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Peripartum changes in the orexigenic CNS peptide neuropeptide-Y in gestational diabetics

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Abstract

<u>Background</u>: Development of relative insulin resistance during pregnancy is a normal physiologic change related to placental lactogen and pregnancy-associated elevated lipid concentrations. Several studies have investigated the role of tissue response, tyrosine kinase insulin receptor phosphorylation, and other cytokines that might contribute to development of such resistance. In this study, we specifically investigate the accompanying changes in concentrations of the orexigenic neurotransmitter neuropeptide Y (NPY) during pregnancy with the development and resolution of insulin resistance in gestational diabetics. Given that increased NPYergic activity is associated with increased eating behavior and body weight, we hypothesize levels of the central nervous system (CNS) peptide NPY will shift, along with other factors such as adiponectin, leptin, and ghrelin, in the development and resolution of gestational diabetes mellitus.

<u>Methods</u>: Antepartum and postpartum plasma concentrations of NPY, leptin, ghrelin, tumor necrosis factor-alpha, glucose, and insulin were measured in gravid patients that were healthy (N=22), class A1 and A2 gestational diabetics (N=8), or type 2 diabetics (N=4). Homeostatic Model Assessment of insulin resistance was also calculated. Data was analyzed using a t-test and considered significant with $p<0.05$.

<u>Results</u>: We demonstrate that during the third trimester, class A2 gestational diabetics display an elevated NPY concentration. This elevated NPY begins to resolve immediately postpartum, normalizing by the patient's 6 week postpartum visit. The associated Homeostatic Model Assessment of insulin resistance index (HOMA) is consistent with these observations showing a concomitant elevation of HOMA intrapartum with improved insulin resistance within 24 hours postpartum.

<u>Discussion</u>: Collectively, these results suggest a role for central nervous system involvement in the development of insulin resistance during gestational diabetes. Additionally, rising NPY levels during pregnancy may warrant routine patient surveillance for the development of occult insulin resistance and the obstetrical management of the same.

<u>Keywords</u>: Neuropeptide Y, insulin resistance, gestational diabetes, pregnancy

Introduction

Pregnancy is a unique physiological state that requires increased energy and nutritional demands in order to promote normal fetal development, increase maternal blood volume, and prepare for lactation postpartum. Various adaptations to both metabolic and food intake pathways must take place in order to maintain a positive energy balance. The increased energy requirement induces a state of hyperphagia and is further supported by the progressive development of relative insulin resistance during pregnancy [1]. Recent research shows that the potent orexigenic neuromodulator Neuropeptide Y (NPY) and the neuroendocrine axis play significant roles in both healthy and unhealthy eating behavior, weight regulation, and the development of insulin resistance [2-6]. When

such resistance reaches heightened levels and control of glycemia is lost, the result is the development of gestational diabetes mellitus (GDM). As with *pre*-existing diabetes, poorly controlled GDM may result in severe perinatal complications due to large for gestational age infants, fetal malformations, birth trauma, and fetal demise, among other conditions. GDM also contributes to poorer maternal health, increasing the risk of type 2 diabetes mellitus 50-70% over 25 years after pregnancy as well as postpartum depression [7-11]. Typically, gestational insulin resistance resolves rapidly in the postpartum period, however the molecular and cellular mechanisms that mediate this are poorly understood. The increasing prevalence of obesity in the population and the sustained risk of developing diabetes mellitus years after GDM further necessitate

investigation of the signaling pathways involved in both healthy and abnormal pregnancies.

