Human placental metabolic adaptation to chronic hypoxia, high altitude:
Hypoxic pre-conditioning.

Martha C. Tissot van Patot 1, Andrew J. Murray 2, Virginia Beckey 1, Tereza Cindrova-Davies 2, Jemma Johns 3, Lisa Zwerdlinger 4, Eric Jauniaux 3, Graham J. Burton 2 and Natalie J. Serkova 1.

1 Department of Anesthesiology, CB 8602, 12700 E. 19th Avenue, University of Colorado Denver Health Sciences Center, Aurora, Colorado, 80045, USA; 2 Department of Physiology, Development and Neuroscience, University of Cambridge, Downing St, Cambridge, CB2 3EG, UK; 3 Academic Department of Obstetrics and Gynaecology, UCL EGA Institute for Women's Health, Royal Free and University College London (UCL Campus), 86-96 Chenes Mews, London WC1E 6HX, UK; 4 Rocky Mountain Family Practice, 35 US Highway 24, Leadville, CO 80461, USA

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Corresponding Author:

M.C. Tissot van Patot, PhD
Department of Anesthesiology, Campus Box 8202
University of Colorado at Denver and Health Sciences Center
PO Box 6511
12631 E. 17th Avenue
Aurora, CO 80045 USA
Phone: 303 724-1762
Fax: 303-724-1761
Martha.TissotvanPatot@UCDenver.EDU

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Abstract

We have previously demonstrated placentas from laboring deliveries at high altitude have lower binding of hypoxia-inducible transcription factor (HIF) to DNA than those from low altitude. It has recently been reported that labor causes oxidative stress in placentas, likely due to ischemic hypoxic insult. We hypothesized that placentas of high altitude residents acquired resistance, in course of their development, to oxidative stress during labor. Full thickness placental tissue biopsies were collected from laboring vaginal and non-laboring C-section term (37 – 41 wks) deliveries from healthy pregnancies at sea-level and 3100 m. After freezing in liquid nitrogen within 5 minutes of delivery, hydrophilic and lipid metabolites were quantified using $^{31}$P and $^1$H nuclear magnetic resonance (NMR) metabolomics. Metabolic markers of oxidative stress, increased glycolysis and free amino acids were present in placentas following labor at sea-level, but not at 3100m. In contrast, at 3100m the equivalent placentas were characterized by the presence of concentrations of stored energy potential (phosphocreatine), anti-oxidants and low free amino acid concentrations. Placentas from pregnancies at sea-level subjected to labor display evidence of oxidative stress. However, equivalent placentas at 3100m have little or no oxidative stress at the time of delivery, suggesting greater resistance to ischemia-reperfusion. We postulate that hypoxic pre-conditioning might occur in placentas that develop at high altitude.

Keywords: oxidative stress, labor, pregnancy, antioxidants, protein metabolism
Introduction

It has been postulated that pregnancy at high altitude induces placental hypoxic stress due to the lowered partial pressure of oxygen prevailing. This stress likely contributes to the greater incidence of preeclampsia reported at high altitude (14). Oxidative stress during hypoxia can activate hypoxia-inducible transcription factor (HIF), a heterodimeric transcription factor that increases the transcription of genes encoding, amongst others, glycolytic enzymes. It thereby promotes anaerobic glycolysis in order to maintain ATP synthesis under low oxygen conditions (19, 24). Reports of elevated HIF in placentas from preeclamptic pregnancies at low altitude (16) support the idea that placentas from high altitude pregnancies may be pushed towards a preeclamptic phenotype.

Surprisingly, we found that HIF activity was lower in normotensive term placentas from high compared with low altitude pregnancies (28). However, all of the placentas studied were from laboring deliveries and, recently, it was recently demonstrated that placentas subjected to labor are oxidatively stressed (4) compared to non-labored placentas delivered by cesarean-section (C-S). Thus, the acute ischemic/hypoxic stress induced by labor appeared to cause HIF activation at low, but not at high, altitude in our earlier study (28). The lower HIF activity in labored placentas at high altitude suggests that the glycolytic response to labor (acute ischemic hypoxia) is blunted in comparison with placentas from sea-level pregnancies.

