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**Research article**

# Endocrine Disruptors and Polycystic Ovary Syndrome: Phthalates

**Akın L et al. Polycystic Ovary Syndrome and Phthalates**

Leyla Akın[1,2], Mustafa Kendirci[1], Figen Narin[3,4], Selim Kurtoglu[1], Nihal Hatipoglu[1], Ferhan Elmalı[4,5]
[1] Erciyes University Faculty of Medicine Department of Pediatric Endocrinology, Kayseri, Turkey
[2] Ondokuz Mayıs University Faculty of Medicine Department of Pediatric Endocrinology, Samsun, Turkey
[3] Erciyes University Faculty of Medicine Department of Biochemistry, Kayseri, Turkey
[4] Katip Çelebi University, İzmir
[5] Erciyes University Faculty of Medicine Department of Bioistatistics, Kayseri, Turkey

**What's known on this subject?**
In animal and in-vitro studies, there are some significant findings suggesting that DEHP and/or MEHP might play a role in the aetiopathogenesis of PCOS. There are only a few human studies investigating the relationship between PCOS and phthalates in the literature.

**What this study adds?**
We found significant correlations among DEHP/MEHP and insulin resistance in PCOS patients suggesting that phthalates might have a possible effect on energy metabolism in this population.

**ABSTRACT**
**Aim:** We aimed to investigate a possible role of endocrine disruptors phthalates, di-2-ethylhexyl phthalate (DEHP) and mono (2-ethylhexyl) phthalate (MEHP), in polycystic ovary syndrome (PCOS) aetiopathogenesis. We also wished to evaluate the relationship between phthalates and metabolic disturbances in adolescents with PCOS.
**Methods:** A total of 124 adolescents (63 PCOS, 61 controls, mean age: 15.2±1.5 age range: 13-19 years) were included in the study. Serum MEHP and DEHP levels were determined with HPLC method. Insulin resistance was evaluated using HOMA-IR, QUICK-I, fasting glucose /insulin ratio, Matsuda index, and total insulin levels during OGTT. Participants were further subdivided into lean and obese subgroups according to body mass index.
**Results:** Serum DEHP and MEHP levels were not significantly different between PCOS and control groups. The mean (95% CI) values of DEHP and MEHP were 2.62 (2.50-2.75) µg/ml vs 2.71 (2.52-2.90) µg/ml and 0.23 (0.19-0.29) µg/ml vs 0.36 (0.18-0.54) µg/ml in PCOS and the control groups respectively, p>0.05). The correlation analysis adjusted for BMI revealed that both phthalates significantly correlated with insulin resistance indices and serum triglycerides in adolescents with PCOS.
**Conclusion:** Serum DEHP and MEHP concentrations were not different between adolescents with or without PCOS. However, these phthalates are associated with metabolic disturbances such as dyslipidemia and insulin resistance, independently of obesity, in girls with PCOS.
**Keywords:** phthalate, di-(2-ethylhexyl)-phthalate, mono-(2-ethylhexyl)-phthalate, endocrine disrupter, polycystic ovary syndrome

**Address correspondence to:** Leyla Akın Yenimahalle Gökkuşağı sitesi B-1 Atakum Samsun
leylabakin@gmail.com
+90 5332401643
23.03.2020
11.05.2020
https://orcid.org/0000-0002-8071-426X

## INTRODUCTION

Phthalates are a group of industrial chemicals that are commonly used as plasticisers in the production of soft toys, flooring, food packages, paints, plastic bags, medical devices, cosmetics, and air fresheners (1). Di-2-ethylhexyl phthalate (DEHP), the most commonly used phthalate, and its metabolites including mono (2-ethylhexyl) phthalate (MEHP) are known to be endocrine disruptors and have been related to some health problems such as obesity (2), abnormal genital development (3), low semen quality (4), precocious puberty (5), and gynaecomastia (6) in humans.
Polycystic ovary syndrome (PCOS) is a common endocrine disorder characterised by menstrual irregularity, hyperandrogenism and polycystic ovaries. The pathogenesis of the disorder has not yet been fully clarified. In several animal studies, DEHP exposure has been shown to result in prolonged oestrous cycles and decreased ovulation rate (7, 8). Davis et al. demonstrated that DEHP exposure caused hypo-oestrogenic, hypo-progestinic anovulatory cycles in previously regularly menstruating rats. Morphologically polycystic ovaries developed in these rats (8).
There are only a few human studies investigating the relationship between PCOS and phthalates in the literature, however, they have conflicting findings (9-12).
In the present study, we aimed to evaluate the serum MEHP and DEHP concentrations in adolescents with PCOS. In addition, we wished to investigate the possible relationship between these endocrine disruptors and metabolic abnormalities in this population.