Investigation of the signaling involved in gestational diabetes and hyperphagia has highlighted NPY, discovered in 1982 by Tatemoto and Mutt [12]. NPY is expressed at very high levels by hypothalamic neurons in the arcuate nucleus as well as in various sympathetic neurons where it functions as a neurotransmitter to mediate appetite, body fat composition, and insulin resistance through its own receptors Y1-5, inhibition of anorexigenic signals, and sympathetic activation of the liver [12-17]. It is also believed to behave as a central nervous system effector for circulating adipocyte- and gastric-derived cytokines, such as leptin and ghrelin. While there appears to be an inverse relationship between concentrations of NPY and both leptin and adiponectin, a causal effect remains unclear [18]. Studies have suggested that leptin inhibits NPY release from the hypothalamus. Accordingly, increased leptin levels concurrent with hyperphagia suggest the development of leptin resistance during pregnancy in addition to insulin resistance [2,19], leading us to investigate differential peripartum levels of this peptide as well. Other regulatory peptides of interest include ghrelin, tumor necrosis factor-alpha (TNF-α), and previously reported adiponectin [20], which all contribute to energy balance, metabolism and insulin resistance through various pathways [21-23]. Recently, TNF-α production has been demonstrated in adipose tissue and when, interacting with the hypothalamus, may also affect appetite. In mice, administration of exogenous TNF-α resulted in insulin resistance while neutralization of TNF-α reduces resistance [24,25]. It is evident that multiple complex feedback mechanisms are involved in the hypothalamic neuroendocrine regulation of eating behavior, energy balance, and consequently, the development of diabetes during pregnancy. It seems likely then that NPY expression might be associated with the pathogenesis of other metabolic conditions, including obesity, type 2 diabetes, and eating disorders, and thus its exact role in eating behavior and body fat composition deserves further investigation.

In an attempt to better expose some of the mechanisms and interactions behind the development and resolution of GDM in a qualitative way, we examined ante-and postpartum plasma levels of NPY, leptin, adiponectin, ghrelin and TNF-α in gestational diabetics, pregnant *pre*-gestational diabetics, and healthy pregnancies. In addition, we assessed insulin resistance and β-cell function to elucidate any correlation between pregnancy condition and orexigenic signals. Gestational diabetes presents a risk to both the patient and fetus, yet the mechanisms behind onset and resolution of the condition are still unclear. The interplay of many hormones in the neuroendocrine axis plays an important role in the regulation of appetite and metabolism, as well as modulating the level of insulin resistance during pregnancy. In the present study, we present differential hormone levels in GDM, type 2 diabetic, and healthy gravid patients.

Methods
Subject selection
This study included 22 healthy term gravid subjects, 8 gravid subjects with gestational diabetes, and 4 gravid subjects with type 2 (adult onset) diabetes. Type 2 diabetics are referred to as class B diabetics (having developed adult onset diabetes prior to pregnancy) based on White's Classification of Diabetes [26]. Patients with normal pre-gestational serum glucose levels were classified as gestational diabetics based on American College of Obstetrics and Gynecologic (ACOG) standard practice criteria with glucose tolerance testing, between 27-29 weeks of gestation as demonstrated by their ability to pass their one hour 50 gram glucose challenge test.. Patients in the class B, *pre*-gestational diabetic group were screened at earlier time points to assess adequate glycemic control. Confirmation of this classification was verified by continued antepartum fasting serum glucose surveillance and management with diet/or medication as warranted to maintain euglycemic levels. Within the gestational diabetics, 4 subjects were subclassified as class A1 (diet-controlled) and 4 subjects as class A2 (medication-controlled) diabetics using White's Classification of Diabetes. Subjects were enrolled at time of admission to labor and delivery for active management of labor [27], which consistently averages delivery within 12 hours of admission [28-30]. Data on glucose levels, insulin levels, and *pre*-gravid BMI were collected from a larger group of gravid patients from each subject group (healthy N=104, class A1 N=20, class A2 N=29, class B N=11) to increase statistical power and analyze whether peripartum changes in insulin resistance could be ascribed to BMI alone. This study was approved by the Institutional Review Board of the Brody School of Medicine at East Carolina University.

Sample collection and analysis
In all subjects, blood plasma concentrations of leptin, ghrelin, and NPY were measured within 24 hours of delivery (*pre*) and 24 hours after delivery (*post*) using standard I125 radioimmunoassay analysis, and using enzyme linked immunoassay for TNF-α. At these times, blood glucose was measured by photometric analysis and insulin levels were measured using a chemiluminescent immunoassay by the hospital clinical laboratory using standard methods and automated equipment. Prior to these tests, patients fasted overnight, typically between 6-12 hours. *Pre*-gravid BMI was also recorded. Blood test samples were not collected postpartum for class B, *pre*-gestational diabetic patients due to logistical issues and the absence of a clinically scheduled blood draw at 24 hours postpartum. Data are presented as means with standard error (±SE). Data was analyzed using a t-test and

Table 1. Patient characteristics.

