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Fatty Acids in Subcutaneous Adipose Tissue of Pregnant Women with and without Polycystic Ovary Syndrome Are Associated with Genes Related to Steroidogenesis: A Case-Control Study

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Abstract

Background: The qualitative analysis of adipose tissue (AT) is an exciting area for research and clinical applications in several diseases and it is emerging along with the quantitative approach to research on overweight and obese people. While the importance of steroid metabolism in women with polycystic ovary syndrome (PCOS) has been reported, limited data exists on the effective roles of AT in pregnant women suffering from PCOS. The aim of this study was to determine association of fatty acid (FA) profiles with expression of 14 steroid genes in abdominal subcutaneous AT of PCOS vs. non-PCOS pregnant women.

Materials and Methods: In this case-control study, the AT samples of 36 non-PCOS pregnant women and 12 pregnant women with PCOS (3:1 ratio control: case) who underwent cesarean section were collected. Relationship of expressing gene targets and different features were performed using Pearson correlation analysis on the R 3.6.2 software. The ggplot2 package in R tool was used to draw the plots.

Results: Age (31.4 and 31.5 years, P=0.99), body mass index (BMI) (prior pregnancy 26 and 26.5 $kg.m^{-2}$, P=0.62) and at delivery day (30.1 and 31, P=0.94), gestational period (264 and 267 days, P=0.70) and parity (1.4 and 1.4, P=0.42) of non-PCOS and PCOS pregnant women were similar. Expression of steroidogenic acute regulator (*STAR*) and 11β-Hydroxysteroid dehydrogenase (*11BHSD2*) in non-PCOS pregnant women showed the highest association with eicosapentaenoic acid (EPA, C20:5 n-3, r=0.59, P=0.001) and (r=0.66, P=0.001), respectively. In the all participants, *STAR* mRNA level showed the greatest association with the EPA fatty acid concentration (P=0.001, r=0.51).

Conclusion: Our results showed a link between the genes involved in steroid metabolism and fatty acids in AT of pregnant women, especially for omega-3 FA and the gene involved in the first step of steroidogenesis in subcutaneous AT. These findings warrant further studies.

Keywords: Adipocyte, Fatty Acids, Polycystic Ovary Syndrome, Steroidogenesis, Subcutaneous Adipose Tissue

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Introduction

Adipose tissue (AT)-also known as fat tissue or fatty tissue – is not only an energy storage organ, but also the largest endocrine organ. It plays an important role in energy balance and reproductive function maintenance (1). It has always been proposed that quantity and amount of AT, commonly referred as obesity and overweight, have significant impacts on many diseases, such as cardiovascular disease, cancers and most importantly infertility as well as polycystic ovary syndrome (PCOS). PCOS is a common endocrine and metabolic disease, occurring in 4-18% of adolescent women and women of childbearing age (2). While some women with PCOS have normal weight, 50% of PCOS patients are suffering from obesity. Although PCOS is considered one of the leading causes of infertility, many women suffering PCOS can get pregnant. So, understanding roles of AT in women with PCOS and monitoring these patients throughout pregnancy and their offspring development is highly relevant (3).

It has been hypothesized that AT plays crucial roles in steroidogenesis and recent data showed that there is an association between fatty acid (FA) profiles and mRNA levels of the genes involved in steroidogenesis (4, 5). Relationship of AT with PCOS is driven by the pivotal role played by the former on the metabolism of steroids, such as sex steroids, including aromatase cytochrome P450 (CYP19A1) and 17 hydroxysteroid dehydrogenase (17BHSD) family.

Additionally, the previous studies mentioned the important roles of mineralocorticoids, including steroid 21-hydroxylase (CYP21) and glucocorticoids (11β-Hydroxysteroid dehydrogenase (*11BHSD1*) metabolism in AT. Profile of the steroids released into circulation from AT are influenced by the adipocyte function and they can possibly affect distribution of AT, modulated by steroids. Hence, metabolic alteration of glucocorticoids has been reported to influence nature of the AT in women with PCOS (6, 7).

