

Metformin increases norepinephrine transporter expression in placenta of patients with polycystic ovary syndrome

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Abstract. – OBJECTIVE: To evaluate the norepinephrine (NE) and placental NE transporter (NET) in women with polycystic ovary syndrome (PCOS) non-treated and treated with metformin during pregnancy.

PATIENTS AND METHODS: We studied sixteen pregnant women with PCOS: 8 without metformin treatment during pregnancy (PCOS-M) and 8 treated with metformin during pregnancy (PCOS+M). Sixteen pregnant women of similar age without PCOS were included as controls (Control). At 24th and 35th weeks of pregnancy, blood samples were obtained. Placentas from full-term pregnancies were collected immediately after delivery. They were divided into two samples representative from the region near the chorionic plate (fetal side) and from the region near the basal plate (maternal side). NE plasma concentrations were measured by HPLC with electrochemical detection, and placental NET protein levels were determined by Western blot.

RESULTS: At week 24 of gestation, PCOS-M had higher NE plasma levels compared to control women ($p < 0.001$). Moreover, NET expression was lower in the maternal side of the placenta of PCOS-M compared to controls ($p < 0.05$). Metformin treatment normalized NE plasma levels at week 24 of gestation and NET expression in the maternal side of the placenta. In the fetal side of the placenta, NET expression was lower in PCOS-M and PCOS+M compared to control women ($p < 0.001$ and < 0.01 , respectively).

CONCLUSIONS: Our results strongly suggest that norepinephrine homeostasis is altered in pregnant women with PCOS. Remarkably, metformin administration during pregnancy decreases circulating norepinephrine levels and increases NET expression in the maternal side of placentas from PCOS women.

Key Words:

Polycystic ovary syndrome, Sympathetic activity, Placenta, Norepinephrine transporter.

Introduction

Polycystic ovary syndrome (PCOS) is the most common ovarian pathology in women of reproductive age. It is characterized by hyperandrogenism, chronic oligo-anovulation and polycystic ovarian morphology. Along with these features, PCOS is strongly associated with metabolic dysfunctions including obesity, insulin resistance and compensatory hyperinsulinemia¹. Moreover, patients with PCOS have evidence of increased sympathetic nerve activity (SNA) contributing to its etiology²⁻⁴. Nevertheless, it is not well established whether the elevated SNA is a primary defect leading to PCOS or a consequence of the hyperandrogenism or the metabolic derangements present in these women⁵. Metformin (1,1-dimethylbiguanide hydrochloride), a biguanide with pleiotropic effects, that improves insulin signaling in different tissues, is currently used in the management of metabolic and reproductive alterations in women with PCOS, reducing insulin resistance, hyperinsulinemia and circulating androgen levels⁶. During pregnancy, increased circulating levels of norepinephrine (NE) have been associated with the presence of pregnancy hypertension and preeclampsia^{7,8}. In this regard, pregnant women with PCOS have a 3-4-fold increased risk to develop these pathologies suggesting a possible hyperactivity of the SNA⁹. Moreover, maternal conditions associated with elevated

androgen levels such as *hyperreactio luteinalis* are related to severe preeclampsia¹⁰. However, to the best of our knowledge, the SNA of pregnant PCOS women has not been studied. Norepinephrine levels are regulated by the activity of the norepinephrine transporter (NET), which clears NE from the circulation. In placental tissues, elevated levels of NET have been documented, which could protect the fetus from high exposure to NE¹¹. Interestingly, animal models have demonstrated that conditions associated with maternal increased SNA affect fetal development, inducing long-term consequences such as ovarian and metabolic dysfunctions and behavioral disorders¹²⁻¹⁴. Therefore, an elevated SNA could impact on maternal and fetal homeostasis. Women with PCOS maintain elevated circulating androgen levels and metabolic alterations during pregnancy, which are reverted with metformin treatment during pregnancy^{15,16}. Of interest, metformin administration to hypertensive patients results in decreased blood pressure, heart rate and SNA¹⁷⁻¹⁹. Then, metformin treatment during pregnancy could improve the metabolic and hyperandrogenic profile and potentially the over activity of SNA in pregnant women with PCOS. Therefore, we propose that pregnant women with PCOS maintain an elevated SNA, which could be reversed with metformin treatment during pregnancy. In order to test this hypothesis, we studied NE plasma levels and NET protein expression in the placenta of pregnant PCOS patients treated and not treated with metformin.