Patient Classification	Healthy Controls	Class A1	Class A2	Class B
N	22	4	4	4
Age (yr)	27.8 ± 5.3	30.3 ± 7.1	30.6 ± 7.0	30.9 ± 3.9
Gestation (wks)	38.7 ± 0.5	39.6 ± 0.2	38.9 ± 1.3	38.1 ± 0.7
Pre-gravid BMI (kg/m^2)	33.0 ± 1.3	30.1 ± 3.1	38.3 ± 3.1	42.7 ± 2.9*
Caucasian	13	1	1	0
Non-Caucasian	9	3	3	4
Glucose (mg/dl)				
Antepartum	90.9 ± 10.3	(A1 and A2 = 65.9 ± 3.1)		113.3 ±27.6
Post partum	87.8 ± 4.8	(A1 and A2 = 103.5 ± 23.4)		ND
Insulin (pmol/L)				
Antepartum	22.5 ± 5.0	(A1 and A2 = 16.1 ± 4.6)		81.8 ± 65.0
Postpartum	14.1 ± 3.1	(A1 and A2 = 16.3 ± 7.2)		ND
HOMA Index				
Antepartum	‡6.2 ± 2.8	(A1 and A2 = 2.50 + 0.8)		4.0 ± 0.4
Postpartum	2.2 ± 0.6	(A1 and A2 = 2.20 ± 1.1)		ND

Patient Data. Blood insulin, blood glucose, and HOMA-IR data for control, class A1 and A2 (combined) gestational diabetics, and class B, pre-gestational diabetics. * Class B, pre-gestational diabetes exhibited higher pre-gravid BMI, p<0.05. Blood samples for class B diabetics were not collected postpartum due to lack of clinically indicated blood draw at this time. Blood insulin in healthy subjects showed a mild decrease post delivery, while blood glucose in class A1 and A2 gestational diabetic subjects demonstrated a marked increase. ‡ Antepartum HOMA in healthy controls is elevated secondary to skewed measurements for 2 patients. We have included these values in our final calculation, which accounts for the increased HOMA value for control patients. When these outliers are excluded, healthy control mean HOMA is 3.5, N=20.
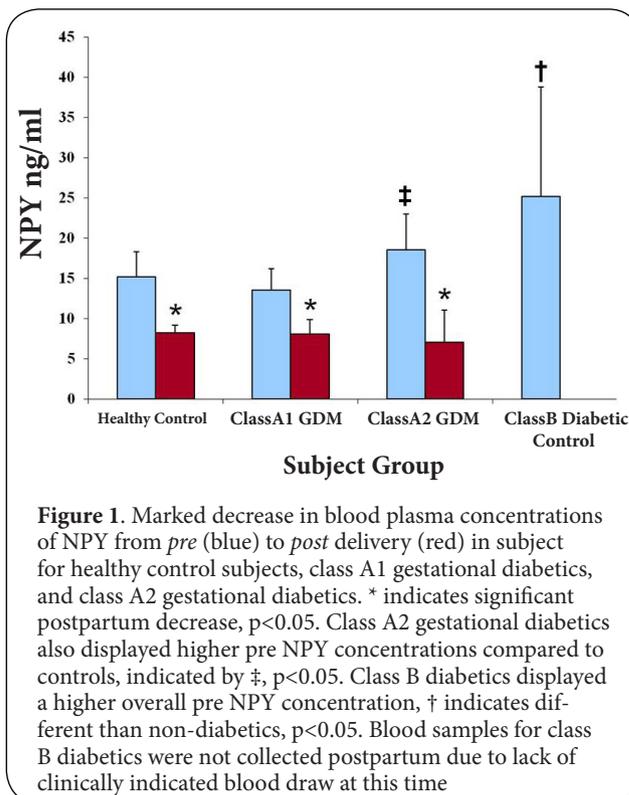

Figure 1. Marked decrease in blood plasma concentrations of NPY from *pre* (blue) to *post* delivery (red) in subject for healthy control subjects, class A1 gestational diabetics, and class A2 gestational diabetics. * indicates significant postpartum decrease, p<0.05. Class A2 gestational diabetics also displayed higher pre NPY concentrations compared to controls, indicated by ‡, p<0.05. Class B diabetics displayed a higher overall pre NPY concentration, † indicates different than non-diabetics, p<0.05. Blood samples for class B diabetics were not collected postpartum due to lack of clinically indicated blood draw at this time

considered significant with *p*<0.05.

