

1 Title:

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

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21 Running head:

22 Adipose tissue microRNA expression and menstrual cycle phase

23

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31

32 Abstract:

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34 Cyclical changes in hormone profiles across the menstrual cycle are associated with
35 alterations in metabolic control. MicroRNAs (miRNA) contribute to regulating metabolic
36 control, including adipose tissue metabolism. How fluctuations in hormonal profiles
37 across the menstrual cycle affect adipose tissue miRNA expression remain unknown.
38 Eleven healthy, regularly menstruating females underwent four sampling visits across
39 their menstrual cycle. Subcutaneous abdominal adipose tissue and venous blood samples
40 were collected at each sampling visit. Luteinizing hormone (LH) tests, calendar counting,
41 and serum hormone concentrations were used to determine menstrual cycle phases:
42 early-follicular (EF); late-follicular (LF); post-ovulatory (PO) and mid-luteal (ML). Serum
43 follicle-stimulating hormone, LH, estrogen, progesterone and testosterone were
44 determined using multiplex magnetic bead panels and enzyme-linked immunosorbent
45 assays. Global adipose tissue miRNA expression levels were determined via microarray
46 in a subset of participants (N=8) and 17 candidate miRNAs validated by RT-qPCR in the
47 whole cohort (N=11). Global analysis of adipose tissue miRNA expression identified 33
48 miRNAs significantly altered across the menstrual cycle; however, no significant
49 differences remained after correcting for multiple testing ($p > 0.05$). RT-qPCR analysis of
50 candidate miRNAs revealed miR-497-5p expression was significantly altered across the
51 menstrual cycle ($\eta_p^2 = 0.18$, $p = 0.03$); however, post-hoc tests did not reveal any significant

52 differences between menstrual cycle phases ($p > 0.05$). miR-30c-5p associated with
53 testosterone concentration ($R^2=0.13$, $p=0.033$). These pilot data indicate differences in
54 adipose tissue miRNAs in healthy women across the menstrual cycle and a weak
55 association with ovarian hormones. Further research in larger sample sizes is required
56 to confirm regulation of miRNA expression across the menstrual cycle.

57

58 Keywords:

59 Ovarian hormones, metabolism, hormone, estradiol, progesterone.

60

61 Introduction:

62

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

75

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

86

87 Adipose tissue is metabolically receptive to changes in the ovarian hormonal milieu.

88 Estrogen, testosterone and progesterone regulate a range of metabolic pathways within

89 adipocytes including lipolysis, lipogenesis and insulin sensitivity (11–15).

90 Correspondingly, acute and chronic alterations to ovarian hormone profiles, including

91 menstrual cycle phase, estrogen supplementation and menopausal status, are associated

92 with alterations in adipose tissue metabolism. These parameters include basal lipolysis,

93 stimulated lipolysis and insulin action (16–18). Thus, hormonal regulation of adipose

94 tissue metabolism across the menstrual cycle may underpin variation in parameters of

95 whole-body metabolic control; however, the associated molecular mechanisms remain

96 uncertain.

97

98 One potential mechanism for the regulation of adipose tissue metabolism is through

99 microRNA (miRNA) expression. miRNAs are short non-coding RNAs that regulate gene

100 expression at a post-transcriptional level through binding to the 3'UTR region of target

101 messenger RNA (mRNA) transcripts (19). Approximately 60% of known protein-coding

102 genes have target sites for miRNA binding, and thus miRNAs are recognized as major

103 regulators of mRNA translation (20). In adipose tissue, miRNAs target components of

104 pathways involved in metabolic control, including lipid transport, lipolysis, lipogenesis

105 and insulin signaling (21–23). Divergent adipose tissue miRNA profiles have been

106 observed in response to chronically altered ovarian hormone profiles, including

107 menopausal status (24), hormone supplementation (24), polycystic ovary syndrome

108 (25) and ovariectomy (26). A recent study reported upregulated adipose tissue

109 expression of miR-16-5p, miR-451a, miR-223-3p, miR-18a-5p, miR-19a-3p, miR-363-3p

110 and miR-486-5p between hormone replacement therapy users and non-users in post-

111 menopausal women (24). However, the few studies that have investigated miRNA
112 expression in response to acute hormone fluctuations across the menstrual cycle have
113 yielded equivocal results (27, 28). Moreover, previous reports are limited to analysis of
114 plasma miRNA expression across the menstrual cycle, which may not be representative
115 of adipose tissue miRNA expression (24, 27, 28). Investigating the effect of menstrual
116 cycle phase on adipose tissue miRNA expression will help unravel mechanisms involved
117 in metabolic control across the menstrual cycle.