Relationship of fat intake with AT (obesity and body weight) has been known for long time and recently, there is awareness of the different types of nutritional fat and oil effects on body fat, metabolism and the related maladies. For example, fat sources, such as omega-3 fatty acids, have been shown to be beneficial to health, in comparison with the other types of fatty acids. Studies showed the beneficial effects of fish oil – a good source of omega-3 fatty acids – on metabolic profiles, biomarkers of inflammation and oxidative stress in PCOS patients (8). Thus, there is growing interest in the use of fish oil for treatment of women with PCOS. This interest has been substantiated by the recent findings of reduction in levels of eicosapentaenoic acid (EPA) and erythrocyte docosahexaenoic acid (DHA) – fatty acids found in fish oil – in infertile women, compared to the controls (9). In addition, a few interventional studies demonstrated the beneficial effects of fish oil supplementation on metabolic profiles in patients with PCOS. In women suffering from PCOS, the earlier study showed that fatty-acid based dietary supplements may be used for improving excessive ROS-caused ovarian disorder and insulin resistance (10). Despite these studies, the mechanisms by which specific fatty acids influence metabolic profiles have not been fully determined yet.

Studies prior to 1990 have not looked at association of fatty with expression of different genes and proteins in different body tissues, especially AT (11). These early studies proposed that lipids only act through altering membrane phospholipid. However, in 1992, Göttlicher et al. (12) pointed out the crucial roles of nuclear receptors, which alter glucocorticoid receptor and gene expression, in fatty acids metabolism. Importantly, activities of these transcription factors are regulated not only by hormones, but also by nutrients and metabolites (13) among which, fatty acids have fundamental roles.

As a scientific background, the previous studies, performed by Vara Prasad et al. (4) and Petrus et al. (5), showed association of one or more limited types of fatty acids with a single gene (11-β-hydroxysteroid-dehydrogenase type 1) in respectively rat and human. Novelty of the present study lies in two approaches – study of the effects of fatty acids on all genes belonging to the steroidogenesis family and assessment of the relationship between fatty acids and gene expression in pregnant women suffering from PCOS. Little information exits about effects and fundamental mechanisms regarding omega-3 polyunsaturated fatty acids (PUFA) function in women suffering from PCOS. Our objective was to determine association between fatty acids and steroidal gene expression in abdominal subcutaneous AT of pregnant women with and without PCOS.

Materials and Methods

Patients and AT samples collection

In this case-control study, after obtaining permission from the Royan Institute Ethics Committee (IR.ACECR.ROYAN.REC.1398.087, Tehran, Iran), demographic data and AT samples were collected. Details of the sample collections, snap freezing and storage at -196°C have been presented in the previous publication (14). This study involved 48 Iranian pregnant women who underwent caesarean section including. Among the 48 women, 12 women had PCOS and 36 did not have PCOS. According to 2003 Rotterdam criteria (2), the diagnostic traits of PCOS are the presence of two or more significant symptoms of the syndrome and patients that met two of three symptoms were chosen as PCOS cases. PCOS diagnosis was done by the medical practitioners affiliated to Royan Institute.

Signed informed consent was obtained from all subjects. Samples were taken from the subcutaneous fat of abdomen of all women. During the time of sampling, the

information about demographic data, life style conditions, drug medical intake, gestational diabetes mellitus (GDM), gestational hypertension (HPT) and metabolic disorders were collected. We confirmed that all subjects do not intake medical drugs affecting lipid metabolism and glucose, while they did not suffer from GDM and HPT (exclusion criteria). Maternal choice is the reason for delivery by caesarean section (90%). Finally, case and control groups were matched in terms of age and body mass index (BMI)-matched.

Fatty acid profiles analysis

At room temperature all samples were thawed. FA methyl esters were prepared with boron trifluoride (BF3) according to ISO 12966-2. A gas chromatograph (Shimadzu GC-2010 PLUS, Shimadzu Corporation, Japan) with a flame ionization detector (FID) was used to separate FA methyl esters. Separation was performed using a 100 m×0.25 mm, 0.2 µm column (Dikmacap 2330, Japan). Hydrogen was used as the carrier gas at a column flow rate of 1.20 ml/minutes. The temperature of the column was increased from 60°C to 240°C within 30 minutes. The injector and detector temperatures were 250°C and 280°C, respectively (14).

