Human Reproduction, Vol.38, No.1, pp. 46–56, 2023
Advance Access Publication on November 9, 2022 https://doi.org/10.1093/humrep/deac243

human reproduction

ORIGINAL ARTICLE *Reproductive biology*

The effect of the menstrual cycle on the circulating microRNA pool in human plasma: a pilot study

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Submitted on July 12, 2022; resubmitted on October 10, 2022; editorial decision on October 21, 2022

STUDY QUESTION: : Do ovarian hormone changes influence the levels of cell-free or circulating microRNA (cf-miRNA) across the menstrual cycle?

SUMMARY ANSWER: This exploratory study suggests that fluctuations in hormonal levels throughout the menstrual cycle may alter cf-miRNAs levels.

WHAT IS KNOWN ALREADY: cf-miRNA levels vary with numerous pathological and physiological conditions in both males and females and are regulated by exogenous and endogenous factors, including hormones.

STUDY DESIGN, SIZE, DURATION: A prospective, monocentric study was conducted between March and November 2021. Since this was a pilot study, the sample size was based on feasibility as well as previous similar human studies conducted in different tissues. A total of 20 participants were recruited for the study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: We conducted an exploratory study where blood samples were collected from 16 eumenorrheic females in the early follicular phase, the ovulation phase and the mid-luteal phase of the menstrual cycle. The levels of oestrogen, progesterone, LH and FSH were measured in serum by electrochemiluminescence. The levels of 174 plasma-enriched miRNAs were profiled using a PCR-based panel, including stringent internal and external controls to account for the potential differences in RNA extraction and reverse-transcription stemming from low-RNA input samples.

MAIN RESULTS AND THE ROLE OF CHANCE: This exploratory study suggests that cf-miRNAs may play an active role in the regulation of the female cycle by mediating the expression of genes during fluctuating hormonal changes. Linear mixed-models, adjusted for the relevant variables, showed associations between phases of the menstrual cycle, ovarian hormones and plasma cf-miRNA levels. Validated gene targets of the cf-miRNAs varying with the menstrual cycle were enriched within female reproductive tissues and are primarily involved in cell proliferation and apoptosis.

LARGE SCALE DATA: All relevant data are available from the Mendeley database: LEGER, Bertrand (2022), 'MiRNA and menstrual cycle', Mendeley Data, V1, doi: 10.17632/2br3zp79m3.1.

LIMITATIONS, REASONS FOR CAUTION: Our study was conducted on a small participant cohort. However, it was tightly controlled for endogenous and exogenous confounders, which is critical to ensure robust and reproducible cf-miRNA research. Both adjusted and non-adjusted *P*-values are presented throughout the article.

WIDER IMPLICATIONS OF THE FINDINGS: Measures of ovarian hormones should be rigorously included in future studies assessing cf-miRNA levels in females and used as time-varying confounders. Our results reinforce the importance of accounting for female-specific biological processes in physiology research by implementing practical or statistical mitigation strategies during data collection and analysis.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by the Clinique romande de réadaptation, Sion, Switzerland. S.L. was supported by an Australian Research Council (ARC) Future Fellowship (FT10100278). D.H. was supported by an Executive Dean's Postdoctoral Research Fellowship from Deakin University. The authors declare no competing interests.

Key words: menstrual cycle / gender / circulating microRNA / ovarian hormones / female-specific biological processes

Introduction

MicroRNAs (miRNAs) are short, non-coding RNA molecules encoded in the introns and exons (Rodriguez *et al.*, 2004) of the nuclear genome (Ha and Kim, 2014) and produced in most human tissues (Ludwig *et al.*, 2016). miRNAs are major regulators of gene and protein expression by their direct or indirect influence on the translation process (Vasudevan *et al.*, 2007; Guo *et al.*, 2010; McGeary *et al.*, 2019), primarily through the specific repression of target mRNA molecules (Guo *et al.*, 2010).

Most physiological fluids including serum, plasma, urine, saliva and breast-milk contain a fraction of the miRNA pool (Weber *et al.*, 2010). These extracellular miRNAs are referred to as cell-free or 'circulating' miRNAs (cf-miRNAs). cf-miRNAs are passively or selectively secreted by cells (Arroyo *et al.*, 2011) into bodily fluids (Weber *et al.*, 2010) and may contribute to cross-tissue communication by exerting their effects on recipient cells (Guescini *et al.*, 2015; Thomou *et al.*, 2017). Although their biological role is not fully elucidated, many pathophysiological conditions, including a number of cancers (Lu *et al.*, 2005), are associated with a dysregulation of the cf-miRNA profile in serum or plasma. Serum and plasma cf-miRNAs present the advantage of being relatively easy to access through venepuncture, making them potentially valuable biological markers (Hiam and Lamon, 2020).

Research into the circulating forms of miRNAs has rapidly evolved over the last decade and methodological approaches have become more stringent (Hiam and Lamon, 2020). In an attempt to reduce participant variability, most studies have focused on male only-cohorts (Nowogrodzki, 2017; Garcia-Sifuentes and Maney, 2021). There is however a global call to include sex as a biological variable in fundamental and applied research studies to broaden their range of applicability (Nowogrodzki, 2017; Bhargava *et al.*, 2021; Garcia-Sifuentes and Maney, 2021). Increasing evidence suggests that the regulation of miRNA expression is sex-specific (Song *et al.*, 2009; Shen *et al.*, 2019) and that males and females display different miRNA profiles in a variety of tissues and disease states (Guo *et al.*, 2017; Shen *et al.*, 2019), including in plasma (Murri *et al.*, 2018). Our group recently suggested that, in parallel to male-female comparisons, researchers should also investigate the effect of female sex hormone fluctuations due to the menstrual cycle and/or oral contraception on their physiological outcome of interest (Knowles *et al.*, 2019; Lamon and Knowles, 2021). This holds true for molecular outcomes, including miRNAs, whose expression levels in reproductive tissues fluctuate throughout the menstrual cycle (Vilella *et al.*, 2015).