118

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

125 Methods:

126

127 *Ethics:*

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

132

133 *Participants:*

134 A total of 11 healthy, pre-menopausal women aged 22-30 yr were recruited from the
135 University of Stirling and surrounding areas via poster and online advertising. Participant
136 characteristics are detailed in **Table 1**. The inclusion criteria were: aged 18-40 yr;
137 recreationally active; no previous diagnosis of a menstrual cycle disorder; no use of
138 hormone based medication within the previous three months; and, not pregnant within
139 the previous three months. Additionally, participants were excluded if they exhibited any
140 symptom of an irregular cycle within the previous three months: menstrual cycle length
141 <21 d or >38 d; abnormal menstrual bleeding; menstrual bleeding ≥ 7 d; abnormal
142 menstrual cramps; or, absence of a period.

143

144 *Experimental design:*

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

150

151 *Experimental protocol:*

152 *Pre- sampling visit:* Participants were provided with a menstrual cycle diary and urinary
153 LH tests (Digital Ovulation Test, Clearblue, Switzerland), to monitor one menstrual cycle
154 prior to the sampling visits. The menstrual cycle diary detailed; menstrual cycle duration,
155 menstrual bleeding duration, date of positive LH test and menstrual cycle symptoms.
156 Based on responses collected in the menstrual cycle diary, participants were excluded at
157 this stage if they reported any symptoms of an irregular cycle, as previously described. In
158 addition, participants were provided with a 48 h food diary to be completed on cycle days
159 3 and 4, which recorded the time, quantity and preparation method of all meals, snacks,
160 and drinks.

161

162 *Sampling visits:* Participants attended the laboratory for four identical sampling visits
163 during the following menstrual cycle phases; early-follicular (EF), late-follicular (LF),
164 post-ovulatory (PO) and mid-luteal (ML). Menstrual cycle phase was determined using
165 the calendar counting method in conjunction with urinary LH tests (Digital Ovulation
166 Test, Clearblue, Switzerland) as follows: EF - cycle day 1-5; LF - cycle day 9-11; PO -
167 positive LH test + 2 d; ML - positive LH test + 8-10 d, dependent on menstrual cycle length.
168 Menstrual cycle phase was retrospectively confirmed via serum hormone analysis. Prior
169 to each sampling visit participants underwent a 10-12 h fast and abstained from alcohol
170 and caffeine for 24 h. To control participants' dietary intake preceding each sampling
171 visit, participants were instructed to repeat food intake as recorded in the 48 h food diary
172 prior to each sampling visit. Participants were instructed to maintain habitual levels of
173 daily living and physical activity throughout the duration of the study, but to abstain from

174 physical exercise in the 48 h preceding each sampling visit. Participants attended the
175 laboratory between 07:00-10:00, with each subsequent sampling visit scheduled for a
176 time within 1 h of the initial sampling visit. Each sampling visit consisted of the following
177 protocol: 15 min rest in a semi-supine position upon arrival at the laboratory; collection
178 of a venous blood sample; and, an adipose tissue biopsy.

179

180 *Blood sampling and adipose tissue biopsies:* Venous blood samples were collected by a
181 trained phlebotomist into 6 ml serum vacutainers (Becton, Dickinson and Company, New
182 Jersey, US). Blood samples were allowed to clot for 60-120 min at room temperature and
183 then centrifuged at 2,500 g for 10 min at 4 °C for serum separation. Serum was aliquoted
184 into 1.5 ml microcentrifuge tubes and stored at -80 °C until analysis. Abdominal adipose
185 tissue biopsies were obtained 5-10 cm lateral of the umbilicus under local anesthesia (2
186 % lidocaine) using a mini-liposuction technique (29). Samples were cleaned of visible
187 contaminants (connective tissue, blood, vasculature) and washed in 0.9 % sodium
188 chloride saline over sterile gauze. Tissue was split into similarly sized pieces (~50-150
189 mg), placed into an RNA-free 1.5 ml microcentrifuge tube containing 5-10 x tissue volume
190 RNAlater (ThermoFisher, UK, cat no. AM7020), flash frozen in liquid nitrogen and stored
191 at -80 °C until analysis. All adipose tissue samples were processed and frozen within 3
192 min of sampling.

