EXAMINING THE RELATIONSHIP BETWEEN MENSTRUAL CYCLE PHASE WITH METABOLIC CONTROL AND ADIPOSE TISSUE MICRORNA EXPRESSION

by

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Declaration

I declare that the contents of this thesis are entirely my own work. The thesis document was composed by myself and under the supervision of Dr. Colin N. Moran and Dr. Iain J. Gallagher, with the following exceptions:

i. Data analysed in Chapter 2 was obtained from the National Health and Nutrition Examination Survey (NHANES).
ii. Data analysed in Chapter 3 was obtained from the UK Biobank.
iii. Dr. Frederick K. Ho and Dr. Carlos A. Celis-Morales provided statistical advice for data analysis in Chapter 3.
iv. Dr. Nicholas Barwell delivered formal training in the adipose tissue biopsy technique required for sample collection in Chapter 4.
v. Dr. Nidia Rodriguez-Sanchez, Dr. Thomas G. Di-Virgilio, Dr. Iain J. Gallagher and Dr. Colin N. Moran undertook the adipose tissue biopsies for Chapter 4.
vi. Ms. Phoebe Lloyd-Evans provided assistance during the experimental trials in Chapter 4.

Neither the thesis, nor the original work contained therein have been submitted to this or any other institution for a higher degree. This thesis does not infringe upon anyone's copyright and any material from the work of others included in this thesis are fully acknowledged in accordance with common reference practices.

Signed:

Kirstin A MacGregor

Abstract:

The menstrual cycle is a fundamental biological rhythm governing physiology in females of a reproductive age. Regulated across an approximately 4-weekly duration, the menstrual cycle is characterised by cyclical fluctuations in ovarian hormones (estradiol, progesterone and testosterone) and pituitary hormones (luteinizing hormone sand follicle stimulating hormone) (1). Ovarian hormones are metabolically active and exert key regulatory roles in metabolic control (2, 3). Correspondingly, a variety of metabolic parameters undergo cyclical rhythmicity across the menstrual cycle, in association with the ovarian hormone milieu (4, 5). However, female physiology is under-researched. Our understanding of variation in metabolic control across the menstrual cycle and the associated molecular mechanisms remains limited. Gaining a full understanding of how metabolic control varies across the menstrual cycle is crucial for the diagnoses, treatment and prevention of metabolic disease in females. Thus, the overall aim of this thesis is to examine cyclical variation in insulin resistance and associated metabolites across the menstrual cycle. Additionally, to examine the role of inflammatory markers and miRNAs as potential molecular mechanisms underpinning variation in metabolic control across the menstrual cycle.

Chapter 2 of this thesis demonstrates that rhythmic variation in insulin sensitivity, insulin, glucose and triglyceride are mediated by body mass index, physical activity and cardiorespiratory fitness. **Chapter 3** extended on these findings to identify indices of body composition, fitness and physical activity levels are key modifiable risk factors mediating the variation in glucose, triglyceride, insulin sensitivity and cholesterol profiles across the menstrual cycle. Additionally, inflammatory markers varied across the

menstrual cycle and associate with metabolite concentration, thereby identifying a potential mechanism which may underpin variation in metabolic control across the menstrual cycle.

To gain further insight into the molecular mechanisms underpinning observed rhythmicity in metabolic control across the menstrual cycle, **Chapter 4** examines the effect of the menstrual cycle on adipose tissue microRNA expression. This determined that miR-495-5p was differentially expressed across menstrual cycle phases and miR-30c-5p was negatively associated with testosterone. Adipose tissue miRNAs with the strongest tendency for differential expression between menstrual cycle phases shared common targets related to insulin signalling pathways.

Overall, this thesis contributes novel data characterising variation in metabolic control across the menstrual cycle. Finally, it identifies inflammation and miRNA expression as potential molecular mechanisms driving observed variation in metabolic control.

Thank you

I would like to express my gratitude to everyone who has supported me during my PhD journey.

Firstly, I would like to thank my academic supervisors. **Dr. Colin Moran**, for your excellent support, constant open-door policy, time and belief during my PhD journey. **Dr. Iain Gallagher**, for your exceptional advice, guidance and thought-provoking discussions (both directly related to this thesis and otherwise!). I am extremely grateful for the opportunities you have both afforded me to develop my academic and research skillset; they far exceeded my expectations of a PhD. Your time and support have been continually appreciated throughout and without it, this research and thesis would certainly not have been possible.

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3'UTR	3 prime untranslated region
Acetyl-CoA	Acetyl coenzyme A
ADIPO-IR	Adipose tissue insulin resistance index
ADP	Adenosine diphosphate
AGO	Argonuate proteins
Akt	Akt kinase
AMPK	AMP-activated protein kinase
ASO	Antisense oligonucleotides
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BMI	Body mass index
C.elegans	Caenorhabditis elegans
Ca2+	Calcium
cAMP	Cyclic adenosine monophosphate
ChReBP	Carbohydrate response element binding protein
CRF	Cardiorespiratory fitness
EF	Early-follicular
ELISA	Enzyme-linked immunosorbent assays
FFA	Free fatty acid
FOXO1	Fork-head transcription factor
FSH	Follicle stimulating hormone
G6P	Glucose-6-phosphate
GAP	GTPase-activating proteins

GK	Glucokinase
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GnRH	Gonadotropin Releasing Hormone
GS	Glycogen synthase
GSK	Glycogen synthase kinase
GSV	Glucose storage vesicle
GTP	Guanosine-5'-triphosphate
HOMA-IR	homeostatic model assessment for insulin resistance
HPO	Hypothalamic-pituitary-axis
HSL	Hormone sensitive lipase
INSR	Insulin receptor
IR	Insulin resistance
IRS	Insulin receptor substrate
K_{ATP}	ATP-sensitive potassium channel
LDL	Low density lipoprotein
LF	Late-follicular
LH	Luteinizing hormone
LPL	Lipoprotein lipase
MEC	Mobile examination survey
MEK1/2	Mitogen-activated protein kinase
MESOR	Rhythm adjusted mean
miRbase	miRNA registry database
miRNA	MicroRNA
ML	Mid-luteal

mRNA	Messenger RNA
mTORC1	Mammalian target of rapamycin complex 1
NHANES	National Health and Nutrition Examination Survey
NICR	NHS, Invasive and Clinical Research
NIH	National institute of health
OGTT	Oral glucose tolerance test
ORWH	Office of research on women's health
P-P	Peak to peak difference
P13K	Phosphoinositide 3-kinases
PDE3	Phosphodiesterase 3
PDK1	Phosphoinositide dependent kinase
PIK	Phosphoinositide 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PLIN1	Perilipin 1
PO	Post-ovulatory
pre-miRNA	Pre-curser miRNA
pri-miRNA	Primary microRNA
PTEN	phosphatase and tensin homolog deleted on chromosome 10
RGC	RAL-GAP-complex
RISC	RNA-induced silencing complex
SABV	Sex as a biological variable
TCA	Tricarboxylic acid
$\dot{V}O_{2max}$	Maximal oxygen consumption

List of publications

Articles published:

MacGregor, K. A., Rodriguez-Sanchez, N., Di Virgilio, T.G., Barwell, N. D., Gallagher, I. J., Moran, C. N. (2021). Changes in adipose tissue microRNA expression across the menstrual cycle in regularly menstruating females: a pilot study. *Physiological genomics,* doi.org/10.1152/physiolgenomics.00088.2021 (**see Appendices**).

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CHAPTER 1: GENERAL INTRODUCTION

The global population is 49.6% female (6). Despite this, historically females have been consistently under-represented in both clinical and non-clinical physiology research (7–10). Whilst sex inclusive participation in research has increased over the last three decades (9, 11), parity has not yet been achieved (7–10, 12). In 2019, only 4.2 % of physiology research studies were conducted in female only participants (9). Accordingly, our understanding of female physiology remains far from complete.

Globally, 52 % of females are of a reproductive age, which equates to approximately 1.8 billion females (6). In females of a reproductive age, the menstrual cycle represents a crucial biological rhythm governing the female physiology. The menstrual cycle is characterised by hormonal fluctuations within the hypothalamic-pituitary-ovarian (HPO) axis; gonadotropin, pituitary hormones (luteinizing hormone (LH) and follicle stimulating hormone (FSH)) and ovarian hormones (estradiol, testosterone and progesterone) across a typical 24-35 d duration (13, 14). The menstrual cycle can be divided into the follicular phase and luteal phase, separated by the occurrence of ovulation (1). Despite being a normal component of female physiology, the effect of the menstrual cycle on physiology is under-researched (6, 7).

Insulin sensitivity refers to the responsiveness of the three main insulin sensitive tissues- adipose tissue, skeletal muscle and the liver- to the metabolic actions of insulin on glucose and lipids (15). Insulin sensitivity is a key component underlying the onset and progression of numerous metabolic disorders and associated co-morbidities (16–18). Clinically impaired insulin sensitivity, termed insulin resistance (IR), refers to a

pathophysiological condition characterised by hyperglycaemia, hyperinsulinemia and dyslipidaemia. The prevalence of IR is exponentially rising and is currently estimated to affect 8.4 % of females globally (19). Without effective interventions it is predicted 9.9 % of females will be diagnosed with IR by 2030 (19, 20). The onset and progression of IR is associated with a variety of non-modifiable (e.g. age, sex, ethnicity) and modifiable (e.g. physical activity, fitness, adiposity) risk factors. Whilst the importance of non-modifiable risk factors in IR is indisputable, evidence demonstrates the onset and/or severity of IR can be prevented by improving non-modifiable risk factors.

Cyclical fluctuations in ovarian hormones across the menstrual cycle are associated with alterations in metabolic control. During the luteal phase, IR, insulin, impaired glycaemic control and dyslipidaemia are increased (4, 5). Moreover, a negative relationship is observed between both estradiol and progesterone with insulin sensitivity, glucose and triglyceride concentrations across the menstrual cycle (4, 5). However, research is inconsistent; others have documented no relationship between menstrual cycle phase and parameters of metabolic control (21–24). Moreover, the influence of modifiable risk factors in the relationship between menstrual cycle phase and metabolic control remains unexplored. Further research is required to fully elucidate rhythmicity in parameters of metabolic control across the menstrual cycle.

The functional importance of adipose tissue in regulating metabolic control extends beyond solely an inert storage site for excess energy; it is a metabolically active endocrine organ which secretes various adipokines involved in metabolic and inflammatory processes (25). Maintaining functional adipose tissue is therefore pertinent to the regulation of whole-body metabolic control. Adipose tissue is metabolically receptive to

concentrations of circulating ovarian hormones (2, 3, 26–28). Accordingly, adipose tissue represents a potential organ through which cyclical fluctuations in ovarian hormones across the menstrual cycle function to mediate variation in metabolic control.

Epigenetic regulation represents one key mechanism in the regulation of adipose tissue metabolic control. Epigenetics is defined as "the study of changes in gene function that do not entail a change in DNA sequence" (29). One form of epigenetic mechanism is microRNA expression (miRNA); short non-coding RNAs which post-transcriptionally regulate messenger RNA (mRNA). Through local and systemic expression, miRNAs are considered major regulators of mRNA expression in the cells of the human body. Within adipose tissue, miRNA interact within pathways integral to regulating metabolic control, including adipogenesis, lipolysis, lipogenesis and insulin sensitivity (30–32). Thus, miRNA expression serves as a key regulator of adipose tissue metabolism.

miRNA expression profiles are hormonally regulated in a range of tissues including; plasma (33), skeletal muscle (34) and endometrium (35). Within adipose tissue, differential miRNA expression has been demonstrated following chronic alterations to the hormonal milieu in females, for example; menopausal status (36), hormone supplementation (36), polycystic ovary syndrome (37) and ovariectomy (38). This suggests miRNA action as a potential mechanism underpinning differences in whole body and adipose tissue specific metabolic control across the menstrual cycle. However, limited literature has examined the effect of acute fluctuations in hormonal profiles across the menstrual cycle on miRNA expression, yielding equivocal results.

This chapter will first discuss the physiology of the menstrual cycle. Followed by a discussion of insulin sensitivity and the mediatory effect of cyclical fluctuations in hormone profiles across the menstrual cycle within the regulation of insulin sensitivity. Finally, the role of miRNA in the regulation of adipose tissue metabolism is discussed, with a focus on the menstrual cycle. The aim of this chapter is to provide the reader with an understanding of the rationale underpinning the research questions presented later.

1.1. Female physiology and the menstrual cycle

1.1.1. Under-representation of females in physiology research:

Within scientific research, the differences between biological sex and gender are an important distinction. Sex refers to the biological classification of male and female based on differences in X and Y chromosomes. Whereas gender refers to socially and culturally constructed roles, behaviours and identities of girls, women, boys, men and gender-diverse individuals. Herein, references to male and female are in the context of biological sex.

Aside from reproductive function, human physiology has been classically defined in the context of the "typical 70 kg man" (39). The under-representation of females within physiology research dates to the 19th and 20th century. At this time society was governed by sociological and cultural ideologies, such as protectionism and paternalism, which excluded female participation from potentially hazardous activities, including research. In 1977, policies and guidelines were formally implemented which excluded females of childbearing potential from early phase clinical research and pregnant females from any clinical research (40). As a result of these policies and guidelines, both clinical and

physiological research was dominated by male participants in human, rodent and cell studies in the 19th and 20th centuries (11, 41).

Since it was widely accepted that males represented suitable physiological proxies for females and thus findings from research conducted in males could be generalised to females, it was not considered erroneous to exclude females from research. However, it has since been concluded that these decades of female exclusion from physiology research had negative consequences for female health. For example, adverse reactions to clinically approved drugs were up to 75% more likely in females than in males (42). Furthermore, over 80% of prescription drug withdrawals from the market were due to adverse reactions in females (42).

During the late 1900's concerns were raised that decisions regarding female health were being made by males and informed by male dominated research. Rising scientific and public concerns lead to greater awareness and publicity surrounding the sex bias in research. In the following decades numerous policies were implemented to reduce the sex bias within research. In 1990, the Office of Research on Women's Health (ORWH) was established within the National Institute of Health (NIH). In 1993, the NIH reversed the 1977 guidance regarding the exclusion of females in clinical research under the Revitalisation Act (43). The Revitalisation Act established legal requirements and guidelines to ensure clinical research, particularly phase III clinical trials, includes females of childbearing potential. In 2001, the Institute of Medicine published a report exploring the biological contribution of sex to human health. This report concluded that sex is a crucial factor in both health and illness and therefore barriers to the advances of knowledge regarding sex differences must be eliminated (44). Most recently, the 'sex as

a biological variable' (SABV) policy was implemented in 2014 by the NIH to ensure the equal inclusion of males and females in NIH funded clinical research within human and animal studies (45). This SABV policy requires researchers to ensure sex is appropriately considered as a biological variable or to provide justification for conducting single sex investigations (45).

In response to changes in policies, guidelines and attitudes towards the inclusion of females in physiology research, there has been a reduction in the sex bias. Female participation has significantly increased following the implementation of the NIH revitalisation act in 1994 (11). Moreover, sex inclusive participation in physiology research studies increased 23% from 2009 to 2019 (9). However, parity has not yet been achieved (7, 8, 12, 46). In 2019, only 4% of physiology research was conducted on females only and 36% of research was conducted in both males and females (9). Furthermore, research including both males and female participants are rarely adequately powered to detect potential differences between sexes. As a result, data from males and females are inappropriately grouped for statistical analyses (7, 8). Further work is required to eliminate the sex-bias in physiology research.

1.1.2. The menstrual cycle

Across the lifespan, the female physiology undergoes a range of changes in reproductive function, which can be divided into four key phases; childhood, sexual maturity, perimenopause and post-menopause (47). During childhood, the reproductive system is quiescent and reproductive hormone concentrations remain low and stable. Childhood continues until the first menstruation, termed menarche, which typically occurs between 8-14 yr and marks the onset of sexual maturity (47). During sexual maturity menstrual

cycles occur approximately monthly, unless disrupted by physiological stressors, pregnancy or the use of hormonal contraceptives. The period of time during which physiologic changes mark the progression towards a final menstrual period is termed the perimenopause (48). The perimenopause typically occurs between 46-52 yr and can last for up to 10 years (49). Following the perimenopausal transition is the onset of the menopause, which refers to the timepoint at which a woman has not had a period in one year (47). The reproductive lifespan is thereafter termed the post-menopausal phase.

The majority of the female lifespan is spent within a reproductive age and thus the menstrual cycle represents a key biological rhythm regulating the female physiology. Globally, approximately 52% of the female population are of reproductive age, equating to approximately 1.8 billion females worldwide (6, 50). The menstrual cycle is comprised of the ovarian and uterine cycles. The ovarian cycle, consisting of follicular, ovulatory and luteal phases is concerned with oocyte maturation and release (1). Whilst the uterine cycle, consisting of menstruation, proliferative and secretory phases, is concerned with preparing the uterine lining for possible oocyte implantation in the event of fertilisation (1). The ovarian cycle and uterine cycle occur in a coordinated and concurrent manner; herein we will refer to menstrual cycle phase in terms of the follicular and luteal phases (1).

1.1.2.1. Hypothalamic-pituitary-ovarian axis

The HPO axis is a tightly controlled regulatory axis governing the female reproductive endocrine system (51). The HPO axis regulates the secretion of gonadotropin releasing hormone (GnRH), pituitary hormones (FSH and LH), and ovarian hormones (estradiol, progesterone and testosterone) across the menstrual cycle (1). Consequently, the HPO

axis regulates cyclic variation in hormonal profiles specific to each menstrual cycle phase, as depicted in **Figure 1**.

Early follicular: The menstrual cycle begins with menstrual bleeding, termed menstruation, which refers to the shedding of the endometrial lining of the uterus. During the early follicular phase the hypothalamic pulse frequency of GnRH is increased (52). In response to rising GnRH concentration, alongside low levels of negative inhibition due to low estradiol and progesterone concentration, FSH is secreted from the anterior pituitary (52). Elevations in the concentration of FSH during the early follicular phase stimulate the ovarian preantral follicles to develop into preovulatory follicles, which contain theca and granulosa cells (53).

Mid follicular: Following increasing ovarian follicle growth and number of FSH receptors on granulosa cells, estradiol secretion from the granulosa cells is elevated (1). The follicle containing the most FSH receptors, and thereby the greatest secretor of estrogen, becomes the dominant follicle. The non-dominant follicles undergo atresia, resulting in apoptosis of cells within those follicles (1). In response to negative inhibition, resultant to increased estradiol and inhibin B secretion from the dominant follicle, FSH concentration begins to decline (54).

Late follicular: Continued growth of the dominant ovarian follicles results in further increases in estradiol secretion, with concentration peaking in the late follicular phase (55). The rise in estradiol concentration stimulates a surge in release of LH from the pituitary gland, typically occurring on cycle day 12 (56). Following the LH surge, the concentration of estradiol declines sharply (55). The LH surge stimulates luteinisation of

the granulosa cells, resulting in small increases in progesterone secretion. The increase in progesterone exerts positive feedback on FSH secretion from the anterior pituitary gland, resulting in an FSH peak (1). Testosterone concentration rises due to increases in secretion from the thecal cells, rising to a peak at the late follicular phase, immediately prior to ovulation (1, 57).

Ovulation: The beginning of the LH surge occurs 34-36 h prior to ovulation, with ovulation occurring 10-12 h following peak LH concentration (58). During ovulation, the secondary oocyte is released from the dominant ovarian follicle.

Early luteal: Immediately following ovulation, the theca and granulosa cells from the dominant follicle differentiate to form the corpus luteum (14). The corpus luteum serves to prepare the endometrium for oocyte implantation.

Mid luteal: Approximately 8 d after ovulation, maximum secretory rates of estradiol and progesterone from the corpus luteum are reached; resulting in a progesterone peak and secondary estradiol peak (55, 59). Elevated estradiol and progesterone concentration during the luteal phase, alongside increased secretion of inhibin B and activins from the corpus luteum, result in reduced secretion of LH and FSH (52).

Pre-menstruation: The function of the corpus luteum is maintained by the local presence of LH and FSH. Resultant to reduced LH and FSH concentrations during the mid-luteal phase, the function of the corpus luteum declines (52). Following reduced corpus luteum function, the secretion of estradiol and progesterone decline, typically occurring ~12 d after formation of the corpus luteum (55). Reduced negative inhibition from estradiol,

progesterone and inhibin B levels, result in rising FSH concentrations prior to the beginning of the next cycle (52).

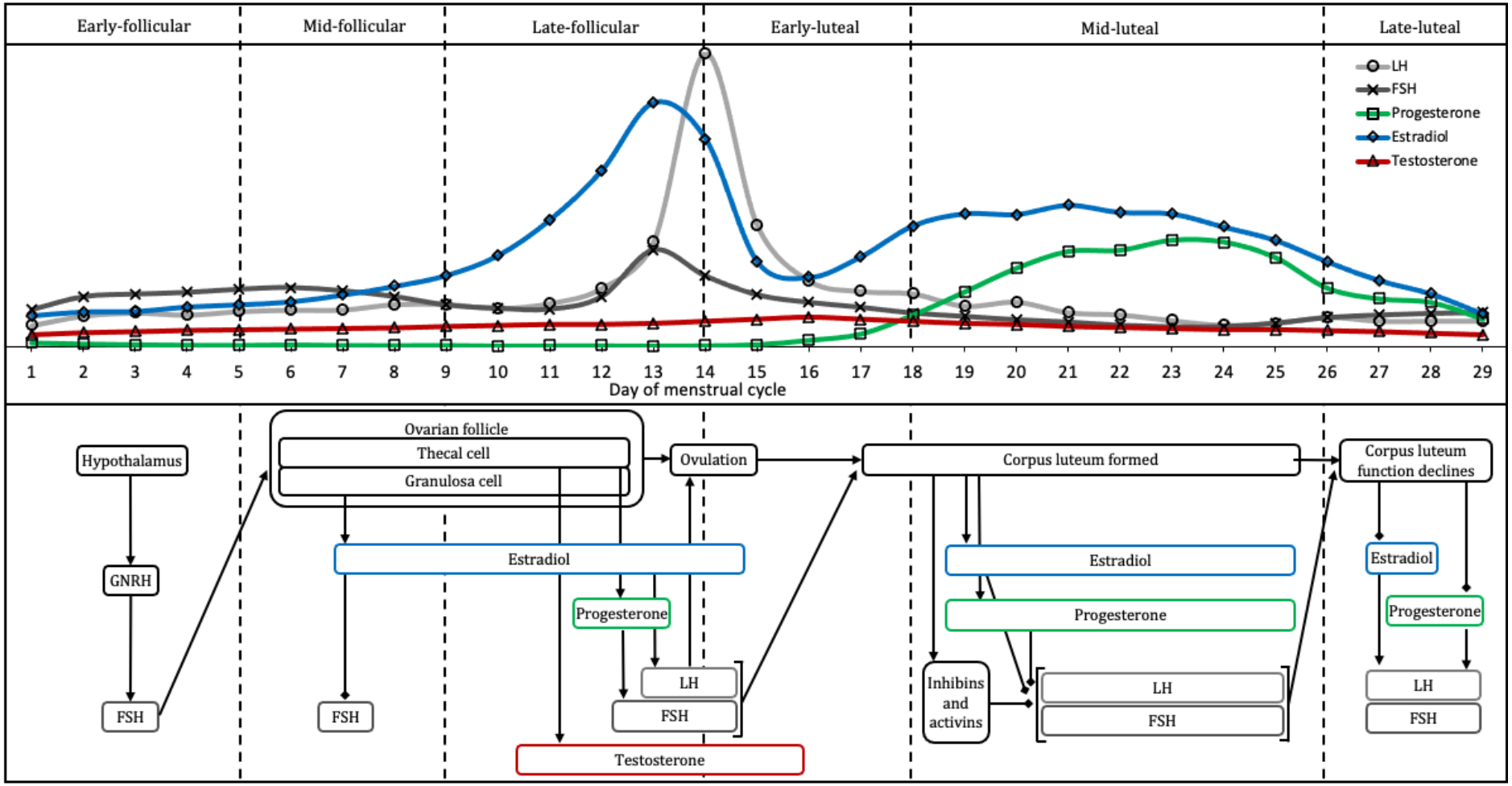

Figure 1: Overview of the HPO axis regulation of pituitary and ovarian hormone levels across the menstrual cycle. Line with arrow end denotes positive relationship. Line with diamond end denotes inhibitory relationship. Follicle stimulating hormone, FSH; gonadotropin releasing hormone, GnRH; luteinizing hormone, LH.

1.1.2.2. Methodological considerations to menstrual cycle testing:

The menstrual cycle adds additional complexity to the female physiology. It affects a broad range of physiological processes throughout the body, including; ventilatory, cardiac, neuromuscular and metabolic pathways (60–63). Accordingly, considering the menstrual cycle as a biological variable is a crucial methodological consideration in female physiology research. Adequate methodological control for the menstrual cycle requires sampling timepoints which accurately and reliably associate with hormonal profiles specific to each menstrual cycle phase (13, 64–66).

Practical recommendations for methodological designs to assess menstrual cycle phase are regulated by various logistical and financial constraints. The most accurate method would require testing individuals on each day of the cycle, then retrospectively confirming menstrual cycle phase via blood hormone concentrations. However, this method is largely unfeasible due to associated increases in time, money and participant burden, alongside methodological issues related to repeated testing. Therefore, a combination of indirect and direct methods are advocated (64–66). Best practice methods recommend using calendar counting and ovulation testing to predict menstrual cycle phase alongside retrospective confirmation via serum hormone concentrations (13, 64–66).

Calendar counting is a cheap, easy, non-invasive technique to predict menstrual cycle phase. Cycle phase is determined by counting a specific number of days forward from the onset of menstrual bleeding or backwards from the predicted start of the next cycle, based on menstrual cycle length (67). Due to the definitive nature of menstruation marking the onset of a new menstrual cycle, the calendar counting method accurately

predicts early phases of the menstrual cycle. However, ovulation has high inter- and intra-individual variability, resulting in low predictive accuracy of ovulatory events and, subsequently, timepoints within the luteal phase (67, 68). Due to its indirect nature, calendar counting cannot detect menstrual cycle irregularities, including anovulatory cycles or abnormal hormone profiles. Therefore, the use of calendar counting methods alongside direct assessment methods is recommended.

Ovulation tests are non-invasive urine-based tests providing a highly accurate, cheap and easy technique to detect ovulation. They identify the occurrence of the LH surge which confirms the presence of ovulatory cycles. Anovulatory cycles are associated with reduced estrogen and progesterone levels during the luteal phase and occur sporadically in 33% of regularly menstruating females (69). A positive ovulation test result accurately detects the onset of ovulation within the following 12-36 h (67, 70). Combining calendar counting and ovulation test accurately predict menstrual cycle phase in ~90% of participants (65). Despite the increased accuracy of combining calendar counting and ovulation tests, menstrual cycle phase should also be confirmed using retrospective analysis of hormone levels (67).

1.2. Insulin sensitivity

In healthy individuals, circulating glucose and triglyceride levels are maintained within narrow ranges, despite periods of prolonged fasting or caloric intake (71, 72). Insulin is the major hormonal regulator of circulating glucose and triglyceride concentration. Insulin functions to regulate an integrated whole-body cross-tissue response to an altered metabolic status. Insulin sensitivity refers to sensitivity of the three main insulin sensitive tissues- the liver, skeletal muscle and adipose tissue- to the glucose and lipid regulatory actions of insulin. Insulin supresses hepatic glucose output and adipose tissue lipolysis, whilst stimulating the uptake and storage of glucose and free fatty acid (FFA) in skeletal muscle, the liver and adipose tissue. These pathways are described in detail in the following sections.

1.2.1. Insulin signalling pathways

Insulin release from pancreatic β-cells

Insulin is released from β-cells. β-cells are clustered in highly vascularised pancreatic islets, which permit rapid nutrient sensing and hormone secretion across the capillary bed (73). β-cells are highly sensitive to changes in plasma nutrient concentration following food ingestion, including glucose, FFA and amino acids. Glucose is the primary stimulus for insulin release from β-cells (74). Glucose-independent stimuli for insulin secretion from β-cells are reviewed in detail elsewhere (75).

Glucose stimulated insulin secretion comprises both intracellular uptake and subsequent metabolic degradation of glucose. Glucose is first sensed by the glucose transporter 2

(GLUT2) (75). GLUT2 translocates to the plasma membrane in an insulin-independent manner and glucose equilibrates in β-cells through GLUT2 mediated facilitated diffusion (76). Once inside the β-cell, glucose is phosphorylated in a reaction catalysed by glucokinase, a rate limiting glycolytic enzyme. The end product of glycolysis is pyruvate, which is subsequently oxidised through the tricarboxylic acid (TCA) cycle within the mitochondria. Increased TCA cycle activity increases the intracellular adenosine triphosphate (ATP)/ Adenosine diphosphate (ADP) ratio (77). Subsequentially, the ATP-sensitive potassium channel (K_{ATP}) channels close, the plasma membrane depolarises and the voltage-dependant Calcium (Ca^{2+}) channels open (77). Following an influx of Ca^{2+}, insulin containing granules fuse with the plasma membrane and exocytosis of granule content ensues (77). Once in circulation, insulin binds to its receptors in the three main target tissues; liver, skeletal muscle and adipose tissue (**Figure 2**). Insulin regulates metabolic changes by activating an intracellular signalling cascade within each target tissue.

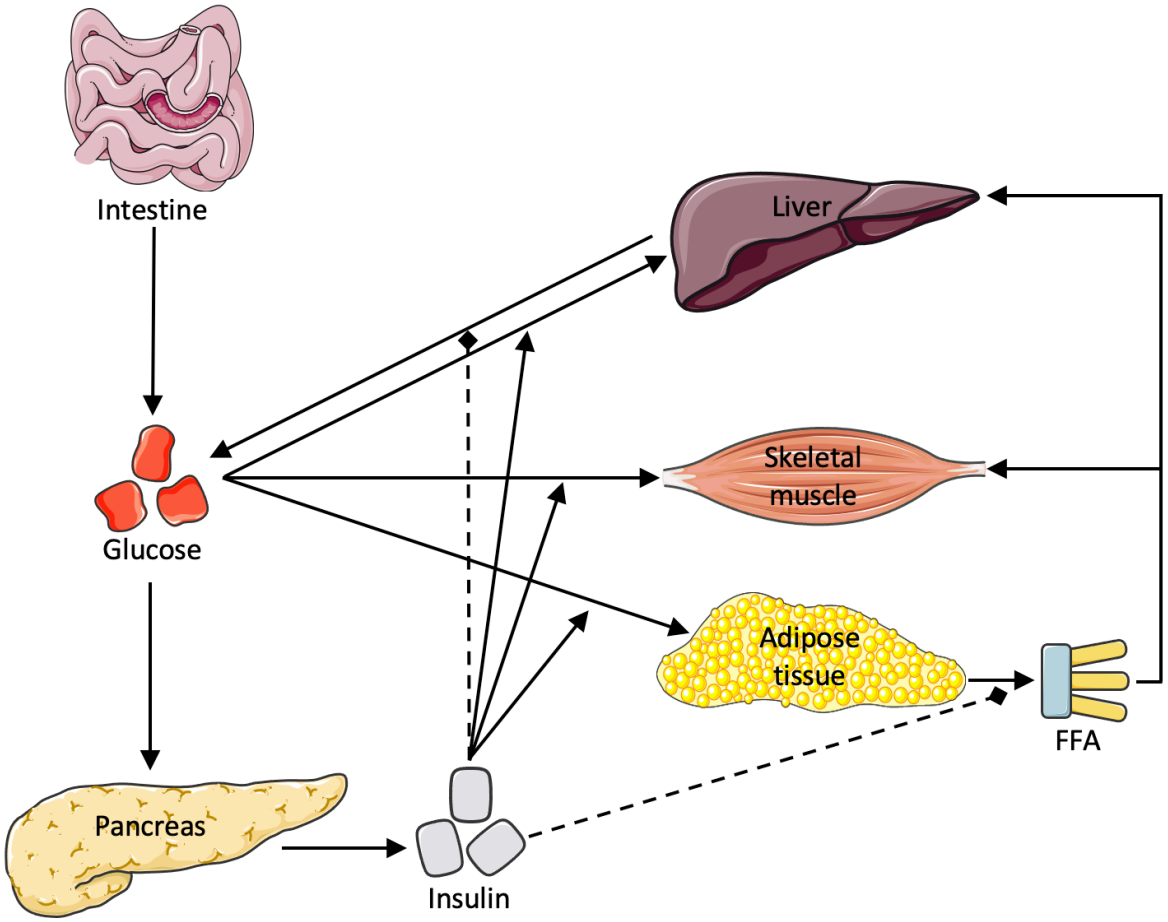

Figure 2: Integrated tissue crosstalk underpinning insulin-stimulated regulation of glucose and lipid control. Insulin is secreted from pancreatic β-cells in response to elevated concentrations of glucose. Insulin acts to stimulate glucose uptake into the liver, skeletal muscle and adipose tissue alongside supressing hepatic glucose production and adipose tissue lipolysis. Complete line represents stimulation, dashed line represents inhibition. FFA, free fatty acid. Schematic was produced using images from Servier Medical art (www.smart.servier.com).

Proximal insulin signalling:

In all insulin sensitive tissues, the insulin signalling cascade begins with the series of molecular events termed proximal insulin signalling; an intracellular signalling cascade resulting in Akt activation. In the first step of proximal insulin signalling, insulin binds to

the insulin receptor (INSR) on the plasma membrane of target cells (78). INSR is a heterotetrameric complex consisting of two extracellular α subunits, which bind to insulin, and two intracellular β subunits, which contain tyrosine kinase domains (78). Insulin binding to the INSR leads to the auto-phosphorylation of intrinsic tyrosine kinases and, in turn, this enables the recruitment of insulin receptor substrates (IRS) (79). Whilst there are multiple IRS isoforms, IRS1 and IRS2 are the predominant isoforms pertinent to mediating INSR activation in humans (15). After IRS binds to the phosphotyrosine-binding domain INSR $pTyr^{972}$, INSR phosphorylates multiple IRS tyrosine residues (78). Phosphorylation of IRS recruits phosphoinositide 3-kinase (P13K) (80). P13K catalyses the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2) in a reaction catalysed by phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (80, 81). PIP_3 recruits Akt kinase (Akt), which is subsequently activated through phosphorylation by phosphoinositide-dependent kinase (PDK1) at Thr^{308} (82). Additional phosphorylation of Akt at Ser^{473} results in its full activation (83). Once activated, Akt phosphorylates numerous substrates within various functional pathways and is thus considered a critical point in insulin signalling.

1.2.1.1. Liver

The liver is a major site of endogenous glucose utilisation and production (84). In the post-prandial state, relative hyperglycaemia promotes the uptake of circulating glucose into the liver via GLUT2 mediated insulin-independent diffusion (85). Once inside the cell, hepatic glucokinase (GK) is released into the cytoplasm and phosphorylates glucose to generate glucose-6-phosphate (G6P) (86). G6P is then converted to glycogen in the final step of glycogenesis, in a reaction catalysed by glycogen synthase (GS) (86).

Insulin released from the pancreatic β-cells is secreted into the portal veins, exposing the liver to two- to three- fold higher insulin concentrations than in systemic circulation (87). Hepatic insulin signalling begins, as in all insulin sensitive tissues, with ISNR activation and subsequent tyrosine kinase phosphorylation of IRS (88). The predominant IRS isoform expressed in hepatocytes is IRS2 (88, 89). Once activated, Akt acts to supress hepatic glucose production through two mechanisms; glycogen synthase kinase (GSK3) and fork-head transcription factor (FOXO1) (90, 91). Firstly, Akt inactivates GSK3 by phosphorylation which in turn activates GS (90). Activated GS stimulates glycogen synthesis; a major direct effect of insulin action in hepatocytes (90). Secondly, Akt phosphorylates FOXO1 which inhibits the transcription of gluconeogenic enzymes (91).

1.2.1.2. Skeletal muscle

Skeletal muscle is the principle site for insulin stimulated glucose disposal, accounting for approximately 65 % of whole-body glucose uptake (92). Numerous previous reviews have extensively discussed insulin signalling pathways in skeletal muscle (15, 93). Within skeletal muscle, proximal insulin signalling begins with ISNR activation and subsequent tyrosine kinase phosphorylation of IRS (88). Whilst IRS1 and IRS2 are both expressed in skeletal muscle, IRS1 is the predominant ISR form (94). Following activation through the proximal insulin signalling pathway, Akt phosphorylates several proteins required for myocellular glucose uptake and its subsequent metabolism.

Upon activation, Akt promotes the translocation, docking and fusion of the GLUT4 containing storage vesicle (GSV) to the cell membrane (95, 96). Once fused to the cell

membrane, GLUT4 permits the facilitated diffusion of glucose into the tissue (96). Thus, a major function of insulin is the promotion of glucose uptake into myocytes.

Following its diffusion into the myocyte, glucose has two primary metabolic outcomes; glycolysis or glycogen synthesis. Insulin upregulates hexokinase and 6-phosphofructokinase activity, key glycolytic enzymes, resulting in increased rates of glycolysis (97, 98). However, the predominant metabolic pathway for myocellular glucose is glycogen synthesis, accounting for ~75% of for insulin-stimulated glucose disposal (99). Akt phosphorylates and inactivates GSK3 at Ser^{21} and Ser^{9}, which in turn dephosphorylates GS resulting in its increased activation (100). Simultaneously, insulin mediated activation of protein phosphatase-1 also promotes the dephosphorylation of GS (101). In addition to increasing GS activity, a concomitant reduction in glycogen phosphorylase is required to result in net glycogenesis (15). Insulin promotes the dephosphorylation and inactivation of phosphorylase kinase, which in turn dephosphorylates and inactivates glycogen phosphorylase (15). These processes combined facilitate the insulin-stimulated upregulation of glycogen synthesis in skeletal muscle (15).

1.2.1.3. Adipose tissue

Adipose tissue is an essential organ required for the maintenance of metabolic homeostasis. Insulin is a potent anti-lipolytic hormone and the major suppressor of lipolysis within white adipose tissue (102). Whilst suppression of adipose tissue lipolysis is a major metabolic function of insulin, our understanding of the associated metabolic mechanisms remains limited (103). The best characterised mechanism for insulin-stimulated suppression of lipolysis is the attenuation of adrenergic signalling through

cyclic adenosine monophosphate (cAMP)/Akt dependant pathways (15). Upon its activation via the proximal insulin signalling cascade, Akt phosphorylates and activates phosphodiesterase 3 (PDE3) (104). Once activated, PDE3 catalyses the hydrolysis of cAMP to 5'AMP (104). As a result, downstream cAMP-stimulated lipolytic signalling pathways are attenuated, namely hormone sensitive lipase (HSL) and perilipin 1 (PLIN1) (104). Additionally, Akt acts to supress lipolysis by decreasing the transcription of adipose triglyceride lipase (ATGL), a key rate limiting enzyme of lipolysis, via the mammalian target of rapamycin complex 1 (mTORC1) mediated pathway (105). Cumulatively, insulin-stimulated actions on adipose tissue function to inhibit the hydrolysis of triglyceride and subsequent release of FFAs into circulation.

Whilst only ~5% of whole-body insulin-stimulated glucose uptake is accounted for by adipose tissue, glucose uptake is a key metabolic function within adipose tissue (92). As in skeletal muscle, insulin-stimulated glucose uptake occurs via the phosphorylation of GLUT4 trafficking proteins subsequent to proximal insulin signalling (96). GLUT4 fuses to the plasma membrane, permitting the facilitated diffusion of glucose into adipocytes (96). Alongside glucose uptake, insulin stimulates FFA uptake through increasing lipoprotein lipase (LPL) activity via phosphoinositide 3-kinase (PIK) and mitogen-activated protein kinase (MEK1/2) pathways (106). Furthermore, insulin stimulates lipogenesis through the increased activity of key transcriptional factors (107) and downstream enzymes (108–110). Insulin-stimulated elevations in glucose and FFA acid uptake increase the available substrates for re-esterification.

1.2.2. Assessment of insulin sensitivity

Studying insulin sensitivity is challenging due to the financial and time costs associated with quantifying insulin-mediated glucose and lipid metabolism. The gold standard measurement of insulin sensitivity is the glucose clamp technique; first introduced in 1979 by De Fronzo and colleagues (111). This technique has two variations: the hyperglycaemic clamp and the euglycaemic clamp. The hyperglycaemic clamp technique uses intravenous infusions of glucose to acutely raise plasma glucose concentration to a hyperglycaemic plateau which is maintained for 2 h (111). The glucose infusion rate is dependent on the rate of insulin secretion and glucose uptake; thus the hyperglycaemic clamp is considered the gold-standard for assessing the β-cell response to glucose (111). The euglycaemic clamp uses intravenous infusions of insulin to acutely raise circulating insulin concentration to ~100 U/mL above basal level which is maintained for 2 h (111). Concurrently, plasma glucose concentration is held constant by variable glucose infusion (111). The euglycaemic clamp quantifies tissue sensitivity to insulin and is therefore considered the gold standard technique for assessing whole-body insulin sensitivity. Whilst these techniques provide precise quantification of measures of IR, they are moderately invasive, laborious and costly. Therefore, the glucose clamp technique is typically unsuitable for large-scale or epidemiological research.

Surrogate indices have been developed which aim to accurately estimate insulin sensitivity, whilst minimising associated participant and analytical burdens. These indices use serum insulin and glucose levels in either the post-prandial state or in response to a glucose challenge (e.g. oral glucose tolerance test (OGTT)). Numerous surrogate measures correlate strongly with the euglycaemic clamp technique and are thus considered valid measures of insulin sensitivity (112). Additionally, quantification

of fasting glucose alone has been demonstrated to provide an accurate and reliable measure of insulin sensitivity (113).

However, fasting blood tests are associated with methodological complications for large-scale or epidemiological trials due to the necessity of a fasting duration > 8 h prior to sampling. Evidence demonstrates strong correlations between semi-fasted (4 h fasting duration) and fasted (8 h fasting duration) samples for levels of glucose, insulin, triglyceride and indices of insulin sensitivity (72, 114). These data suggest that for large-scale or epidemiological trials, semi-fasted blood samples are sufficient for the assessment of insulin sensitivity.

1.2.3. Risk factors for insulin resistance

Clinically impaired insulin sensitivity, termed IR, refers to a pathophysiological condition caused by partial or complete insufficiency of insulin secretion and/or insulin action. As a result, sufferers of IR display impairments in two insulin-mediated metabolic processes: stimulation of glucose uptake and triglyceride synthesis; and, suppression of hepatic glucose output and triglyceride lipolysis. Accordingly, IR is characterised by: hyperglycaemia, hyperinsulinemia and dyslipidaemia with elevated triglyceride, total cholesterol and low density lipoprotein (LDL). IR is a key component underlying the pathophysiology of metabolic disease and associated co-morbidities, including hypertension, cardiovascular disease, hyperlipidaemia, metabolic syndrome and polycystic ovary syndrome (16–18). The global prevalence of IR in the adult population has more than doubled over the last three decades to 451 million, currently affecting 8.4% of females (19, 20). Without effective interventions it is predicted this will further

rise to affect a total of 570.9 million adults by 2030, corresponding to 9.9 % of females (19, 20).

The onset and severity of IR are associated with a complex combination of modifiable and non-modifiable risk factors. Whilst the contribution of non-modifiable risk factors to the predisposition of IR is indisputable (e.g. genetics, ethnicity, age, sex), evidence demonstrates that IR can be prevented by mitigating modifiable risk factors (115). Key modifiable risk factors in IR are obesity, physical activity and physical fitness. These risk factors are discussed in detail below and summarised in **Figure 3.**

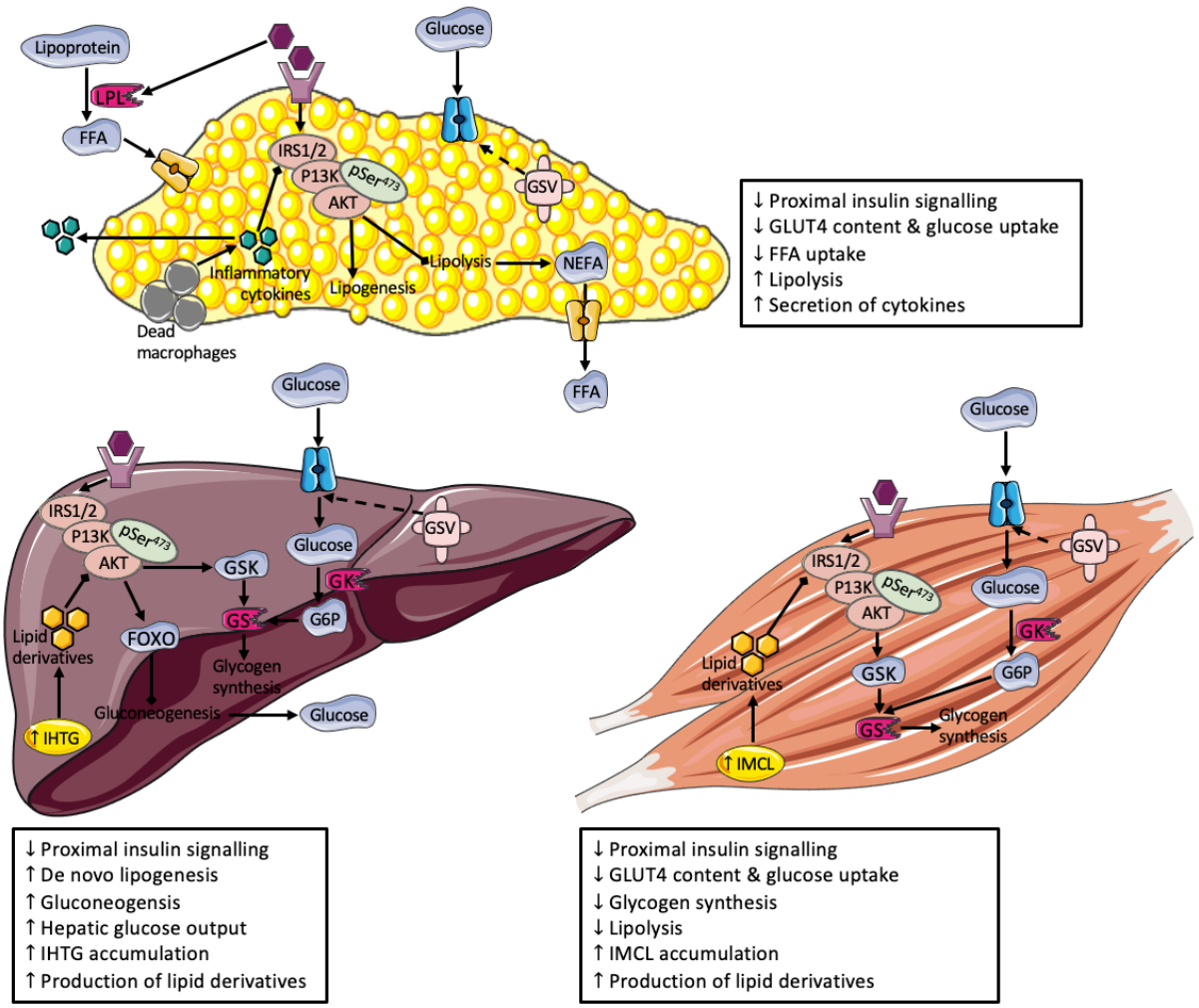

Figure 3: The role of obesity and physical inactivity on insulin-stimulated regulation of glucose and lipid control in adipose tissue (top), the liver (bottom left) and skeletal

muscle (bottom right). Adipose tissue: Proximal insulin signalling is impaired, resulting in reduced insulin-mediated promotion of lipogenesis and suppression of lipolysis. Consequentially elevated FFA are released into circulation. Reduced GLUT4 content reduces glucose uptake. Liver: Elevated FFA availability results in increased de-novo lipogenesis and gluconeogenesis, which increases intra-hepatic triglyceride stores. Elevated intra-hepatic triglyceride stores increased lipid derivative production which contribute to impaired proximal insulin signalling. Skeletal muscle: Elevated FFA availability impair glucose transport and proximal insulin signalling. Intramyocellular lipid (IMCL) accumulation increases the production of lipid derivatives (ceramide, diacyglyceride) which inhibit proximal insulin signalling. GLUT4 content is reduced which impairs glucose uptake and available substrates for glycogen synthesis. Schematic was produced using images from Servier Medical art (www.smart.servier.com).

Adiposity

The global prevalence of obesity has increased more than 5 fold in the last 40 years (116). In 2019 in the UK, 29.1% of females were classified as obese, whilst a further 31.3% were classified as overweight (117). Excess adiposity is a well-established risk factor for IR. Total, abdominal and subcutaneous adipose tissue mass are associated with hyperinsulinemia, hyperglycaemia, glucose disposal rate, endogenous glucose production and dyslipidaemia (118–120). The mechanisms by which obesity causes IR are, at present, incompletely understood. However, evidence demonstrates that impaired IR in obesity is mediated by impaired insulin sensitivity across all three target tissues; adipose tissue, skeletal muscle and the liver.

Reduced insulin sensitivity in adipocytes from obese humans has two main metabolic implications; impaired insulin-stimulated suppression of lipolysis and impaired glucose uptake. Impairments in proximal signalling in obese adipocytes, including INSR binding and IRS phosphorylation, contribute to reduced activation of Akt and downstream signalling pathways (121). Resultantly, insulin stimulated suppression of lipolysis and promotion of adipocyte FFA uptake are impaired (122, 123). Additionally, GLUT4 content is reduced in adipocytes in obesity. As a result, these adipocytes exhibit impaired insulin-stimulated glucose uptake. Whilst adipose tissue is not the major site for insulin-stimulated glucose disposal (< 5 % (92)), the deletion of adipose specific GLUT4 contributes to the onset of IR in the skeletal muscle and liver (124). The mechanism through which reduced adipocyte glucose uptake indirectly regulates peripheral insulin sensitivity remains uncertain, but evidence suggests it may be mediated through carbohydrate response element binding protein (ChReBP), a carbohydrate-signalling transcription factor (125). Glucose activates ChReBP, which in turn promotes lipogenic gene expression (125). This enables adipose tissue to store substrates and decrease substrate delivery to the muscle and liver (125). Consequentially, insulin resistant adipocytes contribute to the elevated circulating FFA and glucose typically observed in IR (123).

Alongside its role as an energy storage depot, adipose tissue functions as an endocrine organ through the secretion of numerous adipokines involved in metabolic regulation and inflammatory processes. Obesity is a pro-inflammatory condition whereby hypertrophied adipocytes and an elevated macrophage content result in increased secretion of pro-inflammatory adipokines (121, 126, 127). Pro-inflammatory adipokines secreted from obese adipose tissue include; interleukins, TNF-*a*, adiponectin, leptin and

resistin (121, 128). These pro-inflammatory cytokines mediate the development of IR locally and systemically, in skeletal muscle and the liver, either directly through regulation within the insulin signalling pathway or indirectly through the stimulation of inflammatory pathways (121, 128).

Elevated circulating FFA concentration is associated with the onset of skeletal muscle IR in a dose dependant manner (129). The mechanism underpinning the association between elevated FFA concentrations and skeletal muscle IR was initially proposed by Randle *et al.* in 1963, termed the glucose-fatty acid cycle (130, 131). The glucose-fatty acid cycle postulates that FFA and glucose compete as substrates for oxidation in skeletal muscle (131). Thus, in the presence of elevated FFA concentrations, skeletal muscle glucose metabolism declines (129, 131). This is accompanied by an associated decrease in skeletal muscle insulin-stimulated glucose uptake and oxidation (129, 131, 132); a phenomenon observed in both healthy and insulin resistant individuals (132, 133). The proposed mechanism was that an increased availability of circulating FFAs produces an increase in intramuscular acetyl coenzyme A (acetyl-CoA), subsequent to elevated rates of fat oxidation via the Krebs cycle (131). Acetyl-CoA inhibits pyruvate dehydrogenase and subsequently glucose oxidation is reduced (131). This in turn elevates intracellular citrate which inhibits phosphofructokinase 1, a rate limiting glycolytic enzyme. Reduced glycolytic flux impairs hexokinase II activity which results in an increase in intracellular glucose concentration and thus a decrease in glucose uptake.

However, others have challenged this conventional hypothesis; the support for this first derived from the work of Shulman and colleagues (102, 134, 135). In response to elevated FFA availability, in contrast to the model proposed by Randal and colleagues, the

reduction in skeletal muscle glycogen synthesis was preceded by a decline in glucose-6-phosphate (135). Moreover, using nuclear magnetic resonance spectroscopy to assess intracellular skeletal muscle glucose concentration, elevated FFA availability reduced the accumulation of intracellular glucose (134). This data indicates that elevated FFA concentration functions to induce skeletal muscle IR initially through impairments in glucose transport and phosphorylation activity. Additional studies demonstrated that elevated FFA availability impairs glucose transport and phosphorylation activity through a reduction in IRS1 associated PI3K activity (102, 134, 135). This body of work indicates that elevated FFA concentrations induce IR initially through impairments in glucose transport or phosphorylation activity, with subsequent impairments in muscle glycogen synthesis and glucose oxidation. Resultantly, circulating glucose levels remain elevated, a key characteristic in the pathophysiology of IR.

Obesity is not only associated with excess lipid accumulation in adipose tissue, but also in non-adipose tissues. The negative metabolic consequences of lipid accumulation in non-adipose tissues are referred to as lipotoxicity. Within skeletal muscle, obesity is associated with elevated levels of intramyocellular lipid (136). Intramyocellular lipid content is a predictor of IR independent of body mass (136). Accumulation of intramyocellular lipid storage increases the production of lipid derivatives, in particular, diacylglycerol, ceramide and long-chain acyl-CoAs. These lipid derivatives are purported to be involved in the onset of IR through the inhibition of insulin stimulated INSR phosphorylation and PI3K activity in skeletal muscle (136–140).

As a consequence of IR in adipose tissue and skeletal muscle, elevated concentrations of glucose and FFA are diverted to the liver. Subsequently, rates of hepatic *de novo*

lipogenesis and gluconeogenesis increase, leading to elevated intra-hepatic triglyceride content (15). Elevated circulating FFA and hepatic lipid accumulation impairs the insulin-stimulated suppression of hepatic glucose output; a key contributor to elevated glucose levels observed in type 2 diabetes (121). Additionally, increased intra-hepatic triglyceride induces IR through abnormal insulin signalling. Cumulatively, lipotoxicity in the liver results in elevated hepatic glucose output.

Physical activity

The beneficial effects of exercise on insulin sensitivity are well characterised. An acute bout of endurance exercise in females increases glucose tolerance during and up to 24 h post exercise (141–143). Correspondingly, habitual physical activity levels are positively associated with incidence of IR in females, independent of adiposity (144). Acute improvements in glucose tolerance following endurance exercise are primarily mediated by two factors. Firstly, elevated skeletal muscle blood flow increases glucose delivery to skeletal muscle, thereby increasing the availability of glucose for myocellular uptake (145). Secondly, contraction induced GLUT4 translocation offers an insulin-independent mechanism of glucose uptake into skeletal muscle (145). Exercise increases AMP-activated protein kinase (AMPK) activation which results in the phosphorylation of GLUT4 trafficking proteins (145, 146). This is turn allows GLUT4 to translocate and fuse to the cell membrane for myocellular glucose uptake (145, 146). Overall, a single bout of endurance exercise acutely improves insulin sensitivity, predominantly through elevated skeletal muscle glucose disposal.

Physical fitness

Following chronic exercise training, frequent and cumulative bouts of exercise elicit beneficial phenotypic adaptations associated with increased insulin sensitivity (147). Accordingly, physical fitness is recognised as a key risk factor for IR (118). In females, both low cardiorespiratory fitness and strength represent independent risk factors for impaired insulin sensitivity (18, 118).

Exercise increases skeletal muscle glucose disposal capacity. Following a period of endurance or resistance exercise training, skeletal muscle GLUT4 content is increased (148). Elevations in GLUT4 content following a period of exercise training are positively associated with rates of glucose uptake (149). Increases in GLUT4 content are skeletal muscle specific and thus facilitate increases in glucose disposal within skeletal muscle, but not in the liver nor adipose tissue (150). Additionally, improvements in glucose uptake are primarily located in the predominant muscle(s) undergoing exercise induced contractions (148, 150). Nonetheless, increases in GLUT4 content following exercise training are positively associated with improvements whole-body insulin sensitivity (149).

Improvements in glucose uptake following endurance and resistance exercise training have been attributed to beneficial adaptations at the level of the insulin signalling (151). Following exercise training, increased activity of Akt, INSR, PI3K and AMPK have been reported (151–154). Increased activity of proteins within the insulin signalling pathways in trained individuals are positively associated with glucose disposal (154). Cumulatively, increases in GLUT4 and proteins involved in up-stream insulin signalling result in elevated glycaemic control.

In addition to impaired glucose tolerance, the insulin resistant phenotype in untrained individuals is associated with elevated circulating FFA and impaired fatty acid oxidation (155). Both an increased supply of FFA to skeletal muscle and a decreased capacity for their subsequent oxidation, results in elevated intramyocellular lipid content. In untrained individuals, elevated intramyocellular lipid content increases the production of lipid derivatives, including ceramide and diacylglycerol (156). Ceramide and diacylglycerol accumulation in skeletal muscle contributes to the onset of IR through impairments in insulin signalling, as previously described. Endurance exercise training decreases accumulation of lipid derivatives in skeletal muscle (156–158). This is likely resultant to a shift in fatty acid partitioning away from *de novo* ceramide synthesis and towards triacylglycerol and or fatty acid oxidation in trained skeletal muscle compared to untrained (156). Additionally, endurance exercise training is associated with an increase in FFA oxidation in basal and exercising conditions (155). Cumulatively, both increases in the capacity for FFA oxidation and reduction in ceramide production contribute to the well documented increases in skeletal muscle insulin sensitivity following endurance exercise training.

In addition to the phenotypic adaptations within skeletal muscle, exercise training is commonly associated with beneficial alterations to body composition. Following a period of endurance or resistance training, typically skeletal muscle mass is increased and/or adipose tissue mass is decreased (159). Increases in skeletal muscle mass increase the capacity for skeletal muscle glucose uptake, through the mechanisms described above. Moreover, a reduction in adipose tissue mass reduces the impact of the negative metabolic consequences of excess adiposity, as discussed in the previous section.

However, given these two morphological adaptations typically occur synonymously, it is hard to ascertain the independent effects of each.

1.3. The menstrual cycle and insulin sensitivity

Approximately 50 % of females globally are of a reproductive age (6). The prevalence of impaired metabolic control is increasing in females of a reproductive age (160). Therefore, it is crucial to understand role of the fluctuations in ovarian hormones across the menstrual cycle on insulin sensitivity and associated metabolites.

1.3.1. The role of ovarian hormones on insulin sensitivity and associated metabolites

Maintaining precise levels of circulating glucose and lipid profiles is crucial for whole-body metabolic homeostasis. Ovarian hormones contribute to the regulation of metabolic pathways mediating glucose and lipid metabolism (2, 28, 161, 162). The metabolic actions of ovarian hormones are mediated through both genomic and non-genomic mechanisms (2).The direct genomic signalling mechanism is considered the classical mechanism of ovarian hormone action (163). Estradiol and progesterone bind to their respective nuclear receptor; estrogen receptors (ER) α and β, and progesterone receptors (PR) α and β. Upon activation, the nuclear receptor complex undergoes a conformational change and binds to the sex steroid response element located in the promotors of target genes, which results in transcriptional regulation of downstream gene expression (163). In the non-genomic mechanism of hormone action, the sex steroid hormone binds to the sex steroid hormone receptor in the cell membrane and regulation of metabolic pathways occur via the actions of a secondary messenger (163). The genomic pathway responds to changes in hormones in the following hours to days,

whereas the non-genomic pathway is quick to respond to changes in hormones, with effects typically occurring within 10 minutes, (163). The effects of estradiol and progesterone on insulin sensitivity in the liver, adipose tissue, skeletal muscle and pancreatic β-cells are discussed in detail below.

Estradiol

At physiological concentrations, estradiol is negatively associated with insulin resistance, hyperglycaemia and dyslipidaemia in pre-menopausal females (4, 5). In rodent models, deficiency in circulating estrogen levels or ER content results in impairments in insulin sensitivity and glycaemic control (164, 165). Estradiol regulates beneficial effects on blood glucose levels through the promotion of skeletal muscle and adipose glucose uptake alongside the suppression of hepatic glucose production (165–167). In skeletal muscle and adipose tissue, estradiol signalling through ERα increases Akt phosphorylation and downstream translocation of the insulin-sensitive glucose transporter GLUT4 to the membrane, subsequently increasing glucose uptake (164, 166–170). Estradiol signalling in the liver through ERα promotes the activation of Akt-FOXO1 signalling and supresses gluconeogenesis (165).

Estradiol promotes the availability and oxidation of FFAs through three main metabolic processes: suppression of FFA storage, mobilisation of adipose tissue triglyceride stores and promotion of skeletal muscle FFA oxidation (2, 161, 171). Estradiol signalling through ERα acts in all insulin sensitive tissues to supress lipogenesis (2, 161, 171). In the skeletal muscle, adipose tissue and the liver, estradiol down-regulates LPL, which promotes the uptake of circulating lipids for triglyceride formation, and the lipogenic genes acetyl-CoA carboxylase -1 and fatty acid synthase, via the transcription factor

SREBP-1c (2, 161, 171). Within adipose tissue, estradiol signalling through ER increases the expression of lipolytic genes, including the rate limiting enzyme HSL, which results in elevated lipolytic rate and subsequent FFA release (2, 26, 161). Estradiol promotes FFA oxidation in skeletal muscle through both genomic (ERα) and non-genomic activation of key transcription factors, PPAR-δ and AMPK, which upregulate downstream targets regulating FFA oxidation, including acyl-CoA oxidase, pyruvate dehydrogenase kinase, uncoupling protein 2 and uncoupling protein 3 (2, 172).

Additionally, estradiol signalling through ERα increases insulin secretion from pancreatic β-cells through activation of PI3k (173). Elevated circulating insulin increases the capacity for the activation of the proximal insulin signalling pathway and downstream targets that regulate glucose and lipid control.

Progesterone

Progesterone is positively associated with insulin resistance in pre-menopausal females (63). Consistent with this, progesterone supplementation using *in vivo* and *in vitro* models is positively associated with elevated insulin resistance and hyperglycaemia (174–178). Progesterone signalling through PR impairs glucose disposal into skeletal muscle and adipocytes through supressing Akt activation and downstream GLUT4 translocation, subsequently impairing glucose uptake (179)(178, 179).

The literature characterising the role of progesterone on lipid metabolism remains inconsistent, but the prevailing view supports a pro-lipogenic and anti-lipolytic role of progesterone (180). Progesterone signalling through PR stimulates adipose tissue lipogenesis through the upregulation of SREBP1, a key lipogenic transcription factor and

subsequent activation of lipogenic enzymes, including fatty acid synthase and LPL (28, 181, 182). Progesterone signalling through PR decreases the rate of lipolysis by inhibiting lipolytic transcription factors and the subsequent activity of rate limiting enzymes (HSL and ATGL) (183). Additionally, some research suggests that progesterone supresses the capacity for skeletal muscle lipid oxidation (172). In rodent models, supplementation of ovariectomised mice with progesterone reduces the activity of key mitochondrial enzymes required for oxidation of FFAs (172).

Interaction between estradiol and progesterone

Estradiol and progesterone exert antagonistic effects on insulin sensitivity. Over and above their absolute concentrations, the ratio of estradiol to progesterone is an additional factor underlying the subsequent metabolic effect(s) on glucose and lipid regulation. Asynchronous fluctuations in estradiol and progesterone across the menstrual cycle lead to diverse estradiol to progesterone ratios which may be an important consideration when examining the effect ovarian hormones on insulin sensitivity and associated metabolites (55).

Limited research in humans has investigated the role of the estrogen to progesterone ratio in insulin sensitivity and associated metabolites. In rodent models, the addition of progesterone attenuated the beneficial effects of estradiol supplementation alone on whole-body insulin sensitivity (184), glucose concentration (3) and glucose uptake (178). Moreover, in ovariectomised mice supplementation with supraphysiological concentrations of estradiol was able to compensate for the inhibitory effect of progesterone supplementation on insulin sensitivity, glucose uptake and GLUT-4 content (178). Similarly, progesterone has been shown to inhibit the stimulatory effect of

estradiol on the maximal activation of several key enzymes involved in skeletal muscle lipid oxidation, including carnitine palmitoyl-transferase 1, citrate synthase and β-3-hydroxyacyl-CoA dehydrogenase (172). Therefore, the relative ratio of estradiol to progesterone is involved in mediating insulin sensitivity, glycaemic control and lipidemic control.