We therefore hypothesized that labor at sea-level results in high oxidative stress and elevated placental glycolytic activity compared to non-labored C-S placentas, and that the same increase would not be seen in a population residing at at 3100m. To test this hypothesis, our approach was to collect placental tissue immediately following delivery from laboring and non-laboring pregnancies at sea-level and 3100 m, and to use quantitative nuclear magnetic resonance (NMR)-based metabolomics to distinguish metabolic markers and pathways involved in the placental stress response. Routine metabolomics $^1$H-NMR analysis will typically detect and quantify all proton-containing smaller molecular weight metabolites (both lipo- and hydrophilic) <1,000 Da and which are present in concentrations above 10 $\mu$M. This means that
various major amino acids, carbohydrates, ketone bodies, glycolysis products, osmolytes, antioxidants, as well as various lipids and phospholipids can be simultaneously assessed by $^1$H-NMR. The addition of $^{31}$P-NMR metabolic analysis on the same hydrophilic samples will provide a quantitative information on energy-reach phosphates (ATP, ADP, phosphocreatine) as well as precursors and catabolic products of phospholipids (phosphomonoesters, phosphodiesters) (23, 24). On average, 30-70 endogenous metabolites can be detected (non-selectively) using NMR-based metabolomics (20-23).

To test the hypothesis that response to the ischemic hypoxia stress of labor is blunted in placentas from high altitude pregnancies we anticipated changes in metabolites that are involved in responses to hypoxic and or ischemic stress. For example, hypoxia/ischemia places extreme demands on tissue energetics, and $^{31}$P-NMR is ideally suited to quantify ATP, ADP, phosphocreatine (PCr) among other phosphor-metabolites. Oxidative stress of hypoxic ischemic insult results in changes in concentrations of endogenous anti-oxidants and osmolytes such as glutathione, inositol and taurine (6, 8, 18, 26), and can affect lipid metabolism (9, 12), which are all detectable in $^1$H-NMR spectra. Protein catabolism occurs in response to acute hypoxic stress (15), and concentrations of a variety of amino acids (including, alanine, aspartate, arginine, glutamine and glutamate) can be analyzed by $^1$H-NMR. Finally, concentrations of placental glucose and lactate are also available from quantitative $^1$H-NMR on placental extracts, providing important information regarding anaerobic glucose utilization in hypoxic tissues. All of the pathways described above are reflective of changes in mitochondrial activity, which is thought to be the ‘backbone’ of the response to ischemia and/or hypoxia.
Methods

Subjects. Informed, written consent was obtained from subjects recruited at St. Vincent’s General Hospital in Leadville, Colorado, USA (3100 m), Rosie Maternity Hospital, Cambridge, UK (sea-level), and University College Hospital, London (sea-level) with the approval of the Colorado Multiple Institutional Review Board, the Cambridge Local Research Ethics Committee, and The University College London Hospitals Committee on the Ethics of Human Research. Exclusion criteria included renal disease, cardiac disease, diabetes, chronic hypertension, pregnancy-induced hypertension or any complication of pregnancy and labor prior to a C-S delivery.

A total of sixteen placentas were collected, four per each of four groups: 1) sea-level, C-S; 2) sea-level, labored; 3) 3100 m, C-S; and 4) 3100 m, labored.

Tissue Collection. The placenta was weighed immediately after delivery, and sampled using a systemic random system by which the placenta was divided into 5 areas. Two full-thickness samples were taken from each area. The samples were immediately (within 5 mins of delivery) frozen in liquid nitrogen to minimize hypoxic/ischemic artifacts (20). They were stored at -80°C until processed for nuclear magnetic resonance spectroscopy (MRS) or molecular analyses.