193

194 *Hormone analysis:*

195 Serum estrogen, testosterone, FSH and LH concentrations were determined using
196 magnetic bead panels (MilliporeSigma, Massachusetts, US, cat no. HPTP1MAG-66k,
197 PTP1MAG-66K-02) analyzed using the Luminex 200 instrument, according to the
198 manufacturer's protocol. Serum progesterone were determined using an Enzyme-linked

199 immunosorbent assay (ELISA) (R&D systems, Minnesota, US, cat no. NBP2-60124),
200 according to the manufacturer's protocol. The intra-assay coefficients of variation (CV)
201 were as follows: FSH= 6.4 %; LH= 8.69 %; estradiol= 12.0 %; progesterone= 8.3 %;
202 testosterone= 10.4 %. These intra-assay CVs are similar to the intra-assay coefficients of
203 variation reported in the manufacturers protocol (FSH < 10 %, LH < 10 %, estradiol < 15
204 %, progesterone < 15 %, testosterone < 10 %). All sample concentrations were above the
205 minimum limit of detection reported in the manufacturers protocol for each assay (FSH=
206 0.01 mIU/mL, LH= 0.01 mIU/mL, estradiol= 0.01, progesterone= 0.5 ng/mL, testosterone
207 0.08 ng/mL).

208

209 *RNA extraction:*

210 Adipose tissue was mechanically homogenized using 1.4 mm ceramic beads (Qiagen,
211 Germany, cat no. 13113-50) and the MagNA Lyser instrument (Roche, Switzerland) with
212 3 x 20 s bouts of homogenization interspersed with 20 s rest. Total RNA was isolated from
213 30-100 mg homogenized adipose tissue in a combined protocol using TRIzol reagent
214 (ThermoFisher Scientific, UK, cat no. 15596026) and the miRNeasy mini lipid kit (Qiagen,
215 Germany, cat no. 217004), as previously described (30). Final elution volume for isolated
216 RNA was 30 μ L. Concentration (μ g/mL) and purity (absorbance ratio 260/280 nm) of
217 isolated RNA samples were assessed using a DS-11 FX + spectrophotometer (DeNovix,
218 Delaware, US). RNA samples were stored at -80 °C until analysis.

219

220 *Global microRNA expression analysis:*

221 Global miRNA expression was examined using microarrays in a subset of participants at
222 all timepoints (N=32; 8 participants, 4 timepoints). Samples were selected from the 8
223 participants with the highest RNA quantity and purity across all timepoints

224 (Concentration: 25-92.6 $\mu\text{g}/\text{mL}$; 260/280: 1.9-2.1 nm). 100 ng RNA were labelled using
225 the Flashtag Biotin HSR labelling kit (ThermoFisher Scientific, UK, cat no. 901910).
226 Hybridization cocktails were prepared using the GeneChip hybridization kit
227 (ThermoFisher Scientific, UK, cat no. 900720). Biotin labelled samples were hybridized
228 overnight onto GeneChip miRNA 4.0 arrays (ThermoFisher Scientific, UK, cat no. 902413)
229 according to manufacturer's instructions. The arrays were washed and stained using the
230 GeneChip wash and stain kit (ThermoFisher, UK, cat no. 902413) and the Affymetrix
231 fluidics station 450, as per manufacturer's instructions. GeneChips were scanned by the
232 Affymetrix scanner 3000 7G. Microarray signal intensities were normalized using the
233 variance stabilization normalization 2 (VSN2) algorithm and probe level summarization
234 conducted using robust multi-chip analysis (RMA) (31). Microarray data has been
235 deposited to the Gene Expression Omnibus (GEO) with the accession number
236 GSE180625.

237

238 *RT-qPCR:*

239 A total of 17 candidate miRNAs were selected for validation using RT-qPCR in the whole
240 cohort (N= 44; 11 participants, 4 timepoints) from the subset of miRNAs differentially
241 expressed across the menstrual cycle, as determined via microarray. Candidate miRNAs
242 were selected based on association with hormones across menstrual cycle phases and
243 reported association with adipose tissue function. Analysis of microarray data via
244 NormFinder (32) reported miR-155-5p, miR-324-3p, miR-331-3p and miR-328-5p had
245 optimal stability values and were selected as potential endogenous qPCR control miRNAs
246 for RT-qPCR analysis (32). RT-qPCR was performed using miRCURY LNA custom PCR
247 panels with pre-coated miRNA primers (Qiagen, Germany, cat no. 339330), according to
248 the manufacturers protocol. Briefly, cDNA synthesis reactions comprised of 2 μL

