DATA SUPPLEMENT

Pregnancy protects hyperandrogenemic female rats from postmenopausal hypertension.

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SUPPLEMENTAL METHODS and MATERIALS

Animal model: Female Sprague–Dawley (SD) rats were obtained at 3 weeks of age from the vendor (Envigo, Indianapolis, IN) and allowed to equilibrate in a temperature-controlled environment with 12-h:12-h light:dark cycle for 1 week. As shown in Figure S1 A and B, rats were randomly selected to be implanted with either 5α-dihydrotestosterone (DHT; 7.5 mg/90 days, Innovative Research, Nivi, MI) or placebo pellets to generate hyperandrogenemic females (HAF) (n=41) or controls (n=23), respectively, as we previously described [1]. Throughout their lives, rats were allowed *ad libitum* access to rat chow (Teklad #8640) and tap water. All protocols followed the ARRIVE Guidelines, and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center and complied with the *Guidelines for the Care and Use of Laboratory Animals* by the National Institutes of Health.

The experimental design is shown in Figure S1.

Body composition prior to and after pregnancy and with aging: In order to determine if there were differences in pre-pregnancy or post-delivery body composition between control and HAF rats, Echo MRI (4 in 1–900 model, Body Composition Analyzer, EchoMRI, Houston, TX), was used to measure body composition in another group of female control and HAF rats (n=3-5/group), prior to mating (~ 9 weeks of age) and then again 48 hours after delivery of the pups, and compared to age-matched virgin HAF rats. In addition at 10 and 16 months of age, body composition was also measured by Echo-MRI.

Proteinuria and Urinary Nitrate/nitrite excretion (UNOxV): HAF rats were placed in metabolism cages and urine was collected for 24 hrs during which they were food-deprived, but allowed *ad libitum* access to water. Proteinuria was measured using commercially available reagents (Bio-Rad) to perform Bradford assay, as previously described [1,2,3]. Urinary nitrate/nitrite excretion (UNOxV) was measured, using Griess reagent, as previously described [4].

Oral glucose tolerance test (OGTT): OGTT was performed on virgin and previously-pregnant HAF rats at both 10 and 16 months, after 5h-fast (7:00 – 12:00), as described [1]. Blood glucose was measured using Contour Next glucometer (Bayer Health Care, Mishawaka, IN) in a drop of blood collected from the tail after making a cut at 1–2 mm from the tail end. Freshly-prepared glucose solution was then administered by gavage (2 g/kg BW), and blood glucose was re-measured at 15, 30, 60 and 120 min. Area under the curve (AUC) was calculated using GraphPad Prism software (GraphPad Software Inc. V6.0c, San Diego, CA, USA). As shown in Figure S3, there were no differences in fasting glucose or oral glucose tolerance between V-HAF and PP-HAF at either 10 or 16 months of age.

Mean arterial pressure (MAP), systolic and diastolic blood pressure: Virgins and previously-pregnant control and HAF rats (10 and 16 months of age) were anesthetized with isoflurane and implanted with radio-telemetry transmitters (HD-

S10; Data Sciences International) in the abdominal aorta below the renal arteries under aseptic techniques, as we previously described [1,2]. The transmitter was secured to the abdominal muscle wall. Rats were placed into individual cages above a receiver (RLA-3000) and allowed to recover for 2 weeks. Thereafter, MAP, systolic (SBP) and diastolic (DBP) blood pressure was monitored continuously in freely-moving, conscious animals. MAP, SBP, DBP were obtained during a 10-second sampling period (500Hz), recorded and averaged every 5 minutes for 24 hours/day for 4 days using Ponemah 6.12 software.

Plasma DHT and insulin: DHT was measured in previously pregnant controls and HAF rats and virgin HAF rats (n=6/grp). Plasma DHT was measured by radioimmunoassay kit (DHT: DSL4900 Active DHT kit; Diagnostic Systems Laboratories, Inc., Beckman Coulter, RRID: AB-2732078), as we previously described [19]. Insulin was measured using ultrasensitive rat insulin ELISA kit (cat# 90060, Crystal Chem, IL, USA), as we previously described [1,2].