## METHODS

The study was approved by the ethical committee of Erciyes University (Approval no: 211-159). Informed consent and assent were obtained from all parents and adolescents, respectively.
A total of **63 adolescent** girls with PCOS who presented to the Paediatric Endocrinology Outpatient Clinic at Erciyes University Faculty of Medicine due to irregular menstrual periods and/or hirsutism between January 2011 and August 2012 were enrolled in the study. (**36 obese, 27 lean**, **mean age:15.3±1.3, range:13-19 years**). The control group consisted of 61 age-matched healthy female adolescents (35 obese and 26 lean) who had regular menses and no hirsutism. Exclusion criteria were the presence of any major disease, history of any antiandrogenic or insulin sensitising drug usage within the past one year and the current use of any medication.
The participants partly served as the study population of another study which was published previously in which the methods of anthropometric measurements, assays, and the definitions of obesity, PCOS and insulin resistance were mentioned in detail (13). In brief, PCOS was diagnosed using modified Rotterdam criteria (14), and obesity was defined as BMI ≥ 95th percentile according to age and sex (15). Subjects required any two of the following three criteria to be diagnosed with PCOS: oligo/anovulation, clinical and/or biochemical evidence of hyperandrogenism and polycystic ovarian morphology (PCOM) on ultrasound, with other endocrinopathies excluded. At the beginning of the study 112 adolescents were diagnosed with PCOS. Twenty-six of the patients had classic phenotype (phenotype 1) with hyperandrogenism (HA), oligo/anovulation (OA) and polycystic ovarian morphology (PCOM). Thirty-seven patients had only OA and HA (phenotype 2), 46 patients had only HA and PCOM (phenotype 3) and 3 patients had only OA and PCOM (phenotype 4). **However, as the current Endocrine Society guidelines (16,17) recommend the presence of both hyperandrogenism and chronic anovulation for PCOS diagnosis in adolescence, we excluded the patients with phenotypes 3 and 4. Therefore, in total 63 girls with PCOS were included in the study**. All subjects were at least 1-year postmenarcheal. In the presence of oligomenorrhoea, a history of symptoms for at least 2 years was required for PCOS diagnosis. Hirsutism was diagnosed in girls when the Ferriman–Gallwey score was eight or more. Total testosterone level was 55 ng/dL or above for the diagnosis of biochemical hyperandrogenism. An adrenocorticotropic hormone stimulation test was performed to exclude congenital adrenal hyperplasia when 17-OH-P levels were above 2 ng/mL. Insulin resistance and sensitivity indexes were estimated by using HOMA-IR (Homeostasis Model Assessment-Insulin Resistance), QUICK-I (Quantitative Insulin Sensitivity Check Index), fasting glucose/ fasting insulin (FGIR), Matsuda index and total insulin levels during oral glucose tolerance test (**18**).
Venous blood samples, for DEHP and MEHP measurement, were taken into glass test tubes to prevent plastic contamination. The tube openings and all surrounding areas were covered by clean aluminium foil to protect the sample from contacts with the screw caps and sunlight. Centrifugation was performed at 800g, serum was separated, and all samples were immediately aliquoted into glass vials and stored in a freezer at -80°C until analysis.
For the hormonal assays, including serum follicle stimulating hormone (FSH), luteinising hormone (LH), oestradiol, 17-hydroxyprogesterone (17-OH-P), dehydroepiandrosterone sulphate (DHEA-S), androstenedione, total and free testosterone and sex hormone binding globulin (SHBG), the blood samples were obtained in the morning during the early follicular phase (second to fifth day) of a spontaneous menstrual cycle or at any time in subjects with amenorrhoea. Progesterone was assayed in the second phase of the period. Serum LH, FSH, oestradiol, prolactin, thyrotropin, free triiodothyronine (FT3), and free thyroxine (FT4) levels were measured by using the chemiluminescence immunoassay method (Siemens Healthcare Diagnostics Products, Llanberis, UK). Serum ACTH, cortisol, and insulin levels were measured by using the chemiluminescence immunoassay method (Siemens Healthcare Diagnostics Inc., Flanders, USA). Serum levels of total testosterone, free testosterone, androstenedione 17-OH-P and DHEA-S were measured by the radioimmunoassay method and SHBG levels by the immunoradiometric assay.
Fasting glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-cholesterol), triglycerides, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined by routine enzymatic methods using Abbott Architect c16000 analyser (Abbott Diagnostics, USA. An OGTT was performed using a dose of 1.75 g glucose/kg body weight (max 75 g) in all PCOS patients and obese controls. Venous blood samples were obtained at 0, 30, 60, 90, 120 min to measure plasma glucose and plasma insulin levels in the morning after an overnight fasting.

