1β in GDM. GDM placental explant tissue cultures (n=12) were treated with 1μM MitoQ and 1mM ERG for 24 hours. Placental secretion of **(A)** IL-18 and **(B)** IL-1β was quantified. Wilcoxon matched-pairs signed rank test was used

to compare treatment groups. Data was normalized to tissue weight. Significance levels are shown as: * $p<0.05$, ** $p<0.01$.

4.3.9 Omental expression of NLRP3 inflammasome cascade members are not altered in GDM

Given the evidence that activation of the NLRP3 inflammasome cascade can equally disrupt insulin signalling and induce insulin resistance in VAT in T2DM (435), we next elucidated if NLRP3 inflammasome cascade expression was altered in GDM. Expression of NLRP3, ASC and pro-caspase-1 were measured in maternal age and BMI-matched visceral omental tissue from NGT and GDM participants (**Figure 4.10A**). Densitometric analysis showed no significant difference in protein expression of NLRP3 (0.22 [0.03, 0.53] vs. 0.19 [0.06, 0.57], p=0.74) (**Figure 4.10B**), ASC (0.26 [0.13, 0.73] vs. 0.3 [0.22, 0.46], p=0.84) (**Figure 4.10C**) or pro-caspase-1 (0.61 [0.38, 0.78] vs. 0.63 [0.38, 0.89], p=0.95) (**Figure 4.10D**) between NGT and GDM omental tissue.

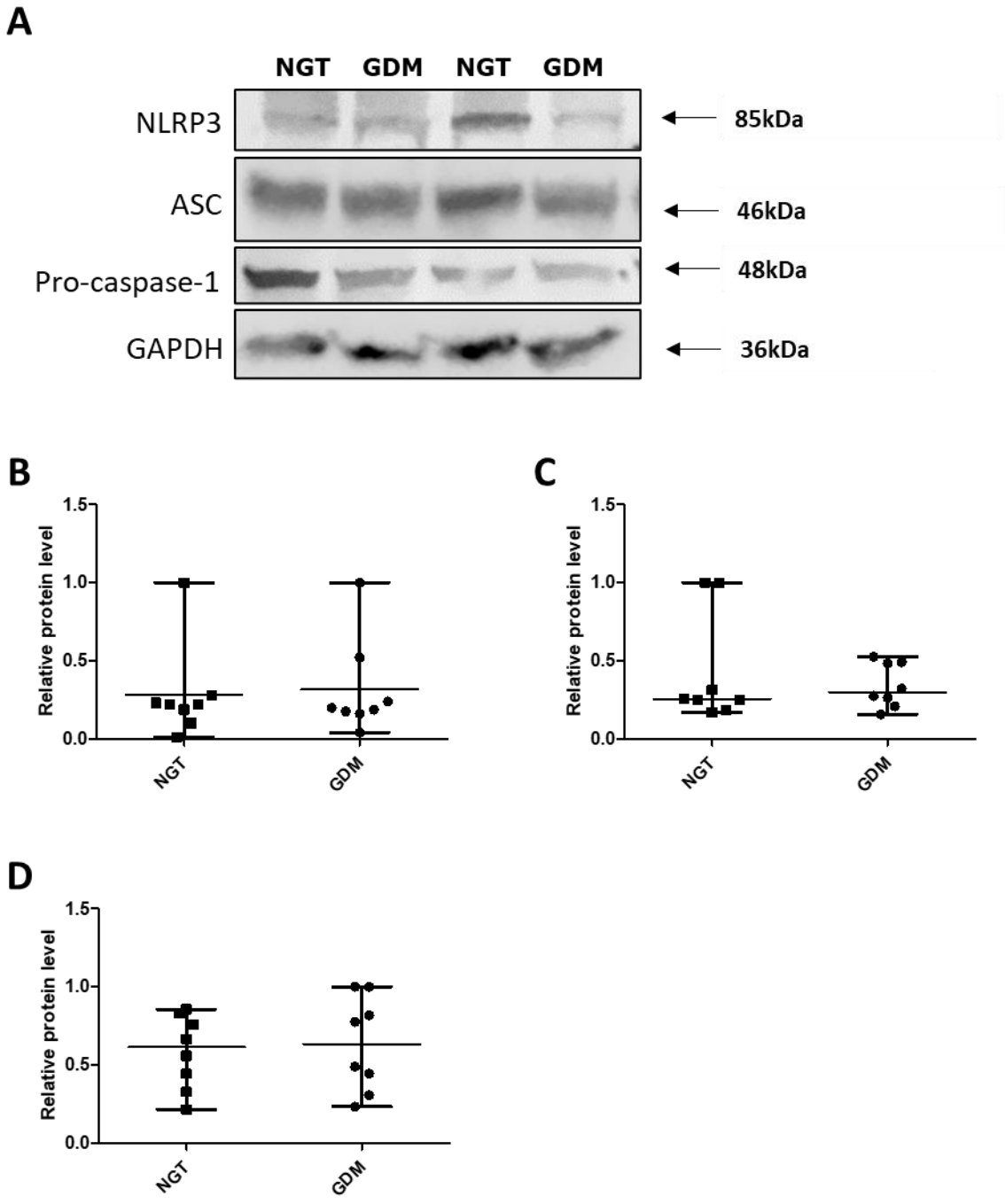


Figure 4.10. Expression of the NLRP3 inflammasome cascade in adipose tissue in GDM. (A) Representative images of NLRP3, ASC and pro-caspase-1 protein expression as measured by Western blotting in omental tissue from NGT (n=8) relative to GDM (n=8), matched by maternal age and BMI. Densitometric analysis was used to quantify expression levels of **(B)** NLRP3, **(C)** ASC and **(D)** pro-caspase-

1, with expression normalised to GAPDH. Wilcoxon matched-pairs signed rank test was used to compare matched samples.

4.3.10 Circulating and omental production of NLRP3-related cytokines IL-1β and IL-18 are not significantly increased in GDM

To investigate if the localised placental increase in NLRP3-related cytokines was mirrored systemically in circulation and locally in VAT, IL-18 and IL-1β were measured in serum and omental adipose tissue explant cultures. These cytokine levels were measured as part of the panel of 13 inflammatory cytokines described in Chapter 2 and 3. As described previously, there were no significant differences in circulating IL-18 (978.7pg/ml ± 514.5pg/ml vs. 847.6pg/ml ± 407.4pg/ml, p=0.39) (**Figure 4.11A**) or IL-1β (29.0pg/ml ± 25.7pg/ml vs. 21.8pg/ml ± 16.0pg/ml, p=0.33) (**Figure 4.11B**) in NGT compared to GDM. Furthermore, there was also no significant increase in IL-18 (244.7pg/ml [137.4, 306.7] vs. 286.4pg/ml [195.9, 494.3], p=0.35) (**Figure 4.11C**) or IL-1β (242.5pg/ml [134.8, 354.3] vs. 293.9pg/ml [178.6, 507.3], p=0.32) (**Figure 4.11D**) in omental adipose tissue explants between study groups. Overall, these results suggest that NLRP3-related inflammation is localised specifically to the placenta in GDM.

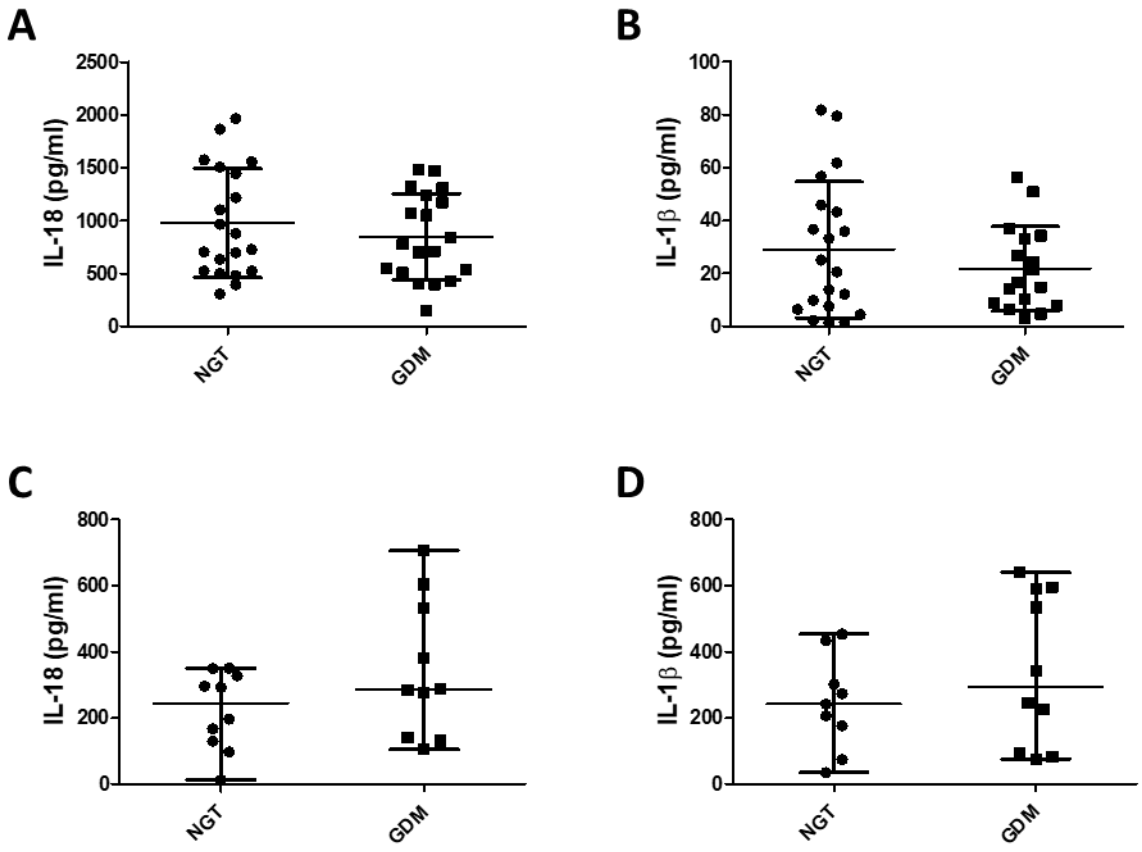


Figure 4.11. Circulating and omental production of NLRP3-related cytokines IL-18 and IL-1β. (A) IL-18 and **(B)** IL-1β levels were quantified in fasting serum samples from healthy NGT (n=20) and GDM (n=20) participants. **(C)** IL-18 and **(D)** IL-1β levels were quantified in omental explant culture supernatant from healthy NGT (n=10) and GDM (n=10) participants. Student's t-test was used to compare cytokine level differences between serum groups. The Mann-Whitney U test was used to compare cytokine level differences between omental explant groups. Grubb's test was used to remove statistical outliers from figure A and B.

4.3.11 Placental inflammation is significantly increased in GDM

Placental macrophages, particularly the M1-like population, substantially contribute to placental inflammation and generally increase in number as pregnancy progresses

(437). Although there was no significant increase in M1-like cells in GDM, this macrophage population produced significantly more mROS, which may impact their production of pro-inflammatory cytokines in the placental microenvironment. Furthermore, we have also provided evidence of systemic inflammation in GDM in Chapter 2, which may include a placental contribution. Using placental tissue explant cultures, 11 key cytokines, in addition to IL-1β (Figure 4.8A) and IL-18 (Figure 4.8B), were measured in NGT and GDM participants (**Table 4.1**). In addition to the increase in the placental production of IL-18 in Figure 4.8B, these findings provide further evidence of placental inflammation in GDM, with significantly elevated placental secretion of IL-6 and TNF-α.

Table 4.1. Placental tissue levels of pro-inflammatory cytokines in NGT and GDM participants.

Cytokine	**NGT (Median [95% CI])(pg/ml)**	**GDM (Median [95% CI])(pg/ml)**	**P value**
IL-6	17476 [10855, 33246]	44108 [27361, 80054]	0.028
TNF-α	11.35 [3.4, 16.97]	35.08 [17.96, 86.7]	0.008
IL-17A	1.4 [0.45, 2.48]	2.19 [0.22, 6.3]	0.57
IL-12p70	20.25 [9.35, 25.44]	22.31 [15.64, 43.1]	0.27
MCP-1	2739 [1469, 5086]	2873 [1979, 4900]	0.60
IFN-α2	5.48 [3.6, 8.8]	10.21 [6.46, 17.36]	0.05
IL-23	17.09 [7.22, 38.92]	42.06 [21.17, 71.98]	0.11
IL-10	53.28 [26.16, 107.0]	102.4 [51.07, 129.0]	0.37
IFN-ɣ	Not detected	Not detected	N/A
IL-8	59485 [23292, 108694]	64632 [41631, 112534]	0.4

IL-33	Not detected	Not detected	N/A

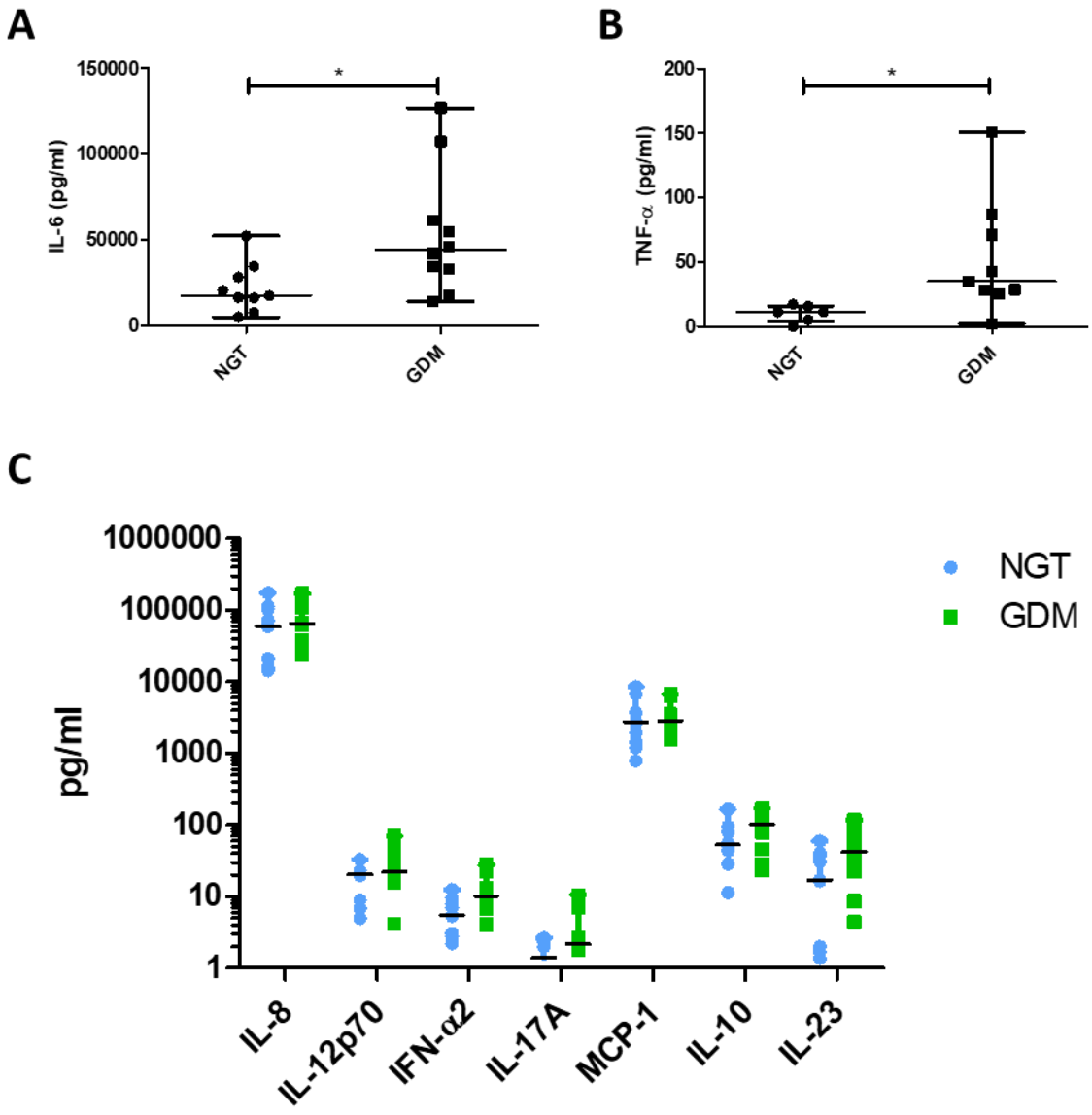


Figure 4.11. Increase in pro-inflammatory cytokines IL-6 and TNF-α in placental tissue in GDM. (A) IL-6, **(B)** TNF-α, **(C)** IL-8, IL-12p70, IFN-α2, IL-17A, MCP-1, IL-10 and IL-23 levels were quantified in placental explant culture supernatants from NGT (n=10) and GDM (n=10) participants. The Mann-Whitney U test was used to compare cytokine levels. Grubb's test was used to remove statistical outliers from figure A, B and C. The Benjamini–Hochberg method with a false

discovery rate of 20% was used for multiple hypothesis testing. Corrected significance levels are shown as: * $p<0.03$.

4.3.12 Dysregulation in placental adipokine production in GDM

In additional to inflammatory cytokines, adipokines and/or their receptors are expressed in placental tissue and contribute to the development of maternal insulin resistance (438). Additionally, in Chapter 2, we provided evidence of a significant reduction in the systemic levels of adiponectin in GDM participants. Four adipokines (leptin, adiponectin, resistin and adipsin) were measured in placental tissue explants from both study groups. Compared to NGT, there was a significant reduction in leptin (0.65ng/ml [0.31, 1.3] vs. 0.26ng/ml [0.11, 0.47], p=0.046) (**Figure 4.12A**) and a significant increase in adipsin (28.25ng/ml [25.35, 33.52] vs. 36.61ng/ml [31.15, 44.38], p=0.028) (**Figure 4.12B**) in GDM participants. There were no significant differences in the placental production of either adiponectin (0.57µg/ml [0.39, 1.07] vs. 0.65µg/ml [0.46, 0.89], p=0.905) (**Figure 4.12C**) or resistin (2.44ng/ml [1.02, 5.84] vs. 2.28ng/ml [1.02, 3.0], p=0.515) (**Figure 4.12D**) between study groups.

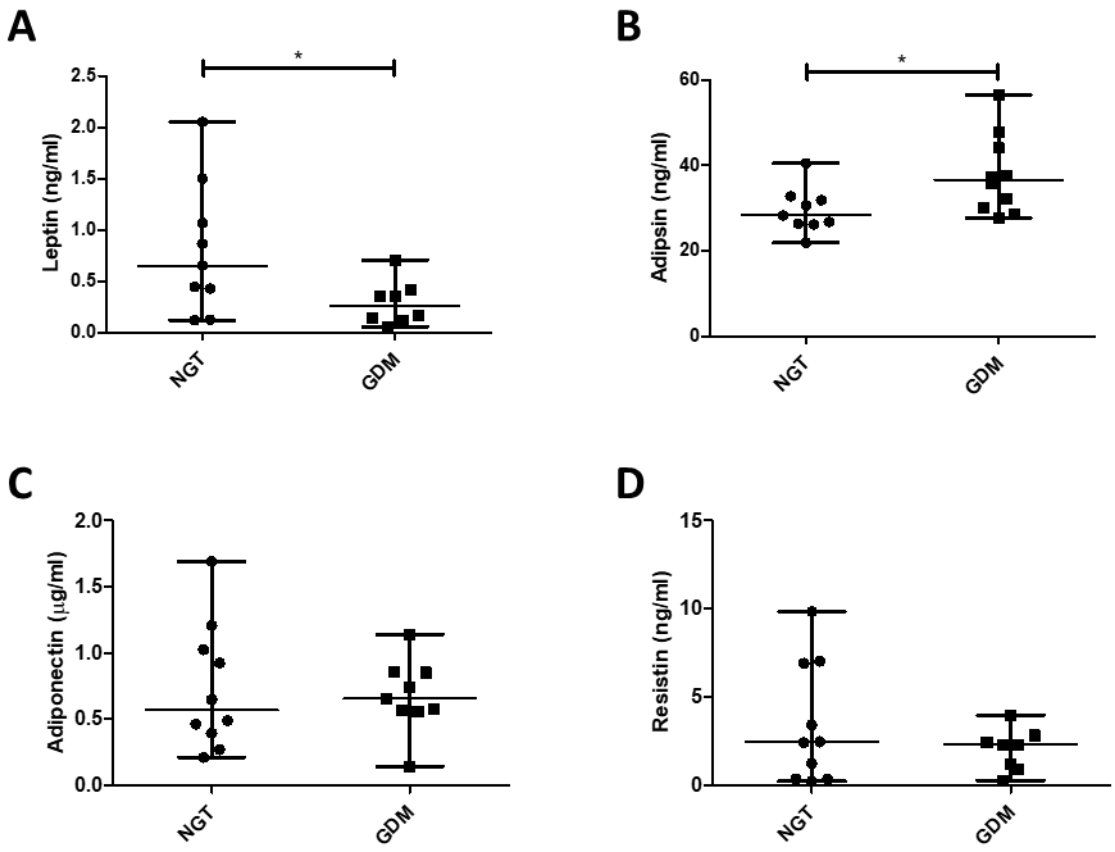


Figure 4.12. Placental adipokine dysregulation in GDM. Adipokine levels of **(A)** leptin, **(B)** adipsin, **(C)** adiponectin and **(D)** resistin were measured in placental tissue explants from NGT (n=10) and GDM (n=10). The Mann-Whitney U test was used to compare differences between groups. Grubb's test was used to remove statistical outliers from figure A, B, C and D. Significance levels are shown as: * $p<0.05$.

4.4 Discussion

In this chapter, we focus on how the placental microenvironment and placental-derived mediators influence systemic metabolic dysfunction in GDM. Primarily, we examined whether mitochondrial dysfunction induces placental inflammation by activating the TLR-9 and/or the NLRP3 inflammasome. In this study, we provide evidence that endogenous and infiltrating placental M1-like macrophages produce significantly increased levels of mitochondrial superoxide in GDM. Furthermore, we showed that increased placental inflammation in GDM is instigated in part by a specific subset of pro-inflammatory cytokines and adipokines. We also determined that the NLRP3 inflammasome may play a regulatory role in the cross-talk between mROS and placental inflammation in GDM. Finally, this study identified that mitochondrial antioxidants ameliorate placental inflammation in GDM.

Initially, we explored placental morphology in study participants. The main structural unit of the placenta are placental villi, which are essential for the exchange of oxygen and nutrients (136). In agreement with previous findings, villous immaturity characterised by a higher intermediate to terminal villi ratio was evident in placental tissue from GDM pregnancies compared to NGT pregnancies. These morphological changes are believed to stem from a hyperglycaemic and hyperinsulinaemic environment, which promotes excessive tissue growth at the expense of sufficient villous maturation (143). This placental pathology equally reduces the efficiency of maternal-fetal exchange and increases the risk of stillbirth, particularly in the third trimester (144). Hyperglycaemia, and a concurrent glucose stress response, induces ROS generation and oxidative stress through an osmotic effect on cells, which can offset endothelial dysfunction and microvascular insufficiency, as is evident in GDM placental terminal villi (439, 440).

To investigate if the hyperglycaemic milieu of GDM pregnancies results in localised placental oxidative damage, secretion of 8-OHdG was quantified in placental explant cultures. 8-OHdG is a marker of oxidative damage to DNA and has been frequently used for quantifying the extent of localised oxidative stress (430). In our study cohort, there was no significant difference in the release of 8-OHdG from NGT and GDM-complicated placental tissue.

Placental tissue macrophages are important homeostatic regulators of the placenta milieu, as well as providing host defence again infections. Recent findings have shown an increase in macrophage activation in GDM. This is largely defined by an increase in the surface expression of CD163 and increased secretion of sCD163, with ambiguous results on functionality and M1/M2 polarity (214, 260-263). Additionally certain pathologies may trigger a phenotypical switch towards pro-inflammatory M1 macrophage polarisation in HBCs (283). In this study, we assessed the abundance of endogenous and infiltrating $CD14^{+}CD86^{+}CD206^{-}$ (M1-like) and $CD14^{+}CD86^{+}CD206^{+}$ (M2-like) macrophage cells in placental tissue. We found no differences in the abundance of either population or in M1/M2 polarity ratio, between study groups. These findings are similar to the characterisation of these monocyte/macrophage populations in maternal circulation in GDM, where there were equally no changes in macrophage abundance but there was an overall increase in production of systemic inflammatory mediators.

Maternal hyperglycaemia has a significant impact on placental metabolism and mitochondrial function. Cytotrophoblasts isolated from GDM placental tissue samples show reduced oxidative phosphorylation, maximal mitochondrial respiratory capacity, lower ATP content and larger and more numerous lipid droplets (441). Furthermore, *in vitro* evidence has demonstrated that a hyperglycaemic

microenvironment can induce placental mitochondrial dysfunction and mROS generation (175). In the current study, we observed that in GDM the pro-inflammatory M1-like ($CD14^{+}CD86^{+}CD206^{-}$) macrophage population in the placenta produce significantly increased levels of mitochondrial superoxide.

Mitochondria are critical regulators of macrophage activity and survival. Excessive intracellular production of mROS in macrophages can induce pro-inflammatory gene expression, cytokine production and DNA damage, potentially increasing mtDNA damage and secretion (442, 443). Our findings demonstrate that, due to the pathological conditions of GDM, placental macrophages are under significant mitochondrial-derived oxidative stress and may be activated to promote a pro-inflammatory response. It has previously been established that mtDNA released from distressed mitochondria can induce inflammation by activating the TLR-9 receptor on the endosomal surface. Given that placental mROS generation may instigate mtDNA release, we subsequently explored if placental-derived mediators released into explant supernatant activated the TLR-9 receptor in GDM. However, no difference was noted between the extent of TLR-9 activation induced by placental-derived mediators from either study group. The lack of TLR-9 activation in GDM is similar to the outcome seen with plasma mediators in Chapter 2. This would suggest that, in GDM, TLR-9 is not involved in mediating the intersection between mitochondrial dysfunction and inflammation.

Another potential nexus between mROS and inflammation is the NLRP3 inflammasome, which is predominantly expressed in macrophages. The NLRP3 inflammasome can be activated by a variety of endogenous DAMPs, such as mtDNA and mROS (15). NLRP3-mediated inflammation is particularly important in diabetic pathology, as activation of this inflammatory cascade drives vascular dysfunction in

T1DM, β-cell damage and obesity-induced insulin resistance in VAT in T2DM (435, 444, 445). Initially, the expression of members of the NLRP3 cascade, namely NLRP3, ASC and pro-caspase-1 were analysed. In the placenta, there were no significant GDM-mediated changes in protein expression of NLRP3, ASC or pro-caspase-1. We also examined the expression of these cascade components in VAT in GDM and NGT participants. Similar to the placenta, there were no significant GDM-mediated changes in protein expression of the NLRP3 signalling components in VAT between study groups. It is important to note that cleaved caspase-1 (20 kDa) was unfortunately not detectable in our tissue lysates using various antibodies, and hence only uncleaved pro-caspase-1 (~48 kDa) was detected and quantified. Cleaved caspase-1 is the critical final step which regulates the cleavage of pro-forms of inflammatory cytokines IL-1β and IL-18.

NLRP3 inflammasome activation leads to cellular pyroptosis and the release of pro-inflammatory cytokines IL-1β and IL-18. As we were unable to quantify cleaved caspase-1 expression in our study groups, we measured the secretion of these cytokines from both placental and omental explant cultures, and well as in the maternal circulation. Although there were no changes in NLRP3-related cytokines IL-18 and IL-1β in both the maternal circulation or locally in VAT between study groups, IL-18 was significantly increased in GDM placental explants compared to NGT. To elucidate if the placental increase in IL-18 was directly related to NLRP3 inflammasome, placental explant cultures were treated with MCC950. This selective small molecule inhibitor of the NLRP3 inflammasome prevents active structural conformation, NLRP3-induced ASC oligomerization and caspase-1 cleavage (446). MCC950 significantly attenuated IL-18 and IL-1β release from GDM placental

tissue, confirming a possible role of the NLRP3 pathway in regulating IL-18 release in GDM.

While we suggest that the increased mROS production by placental M1-like macrophages is the dominant instigator of NLRP3-induced IL-18, another alternative, or co-existing, orchestrator may due to the effect of the hyperglycaemic microenvironment. *In vitro* evidence has shown that elevated glucose can induce NLRP3 activation in vascular endothelial cells, with increased IL-1β and IL-18 mRNA expression (447).

We hypothesized that mitochondrial antioxidant therapy may be a potential avenue to ameliorate placental inflammation driven by mitochondrial dysfunction. Using two mitochondrial antioxidants, GDM placental explants were treated with either MitoQ or ERG. Our lab has previously established that treating a preclinical model of PE with ERG increased placental expression of the mitochondrial orchestrator peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), mitochondrial ROS-detoxifying enzyme uncoupling protein 1 (UCP-1) and nuclear factor erythroid 2–related factor 2 (Nrf2), a regulator of antioxidant gene expression. Furthermore, ERG also reduced mitochondria-specific H_2O_2 production *in vivo* in this preclinical model (448). This evidence demonstrated that ERG has the potential to treat placental dysfunction by specifically targeting mitochondrial health and mROS generation. Our *ex vivo* findings suggest that ERG antioxidant activity can also suppress placental production of NLRP3-related pro-inflammatory cytokines, IL-1β and IL-18, in GDM through the inhibition of mROS production, and may be a therapeutic option to reduce GDM-mediated placental inflammation Previous pharmacokinetic studies idenitfied that administration of 25mg/day of pure ERG, without pharmaceutical modification, elevates human plasma concentrations to >

0.3mM (449), hence our *ex vivo* treatment of 1mM ERG represents a clinically relevant therapeutic model.

In order to examine if additional mediators of local placental inflammation were evident in GDM, a panel of cytokines was measured in placental explant cultures. Both IL-6 and TNF-α, pro-inflammatory cytokines predominantly secreted by T cells and macrophages, were significantly increased in placental explants in GDM. This is in agreement with previous work, which also identified similar increases in placental production of these cytokines in GDM (450). Furthermore, induction of systemic inflammation in pregnant C57BL/6J mice resulted in a significant increase in both placental IL-6 and TNF-α expression. This placental inflammation was maternally driven and may have been mediated by the activation of uterine NK cells, granulocytes and macrophages (451). These findings could be translated to our study as we have provided evidence of systemic chronic low-grade inflammation in maternal circulation in GDM, which equally may be responsible for promoting local placental inflammation. Furthermore, IL-6 was also increased in maternal plasma in GDM participants, demonstrating a potential correlation between placental and circulating levels of these inflammatory mediators. Both IL-6 and TNF-α cytokines promote increased insulin resistance, further orchestrating systemic diabetic pathology (452-454). This evidence suggests that placental inflammation contributes to GDM pathology. Furthermore, this is the first study to characterize this quantity of secreted inflammatory cytokines from GDM placenta, particularly in a multi-organ clinical scenario.

