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In vitro model of perimenopausal depression implicates steroid metabolic and proinflammatory genes

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Abstract

The estimated 20–30% of women who develop perimenopausal depression (PMD) are at an increased risk of cardiovascular and all-cause mortality. The therapeutic benefits of estradiol (E2) and symptom-provoking effects of E2-withdrawal (E2-WD) suggest that a greater sensitivity to changes in E2 at the cellular level contribute to PMD. We developed an *in vitro* model of PMD with lymphoblastoid cell lines (LCLs) derived from participants of a prior E2-WD clinical study. LCLs from women with past PMD (n=8) or control women (n=9) were cultured in three experimental conditions: at vehicle baseline, during E2 treatment, and following E2-WD. Transcriptome analysis revealed significant differences in transcript expression in PMD in all experimental conditions, and significant overlap in genes which were changed in PMD regardless of experimental condition. Of these, chemokine *CXCL10*, previously linked to cardiovascular disease, was upregulated in women with PMD, but most so after E2-WD ($P<1.55\times10^{-5}$). *CYP7B1*, an enzyme intrinsic to DHEA metabolism, was upregulated in PMD across experimental conditions ($F_{(1,45)}=19.93$, $P<0.0001$). These transcripts were further validated via qRT-PCR. Gene networks dysregulated in PMD included inflammatory response, early/late E2-response, and cholesterol homeostasis. Our results provide evidence that differential behavioral responsivity to E2-WD in PMD reflects intrinsic differences in cellular gene expression. Genes such as *CXCL10*, *CYP7B1*, and corresponding proinflammatory and steroid biosynthetic gene networks, may represent biomarkers and molecular targets for intervention in PMD. Finally, this *in vitro* model allows for future investigations into the mechanisms of genes and gene networks involved in the vulnerability to, and consequences of, PMD.

INTRODUCTION

Most women transition through menopause without the occurrence of clinically significant mood changes. However, for some women, perimenopause is a time of increased

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vulnerability for depression. Several prospective, community-based, epidemiologic studies demonstrate that the menopause transition (i.e., perimenopause) is accompanied by an increased risk of both first onset and recurrent depression by 1.5–3-fold[1–5]. Peri- and postmenopausal women who have depressive symptoms during menopause also experience a significantly reduced quality of life[6, 7], and are at a higher risk for serious medical issues including cardiovascular disease[8]. During the menopause transition, estrogen, particularly estradiol (E2), decreases several-fold[9], and longitudinal evidence suggests that perimenopausal depression (PMD) is linked to this decline in ovarian function[10, 11].

Depressive episodes during the perimenopause cluster during the late menopause transition, where there are usually the largest declines in E2 secretion[1, 12, 13]. This observation suggests that a subset of women could have a genetically predisposed sensitivity to rapidly declining E2. Moreover, hormone therapy (HT) can alleviate[14, 15], or prevent[16] depression in perimenopausal women, whereas women who stop HT often have a reoccurrence of depressive symptoms[17, 18]. Intriguingly, ovarian steroid levels (including E2) are normal in PMD compared to reproductively matched women without PMD; thus women with PMD are not simply more estrogen deficient[2, 19].

In a placebo-controlled, double-blind clinical study, we previously demonstrated that asymptomatic postmenopausal women with past PMD who were crossed over from E2-treatment to placebo experienced a significant recurrence of depressive symptoms, whereas women who had never experienced depression during menopause or who continued receiving E2-treatment had no change in symptoms[20]. These findings suggest that women who experience PMD have a differential behavioral response to otherwise identical changes in E2 specifically, and independently, of other events accompanying the menopause transition. As steroids such as E2 traditionally act at the cellular level to alter gene transcription and neural signaling, we hypothesized that we could observe differences in cellular response, both innately (i.e., in the absence of ovarian steroids) and during E2-withdrawal, in PMD.

A cellular basis for PMD has not been established, however, and the relevance of animal models for PMD has not been fully determined. Nonetheless, *in vitro* cellular models facilitate controlled endocrine manipulations and are unperturbed by additional environmental exposures influencing gene expression, thus permitting the examination of innate cellular differences between women with and without PMD under differing conditions of E2 exposure. Lymphoblastoid cell lines (LCLs) express steroid receptors and respond to ovarian steroid administration[21]. Therefore, we used LCLs derived specifically from women with the E2-responsive phenotype (characterized in [20]), and recapitulated E2-withdrawal in cell culture, to test the hypothesis that cells of women with PMD innately differ from those of women without PMD. To identify molecular pathways of altered response, particularly after E2-withdrawal, we compared transcriptomes of LCLs from women with and without PMD, at baseline, during E2-treatment, and immediately following E2-withdrawal.

METHODS

Clinical study participants and identification of E2-responsive PMD phenotype in women

Participants were asymptomatic postmenopausal women with past PMD responsive to hormone therapy and asymptomatic postmenopausal women (controls) with no history of depression, matched for age, body mass index, and reproductive status, who were ascertained and evaluated as outpatients at the NIH Clinical Center. The NIH IRB approved the study protocol. All women provided written informed consent. Women with past PMD experienced a recurrence of depressive symptoms (defined by a score of 10 or greater on the Center for Epidemiologic Studies-Depression Scale [CES-D]) when switched from E2 to placebo, but not when continued on E2. Further details about participants are reported in Schmidt et al., 2015[20] and in the supplemental material, and details on some replication cohort participants are in Steinberg et al., 2008[22]. For all participants, morning blood was drawn during the clinical trial, centrifuged, and frozen until use. DHEA levels were measured by either mass spectroscopy at the NIH Clinical Center or radioimmunoassay by Esoterix (Calabasas Hills, CA).