Evidence around the effect of female sex hormones on the plasma cf-miRNA profile is limited. In females with polycystic ovary syndrome, presenting with higher levels of androgen hormones and lower levels of oestradiol when compared with healthy controls, the levels of 15 cf-miRNAs were closer to that of the average male than to the average healthy female levels (Murri *et al.*, 2018). This dysregulation suggests a pattern of androgenisation associated with sex steroid hormones (Murri *et al.*, 2018) and warrants the investigation of the effect of natural female hormone fluctuations on the cf-miRNA profile. An early human study investigating cf-miRNA levels at four time points across the menstrual cycle (Rekker *et al.*, 2013) suggested that the overall cf-miRNA profile remains stable throughout the cycle. However, no individual data or analysis were made available. In addition, this study failed to control for differences in RNA extraction and reverse-transcription, which considerably increase between-sample variability (Hiam and Lamon, 2020). In contrast, a study conducted in larger mammals (Holstein-Friesian heifers) identified a series of cf-miRNAs whose levels varied during the bovine oestrous cycle (Ioannidis and Donadeu, 2016).

There is an increasing body of research surrounding the role and regulation of cf-miRNAs (Hiam and Lamon, 2020) and their potential to be used as non-invasive, cheap biological markers for a number of disorders including cancers (Lu *et al.*, 2005), cardiovascular disease (Wronska *et al.*, 2015) and liver disease (Szabo and Bala, 2013). This should prompt researchers to broaden the field of applicability of their findings by understanding the effect of the female hormonal environment. This study aims at taking the first step by conducting a stringent, tightly controlled, high-throughput pilot analysis of the plasma cf-miRNA profile across the female menstrual cycle.

Materials and methods

Study population

There were 20 eumenorrheic biological females (defined as a person born with a 46,XX karyotype) recruited among healthcare providers of a rehabilitation clinic in Switzerland. Females aged 18–50 and having had a regular menstrual cycle during the last 18 months were invited to take part in the study. Exclusion criteria included: hormonal contraception (including oral contraceptives, implant and hormonal IUD); pregnancy or attempting to get pregnant; breastfeeding; gynaecological conditions; and/or a history of hepatitis B, C or D or HIV infection. General health characteristics, menstrual anamnesis, use of medication and health events occurring during the menstrual cycle (e.g. menstrual pain, abnormal uterine bleeding or headache) were recorded. Four participants were excluded from the study: two females started hormonal contraception during the sample collection period, and the hormones levels of two other females could not be matched with a specific phase of the menstrual cycle (Elliott-Sale *et al.*, 2021). Therefore, 16 women were included in the final analysis. The study protocol was approved by the local ethics committee (CER-VD 2018-01914) and conducted according to the recommendations of the *Declaration of Helsinki* (World Medical Association, 2004) and its later amendments. Informed written consent was collected from each participant.

Procedures

All participants reported having a regular cycle for the 18 months prior to entering the study, and had monitored their menstrual cycle using their mobile phone app of choice for at least six months to ascertain the regularity of their menstrual cycle. Blood sampling was scheduled at three time-points corresponding to the early follicular phase (T1, ideally defined as the second day of the menstruation), the ovulation phase (T2, ideally defined as one day past the LH surge) and the mid-luteal phase (T3, ideally defined as seven days past T2). Supplementary Figure S1 depicts the outline of the study. Immunochromatographic, urine-based LH detection tests (Livsane LH20M, Pharmapost AG, Switzerland) were used to identify the LH peak according to the manufacturer's instructions. Menstrual cycle phase identification was conducted according to the recommendations from Elliott-Sale *et al.* (2021) modified to account for the ovulation test result and the surge in LH and FSH (Supplementary Table SI). When a resolution could not be met, data were compared to three different clinical reference ranges for oestradiol and progesterone according to Elliott-Sale *et al.* (2021). Data that did not comply with this framework were not considered, resulting in the exclusion of two individual data points.

Blood collection

Peripheral blood samples were collected from the antecubital vein after an overnight fast in 2 × 7.5 ml EDTA and 1 × 7.5 ml Serum-Gel tubes (S-Monovette®, Sarstedt International, Numbrecht, Germany) using a butterfly device. The tubes were immediately centrifuged at 3000 *g* at 4°C for 10 min. Plasma and serum were aliquoted in 1.5 ml RNase-free and DNase-free tubes and stored at −80°C until further processing. Samples with visible signs of haemolysis were excluded from the analysis.

Hormone analysis

Serum levels of ovarian hormones oestrogen, progesterone, LH and FSH were measured by electrochemiluminescence using an Elecsys & Cobas system (Roche, Rotkreuz, Switzerland) at the Clinical Chemistry and Toxicology Department of the Central Institute of the Hospital of Valais (ICHV), Sion, Switzerland.

RNA isolation and cDNA synthesis

Prior to RNA isolation, plasma samples were centrifuged for 5 min at 3000 *g*. Then, 300 µl of supernatant was transferred into a new tube for RNA extraction using the miRNeasy® Serum/plasma advanced kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions. To control for the reproducibility of the extraction, 3, 3×10^{-2} and 3×10^{-5} fmol of UniSp2, UniSp4 and UniSp5 (Qiagen), respectively, were added to the lysis buffer of each sample. The total RNA fraction was eluted in 20 µl RNase-free water. Of the total RNA, 2 µl was used to synthetize cDNA using the miRCURY LNA RT kit (Qiagen) according to the manufacturer's protocol. 2×10^{-3} fmol of the synthetic miRNA cel-miR-39-3p (Qiagen) and 0.15 fmol of the spike-in UniSp6 (Qiagen) were added to the cDNA to control for the reproducibility of the cDNA synthesis.

miRNA profiling

Plasma miRNA analysis was performed using the Human serum/plasma Focus, miRCURY LNA miRNA Focus PCR panel (# 339325) (Qiagen). This sensitive and specific PCR-based method measures the levels of 174 unique serum-enriched miRNAs and also includes 10 internal and external quality controls. These miRNAs have been carefully selected based on their levels in plasma samples reported by previous studies and manufacturer recommendations. Amplification was performed with the LightCycler 480 Real-Time PCR system (Roche) as previously described (Dayer *et al.*, 2019).