1.3.2. The effect of the menstrual cycle on insulin sensitivity and associated metabolites

Insulin sensitivity

Studies examining the variation in insulin sensitivity across the menstrual cycle are summarised in **Table 1**. During the ovulatory and luteal phases of the menstrual cycle, an increase in whole-body IR is observed (4, 185–189). In the largest study to date (N= 259), Yeung and colleagues reported that IR, as assessed via the homeostatic model assessment for IR (HOMA-IR), increased from 1.3 mmol/L in the early-follicular phase to 1.6 mmol/L in the early- and late- luteal phases, respectively (4). This corresponds to an approximately 23% increase in IR observed during the luteal phase (4). Variation in IR across the menstrual cycle is negatively associated with estrogen and progesterone (4). However, peak IR does not occur during the mid-luteal phase, characterised by synchronous rises in estradiol and progesterone (55). Thus, data does not support a cumulative effect of both estradiol and progesterone on the regulation of insulin sensitivity across the menstrual cycle (4). Overall, research indicates insulin sensitivity is reduced during the luteal phase of the menstrual cycle. However, further research to identify the molecular mechanisms underpinning the variation in insulin sensitivity across the menstrual cycle is required.

Insulin

Circulating insulin concentration is predominantly regulated by pancreatic β-cell responsiveness to circulating nutrients (74). However, in concordance with the reported stimulatory effects of estradiol on β-cell insulin secretion, insulin concentration is increased during the ovulatory and luteal phases of the menstrual cycle (4, 173). Moreover, insulin is positively associated with variation in estradiol across the menstrual cycle (4). Some evidence suggests that elevated insulin concentrations during the luteal phase, rather than concomitant variation in glucose concentration, is the predominant factor driving elevated measurements of surrogate measures of IR (i.e. HOMA-IR) during the luteal phase (4).

Glucose and lipid profiles

Circulating glucose and triglyceride represents the balance between glucose uptake and release from metabolically active tissues. Evidence demonstrates glucose and triglyceride concentration decline during the ovulatory and mid-luteal phase (4, 5, 190). Reduced circulating glucose and triglyceride concentration during the luteal phase are likely associated with the elevated resting energy expenditure reported in this menstrual cycle phase (190–192). Correspondingly, reports demonstrate a negative association between estradiol and progesterone levels during the menstrual cycle with glucose and triglyceride concentration (4, 5, 193). Evidence suggests that reductions in triglyceride during the ovulatory and mid-luteal phase are driven by estradiol mediated increases in circulating triglyceride clearance (194).

Favourable cholesterol profiles are observed during the luteal phase, characterised by reduced total cholesterol and LDL concentration alongside increased HDL concentration

(5, 23, 190, 195, 196). Variation in estradiol across the menstrual cycle is negatively associated with cholesterol and LDL and positively associated with HDL (5). These findings are congruent with the reported effects of estradiol on lipoprotein metabolism (197). Additionally, LDL and total cholesterol are the predominant substrates required for estradiol and progesterone biosynthesis in the corpus luteum and maintenance of its endogenous cholesterol reserves (198). Therefore, reductions in total cholesterol and LDL during the mid-luteal phase, may also reflect an increased uptake in the corpus luteum to support steroidogenesis.

Gaps and inconsistencies in the literature

However, the literature is inconsistent; some studies report no change in insulin sensitivity (21–24), glucose concentration (23, 24, 186, 199–204) or lipid concentration (23, 23, 186, 186, 189, 195, 199, 202–206) across the menstrual cycle. Due to the relatively small changes in insulin sensitivity and associated metabolites across the menstrual cycle, inconsistencies may be attributable to discrepancies in sample sizes used in these studies (n= 6-259). Small sample sizes may lack adequate statistical power to robustly detect significant variation between menstrual cycle phases. Moreover, previous studies recruited heterogenous study populations with varying adiposity, physical fitness and physical activity levels, in which limited adjustment or investigation into these potentially confounding factors was conducted (4, 23, 24). Given the association between key metabolic risk factors (e.g. adiposity, cardiovascular fitness, physical activity) with IR and associated metabolites, these factors may mediate the magnitude of fluctuation in metabolic control across the menstrual cycle. Further research is needed to fully elucidate the variation in insulin sensitivity and associated metabolites across the menstrual cycle.

Table 1: Comparison of studies investigating changes in insulin sensitivity across the menstrual cycle in basal conditions.

Study	Participant characteristics	Menstrual cycle phase assessment	Sample collection timepoints	Sampling technique	Main outcomes
Jarvinen *et al.* 1984	N= 7 27-30 yr	Calendar counting	F; cycle day 4-10 L; cycle day 19-25	Plasma sample Euglycaemic clamp technique	Rate of glucose metabolism during clamp were similar between F and L ($p > 0.050$). Co-efficient of variation were 8.5% for follicular and luteal phase and 7.5% for two successive menstrual cycles ($p > 0.050$).
Toth *et al.* 1987	N= 6 25 ± 7 yr 22.5 ± 1.8 kg/ m^2	Calendar counting	EF; cycle day 1-6 MF; cycle day 9-14 L; cycle day 20-28	Plasma sample Oral glucose tolerance test Euglycaemic clamp technique	Insulin and glucose concentration during oral glucose tolerance test similar between EF, MF and L ($p < 0.050$). No significant difference in glucose disposal rates during the euglycaemic insulin clamp between EF, MF and L ($p < 0.050$).
Valdes & Elkind-Hirsch 1991	N= 8 24 yr 21.5 ± 2.0 kg/ m^2	Calendar counting, plasma estradiol test	EF; cycle day 2-3 MF; 1 d post increase in E >150ug/mL ML; 7-9 d after mid-cycle	Plasma sample Intravenous glucose tolerance test	Mean insulin sensitivity reduced in a stepwise fashion across the cycle (highest at EF, reduced at MF and lowest at ML) ($p < 0.007$). Similar glucose effectiveness index between EF, mid-cycle and ML ($p < 0.050$).
Diamond *et al.* 1993	N= 8 28.9 ± 1.2 yr	Calendar counting	MF; cycle day 3-10 ML; cycle day 20-25	Plasma sample Hyperglycaemic clamp technique	Basal and glucose stimulated insulin responses similar between MF and ML ($p > 0.050$). Glucose utilisation increased from 60-120 min during hyperglycaemic clamp in MF compared to ML ($p < 0.010$). Glucose utilisation correlated positively with P ($R = 0.66$, $p < 0.010$), but not E, LH or FSH ($p > 0.050$).
Marsden *et al.* 1996	N= 23 31 ± 5 yr 24.5 ± 4.3 kg/ m^2	Calendar counting, serum progesterone	F; cycle day 2-12 L; cycle day 13-32	Adipose tissue biopsy Plasma sample	Mean adipocyte insulin binding increased in F compared to L ($p < 0.050$). Similar adipocyte glucose uptake and rate of lipolysis in F and L ($p > 0.050$).
Ortiz *et al.* 1997	N= 6 31 ± 2 yr 24.0 ± 2.5 kg/ m^2	Calendar counting	F; cycle day 3-8 L; cycle day 20-25	Serum sample Intravenous insulin tolerance test	Glucose effectiveness index greater in F compared to L ($p = 0.040$). No correlation between serum insulin with E or P ($p > 0.050$).
Pulido & Salazar *et al.* 1999	N= 12 27 ± 3 yr 22.4 ± 1.4 kg/ m^2	Calendar counting	F; cycle day 8 ± 1 L; cycle day 23 ± 1	Serum sample Intravenous glucose tolerance test	Insulin sensitivity greater in F compared to L ($p < 0.001$). Glucose effectiveness similar in F and L ($p > 0.050$). Insulin response to glucose index tended to be greater in F compared to L ($p > 0.050$).
Bingley *et al.* 2008	N= 12 33 ± 7 yr 23.7 ± 4.4 kg/ m^2	Calendar counting, urinary LH test	F; cycle day 7 L; 7d post positive LH test	Plasma sample Intravenous glucose tolerance test	Insulin sensitivity (QUICKI, revised QUICKI, HOMA or Bennets index) similar in L and F ($p > 0.050$). Variability in insulin sensitivity between and within menstrual cycle phases were similar ($p > 0.050$).
Blum *et al.* 2008	N= 8 30 ± 4 yr 21.6 ± 1.9 kg/m^2	Calendar counting, urinary LH test	EF; -15 to -9 d from LH peak MF; -8 to -4 d from LH peak EL; LH peak + 1-3 d ML; LH peak + 4-8 d LL; LH peak + 9-14 d.	Serum sample HOMA_IR	HOMA-IR similar between EF, MF, EL and ML ($p = 0.740$).

Gill *et al.* 2005	N= 13 26 ± 6 yr 22.7 ± 4.3 kg/m2	Calendar counting, BBT	F; cycle day 1-6 L; midway between ovulation (determined via BBT) and the start of next cycle (predicted using calendar counting)	Plasma sample Oral fat tolerance test	TGL concentrations at 2-4 h during the OFTT lower in L compared to F ($p< 0.05$). Glucose concentration at 1 h greater in F compared to L ($p< 0.050$). Insulin concentrations during the OFTT similar between L and F ($p> 0.050$).
Yeung *et al.* 2010	N= 259 27± 8 yr 24 ± 4 kg/ m^2	Calendar counting, fertility monitor (E & LH)	M; cycle day 1 MF; LF; LH surge; peak fertility test result O; 2 d post peak fertility test result EL ML LL	Serum sample HOMA-IR	HOMA-IR increased during the LL, LH surge, O, EL, ML and LL compared to M and MF ($p< 0.001$). Differences in HOMA-IR were primarily due to increases in insulin during LL, LH surge, O, EL, ML, LL. HOMA-IR associated with FSH, E and P across the menstrual cycle ($p< 0.050$).
Zarie *et al.* 2013	N= 30 19 ± 1 yr 20.7 ± 6 kg/ m^2	Calendar counting, urinary LH test	Mid-F; cycle day 7 Late-F; cycle day 13 Mid-L; cycle day 21 Late-L; cycle day 27	Serum sample HOMA-IR	HOMA-IR increased in L compared to F ($p< 0.05$)

BBT, basal body temperature; E, estradiol; EF; early-follicular phase; F, follicular; HOMA-IR; homeostatic model of insulin resistance; L, luteal phase; LH, luteinizing hormone; LF; late-follicular phase; LL, late-luteal phase; M, menstruation; MF, mid-follicular phase; ML; mid-luteal phase; O, ovulatory phase; P, progesterone.

1.4. Role of epigenetics within adipose tissue

The human phenotype is directly influenced by a combination of our genes and environmental stimuli. Metabolic control is associated with high inter-individual variability during basal conditions, which are exacerbated following exposure to physiological challenge (e.g. prolonged fasting, physical exercise, cold exposure) (207). The human genome accounts for 6-47% of observed variance in parameters of metabolic control, including insulin sensitivity, resting metabolic rate, circulating glucose and circulating lipids (208–210). Whilst the biological importance of the genetic contribution to variation in metabolic control is indisputable, an extensive proportion of variance remains unexplained. The epigenome is regulated by physiological and environmental stimuli and accordingly has been proposed as a potential mechanism underpinning inter- and intra- individual variance in metabolic control.

1.4.1. microRNAs

The term epigenetics was coined in 1942 by Conrad Waddington to link the fields of developmental biology and genetics. Epigenetics was initially defined as "the branch of biology that studies the causal interactions between genes and their products which bring the phenotype into being" (211). The present day definition of epigenetics has since evolved and is widely accepted as "the study of changes in gene function that do not entail a change in DNA sequence" (29). The epigenome encompasses numerous epigenetic mechanisms that serve to regulate gene expression- DNA methylation, histone acetylation and non-coding RNAs (212). For the purpose of this thesis, the focus will be on a specific class of non-coding RNAs termed miRNAs.

MiRNAs are endogenous non-coding RNAs approximately 19-24 nucleotides in length which post-transcriptionally regulate mRNA. The first miRNA, lin-4, was discovered in 1993 in the nematode *Caenorhabditis elegans* (*C. elegans*) (213). Lin-4 was found to be essential for temporal control of postembryonic development in *C. elegans*, with the dysregulation of lin-4 expression leading to inappropriately timed progression within developmental stages (213). Lee and colleagues (1993) discovered lin-4 acts to repress the translation of LIN-14 mRNA through binding to complementary sites in the 3'UTR. Although this novel gene regulation was initially thought to be unique to *C. elegans*, the discovery of lin-14 sparked huge research interest. Over the next decade numerous short non-coding RNAs were discovered across multiple species (214, 215).

The miRNA registry database (miRbase) was founded in 2002, initially registering 218 mature miRNAs across 5 species (miRbase 1.0). Since the foundation of miRbase, an exponential rise in miRNA discovery ensued; the latest miRNA register in 2018 registered 38,589 mature miRNAs across 271 species (miRbase 22.1). At present, 1917 pre-curser and 2654 mature human miRNAs are registered (216). The functional importance of miRNAs in regulating mRNA translation is indicated by the conservation of miRNAs across species (217). Despite the relatively small number of miRNAs compared to the human genome, approximately 60% of known human protein-coding genes contain target sites for miRNA binding (217). A single miRNA can regulate the expression of hundreds of target mRNAs and the expression of one mRNA can be modulated by numerous miRNAs (218, 219). As such, miRNAs are now recognised as a major class of mRNA regulatory molecule.

MiRNA biogenesis occurs through canonical and non-canonical pathways (**Figure 4a**). The canonical pathway begins with the transcription of long primary miRNA (pri-miRNA) transcripts containing a 5'cap and 3'polyA tail in the cell nucleus by RNA polymerase II or III (220). Pri-miRNAs are subsequently cleaved by the microprocessor complex (221, 222). The microprocessor complex consists of the RNAse III endonuclease Drosha and the double stranded RNA binding protein DGCR8. DGCR8 binds to the pri-miRNA and guides Drosha into position to cleave the pri-miRNA into a hairpin structured ~70 nt pre-cursor miRNA (pre-miRNA) (221, 222). Alterative non-canonical pathways of pre-miRNA biogenesis without the requirement of pri-miRNAs have now emerged and are discussed in detail elsewhere (223, 224). Once generated, pre-miRNAs are actively transported out of the nucleus into the cytoplasm by exportin-5, a nuclear export receptor (225). In the cytoplasm, pre-cursor miRNAs are cleaved into ~22nt miRNA duplexes by a second RNAse III endonuclease, termed Dicer (221). The strand of this duplex with the greatest 5'cap stability, termed the miRNA* strand, is then targeted for degradation, while the other strand remains as a mature miRNA (226).

MiRNAs regulate gene expression via post-transcriptional translation of mRNA (218) (**Figure 4b**). The mature miRNA loads into Argonuate proteins (AGO), which forms an RNA-induced silencing complex (RISC) (227). The miRNA within the RISC binds to complimentary sequences in the three prime untranslated region (3'UTR) of target mRNAs. Once bound, the RISC induces post-transcriptional gene silencing via inhibition or degradation of the target mRNA (227). When near-perfect complementarity exists between the nucleotide sequence of the miRNA and the target mRNA, the AGO protein cleaves the target mRNA resulting in degradation (228). When there is imperfect complementarity, requiring only 2-7 bases of the target mRNA, translational inhibition

occurs (218). miRNA primarily function as negative regulators of gene expression (218), although some miRNA function to positively regulate gene expression (229).

MiRNAs act to regulate mRNA expression within physiological pathways via concerted expression profiles (230). In this manner, miRNAs regulating functionally related cohorts of genes are synergistically co-expressed in response to a stimuli to act within the same gene regulatory network (230). MiRNAs either act locally within the cells in which they were transcribed or are selectively packaged into extracellular vesicles and secreted into the extracellular space where they circulate until taken-up by specific target cells (231–234). This demonstrates the role of circulating miRNAs in intra-cellular communication (232, 234). Circulating miRNAs exist in a stable manner regardless of the presence of RNases in blood, due to their association with extracellular vesicles, including; exosomes, apoptic bodies, microvesicles, proteins, and HDL (232, 233, 235, 236). Through both local and systemic expression, miRNAs play a key role in regulating mRNA expression throughout the whole body.

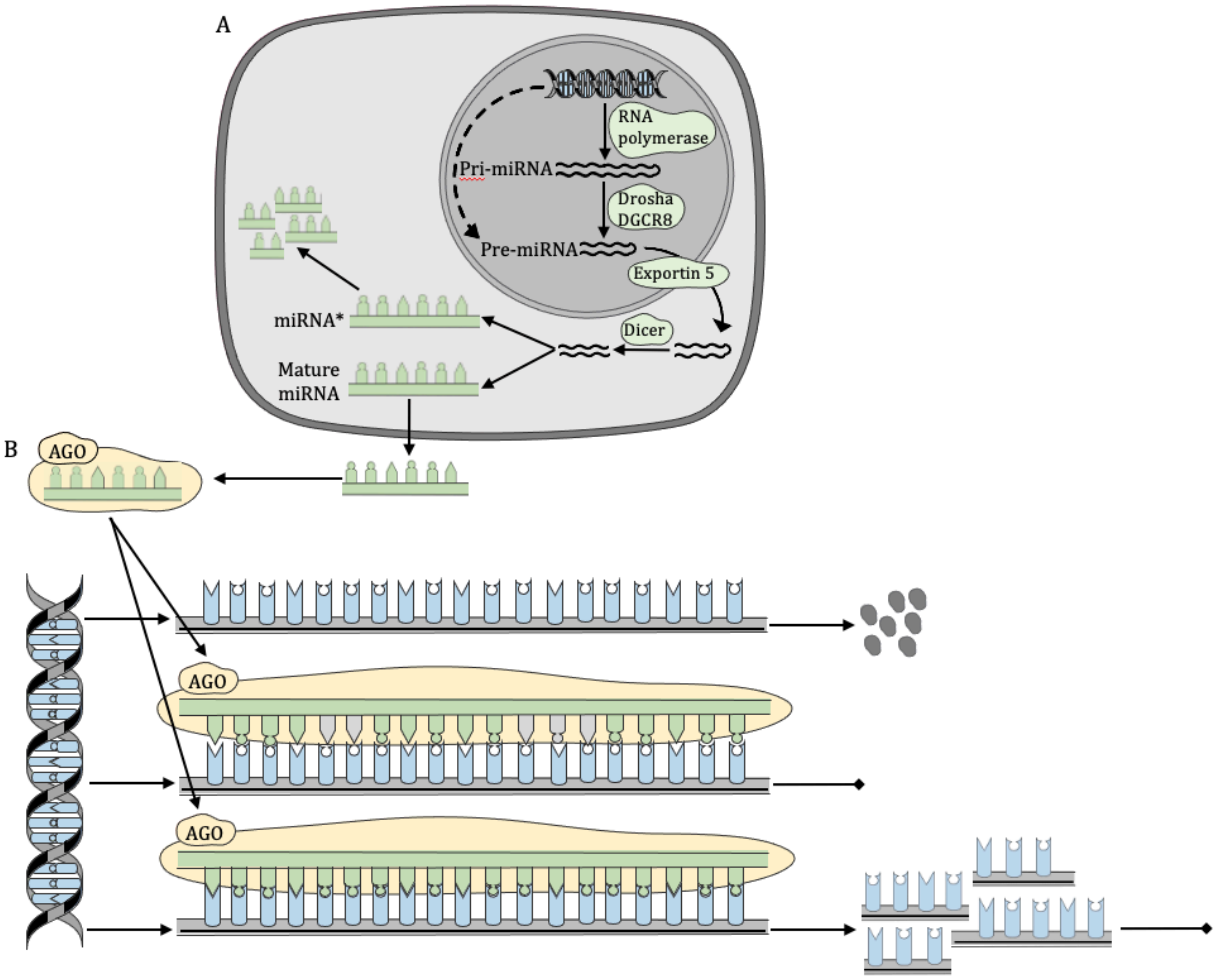

Figure 4: miRNA biogenesis and mechanism of action. (A) Canonical miRNA biogenesis starts with the generation of a primary miRNA (Pri-miRNA) transcript via RNA polymerase in the cell nucleus. Pri-miRNAs are cleaved by the microprocessor complex, comprised of Drosha and DGCR8, into pre-cursor miRNAs (pre-miRNA). Pre-miRNAs can alternatively be generated by non-canonical (dashed arrow). Pre-miRNAs are exported out of the nucleus by Exportin 5. In the cytoplasm, Dicer cleaves the pre-miRNA to create a miRNA duplex. The miRNA* strand is degraded, and the other strand remains as the mature miRNA. The mature miRNA binds with Argonuate (AGO) protein to form the RNA induced silencing complex (RISC). (B) DNA is transcribed into messenger RNA (mRNA), which is translated into protein in the absence of miRNA (top). Near perfect complementarity of miRNA to target mRNA inhibits mRNA translation (middle). Perfect complementarity of miRNA to target mRNA results in mRNA degradation and inhibition

of mRNA translation (bottom). Schematic was created using images from Servier Medical art (https://smart.servier.com).

1.4.2. Function of microRNAs in adipose tissue:

MiRNAs regulate a range of cellular processes throughout the body, including cell development, metabolism and apoptosis. Initial evidence demonstrating miRNA regulation of metabolic control within adipose tissue derived from early loss or gain of function studies in invertebrate organisms. miR-14 deletion in *Drosophila* resulted in enlarged lipid droplets with a 2-fold increase in triglyceride content (237). Similarly, *Drosophila* lacking miR-278 exhibited fat accumulation and reduced adipose tissue insulin sensitivity (238). While these early studies highlight the importance of miRNA expression in lipid control, they do not directly translate to human adipose tissue physiology. The first study investigating the role of miRNA expression within human adipocytes was conducted by Esau and colleagues in 2006 (239). Antisense oligonucleotides (ASO) were used to inhibit 86 miRNAs in human pre-adipocytes prior to differentiation. They observed ASOs targeting miR-143 and miR-9* inhibited adipocyte differentiation and reduced triglyceride accumulation in a concentration dependant manner (239). Numerous other studies now conclusively demonstrate the importance of miRNAs in regulating a variety of pathways pertinent to adipose tissue metabolic control; including adipogenesis, lipolysis, lipogenesis and insulin sensitivity (240–245).

The functional importance of miRNAs in the regulation of adipose tissue metabolic control is exemplified by the differential miRNA profiles observed in metabolic dysfunction. Initial research employing global miRNA expression techniques determined different expression in 11 out of 155 assessed adipose tissue miRNAs between

individuals with normal glucose tolerance and type 2 diabetes (246). Additionally, 15 adipose tissue miRNAs were associated with metabolic parameters, including body composition, plasma glucose and plasma lipids, and adipose tissue morphology, including macrophage infiltration, mean adipocyte volume and maximal adipocyte volume (246). Similarly, miRNA analysis of adipose tissue identified 15 miRNAs exhibiting a step-wise relationship in expression levels between lean, obese-insulin sensitive and obese-IR females (245). Numerous other congruent reports demonstrate the association between miRNA expression and phenotypes of metabolic dysregulation, including obesity, impaired glucose regulation and type 2 diabetes (245–251). Thus, (dys)regulation of miRNA expression in adipose tissue is pertinent to both the maintenance of healthy metabolism and the progression of metabolic disease.

Alongside regulation of metabolic control locally within their tissue of origin, miRNAs also play a role in the systemic regulation of metabolism (234). Adipose tissue is a major site for the secretion of exosomal miRNAs into circulation (234). Selective uptake of circulating miRNAs originating from adipose tissue into distant tissues serve to modulate whole body metabolic control via regulation of mRNA transcription (234, 252). Adipose tissue derived exosomal miRNAs have been shown to regulate glucose metabolism (253), lipid metabolism (252, 253) and whole body, skeletal muscle and liver IR (253–255). The role adipose-derived exosomal miRNAs exert on metabolic control in distant tissues thereby implicate a key role of adipose tissue miRNAs in systemic control of metabolism.

1.4.3. Hormonal regulation of microRNA expression:

As discussed in sections 1.2 and 1.3, cyclical fluctuations in ovarian hormone concentrations across the menstrual cycle are associated with divergences in metabolic

control. However, the associated molecular mechanisms remain uncertain. miRNA profiles are regulated by environmental stimuli and are a key regulator of adipose tissue metabolic control. Thus, adipose tissue miRNA expression offers a potential mechanism through which fluctuations in hormones across the menstrual cycle may regulate metabolic control.

miRNA expression is hormonally regulated in a range of tissues (33, 34, 36–38, 256). Notably, divergences in adipose tissue miRNA expression have been observed in hormonally distinct female populations, e.g. menopausal status (36), hormonal supplementation (36), polycystic ovary syndrome (37) and ovariectomy (38). A recent study from Kangas and colleagues (2018) reported an up-regulation in adipose tissue expression of miR-16-5p, miR-18a-5p, miR-223-3p, miR-363-3p, miR-451a and miR-486-5p in post-menopausal females compared to pre-menopausal females (36). Correspondingly these divergently expressed adipose tissue miRNAs, except miR-223-3p, were negatively correlated with estradiol concentrations (36). Moreover, hormonally regulated adipose tissue miRNAs function within transcriptional networks regulating key metabolic pathways, including; glucose metabolism, lipid metabolism and insulin signalling (36, 38, 240). This relationship between ovarian hormones and adipose tissue miRNA expression suggests a casual mechanism through which an altered hormonal milieu may facilitate divergences in metabolic control.

However, chronically altered ovarian hormone concentrations represents a different physiological stimulus to the acute hormonal fluctuations observed across the menstrual cycle. At present, no research has investigated the effect of the menstrual cycle on adipose tissue miRNA expression. Whilst the body of literature is limited, research examining

circulating miRNA expression across the menstrual cycle can impart some additional insight. Rekker *et al.* (2013) reported no effect of menstrual cycle phase on 375 plasma miRNAs, with miRNA expression similar between early-follicular, mid-follicular, ovulation and mid-luteal phase (257). Whereas Li *et al.* (2017) reported basal expression of serum miRNA-126 was elevated during the mid-luteal phase compared to the mid-follicular phase. miRNA-126 is involved in the regulation of Akt/PI3K signalling (258). Additionally, miR-126 expression exhibited a positive association with serum estradiol concentrations, however the casual nature of this relationship was not examined (258). However, it must be noted that miRNA expression is highly tissue specific and therefore cell-free miRNA expression may not be representative of adipose tissue (36, 259). Discrepancies and limitations within this small body of literature highlight the need for further research. This is crucial to fully elucidate the relationship between adipose tissue miRNA expression and cyclical hormonal fluctuations across the menstrual cycle.

1.5. Conclusions and aims:

The menstrual cycle represents a crucial biological rhythm governing physiology in females of a reproductive age. Variation in parameters of metabolic control, at a whole-body and tissue specific level, are observed across the menstrual cycle. Particularly, evidence demonstrates fluctuations in insulin sensitivity and associated metabolites between menstrual cycle phases in association with ovarian hormones. However, the data gathered so far is inconsistent; conflicting findings are reported regarding the presence of significant fluctuations in insulin sensitivity and associated hormones across the menstrual cycle as well as the magnitude of these fluctuations. As previously discussed, the association between key metabolic risk factors (i.e. adiposity, cardiovascular fitness, physical activity) with IR and associated metabolites is well documented. However, the role of metabolic risk factors in the relationship between metabolic control and menstrual cycle phase remains unclear. From a mechanistic perspective, the specific molecular events underpinning the variation in metabolic control across the menstrual cycle remain to be fully elucidated. Adipose tissue is a metabolically active endocrine organ involved in the secretion of numerous adipokines which regulate metabolic and inflammatory processes. Given that adipose tissue miRNAs regulate metabolic control and are regulated by ovarian hormones, miRNAs offer a potential mechanism through which changes in metabolic control across the menstrual cycle may be mediated.

Overall, this thesis proposes to address these inconsistencies and gaps in the literature through the following aims:

1. Characterise the variation in insulin sensitivity and associated metabolites across the menstrual cycle in healthy women using large prospective cohort studies.
2. Investigate the mediatory role of modifiable risk factors for metabolic disease in the relationship between insulin sensitivity and associated metabolites across the menstrual cycle in healthy women using large prospective cohort studies.
3. Investigate whether variation in insulin sensitivity and associated metabolites across the menstrual cycle in healthy women are consistent across different prospective cohort studies.
4. Investigate whether variation in circulating inflammatory markers play a role in the regulation of metabolic control across the menstrual cycle in healthy women using large prospective cohort studies.
5. Investigate whether variation in adipose tissue miRNA expression play a role in the regulation of metabolic control across the menstrual cycle in healthy females.

Therefore, the overall hypotheses of this thesis are two-fold: Firstly, insulin sensitivity and associated metabolites will vary across the menstrual cycle in large prospective cohort studies and this relationship will be mediated by risk factors for metabolic disease. Secondly, circulating inflammatory markers and adipose tissue miRNA expression will vary across the menstrual cycle and play a role in the regulation of insulin sensitivity and associated metabolites across the menstrual cycle.

CHAPTER 2: The relationship between insulin sensitivity and menstrual cycle is modified by BMI, fitness and physical activity: results from NHANES 1999-2005.

MacGregor, K. A., Gallagher, I. J., & Moran, C. N. (2021). Relationship between insulin sensitivity and menstrual cycle is modified by BMI, fitness and physical activity in NHANES. The Journal of Clinical Endocrinology & Metabolism 106 (10): 2979-2990

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2.1. Abstract

The menstrual cycle is an important biological rhythm in females of reproductive age, characterised by rhythmic variation in hormone levels. Cyclical fluctuations in hormone profiles across the menstrual cycle are associated with alterations in metabolic control (4, 5), with insulin sensitivity reduced during the luteal phase. However, to date, research has yielded inconsistent results. In this study we firstly aimed to characterise the variation in insulin sensitivity and associated metabolites across the menstrual cycle. Secondly, we aimed to investigate the role of BMI, physical activity and cardiovascular fitness on variation in insulin sensitivity and associated metabolites across the menstrual cycle.

Data from 1906 premenopausal females in the National Health and Nutrition Examination Survey (NHANES) cycles 1999 to 2006 were analysed. Participants in NHANES completed an at-home interview and physical examination. Menstrual cycle day was retrospectively assigned using questionnaire responses to “number of days since last period started”. All data for each participant are relevant to this single timepoint within their menstrual cycle. Rhythmic variation of plasma glucose, triglycerides, and insulin,

homeostatic model of IR (HOMA-IR), and adipose tissue IR index (ADIPO-IR) across the menstrual cycle were analysed using cosinor rhythmometry. Cosinor fits a cosine curve to data and calculates the MESOR (rhythm adjusted mean), amplitude (half the predictable variation within a cycle) and acrophase (time of highest value within a cycle). Participants were assigned to low or high categories of BMI, physical activity, and CRF, and category membership was included in cosinor models as covariates.

Rhythmicity was demonstrated by a significant cosine fit for glucose (MESOR: 85.1 ± amplitude: 1.2 mmol/L; p= 0.014), but not triglyceride (87.7 ± 2.8 mg/dL; p= 0.369), insulin (9.8 ± 0.4 mmol/L; p= 0.470), HOMA-IR (2.1 ± 0.1 mmol/L; p= 0.461) or ADIPO-IR, (9.7 ± 0.6 mmol/L; p= 0.335). When covariates were included, rhythmicity was observed when adjusting for: 1. BMI: glucose (p< 0.001), triglyceride (p< 0.001), insulin (p< 0.001), HOMA-IR (p< 0.001) and ADIPO-IR (p< 0.001); 2. Physical activity: glucose (p< 0.001), triglyceride (p= 0.006) and ADIPO-IR (p= 0.038); 3. Cardiorespiratory fitness: triglyceride (p= 0.041), insulin (p= 0.002), HOMA-IR (p= 0.004) and ADIPO-IR (p= 0.004). Triglyceride amplitude, but not acrophase, was lower in low physical activity category compared to high (3.1 vs 7.2 mg/dL, p= 0.018; 12 vs 27 d, p= 0.675). No differences in amplitude nor acrophase were observed for glucose, insulin, HOMA-IR and ADIPO-IR between respective high and low covariate categories (p > 0.05).

In conclusion, our study confirms previous reports showing glucose undergoes small, yet statistically significant, rhythmic cycling across the menstrual cycle. This is the first study to demonstrate a modifying effect of BMI, physical activity and cardiorespiratory fitness on variation in insulin sensitivity and associated metabolites across the menstrual cycle. These findings demonstrate that menstrual cycle phase is an important consideration

when assessing insulin sensitivity in clinical practice or research, especially in populations with high BMI or low cardiorespiratory fitness. Furthermore, this provides direction for investigation into the therapeutic benefit of targeting BMI and physical activity to mitigate disturbances in insulin sensitivity across the menstrual cycle.

2.2. Introduction

The onset and severity of IR are associated with a range of modifiable and non-modifiable risk factors, including, sex, age, adiposity, physical inactivity and cardiovascular fitness (19, 118). Females exhibit lower fasting plasma glucose levels, but greater impairment in glucose tolerance compared to males (260). Within adipose tissue, females have greater insulin stimulated glucose and fatty acid uptake compared to males (261). Whilst BMI and age are positively associated with IR, in females IR typically occurs at a higher BMI and higher age when compared with males (262). Moreover, low fitness has a greater association with IR in overweight females compared with overweight males (118).

Reports have demonstrated a clear mechanistic role of sex hormones underpinning sexual dimorphism in IR (180, 263). Insulin sensitivity has been positively associated with estradiol and negatively associated with progesterone in rats (3). This suggests that hormonal fluctuations across the menstrual cycle in humans may play a role in insulin sensitivity. However, strategies targeting the prevention and treatment of reduced insulin sensitivity rarely consider sex and none consider the role of the menstrual cycle.

The menstrual cycle is a fundamental biological rhythm occurring in females of reproductive age. The average cycle length is 29 d, although this varies between

individuals, with cycle lengths of 24-35 d considered normal and healthy (1, 14). The menstrual cycle is governed by rhythmic fluctuations of hormones within the HPO axis; gonadotropin-releasing hormone, pituitary hormones (FSH and LH) and ovarian hormones (estradiol, progesterone and testosterone) (1). However, the effect of the menstrual cycle on physiology is under-researched and, in fact is frequently cited as a barrier towards the inclusion of females in research projects (7).

Cyclical fluctuations in hormonal profiles across the menstrual cycle have been associated with alterations in metabolic control. During the luteal phase an increase in circulating insulin and reductions in circulating glucose and triglyceride have been observed (4, 5). Correspondingly, insulin sensitivity would be expected to fluctuate across the menstrual cycle. However, studies so far have been equivocal. Reductions in insulin sensitivity during the luteal phase have been reported (4, 185–188, 264, 265). However other studies document no change in insulin sensitivity across the menstrual cycle (21–24). These inconsistencies may be attributable to the relatively small sample sizes used in all but one (4) of these studies (n= 6-30), which lacked statistical power to robustly detect the small, yet clinically meaningful, changes in insulin sensitivity across the menstrual cycle. Yeung *et al.* used a large sample size (n= 259), and reported variation in insulin sensitivity across the menstrual cycle (4). Moreover, previous studies recruited heterogenous study populations with varying BMI and physical activity levels, in which limited adjustment or investigation into the potentially confounding effects of these modifiable risk factors was conducted (4, 23, 24). Examining the role of modifiable risk factors in a large cohort of females is necessary to fully understand rhythmicity in insulin sensitivity across the menstrual cycle.

In this study we firstly aim to characterise the variation in insulin sensitivity and associated metabolites across the menstrual cycle in a large prospective cohort of well characterised females. Secondly, we will investigate the role of BMI, physical activity and CRF on variation in insulin sensitivity and associated metabolites across the menstrual cycle. Thus, we hypothesised that the variation in insulin sensitivity and associated metabolites across the menstrual cycle would be mediated by BMI, physical activity and CRF, and that this variation will be greater in high categories of BMI and low categories of physical activity and CRF.

2.3. Methods

2.3.1. Participants:

NHANES is a national, cross-sectional population-based study representative of the non-institutionalised US civilian population (NHANES, RRID: SCR_013201). Data were collected in 2-year cycles beginning in 1999, with data collection ongoing. NHANES participants completed an at-home interview and a physical examination at a mobile examination centre (MEC). A reproductive health questionnaire was included in data collection cycles between 1999-2006. This questionnaire was completed by 23,569 females. Participants were excluded if they had current diagnoses of metabolic disorder (diabetes, thyroid condition) or were taking medication that altered insulin sensitivity (**Figure 5**). Details of variable description and codes used in this study are provided in **Appendix 1.1.**

2.3.2. Menstrual cycle assessment:

Response to the question "number of days since last period started" was treated as day of menstrual cycle. Responses were collected once for each participant. Unfortunately, data on typical menstrual cycle length were not available within the NHANES database. Participants were excluded from data analysis where reported number of days since last period started was > 35 d. Whilst the typical menstrual cycle length is 29 d, a maximum cycle day value of 35 d was selected to encompass 95% of cycle lengths in females (14). Participants were excluded based on factors that influence the hormonal milieu across the menstrual cycle; < 16 yr, currently taking hormonal contraceptive medication, currently pregnant or gave birth within the previous year. Final analyses were conducted on 1906 participants (**Figure 5**).

2.3.3. Anthropometric assessment:

Stature was measured without shoes using a stadiometer and body mass was measured without shoes and wearing a disposable paper gown using a digital scale, in accordance with standard procedures at the MEC. BMI was calculated using body mass in kilograms divided by stature in metres squared (kg/m^2). Prior to analyses participants were assigned to defined BMI categories based on standard cut-off thresholds (Low BMI- underweight and healthy weight, ≤ 24.9 kg/m^2; high BMI- overweight and obese, >25 kg/m^2) (266).

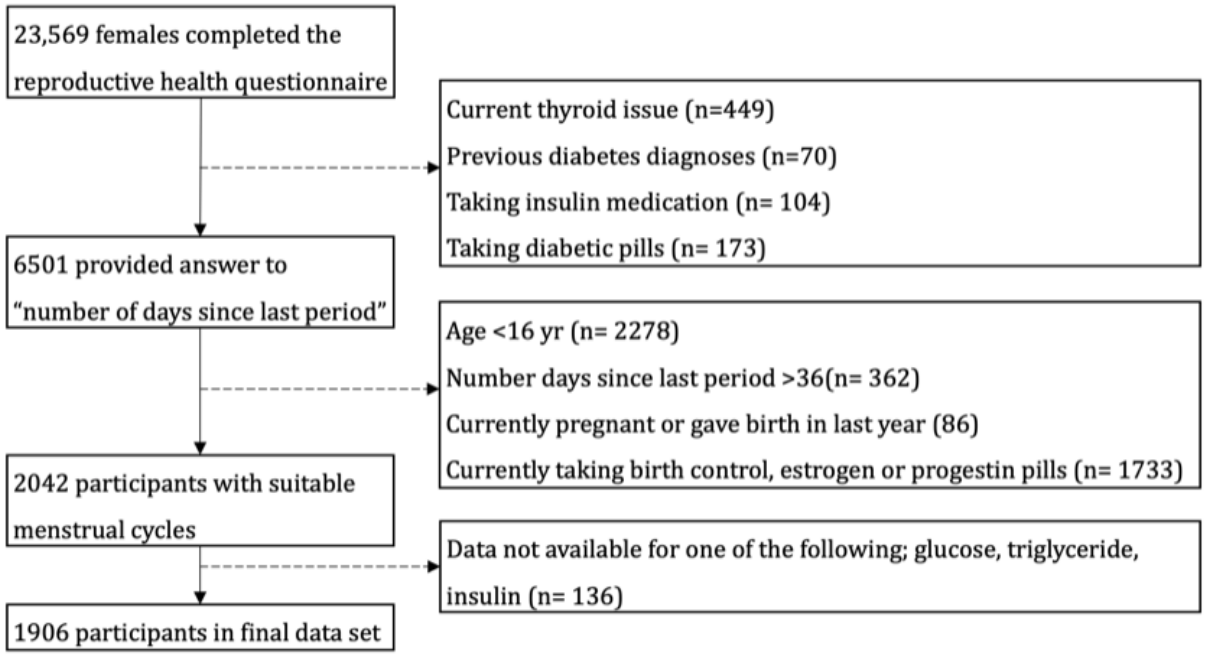

Figure 5: Flow diagram depicting participant inclusion/exclusion in the current study of NHANES participants. Solid lines represent participant inclusion. Dotted lines represent participant exclusion.

2.3.4. Blood sampling and biochemical analysis:

Venous blood samples were collected on the same day as the menstrual cycle questionnaire following a fast of at least 9 h, but not more than 24 h, by a trained phlebotomist at the MEC and processed according to a standardised protocol (267). Serum FSH and LH concentrations were analysed by microparticle Enzyme Immunoassay (Abbot Laboratories, Illinois, US) (268). Plasma glucose and serum triglyceride concentrations were analysed enzymatically (Roche Diagnostic Systems, New Jersey, US) (269, 270). Plasma insulin concentration was assessed via radioimmunoassay (Pharmacia Diagnostics AB, Uppsala, Sweden) (271). Insulin sensitivity was calculated using the HOMA-IR (112) and ADIPO-IR (272) methods.

2.3.5. Physical activity:

Each participant completed a physical activity questionnaire which included questions relating to all physical activity performed in the previous 30 d. Activity type, duration, intensity and number of times performed in the last 30 d were recorded. Moderate intensity activities were defined as inducing light sweating or a slight to moderate increase in breathing or heart rate. Vigorous activities were defined as inducing heavy sweating or large increases in breathing or heart rate. Metabolic equivalent of task (MET) scores for specific activities were calculated using activity type and intensity (273). MET scores were multiplied by the average duration and number of times performed in the last 30 d to calculate MET min/30d for each activity. MET min/30d were summed for each activity then divided by 4.29 to calculate total MET min/wk. Prior to analyses participants were assigned to low and high physical activity categories based on whether they met the national physical activity guidelines (Low physical activity <500 MET/week; high physical activity ≥ 500 MET/ week) (274).

2.3.6. Cardiorespiratory fitness:

Participants underwent a submaximal exercise test on a treadmill to predict maximal oxygen consumption ($\dot{V}O_2max$) (275). Participants were assigned to 1 of 8 protocols, of varying difficulty, based on age, BMI and self-reported physical activity level. Each protocol included a 2 min warm-up, 2 X 2 min stages and a 2 min cool down. Heart rate was recorded throughout using an automated monitor. These exercise protocols aimed to elicit 75% of maximal heart rate by the end of the test. Predicted $\dot{V}O_{2max}$ was estimated by extrapolating age-specific maximal heart-rate responses to the two 2-minute exercise stages, assuming a linear relation between HR and O_2 consumption during exercise (276,

277). Prior to analyses participants were assigned to low and high CRF categories based on whether their $\dot{V}O_{2max}$ score were below or above the age specific 50^{th} percentile (278).

2.3.7. Statistical analysis:

All analyses were conducted in R (V 3.6.3) (279) using the following add-on packages; "Cosinor", "Cosinor2", "Tidyverse" and "ggplot2" (280–283). Participant demographic data are presented as mean ± sd. Number of participants are shown for each analysis; this varies due to missing data. Data were tested for normality using visual inspection of histogram and Shapiro-Wilk test. Non-normal data were log10 transformed. Rhythmicity across the menstrual cycle was detected using Cosinor rhythmometry. Cosinor fits a cosine curve with a free phase to data and calculates MESOR (a rhythm adjusted mean), amplitude (half the predictable variation within a cycle) and acrophase (time of highest value within a cycle). Peak to peak difference (P-P) as percentage was calculated using the following equation: ((2 x amplitude/mean)*100). In separate Cosinor models, we included BMI, physical activity and CRF as a covariate. Inclusion of covariates in the Cosinor model allows the MESOR, amplitude and acrophase to differ between respective high and low covariate categories. Overall significance of the cosine model was established using the zero-amplitude test. Wald tests were conducted to test for differences in the amplitude and acrophase between respective high and low covariate categories. Cosine data are presented as MESOR ± amplitude. Data are shown as conventional boxplots.

2.4. Results

2.4.1. Participant characteristics:

Participant characteristics are outlined in **Table 2**. As would be expected, greater body mass, higher BMI, lower physical activity and lower $\dot{V}O_2$max were observed in the low physical activity category (difference= 2.0 kg, p= 0.020; 1.2 kg/m^2, p< 0.001, 2884.7 MET min/wk p< 0.001; 1.4 ml/min/kg, p= 0.019 respectively), high BMI category (26.6 kg, p< 0.001; 10.3 kg/m^2, p< 0.001; 576.4 MET min/wk, p< 0.001; 2.5 ml/min/kg, p< 0.001) and low CRF category (3.4 kg, p= 0.003; 1.3 kg/m^2, p= 0.001; 403.1 MET min/wk p= 0.028; 10.8 ml/min/kg, p< 0.001 respectively). Age was greater in the low physical activity (3.1 yr, p< 0.001), high BMI (3.6 yr, p< 0.001) and high CRF (5.5 yr, p< 0.001) categories. Stature was greater in the high physical activity category (1.5 cm, p <0.001), but not BMI (0.4 cm, p= 0.166) nor CRF (0.2 cm, p= 0.757). No rhythmic cycling was detected across the menstrual cycle in BMI (MESOR: 26.3 ± amplitude: 0.15 kg/m^2, p= 0.822), physical activity (1527.1± 182.1 MET min/wk, p= 0.199) or CRF (37.5 ± 0.5 ml/min/kg, p= 0.517) (**Appendix 1.2**).

Table 2: Participant characteristics split by demographic category in the current study of NHANES participants.

Characteristic	All	Low PA	High PA	Low BMI	High BMI	Low CRF	High CRF
N	1906	946 (49.6)	960 (50.4)	1021 (53.6)	885 (46.4)	451 (46.7)	514 (53.2)
Age (yr)	25.4 ± 9.4	27.0 ± 10.0	23.9 ± 8.6*	23.8 ± 8.5	27.4 ± 10.1*	21.3 ± 5.9	26.8 ± 10.0*
Stature (cm)	161.8 ± 6.9	161.1 ± 6.9	162.6 ±6.8*	162.0 ± 6.8	161.6 ± 7.0	162.0 ± 6.7	162.2 ± 6.7
Body mass (kg)	69.0 ± 19.0	70.0 ± 19.9	68.0 ± 18.0*	56.6 ± 7.2	83.2 ± 18.3*	69.5 ± 19.5	66.1 ± 15.1*
BMI (kg/m^2)	26.3 ± 6.8	26.9 ± 7.2	25.7 ± 6.3*	21.5 ±2.1	31.8 ± 6.1*	26.4 ± 7.0	25.1 ± 5.4*
$\dot{V}O_{2max}$ (ml/kg/min)	37.5 ± 9.0	36.8 ± 9.5	38.2 ± 8.6*	38.6 ± 8.8	36.1 ± 9.1*	31.8 ± 3.8	42.6 ± 9.3*
PA (MET min/wk)	1548.3 ± 3112.5	95.3 ± 144.7	2980.0 ± 3884.4*	1815.9 ± 3479.9	1239.5 ± 2593.5*	1407.9 ± 2498.3	1811.0 ± 3200.0*

Data are presented as mean ± standard deviation. N-values are presented as total (%) for each demographic category. BMI, body mass index; CRF, cardiorespiratory fitness; MET, metabolic equivalent of task; PA, physical activity; $\dot{V}O_{2max}$, maximal volume of oxygen consumption. * represents $p < 0.05$ following independent samples t test.

2.4.2. Variation in pituitary hormone concentration across the menstrual cycle:

To demonstrate the validity of cosine analysis for analysing cyclic rhythms in variables across the menstrual cycle, pituitary hormones were analysed. Plasma FSH and LH concentrations were available for a subset of participants (**Table 3 & Figure 6**). FSH concentration reached a peak of 8.6 IU at day 9 falling to 3.5 IU at day 26 ($p< 0.001$). LH concentration peaked at 8.1 IU on day 12 and declined to a trough of 2.1 IU on day 29 ($P< 0.001$).

Table 3: Variation in pituitary hormone concentrations across the menstrual cycle.

Variable	N	MESOR (IU)	Amplitude (IU)	P-P (%)	Peak (d)	Trough (d)	p-value
FSH	218	5.5	2.5	91.9	9	26	< 0.001
LH	219	4.2	3.0	144.0	12	29	< 0.001

P-value represents zero amplitude test for model fit. FSH, follicle stimulating hormone; LH, luteinizing hormone; MESOR, rhythm adjusted mean; P-P (%), difference between peak and trough as percentage.

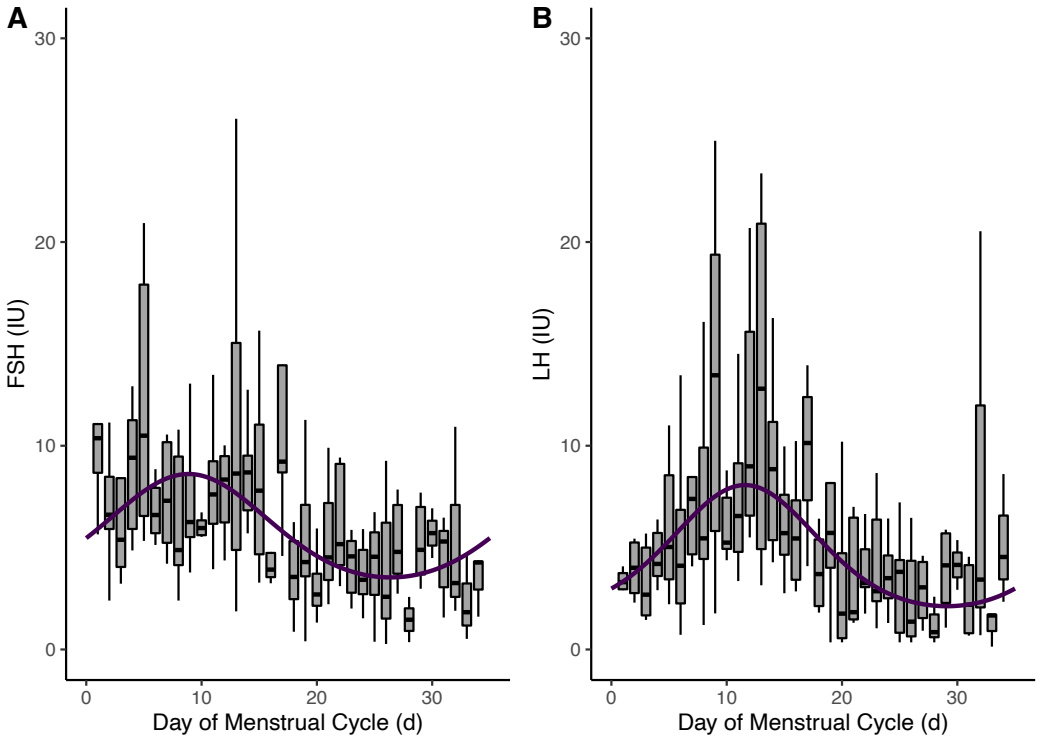

Figure 6: Boxplot with cosine wave showing variation in follicle stimulating hormone (FSH) (N=218) and luteinizing hormone (LH) (N=219) concentration across the menstrual cycle. A, FSH; B, LH.

2.4.3. Variation in insulin sensitivity across the menstrual cycle:

Rhythmicity was demonstrated by a significant cosine fit for glucose (MESOR: 4.72 ± amplitude: 0.06 mmol/L; p= 0.014). Rhythmicity was not observed for triglyceride (0.99 ± 0.03 mmol/L; p= 0.369), insulin (58.52 ± 2.23 pmol /L; p= 0.470), HOMA-IR (2.1 ± 0.1 mmol/L; p= 0.461) or ADIPO-IR, (9.7 ± 0.6 mmol/L; p= 0.335) (**Figure 7, Figure 8 & Table 4).**

2.4.4. Effect of BMI on the variation in insulin sensitivity across the menstrual cycle:

When BMI category was added as a covariate into the cosine model, a significant cosine fit was observed for glucose (p< 0.001), triglyceride (p< 0.001), insulin (p< 0.001), HOMA-IR (p< 0.001) and ADIPO-IR (p< 0.001) (**Figure 7, Figure 8 & Table 4).** There

were no differences in amplitude between low and high BMI categories for glucose (0.04 vs 0.09 mmol/L, p= 0.204), triglyceride (0.004 vs 0.04 mmol/L, p= 0.889), insulin (1.83 vs 6.52 pmol/L, p= 0.486), HOMA-IR (0.1 vs 0.3 mmol/L, p= 0.318) or ADIPOIR (0.2 vs 1.9 mmol/L, p= 0.248). Nor was there a difference in acrophase between low and high BMI categories for glucose (12 vs 16 d, p= 0.335), triglyceride (28 vs 21 d, p= 0.098), insulin (15 vs 23 d, p= 0.180), HOMA-IR (15 vs 22 d, p= 0.267) or ADIPO-IR (23 vs 22 d, p= 0.902).

2.4.5. Effect of physical activity on the variation in insulin sensitivity across the menstrual cycle:

When physical activity category was added as a covariate into the cosine model, a significant cosine fit was observed for glucose (p< 0.001), triglyceride (p= 0.006) and ADIPO-IR (P= 0.038), but not insulin (p= 0.095) or HOMA-IR (p= 0.061) (**Figure 7, Figure 8 & Table 4**). Triglyceride amplitude was lower in the low physical activity category compared to the high physical activity category (0.03 vs 0.08 mmol/L, p= 0.018). No differences were observed in amplitude between low and high physical activity categories across the menstrual cycle for either glucose (0.08 vs 0.06 mmol/L, p= 0.308), insulin (4.02 vs 1.58 pmol/L, p= 0.284), HOMA-IR (0.2 vs 0.1 mmol/L, p= 0.310) or ADIPO-IR (0.7 vs 0.7 mmol/L, p= 0.506). There were no differences in acrophase between low and high BMI categories for glucose (17 vs 12 d, p= 0.236), triglyceride (12 vs 27 d, p= 0.676), insulin (21 vs 14 d, p= 0.571), HOMA-IR (21 vs 14 d, p= 0.577) or ADIPO-IR (18 vs 26 d, p= 0.423).

2.4.6. Effect of CRF on the variation in insulin sensitivity across the menstrual cycle:

When CRF category was added as a covariate into the cosine model, a significant cosine fit was observed for triglyceride (p= 0.041), insulin (p= 0.002), HOMA-IR (p= 0.004) and

ADIPO-IR (p= 0.004), but not glucose (p= 0.225) (**Figure 7**, **Figure 8** & **Table 4**). No differences in amplitude across the menstrual cycle were observed between low and high CRF for glucose (0.02 vs 0.04 mmol/L, p= 0.464), triglyceride (0.08 vs 0.07 mmol/L, p= 0.117), insulin (7.35 vs 4.23 pmol/L, p =0.099), HOMA-IR (0.3 vs 0.2 mmol/L, p= 0.109) or ADIPOIR (1.7 vs 1.0 mmol/L, p= 0.115). There were no differences in acrophase between low and high CRF categories for glucose (20 vs 12 d, p= 0.444), triglyceride (34 vs 24 d, p= 0.270), insulin (27 vs 17 d, p= 0.290), HOMA-IR (27 vs 17 d, p= 0.282) or ADIPO-IR (30 vs 20 d, p= 0.260).

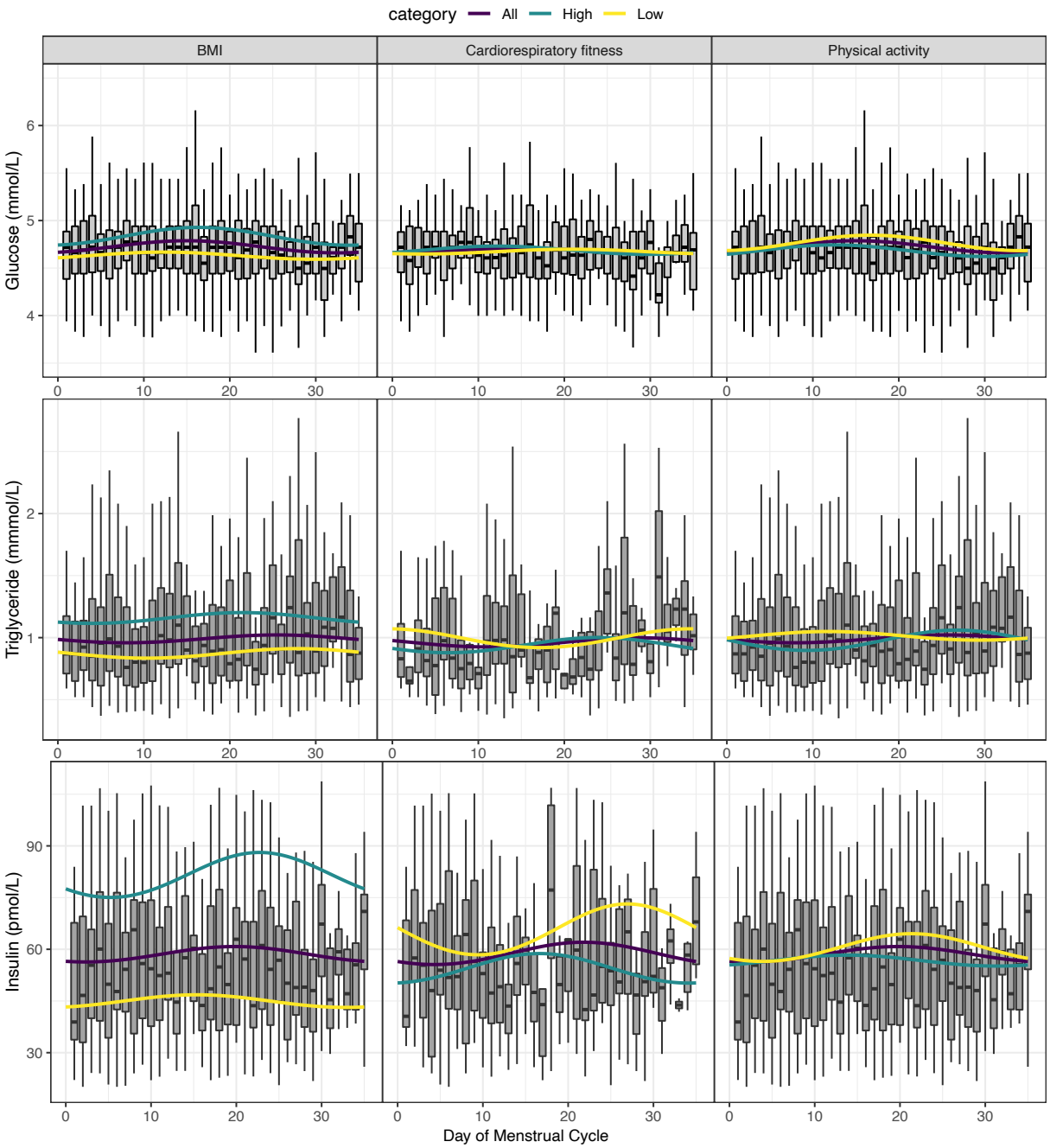

Figure 7: Variation in glucose, triglyceride and insulin across the menstrual cycle in low and high categories of BMI (left), CRF (middle) and physical activity (right). Boxplot represents all participants data for each respective variable. Cosinor model fits are shown for all participants (blue), low covariate category (green) and high covariate category (red). Low BMI, ≤24.9 kg/m^2; high BMI, > 25 kg/m^2; low CRF, ≤50th age specific percentile; high CRF, >50th age specific percentile; low physical activity, ≤500 MET min/wk; high physical activity, > 500 MET min/wk.

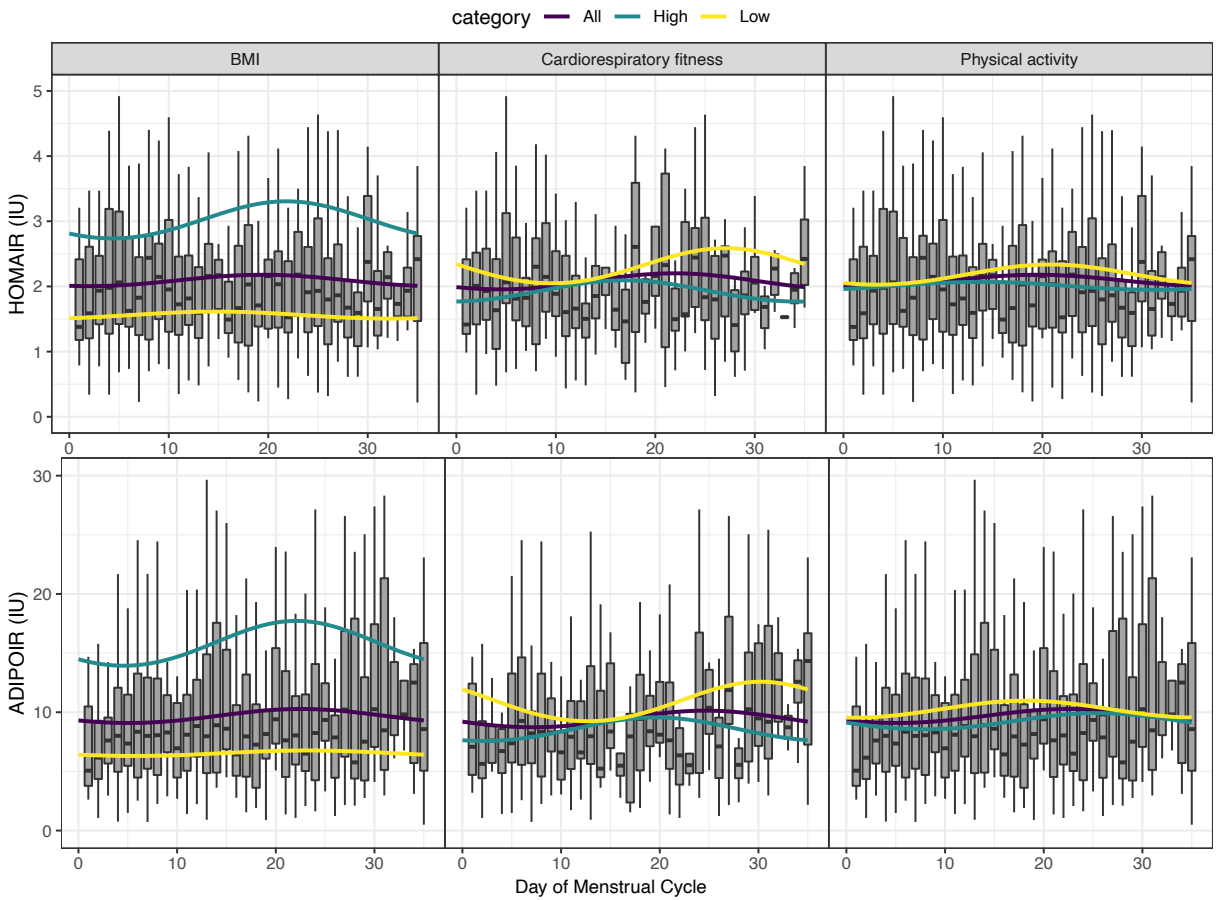

Figure 8: Variation in HOMA-IR and ADIPO-IR across the menstrual cycle in low and high categories of BMI (left), CRF (middle) and physical activity (right). Boxplot represents all participants data for each respective variable. Cosinor model fits are shown for all participants (blue), low covariate category (green) and high covariate category (red). Low BMI, ≤24.9 kg/m^2; high BMI, > 25 kg/m^2; low CRF, ≤50th age specific percentile; high CRF, >50th age specific percentile; low physical activity, ≤500 MET min/wk; high physical activity, > 500 MET min/wk.

Table 4: Variation in insulin sensitivity and associated metabolites across the menstrual cycle.