Isolation of mRNA and cDNA synthesis: Whole kidney samples from virgin and previously-pregnant control and HAF rats, aged 16 months, were obtained under isoflurane anesthesia, and were homogenized to harvest total mRNA. Samples were prepared as previously described [5]. Briefly, whole kidney RNA was extracted with TriReagent (MRC, Cincinnati, OH, Cat# TR-118), re-suspended in Nuclease Free-H_2O, treated with DNase (TURBO™DNAse, Thermo Fisher Scientific, Waltham, MA, Cat#AM2238), and quantified by NanoDrop™ spectrophotometry. Five micrograms of RNA were reverse transcribed (RT) with M-MuLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, Cat #K1642) in a final volume of 20µl. The reaction was performed for 30 min at 50°C and terminated by incubation at 85°C for 5 min.

mRNA expression analysis by real time (RT)-qPCR: Predesigned, gene specific primers and probe (Life technology, Cat No. 4453320) were used to quantify endothelial Nitric Oxide Synthase (eNOS, Rn02132634_S1), angiotensin converting enzyme I (ACE I, Rn00561094_m1), Angiotensin II receptor 1a (AT1aR, Rn02758772_s1), Pre-pro-Endothelin (pre-pro-ET-1, Rn00561129_m1), Endothelin receptor A (ET_AR, Rn00561137_m1), Endothelin receptor B (ET_BR, Rn00569139_m1). In addition, four housekeeping genes were also measured: Eukaryotic Elongation Factor 1 α 1 (EEF1α1, ID: Rn01445547_g1), β-Actin (ID: Rn00667869_m1), Hydroxymethylbilane Synthase (HMBS, ID: Rn00565886_m1) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, ID: Rn99999916_s1). Real-time qPCR sample reaction mix was prepared by adding 1µl of RT product, 1 µl of primer mix, 8 µl of NF water and 10 µl of Luna®Universal Probe qPCR Master Mix (NE Biolab, M3004L) which provided dNTP and Hot Start *Taq* DNA Polymerase. Amplifications were performed in a Bio-Rad CFX thermal cycler (Bio-Rad Laboratories, Hercules, CA). Cycling conditions were: 1 min at 95°C, followed by 50 cycles of 15 sec at 95°C, 30 sec at 60°C. Melting curve analysis and electrophoresis analysis using 2% agarose gel was performed to confirm specificity of PCR product. Results

are expressed as $\Delta\Delta C_t$ normalized for the geometric mean of the expression of the housekeeping genes (see Figure S2A-E).