Data quality assessment

After the initial visual inspection, haemolysis was assessed using the difference in cycle quantification value (Cq) between hsa-miR-23a-3p, which is unaffected by haemolysis, and hsa-miR-451a, which is highly expressed in red blood cells (Blondal *et al.*, 2013). Based on results from the literature, a difference smaller than 7 Cq was considered acceptable (Blondal *et al.*, 2013). According to this criteria, two samples displayed faint signs of haemolysis and were excluded from the analysis.

As recommended by the Biofluids Guidelines from Qiagen and described previously (Nielsen *et al.*, 2014), a pool of exogenous spike-in controls, UniSp2, UniSp4 and UniSp5, and another pool of exogenous spike-in controls, UniSp6 and cel-miR-39-3p, were added prior to the RNA isolation and cDNA synthesis steps, respectively. Six replicates of UniSp3, an exogenous quality control for PCR amplification, were included in the pre-coated PCR panel and UniSp3 was used as an inter-plate calibrator (see data processing below). The between-sample coefficients of variations for UniSp2 (4.44%), UniSp4 (3.45%), UniSp5 (3.02%) and UniSp6 (2.06%) were considered excellent.

Data processing and normalization

Cq values were calculated by applying the second derivative method and transforming into arbitrary units using the formula (2^Ct) × (10^10). Melting curves were examined automatically using the LightCycler 480 software version 1.5.0, and individual data points were manually excluded if a double peak or a 'shoulder' was visible, leading to the exclusion of 181 data points (1.97%). Of the 174 miRNA datasets, 6 datasets presented an average Cq value >35 and were excluded from further analysis. Following this, all individual Cq values >35 remaining in the dataset were excluded from further analysis, leading to the exclusion of an additional 189 data points (2.29%). Of the remaining 168 miRNA datasets, 11 (6.45%) missed more than 20% of their individual data points and were excluded on this basis. For the remaining 157 miRNAs, the arithmetic mean of the transformed Cq values (arbitrary units) of the six endogenous UniSp3 quality controls was calculated for each plate, allocated a random value of 1, and used to account for inter-plate variability. Finally, the geometric mean of the transformed Cq values (arbitrary units) of all miRNAs considered for the final stage of the analysis was used for global normalization (D'Haene *et al.*, 2012).

Statistical analysis

All data were analysed using R studio 4.1.3 (R Core Team, 2021). Missing data were imputed using the mice package (van Buuren and

Groothuis-Oudshoorn, 2011) and iteration three was randomly selected for all analyses. To meet the statistical assumptions of the regression (normally distributed residuals), ovarian hormone levels (oestrogen, progesterone, LH and FSH) were log-transformed. To identify changes in cf-miRNA levels across the menstrual cycle, we used linear mixed models for repeated measures as implemented in the variancePartition package in R (Hoffman and Roussos, 2021). Participant ID was used as the random effect to account for repeated measures and all models were adjusted for age. The model was of the form: $miRNA \sim Timepoint + Age + (1|ID)$. The model was then fitted with contrasts to examine changes in miRNA levels between the different phases of the menstrual cycle. We then added ovarian hormone (oestrogen, progesterone, LH and FSH) concentrations into the model to understand whether hormones explained any of the variability in cf-miRNA levels. Due to co-linearity, models of the form: $miRNA \sim Hormone + Timepoint + Age + (1|ID)$ were run separately for each hormone. We used the Benjamini–Hochberg adjustment to correct for multiple comparisons to minimize the risk of false positive results. However, given the exploratory nature of the study, we decided to report both unadjusted and adjusted *P*-values for all analyses.

Time series clustering analysis was performed to identify similarities in expression patterns across the menstrual cycle. The elbow method and gap statistics were used to identify the optimal number of clusters ($N = 3$) for analysis. The cf-miRNAs were grouped based on the similarities in the directional changes in miR expression across the phases regardless of the distance (level of expression) between them using the *tSCR* R package (Montero and Vilar, 2014). Gene target analysis was conducted using the R package multi-MiR (Ru *et al.*, 2014). Only gene targets identified via luciferase reporter assay or western blotting were included in the gene target and pathway enrichment analysis. Over representation analysis (ORA) was used for gene target enrichment and implemented by the R package clusterProfiler (Wu *et al.*, 2021). The background gene list used for the ORA analysis comprised all validated targets of the c-miRNAs detected in the PCR panel, using the same stringency criteria as described above. Tissue enrichment of the validated gene targets was conducted in the R package TissueEnrich. Finally, transcription factor-microRNA interactions were explored using TransmiR v2.0 (Tong *et al.*, 2019) and compared using two-sample tests of proportion ran in Stata 16.0. *P*-values were deemed statistically significant when $P < 0.05$. Data are presented as mean ± SD unless stated otherwise. The following packages were also used in our analysis; lme4 (Bates *et al.*, 2015), lmerTest (Kuznetsova *et al.*, 2017) and tidyverse (Wickham *et al.*, 2019). The full R code can be found at https://github.com/DaniHiam/menstrual-cycle-circulating-microRNAs.

Results

Participant characteristics

There were 16 eumenorrheic biological females aged 37.8 ± 6.8 years included in the study. Participants were apparently healthy with a BMI of 22.3 ± 3.4 kg/m^2. None of the participants smoked regularly and two participants smoked occasionally. All participants had a regular menstrual cycle for at least six months prior to entering the study. Table I recapitulates participant hormone levels at each sample collection point.