Variable	Category	n-val	MESOR (IU)	Amp-litude (IU)	P-P (%)	Acro-phase (d)	p-value	Amplitude difference p-value	Acrophase difference p-value
Glucose (mmol/L)	All	1903	4.72	0.06	2.70	15	**0.014**		
	Low BMI	1019	4.63	0.04	1.61	12			
	High BMI	884	4.83	0.09	3.88	16	**<0.001**	0.204	0.335
	Low PA	944	4.76	0.08	3.41	17			
	High PA	959	4.68	0.06	2.60	12	**<0.001**	0.308	0.236
	Low CRF	451	4.67	0.02	1.05	20			
	High CRF	513	4.69	0.04	1.85	12	0.225	0.464	0.444
Triglyceride (mmol/L)	All	872	0.99	0.03	6.37	26	0.369		
	Low BMI	482	0.87	0.04	8.79	28			
	High BMI	390	1.16	0.04	7.50	21	**<0.001**	0.889	0.098
	Low PA	404	1.01	0.03	6.87	12			
	High PA	468	0.98	0.08	16.77	27	**0.006**	**0.018**	0.676
	Low CRF	192	1.00	0.08	15.57	34			
	High CRF	254	0.94	0.07	14.50	24	**0.041**	0.117	0.270
Insulin (pmol/L)	All	872	58.52	2.23	7.63	20	0.470		
	Low BMI	483	44.93	1.83	8.14	15			
	High BMI	389	81.31	6.52	16.05	23	**<0.001**	0.486	0.180
	Low PA	405	60.37	4.02	13.32	21			
	High PA	467	56.70	1.58	5.59	14	0.095	0.284	0.571
	Low CRF	194	65.35	7.35	22.48	27			
	High CRF	252	54.27	4.23	15.60	17	**0.002**	0.099	0.290
HOMA-IR (IU)	All	871	2.09	0.09	8.34	20	0.461		
	Low BMI	482	1.56	0.05	6.90	15			
	High BMI	389	3.01	0.28	18.91	22	**<0.001**	0.318	0.267
	Low PA	404	2.18	0.15	14.18	21			
	High PA	467	2.01	0.06	6.03	14	0.061	0.310	0.577
	Low CRF	194	2.30	0.27	23.21	27			
	High CRF	252	1.92	0.16	16.75	17	**0.004**	0.109	0.282
ADIPO-IR (IU)	All	868	9.67	0.59	12.24	23	0.335		
	Low BMI	480	6.53	0.23	7.09	23			
	High BMI	388	15.71	1.89	24.09	22	**<0.001**	0.248	0.902
	Low PA	403	10.24	0.72	14.02	18			
	High PA	465	9.22	0.68	14.82	26	**0.038**	0.506	0.423
	Low CRF	192	10.81	1.71	31.64	30			
	High CRF	252	8.54	1.04	24.47	20	**0.004**	0.115	0.260

Cosine fit p-value represents zero amplitude test for model fit. Amplitude difference p-value represents difference in amplitudes between respective low and high covariate categories. Acrophase difference p-value represents difference in acrophase between respective low and high covariate categories. Bold font indicates a p-value < 0.05. Low BMI, ≤24.9 kg/m2; high BMI, > 25 kg/m2; low CRF, ≤50th age specific percentile; high CRF, >50th age specific percentile; low physical activity, ≤500 MET min/wk; high physical activity, > 500 MET min/wk. BMI, body mass index; CRF, cardiorespiratory fitness; MESOR, rhythm adjusted mean; PA, physical activity; P-P (%), difference between peak and trough as percentage.

2.5. Discussion:

This study aimed to characterise cyclical changes in insulin sensitivity and associated metabolic parameters across the menstrual cycle and their association with BMI, physical activity and CRF. We found rhythmic cycling across the menstrual cycle for glucose, but not triglyceride, insulin, HOMA-IR or ADIPO-IR. Consistent with our hypothesis, when including selected risk factors for impaired metabolic control as covariates, rhythmic cycling was observed across the menstrual cycle for: glucose, triglyceride, insulin, HOMA-IR, and ADIPO-IR when models included BMI; glucose, triglyceride and ADIPO-IR when models included physical activity; and triglyceride, insulin, HOMA-IR and ADIPO-IR when models included CRF. Triglyceride amplitude, but not acrophase, was greater in the high physical activity category compared to the low physical activity category. No differences in amplitude nor acrophase were observed for glucose, insulin, HOMA-IR and ADIPO-IR between respective high and low covariate categories. These findings demonstrate changes in insulin sensitivity and triglyceride levels across the menstrual cycle are modified by BMI, physical activity and CRF status.

Previous literature reports insulin sensitivity is either reduced during the luteal phase (4, 185–188, 264, 265) or remains unchanged across the menstrual cycle (21–24). Reported variation in HOMA-IR across the menstrual cycle is of a relatively small magnitude (0.3 U), although may be clinically meaningful (4). Therefore, some previous studies using small sample sizes may have lacked statistical power to detect significant variation (21–24). In contradiction to a report from another large study, we did not observe rhythmic variation for insulin sensitivity prior to adjusting cosine fit for BMI or cardiorespiratory fitness (4). Participants studied in Yeung *et al.* had an average lower BMI (24.1 vs 26.3 kg/m^2) which may have contributed to discrepancies in findings (4). CRF was not assessed in their study. Following the inclusion of BMI and CRF into models, we observed rhythmic cycling for HOMA-IR with similar variability across the menstrual cycle of 0.2 U (P-P = 7.5%), to that previously reported by Yeung *et al.* (4). This provides evidence that BMI and CRF mediate the variation in HOMA-IR across the menstrual cycle. This mediation effect may underpin inconsistencies reported in the literature.

To our knowledge, this is the first study to investigate ADIPO-IR across the menstrual cycle. We observed rhythmic variation in ADIPO-IR when adjusting for BMI, physical activity and CRF levels. Rhythmic cycling in ADIPO-IR concentration roughly coincided with rhythmic cycling of triglyceride across the menstrual cycle, which peaked at cycle day 23 and declined to a trough at cycle day 5 (**Table 4**). This contradicts previous research, reporting elevated triglyceride concentrations during the follicular phase compared to the luteal phase (5, 190). However, previous studies did not consider BMI, physical activity or CRF, which we determined are mediators of rhythmicity in triglyceride across the menstrual cycle. Additionally, we used a larger sample size in this

study compared previous studies (n= 34 (41) & 259 (5) vs 869). Increases in ADIPO-IR during the luteal phase alongside concurrent elevations in triglyceride concentration, may be underpinned by a decline in insulin stimulated triglyceride uptake or suppression of lipolysis during the luteal phase. Progesterone has been shown to inhibit adipocyte insulin signalling and receptor binding (179, 204). Increased circulating progesterone levels may contribute to increased ADIPO-IR observed during the luteal phase of the menstrual cycle. However, further work is required to elucidate the role progesterone plays in regulating changes in circulating triglyceride and ADIPO-IR cross the menstrual cycle.

We observed lower mean triglyceride concentration alongside greater amplitude across the menstrual cycle in the high physical activity category compared to low. The timing of the peak and trough in triglyceride concentration roughly coincided with the glucose trough and peak, respectively. Regular physical activity increases the capacity for adipose tissue and skeletal muscle lipid uptake and mobilisation (155, 261). Moreover, high physical activity levels are positively associated with increased metabolic flexibility (155). Greater amplitude in triglyceride concentration across the menstrual cycle in the high physical activity category may reflect a coordinated uptake and release of triglyceride in response to fluctuations in glucose concentration.

Whilst BMI and physical activity mediate variation in HOMA-IR and ADIPO-IR across the menstrual cycle, the mechanisms underpinning this relationship are uncertain. Variation in insulin sensitivity across the menstrual cycle has been associated with progesterone and estradiol (4). Differences in BMI and physical activity are known to alter ovarian hormonal profiles; low physical activity levels are associated with higher mean estradiol

levels across the menstrual cycle and higher progesterone levels during the luteal phase (284). High BMI is associated with greater variability of estradiol, but not progesterone (63). Unfortunately, neither estradiol nor progesterone were assessed in NHANES. Future research should investigate the role of sex hormones in the relationship between insulin sensitivity and BMI and physical activity levels.

We observed rhythmicity in HOMA-IR and ADIPO-IR following adjustment for BMI and CRF. This suggests that menstrual cycle phase is an important consideration in the assessment of insulin sensitivity in clinical practice or research, especially in populations with high BMI or low CRF. Additionally, we found greater amplitude across the menstrual cycle for HOMA-IR and glucose in high compared to low BMI and HOMA-IR in low compared to high CRF. Whilst these amplitudes were not statistically significant, these data indicate individuals with high BMI or low CRF may be at greater risk of impaired insulin sensitivity and elevated glucose concentration during the luteal phase. Therefore, therapeutic strategies aiming to reduce disturbances in metabolic control across the menstrual cycle may benefit from targeting a reduction in BMI and increase in CRF. This is of particular clinical importance due to the role of high glucose variability and IR in the development and progression of diabetic complications (285, 286). Future larger studies should further investigate the association between BMI and CRF with the magnitude of variation in insulin sensitivity and glucose concentrations across the menstrual cycle. This research is crucial to further understand the role of the menstrual cycle in IR.

Unexpectedly, some participant demographics were different between respective low and high demographic categories. Therefore, some caution should be applied when interpreting these findings. Age was greater in high BMI, physical activity and CRF

categories compared to low. However, previous research has reported the positive relationship between age and IR is associated with concurrent increases in adiposity and decreases in physical activity (287), which were included in the cosinor analysis as covariates. Stature was greater in the high physical activity group. We performed regression analysis to assess the relationship between stature and metabolic outcome parameters whilst accounting for menstrual cycle day and found no significant associations (**Appendix 1.3**). These statistically significant differences in age and height may simply be due to the number of participants in the study (288). Similarly, a previous large study reported no association between variation in HOMA-IR across the menstrual cycle and stature (4). We would expect there to be overlap in participants within covariate categories, which may have confounding effects. For example, commonality between participants in the high BMI, low physical activity and low CRF categories. Future studies should investigate whether there is a cumulative effect of BMI, physical activity and CRF on rhythmic cycling in insulin sensitivity across the menstrual cycle.

The large, prospective nature of the NHANES data set represents a major strength of this study. Our analyses were conducted in 1906 female participants with detailed questionnaire data available for reproductive and general health. These data permitted the exclusion of females with conditions that alter metabolic control or hormonal concentrations. The indirect assessment of IR using surrogate measures (HOMA-IR and AIDIPO-IR) was a limitation. However, HOMA-IR and ADIPO-IR have been validated against the gold standard hyperinsulinaemic euglycaemic clamp ($r= 0.82$, $p< 0.001$) and the multi-step pancreatic clamp ($r= 0.86$, $p< 0.001$) respectively, demonstrating strong correlations (45, 57). Physical activity levels were determined using questionnaire. However, reports from the NHANES data set demonstrate similar amounts of self-

reported physical activity and objectively measured physical activity via accelerometer in those either meeting or not meeting physical activity guidelines (291). Nonetheless, future studies may benefit from collecting objectively measured physical activity across the menstrual cycle. This study used number of days since last menstrual period started as a proxy for phase of menstrual cycle and was limited by a lack of data regarding participants' typical menstrual cycle length. These data would allow greater accuracy in determining menstrual cycle phase. However, that the analysis of FSH and LH displayed expected rhythmical fluctuations across the menstrual cycle supports the use of "number of days since last menstrual period started" for statistical analysis in this data set. Ovarian hormone concentrations across the menstrual cycle were not measured, which would allow further exploration into the relationship between insulin sensitivity and risk factors for metabolic dysregulation. Future studies should obtain further data to allow thorough characterisation of participants' menstrual cycles, including typical menstrual cycle duration, ovulation date and ovarian hormones.

In conclusion, our study confirms previous reports showing insulin sensitivity undergoes small, yet statistically significant, rhythmic cycling across the menstrual cycle. This is the first study to demonstrate a modifying effect of BMI, physical activity and CRF on variation in insulin sensitivity and associated metabolites across the menstrual cycle. These findings provide a basis for further research to explore the mediatory role of BMI, physical activity and CRF on variation in insulin sensitivity across the menstrual cycle. Furthermore, this provides direction for investigation into the therapeutic benefit of targeting BMI and physical activity to mitigate disturbances in insulin sensitivity across the menstrual cycle.

CHAPTER 3: The association between menstrual cycle status, metabolites and inflammatory markers is mediated by modifiable risk factors for metabolic disease in healthy, regularly menstruating females: Evidence from the UK biobank.

3.1. Abstract

Evidence demonstrates some parameters of metabolic control, including glycaemic control, lipid control and insulin resistance, vary across the menstrual cycle in association with ovarian hormones (4, 5). However, literature is inconsistent, and the underlying mechanisms remain uncertain. This study aimed to investigate the relationship between menstrual cycle status and metabolite concentration, as well as to identify modifiable risk factors mediating this relationship. Additionally, we aimed to investigate whether changes in estradiol and inflammatory markers across the menstrual cycle are associated with metabolite concentration.

Data from 8,694 pre-menopausal regularly menstruating females in the UK Biobank were included in analysis. Menstrual cycle status was assessed using 'days since last period' relative to 'days in usual menstrual cycle'. The outcome variables in this study were; glucose, triglyceride, triglyceride to glucose index (TyG index), total cholesterol, HDL, LDL, total cholesterol:HDL ratio, estradiol, C-reactive protein (CRP) and insulin like growth factor-1 (IGF-1). Generalised additive models (GAM) were used to investigate the relationship between menstrual cycle status and outcome variables, as well as to investigate whether changes in estradiol, CRP and IGF-1 across the menstrual cycle are associated with metabolites. To examine modifiable risk factors mediating the relationship between menstrual cycle status and metabolites, the following covariates

were included in separate models as continuous variables; fat mass, fat-free mass, physical activity, grip strength, CRF and glycated haemoglobin (HbA1c). To further examine modifiable risk factors mediating the relationship between metabolites and menstrual cycle status, sub-group analysis by categories of each covariate were conducted.

Non-linear associations were observed between menstrual cycle status with total cholesterol ($p < 0.001$), HDL ($p< 0.001$), LDL ($p= 0.012$) and total cholesterol:HDL ($p< 0.001$), but not glucose ($p= 0.072$), triglyceride ($p= 0.066$) nor TyG index ($p= 0.100$). When indices of body composition, physical fitness or physical activity levels were included in the models as continuous covariates, we observed a non-linear relationship between menstrual cycle status and all metabolites in at least one of the models ($p< 0.050$). Sub-group analysis determined the magnitude of variation in metabolite across the menstrual cycle was greater in the highest two categories of fat mass and physical activity. In the whole cohort, non-linear associations were observed between menstrual cycle status with estradiol ($p< 0.001$), CRP ($p= 0.006$) and IGF-1 ($p < 0.001$). Estradiol, CRP and IGF-1 were associated with glucose, triglyceride, TyG index, total cholesterol, HDL, LDL and total cholesterol:HDL ($p< 0.050$).

In conclusion, we confirm previous reports demonstrating non-linear associations between cholesterol and menstrual cycle status. Body composition, physical activity and fitness partially explain non-linear associations between menstrual cycle status with glucose, triglyceride, TyG index, total cholesterol, HDL, LDL and total cholesterol:HDL. Variation in metabolites across the menstrual cycle may be underpinned by associated variation in CRP and IGF-1.

3.2. Introduction

The prevalence of impaired metabolic control is increasing in pre-menopausal females (160). Impaired metabolic control is typically characterised by decreased insulin sensitivity, fasting hyperglycaemia and dyslipidaemia (292). Additionally, recent evidence indicates that glycaemic and lipidemic variability are integral components of metabolic control (285, 286, 293, 294). Dysregulation in parameters of metabolic control contribute to the pathophysiology of metabolic disorders, such as metabolic syndrome and type II diabetes (T2D) (16). Therefore, it is crucial to examine factors that affect metabolic control in pre-menopausal females.

The menstrual cycle is a fundamental biological rhythm governing female physiology in pre-menopausal females. Regulated across an approximately 4-weekly duration, the menstrual cycle is characterised by cyclical fluctuations in pituitary (LH and FSH) and ovarian (estradiol and progesterone) hormones (1, 55). Ovarian hormones exert regulatory roles within lipid and glucose homeostasis (2, 3). Correspondingly, previous research has reported cyclical variation in parameters of metabolic control across the menstrual cycle, in association with fluctuations in ovarian hormone profiles. Previous studies have reported divergences in the circulating metabolome across the menstrual cycle, with reductions in glucose, triglyceride, total cholesterol and LDL observed during the mid-luteal phase (4, 5, 190, 193, 195, 196, 295). Additionally, whole-body and adipose tissue insulin sensitivity are reduced during the ovulatory and luteal phase (4, 185–188, 264, 265). However, findings are inconsistent; other studies report no effect of menstrual cycle phase on parameters of metabolic control (22, 23). These inconsistencies may be underpinned by the small sample sizes and heterogeneous female populations recruited

in these studies. Recent research demonstrates that variation in metabolic control across the menstrual cycle is mediated by modifiable risk factors for metabolic disease, including adiposity, cardiorespiratory fitness and physical activity levels (296). However, further research is required to fully elucidate the mediatory effect of these modifiable risk factors on the relationship between metabolites and menstrual cycle phase.

One mechanism which may contribute to the variation in metabolic control across the menstrual cycle is low-grade inflammation. Low grade inflammation is positively associated with impaired metabolic control, including insulin resistance, hyperglycaemia and dyslipidaemia (22, 297–299). Several inflammatory cytokines undergo rhythmic fluctuation across the menstrual cycle, including CRP, interleukin-4, IGF-1 and tumour necrosis alpha (22, 300–303). Together these are suggestive of a potentially causal mechanism underpinning variation in metabolic control across the menstrual cycle; however, further research is needed to fully elucidate this relationship.

This study aimed to investigate the relationship between metabolite concentration and menstrual cycle status. Following this, we aimed to identify modifiable risk factors mediating this relationship. In addition, we aimed to investigate the relationship between estradiol and inflammatory markers with menstrual cycle status. Based on these relationships, we also aimed to investigate whether changes in estradiol and inflammatory markers are associated with metabolite concentration. Thus, our hypothesis were two-fold: Firstly, we hypothesised that the relationship between metabolite concentration and menstrual cycle status would be mediated by body composition, physical activity, fitness and HbA1c; secondly, we hypothesised that

circulating concentration of estradiol, CRP and IGF-1 would vary across the menstrual cycle and associate with metabolite concentration.

3.3. Methods

3.3.1. Study protocol

The UK Biobank is a large prospective, population-based study which recruited 502,682 participants between March 2007 and December 2010 (304). Individuals aged 37-73 yr living within a 10 mile radius of any of the 22 assessment centres across England, Scotland and Wales were invited for participation via postal letter (5.5% response rate (305)). During each assessment visit participants undertook a self-completed touch-screen questionnaire, brief computer-assisted interview, physical and functional measures and sampling of blood, urine and saliva, as described in detail elsewhere (304, 306). The outcomes used in this study were; glucose, triglyceride, TyG index, total cholesterol, HDL, LDL and total cholesterol:HDL, estradiol, CRP and IGF-1. The exposure of the study was menstrual cycle status.

3.3.2. Participant inclusion criteria

Menstrual cycle length was assessed in pre-menopausal females; 66,447 participants answered the question "How many days since your last menstrual period?". Females were excluded from the current analysis based on factors that alter hormonal concentrations across the menstrual cycle: menstrual cycle duration < 21 d or > 36 d; day since last period > 36 d; menstrual bleeding occurring after cycle day 7; estradiol concentration < 31pmol/L or > 2864 pmol/L; < 1 yr since last gave birth, < 1 yr since last used contraceptive pill or < 1 yr since last used hormone replacement therapy.

Participants were further excluded if they reported T2D or current cancer diagnosis. Blood samples were not collected following an overnight fast, therefore participants were excluded from analysis if they recorded a fasting time ≤4 hr. Final analysis was conducted on 8,694 participants (**Figure 9**).

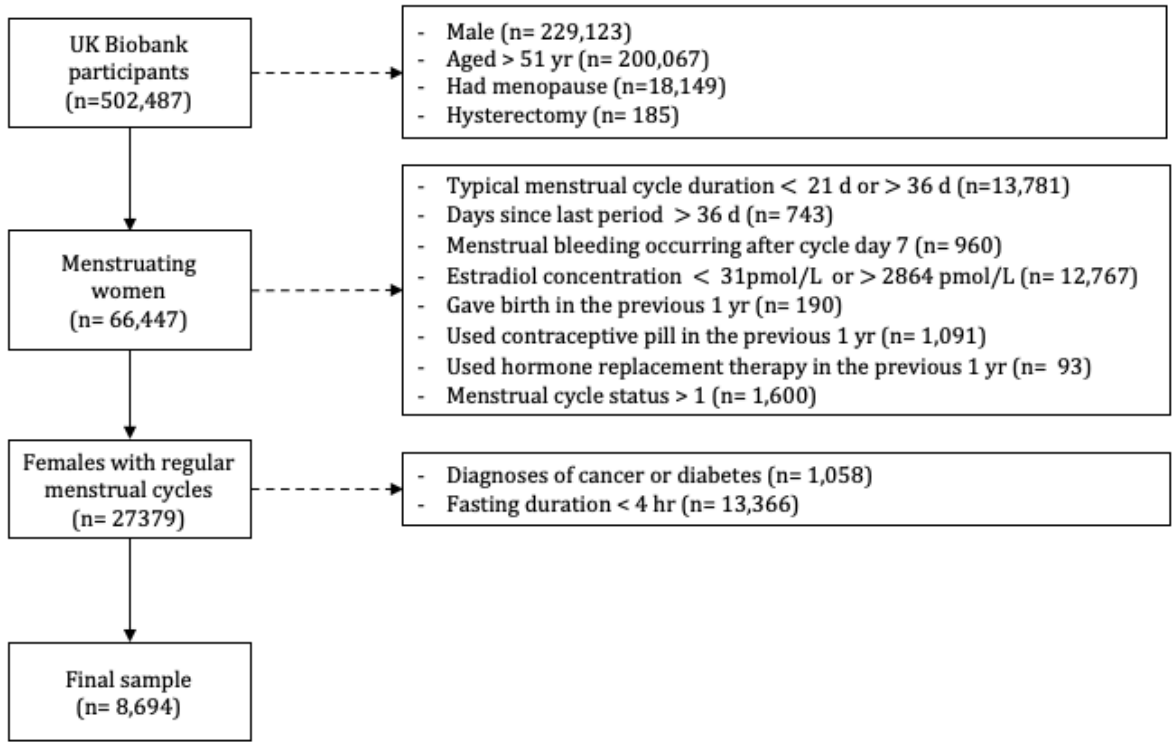

Figure 9: Flow diagram depicting participant inclusion/exclusion in the current study of UK Biobank participants. Solid lines represent participant inclusion. Dotted lines represent participant exclusion.

3.3.3. Exposure variable:

Menstrual cycle status was assessed using self-reported answers to the questions "How many days since your last menstrual period?" and "How many days is your usual menstrual cycle?". Each participant provided responses to these questions at one timepoint. To account for the non-uniformity of participant menstrual cycle lengths, standardised time within menstrual cycle was calculated relative to each participant

using the formula: (Days since last menstrual period / Days in usual menstrual cycle) whereby 0 represents the start of the menstrual cycle and 1 represents the end of the menstrual cycle. This corresponds to the approximate phases; follicular phase 0.00-0.54 and luteal phase 0.54-1.00 (14).

3.3.4. Outcome variable:

The blood sampling protocol and biochemical analysis have previously been outlined and validated elsewhere (307, 308). Serum concentration of glucose, triglyceride, total cholesterol, HDL, LDL and CRP were assessed using the AU5800 (Beckman Coulter, California, US). Serum concentration of estradiol was assessed using the DXI 800 (Beckman Coulter, California, US). Serum concentration of IGF-1 was assessed using the Liaison XL (DiaSorin, Saluggia, Italy). Manufacturers analytical ranges for these analytes are as follows (309, 310): glucose- 0.6-45 mmol/L, triglyceride- 0.1-11.3 mmol/L, total cholesterol- 0.5-18.0 mmol/L, HDL- 0.05-4.65 mmol/L, LDL- 0.26-10.3 mmol/L, CRP- 0.08-80.00 mg/L, estradiol- 72-17621 pmol/L and IGF-1- 1.3-195.0 nmol/L. The triglyceride and glucose index (TyG) was calculated as (Ln[triglyceride (mg/dL) x fasting glucose (mg/dL) /2]) (311). Total cholesterol to HDL ratio (total cholesterol:HDL (mmol/L)) was calculated as (Total cholesterol (mmol/L) / (HDL mmol/L)).

3.3.5. Covariates:

Sociodemographics: Age was calculated from self-reported date of birth. Ethnicity was self-reported and categorised as white, black, mixed, Asian, Chinese or other. Area-level socioeconomic deprivation was assessed by the Townsend score corresponding to self-reported home postcode (312).

Anthropometrics: Anthropometric measurements were obtained by trained personnel according to standard protocol and using calibrated equipment (306). Stature was measured without shoes using a Seca 202 measure. Whole body mass, fat mass and fat-free mass were measured without shoes and outdoor clothing to the nearest 0.1 kg using the Tanita BC-418 MA body composition analyser. Body mass index (BMI) was calculated as mass in kilograms divided by the square of stature in metres. Body composition was expressed as fat mass and fat-free mass in kilograms and percentage of total body mass. Quartiles of fat and fat-free mass percentage were derived.

Fitness: A subset of participants in the UK Biobank underwent CRF testing (N= 1,412). CRF was assessed using a submaximal six-minute cycle ergometer test with workload adjusted for participant age, sex, stature, body mass and resting heart rate (313). Heart rate was measured pre, during and post exercise via a four-lead electrocardiogram. Predicted maximal work rate was calculated by extrapolating heart rate and workload before exercise and at the end of exercise to predicted maximal heart rate (208- (0.7 x age)) (314), assuming a linear relationship (315). Maximal oxygen uptake was predicted using the standard equation for oxygen utilisation during cycle ergometry (METs= (7 + (Work load (W) x 10.8/ body mass (kg)) /3.5). Grip strength was measured using a Jamar J00105 hydraulic hand dynamometer (18). Isometric grip force was assessed from a 3 second maximal grip effort of the left- and right-side arms. The mean of the left- and right-side values were calculated and expressed in kilograms. Quartiles of CRF and grip strength were derived.

Physical activity: Self-reported physical activity was assessed using the International Physical Activity Questionnaire (IPAQ). Data processing guidelines published by IPAQ

were followed (316). Time spent in each level of activity was weighted by the energy expended (Walking- 3.3 metabolic equivalents (METS), moderate intensity- 4.0 METs, vigorous- 8.0 METs) to calculate MET hours per week of summed physical activity. Participants were assigned to low, moderate and high categories of physical activity based on standard data processing guidelines (316).

HbA1c: Serum concentration of glycated haemoglobin ($HbA1_c$) was assessed using the Variant II Turbo (BioRad, California, US). The manufacturers analytical range for $HbA1_c$ is 15-184 mmol/mol. Prior to analysis, participants were assigned to categories of HbA1c; low < 31 mmol/mol, medium = 31-39 mmol/mol, high= >39 mmol/mol (317).

Menstrual cycle symptoms: Physical symptoms associated with the menstrual cycle were assessed via self-reported questionnaire responses. Degree bothered by menstrual cramps or other problem with participants' period during the last 3 months was categorised into "Not bothered at all", "Bothered a little" and "Bothered a lot". Discomfort or pain occurring only during menstrual cycle bleeding during the last 3 months was categorised into "Yes" and "No".

3.3.6. Statistics

All data analysis were conducted using R version 3.6.3 (279), together with the libraries emmeans (318), lme4 (319), mgcv (320), tidymv (321) and tidyverse (283). Participant number varies across analyses due to missing data. Mean and standard deviations were reported for continuous variables.

To assess the relationship between metabolites (glucose, triglyceride, TyG index, total cholesterol, HDL, LDL and total cholesterol:HDL) and menstrual cycle status, spline based model fitting was conducted using generalised additive models (GAM) (320). A cyclic cubic regression spline was fitted to menstrual cycle status, which constrains the start and end points of the smooth to the same value. To examine modifiable risk factors mediating the relationship between metabolites and menstrual cycle status, separate models were adjusted for each covariate as a continuous variable (BMI, fat mass %, fat-free mass %, summed physical activity, HbA1c, grip strength and cardiorespiratory fitness). To further examine modifiable risk factors mediating the relationship between metabolites and menstrual cycle status, sub-group analysis was conducted. Where multiple measures for a similar physiological parameter were available, only one measure from each physiological parameter was selected to avoid multicollinearity. For anthropometric variables, the indices most sensitive to body composition were selected; fat mass (%) and fat-free mass (%). For physical activity levels, IPAQ total physical activity categories were selected because this index incorporates walking, moderate, vigorous and summed physical activity levels and reflects current physical activity recommendations.

To investigate whether changes in estradiol, CRP and IGF-1 are associated with metabolites (glucose, triglyceride, TyG index, total cholesterol, HDL, LDL and total cholesterol:HDL), GAMs were conducted using a thin plate regression spline (320). To avoid over-fitting the parts of the distribution with low N, the top 1 % of values were removed for predictor variables (estradiol >16.23 pmol/L, CRP >16.23 mg/L, IGF-1 >37.9 mmol/L). Four models were considered: Model 1- adjusted for age, ethnicity and deprivation, model 2- adjusted for age, ethnicity, deprivation and fat mass percentage,

model 3- adjusted for age, ethnicity, deprivation and summed physical activity levels, and model 4- adjusted for age, ethnicity, deprivation, estradiol, CRP and IGF-1 (excluding exposure of interest).

In all GAM analyses, the smoothing parameter was estimated by the generalised cross-validation (GCV) method. Non-linearity was tested using likelihood ratio tests. All GAM analyses were adjusted for age, ethnicity and deprivation. A p-value < 0.05 was considered statistically significant in all analyses.

To examine whether the semi-fasted nature of blood samples affected results, sensitivity analysis was conducted by adjusting all GAM models for fasting duration. Additionally, one-way ANOVAs were conducted to examine whether metabolite concentrations differed between reported fasting durations, categorised as 0 to 8 hr in hourly increments Where significant, pairwise comparisons were conducted with Tukey adjustment. Additional sensitivity analysis was conducted to examine if any behavioural or physiological effects of menstrual cycle symptoms affected results by restricting the cohort to females who did not report any menstrual cycle symptoms (abdominal discomfort, abdominal pain, menstrual cramps or other problems with their menstrual cycle).

3.4. Results

3.4.1. Participant characteristics

Participant characteristics are detailed in **Table 5.** Females in this cohort were aged 45 ± 3 yr, 48.3 % were normal body mass and were predominantly of a White ethnicity (89.5

%). As expected, some participant characteristics demonstrated a non-linear relationship with menstrual cycle status (body mass, BMI, fat mass (% and kg), fat-free mass (%), walking physical activity, vigorous physical activity, summed physical activity, grip strength and CRF) (**Appendix 2.1**).

Table 5: Participant characteristics in the current study of UK Biobank participants.

Characteristic	N val	Value
Age (yr)	8694	44.9 ± 2.8
Body mass (kg)	8668	70.4 ± 14.3
Stature (m)	8668	1.6 ± 0.1
BMI (kg/m2)	8668	26.2 ± 5.2
BMI categories (N (%))		
Under-weight, BMI < 18.5		75 (0.9)
Normal-weight, BMI ≤18.5 to < 25		4203 (48.3)
Over-weight, BMI ≤25 to < 30		2800 (32.2)
Obese, BMI ≥30		1590 (18.3)
Fat mass (kg)	8558	25.0 ± 10.3
Fat mass (%)	8563	34.3 ± 7.2
Fat-free mass (kg)	8563	45.4 ± 5.0
Fat-free mass (%)	8555	27.0 ± 3.7
Ethnicity (N (%))		
White		7782 (89.5)
Black		123 (1.4)
Mixed		228 (2.6)
Asian		321 (3.6)
Chinese		67 (0.7)
Other		150 (1.7)
Walking physical activity (MET min/wk)	7357	1050.9 ± 1093.2
Moderate physical activity (MET min/wk)	7357	774.0 ± 1049.1
Vigorous physical activity (MET min/wk)	7357	664.4 ± 1022.8
Summed physical activity (MET min/wk)	7357	2489.4 ± 2405.3
Grip strength (kg)	8669	26.9 ± 6.0
Cardiorespiratory fitness (METs)	1647	9.6 ± 2.4
Estradiol (pmol/L)	8694	560.1 ± 383.8
IGF-1 (mmol/L)	8647	23.8 ± 5.4
CRP (mg/L)	8668	2.0 ± 3.6
HbA1c (mmol/mol)	8558	33.0 ± 3.6
Glucose	7810	4.8 ± 0.5

Triglyceride	8688	1.2 ± 0.7
TyG index	7808	4.5 ± 0.2
Total cholesterol	8686	5.4 ± 0.9
HDL	7809	1.6 ± 0.3
LDL	8673	3.3 ± 0.7
Total cholesterol :HDL	7806	3.6 ± 0.9

Values presented as mean ± SD unless otherwise indicated. BMI, body mass index; CRP, C-reactive protein; HbA1c, glycated haemoglobin; HDL, high density lipoprotein; IGF-1, insulin like growth factor-1; LDL, low density lipoprotein; TyG index, triglyceride to glucose index.

3.4.2. Relationship between metabolites concentration with menstrual cycle status

To analyse variation in concentrations of metabolites across the menstrual cycle GAMs were conducted (**Table 6**). A non-linear relationship with menstrual cycle phase was observed for total cholesterol (Estimated degree of freedom (EDF)= 3.4, p< 0.001), HDL (EDF= 2.9, p< 0.001), LDL (EDF= 3.6, p= 0.012) and total cholesterol:HDL (EDF= 5.4, p< 0.001). Glucose (EDF= 1.3, p= 0.072), triglyceride (EDF= 4.0, p= 0.066) and TyG index (EDF=3.6, p= 0.100) were not associated with menstrual cycle phase. Findings were similar when models were adjusted for fasting duration (**Appendix 2.2**).

Table 6: Relationship between metabolite concentration with menstrual cycle status.

Variable (mmol/L)	N	EDF	p-value	Minimum (IU (MC status)	Maximum (IU (MC status))	Variation (IU (%))
Glucose	7780	1.3	0.072	4.77 (0.46)	4.79 (0.96)	0.02 (0.43)
Triglyceride	8653	4.0	0.066	1.18 (0.92)	1.24 (0.67)	0.06 (5.32)
TyG index	7778	3.6	0.100	4.50 (0.96)	4.52 (0.67)	0.02 (0.45)
Total cholesterol	8651	3.4	**<0.001**	5.32 (0.88)	5.44 (0.25)	0.12 (2.27)
HDL	7779	2.9	**<0.001**	1.53 (0.88)	1.59 (0.46)	0.06 (3.85)
LDL	8638	3.6	**0.012**	3.25 (0.88)	3.32 (0.21)	0.07 (2.15)
Total cholesterol :HDL	7776	5.4	**<0.001**	3.50 (0.50)	3.65 (0.13)	0.15 (4.17)

P-value represents the significance level for smoothed terms in the GAM. Bold face text denotes significant p-value (<0.05). Analyses were adjusted for age, ethnicity and deprivation. Menstrual cycle (MC) status values are shown on a scale of 0-1, this corresponds to the approximate phases: follicular phase, 0-0.54; luteal phase 0.54-1 (14). EDF, estimated degree of Freedom; HDL, high density lipoprotein; LDL, low density lipoprotein; TyG index, triglyceride to glucose index.

3.4.3. Impact of continuous covariates on the relationship between metabolites concentration with menstrual cycle status

To examine whether adjusting models for modifiable risk factors affects the relationship between metabolites and menstrual cycle status, we included covariates in the model as continuous variables (**Table 7**). In summary, triglyceride, TyG index, total cholesterol, HDL, LDL and total cholesterol:HDL, but not glucose, demonstrated a non-linear association with menstrual cycle status when including at least one of the anthropometric variables in separate models (BMI, fat mass % or fat-free mass %). When including summed physical activity in separate models, a non-linear relationship with menstrual cycle phase was observed for total cholesterol (EDF= 3.4, p= < 0.001), HDL (EDF= 2.6, p< 0.001), LDL (EDF=3.5, p= 0.007) and total cholesterol:HDL (EDF= 4.3, p= 0.002). When including grip strength in the model a non-linear relationship with menstrual cycle phase

was observed for total cholesterol (EDF= 3.4, p< 0.001), HDL (EDF=2.9, p< 0.001), LDL (EDF= 3.5, p= 0.015) and total cholesterol:HDL ratio (EDF=5.7, p< 0.001). When including CRF in the model a non-linear relationship with menstrual cycle phase was observed for glucose (EDF=3.6, p= 0.030), triglyceride (EDF=3.5, p= 0.015), TyG index (EDF= 3.5, p= 0.012), HDL (EDF=3.0, p= 0.026). Findings were similar when models were adjusted for fasting duration (**Appendix 2.3**).

Table 7: Effect of continuous covariates on the relationship between metabolite concentration with menstrual cycle status.

Variable	Value	BMI (kg/m^2)	Fat mass (%)	Fat-free mass (%)	Summed PA (MET min/wk)	HbA1c (mmol/ mmol)	Grip strength (kg)	Cardiorespiratory fitness (METs)
Glucose	N	7754	7652	7649	6583	7317	7757	1540
	Dev exp %	1.5	1.7	1.3	0.7	11.4	0.7	4.1
	EDF	1.3	1.4	1.3	1.2	1.4	1.3	3.6
	p-value	0.080	0.060	0.065	0.122	0.065	0.074	**0.030**
Triglyceride	N	8627	8517	8514	7328	8151	8628	1638
	Dev exp %	16.2	14.6	13.6	2.9	5.1	2.1	10.9
	EDF	4.3	4.0	4.0	0.3	4.0	4.0	3.5
	p-value	**0.010**	**0.025**	**0.036**	0.296	0.140	0.079	**0.015**
TyG index	N	7752	7650	7647	6581	7315	7755	1540
	Dev exp %	19.7	18.1	16.8	4.0	7.6	3.1	13.9
	EDF	4.0	3.8	3.7	0.9	3.7	3.5	3.5
	p-value	**0.022**	0.059	0.083	0.209	0.147	0.108	**0.012**
Total cholesterol	N	8625	8515	8512	7326	8149	8626	1639
	Dev exp %	4.3	4.8	5.0	2.5	3.7	2.3	4.4
	EDF	3.4	3.3	3.3	3.4	3.4	3.4	0.4
	p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.001**	**<0.001**	0.292
HDL	N	7753	7651	7648	6581	7316	7756	1541
	Dev exp %	16.5	14.5	13.0	3.2	4.4	2.1	8.9
	EDF	2.8	2.6	2.6	2.6	3.1	2.9	3.0
	p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.026**
LDL	N	8612	8502	8499	7315	8139	8613	1639
	Dev exp %	7.6	8.3	8.4	2.3	4.2	1.6	5.9
	EDF	3.6	3.5	3.4	3.5	3.6	3.5	0.0
	p-value	**0.001**	**<0.001**	**<0.001**	**0.007**	**0.010**	**0.015**	0.953
Total cholesterol: HDL	N	7750	7648	7645	6578	7313	7753	1540
	Dev exp %	21.7	20.2	18.6	3.2	6.8	1.8	12.6
	EDF	5.3	5.9	6.1	4.3	6.0	5.7	3.4
	p-value	**<0.001**	**<0.001**	**<0.001**	**0.002**	**<0.001**	**<0.001**	0.170

P-value represents significance for the smoothed term of menstrual cycle phase in the GAM. Bold face text denotes significant p-value (<0.05). Analyses were adjusted for age, ethnicity and deprivation. Menstrual cycle phase values are shown on a scale of 0-1, this corresponds to the approximate phases: follicular phase, 0-0.54; luteal phase 0.54-1 (14). BMI, body mass index; dev exp %, deviance explained in percentage; EDF, estimated degrees of freedom; HbA1c, glycated haemoglobin; LDL, low density lipoprotein; HDL, high density lipoprotein; TyG index, triglyceride to glucose index.

3.4.4. Impact of categorical covariates on the relationship between metabolites and menstrual cycle status

To further explore the effect of covariates on the relationship between metabolite and menstrual cycle status, sub-group analysis was conducted where covariates were included in the model as categorical factors. Findings are presented in **Figure 10**, **Figure 11** and **Appendix 2.4**. In summary, non-linear relationships between menstrual cycle phase and all metabolites were consistently observed in the highest two quartiles of fat mass. Non-linear relationships between menstrual cycle phase and glucose, total cholesterol, HDL and LDL were consistently observed in low and/or medium physical activity categories. No other covariate categories demonstrated consistent non-linear relationships between menstrual cycle phase and metabolites. Findings were similar when models were adjusted for fasting duration (**Appendix 2.5**).

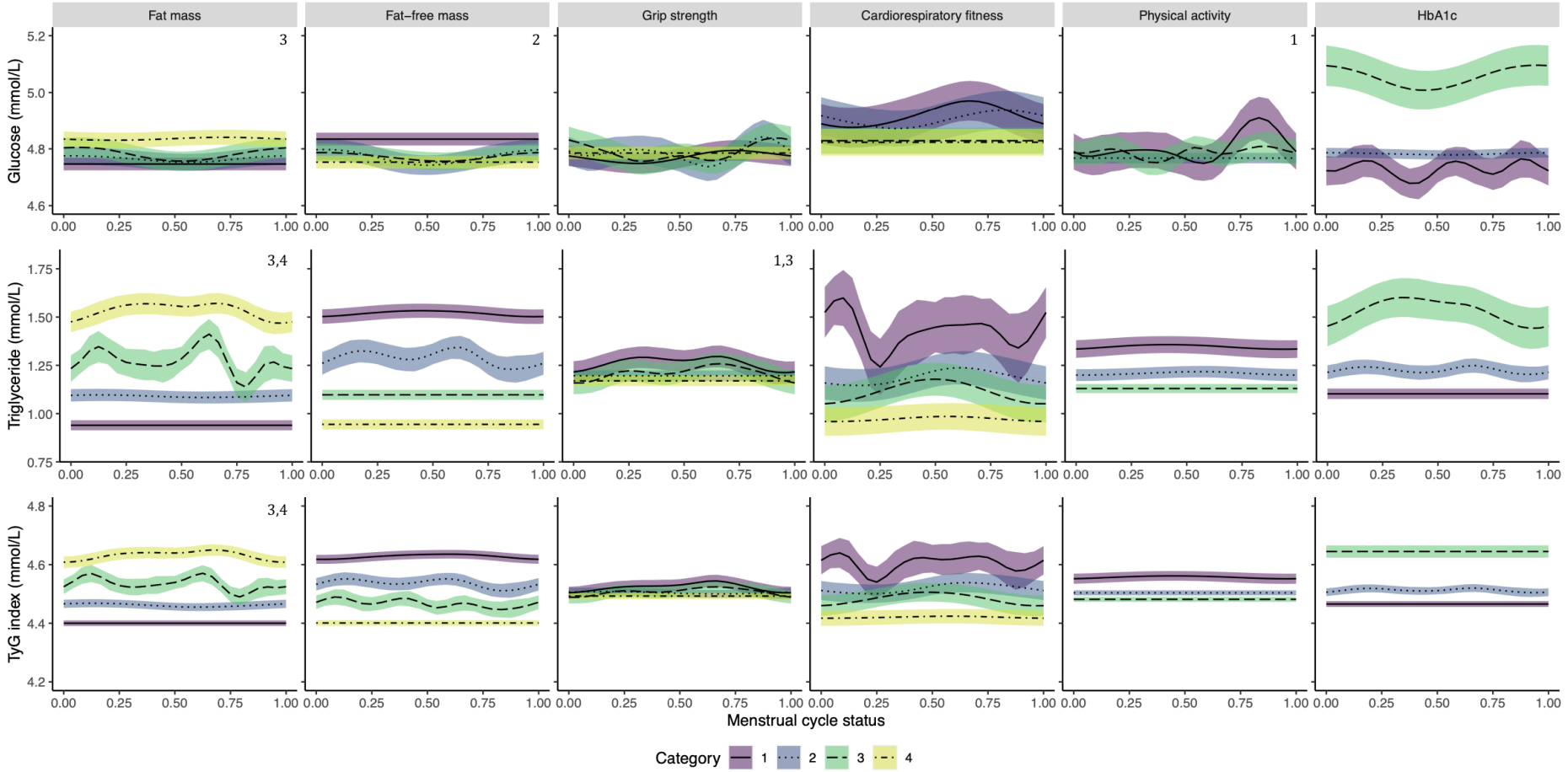

Figure 10: Variation in glucose, triglyceride and TyG index across the menstrual cycle for each model. Fat mass (%), fat-free mass (%), grip strength (kg), and cardiorespiratory fitness (METs) are categorised as quartiles. Physical activity (METs) and HbA1c (mmol/mol)are categorised into low, medium and high according to previously defined criteria. Lines represent GAM estimates using a smoothing spline function. Shaded areas represent 95 % confidence intervals. Menstrual cycle status is shown on a scale of 0-1, this corresponds to the

approximate phases: follicular phase, 0-0.54; luteal phase 0.54-1 (14). Analyses were adjusted for age, ethnicity and deprivation. Significant non-linear relationships for each category level are denoted by the respective numerical at the top right corner. TyG index; triglyceride to glucose index.

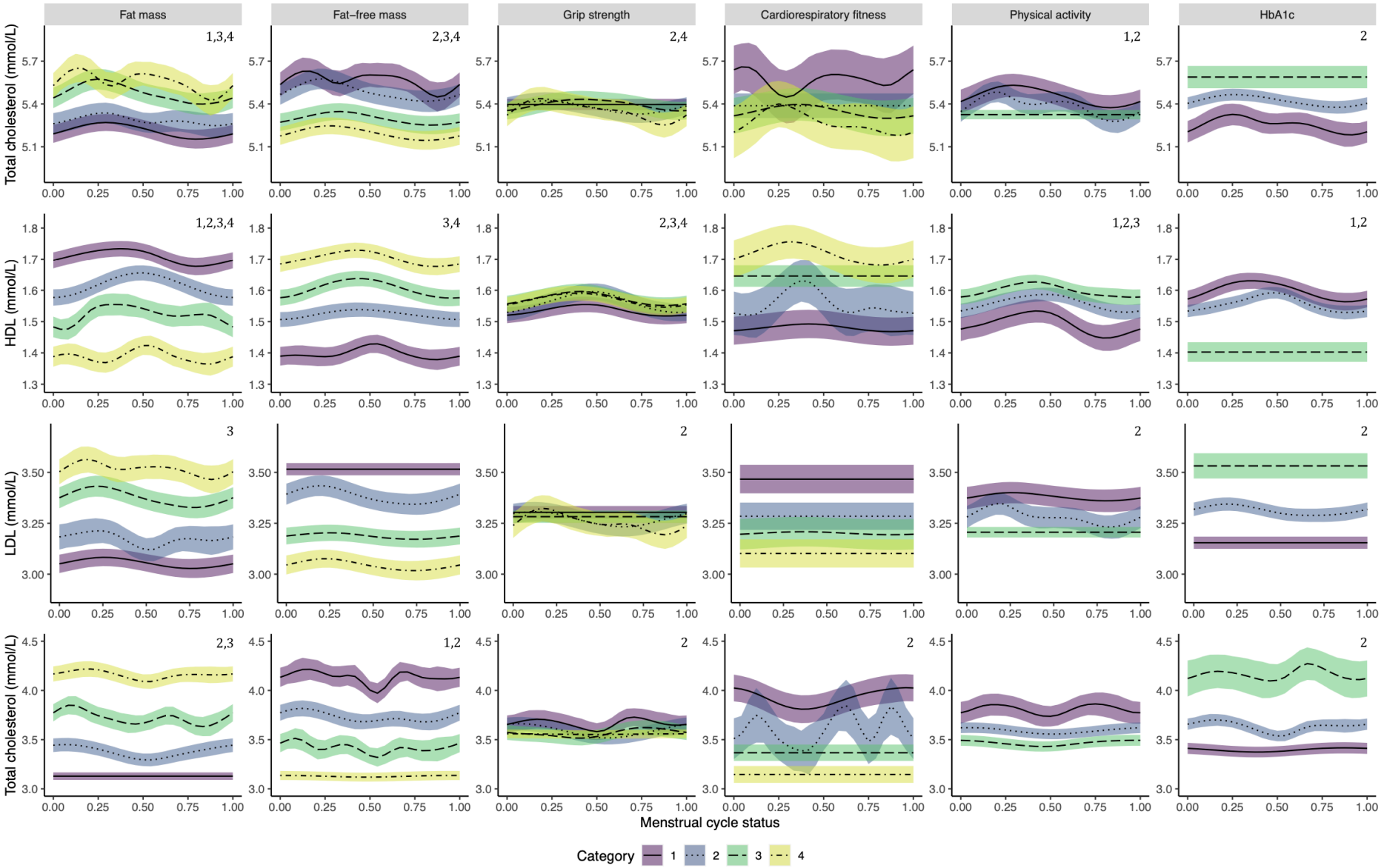

Figure 11: Variation in cholesterol across the menstrual cycle for each model. Fat mass (%), fat-free mass (%), grip strength (kg), and

cardiorespiratory fitness (METs) are categorised as quartiles. Physical activity (METs)and HbA1c (mmol/mol) are categorised into low, medium and high according to previously defined criteria. Lines represent GAM estimates using a smoothing spline function. Shaded areas represent 95 % confidence intervals. Menstrual cycle status is shown on a scale of 0-1, this corresponds to the approximate phases: follicular phase, 0-0.54; luteal phase 0.54-1 (14). Analyses were adjusted for age, ethnicity and deprivation. Significant non-linear relationships for each category level are denoted by the respective numerical at the top right corner. HbA1c, glycated haemoglobin; HDL, high density lipoprotein; LDL, low density lipoprotein.

3.4.5. Relationship between estradiol, IGF-1 and CRP with menstrual cycle status

Markers of low grade inflammation are associated with metabolic control and undergo fluctuations across the menstrual cycle (22). To analyse the relationship between estradiol, CRP and IGF-1 with menstrual cycle GAMs were conducted (**Table 8**). A non-linear relationship with menstrual cycle phase was observed for estradiol (EDF=7.2, p< 0.001), IGF-1 (EDF=6.6, p< 0.001) and CRP (EDF= 6.6, p< 0.001).

Table 8: Relationship between estradiol, IGF-1 and CRP concentration with menstrual cycle status.

Variable	N val	EDF	p-value	Minimum (IU (MC status)	Maximum (IU (MC status))	Variation (IU (%))
Estradiol (pmol/L)	8659	7.2	**<0.001**	407.5 (1.00)	708.3 (0.38)	300.8 (53.90)
IGF-1 (mmol/L)	8612	6.6	**<0.001**	22.7 (0.08)	24.6 (0.63)	1.9 (8.20)
CRP (mg/L)	8633	6.6	**0.006**	1.7 (0.21)	2.2 (0.67)	0.5 (26.90)

P-value represents significance level for smoothed terms in the GAM. Bold face text denotes significant p-value (<0.05). Analyses were adjusted for age, ethnicity and deprivation. Menstrual cycle (MC) status values are shown on a scale of 0-1, this corresponds to the approximate phases: follicular phase, 0-0.54; luteal phase 0.54-1 (14). CRP, C-reactive protein; EDF, estimated degree of Freedom; IGF-1, insulin like growth factor-1.

3.4.6. Relationship between metabolites with hormones and inflammatory markers

Having observed variations in estradiol, CRP and IGF-1 across the menstrual cycle, we examined whether these variations were associated with metabolites (**Table 9, Appendix 2.6, Appendix 2.7**). In model 1, adjusted for age, ethnicity and deprivation, significant relationships were observed between all metabolites with estradiol, CRP or IGF-1, except IGF-1 with glucose and total cholesterol. In model 2, following additional

adjustments for fat mass, significant relationships were no longer observed between: Estradiol with HDL; CRP with glucose; and, IGF-1 with triglyceride, TyG index, HDL or total cholesterol:HDL, when compared to model 1. In model 3, following additional adjustments for physical activity levels, significant relationships were no longer observed between IGF-1 with HDL or LDL when compared to model 1. In model 4, following additional adjustments for remaining hormones or inflammatory markers, significant relationships were no longer observed between estradiol with HDL, and, IGF-1 with triglyceride or HDL when compared to model 1. Findings were generally similar when models were adjusted for fasting duration (**Appendix 2.8).**

Table 9: Relationship between estradiol and inflammatory markers with metabolites.

Variable	Metabolite (IU)	Model 1: SDF			Model 2: SDF + fat mass			Model 3: SDF + physical activity			Model 4: SDF + estradiol, CRP and IGF-1		
		N	Dev exp % (EDF)	p val	N	Dev exp % (EDF)	p val	N	Dev exp % (EDF)	p val	N	Dev exp % (EDF)	p val
Estradiol (pmol/L)	Glucose	7485	2.4 (0.9)	**<0.001**	7368	2.7 (1.6)	**<0.001**	6344	3.3 (1.0	**<0.001**	7485	2.3 (1.0)	**<0.001**
	Triglyceride	8320	1.0 (2.1)	**<0.001**	8195	1.0 (14.7)	**<0.001**	7057	3.0 (3.4)	**<0.001**	8320	2.9 (13.0)	**<0.001**
	TyG index	7483	1.0 (3.2)	**<0.001**	7366	1.0 (17.9)	**<0.001**	6342	1.0 (4.4)	**<0.001**	7483	1.0 (15.2)	**<0.001**
	HDL	8318	4.4 (2.2)	**0.039**	8193	5.2 (4.6)	0.098	7055	4.6 (2.4)	**0.018**	8318	4.0 (3.3)	0.062
	LDL	7484	2.4 (2.1)	**<0.001**	7367	2.3 (14.6)	**<0.001**	6342	2.3 (3.5)	**<0.001**	7484	2.2 (9.6)	**<0.001**
	Total cholesterol	8305	3.8 (1.7)	**0.001**	8180	5.4 (8.2)	**0.048**	7044	4.3 (2.4)	**0.001**	8320	2.9 (13.0)	**<0.001**
	Total cholesterol : HDL	7481	3.6 (1.8)	**<0.001**	7364	4.1 (20.0)	**<0.001**	6339	4.0 (3.6)	**<0.001**	7481	1.0 (12.7)	**<0.001**
CRP (mg/L)	Glucose	7485	1.0 (0.6)	**0.003**	7368	1.1 (1.3)	0.621	6344	1.0 (0.7)	**0.009**	7485	1.0 (1.0)	**0.004**
	Triglyceride	8320	7.7 (12.7)	**<0.001**	8195	7.3 (17.3)	**<0.001**	7057	7.7 (13.2)	**<0.001**	8320	7.7 (13.0)	**<0.001**
	TyG index	7483	7.4 (14.7)	**<0.001**	7366	6.5 (20.2)	**<0.001**	6342	7.1 (15.1)	**<0.001**	7483	7.4 (15.2)	**<0.001**
	HDL	8318	5.4 (3.2)	**<0.001**	8193	3.9 (4.6)	**0.048**	7055	4.9 (3.3)	**<0.001**	8318	5.3 (3.3)	**<0.001**
	LDL	7484	4.5 (8.8)	**<0.001**	7367	2.5 (15.1)	**<0.001**	6342	4.4 (9.8)	**<0.001**	7484	4.6 (9.6)	**<0.001**
	Total cholesterol	8305	5.7 (4.2)	**<0.001**	8180	3.6 (8.1)	**0.035**	7044	5.0 (4.8)	**<0.001**	8320	7.7 (13.0)	**<0.001**
	Total cholesterol : HDL	7481	5.4 (12.1)	**<0.001**	7364	4.0 (21.0)	**<0.001**	6339	5.1 (13.4)	**<0.001**	7481	5.5 (12.7)	**<0.001**
IGF-1 (mmol/L)	Glucose	7485	1.0 (0.5)	0.731	7368	1.0 (1.3)	0.534	6344	1.7 (0.6)	0.761	7485	1.0 (1.0)	0.694
	Triglyceride	8320	2.4 (2.2)	**<0.001**	8195	1.4 (14.6)	0.142	7057	2.5 (3.4)	**<0.001**	8320	1.1 (13.0)	0.145
	TyG index	7483	2.9 (2.9)	**<0.001**	7366	1.0 (17.6)	0.755	6342	3.7 (4.1)	**0.001**	7483	1.0 (15.2)	**<0.001**
	HDL	8318	1.0 (2.1)	**0.033**	8193	2.0 (4.5)	0.404	7055	3.8 (2.3)	0.145	8318	1.4 (3.3)	0.756
	LDL	7484	5.0 (1.7)	**0.025**	7367	5.2 (14.4)	**0.012**	6342	2.0 (2.8)	0.338	7484	1.5 (9.6)	**<0.001**
	Total cholesterol	8305	1.0 (1.5)	0.053	8180	1.6 (8.0)	0.538	7044	1.0 (2.2)	0.053	8320	1.1 (13.0)	0.145
	Total cholesterol : HDL	7481	2.5 (1.5)	**<0.001**	7364	1.3 (19.8)	0.225	6339	2.4 (3.2)	**0.001**	7481	1.5 (13.0)	**<0.001**

P-value represents significance for smoothed term in the GAM. Model parameters shown as deviance explained in percentage (dev exp %) with estimated degrees of freedom (EDF) in brackets. Bold face text denotes significant p-value (<0.05). Sociodemographic factors

(SDF) included were age, ethnicity and deprivation. Model 1 was adjusted for SDF. Model 2 was adjusted for SDF and fat mass (%). Model 3 was adjusted for SDF and summed physical activity (MET min/wk). Model 4 was adjusted for SDF and estradiol, CRP and IGF-1 (except the exposure of interest). CRP, C-reactive protein; EDF, estimated degree of Freedom; IGF-1, insulin like growth factor-1; LDL, low density lipoprotein; HDL, high density lipoprotein; TyG index, triglyceride to glucose index.

3.4.7. Impact of menstrual cycle symptoms in the relationship between metabolites with menstrual cycle status, estradiol and inflammatory markers.

We conducted a sensitivity analysis in the cohort of females who did not report any menstrual cycle symptoms (abdominal discomfort, abdominal pain, menstrual cramps or other problems with their menstrual cycle). Non-linear relationships with menstrual cycle phase were no longer observed for CRP (EDF= 2.0, p= 0.129) (**Appendix 2.9**). In covariate analyses (continuous & sub-group), the relationship between metabolites and menstrual cycle status in this sensitivity analysis were generally similar, except for that of lipids in some models (**Appendix 2.10, Appendix 2.11**). Similarly, the relationship between metabolites with estradiol, CRP and IGF-1 was generally similar in this sensitivity analysis, except for that of lipids in some models (**Appendix 2.12**).

3.5. Discussion

In this population based study using data from 8,694 UK Biobank participants, we observed non-linear associations between menstrual cycle status and total cholesterol, HDL, LDL and total cholesterol:HDL, but not for glucose, triglyceride nor TyG index. When body composition, physical fitness or physical activity levels were included in the models as covariates, we observed a non-linear relationship between menstrual cycle status and all metabolites in at least one of the models. Sub-group analysis determined the magnitude of variation in metabolite across the menstrual cycle varies by categories of covariates. In particular, greater variation in metabolites was consistently observed across the menstrual cycle in highest two quartiles of fat mass and lowest two categories of physical activity. In the whole cohort, non-linear associations were observed between menstrual cycle status and estradiol, CRP and IGF-1. Additionally, estradiol, CRP and IGF-

1 were associated with all metabolites. The associations between estradiol and CRP with metabolites were generally consistent irrespective of fat mass, physical activity and estradiol, CRP and IGF-1 (excluding exposure variable of interest).

During the luteal phase of the menstrual cycle, previous literature has observed a reduction in circulating glucose (4, 190) accompanied by an increase in whole-body insulin resistance (4, 185–188, 264, 265). However, research remains inconsistent (21–24). Our data does not provide evidence supporting a non-linear relationship between menstrual cycle status and glucose or TyG index, an index of whole-body insulin sensitivity prior to considering covariates in the analysis. Females in this study had a higher BMI and age (26.2 ± 5.2 kg/m^2; 44.9 ± 2.8 yr) compared with some previous literature (4, 185–188, 190, 265), which may have contributed to discrepancies in findings.

A non-linear association with menstrual cycle status was observed for cholesterol profiles, but not triglyceride. Total cholesterol, LDL and total cholesterol:HDL were highest in the early-follicular phase and declined during the luteal phase, whereas HDL reached a peak during the late-follicular phase. This finding is consistent with others reporting favourable lipid profiles in the mid-luteal phase and confirms this pattern of rhythmicity exists in a larger population (5, 23, 190, 195). Estradiol was negatively associated with total cholesterol, LDL and total cholesterol:HDL and positively associated with HDL. This is congruent with reported effects of endogenous estradiol on lipoprotein metabolism. Estradiol promotes the synthesis of VLDL and inhibits lipoprotein lipase, thereby reducing LDL and increasing HDL formation (197). Reductions in total cholesterol and LDL during the mid-luteal phase likely also reflect an increased uptake in

the corpus luteum to support steroidogenesis (322). Progesterone may exert antagonistic effects to the actions of estradiol on lipid regulation (197). However, in the UK Biobank, progesterone was not assessed. Thus, we are unable to examine the metabolic actions of synchronous estradiol and progesterone peaks in the mid-luteal phase. Future prospective cohort studies assessing ovarian hormones should endeavour to analyse all ovarian hormones, including progesterone, to support endocrine physiology research in females.

Non-linear relationships between menstrual cycle status and total cholesterol, HDL, LDL and total cholesterol:HDL were detected independent of body composition, physical activity or fitness. Additionally, sub-group analysis determined the magnitude of variation in glucose, TyG index and lipid profiles across the menstrual cycle differed by categories of fat mass and physical activity. Greater variation in all metabolites across the menstrual cycle was consistently observed in the highest two quartiles of fat mass and lowest two categories of physical activity (1-25 % greater, **Appendix 2.4**). Similarly, a recent study identified greater variation in glucose and insulin resistance across the menstrual cycle in individuals with a high BMI (> 25 kg/m^2) and low physical activity levels (< 500 MET min/wk) (323). Consistent with these findings, others report adiposity and physical activity are associated with increased intra-individual variability in glycaemic and lipidemic control in females (324, 325). However, the precise mechanisms that underlie the observed increases in magnitude of variation in metabolites across the menstrual cycle with increasing adiposity and low physical activity remain to be determined.

HbA1c reflects chronic blood glucose levels and is a stable clinical marker for insulin resistance. HbA1c did not exhibit a non-linear relationship with menstrual cycle status and undergoes less variation across the menstrual cycle when compared with glucose and lipid profiles. Therefore, in the clinical assessment of metabolic disease, HbA1c represents a stable biological marker irrespective of menstrual cycle phase in healthy regularly menstruating females. No previous research has examined the role of HbA1c in the variation in metabolites across the menstrual cycle. The relationship between menstrual cycle status and metabolite concentration was independent of HbA1c. In sub-group analysis, no consistent findings were observed between categories of low, medium and high HbA1c levels. These findings suggest HbA1c does not represent a mediatory variable underpinning variation in metabolic control across the menstrual cycle. Others have also reported that intra-individual variation in glucose and insulin resistance is not associated with baseline HbA1c levels (294).

Low grade inflammation is involved in the development of insulin resistance (326–329) and accordingly is an independent risk factor associated with hyperglycaemia (328, 330), hyperinsulinemia (328, 331) and dyslipidaemia (327). CRP is a circulating marker of low-grade systemic inflammation. In agreement with previous reports, CRP exhibited a non-linear relationship with menstrual cycle status, reaching a peak during the mid-luteal phase (22, 300, 332). We were able to expand on previous research by demonstrating levels of CRP across the menstrual cycle were positively associated with glucose, TyG index, total cholesterol, LDL and total cholesterol: HDL and negatively associated with HDL, independent of fat mass percentage and physical activity levels. Additionally, adjustment for estradiol and IGF-1 did not affect the relationship between CRP and metabolites, suggesting that the metabolic actions of CRP may be independent of ovarian

hormones and anti-inflammatory markers. In sub-group analysis, we determined that the magnitude of variation of CRP across the menstrual cycle was greater in higher quartiles of fat mass and low categories of physical activity (**Appendix 2.13**). This may contribute to the greater variation in metabolites across the menstrual cycle observed in individuals with greater adiposity and lower physical activity levels. Taken together, our data supports the hypothesis that elevated low grade-inflammation during the mid-luteal phase may contribute to the decline in metabolic control observed during this menstrual cycle phase. However, future research is required to determine whether the relationship between low-grade inflammation and metabolic control across the menstrual cycle is causal.

A non-linear relationship was observed between IGF-1 and menstrual cycle status, as previously reported (333). Increased IGF-1 during the ovulatory and luteal phase is likely underpinned by elevated estradiol concentration, which increases GH secretion and in turn stimulates hepatic IGF-1 synthesis (334). In agreement with previous literature we observed negative relationships between IGF-1 and glucose, insulin resistance and lipids (335). However, the relationship between IGF-1 and each metabolite did not persist after adjusting for fat mass or estradiol and CRP. This suggests the metabolic effects of IGF-1 across the menstrual cycle may be mediated by simultaneous variations in estradiol and/or CRP.