The Homeostatic Model Assessment of insulin resistance (HOMA) was used to measure insulin resistance (HOMA-IR = [[fasting glucose in mg/dl x 0.05551] x fasting insulin in μIU/ml]/22.5) in gestational diabetic, *pre*-gestational diabetic, and healthy gravid subjects after fasting. Antepartum HOMA in healthy controls is elevated secondary to skewed measurements for 2 patients, with non-physiologic values calculated. We have included these values in our final calculation for **Table 1**, which accounts for the increased antepartum HOMA value for control patients. When these outlier data points are excluded, the mean antepartum HOMA for healthy controls is 3.5, N=20.

Results

Radioimmunoassay analysis showed a marked decrease in postpartum plasma concentrations of NPY in both healthy (**Figure 1**; *pre* 15.2±3.3 ng/ml vs. *post* 8.2±1.2 ng/ml, *P*<0.05) and class A gestational diabetic subjects (A1: *pre* 13.5±2.7 ng/ml vs. *post* 8.0±1.7 ng/ml; A2: *pre* 18.5±4.5 ng/ml vs. *post* 7.0±4.0 ng/ml, *P*<0.05). Class B diabetics had a significantly higher antepartum level of NPY compared to both controls and class A gestational diabetics, while class A2 gestational diabetics also demonstrated significantly higher antepartum NPY concentrations compared to the control group. Contrary to our hypothesis, no differences between *pre* and *post* delivery levels of leptin or ghrelin were observed (data not shown).

Comparison of ante-and postpartum HOMA calculations reveal a 37% reduction in insulin resistance for healthy subjects within 24 hours (when 2 anomolous data points are excluded), as expected for a rapid resolution of gestational diabetes after delivery. Class A1 and A2 gestational diabetics experienced a 12% reduction in insulin resistance (**Table 1**). These results demonstrate, as anticipated, a decreased HOMA postpartum for healthy and class A1 and A2 gestational diabetic patients concurrent with resolution of insulin resistance due to gestational diabetes.

Average BMI was not equal across all subject groups, therefore we also assessed the effect of obesity as a confounding factor for insulin resistance. Data from additional gravid patients from the same clinic were added for statistical analysis examining the relationship between BMI and HOMA, by subject group and by BMI categorization. Correlation of BMI and HOMA in healthy, control patients did not show a significant relationship for all BMI categories of overweight patients (BMI: 25-40+), however patients within the "normal" BMI range (20-25) demonstrated an inverse correlation with HOMA (r= -0.055, p=0.011). Due to the extensive research on relationship between obesity
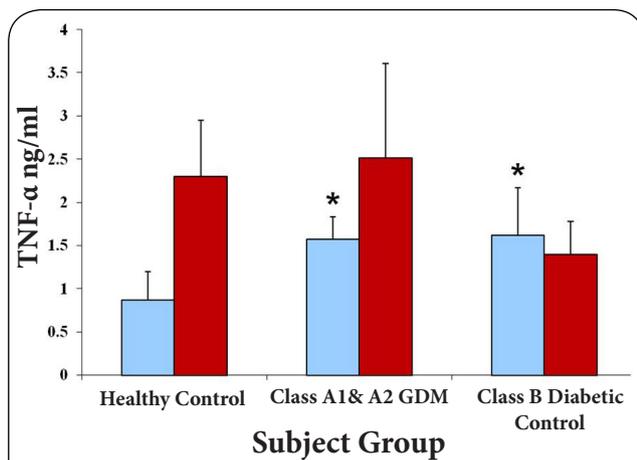
Figure 2. Plasma TNF-α concentrations increased dramatically from *pre* (blue) to *post* delivery (red) in control subjects, and class A1 and type A2 gestational diabetics (combined). Values for Class B diabetics were similar. * indicates different than non-diabetics, P<0.05.
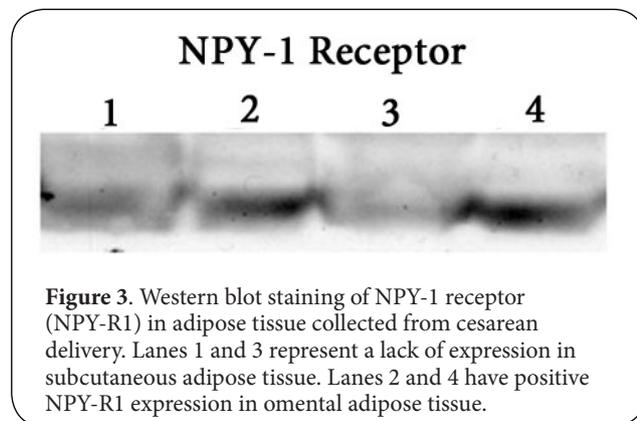

Figure 3. Western blot staining of NPY-1 receptor (NPY-R1) in adipose tissue collected from cesarean delivery. Lanes 1 and 3 represent a lack of expression in subcutaneous adipose tissue. Lanes 2 and 4 have positive NPY-R1 expression in omental adipose tissue.