Selection of women for lymphoblastoid cell lines, LCL culturing and E2-withdrawal

Epstein Barr Virus was used to transform lymphocytes into LCLs, as described[23], additional culturing details in the supplemental material. In brief, two cohorts of LCLs were generated: an RNA-sequencing (RNA-seq) set and a larger replication set. The RNA-seq cohort had 9 matched asymptomatic controls and 8 PMD cases (i.e., women with past PMD who had recurrence of symptoms after E2-withdrawal in the original clinical study[20], Figure S1, representing a specific “E2-sensitive” phenotype). At the time of the clinical (diagnostic) study, there were no significant differences in age (t_{15}=0.463, ns), STRAW Stage (χ^2=0.476, df=1, ns), ethnicity (χ^2=1.022, df=1, ns), and years since last menstrual period (t_{15}=0.585, ns) in the women from whom the RNA-seq LCLs were derived. LCLs were transferred to phenol red-free RPMI[24, 25] supplemented with 15% KOSR[26] at least 5 days prior to experimental treatments.

Experimental treatments to all LCLs were: A) Baseline: one spike of Vehicle Stock every 24 hours, collected after 72 hours; B) E2: one spike (100nM) E2 Stock every 24 hours, collected after 72 hours; C) E2-withdrawal: one spike (100nM) E2 Stock every 24 hours for 72 hours, followed by a media change to completely remove E2 and one spike of Vehicle Stock, collected after 24 hours (96 hours total). Treatments occurred in parallel for each LCL processed (depicted in Figure 1). On the final day of the treatment, 1mL of LCL culture media was collected from approximately half of the samples (randomly selected) and frozen. Media samples were sent to NMS Labs (Willow Grove, PA) for liquid chromatography/tandem mass spectroscopy to analytically validate E2 levels within the media, to confirm levels were in the range expected for each treatment group (Figure S2), and used for CXCL10 cell culture ELISAs.

The independent replication set included 21 control and 18 PMD LCLs, some, but not all of which, were derived from women who participated in the E2-withdrawal protocol[20]. However, all women underwent the same screening and psychiatric assessment, confirming

the presence or absence of PMD or any other Axis I disorder[6, 22]. In our studies, approximately 30% of women with past PMD do not experience a recurrence of depressive symptoms after E2-withdrawal. Thus, the replication set represents an expanded PMD phenotype.

Transcriptome generation and analysis

Total RNA of high quality was isolated from LCL pellets in TRIzol using the Qiagen RNEasy Mini Kit (Qiagen, #15596018) and RNase-free DNase (Qiagen, #79254) according to manufacturer's instructions. Dynabeads® mRNA Purification Kit (ThermoFisher, #61012) and Ion Total RNA-Seq Kit v2 (ThermoFisher, #4479789) were used to generate cDNA libraries for sequencing. RNA-sequencing was performed using an Ion Torrent™ Sequencer. After generation of raw reads, read mapping, quantification, quality control, and analysis was performed with CLC Genomics Workbench 11. Further details of cDNA library creation, Ion Torrent sequencing, and CLC data pipeline can be found in the Supplemental Material.

From these procedures, lists of differentially expressed genes (DEGs) with false discovery rate (FDR) corrected and uncorrected p-values, RPKMs, and log(fold change) were generated. The FDR method used is that of Benjamini and Hochberg[27]. The estimated common dispersion was 4.55×10^{-2}, and coefficient of biological variation was 0.213. While DEGs which were FDR significant and/or differently expressed in all treatment groups (i.e. treatment-independent DEGs) were of primary interest, all $p<0.05$ DEGs were used for pathway/database analyses. Further, given the highly selective phenotype of our LCLs, genes which had high ($p<0.001$) significance in the E2-WD treatment were examined in depth if the function of the gene seemed particularly physiologically relevant to PMD.

Gene validation (qRT-PCR and ELISA)

To validate RNA-seq findings, we used quantitative real-time reverse transcription PCR (qRT-PCR) on the replication cohort with TaqMan® Universal PCR Master Mix, performed using a two-step procedure according to the supplier's manual. Relative gene expression levels were calculated using the ΔΔCT value (normalized detection threshold) with b-Actin as the reference control for normalization. TaqMan primers included (ThermoFisher, #4331182): CXCL10 [Hs00171042_m1], MEST [Hs00853380_g1], IGLL5 [Hs04330879_u1], CYP7B1 [Hs01046431_m1], LYPLA2 [Hs00855445_g1], RHOG [Hs00750922_s1], TRMT112 [Hs00829096_g1], JUP [Hs00158408_m1], b-Actin [HS99999903_m1]. Statistical analyses were performed using Prism8 software (GraphPad, Inc).

Solid Phase ELISA of CXCL10 (R&D systems, #DIP100) was used according to manufacturer's instructions to ascertain CXCL10 levels in the cell culture media; as well as in plasma samples from women who participated in the previously described PMD clinical studies.

Gene network wide analysis of transcriptome data:

Venn Diagrams were generated at http://bioinformatics.psb.ugent.be/webtools/Venn/. Probability distributions were calculated using RStudio3.6 with the package SuperExactTest[28], to calculate the Jaccard intersection probability among multiple sets. The R package qqMan was used to generate Q-Q plots[29]. DEGs with a p-value<0.05 were independently analyzed using Gene Set Enrichment Analysis (GSEA) MSigDB (http://software.broadinstitute.org/gsea/msigdb/index.jsp), Hallmark GSEA[30], and Enrichr[31]. The Library of Integrated Network-based Cellular Signatures (LINCS) was used to explore similarities between known drug transcriptional signatures and E2-withdrawal DEGs[32].

RESULTS:

Treatment-dependent differences in transcript expression in LCLs derived from women with PMD

Principal component analysis (Figure S3A) and hierarchical clustering (Figure S3B) revealed that variations in LCL transcriptomes were driven by diagnosis and/or treatment condition rather than by batch effects. Furthermore, no samples were outliers requiring exclusion from further analyses. The average number of mapped reads per sample was 20.5–29.0 million for all treatment groups.

Differential gene expression and shared transcriptomal dysregulation in PMD across baseline, E2 and E2-withdrawal

At baseline, E2, and E2-withdrawal, 449, 464, and 534 genes were differentially expressed (DEGs) in PMD versus control LCLs, respectively (Table S1, S2, and S3). After FDR correction, a total of 19 independent transcripts were statistically significant in one treatment group or another (Table 1). The scatter and volcano plots of all transcripts differing between each diagnostic group and each treatment group revealed differences of equivalent magnitude, roughly symmetrical distributions of upregulation/downregulation, and large numbers of transcripts differing in expression by 2-fold (Figure S4A–F).