Sensitivity analysis for menstrual cycle symptoms was conducted by excluding females with menstrual cycle symptoms from the cohort. In this analysis, a non-linear association between CRP and menstrual cycle phase was no longer observed (**Appendix 2.9**). This observation is congruent with previous reports observing a positive relationship

between magnitude of variation in CRP across the menstrual cycle and menstrual cycle symptoms (332, 336). Additionally, in the sub-group analysis some differences were observed in the association between menstrual cycle status and lipid profiles, but not glucose or TyG index (**Appendix 2.11**). Given that CRP is an inflammatory hormone involved in the regulation of adipose tissue metabolism (127), increased fluctuation of CRP across the menstrual cycle in females with greater menstrual cycle symptoms may contribute to the differential regulation of lipid profiles.

Intra-individual glucose and lipid variability are independent risk factors for metabolic disease in healthy (337) and T2D females (293, 294). We detected non-linear relationships between menstrual cycle status and lipid profiles, with a variation of 2.2-4.2 % across the menstrual cycle. Additionally, sub-group analysis determined the variation in glucose, triglyceride, TyG index, and lipid profiles across the menstrual cycle was increased in individuals in the highest two quartiles of fat mass and lowest two categories physical activity (up to 25 %). This magnitude of variation in metabolites across the menstrual cycle is greater than reported within cycle phase variation (1-9 %) (23) and analytical technical coefficient of variation (1-2 %) (309). Given the metabolic importance of intra-individual variability in the onset, progression and associated complications of metabolic disorders, this may have important clinical implications to metabolic health in regularly menstruating females.

Our study has several limitations. The cohort of UK Biobank participants included in this study were middle aged (40-50 yr). We ensured any females in the peri-menopausal stage were omitted from the cohort by including irregular menstrual characteristics in the exclusion criteria. Additionally, females with estradiol values < 31 pmol/L or >2864

pmol/L were excluded, as the perimenopausal period of the reproductive cycle is characterised by abnormally high or low ovarian estradiol production (338). Nonetheless, findings from this study must be extrapolated to younger populations with caution. Fasting blood samples were not collected in the UK Biobank. Therefore, any participant with a fasting duration less than 4 hours was excluded from analysis. This cut-off threshold was selected based on evidence demonstrating semi-fasted (> 4 hr) measures of blood glucose and lipids are not significantly different and are closely correlated to fasted measures (> 8 hr) (71, 72, 114, 339). Differences between consecutive hours of fasting were not detected for glucose nor lipids (**Appendix 2.14, Appendix 2.15).** To further ensure fasting duration did not affect results, we conducted sensitivity analysis by adjusting analysis for fasting duration; similar results were obtained in all models (**Appendix 2.2, Appendix 2.3, Appendix 2.5, Appendix 2.8**). Therefore, we are confident that the semi-fasting nature of blood samples did not affect our results. Some variables used for sub-group analysis demonstrated a non-linear relationship with menstrual cycle phase (fat mass, fat-free mass, grip strength, CRF). Therefore, results from sub-group analysis must be interpreted with caution.

The large prospective nature of the UK Biobank data is a substantial strength of this study. We analysed data from 8,694 regularly menstruating females; the largest study to date examining the variation in metabolites across the menstrual cycle. Moreover, detailed information collection on menstrual cycle characteristics allowed the exclusion of females with any symptoms of irregular menstrual cycles and peri-menopausal status.

In conclusion, our study confirms previous findings reporting lipid profiles exhibit a non-linear association with menstrual cycle status. We expanded on previous work to identify

fat mass, fitness and physical activity levels as key modifiable risk factors underpinning the magnitude of variation in glucose, triglyceride, TyG index, total cholesterol, HDL, LDL and total cholesterol:HDL across the menstrual cycle. These findings should be considered in therapeutic strategies to mitigate disturbances in metabolic control across the menstrual cycle. Furthermore, we identified that inflammatory markers (CRP and IGF-1) are associated with variation in metabolites, which may underpin concomitant variation in metabolic control across the menstrual cycle. Further work is required to examine whether these relationships represent a causal mechanism underpinning variation in metabolic control across the menstrual cycle.

CHAPTER 4: Changes in adipose tissue microRNA expression across the menstrual cycle in regularly menstruating females: a pilot study.

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4.1. Abstract

Cyclical changes in hormone profiles across the menstrual cycle are associated with alterations in metabolic control (4, 5). miRNAs contribute to the regulation of metabolic control, including adipose tissue metabolism. Adipose tissue miRNA expression levels are regulated by chronic alterations in the hormonal milieu in females (36, 256). However, the effect of acute fluctuations in ovarian hormone profiles across the menstrual cycle on adipose tissue miRNA expression remain unknown.

Eleven healthy, regularly menstruating females underwent four laboratory sampling visits across their menstrual cycle. Subcutaneous abdominal adipose tissue and venous blood samples were collected at each sampling visit. LH tests, calendar counting, and serum hormone concentrations were used to determine menstrual cycle phases: early-follicular (EF); late-follicular (LF); post-ovulatory (PO) and mid-luteal (ML). Serum FSH, LH, estrogen, progesterone and testosterone were determined using multiplex magnetic bead panels and enzyme-linked immunosorbent assays (ELISA). Global adipose tissue

miRNA expression levels were determined via microarray in a subset of participants (N=8) and 16 candidate miRNAs validated by RT-qPCR in the whole cohort (N=11).

Sampling visits occurred on cycle day; 3 ± 1 (EF), 11 ± 1 (LF), 16 ± 2 (PO) and 24 ± 3 (ML). Concentrations of FSH, LH, estradiol, progesterone and testosterone underwent expected fluctuations across the menstrual cycle. Global analysis of adipose tissue miRNA expression identified 33 miRNAs were differentially expressed across the menstrual cycle; however, no differences remained after correction for multiple testing ($p > 0.05$). RT-qPCR analysis of 16 candidate miRNAs revealed miR-497-5p was differentially expressed across the menstrual cycle ($R^2 = 0.18$, $p = 0.03$); however, post hoc tests did not reveal any significant differences between menstrual cycle phases ($p > 0.05$). miR-30c-5p was negatively associated with testosterone levels ($R^2 = 0.13$, $p = 0.033$).

These pilot data indicate differential expression of adipose tissue miRNAs in healthy females across the menstrual cycle. Additionally, we observed a weak association between adipose tissue miRNAs with ovarian hormones. Further research in larger sample sizes is required to confirm regulation of miRNA expression across the menstrual cycle. Furthermore, miRNAs with the strongest tendency for differential expression between menstrual cycle phases shared common targets related to insulin signalling pathways. Future studies would also benefit from concurrent analysis of mRNA and miRNA expression to gain further mechanistic insight into the molecular consequences of altered miRNA expression. These studies are necessary to fully understand the role of varying miRNA expression across the menstrual cycle on metabolic control.

4.2. Introduction

Approximately 50 % of the female population are of reproductive age, accounting for 1.9 billion females globally (340, 341). The menstrual cycle is a fundamental biological rhythm governing female physiology occurring over a typical duration of 24-35 d (14). The menstrual cycle is characterized by hormonal fluctuations within the HPO axis; gonadotropin, pituitary hormones (FSH and LH) and ovarian hormones (estradiol, progesterone and testosterone) (1). Variation in hormonal profiles across the menstrual cycle increases the complexity of female physiology.

Cyclical fluctuations in hormonal profiles between menstrual cycle phases are associated with variable metabolic control in regularly menstruating females. Studies report elevated insulin during the luteal phase, alongside reduced triglyceride, glucose and cholesterol (4, 5, 190, 296). Correspondingly, glycaemic control, lipid control, IR and energy expenditure differ between menstrual cycle phases (4, 5, 192, 264). Moreover, variation in several metabolic parameters across the menstrual cycle, including insulin sensitivity, glucose and lipoprotein levels, are associated with estradiol and progesterone concentrations (4, 5). However, to fully understand changes in metabolic control across the menstrual cycle, it is crucial to examine metabolic responses at a tissue-specific level.

Adipose tissue is metabolically receptive to changes in the ovarian hormonal milieu. Estrogen, testosterone and progesterone regulate a range of metabolic pathways within adipocytes including lipolysis, lipogenesis and insulin sensitivity (2, 3, 26–28). Correspondingly, acute and chronic alterations to ovarian hormone profiles, including menstrual cycle phase, estrogen supplementation and menopausal status, are associated

with alterations in adipose tissue metabolism (204, 342, 343). These parameters include basal lipolysis, stimulated lipolysis and insulin action (204, 342, 343). Thus, hormonal regulation of adipose tissue metabolism across the menstrual cycle may underpin variation in parameters of whole-body metabolic control; however, the associated molecular mechanisms remain uncertain.

One potential mechanism for the regulation of adipose tissue metabolism is through miRNA expression. miRNAs are short non-coding RNAs that regulate gene expression at a post-transcriptional level through binding to the 3'UTR region of target mRNA transcripts (218). Approximately 60% of known protein-coding genes have target sites for miRNA binding, and thus miRNAs are recognized as major regulators of mRNA translation (217). In adipose tissue, miRNAs target components of pathways involved in metabolic control, including lipid transport, lipolysis, lipogenesis and insulin signalling (30, 31, 344). Divergent adipose tissue miRNA profiles have been observed in response to chronically altered ovarian hormone profiles, including menopausal status (36), hormone supplementation (36), polycystic ovary syndrome (37) and ovariectomy (38). A recent study reported upregulated adipose tissue expression of miR-16-5p, miR-451a, miR-223-3p, miR-18a-5p, miR-19a-3p, miR-363-3p and miR-486-5p between hormone replacement therapy users and non-users in post-menopausal females (36). However, the few studies that have investigated miRNA expression in response to acute hormone fluctuations across the menstrual cycle have yielded equivocal results (257, 258). Moreover, previous reports are limited to analysis of plasma miRNA expression across the menstrual cycle, which may not be representative of adipose tissue miRNA expression (36, 257, 258). Investigating the effect of menstrual cycle phase on adipose tissue miRNA

expression will help unravel mechanisms involved in metabolic control across the menstrual cycle.

In the present pilot study, firstly we aimed to characterize adipose tissue miRNA expression profiles across the menstrual cycle. To achieve this goal, we conducted exploratory analysis utilizing microarrays in a subset of participants followed by RT-qPCR validation of candidate miRNAs in the whole cohort. Secondly, we aimed to investigate the association between ovarian hormone concentration and miRNA expression.

4.3. Methods

4.3.1. Ethical approval

Ethical approval was obtained from the NHS, Invasive and Clinical Research (NICR) ethical committee at the University of Stirling (NICR 17/18 32). This study was conducted in accordance with the Declaration of Helsinki. Following written and verbal explanation of the study procedures, participants provided informed written consent.

4.3.2. Participants

A total of 11 healthy, pre-menopausal females aged 22-30 yr were recruited from the University of Stirling and surrounding areas via poster and online advertising. Participant characteristics are detailed in **Table 10.** The inclusion criteria were: aged 18-40 yr; recreationally active; no previous diagnosis of a menstrual cycle disorder; no use of hormone based medication within the previous three months; and, not pregnant within the previous three months. Additionally, participants completed a menstrual cycle

questionnaire (**Appendix 3.1**) and were excluded if they exhibited any symptoms of an irregular cycle within the previous three months: menstrual cycle length <21 d or >38 d; abnormal menstrual bleeding; menstrual bleeding ≥7 d; abnormal menstrual cramps; or, absence of a period.

4.3.3. Experimental design

All participants attended the laboratory for a pre- sampling visit, followed by four identical sampling visits across the menstrual cycle in a repeated measures design. Four participants completed the sampling visits across two consecutive menstrual cycles either due to failure to obtain a positive ovulation test during the first cycle (n=2) or due to unavailability to attend all scheduled sampling visits during the first cycle (n=2).

4.3.4. Experimental protocol

*Pre-sampling visi*t: Participants were provided with a menstrual cycle diary (**Appendix 3.2** and urinary LH tests (Digital Ovulation Test, Clearblue, Switzerland), to monitor one menstrual cycle prior to the sampling visits. The menstrual cycle diary detailed; menstrual cycle duration, menstrual bleeding duration, date of positive LH test and menstrual cycle symptoms. Based on responses collected in the menstrual cycle diary, participants were excluded at this stage if they reported any symptoms of an irregular cycle, as previously described. In addition, participants were provided with a 48 h food diary to be completed on cycle days 3 and 4, which recorded the time, quantity and preparation method of all meals, snacks, and drinks.

Sampling visits: Participants attended the laboratory for four identical sampling visits during the following menstrual cycle phases; early-follicular (EF), late-follicular (LF),

post-ovulatory (PO) and mid-luteal (ML). Menstrual cycle phase was determined using the calendar counting method in conjunction with urinary LH tests (Digital Ovulation Test, Clearblue, Switzerland) as follows: EF- cycle day 1-5; LF- cycle day 9-11; PO- positive LH test + 2 d; ML- positive LH test + 8-10 d, dependent on menstrual cycle length. Menstrual cycle phase was retrospectively confirmed via serum hormone analysis. Prior to each sampling visit participants underwent a 10-12 h fast and abstained from alcohol and caffeine for 24 h. To control participants' dietary intake preceding each sampling visit, participants were instructed to repeat food intake as recorded in the 48 h food diary prior to each sampling visit. Participants were instructed to maintain habitual levels of daily living and physical activity throughout the duration of the study, but to abstain from physical exercise in the 48 h preceding each sampling visit. Participants attended the laboratory between 07:00-10:00, with each subsequent sampling visit scheduled for a time within 1 h of the initial sampling visit. Each sampling visit consisted of the following protocol: 15 min rest in a semi-supine position upon arrival at the laboratory; collection of a venous blood sample; and, an adipose tissue biopsy.

4.3.5. Blood sampling:

Venous blood samples were collected by a trained phlebotomist into 6 ml serum vacutainers (Becton, Dickinson and Company, New Jersey, US). Blood samples were allowed to clot for 60-120 min at room temperature and then centrifuged at 2,500 g for 10 min at 4 ^{o}C for serum separation. Serum was aliquoted into 1.5 ml microcentrifuge tubes and stored at -80 ^{o}C until analysis.

4.3.6. Adipose tissue biopsy sampling:

Abdominal adipose tissue biopsies were obtained 5-10 cm lateral of the umbilicus under local anesthesia (2 % lidocaine) using a mini-liposuction technique (345). Samples were cleaned of visible contaminants (connective tissue, blood, vasculature) and washed in 0.9 % sodium chloride saline over sterile gauze. Tissue was split into similarly sized pieces (~50-150 mg), placed into an RNA-free 1.5 ml microcentrifuge tube containing 5-10 x tissue volume RNA*later* (ThermoFisher, UK, cat no. AM7020), flash frozen in liquid nitrogen and stored at -80 ^{o}C until analysis. All adipose tissue samples were processed and frozen within 3 min of sampling. Whilst preparing the methodology for this experimental study, my input was instrumental in setting up the subcutaneous adipose tissue biopsy protocol described above within the Physiology, Exercise and Nutrition Research Group at the University of Stirling. Refer to the appendices for the full, published protocol.

4.3.7. Hormone analysis

Serum estrogen, testosterone, FSH and LH concentrations were determined using magnetic bead panels (MilliporeSigma, Massachusetts, US, cat no. HPTP1MAG-66k, PTP1MAG-66K-02) analyzed using the Luminex 200 instrument, according to the manufacturer's protocol. Serum progesterone were determined using an Enzyme-linked immunosorbent assay (ELISA) (R&D systems, Minnesota, US, cat no. NBP2-60124), according to the manufacturer's protocol. The intra-assay coefficients of variation (CV) were as follows: FSH= 6.4 %; LH= 8.69 %; estradiol= 12.0 %; progesterone= 8.3 %; testosterone= 10.4 %. These intra-assay CVs are similar to the intra-assay coefficients of variation reported in the manufacturers protocol (FSH < 10 %, LH < 10 %, estradiol < 15 %, progesterone < 15 %, testosterone < 10 %). All sample concentrations were above the

minimum limit of detection reported in the manufacturers protocol for each assay (FSH= 0.01 mIU/mL, LH= 0.01 mIU/mL, estradiol= 0.01, progesterone= 0.5 ng/mL, testosterone 0.08 ng/mL).

4.3.8. RNA extraction

Adipose tissue was mechanically homogenized using 1.4 mm ceramic beads (Qiagen, Germany, cat no. 13113-50) and the MagNA Lyser instrument (Roche, Switzerland) with 3 x 20 s bouts of homogenization interspersed with 20 s rest. Total RNA was isolated from 30-100 mg homogenized adipose tissue in a combined protocol using TRIzol reagent (ThermoFisher Scientific, UK, cat no. 15596026) and the miRNeasy mini lipid kit (Qiagen, Germany, cat no. 217004), as previously described (346). Final elution volume for isolated RNA was 30 µL. Concentration (µg/mL) and purity (absorbance ratio 260/280 nm) of isolated RNA samples were assessed using a DS-11 FX + spectrophotometer (DeNovix, Delaware, US). RNA samples were stored at -80 ^{o}C until analysis.

4.3.9. Global miRNA expression analysis

Global miRNA expression was examined using microarrays in a subset of participants at all timepoints (N=32; 8 participants, 4 timepoints). Samples were selected from the 8 participants with the highest RNA quantity and purity across all timepoints (Concentration: 25-92.6 µg/mL; 260/280: 1.9-2.1 nm). 100 ng RNA were labelled using the Flashtag Biotin HSR labelling kit (ThermoFisher Scientific, UK, cat no. 901910). Hybridization cocktails were prepared using the GeneChip hybridization kit (ThermoFisher Scientific, UK, cat no. 900720). Biotin labelled samples were hybridized overnight onto GeneChip miRNA 4.0 arrays (ThermoFisher Scientific, UK, cat no. 902413) according to manufacturer's instructions. The arrays were washed and stained using the

GeneChip wash and stain kit (ThermoFisher, UK, cat no. 902413) and the Affymetrix fluidics station 450, as per manufacturer's instructions. GeneChips were scanned by the Affymetrix scanner 3000 7G. Microarray signal intensities were normalized using the variance stabilization normalization 2 (VSN2) algorithm and probe level summarization conducted using robust multi-chip analysis (RMA) (347). Microarray data has been deposited to the Gene Expression Omnibus (GEO) with the accession number GSE180625.

4.3.10. RT-qPCR

A total of 17 candidate miRNAs were selected for validation using RT-qPCR in the whole cohort (N= 44; 11 participants, 4 timepoints) from the subset of miRNAs differentially expressed across the menstrual cycle, as determined via microarray. Candidate miRNAs were selected based on association with hormones across menstrual cycle phases and reported association with adipose tissue function. Analysis of microarray data via NormFinder (348) reported miR-155-5p, miR-324-3p, miR-331-3p and miR-328-5p had optimal stability values and were selected as potential endogenous qPCR control miRNAs for RT-qPCR analysis (348). RT-qPCR was performed using miRCURY LNA custom PCR panels with pre-coated miRNA primers (Qiagen, Germany, cat no. 339330), according to the manufacturers protocol. Briefly, cDNA synthesis reactions comprised of 2 µL miRCURY RT reaction buffer, 4.5 µL RNase-free water, 1 µL 10x miRCURY RT enzyme mix, 0.5 µL UniSp6 RNA spike-in and 2 µL template RNA (5 ng/ µL) in a 10 µL total reaction volume. UniSp6 (Qiagen, UK, cat no. 339390) were added to cDNA synthesis reactions to analyze cDNA synthesis efficiency. Reverse transcription temperature cycling was performed using a LightCycler 480 (Roche, Switzerland) according to manufacturer's instructions. Conditions were 60 min at 42^{o}C, 5 min at 95^{o}C and then immediate cooling

to 4°C. cDNA samples were assayed immediately by qPCR or stored at -20°C until analysis. qPCR reaction volume comprised of 5 µL miRCURY LNA SYBR Green Master Mix (Qiagen, Germany, cat no. 339345), 4 µL cDNA template (diluted 1:80) and 1 µL RNase-free water, in a total reaction volume of 10 µL. UniSp3 were included in miRCURY LNA custom PCR panels to perform inter-plate calibration. miRNA target sequences are supplied in **Appendix 3.3.** qPCR temperature cycling was performed using a LightCycler 480 (Roche, Switzerland) according to manufacturers instructions, consisting of 95°C for 2 min, followed by 45 cycles at 95°C for 10 s then 56°C for 60 s. All PCR cycles were finished with melting curve analysis to confirm specificity of primer annealing. All qPCR reactions were performed in triplicate and analyzed using the $2^{-\Delta\Delta Ct}$ method (349). Analysis of RT-qPCR data using NormFinder identified miR-324-3p and miR-331-3p to be the most stable pair of miRNAs from the four potential endogenous controls (miR-155-5p, miR-324-3p, miR-331-3p and miR-328-5p)(348). Raw Delta Ct (dCt) values were calculated relative to the geometric mean of the endogenous controls (miR-324-3p, miR-331-3p) and DeltaDelta Ct (ddCt) values calculated relative to the EF phase.

4.3.11. miRNA-mRNA pathway analysis

MiRsystem was used to conduct miRNA-mRNA pathway analysis using RT-qPCR results for miRNA expression (350). miRsystem integrates seven miRNA target gene prediction databases (DIANA, miRanda, miRBridge, PicTar, PITA, rna22 and TargetScan) to enable prediction of target genes and functional pathways.

4.3.12. Statistical analysis

A priori power calculations were conducted in G*Power 3.1 (351). miRNA expression was not included in power calculations due to a lack of suitable published data reporting

miRNA expression across similar menstrual cycle phases. Power calculations were conducted using reported mean and standard deviation of estradiol and progesterone concentrations at EF, LF, PO and ML (57, 351, 352) to ensure appropriate statistical power to differentiate between menstrual cycle phases. Testosterone was omitted from power calculations due to relatively small changes between menstrual cycle phases. G*Power reported N = 11 was required to achieve 80% power ($\alpha = 0.05$) to detect changes in estrogen and progesterone between menstrual cycle phases.

All statistical analyses were conducted using R version 3.6.3 (279). Hormone data were log transformed for normality and this was confirmed using the Shapiro-Wilk test. One-way repeated measures ANOVA were used to examine the effect of menstrual cycle phase on hormone concentration. Pairwise comparisons were conducted with Tukey adjustment. To determine differential microarray expression across the menstrual cycle, moderated empirical Bayes ANOVA was conducted and corrected for multiple testing using the false discovery rate (FDR) method, in the 'limma' package (353). Linear mixed effect models were conducted to examine the relationship between menstrual cycle phase and ovarian hormone concentration with candidate miRNA expression using RT-qPCR data, in the 'lme4' package (319). A participant identifier was included in models as a random effect to account for repeated measures. P-values were obtained using the Kenward-Roger approximation for denominator degrees of freedom (354). Pairwise comparisons were conducted with Tukey adjustment. Effect sizes where calculated as adjusted R^2 for fixed effects with Cohen's descriptors used to interpret effect sizes; 0.02 small, 0.13 moderate and 0.26 large (355). In all analyses, miRNA expression was adjusted for age due to the reported association between age and basal adipose miRNA expression in regularly menstruating females (36). Unadjusted candidate miRNA

expression data are presented in **Appendix 3.6**. A p-value < 0.05 was considered statistically significant.

To provide sample size estimations for future studies using data generated in this study, power analysis for linear mixed models were conducted using the package 'simr' in R (356). 'simr' generates power curves for sample size estimation based on Monte Carlo simulations.

4.4. Results

4.4.1. Menstrual cycle characteristics:

Participant characteristics are shown in **Table 10**. All participants reported regular menstrual cycles during the course of the study, as assessed via the menstrual cycle diary. Sampling visits occurred on cycle day; 3 ± 1 (EF), 11 ± 1 (LF), 16 ± 2 (PO) and 24 ± 3 (ML). Serum analysis of hormones showed expected temporal changes in LH, FSH, estradiol, progesterone and testosterone profiles between menstrual cycle phases (**Table 11**). FSH was lower during ML compared to EF, LF and PO ($p< 0.001$; $p= 0.011$; $p< 0.001$). LH was higher during PO compared to EF and ML ($p= 0.006$; $p< 0.001$) and higher during LF compared to ML ($p= 0.005$). Estradiol was greater during LF, PO and ML compared to EF ($p= 0.002$; $p= 0.013$; $p< 0.001$). Progesterone was higher during ML compared to EF, LF and PO (all $p< 0.001$) and higher during PO compared to EF and LF ($p= 0.006$; $p= 0.003$). Testosterone was greater during PO compared to EF ($P= 0.024$).

Table 10: Participant characteristics

Participant characteristic	Value
Age (y)	27.4 ± 3.3
Body mass (kg)	65.8 ± 5.4
Stature (cm)	170.6 ± 5.8
BMI (m/kg^2)	22.6 ± 1.5
Cycle duration (d)	29.1 ± 3.2 (6.3%)
Follicular phase length (d)	15.5 ± 2.2 (9%)
Luteal phase length (d)	13.3 ± 1.3 (11%)

Values are presented as mean ± 1 SD (N=11). Average between cycle intra-subject coefficient of variation is shown in brackets.

Table 11: Ovarian and pituitary hormone concentrations during the early-follicular, late-follicular, post-ovulatory and mid-luteal phases of the menstrual cycle.

Hormone	Early-follicular	Late-follicular	Post-ovulatory	Mid-luteal	P-value
FSH (IU/ml)	4.89 ± 2.45	3.70 ± 2.53	4.81 ± 3.26	2.24 ± 1.63 *†‡	<0.001
LH (IU/ml)	4.90 ± 2.37	7.97 ± 7.05	12.13 ± 8.93 *	4.04 ± 3.08 †‡	<0.001
Estrogen (ng/ml)	0.10 ± 0.03	0.23 ± 0.10 *	0.21 ± 0.13 *	0.32 ± 0.11 *	0.001
Progesterone (ng/ml)	6.80 ± 2.89	7.14 ± 4.66	10.44 ± 3.28 *†	19.2 ± 3.94 *†‡	<0.001
Testosterone (ng/ml)	0.56 ± 0.18	0.63 ± 0.21	0.75 ± 0.31 *	0.62 ± 0.21	0.040

Values are presented as mean ± 1 SD (N=11). P- values represent one-way repeated measures ANOVA between menstrual cycle phases. * Significantly different from early follicular; † significantly different from late-follicular; ‡ significantly different from post-ovulatory. FSH, follicle stimulating hormone; LH, luteinizing hormone.

4.4.2. Global adipose tissue miRNA expression across the menstrual cycle

We analyzed global miRNA expression across the menstrual cycle in a subset of participants (N=32; 8 participants, 4 timepoints). Three arrays did not pass quality

control (PO: n= 2, ML: n= 1) and therefore final analysis was conducted on 29 samples. 729 miRNAs were considered expressed in adipose tissue samples at a median expression level ≥ 1.75. Differential expression across the menstrual cycle was observed in 33/729 miRNAs prior to correction for multiple testing (**Figure 12, Appendix 3.5**). No differences in miRNA expression across the menstrual cycle were observed following FDR correction for multiple testing (**Figure 12, Appendix 3.5**).

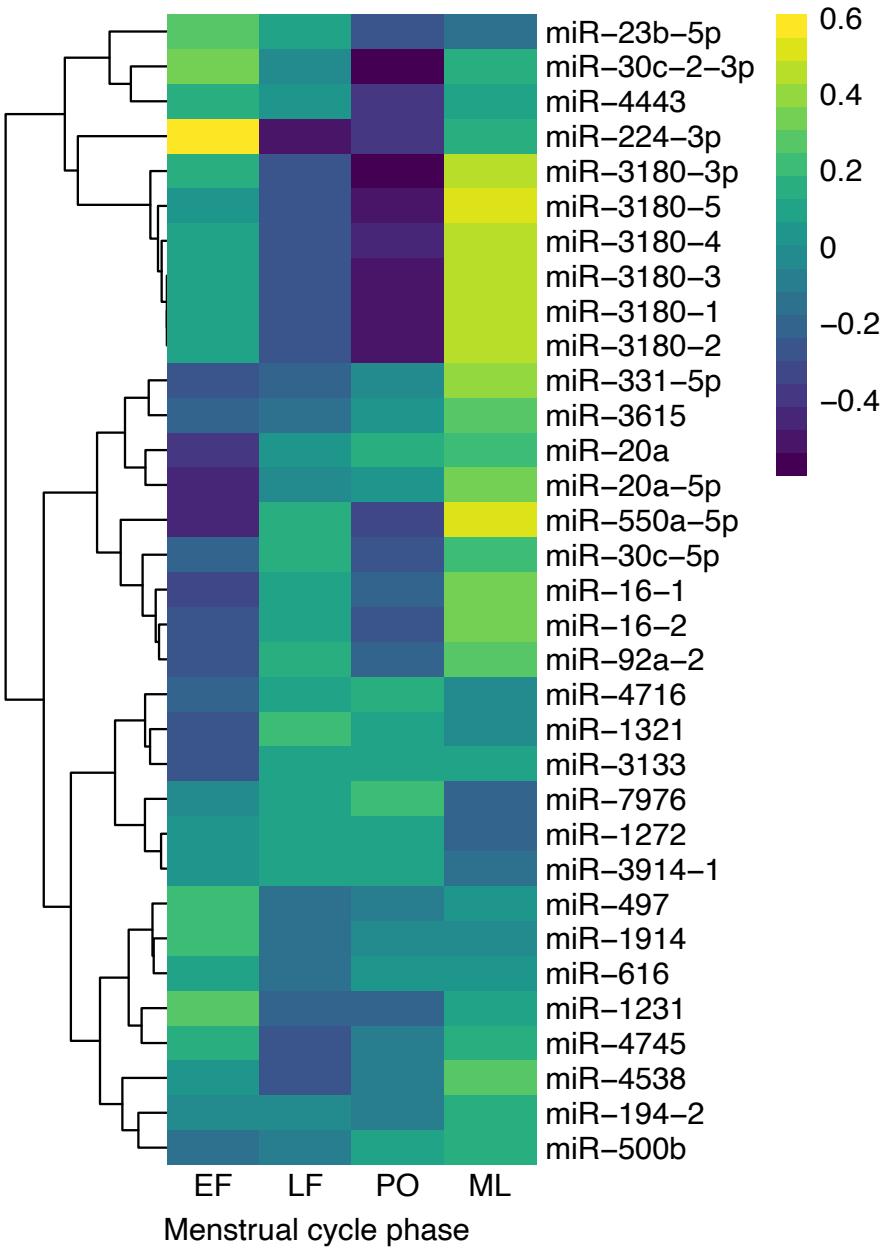

Figure 12: Clustered heatmap of microarray log10 z-scores in differentially expressed adipose tissue miRNAs in the early-follicular (EF), late-follicular (LF), post-ovulatory (PO) and mid-luteal (ML) phases of the menstrual cycle (N= 8). A p-value < 0.05 was considered statistically significant by moderated empirical Bayes ANOVA.

4.4.3. RT-qPCR adipose tissue miRNA expression across the menstrual cycle

A total of 17 candidate miRNAs were analyzed using RT-qPCR in the whole cohort (N=44; 11 participants, 4 timepoints). Out of the 4 miRNAs analyzed for use as potential endogenous controls, the 2 miRNAs which were not selected for use as endogenous controls (miR-155-5p, miR-328-5p) were included in the analysis. 3 miRNAs (miR-1231, miR-1914, miR-3180-3p) were not detected consistently in all participants and were removed from the analysis. **Figure 13** shows mean log fold change in expression level for each of the remaining 16 candidate miRNAs relative to endogenous controls and expression level in EF. miR-497-5p was differentially expressed across the menstrual cycle ($p= 0.030$, $R^2= 0.18$); however, post-hoc testing did not reveal any differences between menstrual cycle phases ($p> 0.05$). There was a tendency for differential expression of miR-224-3p ($p= 0.059$, $R^2= 0.07$) and miR-331-5p ($p= 0.059$, $R^2= 0.11$) across the menstrual cycle,.

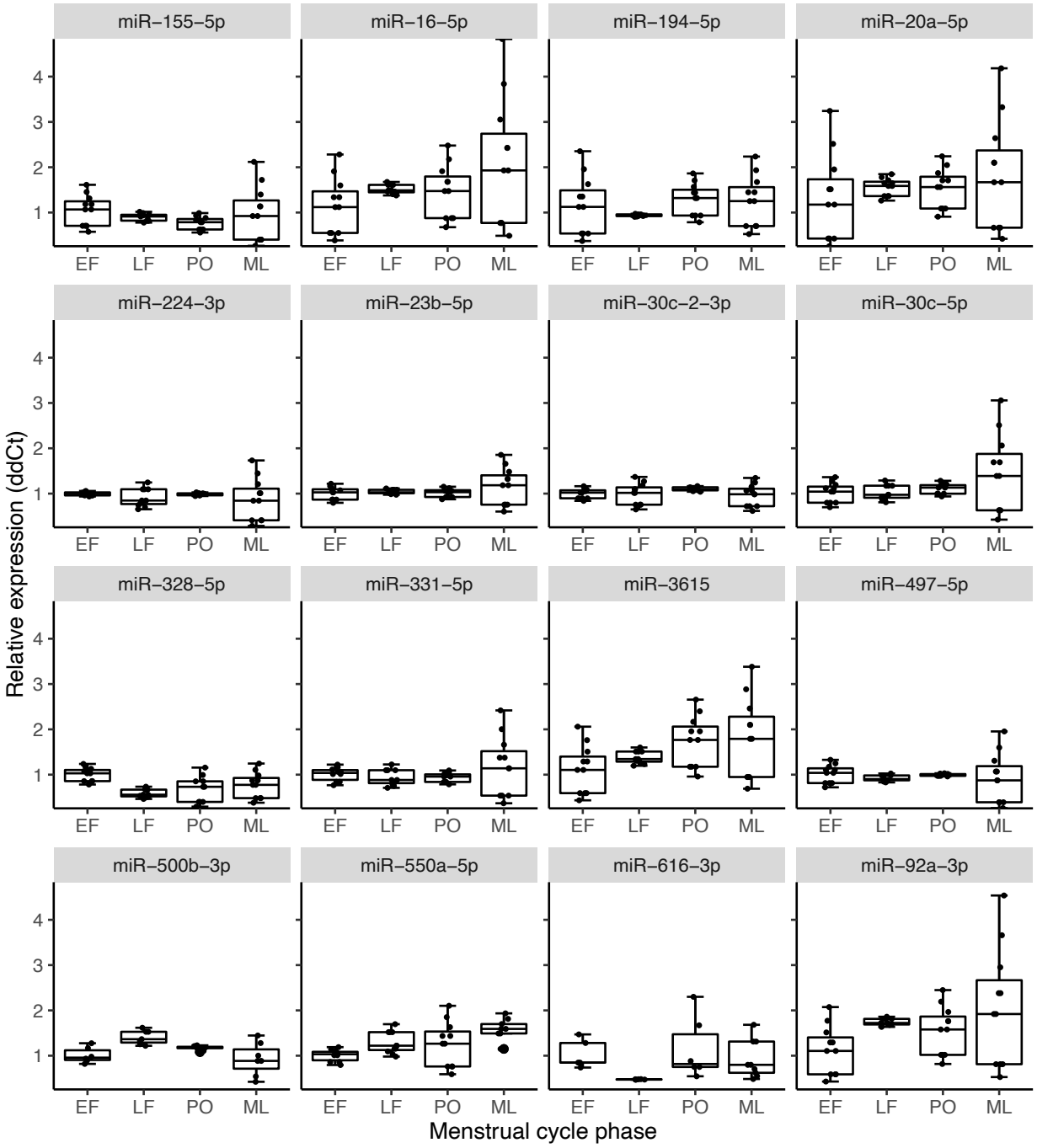

Figure 13: RT-qPCR analysis of adipose tissue miRNA expression in the early-follicular (EF), late-follicular (LF), post-ovulatory (PO) and mid-luteal (ML) phases of the menstrual cycle (N=11). miRNA expression represented as ddCt values adjusted for age. Data are displayed as conventional box and whisker plots with individual data points represented by circles.

4.4.4. Relationship between RT-qPCR miRNA expression and ovarian hormones

Linear mixed effect models were conducted to assess the relationship between hormone concentration and miRNA expression, as determined using RT-qPCR (**Table 12**). miR-30c-5p was negatively associated with serum testosterone (p= 0.033, R^2=0.13). miR-92a-3p had a tendency for a negative association with serum estradiol (p= 0.067, R^2= 0.14).

Table 12: Linear mixed effect models to assess the association between estradiol, progesterone and testosterone with ddCt miRNA expression.

miRNA	Estradiol	Progesterone	Testosterone	Estradiol to progesterone ratio
miR-155-5p	0.07 (0.657)	0.07 (0.726)	0.07 (0.846)	0.07 (0.920)
miR-16-5p	0.17 (0.118)	0.14 (0.315)	0.11 (0.479)	0.13 (0.593)
miR-194-5p	0.10 (0.383)	0.11 (0.286)	0.09 (0.433)	0.09 (0.913)
miR-20a-5p	0.16 (0.267)	0.15 (0.621)	0.12 (0.309)	0.16 (0.596)
miR-224-3p	<0.01 (0.461)	<0.01 (0.476)	<0.01 (0.717)	<0.01 (0.967)
miR-23b-5p	0.03(0.832)	0.04 (0.77)	0.03 (0.350)	0.04 (0.655)
miR-30c-2-3p	0.08 (0.155)	0.03 (0.722)	0.03 (0.826)	0.09 (0.099)
miR-30c-5p	0.04 (0.581)	0.05 (0.492)	**0.13 (0.033)**	0.03 (0.939)
miR-328-5p	0.04 (0.628)	0.07 (0.221)	0.09 (0.146)	0.09 (0.172)
miR-331-5p	0.02 (0.804)	0.03 (0.695)	0.03 (0.591)	0.03 (0.587)
miR-3615	0.09 (0.219)	0.08 (0.216)	0.06 (0.859)	0.07 (0.903)
miR-497-5p	0.08 (0.251)	0.06 (0.517)	0.06 (0.792)	0.06 (0.666)
miR-500b-3p	<0.01 (0.389)	<0.01 (0.376)	<0.01 (0.980)	0.01 (0.146)
miR-550a-5p	<0.01 (0.347)	0.01 (0.233)	<0.01 (0.809)	<0.01 (0.857)
miR-616-3p	<0.01 (0.571)	<0.01 (0.782)	<0.01 (0.943)	<0.01 (0.479)
miR-92a-3p	0.14 (0.067)	0.10 (0.399)	0.06 (0.277)	0.11 (0.395)

Data are presented as adjusted R^2 for fixed effects (p -value) (N=11). miRNA values input as ddCt. Estradiol to progesterone ratio calculated as estradiol in ng/mL divided by progesterone in ng/mL. Estradiol, progesterone, testosterone and estradiol to

progesterone ratio values were log10 transformed. Bold face text represents a statistically significant relationship (P< 0.05).

4.4.5. miRNA-mRNA pathway analysis

miRNA-mRNA pathway analysis was conducted using RT-qPCR results. miRNA-mRNA pathway analysis was performed with miR-497-5p due to differential expression across the menstrual cycle. Additionally, due to the tendency for differential expression across the menstrual cycle in miR-224-3p and miR-331-5p, miRNA-mRNA pathway analysis was also performed for this subset of miRNAs (miR-224-3p and miR-331-5p, miR-497-5p). Data are presented in **Figure 14, Appendix 3.8** and **Appendix 3.9.**

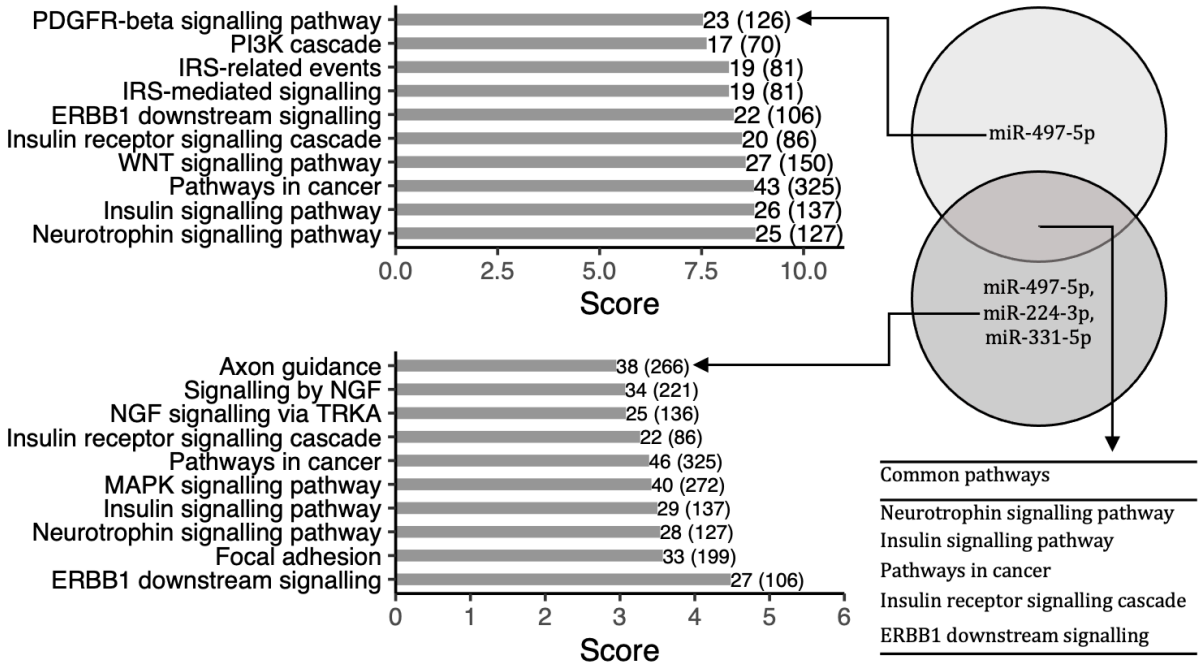

Figure 14: Functional pathway analyses were conducted using RT-qPCR results for miR-497-5p (top left) and miR-224-3p, miR-331-5p and 497-5p (bottom left) (N=11). Score is generated by the weight of miRNA expression (ddCt) times its enrichment -log (p-value). Numbers at the end of bars represent the total number of genes in the indicated term

with total union targets in term encased in brackets. ERRB1, epidermal growth factor; IRS, insulin receptor signaling; MAPK, mitogen activated protein kinase; NGF, nerve growth factor; PDGFR, platelet-derived growth factors; PI3K, phosphoinositide 3-kinase; TRKA, tropomyosin receptor kinase A; WNT, Wingless/Integrated.

4.5. Discussion

To the best of our knowledge, this is the first study to characterize adipose tissue miRNA expression across the menstrual cycle. Initially, we conducted an exploratory approach utilizing microarrays to analyze global miRNA expression in a subset of 8 participants selected from the whole cohort. Based on examination of microarray data distributions we identified 729 miRNAs expressed in adipose tissue samples in healthy, regularly menstruating females. 33 miRNAs were differentially expressed across the menstrual cycle, although no differences remained after correction for multiple testing using FDR. Validation via RT-qPCR was conducted on 16 candidate miRNAs in the whole cohort. RT-qPCR analysis determined miR-497-5p was differentially expressed across the menstrual cycle, whilst miR-224-3p and miR-331-5p exhibited a trend towards differential expression. In addition, we examined the relationship between miRNA expression and circulating hormone concentrations. Testosterone was negatively associated with miR-30c-5p, predicting up to 13 % of the variation in miRNA expression. We also observed a trend for a negative association between miR-92a-3p and estradiol, predicting up to 14 % of the variation in miRNA expression. These pilot data suggest adipose tissue miR-497-5p expression may be altered by menstrual cycle phase and adipose tissue miRNAs exhibit weak associations with ovarian hormones.

The effect of the menstrual cycle on basal miRNA expression has been previously limited to plasma samples, with inconsistent findings reported (257, 357). Li *et al.* observed elevated miR-126 expression during the ML phase compared to the EF and LF phase (258). Whereas Rekker *et al.* observed no change in the expression of 375 miRNAs between four timepoints across the menstrual cycle (257). These studies were conducted in heathy participants with comparable sample sizes to that used in this study (n= 12, (257, 258)). However, miRNA expression is highly tissue specific and miRNA profiles in plasma may not represent adipose tissue (36). In this study, RT-qPCR analysis determined miR-497-5p was differentially across the menstrual cycle. miR-497-5p expression increased 10 % in PO compared with ML and LF; however, pairwise comparisons did not detect any significant differences between phases. Loss of statistical significance between ANOVA and pairwise comparisons can be caused by a weakly significant global effect in ANOVA and insufficient statistical power resultant from a low sample size (358). Additionally, we observed a trend for reduced miR-224-3p during the PO and ML phase (p= 0.059) and a trend for reduced miR-331-5p during the ML phase (p= 0.059). This is congruent with previous studies reporting reduced miR-224 and miR-331 expression following chronic estradiol supplementation in adipose tissue (36) and adipose derived extracellular vesicles (33). Future research should further investigate changes in adipose tissue miRNA expression across the menstrual cycle in a larger study population.

To gain insight into the potential cellular consequences of variation in miRNA expression across the menstrual cycle, we conducted functional pathway analysis using miRsystem. Functional pathways analysis of miR-497-5p revealed 5 of the top 10 pathways target processes related to insulin sensitivity (**Figure 14**). miR-497-5p has previously been

shown to inhibit adipocyte insulin sensitivity and associated gene expression in vivo (359, 360). Small increases in adipose tissue miR-497-5p expression may contribute to the reduction in insulin sensitivity previously reported during the PO phase of the menstrual cycle (4, 186).

We conducted additional functional pathway analysis in a subset of miRNAs demonstrating a trend towards differential expression in addition to miR-497-5p (miR-224-3p, miR-331-5p and miR-497-5p). Similar functional pathways were identified in this subset of miRNAs, which were comparable to miR-495-5p alone, including the insulin signaling pathway and insulin receptor signaling cascade (**Figure 14**). This finding supports the notion that synergistic co-expression of miRNAs regulates functionally related cohorts of genes involved in metabolism (230). These functional pathway predictions suggest that differential co-expression of adipose tissue miRNAs across the menstrual cycle may regulate variation in insulin sensitivity. However, it must be considered that the adipose tissue transcriptome is highly insulin sensitive and, without the use of a background gene list, functional pathway analysis cannot determine which pathways are upregulated over and above the biological bias already present within adipose tissue (361). Nonetheless, these findings suggest that future research should further investigate the role of differential co-expression of subsets of miRNAs across the menstrual cycle on insulin signaling pathways. Concurrent analysis of mRNA-miRNA expression would offer valuable mechanistic insight on the impact of miRNAs on the insulin signaling pathway across the menstrual cycle.

Due to the reported impact of estradiol, progesterone and testosterone on adipocyte metabolism, we examined the relationship between ovarian hormones with adipose

tissue miRNA expression (28, 161, 181). Testosterone was negatively associated with miR-30c-5p, explaining up to 13 % of variation in miRNA expression. No previous studies have investigated the relationship between miR-30c-5p expression and testosterone concentration across the menstrual cycle. However, in females with polycystic ovary syndrome, characterized by elevated testosterone alongside insulin resistance and hyperinsulinemia, studies report an increase (362) or decrease (363) in plasma miR-30c expression. That we observed a negative association between adipose tissue miR-30c-5p and testosterone is in agreement with findings in plasma from Murri *et al.* (363). Moreover, Murri *et al.* reported that plasma miR-30c-5p expression is negatively associated with HOMA-IR, insulin and triglycerides (363). Previous studies demonstrated that testosterone is positively associated with insulin resistance, insulin and triglycerides (364). Cumulatively, this data suggests that the negative association between adipose tissue miR-30c-5p expression and testosterone may contribute to the regulation of insulin resistance and lipids across the menstrual cycle.

Intra-individual variation in ovarian hormones may contribute to the high variance in adipose tissue miRNA expression observed in each menstrual cycle phase (Figure 13). However, associations between miRNA expression and ovarian hormones were not detected in 15 out of the 16 assessed miRNAs, except for miR-30c-5p and testosterone. Moreover, despite expected variation in estradiol to progesterone ratio across the menstrual cycle, (**Appendix 3.10**), we observed no association between the estradiol to progesterone ratio and miRNA expression (**Table 12**). Thus, our data suggests that inter-individual variation in miRNA expression is not underpinned by inter-individual differences in estradiol, progesterone, testosterone nor the estradiol to progesterone ratio.

Adipose tissue is a complex tissue comprised of heterogenous cell populations (365, 366). Correspondingly, miRNA expression profiles are specific to individual cell populations within adipose tissue (367). Thus, detected miRNA signals in this study are reflective of the combined miRNA expression profiles within both mature adipocytes and the stromovascular fraction, including pre-adipocytes, mesenchymal stem cells, endothelial cells and macrophages (366). Additionally, hormonal influences on miRNA expression may be exerted in opposing directions in differing cellular fractions within adipose tissue. This may induce a 'counterbalancing effect' on determined miRNA signal from whole adipose tissue. Therefore, future studies should consider isolating a single adipose tissue cell population (i.e., mature adipocytes) prior to miRNA analysis to reduce variability in expression and gain further mechanistic insight. Additionally, three candidate miRNAs (miR-1231, miR-1914, miR-3180-3p) were detected at low levels via RT-qPCR and were thus removed from statistical analysis. Future studies could consider performing an RNA-Seq study of sufficient sequencing depth or including a cDNA pre-amplification step prior to qPCR analysis of adipose tissue miR-1231, miR-1914 and miR-3180-3p to facilitate the detection of these lowly expressed miRNAs (368, 369).

Females are under-represented in physiological research and therefore, our understanding of physiological changes across the menstrual cycle remains incomplete (7). Understanding how the menstrual cycle affects physiology is important to be able to properly account for these effects in fully inclusive physiology research. Our findings suggest that consideration of menstrual cycle phase is important in research investigating adipose tissue miRNA expression in regularly menstruating females. Additionally, the small-to-large effect sizes detected between ovarian hormones and 12

out of 16 assessed miRNAs highlight the need to consider other situations impacting circulating hormone concentrations in addition to menstrual cycle phase, such as menopausal status and exogenous hormone supplementation.

A major strength of this study is the non-hypothesis driven approach employed to inform candidate miRNA selection. Due to limited literature examining the role of ovarian hormones on miRNA expression and the tissue specific nature of their expression, we performed global microRNA profiling prior to identify candidate miRNAs which we validated using RT-qPCR. We assessed menstrual cycle phase using a three-step method combining calendar counting, urinary ovulation tests and retrospective hormone level confirmation (65, 67). This method ensures accurately timed sample collection across the menstrual cycle, regardless of individual differences in follicular or luteal phase duration.

We conducted power analysis to calculate sample size estimates using effect sizes derived from RT-qPCR data in the 5 miRNAs with the lowest significance levels; miR-497-5p, miR-224-3p, miR-331-5p, miR-30c-5p and miR-32 8-5p. We generated power curves for each of the miRNA under consideration by simulation. Estimates of power from these simulations were accompanied by 95% CI's. The results presented below are those for which the lower limit of that 95% CI was greater than 80% power at $\alpha = 0.05$ to detect differential miRNA expression between menstrual cycle phases (**Appendix 3.11**). Estimated sample sizes are as follows: miR-497-5p, N=90; miR-224-3p, N= 110; miR-331-5p, N= 690; miR-30c-5p, N=150; miR-328-5p, N=30. These sample size estimates provide an important methodological consideration for future studies investigating changes in adipose tissue miRNA expression across the menstrual cycle with defined outcomes. Additionally, it must be noted that ethnicity was not recorded in this study. Ethnicity may

increase inter-individual variation in adipose tissue miRNA expression; we acknowledge that this may be a limitation. Future studies should endeavour to record participants' ethnicity.

This study recruited young, healthy, regularly menstruating females and as such findings must be extrapolated to a broader population with caution. Elevated adiposity and IR are associated with greater perturbations in ovarian hormone concentrations and metabolic control across the menstrual cycle (4, 63, 284, 296). Accordingly, molecular mechanisms underlying metabolic control, such as miRNA expression, may undergo relatively less variation across the menstrual cycle in healthy females compared with populations with obesity or metabolic disorders. Additionally, diverse miRNA expression profiles are observed in individuals with metabolic disorders when compared with healthy controls (245). Therefore, future research should consider investigating changes in miRNA expression across the menstrual cycle in populations with impaired metabolic control.

In conclusion, we present novel pilot data characterizing miRNA expression across the menstrual cycle and associations with ovarian hormone concentrations. We report differential expression of miR-497-5p across the menstrual cycle and a negative association between miR-30c-5p expression and testosterone. Furthermore, miRNAs with the strongest tendency for differential expression across the menstrual cycle shared common targets related to insulin sensitivity pathways. Future studies are warranted to explore miRNA expression across the menstrual cycle in larger sample sizes. Future studies would also benefit from concurrent analysis of miRNA and mRNA expression within insulin signaling pathways to provide further mechanistic insight. Additionally, these findings provide direction for future research to examine changes in miRNA

expression across the menstrual cycle in individuals with impaired metabolic control, such as type 2 diabetes. These studies are necessary to fully elucidate the role of varying hormonal milieu across the menstrual cycle on miRNA expression and associated effects on metabolic control.

CHAPTER 5: General discussion:

Metabolic control has important implications for general health. Derangement of metabolic control is a crucial mechanism underlying the pathophysiology of metabolic disease and associated co-morbidities. Globally, the prevalence of metabolic disease is increasing in females of a reproductive age (160). Despite this, our understanding of how the menstrual cycle regulates cyclical fluctuations in metabolic control in regularly menstruating females remains limited. Research indicates IR, impaired glycaemic control and dyslipidaemia are increased during the luteal phase of the menstrual cycle (4, 5, 190). Given that intra-individual glucose and lipid variability are independent risk factors for the onset and progression of metabolic disease, it is crucial to understand how insulin resistance and associated metabolites fluctuate across the menstrual cycle. However, the literature collectively remains ambiguous, and our understanding of the associated mechanisms remain uncertain. This ambiguity may be underpinned by the small sample sizes used within much of the literature base, which may not be appropriately powered to detect small, yet possibly clinically meaningful, variation in metabolic control across the menstrual cycle. Moreover, previous studies have recruited heterogenous samples, with varying body composition, fitness and physical activity levels, with limited investigation into their potentially confounding effects.

Based on this, the overall aim of this thesis was to add to the current knowledge base by conducting a thorough characterisation of variation in metabolites across the menstrual cycle and the association with modifiable risk factors for metabolic disease. Further, we aimed to examine the variation in inflammatory markers and miRNA expression across the menstrual cycle as potential molecular mechanisms underpinning variation in

metabolic control. After a comprehensive discussion of the relevant literature, the following aims of this doctoral thesis were stated:

1. Characterise the variation in insulin sensitivity and associated metabolites across the menstrual cycle in healthy women using large prospective cohort studies.
2. Investigate the mediatory role of modifiable risk factors for metabolic disease in the relationship between insulin sensitivity and associated metabolites across the menstrual cycle in healthy women using large prospective cohort studies.
3. Investigate whether variation in insulin sensitivity and associated metabolites across the menstrual cycle in healthy women are consistent across different prospective cohort studies.
4. Investigate whether variation in circulating inflammatory markers play a role in the regulation of metabolic control across the menstrual cycle in healthy women using large prospective cohort studies.
5. Investigate whether variation in adipose tissue miRNA expression play a role in the regulation of metabolic control across the menstrual cycle in healthy females.

The primary findings have been discussed in detail within each respective experiential chapter and are summarised below:

1. Variation in glucose and cholesterol profiles were detected across the menstrual cycle, but not for triglyceride nor insulin sensitivity, prior to including risk factors for metabolic disease in models.
2. Variation in insulin sensitivity, insulin, glucose, triglyceride and cholesterol profiles across the menstrual cycle were mediated by body composition, physical activity and fitness levels.

3. Variation in insulin sensitivity and associated metabolites across the menstrual cycle and their relationships with modifiable risk factors for metabolic disease were generally consistent across the two prospective data banks studied (NHANES and UK Biobank).
4. Inflammatory markers (CRP and IGF-1) underwent variation across the menstrual cycle and were associated with metabolite concentration.
5. Adipose tissue miR-495-5p was differentially expressed across the menstrual cycle and miR-30c-5p was negatively associated with testosterone.
6. Adipose tissue miRNAs with the strongest tendency for differential expression between menstrual cycle phases shared common targets related to insulin signalling pathways.

This general discussion presents a critical appraisal of the experimental findings presented in this thesis focusing firstly on the variation in metabolic control across the menstrual cycle and secondly on the potential mechanistic role of inflammatory markers and miRNA expression. Following this is a discussion of the research implications, limitations, strengths and future research directions derived from the experimental findings contained within this thesis.

5.1. The role of the menstrual cycle in metabolic control

Whilst the role of ovarian hormones on metabolic control has been well characterised, the role of cyclical fluctuations in ovarian hormones across the menstrual cycle on metabolic control remained unclear. The evidence base at present can be categorised into two groups of findings; IR and dyslipidaemia are either increased during the luteal phase

(4, 5, 190, 193, 195, 196, 295) or remain unchanged across the menstrual cycle (185–188, 264, 265). In Chapter 2 and Chapter 3 the relationship between menstrual cycle phase with IR and associated metabolites was first examined prior to the inclusion of any potentially mediatory covariates. This is the approach much of the current literature base has taken. Despite the very large sample sizes studied in Chapter 2 (N= 1,906) and Chapter 3 (N= 8,694), we did not detect evidence of rhythmicity in circulating triglyceride, circulating insulin and IR across the menstrual cycle. However, rhythmicity across the menstrual cycle was observed for glucose (Chapter 2) and cholesterol profiles (Chapter 3). Cumulatively, experimental findings in Chapter 2 and Chapter 3 support the presence of rhythmic variation in glucose and cholesterol profiles across the menstrual cycle and confirm that this pattern of rhythmicity exists in a larger population (4, 5).

At a molecular level, our findings are congruent with the molecular effects of estradiol and progesterone on insulin signalling in the liver, skeletal muscle and adipose tissue. Estradiol increases insulin sensitivity through promotion of insulin-signalling pathways, whereas the progesterone opposes these molecular actions (178, 180). Concordant with these reports, we observed reduced insulin resistance in the late-follicular phase, where estradiol is rising and progesterone remains low (Chapter 2 and 3). Further supporting the beneficial role of estradiol on insulin sensitivity, we observed a negative association between TyG index, a marker of insulin resistance, and estradiol (Chapter 3). During the mid-luteal phase, during which both progesterone and estradiol are elevated, whole-body and adipose tissue IR reached its peak (Chapter 2 and 3). In accordance with our findings, data from rodent models demonstrate that the addition of progesterone to estradiol supplementation ameliorates the beneficial effects on insulin sensitivity of estradiol supplementation alone (178). This suggests that reduced estradiol to

progesterone ratios during the luteal phase mediate impairments in whole body and adipose tissue IR. Unfortunately, the assessment of both progesterone and estradiol was not conducted in either NHANES or UK Biobank, so we were unable to explore the role of estradiol to progesterone ratios across the menstrual cycle on IR. Future studies should endeavour to analyse estradiol and progesterone to permit investigation into the effect of varying estradiol to progesterone ratios across the menstrual cycle on IR.

In agreement with the reported stimulatory effects of estradiol on insulin secretion, elevations in serum insulin were observed during the luteal phase (Chapter 2) (173). Estradiol promotes β- cell insulin secretion through activation of P13k, which results in elevated circulating insulin levels (173). However, a failure to observe a concurrent reduction in glucose and/or triglyceride in the presence of elevated insulin during the ovulatory and luteal phases further supports the presence of impaired insulin-stimulated glucose and lipid regulation during these menstrual cycle phases (Chapter 2 and Chapter 3).

Concordant with elevated whole body and adipose tissue IR, glucose concentration was elevated during the mid-luteal phase of the menstrual cycle (Chapter 2 and Chapter 3). Estradiol decreases blood glucose levels through the promotion of GLUT-4 mediated skeletal muscle and adipose tissue glucose uptake and the suppression of hepatic glucose production (165, 178). Whereas progesterone is associated with impaired glucose disposal into skeletal muscle and adipocytes through suppression of the Akt-GLUT-4 pathway (178). Our finding is in agreement with previous data suggesting that concurrent increases in estradiol and progesterone are associated with a decline in glucose uptake and GLUT-4 activity (178). Molecular assays are warranted to examine

the molecular effects of fluctuations in the estradiol to progesterone across the menstrual cycle on insulin-stimulated pathways of glucose disposal.

The decline in triglyceride during the mid-follicular and late-follicular phase (Chapter 2 and Chapter 3) likely reflect the molecular effects of estradiol and progesterone on lipid metabolism. Estradiol signalling through ER increases FFA availability for oxidation by stimulating lipolytic pathways and suppressing lipogenic pathways (2, 172). Subsequently, estradiol promotes FFA oxidation in skeletal muscle through genomic and non-genomic activation of key transcription factors, AMPK and PPAR, which up-regulate down-stream targets mediating FFA oxidation (2, 172). Collectively, the actions of estradiol serve to increase FFA availability and oxidation, which results in reductions in circulating TGL concentration. Further supporting the beneficial effect of estradiol on triglyceride levels, we observed a negative association between estradiol and triglyceride (Chapter 3). However, progesterone down-regulates the capacity for skeletal muscle FFA oxidation, independent of the presence of physiological concentrations of estradiol (172). Therefore, the antagonistic actions of progesterone on FFA oxidation likely underpin the elevated triglyceride concentration was observed during the luteal phase (Chapter 2 and Chapter 3), during which both estradiol and progesterone concentrations are elevated.

5.2. The role of modifiable risk factors on mediating metabolic control across the menstrual cycle

Following this, the role of modifiable risk factors for impaired metabolic control in the relationship between menstrual cycle phase with IR and associated metabolites was considered. When including modifiable risk factors for impaired metabolic control in

models, we observed rhythmicity in all metabolites (Chapter 2 and Chapter 3). Modifiable risk factors identified in Chapter 2 and Chapter 3 were largely consistent. Individuals with high adiposity and low physical activity exhibited greater magnitude of variation in glucose, lipid profiles and IR across the menstrual cycle. These findings are consistent with previous research demonstrating a positive association between physical activity and adiposity with inter-individual variation in glycaemic control, lipidemic control and IR in basal conditions (324, 325). Notably, our findings extend on this evidence base to identify that these modifiable risk factors also mediate variation in the magnitude of fluctuation in metabolic control across the menstrual cycle.

As discussed throughout this thesis, evidence reporting the variation in metabolic control across the menstrual cycle is inconsistent. Notably, previous studies examining variation in metabolic control across the menstrual cycle have recruited heterogenous study populations with varying BMI and physical activity levels, with limited adjustment for the potentially cofounding effects of these modifiable risk factors (4, 23, 24). Based on this, a key aim of this thesis was to identify factors mediating the variation in metabolic control across the menstrual cycle. Experimental findings in Chapter 2 and Chapter 3 suggest that inconsistencies within the current literature base may be underpinned by inter-individual variation in modifiable risk factors metabolic disease (body composition, physical activity, fitness).

We observed divergent variation in IR, glucose and lipids across the menstrual cycle between categories of high and low modifiable risk factors (Chapter 2 and Chapter 3). This finding suggests the presence of differing metabolic responses to fluctuations in estradiol and progesterone across the menstrual cycle. From a molecular perspective,

the underlying mechanisms through which inter-individual variation in modifiable risk factors mediate divergences in the fluctuation of IR, glucose concentration and lipid profiles across the menstrual cycle remain uncertain. One possible explanation surrounds the increased metabolic flexibility observed individuals with low adiposity or increased physical activity levels (155). Increased metabolic flexibility may result in a better ability to coordinate the uptake, release and oxidation of carbohydrate and lipids to maintain homeostasis, in the presence of varying estradiol and progesterone concentration (155). However, future research is required to examine the molecular mechanisms through which adiposity and physical activity levels regulate divergences in metabolic control across the menstrual cycle.

5.3. Role of inflammation in regulating metabolic control across the menstrual cycle

Low grade inflammation is positively associated with insulin resistance, hyperglycaemia and dyslipidaemia (22, 297–299). Whilst variation in low grade inflammation across the menstrual cycle has been well characterised (22, 300–302, 370), it's role in regulating metabolic control across the menstrual cycle remains uncertain. In Chapter 3, we identified that CRP was elevated during the luteal phase and positively associated with glucose, TyG index and lipid profiles, independent of fat mass or physical activity. The CRP peak observed in Chapter 3 roughly coincides with the timing of peak levels of glucose, triglyceride, HOMA-IR and ADIPO-IR identified in chapter 2. Moreover, CRP was negatively associated with glucose, triglyceride and insulin resistance (Chapter 3). Together, our findings are consistent with the notion that CRP may contribute to the regulation of metabolic control across the menstrual cycle.