Patients and Methods

Patients

A case-control study was designed. Sixteen pregnant women with PCOS were included, and 16 normal pregnant women formed the control group. In eight controls we obtained placental and blood samples at term and in the other 8 controls we obtained serum samples at weeks 24 and 35 but no placental samples. We studied two groups of PCOS patients: the first group was instructed to stop metformin treatment upon a positive pregnancy test (PCOS-M); the second group continued metformin treatment at the same dose during the entire pregnancy (PCOS+M). No medications, such as clomiphene citrate or exogenous gonadotropins, were used to induce ovulation. The investigation conforms to the principles outlined in the WMA Declaration of Helsinki – Ethical Principles. The Ethics Committee of San

Juan de Dios Hospital and the University of Chile approved the protocol (Certificate No. 129/28 November 2006). Each patient provided written consent to participate in the study. PCOS was diagnosed according to the criteria for PCOS of the National Institutes of Health (NIH) consensus and the Rotterdam European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine-sponsored PCOS consensus workshop group (phenotype A)²⁰. As part of the initial evaluation, each of the patients underwent a lifestyle assessment and was placed on a diet and exercise treatment program consisting of a 1000-kcal low-fat diet and a daily 30-min walk. In addition, they received 1.500-2.000 mg of standard formulation metformin each, based on bodyweight, medication tolerance, and insulin levels. The women in the control group were recruited from the antenatal care unit of our hospital (San Juan de Dios Hospital) from the 12th week of gestation. Each of these women had a history of regular 28- to 32-day menstrual cycles and absence of hirsutism and other clinical manifestations of hyperandrogenism, including infertility, pregnancy complications, galactorrhea, and thyroid dysfunction. All were healthy, and none were receiving any drug therapy. We excluded patients who had hyperprolactinemia, an androgen-secreting neoplasm, Cushing syndrome, late-onset 21-hydroxylase deficiency, or thyroid disease. Women diagnosed during the current pregnancy with preeclampsia, gestational hypertension, gestational diabetes or preterm delivery and women with severe chronic diseases, such as chronic hypertension or kidney disease, were excluded from the study. Only women who did not smoke, drink alcohol or abuse drugs were included in the study.

Samples

Blood samples were obtained at 24 and 35 weeks of pregnancy. The collected blood was placed in an EDTA solution and was centrifuged; the plasma was frozen at -80°C. Placentas from full-term pregnancies (37-40 weeks of gestation) were collected immediately after delivery. Briefly, each placenta was sectioned transversely using a sterile scalpel near the cord insertion site (approximately 5 cm). It was divided into two horizontal segments from the chorionic surface toward the basal plate²¹. Then, representative samples were obtained from the region near the chorionic plate (fetal side) and the region near the basal plate (maternal side), respectively.

Assays

Serum NE concentrations were determined by high-performance liquid chromatography (HPLC), as previously described²². Briefly, plasma samples (200 μ L each) were precipitated with 0.2 N perchloric acid and the supernatant purified with alumina. NE was measured in the eluate by HPLC coupled to an EICOM ECD-700S electrochemical detector. Inter- and intra-assay coefficients of variation were 7% and 4%, respectively.

Serum insulin, testosterone and androstenedione were assayed by RIA (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). Assay sensitivities were 3.0 μ IU/ml, 0.1 ng/ml and 0.1 ng/ml, respectively. Intra- and inter-assay coefficients of variation were 5.0 and 8.0% for insulin, 9.6 and 8.6% for testosterone and 5.6 and 9.8% for androstenedione, respectively. Serum glucose was determined by the glucose oxidase method (Photometric Instrument 4010; Roche, Basel, Switzerland). The intra-assay coefficient of variation was < 2.0%.

Western Blot Analysis of NET

Proteins were extracted from the maternal or fetal side of the placental tissue by homogenization in 200 μ L of lysis buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM EGTA, 1 mM EDTA, and 1% Triton; pH 7.4) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), 35 mM PMSF and 0.4 mM DTT. The protein samples (30 μ g) were resolved by 8% or 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and blocked

with 5% BSA in TBST for 1 h at room temperature. Then, the membranes were incubated with goat anti-NET (1:500, Santa Cruz Biotechnology, sc-51157, Santa Cruz, CA, USA) at room temperature for 1 h, followed by incubation for 45 min with horseradish peroxidase (HRP)-conjugated donkey anti-goat (1:10.000, Abcam, ab97120). The density of NET was normalized against GAPDH and expressed as fold change to the relative mean density of control group.