**DEHP and MEHP measurements**

Determination of MEHP and DEHP concentrations was conducted by HPLC equipped with an auto-sampler (Hewlett Packard Agilent 1100 Series, Vienna, Austria) using a UV detector (230 nm). A Spherisorb C18 ODS2 column was used (250 mm x 4.6 mm I.D., 5 µm, Waters, Milford, MA, USA). Separations were performed at room temperature. The mobile phase was orthophosphoric acid 0.1% (acetonitrile [90:10, vol/vol]), and the flow rate was 1 mL/min. For the extractions, to a sample of 200 mL serum were added 400 mL of Na OH 1N, 100 mL of 50% H3PO4 and 600 mL of acetonitrile. After each addition, the sample was shaken by vortex for 30 s. After centrifugation for 10 min at 3500 rpm, the supernatant was separated, and the residue was again extracted with 600 mL of acetonitrile. After centrifugation under the same conditions, the collected supernatants were evaporated, reconstituted with 400 mL of mobile phase and injected into the chromatograph. The injection volume was 100 µL. Stock solutions containing DEHP or MEHP (2000 ppm) were prepared by dissolving a weighed amount of substance in acetonitrile. Standard solutions were prepared by dilution of the above stock solutions with the mobile phase and by varying the concentration in the range 0.05–5.0 ppm (6, **19**). The concentrations of DEHP and MEHP in the samples were calculated by using the calibration curve of the peak area prepared for DEHP and MEHP standards. The detection limits were determined as 0.05 ppm for DEHP and as 1 ppm for MEHP.
The retention times for DEHP and MEHP were 23 minutes and 3.7 minutes, respectively. Recovery studies were performed on blank samples of serum, and the average recoveries were found to be (mean ±SD) 92±1.12% for DEHP and 99±1.10% for MEHP on 20 occasions. Between-run precisions were 6.44±0.12% coefficient of variation (CV) for DEHP and 8.03±1.05% CV for MEHP. Within-day precisions were 8.75 ±0.43% CV for DEHP and 4.83±0.21% for MEHP. DEHP and MEHP were purchased from Merck Co (Hohenbrunn, Germany) and Cambridge Isotope Laboratories Inc. (Andover, MA USA), respectively. Acetonitrile (HPLC grade) and all other analytical-grade reagents were obtained from Sigma-Aldrich Co. (St Louis, MO, USA).

**Statistical Analysis**

All statistical analyses were performed by using the IBM SPSS Statistics 21.0 statistics package program. Data are expressed either as frequencies or means with 95% confidence intervals (CIs). Distributions of continuous variables were examined for skewness and kurtosis and logarithmically transformed, when appropriate. Differences between groups were tested by using t-test. Mann-Whitney U was used for variables without normal distributions. The chi-square test was used to compare prevalences. Appropriate correlation tests (Pearson or Spearman) were performed to analyse the relationship between the parameters. The value of $p<0.05$ was taken for statistical significance.