Given the rhythmicity in inflammatory markers across the menstrual cycle observed in Chapter 3, it is tempting to speculate that differentially regulated miRNAs in adipose tissue across the menstrual cycle may function within inflammatory pathways. However, functional pathway analysis in the subset of miRNAs demonstrating a trend towards differential expression across the menstrual cycle (miR-497-5p, miR-224-3p and miR-331-5p) did not reveal any target pathways related to inflammation (Chapter 4). Therefore, it is likely that inflammatory mediated effects on metabolism occur independent of adipose tissue miRNA expression.

In vitro studies have demonstrated that CRP impairs the IRS1-PI3k-Akt pathway in the liver, skeletal muscle and adipose tissue. Resultantly, CRP inhibits numerous key insulin-stimulated pathways including; glucose uptake, GLUT-4 translocation, glycogen synthesis and hepatic gluconeogenesis (371–373). Further work is required to identify the molecular pathways through which inflammatory markers regulate metabolic control across the menstrual cycle. Additionally, CRP is a sensitive marker of systemic inflammation and is positively associated with numerous other pro-inflammatory markers, such as TNFα, IL-6 and IL-10. Therefore, examining the relationship between metabolic control and a larger cohort of pro-inflammatory markers would provide a broader understanding of the molecular role of systemic inflammation in mediating fluctuation in insulin sensitivity across the menstrual cycle.

5.4. Role of epigenetics in regulating metabolic control across the menstrual cycle

Estradiol and progesterone primarily regulate insulin sensitivity through up- or down-regulation of the proximal insulin signalling pathway or downstream targets. However, the molecular mechanisms remain incompletely understood. In chapter 4, miRNAs with

the strongest tendency for differential expression across the menstrual cycle (miR-497-5p, miR-224-3p and miR-331-5p) shared mutual targets related to insulin signalling pathways. This suggests miRNA expression may underpin increases in IR observed during the ovulatory and luteal phase (Chapter 2 and Chapter 3). That miR-497-5p, miR-224-5p and miR-331-5p were increased during the post-ovulatory and mid-luteal phases of the menstrual cycle is consistent with this notion. Therefore, our data supports the hypothesis that differential co-expression of functionally related miRNAs may regulate variation in insulin sensitivity across the menstrual cycle. However, further experimental research to explore the causal nature of this relationship is warranted.

We observed high inter-individual variation in metabolic control (Chapter 2 and Chapter 3) and miRNA expression (Chapter 4) across the menstrual cycle. miRNAs with the greatest tendency towards differential expression across the menstrual cycle target cohorts of genes involved in insulin sensitivity. It remains possible that intra-individual variation in miRNA expression across the menstrual cycle may mediate the magnitude of variation in metabolic control across the menstrual cycle.

Small-to-large effect sizes were determined between ovarian hormones and 12 out of 16 assessed miRNAs (Chapter 4). Previous research has demonstrated that differences in BMI, physical activity and metabolic health are associated with changes in ovarian hormones and miRNA expression (63, 374). Given this association, observed differences in metabolic control in populations with risk factors for metabolic control (Chapter 2 & Chapter 3) may be driven by differential miRNA expression.

5.5. Recommendations for clinical and research practice

In both clinical and non-clinical research, it is neither common practice nor a requirement to assess menstrual cycle status in regularly menstruating females. Data presented in this thesis, and within the broader literature, suggests that menstrual cycle status affects a range of physiological parameters. Based on this, it may be appropriate to adopt a similar approach to that of the 'Sex as a Biological Variable' (SABV) principle when considering the menstrual cycle (45); clinical and non-clinical research must consider menstrual cycle phase as a biological variable or provide justification for not doing so. Certainly, when assessing parameters of metabolic control, inflammatory markers or miRNA expression, data presented in this thesis demonstrates that menstrual cycle phase must be accounted for. Whilst speculative, this data further suggests that the menstrual cycle may mediate other aspects of health. Therefore, menstrual cycle phase may be an important consideration within broader physiological contexts.

However, these findings present a methodological issue regarding the selection of the most appropriate menstrual cycle phase(s) for clinical or research testing. As is the case with most experimental questions, the solution is context dependant. In Chapter 2 and Chapter 3, an average variation in insulin sensitivity and associated metabolites across the menstrual cycle of 2-12 % was observed. Therefore, in repeated or longitudinal assessment of metabolic control in regularly menstruating females, emphasis should be placed on scheduling experimental visits within the same menstrual cycle phase(s) to minimise intra-individual variation. The magnitude of variation in metabolites across the menstrual cycle reported in chapters 2 and 3, and as previously observed (5), is sufficient to cross clinically important boundaries. Mumford and colleagues reported that when

total cholesterol was assessed at 8 timepoints across the menstrual cycle, 20 % of females had values above the clinically acceptable range for at least one, but not all, cycle visits (5, 322). Therefore, when assessing insulin sensitivity or associated metabolites in menstruating females in clinical practice, values close to a clinical cut-off threshold may require additional tests in a different menstrual cycle phase.

Similarly in a research setting, our data demonstrates that consistent menstrual cycle phase(s) must be selected for experimental testing to minimise inter-individual variation. At this point, it is noteworthy to consider that some measures or interventions may differ in effectiveness at different menstrual cycle timepoints, including exercise or nutrition (199, 201). Therefore, a failure to test across multiple menstrual phases may mean statistically significant biological responses are not captured. Previous research has demonstrated that intra-individual variation in parameters of metabolic control is positively associated with the onset and progression of metabolic disorders. Thus, examining the magnitude of metabolic disturbance across the menstrual cycle is of physiological importance. Accordingly, future studies must consider examining scheduling testing in menstrual cycle phases which capture the lowest and highest values of the selected metabolic variable(s) across the menstrual cycle. In Chapter 2 and Chapter 3, we provide data pertinent to selecting the appropriate menstrual cycle phases to capture the peak and trough values of indices of IR and associated metabolites across the menstrual cycle. However, it is not always logistically feasible to examine multiple menstrual cycle phases due to associated financial and participatory burdens. Given the clinical importance of mitigating impairments in metabolic control, if it is only feasible to assess one menstrual cycle phase it would be appropriate to select the timepoint across the menstrual cycle at which the metabolic outcome is most impaired across the

menstrual cycle. For example, scheduling testing during mid-luteal phase when examining the effectiveness of an intervention aiming to reduce IR, whilst selecting the mid-follicular phase when examining the effectiveness of an intervention aiming to improve cholesterol profiles.

The prescription of dietary and exercise interventions is common practice in the therapeutic treatment of metabolic diseases, including type 2 diabetes (375). Reports demonstrate a role for ovarian hormones in the regulation of metabolism and responsiveness to therapeutic treatment (263). Despite this, strategies targeting the treatment of metabolic diseases do not consider how menstrual cycle phase may interact with metabolic responses to interventions. However, evidence in Chapter 2 and 3 suggests that especially during the mid-luteal phase of the menstrual cycle, individuals may benefit from nutritional and exercise strategies to mitigate increases in IR, hyperinsulinemia, and dyslipidaemia. Future research to determine whether exercise and/or nutritional interventions can be effective in mitigating disturbances in metabolic control experienced during the luteal phase is warranted. This may have important clinical implications for the treatment of metabolic conditions in regularly menstruating females.

5.6. Limitations

The data reported in this thesis provide novel findings contributing to our understanding of how the menstrual cycle affects metabolic control. However, there are several noteworthy limitations that warrant discussion.

Gold standard techniques for the assessment of menstrual cycle phase involve combining calendar counting methods with quantitative assessment of ovulation and ovarian hormone concentrations. However, given the nature of the large prospective data banks studied in Chapter 2 and Chapter 3, feasible techniques for identification of menstrual cycle phase were limited to solely calendar counting methods. Therefore, menstrual cycle status was retrospectively assigned based on questionnaire responses to "number of days since last menstrual period?" and "number of days in typical menstrual cycle?". However, that FSH and LH (Chapter 2) and estradiol (Chapter 3) displayed the expected pattern of rhythmicity across the menstrual cycle supports the use of self-reported menstrual cycle questionnaire answers for the assignment of menstrual cycle status. Inaccurate self-reporting of menstrual cycle status would increase data variability and make biological differences in metabolic control across the menstrual cycle harder to detect. Nonetheless, rhythmicity in metabolites across the menstrual cycle were detected in Chapter 2 and chapter 3. More accurate assessment of menstrual cycle status would increase data clarity. Therefore, future large prospective data sets should endeavour to ensure further data is collected regarding menstrual cycle status and irregularities. These include, but are not limited to; menstrual cycle symptoms, length of menstrual bleeding, occurrence of missed menstruation, ovulation testing and analysis of all relevant serum ovarian and pituitary hormones. This is crucial data required to support the future usage of prospective cohort studies for endocrine physiology research in females.

Whilst large prospective data-banks benefit from large sample sizes, a trade-off exists with regards to limited control of external influences on participants, such as prior nutrition, exercise and circadian rhythms. It is well reported that modifiable lifestyle factors (i.e. exercise, nutrition) can influence parameters of metabolism for up to 48 h

(142, 376). However, this would arguably only serve to increase data variability and subsequently make significant differences in outcome variables across the menstrual cycle harder to detect. Nonetheless, appropriately controlled, small scale clinical trials are warranted to definitively confirm the findings presented in Chapter 2 and Chapter 3. Conversely, due to the onerous nature of conducting human physiology trials, the experimental findings in Chapter 4 are limited by a small sample size. However, appropriately powered follow-up studies are permitted by the provision of sample size estimations.

As with all research, findings should be considered within the study context and must be extrapolated to a broader population with caution. It is noteworthy to consider that participants included in Chapter 2 and Chapter 3, whist pre-menopausal, were at different stages within their reproductive lifespan. Chapter 2 included young females (25.4 ± 9.4 yr) whereas females studied in Chapter 3 were older (44.9 ± 2.8 yr). This may underpin some discrepancies in findings, specifically that of variation in glucose across the menstrual cycle. However, overall findings were similar in Chapter 2 and Chapter 3; this suggests that findings can be applied to regularly menstruating females irrespective of age. Moreover, all experimental studies were conducted on a healthy population. Therefore, data may not translate to populations either at risk to or diagnosed with metabolic disease.

5.7. Strengths

The methodologies employed in the experimental chapters benefit from numerous strengths that contributed to the generation of high-quality data.

Experimental findings in chapters 2 and 3 were generated from the analysis of data from prospective cohort studies; NHANES and UK Biobank. These studies are the largest to date to examine variation in insulin sensitivity and associated metabolites across the menstrual cycle. Due to the larger sample size (UK Biobank, N= 500,000; NHANES, N= ~5,000 /yr) and broader inclusion criteria when compared to randomised control trials, results generated from prospective cohort studies are more representative of the population and generalisable to clinical practice. Additionally, prospective cohort studies allow for the assessment of multiple exposures and measures within the one cohort of participants. This allowed for the investigation into the role of numerous modifiable risk factors on the variation in metabolic control across the menstrual cycle, including body composition, physical activity and fitness.

In Chapter 2 and Chapter 3, all data for each participant in NHANES and the UK BioBank are relevant to one single timepoint within their menstrual cycle, as retrospectively assigned using self-reported questionnaire responses. Using cosinor rhythmometry and GAM statistical techniques, we were therefore able to generate novel data which modelled cyclical rhythmicity in outcome variables across one complete menstrual cycle, rather than at each specific phase within the menstrual cycle. Whereas testing at specific menstrual phases may result in 'missing' significant biological responses at certain menstrual cycle timepoints that are not assessed, here we can be certain that biological responses at all timepoints across the menstrual cycle were captured.

In Chapter 4 we utilised a hypothesis generation approach to miRNA analysis. Given the limited previous research examining the role of ovarian hormones on miRNA expression

in adipose tissue, we first conducted global miRNA profiling to identify candidate miRNAs that were differentially regulated across the menstrual cycle. This identified 33 miRNAs that were differentially expressed across the menstrual cycle. A subset from this group of miRNAs was selected for validation using RT-qPCR. Combining microarray and RT-qPCR techniques permitted an un-biased examination of the variation in miRNA expression across the menstrual cycle.

As is commonly discussed within the field, methodological challenges in menstrual cycle physiology are imposed by considerable intra- and inter- individual variation in menstrual cycle lengths, particularly within the follicular phase. Accurate identification of menstrual cycle phases is associated with financial costs and logistical complications. However, for high quality data in regularly menstruating females to be generated, the correct identification of menstrual cycle phases is paramount. This data is crucial to bridge the sex-data gap in physiology. In Chapter 4, we employed the previously validated 'three-step method' for identification of menstrual cycle phase (65, 67). The 'three-step method' combines calendar counting and ovulation testing to predict menstrual cycle phase, followed by retrospective confirmation via serum hormone concentrations (65). This method allowed for the accurate scheduling of experimental visits within each menstrual cycle phase and confirmation of ovulatory cycles in all participants.

5.8. Future directions

Taking together findings from Chapter 2 and Chapter 3, we identified that individuals with high adiposity, low CRF or low physical activity exhibited greater fluctuation in metabolic control across the menstrual cycle. Typically, these characteristics are

observed in individuals with impaired metabolism (118). Additionally, it is well characterised that these individuals exhibit hyperglycaemia and dyslipidaemia alongside increased intra-individual variation in glucose and lipid control. Based on the experimental findings presented in Chapter 2 and Chapter 3, it is tempting to speculate that individuals diagnosed with or at risk of (e.g. pre-T2D) metabolic disease may be more susceptible to increased variation in metabolic control across the menstrual cycle. However, specific research within these populations is required to elucidate the extent to which metabolic control varies across the menstrual cycle. Given the role of intra-individual variation in the onset and progression of type 2 diabetes and associated co-morbidities (294, 325), it is crucial to understand how the menstrual cycle affects metabolism in individuals with impaired metabolism.

Observed fluctuations in IR and associated metabolites across the menstrual cycle suggest menstrual cycle phase is an important consideration in the treatment or prevention of metabolic diseases in regularly menstruating females. However, whether therapeutic interventions to improve metabolic control (e.g. exercise or nutritional interventions) can be periodised across the menstrual cycle to maximise effectiveness remains unexplored. Moreover, it is uncertain whether appropriately timed therapeutic interventions can mitigate observed metabolic disturbances during the luteal phase (Chapter 2 and Chapter 3). Given that individuals with high adiposity, low physical activity levels and low physical fitness exhibited greater fluctuation in metabolic control, this is of particular clinical relevance in these populations. Future research is required to examine whether periodising interventions across the menstrual cycle elicits beneficial effects on IR, glucose and lipid profiles. This knowledge is crucial to maximise the

effectiveness of therapeutic interventions to treat metabolic disease in regularly menstruating females.

All experimental findings contained in Chapter 2 and Chapter 3 were determined from data collected in basal conditions. Some evidence suggests that variation in metabolic control across the menstrual cycle are exacerbated in situations of metabolic stress, such as exercise and nutritional status (189, 199, 201). For example, during and post exercise substrate oxidation, glycaemic control and lipid control vary between menstrual cycle phases (189, 199, 201). However, research has not fully explored the effect of metabolic stress on variation in metabolic control across the menstrual cycle. This gap in the literature is likely underpinned by the notable lack of research conducted in females and the logistical costs and participant burden associated with the complex nature of this research question. Thus, at present our findings do not preclude that in conditions of metabolic stress, significant or exacerbated differences in variation in metabolic control may be observed across the menstrual cycle.

We determined variation in inflammatory markers and miRNA profiles across the menstrual cycle (Chapter 3 and Chapter 4). Whilst these findings identify inflammation and miRNA expression as exciting potential mechanisms underpinning variation in metabolic control across the menstrual cycle, whether, and to what extent, this relationship is causal remains uncertain. To address these questions, a translational approach incorporating *in vitro* cellular models followed by *in vivo* human physiology studies is required. Firstly, cellular models to mimic the menstrual cycle *in vitro* are warranted to ascertain the causal nature of changes in inflammation and miRNA expression across the menstrual cycle on various parameters of metabolic control in

insulin sensitive cells. Secondly, *in vivo* human physiology studies are required to further examine the relationship between variation in parameters of metabolic control with inflammatory markers and miRNA expression.

In Chapter 3 and Chapter 4, inflammation and miRNA expression were identified as potential molecular mechanisms underpinning variation in metabolic control across the menstrual cycle. It is well characterised that both inflammatory and miRNA profiles are divergent in populations with metabolic dysfunction, such as type 2 diabetes or metabolic dysfunction. If, as we speculate, greater variation in metabolic control across the menstrual is observed in individuals with metabolic disease, it remains possible that these metabolic responses are driven by differential inflammatory and miRNA profiles. Future research is required to fully examine whether dysregulated inflammatory markers and miRNA expression mediate the variation in metabolic control across the menstrual cycle in metabolically challenged populations.

miRNA expression levels are tissue specific (259). Experimental findings in Chapter 4 determined differences in adipose tissue miRNAs across the menstrual cycle and a weak association with ovarian hormones. Therefore, it remains possible that miRNA expression may vary across the menstrual cycle in other metabolically active tissues. Skeletal muscle is a highly metabolically and transcriptionally active tissue (15, 377, 378). Exploring whether, and to what extent, miRNA expression is altered in skeletal muscle is crucial towards fully understanding the mechanism(s) underpinning observed variation in whole-body insulin sensitivity across the menstrual cycle (Chapter 2 and Chapter 3).

These future research directions can be summarised by the research questions listed below:

1. Is the magnitude of variation in IR and associated metabolites across the menstrual cycle affected by impaired metabolic control (i.e. type 2 diabetes, metabolic syndrome)? If so, is this relationship mediated by associated changes in miRNA or inflammatory profiles?
2. Can therapeutic strategies to target the prevention and treatment of metabolic disease (e.g. exercise, nutritional interventions) be periodised across the menstrual cycle to maximise effectiveness?
3. Is variation in metabolic control across the menstrual cycle exacerbated in situations of metabolic challenge, such as an exercise bout or nutritional intervention?
4. Is there a causal relationship between variation in inflammatory markers across the menstrual cycle and associated variations in IR and associated metabolites?
5. Is there a causal relationship between differential adipose tissue miRNA expression across the menstrual cycle and associated variations in IR and associated metabolites?
6. Does variation in miRNA expression occur in other insulin-sensitive tissues across the menstrual cycle, such as skeletal muscle?

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CHAPTER 9: APPENDIX

Appendix 1.1: Variable description and NHANES code for all variables included in the study

Category	Variable description	NHANES code	Year
Participant information	Respondent sequence number	SEQN	1999-2000, 2001-2002, 2003-2004, 2005-2006
	Age (yr)	RIDAGEYR	1999-2000, 2001-2002, 2003-2004, 2005-2006
	Height (cm)	BMXHT	1999-2000, 2001-2002, 2003-2004, 2005-2006
	Weight (kg)	BMXWT	1999-2000, 2001-2002, 2003-2004, 2005-2006
	BMI (kg/m2)	BMXBMI	1999-2000, 2001-2002, 2003-2004, 2005-2006
	VO2max (ml/min/kg)	CVDESVO2	1999-2000, 2001-2002, 2003-2004, 2005-2006
Metabolic control	Glucose (mmol.L)	LBXSGL	1999-2000, 2001-2002, 2003-2004, 2005-2006
	Triglyceride (mmol.L)	LBXSTR	1999-2000, 2001-2002, 2003-2004, 2005-2006
	Insulin (IU)	LBXIN	1999-2000, 2001-2002, 2003-2004, 2005-2006
Pituitary hormones	LH (IU)	LBXLH	1999-2000
	FSH (IU)	LBXFSH	1999-2000
Physical activity	Activity type	PADACTIV	1999-2000, 2001-2002, 2003-2004, 2005-2006
	Reported intensity level of activity	PADLEVEL	1999-2000, 2001-2002, 2003-2004, 2005-2006
	Number of times did activity in past 30 d	PADTIMES	1999-2000, 2001-2002, 2003-2004, 2005-2006
	Average duration of activity (minutes)	PADDURAT	1999-2000, 2001-2002, 2003-2004, 2005-2006
	MET score for activity (MET)	PADMETS	1999-2000, 2001-2002, 2003-2004, 2005-2006
	Days since last period (d)	RHD080	1999-2000, 2001-2002, 2003-2004, 2005-2006
	Think that you are pregnant now	RHQ140	1999-2000
		RHQ141	2001-2002
		RHD143	2003-2004, 2005-2006
	Age at last live birth	RHQ190	1999-2000, 2001-2002, 2003-2004, 2005-2006
	Taking birth control pills now?	RHD442	1999-2000, 2001-2002
		RHD440	2003-2004, 2005-2006
	Taking estrogen/progestin now?	RHQ574	1999-2000, 2001-2002, 2003-2004, 2005-2006

	Using estrogen-only patches now?	RHQ584	1999-2000, 2001-2002, 2003-2004, 2005-2006
	Use estrogen/progestin patches now?	RHQ600	1999-2000, 2001-2002, 2003-2004, 2005-2006
Metabolic disorders	Currently have thyroid issue	MCQ170I	1999-2000
		MCQ170M	2001-2002, 2003-2004, 2005-2006
	Age diagnosed with diabetes	DIQ040G	1999-2000, 2001-2002, 2003-2004, 2005-2006
	Taking insulin now	DIQ050	1999-2000, 2001-2002, 2003-2004, 2005-2006
	Take diabetic pills to lower blood sugar	DIQ070	1999-2000, 2001-2002, 2003-2004, 2005-2006

Appendix 1.2: Rhythmicity analysis of BMI, physical activity and cardiorespiratory fitness across the menstrual cycle.

Variable	n-val	Mean (IU)	Amplitude (IU)	P-P (%)	Acrophase (d)	p-value
BMI	1906	26.28	0.15	1.13	13	0.822
PA	1906	1527.05	182.09	23.85	11	0.199
CRF	965	37.48	0.45	2.4	8	0.517

BMI, body mass index; CRF, cardiorespiratory fitness; P-P, peak to peak difference in percentage.

Appendix 1.3: Relationship between stature with metabolic parameters.

Variable	R^2	p-value
Glucose	<0.001	0.358
Triglyceride	0.002	0.165
Insulin	0.002	0.171
HOMA-IR	0.123	0.216
ADIPO-IR	0.004	0.064

Multiple regression models performed with day of menstrual cycle as predictor variable

Appendix 2.1: Relationship between participant characteristics and menstrual cycle status

Variable	N val	EDF	p-value	Minimum (IU (MC status)	Maximum (IU (MC status))	Variation (IU (%))
Participant characteristics						
Age (yr)	8659	0.1	0.354	44.9 (0.63)	44.91 (0.08)	0.0 (0.0)
Body mass (kg)	8633	1.9	**0.008**	70.0 (0.29)	71.01 (0.83)	1.0 (1.5)
Stature (m)	8633	5.1	0.214	1.6 (0.17)	1.7 (0.96)	0.0 (0.3)
BMI (kg/m2)	8633	1.8	**0.016**	26.0 (0.29)	26.3 (0.79)	0.3 (1.3)
Fat mass (kg)	8523	2.1	**0.001**	24.5 (0.29)	25.4 (0.79)	0.9 (3.8)
Fat mass (%)	8523	2.2	**<0.001**	33.8 (0.29)	34.6 (0.79)	0.8 (2.3)
Fat-free mass (kg)	8528	1.2	0.114	45.5 (0.33)	45.7 (0.88)	0.2 (0.4)
Fat-free mass (%)	8520	2.3	**<0.001**	26.8 (0.79)	27.3 (0.29)	0.4 (1.6)
HbA1c (mmol/mol)	8157	3.8	0.336	32.7 (0.00)	32.9 (0.63)	0.2 (0.6)
Physical activity (MET min/wk)						
Walking	7333	5.0	**0.045**	1022.5 (0.04)	1147.0 (0.83)	124.5 (11.5)
Moderate	7333	1.0	0.135	760.8 (0.17)	792.4 (0.67)	31.6 (4.1)
Vigorous	7333	6.2	**0.032**	603.5 (0.29)	734.7 (0.50)	131.3 (19.6)
Summed	7333	5.7	**0.020**	2378.4 (0.33)	2666.2 (0.83)	287.8 (11.4)
Fitness						
Grip strength (kg)	8634	7.1	**0.025**	26.7 (0.21)	27.7 (0.88)	1.0 (3.5)
Cardiorespiratory fitness (METs)	1640	1.9	**0.013**	9.7 (0.92)	10.0 (0.38)	0.4 (3.6)

P-value represents significance level for smoothed terms in the GAM. Bold face text denotes significant p-value (<0.05). Analyses were adjusted for age, ethnicity and deprivation. Menstrual cycle phase values are shown on a scale of 0-1, this corresponds to the approximate phases: follicular phase, 0-0.54; luteal phase 0.54-1 (14). BMI, body mass index; EDF, estimated degrees of freedom; HbA1c, glycated haemoglobin; MC, menstrual cycle.

Appendix 2.2: Relationship between metabolites and menstrual cycle status in model adjusted for fasting duration.

Variable (mmol/L)	N	EDF	p-value	Minimum (Value (MC status))	Maximum (Value (MC status))	Variation (IU (%))
Glucose	7780	1.3	0.077	4.8 (0.46)	4.8 (0.96)	0.0 (0.4)
Triglyceride	8653	4.1	0.063	1.2 (0.92)	1.2 (0.67)	0.1 (5.5)
TyG index	7778	3.6	0.109	4.5 (0.96)	4.5 (0.67)	0.0 (0.4)
Total cholesterol	8651	3.4	**<0.001**	5.3 (0.88)	5.4 (0.25)	0.1 (2.3)
HDL	7779	2.9	**<0.001**	1.5 (0.88)	1.6 (0.46)	0.1 (3.9)
LDL	8638	3.6	**0.013**	3.3 (0.88)	3.3 (0.21)	0.1 (2.1)
Total cholesterol :HDL	7776	5.3	**<0.001**	3.5 (0.50)	3.7 (0.13)	0.1 (4.1)

P-value represents significance level for smoothed terms in the GAM. Bold face text denotes significant p-value (<0.05). Analyses were adjusted for age, ethnicity and deprivation. Menstrual cycle (MC) status are shown on a scale of 0-1, this corresponds to the approximate phases: follicular phase, 0-0.54; luteal phase 0.54-1 (14). EDF, estimated degrees of freedom; HDL, high density lipoprotein; LDL, low density lipoprotein, TyG index; triglyceride to glucose index.

Appendix 2.3: Effect of continuous covariates on variation in metabolites across the menstrual cycle adjusted for fasting duration.

Variable	Value	BMI (kg/m²)	Fat mass (%)	Skeletal muscle mass (%)	Summed PA (MET min/wk)	HbA1c (mmol/ mmol)	Grip strength (kg)	Cardio-respiratory fitness (METs)
Glucose	N	7754	7652	7649	6583	7317	7757	1540
	Dev exp (%)	2.0	2.0	2.0	2.0	2.0	2.0	2.0
	EDF	1.2	1.4	1.3	1.2	1.4	1.3	3.5
	p-value	0.083	0.061	0.066	0.121	0.065	0.079	**0.028**
Triglyceride	N	8627	8517	8514	7328	8151	8628	1638
	Dev exp (%)	16.9	15.3	14.3	3.4	5.4	2.5	11.5
	EDF	4.4	4.2	4.2	0.4	4.1	4.0	3.6
	p-value	**0.009**	**0.021**	**0.031**	0.298	0.126	0.073	**0.025**
TyG index	N	7752	7650	7647	6581	7315	7755	1540
	Dev exp (%)	20.3	18.8	17.5	4.6	7.9	3.4	14.7
	EDF	4.0	3.8	3.8	0.9	3.7	3.5	3.7
	p-value	**0.025**	0.059	0.083	0.199	0.158	**0.115**	**0.022**
Total cholesterol	N	8625	8515	8512	7326	8149	8626	1639
	Dev exp (%)	4.3	4.8	5.0	2.6	3.7	2.3	4.4
	EDF	3.4	3.3	3.3	3.4	3.4	3.4	0.4
	p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.001**	**<0.001**	0.306
HDL	N	7753	7651	7648	6581	7316	7756	1541
	Dev exp (%)	16.6	14.5	13.0	3.3	4.6	2.3	9.0
	EDF	2.7	2.7	2.6	2.6	3.1	2.9	2.9
	p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.026**
LDL	N	8612	8502	8499	7315	8139	8613	1639
	Dev exp (%)	7.7	8.4	8.5	2.5	4.4	1.8	5.9
	EDF	3.6	3.5	3.4	3.5	3.6	3.5	0.0
	p-value	**0.001**	**<0.001**	**<0.001**	**0.008**	**0.011**	**0.017**	0.960
Total cholesterol: HDL	N	7750	7648	7645	6578	7313	7753	1540
	Dev exp (%)	21.8	20.2	18.6	3.4	7.1	2.0	12.7
	EDF	5.3	5.8	6.0	4.4	5.9	5.6	3.4
	p-value	**<0.001**	**<0.001**	**<0.001**	**0.002**	**<0.001**	**<0.001**	0.156

P-value represents significance for the smoothed term of menstrual cycle phase in the GAM. Bold face text denotes significant p-value (<0.05). Analyses were adjusted for age, ethnicity, deprivation and fasting duration. Menstrual cycle phase values are shown on a scale of 0-1, this corresponds to the approximate phases: follicular phase, 0-0.54; luteal phase 0.54-1 (14). BMI, body mass index; EDF, estimated degrees of freedom; HbA1c, glycated haemoglobin LDL, low density lipoprotein; HDL, high density lipoprotein.

Appendix 2.4: Sub-group analysis for variation in metabolites across the menstrual cycle.

Variable	Level	N val	EDF (P val)	Min-max (IU (MC status))	Variation (IU (%))
Fat mass (%)					
Glucose	1	1892	0.0 (0.698)	4.75-4.75 (0.00-0.54)	0.00 (0.00)
	2	1904	0.9 (0.193)	4.75-4.78 (0.04-0.54)	0.02 (0.47)
	3	1909	1.5 (0.044)	4.76-4.81 (0.08-0.54)	0.05 (1.02)
	4	1947	0.4 (0.275)	4.83-4.84 (0.71-0.25)	0.01 (0.21)
Triglyceride	1	2118	0.0 (0.835)	0.94-0.94 (0.50-0.96)	0.00 (0.00)
	2	2109	0.5 (0.269)	1.08-1.10 (0.13-0.58)	0.01 (1.20)
	3	2170	7.1 (**0.001**)	1.14-1.41 (0.63-0.79)	0.27 (21.54)
	4	1892	3.5 (**0.021**)	1.47-1.57 (0.67-0.96)	0.10 (6.80)
TyG index	1	1904	0.0 (0.483)	4.40-4.40 (0.54-0.00)	0.00 (0.00)
	2	1908	1.1 (0.123)	4.46-4.47 (0.13-0.58)	0.01 (0.30)
	3	1946	6.8 (**0.004**)	4.49-4.57 (0.63-0.79)	0.08 (1.78)
	4	2121	3.4 (**0.012**)	4.61-4.65 (0.67-0.00)	0.04 (0.91)
Total cholesterol	1	2115	1.7 (**0.012**)	5.15-5.27 (0.29-0.79)	0.12 (2.23)
	2	2109	2.8 (0.281)	5.25-5.34 (0.29-0.92)	0.09 (1.70)
	3	2170	2.6 (**0.003**)	5.40-5.57 (0.25-0.83)	0.17 (3.19)
	4	1892	4.9 (**0.023**)	5.42-5.65 (0.13-0.88)	0.23 (4.08)
HDL	1	1903	2.2 (**0.006**)	1.68-1.73 (0.38-0.79)	0.06 (3.35)
	2	1909	2.1 (**<0.001**)	1.58-1.66 (0.50-0.00)	0.08 (4.86)
	3	1947	4.5 (**0.007**)	1.48-1.55 (0.33-0.04)	0.08 (5.24)
	4	2118	4.0 (**0.047**)	1.36-1.42 (0.54-0.88)	0.06 (4.26)
LDL	1	2115	1.3 (0.078)	3.03-3.08 (0.25-0.75)	0.05 (1.78)
	2	2105	3.3 (0.136)	3.12-3.21 (0.21-0.50)	0.09 (2.91)
	3	2164	2.1 (**0.010**)	3.33-3.43 (0.21-0.79)	0.10 (3.10)
	4	1892	3.5 (0.256)	3.47-3.56 (0.17-0.88)	0.10 (2.80)
Total cholesterol : HDL	1	2120	0.0 (0.750)	3.13-3.13 (0.88-0.42)	0.00 (0.00)
	2	1901	2.2 (**0.003**)	3.29-3.45 (0.08-0.54)	0.16 (4.61)
	3	1909	4.7 (**0.013**)	3.63-3.85 (0.08-0.83)	0.22 (5.80)
	4	1946	3.2 (0.108)	4.09-4.22 (0.21-0.54)	0.13 (3.08)
Muscle mass (%)					
Glucose	1	1926	0.0 (0.473)	4.83-4.83 (0.71-0.17)	0.00 (0.00)
	2	1887	1.6 (0.029)	4.74-4.80 (0.04-0.50)	0.06 (1.16)
	3	1927	1.1 (0.132)	4.76-4.79 (0.04-0.58)	0.03 (0.65)
	4	1909	0.0 (0.818)	4.75-4.75 (0.00-0.42)	0.00 (0.00)
Triglyceride	1	2150	1.0 (0.173)	1.50-1.53 (0.46-0.96)	0.03 (2.04)
	2	2084	4.2 (0.076)	1.23-1.34 (0.63-0.83)	0.11 (8.78)
	3	2136	0.0 (0.622)	1.10-1.10 (0.25-0.75)	0.00 (0.00)
	4	2144	0.0 (0.939)	0.94-0.94 (0.33-0.96)	0.00 (0.00)

TyG index	1	1925	1.5 (0.109)	4.62-4.64 (0.58-0.04)	0.02 (0.38)
	2	1886	3.9 (0.056)	4.51-4.55 (0.13-0.83)	0.04 (0.90)
	3	1927	5.7 (0.181)	4.45-4.49 (0.08-0.83)	0.04 (0.96)
	4	1909	0.0 (0.670)	4.40-4.40 (0.58-0.04)	0.00 (0.00)
Total cholesterol	1	2150	4.5 (0.088)	5.45-5.63 (0.17-0.88)	0.18 (3.26)
	2	2084	2.6 (**0.008**)	5.42-5.58 (0.21-0.79)	0.16 (2.84)
	3	2135	1.6 (**0.038**)	5.25-5.35 (0.33-0.83)	0.10 (1.79)
	4	2143	1.6 (**0.028**)	5.15-5.25 (0.29-0.79)	0.10 (1.96)
HDL	1	1926	3.2 (0.085)	1.38-1.43 (0.54-0.88)	0.05 (3.58)
	2	1887	1.5 (0.069)	1.51-1.54 (0.46-0.00)	0.03 (2.07)
	3	1926	2.0 (**0.001**)	1.58-1.64 (0.46-0.96)	0.06 (3.87)
	4	1909	2.0 (**0.008**)	1.68-1.73 (0.42-0.88)	0.05 (3.11)
LDL	1	2144	0.0 (0.691)	3.52-3.52 (0.29-0.88)	0.00 (0.00)
	2	2080	1.9 (**0.020**)	3.34-3.43 (0.21-0.75)	0.09 (2.69)
	3	2133	0.9 (0.181)	3.17-3.20 (0.25-0.75)	0.03 (0.99)
	4	2142	1.4 (0.079)	3.02-3.08 (0.21-0.75)	0.06 (1.91)
Total cholesterol : HDL	1	1925	6.3 (**0.032**)	3.97-4.21 (0.17-0.54)	0.24 (5.92)
	2	1887	3.3 (**0.044**)	3.68-3.82 (0.13-0.79)	0.14 (3.66)
	3	1925	5.6 (0.132)	3.32-3.51 (0.08-0.54)	0.19 (5.65)
	4	1908	0.4 (0.289)	3.12-3.14 (0.00-0.46)	0.02 (0.54)
Physical activity					
Glucose	1	1169	4.5 (**0.029**)	4.75-4.91 (0.83-0.58)	0.16 (3.33)
	2	2730	0.0 (0.396)	4.77-4.77 (0.96-0.46)	0.00 (0.00)
	3	2684	4.7 (0.422)	4.75-4.81 (0.88-0.38)	0.06 (1.20)
Triglyceride	1	1300	0.6 (0.250)	1.33-1.36 (0.42-0.96)	0.02 (1.77)
	2	3053	0.7 (0.237)	1.20-1.22 (0.58-0.00)	0.02 (1.47)
	3	2975	0.0 (0.953)	1.13-1.13 (0.04-0.50)	0.00 (0.00)
TyG index	1	1168	0.7 (0.235)	4.55-4.56 (0.46-0.96)	0.01 (0.22)
	2	2729	0.0 (0.429)	4.50-4.50 (0.58-0.04)	0.00 (0.00)
	3	2684	0.0 (0.977)	4.48-4.48 (0.58-0.00)	0.00 (0.00)
Total cholesterol	1	1299	1.8 (**0.014**)	5.37-5.53 (0.29-0.83)	0.16 (2.88)
	2	3052	4.1 (**0.002**)	5.28-5.49 (0.21-0.83)	0.21 (3.83)
	3	2975	0.0 (0.451)	5.33-5.33 (0.29-0.79)	0.00 (0.00)
HDL	1	1169	2.7 (**0.007**)	1.45-1.53 (0.42-0.79)	0.09 (5.79)
	2	2729	2.0 (**0.001**)	1.53-1.59 (0.50-0.92)	0.06 (3.64)
	3	2683	2.4 (**0.009**)	1.58-1.63 (0.42-0.96)	0.05 (3.04)
LDL	1	1296	0.9 (0.209)	3.36-3.40 (0.25-0.79)	0.04 (1.19)
	2	3048	3.5 (**0.035**)	3.23-3.35 (0.17-0.83)	0.11 (3.47)
	3	2971	0.0 (0.430)	3.21-3.21 (0.13-0.54)	0.00 (0.00)
Total cholesterol: HDL	1	1167	3.4 (0.261)	3.74-3.86 (0.75-0.50)	0.13 (3.30)
	2	2728	1.3 (0.073)	3.56-3.62 (0.04-0.50)	0.07 (1.83)
	3	2683	1.4 (0.098)	3.43-3.49 (0.96-0.46)	0.06 (1.83)

HbA1c (mmol/mmol)					
Glucose	1	1962	5.4 (0.240)	4.68-4.76 (0.88-0.38)	0.09 (1.82)
	2	4840	0.5 (0.218)	4.78-4.79 (0.00-0.54)	0.01 (0.16)
	3	480	1.4 (0.076)	5.01-5.10 (0.96-0.46)	0.09 (1.74)
Triglyceride	1	2178	0.0 (0.529)	1.10-1.10 (0.63-0.13)	0.00 (0.00)
	2	5400	3.6 (0.312)	1.20-1.25 (0.67-0.92)	0.05 (3.74)
	3	533	2.3 (0.074)	1.44-1.60 (0.33-0.92)	0.16 (10.47)
TyG index	1	1962	0.0 (0.386)	4.47-4.47 (0.63-0.13)	0.00 (0.00)
	2	4838	3.4 (0.323)	4.50-4.52 (0.67-0.96)	0.02 (0.35)
	3	480	0.0 (0.373)	4.64-4.64 (0.38-0.92)	0.00 (0.00)
Total cholesterol	1	2176	3.1 (0.069)	5.18-5.33 (0.25-0.92)	0.14 (2.72)
	2	5400	2.1 (**0.007**)	5.38-5.47 (0.25-0.83)	0.09 (1.65)
	3	533	0.0 (0.922)	5.59-5.59 (0.29-0.92)	0.00 (0.00)
HDL	1	1961	2.1 (**0.001**)	1.56-1.63 (0.38-0.88)	0.07 (4.19)
	2	4840	3.2 (**<0.001**)	1.53-1.59 (0.50-0.88)	0.06 (4.03)
	3	480	0.0 (0.960)	1.40-1.40 (0.88-0.29)	0.00 (0.00)
LDL	1	2174	0.0 (0.702)	3.15-3.15 (0.25-0.79)	0.00 (0.00)
	2	5393	2.0 (0.026)	3.29-3.34 (0.21-0.67)	0.06 (1.67)
	3	532	0.0 (0.939)	3.53-3.53 (0.75-0.46)	0.00 (0.00)
Total cholesterol: HDL	1	1959	0.9 (0.177)	3.37-3.42 (0.88-0.38)	0.04 (1.26)
	2	4839	4.1 (**0.001**)	3.54-3.70 (0.17-0.50)	0.16 (4.53)
	3	480	3.4 (0.448)	4.10-4.27 (0.67-0.46)	0.18 (4.19)
Cardiorespiratory fitness (METs)					
Glucose	1	463	1.5 (0.054)	4.88-4.97 (0.67-0.13)	0.09 (1.93)
	2	409	1.2 (0.153)	4.87-4.94 (0.83-0.33)	0.07 (1.33)
	3	375	0.0 (0.832)	4.83-4.83 (0.21-0.54)	0.00 (0.00)
	4	290	0.0 (0.420)	4.82-4.82 (0.92-0.42)	0.00 (0.00)
Triglyceride	1	497	5.9 (**0.038**)	1.24-1.60 (0.08-0.25)	0.36 (25.14)
	2	425	1.2 (0.116)	1.15-1.24 (0.63-0.17)	0.09 (7.67)
	3	398	1.4 (**0.048**)	1.05-1.18 (0.50-0.00)	0.13 (11.44)
	4	315	0.4 (0.284)	0.96-0.99 (0.54-0.04)	0.03 (2.75)
TyG index	1	463	5.6 (0.111)	4.54-4.64 (0.08-0.25)	0.10 (2.17)
	2	409	1.4 (0.106)	4.50-4.54 (0.67-0.21)	0.04 (0.84)
	3	375	1.5 (0.058)	4.46-4.51 (0.50-0.96)	0.05 (1.03)
	4	290	0.3 (0.316)	4.42-4.42 (0.58-0.04)	0.01 (0.16)
Total cholesterol	1	497	3.5 (0.373)	5.45-5.66 (0.04-0.29)	0.21 (3.81)
	2	425	0.0 (0.523)	5.39-5.39 (0.54-0.04)	0.00 (0.00)
	3	399	0.9 (0.170)	5.30-5.40 (0.38-0.83)	0.10 (1.82)
	4	315	2.5 (0.194)	5.18-5.40 (0.25-0.92)	0.22 (4.17)
HDL	1	463	0.7 (0.252)	1.47-1.49 (0.42-0.88)	0.02 (1.66)
	2	410	3.5 (0.107)	1.52-1.63 (0.38-0.08)	0.11 (6.99)
	3	375	0.0 (0.845)	1.65-1.65 (0.00-0.54)	0.00 (0.00)

	4	290	1.4 (0.059)	1.68-1.76 (0.33-0.83)	0.08 (4.38)
LDL	1	497	0.0 (0.397)	3.47-3.47 (0.83-0.33)	0.00 (0.00)
	2	424	0.0 (0.794)	3.28-3.28 (0.63-0.13)	0.00 (0.00)
	3	400	0.2 (0.329)	3.19-3.21 (0.38-0.88)	0.01 (0.45)
	4	315	0.0 (0.692)	3.10-3.10 (0.21-0.67)	0.00 (0.0)
Total cholesterol : HDL	1	463	1.8 (**0.037**)	3.81-4.03 (0.96-0.38)	0.22 (5.56)
	2	410	6.7 (0.056)	3.38-3.85 (0.63-0.38)	0.46 (12.8)
	3	374	0.0 (0.470)	3.37-3.37 (0.46-0.96)	0.00 (0.0)
	4	290	0.0 (0.779)	3.15-3.15 (0.75-0.29)	0.00 (0.0)
Grip strength (kg)					
Glucose	1	1919	1.5 (0.087)	4.75-4.79 (0.75-0.29)	0.05 (0.97)
	2	1921	4.3 (0.115)	4.74-4.84 (0.88-0.63)	0.11 (2.22)
	3	1939	3.5 (0.059)	4.76-4.84 (0.92-0.33)	0.08 (1.73)
	4	1978	0.0 (0.426)	4.79-4.79 (0.17-0.63)	0.00 (0.00)
Triglyceride	1	2150	3.1 (0.112)	1.21-1.30 (0.67-0.96)	0.08 (6.69)
	2	2111	0.0 (0.426)	1.20-1.20 (0.13-0.67)	0.00 (0.00)
	3	2158	3.4 (0.097)	1.16-1.26 (0.67-0.04)	0.10 (8.25)
	4	2209	0.0 (0.497)	1.17-1.17 (0.67-0.25)	0.00 (0.00)
TyG index	1	1917	3.1 (0.045)	4.50-4.54 (0.67-0.00)	0.04 (0.89)
	2	1921	0.7 (0.211)	4.50-4.51 (0.17-0.67)	0.01 (0.18)
	3	1939	3.4 (0.111)	4.49-4.52 (0.67-0.04)	0.04 (0.79)
	4	1978	0.0 (0.748)	4.49-4.49 (0.67-0.21)	0.00 (0.00)
Total cholesterol	1	2150	0.0 (0.453)	5.40-5.40 (0.29-0.83)	0.00 (0.00)
	2	2109	1.5 (**0.032**)	5.33-5.42 (0.21-0.71)	0.09 (1.74)
	3	2158	1.6 (0.083)	5.35-5.43 (0.42-0.96)	0.08 (1.50)
	4	2209	3.5 (**0.018**)	5.25-5.44 (0.17-0.88)	0.19 (3.47)
HDL	1	1919	1.7 (0.050)	1.52-1.55 (0.46-0.88)	0.04 (2.39)
	2	1920	2.1 (**0.001**)	1.53-1.60 (0.50-0.96)	0.07 (4.27)
	3	1939	1.7 (**0.018**)	1.55-1.60 (0.42-0.88)	0.05 (2.86)
	4	1978	1.7 (**0.014**)	1.55-1.59 (0.38-0.88)	0.04 (2.84)
LDL	1	2145	0.0 (0.658)	3.30-3.30 (0.17-0.58)	0.00 (0.00)
	2	2105	1.5 (**0.027**)	3.23-3.31 (0.13-0.63)	0.07 (2.27)
	3	2158	0.0 (0.599)	3.28-3.28 (0.50-0.00)	0.00 (0.00)
	4	2205	3.4 (0.054)	3.19-3.32 (0.17-0.88)	0.13 (3.98)
Total cholesterol : HDL	1	1917	4.0 (0.134)	3.58-3.73 (0.71-0.50)	0.15 (4.01)
	2	1920	1.7 (**0.003**)	3.52-3.66 (0.08-0.54)	0.14 (3.99)
	3	1938	2.3 (0.117)	3.51-3.62 (0.75-0.42)	0.11 (3.01)
	4	1978	0.2 (0.333)	3.55-3.56 (0.00-0.50)	0.01 (0.18)

P-value represents significance for the smoothed term of menstrual cycle phase in the GAM. Bold face text denotes significant p-value (<0.05). Analyses were adjusted for age, ethnicity and deprivation. Fat mass, muscle mass, handgrip strength, and fitness are

categorised as quartiles. IPAQ and HbA1c are categorised into low, medium and high according to previously defined criteria. Menstrual cycle phase values are shown on a scale of 0-1, this corresponds to the approximate phases: follicular phase, 0-0.54; luteal phase 0.54-1 (14). EDF, estimated degrees of freedom; HbA1c, glycated haemoglobin; LDL, low density lipoprotein; HDL, high density lipoprotein.

Appendix 2.5: Sub-group analysis for variation in metabolites across the menstrual cycle adjusted for fasting duration

Variable	Level	N val	EDF (P val)	Min-max (IU (MC status))	Variation (IU (%))
Fat mass (%)					
Glucose	1	1892	0.0 (0.676)	4.75-4.75 (0.54-0.00)	0.00 (0.00)
	2	1904	1.0 (0.183)	4.75-4.78 (0.58-0.04)	0.02 (0.50)
	3	1909	1.4 (**0.049**)	4.76-4.80 (0.54-0.08)	0.05 (0.96)
	4	1947	0.4 (0.277)	4.83-4.84 (0.25-0.71)	0.01 (0.19)
Triglyceride	1	2118	0.0 (0.741)	0.94-0.94 (0.96-0.46)	0.00 (0.00)
	2	2109	0.5 (0.273)	1.08-1.10 (0.58-0.13)	0.01 (1.15)
	3	2170	7.2 (**0.001**)	1.14-1.41 (0.79-0.63)	0.27 (21.39)
	4	1892	3.5 (**0.029**)	1.47-1.57 (0.96-0.33)	0.10 (6.49)
TyG index	1	1904	0.0 (0.411)	4.40-4.40 (0.00-0.50)	0.00 (0.00)
	2	1908	1.0 (0.131)	4.45-4.47 (0.58-0.13)	0.01 (0.28)
	3	1946	6.9 (**0.003**)	4.49-4.57 (0.79-0.13)	0.08 (1.80)
	4	2121	3.2 (**0.022**)	4.61-4.65 (0.00-0.67)	0.04 (0.81)
Total cholesterol	1	2115	1.7 (**0.012**)	5.15-5.27 (0.79-0.29)	0.12 (2.22)
	2	2109	0.0 (0.409)	5.29-5.29 (0.83-0.29)	0.00 (0.00)
	3	2170	2.6 (**0.003**)	5.40-5.57 (0.83-0.25)	0.17 (3.18)
	4	1892	4.9 (**0.024**)	5.42-5.65 (0.88-0.13)	0.22 (4.05)
HDL	1	1903	2.2 (**0.005**)	1.68-1.73 (0.79-0.38)	0.06 (3.43)
	2	1909	2.1 (**<0.001**)	1.58-1.66 (0.00-0.50)	0.08 (4.83)
	3	1947	4.4 (**0.007**)	1.48-1.55 (0.04-0.33)	0.08 (5.17)
	4	2118	4.1 (**0.047**)	1.36-1.42 (0.88-0.50)	0.06 (4.25)
LDL	1	2115	1.2 (0.087)	3.03-3.08 (0.75-0.25)	0.05 (1.70)
	2	2105	3.2 (0.141)	3.12-3.21 (0.50-0.21)	0.09 (2.83)
	3	2164	2.0 (**0.010**)	3.33-3.43 (0.79-0.21)	0.10 (3.08)
	4	1892	3.5 (0.263)	3.46-3.56 (0.88-0.17)	0.10 (2.74)
Total cholesterol : HDL	1	2120	0.0 (0.718)	3.13-3.13 (0.42-0.88)	0.00 (0.00)
	2	1901	2.2 (**0.003**)	3.30-3.45 (0.54-0.08)	0.15 (4.56)
	3	1909	4.7 (**0.014**)	3.63-3.85 (0.83-0.08)	0.21 (5.72)
	4	1946	3.2 (**0.114**)	4.09-4.22 (0.54-0.21)	0.13 (3.07)
Fat-free mass (%)					
Glucose	1	1926	0.0 (0.479)	4.83-4.83 (0.17-0.71)	0.00 (0.00)
	2	1887	1.5 (**0.033**)	4.74-4.80 (0.50-0.04)	0.05 (1.11)
	3	1927	1.2 (0.118)	4.76-4.79 (0.58-0.04)	0.03 (0.70)
	4	1909	0.0 (0.812)	4.76-4.76 (0.42-0.00)	0.00 (0.00)
Triglyceride	1	2150	0.91 (0.195)	1.51-1.54 (0.96-0.46)	0.03 (1.78)
	2	2084	4.12 (0.082)	1.23-1.34 (0.83-0.63)	0.11 (8.43)
	3	2136	4.39 (0.559)	1.05-1.12 (0.83-0.08)	0.07 (6.38)

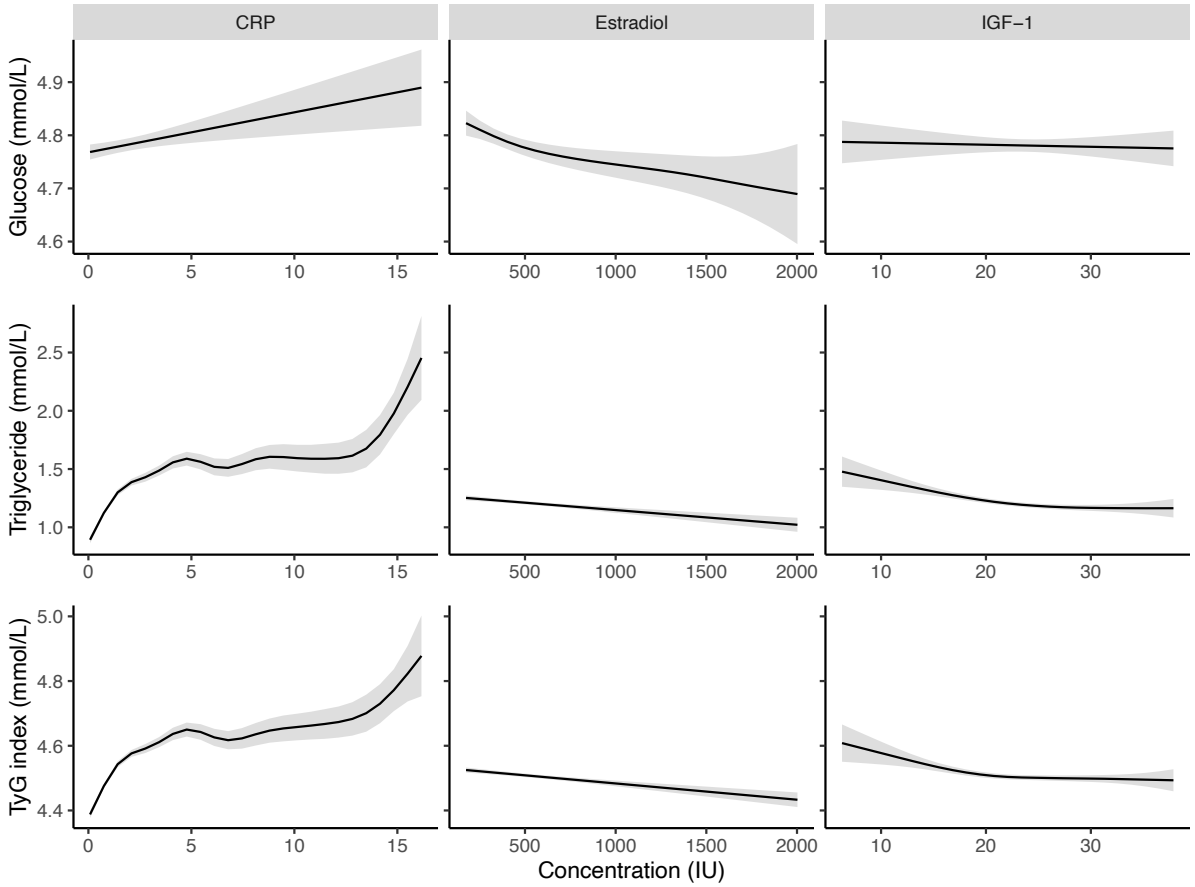
	4	2144	0 (0.904)	0.94-0.94 (0.96-0.33)	0.00 (0.00)
TyG index	1	1925	1.2 (0.146)	4.62-4.64 (0.04-0.58)	0.01 (0.30)
	2	1886	3.9 (0.055)	4.51-4.55 (0.83-0.13)	0.04 (0.93)
	3	1927	5.8 (0.170)	4.45-4.49 (0.83-0.08)	0.04 (0.97)
	4	1909	0.0 (0.644)	4.40-4.40 (0.00-0.58)	0.00 (0.00)
Total cholesterol	1	2150	4.5 (0.089)	5.45-5.63 (0.88-0.17)	0.18 (3.24)
	2	2084	2.6 (**0.009**)	5.42-5.58 (0.79-0.25)	0.16 (2.83)
	3	2135	1.6 (**0.038**)	5.25-5.35 (0.83-0.33)	0.10 (1.80)
	4	2143	1.6 (**0.029**)	5.15-5.25 (0.79-0.29)	0.10 (1.94)
HDL	1	1926	3.3 (0.088)	1.38-1.43 (0.88-0.54)	0.05 (3.58)
	2	1887	1.5 (0.073)	1.51-1.54 (0.00-0.46)	0.03 (2.02)
	3	1926	2.0 (**0.001**)	1.58-1.64 (0.96-0.46)	0.06 (3.85)
	4	1909	2.0 (**0.007**)	1.67-1.73 (0.88-0.42)	0.05 (3.18)
LDL	1	2144	3.2 (0.455)	3.47-3.54 (0.88-0.17)	0.07 (2.01)
	2	2080	1.9 (**0.022**)	3.34-3.43 (0.75-0.21)	0.09 (2.64)
	3	2133	0.9 (0.174)	3.17-3.20 (0.75-0.25)	0.03 (1.03)
	4	2142	1.4 (0.086)	3.02-3.08 (0.71-0.21)	0.06 (1.85)
Total cholesterol : HDL	1	1925	6.3(**0.034**)	3.97-4.21 (0.54-0.17)	0.24 (5.80)
	2	1887	3.3 (**0.048**)	3.68-3.82 (0.79-0.13)	0.14 (3.62)
	3	1925	5.5 (0.144)	3.32-3.51 (0.54-0.08)	0.19 (5.52)
	4	1908	0.5 (0.272)	3.12-3.14 (0.46-0.96)	0.02 (0.66)
Physical activity					
Glucose	1	1169	4.4 (**0.042**)	4.75-4.90 (0.58-0.83)	0.15 (3.13)
	2	2730	0.0 (0.389)	4.77-4.77 (0.46-0.96)	0.00 (0.00)
	3	2684	4.6 (0.445)	4.75-4.81 (0.38-0.88)	0.06 (1.17)
Triglyceride	1	1300	0.2 (0.331)	1.35-1.36 (0.96-0.42)	0.01 (0.56)
	2	3053	0.7 (0.235)	1.20-1.22 (0.00-0.54)	0.02 (1.48)
	3	2975	0.0 (0.964)	1.13-1.13 (0.50-0.08)	0.00 (0.00)
TyG index	1	1168	0.4 (0.297)	4.56-4.56 (0.96-0.46)	0.01 (0.12)
	2	2729	0.0 (0.443)	4.50-4.50 (0.04-0.58)	0.00 (0.00)
	3	2684	0.0 (0.962)	4.48-4.48 (0.00-0.58)	0.00 (0.00)
Total cholesterol	1	1299	1.8 (**0.013**)	5.37-5.53 (0.83-0.29)	0.16 (2.92)
	2	3052	4.2 (**0.002**)	5.28-5.49 (0.83-0.21)	0.21 (3.85)
	3	2975	0.0 (0.457)	5.33-5.33 (0.79-0.29)	0.00 (0.00)
HDL	1	1169	2.5 (**0.009**)	1.45-1.53 (0.79-0.42)	0.08 (5.45)
	2	2729	2.4 (**0.001**)	1.53-1.59 (0.92-0.50)	0.06 (4.12)
	3	2683	2.4 (**0.008**)	1.58-1.63 (0.96-0.42)	0.05 (3.07)
LDL	1	1296	1.0 (0.191)	3.35-3.40 (0.79-0.25)	0.05 (1.37)
	2	3048	3.8 (0.037)	3.23-3.35 (0.83-0.17)	0.12 (3.60)
	3	2971	0.0 (0.413)	3.21-3.21 (0.54-0.08)	0.00 (0.00)
Total cholesterol : HDL	1	1167	3.4 (0.287)	3.74-3.85 (0.50-0.75)	0.12 (3.08)
	2	2728	1.3 (0.077)	3.56-3.62 (0.50-0.04)	0.06 (1.79)
	3	2683	1.5 (0.084)	3.43-3.50 (0.46-0.96)	0.07 (2.00)

HbA1c (mmol/mmol)					
Glucose	1	1962	5.4 (0.241)	4.68-4.76 (0.38-0.88)	0.08 (1.73)
	2	4840	3.3 (0.151)	4.76-4.81 (0.63-0.92)	0.05 (1.01)
	3	480	1.4 (0.084)	5.01-5.10 (0.46-0.96)	0.08 (1.66)
Triglyceride	1	2178	0.0 (0.542)	1.10-1.10 (0.13-0.63)	0.00 (0.00)
	2	5400	3.7 (0.263)	1.20-1.25 (0.92-0.67)	0.05 (4.13)
	3	533	2.7 (0.066)	1.43-1.61 (0.92-0.33)	0.18 (12.17)
TyG index	1	1962	0.0 (0.434)	4.47-4.47 (0.13-0.63)	0.00 (0.00)
	2	4838	3.4 (0.299)	4.50-4.52 (0.96-0.67)	0.02 (0.37)
	3	480	0.0 (0.405)	4.65-4.65 (0.92-0.38)	0.00 (0.00)
Total cholesterol	1	2176	3.2 (0.066)	5.18-5.33 (0.92-0.25)	0.15 (2.77)
	2	5400	2.1 (**0.007**)	5.38-5.47 (0.83-0.25)	0.09 (1.63)
	3	533	0.0 (0.922)	5.59-5.59 (0.92-0.29)	0.00 (0.00)
HDL	1	1961	2.0 (**0.001**)	1.56-1.63 (0.88-0.38)	0.07 (4.11)
	2	4840	3.1 (**<0.001**)	1.53-1.59 (0.88-0.50)	0.06 (4.06)
	3	480	0.0 (0.952)	1.40-1.40 (0.38-0.92)	0.00 (0.00)
LDL	1	2174	0.0 (0.712)	3.15-3.15 (0.79-0.25)	0.00 (0.00)
	2	5393	1.9 (**0.027**)	3.29-3.34 (0.67-0.21)	0.05 (1.64)
	3	532	0.0 (0.945)	3.53-3.53 (0.46-0.75)	0.00 (0.00)
Total cholesterol : HDL	1	1959	0.9 (0.186)	3.38-3.42 (0.38-0.88)	0.04 (1.19)
	2	4839	4.1 (**0.001**)	3.54-3.70 (0.50-0.17)	0.16 (4.50)
	3	480	0.0 (0.799)	4.17-4.17 (0.25-0.71)	0.00 (0.00)
Cardiorespiratory fitness (METs)					
Glucose	1	463	1.5 (0.054)	4.88-4.97 (0.13-0.67)	0.09 (1.93)
	2	409	1.2 (0.153)	4.87-4.94 (0.33-0.83)	0.07 (1.33)
	3	375	0.0 (0.832)	4.83-4.83 (0.54-0.21)	0.00 (0.00)
	4	290	0.0 (0.420)	4.82-4.82 (0.42-0.92)	0.00 (0.00)
Triglyceride	1	497	5.9 (0.038)	1.24-1.60 (0.25-0.08)	0.36 (25.14)
	2	425	1.3 (0.116)	1.15-1.24 (0.17-0.63)	0.09 (7.67)
	3	398	1.4 (**0.048**)	1.05-1.18 (0.00-0.50)	0.13 (11.44)
	4	315	0.4 (0.284)	0.96-0.99 (0.04-0.54)	0.03 (2.75)
TyG index	1	463	5.6 (0.111)	4.54-4.64 (0.25-0.08)	0.10 (2.17)
	2	409	1.4 (0.106)	4.50-4.54 (0.21-0.67)	0.04 (0.84)
	3	375	1.5 (0.058)	4.46-4.51 (0.96-0.50)	0.05 (1.03)
	4	290	0.3 (0.316)	4.42-4.42 (0.04-0.58)	0.01 (0.16)
Total cholesterol	1	497	3.5 (0.373)	5.45-5.66 (0.29-0.04)	0.21 (3.81)
	2	425	0.0 (0.523)	5.39-5.39 (0.04-0.54)	0.00 (0.00)
	3	399	0.9 (0.170)	5.30-5.40 (0.83-0.38)	0.10 (1.82)
	4	315	2.5 (0.194)	5.18-5.40 (0.92-0.25)	0.22 (4.17)
HDL	1	463	0.7 (0.252)	1.47-1.49 (0.88-0.42)	0.02 (1.66)
	2	410	3.5 (0.107)	1.52-1.63 (0.08-0.38)	0.11 (6.99)
	3	375	0.0 (0.845)	1.65-1.65 (0.54-0.00)	0.00 (0.00)
	4	290	1.4 (0.059)	1.68-1.76 (0.83-0.33)	0.08 (4.38)

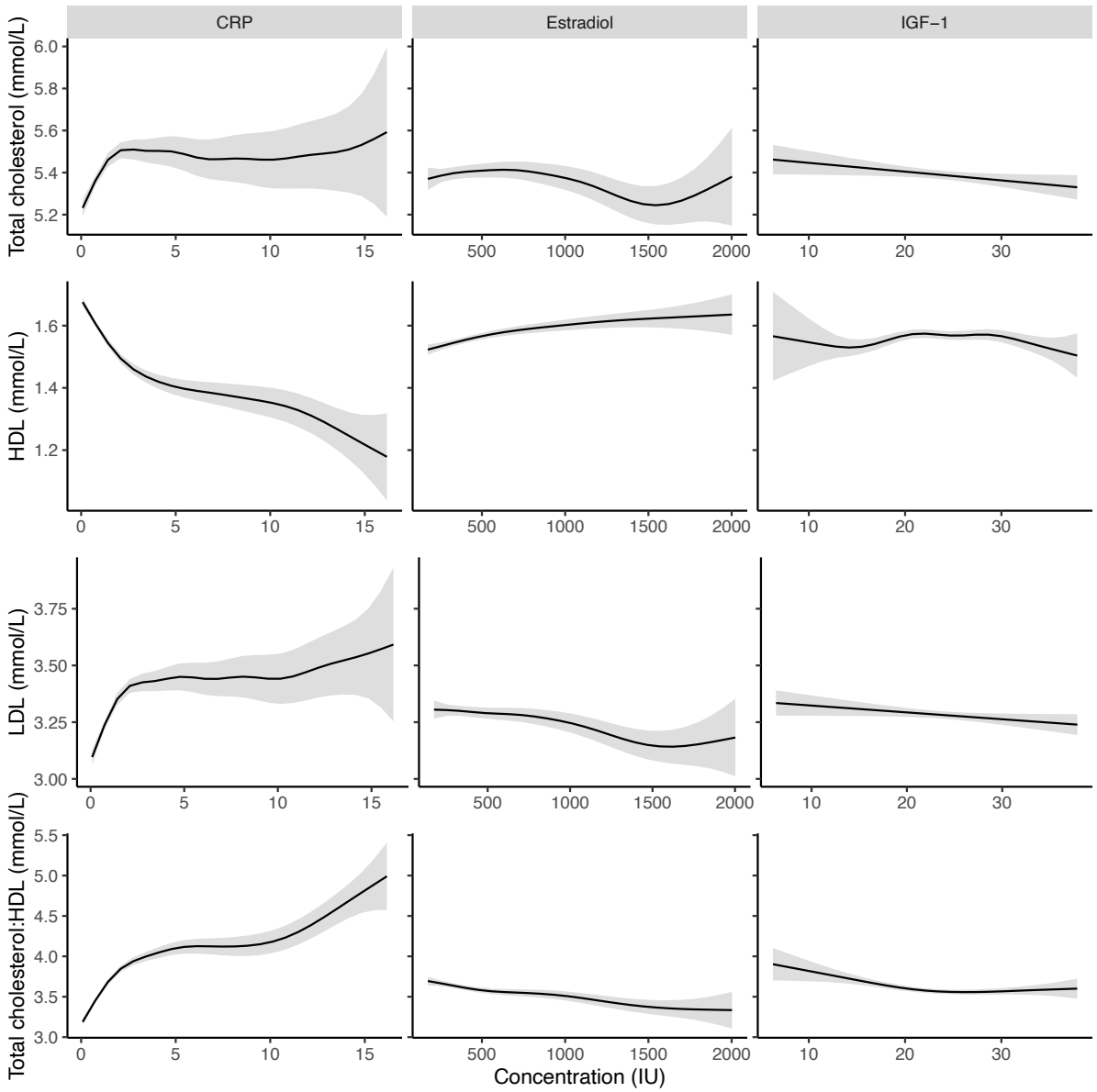
LDL	1	497	0.0 (0.397)	3.47-3.47 (0.33-0.83)	0.00 (0.00)
	2	424	0.0 (0.794)	3.28-3.28 (0.13-0.63)	0.00 (0.00)
	3	400	0.2 (0.329)	3.19-3.21 (0.88-0.38)	0.01 (0.45)
	4	315	0.0 (0.692)	3.10-3.10 (0.67-0.21)	0.00 (0.00)
Total cholesterol : HDL	1	463	1.8 (0.037)	3.81-4.03 (0.38-0.96)	0.22 (5.56)
	2	410	6.7 (0.056)	3.38-3.85 (0.38-0.63)	0.46 (12.8)
	3	374	0.0 (0.470)	3.37-3.37 (0.96-0.46)	0.00 (0.00)
	4	290	0.0 (0.779)	3.15-3.15 (0.29-0.75)	0.00 (0.00)
Grip strength (kg)					
Glucose	1	1919	1.5 (0.089)	4.75-4.79 (0.29-0.75)	0.05 (0.95)
	2	1921	4.3 (0.108)	4.74-4.85 (0.63-0.88)	0.11 (2.26)
	3	1939	3.8 (0.053)	4.75-4.84 (0.33-0.92)	0.09 (1.80)
	4	1978	0.0 (0.447)	4.79-4.79 (0.67-0.17)	0.00 (0.00)
Triglyceride	1	2150	3.2 (0.104)	1.21-1.30 (0.96-0.67)	0.09 (6.89)
	2	2111	0.0 (0.407)	1.20-1.20 (0.67-0.13)	0.00 (0.00)
	3	2158	3.4 (0.078)	1.16-1.26 (0.04-0.67)	0.10 (8.51)
	4	2209	0.0 (0.532)	1.17-1.17 (0.25-0.71)	0.00 (0.00)
TyG index	1	1917	3.1 (0.051)	4.50-4.54 (0.00-0.67)	0.04 (0.88)
	2	1921	0.7 (0.215)	4.50-4.51 (0.67-0.17)	0.01 (0.18)
	3	1939	3.3 (0.100)	4.49-4.52 (0.04-0.67)	0.04 (0.78)
	4	1978	0.0 (0.790)	4.49-4.49 (0.25-0.67)	0.00 (0.00)
Total cholesterol	1	2150	0.0 (0.466)	5.04-5.40 (0.83-0.29)	0.00 (0.00)
	2	2109	1.5 (**0.032**)	5.33-5.42 (0.71-0.21)	0.09 (1.74)
	3	2158	1.6 (0.084)	5.35-5.43 (0.96-0.42)	0.08 (1.49)
	4	2209	3.4 (0.019)	5.25-5.44 (0.88-0.21)	0.18 (3.42)
HDL	1	1919	1.7 (0.051)	1.52-1.55 (0.88-0.42)	0.04 (2.40)
	2	1920	2.1 (**0.001**)	1.53-1.60 (0.96-0.50)	0.07 (4.24)
	3	1939	1.7 (**0.017**)	1.55-1.60 (0.88-0.42)	0.04 (2.86)
	4	1978	1.7 (**0.013**)	1.55-1.59 (0.88-0.38)	0.05 (2.90)
LDL	1	2145	0.0 (0.688)	3.30-3.30 (0.58-0.17)	0.00 (0.00)
	2	2105	1.5 (**0.029**)	3.23-3.31 (0.63-0.13)	0.07 (2.24)
	3	2158	0.0 (0.615)	3.28-3.28 (0.00-0.50)	0.00 (0.00)
	4	2205	3.4 (0.063)	3.19-3.32 (0.88-0.17)	0.12 (3.83)
Total cholesterol : HDL	1	1917	4.1 (0.130)	3.58-3.73 (0.50-0.71)	0.15 (4.09)
	2	1920	1.8 (**0.003**)	3.52-3.66 (0.54-0.08)	0.14 (4.04)
	3	1938	2.0 (0.126)	3.52-3.62 (0.42-0.79)	0.10 (2.71)
	4	1978	6.3 (0.209)	3.46-3.65 (0.54-0.13)	0.19 (5.27)

P-value represents significance for the smoothed term of menstrual cycle phase in the GAM. Bold face text denotes significant p-value (<0.05). Analyses were adjusted for age, ethnicity, deprivation and fasting duration. Fat mass, fat-free mass, grip strength, and fitness are categorised as quartiles. IPAQ and HbA1c are categorised into low, medium

and high according to previously defined criteria. Menstrual cycle phase values are shown on a scale of 0-1, this corresponds to the approximate phases: follicular phase, 0-0.54; luteal phase 0.54-1 (14). EDF, estimated degrees of freedom; HbA1c, glycated haemoglobin LDL, low density lipoprotein; HDL, high density lipoprotein.