The effect of the menstrual cycle on circulating miRNA levels

Of the 174 endogenous plasma-enriched miRNAs included on each plate, 157 (92%) met our minimal detection criteria and were considered for further analysis. An unsupervised, clustered heat map of the z-scored levels of all analysed miRNAs grouped by time point is presented in Fig. 1, with clustered heat maps of the z-scored levels of all analysed miRNAs clustered by individual miRNA levels and participant ID, respectively, presented in Supplementary Figs S2 and S3.

The levels of six individual miRNAs, hsa-miR-99b-5p, hsa-miR-1260a, hsa-let-7e-5p, hsa-miR-30c-5p, hsa-miR-766-3p and hsa-miR-326, displayed significant differences with the menstrual cycle ($P < 0.05$ prior to the false discovery rate (FDR) adjustment) (Fig. 2). Contrasts were then added in the model. Additional miRNAs presented significant changes between individual time points (T1 vs T2: hsa-miR-30e-5p, hsa-miR-155-5p, hsa-miR-27a-3p, hsa-miR-382-5p, hsa-miR-590-5p, hsa-miR-374a-5p, hsa-miR-32-5p, hsa-let-7d-5p; T1 vs T3: hsa-miR-30c-5p; T2 vs T3: hsa-miR-374a-5p, hsa-miR-454-3p, hsa-miR-32-5p, hsa-miR-590-5p, hsa-miR-30e-5p, hsa-miR-424-5p, hsa-miR-126-5p, hsa-miR-1260a, hsa-miR-154-5p, hsa-miR-136-3p, hsa-miR-324-5p, hsa-miR-382-5p, all $P < 0.05$ prior to FDR adjustment). All *P*-values adjusted for multiple comparisons were non-significant and are available in Supplementary Table SII. Since our study is exploratory, we elected to report non-adjusted *P*-values. Between-participant variability explained a greater portion of the variance than any other variable including timepoint in the menstrual cycle (Supplementary Fig. S4), indicating that, in future studies, a larger cohort may help overcoming the issue of small effect sizes.

In an attempt to identify similarities in patterns across the menstrual cycle, three clusters containing 26, 4 and 127 miRNAs, respectively, were identified based on similarities in their slopes (Montero and Vilar, 2014). None of these clusters however displayed significant changes in miRNA patterns across time (Supplementary Fig. S5).

Instead, we ran gene target prediction and pathway analysis on the six above-mentioned miRNAs that significantly varied with time in our exploratory analysis to identify potential common biological pathways. Tissue enrichment was conducted on the premises that each miRNA gene target had been experimentally confirmed either by luciferase reporter assay or western blot. Gene targets were significantly enriched in the endometrium, the cervix and uterine tissue, the placenta and the ovary (all $P < 0.05$) (Fig. 3).

This analysis was then repeated with the complete list of miRNAs presenting significant contrasts between any two time points and yielded similar results (Supplementary Fig. S6). Hierarchical clustering of enriched terms was conducted on the same list of genes. The most significantly enriched biological pathways pertained to cellular responses to development. Amongst the four pathways demonstrating the highest number of gene targets and levels of statistical significance were: epithelium development; regulation of organismal development; positive regulation of cell population proliferation; and positive regulation of cell death (Fig. 4).

Table I Hormone levels at each blood collection point.

	T1 (EF)	T2 (O)	T3 (ML)	P
Oestradiol (pmol/l) [mean (SD)]	133.40 (50.56)	634.94 (367.17)	587.06 (210.16)	*<0.01*
Progesterone (nmol/l) [mean (SD)]	0.94 (0.61)	7.60 (6.40)	49.14 (19.40)	*<0.01*
LH (UI/l) [mean (SD)]	7.22 (4.02)	22.77 (16.83)	6.39 (5.13)	*<0.01*
FSH (UI/l) [mean (SD)]	9.19 (4.12)	11.49 (6.06)	4.06 (1.93)	*<0.01*
LH/FSH ratio [mean (SD)]	0.86 (0.41)	2.14 (1.38)	1.70 (1.16)	*<0.01*

T1 = early follicular (EF) phase, ideally defined as the second day of the menstruation; T2 = ovulation (O) phase, ideally defined as 1 day past the LH surge; T3 = mid-luteal phase T3 (ML), ideally defined as 7 days past T2.

