# Insights from the Menstrual Cycle in Pulmonary Arterial Hypertension

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## Online Data Supplement

## Supplemental Data

### Sex Hormone and Estradiol Metabolite Levels

Serum estradiol (E2), dehydoepiandrosterone-sulfate (DHEA-S), luteinizing hormone (LH), and testosterone were measured by electrochemiluminescence immunoassay at the Laboratory for Clinical Biochemical Research at the University of Vermont (all Roche, Indianapolis, IN). The E2 assay has an analytical range of 5 pg/mL – 3000 pg/mL and coefficients of variation (CVs) of < 6.8%; DHEA-S has detection limits between 0.1 – 1000 μg/dL and CVs < 8.3%. All CVs for remaining hormones were < 8.3%. Progesterone was measured by high-pressure liquid chromatography-mass spectrometry (LC-MS) (Quest Diagnostics, Chantilly VA and Esoterix, Austin, TX). E2 metabolites were measured from plasma by LC-MS by the Mass Spectrometry Core at the Edinburgh Clinical Research Facility, Queen's Medical Research Institute in Edinburgh, Scotland using previously published methods (1).

### Markers of Angiogenesis

### Extracellular Vesicles

Extracellular vesicles (EVs) were isolated in a series of steps to first remove cells by centrifugation (1,900g x 5 minutes at 4°C). The cell-free supernatant was transferred to 5 ml polypropylene tubes and centrifuged at 5,000g for 10 min at 4°C to remove platelets. The supernatant (platelet free plasma) was transferred into a capped ultracentrifuge tube and ultracentrifuged at 10,000g for 1 hour to remove cellular debris. The supernatant was then ultracentrifuged at 100,000g for 1 hour at 4°C. Ultracentrifuged pelleted material, containing EVs, was resuspended in 500ul of

PBS and 250ul of each sample was added to TRIzol (Invitrogen) for total RNA extraction. With the remaining 250 ul, total numbers of EVs were assessed by NanoSight utilizing laser illuminated microscopical technique and quantified as EV protein (mg/mL).

MicroRNAs were isolated from EVs in order to gain further insight into specific mechanistic pathways linked to sex hormone fluctuations. Total RNA was measured for quantity and quality using an Agilent 2100 bioanalyzer. Approximately 0.25 – 0.5% of the total RNA fraction extracted were miRNA species. Ten nanograms of total RNA per sample, isolated from 5 to 10 µg of EVs, was used to amplify cDNA using the High Capacity cDNA transcription kit (Applied Biosystems, all equipment/reagents). cDNA pre-amplification reactions were performed using a pooled mixture of all primer assays and consisted of one 10 min cycle, 95°C; 14 cycles, 95°C, 15 s; one 4 min cycle, 60°C. RT–PCR reactions were performed in with the 7900HT Fast RT PCR System using 384-well TaqMan® Human MicroRNA Array Cards. The $2^{-\Delta\Delta CT}$ method was used to calculate relative expression of each target miRNA,[17] using RNU6B (human array) as control.

**Vascular Endothelial Growth Factor Receptor 2 and Angiopoietin-2**

VEGF-R2 and Angiopoietin (Ang)-2 were measured by electrochemiluminescence at the Laboratory for Clinical Biochemical Research at the University of Vermont (Meso Scale Diagnostics, Rockville, Maryland). The inter-assay CV for VEGF-R2 was 2.3%

and for Ang 2 was 4.1 – 8.4%.

### Markers of Cardiopulmonary Function

#### Six Minute Walk Test

Weekly six-minute walk tests (6MWTs) were performed according to standardized procedures (2). Variability was minimized by administering the test in the same corridor for all study visits (and all participants), the avoidance of a “warm-up” period, and using standard phrases of encouragement. Intra-class correlation coefficients were 0.95 – 0.98 from a completed randomized clinical trial in our center (3).

#### Diffusing Capacity of the Lung for Carbon Monoxide

Weekly $DL_{CO}$ measurements were performed by trained staff at the Rhode Island Hospital Pulmonary Function Testing (PFT) Laboratory according to standardized procedures (4). $DL_{CO}$ was obtained via single-breath method. PFT staff were blinded to case-control status and to menstrual phase.