249 miRCURY RT reaction buffer, 4.5 μ L RNase-free water, 1 μ L 10x miRCURY RT enzyme mix,
250 0.5 μ L UniSp6 RNA spike-in and 2 μ L template RNA (5 ng/ μ L) in a 10 μ L total reaction
251 volume. UniSp6 (Qiagen, UK, cat no. 339390) were added to cDNA synthesis reactions to
252 analyze cDNA synthesis efficiency. Reverse transcription temperature cycling was
253 performed using a LightCycler 480 (Roche, Switzerland) according to manufacturer
254 instructions. Conditions were 60 min at 42°C, 5 min at 95°C and then immediate cooling
255 to 4°C. cDNA samples were assayed immediately by qPCR or stored at -20°C until analysis.
256 qPCR reaction volume comprised of 5 μ L miRCURY LNA SYBR Green Master Mix (Qiagen,
257 Germany, cat no. 339345), 4 μ L cDNA template (diluted 1:80) and 1 μ L RNase-free water,
258 in a total reaction volume of 10 μ L. UniSp3 were included in miRCURY LNA custom PCR
259 panels to perform inter-plate calibration. miRNA target sequences are supplied in
260 supplementary table 1 (33). qPCR temperature cycling was performed using a
261 LightCycler 480 (Roche, Switzerland) according to manufacturers instructions,
262 consisting of 95°C for 2 min, followed by 45 cycles at 95°C for 10 s then 56°C for 60 s and
263 finished with melting curve analysis. All qPCR reactions were performed in triplicate and
264 analyzed using the $2^{-\Delta\Delta C_t}$ method (34). Analysis of RT-qPCR data using NormFinder
265 identified miR-324-3p and miR-331-3p to be the most stable pair of miRNAs from the
266 four potential endogenous controls (miR-155-5p, miR-324-3p, miR-331-3p and miR-
267 328-5p)(32). Raw Delta Ct (dCt) values were calculated relative to the geometric mean of
268 the endogenous controls (miR-324-3p, miR-331-3p) and DeltaDelta Ct (ddCt) values
269 calculated relative to the EF phase.

270

271 *miRNA-mRNA pathway analysis:*

272 miRsystem was used to conduct miRNA-mRNA pathway analysis using RT-qPCR results
273 for miRNA expression (35). miRsystem integrates seven miRNA target gene prediction

274 databases (DIANA, miRanda, miRBridge, PicTar, PITA, rna22 and TargetScan) to enable
275 prediction of target genes and functional pathways.

276

277 *Statistical analysis:*

278 A priori power calculations were conducted in G*Power 3.1 (36). miRNA expression was
279 not included in power calculations due to a lack of suitable published data reporting
280 miRNA expression across similar menstrual cycle phases. Power calculations were
281 conducted using reported mean and standard deviation of estradiol and progesterone
282 concentrations at EF, LF, PO and ML (36–38) to ensure appropriate statistical power to
283 differentiate between menstrual cycle phases. Testosterone was omitted from power
284 calculations due to relatively small changes between menstrual cycle phases. G*Power
285 reported $n = 11$ was required to achieve 80% power ($\alpha = 0.05$) to detect changes in
286 estrogen and progesterone between menstrual cycle phases.

287

288 All statistical analyses were conducted using R version 3.6.3 (39). Hormone data were log
289 transformed for normality and this was confirmed using the Shapiro-Wilk test. One-way
290 repeated measures ANOVA were used to examine the effect of menstrual cycle phase on
291 hormone concentration. Pairwise comparisons were conducted with Tukey adjustment.
292 To determine differential microarray expression across the menstrual cycle, moderated
293 empirical Bayes ANOVA was conducted and corrected for multiple testing using the false
294 discovery rate (FDR) method, in the 'limma' package (40). Linear mixed effect models
295 were conducted to examine the relationship between menstrual cycle phase and ovarian
296 hormone concentration with candidate miRNA expression using RT-qPCR data, in the
297 'lme4' package in R (41). A participant identifier was included in models as a random
298 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 were 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 $p <$

324 0.001) and higher during PO compared to EF and LF (p= 0.006; p= 0.003). Testosterone
325 was significantly greater during PO compared to EF (P= 0.024).

326

327 *Global miRNA expression:*

328 We analyzed global miRNA expression across the menstrual cycle in a subset of
329 participants (N=32; 8 participants, 4 timepoints). Three arrays did not pass quality
330 control (PO: n= 2, ML: n= 1) and therefore final analysis was conducted on 29 samples.
331 729 miRNAs were considered significantly expressed in adipose tissue samples at a
332 median expression level ≥ 1.75 . Differential expression across the menstrual cycle was
333 observed in 33/729 miRNAs prior to correction for multiple testing (**Figure 1,**
334 **Supplementary table 2** (33)). No significant differences were observed following FDR
335 correction for multiple testing (**Figure 1, Supplementary table 2** (33)).