Nuclear Magnetic Resonance (NMR) Spectroscopy

Sample Preparation for $^1$H- and $^{31}$P-NMR Spectroscopy. Placental tissues were extracted using 8% perchloric acid (Sigma-Aldrich Co., St. Louis, MO) as previously described (22-24). Briefly: ~0.07 – 0.15 g of frozen tissues were powdered in a mortar in the presence of liquid nitrogen and added to 4 mL of ice-cold perchloric acid. After centrifugation for 20 min at 1300g at 4°C, the supernatants were collected and the pellets were re-dissolved with 2 mL perchloric acid, vortexed and centrifuged. Both supernatants, containing the hydrophilic fraction of the extract, were combined, and the mixture was neutralized (pH 7.0) using KOH before centrifuging again to remove potassium perchlorate. Supernatants with their water-soluble metabolites (polar hydrophilic compounds) were then lyophilized overnight to remove
water for NMR experiments. The extracted hydrophilic metabolites were dissolved in 0.55 mL of
deuterium oxide (D$_2$O) prior to NMR analysis. The pellets from the second centrifugation containing the
lipid fraction were dissolved in 4 mL ice-cold water and adjusted to pH 7.0 using KOH, then lyophilized
overnight to remove water for MRS experiments. The lipids were dissolved in 1.2 mL of deuterated
chloroform/methanol mixture (2:1, vol/vol) prior to $^1$H-MRS. All deuterated compounds were purchased
from Cambridge Isotope Inc. (Andover, MA).

Quantitative $^1$H-NMR and $^{31}$P-NMR Analysis. All NMR analyses were performed by the MR scientist
(NJS) who was blinded to the group assignment of the samples. For NMR analysis, the dissolved
hydrophilic and lipophilic extracts were transferred into 5-mm NMR glass tubes (Wilmad LabGlass,
Buena, NJ). The hydrophilic extracts were analyzed by high-resolution $^1$H-NMR using a 500 MHz high-
resolution Bruker DRX system equipped with Bruker TopSpin software (Bruker Biospin Inc., Fremont,
CA) (22). An inverse TXI 5-mm probe was used for all $^1$H-NMR experiments. In order to suppress water
residue in the extracts, a standard Bruker water presaturation sequence was used (“zgpr”). An external
reference, trimethylsilyl propionic-2,2,3,3,-d$_4$ acid (TMSP, 0.5 mmol/L for hydrophilic and 1.2 mmol/L
for lipid extracts), was used for metabolite quantification of fully relaxed $^1$H-MRS spectra and as a $^1$H
chemical shift reference (0 ppm). For metabolite identification in water-soluble and lipid extracts, a two-
dimensional (2D)-$^1$H, $^{13}$C-HSQC (heteronuclear single quantum correlation) NMR sequence was used.
The $^1$H-NMR peaks for single metabolites were identified and referred to a metabolite chemical shift
library. After performing Fourier transformation and making phase and baseline corrections, each $^1$H peak
was integrated using 1D WINNMR software (Bruker Biospin Inc.). The absolute concentrations of single
metabolites were then referred to the TMSP integral and calculated according to the equation:

$$\
C_x = \frac{I_x \cdot N_x \cdot C}{1:9} \times V : M
$$

where:  $C_x$ = metabolite concentration

$I_x$ = integral of metabolite $^1$H peak
N_x = number of protons in metabolite $^1$H peak (from CH, CH$_2$, CH$_3$, etc.)

C = TMSP concentration

I = integral of TMSP $^1$H peak at 0 ppm (9 since TMSP has 9 protons)

V = volume of the extract

M = weight of placental tissue sample

The final metabolite concentrations were expressed as μmol per g placental tissue.

The water-soluble (hydrophilic) placental extracts were additionally analyzed by $^{31}$P-NMR spectroscopy immediately after $^1$H-NMR and addition of 100 mmol/l EDTA to chelate divalent ions bound to ATP (22). Phosphorous spectra were obtained on a Bruker 300 MHz Advance spectrometer ($^{31}$P-NMR frequency: 121.5 MHz) equipped with a 5-mm QNP $^{31}$P/$^{13}$C/$^{19}$F/$^1$H probe using a composite pulse-decoupling (CPD) program. An external standard in a thin capillary, methyl diposphoric acid (MDP, 2.3 mmol/L D$_2$O, Sigma-Aldrich), was placed into the NMR tube to serve as a reference for both chemical shift (18.6 ppm) and phosphor metabolite quantification (see above).