RNA extraction, cDNA synthesis and quantitative reverse-transcription polymerase chain reaction procedures

Total RNA was extracted using the mini kit for RNeasy AT (Qiagen, Germany). A spectrophotometer (Bio-Rad, Stanford, USA) was used for evaluation of the quality of the extracted RNA in terms of the A260/280 ratio. Then, for cDNA synthesis on RNA samples, Prime Script RT Reagent Kit (Takara, Japan) was used and all experiments included negative controls (without cDNA) and RT controls. Gel electrophoresis were used for analysis of PCR products. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed on the Step-One PCR system (Applied Biosystems, USA), mRNA quantification was conducted and each reaction was run in duplicate. The NCBI primer Blast and Perl primer Software (version1.1.21) were used for primer design of all steroids target genes. Ultimately, *GAPDH* as the housekeeping gene was used and messenger RNA expression levels of all genes were analyzed by Qrt-pcr ($2^{-\Delta\Delta ct}$). We analyzed expression level of 14 genes (7): steroidogenic acute regulator (*STAR*), cytochrome P450 monooxygenase (*CYP11A1*), 17α-hydroxylase (*CYP17A1*), steroid 21-hydroxylase (*CYP21*), 11β-Hydroxysteroid dehydrogenase (*11BHSD1* and *11BHSD2*), aromatase cytochrome P450 (*CYP19A1*), 3β-hydroxysteroid dehydrogenase (*3BHSD1* and *3BHSD2*) and 17 hydroxysteroid dehydrogenase family (*17BHSD* types 1, 3, 5, 7 and 12).

Statistical analysis

Kolmogorov-Smirnov test was used for the normal distribution test. t test and Mann-Whitney U test were used for the analyzed data with normal and abnormal distribution, respectively. Relationships among the expressions of gene targets and the different features were analyzed using Pearson correlation analysis on the R 3.6.2 software (University of Auckland, New Zealand). The ggplot2 package in R tool was used to draw the plots. The differences were considered significant at $P \leq 0.05$.

Supplementary information

The manuscript contains supplementary material available at the enclosed files (Figs.S1-S28, See Supplementary Online Information at www.ijfs.ir). In the supplemented figures, we showed correlation among different traits and dot plot of correlations between expression of gens and FA in women with and without PCOS. The ggplot2 package in R tool was used to draw the plots.

Results

Characteristics of PCOS patients and non-PCOS pregnant women

Age, BMI (prior pregnancy and at delivery day), gestational period and parity among 36 non-PCOS and 12 PCOS pregnant women were similar, as the case and control groups (Table 1).

Table 1: General characteristics of participants

Variables	PCOS (n=12)	Non-PCOS (n=32)	P value
Age (Y)	31.4 ± 3.57	31.5 ± 5.42	0.99
BMI ($kg.m^{-2}$) before pregnancy	26 ± 5.92	26.5 ± 4.93	0.62
BMI ($kg.m^{-2}$) on the day of delivery	30.1 ± 5.35	31.5 ± 5.33	0.94
Gestational period (days)	267 ± 5	264 ± 13	0.70
Parity (n)	1.4 ± 0.5	1.4 ± 0.62	0.42

Data with normal distribution were analyzed using t test. PCOS; Polycystic ovary syndrome and BMI; Body mass index.

Relationship between fatty acids and gene expression

Results showed that C20-5n3 FA was the most associated factor with gene expression. Expression of *STAR* and *11BHSD1* genes in non-PCOS pregnant women showed highest association with C20-5n3 FA (P=0.001, r=0.66 for *STAR* and P=0.001, r=0.59 for *11BHSD1*). In the all participants, *STAR* gene expression had highest association with the C20-5n3 FA concentration (P=0.001, r=0.51). Significant items are listed in Table 2 and non-significant items are not included. Additionally, the figures were mentioned as supplementary files. They are figures of heat plot of pairwise correlation among different traits and dot plot of correlations between expression of gens and fatty acids in women with and without PCOS.