#### Echocardiography

In PAH cases, echocardiography was performed weekly in accordance with the American Society of Echocardiography guidelines (5). Tricuspid annular plane systolic excursion (TAPSE) was obtained by aligning an M-mode cursor through the anterior tricuspid annulus in the apical 4-chamber view and longitudinal annular displacement during systole was recorded. Trained echosonographers were blinded to menstrual cycle phase, as was a single reader (author A.P.) who batched all study reads.

**N-Terminal Prohormone of Brain Natriuretic Peptide (NT-proBNP)**

NT-proBNP was measured by electrochemiluminescence at the Laboratory for Clinical Biochemical Research at the University of Vermont (Roche, Indianapolis, IN) with a detectable range of 5 – 70K pg/mL and CVs of 1.7 – 7.8%.

## References


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5. Rudski LG, Lai WW, Afilalo J, Hua L, Handschumacher MD, Chandrasekaran K, Solomon SD, Louie EK, Schiller NB. Guidelines for the echocardiographic assessment of the right heart in adults: a report from the American Society of Echocardiography endorsed by the European Association of Echocardiography, a registered branch of the European Society of Cardiology, and the Canadian Society of Echocardiography. *J Am Soc Echocardiogr* 2010; 23: 685-713.

**Figure E1.** 2-methoxyestrone over the course of the menstrual cycle. Mean Ln(2-methoxyestrone) levels over the menstrual cycle (visit 1, 2, 3, and 4) between pulmonary arterial hypertension cases (red) and controls (blue) with 95% confidence intervals.

**Figure E2.** 2-methoxyestrone levels during the menstrual cycle (visit 1, 2, 3, and 4) and the relationship to tricuspid annular plane systolic excursion (TAPSE), Ln(centimeters)(cm) in pulmonary arterial hypertension cases only.

**Figure E3.** Estradiol levels during the menstrual cycle (visit 1, 2, 3, and 4) and the relationship to fold changes in microRNA expression in pulmonary arterial hypertension cases (red) and controls (blue) with 95% confidence bands. Panel A) microRNA-29c expression. Panel B) microRNA-376a expression. Panel C) microRNA-21 expression. $2^{-\Delta\Delta CT}$ method was used to calculate relative expression of each target miRNA using RNU6B (human array) as control.

**Figure E4.** Dehydroepiandrosterone-sulfate (DHEA-S) levels during the menstrual cycle (visit 1, 2, 3, and 4) and the relationship to fold changes in microRNA expression in pulmonary arterial hypertension cases (red) and controls (blue) with 95% confidence bands. Panel A) microRNA-29c expression. Panel B) microRNA-376a expression. Panel C) microRNA-21 expression. $2^{-\Delta\Delta CT}$ method was used to calculate relative expression of each target miRNA using RNU6B (human array) as control.