Statistical Analysis

The results are presented as the mean \pm SEM. Comparisons between multiple groups (such as for NET and NE levels) were performed using ANOVA followed by Tukey's multiple comparisons test. Categorical variables were compared by χ^2 -test. The significance level was set at 5%. The sample size was calculated assuming a difference between the control and PCOS groups of 30 arbitrary units of NET expression with a type I error of 0.05 and a power of 80%; the number of samples required was 8 per group.

Results

Clinical and Endocrine Characteristics

The clinical characteristics of the three groups of pregnant women and their newborns are presented in Table I. Age, initial weight, initial BMI, weight and BMI at term, and overall pregnancy weight gain were not different between groups. Cesarean section rate was comparable among the groups (Con-

Table I. Clinical characteristics of the mothers and their newborns.

	Control (n = 16)	PCOS-M (n = 8)	PCOS+M (n = 8)
Mothers			
Age (years)	27.80 \pm 1.39	32.88 \pm 1.87	27.34 \pm 2.63
Height (m)	1.58 \pm 0.01	1.62 \pm 0.02	1.60 \pm 0.01
Initial weight (kg)	68.72 \pm 2.85	66.58 \pm 3.91	75.75 \pm 5.46
Initial body mass index (kg/m ²)	27.60 \pm 1.14	25.30 \pm 1.29	29.67 \pm 2.05
Weight at term of pregnancy (kg)	79.69 \pm 2.82	75.56 \pm 3.23	83.21 \pm 5.44
BMI at term of pregnancy (kg/m ²)	31.98 \pm 1.06	28.74 \pm 1.05	32.64 \pm 2.12
Weight gain during pregnancy (kg)	10.97 \pm 1.30	8.97 \pm 1.66	9.15 \pm 1.38
Newborns			
Sex (F/M)	7/9	4/4	3/5
Gestational age (weeks)	38.87 \pm 0.26	38.87 \pm 0.48	38.88 \pm 0.35
Birth weight (kg)	3.50 \pm 0.07	3.49 \pm 0.09	3.32 \pm 0.10
Length (cm)	49.20 \pm 0.60	48.68 \pm 0.38	50.50 \pm 0.66

Values are mean \pm SEM. Differences were calculated by One-way ANOVA. Categorical data were analyzed by χ^2 test. PCOS-M: Polycystic ovary syndrome patients without metformin treatment during pregnancy; PCOS+M: Polycystic ovary syndrome patients who were treated with metformin during pregnancy.

trol: 32.3% (5/16); PCOS-M: 25.0% (2/8); PCOS+M: 37.5% (3/8, $p = 0.864$). Overall, 56.5%, 62.5% and 75% of the women in the control, PCOS-M and PCOS+M groups ($p = 0.670$), were primiparous, respectively. The newborns of the three groups of pregnant women were comparable in gestational age, weight and length. The proportion of female fetuses was comparable among the groups ($p = 0.956$). At week 24 of gestation, fasting glucose and insulin were comparable between groups (Table II). Circulating testosterone levels were higher in PCOS-M compared to control and PCOS+M ($p = 0.028$ and 0.039 , respectively), whereas androstenedione was higher in PCOS-M and PCOS+M compared to control ($p < 0.0001$ and 0.035 , respectively) (Table II). At week 35, fasting insulin was lower in controls compared to PCOS-M ($p = 0.007$) and PCOS+M ($p = 0.036$). Androstenedione was higher in PCOS-M and PCOS+M compared to the control group ($p < 0.001$, respectively), whereas testosterone was higher in PCOS-M compared to control and PCOS+M ($p = 0.002$ and 0.029 , respectively), but no differences were found between control and PCOS+M groups (Table II).

Plasma NE Levels During Pregnancy

At 24 weeks of pregnancy, plasma NE concentrations were significantly higher in the PCOS-M group compared to the control and PCOS+M groups ($p < 0.001$ and 0.006 , respectively) (Figure 1). PCOS+M patients showed similar NE plasma levels as controls. At 35 weeks of pregnancy, no differences were observed between groups (Figure 1).

