ONLINE SUPPLEMENT

Vasopressin in Preeclampsia: A Novel Very-Early Human Pregnancy Biomarker and Clinically-Relevant Mouse Model.

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SUPPLEMENTAL METHODS

Biosample and Clinical Data Acquisition:
Coded maternal plasma with annotated clinical patient information were obtained through the University of Iowa IRB-approved (IRB# 200910784) Maternal Fetal Tissue Bank (MFTB). In this biorepository, pregnant women are prospectively recruited from the beginning of their prenatal care. MFTB inclusion criteria include any women \( \geq 18 \) years old receiving prenatal care at the University of Iowa Hospitals & Clinics who are English-speaking. The MFTB exclusion criteria include human immunodeficiency virus or hepatitis C positive women. Women who enroll into the MFTB provide a maternal blood sample for research whenever they have clinically indicated blood draws throughout pregnancy. All maternal blood in the MFTB is uniformly processed. Maternal whole blood is collected in Acid Citrate Dextrose-A (ACD-A) Vacutainer tubes (Becton Dickenson) and centrifuged at 1300 \( \text{g} \). Maternal plasma and the buffy coat are isolated, aliquoted, and stored at -80\( ^\circ \text{C} \). Maternal and neonatal clinical data obtained by the MFTB are obtained via data extraction from the electronic medical record using standardized data extraction forms. Extracted clinical data are routinely monitored for accuracy and completeness by two of the authors (MKS and DAS). Additional data are also extracted by bioinformatics collaborators from the University of Iowa Institute for Clinical and Translational Science who are able to query the central electronic medical record database. For comparisons to nonpregnant patients, clinical data and patient plasma was obtained through the University of Iowa Women’s Health Tissue Repository (IRB# 201010769, 201202714) focused on recruiting women who seek gynecologic care. These biorepositories are run by two of the authors (DAS and MKS) ensuring uniform sample processing and clinical data extraction to maintain high fidelity clinical data and samples for all the biobanks. The human study was IRB approved as the use of these deidentified and coded samples and clinical data from these biorepositories were deemed non-human subjects research (IRB # 201405808) by the University of Iowa IRB. Further elaboration of the mechanisms of this bank and the quality assurance measures of the biosamples and clinical data have been recently reported\(^1\).

Cohort Assembly:
Inclusion criteria for preeclampsia cases included women who delivered at UIHC, were enrolled in the MFTB, and carried the diagnosis of preeclampsia at the time of delivery. The diagnosis and classification of cases of mild preeclampsia, severe preeclampsia and chronic hypertension with superimposed preeclampsia were based on the standard American College of Obstetrics and Gynecology (ACOG) definitions for analysis with a minimal clinical presentation of elevated blood pressures of greater than 140/90 at least 2 occasions 6 hours apart and proteinuria\(^2\). These cases were identified by cross-referencing the MFTB database with the bioinformatics query of mild and severe preeclampsia and chronic hypertension with superimposed preeclampsia ICD-9 codes (642.4x, 642.5x, 642.7x, 642.9x) of bank participants at the time of delivery. The electronic medical record of each potential case was evaluated to confirm the diagnosis of preeclampsia by the ACOG definitions. Maternal age-matched plasma samples and corresponding clinical data for the control population were obtained by utilizing the MFTB database. Control pregnancies were pregnant women who did not develop...
preeclampsia. The gestational age at the time of the collection of the samples was classified by trimesters: first trimester (< 13 completed gestational weeks), second trimester (13-26 completed gestational weeks), and third trimester (>26 weeks). To compare pregnant and non-pregnant plasma copeptin concentrations, a cohort of clinical data and plasma from non-pregnant, age-matched patients was assembled. These patients were recruited from the gynecologic clinics at our institution. A sample of blood was obtained at the time of recruitment which was processed in the same way as the maternal blood for plasma isolation. Control and case assignment was performed by a trained high risk obstetrician (MKS).