Sets of DEGs between treatments were partially overlapping and we observed that the intersection of genes that differed between PMD and controls regardless of LCL treatment was highly significant (probability density $p<6.49e^{-41}$, Figure 2A). This intersection contained 38 genes that were differentially expressed due to PMD diagnosis alone (i.e. “treatment-independent” DEGs). Many, but not all, genes were differentially expressed in the same direction in certain treatments. For instance, 37 of the 38 genes that overlapped in all treatment groups were dysregulated in the same direction (sign test, $p<10^{-7}$), with 17 genes being consistently upregulated (Figure 2B, $p<1.00e^{-40}$) and 20 being consistently downregulated (Figure 2C, $p<4.07e^{-31}$). Genes represented in each overlapping region are documented in Table S4.

Replication cohort confirms that CXCL10 shows significant upregulation in PMD

Many genes changed significantly in the same direction in all three treatment groups (i.e. treatment-independent DEGs). Of this subset of DEGs, we first followed up on genes that were FDR significant after E2-withdrawal (where symptoms were evoked in the clinical

study). Our goal was to determine if these genes showed the same patterns in a larger clinical population in which E2-senstivity was not determined. Three treatment-independent DEGs met this criteria, and were therefore selected for close investigation: *CXCL10* (Figure 3A), *IGLL5* (Figure S5A), and *MEST* (Figure S5B).

qRT-PCR using a larger, independent cohort of LCLs with a broader PMD phenotype (methods) confirmed that *CXCL10,* a chemokine and proinflammatory biomarker, was significantly increased in PMD at baseline ($t_{(37)}$=2.155, p=0.038, Figure 3B), E2 ($t_{(36)}$=2.046, p=0.048, Figure 3C,) and E2-withdrawal ($t_{(37)}$=2.757, p<0.009, Figure 3D) conditions. CXCL10 protein levels in cell culture supernatant were also significantly upregulated in PMD compared to controls both at baseline ($t_{(9)}$=3.438, p=0.007, Figure 3E) and E2-withdrawal ($t_{(9)}$=2.984, p=0.015, Figure 3F). However, CXCL10 levels in the plasma of women with past PMD were not significantly different ($t_{(68)}$=0.537, p=0.59, ns) from control women (Figure S6).

In the RNA-seq discovery sample, three of eight women showed a >10-fold increase in *IGLL5* compared to controls after E2-withdrawal. The significant difference in *IGLL5* did not reoccur in the qRT-PCR independent cohort, but showed a trend consistent with RNA-seq observations, with approximately 10% of women with PMD showing strikingly higher (>12-fold) *IGLL5* compared to controls after E2-withdrawal (Figure S6C, D). The significant upregulation of *MEST* observed via RNA-Seq did not statistically replicate in the expanded cohort (Figure S6E, F). Other E2-withdrawal genes tested via qRT-PCR did not show statistically significant differences with the expanded phenotype cohort (Figure S7A–D).

CYP7B1 shows a significant upregulation in PMD, and correlates to DHEA levels in plasma

Of the 34 remaining treatment-independent DEGs, *CYP7B1* was noted to be of physiologic significance despite not surviving FDR statistical correction, and was further explored (Figure 4A). *CYP7B1* is an enzyme involved in the metabolism of the neurosteroids dehydroepiandrosterone (DHEA) and pregnenolone, and consequently could play a role in the pathogenesis of PMD. qRT-PCR analysis in the replication cohort confirmed that *CYP7B1* expression was significantly elevated in LCLs of women with PMD at baseline ($t_{(37)}$=2.345, p=0.025, Figure 4B), as well as after E2-withdrawal ($t_{(35)}$=2.378, p=0.023, Figure 4D), and trended toward significance with E2 ($t_{(35)}$=2.02, p=0.051, ns, Figure 4C). Grubbs test for statistical outliers was run on all qRT-PCR data sets, which prompted removal of one outlier from the control group in the E2 (G=3.318, alpha=0.01) and E2-withdrawal (G=3.887, alpha=0.01) comparisons. Plasma DHEA levels available on a subset (n=7 control, n=7 PMD) of women whose LCLs were RNA-sequenced revealed significantly decreased DHEA in PMD women ($t_{(13)}$=3.322, p=0.006, Figure 4E), and a significant negative correlation (r^2=0.46, p<0.008) to *CYP7B1* E2-withdrawal RPKMs (Figure 4F).

Dysregulation of proinflammatory pathways in PMD LCLs

Gene Set Enrichment Analysis (GSEA) of 52 Hallmark pathways identified processes significantly dysregulated (Q_{FDR}<0.05) in the transcriptome at baseline (n=31, Table S5),

E2 (n=26, Table S6), and after E2-withdrawal (n=32, Table S7), indicative of widespread alteration of well-characterized cellular processes in LCLs of PMD women, regardless of treatment-condition. E2-response, inflammation, cell cycle dysregulation/apoptosis, and steroid metabolism-related gene networks were strongly represented among the top significant Hallmark pathways after E2-withdrawal. Interestingly, the same processes tended to be implicated in the different conditions ($p<1.56e^{-04}$, Table S8 and Figure S8), and among the 17 overlapping enriched pathways were many inflammatory and steroid-responsive gene networks.

Enrichr analysis was run on a E2-withdrawal weighted gene list of all expressed genes. With this approach, one pathway survived statistical correction ($p_{cor}=0.03$, $z=-2.43$), Type II Interferon signaling (IFN-γ), of which *CXCL10* is a marker of proinflammatory activation (Table S9).