Appendix 2.6: Association between metabolites and estradiol, IGF-1, SHBG and CRP in model 1. Lines represent GAM estimates using a smoothing spline function. Shaded areas represent 95 % confidence intervals. Analyses were adjusted for age, ethnicity and deprivation.



Appendix 2.7: Association between metabolites and estradiol, IGF-1, SHBG and CRP in model 1. Lines represent GAM estimates using a smoothing spline function. Shaded areas represent 95 % confidence intervals. Analyses were adjusted for age, ethnicity and deprivation.

Appendix 2.8: Relationship between metabolites with estradiol and inflammatory markers adjusted for fasting duration

Variable	Metabolite	Model 1: SDF			Model 2: SDF +fat mass			Model 3: SDF+ physical activity			Model 4: SDF + estradiol, CRP and IGF-1		
		N	Dev exp (EDF)	p val	N	Dev exp (EDF)	p val	N	Dev exp (EDF)	p val	N	Dev exp (EDF)	p val
Estradiol	Glucose	7485	1.2 (2.4)	**<0.001**	7368	1.9 (2.6)	**<0.001**	6344	1.4 (3.2)	**<0.001**	7485	1.3 (1.0)	**0.005**
	Triglyceride	8320	2.5 (1.0)	**<0.001**	8195	15.4 (1.0)	**<0.001**	7057	3.9 (2.8)	**<0.001**	8320	13.4 (7.7)	**<0.001**
	TyG index	7483	3.4 (1.0)	**<0.001**	7366	18.6 (1.0)	**<0.001**	6342	4.8 (1.0)	**<0.001**	8318	2.3 (4.4)	**0.037**
	HDL	8193	4.6 (5.3)	0.075	7055	2.5 (4.6)	**0.016**	8318	3.4 (5.4)	**<0.001**	7484	2.3 (2.4)	**<0.001**
	LDL	7367	14.6 (2.3)	**<0.001**	6342	3.6 (2.3)	**<0.001**	7484	9.8 (4.6)	**<0.001**	8305	1.9 (3.9)	**0.001**
	Total cholesterol	8180	8.3 (5.3)	**0.045**	7044	2.7 (4.5)	**0.001**	8305	4.5 (5.9)	**<0.001**	7481	2.1 (3.5)	**<0.001**
	Total cholesterol : HDL	7364	20.0 (4.0)	**<0.001**	6339	3.8 (4.0)	**<0.001**	7481	12.9 (5.5)	**<0.001**	7483	15.6 (7.4)	**<0.001**
CRP	Glucose	7485	0.9 (1.0)	0.003	7368	1.6 (1.2)	0.702	6344	1.0 (1.0)	**0.010**	7485	1.3 (1.0)	0.581
	Triglyceride	8320	13.1 (7.7)	**<0.001**	8195	17.9 (7.3)	**<0.001**	7057	13.7 (7.7)	**<0.001**	8320	13.4 (1.2)	0.300
	TyG index	7483	15.1 (7.3)	**<0.001**	7366	20.9 (6.4)	**<0.001**	6342	15.7 (7.0)	**<0.001**	8318	3.2 (5.4)	**<0.001**
	HDL	8193	4.6 (3.9)	**0.048**	7055	3.3 (4.9)	**<0.001**	8318	3.4 (1.4)	0.771	7484	8.9 (4.5)	**<0.001**
	LDL	7367	15.2 (2.5)	**<0.001**	6342	9.9 (4.4)	**<0.001**	7484	9.8 (1.4)	**<0.001**	8305	4.3 (5.7)	**<0.001**
	Total cholesterol	8180	8.2 (3.7)	**0.035**	7044	5.0 (5.48)	**<0.001**	8305	4.5 (1.0)	0.140	7481	12.2 (5.5)	**<0.001**
	Total cholesterol : HDL	7364	21.1 (4.0)	**<0.001**	6339	13.6 (5.1)	**<0.001**	7481	12.9 (1.4)	**<0.001**	7483	15.6 (1.0)	**0.001**
IGF-1	Glucose	7485	0.8 (1.0)	0.861	7368	1.6 (1.0)	0.456	6344	1.0 (1.5)	0.822	7485	1.3 (2.3)	**<0.001**
	Triglyceride	8320	2.6 (2.5)	**<0.001**	8195	15.3 (1.5)	0.073	7057	3.9 (2.5)	**<0.001**	8320	13.4 (2.8)	**<0.001**
	TyG index	7483	3.3 (2.9)	**<0.001**	7366	18.3 (2.5)	0.677	6342	4.6 (3.8)	**0.001**	8318	2.1 (1.0)	**0.040**
	HDL	8193	4.5 (2.0)	0.409	7055	2.4 (3.7)	0.175	8318	3.3 (4.1)	0.060	7484	1.8 (5.0)	**0.030**
	LDL	7367	14.5 (5.2)	**0.009**	6342	3.0 (1.9)	0.355	7484	9.8 (2.2)	**<0.001**	8305	1.7 (1.0)	0.081
	Total cholesterol	8180	8.1 (1.6)	0.502	7044	2.3 (1.0)	0.082	8305	4.5 (1.1)	**0.001**	7481	1.7 (2.5)	**<0.001**
	Total cholesterol : HDL	7364	19.7 (1.3)	0.186	6339	3.4 (2.4)	**0.003**	7481	12.9 (1.0)	**<0.001**	7483	15.6 (1.0)	**<0.001**

P-value represents significance for smoothed term in the GAM. Model parameters shown as deviance explained in percentage (dev exp) with estimated degrees of freedom (EDF) in brackets. Bold face text denotes significant p-value (<0.05). Sociodemographic factors (SDF) included were age, ethnicity and deprivation. Model 1 was adjusted for SDF and fasting duration. Model 2 was adjusted for SDF, fasting duration and fat mass (%). Model 3 was adjusted for SDF, fasting duration and summed physical activity (MET min/wk). Model 4 was adjusted for SDF, fasting duration, estradiol, CRP and IGF-1 (except the exposure of interest). EDF, Estimated degree of Freedom; LDL, low density lipoprotein; HDL, high density lipoprotein; TyG index, triglyceride to glucose index.

Appendix 2.9: Variation in outcome variables across the menstrual cycle in participants without reported menstrual cycle symptoms.

Variable	N val	Mean ± SD	EDF	p-value	Minimum (IU (MC status)	Variation
Estradiol (pmol/L)	7788	564.3 ± 387.9	7.4	**<0.001**	377.33-705.7 (0.00-0.38)	328.37 (60.64)
IGF-1 (mmol/L)	7743	23.7 ± 5.4	2.8	**<0.001**	22.37-24.29 (0.00-0.67)	1.93 (8.25)
SHBG (mmol/L)	6970	69.7 ± 30.6	5.8	**0.002**	66.91-73.48 (0.21-0.00)	6.58 (9.37)
CRP (mg/L)	7763	2.0 ± 3.6	2.0	0.129	1.89-2.22 (0.29-1.00)	0.33 (15.84)
Walking	6566	1061.9 ± 1106.2	1.0	0.116	1041.68-1117.95 (0.00-1.00)	76.27 (7.06)
Moderate	6566	775.5 ± 1050.8	1.0	0.105	745.42-820.21 (0.00-1.00)	74.79 (9.55)
Vigorous	6566	662.7 ± 1024.6	1.2	0.152	618.33-691.69 (0.00-1.00)	73.35 (11.2)
Summed	6566	2500.1 ± 2411.9	1.1	**0.044**	2407.13-2628.93 (0.00-1.00)	221.8 (8.81)
Fitness						
Grip strength	7764	26.9 ± 6.0	7.0	**0.016**	26.64-27.62 (0.21-0.88)	0.97 (3.59)
CRF (METS)	1409	9.5 ± 2.4	1.80	**0.012**	9.55-9.92 (0.88-0.33)	0.37 (3.80)
Metabolites						
Glucose	6985	4.8 ± 0.5	2.0	0.176	4.77-4.81 (0.42-1.00)	0.04 (0.90)
Triglyceride	7782	1.2 ± 0.7	2.0	0.488	1.19-1.23 (1.00-0.54)	0.03 (2.67)
TyG index	6983	4.5 ± 0.2	1.7	0.432	4.50-4.51 (0.00-0.58)	0.01 (0.20)
Total cholesterol	7780	5.4 ± 0.9	4.1	**0.006**	5.33-5.45 (0.00-0.25)	0.12 (2.15)
HDL	6984	1.6 ± 0.4	4.0	**<0.001**	1.53-1.59 (0.00-0.46)	0.06 (3.61)
LDL	7769	3.3 ± 0.7	4.3	0.168	3.26-3.33 (0.00-0.21)	0.07 (2.03)
Total cholesterol: HDL	6981	3.6 ± 0.9	6.2	0.029	3.53-3.66 (0.50-0.13)	0.14 (3.76)

P-value represents significance level for smoothed terms in the GAM. Bold face text denotes significant p-value (<0.05). Participants reporting any physical symptoms associated with the menstrual cycle (abdominal discomfort, abdominal pain, menstrual cramps or other

problems) were excluded. Analyses were adjusted for age, ethnicity and deprivation. Menstrual cycle phase values are shown on a scale of 0-1, this corresponds to the approximate phases: follicular phase, 0-0.54; luteal phase 0.54-1 (14). CRF, cardiorespiratory fitness; EDF, estimated degree of Freedom; HDL, high density lipoprotein; LDL, low density lipoprotein, TyG index; triglyceride to glucose index.

Appendix 2.10: Effect of continuous covariates on variation in metabolites across the menstrual cycle excluding menstrual cycle symptoms

Variable	Value	BMI (kg/m²)	Fat mass (%)	Skeletal muscle mass (%)	Summed PA (MET min/wk)	HbA1c (mmol/ mmol)	Grip strength (kg)	Cardiorespiratory fitness (METs)
Glucose	N	6960	6866	6863	5884	6569	6963	1319
	Dev exp	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	0.1
	EDF	1.2	1.3	1.3	1.2	1.4	1.3	4.3
	p-value	0.085	0.065	0.068	0.116	0.058	0.077	**0.006**
Triglyceride	N	7757	7657	7654	6561	7330	7758	1407
	Dev exp	0.2	0.1	0.1	<0.1	0.1	<0.1	0.1
	EDF	4.4	4.1	4.1	0.9	4.5	4.0	3.6
	p-value	**0.009**	**0.020**	**0.030**	0.209	0.145	0.093	**0.019**
TyG index	N	6958	6864	6861	5882	6567	6961	1319
	Dev exp	0.2	0.2	0.2	<0.1	0.1	<0.1	0.1
	EDF	4.0	3.8	3.7	1.2	3.8	3.6	3.3
	p-value	**0.018**	0.051	0.077	0.141	0.155	0.096	**0.018**
Total cholesterol	N	7755	7655	7652	6559	7328	7756	1408
	Dev exp	<0.1	0.1	0.1	<0.1	<0.1	<0.1	<0.1
	EDF	3.2	3.1	3.0	3.1	3.4	3.2	1.2
	p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.002**	**<0.001**	0.108
HDL	N	6959	6865	6862	5882	6568	6962	1320
	Dev exp	0.2	0.2	0.1	<0.1	<0.1	<0.1	0.1
	EDF	2.4	2.4	2.3	2.3	2.5	2.6	4.2
	p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.021**
LDL	N	7744	7644	7641	6550	7320	7745	1408
	Dev exp	0.1	0.1	0.1	<0.1	<0.1	<0.1	0.1
	EDF	3.5	3.4	3.2	3.3	3.6	3.4	0.0
	p-value	**0.007**	**0.002**	**0.003**	**0.031**	**0.042**	0.053	0.651
Total cholesterol: HDL	N	6956	6862	6859	5879	6565	6959	1319
	Dev exp	0.2	0.2	0.2	<0.1	0.1	<0.1	0.1
	EDF	5.2	5.8	6.0	4.2	6.2	5.7	4.1
	p-value	**0.001**	**0.001**	**0.001**	**0.025**	**<0.001**	**0.002**	0.158

P-value represents significance for the smoothed term of menstrual cycle phase in the GAM. Bold face text denotes significant p-value (<0.05). Participants reporting any physical symptoms associated with the menstrual cycle (abdominal discomfort, abdominal pain, menstrual cramps or other problems) were excluded. Analyses were adjusted for age, ethnicity and deprivation. Menstrual cycle phase values are shown on a scale of 0-1, this corresponds to the approximate phases: follicular phase, 0-0.54; luteal phase 0.54-1 (14). EDF, estimated degree of Freedom; HbA1c, glycated haemoglobin LDL, low density lipoprotein; HDL, high density lipoprotein.

Appendix 2.11: Sub-group analysis for variation in metabolites across the menstrual cycle excluding women with menstrual cycle symptoms.

Variable	Level	N val	EDF (P val)	Min-max (IU (MC status))	Variation (IU (%))
Fat mass (%)					
Glucose	1	1660	<0.1 (0.64)	4.74-4.74 (0.38-0.96)	0.00 (0.00)
	2	1710	0.7 (0.22)	4.75-4.76 (0.58-0.08)	0.02 (0.39)
	3	1709	2.1 (0.02)	4.74-4.81 (0.54-0.13)	0.07 (1.56)
	4	1787	0.9 (0.17)	4.82-4.85 (0.25-0.71)	0.02 (0.50)
HDL	1	1660	2.0 (0.01)	1.68-1.73 (0.79-0.38)	0.05 (3.18)
	2	1709	2.0 (0.00)	1.58-1.65 (0.00-0.46)	0.07 (4.15)
	3	1709	3.6 (0.02)	1.48-1.55 (0.04-0.38)	0.07 (4.52)
	4	1787	3.9 (0.11)	1.37-1.42 (0.88-0.50)	0.05 (3.93)
LDL	1	1863	1.3 (0.06)	3.03-3.10 (0.83-0.29)	0.06 (2.07)
	2	1900	3.0 (0.45)	3.14-3.20 (0.50-0.25)	0.06 (1.90)
	3	1894	2.5 (0.01)	3.33-3.45 (0.79-0.21)	0.12 (3.51)
	4	1987	3.2 (0.37)	3.47-3.55 (0.88-0.17)	0.08 (2.22)
Total cholesterol	1	1866	1.7 (0.01)	5.16-5.29 (0.83-0.33)	0.13 (2.52)
	2	1899	<0.1 (0.47)	5.29-5.29 (0.92-0.33)	0.00 (0.00)
	3	1898	3.1 (0.01)	5.42-5.60 (0.88-0.25)	0.19 (3.38)
	4	1992	4.7 (0.04)	5.44-5.64 (0.88-0.13)	0.20 (3.64)
Total cholesterol : HDL	1	1660	<0.1 (0.95)	3.13-3.13 (0.42-0.79)	0.00 (0.00)
	2	1707	1.6 (0.04)	3.33-3.43 (0.54-0.04)	0.09 (2.80)
	3	1709	4.7 (0.02)	3.63-3.86 (0.83-0.13)	0.24 (6.30)
	4	1786	2.8 (0.19)	4.10-4.21 (0.54-0.21)	0.11 (2.60)
Triglyceride	1	1865	<0.1 (0.89)	0.94-0.94 (0.92-0.38)	0.00 (0.00)
	2	1902	<0.1 (0.46)	1.10-1.10 (0.63-0.17)	0.00 (0.00)
	3	1898	7.2 (0.00)	1.14-1.43 (0.79-0.63)	0.28 (22.17)
	4	1992	4.7 (0.01)	1.44-1.59 (0.92-0.33)	0.15 (10.12)
TyG index	1	1660	<0.1 (0.80)	4.40-4.40 (0.92-0.50)	0.00 (0.00)
	2	1710	0.2 (0.32)	4.46-4.46 (0.67-0.17)	0.00 (0.05)
	3	1708	7.0 (0.01)	4.49-4.58 (0.79-0.63)	0.08 (1.85)
	4	1786	4.0 (0.01)	4.60-4.65 (0.96-0.71)	0.05 (1.04)
Fat-free mass (%)					
Glucose	1	1768	<0.1 (0.37)	4.83-4.83 (0.17-0.71)	0.00 (0.00)
	2	1703	1.9 (0.02)	4.72-4.79 (0.50-0.08)	0.07 (1.49)
	3	1717	1.1 (0.15)	4.75-4.78 (0.58-0.04)	0.03 (0.65)
	4	1675	<0.1 (0.78)	4.75-4.75 (0.38-0.96)	0.00 (0.00)
HDL	1	1768	2.7 (0.16)	1.38-1.42 (0.88-0.54)	0.04 (2.95)
	2	1703	0.9 (0.20)	1.52-1.53 (0.00-0.42)	0.02 (1.06)
	3	1716	1.9 (0.00)	1.58-1.64 (0.96-0.42)	0.06 (3.53)
	4	1675	1.8 (0.02)	1.68-1.73 (0.88-0.42)	0.05 (2.79)

LDL	1	1971	<0.1 (0.67)	3.52-3.52 (0.88-0.42)	0.00 (0.00)
	2	1884	1.8 (0.05)	3.36-3.44 (0.75-0.21)	0.08 (2.41)
	3	1906	<0.1 (0.52)	3.19-3.19 (0.79-0.29)	0.00 (0.00)
	4	1880	1.4 (0.08)	3.02-3.08 (0.79-0.29)	0.06 (1.98)
Total cholesterol	1	1976	4.0 (0.13)	5.47-5.62 (0.88-0.50)	0.15 (2.67)
	2	1888	2.8 (0.02)	5.44-5.60 (0.88-0.25)	0.16 (2.90)
	3	1907	1.2 (0.10)	5.27-5.34 (0.88-0.38)	0.07 (1.28)
	4	1881	1.7 (0.02)	5.14-5.26 (0.83-0.33)	0.12 (2.26)
Total cholesterol : HDL	1	1767	6.5 (0.05)	3.96-4.21 (0.54-0.13)	0.25 (6.20)
	2	1703	3.0 (0.12)	3.69-3.81 (0.79-0.13)	0.12 (3.17)
	3	1715	5.1 (0.33)	3.35-3.50 (0.54-0.08)	0.16 (4.56)
	4	1674	<0.1 (0.61)	3.13-3.13 (0.50-0.00)	0.00 (0.00)
Triglyceride	1	1976	1.56 (0.08)	1.48-1.54 (0.92-0.38)	0.06 (3.97)
	2	1888	3.60 (0.17)	1.25-1.34 (0.88-0.63)	0.10 (7.56)
	3	1908	<0.1 (0.72)	1.11-1.11 (0.79-0.33)	0.00 (0.00)
	4	1882	<0.1 (0.92)	0.95-0.95 (0.92-0.29)	0.00 (0.00)
TyG index	1	1767	2.07 (0.09)	4.61-4.64 (0.00-0.42)	0.02 (0.52)
	2	1702	3.69 (0.13)	4.52-4.55 (0.88-0.58)	0.04 (0.85)
	3	1717	5.59 (0.26)	4.45-4.49 (0.83-0.42)	0.04 (0.93)
	4	1675	<0.1 (0.88)	4.40-4.40 (0.96-0.58)	0.00 (0.00)
Physical activity					
Glucose	1	1054	3.9 (0.04)	4.73-4.88 (0.54-0.83)	0.14 (2.97)
	2	2437	<0.1 (0.37)	4.77-4.77 (0.42-0.92)	0.00 (0.00)
	3	2393	5.1 (0.44)	4.75-4.81 (0.38-0.83)	0.06 (1.33)
HDL	1	1054	2.2 (0.01)	1.45-1.53 (0.83-0.42)	0.08 (5.39)
	2	2436	2.5 (0.00)	1.53-1.59 (0.92-0.50)	0.06 (3.77)
	3	2392	2.9 (0.03)	1.58-1.63 (0.00-0.42)	0.05 (2.95)
LDL	1	1171	0.4 (0.29)	3.38-3.40 (0.83-0.29)	0.02 (0.56)
	2	2720	3.3 (0.09)	3.24-3.34 (0.83-0.17)	0.10 (3.17)
	3	2659	0.0 (0.80)	3.21-3.21 (0.58-0.25)	0.00 (0.00)
Total cholesterol	1	1173	1.7 (0.02)	5.39-5.54 (0.83-0.33)	0.14 (2.63)
	2	2723	3.8 (0.01)	5.29-5.48 (0.88-0.21)	0.19 (3.61)
	3	2663	3.0 (0.17)	5.29-5.39 (0.00-0.33)	0.10 (1.87)
Total cholesterol : HDL	1	1052	3.1 (0.29)	3.74-3.87 (0.50-0.79)	0.13 (3.30)
	2	2435	0.3 (0.30)	3.59-3.60 (0.54-0.04)	0.01 (0.34)
	3	2392	<0.1 (0.37)	3.48-3.48 (0.46-0.96)	0.00 (0.00)
Triglyceride	1	1174	0.1 (0.36)	1.35-1.35 (0.88-0.33)	0.00 (0.21)
	2	2724	1.0 (0.18)	1.20-1.23 (0.00-0.54)	0.03 (2.24)
	3	2663	<0.1 (0.99)	1.14-1.14 (0.92-0.29)	0.00 (0.00)
TyG index	1	1053	<0.1 (0.45)	4.56-4.56 (0.92-0.42)	0.00 (0.00)
	2	2436	<0.1 (0.37)	4.51-4.51 (0.04-0.54)	0.00 (0.00)
	3	2393	<0.1 (0.73)	4.48-4.48 (0.00-0.54)	0.00 (0.00)
HbA1c (mmol/mmol)					

Glucose	1	1749	5.3 (0.46)	4.67-4.76 (0.42-0.88)	0.09 (1.84)
	2	4346	0.8 (0.22)	4.77-4.79 (0.54-0.96)	0.01 (0.26)
	3	444	0.8 (0.24)	5.01-5.05 (0.46-0.04)	0.04 (0.77)
HDL	1	1748	2.1 (0.00)	1.56-1.63 (0.88-0.33)	0.07 (4.69)
	2	4346	2.7 (0.00)	1.54-1.59 (0.88-0.46)	0.05 (3.28)
	3	444	<0.1 (0.90)	1.40-1.40 (0.13-0.75)	0.00 (0.00)
LDL	1	1943	3.0 (0.29)	3.11-3.19 (0.96-0.25)	0.07 (2.37)
	2	4849	1.4 (0.10)	3.31-3.34 (0.71-0.21)	0.04 (1.08)
	3	493	<0.1 (0.96)	3.54-3.54 (0.00-0.71)	0.00 (0.00)
Total cholesterol	1	1945	3.6 (0.01)	5.15-5.35 (0.96-0.29)	0.20 (3.72)
	2	4854	1.7 (0.04)	5.40-5.47 (0.83-0.29)	0.07 (1.21)
	3	494	<0.1 (0.87)	5.60-5.60 (0.96-0.38)	0.00 (0.00)
Total cholesterol : HDL	1	1746	0.9 (0.19)	3.38-3.43 (0.33-0.79)	0.05 (1.33)
	2	4345	4.1 (0.00)	3.55-3.71 (0.50-0.17)	0.15 (4.26)
	3	444	<0.1 (0.91)	4.19-4.19 (0.42-0.75)	0.00 (0.00)
Triglyceride	1	1947	0.1 (0.35)	1.11-1.11 (0.08-0.58)	0.00 (0.24)
	2	4854	3.9 (0.30)	1.21-1.26 (0.88-0.67)	0.05 (4.34)
	3	494	1.4 (0.10)	1.49-1.60 (0.92-0.38)	0.11 (7.24)
TyG index	1	1749	0.5 (0.25)	4.46-4.47 (0.13-0.63)	0.01 (0.15)
	2	4344	3.4 (0.37)	4.51-4.52 (0.92-0.67)	0.02 (0.35)
	3	444	0.1 (0.34)	4.65-4.65 (0.88-0.33)	0.00 (0.03)
Cardiorespiratory fitness (METs)					
Glucose	1	401	2.0 (0.02)	4.85-4.98 (0.13-0.67)	0.13 (2.67)
	2	350	<0.1 (0.54)	4.89-4.89 (0.33-0.79)	0.00 (0.00)
	3	325	<0.1 (0.67)	4.82-4.82 (0.58-0.17)	0.00 (0.00)
	4	240	3.4 (0.19)	4.73-4.88 (0.46-0.71)	0.15 (3.14)
HDL	1	401	<0.1 (0.44)	1.47-1.47 (0.88-0.42)	0.00 (0.00)
	2	351	<0.1 (0.37)	1.57-1.57 (0.92-0.38)	0.00 (0.00)
	3	325	0.4 (0.28)	1.64-1.66 (0.63-0.13)	0.02 (1.01)
	4	240	5.6 (0.08)	1.63-1.83 (0.13-0.33)	0.20 (11.65)
LDL	1	432	<0.1 (0.64)	3.47-3.47 (0.38-0.92)	0.00 (0.00)
	2	364	<0.1 (0.59)	3.29-3.29 (0.08-0.58)	0.00 (0.00)
	3	348	1.1 (0.11)	3.17-3.28 (0.83-0.33)	0.11 (3.51)
	4	261	<0.1 (0.87)	3.12-3.12 (0.92-0.33)	0.00 (0.00)
Total cholesterol	1	432	3.7 (0.41)	5.47-5.70 (0.29-0.08)	0.23 (4.10)
	2	365	<0.1 (0.39)	5.40-5.40 (0.04-0.54)	0.00 (0.00)
	3	347	1.6 (0.02)	5.25-5.51 (0.83-0.33)	0.26 (4.82)
	4	261	<0.1 (0.38)	5.31-5.31 (0.88-0.38)	0.00 (0.00)
Total cholesterol : HDL	1	401	1.7 (0.08)	3.85-4.03 (0.42-0.00)	0.19 (4.75)
	2	351	6.8 (0.05)	3.36-3.87 (0.38-0.63)	0.51 (14.18)
	3	324	0.5 (0.26)	3.35-3.41 (0.00-0.50)	0.05 (1.60)
	4	240	<0.1 (0.72)	3.17-3.17 (0.25-0.71)	0.00 (0.00)
Triglyceride	1	432	6.3 (0.01)	1.23-1.68 (0.25-0.08)	0.45 (31.13)

	2	365	<0.1 (0.39)	1.18-1.18 (0.13-0.63)	0.00 (0.00)
	3	346	1.5 (0.04)	1.05-1.20 (0.00-0.50)	0.15 (13.5)
	4	261	0.9 (0.14)	0.94-1.02 (0.08-0.58)	0.08 (8.29)
TyG index	1	401	5.9 (0.04)	4.54-4.66 (0.25-0.08)	0.12 (2.68)
	2	350	4.8 (0.23)	4.48-4.57 (0.38-0.58)	0.09 (2.00)
	3	325	1.4 (0.05)	4.46-4.51 (0.96-0.5)	0.05 (1.06)
	4	240	1.0 (0.16)	4.41-4.44 (0.08-0.63)	0.03 (0.66)
Grip strength (kg)					
Glucose	1	1731	1.7 (0.06)	4.74-4.80 (0.29-0.75)	0.06 (1.21)
	2	1726	<0.1 (0.66)	4.78-4.78 (0.54-0.96)	0.00 (0.00)
	3	1745	4.6 (0.03)	4.73-4.85 (0.33-0.92)	0.11 (2.38)
	4	1761	0.5 (0.27)	4.78-4.79 (0.67-0.17)	0.01 (0.27)
HDL	1	1731	0.5 (0.24)	1.53-1.54 (0.92-0.46)	0.01 (0.60)
	2	1725	2.0 (0.00)	1.53-1.59 (0.96-0.46)	0.07 (4.22)
	3	1745	1.5 (0.03)	1.55-1.59 (0.88-0.38)	0.04 (2.66)
	4	1761	1.5 (0.02)	1.55-1.59 (0.88-0.38)	0.04 (2.62)
LDL	1	1946	<0.1 (0.91)	3.31-3.31 (0.58-0.21)	0.00 (0.00)
	2	1893	1.1 (0.09)	3.25-3.30 (0.67-0.13)	0.05 (1.56)
	3	1951	<0.1 (0.39)	3.30-3.30 (0.00-0.50)	0.00 (0.00)
	4	1955	3.7 (0.08)	3.19-3.32 (0.92-0.21)	0.13 (4.07)
Total cholesterol	1	1950	<0.1 (0.65)	5.40-5.40 (0.92-0.38)	0.00 (0.00)
	2	1896	1.3 (0.06)	5.34-5.42 (0.75-0.25)	0.08 (1.54)
	3	1951	1.7 (0.05)	5.37-5.47 (0.96-0.42)	0.10 (1.83)
	4	1959	3.4 (0.03)	5.26-5.44 (0.92-0.21)	0.17 (3.23)
Total cholesterol : HDL	1	1729	3.9 (0.20)	3.61-3.74 (0.50-0.71)	0.13 (3.58)
	2	1725	1.7 (0.01)	3.53-3.66 (0.54-0.08)	0.14 (3.80)
	3	1744	0.9 (0.21)	3.56-3.61 (0.33-0.79)	0.04 (1.24)
	4	1761	6.9 (0.13)	3.45-3.70 (0.54-0.67)	0.25 (6.92)
Triglyceride	1	1950	2.6 (0.08)	1.22-1.30 (0.96-0.33)	0.09 (6.88)
	2	1898	0.5 (0.27)	1.19-1.20 (0.63-0.13)	0.01 (1.21)
	3	1951	1.3 (0.11)	1.20-1.25 (0.08-0.63)	0.05 (4.04)
	4	1959	<0.1 (0.53)	1.18-1.18 (0.25-0.67)	0.00 (0.00)
TyG index	1	1729	3.0 (0.06)	4.50-4.54 (0.96-0.63)	0.04 (0.87)
	2	1726	0.7 (0.21)	4.50-4.51 (0.67-0.17)	0.01 (0.19)
	3	1745	1.11 (0.14)	4.51-4.52 (0.08-0.63)	0.02 (0.34)
	4	1761	<0.1 (0.80)	4.50-4.50 (0.21-0.63)	0.00 (0.00)

P-value represents significance for the smoothed term of menstrual cycle phase in the GAM. Bold face text denotes significant p-value (<0.05). Analyses were adjusted for age, ethnicity, deprivation. Fat mass, muscle mass, handgrip strength, and fitness are categorised as quartiles. IPAQ and HbA1c are categorised into low, medium and high

according to previously defined criteria. Menstrual cycle phase values are shown on a scale of 0-1, this corresponds to the approximate phases: follicular phase, 0-0.54; luteal phase 0.54-1 (1). EDF, estimated degrees of freedom; HbA1c, glycated haemoglobin; LDL, low density lipoprotein; HDL, high density lipoprotein.

Appendix 2.12: Relationship between metabolites with estradiol and inflammatory markers excluding women with menstrual cycle symptoms.

Variable	Metabolite	Model 1: SDF			Model 2: SDF +f at mass			Model 3: SDF+ physical activity			Model 4: SDF + estradiol, CRP and IGF-1		
		N	Dev exp (EDF)	p val	N	Dev exp (EDF)	p val	N	Dev exp (EDF)	p val	N	Dev exp (EDF)	p val
Estradiol	Glucose	6705	1.1 (2.6)	**<0.001**	6597	1.9 (2.8)	**<0.001**	5658	1.1 (3.2)	**<0.001**	6705	1.4 (2.5)	**<0.001**
	Triglyceride	7466	2.1 (3.1)	**<0.001**	7352	14.3 (3.2)	**0.001**	6305	3.5 (3.8)	**<0.001**	7466	12.8 (4.0)	**<0.001**
	TyG index	6703	3.1 (1.0)	**<0.001**	6595	17.6 (1.0)	**<0.001**	5656	4.4 (1.0)	**<0.001**	6703	15.2 (3.1)	**<0.001**
	HDL	7464	2.1 (3.4)	0.051	7350	4.3 (3.9)	0.130	6303	2.2 (3.6)	**0.028**	7464	3.2 (3.3)	0.075
	LDL	6704	2.3 (2.0)	**<0.001**	6596	14.7 (2.1)	**<0.001**	5656	3.6 (1.8)	**<0.001**	6704	9.9 (1.9)	**<0.001**
	Total cholesterol	7453	1.5 (1.4)	**0.001**	7339	7.8 (3.3)	0.105	6294	2.2 (3.0)	**0.003**	7453	4.2 (1.6)	**0.009**
	Total cholesterol : HDL	6701	1.7 (1.0)	**<0.001**	6593	19.6 (1.0)	**<0.001**	5653	3.4 (1.0)	**<0.001**	6701	12.7 (1.0)	**<0.001**
CRP	Glucose	6705	0.8 (1.0)	**0.001**	6597	1.6 (1.1)	0.738	5658	1.1 (8.5)	**0.003**	6705	1.4 (8.4)	**0.016**
	Triglyceride	7466	12.4 (7.4)	**<0.001**	7352	16.7 (6.7)	**<0.001**	6305	13.0 (7.4)	**<0.001**	7466	12.8 (7.4)	**<0.001**
	TyG index	6703	14.6 (7.2)	**<0.001**	6595	19.9 (6.3)	**<0.001**	5656	15.2 (7.0)	**<0.001**	6703	15.2 (7.3)	**<0.001**
	HDL	7464	3.0 (5.2)	**<0.001**	7350	4.3 (3.9)	0.110	6303	3.1 (4.9)	**<0.001**	7464	3.2 (5.2)	**<0.001**
	LDL	6704	9.1 (4.4)	**<0.001**	6596	15.3 (2.6)	**<0.001**	5656	10.1 (4.2)	**<0.001**	6704	9.9 (4.6)	**<0.001**
	Total cholesterol	7453	4.0 (5.6)	**<0.001**	7339	7.8 (3.6)	0.064	6294	4.6 (5.1)	**<0.001**	7453	4.2 (5.6)	**<0.001**
	Total cholesterol : HDL	6701	12.1 (5.4)	**<0.001**	6593	20.8 (3.9)	**<0.001**	5653	13.4 (4.9)	**<0.001**	6701	12.7 (5.4)	**<0.001**
IGF-1	Glucose	6705	0.7 (1.0)	0.492	6597	1.6 (1.0)	0.762	5658	0.6 (1.0)	0.592	6705	1.4 (1.0)	0.932
	Triglyceride	7466	2.2 (2.7)	**<0.001**	7352	14.1 (1.8)	0.203	6305	3.4 (2.8)	**<0.001**	7466	12.8 (1.6)	0.193
	TyG index	6703	3.0 (3.1)	**<0.001**	6595	17.3 (2.7)	0.505	5656	4.2 (3.7)	**0.001**	6703	15.2 (2.5)	**0.005**
	HDL	7464	2.0 (4.1)	0.233	7350	4.3 (4.3)	0.441	6303	2.2 (4.5)	0.196	7464	3.2 (4.1)	0.484
	LDL	6704	1.9 (5.6)	**0.032**	6596	14.5 (1.0)	**0.002**	5656	3.0 (1.9)	0.402	6704	9.9 (1.0)	**<0.001**
	Total cholesterol	7453	1.3 (1.0)	0.184	7339	7.7 (1.0)	0.345	6294	2.1 (3.2)	0.334	7453	4.2 (1.0)	0.090
	Total cholesterol : HDL	6701	1.5 (2.6)	**<0.001**	6593	19.4 (1.7)	0.155	5653	3.1 (2.6)	**0.002**	6701	12.7 (1.8)	**<0.001**

P-value represents significance for smoothed term in the GAM. Model parameters shown as deviance explained in percentage (dev exp) with estimated degrees of freedom (EDF) in brackets. Bold face text denotes significant p-value (<0.05). Sociodemographic factors (SDF) included were age, ethnicity and deprivation. Model 1 was adjusted for SDF. Model 2 was adjusted for SDF and fat mass (%). Model 3 was adjusted for SDF and summed physical activity (MET min/wk). Model 4 was adjusted for SDF and estradiol, CRP and IGF-1 (except the exposure of interest). EDF, estimated degree of Freedom; LDL, low density lipoprotein; HDL, high density lipoprotein; TyG index, triglyceride to glucose index.

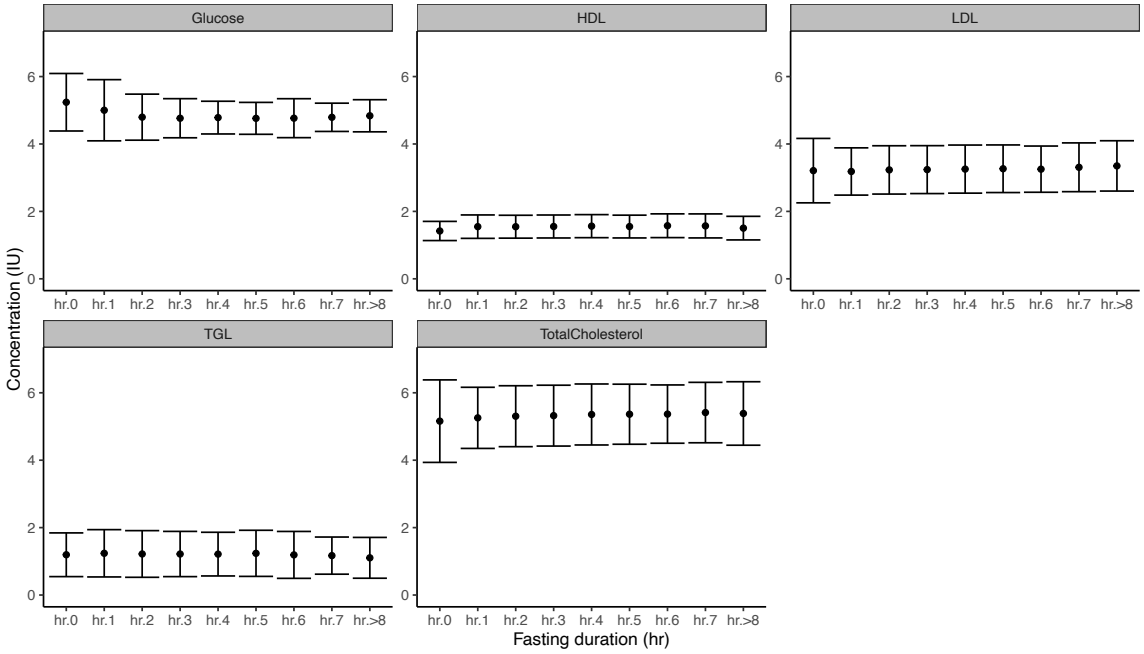
Appendix 2.13: Sub-group analysis for variation in estradiol, CRP and IGF-1 across the menstrual cycle.

Variable	Level	N val	EDF (P val)	Min-max (IU (MC status))	Variation (IU (%))
Fat mass (%)					
Estradiol	1	2122	4.5 (**<0.001**)	416.48-728.39 (0.38-1.00)	311.91 (54.49)
Estradiol	2	2120	5.4 (**<0.001**)	440.88-755.27 (0.38-0.00)	314.39 (52.57)
Estradiol	3	2110	7.1 (**<0.001**)	408.61-694.24 (0.42-0.00)	285.63 (51.80)
Estradiol	4	2171	4.5 (**<0.001**)	395.51-605.03 (0.33-0.00)	209.52 (41.88)
CRP	1	2113	0.0 (0.628)	0.92-0.92 (0.88-0.38)	0.00 (0.00)
CRP	2	2113	0.0 (0.846)	1.39-1.39 (0.33-0.83)	0.00 (0.00)
CRP	3	2106	0.0 (0.84)	1.73-1.73 (0.75-0.29)	0.00 (0.00)
CRP	4	2167	5.4 (**0.022**)	3.45-4.22 (0.00-0.21)	0.77 (20.17)
IGF-1	1	2113	2.8 (**<0.001**)	22.97-25.13 (0.67-0.13)	2.16 (8.96)
IGF-1	2	2108	6.5 (**<0.001**)	23.31-25.40 (0.63-0.08)	2.08 (8.56)
IGF-1	3	2097	6.0 (**<0.001**)	23.11-24.99 (0.54-0.08)	1.88 (7.82)
IGF-1	4	2159	3.3 (**<0.001**)	21.57-23.40 (0.67-0.13)	1.83 (8.12)
Muscle mass (%)					
Estradiol	1	2122	4.6 (**<0.001**)	389.59-606.46 (0.38-0.00)	216.87 (43.55)
Estradiol	2	2120	4.4 (**<0.001**)	435.12-667.01 (0.38-0.00)	231.89 (42.08)
Estradiol	3	2110	6.4 (**<0.001**)	428.77-755.91 (0.38-0.00)	327.14 (55.23)
Estradiol	4	2171	4.4 (**<0.001**)	414.71-733.39 (0.38-0.00)	318.69 (55.52)
CRP	1	2113	1.4 (0.109)	3.56-3.82 (0.00-0.54)	0.26 (7.12)
CRP	2	2113	5.5 (**0.016**)	1.47-2.38 (0.63-0.38)	0.91 (47.39)
CRP	3	2106	0.0 (0.934)	1.43-1.43 (0.33-0.79)	0.00 (0.00)
CRP	4	2167	0.0 (0.992)	0.91-0.91 (0.88-0.21)	0.00 (0.00)
IGF-1	1	2113	3.8 (**<0.001**)	21.59-23.15 (0.75-0.13)	1.56 (6.95)
IGF-1	2	2108	5.7 (**<0.001**)	22.97-24.97 (0.54-0.04)	2.00 (8.36)
IGF-1	3	2097	2.3 (**<0.001**)	23.67-24.90 (0.63-0.13)	1.23 (5.06)
IGF-1	4	2159	2.9 (**<0.001**)	23.13-25.32 (0.67-0.13)	2.19 (9.04)
Physical activity					
Estradiol	1	1302	4.8 (**<0.001**)	425.69-692.66 (0.38-0.04)	266.97 (47.74)
Estradiol	2	3054	5.0 (**<0.001**)	420.95-697.29 (0.33-0.00)	276.34 (49.42)
Estradiol	3	2977	5.5 (**<0.001**)	418.33-696.91 (0.38-0.00)	278.58 (49.96)
CRP	1	1299	7.3 (**0.007**)	1.95-3.44 (0.67-0.21)	1.49 (55.38)
CRP	2	3045	0.6 (0.25)	1.77-1.85 (0.92-0.42)	0.08 (4.19)
CRP	3	2971	0.0 (0.399)	1.63-1.63 (0.58-0.17)	0.00 (0.00)
IGF-1	1	1299	2.6 (**<0.001**)	22.73-24.19 (0.54-0.13)	1.45 (6.20)
IGF-1	2	3037	5.0 (**<0.001**)	22.78-24.60 (0.63-0.13)	1.82 (7.67)
IGF-1	3	2957	2.8 (**<0.001**)	23.23-24.82 (0.67-0.13)	1.59 (6.60)
HbA1c (mmol/mmol)					

Estradiol	1	2179	5.7 (**<0.001**)	408.25-759.85 (0.38-0.04)	351.6 (60.2)
Estradiol	2	5405	5.7 (**<0.001**)	407.92-680.60 (0.38-0.00)	272.67 (50.1)
Estradiol	3	533	1.9 (**0.005**)	487.49-604.67 (0.42-0.96)	117.18 (21.46)
CRP	1	2171	0.0 (0.903)	1.58-1.58 (0.79-0.25)	0.00 (0.00)
CRP	2	5389	4.9 (0.112)	1.81-2.20 (0.00-0.21)	0.39 (19.43)
CRP	3	532	5.0 (**0.011**)	3.18-4.96 (0.67-0.13)	1.78 (43.77)
IGF-1	1	2170	3.6 (**<0.001**)	22.62-24.57 (0.67-0.08)	1.96 (8.29)
IGF-1	2	5374	6.6 (**<0.001**)	22.79-24.84 (0.88-0.04)	2.04 (8.58)
IGF-1	3	529	4.7 (0.181)	21.95-23.66 (0.54-0.96)	1.71 (7.48)
Cardiorespiratory fitness (METs)					
Estradiol	1	497	1.8 (**0.026**)	494.4-601.68 (0.42-0.96)	107.29 (19.58)
Estradiol	2	425	2.4 (**<0.001**)	478.01-672.15 (0.38-0.92)	194.15 (33.76)
Estradiol	3	400	3.9 (**<0.001**)	448.29-698.25 (0.38-0.04)	249.96 (43.6)
Estradiol	4	315	6.1 (**<0.001**)	395.95-796.11 (0.33-0.96)	400.16 (67.14)
CRP	1	497	6.9 (**0.001**)	2.27-5.70 (0.63-0.21)	3.43 (86.13)
CRP	2	424	0.1 (0.343)	1.90-1.96 (0.67-0.21)	0.06 (2.99)
CRP	3	400	0.0 (0.906)	1.22-1.22 (0.92-0.38)	0.00 (0.00)
CRP	4	314	0.7 (0.24)	1.12-1.42 (0.42-0.92)	0.30 (23.48)
IGF-1	1	494	1.2 (0.105)	22.57-23.37 (0.79-0.29)	0.80 (3.50)
IGF-1	2	425	7.3 (**0.020**)	22.32-26.41 (0.67-0.00)	4.09 (16.77)
IGF-1	3	400	1.7 (**0.031**)	24.03-25.35 (0.63-0.08)	1.32 (5.36)
IGF-1	4	312	5.4 (**0.001**)	23.01-26.43 (0.83-0.04)	3.42 (13.83)
Grip strength (kg)					
Estradiol	1	2153	7.0 (**<0.001**)	432.43-741.49 (0.33-0.00)	309.06 (52.66)
Estradiol	2	2112	6.1 (**<0.001**)	394.89-710.67 (0.38-0.00)	315.78 (57.13)
Estradiol	3	2160	4.7 (**<0.001**)	397.55-680.93 (0.38-0.96)	283.37 (52.55)
Estradiol	4	2209	4.5 (**<0.001**)	427.89-676.87 (0.38-0.00)	248.99 (45.07)
CRP	1	2147	7.1 (0.135)	1.83-2.73 (0.67-0.25)	0.89 (39.16)
CRP	2	2107	4.8 (0.138)	1.77-2.41 (0.92-0.50)	0.64 (30.41)
CRP	3	2153	0.6 (0.247)	1.71-1.81 (0.92-0.42)	0.10 (5.77)
CRP	4	2201	0 (0.456)	1.74-1.74 (0.71-0.25)	0.00 (0.00)
IGF-1	1	2138	6.0 (**<0.001**)	22.10-24.08 (0.54-0.04)	1.99 (8.60)
IGF-1	2	2099	2.4 (**<0.001**)	22.81-24.14 (0.58-0.13)	1.33 (5.68)
IGF-1	3	2153	2.7 (**<0.001**)	23.04-25.02 (0.58-0.13)	1.98 (8.24)
IGF-1	4	2197	5.6 (**<0.001**)	23.43-25.23 (0.67-0.04)	1.80 (7.39)

P-value represents significance for the smoothed term of menstrual cycle phase in the GAM. Bold face text denotes significant p-value (<0.05). Analyses were adjusted for age, ethnicity, deprivation. Fat mass, muscle mass, handgrip strength, and fitness are categorised as quartiles. IPAQ and HbA1c are categorised into low, medium and high according to previously defined criteria. Menstrual cycle phase values are shown on a

scale of 0-1, this corresponds to the approximate phases: follicular phase, 0-0.54; luteal phase 0.54-1 (1). CRP, C-reactive protein; EDF, estimated degrees of freedom; HbA1c, glycated haemoglobin; IGF-1, insulin like growth factor-1.



Appendix 2.14: Changes in metabolite concentration across hourly fasting durations.

Appendix 2.15: Metabolite concentration across fasting durations.

Fasting duration (hr)	Glucose		Triglyceride		Total cholesterol		HDL		LDL	
	Value (mmol/L)	P-val	Value (mmol/L)	P-val	Value (mmol/L)	P-val	Value (mmol/L)	P-val	Value (mmol/L)	P-val
0	5.24 ± 0.85	0.147	1.19 ± 0.65	1.000	5.16 ± 1.22	0.608	1.42 ± 0.28	0.660	3.21 ± 0.96	0.833
1	5.00 ± 0.91	**<0.001**	1.24 ± 0.70	0.923	5.26 ± 0.91	0.992	1.55 ± 0.35	0.178	3.18 ± 0.70	1.000
2	4.80 ± 0.68	0.052	1.22 ± 0.69	1.000	5.30 ± 0.90	0.371	1.55 ± 0.34	1.000	3.23 ± 0.72	0.169
3	4.76 ± 0.58	0.665	1.22 ± 0.67	1.000	5.32 ± 0.90	0.908	1.55 ± 0.34	0.932	3.24 ± 0.71	0.991
4	4.78 ± 0.49	0.770	1.21 ± 0.65	0.758	5.36 ± 0.90	0.313	1.56 ± 0.34	0.655	3.26 ± 0.71	0.875
5	4.76 ± 0.47	1.000	1.24 ± 0.68	0.398	5.36 ± 0.89	1.000	1.55 ± 0.34	0.762	3.27 ± 0.71	0.997
6	4.77 ± 0.58	0.997	1.19 ± 0.70	0.999	5.37 ± 0.86	1.000	1.57 ± 0.35	0.415	3.25 ± 0.69	0.999
7	4.79 ± 0.42	0.915	1.17 ± 0.55	0.703	5.41 ± 0.90	0.985	1.57 ± 0.36	1.000	3.31 ± 0.72	0.863
≥ 8	4.84 ± 0.48	NA	1.10 ± 0.61	NA	5.39 ± 0.94	NA	1.50 ± 0.35	NA	3.35 ± 0.75	NA

Values are represented as mean ± 1 SD. P-value represents paired t-test between consecutive hours of fasting duration (i.e. 0-1, 1-2, etc). Bold type font represents a significant P-value (< 0.05). HDL, high density lipoprotein; LDL, low density lipoprotein. Note: significant p-value for glucose at 0 hr vs 1 hr is at a timepoint prior to the 4 hr threshold for fasting duration implemented as a participant exclusion criteria.

Appendix 3.1: Menstrual cycle questionnaire

01/05/2018 [V2.0]

UNIVERSITY of STIRLING

Participant number:

Date:

Menstrual cycle questionnaire.

Have you ever had/experienced any of the following in the previous 3 months: (Please mark an X the appropriate box)	**YES**	**NO**
Current pregnancy or pregnancy within the previous 3 months?		
Use of any hormone based medication (Including any hormone based contraception, i.e. contraceptive pill or contraceptive implant)?		
Previous diagnoses of a menstrual cycle disorder?		
Absence of a period?		
Severe painful cramps during menstruation?		
Heavy menstrual bleeding (including bleeding >7d)?		
Bleeding between periods?		

Appendix 3.2: Menstrual cycle diary

01/05/2018 [V2.0]

UNIVERSITY of STIRLING

Participant number:

Date:

Menstrual cycle diary

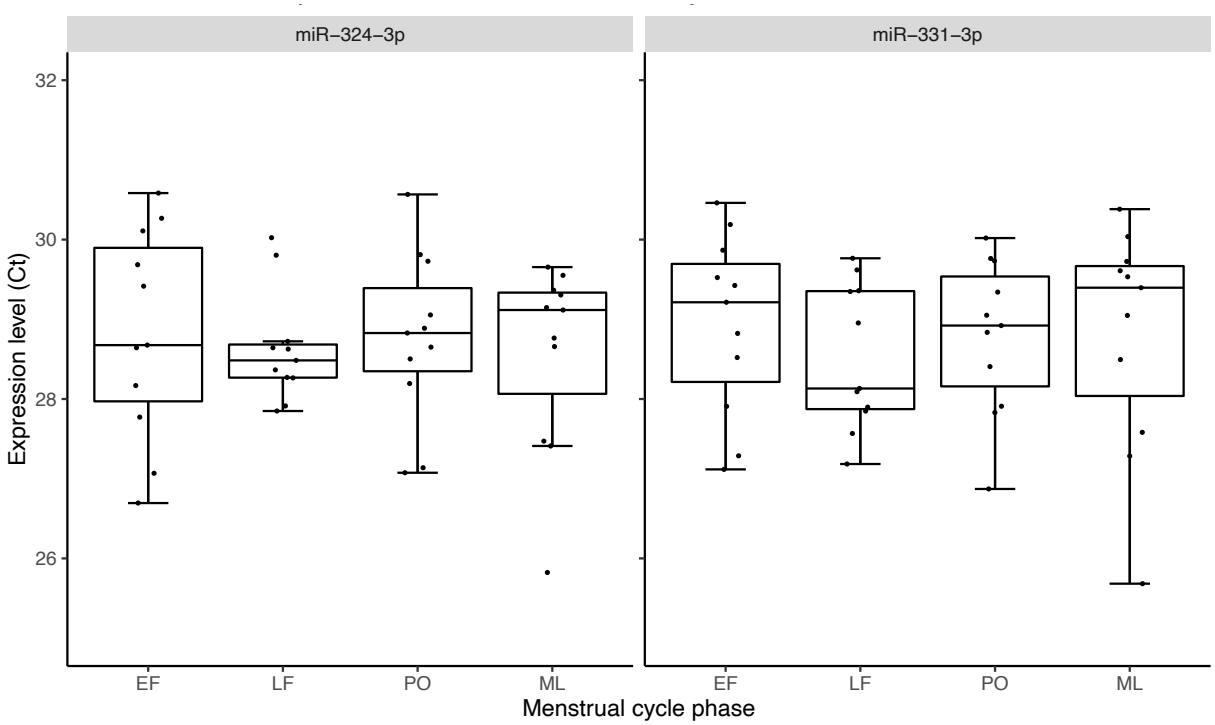
The questions below are about your current menstrual cycle.

- Please answer question 5-6 during the occurrence of menstrual bleeding.
- Date period begins is defined as the first day of menstrual bleeding.

1. Date last period began:									
2. Date this period began:									
3. Date of positive luteinizing hormone test:									
4. Symptoms of this period have been:									
1	2	3	4	5	6	7	8	9	10
Least symptomatic									Most symptomatic
5. This period compared to previous cycles has been									
1	2	3	4	5	6	7	8	9	10
Less symptomatic					No change				More symptomatic
6. Did this cycle exhibit any of the following? (Place an X in the appropriate box)								YES	NO
Heavy menstrual bleeding (including bleeding duration >7d)									
Severe cramps/unusually painful cramps during menstruation									

Appendix 3.3: miRNA target sequence in Qiagen custom LNA miRNA RT-qPCR panels.

Catalogue number	miRNA ID	Target sequence
YP00204308	hsa-miR-155-5p	UUAAUGCUAAUCGUGAUAGGGGU
YP00206046	hsa-miR-331-3p	GCCCCUGGGCCUAUCCUAGAA
YP00204303	hsa-miR-324-3p	ACUGCCCCAGGUGCUGCUGG
YP02113205	hsa-miR-328-5p	GGGGGGGCAGGAGGGGCUCAGGG
YP00205632	hsa-miR-30c-2-3p	CUGGGAGAAGGCUGUUUACUCU
YP00204783	hsa-miR-30c-5p	UGUAAACAUCCUACACUCUCAGC
YP00204292	hsa-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG
YP00205873	hsa-miR-23b-5p	UGGGUUCCUGGCAUGCUGAUUU
YP00204629	hsa-miR-224-3p	AAAAUGGUGCCCUAGUGACUACA
YP00204638	hsa-miR-550a-5p	AGUGCCUGAGGGAGUAAGAGCCC
YP00204423	hsa-miR-331-5p	CUAGGUAUGGUCCCAGGGAUCC
YP02104649	hsa-miR-3180-3p	UGGGGCGGAGCUUCCGGAGGCC
YP00205702	hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG
YP00204080	hsa-miR-194-5p	UGUAACAGCAACUCCAUGUGGA
YP00204258	hsa-miR-92a-3p	UAUUGCACUUGUCCCGGCCUGU
YP00204116	hsa-miR-616-3p	AGUCAUUGGAGGGUUUGAGCAG
YP00204354	hsa-miR-497-5p	CAGCAGCACACUGUGGUUUGU
YP02105743	hsa-miR-500b-3p	GCACCCAGGCAAGGAUUCUG
YP02114733	hsa-miR-1231	GUGUCUGGGCGGACAGCUGC
YP00204644	hsa-miR-1914-3p	GGAGGGGUCCCGCACUGGGAGG
YP02106599	hsa-miR-3615	UCUCUCGGCUCCUCGCGGCUC



Appendix 3.4: Raw cycle threshold (Ct) values for RT-qPCR endogenous controls (miR-324-3p & miR-331-3p) in the early-follicular (EF), late-follicular (LF), post-ovulatory (PO) and mid-luteal (ML) phases of the menstrual cycle (N=11). Data are displayed as conventional box and whisker plot with individual data points.

