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The effect of exercise and metformin treatment on circulating free DNA in pregnancy

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ABSTRACT

Introduction: Some pregnancy complications are characterized by increased levels of cell-free fetal (cffDNA) and maternal DNA (cfmDNA), the latter may also be elevated during physical strain. This study aims at assessing the impact of exercise and metformin intervention in pregnancy, and to compare the levels of cell free DNA in pregnant women with or without PCOS diagnosis.
Methods: Consecutive women from two previous randomized controlled trials in pregnancy were included. Women came from a trial with organized exercise vs. standard antenatal care in pregnancy and a trial of metformin vs. placebo in PCOS women. Levels of cffDNA, cfmDNA and cell-free total DNA (cftDNA) were measured by qPCR.
Results: Training in pregnancy did not affect the levels of cffDNA, cfmDNA or cftDNA.
PCOS-women treated with metformin had lower levels of cfmDNA and cftDNA at week 32 (mean ± SD: 301 ± 162 versus 570 ± 337, $p = 0.012$, 345 ± 173 versus 635 ± 370, $p = 0.019$); otherwise the levels were comparable to PCOS-controls. Metformin-treated PCOS-women had higher cffDNA at inclusion, in the 1st trimester; later on in pregnancy the levels in the metformin and placebo groups were equal.
A comparison of pregnant women in the exercise study (TRIP) to placebo-treated pregnant PCOS-women, showed the levels of cffDNA, cfmDNA or cftDNA during mid-pregnancy (weeks 18–36) to be equal.
Discussion: Training during pregnancy was not associated with altered levels of cffDNA cfmDNA or cftDNA, but metformin treatment may reduce cfmDNA and cftDNA in pregnant PCOS women.

1. Introduction

From the 5–7th gestational week cell free fetal DNA (cffDNA) is detectable in the maternal circulation and increases during gestation [1,2]. In the first trimester cffDNA may increase as much as 21% per week [3]. After delivery it rapidly disappears, indicating that placenta is the main source of origin [2].

Elevated levels of cffDNA in maternal blood have been associated with gestational complications such as preeclampsia, preterm birth, intrauterine growth restriction and miscarriages. The main source under such circumstances is postulated to be leakage from damaged apoptotic fetal cells [4–9]. Maternal age, race, parity, placental volume and type of conception (in vitro fertilization versus natural conception) do not seem to influence cffDNA levels [3,10]. Reports on the influence of maternal BMI on cffDNA are conflicting [3,11,12]. A recent study in 406 women with low risk pregnancies found no relation between maternal weight and cffDNA in mid-pregnancy [11].

Abbreviations: TRIP, Training in Pregnancy study; PregMet, Metformin treatment in pregnant PCOS women study; cffDNA, cell-free fetal DNA; cfmDNA, cell-free maternal DNA; cftDNA, cell-free total DNA; qPCR, quantitative real-time polymerase chain reaction; PCOS, polycystic ovary syndrome; BMI, body mass index.

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Physical strain in men has been shown to increase the levels of cell free total DNA immediately after exercise. Recently a similar increase in maternal cell free DNA (cfmDNA) was observed in 9 pregnant women following physical strain, but there were no significant elevations of cffDNA 30 min after the end of the exercise, where some participants had increases in cffDNA whereas others had a decrease [13,14].

Pregnant women with polycystic ovary syndrome (PCOS) have higher risk of preeclampsia (3.5-fold) and preterm delivery (1.8-fold) [15–18]. Metformin treatment may reduce preterm delivery in PCOS women [19]. Although, no effect was found when metformin was tried out in a randomized controlled trial, an epi-analysis combining two studies achieved sufficient power to report a significantly lower rate of preterm delivery/second trimester miscarriages in metformin-treated women with PCOS [19,20].

It is assumed that PCOS-women may have impaired decidua trophoblast invasion related to testosterone levels and insulin-resistance [21]. If this is followed by a breakdown of the placental barrier, trophoblasts and cffDNA may pass into the maternal circulation. No previous study has addressed the levels of cfmDNA and cffDNA in PCOS women with or without metformin treatment in pregnancy.

The aims of the present study was to investigate whether a moderate 12-week exercise program during pregnancy had impact on the levels of cffDNA, cfmDNA and cftDNA during the intervention period, and to study any associations between levels of cffDNA, cfmDNA and cftDNA and metformin treatment of pregnant PCOS women.

2. Material and methods

2.1. Study population

The present study included the first 51 women who participated in the "Training in Pregnancy" (TRIP) study [22]. The TRIP study included 855 women older than 18 years with singleton pregnancies at around 18 weeks of gestation. Women in the TRIP study were randomized to a 12 week exercise program (intervention group) or standard antenatal care (control group) [22]. The intervention included 1 h of organized exercise weekly, composed as follows: 30–35 min of low-impact aerobics, 20–25 min of strength exercises, 5–10 min of light stretching, body awareness, breathing, and relaxation exercises. In addition participants in the intervention group were urged to follow a written 45-min home exercise program at least twice per week (30 min of endurance training and 15 min of strength and balance exercises). Control group women were offered standard antenatal care and were not discouraged from training during pregnancy. All study women were re-examined at gestational week 32–36.

Women with high risk pregnancies and/or diseases that could interfere with participation or were living to far from the hospital to take part in the exercise program were formerly excluded.