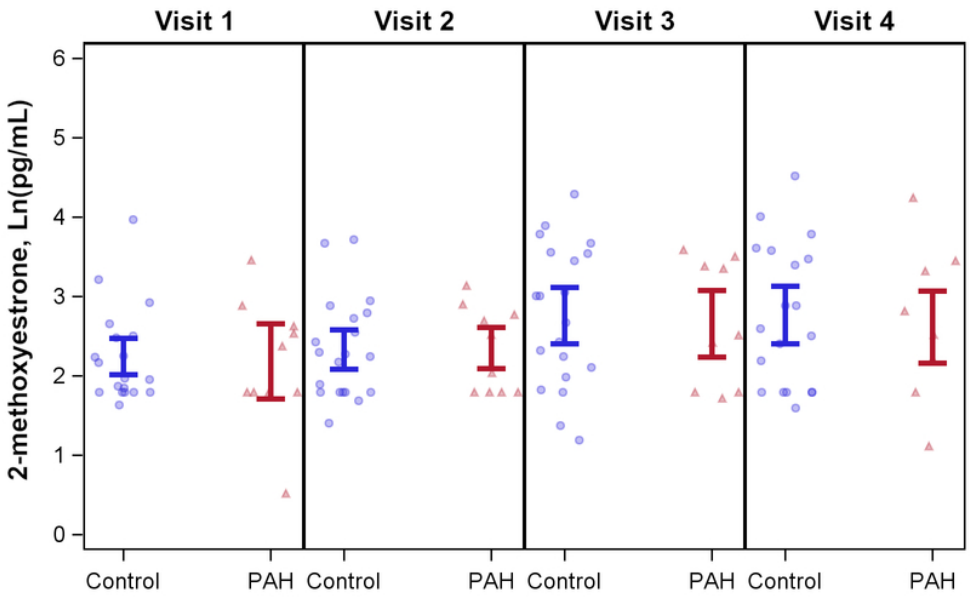


Figure E1. 2-methoxyestrone over the course of the menstrual cycle. Mean Ln(2-methoxyestrone) levels over the menstrual cycle (visit 1, 2, 3, and 4) between pulmonary arterial hypertension cases (red) and controls (blue) with 95% confidence intervals.

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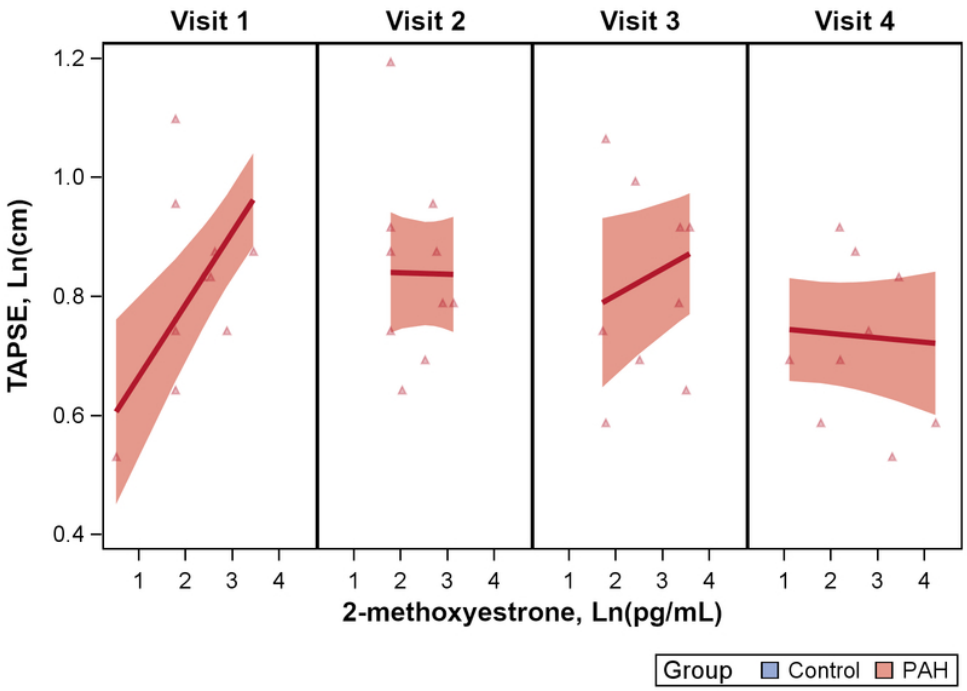


Figure E2. 2-methoxyestrone levels during the menstrual cycle (visit 1, 2, 3, and 4) and the relationship to tricuspid annular plane systolic excursion (TAPSE), Ln(centimeters)(cm) in pulmonary arterial hypertension cases only.