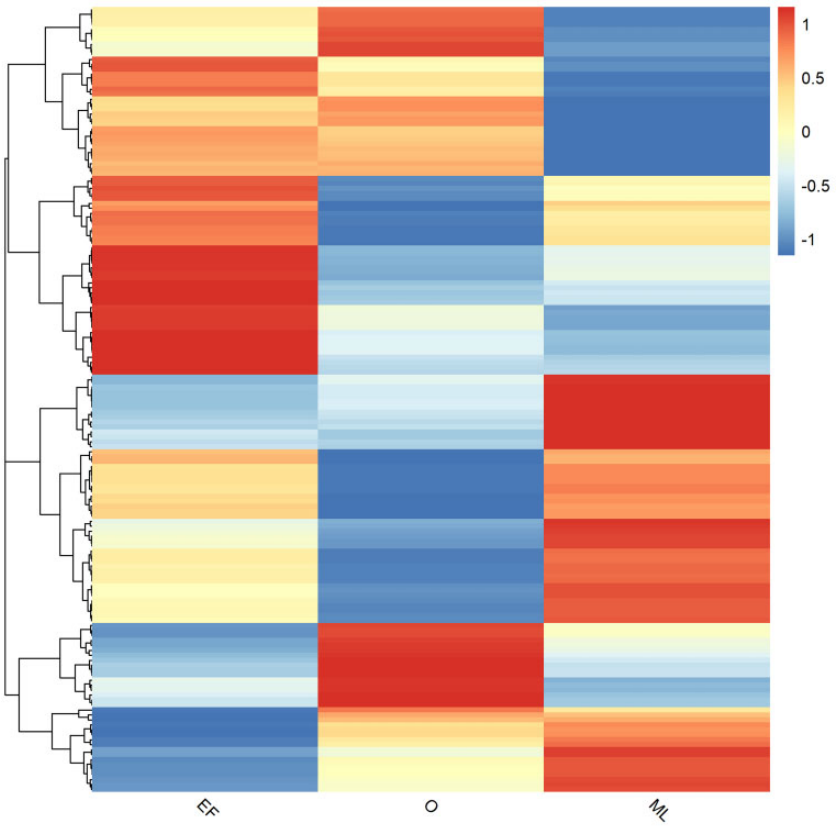

Figure 1. Unsupervised clustered heat map of the z-scored levels of plasma-enriched miRNAs across the menstrual cycle. The 157 plasma-enriched miRNAs met the minimal expression criteria in the early follicular (EF), ovulatory (O) and mid-luteal (ML) phases of the menstrual cycle (N = 16).

The effect of female sex hormones on circulating miRNA levels

We then investigated whether plasma levels of oestradiol, progesterone, LH and FSH explained any of the variance in miRNA levels across time once adjusted for age. The levels of 49 individual miRNAs were significantly associated with progesterone, oestradiol, LH and/or FSH levels prior to FDR adjustment (all $P < 0.05$ prior to FDR adjustment) (Fig. 5). *P*-values adjusted for multiple comparisons were non-significant and are available in Supplementary Table SII.

Finally, we investigated whether specific transcription factors were more likely to regulate the levels of the 49 miRNAs associated with progesterone, oestradiol, LH and/or FSH levels. Numerous

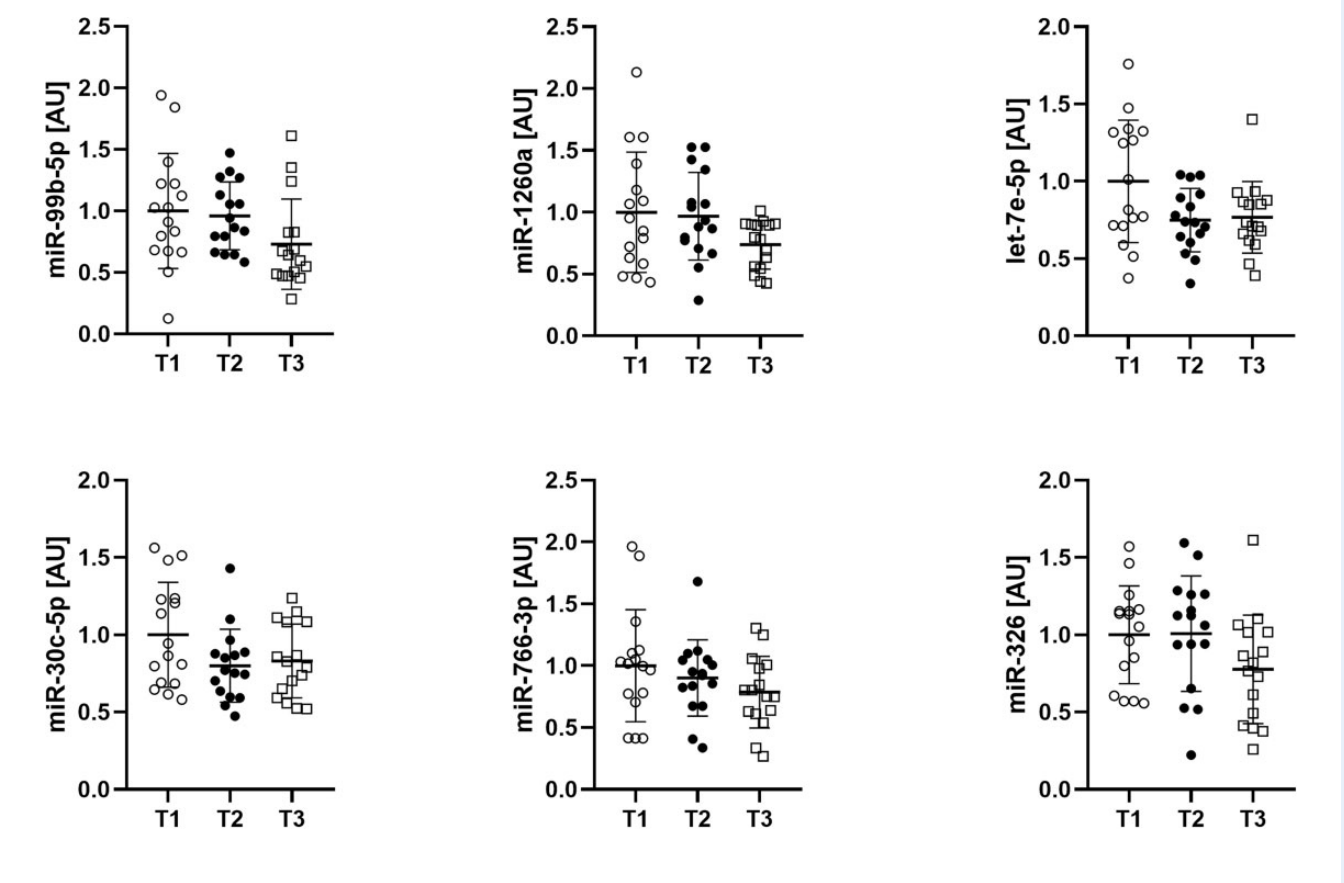

Figure 2. Individual miRNA levels across the menstrual cycle. Levels of hsa-miR-99b-5p ($P = 0.007$, $P = 0.704$ after the false discovery rate (FDR) adjustment, T3 different from T1 and T2), hsa-miR-1260a ($P = 0.013$, $P = 0.704$ after FDR adjustment, T3 different from T2), hsa-let-7e-5p ($P = 0.013$, $P = 0.704$ after FDR adjustment, T3 different from T1), hsa-miR-30c-5p ($P = 0.032$, $P = 0.847$ after FDR adjustment), hsa-miR-766-3p ($P = 0.033$, $P = 0.847$ after FDR adjustment) and hsa-miR-326 ($P = 0.044$, $P = 0.847$ after FDR adjustment) varied across the menstrual cycle.