Finally, we explored the contribution of placental adipokines, namely adiponectin, leptin, resistin and adipsin, to the pathophysiology of GDM. We previously identified significantly lower levels of circulating adiponectin in our GDM

participants. At a placental level, there was no change in adiponectin, but placental leptin was significantly reduced while placental production of adipsin was significantly increased in GDM. Despite some conflicting evidence regarding the regulation of leptin, elevated placental leptin expression is predominantly reported in GDM and may be associated with increased placental production of IL-6 and TNF-α (98, 455, 456). As such, our findings are contrary to those from previous research. As insulin and leptin receptors share common signalling pathways, including MAPK and PI3K, Pérez-Pérez et al. established that increased activation of leptin and insulin receptors in GDM placental tissue had a positive cross-talk effect on these shared signalling pathways. However, they equally established that placental leptin signalling was inhibited by *in vitro* stimulation with insulin and leptin (109). These findings show that a negative feedback mechanism exists in leptin signalling when elevated insulin or leptin levels are present. In our study, systemic insulin levels were significantly increased in our GDM cohort, which may in part be responsible for downregulation of placental leptin release, particularly in term placental tissue.

Although no previous studies have examined the placental secretion of adipsin in GDM, higher circulating levels have been reported in women with GDM (118). Additionally, increased placental secretion of adipsin has been observed in pregnant obese women (112, 457). In terms of a potential role in GDM, increased peripheral insulin resistance seen in late gestation is associated with increased circulating adipsin/acylation-stimulating protein (ASP) levels (458). Adipsin promotes lipid storage in adipose tissue via the formation of ASP, hence increased placental release of adipsin may be a homeostatic response to enhance triglyceride storage in conditions of impaired insulin sensitivity.

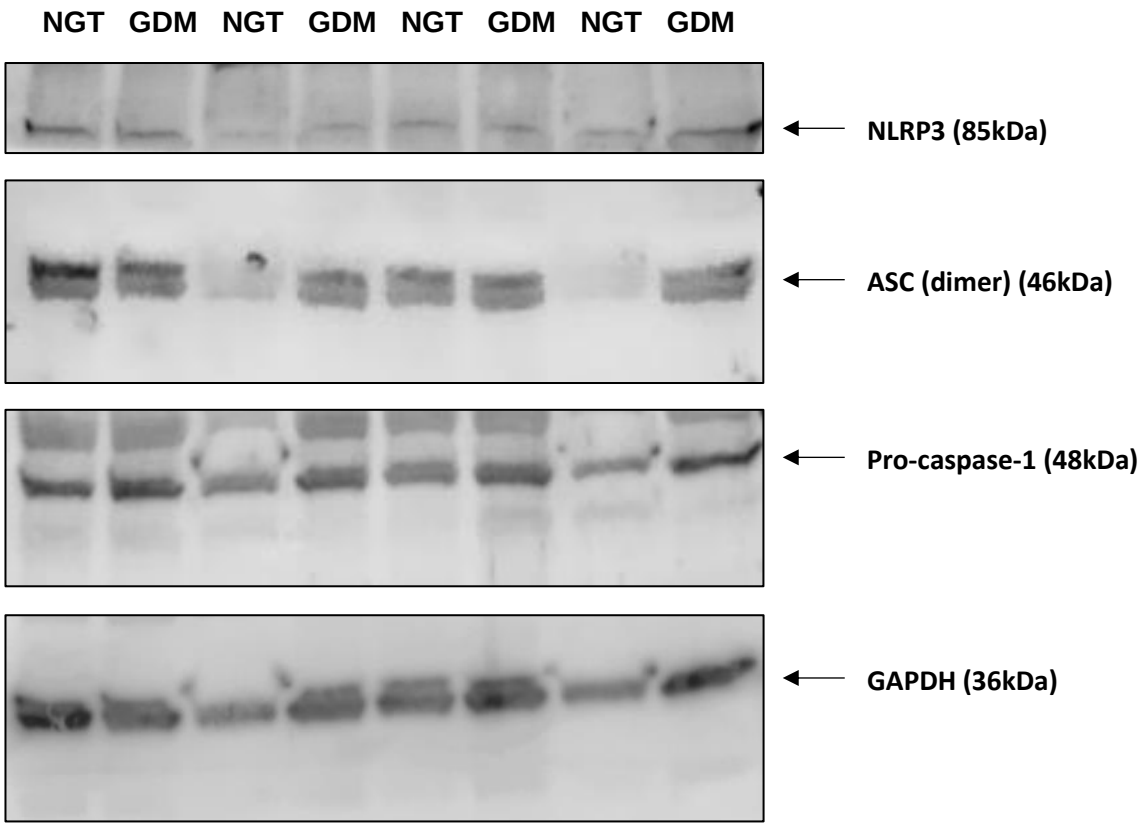
The strengths of this study include strict participant exclusion criteria and consistent sample collection methods. Limitations of this study include its cross-sectional nature. Also, it is a single site study that recruited a predominantly white Irish cohort, thereby limiting the extrapolation of our findings to other clinical settings or ethnicities. Overall, this study provided evidence of placental dysfunction and increased placental inflammation, partly orchestrated by elevated mitochondrial superoxide production by M1-like macrophages. Finally, we demonstrated that antioxidants which neutralize the pathogenic effects of mROS can reduce placental inflammation in GDM.

4.5 Conclusion

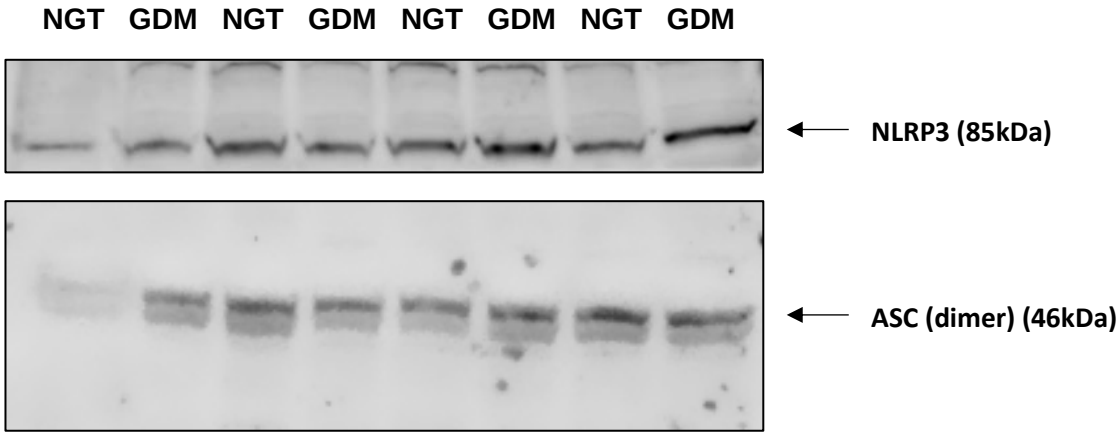
The important metabolic role of the placenta in the pathophysiology of GDM, and the subsequent effect on maternal and fetal outcomes, cannot be underestimated. Firstly, this study confirms that placental villous immaturity is the dominant morphological feature in GDM. This structural defect may be a result of systemic insulin resistance and hyperglycaemia, resulting in pathological placental insufficiency in GDM. This study shows for the first time that aberrant mitochondrial dysfunction is evident in placental $CD14^+$ and M1-like macrophages in GDM. Furthermore, the increase in mROS stimulates a macrophage-mediated inflammatory response, which may be regulated by the NLPR3 inflammasome. Further evidence of immune and metabolic dysregulation in placental tissue in GDM was provided with increased placental production of IL-18, IL-6, TNF-α and adipsin. The increase in placental inflammation may equally contribute to systemic inflammation and insulin resistance evident in our GDM cohort. Finally, it was demonstrated that directly targeting mROS production with ERG dampens placental inflammation in GDM, identifying a potential therapeutic role for this nutraceutical antioxidant.

Supplementary material related to Chapter 4

A



B



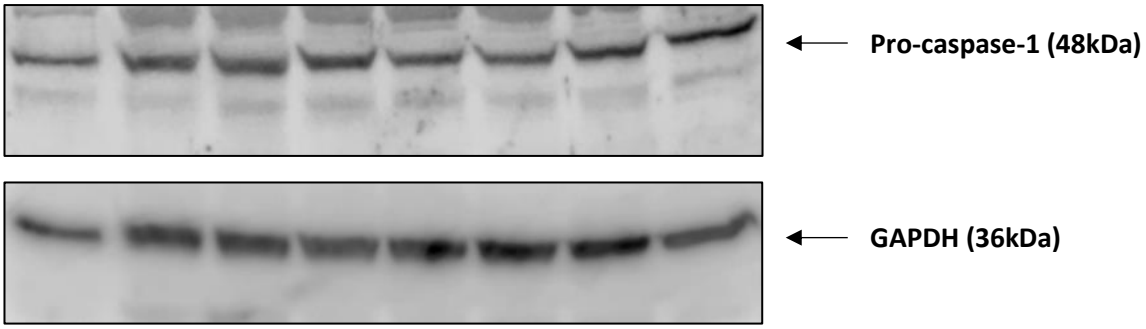
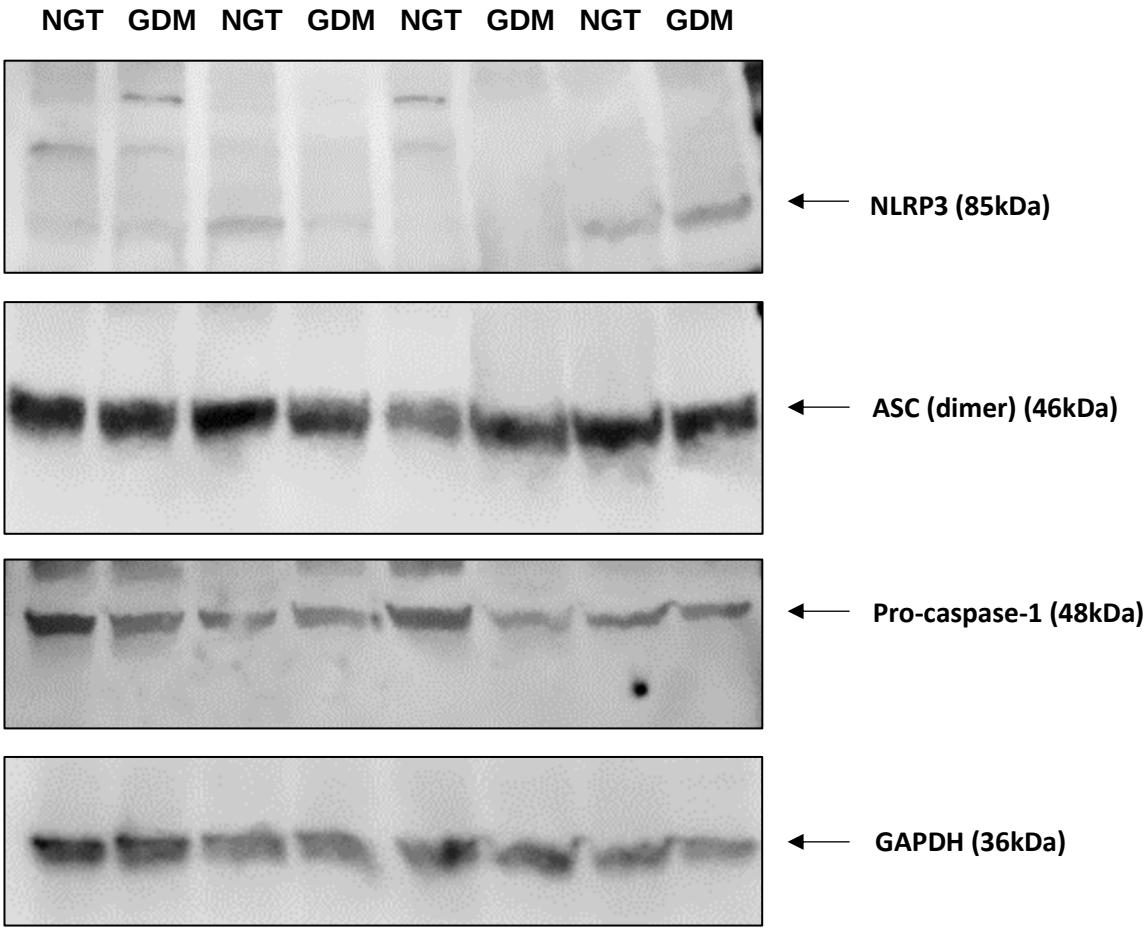


Figure S4.1. Placental tissue protein expression of NLRP3, ASC and pro-caspase-1 was compared in NGT placenta (n=8) relative to GDM placenta (n=8) matched by maternal age and BMI. Matched pairs were loaded adjacent to each other over two separate blots **(A + B).**

A



B

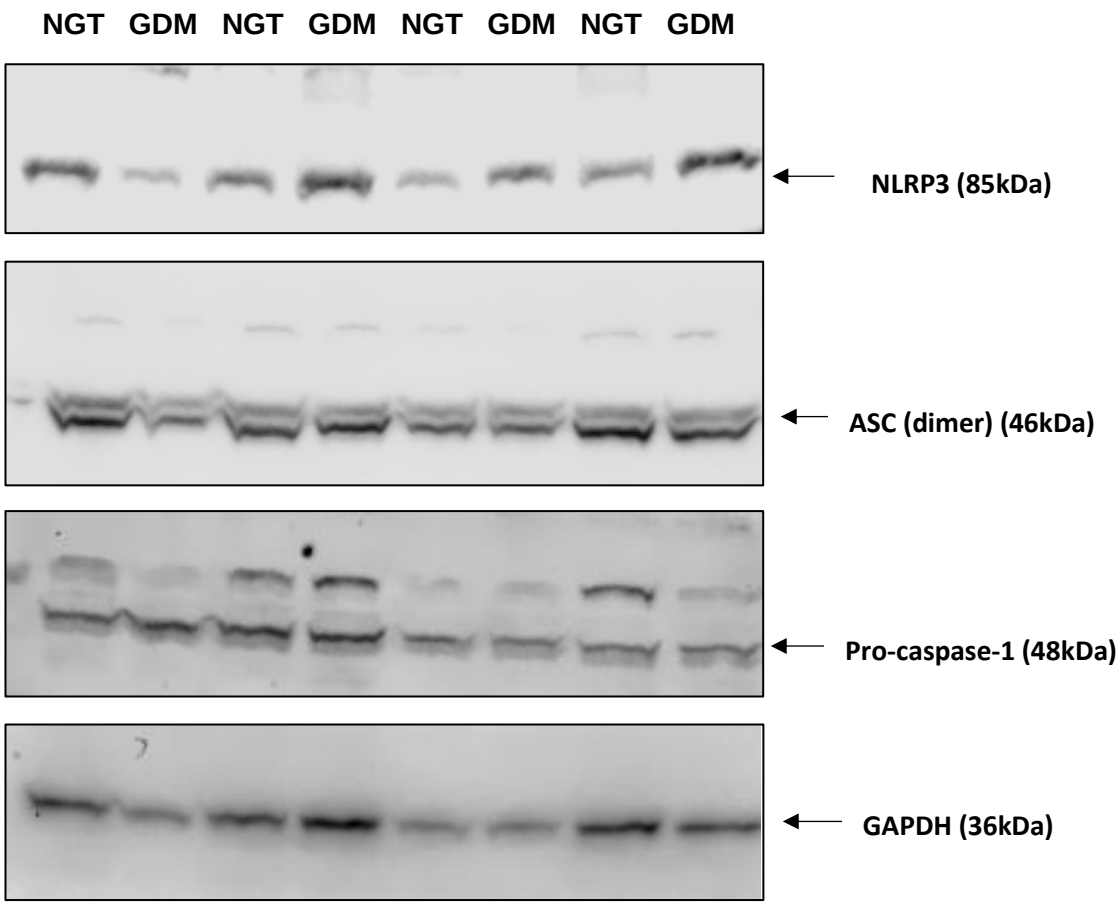


Figure S4.2. Omental tissue protein expression of NLRP3, ASC and pro-caspase-1 was compared in NGT adipose tissue (n=8) relative to GDM adipose tissue (n=8), matched by maternal age and BMI. Matched pairs were loaded adjacent to each other over two separate blots **(A + B).**

Chapter 5

Investigating adipose-placental cross-talk in mediating the pathophysiology of GDM

In the previous three chapters we have characterised substantial meta-inflammation in GDM, both peripherally and locally in omental VAT and placental tissue, linking tissue-mediated inflammation and metabolic insufficiency to systemic insulin resistance. This chapter will investigate the intersection of adipose-placental signalling in mediating GDM pathology. Using *in vitro* cellular models, this chapter will interrogate if the metabolic-inflammatory interactions evident in Chapters 3 and 4 are involved in a mutually dependent regulatory system. Our hypothesis is that experimental findings will potentially uncover a bidirectional relationship between adipose and placental tissue signalling, providing evidence of inter-organ communication which may exacerbate GDM pathology.

Abstract

Background

During a healthy pregnancy, the placental secretome plays a crucial role in promoting healthy maternal adaptation, through the release of hormones and other metabolic mediators. The discovery that insulin receptors and adipokine production, key players in insulin regulation, are active in the placenta suggests potential placenta-adipose communication. Both tissues provide sources of inflammatory mediators, emphasising the role of the immune-metabolic network in pregnancy. In this chapter we tested our hypothesis that inter-organ signalling between adipose and placental tissue in GDM may contribute to local tissue metabolic dysregulation, and subsequently to systemic meta-inflammation, as characterised in the earlier chapters.

Methods

Placental and omental tissue was collected from nulliparous women with normal glucose tolerant (NGT) pregnancies and women diagnosed with GDM post-elective caesarean section. Placental and omental explants from GDM (n=10) and NGT (n=10) participants were cultured for 24 hours and supernatant collected for *in vitro* culture assays. Primary omental preadipocyte differentiation was assessed with the AdipoRed fluorescent assay following treatment with 5% placental explant conditioned supernatant. Adipocyte lipid depletion in mature omental adipocytes was measured with the AdipoRed fluorescent assay. Glucose uptake was measured with a Glucose Uptake-Glo luminescence assay and mitochondrial-specific superoxide production was determined using MitoSOX/MitoTracker fluorescent staining in mature omental adipocytes following treatment with 5% placental explant conditioned supernatant. Immunoblotting was used to quantify adipocyte expression

of IGFBP-3, IGFBP-4, IRS-1 and phospho-IRS-1(ser312). Mitochondrial-specific superoxide production using MitoSOX/MitoTracker fluorescent staining was measured in the HTR8/SVneo trophoblast cell line following exposure to 5% omental explant conditioned supernatant. Finally, placental production of inflammatory mediators (IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33) were measured in the HTR8/SVneo trophoblast cell line following exposure to 5% omental explant conditioned supernatant by multiplex ELISA.

Results

In this study, there was no evidence of a placental-mediated effect on adipogenesis in primary preadipocytes following exposure to 5% placental explant conditioned supernatant from NGT and GDM. However, treatment with GDM placental supernatant promoted a significant increase in preadipocyte differentiation capacity relative to untreated culture (p=0.01), which was not evident with NGT supernatant treatment (p=0.14). There was no significant difference in protein expression of IGFBP-3 (p=1.00) or IGFBP-4 (p=0.89) in mature adipocytes following treatment with GDM and NGT placental supernatant. Furthermore, there were no significant differences in adipocyte IC lipid content (p=0.12), glucose uptake capacity (p=0.95), protein expression of IRS-1 (p=0.89) or phospho-IRS-1(ser312)/IRS-1 (p=0.89), or mitochondrial-specific superoxide production (p=0.51) in mature adipocytes following treatment with GDM and NGT placental supernatant. Finally, there were no significant differences in placental mitochondrial-specific superoxide production (p=0.15) or inflammatory cytokine production in HTR8 trophoblasts following treatment with GDM and NGT omental supernatant.

Conclusion

This study looked at the potential intersection of communication between the placental and adipose tissue in the development of GDM. Treatment with placental supernatant from GDM pregnancies did not significantly affect adipogenesis of omental preadipocytes or have a significant functional impact on mature omental adipocytes in terms of glucose uptake or mROS production. Furthermore, co-culture of placental trophoblasts with supernatant from omental VAT from GDM pregnancies did not significantly affect their mitochondrial or inflammatory profile. While these findings suggest a lack of direct *in vitro* communication between the placenta and visceral adipose tissue in GDM, indirect effects of each metabolic organ, mediated in part by systemic circulating mediators, cannot be ruled out.

5.1 Introduction

During healthy pregnancy the placental secretome is vital for maintaining a healthy maternal adaption, orchestrated by the release of placental hormones and other endocrine mediators. The placenta is a substantial source of circulating leptin, which coordinates communication with AT during pregnancy. In healthy pregnancy, placental leptin promotes endocrine-mediated alterations in energy balance including the mobilization of maternal fat, which occurs during the second half of pregnancy (459). However, altered leptin secretion can have deleterious effects on distal maternal organs including the maternal VAT depots. While elevated leptin levels can increase the production of adipogenesis and lipogenesis proteins in preadipocytes, it can also induce a pro-inflammatory cytokine profile with elevated TNF-α and IL-6, and reduced IL-10 production in VAT (460). Placental dysfunction leads to aberrant local and systemic adaptions to pregnancy and is a prominent cause of obstetric complications such as PE, IUGR and GDM.

Napso et al. recently established a comprehensive secretome map of the placenta and found 18 secreted placental proteins that were specifically altered in women with GDM. One of these proteins, soluble fms-like tyrosine kinase-1 (sFlt-1), was significantly increased in maternal circulation of women who developed GDM. SFlt-1 has been shown to cause systemic maternal vascular pathology and immune dysregulation in obstetric complications (461, 462). This increase in sFlt-1 was evident as early as 12 weeks' gestation, proving that placental mediators have a profound effect on the development of GDM (463). Another potential mechanism of placental signalling may be via placenta-derived exosomes (PdEs), which facilitate the transportation of signalling motifs to other maternal organs. A recent prospective cohort study found that the concentration of PdEs in maternal plasma was

significantly higher in GDM pregnancies compared to normoglycaemic pregnancies. Furthermore, GDM-derived placental exosomes significantly increased the release of granulocyte macrophage colony-stimulating factor (GM-CSF), IL-4, IL-6, IL-8, IFN-γ, TNF-α from endothelial cells, relative to PdEs from NGT pregnancies (464). These findings are supported by Rice et al. who found that hyperglycaemic conditions significantly increased the release of exosomes from first trimester primary trophoblast cells (465). Therefore, dysregulated placental signalling can substantially impact the maternal physiological response during pregnancy, stimulating metabolic imbalances and adverse maternal outcomes.

Inversely, an increase in maternal adiposity and adipocyte dysregulation, key factors in GDM pathophysiology, have been linked to placental dysfunction through increased lipid accumulation, inflammation and oxidative stress (466). Placental tissue of obese women had 17.5% more lipid content than that of lean women and had increased expression of PPAR-γ and acetyl-CoA carboxylase, key regulators of fatty acid (FA) esterification. A reduction in lipid transport intermediates, specifically acylcarnitines, was also noted in placental tissue of obese women (466, 467). This increase in placental lipotoxicity is not resultant of increased lipid uptake from the maternal circulation, as FA uptake has been shown to be decreased in cases of maternal obesity (468), but is the result of an increase in lipid esterification and storage, and dysregulation in FA β-oxidation (FAO). Furthermore, extra-mitochondrial (peroxisomal) FAO is also upregulated in placental tissue of obese mothers, compensating for an impaired mitochondrial function (466). This pathway not only presents a rationale for AT-driven placental dysfunction in GDM, but enhances placental ROS production and pro-inflammatory signalling in obese pregnancies, through immune cell infiltration and mitochondrial dysfunction.

Another mediator of lipid-driven placental inflammation may be palmitic acid (PA). In a preclinical model of maternal obesity, pregnant mice fed a high-fat diet had increased levels of circulating PA, increased placental expression of IL-1β protein and mRNA expression of NLRP3, ASC and caspase-1. These findings proposed that transient increases in circulating PA levels may activate the NLRP3 inflammasome and induce placental inflammation (469). This aberrant NLRP3 activation may then further propagate obesity-induced VAT insulin resistance and metabolic syndrome, presenting a potential deleterious feedback loop in adipose-placental communication (435).

The aim of this chapter was to investigate the potential of direct placental-adipose cross-talk in mediating metabolic dysfunction and inflammation in GDM. This work focused on the pathological pathways we identified in placental tissue and omental VAT of GDM participants in this study.

5.2 Methods

5.2.1 Study design and participant recruitment

GDM-diagnosed (n=30) and healthy, NGT control (n=30) participants were recruited from Cork University Maternity Hospital (CUMH), Ireland between 2019 and 2022 as part of the COMRADES study, a non-interventional cohort study of nulliparous singleton pregnancies with the aim of characterizing mitochondrial dysfunction and meta-inflammation in GDM. The COMRADES study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all the procedures were approved by the Clinical Research Ethics Committee of the Cork Teaching (ECM4 (ff) 04/12/18). Inclusion criteria for all study participants included; women aged between 18-50 years, nulliparous pregnancy, singleton pregnancy as determined by ultrasound scan, opted for an elective caesarean section, and had provided written informed consent.

After receiving participant consent, samples were collected from nulliparous, singleton pregnancies undergoing elective pre-labour caesarean section. As per national HSE guidelines, selective screening for GDM was carried out on identified at-risk pregnant women between 24-28 weeks gestation using the 75g oral glucose tolerance test (OGTT). A diagnosis of GDM was confirmed if one of the following values were met or exceeded: fasting blood glucose levels of 5.1mmol/L, blood glucose levels of 10.0mmol/L 1 hour post-OGTT or blood glucose levels of 8.5mmol/L 2 hours post-OGTT. Healthy, NGT control participants were defined as pregnant women who had not been diagnosed with GDM, as per the above guidelines, and did not present with any other diagnosed comorbidities.

Exclusion criteria included women who: had a multiple pregnancy; were multiparous; had already started to labour, who were outside the age range of 18-50

years of age or were an emergency caesarean section. Women with known pregnancy or other health related conditions namely: pre pregnancy essential hypertension, known liver or renal diseases, known Human Immunodeficiency Viruses (HIV), Hepatitis B or Hepatitis C positive, known Systemic Lupus Erythematous, known antiphospholipid syndrome and known or suspected fetal anomalies were excluded from the study.

5.2.2 Sample collection

Omentum and placental tissue was obtained during the caesarean section and processed within 30 minutes post-surgery.

5.2.3 Placental and omental explant culture

Tissue explants were prepared from placental samples collected from both NGT and GDM participants. Following the removal of outer membrane and connective tissue, 200mg ± 20mg of placental tissue (combined from three regions of the placenta) were cultured in 12-well plates, containing 2ml of culture media. Placental explant media consisted of RPMI-1640 1x (Gibco 21875034), FBS (heat-inactivated) 10% (Gibco 16140071), penicillin-streptomycin (10,000 U/mL) 1% (Gibco 15140122), insulin 1µg/ml (SAFC 91077C), hydrocortisone 0.1µg/ml (Sigma H0888), retinol acetate 0.1µg/ml (Sigma R0300000), gentamicin 0.05µg/ml (Sigma G1397). Tissue explants were also prepared from omental AT samples collected from both NGT and GDM participants. 100mg ± 10mg of omental tissue were cultured in 12-well plates, containing 2ml of culture media containing Medium 199 1x (Gibco 31150022), FBS (heat-inactivated) 10% (Gibco 16140071), penicillin-streptomycin (10,000 U/mL) 1% (Gibco 15140122), amphotericin B 0.25µg/ml (Gibco 15290018). Supernatants were subsequently stored at -80°C for further analysis.

5.2.4 Cell culture

Primary omental preadipocytes at passage 4 were obtained from Caltag Medsystems (Zenbio OP-F-SL). These cells were a pool from non-diabetic, non-smoker, non-pregnant female donors with a mean age of 37.7, a mean BMI of 46.5 and mixed ethnicity including African American, Caucasian and Hispanic ethnic groups. Before culture, all plates were coated with poly-l-lysine (PLL) (Sigma P4832) to improve cell adherence and proliferation. Culture plate/flask surfaces were aseptically coated with 1.0ml/25cm^2 PLL and plates were gently rocked to ensure even coating of the culture surface. After 2 hours, PPL solution was removed by aspiration and surfaces were rinsed with sterile PBS (Sigma P4417) (x3 the volume of PLL added). PLL-treated surfaces were allowed to dry for at least 2 hours before seeding cells.

Preadipocytes were seeded at 5,000 cells/cm² in preadipocyte growth medium (PromoCell C-27410) until they reached 100% confluency. Subculturing was carried out by removing growth medium, washing with sterile PBS (Sigma P4417) and adding sterile trypsin-EDTA solution (Gibco R001100). Once detached, complete growth media was added and the preadipocyte cell suspension was centrifuged at 220g for 3 minutes. The cell pellet was then resuspended in fresh growth medium for further culture. Media change was performed after 24 hours and then every 2-3 days until preadipocytes became confluent (approximately 7 days). Once confluent, preadipocytes were differentiated by aspirating preadipocyte growth medium and adding preadipocyte differentiation media (PromoCell C-27436) to preadipocyte cultures for 72 hours. After 72 hours, differentiation media was aspirated and adipocyte nutrition media (PromoCell C-27438) was added. Cells were maintained in nutrition media, with media change every 2-3 days, until adipocytes were fully differentiated (approximately 14 days).

HTR-8/SVneo cells (ATCC CRL-3271) are a cell line derived from explants from first trimester placenta and are used for the study of trophoblast biology and placental function. HTR8/SVneo cells at passage 14 were seeded at 15,000 cells/cm² in complete RPMI-1640 medium (Gibco 21875034) containing FBS (heat-inactivated) 10% (Gibco 16140071) and penicillin-streptomycin (10,000 U/mL) 1% (Gibco 15140122). Cells were cultured (37°C, 5% CO_2) until they reached 70-90% confluency (approximately 5 days) and subcultured. Subculturing was carried out by removing growth medium and trypsinizing cells with trypsin-EDTA solution (Gibco R001100).

5.2.5 Adipogenesis assay

To assess the effect of placental mediators on the differentiation of preadipocytes, an adipogenesis assay was performed using the AdipoRed fluorescent assay (Lonza PT-7009). Primary preadipocyte cells were cultured as above in a 96-well plate with opaque walls (Corning 3610). Once preadipocytes reached confluency, differentiation medium was added plus 5% placental explant culture supernatant from either NGT or GDM participants. Untreated control wells and blank wells were included in the assay protocol. After 72 hour culture, media was aspirated and fluorescent staining with AdipoRed was performed as per the manufacturer's protocol. Briefly, the cell culture plate was cooled to room temperature and culture supernatant was carefully removed. Wells were washed with 200µl PBS (Sigma P4417). Each well was then filled with 200µl PBS and 5µl AdipoRed reagent. After 10 minutes, fluorescence was measured with excitation at 485nm and emission at 595nm on a Tecan Spark spectrophotometer. 9 measurements were recorded in each well in a 3x3 pattern, covering the total well surface area. RIPA 1x (Millipore 20-188) was then added to each well for protein extraction for assay normalisation. Cell

protein lysate content was quantified using the Pierce™ BCA Protein assay kit (Thermo Scientific 23227) and used for normalisation.