Statistics. A prospective power test indicated that in order to detect a 50% difference between means in 4 groups, at 80% power ($\alpha \leq 0.05$, STD = 0.5), a total n = 16 or n = 4 per group was needed.

Differences between group means (altitude and/or labor) were determined by one-way analysis of variance with Sheffe’s post hoc analyses. Significance was set at $p < 0.05$ for all statistical analyses.
**Results**

Women from sea-level and 3100m were of similar age and body size, had similar gravidity and parity, and there were no significant differences in gestational age, birthweight, blood pressure or length of labor (Table 1). All neonates had APGAR scores between 7 and 9.

**Energetic State.**

There were no differences in ATP or ADP with altitude, although the ATP/ADP ratio was lower at high altitude (Figure 1). Glucose and lactate concentrations were similar between non-labored placentas at the two-altitudinal levels, indicating no change in the balance of aerobic and anaerobic metabolism prior to delivery (Figure 2). Phosphocreatine (PCr) trended higher with increased altitude.

Labor at sea-level was associated with 2.5 fold higher placental concentrations of ATP, ADP, glucose, lactate, and PCr compared to the C-S controls (p < 0.0001, and = 0.014, 0.0007, 0.015, and 0.022, respectively) but there was no increase in the ATP/ADP ratio (Figure 1). In contrast, glucose was only slightly elevated following labor at high altitude compared to the C-S controls, as was the ATP/ADP ratio (Figure 1; p < 0.0001, and = 0.015, and 0.004, respectively). There were, however, no changes in ADP, lactate, or PCr.

Despite variable changes in ATP and ADP concentrations, the adenylate energy charge remained equivalent between all groups.

**Oxidative Stress Markers.**

The concentrations of the placental osmolytes and antioxidants taurine and inositol, were greater in non-labored placentas at 3100 m vs. sea-level (Figure 2; p = 0.023), but there was no difference in glutathione. The concentration of MUFA trended lower at 3100m, while concentrations of PUFA and cholesterol were greater. Accordingly, the PUFA/MUFA ratio was greater at 3100m compared with sea-level.

Labor was associated with a 2-3 fold greater level of glutathione at sea-level and higher cholesterol, with no change in placentas at high altitude (Figure 2). At 3100 m the concentrations of taurine and inositol
were lower following labor, whilst the level of PUFA was greater (p = 0.023, 0.014, < 0.0001 and < 0.0001, respectively).

*Amino Acids.* The concentrations of amino acids were similar at the two-altitudinal levels, with the exception of glutamine, which was greater at high altitude (Figure 3, p = 0.002).

Placentas laboring at sea-level had greater concentrations of alanine, arginine, glutamate, glutamine and aspartate than C-S placentas (Figure 3; p = 0.002, 0.007, 0.0003, 0.0002, and 0.0005, respectively). However at 3100 m there were no differences in amino acid concentrations between labored and non-labored placentas. Consequently, labored placentas at sea-level had consistently greater amino acid concentrations than the labored placentas from pregnancies at 3100 m.
Discussion

Our results demonstrate that placentas from 3100 m do not show metabolic evidence of hypoxia-induced stress, irrespective of whether they are labored or delivered by C-S. In contrast, placentas from sea-level had significant evidence of glycolysis, oxidative stress and stress-induced protein metabolism following labor. In their review, Hochachka et al. (11) concluded that metabolic adaptation to chronic hypoxia is characterized by cells and tissues altering their metabolism to reduce production of, and demand for, ATP. In the current study, term placentas from all pregnancies at 3100m had metabolic profiles that differed from those at sea-level, suggesting that adaptation to chronic hypoxia had occurred. Further, when challenged with acute ischemic hypoxia (labor) the metabolic profiles resembled that of tissue preconditioned to hypoxia (11).

Placental Energetics. In comparing C-S and laboring placentas at sea-level, it is apparent that the labor process produced greater ATP and ADP concentrations and elevated glycolytic activity. This probably reflects a stress-induced activation of anaerobic glycolysis in order to compensate for high-energy demand. Indeed, ATP/ADP ratio and adenylate energy charge were maintained. These findings support the premise that labored placentas have experienced a hypoxic ischemic insult, consistent with the findings in a recent report from Cindrova-Davies et al. (4).