Table 2: Relationship between eicosapentaenoic acid (EPA) C20-5n3 and gene expression in women with and without polycystic ovary syndrome (PCOS)

Variables	EPA C20-5n3		
	Non-PCOS	PCOS	Total
	r (P value)	r (P value)	r (P value)
STAR	0.66 (P=0.001)	-0.58 (P=0.09)	0.51 (P=0.001)
11HSDB2	0.59 (P=0.001)	0.-44 (P=0.15)	0.19 (P=0.15)

Relationship of the expression of gene targets with the different features was performed using Pearson correlation analysis on the R software.

Discussion

PCOS, which is one of the most common causes of unhealthy condition in women and accompanied by metabolic disorders, can be treated or reduced by changing lifestyle. It was suggested that consumption of omega-3 is a typical recommendation for women suffering from PCOS (15, 16). Our aim in this study was not to investigate potential pathways whereby fatty acids affect expression of steroid genes, but only to investigate relationship between fatty acids and expression of steroid genes. Our results showed that expression of *STAR* and *11BHSD2* genes have highest association with EPA in non-PCOS pregnant women (EPA, c20:5 n-3). It was surprising to find that in the initial step of steroidogenesis and in all participants, *STAR* mRNA level had the greatest association with the omega-3 FA concentration.

This is the first experiment, reposting association between the fatty acids in subcutaneous AT of pregnant women with PCOS and the expression of 14 steroid genes. There have been a few studies investigated effects of this type of fat on gene expression. The first study conducted by Vara Prasad et al. (4) in 2010, showing that dietary FA composition altered *11bhsd type 1* gene expression in retroperitoneal rat white AT. The most relevant study in this area, carried out by Petrus et al. (5). They compared relationship between saturated and unsaturated fatty acids with the expression of *11BHSD1* gene and 11BHSD1 protein in two areas of visceral and subcutaneous women AT. Overall, they showed that there was a significant and strong relationship between expression of the gene and the protein in visceral adipose tissue. They also indicated that mono unsaturated FA (MUFA) fatty acids reduced expression of this gene, while saturated FA (SFA) in visceral AT increased expression of *11BHSD1*; the latter has Irrefutable role in cortisol metabolism. It seems that difference in expression of this gene within two areas of AT may be due to differences in LMO3 transcription factor, and possible signals may be due to Toll-like receptors. This protein complex can act as a transcription regulator for genes in the pathways of the nervous system, and the endocrine system, both individually and in conjunction with other molecules (17). Using animal and *in vitro* models, several studies demonstrated that saturated fatty acids were potent activators of the NLRP3 inflammasome (18). On the other hand, it seems that unsaturated fatty acids, particularly animal omega-3 source such as fish oil, inhibit the inflammasome (19).

There are two putative mechanisms to improve PCOS in women via omega-3 fatty acids: the first, relation of omega-3 fatty acids with inflammation and the second possible mechanism, diminished effects of omega-3 on Testosterone levels in women with PCOS. On the basis of this evidence, a study conducted by Calder (20) reported that PUFAs and their metabolites activated PPARs to inhibit activity of NFKβ signals, while they were effective in reducing cytokines, inflammatory processes and homeostasis. This pointed role of PUFA in regulating steroidogenesis and lipogeneses. EPA and DHA also provided substrates for synthesis of the pro-inflammatory lipid mediator protectins, resolvins and decreased adipokines through inhibition of NFKβ signaling. Another study in the same field, confirmed the last statements, indicating that *CYP51* mRNA levels is up-regulated in granulosa cells by omega-3 in PCOS rat (21). Although in the current study, the goal was not to reach possible pathways whereby FA affects genes, but many studies have shown that fatty acids either activate or inhibit inflammatory pathways. Our previous study confirmed higher level of *11BHSD1* in women with PCOS and there was association between inflammatory and cortisol (7). In line with the current research, an interesting study proposed that supplementation of overweight/obese pregnant women, with dietary omega-3 fatty acids for more than 25 weeks, reduced inflammation in maternal adipose and the placental tissue (22).