5.2.6 Immunoblotting

To assess the effect of placental mediators on markers of adipogenesis and the insulin signalling cascade in mature adipocytes, immunoblotting analysis was performed. Fully differentiated adipocytes as described in section 5.2.4 were cultured in adipocyte nutrition media (PromoCell C-27438) with 5% treatment of placental explant culture supernatant from NGT and GDM participants for 24 hours. Protein isolation was performed using RIPA 1x (Millipore 20-188) supplemented with phosphatase inhibitor (Roche, PhosSTOP™) and protease inhibitor (Roche). Protein (15µg) was separated at 100V by SDS-PAGE on a 7.5% Tris-Glycine polyacrylamide gel and transferred to a methanol activated PVDF membrane (Amersham™ Hybond® Sigma-Aldrich) at 400mA on ice for 2 hours. Protein ladder (Thermo Scientific 26634) was included in the run to confirm protein band kDa size. Membranes were blocked in 5% BSA in 0.1% TBS-T (tris-buffered saline with 0.1% Tween detergent) for 1 hour at room temperature and incubated overnight at 4°C with the following primary antibodies: IGFBP3 1:1000 (R&D Systems AF675), IGFBP4 1:3000 (R&D Systems AF804), IRS1 1:1000 (Cell Signalling #3407), phospho-IRS1(ser312) 1:1000 (Cell Signalling #2381) and GAPDH 1:1000 (Cell Signalling #97166). After overnight incubation, membranes were washed and incubated at room temperature for 1 hour with secondary HRP-linked antibody, either anti-rabbit 1:2000 (Cell Signalling #7074), anti-mouse 1:2000 (Santa Cruz 516102) or anti-goat 1:1000 (R&D Systems HAF019). Membranes were washed in 0.1% TBS-T and developed using Pierce™ ECL Western Blotting Substrate

(Thermo Scientific 32209) and LICOR Odyssey image analyser. Densitometry was performed using LICOR Image Studio Lite Version 5.2.

5.2.7 Lipid utilisation assay

To assess the effect of placental mediators on the depletion of intracellular triglycerides from terminally differentiated mature adipocytes, a lipid utilisation assay was performed using the AdipoRed fluorescent assay reagent (Lonza PT-7009). Adipocytes were cultured as in section 5.2.4. in a 96-well plate with opaque walls (Corning 3610). Prior to the assay, fully differentiated mature adipocytes were cultured in adipocyte nutrition media (PromoCell C-27438) with 5% treatment of placental explant culture supernatant from NGT and GDM participants for 24 hours. Untreated control wells and blank wells were included in the assay protocol. Fluorescent staining with AdipoRed was carried out as per section 5.2.5.. Fluorescence was measured with excitation at 485nm and emission at 595nm on a Tecan Spark spectrophotometer. 9 measurements were recorded in each well in a 3x3 pattern, covering the total well surface area. RIPA 1x (Millipore 20-188) was then added to each well for protein extraction for assay normalisation. Cell protein lysate content was quantified using the Pierce™ BCA Protein assay kit (Thermo Scientific 23227).

5.2.8 Glucose uptake assay

To investigate the effect of placental mediators on glucose uptake in mature adipocytes, the Glucose Uptake-Glo assay (Promega J1342) was used. Adipocyte cells were cultured as in section 5.2.4. in a 96-well plate with opaque walls (Corning 3610). Prior to the assay, fully differentiated mature adipocytes were cultured in adipocyte nutrition media (PromoCell C-27438) with 5% treatment of placental explant culture supernatant from NGT and GDM participants for 24 hours. No

reagent and blank wells were included in the assay protocol. The Glucose Uptake-Glo assay was performed as per the manufacturer's protocol. In short, media was removed and cells were washed with 100µl PBS (Sigma P4417). 50µl of 1mM 2-deoxyglucose was added to each well, briefly shaken, and incubated for 10 minutes at room temperature. 25µl of stop buffer was added followed by 25µl of neutralization buffer. 100µl of 2-deoxyglucose-6-phosphate detection reagent was added and the plate was agitated. Cells were incubated at room temperature for 2 hours and luminescence was measured on an Orion L microplate luminometer at 1 second/well.

5.2.9 Mitochondrial superoxide assay

To measure induced mROS generation in both omental and placental explant supernatant treated cell cultures, mitochondrial-specific superoxide production was measured. Adipocytes were cultured as in section 5.2.4. in a 96-well plate with opaque walls (Corning 3610). Prior to the assay, fully differentiated mature adipocytes were cultured in adipocyte nutrition media (PromoCell C-27438) with 5% treatment of placental explant culture supernatant from NGT and GDM participants for 24 hours. Untreated control wells and blank wells were included in the assay protocol. Post-treatment, media was removed and 50µl of phenol-red free RPMI-1640 (Gibco 32404014) containing 5µM MitoSOX (mitochondrial superoxide stain)(Invitrogen M36008) and 100nM MitoTracker (live cell mitochondrial stain)(Invitrogen M22426) was added to each well. MitoTracker staining of live mitochondria was used to normalise MitoSOX fluorescent staining for mROS. Cells were incubated in the dark at 37°C for 20 minutes. Mitochondrial staining cocktail was removed and 50µl of fresh phenol-red free RPMI-1640 (Gibco 32404014) was added to each well. Fluorescence was measured for MitoTracker staining

(excitation/emission 620nm/680nm) and MitoSOX staining (excitation/emission 340nm/595nm) on a Tecan Spark spectrophotometer. 9 measurements were recorded in each well in a 3x3 pattern, covering the total well surface area.

HTR8 cells were cultured as in section 5.2.4. in a 96-well plate (Corning 3610). Prior to the assay, cells were cultured in completed RPMI-1640 medium with 5% treatment of omental explant culture supernatant from NGT and GDM participants for 24 hours. Untreated control wells and blank wells were included in the assay protocol. Post-treatment, supernatant was removed and cells were washed with sterile PBS (Sigma P4417). Fresh, completed RPMI-1640 medium was added for 24 hours. Media was removed and 50µl/well was added of phenol-red free RPMI-1640 (Gibco 32404014) containing 5µM MitoSOX (mitochondrial superoxide stain) (Invitrogen M36008) and 100nM MitoTracker (live cell mitochondrial stain) (Invitrogen M22426). Fluorescent staining was completed as described.

5.2.10 Omental-stimulated HTR8 cell cytokine release

To assess the effect of omental VAT mediators on the inflammatory profile of HTR8 trophoblasts, HTR8 cells were cultured as per section 5.2.4. in a 96-well plate (Corning 3610). Prior to the assay, cells were cultured in completed RPMI-1640 medium with 5% treatment of omental explant culture supernatant from NGT and GDM participants for 24 hours. Post-treatment, supernatant was removed and cells were washed with sterile PBS (Sigma P4417). Fresh complete RPMI-1640 medium was added for 24 hours and supernatant removed and used for cytokine quantification. A cytokine panel including IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33 was measured using the LEGENDPlex Human Inflammation Panel 1 kit (BioLegend 740809). The assay was performed as per the manufacturer protocol. RIPA 1x (Millipore 20-188) was added

to each well for protein extraction for assay normalisation. Protein lysate was quantified using the Pierce™ BCA Protein assay kit (Thermo Scientific 23227).

5.2.11 Statistics

Analysis was performed using GraphPad Prism 5.03. Where applicable, data is represented as mean ± standard deviation for parametric analysis and median [95% confidence interval] for non-parametric analysis. P values < 0.05 were considered as statistically significant. The Kolmogorov–Smirnov test was used to test for data normality. For statistical comparisons, either the Student's t-test or the Mann-Whitney U test was used depending on data normality and test suitability. The Benjamini–Hochberg method with a 20% false discovery rate was used for multiple hypothesis in cytokine (n=13) panels testing to decrease the false discovery rate. Grubbs's test was used to detect outliers in data sets.

5.3 Results

5.3.1 Lack of evidence of placental-mediated increase in adipogenesis in GDM

Omental adipocyte hypoplasia was established in GDM participants in Chapter 3. Hypoplasia can limit the protective effect that adipocyte proliferation has on glucose and lipid metabolism in AT. As a result women with GDM are exposed to increased metabolic dysfunction and insulin resistance (391, 392). Adipocyte hypoplasia may partly result from a deficiency in preadipocyte differentiation to mature adipocytes. To examine the effect that placental-derived mediators have on adipogenic differentiation, preadipocytes cultured in a differentiation cocktail were treated with 5% placental explant culture supernatant from NGT and GDM participants during a 3-day differentiation protocol. Untreated preadipocytes were equally cultured in the differentiation cocktail to induce physiological differentiation. Preadipocyte differentiation is characterised by increased lipid uptake and was assessed by AdipoRed fluorescent staining. Although exposure to placental supernatants from both study groups increased preadipocyte differentiation compared to untreated preadipocytes, particularly in GDM (11958 RFU (relative fluorescence units) [10693, 13293] vs. 16031 RFU [14219, 18253], p=0.01) compared to NGT (11958 RFU [10693, 13293] vs. 15067 RFU [12180, 16525], p=0.14), there was no significant difference between NGT and GDM study groups (p=0.32) (**Figure 5.1**).

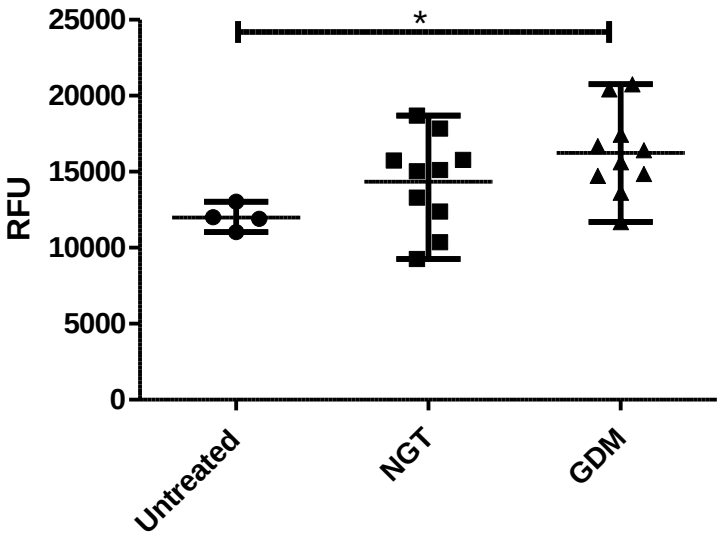


Figure 5.1. Placental-derived mediators in GDM do not increase adipogenesis relative to NGT. Adipogenesis was measured by AdipoRed fluorescent assay in differentiating preadipocytes treated with NGT (n=10) and GDM (n=10) placental explant culture supernatant, in addition to an untreated control. Total protein content was used for normalisation. Analysis groups were compared with Kruskel-Wallis test and Dunn's Multiple Comparison Test. Significance levels are shown as: * $p<0.05$.

5.3.2 Placental-derived mediators in GDM do not affect adipocyte IGFBP expression

Further to a reduction in adipocyte count in omental VAT in GDM in our study, there was also a significant reduction in protein expression of IGFBP-4 in VAT in GDM. To investigate if placental-derived mediators modulate IGFBP expression in GDM, the expression of IGFBP-3 and IGFBP-4 was measured in primary mature adipocytes treated with 5% NGT (n=4) and GDM (n=4) placental explant supernatant (**Figure 5.2A**). Densitometric analysis showed no significant difference in IGFBP-3 (0.072 [0.026, 0.11] vs. 0.072 [0.047, 0.098], p=1.00) (**Figure 5.2B**) or

IGFBP-4 (0.022 [0.013, 0.036] vs. 0.024 [0.017, 0.032], p=0.89) (**Figure 5.2C**) protein expression between treatment groups.

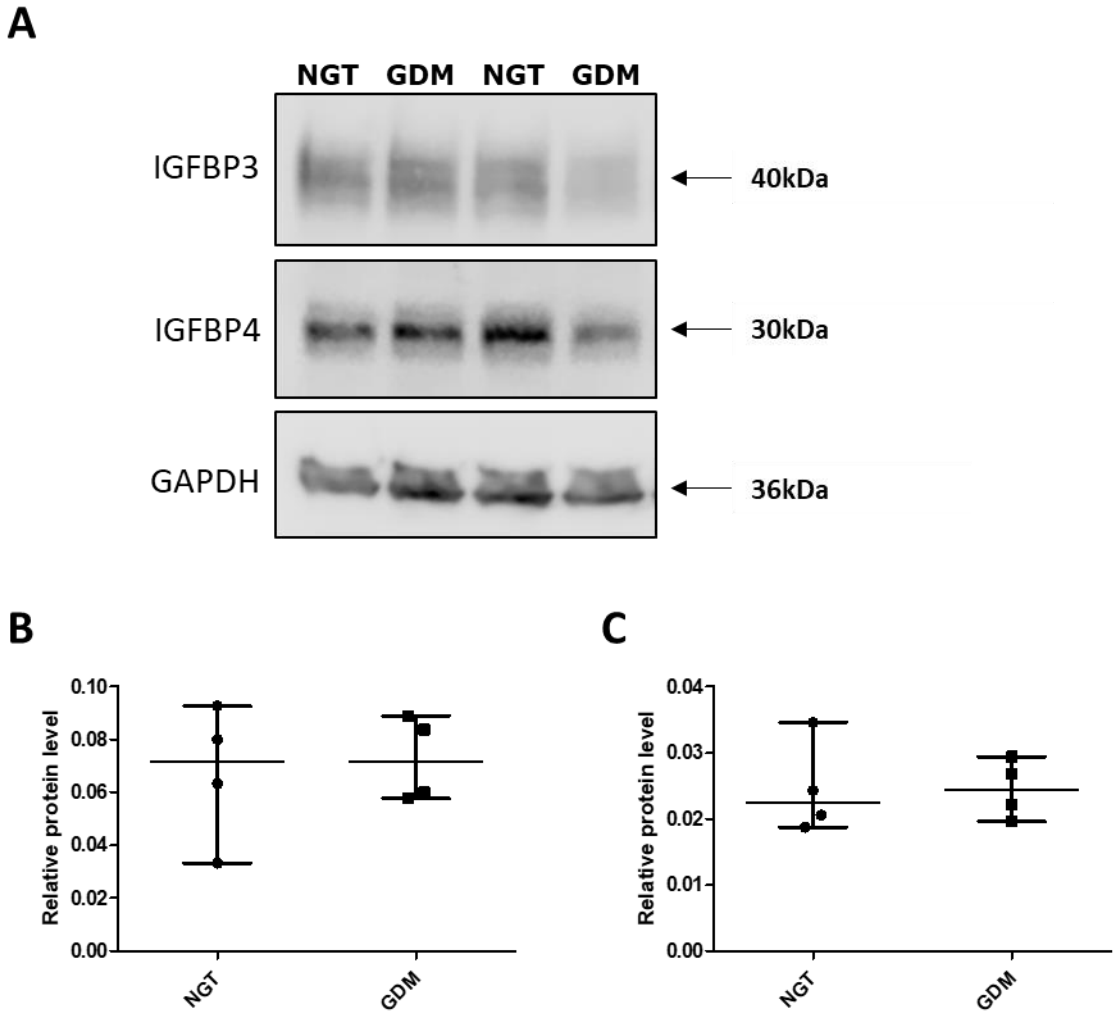


Figure 5.2. Primary mature adipocyte expression of IGFBP-3 and IGFBP-4 following treatment with placental explant supernatant. (A) Representative images of IGFBP-3, IGFBP-4 and GAPDH protein expression as measured by Western blotting in primary mature adipocytes following treatment for 24 hours with 5% placental explant supernatant from NGT (n=4) and GDM (n=4). Densitometric analysis was used to quantify protein expression of **(B)** IGFBP-3 and **(C)** IGFBP-4. Mann-Whitney U test was used to compare protein expression between treatment groups.

5.3.3 Adipocyte IC lipid content is not altered by placental mediators in GDM

Excessive lipolysis, specifically the breakdown of triglycerides into FFAs and glycerol, is a major risk factor for the development of insulin resistance and T2DM (470). There is evidence that many of the adverse metabolic effects of glucose intolerance may be mediated by lipotoxicity as a result of elevated circulating FFAs (471). Furthermore, lipolysis is particularly sensitive to insulin activity, as insulin receptor activation promotes PI3K activation and downstream suppression of lipolysis (472). While we did not see any significant increase in circulating levels of triglycerides or FFAs in GDM, as described in Chapter 2, we did establish, in Chapter 3, that GDM participants had significantly impaired insulin signalling capacity in omental VAT. Therefore, we wanted to investigate if placental signalling in GDM may, in part, alter adipocyte lipolysis and drive local AT dysfunction in GDM.

To assess if placental mediators in GDM affect adipocyte lipolysis, adipocyte intracellular lipid content was quantified in terminally differentiated mature adipocytes using fluorescent AdipoRed staining, following 24-hour treatment with 5% NGT (n=10) and GDM (n=10) placental explant supernatant. There were no significant differences in intracellular lipid content between the untreated, NGT or GDM treated groups (70637 RFU [52285, 104838] vs. 119924 RFU [86463, 135276] vs. 94056 RFU [81006, 110157], respectively, p=0.12) (**Figure 5.3**).

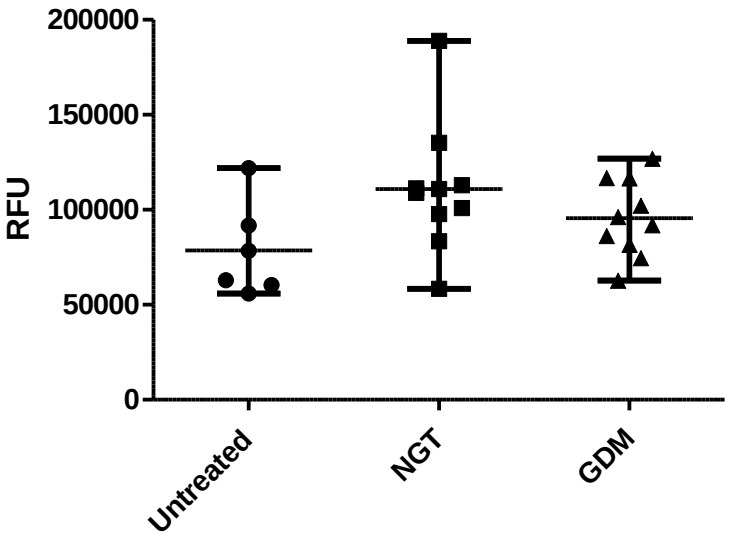


Figure 5.3. Placental-derived mediators in GDM do not significantly alter adipocyte lipolysis. Intracellular lipid content was quantified using the AdipoRed fluorescent assay in terminally differentiated mature adipocytes treated with NGT (n=10) and GDM (n=10) placental explant culture supernatant, in addition to an untreated control. Total protein content was used for normalisation. Analysis groups were compared with Kruskel-Wallis test.

5.3.4 Placental-derived mediators in GDM do not affect adipocyte glucose uptake

In this study, we found substantial evidence of insulin resistance and concurrent hyperglycaemia in GDM. Participants with GDM had altered circulating levels of both glucose and insulin and impaired insulin signalling in VAT. As such, we investigated whether placental mediators released in response to GDM pathology alter glucose uptake in mature adipocytes. Glucose uptake was measured using the bioluminescent detection of 2-deoxyglucose-6-phosphate (2DG6P) in mature adipocytes following 24-hour treatment with 5% NGT (n=10) and GDM (n=10) placental explant supernatant. There was no significant differences in glucose uptake between the untreated, NGT or GDM treated groups (246231 RLU (relative light

units) [-208580, 821191] vs. 236486 RLU [205278, 287085] vs. 229823 RLU [189879, 287685] respectively, p=0.95) (**Figure 5.4**).

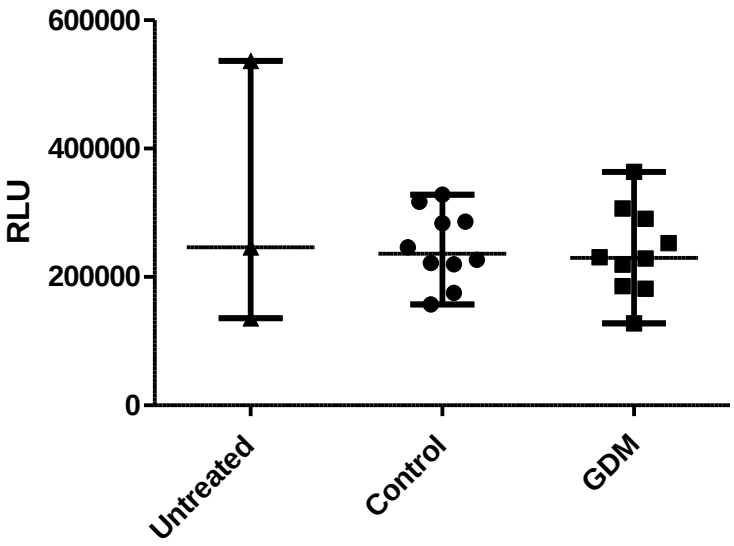


Figure 5.4. Placental-derived mediators in GDM do not affect adipocyte glucose uptake. Glucose uptake was measured in terminally differentiated mature adipocytes treated with NGT (n=10) and GDM (n=10) placental explant culture supernatant. Analysis groups were compared with Kruskel-Wallis test.

5.3.5 Insulin signalling in adipocytes is not significantly altered by placental-derived mediators in GDM

The IRS-1 signalling cascade is responsible for transmitting signals following activation of the insulin receptor, while IRS-1 phosphorylation at serine/threonine residues, such as ser312, results in insulin resistance by impairing signal transduction (473, 474). To further decipher the potential role of placental-derived mediators in modifying insulin signalling in VAT as seen in GDM participants in this study, protein expression of IRS-1 and phospho-IRS-1(ser312) were measured by immunoblotting in mature adipocytes following 24-hour treatment with 5% NGT (n=4) and GDM (n=4) placental explant supernatant (**Figure 5.5A**). Densitometric

analysis showed no significant difference in total IRS-1 (0.15 [0.10, 0.19] vs. 0.15 [0.09, 0.21], p=0.89) (**Figure 5.5B**), phospho-IRS-1(ser312) (0.008 [0.007, 0.008] vs. 0.008 [0.004, 0.013], p=1.00) (**Figure 5.5C**) or phospho-IRS-1(ser312)/IRS-1 (0.054 [0.034, 0.074] vs. 0.062 [0.038, 0.075], p=0.89) (**Figure 5.5D**) protein expression between treatment groups.

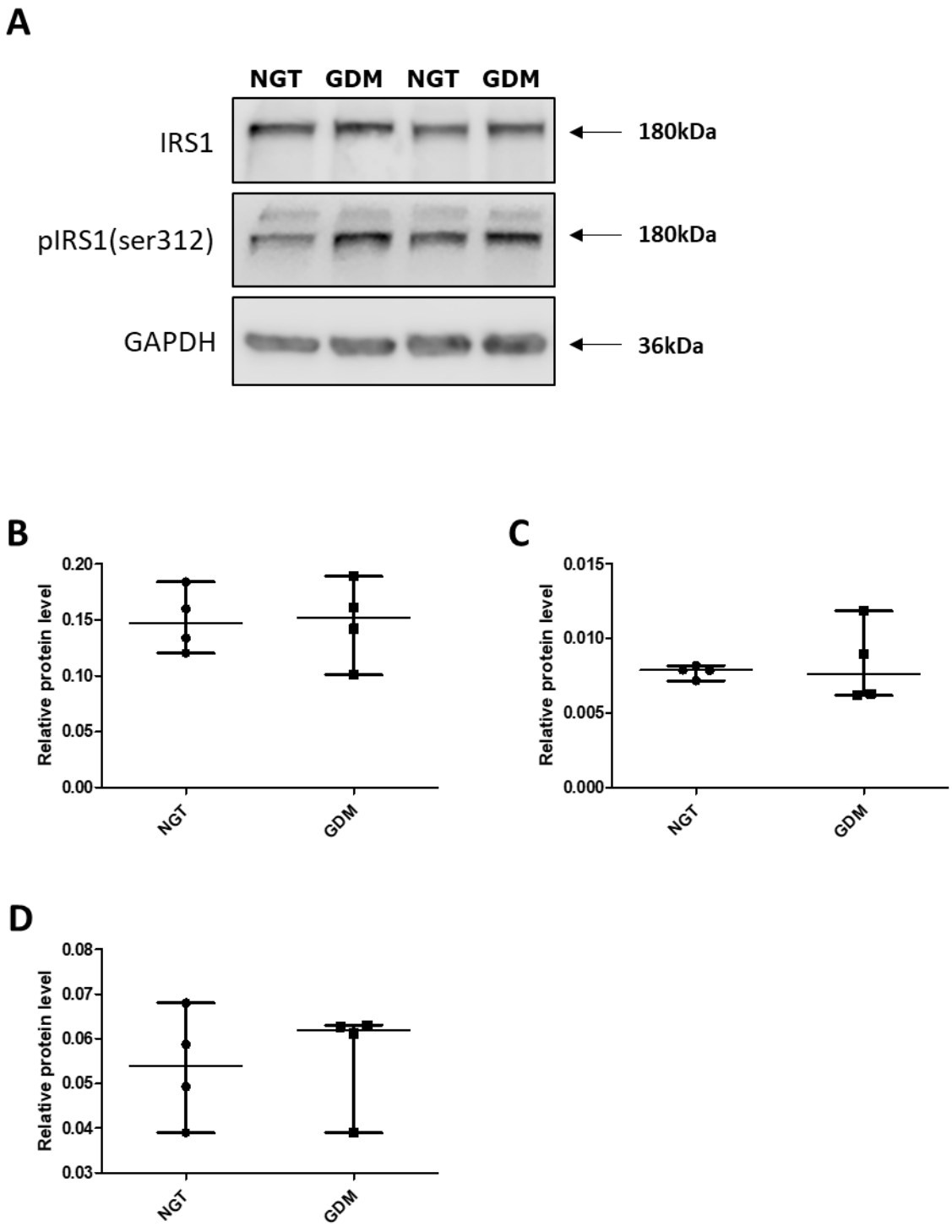


Figure 5.5. Primary adipocyte expression of IRS-1 and phospho-IRS-1(ser312) following treatment with placental explant supernatant. (A) Representative images of IRS-1, phospho-IRS-1(ser312) and GAPDH protein expression as measured by Western blotting in primary mature adipocytes following treatment for

24 hours with 5% placental explant supernatant from NGT (n=4) and GDM (n=4). Densitometric analysis was used to quantify protein expression of **(B)** IRS-1, **(C)** phospho-IRS-1(ser312) and **(D)** phospho-IRS-1(ser312)/IRS-1, with expression normalised to GAPDH. Mann-Whitney U test was used to compare protein expression between treatment groups.

5.3.6 Placental-derived mediators in GDM have no significant impact on adipocyte mROS production

In this study we have shown that placental tissue from GDM pregnancies secretes a plethora of pro-inflammatory motifs, including IL-18, IL-6 and TNF-α. Additionally, endogenous and infiltrating $CD14^+$ cells, particularly M1-like macrophages, demonstrated significant mROS production in GDM placental tissue. Although we did not see similar levels of mitochondrial superoxide production in omental VAT $CD14^+$ populations in GDM in Chapter 3, localized adipocyte-mROS production can exacerbate insulin resistance. Oxidative stress, localized to mitochondria in insulin-resistant adipocytes, has been shown to directly impair insulin-stimulated glucose uptake and GLUT4 translocation to the plasma membrane (347). To assess if placental-derived mediators in GDM may stimulate localised mROS production in adipocytes, mitochondrial-specific superoxide production (MitoSOX) was measured in primary mature adipocytes and normalized with MitoTracker Deep Red (live cell mitochondrial stain), following 24-hour treatment with 5% NGT (n=10) and GDM (n=10) placental explant supernatant. There was no significant differences in mROS production between the untreated, NGT or GDM treated groups (0.34 RFU [0.23, 0.42] vs. 0.37 RFU [0.31, 0.40] vs. 0.36 RFU [0.31, 0.38] respectively, p=0.51) (**Figure 5.6**).

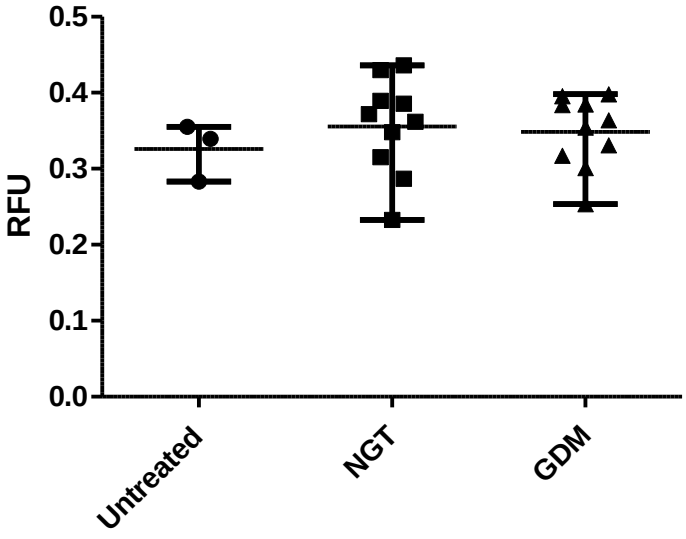


Figure 5.6. Placental-derived mediators in GDM do not affect adipocyte mROS production. Mitochondrial superoxide production was measured in terminally differentiated adipocyte cultures treated with NGT (n=10) and GDM (n=10) placental explant culture supernatant. Analysis groups were compared with the Kruskel-Wallis test.

5.3.7 Effect of omental-derived mediators in GDM on the placental production of mROS

The results to date in this chapter suggest that placental signalling in GDM does not directly impact the function or metabolic activity of primary adipocytes *in vitro*. These findings also indicate that the *in vivo* alterations evident in VAT in this study may not be directly regulated by placental cross-talk. To further examine the potential bidirectional role of the adipose-placental axis in mediating meta-inflammation in GDM, we interrogated the role of secreted omental mediators on placental mitochondrial and inflammatory profiles. Specifically, HTR8 trophoblast cells were treated with 5% NGT (n=10) and GDM (n=10) omental explant supernatant for 24 hours and mitochondrial-specific superoxide production (MitoSOX) was measured by fluorescence and normalized with MitoTracker Deep

Red (live cell mitochondrial stain). There were no significant differences in mROS production between the untreated, NGT or GDM treated groups (0.25 RFU [0.13, 0.31] vs. 0.26 RFU [0.24, 0.28] vs. 0.28 RFU [0.26, 0.31] respectively, p=0.15) (**Figure 5.7**).

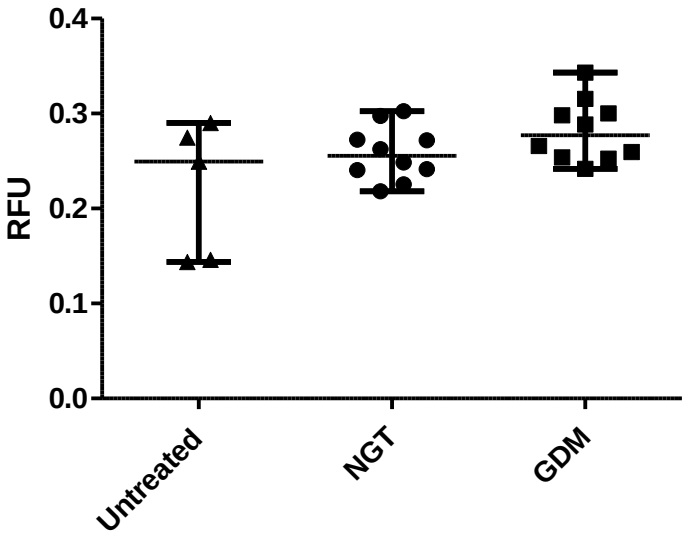


Figure 5.7. Omental-derived mediators in GDM do not affect trophoblast mROS production. Mitochondrial superoxide production was quantified in HTR8 trophoblast cells treated with NGT (n=10) and GDM (n=10) omental explant culture supernatant. Analysis groups were compared with Kruskel-Wallis test.