Placentas from non-labored high altitude pregnancies had lower ATP/ADP ratios than those at sea-level. However, labor at 3100m generated greater ATP and ATP/ADP ratios, without large changes in glucose or lactate. The trend toward higher concentrations of phosphocreatine (PCr) at 3100m suggests that placentas were pre-conditioned to store energy for use during ischemic/hypoxic stress. Analogous to our findings in high altitude placentas, chronic hypoxia results in increased creatine phosphate concentrations in rat hippocampal slices (17). PCr, used as an energetic reserve to maintain ATP levels, can inhibit ADP-stimulated mitochondrial respiratory activity (29). Thus the greater PCr concentration at 3100 m, most likely plays a role in lowering glycolytic activity, and reducing ATP production both prior to and during labor.
Lactate Paradox. Following acclimatization to high altitude, a phenomenon, known as the lactate paradox, describes a lower circulating lactate concentration in individuals following exercise at a given work load than that seen in the same individual when acutely hypoxic and unacclimatised (30). In the current study, we observed a similar pattern in acclimatized placentas from pregnancies at 3100 m, which, when stressed by labor, did not yield the elevated lactate concentrations seen in stressed sea-level placentas. This is possibly explained by the notion that acclimatization to hypoxia leads to tighter coupling of ATP demand and production, possibly via mitochondrial adaptation, leading to a decreased reliance on anaerobic glycolysis (10). In the current study, higher ADP at sea-level may have stimulated mitochondrial respiration and thus ATP production (29). Conversely, at 3100 m, the lower ADP concentration may have played a role in blunting the stimulation of mitochondrial respiration, as evidenced by decreased glycolysis and ATP production in response to labor stress.

Overall, it appears that labor resulted in a standard metabolic response to ischemia and/or hypoxia characterized by glycolysis and increased mitochondrial respiration at sea-level. In contrast, the metabolic stress response was blunted in placentas from pregnancies at 3100 m, yet ATP/ADP ratios were maintained and even elevated (Figure 4).

Oxidative stress. Non-stressed placentas from high altitude vs. sea-level pregnancies had greater, or trends towards greater, concentrations of taurine and inositol, suggesting greater potential antioxidant capacity and volume regulation. Labor at sea-level produced greater total glutathione (Unfortunately, NMR only detects total glutathione and the ratio of GSH/GSSG is unknown), suggesting the need for antioxidants during ischemic hypoxic insult. Interestingly, cholesterol elevated with labor at sea-level, but not 3100m. Cholesterol is elevated following renal ischemic reperfusion injury in association with mitochondrial damage and apoptosis (13, 31, 32). Reports suggest that increasing cholesterol in response to mitochondrial stress may be a protective mechanism during ischemic reperfusion injury (13, 31). Our data indicating greater cholesterol at altitude may reflect another preconditioning effect in response to gestation at 3100m.
Overall, MUFA was greater at sea-level while PUFA was greater at 3100m, resulting in higher PUFA/MUFA ratios at 3100m. High [PUFA/MUFA] ratios represent a saturation index which inversely correlates with lipid peroxidation (low LPO). Greater concentrations of PUFA suggest a pre-conditioning protective adaptation against LPO. In chronically hypoxic tissue high PUFA concentrations protect membrane integrity (12). In response to an acute hypoxic ischemic insult, such as in kidney transplant tissue, damage from LPO lowers PUFA concentrations (21).

Protein metabolism. Concentrations of placental amino acids, with the exception of glutamine, were the same at sea-level and 3100 m. Placentas stressed by labor at sea-level had consistently greater amino acid concentrations than non-stressed placentas, suggesting protein catabolism was utilized as an energy source, as generally occurs during acute hypoxic stress (15). However, at 3100 m amino acid concentrations were not different between laboring and non-laboring placentas. These data suggest that proteins are not utilized as a significant fuel source during acute ischemic hypoxic stress in placentas adapted to 3100 m.

