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Title:

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

Authors:

Kirstin A MacGregor[1], Nidia Rodriguez-Sanchez[1], Thomas G Di Virgilio[1], Nick D Barwell[2], Iain J Gallagher[1], Colin N Moran[1]*

Affiliation:

[1]Physiology, Exercise and Nutrition Research Group, University of Stirling, Scotland, UK.

[2]Department of Diabetes and Endocrinology, Forth Valley Royal Hospital, Scotland, UK

ORCID numbers:

KAM: 0000-0001-9269-9004

NRS 0000-0003-3960-5704

TDV: 0000-0002-4520-0423

NDB: 0000-0001-8870-8123

IJG: 0000-0002-8630-7235

CNM: 0000-0001-6226-8131

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Adipose tissue microRNA expression and menstrual cycle phase

Correspondence:

Colin Moran,

University of Stirling,

Stirling,

FK9 4LA,

colin.moran@stir.ac.uk

Abstract:

Cyclical changes in hormone profiles across the menstrual cycle are associated with alterations in metabolic control. MicroRNAs (miRNA) contribute to regulating metabolic control, including adipose tissue metabolism. How fluctuations in hormonal profiles across the menstrual cycle affect adipose tissue miRNA expression remain 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); post-ovulatory (PO) and mid-luteal (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 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 ($\eta_p^2=0.18$, $p=0.03$); however, post-hoc tests did not reveal any significant

differences between menstrual cycle phases ($p > 0.05$). miR-30c-5p 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.

Keywords:

Ovarian hormones, metabolism, hormone, estradiol, 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 d (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 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 non-coding 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 non-users in post-

menopausal 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, 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.

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 informed written consent.

Participants:

A total of 11 healthy, pre-menopausal 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 three months; and, not pregnant within the previous three months. Additionally, participants were excluded if they exhibited any symptom 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.

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 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.

Blood sampling and adipose tissue biopsies: 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. 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, 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.

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).

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 (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, Delaware, US). RNA samples were stored at -80 ^{o}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 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) (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 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 (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 (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 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 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 supplementary table 1 (33). 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 and finished with melting curve analysis. All qPCR reactions were performed in triplicate and analyzed using the $2^{-\Delta\Delta Ct}$ method (34). 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 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.

miRNA-mRNA pathway analysis:

miRsystem was used to conduct miRNA-mRNA pathway analysis using RT-qPCR results for miRNA expression (35). 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 (36). 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 (36–38) 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 (39). 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 (40). 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 (41). A participant identifier was included in models as a random effect to account for repeated measures. P-values were obtained using the Kenward-

299 Roger approximation for denominator degrees of freedom (42). Pairwise comparisons
300 were conducted with Tukey adjustment. Effect sizes where calculated as adjusted R^2 for
301 fixed effects with Cohen's descriptors used to interpret effect sizes; 0.02 small, 0.13
302 moderate and 0.26 large (43). In all analyses, miRNA expression was adjusted for age due
303 to the reported association between age and basal adipose miRNA expression in regularly
304 menstruating women (24). Unadjusted candidate miRNA expression data are presented
305 in supplementary table 3 (33). A p-value < 0.05 was considered statistically significant.
306
307 To provide sample size estimations for future studies using data generated in this study,
308 power analysis for linear mixed models were conducted using the package 'simr' in R
309 (44). 'simr' generates power curves for sample size estimation based on Monte Carlo
310 simulations.
311
312 Results:

313 *Menstrual cycle characteristics:*
314 Participant characteristics are shown in **Table 1.** All participants reported regular
315 menstrual cycles during the course of the study, as assessed via the menstrual cycle diary.
316 Sampling visits occurred on cycle day; 3 ± 1 (EF), 11 ± 1 (LF), 16 ± 2 (PO) and 24 ± 3 (ML).
317 Serum analysis of hormones showed expected temporal changes in LH, FSH, estradiol,
318 progesterone and testosterone profiles between menstrual cycle phases (**Table 2**). FSH
319 was significantly lower during ML compared to EF, LF and PO (p< 0.001; p= 0.011; p<
320 0.001). LH was significantly higher during PO compared to EF and ML (p= 0.006; p<
321 0.001) and higher during LF compared to ML (p= 0.005). Estradiol was significantly
322 greater during LF, PO and ML compared to EF (p= 0.002; p= 0.013; p< 0.001).
323 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. 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 prior to correction for multiple testing (**Figure 1, Supplementary table 2** (33)). No significant differences were observed following FDR correction for multiple testing (**Figure 1, Supplementary table 2** (33)).