5.3.8 Omental-derived mediators in GDM do not alter placental cytokine release

We have provided evidence of systemic inflammation in GDM in Chapter 2, with increased circulating levels of pro-inflammatory cytokines IL-6, IL-17A, IL-12p70 and MCP-1, and increased placental production of IL-18, IL-6 and TNF-α in GDM placental tissue in Chapter 4. To elucidate if dysfunctional omental metabolism in GDM may exacerbate local placental inflammation, HTR8 trophoblast cells were treated with 5% NGT (n=10) and GDM (n=10) omental explant supernatant for 24

hours. A panel of 13 key cytokines were measured in HTR8 cell supernatants following a 24-hour wash-out period. There were no significant differences, between treatment groups, in the placental production of cytokines (**Table 5.1**).

Table 5.1. HTR8 secretion of pro-inflammatory cytokines following treatment with NGT and GDM omental supernatant

Cytokine	**NGT (Median [95% CI])(pg/ml)**	**GDM (Median [95% CI])(pg/ml)**	**P value**
IL-6	105799 [77139, 134187]	97096 [88343, 142867]	0.63
IL-17A	1.86 [1.59, 2.23]	1.79 [1.68, 2.09]	1.00
IL-12p70	81.79 [65.86, 91.62]	75.77 [70.15, 89.34]	0.91
MCP-1	13248 [10350, 14859]	13327 [12179, 15242]	0.44
TNF-α	55.14 [48.79, 64.68]	61.08 [52.41, 64.46]	0.68
IFN-α2	32.39 [25.8, 36.37]	30.39 [27.64, 35.44]	0.63
IL-23	118.2 [100.2, 139.3]	116.1 [103.6, 132.6]	0.97
IL-10	171.3 [135.7, 195.4]	164.4 [147.4, 195.5]	0.8
IFN-ɣ	64.63 [51.23, 71.92]	57.95 [48.93, 67.98]	0.68
IL-8	85759 [64330, 96505]	75769 [69518, 94031]	0.8
IL-33	97.71 [81.16, 115.4]	94.19 [80.41, 103.1]	0.68
IL-1β	165.1 [140.4, 204.2]	165.1 [149.1, 197.7]	1.00
IL-18	196.6 [157.8, 214.7]	183.7 [166.5, 216.7]	0.85

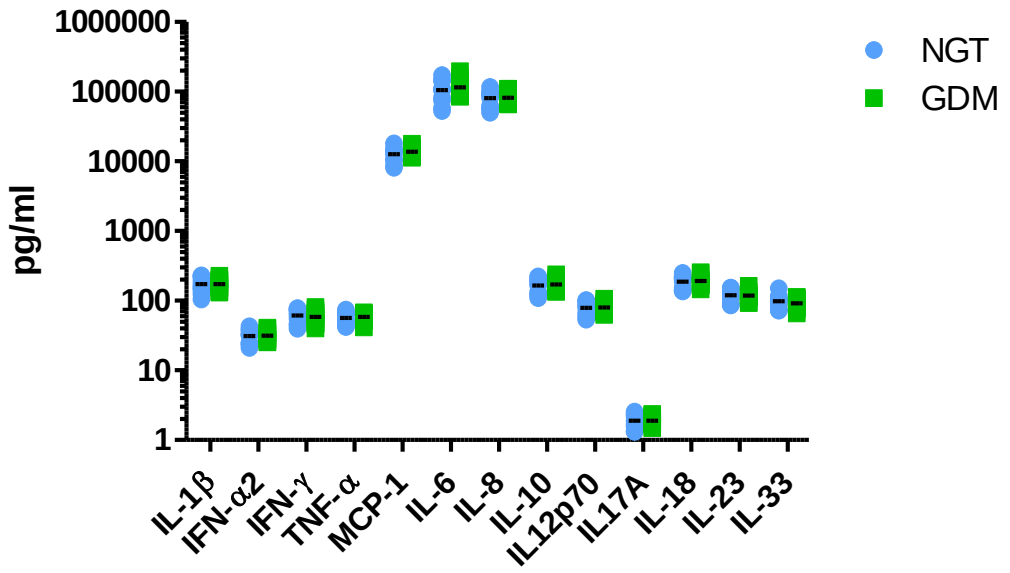


Figure 5.8. No change in omental-mediated cytokine release from HTR8 cells. Trophoblast production of IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33 levels were quantified following 24-hour treatment with NGT (n=10) and GDM (n=10) omental explant culture supernatant. The Mann-Whitney U test was used to compare cytokine levels.

5.4 Discussion

In this chapter, we investigated the potential intersection of placental-adipose signalling in contributing to the pathophysiology of GDM. Primarily we were interested in interrogating the metabolic and inflammatory processes which were dysregulated *in vivo* in Chapters 2, 3 and 4 to elucidate if there was an interdependent pathogenic link between the placenta and omental adipose tissue in GDM. In this study we used both omental VAT and placental explant supernatant cultures from nulliparous NGT and GDM participants at term pregnancy to mimic the GDM condition *in vitro*. Using both adipocyte and placental *in vitro* cellular models, the impact of placental mediators on adipogenesis in primary undifferentiated preadipocytes, intracellular lipid content, glucose uptake and mROS production in primary terminally differentiated adipocytes was explored. Finally, the mitochondrial and inflammatory profile was determined in the HTR-8/SVneo trophoblast cell line, upon exposure to omental mediators in GDM. Overall, there was no direct evidence that cross-talk between placental-adipose tissue contributes to a mutually dependent regulatory relationship in GDM.

Firstly, this study focused on exploring if the placental secretome instigates adipocyte dysfunction in GDM. In Chapter 4, we reported increased macrophage-induced inflammation in placental tissue in GDM, with increased secretion of IL-6, TNF-α and IL-18 cytokines. Additionally, GDM participants had significant omental VAT adipocyte hypoplasia which correlated with insulin resistance. One potential cause of a reduction in mature adipocyte cell count may be as a result of a deficiency in preadipocyte differentiation (adipogenesis) to mature adipocytes. In order to elucidate if placental-derived mediators impact adipogenesis, primary preadipocytes were treated with placental explant supernatant from NGT and GDM participants

over a 72 hour differentiation period. However, while treatment with placental mediators from each study group increased adipogenesis compared to untreated controls, we found no significant difference in adipogenesis between treatment groups as characterised by preadipocyte lipid uptake. It is important to highlight that all preadipocytes were equally cultured in a cocktail of potent differentiation factors during the treatment period, a process which may be difficult to reverse *in vitro*. Nonetheless, when compared to untreated control preadipocytes, placental-derived mediators in GDM stimulated significantly increased differentiation, which was not significant when compared to NGT treated cells. While this result should not be misinterpreted as a major difference between treatment groups, it indicates that placental signalling in GDM promotes adipogenesis, potentially in a homeostatic manner. One possible mediator of placental-induced adipogenesis may be the increased secretion of adipsin from placental tissue in GDM. Adipsin has previously been shown to promote lipid accumulation and adipocyte differentiation *in vitro* (475). Additionally, this finding also indicates that adipocyte hypoplasia evident in VAT in GDM in our study is possibly not due to a lack of placental-derived adipogenic signals but may be related to an earlier deficiency in commitment of progenitor stem cells towards an adipogenic lineage.

To further delineate the placental influence on adipogenic differentiation in GDM, IGFBP-3 and IGFBP-4 protein expression was determined in terminally differentiated mature adipocytes after treatment with placental explant supernatant from NGT and GDM participants. In Chapter 3, we identified that omental VAT expression of IGFBP-4 was significantly reduced in GDM participants. IGFBP-4 is an important binding protein which modulates IGF-1 activity by increasing local retention and driving adipogenesis (394). However, placental signalling in GDM

does not appear to directly limit IGFBP expression in adipocytes, as no significant difference in IGFBP-3 or IGFBP-4 expression between NGT and GDM treatments was evident.

Adipocyte lipolysis is the process by which adipocytes break down stored lipids into FFAs and glycerol, which can then be used as energy sources. In healthy individuals, adipocyte lipolysis is regulated by hormones such as insulin, which help to control the release of FFAs into the bloodstream. However, insulin resistance can lead to an abnormal regulation of adipocyte lipolysis, leading to an excess of FFAs in the bloodstream. FFAs contribute to inflammation and oxidative stress, and increase the risk of cardiometabolic diseases. In addition, excess FFAs can disrupt insulins functional roles and glucose uptake into cells, leading to hyperglycaemia-related complications (476). Previous findings have shown that placenta-secreted hormones such as hPL and estrogen regulate lipolysis during healthy pregnancy, particularly during the third trimester (149). In GDM, lipolysis is increased as a result of insulin resistance and subsequent hyperinsulinaemia. As a result, there is an increase in circulating FFA levels which can propagate insulin resistance and systemic inflammation (311).

Although we did not find significantly elevated circulating levels of triglycerides or FFAs in GDM participants, we wanted to explore if the placental secretome may offset local adipocyte lipolysis which may contribute to metabolic dysfunction in GDM. To assess whether placental mediators in GDM may affect adipocyte lipolysis and the rate of IC triglyceride depletion, terminally differentiated mature adipocytes were treated for 24 hours with placental explant supernatant from NGT and GDM participants and IC lipid content was quantified by AdipoRed fluorescence. Placental signalling from either treatment group did not significantly deplete adipocyte lipid

content compared with untreated control adipocytes. This suggests that placental-derived mediators do not directly impact adipocyte lipolysis in GDM.

Our previous results highlighted significant systemic insulin resistance in GDM participants at term, characterised by hyperinsulinaemia, hyperglycaemia and significantly higher HOMA-IR scores. Locally in omental VAT, we also found significant evidence of disruption in the insulin signalling cascade in GDM. This disruption resulted from reduced omental expression of IRS-2 and increased phosphorylation of IRS-1 at the ser312 residue, thereby inhibiting insulin signal transduction and subsequent glucose uptake. Previous research has confirmed that placental-secreted hormones, including estrogen, progesterone, leptin, cortisol, placental lactogen and placental growth hormone can limit glucose uptake and promote insulin resistance (477). These placental hormones promote systemic insulin resistance in healthy pregnancy but may drive pathological insulin resistance in placental dysregulation.

To further examine the potential contribution of placental-derived mediators to this insulin resistant phenotype in GDM, we measured glucose uptake and quantified members of the insulin signalling cascade in primary mature adipocytes upon exposure to placental explant culture supernatant. There was no significant difference in either adipocyte glucose uptake or protein expression of IRS-1 or phospho-IRS1(ser312) upon exposure to placental-derived mediators. Despite the increased abundance of inflammatory cytokines and alterations in leptin and adipsin levels in the placental secretome, these findings suggest that placental mediators do not directly impair AT insulin sensitivity and systemic insulin resistance as was observed in GDM participants.

Exaggerated production of mROS in adipocytes can inhibit adipocyte differentiation by promoting the expression of the adipogenic repressor C/EBP Homologous Protein-10 (CHOP-10) (478). Furthermore, increased mROS production in 3T3-L1 adipocytes, via knockdown of the mitochondrial antioxidant protein peroxiredoxin 3 (Prx3), reduced adiponectin expression *in vitro* and impaired glucose tolerance in Prx3 KO mice (479). We next wanted to characterise if placental derived mediators induce mitochondrial superoxide in mature adipocytes which may contribute to impaired adipogenesis, lower circulating adiponectin levels and increased insulin resistance, as observed in GDM participants in this study. Similar to our results in Chapter 3, we found no evidence of increased mROS production in adipocytes upon exposure to placental explant supernatant culture from either study group. This finding would suggest that localised mitochondrial metabolism in adipocytes is not directly responsible for the VAT phenotype evident in GDM.

Our next step was to elucidate if there was bidirectional communication between both omental adipose tissue and placenta in GDM. In Chapter 4, we identified increased mROS production in endogenous and infiltrating placental M1-like macrophages and increased placental inflammation in GDM participants. In this study we used the HTR-8/SVneo cell line, an immortalized cell line that is a well-established model of first trimester human trophoblasts, to investigate if omental signalling contributes to the meta-inflammatory phenotype evident in placental tissue in GDM. Previous research has established that primary cytotrophoblasts isolated from GDM term placental tissues had decreased oxidative phosphorylation capacity and significantly lower ATP content, suggesting that mitochondrial activity is hindered in GDM placental tissue (441). HTR8 cells were treated with omental supernatant from NGT and GDM participants for 24 hours but no significant

differences in mitochondrial superoxide production were found between study groups as determined by MitoSOX staining. This result shows that omental-derived mediators do not directly contribute to placental trophoblast mROS production in GDM, which may in fact be mediated by infiltrating macrophages.

Additionally, we provided evidence of placental inflammation in GDM participants in Chapter 4, with increased production of pro-inflammatory cytokines IL-6, TNF-α and IL-18. We equally want to determine if omental-derived mediators contributed to this placental inflammatory phenotype. A panel of 13 key cytokines were measured in HTR8 trophoblasts exposed to omental explant supernatant for 24 hours followed by a washout period of 24 hours. However, there were no significant differences seen in the trophoblast cytokine profile between NGT and GDM treatment groups. This result may be partly explained by the fact that FFAs released from VAT, known instigators of inflammation, were not significantly increased in GDM participants. Therefore, from these findings, we can conclude that omental signalling does not directly impact trophoblast inflammation in GDM.

This chapter allowed us to investigate the potential bidirectional communication pathway between key metabolic organs in GDM, namely VAT and the placenta. The main strengths of our work include the use of primary human preadipocytes, thereby improving the external validity of our *in vitro* findings. These preadipocytes were commercially acquired from non-smoking female donors with a mean age of 37.7, mean BMI of 46.5 and mixed ethnicity including African American, Caucasian and Hispanic ethnic groups. These donors were chosen to match with our study participants as closely as possible. The mixed ethnicity of the cell donors improves the relevance of our research to the general population. The same omental and placental supernatant cultures were used for our *in vitro* work as was used

throughout this thesis for clinical characterisation, allowing us to correlate our results in this chapter with other findings in earlier chapters from these same participants.

Limitations of the research in this chapter include the use of the HTR8/SVneo cell line, rather than primary trophoblasts, for our placental investigations. Additionally, while the preadipocytes used in this study were carefully match for gender, age and BMI, they were not from a pregnant population which may have influenced some of our results, given the significant impact pregnancy has on maternal cellular adaption. Furthermore, the mean BMI of cell donors was 46.5, which represents a particularly obese population. Apart from the 72-hour treatment for the investigation of preadipocyte differentiation, all other *in vitro* treatments were 24 hours. This timeline was chosen to reduce the confounding effect of general exogenous stress on cell cultures. Primary cells are particularly sensitive to changes in media composition or external stimuli and we wanted to ensure that our results were directly related to signalling components in the tissue explant supernatants rather than due to changes in culture conditions. However, this 24-hour treatment may not have been sufficient to represent the chronic exposure of placental-omental signalling throughout the gestation of GDM pregnancies.

Overall, this study showed that placental-derived mediators in GDM do not directly affect adipocyte differentiation capacity, lipolysis, glucose uptake or disrupt the insulin signalling cascade in adipocytes. Furthermore, omental-derived mediators in GDM do not appear to directly exacerbate mitochondrial superoxide production or inflammation in placental trophoblasts.

5.5 Conclusion

As described in earlier chapters, both VAT and the placenta are integral metabolic mediators involved in the pathophysiology of GDM. Furthermore, we have identified specific meta-inflammatory processes which were dysregulated *in vivo* in each organ and which may contribute to systemic insulin resistance in GDM. In this chapter we investigated the potential interdependent role of placental-adipose signalling in the development of GDM. Firstly, while this study found that placental-derived mediators in GDM stimulated increased differentiation of preadipocytes compared to untreated control, there was no significant difference in adipogenesis compared to NGT treatment. Additionally, placental-derived mediators in GDM did not increase the expression of IGFBP-3 or IGFBP-4, key mediators of adipogenesis in terminally differentiated adipocytes. Furthermore, we found that placental-derived mediators in GDM did not significantly impact lipolysis, glucose uptake capacity, IRS-1 functionality or mROS production of mature adipocytes. Finally, this study established that omental-derived mediators in GDM did not increase mROS production or cytokine secretion in HTR-8/SVneo trophoblasts. While these findings indicate a lack of inter-organ communication between adipose and placental tissues in GDM, indirect effects mediated by systemic circulating factors cannot be excluded.

Supplementary material related to Chapter 5

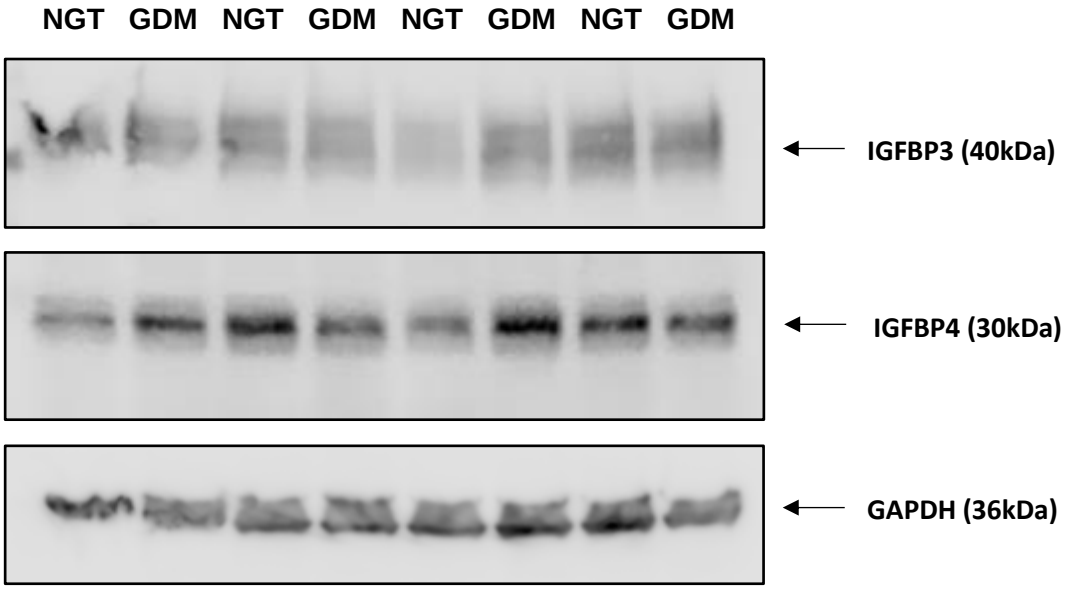
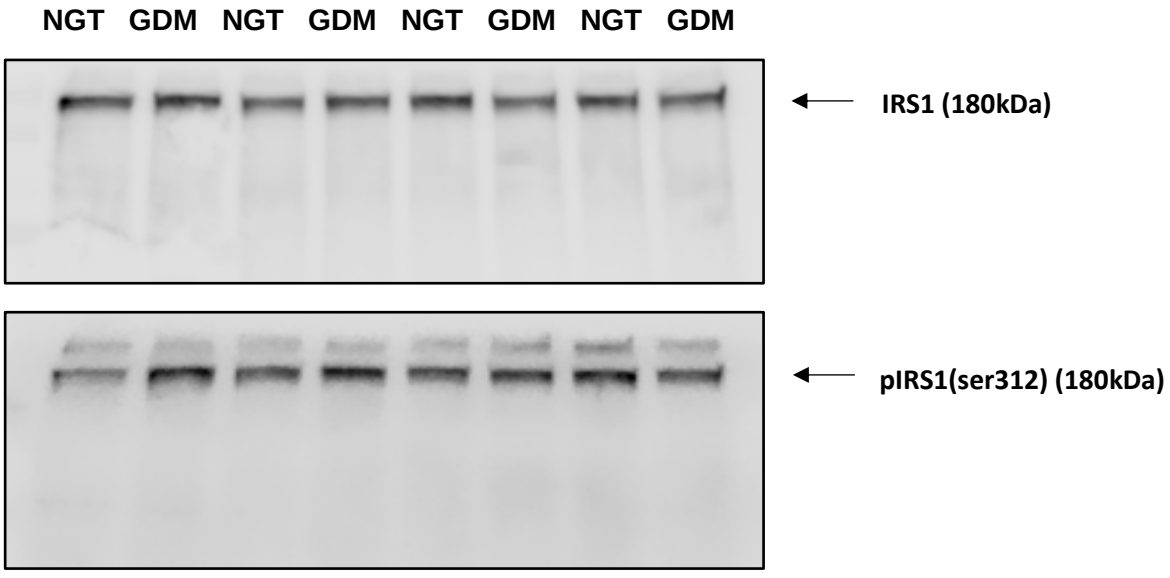


Figure S5.1. Primary adipocyte protein expression of IGFBP-3 and IGFBP-4 was compared after 24-hour treatment with NGT (n=4) and GDM (n=4) placental explant culture supernatant. NGT and GDM-treated protein lysates were loaded in alternating order.



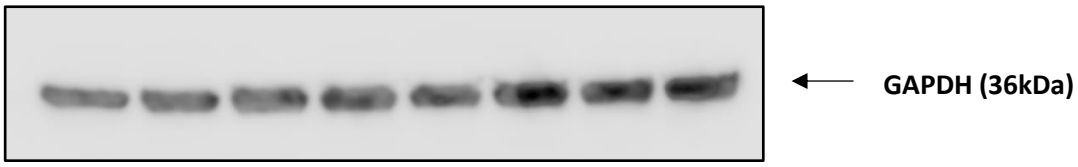


Figure S5.2. Primary adipocyte protein expression of IRS-1 and phospho-IRS-1(ser312) was compared after 24-hour treatment with NGT (n=4) and GDM (n=4) placental explant culture supernatant. NGT and GDM-treated protein lysates were loaded in alternating order.

Chapter 6

General discussion

6.1 Overview and summary of key findings

Chapter 1 reviewed the current literature on the clinical considerations and pathophysiology of GDM. The development of GDM during pregnancy is considered an interplay between genetic risk factors and environmental influence. Identified genetic risk factors include those associated with susceptibility to obesity and metabolic diseases such as T2DM (16). However, maternal obesity remains the most significant modifiable risk factor for GDM diagnosis (4). Adverse outcomes of GDM pathology include chronic risk of maternal T2DM development, recurrent GDM, metabolic syndrome and cardiovascular disease (25). GDM is also associated with an increased risk of adverse perinatal complications such as macrosomia, respiratory distress syndrome and fetal hyperinsulinaemia (37). GDM stems from an insufficient response in regulating the physiological insulin resistance of healthy pregnancy, resulting in aberrant, exaggerated insulin resistance with associated systemic inflammation. The exact pathophysiological origins of GDM are not fully understood, however several key biological factors likely contribute to instigate and promote the diabetic syndrome, including pancreatic β-cell dysfunction, disruption in insulin signalling, inflammation and mitochondrial dysfunction.

By reviewing these findings, we hypothesised that a link exists between local tissue meta-inflammation and the release of mitochondrial-derived DAMPs, which orchestrate inflammatory pathways locally and systemically and ultimately contribute to GDM pathology. The main aims of this thesis were to characterise GDM-mediated meta-inflammation and mitochondrial dysfunction locally in both omental VAT and the placenta, and systemically in circulation (see Table 6.1).. In addition, we aimed to investigate the bidirectional communication between placental and adipose tissue which may contribute to systemic insulin resistance in GDM.

In **Chapter 2**, we characterised metabolic, mitochondrial and inflammatory mediators in the maternal circulation, to elucidate if meta-inflammation was involved in exaggerated insulin resistance in GDM. Firstly, significant insulin resistance was established in GDM participants, as determined by HOMA-IR score calculation. Stratification based on clinical intervention established that insulin resistance was significantly increased in women who required pharmacological intervention but not in women whose hyperglycaemia could be managed through lifestyle modification alone. Furthermore, HOMA-IR score correlated with maternal BMI in GDM participants, who had significantly higher BMI values relative to NGT participants. However, subgrouping GDM participants by BMI uncovered two distinct phenotypes, namely lean, insulin-deficient and obese, insulin-resistant GDM pathologies. Cf-mtDNA levels were also significantly increased in GDM, suggesting a possible pathogenic role for mitochondrial dysfunction. Despite the lack of alteration in monocyte/macrophage populations between NGT and GDM study groups, there was significant evidence that GDM is associated with a chronic inflammatory state, with increased pro-inflammatory cytokine production of IL-6, IL-17A, IL-12p70, MCP-1 and lower adiponectin levels, an anti-inflammatory, insulin-sensitizing adipokine.

Chapter 3 took a closer look at participant omental VAT to determine if local adipocyte dysfunction and meta-inflammation may instigate insulin resistance in GDM. We provided evidence of reduced adipocyte count and reduced protein expression of IGFBP-4 and IRS-2 in the omental VAT of GDM participants. Reduced IRS-2 expression in GDM omental VAT correlated with hypoplasia. Given the integral role of these key regulators in mediating adipogenesis, their reduction in omental expression could partly contribute to VAT adipocyte hypoplasia in GDM.

Furthermore, adipocyte hypoplasia correlated with higher HOMA-IR scores in GDM, suggesting that adipocyte hypoplasia may impair insulin sensitivity in omental VAT depots. We also found evidence of disrupted insulin signalling in GDM participants, including reduced expression of IRS-2 and increased phosphorylation of the ser312 residue of the critical signalling mediator IRS-1, inhibiting IRS-1 mediated insulin signal transduction. These findings suggest that impaired metabolic flexibility in the omental visceral tissue may contribute to the previously established systemic insulin resistance in GDM.

Chapter 4 examined placental tissue in GDM, focusing on whether mitochondrial dysfunction may activate the NLRP3 inflammasome and promote placental inflammation. Initially, we found significant evidence of placental villous immaturity in GDM, potentially as a result of systemic insulin resistance and hyperglycaemia. M1-like macrophages infiltrating placental tissue produced significantly increased levels of mROS in GDM. Additional evidence of immune and metabolic dysregulation in the placental milieu in GDM was provided with increased secretion of IL-18, IL-6, TNF-α and adipsin and lower leptin secretion. We also identified that the NLRP3 inflammasome may play a regulatory role in the cross-talk between mROS and placental inflammation in GDM. Directly targeting mROS production with ERG diminished placental inflammation in GDM, establishing a role of mROS production in driving placental inflammation and identifying a potential therapeutic role for this nutraceutical antioxidant.

In **Chapter 5,** we used *in vitro* cellular models to elucidate the potential role of placental-adipose tissue cross-talk in mediating GDM pathology. Using supernatant from NGT and GDM placental and omental VAT explant cultures, we examined the response of primary human adipocytes and HTR8/SVneo trophoblasts to placental

and omental signalling, respectively. This study showed that placental signalling in GDM does not directly affect adipocyte differentiation capacity, IGFBP expression, adipocyte lipolysis, insulin signalling or adipocyte mROS production. Furthermore, omental signalling in GDM does not increase trophoblast mROS production or elevate the placental release of inflammatory cytokines that we observed in GDM participant tissue. These findings suggest a lack of direct communication between the placenta and VAT in GDM.

6.2 Interpretation of key findings

The first important finding of this study was to consolidate the key role that maternal BMI plays as a risk factor in the development of GDM. Comparison of participant clinical correlations in this study established that women diagnosed with GDM had a significantly higher BMI, relative to NGT pregnant women. Furthermore, maternal BMI significantly and positively correlated with HOMA-IR scores in this GDM cohort, suggesting that obesity-driven insulin resistance may contribute to pathology in GDM participants, as has been previously identified in T2DM (480). However, GDM equally develops in lean individuals. In this study, 13.3% of GDM participants had a BMI < 25 kg/m^2 and 40% had a BMI < 30 kg/m^2. These anthropometric findings suggest that obesity alone does not account for all cases of GDM diagnosis, as only 60% of GDM participants in this study had a BMI >30 kg/m^2.

The second prominent finding of this study was the identification of a reduced omental adipocyte count in GDM, particularly in women with severe GDM requiring insulin therapy. Interestingly, adipocyte hypoplasia correlated with an increase in systemic insulin resistance. Adipocyte hypoplasia was significantly evident in obese GDM participants (BMI > 29 kg/m^2) relative to both NGT and lean GDM participants, showing evidence for the first time that deficient adipogenesis and concurrent insulin resistance are prominent pathogenic mediators in this subgroup. Visceral WAT expands through both hypertrophy and hyperplasia. While hypertrophy is an adaptive response to nutrient excess, hyperplasia, influenced by various transcription factors including PPAR-ɣ, acts to balance hypertrophic responses and restore AT homeostasis. Here, we found no significant increase in adipocyte hypertrophy in GDM, despite the increase in maternal BMI. Muir et al. found that patients with T2DM had a smaller depot of adipocyte progenitors in

omental VAT, which may increase hypertrophy by limiting the generation of new adipocytes in diabetic patients (391). This same predisposition to impaired adipogenesis may explain how adipocyte hypoplasia in GDM participants may limit the preadipocyte proliferation capacity necessary to maintain adipose tissue homeostasis in obesity-driven metabolic dysfunction.

Accumulation of VAT is a critical predictor of metabolic disorders such as insulin resistance, however some findings suggest that, in obese physiology, hyperplasia is predominant in SC adipose depots rather than the omental depots and that hypertrophic responses are more relevant when examining omental VAT response to obesity (481). However, these findings were based on anthropometric characteristics of non-pregnant healthy women. During pregnancy, placental hormones stimulate enhanced expansion of adipose tissue depots to support both maternal and fetal nutritional requirements. Importantly, AT is preferentially deposited in the more metabolically active visceral depot as pregnancy progresses (482). Therefore, we can consider that AT dysregulation during GDM pregnancy is a unique pathology, which is underpinned by alterations in normal physiological responses.

The next step was to investigate the mechanistic pathways contributing to adipocyte hypoplasia in GDM. Initially, we looked at the IGF-IGFBP axis, which is an important regulator of adipocyte differentiation (483). IGF-1 is a hormone responsible for promoting normal bone and tissue development, with a specific effect on stimulating the differentiation of adipocyte precursor cells (484). Although we did not find any changes in maternal circulating IGF-1 levels in GDM, omental protein expression of IGFBP-4 was significantly reduced in GDM. As IGFBP-4 plays a role in potentiating IGF-1 activity and adipogenesis by increasing local retention, reduced expression may in part explain the hypoplasia evident in GDM omental VAT (394).

In this way, insufficient IGFBP-4 expression in GDM may contribute to a reduction in tissue expandability which is required to accommodate the metabolic changes that occur in pregnancy.

Adipokines equally have important roles in regulating local and systemic metabolic responses, including the induction of adipocyte differentiation and AT expansion. As such, leptin, adiponectin, adipsin and resistin levels were measured in the maternal circulation and in the supernatant of omental and placental tissue explant cultures Despite the reduction in adipocyte count, omental secretion of these adipokines were not significanty altered in GDM, however circulating adiponectin levels were significantly lower in GDM participants. Adiponectin also plays a role in promoting preadipocyte proliferation and differentiation into adipocytes, in addition to exerting beneficial effects on adipocyte function including the stimulation of glucose uptake by increased GLUT4 translocation (485). Therefore, reduced circulating adiponectin levels in this study may equally contribute to adipocyte pathology. These findings add to the current body of evidence that reduced adiponectin levels have a pathogenic role in GDM.

In exploring the concept of reduced adipogenesis further, we identified in Chapter 4 that placental secretion of adipsin, a promoter of adipogenesis, was elevated in GDM relative to NGT. Additionally, in Chapter 5, there was evidence that placental-derived mediators from GDM had an increased capacity for promoting adipogenesis when compared to untreated primary VAT adipocytes, an effect not evident in NGT. This somewhat conflicting evidence hints that while placental-derived mediators in GDM may be pro-adipogenic, the inherent potential of preadipocytes to proliferate and undergo adipogenesis may be deficient, which could lead to the hypoplasia phenotype evident in VAT in GDM.