DISCUSSION:

Clinically, estradiol withdrawal precipitates symptoms of depression in otherwise asymptomatic postmenopausal women with a past PMD, but not in women without a past PMD[20]. In this study, we investigated whether the PMD behavioral endophenotype of E2-sensitivity was linked to cellular differences in genes and gene networks in women with PMD compared with control women, and if these differences were exaggerated by acute withdrawal from E2. To test these hypotheses, we devised an *in vitro* strategy that recapitulated E2-withdrawal in derived LCLs from women with PMD and matched controls, in which we assayed LCLs not only at baseline, but after 72 hours of E2-treatment and subsequent withdrawal. We used RNA-sequencing of LCLs derived from a highly selected, E2-sensitive PMD phenotype, and then further probed the significance of particular genes from the RNA-seq findings with a larger, phenotypically broader qRT-PCR replication cohort. These experiments supported the hypothesis that there were significant differences in the transcriptomes of PMD LCLs and implicate at least two new individual molecular targets and numerous gene networks that could cause, or are a consequence of, PMD. Pro-inflammatory genes (i.e., *CXCL10*) and networks involved in inflammatory response were hyperactivated in PMD, particularly following E2-withdrawal. *CYP7B1*, a steroid enzyme which metabolizes DHEA, was consistently upregulated in PMD regardless of experimental treatment, and, correspondingly, we found a significant decrease in plasma DHEA levels in women with PMD.

Depression during perimenopause has been associated with heightened risk of stroke, metabolic conditions, osteoporosis, and cardiovascular dysfunction[10]. Even in women with no prior history of cardiovascular disease, a single episode of depression increases risk of cardiovascular death in postmenopausal women by almost 50%[8]. Depression outside of perimenopause has been widely linked to dysfunction of the immune system (reviewed in [33, 34]). Thus, finding that *CXCL10* is significantly increased in women with PMD, and most so after E2-withdrawal, establishes a novel cellular link between PMD and inflammation.

CXCL10, also known as IFN-γ inducible protein-10 (IP-10), is a chemokine in a well-established pro-inflammatory molecular cascade linked to cardiovascular disease

risk[35, 36]. Following IFN-γ binding to IFN-γR, the JAK-STAT signaling pathway is activated, triggering transcription factors to induce expression of *CXCL10* as well as other chemokines. CXCL10 has been distinguished clinically as a biomarker for inflammation in several other disorders (reviewed in [35, 37–39]). Generally, elevated levels of CXCL10 predict poorer prognoses, with a major exception being breast cancer[40]. Nevertheless, levels of CXCL10 can rise and fall in a matter of hours, and elevated levels in cells are not always evident in the serum or plasma of patients[37, 41]. This suggests there is a limited window in which CXCL10 levels on their own are clinically useful.

However, CXCL10 is a part of a larger molecular cascade, which can be captured by gene network-wide approaches. In our study, gene enrichment analyses identified several inflammatory networks as dysregulated in PMD compared to controls, including: IL6-JAK-STAT3 signaling, IL2-STAT5 signaling, TNFα signaling, and IFN-γ. Therefore, the larger CXCL10 signaling cascade, especially other ligands for its receptor, CXCR3, may yield better therapeutic targets for PMD. CXCR3 can have both pro- and anti-inflammatory signaling properties[42], depending on which chemokine activates it. For instance, CXCL11 has been shown to be primarily anti-inflammatory and has a higher binding affinity to CXCR3 than CXCL10. Thus, CXCR3 is a manipulable target, where increasing levels of CXCL11 could mitigate the pro-inflammatory effects CXCL10.

Previous studies have also linked changes in reproductive steroids to CXCL10. Endometrial stromal cells given 12 days of E2 and progesterone followed by four days of withdrawal (to model menstruation) have significant increases in CXCL10[43]. It appears to be cell-type specific as to whether estrogen inhibits[44, 45] or increases[46] CXCL10 secretion. However, we consistently observed the lowest levels of *CXCL10* in E2-treated LCLs compared to those which underwent E2-withdrawal. Future studies in women could quantify CXCL-levels throughout perimenopause to link *in vitro* cell differences with *in vivo* measures and establish whether these levels are relevant as biomarkers in PMD.

Among genes that could contribute to the risk for (or ontogeny of) PMD is *CYP7B1*. In our study, *CYP7B1* expression was not directly related to E2-withdrawal but was associated with PMD diagnosis. *CYP7B1* (25-hydroxycholesterol-7-alpha-hydroxylase) encodes an enzyme of the cytochrome P450 superfamily responsible for DHEA and pregnenolone metabolism via 7α-hydroxylation into 7α-hydroxy-DHEA and 7α-hydroxy-pregnenolone[47, 48], as well as 3βdiol metabolism[49, 50]. DHEA itself is anxiolytic[51], anti-depressive[52, 53], and its levels can be modulated by E2[54]; thus, elevated *CYP7B1* could decrease available DHEA and contribute to depression symptomology. We also observed a significant decrease in DHEA levels in the plasma of PMD women. This finding is consistent with some previous studies in PMD showing significantly lower morning plasma DHEA and by reports showing either higher or lower levels of the sulphated metabolite DHEA-(S)[19, 55]. Conversely, the metabolite 7α-hydroxy-DHEA can bind to estrogen receptor (ER) β, exerting agonistic activity[56, 57], whereas the substrate 3βdiol itself is an ERβ agonist[50], suggesting that CYP7B1 could instead function in a compensatory manner to increase or decrease ER signaling during E2-withdrawal. *CYP7B1* has recently been shown to be a genome-wide significant gene in unipolar depression[58], neuroticism[59] and other related psychiatric phenotypes[60].

This finding suggests that cellular-based, aberrant steroid signaling could play a role in priming cells for a dysregulated response in PMD. Further, it appears that the specific E2-sensitivity endophenotype perhaps represents an additional level of irregular inflammatory response. Indeed, mounting evidence supports the idea that declining ovarian function, i.e. decreases and fluctuations in E2, during menopause is associated with increases in inflammasome activity[61], which include proinflammatory cytokines and changes in immune cell function[62]. Although *CYP7B1* transcription did not respond to changes in E2, both CYP7B1 and 7α-hydroxy-DHEA levels reportedly increase during inflammation[47, 63]. *CXCL10*, however, did increase after E2-withdrawal, suggesting that symptoms may stem from a combination of these two gene networks, and perhaps increase a woman's vulnerability to both depression and cardiovascular disease if untreated with HT.