Antepartum concentrations of the cytokine TNF-α were 158% higher in class A1 and A2 gestational diabetic subjects relative to controls (**Figure 2**). In both groups, a marked postpartum increase in TNF-α concentration was observed. In class B diabetics, there was no change between *pre-* and postpartum TNF-α.

Western blot staining for NPY-1 receptor (NPY-R1) was performed on both subcutaneous and omental adipose tissue collected from a Cesarean delivery. NPY-R1 proteins were detected only in omental adipose tissue (**Figure 3**).

Discussion

NPY concentrations function as a neuroendocrine signal for appetite regulation and also appear to influence insulin resistance via the hypothalamic axis based on this preliminary study. Serum levels of NPY are markedly reduced 24 hours postpartum in both gravid class A1 and A2 gestational diabetics and control subjects. It does not appear that the mere expulsion of the placenta would account for the dramatic decrease in NPY observed in these studies, as placental expression of NPY sharply decreases as pregnancy progresses past the initial period [36]. This suggests instead an acute decrease in maternal production of the peptide. This reduction seems consistent with a postpartum change in nutritional status. It likely represents a physiologic indicator that the elevated nutritional demands of pregnancy are no longer required, signaled by CNS regulation of appetite and energy balance. This preliminary study demonstrates that the clinically observed elevation in serum concentrations of neuropeptide Y in gestational diabetic patients decreases postpartum in coordination with the resolution of insulin resistance.

The detection of NPY receptors in omental adipocytes demonstrates the ability of non-CNS target tissue to respond to changing levels of NPY. The fact that these receptors are located on non-neuronal target cells suggests the presence of neuroendocrine regulation of target tissue responses in addition to interactions with other mediators of body weight, metabolism and insulin resistance. Transient dysregulation of normal tissue response or feedback mechanism may play a role in the pathophysiology of insulin resistance and gestational diabetes.

A unique feature of the pathophysiology of gestational diabetes is the relatively rapid intrapartum onset and postpartum resolution of insulin resistance. This implies reversible changes to the normal endocrine pathways that regulate homeostatic insulin responses in the body. Patients were followed at least 6 weeks postpartum and noted to return to normal fasting serum glucose concentrations. The observation of an analogous, larger NPY decrease in healthy pregnancies suggests that the pathophysiology of insulin resistance in class A1 and A2 gestational diabetes may in part be due to an aberrant feedback mechanism to the hypothalamus. This could prevent normal adjustment to the increased nutritional and energy needs of pregnancy.

and diabetes, one would expect a positive correlation between BMI and HOMA in both non-pregnant subjects [31-33] and in pregnant subjects [34,35]. Thus, this finding appears anomalous and might be due to the small sample size for "normal" BMI patients (n=19) or the regional selectivity of the sample. The only group that demonstrated a correlation that approached significance (r=0.292, p=0.094) was that of diabetic subjects with BMIs in the morbidly obese range (40+). All other BMI categories for diabetics were not significantly correlated. This suggests that variations in HOMA calculations could not be ascribed solely to patient BMI, although it is likely a contributing factor. Instead HOMA was likely influenced by the individual's progression of insulin resistance due to gestational diabetes. Thus the differential reduction in insulin resistance post-partum in healthy and diabetic subjects appears to be a true disparate physiological response to delivery.

Curiously, the control patients in our study had elevated HOMA indices, but normal postprandial glucose levels as well as euglycemic indices throughout their pregnancy. This finding may be a consequence of the small control sample size, aberrant data points in the healthy control group (refer to **Table 1**), range in patient BMIs (33.0±1.3), and differences in diet. For example, it is of note that the gestational diabetic patients were strictly diet-regulated unlike the gravid healthy controls. However, using each patient's antepartum HOMA calculation as her own control in comparison to postpartum HOMA index provides insight into the relative change in insulin resistance. From this perspective, the resolution of insulin resistance was attenuated in class A1 and A2 gestational diabetic patients compared to healthy patients, independent of the absolute value of the antepartum HOMA index. This too supports the hypothesis of inadequate metabolic adjustments to pregnancy and delivery. The lack of change in ghrelin and leptin *pre*-and *post*-delivery reflects dysregulation of normal endocrine function and feedback mechanisms while highlighting the need for added emphasis on the role of NPY in gestational diabetes. A larger prospective cohort study would help better understand these findings for this specific demographic of patients. Despite these results, measured serum NPY concentrations antepartum and postpartum were markedly and consistently different.