336

337

338 *Candidate RT-qPCR miRNA expression:*

339 A total of 19 miRNAs were selected for validation using RT-qPCR (17 candidate miRNAs,
340 2 unused potential endogenous control miRNAs) in the whole cohort (N=44; 11
341 participants, 4 timepoints). 3 miRNAs (miR-1231, miR-1914, miR-3180-3p) were not
342 detected consistently in all participants and were removed from the analysis. **Figure 2**
343 shows mean log fold change in expression level for each of the remaining 16 candidate
344 miRNAs relative to endogenous controls and expression level in EF. miR-497-5p
345 expression was significantly altered across the menstrual cycle ($p= 0.030$, $R^2= 0.18$);
346 however, post-hoc testing did not reveal any significant differences between menstrual
347 cycle phases ($p> 0.05$). There was a tendency for altered expression of miR-224-3p ($p=$
348 0.059 , $R^2= 0.07$) and miR-331-5p ($p= 0.059$, $R^2= 0.11$) across the menstrual cycle,
349 although they did not reach statistical significance.

350

351 *Association between RT-qPCR miRNA expression and ovarian hormones:*

352 Linear mixed effect models were conducted to assess the relationship between hormone
353 concentration and miRNA expression, as determined using RT-qPCR (**Table 3**). miR-30c-
354 5p was significantly, negatively associated with serum testosterone ($p= 0.033$, $R^2=0.13$).
355 miR-92a-3p had a tendency for a negative association with serum estradiol ($p= 0.067$,
356 $R^2= 0.14$).

357

358 *miRNA-mRNA pathway analysis:*

359 miRNA-mRNA pathway analysis was conducted using RT-qPCR results. miRNA-mRNA
360 pathway analysis was performed with miR-497-5p due to significant variation in
361 expression across the menstrual cycle. Additionally, due to the tendency for alteration

362 across the menstrual cycle in miR-224-3p and miR-331-5p, miRNA-mRNA pathway
363 analysis was also performed for this subset of miRNAs (miR-224-3p and miR-331-5p,
364 miR-497-5p). Data are presented **Figure 3, Supplementary table 5 & 6 (33)**.

365

366 Discussion:

367

368 To the best of our knowledge, this is the first study to characterize adipose tissue miRNA
369 expression across the menstrual cycle. Initially, we conducted an exploratory approach
370 utilizing microarrays to analyze global miRNA expression in a subset of 8 participants
371 selected from the whole cohort. Based on examination of microarray data distributions
372 we identified 729 miRNAs expressed in adipose tissue samples in healthy, regularly
373 menstruating women. Expression levels of 33 miRNAs were significantly altered across
374 the menstrual cycle, although no significant differences remained after correction for
375 multiple testing using FDR. Validation via RT-qPCR was conducted on 16 candidate
376 miRNAs in the whole cohort. RT-qPCR analysis determined miR-497-5p was significantly
377 altered across the menstrual cycle, whilst miR-224-3p and miR-331-5p exhibited a trend
378 towards differential expression. In addition, we examined the relationship between
379 miRNA expression and circulating hormone concentrations. Testosterone was
380 significantly associated with miR-30c-5p expression, predicting up to 13 % of the
381 variation in expression. We also observed a trend for a negative association between miR-
382 92a-3p and estradiol, predicting up to 14 % of the variation in miRNA expression. These
383 pilot data suggest adipose tissue miR-497-5p expression may be altered by menstrual
384 cycle phase and adipose tissue miRNAs exhibit weak associations with ovarian hormones.

385

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 healthy
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

436 on adipocyte metabolism, we examined the relationship between serum estradiol,
437 progesterone and testosterone with adipose tissue miRNA expression (14, 53, 54). We
438 observed a significant negative association between testosterone and miR-30c-5p,
439 explaining up to 13 % of the variation in miRNA expression level. This is concordant with
440 previous research demonstrating lower plasma miR-30c-5p expression in women with
441 polycystic ovary syndrome, whose testosterone levels are significantly greater, compared
442 to healthy controls (55).

443

444 Adipose tissue is a complex tissue comprised of heterogenous cell populations (56, 57).
445 Correspondingly, miRNA expression profiles are specific to individual cell populations
446 within adipose tissue (58). Thus, detected miRNA signals in this study are reflective of
447 the combined miRNA expression profiles within both mature adipocytes and the
448 stromovascular fraction, including pre-adipocytes, mesenchymal stem cells, endothelial
449 cells and macrophages (57). Additionally, hormonal influences on miRNA expression may
450 be exerted in opposing directions in differing cellular fractions within adipose tissue. This
451 may induce a 'counterbalancing effect' on determined miRNA signal from whole adipose
452 tissue. Therefore, future studies should consider isolating a single adipose tissue cell
453 population (i.e., mature adipocytes) prior to miRNA analysis to reduce variability in
454 expression and gain further mechanistic insight. Additionally, three candidate miRNAs
455 (miR-1231, miR-1914, miR-3180-3p) were detected at low levels via RT-qPCR and were
456 thus removed from statistical analysis. Future studies could consider performing an RNA-
457 Seq study of sufficient sequencing depth or including a cDNA pre-amplification step prior
458 to qPCR analysis of adipose tissue miR-1231, miR-1914 and miR-3180-3p to facilitate the
459 detection of these lowly expressed miRNAs (59, 60).