transcriptions factors presented a significant interaction with the 49 miRNAs of interest ($P < 0.05$), but none of them was specific to any of the female sex hormones. Only three transcription factors, the Aryl hydrocarbon receptor nuclear translocator, the Myb proto-oncogene protein and Upstream stimulatory factor 1 were significantly over-represented as regulators of the list of miRNAs of interest when compared with the complete list of miRNAs detected in the PCR panel ($P < 0.05$) (Supplementary Table SIII).

Discussion

Here, we report the results of an exploratory study investigating the effects of the menstrual cycle on plasma cf-miRNA levels in 16 eumenorrheic females. One prior study conducted in nine young females suggested that the overall cf-miRNA profile remained stable throughout the menstrual cycle. However, this study critically failed to control for differences in RNA extraction and reverse-transcription, and individual data or analysis were not made available (Rekker *et al.*, 2013). A more recent study investigating a suite of serum cf-miRNAs as potential markers for endometriosis found that the phase of the menstrual cycle, which was recorded but not controlled for, did not influence the levels of the six miRNAs used in their diagnosis algorithm (Moustafa *et al.*, 2020). Again, differences in RNA extraction and reverse-transcription were not accounted for. In contrast, our study, while conducted on a small cohort, was tightly controlled for endogenous and exogenous confounders, which is critical to ensure robust and reproducible cf-miRNA research (Hiam and Lamon, 2020). Our data highlight numerous associations between phases of the menstrual cycle, female sex hormone levels and plasma cf-miRNA levels.

The idea that sex hormones influence miRNA expression in human tissue is not new (Nielsen *et al.*, 2013), but little research has focused on the menstrual cycle and its influence on cf-miRNA levels, maybe because of the intrinsic complexity of these two systems (Hiam and Lamon, 2020; Lamon and Knowles, 2021). In larger mammals, Ioannidis et al. reported a series of plasma cf-miRNAs whose levels varied during the oestrous cycle of Holstein-Friesian heifers (Ioannidis and Donadeu, 2016). Interestingly, we identified two conserved miRNAs, hsa-miR-99b-5p (homologous to bta-miR-99b) and hsa-miR-155-5p (homologous to bta-miR-155) that also displayed significant contrasts between time points in humans. While these contrasts did not retain their significance once adjusted for FDR in either study, the

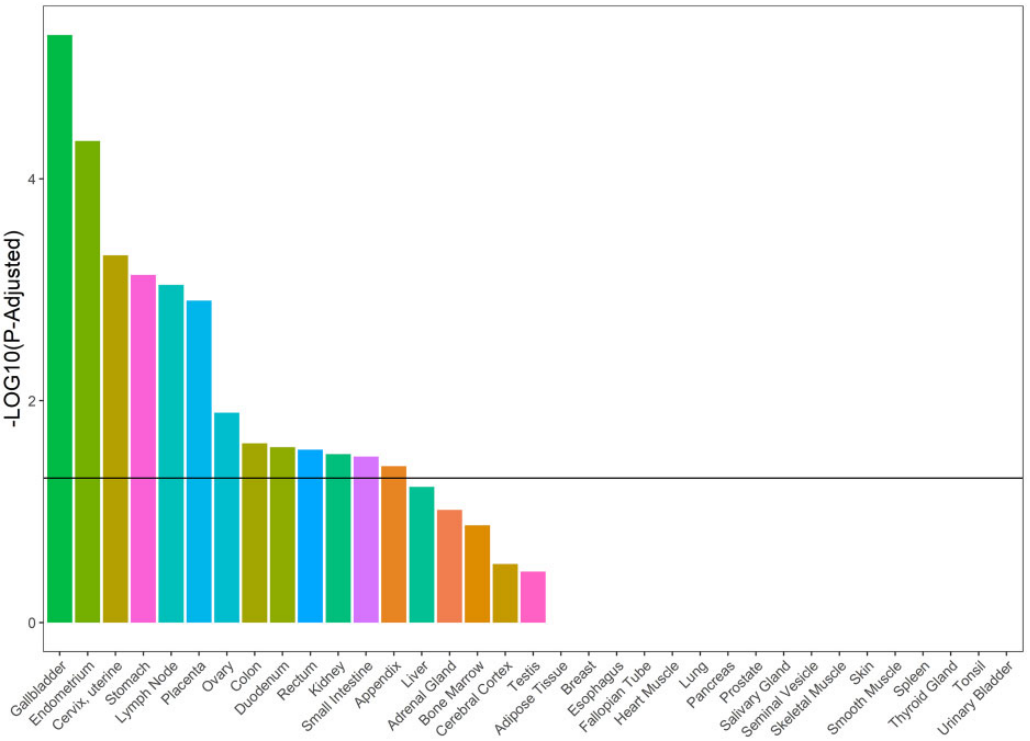

Figure 3. Tissue enrichment of the validated gene targets of the cf-miRNAs varying across the menstrual cycle. A solid black line indicates significance ($P < 0.05$).

Figure 4. Hierarchical clustering of enriched terms for the validated gene targets of the cf-miRNAs varying across the menstrual cycle.