Appendix 3.5: Differentially expressed adipose tissue miRNA across the menstrual cycle determined using microarray analysis.

miRNA	Early-follicular	Late-follicular	Post-ovulatory	Mid- luteal	P-value	Adjusted P-value
mir-16-1	2.43 ± 0.72	2.03 ± 0.44	1.4 ± 0.67	2.32 ± 0.33	0.039	0.68
mir-16-2	4.67 ± 0.89	5.18 ± 0.95	4.8 ± 1.73	5.57 ± 1.43	0.046	0.68
mir-20a	2.12 ± 0.19	1.74 ± 0.24	1.91 ± 0.29	1.86 ± 0.26	0.046	0.68
mir-20a-5p	2.35 ± 0.36	2.15 ± 0.37	1.73 ± 0.47	1.85 ± 0.46	0.039	0.68
mir-23b-5p	1.84 ± 0.21	1.82 ± 0.31	1.77 ± 0.43	2.11 ± 0.31	0.045	0.68
mir-30c-2-3p	1.71 ± 0.26	2 ± 0.23	2.07 ± 0.31	1.91 ± 0.28	0.015	0.68
mir-30c-5p	1.76 ± 0.2	1.89 ± 0.26	2.06 ± 0.42	1.5 ± 0.2	0.004	0.68
mir-92a-2	1.8 ± 0.32	1.89 ± 0.19	1.85 ± 0.2	1.51 ± 0.07	0.048	0.68
mir-194-2	1.81 ± 0.2	1.9 ± 0.26	1.92 ± 0.36	1.54 ± 0.23	0.020	0.68
mir-224-3p	2.1 ± 0.3	1.7 ± 0.35	1.8 ± 0.35	1.94 ± 0.26	0.012	0.68
mir-331-5p	3.59 ± 0.88	4.05 ± 0.91	3.66 ± 1.55	4.33 ± 1.13	0.001	0.67
mir-497	1.82 ± 0.56	2.23 ± 0.63	2.36 ± 1.07	2.53 ± 1.23	0.043	0.68
mir-500b	2.73 ± 0.84	3.38 ± 1.26	2.88 ± 1.27	3.95 ± 1.56	0.006	0.68
mir-550a-5p	1.62 ± 0.17	2.04 ± 0.17	2.03 ± 0.18	2.05 ± 0.43	0.032	0.68
mir-616	1.78 ± 0.43	1.87 ± 0.57	2.02 ± 0.73	2.66 ± 0.47	0.023	0.68
mir-1231	3.17 ± 0.79	2.75 ± 0.67	2.54 ± 1.2	3.77 ± 0.67	0.024	0.68
mir-1272	1.76 ± 0.2	1.79 ± 0.22	2.01 ± 0.29	2.11 ± 0.21	0.009	0.68
mir-1321	1.65 ± 0.34	2.04 ± 0.2	1.59 ± 0.52	2.21 ± 0.32	0.037	0.68

mir-1914	2.3 ± 0.43	1.76 ± 0.42	1.74 ± 0.56	2.17 ± 0.21	0.049	0.68
mir-3133	2.01 ± 0.21	1.71 ± 0.21	1.9 ± 0.25	1.94 ± 0.17	0.004	0.68
mir-3180-1	2.97 ± 0.66	2.58 ± 0.63	2.31 ± 1.1	3.56 ± 0.71	0.025	0.68
mir-3180-2	2.97 ± 0.66	2.58 ± 0.63	2.31 ± 1.1	3.56 ± 0.71	0.025	0.68
mir-3180-3	2.97 ± 0.66	2.58 ± 0.63	2.31 ± 1.1	3.56 ± 0.71	0.025	0.68
mir-3180-3p	2.91 ± 0.74	2.55 ± 0.66	2.27 ± 1.14	3.61 ± 0.68	0.024	0.68
mir-3180-4	5.17 ± 0.72	3.93 ± 1.4	4.11 ± 1.52	4.77 ± 1.55	0.040	0.68
mir-3180-5	3.45 ± 0.7	3.38 ± 0.34	2.83 ± 0.99	3.46 ± 0.37	0.019	0.68
mir-3615	2.02 ± 0.39	1.56 ± 0.41	1.77 ± 0.86	2.03 ± 0.26	0.014	0.68
mir-3914-1	3.65 ± 0.64	4.12 ± 0.93	4.16 ± 1.66	4.62 ± 1.7	0.040	0.68
mir-4443	2.68 ± 0.54	2.32 ± 0.7	2.51 ± 0.55	3.07 ± 0.21	0.016	0.68
mir-4538	2.23 ± 0.49	2.27 ± 0.31	2.49 ± 0.49	2.88 ± 0.38	0.022	0.68
mir-4716	4.8 ± 0.95	5.24 ± 1.04	4.8 ± 1.98	5.58 ± 1.62	0.046	0.68
mir-4745	1.48 ± 0.39	2.02 ± 0.38	1.87 ± 0.28	1.79 ± 0.36	0.048	0.68
mir-7976	3.05 ± 0.81	2.58 ± 0.68	2.22 ± 1.21	3.58 ± 0.87	0.025	0.68

Data are expressed as mean ± SD (N=8). P-value represents moderated empirical Bayes ANOVA. Adjusted p-value represents FDR correction for multiple testing. P-value < 0.05 considered statistically significant.

Appendix 3.6: miRNA expression in the early-follicular, late-follicular, post-ovulatory and mid-luteal phase of the menstrual cycle.

miRNA	Early-follicular	Late-follicular	Post-ovulatory	Mid-luteal	p-value	R^2
miR-155-5p	1.37 ± 1.08	1.03 ± 0.52	1.15 ± 1.13	1.72 ± 2.49	0.882	<0.01
miR-16-5p	1.83 ± 2.54	1.73 ± 0.85	2.12 ± 2.47	3.71 ± 5.09	0.577	<0.01
miR-194-5p	1.85 ± 2.61	1.09 ± 0.64	1.7 ± 1.35	2 ± 2.06	0.856	<0.01
miR-20a-5p	2.2 ± 3.4	1.83 ± 1.05	2.41 ± 2.56	3.6 ± 5.59	0.714	<0.01
miR-224-3p	1.18 ± 0.65	1.03 ± 0.6	1.11 ± 0.5	1.19 ± 1.37	0.769	<0.01
miR-23b-5p	1.07 ± 0.38	1.16 ± 0.57	1.08 ± 0.41	1.4 ± 1.13	0.953	<0.01
miR-30c-2-3p	1.18 ± 0.75	1.07 ± 0.52	1.17 ± 0.4	1.16 ± 0.97	0.896	<0.01
miR-30c-5p	1.2 ± 0.78	1.23 ± 0.99	1.43 ± 1.23	2.17 ± 3.39	0.883	<0.01
miR-328-5p	1.09 ± 0.45	0.73 ± 0.51	0.67 ± 0.3	1.02 ± 1.3	0.158	0.05
miR-331-5p	1.29 ± 0.99	1.04 ± 0.56	1.09 ± 0.62	1.44 ± 1.61	0.982	<0.01
miR-3615	1.64 ± 2.13	1.56 ± 0.8	2.51 ± 2.53	2.9 ± 3.08	0.510	<0.01
miR-497-5p	1.24 ± 0.84	1.07 ± 0.65	1.1 ± 0.46	1.02 ± 0.86	0.756	<0.01
miR-500b-3p	1.14 ± 0.69	1.55 ± 0.82	1.31 ± 0.63	1.15 ± 0.87	0.537	<0.01
miR-550a-5p	1.53 ± 1.62	1.58 ± 0.94	1.59 ± 1.5	2.4 ± 2.27	0.785	<0.01
miR-616-3p	1.05 ± 0.36	0.6 ± 0.48	1.34 ± 0.95	0.98 ± 0.58	0.200	0.07
miR-92a-3p	1.98 ± 3.2	2.02 ± 1.04	2.58 ± 3.06	3.54 ± 5.2	0.458	<0.01

Data are presented as mean ± 1 SD (N=11). Early-follicular, EF; late-follicular, LF; post-ovulatory, PO; mid-luteal, ML. p-values represent repeated measures ANOVA between menstrual cycle phases. Effect size represented as adjusted R^2 for fixed effects. P-value < 0.05 considered statistically significant.

Appendix 3.7: miRNA expression in the early-follicular, late-follicular, post-ovulatory and mid-luteal phase of the menstrual cycle, adjusted for age.

miRNA	Early-follicular	Late-follicular	Post-ovulatory	Mid-luteal	p-value	R^2
miR-155-5p	1.05 ± 0.34	0.9 ± 0.08	0.77 ± 0.14	0.98 ± 0.60	0.435	0.02
miR-16-5p	1.16 ± 0.62	1.51 ± 0.1	1.47 ± 0.59	2.11 ± 1.40	0.187	0.15
miR-194-5p	1.17 ± 0.64	0.94 ± 0.02	1.29 ± 0.36	1.26 ± 0.56	0.395	0.06
miR-20a-5p	1.33 ± 0.96	1.56 ± 0.19	1.53 ± 0.44	1.83 ± 1.21	0.260	0.14
miR-224-3p	1.00 ± 0.04	0.90 ± 0.2	0.99 ± 0.03	0.87 ± 0.47	0.059	0.07
miR-23b-5p	1.01 ± 0.14	1.04 ± 0.05	1.02 ± 0.09	1.17 ± 0.41	0.195	0.01
miR-30c-2-3p	1.01 ± 0.11	1.00 ± 0.24	1.10 ± 0.04	0.97 ± 0.24	0.489	<0.01
miR-30c-5p	1.02 ± 0.22	1.01 ± 0.16	1.11 ± 0.11	1.46 ± 0.85	0.064	0.10
miR-328-5p	1.01 ± 0.15	0.58 ± 0.09	0.68 ± 0.29	0.77 ± 0.28	0.069	0.17
miR-331-5p	1.01 ± 0.15	0.93 ± 0.17	0.94 ± 0.10	1.19 ± 0.66	0.059	0.11
miR-3615	1.12 ± 0.53	1.38 ± 0.13	1.74 ± 0.56	1.82 ± 0.88	0.339	0.07
miR-497-5p	1.02 ± 0.20	0.92 ± 0.07	1.00 ± 0.02	0.92 ± 0.55	**0.030**	0.18
miR-500b-3p	1.01 ± 0.17	1.41 ± 0.15	1.17 ± 0.05	0.92 ± 0.37	0.349	<0.01
miR-550a-5p	1.01 ± 0.13	1.28 ± 0.24	1.26 ± 0.49	1.55 ± 0.27	0.535	<0.01
miR-616-3p	1.04 ± 0.32	0.48 ± 0.01	1.15 ± 0.68	0.92 ± 0.42	0.151	0.24
miR-92a-3p	1.12 ± 0.54	1.74 ± 0.07	1.56 ± 0.54	2.06 ± 1.29	0.253	0.11

Data were adjusted for age and presented as mean ± 1 SD (N=11). Early-follicular, EF; late-follicular, LF; post-ovulatory, PO; mid-luteal, ML. p-values represent repeated measures ANOVA between menstrual cycle phases. Effect size represented as adjusted R^2 for fixed effects. Bold face text represents a statistically significant relationship (P <0.05).

Appendix 3.8: Functional pathway analysis for miR-497-5p.

Category	Term	Total genes of term	Score
KEGG	Neurotrophin signaling pathway	127 (25)	8.821
KEGG	Insulin signaling pathway	137 (26)	8.791
KEGG	Pathways in cancer	325 (43)	8.783
KEGG	WNT signaling pathway	150 (27)	8.58
Reactome	Insulin receptor signaling cascade	86 (20)	8.494
Pathway interaction database	ERBB1 downstream signaling	106 (22)	8.292
Reactome	IRS-mediated signaling	81 (19)	8.171
Reactome	IRS-related events	81 (19)	8.171
Reactome	PI3K cascade	70 (17)	7.624
Pathway interaction database	PDGFR-beta signaling pathway	126 (23)	7.538

Score is generated by the weight of RT-qPCR miRNA expression (ddCt) times its enrichment -log (p-value) (N=11).

Appendix 3.9: Functional pathway analysis for miR-224-3p, miR-331-5p and miR-497-5p.

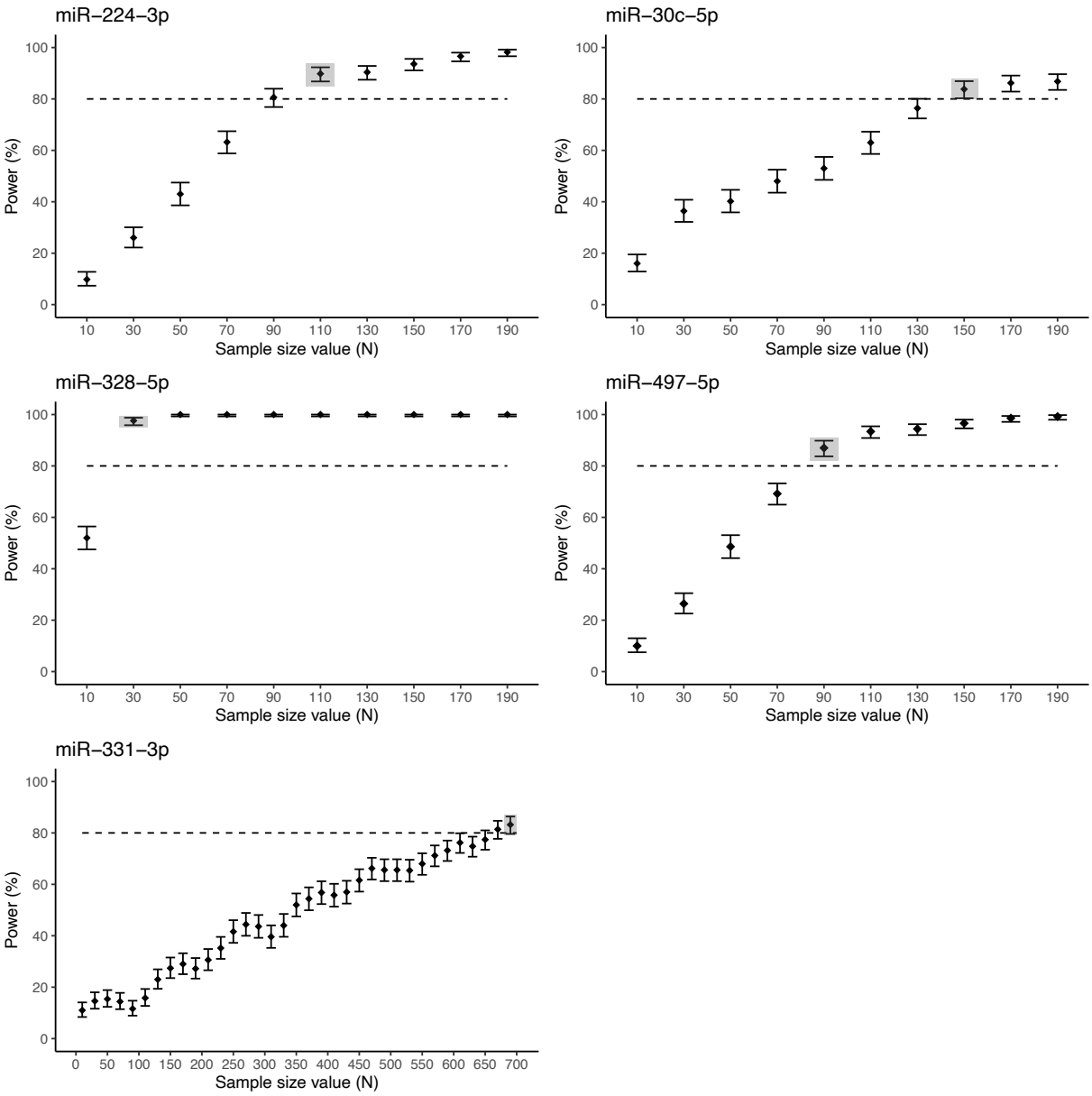
Category	Term	Total genes of term	Score
Pathway interaction database	ERBB1 downstream signaling	106 (27)	4.481
KEGG	Focal adhesion	199 (33)	3.572
KEGG	Neurotrophin signaling pathway	127 (28)	3.537
KEGG	Insulin signaling pathway	137 (29)	3.5
KEGG	MAPK signaling pathway	272 (40)	3.42
KEGG	Pathways in cancer	325 (46)	3.389
Reactome	Insulin receptor signaling cascade	86 (22)	3.269
Reactome	NGF signaling via TRKA from the plasma membrane	136 (25)	3.079
Reactome	Signaling by NGF	221 (34)	3.069
Reactome	Axon guidance	266 (38)	2.951

Score is generated by the weight of RT-qPCR miRNA expression (ddCt) times its enrichment -log (p-value) (N=11).

Appendix 3. 10: Estradiol to progesterone ratio during the early-follicular, late-follicular, post-ovulatory and mid-luteal phases of the menstrual cycle.

Value	Early-follicular	Late-follicular	Post-ovulatory	Mid-luteal	P-value
E:P (ng/mL)	0.017 (0.009)	0.045 (0.029) *	0.017 (0.006) *†	0.023 (0.019) †‡	< 0.001

Values are presented as mean ± 1 SD (N=11). P- values represent one-way repeated measures ANOVA between menstrual cycle phases. E:P, estradiol to progesterone ratio. * Significantly different from early follicular; † significantly different from late-follicular; ‡ significantly different from post-ovulatory. FSH, follicle stimulating hormone; LH, luteinizing hormone.



Appendix 3.11: Power curves for detecting differences in miRNA expression across the menstrual cycle. Power curves were generated by simulation using RT-qPCR data (N=11). Estimated power for a given sample size is shown with a 95 % confidence interval for that power estimate. Data represented as estimated value with 95 % confidence intervals. Dashed line represents level of 80 % power. Grey box represents the minimum sample size required at the lower 95 % confidence interval to achieve 80% power

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RESEARCH ARTICLE

Changes in adipose tissue microRNA expression across the menstrual cycle in regularly menstruating females: a pilot study

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Abstract

Cyclical changes in hormone profiles across the menstrual cycle are associated with alterations in metabolic control. MicroRNAs (miRNAs) contribute to regulating metabolic control, including adipose tissue metabolism. How fluctuations in hormonal profiles across the menstrual cycle affect adipose tissue miRNA expression remains unknown. Eleven healthy, regularly menstruating females underwent four sampling visits across their menstrual cycle. Subcutaneous abdominal adipose tissue and venous blood samples were collected at each sampling visit. Luteinizing hormone (LH) tests, calendar counting, and serum hormone concentrations were used to determine menstrual cycle phases: early-follicular (EF), late-follicular (LF), postovulatory (PO), and midluteal (ML). Serum follicle-stimulating hormone, LH, estrogen, progesterone, and testosterone were determined using multiplex magnetic bead panels and enzyme-linked immunosorbent assays. Global adipose tissue miRNA expression levels were determined via microarray in a subset of participants (n = 8) and 17 candidate miRNAs were validated by RT-qPCR in the whole cohort (n = 11). Global analysis of adipose tissue miRNA expression identified 33 miRNAs significantly altered across the menstrual cycle; however, no significant differences remained after correcting for multiple testing ($P > 0.05$). RT-qPCR analysis of candidate miRNAs revealed miR-497-5p expression was significantly altered across the menstrual cycle (η_p^2 = 0.18, P = 0.03); however, post hoc tests did not reveal any significant differences between menstrual cycle phases ($P > 0.05$). miR-30c-5p was associated with testosterone concentration (R^2 = 0.13, P = 0.033). These pilot data indicate differences in adipose tissue miRNAs in healthy women across the menstrual cycle and a weak association with ovarian hormones. Further research in larger sample sizes is required to confirm regulation of miRNA expression across the menstrual cycle.

estradiol; hormone; metabolism; ovarian hormones; progesterone

INTRODUCTION

Approximately 50% of the female population are of reproductive age, accounting for 1.9 billion women globally (1, 2). The menstrual cycle is a fundamental biological rhythm governing female physiology and encompasses the ovarian cycle and the uterine cycle. The ovarian cycle relates to oocyte maturation and release across the follicular, ovulatory, and luteal phases, whereas the uterine cycle relates to the changes in the uterine lining across the menstrual, proliferative, and secretory phases (3). These cycles occur simultaneously and are coordinated across a typical duration of 24–35 days (4). The menstrual cycle is characterized by hormonal fluctuations within the hypothalamic-pituitary-ovarian axis; gonadotropin, pituitary hormones [follicle stimulating hormone (FSH) and luteinizing hormone (LH)], and ovarian hormones (estradiol, progesterone, and testosterone) (3). Variation in hormonal profiles across the menstrual cycle increases the complexity of female physiology.

Cyclical fluctuations in hormonal profiles between menstrual cycle phases are associated with variable metabolic control in regularly menstruating women. Studies report elevated glucose and insulin during the luteal phase, alongside reduced triglyceride and cholesterol (5–8). Correspondingly, glycemic control, lipid control, insulin resistance, and energy expenditure differ between menstrual cycle phases (6, 7, 9, 10). Moreover, variation in several metabolic parameters across the menstrual cycle, including insulin sensitivity, glucose, and lipoprotein levels, are positively associated with estradiol and progesterone concentrations (6, 7). However, to fully understand changes in metabolic control across the menstrual cycle, it is crucial to examine metabolic responses at a tissue-specific level.

Adipose tissue is metabolically receptive to changes in the ovarian hormonal milieu. Estrogen, testosterone, and progesterone regulate a range of metabolic pathways within adipocytes, including lipolysis, lipogenesis, and insulin sensitivity (11–15). Correspondingly, acute and chronic alterations to ovarian hormone profiles, including menstrual cycle phase, estrogen supplementation, and menopausal status, are associated with alterations in adipose tissue metabolism. These parameters include basal lipolysis, stimulated

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lipolysis, and insulin action (16–18). Thus, hormonal regulation of adipose tissue metabolism across the menstrual cycle may underpin variation in parameters of whole body metabolic control; however, the associated molecular mechanisms remain uncertain.

One potential mechanism for the regulation of adipose tissue metabolism is through microRNA (miRNA) expression. miRNAs are short noncoding RNAs that regulate gene expression at a post-transcriptional level through binding to the 3'UTR region of target messenger RNA (mRNA) transcripts (19). Approximately 60% of known protein-coding genes have target sites for miRNA binding, and thus miRNAs are recognized as major regulators of mRNA translation (20). In adipose tissue, miRNAs target components of pathways involved in metabolic control, including lipid transport, lipolysis, lipogenesis, and insulin signaling (21–23). Divergent adipose tissue miRNA profiles have been observed in response to chronically altered ovarian hormone profiles, including menopausal status (24), hormone supplementation (24), polycystic ovary syndrome (25), and ovariectomy (26). A recent study reported upregulated adipose tissue expression of miR-16-5p, miR-451a, miR-223-3p, miR-18a-5p, miR-19a-3p, miR-363-3p, and miR-486-5p between hormone replacement therapy users and nonusers in postmenopausal women (24). However, the few studies that have investigated miRNA expression in response to acute hormone fluctuations across the menstrual cycle have yielded equivocal results (27, 28). Moreover, previous reports are limited to analysis of plasma miRNA expression across the menstrual cycle, which may not be representative of adipose tissue miRNA expression (24, 27, 28). Investigating the effect of menstrual cycle phase on adipose tissue miRNA expression will help unravel mechanisms involved in metabolic control across the menstrual cycle.

In the present pilot study, first, we aimed to characterize adipose tissue miRNA expression profiles across the menstrual cycle. To achieve this goal, we conducted exploratory analysis using microarrays in a subset of participants followed by RT-qPCR validation of candidate miRNAs in the whole cohort. Second, we aimed to investigate the association between ovarian hormone concentration and miRNA expression.

METHODS

Ethics

Ethical approval was obtained from the NHS, Invasive and Clinical Research (NICR) Ethical Committee at the University of Stirling (NICR 17/18 32). This study was conducted in accordance with the Declaration of Helsinki. Following written and verbal explanation of the study procedures, participants provided written informed consent.

Participants

A total of 11 healthy, premenopausal women aged 22–30 yr were recruited from the University of Stirling and surrounding areas via poster and online advertising. Participant characteristics are detailed in Table 1. The inclusion criteria were: aged 18–40 yr; recreationally active; no previous diagnosis of a menstrual cycle disorder; no use of hormone-based medication within the previous 3 mo; and, not pregnant within the previous 3 mo. Additionally, participants were excluded if they exhibited any symptom of an irregular cycle within the previous 3 mo: menstrual cycle length <21 days or >38 days; abnormal menstrual bleeding; menstrual bleeding ≥7 days; abnormal menstrual cramps; or absence of a period.

Table 1. *Participant characteristics*

	Value
Age, yr	27.4 ± 3.3
Body mass, kg	65.8 ± 5.4
Height, cm	170.6 ± 5.8
BMI, m/kg^2	22.6 ± 1.5
Cycle duration, days	29.1 ± 3.2 (6.3%)
Follicular phase length, days	15.5 ± 2.2 (9%)
Luteal phase length, days	13.3 ± 1.3 (11%)

Values are means ± 1 SD (n = 11). Average between cycle intra-subject coefficient of variation is shown in brackets.

Experimental Design

All participants attended the laboratory for a pre-sampling visit, followed by four identical sampling visits across the menstrual cycle in a repeated measures design. Four participants completed the sampling visits across two consecutive menstrual cycles either due to failure to obtain a positive ovulation test during the first cycle (n = 2) or due to unavailability to attend all scheduled sampling visits during the first cycle (n = 2).

Experimental Protocol

Pre-sampling visit.

Participants were provided with a menstrual cycle diary and urinary LH tests (Digital Ovulation Test, Clearblue, Switzerland) to monitor one menstrual cycle before the sampling visits. The menstrual cycle diary detailed menstrual cycle duration, menstrual bleeding duration, date of positive LH test, and menstrual cycle symptoms. Based on responses collected in the menstrual cycle diary, participants were excluded at this stage if they reported any symptoms of an irregular cycle, as described earlier. In addition, participants were provided with a 48 h food diary to be completed on *cycle days 3* and *4*, which recorded the time, quantity, and preparation method of all meals, snacks, and drinks.

Sampling visits.

Participants attended the laboratory for four identical sampling visits during the following menstrual cycle phases: early-follicular (EF), late-follicular (LF), postovulatory (PO), and mid-luteal (ML). Menstrual cycle phase was determined using the calendar counting method in conjunction with urinary LH tests (Digital Ovulation Test, Clearblue, Switzerland) as follows: EF, *cycle days 1–5*; LF, *cycle days 9–11*; PO, positive LH test + 2 days; ML, positive LH test + 8–10 days, dependent on menstrual cycle length. Menstrual cycle phase was retrospectively confirmed via serum hormone analysis. Before each sampling visit, participants underwent a 10–12 h fast and abstained from alcohol and caffeine for 24 h. To control participants' dietary intake preceding each sampling visit, participants were instructed to repeat food intake as recorded in the 48 h food diary before each sampling visit. Participants were instructed to maintain habitual levels of daily living and

physical activity throughout the duration of the study, but to abstain from physical exercise in the 48 h preceding each sampling visit. Participants attended the laboratory between 07:00 AM and 10:00 AM, with each subsequent sampling visit scheduled for a time within 1 h of the initial sampling visit. Each sampling visit consisted of the following protocol: 15 min rest in a semisupine position upon arrival at the laboratory; collection of a venous blood sample; and an adipose tissue biopsy.

Blood sampling and adipose tissue biopsies. Venous blood samples were collected by a trained phlebotomist into 6 mL serum vacutainers (Becton, Dickinson and Company, NJ). Blood samples were allowed to clot for 60–120 min at room temperature and then centrifuged at 2,500 *g* for 10 min at 4°C for serum separation. Serum was aliquoted into 1.5 mL microcentrifuge tubes and stored at −80°C until analysis. Abdominal adipose tissue biopsies were obtained 5–10 cm lateral of the umbilicus under local anesthesia (2% lidocaine) using a mini-liposuction technique (29). Samples were cleaned of visible contaminants (connective tissue, blood, and vasculature) and washed in 0.9% sodium chloride saline over sterile gauze. Tissue was split into similarly sized pieces (~50–150 mg), placed into an RNA-free 1.5 mL microcentrifuge tube containing 5–10 × tissue volume RNA*later* (Cat No. AM7020, Thermo Fisher Scientific, UK), flash frozen in liquid nitrogen and stored at −80°C until analysis. All adipose tissue samples were processed and frozen within 3 min of sampling.

Hormone Analysis

Serum estrogen, testosterone, FSH, and LH concentrations were determined using magnetic bead panels (Cat Nos. HPTP1MAG-66k and PTP1MAG-66K-02, MilliporeSigma, MA) analyzed using the Luminex 200 instrument, according to the manufacturer's protocol. Serum progesterone were determined using an enzyme-linked immunosorbent assay (ELISA) (Cat No. NBP2-60124, R&D Systems, MN), according to the manufacturer's protocol. The intra-assay coefficients of variation (CVs) were as follows: FSH = 6.4%; LH = 8.69%; estradiol = 12.0%; progesterone = 8.3%; testosterone = 10.4%. These intra-assay CVs are similar to the intra-assay CVs reported in the manufacturers protocol (FSH <10%, LH <10%, estradiol <15%, progesterone <15%, testosterone <10%). All sample concentrations were above the minimum limit of detection reported in the manufacturers protocol for each assay (FSH = 0.01 mIU/mL, LH = 0.01 mIU/mL, estradiol = 0.01, progesterone = 0.5 ng/mL, testosterone = 0.08 ng/mL).

RNA Extraction

Adipose tissue was mechanically homogenized using 1.4 mm ceramic beads (Cat No. 13113-50, Qiagen, Germany) and the MagNA Lyser instrument (Roche, Switzerland) with 3 × 20 s bouts of homogenization interspersed with 20 s rest. Total RNA was isolated from 30–100 mg homogenized adipose tissue in a combined protocol using TRIzol reagent (Cat No. 15596026, Thermo Fisher Scientific, UK) and the miRNeasy mini lipid kit (Cat No. 217004, Qiagen, Germany,), as previously described (30). Final elution volume for isolated RNA was 30 μL. Concentration (μg/mL) and purity (absorbance ratio 260/280 nm) of isolated RNA samples were assessed using a DS-11 FX + spectrophotometer (DeNovix, DE). RNA samples were stored at −80°C until analysis.

Global microRNA Expression Analysis

Global miRNA expression was examined using microarrays in a subset of participants at all timepoints (n = 32; 8 participants, 4 time points). Samples were selected from the eight participants with the highest RNA quantity and purity across all timepoints (Concentration: 25–92.6 μg/mL; absorbance ratio 260/280: 1.9–2.1 nm). Then 100 ng RNA were labeled using the Flashtag Biotin HSR labeling kit (Cat No. 901910, Thermo Fisher Scientific, UK). Hybridization cocktails were prepared using the GeneChip hybridization kit (Cat No. 900720, Thermo Fisher Scientific, UK). Biotin labeled samples were hybridized overnight onto GeneChip miRNA 4.0 arrays (Cat No. 902413, Thermo Fisher Scientific, UK) according to the manufacturer's instructions. The arrays were washed and stained using the GeneChip wash and stain kit (Cat No. 902413, Thermo Fisher Scientific, UK) and the Affymetrix fluidics station 450, as per the manufacturer's instructions. GeneChips were scanned by the Affymetrix scanner 3000 7G. Microarray signal intensities were normalized using the variance stabilization normalization 2 (VSN2) algorithm and probe-level summarization conducted using robust multichip analysis (RMA) (31). Microarray data has been deposited to the Gene Expression Omnibus (GEO) with the accession number GSE180625.

RT-qPCR

A total of 17 candidate miRNAs were selected for validation using RT-qPCR in the whole cohort (n = 44; 11 participants, 4 time points) from the subset of miRNAs differentially expressed across the menstrual cycle, as determined via microarray. Candidate miRNAs were selected based on association with hormones across menstrual cycle phases and reported association with adipose tissue function. Analysis of microarray data via NormFinder (32) reported miR-155-5p, miR-324-3p, miR-331-3p, and miR-328-5p had optimal stability values and were selected as potential endogenous qPCR control miRNAs for RT-qPCR analysis (32). RT-qPCR was performed using miRCURY LNA custom PCR panels with pre-coated miRNA primers (Cat No. 339330, Qiagen, Germany), according to the manufacturer's protocol. Briefly, cDNA synthesis reactions comprised 2 μL miRCURY RT reaction buffer, 4.5 μL RNase-free water, 1 μL 10× miRCURY RT enzyme mix, 0.5 μL UniSp6 RNA spike-in and 2 μL template RNA (5 ng/μL) in a 10 μL total reaction volume. UniSp6 (Cat No. 339390, Qiagen, UK) were added to cDNA synthesis reactions to analyze cDNA synthesis efficiency. Reverse transcription temperature cycling was performed using a LightCycler 480 (Roche, Switzerland) according to manufacturer instructions. Conditions were 60 min at 42°C, 5 min at 95°C, and then immediate cooling to 4°C. cDNA samples were assayed immediately by qPCR or stored at −20°C until analysis. qPCR reaction volume comprised 5 μL miRCURY LNA SYBR Green Master Mix (Cat No. 339345, Qiagen, Germany), 4 μL cDNA template (diluted 1:80), and 1 μL RNase-free water, in a total reaction volume of 10 μL.

UniSp3 were included in miRCURY LNA custom PCR panels to perform inter-plate calibration. miRNA target sequences are supplied in Supplemental Table S1 (all Supplemental Material is available at https://doi.org/10.6084/m9.figshare.15059688). qPCR temperature cycling was performed using a LightCycler 480 (Roche, Switzerland), according to the manufacturer's instructions, consisting of 95°C for 2 min, followed by 45 cycles at 95°C for 10 s, then 56°C for 60 s, and finished with melting curve analysis. All qPCR reactions were performed in triplicate and analyzed using the $2^{-\Delta\Delta Ct}$ method (33). Analysis of RT-qPCR data using NormFinder identified miR-324-3p and miR-331-3p to be the most stable pair of miRNAs from the four potential endogenous controls (miR-155-5p, miR-324-3p, miR-331-3p, and miR-328-5p) (32). Raw ΔCt values were calculated relative to the geometric mean of the endogenous controls (miR-324-3p, miR-331-3p) and ΔΔCt values calculated relative to the EF phase. Raw ΔCt values for endogenous controls are shown in Supplemental Fig. S1.

miRNA-mRNA Pathway Analysis

miRsystem was used to conduct miRNA-mRNA pathway analysis using RT-qPCR results for miRNA expression (34). miRsystem integrates seven miRNA target gene prediction databases (DIANA, miRanda, miRBridge, PicTar, PITA, rna22, and TargetScan) to enable prediction of target genes and functional pathways.

Statistical Analysis

A priori power calculations were conducted in G*Power 3.1 (35). miRNA expression was not included in power calculations due to a lack of suitable published data reporting miRNA expression across similar menstrual cycle phases. Power calculations were conducted using reported means and standard deviation of estradiol and progesterone concentrations at EF, LF, PO, and ML (35–37) to ensure appropriate statistical power to differentiate between menstrual cycle phases. Testosterone was omitted from power calculations due to relatively small changes between menstrual cycle phases. G*Power reported $n = 11$ was required to achieve 80% power ($\alpha = 0.05$) to detect changes in estrogen and progesterone between menstrual cycle phases.

All statistical analyses were conducted using R v. 3.6.3 (38). Hormone data were log transformed for normality and this was confirmed using the Shapiro–Wilk test. One-way repeated measures ANOVA were used to examine the effect of menstrual cycle phase on hormone concentration. Pairwise comparisons were conducted with Tukey adjustment. To determine differential microarray expression across the menstrual cycle, moderated empirical Bayes ANOVA was conducted and corrected for multiple testing using the false discovery rate (FDR) method, in the "limma" package (39). Linear mixed-effect models were conducted to examine the relationship between menstrual cycle phase and ovarian hormone concentration with candidate miRNA expression using RT-qPCR data, in the "lme4" package in R (40). A participant identifier was included in models as a random effect to account for repeated measures. P values were obtained using the Kenward–Roger approximation for denominator degrees of freedom (41). Pairwise comparisons were conducted with Tukey adjustment. Effect sizes where calculated as adjusted R^2 for fixed effects with Cohen's descriptors used to interpret effect sizes; 0.02 small, 0.13 moderate, and 0.26 large (42). In all analyses, miRNA expression was adjusted for age due to the reported association between age and basal adipose miRNA expression in regularly menstruating women (24). Unadjusted candidate miRNA expression data are presented in Supplemental Table S3. $P < 0.05$ was considered statistically significant.

To provide sample size estimations for future studies using data generated in this study, power analysis for linear mixed-effect models were conducted using the package "simr" in R (43). "simr" generates power curves for sample-size estimation based on Monte Carlo simulations.

RESULTS

Menstrual Cycle Characteristics

Participant characteristics are shown in Table 1. All participants reported regular menstrual cycles during the course of the study, as assessed via the menstrual cycle diary. Sampling visits occurred on cycle day; 3 ± 1 (EF), 11 ± 1 (LF), 16 ± 2 (PO), and 24 ± 3 (ML). Serum analysis of hormones showed expected temporal changes in LH, FSH, estradiol, progesterone, and testosterone profiles between menstrual cycle phases (Table 2). FSH was significantly lower during ML compared to EF, LF, and PO ($P < 0.001$; $P = 0.011$; $P < 0.001$). LH was significantly higher during PO compared to EF and ML ($P = 0.006$; $P < 0.001$) and higher during LF compared to ML ($P = 0.005$). Estradiol was significantly greater during LF, PO, and ML compared to EF ($P = 0.002$; $P = 0.013$; $P < 0.001$). Progesterone was significantly higher during ML compared to EF, LF, and PO (all $P < 0.001$) and higher during PO compared to EF and LF ($P = 0.006$; $P = 0.003$). Testosterone was significantly greater during PO compared to EF ($P = 0.024$).

Global miRNA Expression

We analyzed global miRNA expression across the menstrual cycle in a subset of participants ($n = 32$; 8 participants, 4 timepoints). Three arrays did not pass quality control (PO: $n = 2$, ML: $n = 1$) and therefore final analysis was conducted on 29 samples. Total 729 miRNAs were considered significantly expressed in adipose tissue samples at a median expression level ≥1.75. Differential expression across the menstrual cycle was observed in 33/729 miRNAs before correction for multiple testing (Fig. 1 and Supplemental Table S2). No significant differences were observed following FDR correction for multiple testing (Fig. 1 and Supplemental Table S2).

Candidate RT-qPCR miRNA Expression

A total of 19 miRNAs were selected for validation using RT-qPCR (17 candidate miRNAs, 2 unused potential endogenous control miRNAs) in the whole cohort ($n = 44$; 11 participants, 4 time points). Three miRNAs (miR-1231, miR-1914, miR-3180-3p) were not detected consistently in all participants and were removed from the analysis. Mean log fold change in expression level for each of the remaining 16 candidate miRNAs relative to endogenous controls and expression level in EF are shown in Fig. 2 and Supplemental Table S4. miR-497-5p expression was significantly altered across the menstrual cycle ($P = 0.030$, R^2=

Table 2. *Ovarian and pituitary hormone concentrations during the early-follicular, late-follicular, postovulatory and mid-luteal phases of the menstrual cycle*

Hormone	Early-follicular	Late-follicular	Postovulatory	Midluteal	*P* Value
FSH, IU/mL	4.89 ± 2.45	3.70 ± 2.53	4.81 ± 3.26	2.24 ± 1.63*†‡	<0.001
LH, IU/mL	4.90 ± 2.37	7.97 ± 7.05	12.13 ± 8.93*	4.04 ± 3.08†‡	<0.001
Estrogen, ng/mL	0.10 ± 0.03	0.23 ± 0.10*	0.21 ± 0.13*	0.32 ± 0.11*	0.001
Progesterone, ng/mL	6.80 ± 2.89	7.14 ± 4.66	10.44 ± 3.28*†	19.2 ± 3.94*†‡	<0.001
Testosterone, ng/mL	0.56 ± 0.18	0.63 ± 0.21	0.75 ± 0.31*	0.62 ± 0.21	0.040

Values are means ± 1 SD (n = 11). P values represent one-way repeated measures ANOVA between menstrual cycle phases. FSH, follicle stimulating hormone; LH, luteinizing hormone. *Significantly different from early follicular; †significantly different from late-follicular; ‡significantly different from postovulatory.

0.18); however, post-hoc testing did not reveal any significant differences between menstrual cycle phases ($P > 0.05$). There was a tendency for altered expression of miR-224-3p ($P = 0.059$, $R^2 = 0.07$) and miR-331-5p ($P = 0.059$, $R^2 = 0.11$) across the menstrual cycle, although they did not reach statistical significance.

Association between RT-qPCR miRNA Expression and Ovarian Hormones

Linear mixed-effect models were conducted to assess the relationship between hormone concentration and miRNA expression, as determined using RT-qPCR (Table 3). miR-30c-5p was significantly, negatively associated with serum testosterone ($P = 0.033$, $R^2 = 0.13$). miR-92a-3p had a tendency for a negative association with serum estradiol ($P = 0.067$, $R^2 = 0.14$).

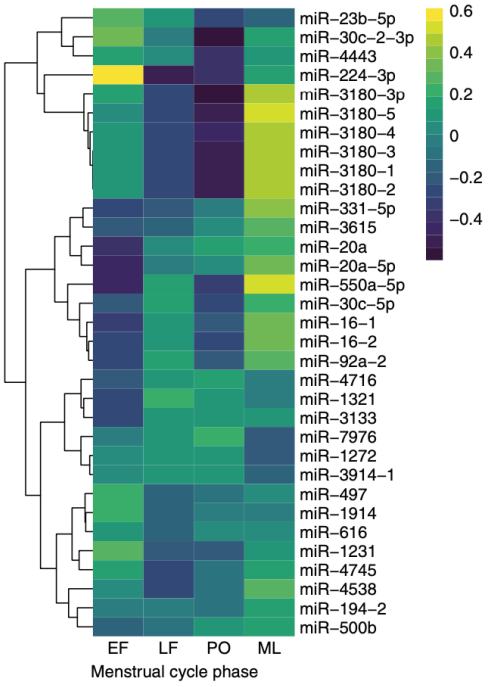

Figure 1. Clustered heatmap of microarray log10 z-scores in differentially expressed adipose tissue miRNAs in the early-follicular (EF), late-follicular (LF), postovulatory (PO), and mid-luteal (ML) phases of the menstrual cycle (n = 8). $P < 0.05$ was considered statistically significant by moderated empirical Bayes ANOVA.

miRNA-mRNA Pathway Analysis

miRNA-mRNA pathway analysis was conducted using RT-qPCR results. miRNA-mRNA pathway analysis was performed with miR-497-5p due to significant variation in expression across the menstrual cycle. In addition, due to the tendency for alteration across the menstrual cycle in miR-224-3p and miR-331-5p, miRNA-mRNA pathway analysis was also performed for this subset of miRNAs (miR-224-3p and miR-331-5p, miR-497-5p). Data are presented in Fig. 3 and Supplemental Tables S5 and S6.

DISCUSSION

To the best of our knowledge, this is the first study to characterize adipose tissue miRNA expression across the menstrual cycle. Initially, we conducted an exploratory approach utilizing microarrays to analyze global miRNA expression in a subset of eight participants selected from the whole cohort. Based on examination of microarray data distributions, we identified 729 miRNAs expressed in adipose tissue samples in healthy, regularly menstruating women. Expression levels of 33 miRNAs were significantly altered across the menstrual cycle, although no significant differences remained after correction for multiple testing using FDR. Validation via RT-qPCR was conducted on 16 candidate miRNAs in the whole cohort. RT-qPCR analysis determined miR-497-5p was significantly altered across the menstrual cycle, whereas miR-224-3p and miR-331-5p exhibited a trend toward differential expression. In addition, we examined the relationship between miRNA expression and circulating hormone concentrations. Testosterone was significantly associated with miR-30c-5p expression, predicting up to 13% of the variation in expression. We also observed a trend for a negative association between miR-92a-3p and estradiol, predicting up to 14% of the variation in miRNA expression. These pilot data suggest adipose tissue miR-497-5p expression may be altered by menstrual cycle phase and adipose tissue miRNAs exhibit weak associations with ovarian hormones.

The effect of the menstrual cycle on basal miRNA expression has been previously limited to plasma samples, with inconsistent findings reported (28, 44). Li et al. (27) observed elevated miR-126 expression during the ML phase compared

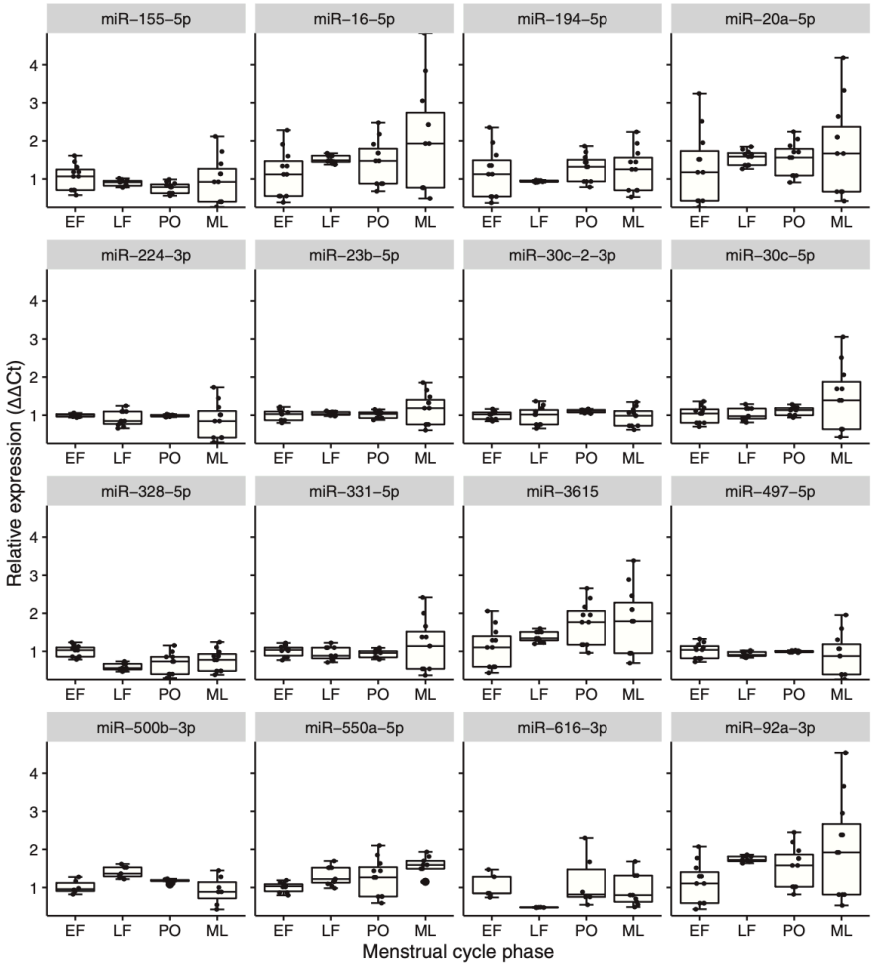

Figure 2. RT-qPCR analysis of adipose tissue miRNA expression in the early-follicular (EF), late-follicular (LF), postovulatory (PO), and mid-luteal (ML) phases of the menstrual cycle (n =11). miRNA expression represented as ΔΔCt values adjusted for age. Data are displayed as conventional box and whisker plots with individual data points represented by circles.

to the EF and LF phases, whereas Rekker et al. (28) observed no change in the expression of 375 miRNAs between four time points across the menstrual cycle. These studies were conducted in healthy participants with comparable sample sizes to that used in this study (n = 12) (27, 28). However, miRNA expression is highly tissue specific and miRNA profiles in plasma may not represent adipose tissue (24). In this study, RT-qPCR analysis determined miR-497-5p expression was significantly altered across the menstrual cycle. miR-497-5p expression increased 10% in PO compared with ML and LF; however, pairwise comparisons did not detect any significant differences between phases. Loss of statistical significance between ANOVA and pairwise comparisons can be caused by a weakly significant global effect in ANOVA and insufficient statistical power resultant from a low sample size (45). In addition, we observed a trend for reduced miR-224-3p during the PO and ML phase (P = 0.059) and a trend for reduced miR-331-5p during the ML phase (P = 0.059). This is congruent with previous studies reporting reduced miR-224 and miR-331 expression following chronic estradiol supplementation in adipose tissue (24) and adipose-derived extracellular vesicles (46). Future research should further investigate changes in adipose tissue miRNA expression across the menstrual cycle in a larger study population.

Table 3. *Linear mixed-effect models to assess the association between estradiol, progesterone, and testosterone with ΔΔCt miRNA expression*

miRNA	Estradiol	Progesterone	Testosterone
miR-155-5p	0.07 (0.657)	0.07 (0.726)	0.07 (0.846)
miR-16-5p	0.17 (0.118)	0.14 (0.315)	0.11 (0.479)
miR-194-5p	0.10 (0.383)	0.11 (0.286)	0.09 (0.433)
miR-20a-5p	0.16 (0.267)	0.15 (0.621)	0.12 (0.309)
miR-224-3p	<0.01 (0.461)	<0.01 (0.476)	<0.01 (0.717)
miR-23b-5p	0.03(0.832)	0.04 (0.77)	0.03 (0.35)
miR-30c-2-3p	0.08 (0.155)	0.03 (0.722)	0.03 (0.826)
miR-30c-5p	0.04 (0.581)	0.05 (0.492)	**0.13 (0.033)**
miR-328-5p	0.04 (0.628)	0.07 (0.221)	0.09 (0.146)
miR-331-5p	0.02 (0.804)	0.03 (0.695)	0.03 (0.591)
miR-3615	0.09 (0.219)	0.08 (0.216)	0.06 (0.859)
miR-497-5p	0.08 (0.251)	0.06 (0.517)	0.06 (0.792)
miR-500b-3p	<0.01 (0.389)	<0.01 (0.376)	<0.01 (0.98)
miR-550a-5p	<0.01 (0.347)	0.01 (0.233)	<0.01 (0.809)
miR-616-3p	<0.01 (0.571)	<0.01 (0.782)	<0.01 (0.943)
miR-92a-3p	0.14 (0.067)	0.10 (0.399)	0.06 (0.277)

Values are adjusted R^2 for fixed effects (P value) (n = 11). miRNA values input as ΔΔCt. All hormone values were log transformed. Bold face text represents a statistically significant relationship ($P < 0.05$).

To gain insight into the potential cellular consequences of variation in miRNA expression across the menstrual cycle, we conducted functional pathway analysis using miRsystem. Functional pathways analysis of miR-497-5p revealed 5 of the top 10 pathways target processes related to insulin sensitivity (Fig. 3). miR-497-5p has previously been shown to inhibit adipocyte insulin sensitivity and associated gene expression in vivo (47, 48). Small increases in adipose tissue miR-497-5p expression may contribute to the reduction in insulin sensitivity previously reported during the PO phase of the menstrual cycle (6, 49).

We conducted additional functional pathway analysis in a subset of miRNAs demonstrating a trend toward differential expression in addition to miR-497-5p (miR-224-3p, miR-331-5p, and miR-497-5p). Similar functional pathways were identified in this subset of miRNAs, which were comparable to miR-495-5p alone, including the insulin signaling pathway and insulin receptor signaling cascade (Fig. 3). This finding supports the notion that synergistic co-expression of miRNAs regulates functionally related cohorts of genes involved in metabolism (50). These functional pathway predictions suggest that differential co-expression of adipose tissue miRNAs across the menstrual cycle may regulate variation in insulin sensitivity. However, it must be considered that the adipose tissue transcriptome is highly insulin sensitive and, without the use of a background gene list, functional pathway analysis cannot determine which pathways are upregulated over and above the biological bias already present within adipose tissue (51). Nonetheless, these findings suggest that future research should further investigate the role of differential co-expression of subsets of miRNAs across the menstrual cycle on insulin signaling pathways. Concurrent analysis of mRNA-miRNA expression would offer valuable mechanistic insight on the impact of miRNAs on the insulin signaling pathway across the menstrual cycle.

Cyclical fluctuations in metabolic control across the menstrual cycle are associated with ovarian hormone concentrations (6, 49). Due to the reported impact of ovarian hormones on adipocyte metabolism, we examined the relationship between serum estradiol, progesterone, and testosterone with adipose tissue miRNA expression (14, 52, 53). We observed a significant negative association between testosterone and miR-30c-5p, explaining up to 13% of the variation in miRNA expression level. This is concordant with previous research demonstrating lower plasma miR-30c-5p expression in

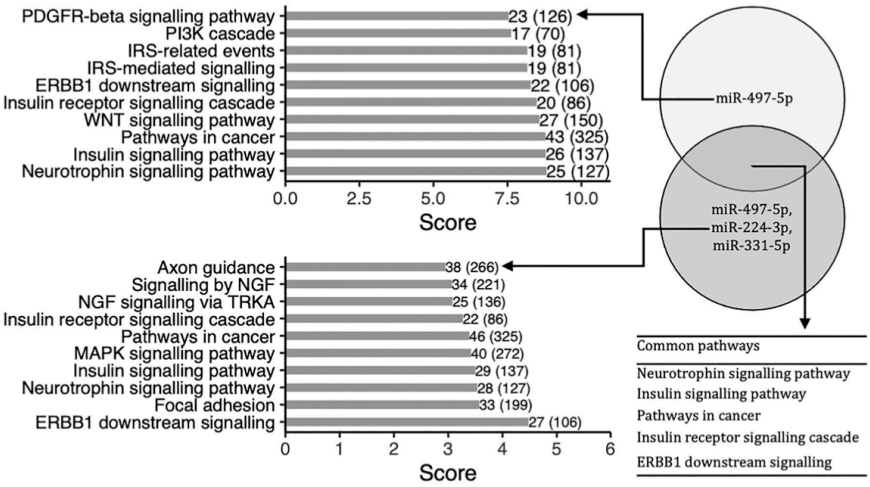

Figure 3. Functional pathway analyses were conducted using RT-qPCR results for miR-497-5p (*top left*) and miR-224-3p, miR-331-5p, and 497-5p (*bottom left*) (n = 11). Score is generated by the weight of miRNA expression (ΔΔCt) times its enrichment −log (P value). Numbers at the end of bars represent the total number of genes in the indicated term with total union targets in term encased in brackets.

women with polycystic ovary syndrome, whose testosterone levels are significantly greater, compared to healthy controls (54).

Adipose tissue is a complex tissue that comprises heterogenous cell populations (55, 56). Correspondingly, miRNA expression profiles are specific to individual cell populations within adipose tissue (57). Thus, detected miRNA signals in this study are reflective of the combined miRNA expression profiles within both mature adipocytes and the stromovascular fraction, including pre-adipocytes, mesenchymal stem cells, endothelial cells, and macrophages (56). In addition, hormonal influences on miRNA expression may be exerted in opposing directions in differing cellular fractions within adipose tissue. This may induce a "counterbalancing effect" on determined miRNA signal from whole adipose tissue. Therefore, future studies should consider isolating a single adipose tissue cell population (i.e., mature adipocytes) before miRNA analysis to reduce variability in expression and gain further mechanistic insight. In addition, three candidate miRNAs (miR-1231, miR-1914, miR-3180-3p) were detected at low levels via RT-qPCR and were thus removed from statistical analysis. Future studies can consider performing an RNA-Seq study of sufficient sequencing depth or including a cDNA pre-amplification step before qPCR analysis of adipose tissue miR-1231, miR-1914, and miR-3180-3p to facilitate the detection of these less expressed miRNAs (58, 59).

Females are significantly under-represented in physiological research and therefore our understanding of physiological changes across the menstrual cycle remains incomplete (60). Understanding how the menstrual cycle affects physiology is important to be able to properly account for these effects in fully inclusive physiology research. Our findings suggest that consideration of menstrual cycle phase is important in research investigating miRNA expression in regularly menstruating women. Associations between miRNA expression and reproductive hormone levels highlight the need to consider other situations impacting circulating hormone concentrations in addition to menstrual cycle phase, such as menopausal status and exogenous hormone supplementation.

This study was conducted with 11 participants and high interindividual variability of miRNA expression and ovarian hormone concentration was observed. Analysis of miRNA expression across the menstrual cycle obtained multiple P values close to the significance threshold, alongside small-to-moderate effect sizes in 12 out of 16 measured miRNAs. We did not perform post-hoc power calculations as this technique is considered analytically misleading (61).

We conducted power analysis to calculate sample size estimates using effect sizes derived from RT-qPCR data in the five miRNAs with the lowest significance levels; miR-497-5p, miR-224-3p, miR-331-5p, miR-30c-5p, and miR-32 8-5p. We generated power curves for each of the miRNA under consideration by simulation. Estimates of power from these simulations were accompanied by 95% confidence intervals (CIs). The results presented below are those for which the lower limit of that 95% CI was greater than 80% power at $\alpha = 0.05$ to detect differential miRNA expression between menstrual cycle phases (Supplemental Fig. S2). Estimated sample sizes are as follows: miR-497-5p, $n = 90$; miR-224-3p, $n = 110$; miR-331-5p, $n = 690$; miR-30c-5p, $n = 150$; miR-328-5p, $n = 30$. These sample size estimates provide an important methodological consideration for future studies investigating changes in adipose tissue miRNA expression across the menstrual cycle with defined outcomes. In addition, it must be noted that ethnicity was not recorded in this study. Ethnicity may increase interindividual variation in adipose tissue miRNA expression; we acknowledge that this may be a limitation. Future studies should endeavor to record participants' ethnicity.

This study recruited young, healthy, regularly menstruating women and as such findings must be extrapolated to a broader population with caution. Elevated adiposity and insulin resistance are associated with greater perturbations in ovarian hormone concentrations and metabolic control across the menstrual cycle (5, 6, 62, 63). Accordingly, molecular mechanisms underlying metabolic control, such as miRNA expression, may undergo relatively less variation across the menstrual cycle in healthy women compared with populations with obesity or metabolic disorders. In addition, diverse miRNA expression profiles are observed in individuals with metabolic disorders when compared with healthy controls (64). Therefore, future research should consider investigating changes in miRNA expression across the menstrual cycle in populations with impaired metabolic control.

In conclusion, we present novel pilot data characterizing miRNA expression across the menstrual cycle and associations with ovarian hormone concentrations. We report significant variation in miR-497-5p across the menstrual cycle alongside small-to-large effect sizes in 12 out of 16 assessed miRNAs. Furthermore, miRNAs with the strongest tendency for differential expression across the menstrual cycle shared common targets related to insulin sensitivity pathways. However, future studies should explore miRNA expression across the menstrual cycle in larger sample sizes. Future studies would also benefit from concurrent analysis of miRNA and mRNA expression within insulin signaling pathways to provide further mechanistic insight. In addition, these findings provide direction for future research to examine changes in miRNA expression across the menstrual cycle in individuals with impaired metabolic control, such as type 2 diabetes. These studies are necessary to fully elucidate the role of varying hormonal milieu across the menstrual cycle on miRNA expression and associated effects on metabolic control.

SUPPLEMENTAL DATA

Supplemental Figs. S1 and S2 and Tables S1–S6: https://doi.org/10.6084/m9.figshare.15059688.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.A.M., I.J.G., and C.N.M. conceived and designed research; K.A.M., N.R.-S., T.G.D.V., N.D.B., I.J.G., and C.N.M. performed

experiments; K.A.M., and I.J.G. analyzed data; K.A.M., I.J.G., and C.N.M. interpreted results of experiments; K.A.M. prepared figures; K.A.M. drafted manuscript; K.A.M., N.R.-S., T.G.D.V., N.D.B., I.J.G., and C.N.M. edited and revised manuscript; K.A.M., N.R.-S., T.G.D.V., N.D.B., I.J.G., and C.N.M. approved final version of manuscript.

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Human Subcutaneous Adipose Tissue Sampling using a Mini-liposuction Technique

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Abstract

Studies on adipose tissue are useful in understanding metabolic and other conditions. Human subcutaneous adipose tissue is accessible. With appropriate training and strict adherence to aseptic technique, subcutaneous adipose samples can be safely and efficiently obtained in a non-clinical setting by researchers. Following the administration of local anesthetic lateral to the umbilicus, a 14 G needle attached to a 5 or 10 mL syringe is inserted through the skin into the subcutaneous tissue. Under suction, the syringe is moved in a reciprocating, slicing motion to isolate fragments of adipose tissue. Withdrawing the plunger is enough to ensure that adipose tissue fragments are aspirated through the needle into the syringe. A single biopsy can collect about 200 mg of tissue. This biopsy technique is very safe for both participants and research staff. Following the biopsy, participants can resume most everyday activities, although they should avoid swimming and overly strenuous activities for 48 h to avoid excessive bleeding. Participants can safely undergo 2 biopsies within a single day, meaning that the technique can be applied in before-after acute intervention studies.

Introduction

Adipose tissue can provide useful information on the metabolic function of humans. Human subcutaneous adipose tissue is readily accessible. A technique for subcutaneous adipose tissue extraction was first described in the mid-80s[1]; since then, the initial protocol has been improved to increase the yield and improve study participant tolerability. Subcutaneous adipose tissue can be obtained from numerous sites, most commonly from the glutei[1] and abdominal area[2]. Samples from the latter may be more desirable as they provide more valuable information in metabolic disease-related contexts[3].

Subcutaneous adipose tissue biopsy using the mini-liposuction method can be safely and efficiently performed in a non-clinical setting. Following appropriate training by a board-certified physician and using strict aseptic technique, researchers can routinely perform these biopsies with minimal risk to both participant and investigators. The biopsy

team must consist of at least 2 individuals: the person who will perform the biopsy and an assistant.

The person responsible for the biopsy is tasked with confirming the participant's identity, checking the participant can safely undergo the procedure (see protocol steps 2.1-2.4 below), ensuring the participant is comfortable throughout the procedure, ensuring sterile technique is maintained throughout the procedure, carrying out the procedure, and providing the participant with verbal and written after-care procedures. The assistant's role is to handle and rapidly process the adipose tissue obtained for later analysis and/or storage. The assistant also helps by being the "non-sterile hands" and ensuring the participant is at ease throughout the procedure. The purpose of this video and paper is to describe the step-by-step biopsy procedure to safely obtain subcutaneous adipose tissue from the abdominal area.

Protocol

NOTE: The University of Stirling NHS, Invasive, or Clinical Research Committee approved the biopsy procedure described below. All research studies using this procedure must be approved by the appropriate independent ethics committee. The biopsy taker must have completed formal training in the described technique in accordance with their institution's requirements. Typically, this involves observing a demonstration of the described adipose tissue biopsy technique by a board-certified physician, followed by supervised practice. Once the trainee has performed 10 practice adipose tissue biopsies on volunteer subjects under supervision, they will be examined by a board-certified physician to ensure good knowledge and practice of the procedure. The board-certified physician then provides the individual with a signed examination form.

1. Laboratory room preparation

1. Ensure that the laboratory has an appropriately private room with clean, wipeable non-porous surfaces and a clean, comfortable (preferably non-porous) bed on which the participant may lie supine. Clean all required surfaces for the biopsy procedure using 70% ethanol spray and clean paper towels. Provide clean pillows or cushions to support the participant if required.
2. Keep appropriate sharps disposal bins and biohazard waste bags within easy reach of the area where the biopsy is being performed and within easy reach of the person taking the biopsy.
3. Prepare the equipment required for the procedure and set up on a freshly cleaned general medical trolley prior to the participant arriving to the laboratory (**Figure 1**). For a complete list of consumables required, see the **Table of Materials**.

2. Participant preparation

1. Ensure that all participants provide written informed consent prior to undergoing the procedure in accordance with protocols required by the institution's independent ethics committee. Additionally, ask the participants to complete a written questionnaire to ensure they are not allergic to any materials used in the procedure (namely, nickel, chromium, local anesthetic, iodine, shellfish, and plasters).
2. Confirm the identity of the participant. Ensure the participant understands the procedure to be carried out and potential secondary effects, including bruising, pain, and infection (**Table 1**). Gather verbal consent in addition to previously obtained written informed consent.

3. Describe to the participant how the procedure will be carried out, with emphasis on how the administration of the anesthetic and biopsy itself will feel. Ensure that the participant is comfortable with proceeding.
   NOTE: Local subcutaneous anesthetic will produce a stinging sensation, similar to a bee sting of short duration. Many participants report the anesthetic administration as the most uncomfortable part of the technique. Once the anesthetic has taken effect, the participant should feel no more than a slight tugging sensation during the biopsy.
4. Ensure that the participant has no allergies to the local anesthetic (specifically from the amino-amide type, if using lidocaine or similar), certain metals (nickel and chromium), and shellfish (if using iodine-based solutions). Additionally, ensure that the participants are not taking any form of anticoagulant medication.
5. Provide the participant with an opportunity to go and empty their bladder if required, to ensure they do not have to interrupt the procedure or experience undue discomfort in step 4.1.