460

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

470

471 This study was conducted with 11 participants and high inter-individual variability of
472 miRNA expression and ovarian hormone concentration was observed. Analysis of miRNA
473 expression across the menstrual cycle obtained multiple p-values close to the significance
474 threshold, alongside small-to-moderate effect sizes in 12 out of 16 measured miRNAs. We
475 did not perform post-hoc power calculations as this technique is considered analytically
476 misleading (62).

477

478 We conducted power analysis to calculate sample size estimates using effect sizes derived
479 from RT-qPCR data in the 5 miRNAs with the lowest significance levels; miR-497-5p, miR-
480 224-3p, miR-331-5p, miR-30c-5p and miR-32 8-5p. We generated power curves for each
481 of the miRNA under consideration by simulation. Estimates of power from these
482 simulations were accompanied by 95% CI's. The results presented below are those for
483 which the lower limit of that 95% CI was greater than 80% power at $\alpha = 0.05$ to detect
484 differential miRNA expression between menstrual cycle phases (Supplementary figure
485 2). Estimated sample sizes are as follows: miR-497-5p, N=90; miR-224-3p, N= 110; miR-

486 331-5p, N= 690; miR-30c-5p, N=150; miR-328-5p, N=30. These sample size estimates
487 provide an important methodological consideration for future studies investigating
488 changes in adipose tissue miRNA expression across the menstrual cycle with defined
489 outcomes. Additionally, it must be noted that ethnicity was not recorded in this study.
490 Ethnicity may increase inter-individual variation in adipose tissue miRNA expression; we
491 acknowledge that this may be a limitation. Future studies should endeavour to record
492 participants' ethnicity.

493

494 This study recruited young, healthy, regularly menstruating women and as such findings
495 must be extrapolated to a broader population with caution. Elevated adiposity and
496 insulin resistance are associated with greater perturbations in ovarian hormone
497 concentrations and metabolic control across the menstrual cycle (5, 6, 63, 64).
498 Accordingly, molecular mechanisms underlying metabolic control, such as miRNA
499 expression, may undergo relatively less variation across the menstrual cycle in healthy
500 women compared with populations with obesity or metabolic disorders. Additionally,
501 diverse miRNA expression profiles are observed in individuals with metabolic disorders
502 when compared with healthy controls (65). Therefore, future research should consider
503 investigating changes in miRNA expression across the menstrual cycle in populations
504 with impaired metabolic control.

505

506 In conclusion, we present novel pilot data characterizing miRNA expression across the
507 menstrual cycle and associations with ovarian hormone concentrations. We report
508 significant variation in miR-497-5p across the menstrual cycle alongside small-to-large
509 effect sizes in 12 out of 16 assessed miRNAs. Furthermore, miRNAs with the strongest
510 tendency for differential expression across the menstrual cycle shared common targets

511 related to insulin sensitivity pathways. However future studies should explore miRNA
512 expression across the menstrual cycle in larger sample sizes. Future studies would also
513 benefit from concurrent analysis of miRNA and mRNA expression within insulin signaling
514 pathways to provide further mechanistic insight. Additionally, these findings provide
515 direction for future research to examine changes in miRNA expression across the
516 menstrual cycle in individuals with impaired metabolic control, such as type 2 diabetes.
517 These studies are necessary to fully elucidate the role of varying hormonal milieu across
518 the menstrual cycle on miRNA expression and associated effects on metabolic control.