Next, we wanted to determine whether the functionality of adipocyte insulin signalling capacity may be impaired in GDM and if this impairment contributes to systemic insulin resistance evident in these participants. In the omental VAT of GDM participants, we found that increased phosphorylation of the IRS-1 serine 312 residue hindered IRS-1 mediated insulin signalling while total IRS-2 expression was reduced, thereby impairing IRS-2 activity. Phosphorylation of IRS-1 serine residues can be initiated in response to TNF-α and IL-6 inflammatory signalling. IL-6 levels were significantly higher in both serum and placenta of GDM participants while TNF-α release was also significantly elevated from GDM placental tissue, relative to NGT participants. Adiponectin, which was systemically depleted in GDM participants in this study, protects IRS-1 from these inflammatory-induced effects and preserves insulin sensitivity (401-403). Therefore, deleterious IRS-1 phosphorylation in GDM participants may be promoted by systemic low-grade inflammation, paired with reduced adiponectin-mediated protection.

The significance of IRS-2 insufficiency in mediating insulin signalling is not as well defined as IRS-1. Knockdown of IRS-2 in murine preadipocytes severely impairs glucose uptake, hinders preadipocyte differentiation and reduces secretion of adiponectin (399). In this study, reduced total IRS-2 expression in the VAT of GDM participants significantly correlated with reduced adipocyte count. Furthermore, circulating adiponectin levels were significantly reduced in these GDM participants. It is therefore plausible that depleted IRS-2 expression in the VAT of GDM participants could promote insulin resistance on three levels, by diminishing insulin signalling transduction, preventing homeostatic adipogenesis and reducing adiponectin production. These are the first clinical findings of VAT insulin signalling dysfunction in GDM participants.

The next integral finding of this thesis was the possible pathogenic role of maternal inflammation in GDM. We had initially hypothesised that localised tissue meta-inflammation and the release of mitochondrial-derived DAMPs may increase systemic inflammation and exacerbate insulin resistance in GDM. Evidence presented in this thesis suggests that this hypothesis is partially true, as we identified localised placental dysregulation of metabolic adipokines and increased placental inflammation. However, there was no evidence of increased infiltration of macrophages or localised inflammation in omental VAT in GDM, unlike the consistent evidence presented in T2DM (see Table 6.1). Nonetheless, there was very strong evidence of systemic inflammation in the maternal circulation in GDM participants, although this increase in inflammatory mediators was not directly related to an increase in abundance of circulating monocyte/macrophages.

Additionally, elevated circulating cf-mtDNA numbers were quantified in GDM participants, suggesting mitochondrial dysfunction is a feature of GDM pathophysiology. Peripheral cf-mtDNA levels did not correlate with maternal age or BMI in either study group. Furthermore, the origin of cf-mtDNA in circulation was not elucidated. However, previous findings from our group have found that, at 20 weeks' gestation, circulating cf-mtDNA levels are significantly elevated in GDM and may be linked to maternal BMI (194). Indirectly, we have previously shown that maternal plasma (containing elevated cf-mtDNA) increases inflammation in PE via activation of the TLR-9 receptor (192). However this was not evident in GDM, with no increase in activation of TLR-9 *in vitro* upon exposure to GDM plasma. One possible reason for this outcome is that the amount of cf-mtDNA in maternal plasma in GDM may have been below the threshold required to activate TLR-9 in this cellular assay. Future studies with purified cf-mtDNA isolated from maternal plasma

are required to determine if this DAMP directly instigates maternal inflammation in GDM.

As described earlier, there was evidence of localised placental inflammation in GDM. Similar to the maternal circulation, this increase was not directly related to an increase in macrophage abundance. However, this pro-inflammatory state is possibly related to an increased production of mitochondrial-specific ROS, which was particularly evident in the placental M1-like pro-inflammatory macrophage population in GDM. We now propose that an infiltrating macrophage population, rather than endogenous placental macrophages, are responsible for increased placental inflammation as there was no evidence of oxidative damage in placental tissue.

My original hypothesis suggested that the NLRP3 inflammasome acts a a nexus, co-ordinating mitochondrial DAMP-induced inflammation in GDM. In this thesis, placental secretion of IL-18, a key cytokine released downstream of NLRP3 activation, was significantly increased in GDM placental tissue. Additionally, treatment of placental explants with MCC950, a selective inhibitor of NLRP3 activation, significantly attenuated exaggerated placental release of IL-18. Activation of the NLRP3 inflammasome in GDM was specific to the placenta as there was no evidence of NLRP3 activation locally in adipose tissue or systemically. Furthermore, the NLRP3 inflammasome is predominantly expressed by macrophages (486). Interestingly, elevated placental IL-18 production was significantly reduced by treatment with ERG, a potent antioxidant, which suggests that exaggerated macrophage prodution of mROS subsequently activates the NLRP3 inflammasome and increases placental inflammation. It is important to note that the protein expression of NLRP3, ASC or pro-caspase-1 were not increased in GDM placental

tissue in this study. This suggests that, in term GDM placenta, exaggerated mROS production may promote activation and downstream cleavage of caspase-1 with the subsequent release of active IL-18, rather than regulating NLRP3 priming and expression. Unfortunately, we were unable to successfully detect cleaved caspase-1 by immunoblotting in our samples to further delineate this theory. This thesis adds to growing evidence for the pathogenic role of inflammasomes in mediating placental inflammation in obstetric complications.

While the systemic inflammatory profile differed somewhat from the identified placental profile, IL-6 was increased in both locations in GDM, suggesting that placental tissue contributes to the circulating inflammatory phenotype in GDM. A recent systematic review identified that peripheral IL-6 may be a possible predictive marker of GDM (487). Maternal inflammation may not only exacerbate maternal insulin resistance during pregnancy, but may equally represent a pathophysiological link between GDM and future maternal risk of cardiometabolic complications. Therefore, adopting glucose-centric therapeutic approaches alone in GDM may not be sufficient for optimising acute and chronic maternal health outcomes.

Finally, this thesis recognised a possible therapeutic avenue for antioxidant therapy, such as ERG, in ameliorating localised placental inflammation in GDM. ERG has the advantage of a favourable pharmacokinetic profile, with avid uptake and retention in plasma after oral administration, relatively low excretion rates and active transfer and accumulation in sites that express the transporter OCTN1, which includes placental tissue. Also, minimal adverse effects have been reported with ERG administration in animal or human studies (449, 488). There are current interventional trials underway to assess the health benefits of ERG supplementation in people with metabolic syndrome (489), however future investigations are required

to explore the effects of this nutraceutical in modifiying insulin resistance, particularly in GDM.

Table 6.1. Summary of macrophage phenotype, cytokine and adipokine findings in maternal fasting blood, omental VAT and placental tissue.

Analytes measured	**Tissue**	**GDM vs. NGT participants**
Macrophage phenotype (M1/M2 polarization, mROS production)	Fasting blood	No change
	Omentum	No change
	Placenta	Elevated mROS production in $CD14^+$ and M1-like macrophages
Cytokines (IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33)	Fasting blood	Elevated IL-6, IL-17A, IL-12p70, MCP-1 levels
	Omentum	No change
	Placenta	Elevated secretion of IL-18, IL-6 and TNF-α
Adipokines (adiponectin, adipsin, leptin, resistin)	Fasting blood	Reduced adiponectin levels
	Omentum	No change
	Placenta	Elevated adipsin and reduced leptin secretion

6.3 Strengths and limitations

One of the primary strengths of this thesis is that it is one of the first studies to employ a systemic multi-organ characterisation of GDM pathophysiology. Furthermore, this study recruited nulliparous women which allowed us to explore the pathophysiology of GDM, without the confounding effects of adaptive maternal physiological responses to previous pregnancies. The analysis of biological mediators of GDM were further supported by correlating outcomes with clinical variables for each participant in the study. The experimental design of this thesis was devised to limit confounding variables wherever possible. These efforts included consistent methods for participant recruitment and sample processing throughout the study and employing strict inclusion and exclusion criteria for participant enrolment. Where possible, samples were matched by maternal BMI and/or age to limit the effect of these variables on biochemical analysis.

There are equally limitations to this research. Although the single-site nature of our study removes the variability that can occur with pooling data from multiple sites and various research teams, this also limits the external validity of our findings. Additionally, all participants in this study were recruited in Cork University Maternity Hospital (CUMH), resulting in a predominantly white Irish study cohort. All biological samples were collected at a single timepoint at the end of pregnancy, which makes it difficult to accurately determine if the identified pathogenic mediators are contributing or consequential factors in GDM. However, the extent of our findings in term GDM pregnancy allows for future prospective studies to investigate these pathways in greater detail earlier in pregnancy.

6.4 Implications for Future Research

This thesis has characterised the role of mitochondrial dysfunction and meta-inflammation as a shared pathogenic network in multiple tissues in GDM. While these findings have enhanced our understanding of the complex interplay between immune-endocrine mediators in GDM, there are a number of additional research questions that warrant further investigation.

- In the current clinical care pathways for women with GDM, a one-size-fits-all approach is applied, yet in this study there is obvious heterogeneity evident in this population. Recent work by Powe et al. (320) has begun to adopt a personalised medicine approach to GDM by stratifying participants based on the contribution of insulin resistance and insulin deficiency to GDM heterogeneity. Three specific subpopulations were identified, namely lean GDM patients who lack evidence of insulin resistance but instead are insulin-deficient, obese GDM patients who are hyperinsulinaemic and whose hyperglycaemic state is driven by insulin resistance alone and those GDM patients who portray a mix of these two phenotypes. In this study this heterogeneity within GDM participants is equally evident. When insulin-treated participants were excluded for confounding reasons, women with insulin-resistant GDM (64%), had increased maternal BMI, hyperinsulinemia and significant VAT adipocyte hypoplasia, relative to insulin-deficient GDM participants (36%). This study was unable to identify women who may have a mix of insulin-deficient and insulin-resistant pathologies. Future work is required to further characterise these phenotypes and examine their utility in the clinical management of GDM, with the aim of providing precision medicine to the obstetric population.

- While VAT is widely recognised as a pathogenic mediator of T2DM, this is the first study to comprehensively characterise its potential role in GDM. Furthermore, while there has been limited evidence of adipocyte hypertrophy in subcutaneous depots in GDM, this is the first study to identify adipocyte hypoplasia in VAT in GDM and equally highlight its potentially important relationship with insulin resistance, as reported in Chapter 3. Dysregulation of the molecular cues that stimulate the physiological expansion of adipose tissue depots during pregnancy may have a pathogenic role in the development of insulin resistance, yet the mechanisms involved are yet to be fully elucidated. One possible avenue of investigation would be to employ single cell RNA sequencing technology to characterise the adipocyte progenitor cells (APCs) in the stromal vascular fraction of VAT. This would help decipher the differentiation trajectory of APCs and whether they regulate visceral adipose tissue plasticity and contribute to metabolic inflexibility in GDM.
- Despite the lack of a significant difference in circulating triglyceride levels in term participants with GDM in Chapter 2, a recent meta-analysis of 97,880 pregnant women (322) established that women with GDM had significantly higher triglycerides levels compared to NGT. Interestingly, this increase was most significant in both first and second trimester, prior to clinical diagnosis of GDM. Further studies are required to delineate the contribution of triglycerides to the pathophysiology of GDM, in particular their potential lipotoxic effects on maternal organs.
- While this thesis specifically characterised monocyte/macrophage subpopulations, more comprehensive immunophenotyping is needed,

particularly in the maternal circulation, given the significant evidence of systemic inflammation evident in GDM. Upon futher examination of the circulating cytokine profile of GDM in Chapter 2, it would appear that these mediators represent a T-cell, specifically a Th-17, signature due to the increase in circulating IL-6 and IL-17 levels in GDM. IL-6 not only functions upstream of IL-17 but also acts as a critical downstream target of IL-17. Equally, IL-17 together with IL-6 triggers a positive-feedback loop of IL-6 expression through the activation of NF-κB and STAT-3 signalling pathways. Future studies should elucidate if peripheral Th-17 cells are present at the onset of GDM and contribute to the systemic symptoms or develop as a consequence of GDM-mediated metabolic dysfunction.

- As with all translational research, one of the primary objectives of this study was to define areas that could be therapeutically targeted to improve patient outcomes. Unfortunately, therapeutic options for obstetric complications such as GDM are limited, largely due to the reluctance and ethical complications with including pregnant participants in clinical trials. As such, novel therapeutic avenues must be explored to improve patient outcomes in pregnancy. One such potential therapeutic strategy may be to attenuate localised tissue inflammation and limit subsequent systemic metabolic dysregulation through the inhibition of the NLRP3 inflammasome pathway. As described in Chapter 4, the NLRP3 inflammasome is activated by a diverse range of stimuli, including products of mitochondrial dysfunction and ROS (413). However, there remains a question over whether the increase in mitochondrial dysfunction and exaggerated ROS generation precedes or proceeds the GDM condition, as the physiological criteria required may often

be present long before a clinical diagnosis is confirmed. Either way, targeting mitochondrial dysfunction and subsequent NLRP3 inflammasome activation with mitochondrial-specific antioxidants may be a promising therapeutic avenue to alleviate both the acute and chronic inflammatory and metabolic symptoms of GDM.

6.5 Conclusion

GDM is one of the most common medical complications of pregnancy and remains a leading cause of adverse maternal and fetal health outcomes in obstetrics. The global prevalence of GDM continues to rise due to increases in the rates of obesity in women of reproductive age (490). In this thesis, we have provided substantial evidence of metabolic dysfunction and inflammation in the maternal circulation, omental VAT depot and placental tissue of women with GDM. This includes direct findings of defects in adipocyte expansion and impaired insulin signalling transduction, in addition to placental and systemic mitochondrial dysfunction and inflammation, which may ultimately orchestrate maternal insulin resistance. Prospective research interrogating the coordinated influence these mechanistic pathways have on the development of increased insulin resistance in GDM-complicated pregnancies may uncover alternative therapeutic strategies with the hope of improving maternal and fetal health outcomes.

References

1. Kampmann U, Madsen LR, Skajaa GO, Iversen DS, Moeller N, Ovesen P. Gestational diabetes: A clinical update. World J Diabetes. 2015;6(8):1065-72.
2. Behboudi-Gandevani S, Amiri M, Bidhendi Yarandi R, Ramezani Tehrani F. The impact of diagnostic criteria for gestational diabetes on its prevalence: a systematic review and meta-analysis. Diabetol Metab Syndr. 2019;11:11.
3. Simjak P, Cinkajzlova A, Anderlova K, Parizek A, Mraz M, Krsek M, et al. The role of obesity and adipose tissue dysfunction in gestational diabetes mellitus. J Endocrinol. 2018;238(2):R63-R77.
4. Paulo MS, Abdo NM, Bettencourt-Silva R, Al-Rifai RH. Gestational Diabetes Mellitus in Europe: A Systematic Review and Meta-Analysis of Prevalence Studies. Front Endocrinol (Lausanne). 2021;12:691033.
5. Rani PR, Begum J. Screening and Diagnosis of Gestational Diabetes Mellitus, Where Do We Stand. J Clin Diagn Res. 2016;10(4):QE01-4.
6. Benhalima K, Mathieu C, Van Assche A, Damm P, Devlieger R, Mahmood T, et al. Survey by the European Board and College of Obstetrics and Gynaecology on screening for gestational diabetes in Europe. Eur J Obstet Gynecol Reprod Biol. 2016;201:197-202.
7. Jamieson EL, Spry EP, Kirke AB, Atkinson DN, Marley JV. Real-World Gestational Diabetes Screening: Problems with the Oral Glucose Tolerance Test in Rural and Remote Australia. Int J Environ Res Public Health. 2019;16(22).
8. Aroda VR, Christophi CA, Edelstein SL, Zhang P, Herman WH, Barrett-Connor E, et al. The effect of lifestyle intervention and metformin on preventing or delaying diabetes among women with and without gestational diabetes: the Diabetes Prevention Program outcomes study 10-year follow-up. J Clin Endocrinol Metab. 2015;100(4):1646-53.
9. HSE. Guidelines for the Management of Pre-gestational and Gestational Diabetes Mellitus from Pre-conception to the Postnatal period.2010.
10. Li X, Liu X, Zuo Y, Gao J, Liu Y, Zheng W. The risk factors of gestational diabetes mellitus in patients with polycystic ovary syndrome: What should we care. Medicine (Baltimore). 2021;100(31):e26521.
11. Shapiro GD, Dodds L, Arbuckle TE, Ashley-Martin J, Fraser W, Fisher M, et al. Exposure to phthalates, bisphenol A and metals in pregnancy and the association with impaired glucose tolerance and gestational diabetes mellitus: The MIREC study. Environ Int. 2015;83:63-71.
12. Eslami B, Naddafi K, Rastkari N, Rashidi BH, Djazayeri A, Malekafzali H. Association between serum concentrations of persistent organic pollutants and gestational diabetes mellitus in primiparous women. Environ Res. 2016;151:706-12.
13. Hinkle SN, Buck Louis GM, Rawal S, Zhu Y, Albert PS, Zhang C. A longitudinal study of depression and gestational diabetes in pregnancy and the postpartum period. Diabetologia. 2016;59(12):2594-602.
14. McMahon LE, O'Malley EG, Reynolds CME, Turner MJ. The impact of revised diagnostic criteria on hospital trends in gestational diabetes mellitus rates in a high income country. BMC Health Serv Res. 2020;20(1):795.
15. Shaat N, Groop L. Genetics of gestational diabetes mellitus. Curr Med Chem. 2007;14(5):569-83.
16. Cao M, Zhang L, Chen T, Shi A, Xie K, Li Z, et al. Genetic Susceptibility to Gestational Diabetes Mellitus in a Chinese Population. Front Endocrinol (Lausanne). 2020;11:247.
17. Cho YM, Kim TH, Lim S, Choi SH, Shin HD, Lee HK, et al. Type 2 diabetes-associated genetic variants discovered in the recent genome-wide association studies are related to gestational diabetes mellitus in the Korean population. Diabetologia. 2009;52(2):253-61.

18. Lauenborg J, Grarup N, Damm P, Borch-Johnsen K, Jorgensen T, Pedersen O, et al. Common type 2 diabetes risk gene variants associate with gestational diabetes. J Clin Endocrinol Metab. 2009;94(1):145-50.
19. Benny P, Ahn HJ, Burlingame J, Lee MJ, Miller C, Chen J, et al. Genetic risk factors associated with gestational diabetes in a multi-ethnic population. PLoS One. 2021;16(12):e0261137.
20. Yahaya TO, Salisu T, Abdulrahman YB, Umar AK. Update on the genetic and epigenetic etiology of gestational diabetes mellitus: a review. Egyptian J Med Human. 2020;21(1).
21. Chu SY, Callaghan WM, Kim SY, Schmid CH, Lau J, England LJ, et al. Maternal obesity and risk of gestational diabetes mellitus. Diabetes Care. 2007;30(8):2070-6.
22. Amorim AR, Rossner S, Neovius M, Lourenco PM, Linne Y. Does excess pregnancy weight gain constitute a major risk for increasing long-term BMI? Obesity (Silver Spring). 2007;15(5):1278-86.
23. Miao M, Dai M, Zhang Y, Sun F, Guo X, Sun G. Influence of maternal overweight, obesity and gestational weight gain on the perinatal outcomes in women with gestational diabetes mellitus. Sci Rep. 2017;7(1):305.
24. Kim SY, Sharma AJ, Callaghan WM. Gestational diabetes and childhood obesity: what is the link? Curr Opin Obstet Gynecol. 2012;24(6):376-81.
25. Farahvar S, Walfisch A, Sheiner E. Gestational diabetes risk factors and long-term consequences for both mother and offspring: a literature review. Expert Rev Endocrinol Metab. 2019;14(1):63-74.
26. Mordwinkin NM, Ouzounian JG, Yedigarova L, Montoro MN, Louie SG, Rodgers KE. Alteration of endothelial function markers in women with gestational diabetes and their fetuses. J Matern Fetal Neonatal Med. 2013;26(5):507-12.
27. Guimaraes MF, Brandao AH, Rezende CA, Cabral AC, Brum AP, Leite HV, et al. Assessment of endothelial function in pregnant women with preeclampsia and gestational diabetes mellitus by flow-mediated dilation of brachial artery. Arch Gynecol Obstet. 2014;290(3):441-7.
28. Shostrom DCV, Sun Y, Oleson JJ, Snetselaar LG, Bao W. History of Gestational Diabetes Mellitus in Relation to Cardiovascular Disease and Cardiovascular Risk Factors in US Women. Front Endocrinol (Lausanne). 2017;8:144.
29. Thirunavukarasu S, Ansari F, Cubbon R, Forbes K, Chowdhary A, Jex N, et al. Gestational Diabetes, Preeclampsia and the Maternal Heart. Heart. 2022;108:A103-A4.
30. Hauspurg A, Ying W, Hubel CA, Michos ED, Ouyang P. Adverse pregnancy outcomes and future maternal cardiovascular disease. Clin Cardiol. 2018;41(2):239-46.
31. Ormazabal V, Nair S, Elfeky O, Aguayo C, Salomon C, Zuniga FA. Association between insulin resistance and the development of cardiovascular disease. Cardiovascular Diabetology. 2018;17.
32. Jia M, Zhong WT, Cai P, Peng Y, Zhang B, Wang Y, et al. Correlation between insulin-induced estrogen receptor methylation and atherosclerosis. Cardiovascular Diabetology. 2016;15.
33. Cersosimo E, DeFronzo RA. Insulin resistance and endothelial dysfunction: the road map to cardiovascular diseases. Diabetes Metab Res Rev. 2006;22(6):423-36.
34. Ajmera VH, Gunderson EP, VanWagner LB, Lewis CE, Carr JJ, Terrault NA. Gestational Diabetes Mellitus Is Strongly Associated With Non-Alcoholic Fatty Liver Disease. Am J Gastroenterol. 2016;111(5):658-64.
35. Lee KW, Ching SM, Devaraj NK, Chong SC, Lim SY, Loh HC, et al. Diabetes in Pregnancy and Risk of Antepartum Depression: A Systematic Review and Meta-Analysis of Cohort Studies. Int J Environ Res Public Health. 2020;17(11).

36. Dodd JM, Crowther CA, Antoniou G, Baghurst P, Robinson JS. Screening for gestational diabetes: the effect of varying blood glucose definitions in the prediction of adverse maternal and infant health outcomes. Aust N Z J Obstet Gynaecol. 2007;47(4):307-12.
37. Mitanchez D, Yzydorczyk C, Simeoni U. What neonatal complications should the pediatrician be aware of in case of maternal gestational diabetes? World J Diabetes. 2015;6(5):734-43.
38. Coustan DR. Gestational diabetes mellitus. Clin Chem. 2013;59(9):1310-21.
39. Maher GM, McCarthy FP, McCarthy CM, Kenny LC, Kearney PM, Khashan AS, et al. A perspective on pre-eclampsia and neurodevelopmental outcomes in the offspring: Does maternal inflammation play a role? Int J Dev Neurosci. 2019;77:69-76.
40. Blotsky AL, Rahme E, Dahhou M, Nakhla M, Dasgupta K. Gestational diabetes associated with incident diabetes in childhood and youth: a retrospective cohort study. CMAJ. 2019;191(15):E410-E7.
41. Wu CS, Nohr EA, Bech BH, Vestergaard M, Catov JM, Olsen J. Health of children born to mothers who had preeclampsia: a population-based cohort study. Am J Obstet Gynecol. 2009;201(3):269 e1- e10.
42. Cox NJ. Maternal component in NIDDM transmission. How large an effect? Diabetes. 1994;43(1):166-8.
43. Crowther CA, Hiller JE, Moss JR, McPhee AJ, Jeffries WS, Robinson JS, et al. Effect of treatment of gestational diabetes mellitus on pregnancy outcomes. N Engl J Med. 2005;352(24):2477-86.
44. Behboudi-Gandevani S, Bidhendi-Yarandi R, Panahi MH, Vaismoradi M. The Effect of Mild Gestational Diabetes Mellitus Treatment on Adverse Pregnancy Outcomes: A Systemic Review and Meta-Analysis. Front Endocrinol (Lausanne). 2021;12:640004.
45. Plows JF, Stanley JL, Baker PN, Reynolds CM, Vickers MH. The Pathophysiology of Gestational Diabetes Mellitus. Int J Mol Sci. 2018;19(11).
46. Parsons JA, Brelje TC, Sorenson RL. Adaptation of islets of Langerhans to pregnancy: increased islet cell proliferation and insulin secretion correlates with the onset of placental lactogen secretion. Endocrinology. 1992;130(3):1459-66.
47. Leguizamon G, Trigubo D, Pereira JI, Vera MF, Fernandez JA. Vascular complications in the diabetic pregnancy. Curr Diab Rep. 2015;15(4):22.
48. Hill DJ. Placental control of metabolic adaptations in the mother for an optimal pregnancy outcome. What goes wrong in gestational diabetes? Placenta. 2018;69:162-8.
49. Skajaa GO, Fuglsang J, Knorr S, Moller N, Ovesen P, Kampmann U. Changes in insulin sensitivity and insulin secretion during pregnancy and post partum in women with gestational diabetes. BMJ Open Diabetes Res Care. 2020;8(2).
50. Tumurbaatar B, Poole AT, Olson G, Makhlouf M, Sallam HS, Thukuntla S, et al. Adipose Tissue Insulin Resistance in Gestational Diabetes. Metab Syndr Relat Disord. 2017;15(2):86-92.
51. Sonagra AD, Biradar SM, K D, Murthy DSJ. Normal pregnancy- a state of insulin resistance. J Clin Diagn Res. 2014;8(11):CC01-3.
52. Butler AE, Cao-Minh L, Galasso R, Rizza RA, Corradin A, Cobelli C, et al. Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy. Diabetologia. 2010;53(10):2167-76.
53. Moyce BL, Dolinsky VW. Maternal beta-Cell Adaptations in Pregnancy and Placental Signalling: Implications for Gestational Diabetes. Int J Mol Sci. 2018;19(11).
54. Kaaja R, Ronnemaa T. Gestational diabetes: pathogenesis and consequences to mother and offspring. Rev Diabet Stud. 2008;5(4):194-202.

55. Ernst S, Demirci C, Valle S, Velazquez-Garcia S, Garcia-Ocana A. Mechanisms in the adaptation of maternal beta-cells during pregnancy. Diabetes Manag (Lond). 2011;1(2):239-48.
56. Retnakaran R, Ye C, Kramer CK, Connelly PW, Hanley AJ, Sermer M, et al. Evaluation of Circulating Determinants of Beta-Cell Function in Women With and Without Gestational Diabetes. J Clin Endocrinol Metab. 2016;101(7):2683-91.
57. Kenna LA, Olsen JA, Spelios MG, Radin MS, Akirav EM. beta-Cell death is decreased in women with gestational diabetes mellitus. Diabetol Metab Syndr. 2016;8(1):60.
58. Buchanan TA. Pancreatic B-cell defects in gestational diabetes: implications for the pathogenesis and prevention of type 2 diabetes. J Clin Endocrinol Metab. 2001;86(3):989-93.
59. Ryan EA. Hormones and insulin resistance during pregnancy. Lancet. 2003;362(9398):1777-8.
60. McLachlan KA, O'Neal D, Jenkins A, Alford FP. Do adiponectin, TNFalpha, leptin and CRP relate to insulin resistance in pregnancy? Studies in women with and without gestational diabetes, during and after pregnancy. Diabetes Metab Res Rev. 2006;22(2):131-8.
61. Hotamisligil GS. Inflammation and metabolic disorders. Nature. 2006;444(7121):860-7.
62. Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Bio. 2008;9(5):367-77.
63. Lin J, Jin H, Chen L. Associations between insulin resistance and adverse pregnancy outcomes in women with gestational diabetes mellitus: a retrospective study. BMC Pregnancy Childbirth. 2021;21(1):526.
64. Leto D, Saltiel AR. Regulation of glucose transport by insulin: traffic control of GLUT4. Nat Rev Mol Cell Biol. 2012;13(6):383-96.
65. De Meyts P. The Insulin Receptor and Its Signal Transduction Network. In: Feingold KR, Anawalt B, Boyce A, Chrousos G, de Herder WW, Dhatariya K, et al., editors. Endotext. South Dartmouth (MA)2000.
66. Huang X, Liu G, Guo J, Su Z. The PI3K/AKT pathway in obesity and type 2 diabetes. Int J Biol Sci. 2018;14(11):1483-96.
67. Shaw LM. The insulin receptor substrate (IRS) proteins: at the intersection of metabolism and cancer. Cell Cycle. 2011;10(11):1750-6.
68. Catalano PM. Trying to understand gestational diabetes. Diabet Med. 2014;31(3):273-81.
69. Friedman JE, Ishizuka T, Shao J, Huston L, Highman T, Catalano P. Impaired glucose transport and insulin receptor tyrosine phosphorylation in skeletal muscle from obese women with gestational diabetes. Diabetes. 1999;48(9):1807-14.
70. Friedman JE, Kirwan JP, Jing M, Presley L, Catalano PM. Increased skeletal muscle tumor necrosis factor-alpha and impaired insulin signaling persist in obese women with gestational diabetes mellitus 1 year postpartum. Diabetes. 2008;57(3):606-13.
71. Alharbi KK, Khan IA, Abotalib Z, Al-Hakeem MM. Insulin receptor substrate-1 (IRS-1) Gly927Arg: correlation with gestational diabetes mellitus in Saudi women. Biomed Res Int. 2014;2014:146495.
72. Barbour LA, McCurdy CE, Hernandez TL, Friedman JE. Chronically increased S6K1 is associated with impaired IRS1 signaling in skeletal muscle of GDM women with impaired glucose tolerance postpartum. J Clin Endocrinol Metab. 2011;96(5):1431-41.
73. Nguyen-Ngo C, Jayabalan N, Salomon C, Lappas M. Molecular pathways disrupted by gestational diabetes mellitus. J Mol Endocrinol. 2019;63(3):R51-R72.