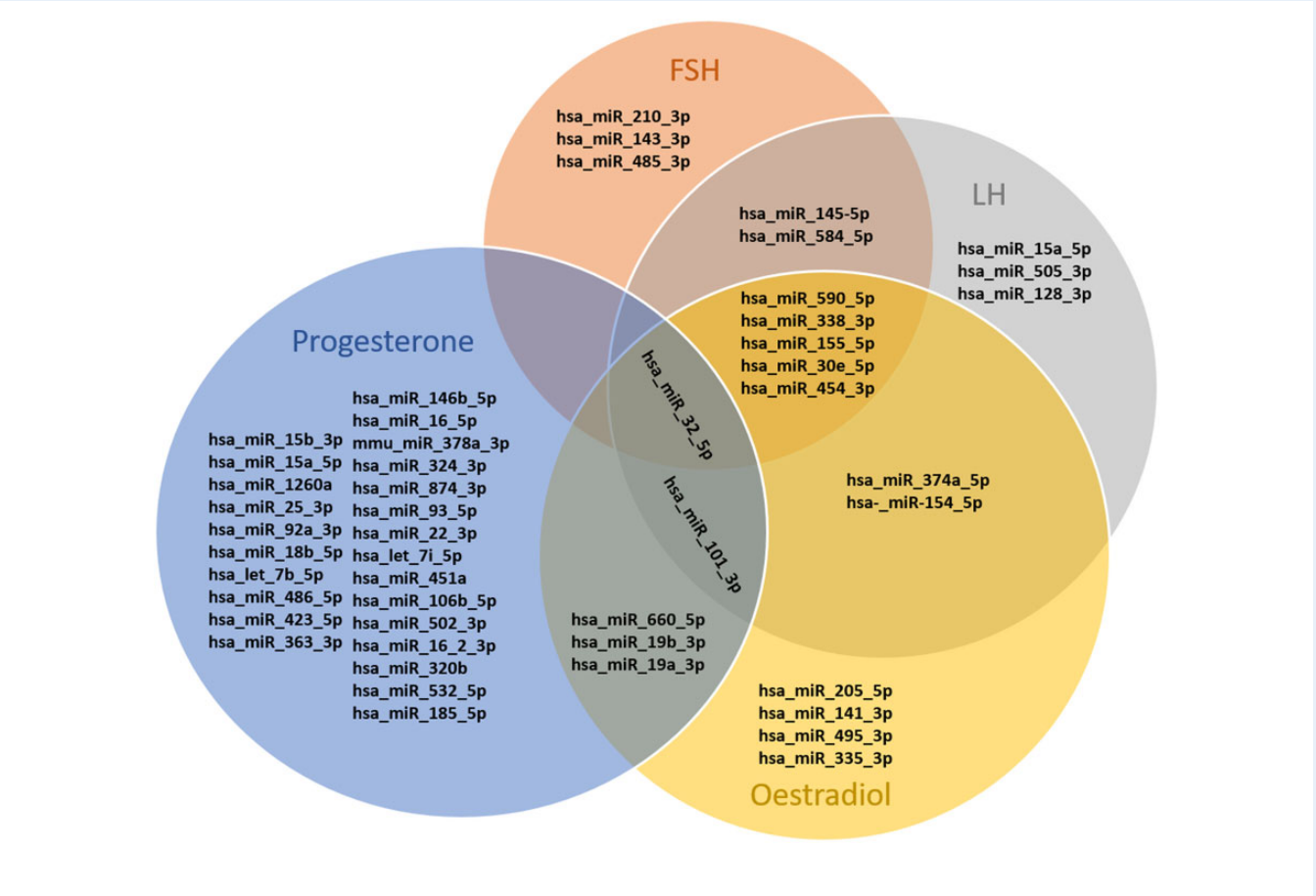

Figure 5. Individual miRNAs that were significantly associated with progesterone, oestradiol, LH and/or FSH plasma levels in age-adjusted regression models. Progesterone: 30 miRNAs (blue), oestradiol: 16 miRNAs (yellow), LH: 14 miRNAs (grey) and/or FSH: 11 miRNAs (orange).

pattern of change remained similar between both species. The levels of hsa-miR-99b-5p decreased by 27% (humans) and 25% (bovines) (10), respectively, in the mid-luteal phase of the menstrual cycle when compared with the early-follicular phase. MiR-99b-5p is a well-studied miRNA that is mechanistically associated with the AKT/mTOR pathway and a direct target of mTOR, RPTOR and IGF1 3′-UTRs (Zacharewicz *et al.*, 2020). This pathway retains its homology in the mouse and mediates oestradiol-induced protein synthesis in mouse reproductive tissue (Wang *et al.*, 2015). In humans, miR-99b clusters with hsa-let-7e on chromosome 19, which also demonstrated a 25% decrease between the early-follicular and mid-luteal phases. Supporting a role for this cluster in human reproductive tissues, hsa-let-7e expression significantly increased by about 2-fold when treated with oestradiol, but not progesterone, in human endometrial stromal cells (Reed *et al.*, 2018).

Similarly, the levels of hsa-miR-155-5p increased by 45% (humans) (MacGregor, 2022) and 32% (bovines) (Ioannidis and Donadeu, 2016) in the ovulatory when compared with the early-follicular phase. MiR-155-5p is a well-studied oncomiR that is upregulated in different types of breast cancers (Iorio *et al.*, 2005; Blenkiron *et al.*, 2007; Kong *et al.*, 2014) and associated with reduced expression of the oestrogen receptor and progesterone receptor in human primary breast tumours (Pasculli *et al.*, 2020).