Procedures:

All maternal plasma copeptin concentrations were measured in duplicate using a commercial enzyme-linked immunosorbent assay (ELISA) specific for human copeptin (USCN Life Science, Inc, Houston, TX). The assay was performed according to the manufacturer's instructions. The minimum detectible dose of human copeptin for this assay was 5.4 pg/mL. The intra-assay coefficient of variation is < 10% and the inter-assay coefficient of variation is < 12%. To examine if renal function or AVP degradation throughout pregnancy affected copeptin concentration, plasma Cystatin C (Sigma-Aldrich, St. Louis, MO) and vasopressinase (LNPEP, USCN Life Science, Inc, Houston, TX) were measured in duplicate in all samples utilizing commercial ELISA kits. Cystatin C is a potent marker of renal function that is uniform across multiple populations and is not altered by muscle mass, meat consumption, physical activity, and overall health status in comparison to serum creatinine and glomerular filtration rate (GFR). Given the superiority of cystatin C in estimating renal function in multiple studies, the 2012 KDIGO (Kidney Disease: Improving Global Outcomes) guideline statements suggest using cystatin C to estimate glomerular filtration rate\(^3\), \(^4\). For these reasons, cystatin-C was used to estimate renal function in this study.

Animal Studies:

Wildtype C57BL/6J male and female mice were obtained from the Jackson Laboratories, and maintained on standard chow (Teklad 7013) at standard room temperature (22°C) with a 12:12 light:dark cycle. Virgin female mice were implanted with subcutaneous osmotic minipumps (Alzet Model # 1004) to deliver AVP (24 ng/hr) or saline vehicle. Three days after implantation, mice were individually mated for a single overnight period. Blood pressure was assessed by tail-cuff (Visitech, as previously \(^5\)) for three weeks preceding mating, and through gestational day (GD) 16. Urine was collected using single-mouse sized metabolic cages (Nalgene, as previously \(^6\)) overnight on GD17 and GD18, and protein content was assessed using commercially-available BCA assay kit (Pierce). Mice were sacrificed on GD18, and organs and fetuses were harvested for subsequent analyses. Electron microscopic examinations of maternal kidneys were performed by the University of Iowa Department of Pathology using a JEOL JEM-1011 Transmission Electron Microscope. All procedures were approved by the University of Iowa Animal Care and Use Committee (protocols 1211239 and 1311213). A nonpregnant cohort of female mice was processed in the same way.
Statistical Analyses:

A major aim of this study was to determine if there were differences in first-trimester copeptin concentrations between pregnant women who did and did not develop preeclampsia and if these predicted the development of preeclampsia. Using the smallest effect size in late gestation, maternal plasma copeptin concentrations from Zulfikaroglu et al. between control (310 pg/mL) and mild preeclamptics (620 pg/mL)\(^7\) with the largest reported standard deviation of 180 pg/mL, power of 80% and \(\alpha = 0.05\), only 7 participants per group were required. However, in order to account for a parsimonious, mixed effects regression model of 3 variables, a minimum of 30 samples per group was necessary.

All statistical analyses were performed with SigmaPlot 12.0 software (Systat Software, Inc, California) and confirmed using SAS 9.1 software (SAS Institute Inc, Cary, NC). Stepwise regression was used to identify potential confounding variables. Logistic regression models were constructed using regression identified and clinically significant confounding variables. Receiver operating characteristic curves were constructed for regression diagnostics. Trimester specific cutoff values were determined by graphing the sensitivity and specificity for each trimester. The cutoff was determined at the intersection of these curves. Positive and negative predictive values were also calculated. In addition, chi square or Fisher exact test was utilized for categorical variables. For continuous variables, the Student’s t-test or ANOVA was utilized. If criteria for normality were not met, Mann-Whitney test or ANOVA on Ranks was utilized. All variables were tested at significance level of 0.05.
SUPPLEMENTAL REFERENCES


SUPPLEMENTAL DATA

Figure S1. Induction of hypertension during pregnancy with AVP infusion. (A) Timeline of procedures. (B) Systolic blood pressure (SBP) increased over time during pregnancy in C57BL/6J mice infused with arginine vasopressin (AVP, 24 ng/hr), achieving a significant increase over baseline (pre-pregnancy) values by gestational days 15-16. In contrast, saline-infused dams exhibited a significant reduction in SBP during gestational days 8-12, which returned to baseline levels by gestational days 15-16. These data are calculated and redrawn from the same mice and recordings as presented in Figure 3A. Saline n=16, AVP n=11. * P<0.05 vs saline-infused, † P<0.05 vs baseline.