74. Vazirani RP, Verma A, Sadacca LA, Buckman MS, Picatoste B, Beg M, et al. Disruption of Adipose Rab10-Dependent Insulin Signaling Causes Hepatic Insulin Resistance. Diabetes. 2016;65(6):1577-89.
75. Colomiere M, Permezel M, Riley C, Desoye G, Lappas M. Defective insulin signaling in placenta from pregnancies complicated by gestational diabetes mellitus. Eur J Endocrinol. 2009;160(4):567-78.
76. Park A, Kim WK, Bae KH. Distinction of white, beige and brown adipocytes derived from mesenchymal stem cells. World J Stem Cells. 2014;6(1):33-42.
77. Suarez-Cuenca JA, De La Pena-Sosa G, De La Vega-Moreno K, Banderas-Lares DZ, Salamanca-Garcia M, Martinez-Hernandez JE, et al. Enlarged adipocytes from subcutaneous vs. visceral adipose tissue differentially contribute to metabolic dysfunction and atherogenic risk of patients with obesity. Sci Rep. 2021;11(1):1831.
78. Trivett C, Lees ZJ, Freeman DJ. Adipose tissue function in healthy pregnancy, gestational diabetes mellitus and pre-eclampsia. Eur J Clin Nutr. 2021;75(12):1745-56.
79. Rojas-Rodriguez R, Lifshitz LM, Bellve KD, Min SY, Pires J, Leung K, et al. Human adipose tissue expansion in pregnancy is impaired in gestational diabetes mellitus. Diabetologia. 2015;58(9):2106-14.
80. Ebbert JO, Jensen MD. Fat depots, free fatty acids, and dyslipidemia. Nutrients. 2013;5(2):498-508.
81. Catalano PM, Nizielski SE, Shao J, Preston L, Qiao L, Friedman JE. Downregulated IRS-1 and PPARgamma in obese women with gestational diabetes: relationship to FFA during pregnancy. Am J Physiol Endocrinol Metab. 2002;282(3):E522-33.
82. Ibrahim MM. Subcutaneous and visceral adipose tissue: structural and functional differences. Obes Rev. 2010;11(1):11-8.
83. Rocha ADS, Bernardi JR, Matos S, Kretzer DC, Schoffel AC, Goldani MZ, et al. Maternal visceral adipose tissue during the first half of pregnancy predicts gestational diabetes at the time of delivery - a cohort study. PLoS One. 2020;15(4):e0232155.
84. Allard JB, Duan C. IGF-Binding Proteins: Why Do They Exist and Why Are There So Many? Front Endocrinol (Lausanne). 2018;9:117.
85. Kaur H, Muhlhausler BS, Roberts CT, Gatford KL. The growth hormone-insulin like growth factor axis in pregnancy. J Endocrinol. 2021.
86. Matuszek B, Lenart-Lipinska M, Burska A, Paszkowski T, Smolen A, Nowakowski A. Increased serum insulin-like growth factor-1 levels in women with gestational diabetes. Adv Med Sci. 2011;56(2):200-6.
87. Zhao Y. Expression of IGF-1 and GDF-15 in patients with gestational diabetes. Int J Clin Exp Med. 2020;13(12):9699-705.
88. Wang XR, Wang WJ, Yu XD, Hua XL, Ouyang FX, Luo ZC. Insulin-Like Growth Factor Axis Biomarkers and Gestational Diabetes Mellitus: A Systematic Review and Meta-Analysis. Frontiers in Endocrinology. 2019;10.
89. Gutaj P, Sibiak R, Jankowski M, Awdi K, Bryl R, Mozdziak P, et al. The Role of the Adipokines in the Most Common Gestational Complications. Int J Mol Sci. 2020;21(24).
90. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, et al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature. 2003;423(6941):762-9.
91. Pheiffer C, Dias S, Jack B, Malaza N, Adam S. Adiponectin as a Potential Biomarker for Pregnancy Disorders. Int J Mol Sci. 2021;22(3).
92. Choi HM, Doss HM, Kim KS. Multifaceted Physiological Roles of Adiponectin in Inflammation and Diseases. Int J Mol Sci. 2020;21(4).
93. Wang F, Liu Y, Yang W, Yuan J, Mo Z. Adiponectin inhibits NLRP3 inflammasome by modulating the AMPK-ROS pathway. Int J Clin Exp Pathol. 2018;11(7):3338-47.

94. Xu X, Huang X, Zhang L, Huang X, Qin Z, Hua F. Adiponectin protects obesity-related glomerulopathy by inhibiting ROS/NF-kappaB/NLRP3 inflammation pathway. BMC Nephrol. 2021;22(1):218.
95. Dong Z, Zhuang Q, Ye X, Ning M, Wu S, Lu L, et al. Adiponectin Inhibits NLRP3 Inflammasome Activation in Nonalcoholic Steatohepatitis via AMPK-JNK/ErK1/2-NFkappaB/ROS Signaling Pathways. Front Med (Lausanne). 2020;7:546445.
96. Aye IL, Powell TL, Jansson T. Review: Adiponectin--the missing link between maternal adiposity, placental transport and fetal growth? Placenta. 2013;34 Suppl:S40-5.
97. Chen J, Tan B, Karteris E, Zervou S, Digby J, Hillhouse EW, et al. Secretion of adiponectin by human placenta: differential modulation of adiponectin and its receptors by cytokines. Diabetologia. 2006;49(6):1292-302.
98. Ategbo JM, Grissa O, Yessoufou A, Hichami A, Dramane KL, Moutairou K, et al. Modulation of adipokines and cytokines in gestational diabetes and macrosomia. J Clin Endocrinol Metab. 2006;91(10):4137-43.
99. Iliodromiti S, Sassarini J, Kelsey TW, Lindsay RS, Sattar N, Nelson SM. Accuracy of circulating adiponectin for predicting gestational diabetes: a systematic review and meta-analysis. Diabetologia. 2016;59(4):692-9.
100. Henson MC, Castracane VD. Leptin in pregnancy: an update. Biol Reprod. 2006;74(2):218-29.
101. Moschos S, Chan JL, Mantzoros CS. Leptin and reproduction: a review. Fertil Steril. 2002;77(3):433-44.
102. Kelesidis T, Kelesidis I, Chou S, Mantzoros CS. Narrative review: the role of leptin in human physiology: emerging clinical applications. Ann Intern Med. 2010;152(2):93-100.
103. Obradovic M, Sudar-Milovanovic E, Soskic S, Essack M, Arya S, Stewart AJ, et al. Leptin and Obesity: Role and Clinical Implication. Front Endocrinol (Lausanne). 2021;12:585887.
104. La Cava A, Matarese G. The weight of leptin in immunity. Nat Rev Immunol. 2004;4(5):371-9.
105. Perez-Perez A, Toro A, Vilarino-Garcia T, Maymo J, Guadix P, Duenas JL, et al. Leptin action in normal and pathological pregnancies. J Cell Mol Med. 2018;22(2):716-27.
106. Soheilykhah S, Mojibian M, Rahimi-Saghand S, Rashidi M, Hadinedoushan H. Maternal serum leptin concentration in gestational diabetes. Taiwan J Obstet Gynecol. 2011;50(2):149-53.
107. Santos-Alvarez J, Goberna R, Sanchez-Margalet V. Human leptin stimulates proliferation and activation of human circulating monocytes. Cell Immunol. 1999;194(1):6-11.
108. Perez-Perez A, Maymo JL, Gambino YP, Guadix P, Duenas JL, Varone CL, et al. Activated translation signaling in placenta from pregnant women with gestational diabetes mellitus: possible role of leptin. Horm Metab Res. 2013;45(6):436-42.
109. Perez-Perez A, Guadix P, Maymo J, Duenas JL, Varone C, Fernandez-Sanchez M, et al. Insulin and Leptin Signaling in Placenta from Gestational Diabetic Subjects. Horm Metab Res. 2016;48(1):62-9.
110. Klein S, Coppack SW, Mohamed-Ali V, Landt M. Adipose tissue leptin production and plasma leptin kinetics in humans. Diabetes. 1996;45(7):984-7.
111. Martinez-Sanchez N. There and Back Again: Leptin Actions in White Adipose Tissue. Int J Mol Sci. 2020;21(17).
112. Sivakumar K, Bari MF, Adaikalakoteswari A, Guller S, Weickert MO, Randeva HS, et al. Elevated fetal adipsin/acylation-stimulating protein (ASP) in obese pregnancy: novel placental secretion via Hofbauer cells. J Clin Endocrinol Metab. 2013;98(10):4113-22.
113. Lo JC, Ljubicic S, Leibiger B, Kern M, Leibiger IB, Moede T, et al. Adipsin is an adipokine that improves beta cell function in diabetes. Cell. 2014;158(1):41-53.

114. Poveda NE, Garces MF, Ruiz-Linares CE, Varon D, Valderrama S, Sanchez E, et al. Serum Adipsin Levels throughout Normal Pregnancy and Preeclampsia. Sci Rep. 2016;6:20073.
115. Milek M, Moulla Y, Kern M, Stroh C, Dietrich A, Schon MR, et al. Adipsin Serum Concentrations and Adipose Tissue Expression in People with Obesity and Type 2 Diabetes. Int J Mol Sci. 2022;23(4).
116. Zhou Q, Ge Q, Ding Y, Qu H, Wei H, Wu R, et al. Relationship between serum adipsin and the first phase of glucose-stimulated insulin secretion in individuals with different glucose tolerance. J Diabetes Investig. 2018;9(5):1128-34.
117. Vejrazkova D, Lischkova O, Vankova M, Stanicka S, Vrbikova J, Lukasova P, et al. Distinct response of fat and gastrointestinal tissue to glucose in gestational diabetes mellitus and polycystic ovary syndrome. Physiol Res. 2017;66(2):283-92.
118. Saucedo R, Valencia J, Moreno-Gonzalez LE, Pena-Cano MI, Aranda-Martinez A, Garcia Y, et al. Maternal serum adipokines and inflammatory markers at late gestation and newborn weight in mothers with and without gestational diabetes mellitus. Ginekol Pol. 2022;93(2):126-33.
119. Kunnari AM, Savolainen ER, Ukkola OH, Kesaniemi YA, Jokela MA. The expression of human resistin in different leucocyte lineages is modulated by LPS and TNFalpha. Regul Pept. 2009;157(1-3):57-63.
120. Li Y, Yang Q, Cai D, Guo H, Fang J, Cui H, et al. Resistin, a Novel Host Defense Peptide of Innate Immunity. Front Immunol. 2021;12:699807.
121. Harris TA, Gattu S, Propheter DC, Kuang Z, Bel S, Ruhn KA, et al. Resistin-like Molecule alpha Provides Vitamin-A-Dependent Antimicrobial Protection in the Skin. Cell Host Microbe. 2019;25(6):777-88 e8.
122. Propheter DC, Chara AL, Harris TA, Ruhn KA, Hooper LV. Resistin-like molecule beta is a bactericidal protein that promotes spatial segregation of the microbiota and the colonic epithelium. Proc Natl Acad Sci U S A. 2017;114(42):11027-33.
123. Jamaluddin MS, Weakley SM, Yao Q, Chen C. Resistin: functional roles and therapeutic considerations for cardiovascular disease. Br J Pharmacol. 2012;165(3):622-32.
124. Su KZ, Li YR, Zhang D, Yuan JH, Zhang CS, Liu Y, et al. Relation of Circulating Resistin to Insulin Resistance in Type 2 Diabetes and Obesity: A Systematic Review and Meta-Analysis. Front Physiol. 2019;10:1399.
125. Sadashiv, Tiwari S, Paul BN, Kumar S, Chandra A, Dhananjai S, et al. Over expression of resistin in adipose tissue of the obese induces insulin resistance. World J Diabetes. 2012;3(7):135-41.
126. Nien JK, Mazaki-Tovi S, Romero R, Kusanovic JP, Erez O, Gotsch F, et al. Resistin: a hormone which induces insulin resistance is increased in normal pregnancy. J Perinat Med. 2007;35(6):513-21.
127. Hu SM, Chen MS, Tan HZ. Maternal serum level of resistin is associated with risk for gestational diabetes mellitus: A meta-analysis. World J Clin Cases. 2019;7(5):585-99.
128. Megia A, Vendrell J, Gutierrez C, Sabate M, Broch M, Fernandez-Real JM, et al. Insulin sensitivity and resistin levels in gestational diabetes mellitus and after parturition. Eur J Endocrinol. 2008;158(2):173-8.
129. Tagoma A, Haller-Kikkatalo K, Oras A, Roos K, Kirss A, Uibo R. Plasma cytokines during pregnancy provide insight into the risk of diabetes in the gestational diabetes risk group. J Diabetes Investig. 2022;13(9):1596-606.
130. Al-Musharaf S, Sabico S, Hussain SD, Al-Tawashi F, AlWaily HB, Al-Daghri NM, et al. Inflammatory and Adipokine Status from Early to Midpregnancy in Arab Women and Its Associations with Gestational Diabetes Mellitus. Dis Markers. 2021;2021:8862494.

131. Dong Y, Chauhan M, Betancourt A, Belfort M, Yallampalli C. Adipose Tissue Inflammation and Adrenomedullin Overexpression Contribute to Lipid Dysregulation in Diabetic Pregnancies. J Clin Endocrinol Metab. 2018;103(10):3810-8.
132. Saghian R, Bogle G, James JL, Clark AR. Establishment of maternal blood supply to the placenta: insights into plugging, unplugging and trophoblast behaviour from an agent-based model. Interface Focus. 2019;9(5):20190019.
133. Burton GJ, Fowden AL. The placenta: a multifaceted, transient organ. Philos Trans R Soc Lond B Biol Sci. 2015;370(1663):20140066.
134. Carrasco-Wong I, Moller A, Giachini FR, Lima VV, Toledo F, Stojanova J, et al. Placental structure in gestational diabetes mellitus. Biochim Biophys Acta Mol Basis Dis. 2020;1866(2):165535.
135. Gauster M, Moser G, Wernitznig S, Kupper N, Huppertz B. Early human trophoblast development: from morphology to function. Cell Mol Life Sci. 2022;79(6):345.
136. Wang Y, Zhao S. Vascular Biology of the Placenta. Integrated Systems Physiology: from Molecules to Function to Disease. San Rafael (CA)2010.
137. Persson G, Staehr CS, Klok FS, Lebech M, Hviid TVF. Evidence for a shift in placental HLA-G allelic dominance and the HLA-G isoform profile during a healthy pregnancy and preeclampsiadagger. Biol Reprod. 2021;105(4):846-58.
138. Apps R, Murphy SP, Fernando R, Gardner L, Ahad T, Moffett A. Human leucocyte antigen (HLA) expression of primary trophoblast cells and placental cell lines, determined using single antigen beads to characterize allotype specificities of anti-HLA antibodies. Immunology. 2009;127(1):26-39.
139. Makhseed M, Musini VM, Ahmed MA, Al-Harmi J. Placental pathology in relation to the White's classification of diabetes mellitus. Arch Gynecol Obstet. 2002;266(3):136-40.
140. Abdelhalim NY, Shehata MH, Gadallah HN, Sayed WM, Othman AA. Morphological and ultrastructural changes in the placenta of the diabetic pregnant Egyptian women. Acta Histochem. 2018;120(5):490-503.
141. Daskalakis G, Marinopoulos S, Krielesi V, Papapanagiotou A, Papantoniou N, Mesogitis S, et al. Placental pathology in women with gestational diabetes. Acta Obstet Gynecol Scand. 2008;87(4):403-7.
142. Arizawa M, Nakayama M, Kidoguchi K. [Correlation of placental villous immaturity and dysmaturity with clinical control of maternal diabetes]. Nihon Sanka Fujinka Gakkai Zasshi. 1991;43(6):595-602.
143. Redline R. Distal villous immaturity. Diagnostic Histopathology. 2012;18(5):189-94.
144. Jarmuzek P, Wielgos M, Bomba-Opon D. Placental pathologic changes in gestational diabetes mellitus. Neuro Endocrinol Lett. 2015;36(2):101-5.
145. El-Tarhouny SA, Almasry SM, Elfayomy AK, Baghdadi H, Habib FA. Placental growth factor and soluble Fms-like tyrosine kinase 1 in diabetic pregnancy: A possible relation to distal villous immaturity. Histol Histopathol. 2014;29(2):259-72.
146. Di Cianni G, Miccoli R, Volpe L, Lencioni C, Del Prato S. Intermediate metabolism in normal pregnancy and in gestational diabetes. Diabetes Metab Res Rev. 2003;19(4):259-70.
147. Kampmann U, Knorr S, Fuglsang J, Ovesen P. Determinants of Maternal Insulin Resistance during Pregnancy: An Updated Overview. J Diabetes Res. 2019;2019:5320156.
148. Newbern D, Freemark M. Placental hormones and the control of maternal metabolism and fetal growth. Curr Opin Endocrinol Diabetes Obes. 2011;18(6):409-16.
149. Napso T, Yong HEJ, Lopez-Tello J, Sferruzzi-Perri AN. The Role of Placental Hormones in Mediating Maternal Adaptations to Support Pregnancy and Lactation. Front Physiol. 2018;9:1091.
150. Li M, Song Y, Rawal S, Hinkle SN, Zhu Y, Tekola-Ayele F, et al. Plasma Prolactin and Progesterone Levels and the Risk of Gestational Diabetes: A Prospective and Longitudinal Study in a Multiracial Cohort. Front Endocrinol (Lausanne). 2020;11:83.

151. Ngala RA, Fondjo LA, Gmagna P, Ghartey FN, Awe MA. Placental peptides metabolism and maternal factors as predictors of risk of gestational diabetes in pregnant women. A case-control study. PLoS One. 2017;12(7):e0181613.
152. Lolis D, Tzingounis V, Kaskarelis D. Maternal serums and amniotic fluid levels of human placental lactogen in gestational diabetes. Eur J Clin Invest. 1978;8(4):259-60.
153. Lappas M, Yee K, Permezel M, Rice GE. Release and regulation of leptin, resistin and adiponectin from human placenta, fetal membranes, and maternal adipose tissue and skeletal muscle from normal and gestational diabetes mellitus-complicated pregnancies. J Endocrinol. 2005;186(3):457-65.
154. Meller M, Qiu C, Vadachkoria S, Abetew DF, Luthy DA, Williams MA. Changes in placental adipocytokine gene expression associated with gestational diabetes mellitus. Physiol Res. 2006;55(5):501-12.
155. Zhang C, Li Y, Wang J, Liu C, Chen Y. Association between levels of aquaporin 3 in the placenta and adiponectin in the umbilical cord blood with gestational diabetes mellitus and pregnancy outcome. Mol Med Rep. 2020;22(2):1498-506.
156. Mohan Shrestha M, Wermelin S, Stener-Victorin E, Wernstedt Asterholm I, Benrick A. Adiponectin Deficiency Alters Placenta Function but Does Not Affect Fetal Growth in Mice. Int J Mol Sci. 2022;23(9).
157. Schanton M, Maymo JL, Perez-Perez A, Sanchez-Margalet V, Varone CL. Involvement of leptin in the molecular physiology of the placenta. Reproduction. 2018;155(1):R1-R12.
158. Park MJ, Lee DH, Joo BS, Lee YJ, Joo JK, An BS, et al. Leptin, leptin receptors and hypoxia-induced factor-1alpha expression in the placental bed of patients with and without preeclampsia during pregnancy. Mol Med Rep. 2018;17(4):5292-9.
159. Lacroix M, Battista MC, Doyon M, Moreau J, Patenaude J, Guillemette L, et al. Higher maternal leptin levels at second trimester are associated with subsequent greater gestational weight gain in late pregnancy. BMC Pregnancy Childbirth. 2016;16:62.
160. Yan B, Yu Y, Lin M, Li Z, Wang L, Huang P, et al. High, but stable, trend in the prevalence of gestational diabetes mellitus: A population-based study in Xiamen, China. J Diabetes Investig. 2019;10(5):1358-64.
161. Stefaniak M, Dmoch-Gajzlerska E, Mazurkiewicz B, Gajzlerska-Majewska W. Maternal serum and cord blood leptin concentrations at delivery. PLoS One. 2019;14(11):e0224863.
162. Saleh J, Al-Riyami HDS, Chaudhary TA, Cianflone K. Cord blood ASP is predicted by maternal lipids and correlates with fetal birth weight. Obesity. 2008;16(6):1193-8.
163. Yura S, Sagawa N, Itoh H, Kakui K, Nuamah MA, Korita D, et al. Resistin is expressed in the human placenta. J Clin Endocrinol Metab. 2003;88(3):1394-7.
164. Yu J, Su XL, Jia J, Zeng Y, Zhang JY, Wang SS, et al. The Relationship Between the Expression of Resistin and Apoptosis Factors in Placenta and the Pathogenesis of Gestational Diabetes Mellitus. Matern-Fetal Med. 2020;2(2):80-3.
165. Shang M, Dong X, Hou L. Correlation of adipokines and markers of oxidative stress in women with gestational diabetes mellitus and their newborns. J Obstet Gynaecol Res. 2018;44(4):637-46.
166. Bischof P. 3 Pregnancy Proteins (Pp12, Pp14, and Papp-a) - Their Biological and Clinical Relevance. Am J Perinat. 1989;6(2):110-6.
167. Byun D, Mohan S, Yoo M, Sexton C, Baylink DJ, Qin X. Pregnancy-associated plasma protein-A accounts for the insulin-like growth factor (IGF)-binding protein-4 (IGFBP-4) proteolytic activity in human pregnancy serum and enhances the mitogenic activity of IGF by degrading IGFBP-4 in vitro. J Clin Endocrinol Metab. 2001;86(2):847-54.
168. Caliskan R, Atis A, Aydin Y, Acar D, Kiyak H, Topbas F. PAPP-A concentrations change in patients with gestational diabetes. J Obstet Gynaecol. 2020;40(2):190-4.

169. Ramezani S, Doulabi MA, Saqhafi H, Alipoor M. Prediction of Gestational Diabetes by Measuring the Levels of Pregnancy Associated Plasma Protein-A (PAPP-A) During Gestation Weeks 11-14. J Reprod Infertil. 2020;21(2):130-7.
170. Rojas-Rodriguez R, Ziegler R, DeSouza T, Majid S, Madore AS, Amir N, et al. PAPPA-mediated adipose tissue remodeling mitigates insulin resistance and protects against gestational diabetes in mice and humans. Sci Transl Med. 2020;12(571).
171. Roberts VH, Smith J, McLea SA, Heizer AB, Richardson JL, Myatt L. Effect of increasing maternal body mass index on oxidative and nitrative stress in the human placenta. Placenta. 2009;30(2):169-75.
172. Snezhkina AV, Kudryavtseva AV, Kardymon OL, Savvateeva MV, Melnikova NV, Krasnov GS, et al. ROS Generation and Antioxidant Defense Systems in Normal and Malignant Cells. Oxid Med Cell Longev. 2019;2019:6175804.
173. Qiu C, Hevner K, Abetew D, Sedensky M, Morgan P, Enquobahrie DA, et al. Mitochondrial DNA copy number and oxidative DNA damage in placental tissues from gestational diabetes and control pregnancies: a pilot study. Clin Lab. 2013;59(5-6):655-60.
174. Wu F, Tian FJ, Lin Y. Oxidative Stress in Placenta: Health and Diseases. Biomed Res Int. 2015;2015:293271.
175. Sarina, Li DF, Feng ZQ, Du J, Zhao WH, Huang N, et al. Mechanism of Placenta Damage in Gestational Diabetes Mellitus by Investigating TXNIP of Patient Samples and Gene Functional Research in Cell Line. Diabetes Ther. 2019;10(6):2265-88.
176. Sferruzzi-Perri AN. Placental mitochondria central to gestational diabetes pathogenesis? J Physiol. 2021;599(4):1019-20.
177. Coughlan MT, Vervaart PP, Permezel M, Georgiou HM, Rice GE. Altered placental oxidative stress status in gestational diabetes mellitus. Placenta. 2004;25(1):78-84.
178. Aroor AR, DeMarco VG. Oxidative stress and obesity: the chicken or the egg? Diabetes. 2014;63(7):2216-8.
179. Li J, Shen X. Oxidative stress and adipokine levels were significantly correlated in diabetic patients with hyperglycemic crises. Diabetol Metab Syndr. 2019;11:13.
180. Incalza MA, D'Oria R, Natalicchio A, Perrini S, Laviola L, Giorgino F. Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases. Vascul Pharmacol. 2018;100:1-19.
181. Gu F, Chauhan V, Kaur K, Brown WT, LaFauci G, Wegiel J, et al. Alterations in mitochondrial DNA copy number and the activities of electron transport chain complexes and pyruvate dehydrogenase in the frontal cortex from subjects with autism. Transl Psychiatry. 2013;3:e299.
182. Thurairajah K, Briggs GD, Balogh ZJ. The source of cell-free mitochondrial DNA in trauma and potential therapeutic strategies. Eur J Trauma Emerg Surg. 2018;44(3):325-34.
183. Sanchez-Guerra M, Peng C, Trevisi L, Cardenas A, Wilson A, Osorio-Yanez C, et al. Altered cord blood mitochondrial DNA content and pregnancy lead exposure in the PROGRESS cohort. Environ Int. 2019;125:437-44.
184. Williamson RD, McCarthy FP, Khashan AS, Totorika A, Kenny LC, McCarthy C. Exploring the role of mitochondrial dysfunction in the pathophysiology of pre-eclampsia. Pregnancy Hypertens. 2018;13:248-53.
185. Bernardi P. The mitochondrial permeability transition pore: a mystery solved? Front Physiol. 2013;4:95.
186. Kaarniranta K, Pawlowska E, Szczepanska J, Jablkowska A, Blasiak J. Role of Mitochondrial DNA Damage in ROS-Mediated Pathogenesis of Age-Related Macular Degeneration (AMD). Int J Mol Sci. 2019;20(10).
187. Lee JH, Park A, Oh KJ, Lee SC, Kim WK, Bae KH. The Role of Adipose Tissue Mitochondria: Regulation of Mitochondrial Function for the Treatment of Metabolic Diseases. Int J Mol Sci. 2019;20(19).

188. Bronner DN, O'Riordan MX. Measurement of Mitochondrial DNA Release in Response to ER Stress. Bio Protoc. 2016;6(12).
189. Kuleva M, Ben Miled S, Steffann J, Bonnefont JP, Rondeau S, Ville Y, et al. Increased incidence of obstetric complications in women carrying mitochondrial DNA mutations: a retrospective cohort study in a single tertiary centre. BJOG. 2018.
190. Roh JS, Sohn DH. Damage-Associated Molecular Patterns in Inflammatory Diseases. Immune Netw. 2018;18(4):e27.
191. Suzumori N, Ebara T, Yamada T, Samura O, Yotsumoto J, Nishiyama M, et al. Fetal cell-free DNA fraction in maternal plasma is affected by fetal trisomy. J Hum Genet. 2016;61(7):647-52.
192. Williamson RD, McCarthy FP, Kenny LC, McCarthy CM. Activation of a TLR9 mediated innate immune response in preeclampsia. Sci Rep. 2019;9(1):5920.
193. McCarthy CG, Wenceslau CF, Goulopoulou S, Ogbi S, Baban B, Sullivan JC, et al. Circulating mitochondrial DNA and Toll-like receptor 9 are associated with vascular dysfunction in spontaneously hypertensive rats. Cardiovasc Res. 2015;107(1):119-30.
194. McElwain C, McCarthy CM. Investigating mitochondrial dysfunction in gestational diabetes mellitus and elucidating if BMI is a causative mediator. Eur J Obstet Gynecol Reprod Biol. 2020;251:60-5.
195. Lindinger A, Peterli R, Peters T, Kern B, von Flue M, Calame M, et al. Mitochondrial DNA content in human omental adipose tissue. Obes Surg. 2010;20(1):84-92.
196. Kusminski CM, Scherer PE. Mitochondrial dysfunction in white adipose tissue. Trends Endocrinol Metab. 2012;23(9):435-43.
197. Gomez-Lopez N, Motomura K, Miller D, Garcia-Flores V, Galaz J, Romero R. Inflammasomes: Their Role in Normal and Complicated Pregnancies. J Immunol. 2019;203(11):2757-69.
198. Zhao H, Liao X, Kang Y. Tregs: Where We Are and What Comes Next? Front Immunol. 2017;8:1578.
199. Mjosberg J, Berg G, Ernerudh J, Ekerfelt C. CD4+ CD25+ regulatory T cells in human pregnancy: development of a Treg-MLC-ELISPOT suppression assay and indications of paternal specific Tregs. Immunology. 2007;120(4):456-66.
200. Fu B, Li X, Sun R, Tong X, Ling B, Tian Z, et al. Natural killer cells promote immune tolerance by regulating inflammatory TH17 cells at the human maternal-fetal interface. Proc Natl Acad Sci U S A. 2013;110(3):E231-40.
201. Holdsworth SR, Gan PY. Cytokines: Names and Numbers You Should Care About. Clin J Am Soc Nephrol. 2015;10(12):2243-54.
202. Szukiewicz D. Cytokines in placental physiology and disease. Mediators Inflamm. 2012;2012:640823.
203. Lorenzo-Almoros A, Hang T, Peiro C, Soriano-Guillen L, Egido J, Tunon J, et al. Predictive and diagnostic biomarkers for gestational diabetes and its associated metabolic and cardiovascular diseases. Cardiovasc Diabetol. 2019;18(1):140.
204. Sheu A, Chan Y, Ferguson A, Bakhtyari MB, Hawke W, White C, et al. A proinflammatory CD4(+) T cell phenotype in gestational diabetes mellitus. Diabetologia. 2018;61(7):1633-43.
205. Hardy OT, Czech MP, Corvera S. What causes the insulin resistance underlying obesity? Curr Opin Endocrinol Diabetes Obes. 2012;19(2):81-7.
206. Berbudi A, Rahmadika N, Tjahjadi AI, Ruslami R. Type 2 Diabetes and its Impact on the Immune System. Curr Diabetes Rev. 2020;16(5):442-9.
207. Correa-Silva S, Alencar AP, Moreli JB, Borbely AU, de SLL, Scavone C, et al. Hyperglycemia induces inflammatory mediators in the human chorionic villous. Cytokine. 2018;111:41-8.

208. Pan X, Jin X, Wang J, Hu Q, Dai B. Placenta inflammation is closely associated with gestational diabetes mellitus. Am J Transl Res. 2021;13(5):4068-79.
209. Santalahti K, Maksimow M, Airola A, Pahikkala T, Hutri-Kahonen N, Jalkanen S, et al. Circulating Cytokines Predict the Development of Insulin Resistance in a Prospective Finnish Population Cohort. J Clin Endocrinol Metab. 2016;101(9):3361-9.
210. Mariusz Kuzmicki BT, Anna Zonenberg, Jacek Szamatowicz, Adam Kretowski, Agnieszka Nikolajuk, Maria Gorska. Circulating pro- and anti-inflammatory cytokines in women with gestational diabetes. Endocrine Abstracts. 2007;14.
211. Xu J, Zhao YH, Chen YP, Yuan XL, Wang J, Zhu H, et al. Maternal circulating concentrations of tumor necrosis factor-alpha, leptin, and adiponectin in gestational diabetes mellitus: a systematic review and meta-analysis. ScientificWorldJournal. 2014;2014:926932.
212. Gomes CP, Torloni MR, Gueuvoghlanian-Silva BY, Alexandre SM, Mattar R, Daher S. Cytokine levels in gestational diabetes mellitus: a systematic review of the literature. Am J Reprod Immunol. 2013;69(6):545-57.
213. Sisino G, Bouckenooghe T, Aurientis S, Fontaine P, Storme L, Vambergue A. Diabetes during pregnancy influences Hofbauer cells, a subtype of placental macrophages, to acquire a pro-inflammatory phenotype. Biochim Biophys Acta. 2013;1832(12):1959-68.
214. Schliefsteiner C, Peinhaupt M, Kopp S, Logl J, Lang-Olip I, Hiden U, et al. Human Placental Hofbauer Cells Maintain an Anti-inflammatory M2 Phenotype despite the Presence of Gestational Diabetes Mellitus. Front Immunol. 2017;8:888.
215. Challier JC, Basu S, Bintein T, Minium J, Hotmire K, Catalano PM, et al. Obesity in pregnancy stimulates macrophage accumulation and inflammation in the placenta. Placenta. 2008;29(3):274-81.
216. Pantham P, Aye IL, Powell TL. Inflammation in maternal obesity and gestational diabetes mellitus. Placenta. 2015;36(7):709-15.
217. Zhang G, Sun Q, Liu C. Influencing Factors of Thermogenic Adipose Tissue Activity. Front Physiol. 2016;7:29.
218. Fantuzzi G. Adiponectin in inflammatory and immune-mediated diseases. Cytokine. 2013;64(1):1-10.
219. Wronkowitz N, Romacho T, Sell H, Eckel J. Adipose tissue dysfunction and inflammation in cardiovascular disease. Front Horm Res. 2014;43:79-92.
220. Lau WB, Ohashi K, Wang Y, Ogawa H, Murohara T, Ma XL, et al. Role of Adipokines in Cardiovascular Disease. Circ J. 2017;81(7):920-8.
221. Coppack SW. Pro-inflammatory cytokines and adipose tissue. Proc Nutr Soc. 2001;60(3):349-56.
222. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature. 1997;389(6651):610-4.
223. Tao C, Holland WL, Wang QA, Shao M, Jia L, Sun K, et al. Short-Term Versus Long-Term Effects of Adipocyte Toll-Like Receptor 4 Activation on Insulin Resistance in Male Mice. Endocrinology. 2017;158(5):1260-70.
224. Mor G, Cardenas I, Abrahams V, Guller S. Inflammation and pregnancy: the role of the immune system at the implantation site. Ann N Y Acad Sci. 2011;1221:80-7.
225. McElwain CJ, McCarthy FP, McCarthy CM. Gestational Diabetes Mellitus and Maternal Immune Dysregulation: What We Know So Far. Int J Mol Sci. 2021;22(8).
226. Stoikou M, Grimolizzi F, Giaglis S, Schafer G, van Breda SV, Hoesli IM, et al. Gestational Diabetes Mellitus Is Associated with Altered Neutrophil Activity. Front Immunol. 2017;8:702.