Toll-like receptor 4 (TLR4) appears to be a central target of the anti-inflammatory effects at the cellular level. AT and placenta of the treated women exhibited significant decrease in TLR4 adipose and placental expression as well as IL6, IL8 and TNFα. *In vitro*, EPA and DHA suppressed activation of TLR4, IL6 and IL8 in cultures of adipocyte and trophoblast cells, induced by palmitate (22). In support of this study, another study by Mansoori et al showed that DHA-rich fish oil supplementation for eight weeks increased *CD36* expression in hypertriglyceridemia compared to normotriglyceridemic subjects (23). It is typical that animal source of omega-3 FA (DHA and EPA) affect inflammation responses through down-regulation of the genes involved in inflammation process. Therefore, it is an expected response when fish oil supplements reduced expression of the adipose inflammatory genes *(IL-18* and *IL-1β* and *circulating IL-18* levels) in AT (24).

On the other hand, studies performed by Forouhi et al. (25), Hajishafiee et al. (16) and Nadjarzadeh et al. (26) determined effect of omega-3 on diminished testosterone levels in PCOS women, pointed out the importance of omega-3 fatty acids in PCOS. We focused on pregnant women with PCOS and according to our findings and previous studies, omega-3 might associate with hormonal changes by affecting steroid genes expression in AT. Additionally, a study performed in rat ovaries by Ma et al. (27) showed that unsaturated fatty acids ratio is important, apart from its concentration. These findings provided evidence that a balanced n-3/n-6 PUFA ratio is beneficial to improve fertility of women with PCOS.

Ratio of n-3/n-6 about 1/15 in rat food altered expression of Cyp19, Star, 3bhsd and Cyp11 proteins, which may indicate the importance of these fatty acids as a transcription factor for the above genes. Similarly, a study showed that consumption of *Linum usitatissimum* oil for 21 days could regulate steroid genes (*Star* and *Cyp11*) expression involved in initial steps of steroidogenesis as well as testosterone metabolism (28). In this regard, our findings illustrated relationship of omega-3 FA with the gens involved in initial steps of steroidogenesis in AT.

Altogether, it seems that complications and dangers of PCOS are from activation of inflammatory pathways and high testosterone levels, while omega-3 fatty acids affect expression of the genes in AT of pregnant women by reducing inflammation and diminishing testosterone. We need more investigation regarding mothers suffering from PCOS.

This study can hypothesize that type of fatty acids in AT is involved in activating inflammatory and activation pathways or turning off many genes, including steroid genes, and finally it can determine important association between fatty acids and gene expression in AT.

Conclusion

Fatty acids, directly or metabolically, can play a role in many diseases through extensive signaling pathways, specifically in exacerbating PCOS, although pregnancy can double the role of nutrition in exacerbating these effects. This is the first study in humans that has investigated association between FA types and gene expression of steroids in AT of pregnant women with and without PCOS. We showed distinct relationships between polyunsaturated fatty acids with regard to the genes of fourteen steroids, especially for omega-3 fatty acids and the genes involved in initial steps of steroidogenesis.

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Authors' Contributions

N.E.; Main project contributor, investigation, data collection, analysis, and writing the original draft preparation. A.M.; Project advisor, head of surgical team, methodology and participants selection, resources, writing, review, and editing. M.R.B.; Methodology, statistical analysis, writing, review and editing. P.Y.; Project advisor, investigation, methodology, writing, review, and editing. M.Sh.; Projects supervisor, methodology, validation, writing, reviewing, and visualization. A.A.; Project supervisor and designing, project management, contributed to data interpretation, writing-original draft preparation, writing and reviewing final version, and visualization. All authors read and approved the final manuscript.

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