The second half of the study population comprised 31 pregnant women with PCOS from the "Metformin treatment in pregnant PCOS women" (PregMet) [23,24]. The PregMet study included 257 women 18–42 years of age with singleton pregnancies at 5th–12th week of gestation. Women were randomized to either 2000 mg of metformin daily or placebo to be taken until delivery [23,24]. PCOS was diagnosed before the pregnancy by a gynecologist according to the Rotterdam Criteria. Exclusion criteria were elevated liver enzymes or serum creatinine, alcohol abuse, known diabetes, use of glucocorticoids, or drugs known to interfere with the use of metformin.

In the present study only women from the PregMet study and the TRIP study who delivered a boy were included. The laboratory staff performing the analyses was blinded to whether the participants were allocated to treatment with metformin, but not blinded for whether the women were in the exercise group or not.

No power calculation was done prior to the study. We aimed to analyze around 50 samples from each of the two studies (TRIP + Pregmet).

2.2. Laboratory procedures

2.2.1. Blood sampling

EDTA blood samples were collected after an overnight fast; in PCOS women at gestational weeks 5–12, 19, 32 and 36, and in TRIP women at gestational weeks 18–22 and 32–36. The samples were centrifuged at 200 G for 10 min, then at 1600 G for 10 min. Thereafter the supernatant was transferred to a new tube and centrifuged for another 10 min at 1600 G and the plasma stored in clean polypropylene tubes at −80 °C [25]. To secure a safe plasma treatment, the acceleration and deceleration of the centrifuge was set to be zero.

2.2.2. DNA extraction

After thawing, the samples were centrifuged at 1100 g for 3 min. Plasma DNA was isolated from 300 to 800 μl of the plasma samples using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, Spain) according to the blood and body fluid protocol supplied by the manufacturer. Extracted DNA was run the same day as the extraction [26].

2.2.3. Real-time quantitative polymerase chain reaction (PCR

A quantitative real-time polymerase chain reaction (qPCR) was used to measure cftDNA and cffDNA, the latter using DYS14 [27–29]. The DYS14 sequence is a Y-specific multi-copied sequence and therefore increases the qPCR sensitivity for detection of the Y chromosome at low concentration [28]. DYS14 gene primers and probe were obtained from Applied Biosystems®, and was reconstituted with TE-buffer (Sigma, pH 8) to 100 μM and then diluted with distilled water to 10 μM. We used the following DYS14 primers: forward 5′-GGG CCA ATG TTG TAT CCT TCT C-3′, reverse 5′-GCC CAT CGG TCA CTT ACA CTT C-3′and probe: 5′(FAM)-TCT AGT GGA GAG GTG CTC-3′ [28,29].

β-globin gene primers and probe were obtained from Sigma and were reconstituted with distilled water to 100 μM and then diluted with distilled water to 10 μM. We used the following β-globin primers: forward 5′-GTG CAC CTG ACT CCT GAG GAG A-3′, reverse 5′-CCT TGA TAC CA ACCT GCC CAG-3′and probe: 5′(HEX)-AAG GTG AAC GTG GAT GAA GTT GGT GG-3′ [28,29].

2.2.4. Standard curves

A human male control DNA (Promega) was used to prepare an eight-point standard curve in the range 0.5–2500 genome-equivalents/μl. Each dilution was aliquoted into small volumes, and stored at −80 °C [26]. Standard curves were constructed by plotting the C_T as a function of the log of the template copy number: DYS14: $C_T = -\text{slope } x \log(\text{copies}) + b$; and β-globin: $C_T = -\text{slope } x \log(\text{copies}) + b$.

The concentrations of DNA are presented in genome-equivalents/ml plasma (GE/ml). One genome-equivalent counts for 6.6 pg of DNA, corresponding to the amount of DNA from one diploid male cell [29].

The initial quantity of DNA in each unknown sample was calculated based on the standard curve, the elution volume from the DNA extraction column and the starting volume of plasma.

Each run for both DYS14 and β-globin included an eight-point standard curve (0.5 GE to 2500 GE), one positive control (DNA isolated from plasma from a pregnant woman carrying a male fetus), one negative control (DNA isolated from plasma from a pregnant woman carrying a female fetus), DNA from a male, blanks and samples. All samples were analyzed in triplicates.

A result was regarded as positive only if all triplicates were positive for DYS14. The mean of each triplicate was used for further concentration calculations. Absolute quantification of DNA was obtained by interpolation of the experimental data on a standard curve generated with reference genomic DNA [30].

2.3. Statistical analyses

All statistical procedures were performed using the PASW version 20 (IBM, SPSS, Armonk, NY, USA). The differences between the study groups were compared with two-tailed Mann–Whitney *U* tests for independent samples. Linear regression analyses were used to adjust for time point of blood sampling. Values are reported as means ± SD. *p*-values <0.05 were considered significant. All together 30 comparisons are reported with respective *p*-values. No adjustments for multiple comparisons were performed.

3. Results

We found no association between cffDNA, cfmDNA and cftDNA levels and training during pregnancy. The DNA levels increased with increasing gestational age, but there was no difference between training and control group women at 32–36 weeks gestation after a 12-week training program during pregnancy (Table 1).

PCOS-women randomized to metformin in pregnancy, had significantly higher levels of cffDNA at inclusion compared to those randomized to placebo (22.6 ± 13.5 versus 13.5 ± 7.2, $p = 0.037$). Later in pregnancy the levels were comparable between the groups (Table 2).

At gestational week 32, placebo treated PCOS-women had higher levels of cfmDNA (570 ± 337 GE/ml vs. 301 ± 162 GE/ml, $p = 0.012$) and cftDNA (635 ± 370 GE/ml vs. 345 ± 173 GE/ml, $p = 0.019$) than women in the metformin group.

After adjusting for gestational length, there were no significant differences between mean cffDNA in placebo-treated pregnant