**RESULTS**

The clinical and laboratory characteristics of the study population are shown in **Tables 1 and 2.** Serum DEHP and MEHP concentrations were not different between adolescent girls with or without PCOS **(Table 2).** In bivariate correlation analysis, we tested correlations between the phthalates DEHP and MEHP, and the following parameters: age, FSH, LH, oestradiol, progesterone, total and free testosterone, androstenedione, 17-OH-P, DHEA-S, BMI, waist circumference, glucose, triglyceride, HDL, LDL, total cholesterol, ALT, AST, insulin, HOMA-IR, QUICKI, Matsuda index, FGIR, and total insulin levels during OGTT. Serum MEHP and DEHP levels were correlated ($r=0.32$, $p=0.02$). We did not find any correlations between DEHP or MEHP and androgens, sex steroids and gonadotropins in either the entire group or in the PCOS subgroup. On the other hand, we found significant correlations between MEHP or DEHP and insulin resistance indices as well as serum lipids in patients with PCOS. The parameters with statistically significant correlations with MEHP or DEHP in the PCOS group are shown in **Table 3**. The correlations were even more pronounced in the obese PCOS subgroup (**Table 4).** To eliminate the possible effect of obesity, we performed correlation analysis after adjustment for BMI in adolescents with PCOS which revealed that both phthalates remained to be significantly correlated with insulin resistance indices and serum triglycerides (**Table 5).** There was no correlation among DEHP or MEHP and any parameters neither in the control group, nor in the obese non-PCOS subgroup separately.

**DISCUSSION**

Although the evidence is limited, accumulating data are indicating the potential role of endocrine disruptors in pathogenesis of adipogenesis and diabetes (**20**). It has been reported that DEHP exposure has been related with insulin resistance in adolescents (**21**). Insulin resistance, which is a well-known factor in PCOS development, is reported in 50-80 % of women with PCOS (**22, 23**). To our knowledge, this is the first report of an association of phthalates with insulin resistance in a PCOS cohort in the English literature.

In the current study, we found significant correlations between DEHP and insulin resistance and dyslipidaemia suggesting that DEHP might have a direct or indirect effect on energy metabolism. This association was even stronger in PCOS group and absent in the control group. Interestingly, in PCOS group, after adjustment for BMI, the correlations of both DEHP and MEHP with insulin resistance indices and serum triglycerides were remained to be strikingly significant. These findings lead us to think that there might be another mechanism other than obesity by which phthalates affect insulin resistance -one of the key pathologies in PCOS development- and dyslipidaemia in patients with PCOS.

In animal and in vitro studies, there are some significant findings suggesting that phthalates might have a role in the aetiopathogenesis of PCOS (7,8, 24-30). Previous studies showed that DEHP exposure in rats results in prolonged oestrous cycles and decreased ovulation rates, altered circulating FSH, LH, testosterone, and progesterone levels (7,8,**24,25).** DEHP exposure resulted in suppressed oestradiol levels in granulosa cells which could not stimulate the LH surge necessary for ovulation; this, consequently, caused hypo-estrogenic anovulatory cycles and polycystic ovaries in adult female rats (8). Moreover, MEHP stimulates basal steroidogenesis (**25**) inhibits progesterone production in rat granulosa cells and decreases aromatase causing a hyperandrogenaemic, hypo-progestinic situation which is like the picture in PCOS (**29**). In humans, in a study on granulosa-lutein cells from women planning in vitro fertilisation, MEHP was reported to inhibit oestradiol production and affected steroidogenesis similarly in rats (**31**).

Based on the results of these studies and given the fact that hyperfunctioning of theca and relative hypofunctioning of granulosa cells accompany the acyclicity of PCOS in humans, we thought that it is worthwhile to investigate the PCOS-phthalate relationship in humans. In another paper inspiring us to conduct this study, Svechnikova et al (**32**) reported that DEHP exposure in female rats caused increased LH response to GnRH by pituitary and inhibition of progesterone production in granulosa cells.

There are only four studies in the literature, investigating PCOS-phthalate relationship in humans, which have conflicting results (9-12). In this study, we did not find any relations between DEHP or MEHP and gonadotropins or sex hormones. It is known that the effects of phthalates on hormones are both complex and multifactorial. Hence, considering the results of experimental studies, one might suggest that random measurements of serum concentrations of these compounds are not enough to elucidate a causal relationship between DEHP/MEHP and the disorder. Phthalates have been reported to be associated with gynecomastia and the risk of shortened anogenital distance (6, 33). It may be argued that higher levels of DEHP/MEHP could conceal PCOS findings due to its antiandrogenic effects which could be investigated in a non-hyperandrogenic PCOS group such as phenotype 4 according to Rotterdam criteria (14).