Energetic, oxidative stress and amino acid metabolic pathways are complex and inter-related. Examining the data from the current study in these simplified metabolic systems provides insight into specific pathways that may be important to placental adaptation to chronic hypoxia. The current data provide a snapshot in time of the metabolic processes occurring, and because birth is a time of great physiologic change these metabolites are likely in continual flux. Across the differing conditions of altitude and labor, three primary metabolic pathways (energy, oxidative stress and protein metabolism) are consistently affected and appear to play a role in placental adaptation to chronic hypoxia.

In general, placentas supporting healthy fetuses at altitude develop mechanisms that efficiently handle acute ischemic/hypoxic stress. Our data suggest that metabolic adaptations include developing energy reserves, elevating antioxidant capacity, and reducing the stress-induced inhibition of protein synthesis (Figure 5). Mitochondrial activity is integral to all of these processes (1, 2, 5, 25, 27), critical to cellular
response to hypoxia (3, 7) and is likely a large contributor to the preconditioning in placentas at high altitude observed in this study.

Conclusion

Placentas from high altitude normotensive pregnancies are exposed to a lower maternal arterial PO$_2$ and oxygen saturation throughout development yet support healthy fetuses. The lack of oxidative stress in response to ischemia/hypoxia (labor) suggests that adaptation blunts the stress response of placental tissues to acute hypoxia. It seems unlikely that a placenta could support a healthy fetus for nine months while in a state of chronic hypoxic stress that would induce strong glycolytic metabolism. Thus, our data suggest that by term, the placenta at high altitude has moderated its response to low oxygen delivery in order to sustain a healthy fetus.

Acknowledgements

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References


Figure Legends

**Figure 1.** Energy metabolites in term placental tissue from pregnancies delivered by non-laboring C-section (C-S) and laboring vaginal procedures at sea-level and 3100 m. When letters above the columns differ, columns are significantly different (p < 0.05). Data expressed as mean ± standard deviation.

**Figure 2.** Anti-oxidant reserves and metabolic markers of hypoxia in term placental tissue from pregnancies delivered by non-laboring C-section (C-S) and laboring vaginal procedures at sea-level and 3100 m. When letters above the columns differ, columns are significantly different (p < 0.05). Data expressed as mean ± standard deviation.

**Figure 3.** Amino acid concentrations in term placental tissue from pregnancies delivered by non-laboring C-section (C-S) and laboring vaginal procedures at sea-level and 3100 m. When letters above the columns differ, columns are significantly different (p < 0.05). Data expressed as mean ± standard deviation.

**Figure 4.** Potential mechanism of placental adaptation to pregnancy at 3100 m, and the metabolic responses to labor stress based on the results of the current study.

Table Legend

**Table 1.** Characteristics of pregnancies

Data are presented as mean (± standard deviation (SD), * indicates significantly different (α < 0.05) from normotensive pregnancies, BMI = Basal Metabolic Index, NA not applicable
Figure 4. Potential mechanism of placental adaptation to pregnancy at 3100 m, and the metabolic responses to labor stress based on the results of the current study.
Table 1. Characteristics of pregnancies

<table>
<thead>
<tr>
<th></th>
<th>Sea-level C-S Normotensive</th>
<th>Sea-level Labor Normotensive</th>
<th>3100 m C-S Normotensive</th>
<th>3100 m Labor Normotensive</th>
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<tr>
<td>Birth Weight (kg)</td>
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<td>3.48 (0.403)</td>
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<td>Maternal Age (yrs)</td>
<td>30 (6)</td>
<td>34 (3)</td>
<td>31 (5)</td>
<td>27 (9)</td>
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<td>BMI (kg/m²)</td>
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<td>23 (3.7)</td>
<td>26 (4.9)</td>
<td>22 (6.6)</td>
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<td>Systolic</td>
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<td>126 (11)</td>
<td>116 (12)</td>
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<td>76 (6)</td>
<td>74 (6)</td>
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<td>2.5 /1.8</td>
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<td>Gestational Age (wks)</td>
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<td>39 (3.0)</td>
<td>40 (0.5)</td>
<td>39 (0.9)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (SD), * indicates significantly different (α < 0.05) from normotensive pregnancies, BMI = Basal Metabolic Index, NA not applicable