519

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522

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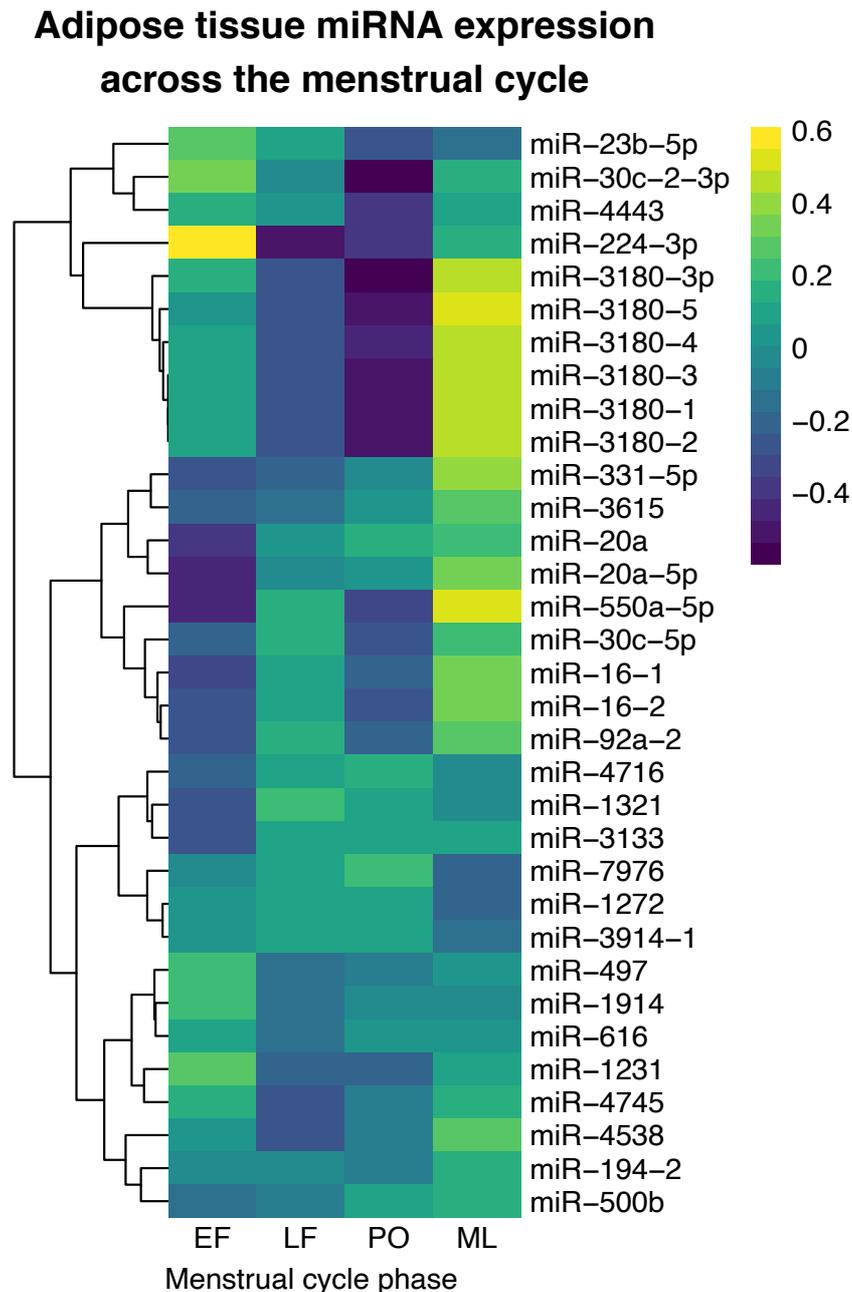
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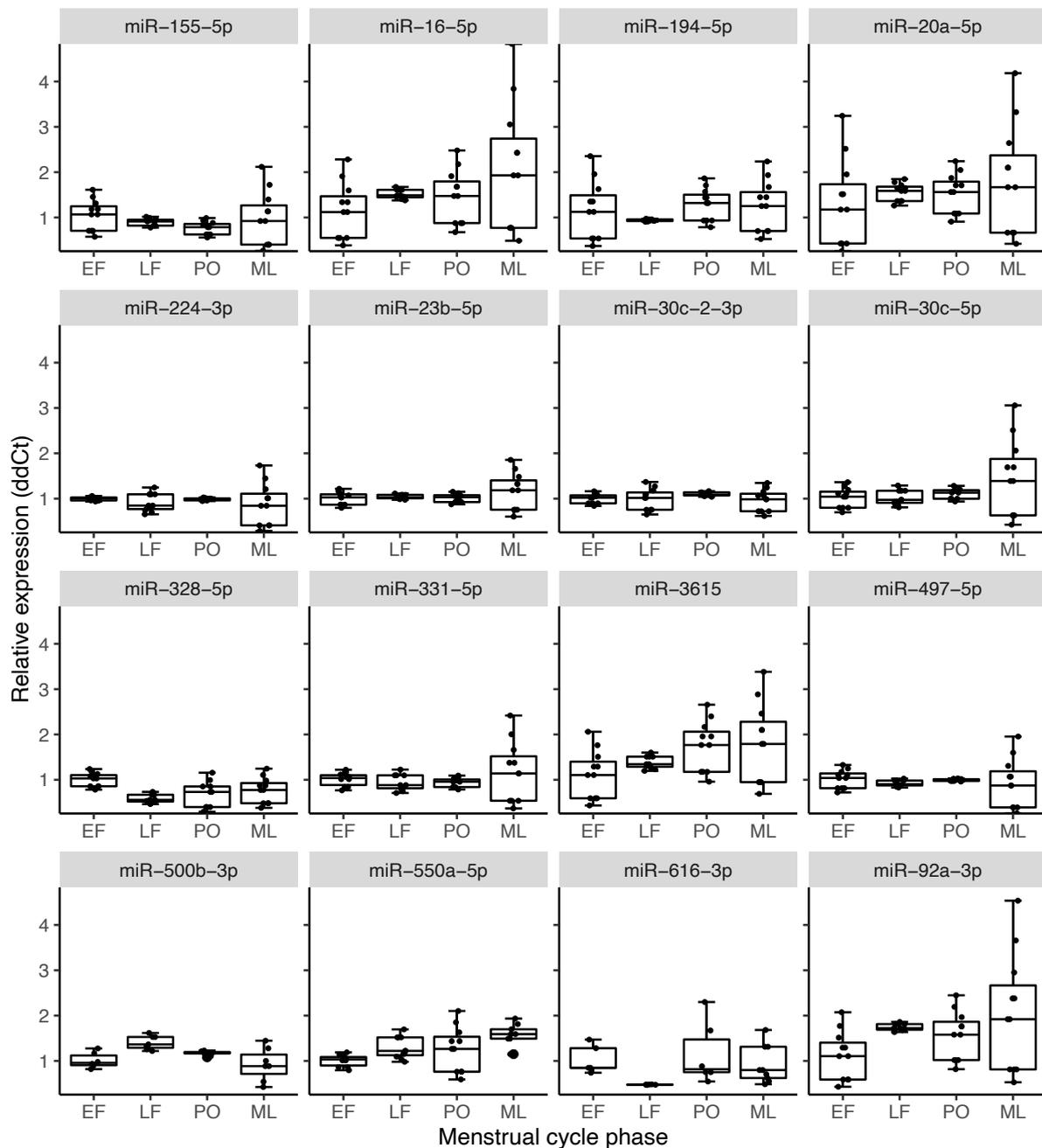
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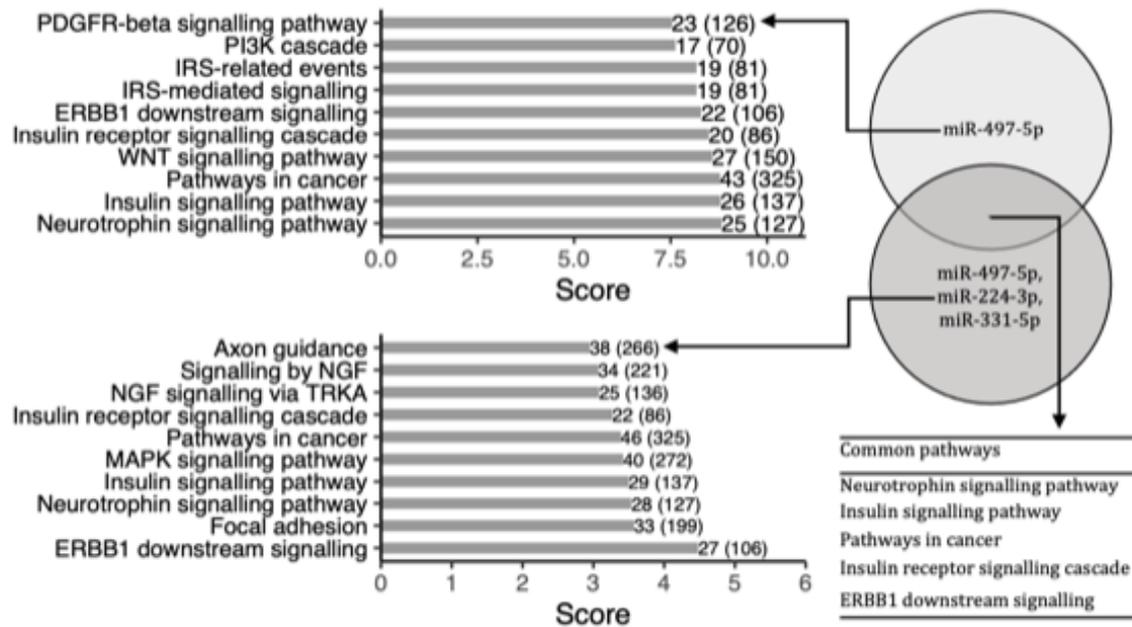
725 Figures:

726 **Figure 1:** Clustered heatmap of microarray log₁₀ z-scores in differentially expressed
 727 adipose tissue miRNAs in the early-follicular (EF), late-follicular (LF), post-ovulatory
 728 (PO) and mid-luteal (ML) phases of the menstrual cycle (N= 8). A p-value < 0.05 was
 729 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 $-\log(p\text{-value})$.
 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