In a very recent study, Jin et al (10) reported significantly increased DEHP levels in follicular fluid of women with PCOS compared to controls. They also showed that DEHP is associated with lower pregnancy rate and DEHP exposure resulted in significant increase in androgen production in human granulosa cells. We speculate that, in our cohort, DEHP might have a role in PCOS development through insulin resistance at the follicular level, which is not reflected in the serum concentrations of the participants.

**Study limitations**

We are aware of the limitations of this study. First, because of its cross-sectional design, we cannot conclude a causal relationship. The second point is that we measured serum FSH, LH, progesterone, and phthalates levels at different times of the menstrual cycle in patients which might have caused to overlook a relationship.

**Conclusion**

In conclusion, in this study, serum DEHP and MEHP concentrations in adolescents with PCOS were not different from those in their non-PCOS controls. However, both DEHP and MEHP significantly correlated with insulin resistance and metabolic

disturbances in patients with PCOS. We believe that further well-designed detailed studies are needed to evaluate the possible role of phthalates in PCOS development in humans.

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Table 1: Clinical characteristics of adolescents with and without PCOS

| | Entire group (*n*=124) | | *p* |
|---|---|---|---|
| | PCOS | Controls | |
| Demographic variables | | | |
| Age, (*years*) | 15.4 (15.1-15.7) | 14.9 (14.5-15.4) | ns |
| Ferriman-Gallwey score* | 14.2 (12.4-16.4) | 2.69 (2.20-3.31) | <0.001 |
| Acne (%) | 38.1 | 19.7 | 0.030 |
| Acanthosis nigricans (%) | 31.1 | 18.0 | ns |
| Anthropometric measurements | | | |
| BMI ($kg/m^2$) | 27.3 (25.5-28.9) | 25.6 (24.1-27.1) | ns |
| Waist circumference (*cm*) | 87.1 (82.9-86.6) | 83.5 (79.9-87.2) | ns |
| Body fat percentage (%) | 36.2 (33.4-38.6) | 34.6 (30.3-38.9) | ns |
| Systolic BP (*mmHg*) | 115.2 (111.5-119.1) | 109.7 (105.1-114.5) | ns |
| Diastolic BP (*mmHg*) | 72.6 (69.9-75.2) | 73.8 (69.7-78.2) | ns |

Data are expressed as (%) , mean (95% CI), or *geometric mean (95% CI) for log-transformed variables, ns: not significant

Table 2: Laboratory characteristics and serum DEHP and MEHP levels in adolescents with and without PCOS

| | | | |
|---|---|---|---|
| | PCOS (*n*=63) | Controls (*n*=61) | *p* |
| Phthalates and Hormone profiles | | | |
| DEHP (*µg/ml*) | 2.62 (2.50-2.75) | 2.71 (2.52-2.90) | ns |
| MEHP (*µg/ml*)* | 0.23 (0.19-0.29) | 0.36 (0.18-0.54) | ns |
| FSH (*mIU/ml*)* | 5.51 (4.96-6.12) | 5.4 (4.3-6.5) | ns |
| LH (*mIU/ml*) | 10.73 (9.32-12.15) | 4.4 (2.5-7.8) | **0.009** |
| Estradiol (*pg/ml*)* | 54.89 (49.21-61.23) | 65.7 (45.9-94.1) | ns |
| Progesterone (*ng/ml*)* | 0.66 (0.52-0.84) | 0.6 (0.2-1.8) | ns |
| 17-OH-Progesterone (*ng/ml*) | 2.01 (1.71-2.31) | 1.0 (0.8-1.4) | ns |
| Androstenedione (*ng/ml*) | 2.53 (2.18-2.88) | 1.3 (1.1-1.6) | **<0.001** |
| DHEA-S (*ng/ml*) | 2517.9 (2246.2-2789.5) | 1909.1 (1612.3-2206.0) | **0.006** |
| Total testosterone (*ng/dl*) | 101.6 (89.5-113.6) | 40.5 (33.7-47.2) | **<0.001** |
| Free testosterone (*pg/ml*) | 2.78 (2.32-3.23) | 1.9 (1.7-2.1) | **0.009** |
| SHBG (*nmol/l*)* | 24.11 (18.73-31.02) | 26.4 (19.5-33.4) | ns |
| Liver enzymes | | | |
| Aspartate aminotransferase (*U/L*)* | 22.80 (20.74-25.07) | 22.3 (20.6-23.9) | ns |
| Alanine aminotransferase (*U/L*)* | 20.66 (18.09-23.61) | 19.2 (17.2-21.5) | ns |
| Serum lipids | | | |
| Triglycerides (*mg/dl*)* | 96.54 (85.65-108.81) | 108.7 (95.3-122.2) | ns |
| HDL-Cholesterol (*mg/dl*) | 43.83 (41.25-46.42) | 46.1 (42.4-49.8) | ns |
| LDL-Cholesterol (*mg/dl*) | 86.47 (80.33-92.61) | 86.2 (77.9-94.4) | ns |