227. Sun T, Meng F, Zhao H, Yang M, Zhang R, Yu Z, et al. Elevated First-Trimester Neutrophil Count Is Closely Associated With the Development of Maternal Gestational Diabetes Mellitus and Adverse Pregnancy Outcomes. Diabetes. 2020;69(7):1401-10.
228. Aktulay A, Engin-Ustun Y, Ozkan MS, Erkaya S, Kara M, Kaymak O, et al. Gestational Diabetes Mellitus Seems to Be Associated with Inflammation. Acta Clin Croat. 2015;54(4):475-8.
229. Yilmaz H, Celik HT, Namuslu M, Inan O, Onaran Y, Karakurt F, et al. Benefits of the neutrophil-to-lymphocyte ratio for the prediction of gestational diabetes mellitus in pregnant women. Exp Clin Endocrinol Diabetes. 2014;122(1):39-43.
230. Liu W, Lou X, Zhang Z, Chai Y, Yu Q. Association of neutrophil to lymphocyte ratio, platelet to lymphocyte ratio, mean platelet volume with the risk of gestational diabetes mellitus. Gynecol Endocrinol. 2020:1-3.
231. Sahin Uysal N, Eroglu H, Ozcan C, Sahin D, Yucel A. Is the serum delta neutrophil index level different in gestational diabetic women? J Matern Fetal Neonatal Med. 2020;33(19):3349-54.
232. Yang H, Zhu C, Ma Q, Long Y, Cheng Z. Variations of blood cells in prediction of gestational diabetes mellitus. J Perinat Med. 2015;43(1):89-93.
233. Lobo TF, Borges CM, Mattar R, Gomes CP, de Angelo AGS, Pendeloski KPT, et al. Impaired Treg and NK cells profile in overweight women with gestational diabetes mellitus. Am J Reprod Immunol. 2018;79(3).
234. Chiba H, Fukui A, Fuchinoue K, Funamizu A, Tanaka K, Mizunuma H. Expression of Natural Cytotoxicity Receptors on and Intracellular Cytokine Production by NK Cells in Women with Gestational Diabetes Mellitus. Am J Reprod Immunol. 2016;75(5):529-38.
235. Hara Cde C, Franca EL, Fagundes DL, de Queiroz AA, Rudge MV, Honorio-Franca AC, et al. Characterization of Natural Killer Cells and Cytokines in Maternal Placenta and Fetus of Diabetic Mothers. J Immunol Res. 2016;2016:7154524.
236. Mahmoud F, Abul H, Dashti A, Al-Jassar W, Omu A. Trace elements and cell-mediated immunity in gestational and pre-gestational diabetes mellitus at third trimester of pregnancy. Acta Med Acad. 2012;41(2):175-85.
237. Pendeloski KP, Mattar R, Torloni MR, Gomes CP, Alexandre SM, Daher S. Immunoregulatory molecules in patients with gestational diabetes mellitus. Endocrine. 2015;50(1):99-109.
238. Angelo AGS, Neves CTC, Lobo TF, Godoy RVC, Ono E, Mattar R, et al. Monocyte profile in peripheral blood of gestational diabetes mellitus patients. Cytokine. 2018;107:79-84.
239. Wang J, Zhu QW, Cheng XY, Sha CX, Cui YB. Clinical significance of neutrophil-lymphocyte ratio and monocyte-lymphocyte ratio in women with hyperglycemia. Postgrad Med. 2020;132(8):702-8.
240. Friebe-Hoffmann U, Antony L, Kruessel JS, Pawlowski B, Hoffmann TK. Peripheral Immunological Cells in Pregnant Women and their Change during Diabetes. Exp Clin Endocrinol Diabetes. 2017;125(10):677-83.
241. Schober L, Radnai D, Spratte J, Kisielewicz A, Schmitt E, Mahnke K, et al. The role of regulatory T cell (Treg) subsets in gestational diabetes mellitus. Clin Exp Immunol. 2014;177(1):76-85.
242. Yang Y, Liu L, Liu B, Li Q, Wang Z, Fan S, et al. Functional Defects of Regulatory T Cell Through Interleukin 10 Mediated Mechanism in the Induction of Gestational Diabetes Mellitus. DNA Cell Biol. 2018;37(3):278-85.
243. Sifnaios E, Mastorakos G, Psarra K, Panagopoulos ND, Panoulis K, Vitoratos N, et al. Gestational Diabetes and T-cell (Th1/Th2/Th17/Treg) Immune Profile. In Vivo. 2019;33(1):31-40.

244. Zhao Y, Zhang X, Du N, Sun H, Chen L, Bao H, et al. Immune checkpoint molecules on T cell subsets of pregnancies with preeclampsia and gestational diabetes mellitus. J Reprod Immunol. 2020;142:103208.
245. Mahmoud FF, Haines DD, Abul HT, Omu AE, Abu-Donia MB. Butyrylcholinesterase activity in gestational diabetes: correlation with lymphocyte subpopulations in peripheral blood. Am J Reprod Immunol. 2006;56(3):185-92.
246. Lapolla A, Sanzari M, Betterle C, Dalfra MG, Masin M, Zanchetta R, et al. Evaluation of T-cell receptor CD3+ gamma delta in gestational diabetes mellitus. Acta Diabetol. 2000;37(4):207-11.
247. Lapolla A, Dalfra MG, Sanzari M, Fedele D, Betterle C, Masin M, et al. Lymphocyte subsets and cytokines in women with gestational diabetes mellitus and their newborn. Cytokine. 2005;31(4):280-7.
248. Zhuang Y, Zhang J, Li Y, Gu H, Zhao J, Sun Y, et al. B Lymphocytes Are Predictors of Insulin Resistance in Women with Gestational Diabetes Mellitus. Endocr Metab Immune Disord Drug Targets. 2019;19(3):358-66.
249. Erikci AA, Muhcu M, Dundar O, Ozturk A. Could mean platelet volume be a predictive marker for gestational diabetes mellitus? Hematology. 2008;13(1):46-8.
250. Iyidir OT, Degertekin CK, Yilmaz BA, Toruner FB, Akturk M, Arslan M. Elevated mean platelet volume is associated with gestational diabetes mellitus. Gynecol Endocrinol. 2014;30(9):640-3.
251. Yin SM, Li YQ, Xie SF, Ma LP, Wu YD, Nie DN, et al. [Study on the variation of platelet function in pregnancy induced hypertension and gestational diabetes mellitus]. Zhonghua Fu Chan Ke Za Zhi. 2005;40(1):25-8.
252. Basu J, Datta C, Chowdhury S, Mandal D, Mondal NK, Ghosh A. Gestational Diabetes Mellitus in a Tertiary Care Hospital of Kolkata, India: Prevalence, Pathogenesis and Potential Disease Biomarkers. Exp Clin Endocrinol Diabetes. 2020;128(4):216-23.
253. Gorar S, Abanonu GB, Uysal A, Erol O, Unal A, Uyar S, et al. Comparison of thyroid function tests and blood count in pregnant women with versus without gestational diabetes mellitus. J Obstet Gynaecol Res. 2017;43(5):848-54.
254. Gur EB, Karadeniz M, Genc M, Eskicioglu F, Yalcin M, Hepyilmaz I, et al. Relationship between mean platelet volume and vitamin D deficiency in gestational diabetes mellitus. Arch Endocrinol Metab. 2015;59(5):448-54.
255. Erdogan S, Ozdemir O, Dogan HO, Sezer S, Atalay CR, Meric F, et al. Liver enzymes, mean platelet volume, and red cell distribution width in gestational diabetes. Turk J Med Sci. 2014;44(1):121-5.
256. Bozkurt N, Yilmaz E, Biri A, Taner Z, Himmetoglu O. The mean platelet volume in gestational diabetes. J Thromb Thrombolysis. 2006;22(1):51-4.
257. Maconi M, Cardaropoli S, Cenci AM. Platelet parameters in healthy and pathological pregnancy. J Clin Lab Anal. 2012;26(1):41-4.
258. Lao TT, Ho LF. Gestational diabetes and maternal third-trimester blood count. J Reprod Med. 2002;47(4):309-12.
259. Sahbaz A, Cicekler H, Aynioglu O, Isik H, Ozmen U. Comparison of the predictive value of plateletcrit with various other blood parameters in gestational diabetes development. J Obstet Gynaecol. 2016;36(5):589-93.
260. Barke TL, Goldstein JA, Sundermann AC, Reddy AP, Linder JE, Correa H, et al. Gestational diabetes mellitus is associated with increased CD163 expression and iron storage in the placenta. Am J Reprod Immunol. 2018;80(4):e13020.
261. Bari MF, Weickert MO, Sivakumar K, James SG, Snead DR, Tan BK, et al. Elevated soluble CD163 in gestational diabetes mellitus: secretion from human placenta and adipose tissue. PLoS One. 2014;9(7):e101327.

262. Yu J, Zhou Y, Gui J, Li AZ, Su XL, Feng L. Assessment of the number and function of macrophages in the placenta of gestational diabetes mellitus patients. J Huazhong Univ Sci Technolog Med Sci. 2013;33(5):725-9.
263. Stirm L, Kovarova M, Perschbacher S, Michlmaier R, Fritsche L, Siegel-Axel D, et al. BMI-Independent Effects of Gestational Diabetes on Human Placenta. J Clin Endocrinol Metab. 2018;103(9):3299-309.
264. Harlev A, Aricha-Tamir B, Shaco-Levy R, Tarnovscki T, Bashan N, Rudich A, et al. Macrophage infiltration and stress-signaling in omental and subcutaneous adipose tissue in diabetic pregnancies. J Matern Fetal Neonatal Med. 2014;27(12):1189-94.
265. Unamuno X, Gomez-Ambrosi J, Rodriguez A, Becerril S, Fruhbeck G, Catalan V. Adipokine dysregulation and adipose tissue inflammation in human obesity. Eur J Clin Invest. 2018;48(9):e12997.
266. Gehrke N, Schattenberg JM. Metabolic Inflammation-A Role for Hepatic Inflammatory Pathways as Drivers of Comorbidities in Nonalcoholic Fatty Liver Disease? Gastroenterology. 2020;158(7):1929-47 e6.
267. Talukdar S, Oh DY, Bandyopadhyay G, Li D, Xu J, McNelis J, et al. Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase. Nat Med. 2012;18(9):1407-12.
268. Tsuhako R, Yoshida H, Sugita C, Kurokawa M. Naringenin suppresses neutrophil infiltration into adipose tissue in high-fat diet-induced obese mice. J Nat Med. 2019.
269. Sampath P, Moideen K, Ranganathan UD, Bethunaickan R. Monocyte Subsets: Phenotypes and Function in Tuberculosis Infection. Front Immunol. 2018;9:1726.
270. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, et al. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. Nat Rev Immunol. 2014;14(8):571-8.
271. Chiu S, Bharat A. Role of monocytes and macrophages in regulating immune response following lung transplantation. Curr Opin Organ Transplant. 2016;21(3):239-45.
272. Melgert BN, Spaans F, Borghuis T, Klok PA, Groen B, Bolt A, et al. Pregnancy and preeclampsia affect monocyte subsets in humans and rats. PLoS One. 2012;7(9):e45229.
273. Luppi P, Haluszczak C, Betters D, Richard CA, Trucco M, DeLoia JA. Monocytes are progressively activated in the circulation of pregnant women. J Leukoc Biol. 2002;72(5):874-84.
274. Shive CL, Jiang W, Anthony DD, Lederman MM. Soluble CD14 is a nonspecific marker of monocyte activation. AIDS. 2015;29(10):1263-5.
275. Telejko B, Kuzmicki M, Zonenberg A, Niedziolko-Bagniuk K, Nikolajuk A, Szamatowicz J, et al. Circulating monocyte chemoattractant protein-1 in women with gestational diabetes. Folia Histochem Cytobiol. 2007;45 Suppl 1:S153-6.
276. Saqib U, Sarkar S, Suk K, Mohammad O, Baig MS, Savai R. Phytochemicals as modulators of M1-M2 macrophages in inflammation. Oncotarget. 2018;9(25):17937-50.
277. Jayasingam SD, Citartan M, Thang TH, Mat Zin AA, Ang KC, Ch'ng ES. Evaluating the Polarization of Tumor-Associated Macrophages Into M1 and M2 Phenotypes in Human Cancer Tissue: Technicalities and Challenges in Routine Clinical Practice. Front Oncol. 2019;9:1512.
278. Muir LA, Cho KW, Geletka LM, Baker NA, Flesher CG, Ehlers AP, et al. Human CD206+ macrophages associate with diabetes and adipose tissue lymphoid clusters. JCI Insight. 2022;7(3).
279. Finlin BS, Memetimin H, Confides AL, Zhu B, Westgate PM, Dupont-Versteegden EE, et al. Macrophages expressing uncoupling protein 1 increase in adipose tissue in response to cold in humans. Sci Rep. 2021;11(1):23598.
280. Liu S, Diao L, Huang C, Li Y, Zeng Y, Kwak-Kim JYH. The role of decidual immune cells on human pregnancy. J Reprod Immunol. 2017;124:44-53.

281. Zulu MZ, Martinez FO, Gordon S, Gray CM. The Elusive Role of Placental Macrophages: The Hofbauer Cell. J Innate Immun. 2019;11(6):447-56.
282. Young OM, Tang Z, Niven-Fairchild T, Tadesse S, Krikun G, Norwitz ER, et al. Toll-like receptor-mediated responses by placental Hofbauer cells (HBCs): a potential pro-inflammatory role for fetal M2 macrophages. Am J Reprod Immunol. 2015;73(1):22-35.
283. Azari S, Johnson LJ, Webb A, Kozlowski SM, Zhang X, Rood K, et al. Hofbauer Cells Spread Listeria monocytogenes among Placental Cells and Undergo Pro-Inflammatory Reprogramming while Retaining Production of Tolerogenic Factors. mBio. 2021;12(4):e0184921.
284. Reyes L, Golos TG. Hofbauer Cells: Their Role in Healthy and Complicated Pregnancy. Front Immunol. 2018;9:2628.
285. Dige A, Stoy S, Thomsen KL, Hvas CL, Agnholt J, Dahlerup JF, et al. Soluble CD163, a specific macrophage activation marker, is decreased by anti-TNF-alpha antibody treatment in active inflammatory bowel disease. Scand J Immunol. 2014;80(6):417-23.
286. Ueland T, Michelsen AE, Aukrust P, Henriksen T, Bollerslev J, Lekva T. Adipokines and macrophage markers during pregnancy-Possible role for sCD163 in prediction and progression of gestational diabetes mellitus. Diabetes Metab Res Rev. 2019;35(3):e3114.
287. Luo N, Liu J, Chung BH, Yang Q, Klein RL, Garvey WT, et al. Macrophage adiponectin expression improves insulin sensitivity and protects against inflammation and atherosclerosis. Diabetes. 2010;59(4):791-9.
288. Yilmaz O, Kucuk M, Kebapcilar L, Altindag T, Yuksel A, Yuvanc HO, et al. Macrophage migration-inhibitory factor is elevated in pregnant women with gestational diabetes mellitus. Gynecol Endocrinol. 2012;28(1):76-9.
289. Calandra T, Roger T. Macrophage migration inhibitory factor: a regulator of innate immunity. Nat Rev Immunol. 2003;3(10):791-800.
290. Aslani S, Hossein-nezhad A, Maghbooli Z, Mirzaei K, Karimi F. Genetic variation in macrophage migration inhibitory factor associated with gestational diabetes mellitus and metabolic syndrome. Horm Metab Res. 2011;43(8):557-61.
291. Zhan Y, Li C, Chen J, Yu S, Gao Q, Wang YP, et al. Association between macrophage migration inhibitory factor rs1007888 and GDM. Genet Mol Res. 2015;14(1):797-804.
292. Zheng L, Li C, Qi WH, Qiao BL, Zhao H, Zhou YB, et al. [Expression of macrophage migration inhibitory factor gene in placenta tissue and its correlation with gestational diabetes mellitus]. Zhonghua Yi Xue Za Zhi. 2017;97(43):3388-91.
293. Quintanilla Rodriguez BS, Mahdy H. Gestational Diabetes. StatPearls. Treasure Island (FL): StatPearls Publishing

Copyright © 2022, StatPearls Publishing LLC.; 2022.

294. Petersen MC, Shulman GI. Mechanisms of Insulin Action and Insulin Resistance. Physiol Rev. 2018;98(4):2133-223.
295. Ghanim H, Mohanty P, Deopurkar R, Sia CL, Korzeniewski K, Abuaysheh S, et al. Acute modulation of toll-like receptors by insulin. Diabetes Care. 2008;31(9):1827-31.
296. Leffler M, Hrach T, Stuerzl M, Horch RE, Herndon DN, Jeschke MG. Insulin attenuates apoptosis and exerts anti-inflammatory effects in endotoxemic human macrophages. J Surg Res. 2007;143(2):398-406.
297. Dandona P, Ghanim H, Green K, Sia CL, Abuaysheh S, Kuhadiya N, et al. Insulin infusion suppresses while glucose infusion induces Toll-like receptors and high-mobility group-B1 protein expression in mononuclear cells of type 1 diabetes patients. Am J Physiol Endocrinol Metab. 2013;304(8):E810-8.
298. van Niekerk G, Christowitz C, Conradie D, Engelbrecht AM. Insulin as an immunomodulatory hormone. Cytokine Growth Factor Rev. 2020;52:34-44.
299. Singh AK, Singh R. Metformin in gestational diabetes: An emerging contender. Indian J Endocrinol Metab. 2015;19(2):236-44.

300. Rowan JA, Rush EC, Plank LD, Lu J, Obolonkin V, Coat S, et al. Metformin in gestational diabetes: the offspring follow-up (MiG TOFU): body composition and metabolic outcomes at 7-9 years of age. BMJ Open Diabetes Res Care. 2018;6(1):e000456.
301. Given JE, Loane M, Garne E, Addor MC, Bakker M, Bertaut-Nativel B, et al. Metformin exposure in first trimester of pregnancy and risk of all or specific congenital anomalies: exploratory case-control study. BMJ. 2018;361:k2477.
302. Schuiveling M, Vazirpanah N, Radstake T, Zimmermann M, Broen JCA. Metformin, A New Era for an Old Drug in the Treatment of Immune Mediated Disease? Curr Drug Targets. 2018;19(8):945-59.
303. Thomson AW, Turnquist HR, Raimondi G. Immunoregulatory functions of mTOR inhibition. Nat Rev Immunol. 2009;9(5):324-37.
304. Jing Y, Wu F, Li D, Yang L, Li Q, Li R. Metformin improves obesity-associated inflammation by altering macrophages polarization. Mol Cell Endocrinol. 2018;461:256-64.
305. Vial G, Detaille D, Guigas B. Role of Mitochondria in the Mechanism(s) of Action of Metformin. Front Endocrinol (Lausanne). 2019;10:294.
306. Kalra B, Gupta Y, Singla R, Kalra S. Use of oral anti-diabetic agents in pregnancy: a pragmatic approach. N Am J Med Sci. 2015;7(1):6-12.
307. Guo L, Ma J, Tang J, Hu D, Zhang W, Zhao X. Comparative Efficacy and Safety of Metformin, Glyburide, and Insulin in Treating Gestational Diabetes Mellitus: A Meta-Analysis. J Diabetes Res. 2019;2019:9804708.
308. Koh GC, Maude RR, Schreiber MF, Limmathurotsakul D, Wiersinga WJ, Wuthiekanun V, et al. Glyburide is anti-inflammatory and associated with reduced mortality in melioidosis. Clin Infect Dis. 2011;52(6):717-25.
309. Lin YW, Liu PS, Pook KA, Wei LN. Glyburide and retinoic acid synergize to promote wound healing by anti-inflammation and RIP140 degradation. Sci Rep. 2018;8(1):834.
310. Rahnemaei FA, Pakzad R, Amirian A, Pakzad I, Abdi F. Effect of gestational diabetes mellitus on lipid profile: A systematic review and meta-analysis. Open Med (Wars). 2022;17(1):70-86.
311. Villafan-Bernal JR, Acevedo-Alba M, Reyes-Pavon R, Diaz-Parra GA, Lip-Sosa DL, Vazquez-Delfin HI, et al. Plasma Levels of Free Fatty Acids in Women with Gestational Diabetes and Its Intrinsic and Extrinsic Determinants: Systematic Review and Meta-Analysis. J Diabetes Res. 2019;2019:7098470.
312. Lappas M, Hiden U, Desoye G, Froehlich J, Hauguel-de Mouzon S, Jawerbaum A. The role of oxidative stress in the pathophysiology of gestational diabetes mellitus. Antioxid Redox Signal. 2011;15(12):3061-100.
313. Li X, Fang P, Mai J, Choi ET, Wang H, Yang XF. Targeting mitochondrial reactive oxygen species as novel therapy for inflammatory diseases and cancers. J Hematol Oncol. 2013;6:19.
314. Moya GE, Rivera PD, Dittenhafer-Reed KE. Evidence for the Role of Mitochondrial DNA Release in the Inflammatory Response in Neurological Disorders. Int J Mol Sci. 2021;22(13).
315. Lauterbach MA, Wunderlich FT. Macrophage function in obesity-induced inflammation and insulin resistance. Pflugers Arch. 2017;469(3-4):385-96.
316. Cruz-Pineda WD, Parra-Rojas I, Rodriguez-Ruiz HA, Illades-Aguiar B, Matia-Garcia I, Garibay-Cerdenares OL. The regulatory role of insulin in energy metabolism and leukocyte functions. J Leukoc Biol. 2022;111(1):197-208.
317. Korda M, Kubant R, Patton S, Malinski T. Leptin-induced endothelial dysfunction in obesity. Am J Physiol Heart Circ Physiol. 2008;295(4):H1514-21.
318. Palanivel R, Maida A, Liu Y, Sweeney G. Regulation of insulin signalling, glucose uptake and metabolism in rat skeletal muscle cells upon prolonged exposure to resistin. Diabetologia. 2006;49(1):183-90.

319. Ajaz S, Czajka A, Malik A. Accurate measurement of circulating mitochondrial DNA content from human blood samples using real-time quantitative PCR. Methods Mol Biol. 2015;1264:117-31.
320. Powe CE, Hivert MF, Udler MS. Defining Heterogeneity Among Women With Gestational Diabetes Mellitus. Diabetes. 2020;69(10):2064-74.
321. Couch SC, Philipson EH, Bendel RB, Pujda LM, Milvae RA, Lammi-Keefe CJ. Elevated lipoprotein lipids and gestational hormones in women with diet-treated gestational diabetes mellitus compared to healthy pregnant controls. J Diabetes Complications. 1998;12(1):1-9.
322. Hu J, Gillies CL, Lin S, Stewart ZA, Melford SE, Abrams KR, et al. Association of maternal lipid profile and gestational diabetes mellitus: A systematic review and meta-analysis of 292 studies and 97,880 women. EClinicalMedicine. 2021;34:100830.
323. Sandhu MS, Heald AH, Gibson JM, Cruickshank JK, Dunger DB, Wareham NJ. Circulating concentrations of insulin-like growth factor-I and development of glucose intolerance: a prospective observational study. Lancet. 2002;359(9319):1740-5.
324. Andersson DP, Laurencikiene J, Acosta JR, Ryden M, Arner P. Circulating and Adipose Levels of Adipokines Associated With Insulin Sensitivity in Nonobese Subjects With Type 2 Diabetes. J Clin Endocrinol Metab. 2016;101(10):3765-71.
325. Youn BS, Yu KY, Park HJ, Lee NS, Min SS, Youn MY, et al. Plasma resistin concentrations measured by enzyme-linked immunosorbent assay using a newly developed monoclonal antibody are elevated in individuals with type 2 diabetes mellitus. J Clin Endocrinol Metab. 2004;89(1):150-6.
326. Kim JA, Wei Y, Sowers JR. Role of mitochondrial dysfunction in insulin resistance. Circ Res. 2008;102(4):401-14.
327. Zheng LD, Linarelli LE, Liu L, Wall SS, Greenawald MH, Seidel RW, et al. Insulin resistance is associated with epigenetic and genetic regulation of mitochondrial DNA in obese humans. Clin Epigenetics. 2015;7:60.
328. Bao W, Xia H, Liang Y, Ye Y, Lu Y, Xu X, et al. Toll-like Receptor 9 Can be Activated by Endogenous Mitochondrial DNA to Induce Podocyte Apoptosis. Sci Rep. 2016;6:22579.
329. Puschel GP, Klauder J, Henkel J. Macrophages, Low-Grade Inflammation, Insulin Resistance and Hyperinsulinemia: A Mutual Ambiguous Relationship in the Development of Metabolic Diseases. J Clin Med. 2022;11(15).
330. Cruz CM, Rinna A, Forman HJ, Ventura AL, Persechini PM, Ojcius DM. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. J Biol Chem. 2007;282(5):2871-9.
331. Horakova D, Stepanek L, Janout V, Janoutova J, Pastucha D, Kollarova H, et al. Optimal Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) Cut-Offs: A Cross-Sectional Study in the Czech Population. Medicina (Kaunas). 2019;55(5).
332. Wang X, Yang T, Miao J, Liu H, Wu K, Guo J, et al. Correlation Between Maternal and Fetal Insulin Resistance in Pregnant Women with Gestational Diabetes Mellitus. Clin Lab. 2018;64(6):945-53.
333. Okura T, Nakamura R, Fujioka Y, Kawamoto-Kitao S, Ito Y, Matsumoto K, et al. Body mass index >/=23 is a risk factor for insulin resistance and diabetes in Japanese people: A brief report. PLoS One. 2018;13(7):e0201052.
334. Martinez KE, Tucker LA, Bailey BW, LeCheminant JD. Expanded Normal Weight Obesity and Insulin Resistance in US Adults of the National Health and Nutrition Examination Survey. J Diabetes Res. 2017;2017:9502643.
335. Jiang J, Cai X, Pan Y, Du X, Zhu H, Yang X, et al. Relationship of obesity to adipose tissue insulin resistance. BMJ Open Diabetes Res Care. 2020;8(1).
336. Denley A, Cosgrove LJ, Booker GW, Wallace JC, Forbes BE. Molecular interactions of the IGF system. Cytokine Growth Factor Rev. 2005;16(4-5):421-39.

337. Vatten LJ, Nilsen TI, Juul A, Jeansson S, Jenum PA, Eskild A. Changes in circulating level of IGF-I and IGF-binding protein-1 from the first to second trimester as predictors of preeclampsia. Eur J Endocrinol. 2008;158(1):101-5.
338. Asvold BO, Eskild A, Jenum PA, Vatten LJ. Maternal concentrations of insulin-like growth factor I and insulin-like growth factor binding protein 1 during pregnancy and birth weight of offspring. Am J Epidemiol. 2011;174(2):129-35.
339. Antsaklis P, Fasoulakis Z, Theodora M, Diakosavvas M, Kontomanolis EN. Association of Low Maternal Pregnancy-associated Plasma Protein A with Adverse Perinatal Outcome. Cureus. 2019;11(6):e4912.
340. Tsiotra PC, Halvatsiotis P, Patsouras K, Maratou E, Salamalekis G, Raptis SA, et al. Circulating adipokines and mRNA expression in adipose tissue and the placenta in women with gestational diabetes mellitus. Peptides. 2018;101:157-66.
341. Bhograj A, Suryanarayana KM, Nayak A, Murthy NS, Dharmalingam M, Kalra P. Serum adiponectin levels in gestational diabetes mellitus. Indian J Endocrinol Metab. 2016;20(6):752-5.
342. Ziemke F, Mantzoros CS. Adiponectin in insulin resistance: lessons from translational research. Am J Clin Nutr. 2010;91(1):258S-61S.
343. Mohammadi T, Paknahad Z. Adiponectin Concentration in Gestational Diabetic Women: a Case-Control Study. Clin Nutr Res. 2017;6(4):267-76.
344. Ranheim T, Haugen F, Staff AC, Braekke K, Harsem NK, Drevon CA. Adiponectin is reduced in gestational diabetes mellitus in normal weight women. Acta Obstet Gynecol Scand. 2004;83(4):341-7.
345. Francis EC, Li M, Hinkle SN, Cao Y, Chen J, Wu J, et al. Adipokines in early and mid-pregnancy and subsequent risk of gestational diabetes: a longitudinal study in a multiracial cohort. BMJ Open Diabetes Res Care. 2020;8(1).
346. Fisher JJ, Vanderpeet CL, Bartho LA, McKeating DR, Cuffe JSM, Holland OJ, et al. Mitochondrial dysfunction in placental trophoblast cells experiencing gestational diabetes mellitus. J Physiol. 2021;599(4):1291-305.
347. Fazakerley DJ, Minard AY, Krycer JR, Thomas KC, Stockli J, Harney DJ, et al. Mitochondrial oxidative stress causes insulin resistance without disrupting oxidative phosphorylation. J Biol Chem. 2018;293(19):7315-28.
348. Iacobazzi V, Castegna A, Infantino V, Andria G. Mitochondrial DNA methylation as a next-generation biomarker and diagnostic tool. Mol Genet Metab. 2013;110(1-2):25-34.
349. Yuzefovych LV, Pastukh VM, Ruchko MV, Simmons JD, Richards WO, Rachek LI. Plasma mitochondrial DNA is elevated in obese type 2 diabetes mellitus patients and correlates positively with insulin resistance. PLoS One. 2019;14(10):e0222278.
350. Stump CS, Short KR, Bigelow ML, Schimke JM, Nair KS. Effect of insulin on human skeletal muscle mitochondrial ATP production, protein synthesis, and mRNA transcripts. Proc Natl Acad Sci U S A. 2003;100(13):7996-8001.
351. Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, et al. Mitochondrial dysfunction in the elderly: possible role in insulin resistance. Science. 2003;300(5622):1140-2.
352. Prins JR, Gomez-Lopez N, Robertson SA. Interleukin-6 in pregnancy and gestational disorders. J Reprod Immunol. 2012;95(1-2):1-14.
353. Kuzmicki M, Telejko B, Lipinska D, Pliszka J, Wilk J, Wawrusiewicz-Kurylonek N, et al. The IL-6/IL-6R/sgp130 system and Th17 associated cytokines in patients with gestational diabetes. Endokrynol Pol. 2014;65(3):169-75.
354. Lagathu C, Bastard JP, Auclair M, Maachi M, Capeau J, Caron M. Chronic interleukin-6 (IL-6) treatment increased IL-6 secretion and induced insulin resistance in adipocyte: prevention by rosiglitazone. Biochem Biophys Res Commun. 2003;311(2):372-9.