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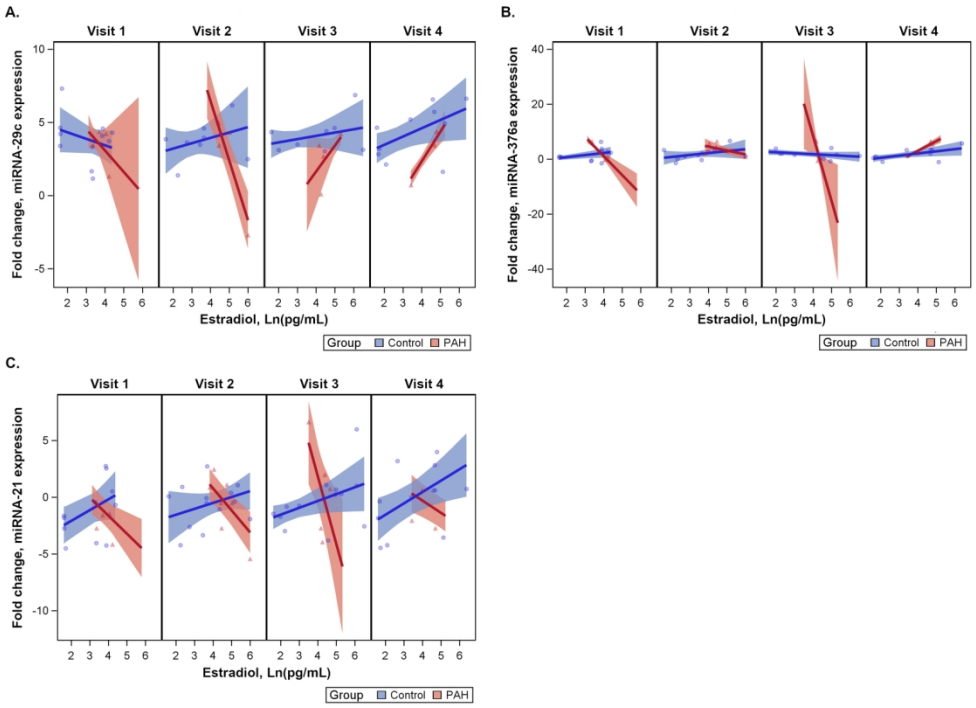


Figure E3. Estradiol levels during the menstrual cycle (visit 1, 2, 3, and 4) and the relationship to fold changes in microRNA expression in pulmonary arterial hypertension cases (red) and controls (blue) with 95% confidence bands. Panel A) microRNA-29c expression. Panel B) microRNA-376a expression. Panel C) microRNA-21 expression. 2−ΔΔCT method was used to calculate relative expression of each target miRNA using RNU6B (human array) as control.

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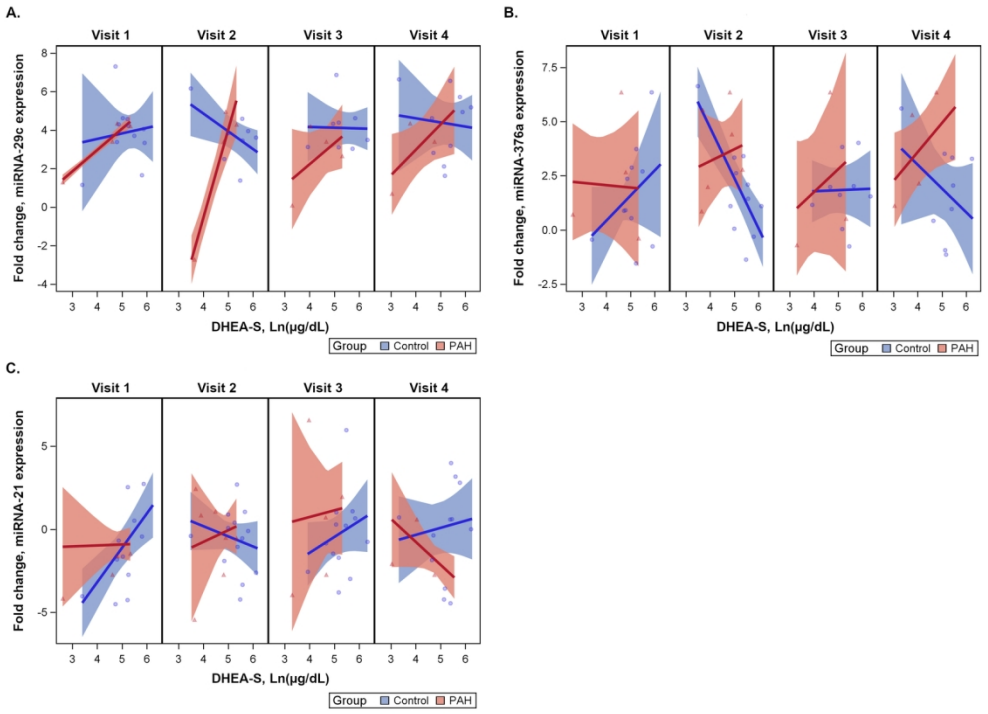


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