A goal of biological studies of PMD is to identify potential novel therapeutics. The LINCs database, which finds commonalities in gene expression signatures across multiple cell lines and pharmacologic exposures in response to different perturbations[32], suggested molecules that might either mimic or reverse the molecular signature seen during E2-withdrawal in PMD, and warrant further study. The top 50 Rank Scores for mimicking or reversing the PMD E2-withdrawal transcriptomic signature are in Table S10. Geldanamycin, which inhibits Hsp90, was predicted to have the most similar changes in transcript expression, comprising 12 of the top 50 hits to the database (Figure S9A). Geldanamycin, an antibiotic associated with depression and anxiety[64] inhibits the function of Heat Shock Protein 90 (Hsp90). Hsp90 is an FKBP5 co-chaperone that works to regulate the glucocorticoid receptor's sensitivity to cortisol[65], and accordingly has been implicated in the pathophysiology of several psychiatric disorders[66] and in rodent models of menopause[67]. Future studies should investigate the role inhibition of Hsp90 signaling could play in PMD.

MLN2238, a proteasome inhibitor, and Curcumin, the active anti-inflammatory ingredient in turmeric, were the top compounds projected to reverse the E2-withdrawal DEG signature (Figure S9B), with Curcumin comprising 5 of the top 50 hits to the database. Curcumin has a growing literature that suggests it can decrease anxiety or alleviate depression-like symptoms in animal models for depression[68, 69], neurological disease[70], and schizophrenia[71]. A recent meta-analysis of several studies on curcumin, including placebo-controlled trials, concluded that curcumin is safe and effective in helping alleviate symptoms of depression in patients[72]. However, these are just predicted compounds that have not been experimentally confirmed, so further study is required to know if any of these molecules could play a role in PMD.

While studying LCLs derived from postmenopausal women ensured that the highly selective E2-sensitive depression phenotype was not confounded by environmental factors, we do not have prospective, longitudinal *in vivo* data directly related to some of our findings, an obvious goal for future studies. Further, while LCLs have been shown to express hormone receptors[21] and retain a high percentage of their genomic architecture during transformation[73], LCLs do not replicate functional consequences of hormone withdrawal within the cells and circuits of the brain. However, at this point the critical cells and circuits that may drive PMD are unknown.

In conclusion, our *in vitro* cellular model has identified several gene networks as well as implicated two specific genes in the potential pathophysiology of PMD. Molecular mechanisms of PMD appear to be at least partly distinct from other endocrine-related mood disorders such as Premenstrual Dysphoric Disorder[21], as might be expected from the weak clinical comorbidity[74]. Existence of both diagnostic and E2-withdrawal induced transcriptome differences in LCLs support the hypothesis that there is a genetic susceptibility to PMD that is exacerbated by E2-withdrawal occurring during perimenopause. Future studies may link dysregulated transcripts during E2-withdrawal to specific symptoms or co-morbid risks of PMD such as cardiovascular disease[75]. Clinically, genes and gene networks implicated by transcriptomics may represent druggable targets that contribute to the causes and consequences of PMD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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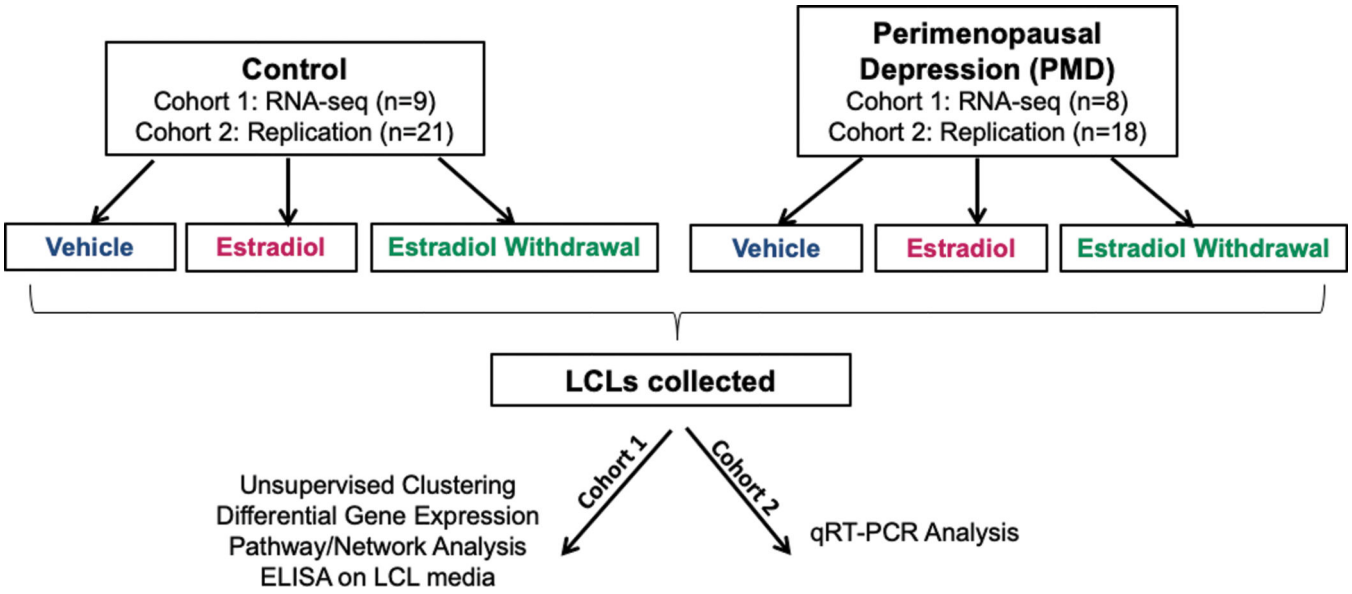

Figure 1 –. PMD vs. Control LCLs: *in vitro* estradiol (E2) exposures

LCLs were subjected to the following experimental treatment time course, creating three treatment groups: baseline, E2, and E2-withdrawal, per diagnostic category (control or PMD). 100nM E2 or vehicle (DMSO) was spiked into the cultures every 24 hours for a total of 72 hours of exposure, with an additional 24 hours of vehicle for LCLs in the E2-withdrawal treatment group. Two LCL cohorts were used: Cohort 1 was a smaller, subject-matched discovery cohort in which RNA-sequencing was performed with resulting differential gene expression (DEG) and gene network analysis, E2 quantification, and ELISAs on LCL culture media. Cohort 2 was an independent replication cohort that was used for qRT-PCR. For Cohort 1, all 8 PMD LCLs were derived from women with past PMD that was demonstrated to be E2-sensitive. For Cohort 2, all 18 PMD LCLs were derived from women with past PMD that underwent a similar psychiatric screening to the Cohort 1 PMD women but were not tested for E2-sensitivity. Thus, Cohort 2 represents an independent and broader PMD phenotype.