References

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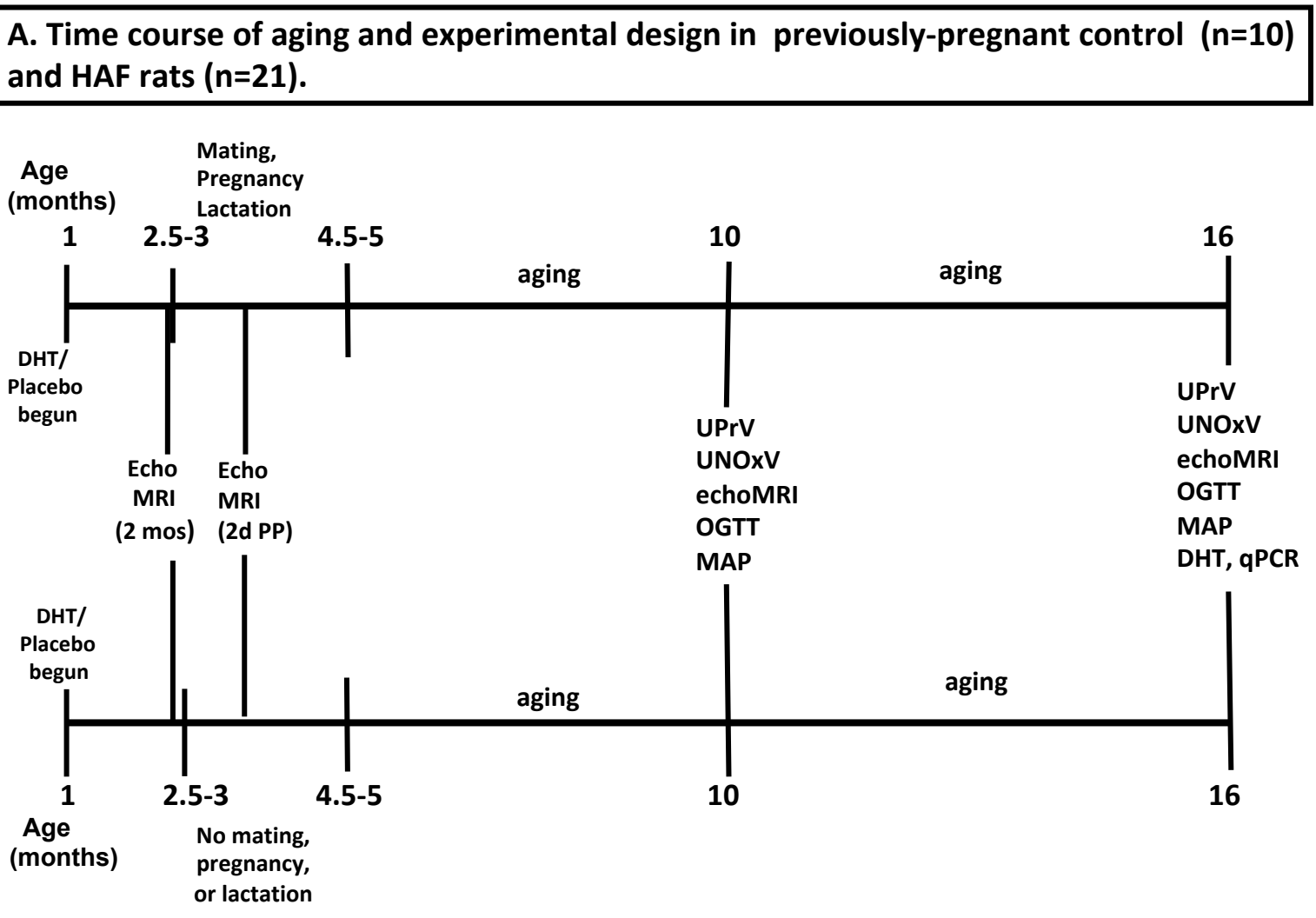

Figure S1: Schematic diagram of experimental design.

Female rats were implanted with dihydrotestosterone (DHT) or placebo pellet beginning at 1 month of age, and were reimplanted every 85 days (q 85d) throughout their lives. Prior to mating at 2 months of age, body composition was measured via Echo MRI, as described in Methods. Rats were either mated at 2.5 - 3 months of age (A) or remained virgins (B). Within 12 hours of birth, pups numbers and weights were determined. In the groups that had pre-pregnancy Echo-MRI performed, body composition was again measured using Echo-MRI 48 hrs postpartum. Following pregnancy, lactation, and weaning of offspring, dams and virgins were allowed to age to either 10 months or 16 months. At 10 months of age, One group of rats was used for mean arterial pressure (MAP), and one group was used for excretion of protein (UPrV) and nitrate/nitrite excretion (UNOxV), body composition (EchoMRI) and oral glucose tolerance (OGTT). At 16 months of age, one group of rats was used for MAP; and one group was used for UPrV, UNOxV, body composition, plasma insulin, DHT and renal tissue for qPCR.