While the data presented here is a compelling argument warranting further investigation of this observation related to serum NPY changes, a limitation of this study arises from the small sample size and the wide HOMA indices range noted in control patients. The euglycemic insulin clamp [37] remains the ideal standard for accurately measuring insulin resistance, however is not feasible in the setting of clinical practice, especially obstetrical care, which is often constrained by the changing dynamics of labor and delivery. The oral glucose tolerance test, insulin sensitivity index, and HOMA indices have been proposed to play a reliable role in the diagnosis and surveillance of insulin resistance [38-40]. Nevertheless, in the clinical setting these approaches can have limitations based on individual patient characteristics which include BMI, diet, and presence of polycystic ovarian syndrome. Indeed at least one comparative analyses of the Insulin Sensitivity Index [38] suggests that the clinical oral glucose tolerance test insulin/glucose measurements may better correlate with ISI calculations than HOMA indices.

Several studies have shown that rising leptin concentrations decrease both the synthesis and the function of neuropeptide Y, a relationship that is consistent with the orexigenic nature of NPY. However in severely obese, polycystic ovarian syndrome (PCOS) patients it has been observed that this inverse relationship between leptin and NPY is lost. NPY levels are often elevated, yet some PCOS patients exhibit equal to or higher than expected leptin levels likely due to endocrine dysregulation [41-43] which is consistent with our observations in this study. Furthermore, NPY response to ghrelin is also defective in PCOS patients [41]. This evidence, taken together with our current study, suggests a general uncoupling of hormonal feedback mechanisms. Further investigation is warranted to elucidate these putative mechanisms.

This study's findings also imply underlying alternate mechanisms for regulating NPY concentrations related to the HOMA index. One such endocrine response is the low levels of adiponectin that were measured in these same patients [20], proposing a potential regulatory function for adiponectin on NPY concentrations independent of the leptin feedback. Adiponectin has previously been shown modulate other adiposity- and reproduction-related hormones including inhibit luteinizing hormone (LH) and gonadotropin-releasing hormone (GnRH) [44, 45] and affect insulin sensitivity [1]. Additional effectors of insulin resistance such as inflammatory cytokines may also contribute to our observations as demonstrated by the higher concentrations of TNF-α in this patient population. The role TNF-α in inflammation, acute phase reaction, and immune response are also a possible explanation as these could be an effect of labor and delivery. However, the significantly higher antepartum TNF-α levels in class A1 and A2 gestational diabetics group compared to healthy controls are consistent with the progressive development of insulin resistance during pregnancy [23].

Conclusions

In summary, these preliminary results suggest an intriguing putative function for CNS neuropeptide Y-mediated regulation of insulin resistance and metabolism during pregnancy, diabetes and even obesity. Due to the small sample size of the present study, further studies will be needed to investigate whether rising NPY serum concentrations might prove useful to monitor the early development of occult insulin resistance during pregnancy before patients manifest symptoms of gestational diabetes. This could prove useful in improving obstetrical care and follow-up for these patients.

List of abbreviations
CNS: Central nervous system
NPY: Neuropeptide Y
HOMA: Homeostatic Model Assessment
GDM: Gestational diabetes mellitus
TNF-α: Tumor necrosis factor-alpha
BMI: Body mass index
Pre: Antepartum
Post: Postpartum
NPY-R1: Neuropeptide Y-1 receptor
PCOS: Polycystic ovarian syndrome
LH: Luteinizing hormone
GnRH: Gonadotropin-releasing hormone

Competing interests
The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	RMA	JL	EN	JT	JD
Research concept and design	√	√	√	√	√
Collection and/or assembly of data	√	--	--	√	√
Data analysis and interpretation	√	--	--	√	√
Writing the article	√	√	√	√	√
Critical revision of the article	√	√	√	√	√
Final approval of article	√	√	√	√	√
Statistical analysis	--	--	--	--	--

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