355. Saini C, Srivastava RK, Tarique M, Kurra S, Khanna N, Ramesh V, et al. Elevated IL-6R on CD4(+) T cells promotes IL-6 driven Th17 cell responses in patients with T1R leprosy reactions. Sci Rep. 2020;10(1):15143.
356. Chen J, Liao MY, Gao XL, Zhong Q, Tang TT, Yu X, et al. IL-17A induces pro-inflammatory cytokines production in macrophages via MAPKinases, NF-kappaB and AP-1. Cell Physiol Biochem. 2013;32(5):1265-74.
357. Pestel J, Chehimi M, Bonhomme M, Robert M, Vidal H, Eljaafari A. IL-17A contributes to propagation of inflammation but does not impair adipogenesis and/or insulin response, in adipose tissue of obese individuals. Cytokine. 2020;126:154865.
358. Moore TA, Case AJ, Mathews TL, Epstein CM, Kaiser KL, Zimmerman MC. Interleukin-17A and Chronic Stress in Pregnant Women at 24-28 Weeks Gestation. Nurs Res. 2019;68(2):167-73.
359. El Shahaway AA, Abd Elhady RR, Abdelrhman AA, Yahia S. Role of maternal serum interleukin 17 in preeclampsia: diagnosis and prognosis. J Inflamm Res. 2019;12:175-80.
360. Randeria SN, Thomson GJA, Nell TA, Roberts T, Pretorius E. Inflammatory cytokines in type 2 diabetes mellitus as facilitators of hypercoagulation and abnormal clot formation. Cardiovasc Diabetol. 2019;18(1):72.
361. Wegner M, Winiarska H, Bobkiewicz-Kozlowska T, Dworacka M. IL-12 serum levels in patients with type 2 diabetes treated with sulphonylureas. Cytokine. 2008;42(3):312-6.
362. Memon AA, Sundquist J, Wang X, Palmer K, Sundquist K, Bennet L. The association between cytokines and insulin sensitivity in Iraqi immigrants and native Swedes. BMJ Open. 2013;3(11):e003473.
363. Ye J, Wang Y, Wang Z, Liu L, Yang Z, Wang M, et al. Roles and Mechanisms of Interleukin-12 Family Members in Cardiovascular Diseases: Opportunities and Challenges. Front Pharmacol. 2020;11:129.
364. Klein K, Satler M, Elhenicky M, Brix J, Krzyzanowska K, Schernthaner G, et al. Circulating levels of MCP-1 are increased in women with gestational diabetes. Prenat Diagn. 2008;28(9):845-51.
365. Yanchun Fan YW, Xia Du, Wenju Yang, Ningning Cao, Yaqiang Tian, Ming Li. A study on serum IGF-1, VEGF, MCP-1 levels in patients with GDM and its clinical significance. Int J Clin Exp Med. 2020;13(5):3616-22.
366. Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. Proc Natl Acad Sci U S A. 2003;100(12):7265-70.
367. Hedderson MM, Darbinian JA, Ferrara A. Disparities in the risk of gestational diabetes by race-ethnicity and country of birth. Paediatr Perinat Epidemiol. 2010;24(5):441-8.
368. Emont MP, Jacobs C, Essene AL, Pant D, Tenen D, Colleluori G, et al. A single-cell atlas of human and mouse white adipose tissue. Nature. 2022;603(7903):926-33.
369. Santomauro AT, Boden G, Silva ME, Rocha DM, Santos RF, Ursich MJ, et al. Overnight lowering of free fatty acids with Acipimox improves insulin resistance and glucose tolerance in obese diabetic and nondiabetic subjects. Diabetes. 1999;48(9):1836-41.
370. Fruhbeck G, Catalan V, Rodriguez A, Gomez-Ambrosi J. Adiponectin-leptin ratio: A promising index to estimate adipose tissue dysfunction. Relation with obesity-associated cardiometabolic risk. Adipocyte. 2018;7(1):57-62.
371. Semnani-Azad Z, Connelly PW, Bazinet RP, Retnakaran R, Jenkins DJA, Harris SB, et al. Adipose Tissue Insulin Resistance Is Longitudinally Associated With Adipose Tissue Dysfunction, Circulating Lipids, and Dysglycemia: The PROMISE Cohort. Diabetes Care. 2021;44(7):1682-91.

372. Pasarica M, Xie H, Hymel D, Bray G, Greenway F, Ravussin E, et al. Lower total adipocyte number but no evidence for small adipocyte depletion in patients with type 2 diabetes. Diabetes Care. 2009;32(5):900-2.
373. Hoffstedt J, Arner E, Wahrenberg H, Andersson DP, Qvisth V, Lofgren P, et al. Regional impact of adipose tissue morphology on the metabolic profile in morbid obesity. Diabetologia. 2010;53(12):2496-503.
374. Ott R, Melchior K, Stupin JH, Ziska T, Schellong K, Henrich W, et al. Reduced Insulin Receptor Expression and Altered DNA Methylation in Fat Tissues and Blood of Women With GDM and Offspring. J Clin Endocrinol Metab. 2019;104(1):137-49.
375. Ma M, Quan Y, Li Y, He X, Xiao J, Zhan M, et al. Bidirectional modulation of insulin action by reactive oxygen species in 3T3-L1 adipocytes. Mol Med Rep. 2018;18(1):807-14.
376. Kleiblova P, Dostalova I, Bartlova M, Lacinova Z, Ticha I, Krejci V, et al. Expression of adipokines and estrogen receptors in adipose tissue and placenta of patients with gestational diabetes mellitus. Mol Cell Endocrinol. 2010;314(1):150-6.
377. Kuzmicki M, Telejko B, Wawrusiewicz-Kurylonek N, Citko A, Lipinska D, Pliszka J, et al. The expression of suppressor of cytokine signaling 1 and 3 in fat and placental tissue from women with gestational diabetes. Gynecol Endocrinol. 2012;28(11):841-4.
378. Osman OS, Selway JL, Kepczynska MA, Stocker CJ, O'Dowd JF, Cawthorne MA, et al. A novel automated image analysis method for accurate adipocyte quantification. Adipocyte. 2013;2(3):160-4.
379. Vishvanath L, Gupta RK. Contribution of adipogenesis to healthy adipose tissue expansion in obesity. J Clin Invest. 2019;129(10):4022-31.
380. Marcelin G, Gautier EL, Clement K. Adipose Tissue Fibrosis in Obesity: Etiology and Challenges. Annu Rev Physiol. 2022;84:135-55.
381. Rajan MR, Fagerholm S, Jonsson C, Kjolhede P, Turkina MV, Stralfors P. Phosphorylation of IRS1 at serine 307 in response to insulin in human adipocytes is not likely to be catalyzed by p70 ribosomal S6 kinase. PLoS One. 2013;8(4):e59725.
382. Copps KD, White MF. Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. Diabetologia. 2012;55(10):2565-82.
383. Apovian CM, Bigornia S, Mott M, Meyers MR, Ulloor J, Gagua M, et al. Adipose macrophage infiltration is associated with insulin resistance and vascular endothelial dysfunction in obese subjects. Arterioscler Thromb Vasc Biol. 2008;28(9):1654-9.
384. Tordjman J, Poitou C, Hugol D, Bouillot JL, Basdevant A, Bedossa P, et al. Association between omental adipose tissue macrophages and liver histopathology in morbid obesity: influence of glycemic status. J Hepatol. 2009;51(2):354-62.
385. Sada K, Nishikawa T, Kukidome D, Yoshinaga T, Kajihara N, Sonoda K, et al. Hyperglycemia Induces Cellular Hypoxia through Production of Mitochondrial ROS Followed by Suppression of Aquaporin-1. PLoS One. 2016;11(7):e0158619.
386. Masschelin PM, Cox AR, Chernis N, Hartig SM. The Impact of Oxidative Stress on Adipose Tissue Energy Balance. Front Physiol. 2019;10:1638.
387. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest. 2004;114(12):1752-61.
388. Stafeev I, Podkuychenko N, Michurina S, Sklyanik I, Panevina A, Shestakova E, et al. Low proliferative potential of adipose-derived stromal cells associates with hypertrophy and inflammation in subcutaneous and omental adipose tissue of patients with type 2 diabetes mellitus. J Diabetes Complications. 2019;33(2):148-59.
389. Kim JI, Huh JY, Sohn JH, Choe SS, Lee YS, Lim CY, et al. Lipid-overloaded enlarged adipocytes provoke insulin resistance independent of inflammation. Mol Cell Biol. 2015;35(10):1686-99.

390. Hammarstedt A, Graham TE, Kahn BB. Adipose tissue dysregulation and reduced insulin sensitivity in non-obese individuals with enlarged abdominal adipose cells. Diabetol Metab Syndr. 2012;4(1):42.
391. Muir LA, Neeley CK, Meyer KA, Baker NA, Brosius AM, Washabaugh AR, et al. Adipose tissue fibrosis, hypertrophy, and hyperplasia: Correlations with diabetes in human obesity. Obesity (Silver Spring). 2016;24(3):597-605.
392. Sorisky A, Molgat AS, Gagnon A. Macrophage-induced adipose tissue dysfunction and the preadipocyte: should I stay (and differentiate) or should I go? Adv Nutr. 2013;4(1):67-75.
393. Wang F, Li H, Lou Y, Xie J, Cao D, Huang X. Insulin-like growth factor I promotes adipogenesis in hemangioma stem cells from infantile hemangiomas. Mol Med Rep. 2019;19(4):2825-30.
394. Maridas DE, DeMambro VE, Le PT, Mohan S, Rosen CJ. IGFBP4 Is Required for Adipogenesis and Influences the Distribution of Adipose Depots. Endocrinology. 2017;158(10):3488-500.
395. Chan SS, Twigg SM, Firth SM, Baxter RC. Insulin-like growth factor binding protein-3 leads to insulin resistance in adipocytes. J Clin Endocrinol Metab. 2005;90(12):6588-95.
396. Mohanraj L, Kim HS, Li W, Cai Q, Kim KE, Shin HJ, et al. IGFBP-3 inhibits cytokine-induced insulin resistance and early manifestations of atherosclerosis. PLoS One. 2013;8(1):e55084.
397. de Silva HC, Firth SM, Twigg SM, Baxter RC. Interaction between IGF binding protein-3 and TGFbeta in the regulation of adipocyte differentiation. Endocrinology. 2012;153(10):4799-807.
398. Davidge-Pitts C, Escande CJ, Conover CA. Preferential expression of PAPPA in human preadipocytes from omental fat. J Endocrinol. 2014;222(1):87-97.
399. Groeneveld MP, Brierley GV, Rocha NM, Siddle K, Semple RK. Acute knockdown of the insulin receptor or its substrates Irs1 and 2 in 3T3-L1 adipocytes suppresses adiponectin production. Sci Rep. 2016;6:21105.
400. Chylikova J, Dvorackova J, Cizkova K, Lacey H, Kamarad V. Macrophages of the subcutaneous and omental fatty tissue in obese patients: Immunohistochemical phenotyping of M2 subtypes in relation to type 2 diabetes. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2020;164(2):133-7.
401. Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, et al. Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. Circulation. 2000;102(11):1296-301.
402. Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ, et al. Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. J Biol Chem. 2002;277(50):48115-21.
403. Andreozzi F, Laratta E, Procopio C, Hribal ML, Sciacqua A, Perticone M, et al. Interleukin-6 impairs the insulin signaling pathway, promoting production of nitric oxide in human umbilical vein endothelial cells. Mol Cell Biol. 2007;27(6):2372-83.
404. Augustine G, Pulikkathodi M, S R, TK J. A study of placental histological changes in gestational diabetes mellitus on account of fetal hypoxia. International Journal of Medical Science and Public Health. 2016;5(12).
405. Tirosh A, Potashnik R, Bashan N, Rudich A. Oxidative stress disrupts insulin-induced cellular redistribution of insulin receptor substrate-1 and phosphatidylinositol 3-kinase in 3T3-L1 adipocytes - A putative cellular mechanism for impaired protein kinase B activation and GLUT4 translocation. J Biol Chem. 1999;274(15):10595-602.
406. Tschopp J, Schroder K. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? Nat Rev Immunol. 2010;10(3):210-5.

407. Zhou W, Chen C, Chen Z, Liu L, Jiang J, Wu Z, et al. NLRP3: A Novel Mediator in Cardiovascular Disease. J Immunol Res. 2018;2018:5702103.
408. Zhong Z, Liang S, Sanchez-Lopez E, He F, Shalapour S, Lin XJ, et al. New mitochondrial DNA synthesis enables NLRP3 inflammasome activation. Nature. 2018;560(7717):198-203.
409. Mangan MSJ, Olhava EJ, Roush WR, Seidel HM, Glick GD, Latz E. Targeting the NLRP3 inflammasome in inflammatory diseases. Nat Rev Drug Discov. 2018;17(9):688.
410. Dixit VD. Nlrp3 Inflammasome Activation in Type 2 Diabetes: Is It Clinically Relevant? Diabetes. 2013;62(1):22-4.
411. Huang Y, Xu W, Zhou R. NLRP3 inflammasome activation and cell death. Cell Mol Immunol. 2021;18(9):2114-27.
412. Wu B, Ni H, Li J, Zhuang X, Zhang J, Qi Z, et al. The Impact of Circulating Mitochondrial DNA on Cardiomyocyte Apoptosis and Myocardial Injury After TLR4 Activation in Experimental Autoimmune Myocarditis. Cell Physiol Biochem. 2017;42(2):713-28.
413. Kelley N, Jeltema D, Duan Y, He Y. The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. Int J Mol Sci. 2019;20(13).
414. Xie F, Hu Y, Turvey SE, Magee LA, Brunham RM, Choi KC, et al. Toll-like receptors 2 and 4 and the cryopyrin inflammasome in normal pregnancy and pre-eclampsia. BJOG. 2010;117(1):99-108.
415. Han CS, Herrin MA, Pitruzzello MC, Mulla MJ, Werner EF, Pettker CM, et al. Glucose and metformin modulate human first trimester trophoblast function: a model and potential therapy for diabetes-associated uteroplacental insufficiency. Am J Reprod Immunol. 2015;73(4):362-71.
416. Rogers LM, Serezani CH, Eastman AJ, Hasty AH, Englund-Ogge L, Jacobsson B, et al. Palmitate induces apoptotic cell death and inflammasome activation in human placental macrophages. Placenta. 2020;90:45-51.
417. Yang Y, Wang H, Kouadir M, Song H, Shi F. Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors. Cell Death Dis. 2019;10(2):128.
418. Cao J, Peng Q. NLRP3 Inhibitor Tranilast Attenuates Gestational Diabetes Mellitus in a Genetic Mouse Model. Drugs R D. 2022;22(1):105-12.
419. Gottwald EM, Duss M, Bugarski M, Haenni D, Schuh CD, Landau EM, et al. The targeted anti-oxidant MitoQ causes mitochondrial swelling and depolarization in kidney tissue. Physiol Rep. 2018;6(7):e13667.
420. Gioscia-Ryan RA, LaRocca TJ, Sindler AL, Zigler MC, Murphy MP, Seals DR. Mitochondria-targeted antioxidant (MitoQ) ameliorates age-related arterial endothelial dysfunction in mice. J Physiol. 2014;592(12):2549-61.
421. Rossman MJ, Santos-Parker JR, Steward CAC, Bispham NZ, Cuevas LM, Rosenberg HL, et al. Chronic Supplementation With a Mitochondrial Antioxidant (MitoQ) Improves Vascular Function in Healthy Older Adults. Hypertension. 2018;71(6):1056-63.
422. Murphy MP. Understanding and preventing mitochondrial oxidative damage. Biochem Soc Trans. 2016;44(5):1219-26.
423. Morillon AC, Williamson RD, Baker PN, Kell DB, Kenny LC, English JA, et al. Effect of L-Ergothioneine on the metabolic plasma profile of the RUPP rat model of pre-eclampsia. PLoS One. 2020;15(3):e0230977.
424. Kerley RN, McCarthy C, Kell DB, Kenny LC. The potential therapeutic effects of ergothioneine in pre-eclampsia. Free Radic Biol Med. 2018;117:145-57.
425. Crossland J, Mitchell J, Woodruff GN. The presence of ergothioneine in the central nervous system and its probable identity with the cerebellar factor. J Physiol. 1966;182(2):427-38.

426. Paul BD, Snyder SH. The unusual amino acid L-ergothioneine is a physiologic cytoprotectant. Cell Death Differ. 2010;17(7):1134-40.
427. Avagliano L, Massa V, Bulfamante GP. Histology of Human Placenta. In: SMGroup, editor. Histopathology2016.
428. Redline RW. Distal villous immaturity. Diagnostic Histopathology. 2012;18(5):189-94.
429. Mezouar S, Katsogiannou M, Ben Amara A, Bretelle F, Mege JL. Placental macrophages: Origin, heterogeneity, function and role in pregnancy-associated infections. Placenta. 2021;103:94-103.
430. Valavanidis A, Vlachogianni T, Fiotakis C. 8-hydroxy-2' -deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 2009;27(2):120-39.
431. Fukushima K, Murata M, Tsukimori K, Eisuke K, Wake N. 8-Hydroxy-2-deoxyguanosine staining in placenta is associated with maternal serum uric acid levels and gestational age at diagnosis in pre-eclampsia. Am J Hypertens. 2011;24(7):829-34.
432. Zhang JZ, Liu Z, Liu J, Ren JX, Sun TS. Mitochondrial DNA induces inflammation and increases TLR9/NF-kappaB expression in lung tissue. Int J Mol Med. 2014;33(4):817-24.
433. Goulopoulou S, Matsumoto T, Bomfim GF, Webb RC. Toll-like receptor 9 activation: a novel mechanism linking placenta-derived mitochondrial DNA and vascular dysfunction in pre-eclampsia. Clin Sci (Lond). 2012;123(7):429-35.
434. Swanson KV, Deng M, Ting JP. The NLRP3 inflammasome: molecular activation and regulation to therapeutics. Nat Rev Immunol. 2019;19(8):477-89.
435. Vandanmagsar B, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL, et al. The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. Nat Med. 2011;17(2):179-88.
436. Pizzicannella J, Fonticoli L, Guarnieri S, Marconi GD, Rajan TS, Trubiani O, et al. Antioxidant Ascorbic Acid Modulates NLRP3 Inflammasome in LPS-G Treated Oral Stem Cells through NFkappaB/Caspase-1/IL-1beta Pathway. Antioxidants (Basel). 2021;10(5).
437. Pavlov OV, Selutin AV, Pavlova OM, Selkov SA. Two patterns of cytokine production by placental macrophages. Placenta. 2020;91:1-10.
438. D'Ippolito S, Tersigni C, Scambia G, Di Simone N. Adipokines, an adipose tissue and placental product with biological functions during pregnancy. Biofactors. 2012;38(1):14-23.
439. Stratton IM, Adler AI, Neil HA, Matthews DR, Manley SE, Cull CA, et al. Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. BMJ. 2000;321(7258):405-12.
440. Byford A, Baird-Rayner C, Forbes K. Don’t sugar coat it: the effects of gestational diabetes on the placental vasculature. The Biochemist. 2021;43(2):34-9.
441. Valent AM, Choi H, Kolahi KS, Thornburg KL. Hyperglycemia and gestational diabetes suppress placental glycolysis and mitochondrial function and alter lipid processing. FASEB J. 2021;35(3):e21423.
442. Mills EL, Kelly B, Logan A, Costa ASH, Varma M, Bryant CE, et al. Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. Cell. 2016;167(2):457-70 e13.
443. Forrester SJ, Kikuchi DS, Hernandes MS, Xu Q, Griendling KK. Reactive Oxygen Species in Metabolic and Inflammatory Signaling. Circ Res. 2018;122(6):877-902.
444. Pereira CA, Carlos D, Ferreira NS, Silva JF, Zanotto CZ, Zamboni DS, et al. Mitochondrial DNA Promotes NLRP3 Inflammasome Activation and Contributes to Endothelial Dysfunction and Inflammation in Type 1 Diabetes. Front Physiol. 2019;10:1557.
445. Hu C, Ding H, Li Y, Pearson JA, Zhang X, Flavell RA, et al. NLRP3 deficiency protects from type 1 diabetes through the regulation of chemotaxis into the pancreatic islets. Proc Natl Acad Sci U S A. 2015;112(36):11318-23.

446. Coll RC, Hill JR, Day CJ, Zamoshnikova A, Boucher D, Massey NL, et al. MCC950 directly targets the NLRP3 ATP-hydrolysis motif for inflammasome inhibition. Nat Chem Biol. 2019;15(6):556-9.
447. Wang J, Shen X, Liu J, Chen W, Wu F, Wu W, et al. High glucose mediates NLRP3 inflammasome activation via upregulation of ELF3 expression. Cell Death Dis. 2020;11(5):383.
448. Williamson RD, McCarthy FP, Manna S, Groarke E, Kell DB, Kenny LC, et al. L-(+)-Ergothioneine Significantly Improves the Clinical Characteristics of Preeclampsia in the Reduced Uterine Perfusion Pressure Rat Model. Hypertension. 2020;75(2):561-8.
449. Cheah IK, Tang RM, Yew TS, Lim KH, Halliwell B. Administration of Pure Ergothioneine to Healthy Human Subjects: Uptake, Metabolism, and Effects on Biomarkers of Oxidative Damage and Inflammation. Antioxid Redox Signal. 2017;26(5):193-206.
450. Zhang J, Chi H, Xiao H, Tian X, Wang Y, Yun X, et al. Interleukin 6 (IL-6) and Tumor Necrosis Factor alpha (TNF-alpha) Single Nucleotide Polymorphisms (SNPs), Inflammation and Metabolism in Gestational Diabetes Mellitus in Inner Mongolia. Med Sci Monit. 2017;23:4149-57.
451. Hsiao EY, Patterson PH. Activation of the maternal immune system induces endocrine changes in the placenta via IL-6. Brain Behav Immun. 2011;25(4):604-15.
452. Winkler G, Cseh K, Baranyi E, Melczer Z, Speer G, Hajos P, et al. Tumor necrosis factor system in insulin resistance in gestational diabetes. Diabetes Res Clin Pract. 2002;56(2):93-9.
453. Kirwan JP, Hauguel-De Mouzon S, Lepercq J, Challier JC, Huston-Presley L, Friedman JE, et al. TNF-alpha is a predictor of insulin resistance in human pregnancy. Diabetes. 2002;51(7):2207-13.
454. Kurauti MA, Costa-Junior JM, Ferreira SM, Santos GJ, Sponton CHG, Carneiro EM, et al. Interleukin-6 increases the expression and activity of insulin-degrading enzyme. Sci Rep. 2017;7:46750.
455. Lea RG, Howe D, Hannah LT, Bonneau O, Hunter L, Hoggard N. Placental leptin in normal, diabetic and fetal growth-retarded pregnancies. Mol Hum Reprod. 2000;6(8):763-9.
456. Lepercq J, Cauzac M, Lahlou N, Timsit J, Girard J, Auwerx J, et al. Overexpression of placental leptin in diabetic pregnancy: a critical role for insulin. Diabetes. 1998;47(5):847-50.
457. Lekva T, Lyle R, Roland MC, Friis C, Bianchi DW, Jaffe IZ, et al. Gene expression in term placentas is regulated more by spinal or epidural anesthesia than by late-onset preeclampsia or gestational diabetes mellitus. Sci Rep. 2016;6:29715.
458. Saleh J, Cianflone K, Chaudhary T, Al-Riyami H, Al-Abri AR, Bayoumi R. Increased plasma acylation-stimulating protein correlates with hyperlipidemia at late gestation. Obesity (Silver Spring). 2007;15(3):646-52.
459. Masuzaki H, Ogawa Y, Sagawa N, Hosoda K, Matsumoto T, Mise H, et al. Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans. Nat Med. 1997;3(9):1029-33.
460. Palhinha L, Liechocki S, Hottz ED, Pereira J, de Almeida CJ, Moraes-Vieira PMM, et al. Leptin Induces Proadipogenic and Proinflammatory Signaling in Adipocytes. Front Endocrinol (Lausanne). 2019;10:841.
461. Aplin JD, Myers JE, Timms K, Westwood M. Tracking placental development in health and disease. Nat Rev Endocrinol. 2020;16(9):479-94.
462. Weel IC, Baergen RN, Romao-Veiga M, Borges VT, Ribeiro VR, Witkin SS, et al. Association between Placental Lesions, Cytokines and Angiogenic Factors in Pregnant Women with Preeclampsia. PLoS One. 2016;11(6):e0157584.

463. Napso T, Zhao X, Lligona MI, Sandovici I, Kay RG, George AL, et al. Placental secretome characterization identifies candidates for pregnancy complications. Commun Biol. 2021;4(1):701.
464. Salomon C, Scholz-Romero K, Sarker S, Sweeney E, Kobayashi M, Correa P, et al. Gestational Diabetes Mellitus Is Associated With Changes in the Concentration and Bioactivity of Placenta-Derived Exosomes in Maternal Circulation Across Gestation. Diabetes. 2016;65(3):598-609.
465. Rice GE, Scholz-Romero K, Sweeney E, Peiris H, Kobayashi M, Duncombe G, et al. The Effect of Glucose on the Release and Bioactivity of Exosomes From First Trimester Trophoblast Cells. J Clin Endocrinol Metab. 2015;100(10):E1280-8.
466. Calabuig-Navarro V, Haghiac M, Minium J, Glazebrook P, Ranasinghe GC, Hoppel C, et al. Effect of Maternal Obesity on Placental Lipid Metabolism. Endocrinology. 2017;158(8):2543-55.
467. Saben J, Lindsey F, Zhong Y, Thakali K, Badger TM, Andres A, et al. Maternal obesity is associated with a lipotoxic placental environment. Placenta. 2014;35(3):171-7.
468. Brass E, Hanson E, O'Tierney-Ginn PF. Placental oleic acid uptake is lower in male offspring of obese women. Placenta. 2013;34(6):503-9.
469. Sano M, Shimazaki S, Kaneko Y, Karasawa T, Takahashi M, Ohkuchi A, et al. Palmitic acid activates NLRP3 inflammasome and induces placental inflammation during pregnancy in mice. J Reprod Dev. 2020;66(3):241-8.
470. Morigny P, Houssier M, Mouisel E, Langin D. Adipocyte lipolysis and insulin resistance. Biochimie. 2016;125:259-66.
471. Stefanovski D, Punjabi NM, Boston RC, Watanabe RM. Insulin Action, Glucose Homeostasis and Free Fatty Acid Metabolism: Insights From a Novel Model. Front Endocrinol (Lausanne). 2021;12:625701.
472. Nielsen TS, Jessen N, Jorgensen JO, Moller N, Lund S. Dissecting adipose tissue lipolysis: molecular regulation and implications for metabolic disease. J Mol Endocrinol. 2014;52(3):R199-222.
473. Seow KM, Juan CC, Hsu YP, Hwang JL, Huang LW, Ho LT. Amelioration of insulin resistance in women with PCOS via reduced insulin receptor substrate-1 Ser312 phosphorylation following laparoscopic ovarian electrocautery. Hum Reprod. 2007;22(4):1003-10.
474. Liu T, Yu B, Kakino M, Fujimoto H, Ando Y, Hakuno F, et al. A novel IRS-1-associated protein, DGKzeta regulates GLUT4 translocation in 3T3-L1 adipocytes. Sci Rep. 2016;6:35438.
475. Song NJ, Kim S, Jang BH, Chang SH, Yun UJ, Park KM, et al. Small Molecule-Induced Complement Factor D (Adipsin) Promotes Lipid Accumulation and Adipocyte Differentiation. PLoS One. 2016;11(9):e0162228.
476. Kojta I, Chacinska M, Blachnio-Zabielska A. Obesity, Bioactive Lipids, and Adipose Tissue Inflammation in Insulin Resistance. Nutrients. 2020;12(5).
477. Stern C, Schwarz S, Moser G, Cvitic S, Jantscher-Krenn E, Gauster M, et al. Placental Endocrine Activity: Adaptation and Disruption of Maternal Glucose Metabolism in Pregnancy and the Influence of Fetal Sex. Int J Mol Sci. 2021;22(23).
478. Carriere A, Carmona MC, Fernandez Y, Rigoulet M, Wenger RH, Penicaud L, et al. Mitochondrial reactive oxygen species control the transcription factor CHOP-10/GADD153 and adipocyte differentiation: a mechanism for hypoxia-dependent effect. J Biol Chem. 2004;279(39):40462-9.
479. Huh JY, Kim Y, Jeong J, Park J, Kim I, Huh KH, et al. Peroxiredoxin 3 is a key molecule regulating adipocyte oxidative stress, mitochondrial biogenesis, and adipokine expression. Antioxid Redox Signal. 2012;16(3):229-43.

480. Wondmkun YT. Obesity, Insulin Resistance, and Type 2 Diabetes: Associations and Therapeutic Implications. Diabetes Metab Syndr Obes. 2020;13:3611-6.
481. Drolet R, Richard C, Sniderman AD, Mailloux J, Fortier M, Huot C, et al. Hypertrophy and hyperplasia of abdominal adipose tissues in women. Int J Obes (Lond). 2008;32(2):283-91.
482. Straughen JK, Trudeau S, Misra VK. Changes in adipose tissue distribution during pregnancy in overweight and obese compared with normal weight women. Nutr Diabetes. 2013;3(8):e84.
483. Scavo LM, Karas M, Murray M, Leroith D. Insulin-like growth factor-I stimulates both cell growth and lipogenesis during differentiation of human mesenchymal stem cells into adipocytes. J Clin Endocrinol Metab. 2004;89(7):3543-53.
484. Wabitsch M, Hauner H, Heinze E, Teller WM. The role of growth hormone/insulin-like growth factors in adipocyte differentiation. Metabolism. 1995;44(10 Suppl 4):45-9.
485. Fu Y, Luo N, Klein RL, Garvey WT. Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation. J Lipid Res. 2005;46(7):1369-79.
486. Lebreton F, Berishvili E, Parnaud G, Rouget C, Bosco D, Berney T, et al. NLRP3 inflammasome is expressed and regulated in human islets. Cell Death Dis. 2018;9(7):726.
487. Amirian A, Mahani MB, Abdi F. Role of interleukin-6 (IL-6) in predicting gestational diabetes mellitus. Obstet Gynecol Sci. 2020;63(4):407-16.
488. Tamai I, Yabuuchi H, Nezu J, Sai Y, Oku A, Shimane M, et al. Cloning and characterization of a novel human pH-dependent organic cation transporter, OCTN1. FEBS Lett. 1997;419(1):107-11.
489. Tian X, Cioccoloni G, Sier JH, Naseem KM, Thorne JL, Moore JB. Ergothioneine supplementation in people with metabolic syndrome (ErgMS): protocol for a randomised, double-blind, placebo-controlled pilot study. Pilot Feasibility Stud. 2021;7(1):193.
490. Eades CE, Cameron DM, Evans JMM. Prevalence of gestational diabetes mellitus in Europe: A meta-analysis. Diabetes Res Clin Pract. 2017;129:173-81.