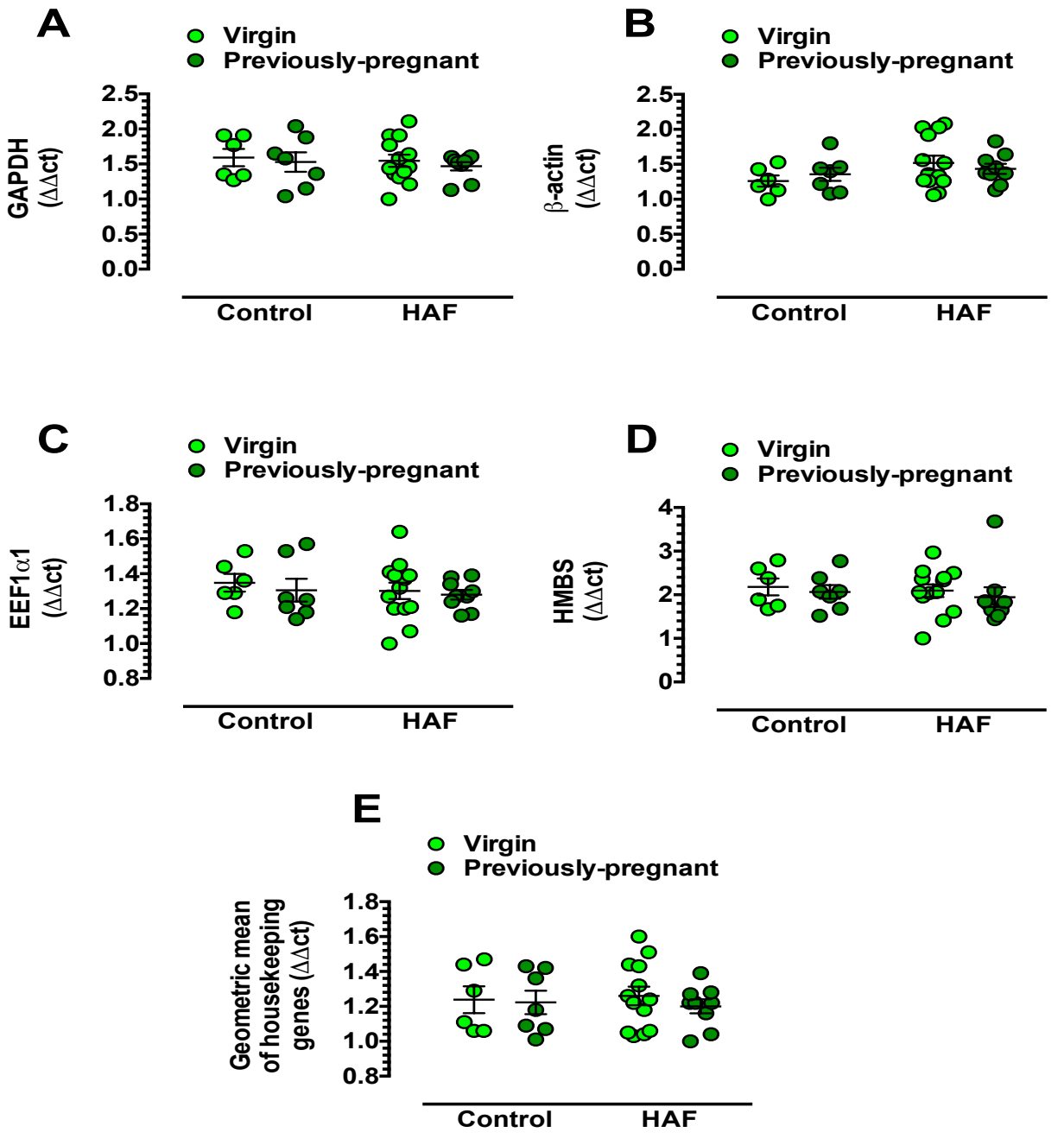

Figure S2: Comparison of the intrarenal mRNA expression by qPCR of 4 house-keeping genes and geometric mean of the four:

A) glyceraldehyde 3-phosphate dehydrogenase (GAPDH); B) β-actin; C) Eukaryotic Elongation Factor 1α1 (EEF1α1); D) hydroxymethylbilane synthase (HMBS). E) The geometric mean of the four housekeeping genes that was used for comparison of the individual mRNAs of interest. There were no statistical differences in the mRNA expressions of the housekeeping genes in HAF rats versus age-matched controls, regardless of pregnancy status.