Further relevant comparisons can be drawn with the recent pilot study by McGregor *et al.* describing the fluctuations of miRNAs in human adipose tissue at four points of the menstrual cycle (MacGregor *et al.*, 2022). The time points used in the McGregor study do not fully align with ours (they collected a late-follicular and a post-ovulatory time point, while we collected a peri-ovulatory time point instead), but some of the miRNAs differentially expressed in adipose tissue were also differentially expressed in plasma, again with the limitation of FDR adjustment. Surprisingly, we report opposite patterns for hsa-miR-155-5p, which tends to increase around ovulation in plasma, but to decrease in adipose tissue, and hsa-miR-30c-5p, which tends to increase during the mid-luteal phase in plasma but to increase in adipose tissue. cf-miRNAs are passively or selectively secreted by cells (Arroyo *et al.*, 2011) into bodily fluids (Weber *et al.*, 2010). It is therefore possible that adipose tissue acts as a reservoir for a pool of miRNAs that are secreted into plasma at specific time points to exert their effect on recipient cells (Guescini *et al.*, 2015; Thomou *et al.*, 2017).

The gene targets of the miRNAs fluctuating with the menstrual cycle were significantly enriched within the female reproductive tissues

endometrium, cervix, uterine, placenta and ovary. Despite an exponential increase in circulating miRNA research over the last decade (Hiam and Lamon, 2020), insights into the role and regulation of these molecules remain elusive. How specific cf-miRNA transport processes are is unclear, and the mechanisms governing their uptake by recipient cells remain mostly unknown. A number of studies suggest highly selective and tightly regulated processes (Alexander *et al.*, 2015; Thomou *et al.*, 2017; Whitham *et al.*, 2018), which may underpin a whole level of cross-tissue communication (Guescini *et al.*, 2015; Whitham *et al.*, 2018). Our findings support this hypothesis, where the menstrual cycle may control the tissue-specific secretion of cf-miRNAs targeting the female reproductive apparatus. In addition, the gene targets of these cf-miRNAs primarily regulated the regulation of epithelium development. Endometrial epithelial cell proliferation peaks in the follicular or 'proliferative' phase of the menstrual cycle, in preparation for a potential implantation, while apoptosis occurs later in the luteal or 'secretory' phase (Monis and Tetrokalashvili, 2022). These results indicate that cf-miRNAs may be intrinsic regulators of these processes by mediating the expression of key genes fluctuating throughout the menstrual cycle in female reproductive tissues. This regulation may however not specifically result from sex-hormone specific transcription factors, such as the oestrogen receptors (ESR1 and ESR2) or the progesterone receptor (PR), acting on miRNA transcription. Future studies may therefore focus on investigating indirect mechanisms.

Our article presents several limitations that must be mentioned. Firstly, our study is exploratory. The one prior study reporting human cf-miRNA levels across the menstrual cycle did not make individual data or analysis available (Rekker *et al.*, 2013). To the best of our knowledge, no other suitable data set was available in the literature to base our power calculation on. We therefore established our sample size based on feasibility as well as previous similar human studies conducted in different tissues (MacGregor *et al.*, 2022). Bearing the restrictions of this approach in mind, no participant was added to the original cohort past the end of data collection. Our analysis was further limited by the stringent Benjamini–Hochberg adjustment, which was applied to our *P*-values to account for the multiple cf-miRNAs measured on the same assay plate. While none of the adjusted *P*-values reached statistical significance, unadjusted *P*-values indicate that our study would have been adequately powered for more targeted analysis and may therefore be considered as hypothesis-generating. Unadjusted *P*-values suggest a range of likely associations between cf-miRNA levels and serum sex hormone levels. We therefore recommend that future larger studies investigating the individual or collective levels of miRNAs in female plasma should (i) include measures of serum progesterone, oestrogen, FSH and LH levels, and (ii) adjust their mixed-models by accounting for these hormone levels as time-varying confounders. The full code based on R language is available for use at https://github.com/DaniHiam/menstrual-cycle-circulating-microRNAs. Finally, it should be noted that our model could not identify all sources of unexplained variance, and that other participant variables, including sleep or physical activity levels, may potentially explain a small proportion of miRNA fluctuations throughout the cycle and should be investigated in future studies. Furthermore, a direct extrapolation of these findings to females without a regular menstrual cycle should be considered carefully. Investigating confounding factors specific to these cohorts might be necessary in order to translate our proposed model to the whole female population.

In conclusion, we report the results from a pilot study investigating the effects of the menstrual cycle on the plasma cf-miRNA levels profile in humans. We found numerous associations between cf-miRNAs levels, phases of the menstrual cycle and ovarian hormone levels prior to adjusting our results for multiple comparisons. Stringent bioinformatics analysis indicates that the levels of a series of cf-miRNAs targeting cell development and proliferation in female reproductive tissues fluctuate with the menstrual cycle. Our results reinforce the importance of accounting for female-specific biological processes in physiology research by implementing practical or statistical mitigation strategies during data collection and analysis.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

All relevant data are available from the Mendeley database: LEGER, Bertrand (2022), 'MiRNA and menstrual cycle', Mendeley Data, V1, doi: 10.17632/2br3zp79m3.1.

Acknowledgements

The authors would like to thank Dr Michel Rossier from the Institut Central des Hôpitaux Valaisans (ICHV) for his technical support.

Authors' roles

S.L.: conception and design, analysis and interpretation of data, drafting the article, final approval of the version to be published. J.L.C.: conception and design, acquisition of data, critical revision for important intellectual content, final approval of the version to be published. G.P.: acquisition of data, critical revision for important intellectual content, final approval of the version to be published. H.P.D.: analysis and interpretation of data, critical revision for important intellectual content, final approval of the version to be published. F.L.: analysis and interpretation of data, critical revision for important intellectual content, final approval of the version to be published. D.H.: analysis and interpretation of data, drafting the article, final approval of the version to be published. B.L.: conception and design, analysis and interpretation of data, critical revision for important intellectual content, final approval of the version to be published. All authors agree to be accountable for all aspects of the work in ensuring that any questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Funding

This study was supported by the Clinique romande de réadaptation, Sion, Switzerland. S.L. was supported by an Australian Research Council Future Fellowship (FT10100278). D.H. was supported by an Executive Dean's Postdoctoral Research Fellowship from Deakin University.

Conflict of interest

The authors declare no competing interests.

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