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 timepoints). 3 miRNAs (miR-1231, miR-1914, miR-3180-3p) were not detected consistently in all participants and were removed from the analysis. **Figure 2** 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 expression was significantly altered across the menstrual cycle ($p= 0.030$, $R^2= 0.18$); 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 ($p= 0.067$, $R^2= 0.14$).

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. Additionally, 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 **Figure 3, Supplementary table 5 & 6** *(33)*).

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 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, 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 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.

386 The effect of the menstrual cycle on basal miRNA expression has been previously limited
387 to plasma samples, with inconsistent findings reported (28, 45). Li *et al.* observed
388 elevated miR-126 expression during the ML phase compared to the EF and LF phase (27).
389 Whereas Rekker *et al.* observed no change in the expression of 375 miRNAs between four
390 timepoints across the menstrual cycle (28). These studies were conducted in heathy
391 participants with comparable sample sizes to that used in this study (n= 12 (27, 28)).
392 However, miRNA expression is highly tissue specific and miRNA profiles in plasma may
393 not represent adipose tissue (24). In this study, RT-qPCR analysis determined miR-497-
394 5p expression was significantly altered across the menstrual cycle. miR-497-5p
395 expression increased 10 % in PO compared with ML and LF; however, pairwise
396 comparisons did not detect any significant differences between phases. Loss of statistical
397 significance between ANOVA and pairwise comparisons can be caused by a weakly
398 significant global effect in ANOVA and insufficient statistical power resultant from a low
399 sample size (46). Additionally, we observed a trend for reduced miR-224-3p during the
400 PO and ML phase (p= 0.059) and a trend for reduced miR-331-5p during the ML phase
401 (p= 0.059). This is congruent with previous studies reporting reduced miR-224 and miR-
402 331 expression following chronic estradiol supplementation in adipose tissue (24) and
403 adipose derived extracellular vesicles (47). Future research should further investigate
404 changes in adipose tissue miRNA expression across the menstrual cycle in a larger study
405 population.
406
407 To gain insight into the potential cellular consequences of variation in miRNA expression
408 across the menstrual cycle, we conducted functional pathway analysis using miRsystem.
409 Functional pathways analysis of miR-497-5p revealed 5 of the top 10 pathways target
410 processes related to insulin sensitivity (Error! Reference source not found.). miR-497-5p

411 has previously been shown to inhibit adipocyte insulin sensitivity and associated gene
412 expression in vivo (48, 49). Small increases in adipose tissue miR-497-5p expression may
413 contribute to the reduction in insulin sensitivity previously reported during the PO phase
414 of the menstrual cycle (6, 50).
415
416 We conducted additional functional pathway analysis in a subset of miRNAs
417 demonstrating a trend towards differential expression in addition to miR-497-5p (miR-
418 224-3p, miR-331-5p and miR-497-5p). Similar functional pathways were identified in
419 this subset of miRNAs, which were comparable to miR-495-5p alone, including the insulin
420 signaling pathway and insulin receptor signaling cascade (**Error! Reference source not**
421 **found.**). This finding supports the notion that synergistic co-expression of miRNAs
422 regulates functionally related cohorts of genes involved in metabolism (51). These
423 functional pathway predictions suggest that differential co-expression of adipose tissue
424 miRNAs across the menstrual cycle may regulate variation in insulin sensitivity.
425 However, it must be considered that the adipose tissue transcriptome is highly insulin
426 sensitive and, without the use of a background gene list, functional pathway analysis
427 cannot determine which pathways are upregulated over and above the biological bias
428 already present within adipose tissue (52). Nonetheless, these findings suggest that
429 future research should further investigate the role of differential co-expression of subsets
430 of miRNAs across the menstrual cycle on insulin signaling pathways. Concurrent analysis
431 of mRNA-miRNA expression would offer valuable mechanistic insight on the impact of
432 miRNAs on the insulin signaling pathway across the menstrual cycle.
433
434 Cyclical fluctuations in metabolic control across the menstrual cycle are associated with
435 ovarian hormone concentrations (6, 50). 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, 53, 54). 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 women with polycystic ovary syndrome, whose testosterone levels are significantly greater, compared to healthy controls (55).