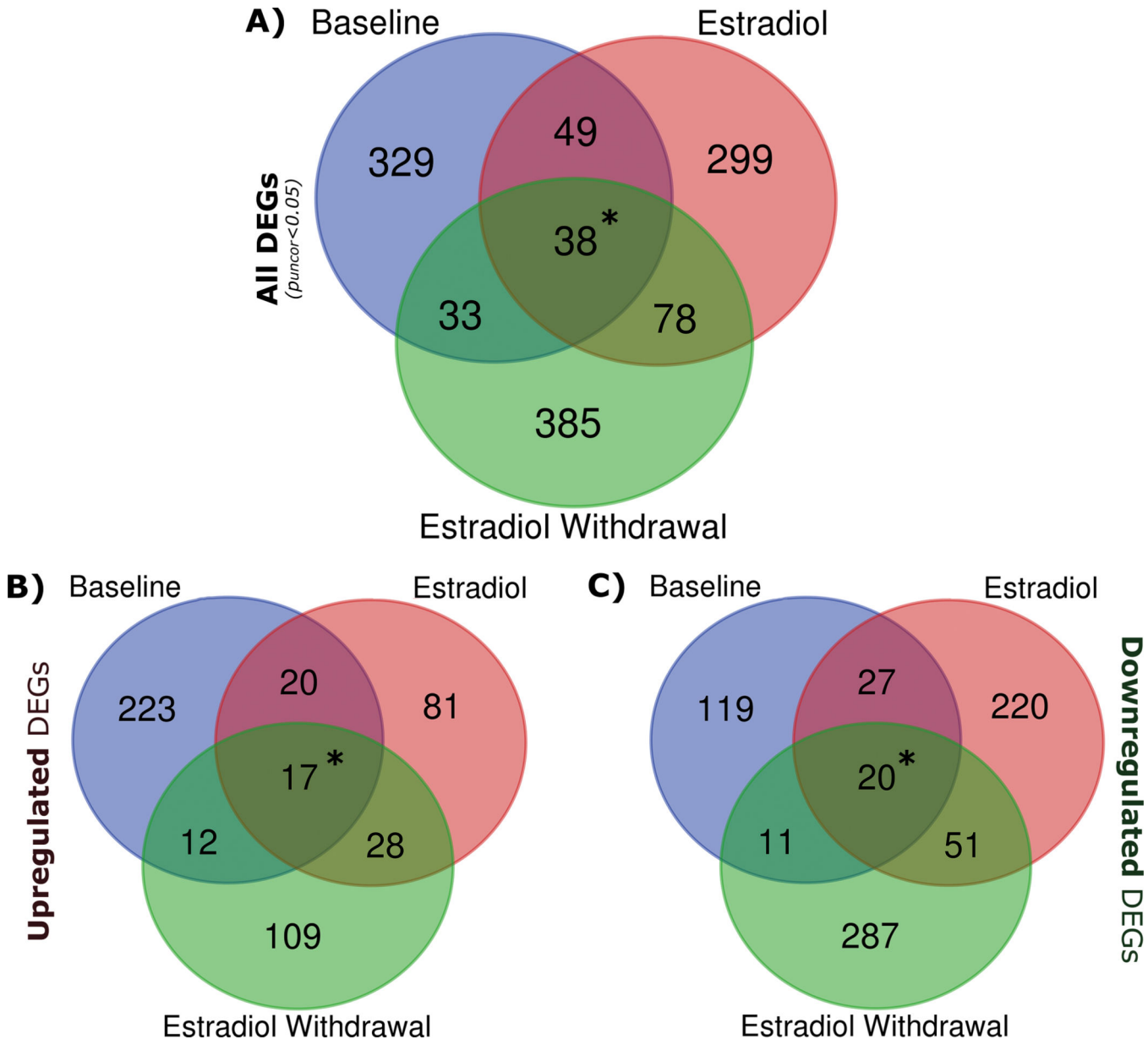

Figure 2 –. Significant Overlap between DEGs in all experimental treatment groups

Venn Diagrams indicating the overlap between genes that are differentially expressed (either up- or down- regulated) ($p<0.05$) in PMD at baseline, E2, and E2-withdrawal compared to control. Upregulated means significantly higher expression in PMD vs controls, downregulated means significantly lower expression in PMD vs controls. Not every gene changed significantly in the same direction in each treatment.

A) Overlap of DEGs between treatment groups, regardless of the direction of change. The intersection of DEGs between all treatment-groups (treatment-independent, or diagnosis-dependent DEGs) is significantly higher than expected by chance (*, $p < 6.49e^{-41}$, Jaccard Exact Test).

B) Overlap between transcripts that are upregulated (DEGs $p<0.05$) in PMD at baseline, E2, and E2-withdrawal LCLs compared to control LCLs. The overlap in DEGs between groups is significantly higher than expected by chance (*, $p < 1.00e^{-40}$, Jaccard Exact Test).
C) Overlap between transcripts that are downregulated (DEGs $p<0.05$) in PMD at baseline, E2, and E2-withdrawal LCLs compared to control LCLs. Again, the overlap in DEGs between groups is significantly higher than expected by chance (*, $p < 4.07e^{-31}$, Jaccard Exact Test).