| | | | |
|---|---|---|---|
| Total cholesterol (*mg/dl*) | 150.8 (143.5-158.2) | 155.5 (146.7-164.4) | ns |
| Insulin resistance | | | |
| Fasting glucose (*mg/dl*) | 82.72 (80.45-84.98) | 86.1 (82.9-89.3) | ns |
| HOMA-IR * | 2.95 (2.33-3.73) | 3.3 (2.5-4.3) | ns |
| Matsuda index* | 3.57 (2.87-4.43) | 3.3 (2.7-4.1) | ns |
| QUICKI | 0.33 (0.326-0.35) | 0.32 (0.31-0.33) | ns |
| FGIR* | 5.36 (4.32-6.65) | 5.16 (4.05-6.56) | ns |

Data are expressed as mean (95% CI), or *geometric mean (95% CI) for log-transformed variables, ns: not significant

Table 3: Correlations between MEHP or DEHP and metabolic parameters in adolescents with PCOS

| | **DEHP** | | **MEHP** | |
|---|---|---|---|---|
| | *r* | *p* | *r* | *p* |
| Triglyceride | **0,417** | **0,020** | -0,050 | 0,797 |
| Glucose | 0,168 | 0,359 | **0,443** | **0,014** |
| Matsuda index | **-0,405** | **0,018** | 0,024 | 0,896 |
| HOMA-IR | **0,515** | **0,003** | 0,216 | 0,251 |
| QUICK-I | **-0,496** | **0,003** | 0,056 | 0,759 |
| Total insulin during OGTT | **0,386** | **0,024** | 0,078 | 0,672 |
| Cholesterol | **0,405** | **0,024** | -0,052 | 0,788 |

Table 4: Correlations between MEHP or DEHP and metabolic parameters in obese adolescents with PCOS

| | DEHP | | MEHP | |
|---|---|---|---|---|
| | *r* | *p* | *r* | *p* |
| Glucose | 0,155 | 0,492 | **0,454** | **0,034** |
| Triglyceride | **0,493** | **0,020** | -0,221 | 0,323 |
| Insulin | **0,683** | **<0,001** | 0,063 | 0,782 |
| Matsuda index | **-0,508** | **0,016** | 0,058 | 0,797 |
| HOMA-IR | **0,683** | **<0,001** | 0,124 | 0,584 |
| QUICK-I | **-0,635** | **0,001** | 0,058 | 0,797 |
| Total insulin during OGTT | **0,482** | **0,023** | 0,067 | 0,766 |
| Cholesterol | **0,524** | **0,012** | -0,101 | 0,654 |
| FGIR | **-0,653** | **0,001** | -0,257 | 0,248 |

Table 5: Correlations of MEHP and DEHP with metabolic parameters adjusted for BMI in adolescents with PCOS

| | **DEHP** | | **MEHP** | |
|---|---|---|---|---|
| | *r* | *p* | *r* | *p* |
| Waist circumference | 0,365 | 0,056 | 0,337 | 0,079 |
| Glucose | **0,460** | **0,014** | **0,465** | **0,013** |
| Triglyceride | **0,521** | **0,004** | 0,169 | 0,391 |
| Insulin | **0,495** | **0,007** | 0,298 | 0,123 |
| Matsuda index | **-0,439** | **0,020** | -0,133 | 0,501 |
| HOMA-IR | **0,558** | **0,002** | **0,631** | **<0,001** |
| Total insulin during OGTT | **0,548** | **0,003** | 0,275 | 0,157 |