Adipose tissue is a complex tissue comprised of heterogenous cell populations (56, 57). Correspondingly, miRNA expression profiles are specific to individual cell populations within adipose tissue (58). 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 (57). 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 (59, 60).

Females are significantly under-represented in physiological research and therefore, our understanding of physiological changes across the menstrual cycle remains incomplete (61). 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 inter-individual 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 (62).

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 (Supplementary figure 2). 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 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, 63, 64). 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. Additionally, diverse miRNA expression profiles are observed in individuals with metabolic disorders when compared with healthy controls (65). 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. 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.

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Figures:

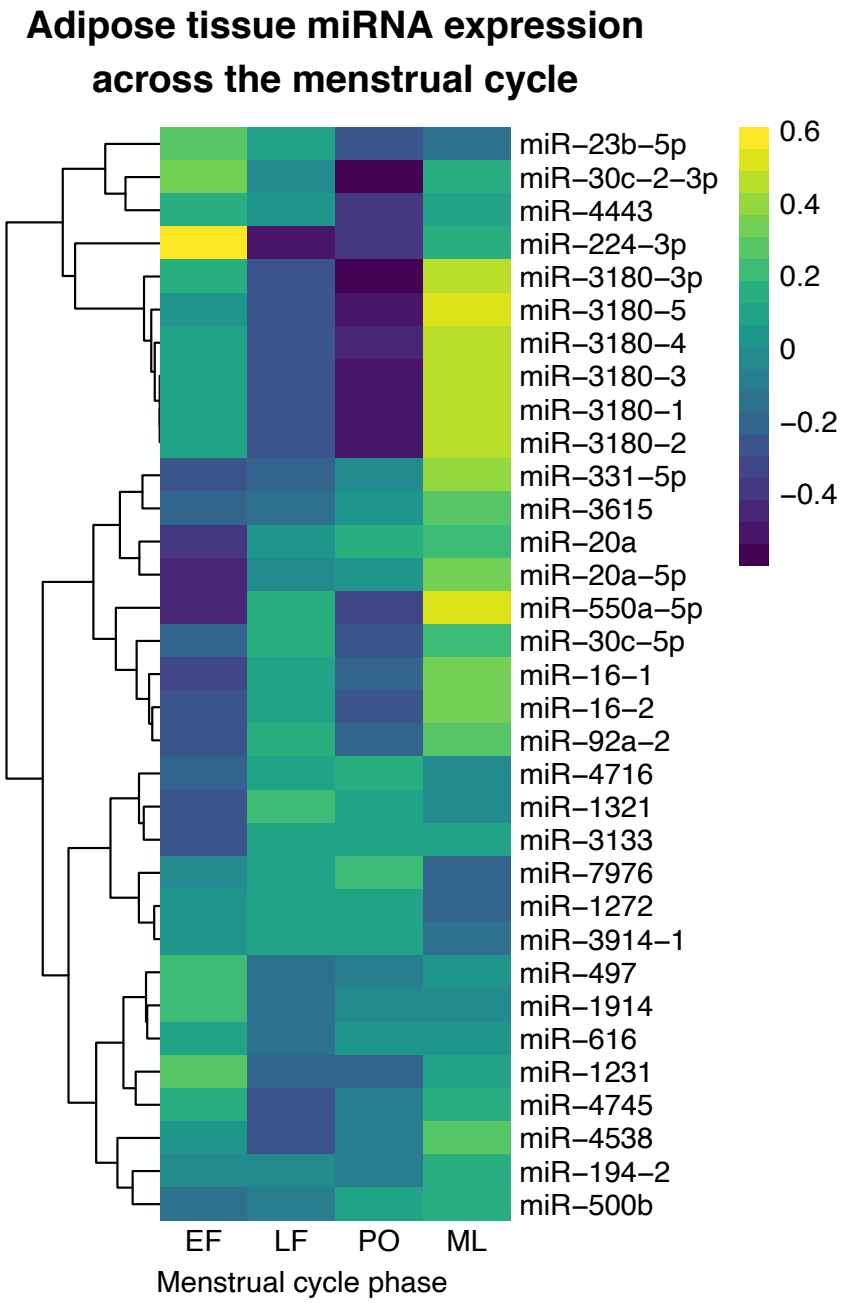
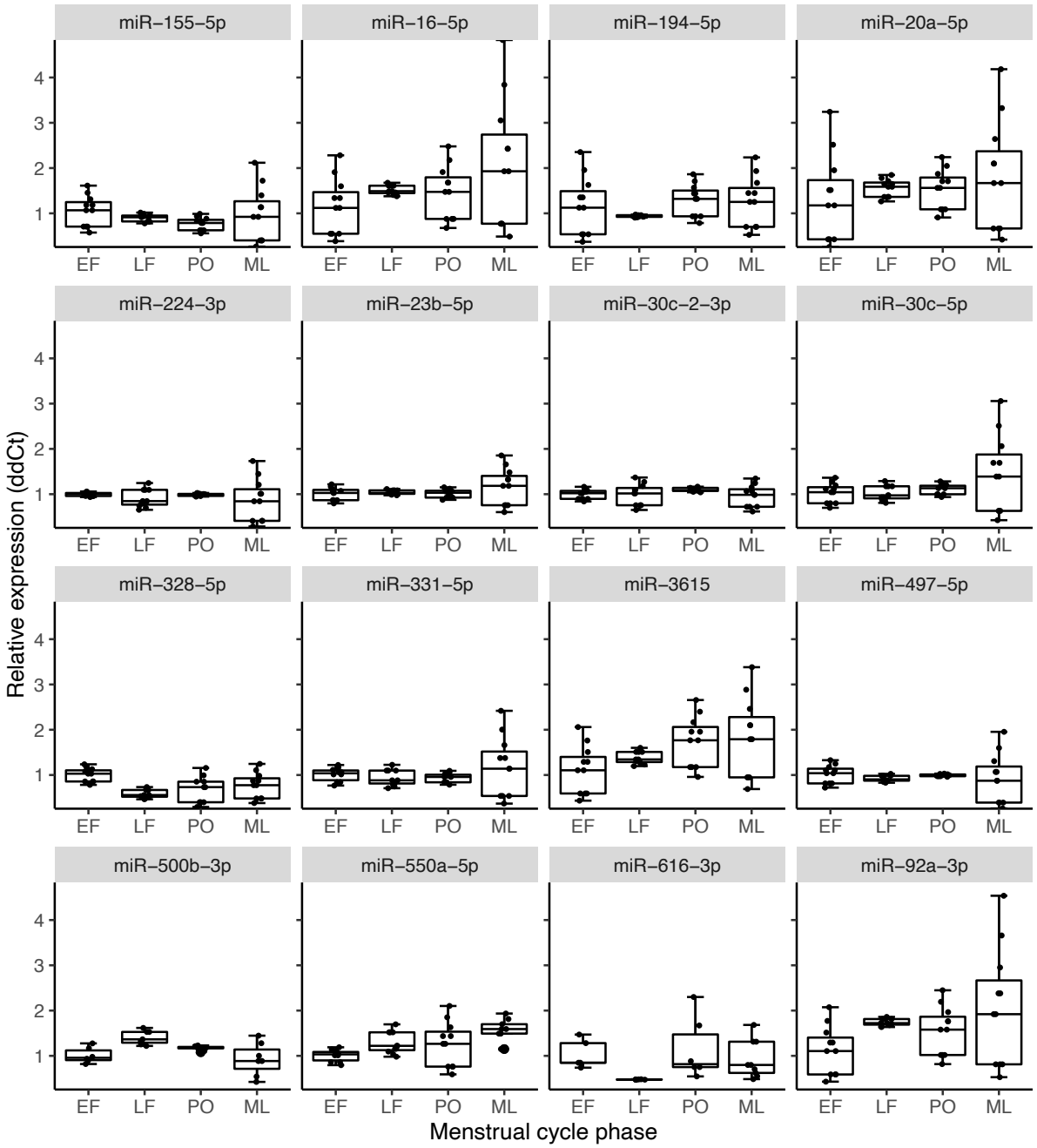
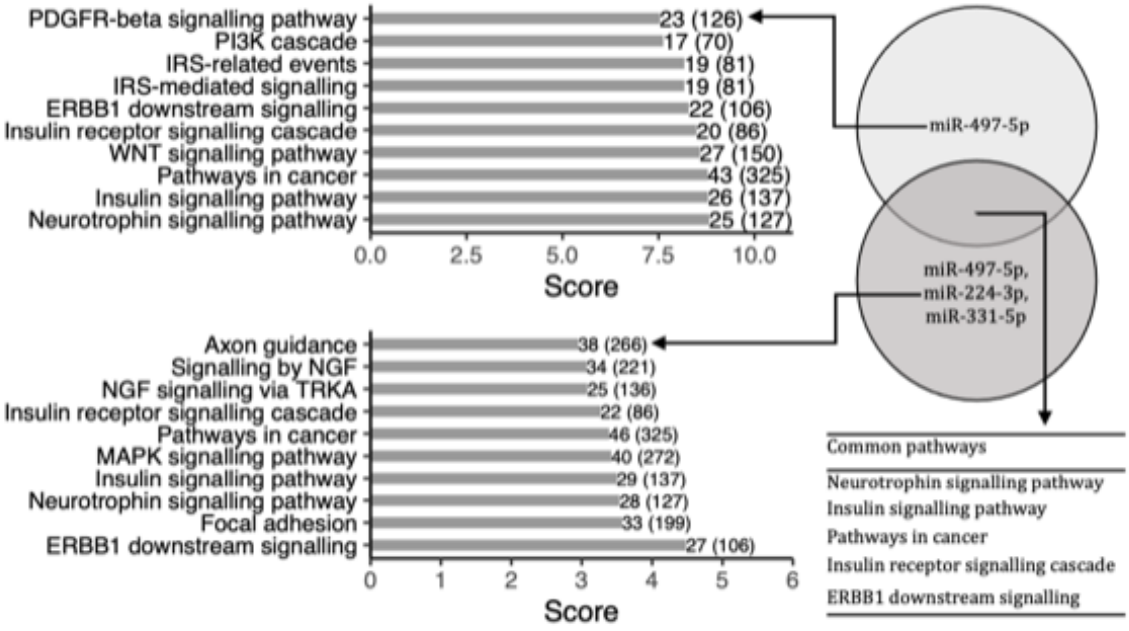


Figure 1: Clustered heatmap of microarray log10 z-scores in differentially expressed adipose tissue miRNAs in the early-follicular (EF), late-follicular (LF), post-ovulatory (PO) and mid-luteal (ML) phases of the menstrual cycle (N= 8). A p-value < 0.05 was considered statistically significant by moderated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



730 **Figure 2:** RT-qPCR analysis of adipose tissue miRNA expression in the early-follicular
731 (EF), late-follicular (LF), post-ovulatory (PO) and mid-luteal (ML) phases of the
732 menstrual cycle (N=11). miRNA expression represented as ddCt values adjusted for age.
733 Data are displayed as conventional box and whisker plots with individual data points
734 represented by circles.

735



736 **Figure 3:** Functional pathway analyses were conducted using RT-qPCR results for miR-
737 497-5p (top left) and miR-224-3p, miR-331-5p and 497-5p (bottom left) (N=11). Score is
738 generated by the weight of miRNA expression (ddCt) times its enrichment -log (p-value).
739 Numbers at the end of bars represent the total number of genes in the indicated term
740 with total union targets in term encased in brackets.

741