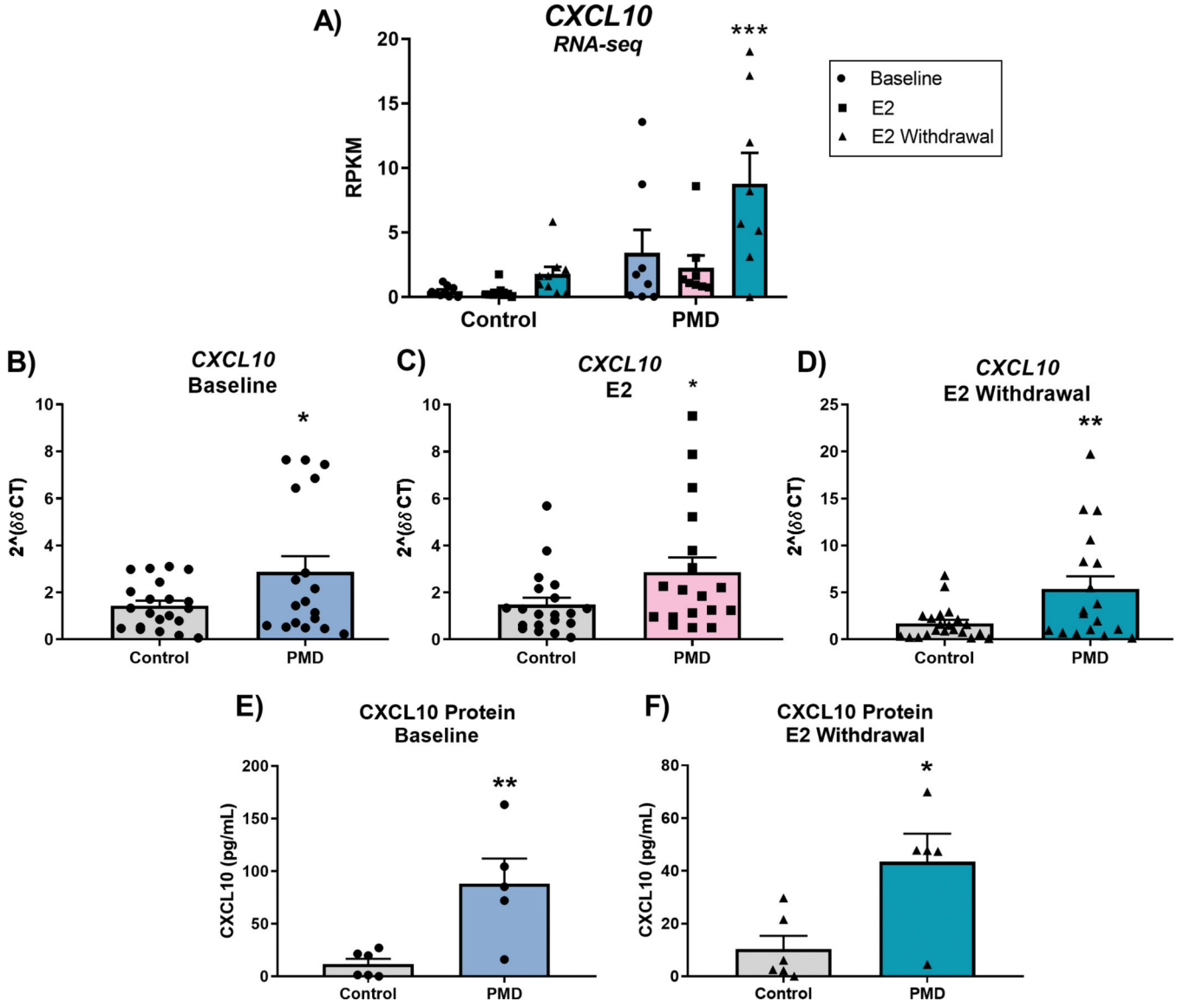

Figure 3 –. CXCL10, a proinflammatory chemokine, is significantly increased in LCLs from women with PMD, particularly after E2-Withdrawal

A) Two-way ANOVA of LCL gene expression (RNA-seq); diagnosis x experimental treatment. *CXCL10* expression significantly increased in PMD LCLs compared to controls ($F_{1,45}$=15.82, p=0.0003), and in response to E2 treatment ($F_{2,45}$=6.041, p=0.0047). Sidak posthoc t-tests show a significant increase specifically after E2-withdrawal (t_{45}=4.06, p<0.0006, ***).

B, C, D) qRT-PCR validation of RNAseq *CXCL10* expression confirmed significant increases in transcript expression in PMD LCLs at baseline ($t_{(37)}$=2.155, p=0.038, *), E2 ($t_{(36)}$=2.046, p=0.048, *) and E2-withdrawal ($t_{(37)}$=2.757, p<0.009, **).

E, F) ELISA on the cell culture media on the day of LCL collection from a subset (n=6 controls, n=5 PMD) of the RNA-seq samples. Levels of the chemokine CXCL10 were significantly upregulated in the cell culture supernatant of LCLs from women with

PMD compared to controls at baseline ($t_{(9)}$=3.438, p=0.007, **) and after E2-withdrawal ($t_{(9)}$=2.984, p=0.015, *).