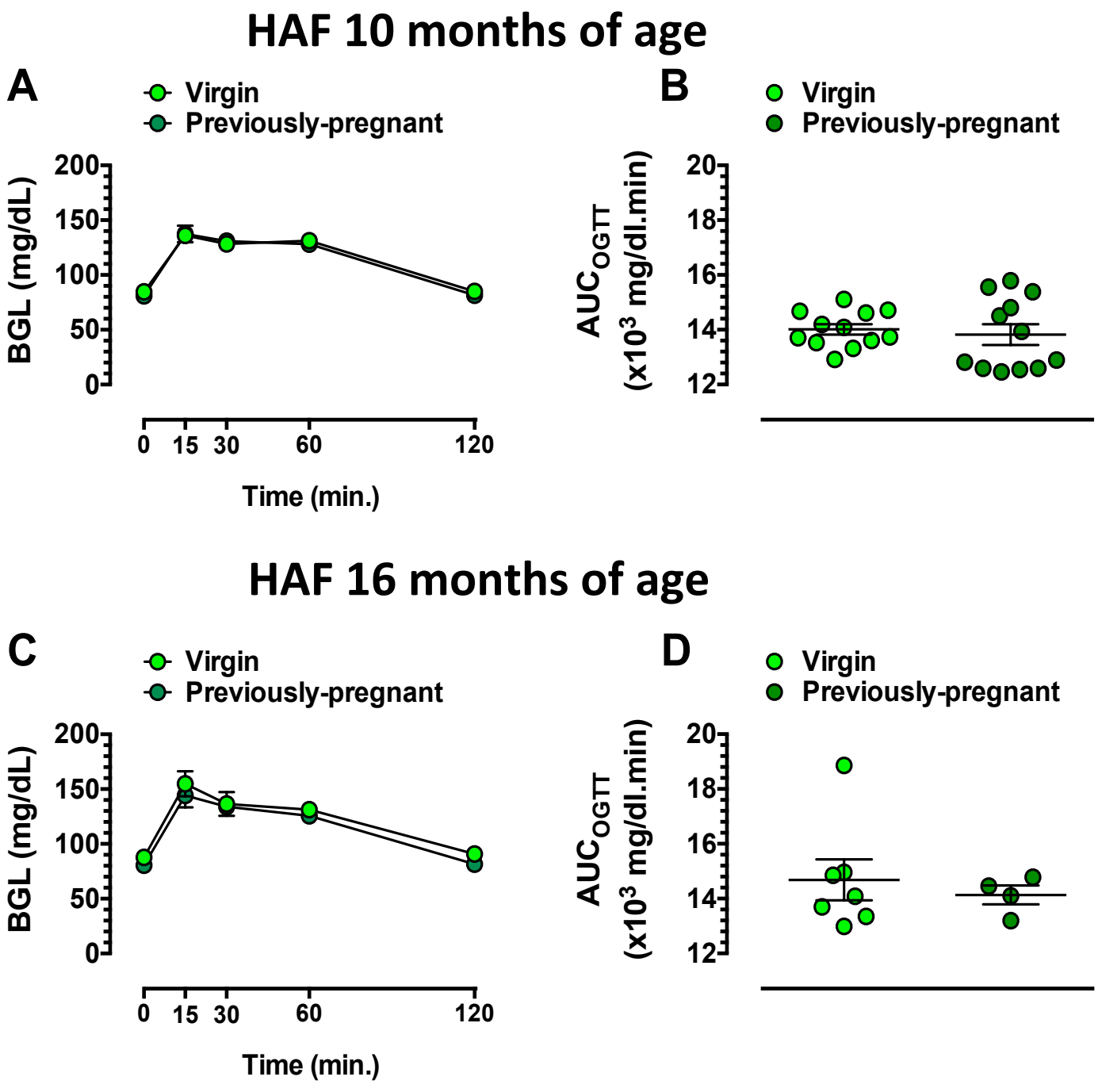

Figure S3:

Oral glucose tolerance test (blood glucose levels (BGL) with time) and area under the curves (AUC_{OGGT}) in virgin and previously-pregnant HAF rats at 10 (A, B) and 16 (C, D) months of age.

There were no differences in fasting blood glucose or response to glucose challenge between virgin and previously-pregnant HAF rats at either age nor were there differences in AUC_{OGTT}.