3. Biopsy procedure - instructions for the biopsy taker

1. Once the participant is lying in a supine position, identify the biopsy site approximately 5-10 cm lateral to the umbilicus.
   NOTE: If the participant is to undergo multiple biopsies on the same day, identify biopsy sites on opposing sides of the umbilicus for each biopsy. This will ensure maximal distance between each biopsy site.
2. Wash hands with soap and warm water according to standard medical guidelines[4].
3. Place the sterile sheet on the cleaned trolley or work area, taking care to only touch the outer edges of the sheet.
4. Put on sterile surgical gloves using proper aseptic technique. Have the assistant open the rest of the equipment in such a way that it drops onto the prepared sterile sheet without touching/contaminating the equipment. Ensure that the assistant takes care not to touch items when removing tools from their sterile wrappings.
5. Instruct the assistant to dispense a small amount of iodine-based solution on some sterile gauze (without oversaturating the gauze) on the work surface.
6. Sterilize approximately 5-10 cm^2 around the chosen biopsy site using the sterile gauze and iodine-based solution. Ensure the skin is cleaned in a spiraling motion moving outward from the proposed biopsy site. Repeat the skin cleaning procedure twice. Remove excess liquid (e.g., running off sterile area) by wiping with fresh sterile gauze.
7. Along with the assistant, verbally confirm the content of the local anesthetic vial (2% lidocaine in this protocol) and that this is within its expiry date. Instruct the assistant to hold the opened vial upside down and draw 5 mL of local anesthetic into a syringe, using a 21 G needle. Dispose of the needle into the sharps bin, and ensure the syringe is free of air bubbles.
8. Apply a 26 G needle to the syringe and expel any air bubbles. Gently pinch the abdominal skin and adipose tissue, moving it away from the abdominal wall. Then, insert the needle horizontally into the subcutaneous tissue at an angle no greater than 10° relative to the surface of the skin.

1. Withdraw the syringe's plunger an additional 0.5 mL (to ensure the needle is not in a blood vessel). If blood appears in the syringe, withdraw and reinsert the needle at a different angle.
2. Raise a bleb of 2-4 mm diameter to anesthetize the insertion area.
3. Advance the needle into the subcutaneous tissue and administer ~1 mL of lidocaine in a fan-shaped pattern (**Figure 2**), taking care to withdraw the plunger each time before injecting the anesthetic.
4. Remove and dispose of the 26 G needle, apply a 21 G needle to the syringe, expel any air bubbles, and administer the remaining ~4 mL of lidocaine in a fan-shaped pattern (**Figure 2**), taking care to withdraw the plunger each time before injecting the anesthetic.

9. Wait approximately 5 min for the local anesthetic to take effect. Use a sterile scalpel to gently prod the biopsy area to i) ensure the local anesthetic has taken effect and ii) identify the boundaries of the anesthetized area. If necessary, wait an additional minute or two and reassess.
10. Once satisfied that the local anesthetic is working, gently pinch the skin and adipose tissue (as in step 3.8) and use a sterile scalpel to make a small 1-2 mm puncture in the skin.
    NOTE: This only needs to be large enough to ease the entry of the 14 G needle and must be small enough that no suture is required to close it. It is common for some bleeding to occur from this point onwards, which can be controlled with a piece of sterile gauze.
11. First, apply a 14 G needle to a 5 or 10 mL syringe. Then, while gently pinching the skin and adipose tissue, gradually insert the needle through the puncture into the adipose tissue approximately centrally in the anesthetized area and at an angle no greater than 10° relative to the surface of the skin.
    NOTE: For all cases of needle advancement in step 3.11, a syringe angle of no greater than 10° must be maintained.
    1. Apply suction by withdrawing the plunger to approximately the 2.5 mL mark. Take the biopsy by moving the needle in a quick backwards and forwards motion to slice fragments of adipose tissue. After approximately 30 s, twist the needle and syringe through 90° and repeat this procedure to break up the fragments of adipose tissue, which are then aspirated into the syringe by the suction.
       NOTE: Other syringe sizes can be used. It is essential that the researcher selects a syringe size that permits both a good grip on the syringe and to comfortably maintain plunger retraction for maintenance of the vacuum. Locking syringes are available that maintain the vacuum, which can improve needle control and reduce perceived difficulty for the biopsy taker [5].
    2. After approximately 45-60 s of step 3.11.1, remove the needle and empty the syringe content onto a layer of gauze covering a weighing boat. Ensure that the lumen of the needle is facing down to avoid potential blood spatter.
    3. Repeat steps 3.11.1 and 3.11.2 for a maximum of 3 times. Check that the participant is content to proceed before each repeat of the above procedure.

4. Whilst performing steps 3.11.1 and 3.11.2, instruct the assistant to process and prepare the samples for analysis/storage (see section 5).

4. Post-biopsy procedure

1. Once a satisfactory sample (i.e., ~200 mg) of adipose tissue has been obtained, place 1-2 layers of sterile gauze over the puncture wound, then place an ice pack over these, and apply firm pressure for approximately 10 min to induce hemostasis.
2. When hemostasis has occurred, wipe away any iodine-based solution/dried blood with sterile gauze, and apply an adhesive wound dressing with absorbent pad to the site. Check that the participant feels well and provide verbal and written instructions on biopsy site aftercare.
    1. Emphasize that the participants will likely exhibit some bruising for the next few days. Inform them that this may be substantial, although it is minimized by the ice pack in step 4.1 and will resolve without lasting effects.
    2. Recommend that should the participants feel any discomfort/pain once the anesthetic has worn off, they should take analgesics such as paracetamol following the instructions on the packet but refrain from taking analgesics that have anticoagulant activities (e.g., ibuprofen or aspirin).
    3. Explain that swelling, redness, or discharge from the biopsy site are indications of infection. In the unlikely event that these signs or symptoms occur, instruct the participant to urgently seek medical advice from a doctor or local Accident & Emergency unit. Inform the participant that if they seek medical advice, they must also notify the research team.

        NOTE: As research staff, neither the biopsy taker nor the assistant can provide medical advice or treatment; however, it is important that the research team are aware of and record all instances of complications resulting from the biopsy procedure.
    4. Recommend that participants should avoid swimming or overly strenuous activity for 48 h until the site of incision has closed.
3. Clear away any used sharps and contaminated materials into designated sharps and/or clinical waste containers.
4. Clean all surfaces used in the biopsy procedure using 70% ethanol spray and clean paper towels. Place disposable and non-disposable items of bedding in appropriate clinical bags for disposal or cleaning, respectively.

5. Sample processing - instructions for the assistant

1. Use sterile tweezers and 0.9% saline to rinse the adipose tissue sample to remove visible contaminants (i.e., blood, vasculature). Then, weigh the adipose tissue samples using digital scales. Split the tissue into appropriately sized pieces for downstream analysis and place them into appropriate storing tubes using sterile tweezers. Immerse the tubes containing the adipose tissue biopsies in liquid nitrogen at -190 °C to flash-freeze until the samples are stored at -80 °C.

    NOTE: The assistant must complete sample processing as quickly as possible, typically within 3 min of sample aspiration, to minimize potential sample degradation.

Representative Results

The described adipose tissue biopsy procedure is an efficient and low-risk technique for researchers to

obtain subcutaneous adipose tissue samples from human volunteers. We performed 39 subcutaneous adipose tissue biopsies using the described procedure in 11 healthy, normal weight females (age, 27.4 ± 3.3 years; body mass index (BMI), 22.6 ± 1.5 kg.m^2). All participants attended the laboratory between 07:00 and 10:00 following an 8-12 h fasting period. Sample yield using this adipose tissue biopsy procedure was 192.0 ± 97.1 mg (range = 32.8-393.6 mg) (**Figure 4**). We observed no relationship between the biopsy yield and participant BMI (p= 0.643), although the participants' BMI were all within the healthy weight range (range= 21.1-25.4 kg/m^2). Adequate sample weight was typically obtained following 2-3 bouts of tissue collection (i.e., number of repetitions of steps 3.11.1 and 3.11.2). Following adipose tissue biopsies, all participants experienced a bruise, but none experienced excessive pain that was not alleviated by painkillers. Nor were there any other adverse reactions (**Table 1**). This is consistent with previously reported complication rates for adipose tissue biopsies[1,5].

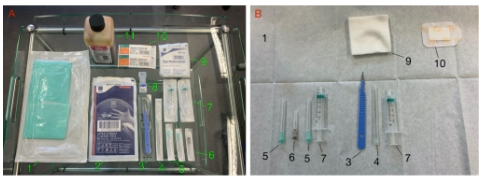

Figure 1: Materials required for the procedure. (**A**) The trolley laid out with the materials required for the procedure. (**B**) Materials arranged on the sterile field. 1: sterile field; 2: sterile gloves; 3: scalpel; 4: 14 G needle; 5: 21 G needle; 6: 26 G needle; 7: 5mL syringe; 8: lidocaine 2%; 9: sterile gauze; 10: adhesive wound dressing; 11: iodine-based solution. Please click here to view a larger version of this figure.

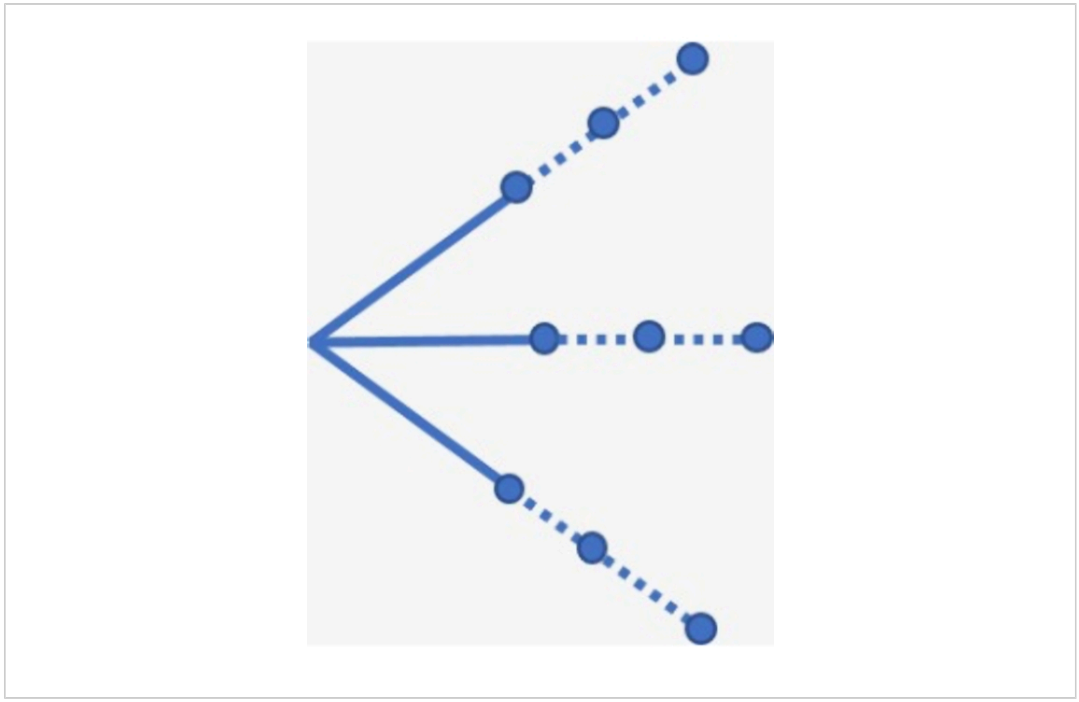

Figure 2: Schematic of the fan-shaped injection sites for administering the local anesthetic. The solid and dotted lines represent where the anesthetic should be administered using the 26 G and 21 G needle, respectively. Please click here to view a larger version of this figure.

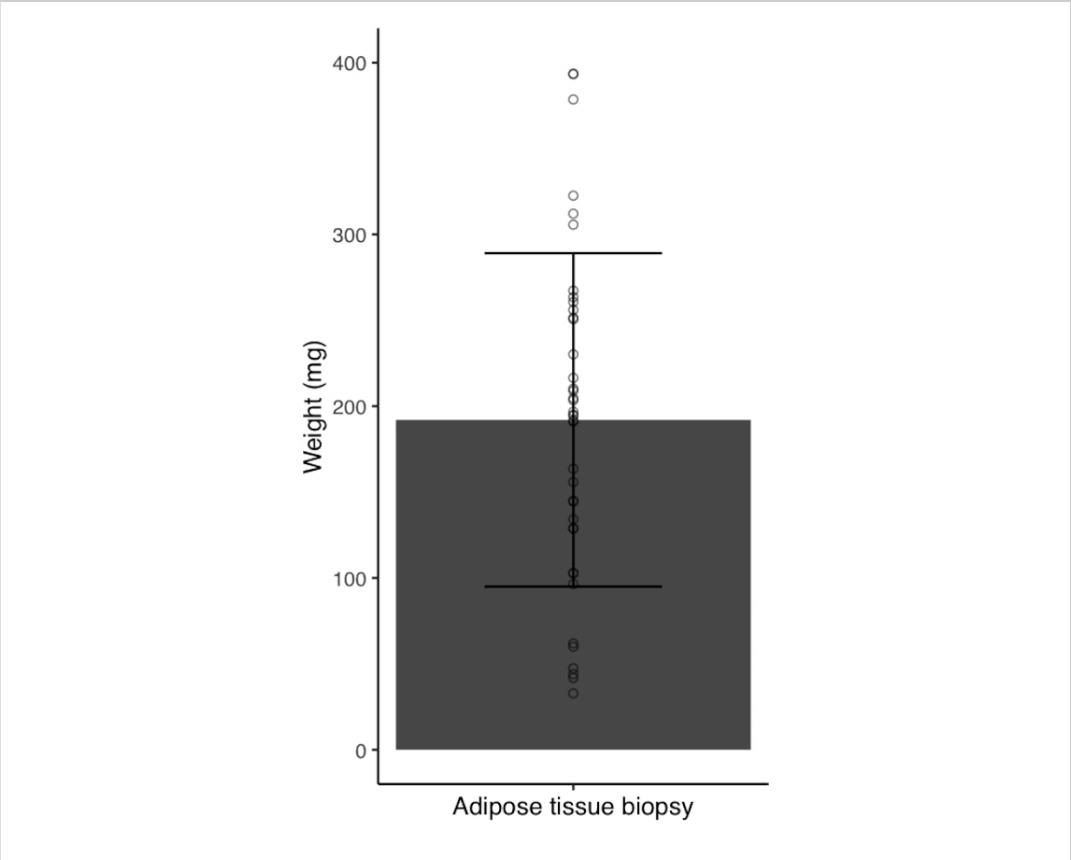

Figure 3: Adipose tissue sample yield from healthy, normal weight women (n= 39). Bar chart with error bars represent mean ± standard deviation. Circles represent individual data points. Please click here to view a larger version of this figure.

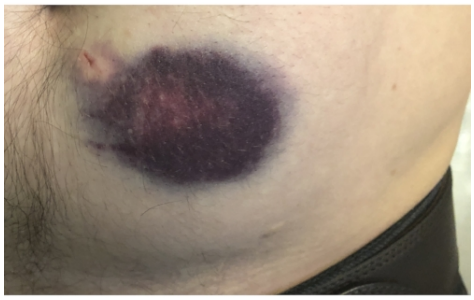

Figure 4: An example of a bruise resulting from an early training attempt. Please click here to view a larger version of this figure.

Complication	Response
Pain	Participant may take analgesics if necessary, following the instructions on the packet (e.g., paracetamol). Participants must refrain from taking analgesics that have anticoagulant activities.
Bleeding	Participant is to be advised that some bleeding is to be expected.
Bruising	Participant is to be advised that bruising is to be expected.
Scar tissue	Participant is to be advised that the development of some scar tissue at the biopsy site is to be expected.
Infection	Participant must be informed of all symptoms of an infection at the biopsy site prior to the biopsy. Participants must be instructed to seek medical advice from a doctor or local Accident and Emergency unit should these symptoms occur and notify the research team retrospectively.

Table 1: List of complications that may be experienced by participants.

Discussion

The described protocol and associated video provide a step-by-step overview of a mini-liposuction technique to obtain subcutaneous adipose tissue samples from the abdominal area. This research group has performed a total of 124 biopsies over the course of 19 months with no adverse effects in participants. The procedure is safe and associated with minimum risk to participants or the biopsy team, provided that the described safety measures are followed. Aseptic technique (including opening and dispensing of

sterile equipment without contaminating them, appropriately donning/removing sterile gloves, general hand hygiene) must be maintained at all times by the researchers performing the procedure (to minimize the risk of infection to the participant)[6]. Additionally, disposal of used sharps in an appropriate manner ensures the safety of the researcher and others who handle this waste by reducing the risk of needle-stick injuries[7].

Although the procedure can be classed as "low-risk", there are several critical steps in addition to aseptic technique and appropriate waste disposal that need to be followed to minimize adverse effects. Primarily, participants should confirm that they have no allergies to local anesthetics in the amino amide family (e.g., lidocaine) or the drug family of the local anesthetic used, certain metals that may be contained in needles (chromium, nickel, and cobalt), and shellfish/iodine if using an iodine-based skin disinfectant solution (step 2.4). As participants may not be familiar with the name of the anesthetic, and as lidocaine is commonly used in dental procedures, it might be helpful to ask whether they have had a reaction to anesthetic administration in that context. Similarly, participants can be asked whether they had allergic reactions to any jewelry/piercings rather than specifically chromium and nickel. Individuals currently on anticoagulants should not undergo the procedure as they are at increased risk of excessive bleeding. Participants routinely taking low-dose aspirin would not preclude participation in the biopsy protocol; however, participants must inform the biopsy taker as this may affect rate of hemostasis[8]. While omega-3 fatty acids supplementation would not preclude the biopsy from being performed, participants should confirm whether such supplements (or fatty-rich fish) are part of their routine diet as this may affect blood viscosity[9]. Prior to commencing the procedure, participants should also be asked whether they have any conditions that might otherwise affect the biopsy. For example, cosmetic surgery (i.e., liposuction) would affect the quantity/quality of tissue sample, and previous scars/ tattoo sites should be avoided. Lastly, the biopsy team may want to consider shaving participants with substantial amounts of body hair to make the biopsy area more visible.

When selecting the biopsy area (step 3.1), the researcher should make sure that the site is sufficiently far from the navel (approximately 5-10 cm) as the proximal area is very vascular. Choosing a biopsy site too close to the umbilicus can lead to unnecessarily extensive bruising (e.g., **Figure 4**). While excessive bruising can be limited by an appropriate choice of biopsy area and the application of an ice pack following the procedure, participants should be informed that some degree of bruising is likely to occur. Within this research group, we anecdotally observed that such contusions dissipate within 3-5 days. In addition, some participants may develop some scar tissue at the biopsy site, presenting as a lump of tissue hard to the touch. Anyone undergoing the biopsy procedure should be made aware that the scar tissue is transient and will resolve itself within 2-3 weeks. To maximize patient tolerability, the researcher should identify the area affected by the local anesthetic (step 3.9): by using a scalpel and gently prodding the biopsy area, the researcher can verbally confirm with the participant that the area has been successfully anesthetized. The limits of the anesthetized area should be confirmed by going beyond the area. Inform the participant that this will be done and that they may feel some very slight discomfort. This is a particularly important step, as placing the biopsy needle in non-anesthetized areas will cause participant discomfort.

The mini-liposuction biopsy technique described here is a low-cost alternative to surgical procedures and does not

require specialist tools. Owing to their straightforwardness, these biopsies can be performed routinely with little-to-no problems. The most common issue encountered when performing the adipose tissue sampling is that the shaft of the 14 G needle can become obstructed, preventing adipose tissue aspiration into the syringe. An experienced individual trained in the described biopsy technique will notice the obstruction through changes in the responsiveness of the syringe's plunger (i.e., it "sticks" in place). Should a needle obstruction occur, the researcher is advised *in primis* to attempt removing the obstruction by forcefully depressing the plunger while the needle bevel is over the weighing boat. If the obstruction is firmly lodged, the second option is to replace the needle and syringe. After the procedure, tissue lodged in a needle can be retrieved by pushing sterile saline through the needle. To prevent sample degradation, the obtained tissue should be cleaned, processed, and stored as soon as possible following the procedure[10]. To minimize RNA degradation, a stabilization solution can be utilized at the sample processing step[17] (please refer to the Table of Materials).

The main limitation of this technique is that while it is relatively fast (~15 min for a trained and experienced individual) and cost-effective, it results in only a moderately sized sample (~200 mg). Whilst this sample size is typically adequate for various metabolic assays, it is recommended that the researcher ensures the expected sample yield is sufficient for the intended sample analysis. The sample yield obtained using the described technique is typically lower than that of surgical techniques[11]; however, larger incision sites used in surgical biopsies cause more discomfort to participants and may prevent them from engaging in certain day-to-day activities until fully healed[11]. These techniques are also more likely to discourage participants from enrolling in research studies and require a trained clinician. A key advantage of the mini-liposuction biopsy described in this video is that it can be quickly performed in a non-clinical setting by non-medical researchers. Furthermore, being able to complete multiple biopsies on one participant within the same day enables researchers to perform acute before-after nutritional/exercise intervention studies. It should be noted that in the UK, lidocaine administration requires a prescription; a member of our team is qualified in non-medical prescribing. Local regulations should be checked before the administration of local anesthetic.

Many research groups have applied the mini-liposuction technique for a variety of research questions. These include, but are not limited to, providing adipose tissue hormone profiles in participants with diabetes[2], quantifying the variation of adipose tissue miRNA expression in patients with metabolic dysfunction[12], and assessing nutritional and exercise interventions in overweight populations[13,14]. Additionally, the immediate processing of adipose tissue samples permits; isolation of pre-adipocytes for cell culture[15]; and analysis of *ex vivo* metabolic parameters, such as lipolytic rate[16], hormone secretion[13], and mitochondrial respiration[14]. It must be noted that adipose tissue samples obtained via the described technique have high levels of fragmentation when compared to samples obtained via surgical techniques using a cutting needle or scalpel[5]. This precludes the successful usage of analytical techniques for the assessment of architectural and morphological parameters[5]. Should researchers intend to obtain adipose tissue samples for analysis of architectural and morphological parameters, alternative methods are associated with reduced tissue fragmentation[18]. Nonetheless, obtaining adipose

tissue samples via the described technique permits the investigation of a broad range of key physiological processes.

In summary, the present video and paper describe a non-clinical mini-liposuction biopsy technique to obtain subcutaneous abdominal adipose tissue. With appropriate controls in place, the method is relatively pain-free, safe, and time-/cost-effective. This biopsy method is particularly well suited for studies that implement a before-after study design and do not require large amounts of tissue sample.

Disclosures

The authors have no conflicts of interest to declare.

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Clinical Research Article

Clinical Research Article

Relationship Between Insulin Sensitivity and Menstrual Cycle Is Modified by BMI, Fitness, and Physical Activity in NHANES

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Abbreviations: ADIPO-IR, adipose tissue insulin resistance index; BMI, body mass index; CRF, cardiorespiratory fitness; FSH, follicle-stimulating hormone; HOMA-IR, homeostatic model of insulin resistance; HR, heart rate; LH, luteinizing hormone; MEC, mobile examination center; MESOR, a rhythm-adjusted mean; MET, metabolic equivalent; NHANES, National Health and Nutrition Examination Survey; VO_{2max}, maximal oxygen consumption.

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Abstract

Context: There is evidence demonstrating variation in insulin sensitivity across the menstrual cycle. However, to date, research has yielded inconsistent results.
Objective: This study investigated variation in insulin sensitivity across the menstrual cycle and associations with body mass index (BMI), physical activity, and cardiorespiratory fitness (CRF).
Methods: Data from 1906 premenopausal women in NHANES cycles 1999 to 2006 were analyzed. Menstrual cycle day was assessed using questionnaire responses recording days since last period. Rhythmic variation of plasma glucose, triglycerides, and insulin, homeostatic model of insulin resistance (HOMA-IR), and adipose tissue insulin resistance index (ADIPO-IR) across the menstrual cycle were analyzed using cosinor rhythmometry. Participants were assigned low or high categories of BMI, physical activity, and CRF, and category membership included in cosinor models as covariates.
Results: Rhythmicity was demonstrated by a significant cosine fit for glucose ($P = .014$) but not triglycerides ($P = .369$), insulin ($P = .470$), HOMA-IR ($P = .461$), and ADIPO-IR ($P = .335$). When covariates were included, rhythmicity was observed when adjusting for: 1) BMI: glucose ($P < .001$), triglycerides ($P < .001$), insulin ($P < .001$), HOMA-IR ($P < .001$), and ADIPO-IR ($P < .001$); 2) physical activity: glucose ($P < .001$), triglycerides ($P = .006$), and ADIPO-IR ($P = .038$); and 3) CRF: triglycerides ($P = .041$), insulin ($P = .002$), HOMA-IR

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($P = .004$), and ADIPO-IR ($P = .004$). Triglyceride amplitude, but not acrophase, was greater in the high physical activity category compared to low ($P = .018$).
Conclusion: Rhythmicity in insulin sensitivity and associated metabolites across the menstrual cycle are modified by BMI, physical activity, and CRF.

Key Words: menstrual cycle, insulin, glucose, triglyceride, insulin sensitivity, NHANES

The onset and severity of insulin resistance are associated with a range of modifiable and nonmodifiable risk factors, including, sex, age, adiposity, physical inactivity, and cardiovascular fitness (1, 2). Women exhibit lower fasting plasma glucose levels, but greater impairment in glucose tolerance compared to men (3). Within adipose tissue, women have greater insulin-stimulated glucose and fatty acid uptake compared to men (4). While body mass index (BMI) and age are positively associated with insulin resistance, in women insulin resistance typically occurs at a higher BMI and higher age compared with men (5). Moreover, low fitness has a greater association with insulin resistance in overweight women compared with overweight men (2).

Reports have demonstrated a clear mechanistic role of sex hormones underpinning sexual dimorphism in insulin resistance (6). Insulin sensitivity has been positively associated with estradiol and negatively associated with progesterone in rats (7). This suggests that hormonal fluctuations across the menstrual cycle in humans may play a role in insulin sensitivity. However, strategies targeting the prevention and treatment of reduced insulin sensitivity rarely consider sex and none consider the role of the menstrual cycle.

The menstrual cycle is a fundamental biological rhythmic cycle occurring in females of reproductive age, composed of the ovarian and uterine cycles. The ovarian cycle, consisting of follicular, ovulatory, and luteal phases, is concerned with oocyte maturation and release, while the uterine cycle, consisting of menstruation, proliferative, and secretory phases, is concerned with preparing the uterine lining for possible oocyte implantation in the event of fertilization (8). The ovarian cycle and uterine cycle occur in a coordinated and concurrent manner; herein we will refer to menstrual cycle phase in terms of the follicular and luteal phases (8). The average cycle length is 29 days, although this varies between individuals, with cycle lengths of 24 to 35 days considered normal and healthy (8, 9). Within an individual, typical cycle length declines as age increases (9). The menstrual cycle is governed by rhythmic fluctuations of hormones within the hypothalamic-pituitary-gonadal axis; gonadotropin-releasing hormone, pituitary hormones (follicle-stimulating hormone [FSH] and luteinizing hormone [LH]), and ovarian hormones (estradiol, progesterone, and testosterone) (8). However, the effect of the menstrual cycle on physiology is underresearched and in fact is frequently cited as a barrier toward the inclusion of women in research projects (10).

Cyclical fluctuations in hormonal profiles across the menstrual cycle have been associated with alterations in metabolic control. During the luteal phase an increase in circulating insulin and reductions in circulating glucose and triglycerides have been observed (11, 12). Correspondingly, insulin sensitivity would be expected to fluctuate across the menstrual cycle. However, studies so far have been equivocal. Reductions in insulin sensitivity during the luteal phase have been reported (12-18). However, other studies have documented no change in insulin sensitivity across the menstrual cycle (19-22). These inconsistencies may be attributable to the relatively small sample sizes used in all but one (12) of these studies (n = 6-30), which lacked statistical power to robustly detect the small, yet clinically meaningful, changes in insulin sensitivity across the menstrual cycle. Yeung et al used a large sample size (n = 259) and reported significant variation in insulin sensitivity across the menstrual cycle (12). Moreover, previous studies recruited heterogeneous study populations with varying BMI and physical activity levels, in which limited adjustment or investigation into the potentially confounding effects of these modifiable risk factors was conducted (12, 21, 22). Examining the role of modifiable risk factors in a large cohort of women is necessary to fully understand rhythmicity in insulin sensitivity across the menstrual cycle.

In this study we first aim to characterize the variation in insulin sensitivity and associated metabolites across the menstrual cycle in a large cohort of well-characterized female participants. Second, we will investigate the role of BMI, physical activity, and cardiovascular fitness on the variation in insulin sensitivity and associated metabolites across the menstrual cycle.

Materials and Methods

Participants

The National Health and Nutrition Examination Survey (NHANES) is a national, cross-sectional, population-based study representative of the noninstitutionalized US civilian population (NHANES, RRID: SCR_013201). Data were collected in 2-year cycles beginning in 1999, with data collection ongoing. NHANES participants completed an at-home

interview and a physical examination at a mobile examination center (MEC). A reproductive health questionnaire was included in data collection cycles between 1999 and 2006. This questionnaire was completed by 23 569 females. Participants were excluded if they had current diagnoses of metabolic disorder (diabetes, thyroid condition) or were taking medication that altered insulin sensitivity (Fig. 1). Details of variable descriptions and codes used in this study are provided in data repository (23).

Menstrual Cycle Assessment

The response to the question "number of days since last period started" was treated as the day of menstrual cycle. Responses were collected once for each participant. Unfortunately, data on typical menstrual cycle length were not available within the NHANES database. Participants were excluded from data analysis if the reported number of days since their last period started more than 35 days earlier. While the typical menstrual cycle length is 29 days, a maximum cycle day value of 35 days was selected to encompass 95% of the cycle lengths in women (9). Participants were excluded based on factors that influence the hormonal milieu across the menstrual cycle: age younger than 16 years, currently taking hormonal contraceptive medication, or currently pregnant or gave birth within the previous year. Final analyses were conducted on 1906 participants (see Fig. 1).

Anthropometric Assessment

Height was measured using a stadiometer, and weight was measured using a digital scale following standard procedures at the MEC. BMI was calculated using weight in kilograms divided by height in meters squared. Prior to analyses, participants were assigned to BMI-defined categories based on standard cutoff thresholds (low BMI, underweight and healthy weight ≤ 24.9; high BMI, overweight and obese > 25) (24).

Blood Sampling and Biochemical Analysis

Venous blood samples were collected the same day as the menstrual cycle questionnaire following a fast of at least 9 hours, but not more than 24 hours, by a trained phlebotomist at the MEC and processed according to a standardized protocol (25). Serum FSH and LH concentrations were analyzed by microparticle Enzyme Immunoassay (Abbot Laboratories) (26). Plasma glucose and triglyceride concentrations were analyzed enzymatically (Roche Diagnostic Systems) (27, 28). Plasma insulin concentration was assessed via radioimmunoassay (Pharmacia Diagnostics AB) (29). Insulin sensitivity was calculated using the HOMA-IR (30) and ADIPO-IR (31) methods.

Physical Activity

Each participant completed a physical activity questionnaire that included questions relating to all physical activity performed in the previous 30 days. Activity type, duration, intensity, and number of times performed in the last 30 days were recorded. Moderate-intensity activities were defined as inducing light sweating or a slight to moderate increase in breathing or heart rate (HR). Vigorous activities were defined as inducing

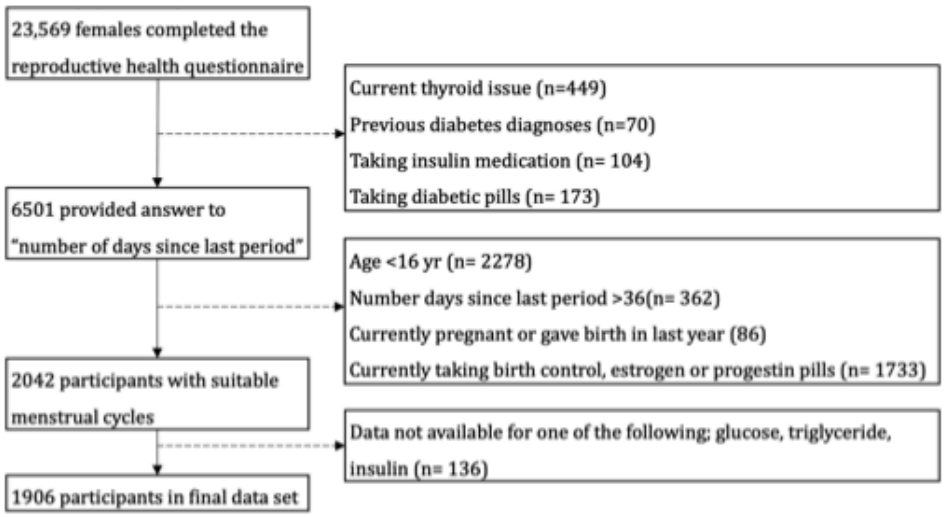

Figure 1. Flowchart depicting participant selection in the study. The dotted lines represent participant exclusion.

heavy sweating or large increases in breathing or HR. Metabolic equivalent (MET) scores for specific activities were calculated based on the activity type and intensity (32). MET scores were multiplied by the average duration and number of times performed in the last 30 days to calculate MET minutes per 30 days (MET min/30d) for each activity. MET min/30d were summed for each activity then divided by 4.29 to calculate total MET minutes per week. Prior to analyses, participants were assigned to low and high physical activity categories based on whether they met the national physical activity guidelines (low physical activity < 500 MET/wk; high physical activity ≥ 500 MET/wk) (33).

Cardiorespiratory Fitness

Participants underwent a submaximal exercise test on a treadmill to predict maximal oxygen consumption ($\dot{V}O_{2max}$) (34). Participants were assigned to 1 of 8 protocols, of varying difficulty, based on age, BMI, and self-reported physical activity level. Each protocol included a 2-minute warmup, 2 × 2-minute stages, and a 2-minute cool down. Heart rate was recorded throughout using an automated monitor. These exercise protocols aimed to elicit 75% of maximal HR by the end of the test. Predicted $\dot{V}O_{2max}$ was estimated by extrapolating age-specific maximal HR responses to the two 2-minute exercise stages, assuming a linear relation between HR and O_2 consumption during exercise (35, 36). Prior to analyses, participants were assigned to low and high cardiorespiratory fitness categories based on whether their $\dot{V}O_{2max}$ scores were below or above the age-specific 50th percentile (37).

Statistical Analysis

All analyses were conducted in R (version 3.6.3) (38). Participant demographic data are presented as mean ± SD. The number of participants are shown for each analysis; this varies because of missing data. Data were tested for normality using visual inspection of histogram and Shapiro-Wilk test. Nonnormal data were log10-transformed. Rhythmicity across the menstrual cycle was detected using the "Cosinor" and "Cosinor2" packages (39, 40). Cosinor fits a cosine curve with a free phase to data and calculates MESOR (a rhythm-adjusted mean), amplitude (half the predictable variation within a cycle), and acrophase (time of highest value within a cycle). Peak-to-peak difference (%) was calculated using the following equation: (2 × amplitude/mean)*100. In separate cosinor models, we included the BMI, physical activity, and cardiorespiratory fitness category as a covariate. Inclusion of covariates in the cosinor model allows the MESOR, amplitude, and acrophase to differ between respective high and low covariate categories. Overall significance of the cosine model was established using the zero-amplitude test. Wald tests were conducted to test for differences in the amplitude and acrophase between respective high and low covariate categories. Cosine data are presented as MESOR ± amplitude. Data are shown as conventional box plots.

Results

Participant Characteristics

Participant characteristics are outlined in Table 1. As would be expected, greater weight, higher BMI, lower physical activity, and lower $\dot{V}O_{2max}$ were observed in the low physical activity category (difference = 2.0 kg, $P = .020$; 1.2, $P < .001$; 2884.7 MET min/wk $P < .001$; 1.4 mL/min/kg, $P = .019$, respectively), high BMI category (26.6 kg, $P < .001$; 10.3, $P < .001$; 576.4 MET min/wk, $P < .001$; 2.5 mL/min/kg, $P < .001$), and low cardiorespiratory fitness category (3.4 kg, $P = .003$; 1.3, $P = .001$; 403.1 MET min/wk, $P = .028$; 10.8 mL/min/kg, $P < .001$, respectively). Age was significantly greater for the low physical activity (3.1 years, $P < .001$), high BMI (3.6 years, $P < .001$), and high cardiorespiratory fitness (5.5 years, $P < .001$) categories. Height was significantly greater in the high physical activity category (1.5 cm, $P < .001$), but not BMI (0.4 cm, $P = .166$) nor cardiorespiratory fitness (0.2 cm, $P = .757$). No rhythmic cycling was detected across the menstrual cycle in BMI (MESOR: 26.3 ± amplitude: 0.15, $P = .822$), physical activity (1527.1 ± 182.1 MET min/wk, $P = .199$), or cardiorespiratory fitness (37.5 ± 0.5 mL/min/kg, $P = .517$) (23).

Pituitary Hormone Concentration Across the Menstrual Cycle

To demonstrate the validity of cosine analysis for analyzing cyclic rhythms in variables across the menstrual cycle, pituitary hormones were analyzed. Plasma FSH and LH concentrations were available for a subset of participants (Table 2 and Fig. 2). FSH concentration reached a peak of 8.6 IU on day 9 falling to 3.5 IU on day 26 ($P < .001$). LH concentration peaked at 8.1 IU on day 12 and declined to a trough of 2.1 IU on day 29 ($P < .001$).

Table 1. Participant characteristics split by demographic category

Demographic	All	Low MET	High MET	Low BMI	High BMI	Low CRF	High CRF
No.	1906	946 (49.6)	960 (50.4)	1021 (53.6)	885 (46.4)	451 (46.7)	514 (53.2)
Age, y	25.4 ± 9.4	27.0 ± 10.0	23.9 ± 8.6[a]	23.8 ± 8.5	27.4 ± 10.1[a]	21.3 ± 5.9	26.8 ± 10.0[a]
Height, cm	161.8 ± 6.9	161.1 ± 6.9	162.6 ± 6.8[a]	162.0 ± 6.8	161.6 ± 7.0	162.0 ± 6.7	162.2 ± 6.7
Weight, kg	69.0 ± 19.0	70.0 ± 19.9	68.0 ± 18.0[a]	56.6 ± 7.2	83.2 ± 18.3[a]	69.5 ± 19.5	66.1 ± 15.1[a]
BMI	26.3 ± 6.8	26.9 ± 7.2	25.7 ± 6.3[a]	21.5 ± 2.1	31.8 ± 6.1[a]	26.4 ± 7.0	25.1 ± 5.4[a]
VO_{2max}, mL/kg/min	37.5 ± 9.0	36.8 ± 9.5	38.2 ± 8.6[a]	38.6 ± 8.8	36.1 ± 9.1[a]	31.8 ± 3.8	42.6 ± 9.3[a]
MET, min/wk	1548.3 ± 3112.5	95.3 ± 144.7	2980.0 ± 3884.4[a]	1815.9 ± 3479.9	1239.5 ± 2593.5[a]	1407.9 ± 2498.3	1811.0 ± 3200.0[a]

Data are presented as mean ± SD. No. values are presented as total (%) for each demographic category.
Abbreviations: BMI, body mass index; CRF, cardiorespiratory fitness; MET, metabolic equivalent; PA, physical activity; VO_{2max}, maximal oxygen consumption.
[a]*P* less than .05 following independent samples *t* test.

What Is the Effect of the Menstrual Cycle on Insulin Sensitivity?

Rhythmicity was demonstrated by a significant cosine fit for glucose (MESOR: 85.1 ± amplitude: 1.2 mg/dL; $P = .014$). No significant fit was observed for triglycerides (87.7 ± 2.8 mg/dL; $P = .369$), insulin (9.8 ± 0.4 uU/mL; $P = .470$), HOMA-IR (2.1 ± 0.1 mmol/L; $P = .461$), or ADIPO-IR (9.7 ± 0.6 mmol/L; $P = .335$) (Table 3; Figs. 3 and 4).

How Does Body Mass Index Affect Insulin Sensitivity Across the Menstrual Cycle?

When the BMI category was added as a covariate into the cosine model, significant cosine fit was observed for glucose ($P < .001$), triglycerides ($P < .001$), insulin ($P < .001$), HOMA-IR ($P < .001$), and ADIPO-IR ($P < .001$) (see Table 3; Figs. 3 and 4). There were no significant differences in amplitude between low and high BMI categories for glucose (0.7 vs 1.7 mg/dL, $P = .205$), triglycerides (3.4 vs 3.9 mg/dL, $P = .889$), insulin (0.3 vs 1.1 uU/mL, $P = .486$), HOMA-IR (0.1 vs 0.3 mmol/L, $P = .318$), or ADIPOIR (0.2 vs 1.9 mmol/L, $P = .248$). Nor was there a significant difference in acrophase between low and high BMI categories for glucose (12 vs 16 days, $P = .335$), triglycerides (28 vs 21 days, $P = .098$), insulin (15 vs 23 days, $P = .180$), HOMA-IR (15 vs 22 days, $P = .267$), or ADIPO-IR (23 vs 22 days, $P = .902$).

How Does Physical Activity Affect Insulin Sensitivity Across the Menstrual Cycle?

When the physical activity category was added as a covariate into the cosine model, significant cosine fit was observed for glucose ($P < .001$), triglycerides ($P = .006$), and ADIPO-IR ($P = .038$), but not insulin ($P = .095$) or HOMA-IR ($P = .061$) (see Table 3; Figs. 3 and 4). Triglyceride amplitude was significantly lower in the low physical activity category compared to the high physical activity category (3.1 vs 7.2 mg/dl, $P = .018$). No significant differences were observed in amplitude between low and high physical activity categories across the menstrual cycle for either glucose (1.5 vs 1.1 mg/dL, $P = .308$), insulin (0.7 vs 0.3 uU/mL, $P = .284$), HOMA-IR (0.2 vs 0.1 mmol/L, $P = .310$) or ADIPO-IR (0.7 vs 0.7 mmol/L, $P = .506$). There were no significant differences in acrophase between low and high BMI categories for glucose (17 vs 12 days, $P = .235$), triglycerides (12 vs 27 days, $P = .675$), insulin (21 vs 14 days, $P = .571$), HOMA-IR (21 vs 14 days, $P = .577$), or ADIPO-IR (18 vs 26 days, $P = .423$).

Table 2. Pituitary hormone concentrations across the menstrual cycle

Variable	No.	MESOR, IU	Amplitude, IU	P-P, %	Peak, d	Trough, d	*P*
FSH	218	6.1	2.5	91.9	9	26	< .001
LH	219	5.1	3.0	144.0	12	29	< .001

P value from zero amplitude test for model fit.
Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; MESOR, rhythm-adjusted mean; P-P, %, difference between peak and trough.

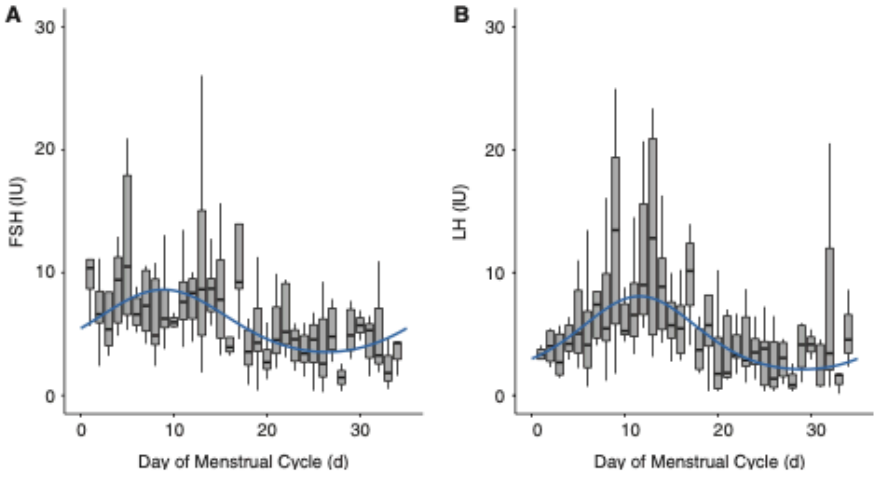

Figure 2. Box plot with cosine wave showing pituitary hormone concentration across the menstrual cycle. A, Follicle-stimulating hormone (FSH). B, Luteinizing hormone (LH).

How does Cardiorespiratory Fitness Affect Insulin Sensitivity across the Menstrual Cycle?

When the cardiorespiratory fitness category was added as a covariate into the cosine model, significant cosine fit was observed for triglycerides ($P = .041$), insulin ($P = .002$), HOMA-IR ($P = .004$), and ADIPO-IR ($P = .004$), but not glucose ($P = .223$) (see Table 3; Figs. 3 and 4). No significant differences in amplitude across the menstrual cycle were observed between low and high cardiorespiratory fitness for glucose (0.4 vs 0.8 mg/dL, $P = .460$), triglycerides (6.9 vs 6.0 mg/dL, $P = .116$), insulin (1.2 vs 0.7 uU/mL, $P = .099$), HOMA-IR (0.3 vs 0.2 mmol/L, $P = .109$), or ADIPOIR (1.7 vs 1.0 mmol/L, $P = .115$). There were no significant differences in acrophase between low and high BMI categories for glucose (20 vs 12 days, $P = .443$), triglycerides (34 vs 24 days, $P = .271$), insulin (27 vs 17 days, $P = .290$), HOMA-IR (27 vs 17 days, $P = .282$), or ADIPO-IR (30 vs 20 days, $P = .260$).

Discussion

This study aimed to characterize cyclical changes in insulin sensitivity and associated metabolic parameters across the menstrual cycle and their association with BMI, physical activity, and cardiorespiratory fitness. We found rhythmic cycling across the menstrual cycle for glucose, but not triglycerides, insulin, HOMA-IR, or ADIPO-IR. When including selected risk factors for insulin resistance as covariates, rhythmic cycling was observed across the menstrual cycle for glucose, triglycerides, insulin, HOMA-IR, and ADIPO-IR when models included BMI; glucose, triglycerides, and ADIPO-IR when models included physical activity; and triglycerides, insulin, HOMA-IR, and ADIPO-IR when models included cardiorespiratory fitness. Triglyceride amplitude, but not acrophase, was significantly greater in the high physical activity category compared to the low physical activity category. No significant differences in amplitude nor acrophase were observed for glucose, insulin, HOMA-IR, or ADIPO-IR between respective high and low covariate categories. These findings demonstrate changes in insulin sensitivity and triglyceride levels across the menstrual cycle are modified by BMI, physical activity, and cardiorespiratory fitness status.

Previous literature reports insulin sensitivity is either reduced during the luteal phase (12-18) or remains unchanged

Table 3. Variation in insulin sensitivity and associated metabolites across the menstrual cycle

Variable	Category	*n* value	Mean, IU	Amplitude, IU	P-P, %	Acrophase, d	*P*	Amplitude difference *P*	Acrophase difference *P*
Glucose	All	1903	85.09	1.15	2.70	15	**.014**		
	Low BMI	1019	83.41	0.67	1.62	12			
	High BMI	884	87.07	1.69	3.88	16	**.000**	.205	.335
	Low PA	944	85.82	1.46	3.41	17			
	High PA	959	84.35	1.10	2.61	12	**.000**	.308	.235
	Low CRF	451	84.16	0.44	1.05	20			
	High CRF	513	84.44	0.78	1.86	12	.223	.460	.443
Triglycerides	All	872	87.67	2.79	6.37	26	0369		
	Low BMI	482	77.26	3.39	8.78	28			
	High BMI	390	102.55	3.85	7.51	21	**.000**	.889	.098
	Low PA	404	89.83	3.07	6.85	12			
	High PA	468	86.39	7.22	16.73	27	**.006**	**.018**	.675
	Low CRF	192	88.14	6.87	15.58	34			
	High CRF	254	83.39	6.03	14.47	24	**.041**	.116	.271
Insulin	All	872	9.75	0.37	7.63	20	.470		
	Low BMI	483	7.49	0.30	8.14	15			
	High BMI	389	13.55	1.09	16.05	23	**.000**	.486	.180
	Low PA	405	10.06	0.67	13.32	21			
	High PA	467	9.45	0.26	5.59	14	.095	.284	.571
	Low CRF	194	10.89	1.22	22.49	27			
	High CRF	252	9.04	0.71	15.60	17	**.002**	.099	.290
HOMA-IR	All	871	2.09	0.09	8.34	20	.461		
	Low BMI	482	1.56	0.05	6.90	15			
	High BMI	389	3.01	0.28	18.91	22	**.000**	.318	.267
	Low PA	404	2.18	0.15	14.18	21			
	High PA	467	2.01	0.06	6.03	14	.061	.310	.577
	Low CRF	194	2.30	0.27	23.21	27			
	High CRF	252	1.92	0.16	16.75	17	**.004**	.109	.282
ADIPO-IR	All	868	9.67	0.59	12.24	23	.335		
	Low BMI	480	6.53	0.23	7.09	23			
	High BMI	388	15.71	1.89	24.09	22	**.000**	.248	.902
	Low PA	403	10.24	0.72	14.02	18			
	High PA	465	9.22	0.68	14.82	26	**.038**	.506	.423
	Low CRF	192	10.81	1.71	31.64	30			
	High CRF	252	8.54	1.04	24.47	20	**.004**	.115	.260

Cosine fit *P* value represents zero amplitude test for model fit. Amplitude difference *P* value represents difference in amplitudes between respective low and high covariate categories. Acrophase difference *P* value represents difference in acrophase between respective low and high covariate categories. Bold font indicates a *P* value of less than .05.

Low BMI, 24.9 or less; high BMI, greater than 25; low CRF, less than or equal to 50th age-specific $\dot{V}O_{2max}$ percentile; high CRF, greater than 50th specific $\dot{V}O_{2max}$ percentile; low PA, less than or equal to 500 MET min/wk; high PA, greater than 500 MET min/wk.

Abbreviations: ADIPO-IR, adipose tissue insulin resistance index; BMI, body mass index; CRF, cardiorespiratory fitness; HOMA-IR, homeostatic model of insulin resistance; PA, physical activity.

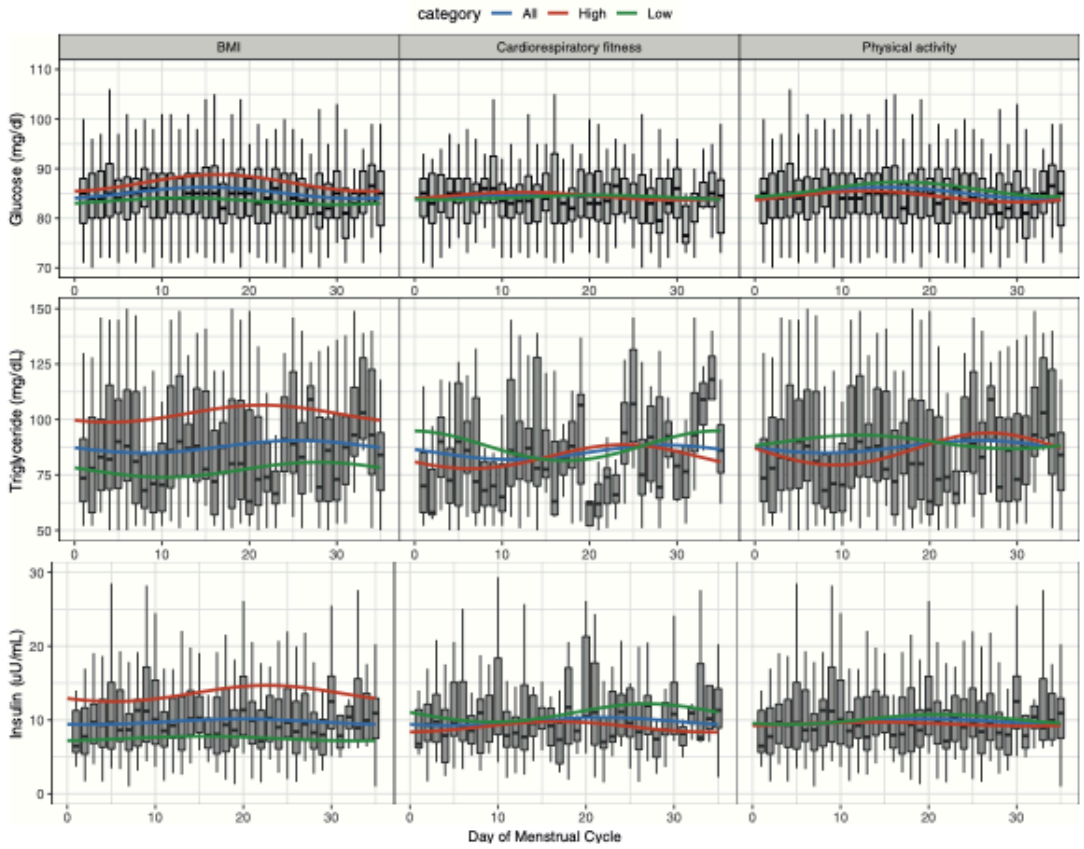

Figure 3. Changes in glucose, triglycerides, and insulin across the menstrual cycle with low and high categories of left, body mass index (BMI); middle, cardiorespiratory fitness; and right, physical activity. Box plot represents all participants data for each respective variable. Cosinor model fits are shown for (blue) all participants, (green) low covariate category, and (red) high covariate category. Low BMI, 24.9 or less; high BMI, greater than 25; low cardiorespiratory fitness, 50th or lower age-specific percentile; high cardiorespiratory fitness, greater than 50th age-specific percentile; low physical activity, less than or equal to 500 metabolic equivalent (MET) min/wk; high physical activity, greater than 500 MET min/wk.

across the menstrual cycle (19-22). Reported variation in HOMA-IR across the menstrual cycle is of a relatively small magnitude (0.3 U), although it may be clinically meaningful (12). Therefore, some previous studies using small sample sizes may have lacked statistical power to detect significant variation (19-22). In contradiction to a report from another large study, we did not observe rhythmic variation for insulin sensitivity prior to adjusting cosine fit for BMI or cardiorespiratory fitness (12). Participants studied by Yeung et al had an average lower BMI (24.1 vs 26.3), which may have contributed to discrepancies in findings (12). Cardiorespiratory fitness was not assessed in their study. Following the inclusion of BMI and cardiorespiratory fitness into our models, we observed rhythmic cycling for HOMA-IR with variability across the menstrual cycle of 0.3 U, similar to that previously reported by Yeung and colleagues. This provides evidence that BMI and cardiorespiratory fitness mediate the variation in HOMA-IR across the menstrual cycle. This mediation effect may underpin inconsistencies reported in the literature.

To our knowledge, this is the first study to investigate ADIPO-IR across the menstrual cycle. We observed rhythmic variation in ADIPO-IR when adjusting for BMI, physical activity, and cardiorespiratory fitness levels. Rhythmic cycling in ADIPO-IR concentration roughly coincided with rhythmic cycling of triglycerides across the menstrual cycle, which peaked at cycle day 23, declining to a trough at cycle day 5 (see Table 3). This contradicts previous research reporting elevated triglyceride concentrations during the follicular phase compared to the luteal phase (11, 41). However, previous studies did not consider BMI, physical activity, or cardiorespiratory fitness, which

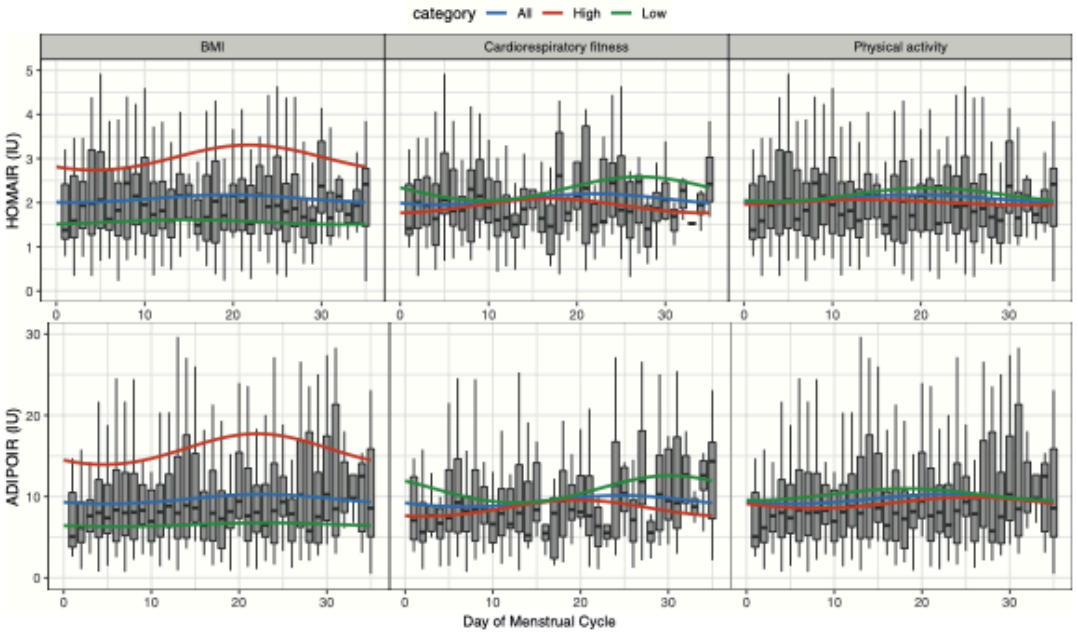

Figure 4. Changes in homeostatic model of insulin resistance (HOMA-IR) and adipose tissue insulin resistance index (ADIPO-IR) across the menstrual cycle with low and high categories of left, body mass index (BMI); middle, cardiorespiratory fitness; and right, physical activity. Box plot represents all participant data for each respective variable. Cosinor model fits are shown for (blue) all participants, (green) low covariate category, and (red) high covariate category. Low BMI, 24.9 or less; high BMI, greater than 25; low cardiorespiratory fitness, 50th or lower age-specific percentile; high cardiorespiratory fitness, greater than 50th age-specific percentile; low physical activity, less than or equal to 500 metabolic equivalent (MET) min/wk; high physical activity, greater than 500 MET min/wk.

we found significantly mediated rhythmicity in triglycerides across the menstrual cycle. Additionally, we used a larger sample size in this study compared with previous studies (n = 34 (41) and 259 (11) vs 869). Increases in ADIPO-IR during the luteal phase alongside concurrent elevations in triglyceride concentration may be underpinned by a decline in insulin-stimulated triglyceride uptake or suppression of lipolysis during the luteal phase. Progesterone has been shown to inhibit adipocyte insulin signaling and receptor binding (42, 43). Increased circulating progesterone levels may contribute to the increased ADIPO-IR observed during the luteal phase of the menstrual cycle. However, further work is required to elucidate the role progesterone plays in regulating changes in circulating triglycerides and ADIPO-IR across the menstrual cycle.

We observed lower mean triglyceride concentration alongside significantly greater amplitude across the menstrual cycle in the high physical activity category compared to low. The timing of the peak and trough in triglyceride concentration roughly coincided with the glucose trough and peak, respectively. Regular physical activity increases the capacity for adipose tissue and skeletal muscle lipid uptake and mobilization (4, 44). Moreover, high physical activity levels are positively associated with increased metabolic flexibility (44). Greater amplitude in triglyceride concentration across the menstrual cycle in the high physical activity category may reflect a coordinated uptake and release of triglycerides in response to fluctuations in glucose concentration.

While BMI and physical activity are significantly associated with variation in HOMA-IR and ADIPO-IR across the menstrual cycle, the mechanisms underpinning this relationship are uncertain. Variation in insulin sensitivity across the menstrual cycle has been associated with progesterone and estradiol (12). Differences in BMI and physical activity are known to alter ovarian hormonal profiles. Low physical activity levels are associated with higher mean estradiol levels across the menstrual cycle and higher progesterone levels during the luteal phase (45). High BMI is associated with greater variability of estradiol, but not progesterone (46). Unfortunately, neither estradiol nor progesterone was assessed in NHANES. Future research should investigate the role of sex hormones in the relationship between insulin sensitivity and BMI and physical activity levels.

We observed significant rhythmicity in HOMA-IR and ADIPO-IR following adjustment for BMI and

cardiorespiratory fitness. This suggests that the menstrual cycle phase is an important consideration in the assessment of insulin sensitivity in clinical practice or research, especially in populations with high BMI or low cardiorespiratory fitness. Additionally, we found greater amplitude across the menstrual cycle for HOMA-IR and glucose in high compared to low BMI and HOMA-IR in low compared to high cardiorespiratory fitness. Although these amplitudes were not statistically significant, these data indicate individuals with high BMI or low cardiorespiratory fitness may be at greater risk of impaired insulin sensitivity and elevated glucose concentration during the luteal phase. Therefore, therapeutic strategies aiming to reduce disturbances in metabolic control across the menstrual cycle may benefit from targeting a reduction in BMI and increase in cardiovascular fitness. This is of particular clinical importance because of the role of high glucose variability and insulin resistance in the development and progression of diabetic complications (47, 48). Future larger studies should further investigate the association between BMI and cardiorespiratory fitness with the magnitude of variation in insulin sensitivity and glucose concentrations across the menstrual cycle. This research is crucial to further understand the role of the menstrual cycle in diabetes.

Unexpectedly, some participant demographics were significantly different between respective low and high demographic categories. Therefore, some caution should be applied when interpreting these findings. Age was significantly greater in the high BMI, physical activity, and cardiorespiratory fitness categories compared to low. However, previous research has reported the positive relationship between age and insulin resistance is associated with concurrent increases in adiposity and decreases in physical activity (49), which were included in the cosinor analysis as covariates. Height was significantly greater in the high physical activity group. We performed a regression analysis to assess the relationship between height and metabolic outcome parameters while accounting for menstrual cycle day and found no significant associations (23). This statistically significant effect may simply be due to the number of participants in the study (50). Similarly, a previous large study reported no association between variation in HOMA-IR across the menstrual cycle and height (12). We would expect there to be overlap in participants within covariate categories, which may have confounding effects, for example, commonality between participants in the high BMI, low physical activity, and low cardiorespiratory categories. Future studies should investigate whether there is a cumulative effect of BMI, physical activity, and cardiorespiratory on rhythmic cycling in insulin sensitivity across the menstrual cycle.

The large, prospective nature of the NHANES data set represents a major strength of this study. Our analyses were conducted in 1906 female participants with detailed questionnaire data available for reproductive and general health. These data permitted the exclusion of women with conditions that alter metabolic control or hormonal concentrations. The indirect assessment of insulin resistance using surrogate measures (HOMA-IR and AIDIPO-IR) was a limitation. However, HOMA-IR and ADIPO-IR have been validated against the gold-standard hyperinsulinemic euglycemic clamp ($r = 0.82$, $P < .001$) and the multistep pancreatic clamp ($r = 0.86$, $P < .001$) respectively, demonstrating strong correlations (51, 52). Physical activity levels were determined using a questionnaire. However, reports from the NHANES data set demonstrate similar amounts of self-reported physical activity and objectively measured physical activity via accelerometer in those either meeting or not meeting physical activity guidelines (53). Nonetheless, future studies may benefit from collecting objectively measured physical activity across the menstrual cycle. This study used the number of days since the last menstrual period started as a proxy for phase of menstrual cycle and was limited by a lack of data regarding participants' typical menstrual cycle length. These data would allow greater accuracy in determining menstrual cycle phase. However, that the analysis of FSH and LH displayed expected fluctuations with significant rhythmicity across the menstrual cycle supports the use of "number of days since last menstrual period started" for statistical analysis in this data set. Ovarian hormone concentrations across the menstrual cycle were not measured, which would allow further exploration into the relationship between insulin sensitivity and risk factors for metabolic dysregulation. Future studies should obtain further data to allow a thorough characterization of participants' menstrual cycles, including typical menstrual cycle duration, ovulation date, and ovarian hormones.

In conclusion, our study confirms previous reports showing insulin sensitivity undergoes small yet statistically and clinically significant rhythmic cycling across the menstrual cycle. This is the first study to demonstrate a modifying effect of BMI, physical activity, and cardiorespiratory fitness on variation in insulin sensitivity and associated metabolites across the menstrual cycle. These findings provide a basis for further research to explore the mediatory role of BMI, physical activity, and cardiorespiratory fitness on variation in insulin sensitivity across the menstrual cycle. Furthermore, this provides direction for investigation into the therapeutic benefit of targeting BMI and physical activity to mitigate disturbances in insulin sensitivity across the menstrual cycle.

Additional Information

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Disclosures: The authors have nothing to disclose.

Data Availability: All data generated or analyzed during this study are included in this published article or in the data repositories listed in "References" (54, 55).

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