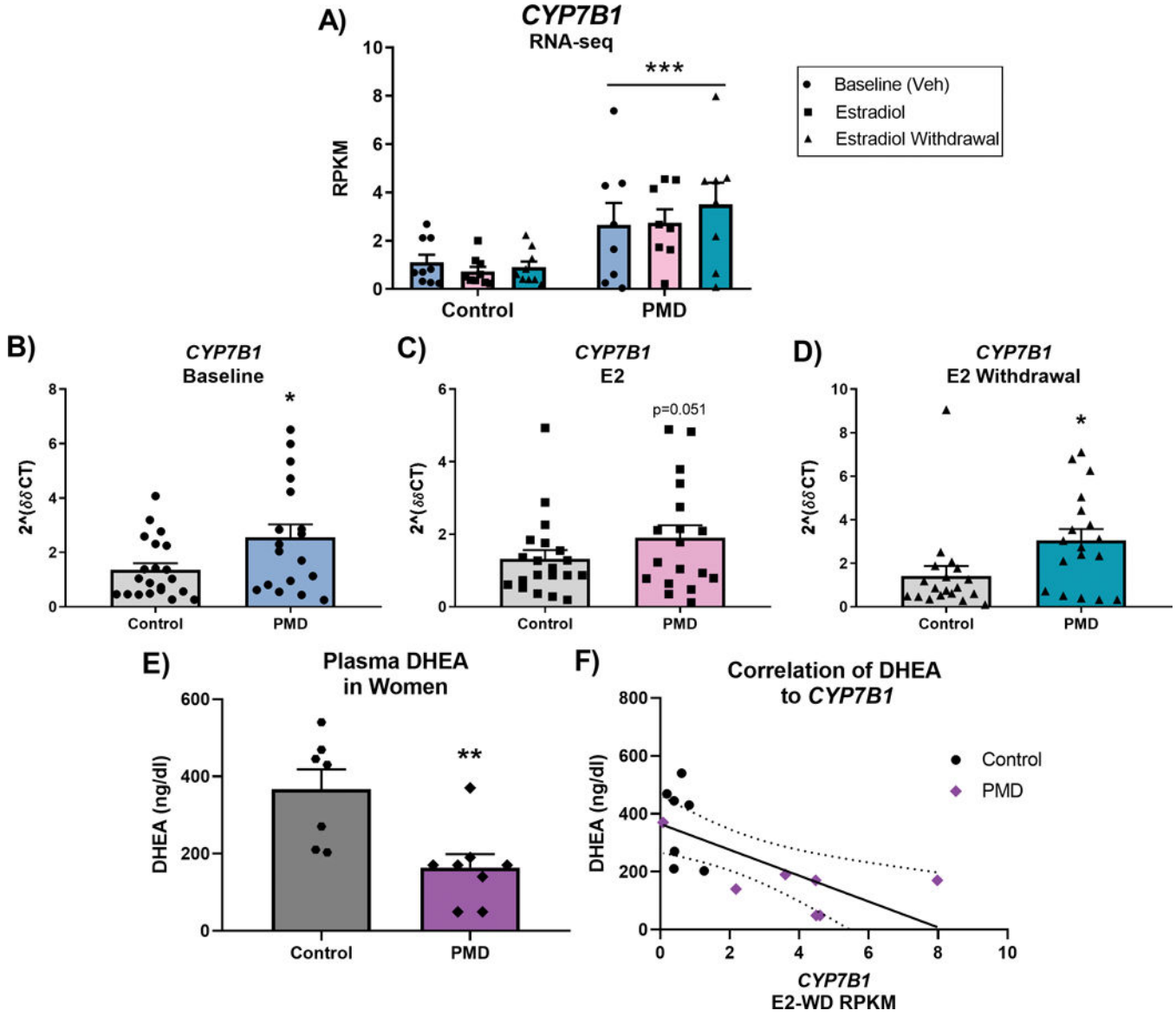

Figure 4 –. *CYP7B1*, the gene encoding the enzyme involved in DHEA metabolism, is significantly increased in LCLs from women with PMD, and significantly correlates to decreased plasma DHEA levels

A) A two-way ANOVA of gene expression (RNA-seq) demonstrated a significant main effect of diagnosis in PMD vs controls ($F_{1,45}$=19.93, p<0.0001, ***), but showed no significant main effect specifically in response to E2-treatment ($F_{2,45}$=0.358, p=0.701, ns), nor a significant interaction ($F_{2,45}$=0.437, p=0.648, ns).

B, C, D) qRT-PCR validation of the expression levels revealed that *CYP7B1* was significantly increased at baseline ($t_{(37)}$=2.345, p=0.025, *), as well as after E2-withdrawal ($t_{(35)}$=2.378, p=0.023, *), and trended toward significance with E2 ($t_{(35)}$=2.02, p=0.051, ns).

E) Available plasma DHEA levels on a subset (n=7 control, n=7 PMD) of women whose LCLs were in the RNA-sequencing cohort revealed significantly decreased DHEA in PMD women ($t_{(13)}$=3.322, p=0.006, **)

F) *CYP7B1* E2-withdrawal RPKMs (Reads Per Kilobase per Million mapped reads from RNA-seq) demonstrated a significant negative correlation to plasma DHEA levels (r^2=0.46, p<0.008). Small black dots represent the 95% confidence intervals of the linear regression line.

Table 1 –

RNA-seq analysis reveals FDR significant differentially expressed genes in LCLs of women with PMD within each experimental treatment

EDGE test p values, FDR corrected p values, and Fold Change relative to control of FDR significant genes of PMD and control LCLs after Vehicle (4 genes), E2 (9 genes), and E2-withdrawal (7 genes) treatments. A positive fold change indicates significantly increased expression in PMD compared to control.

Experimental Treatment Group	Gene ID	EDGE test: P-value	EDGE test: FDR p-value correction	Fold change compared to Control
Baseline *(72hrs DMSO)*	*IGLL5*	1.53E-08	0.0001	28.14
	LAPTM4B	9.52E-07	0.004	3.06
	ITGAL	1.79E-05	0.045	2.24
	CCL17	2.10E-05	0.045	5.23
E2 continued *(72hrs E2)*	*UGT2B17*	4.01E-07	0.003	3.69
	MAP4K1	6.22E-06	0.017	−1.95
	DEXI	7.30E-06	0.017	−2.39
	NDNL2	9.53E-06	0.017	−1.97
	ATF5	9.72E-06	0.017	−5.58
	TNFRSF13C	1.20E-05	0.017	−2.22
	PIM3	1.62E-05	0.020	−1.80
	GADD45B	1.94E-05	0.021	−1.96
	TREML2	5.27E-05	0.050	−3.11
E2 Withdrawal *(72hrs E2; 24hrs DMSO)*	*IGLL5*	2.53E-07	0.002	16.60
	LYPLA2	6.89E-06	0.024	−3.57
	MEST	1.06E-05	0.024	5.79
	RHOG	1.12E-05	0.024	−3.61
	CXCL10	1.55E-05	0.024	4.51
	HIST1H4A	1.78E-05	0.024	−4.94
	TRMT112	1.93E-05	0.024	−1.77