Placental NET Expression

NET levels in the maternal side of the placental tissue from PCOS-M patients was sig-

nificantly decreased compared to that of control women ($p = 0.035$) (Figure 2A). On the other hand, PCOS+M exhibited significantly increased NET expression compared with PCOS-M ($p = 0.002$) but no differences were observed between PCOS+M and control women (Figure 2A). In the fetal side, NET expression was lower in PCOS-M compared to control women ($p < 0.001$). However, metformin treatment failed to recover NET expression ($p = 0.002$ between controls vs. PCOS+M) (Figure 2B).

Discussion

To our knowledge, the present investigation is the first to show that pregnant PCOS women exhibits elevated plasma concentrations of NE at 24 weeks of gestation and lower expression of placental NET at term. Of interest, treatment with metformin decreased circulating norepinephrine levels and increased NET expression on the maternal side of placental tissue from women with PCOS. Several studies have shown that pregnant women with PCOS maintain elevated circulating androgen levels and insulin concentrations^{15,23,24}, which could account for their pregnancy outcomes such as gestational diabetes, preeclampsia and pregnancy-induced hypertension, which have a direct impact on fetal health^{25,26}. The management of pregnant PCOS women with metformin during pregnancy has shown to reduce androgen and insulin levels¹⁶. In the present trial, we did not observe a reduction in fasting insulin concentrations in pregnant women with PCOS treated with metformin probably due to the small sample size. Nevertheless, metformin treatment reduced testosterone levels at 24 and 35 weeks of gestation.

Table II. Clinical characteristics of the mothers and their newborns.

	Control	PCOS-M	PCOS+M
Week 24	(n = 16)	(n = 8)	(n = 8)
Fasting Glucose (mg/dl)	78.60 ± 3.71	85.13 ± 4.57	79.33 ± 3.60
Fasting Insulin (μIU/ml)	10.79 ± 1.03	11.65 ± 2.13	8.91 ± 1.58
Androstenedione (ng/ml)	1.98 ± 0.41	5.62 ± 0.57 ^a	3.75 ± 0.45 ^a
Testosterone (ng/ml)	0.85 ± 0.22	1.73 ± 0.24 ^a	0.77 ± 0.11 ^b
Week 35	(n = 8)	(n = 8)	(n = 8)
Fasting Glucose (mg/dl)	72.20 ± 2.81	79.50 ± 5.36	70.50 ± 4.67
Fasting Insulin (μIU/ml)	9.43 ± 0.44	20.13 ± 3.50 ^a	17.65 ± 1.46 ^a
Androstenedione (ng/ml)	1.34 ± 0.13	4.54 ± 0.67 ^a	3.91 ± 0.46 ^a
Testosterone (ng/ml)	0.56 ± 0.05	1.51 ± 0.26 ^a	0.86 ± 0.11 ^b

Values are mean ± SEM. Differences were calculated by One-way ANOVA. ^a $p < 0.05$ vs. Control group; ^b $p < 0.05$ vs. PCOS-M.

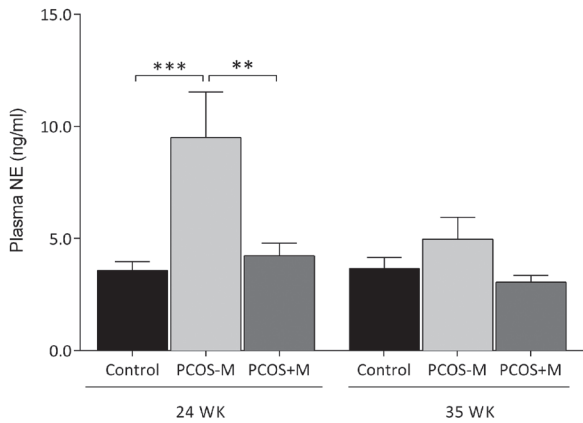


Figure 1. NE plasma levels at 24 and 35 weeks of gestation in controls, non-metformin treated PCOS pregnant women (PCOS-M) and metformin treated PCOS pregnant women (PCOS+M). Data are expressed as the mean \pm SEM. At week 24, 16 controls, 8 PCOS-M and 8 PCOS+M were included. At week 35, 8 controls, 8 PCOS-M and 8 PCOS+M were included. *** $p < 0.001$ and ** $p < 0.01$ between the respective bars in the chart. NE: Norepinephrine

Changes in NE Plasma Levels and the Effect of Metformin Treatment

Previous studies strongly suggest that ovarian sympathetic nerves are more active in patients with PCOS^{5,27-29}. However, this is the first report describing higher plasma levels of NE in pregnant PCOS women. Increased NE plasma levels were present at 24 weeks of gestation but had normalized by the 35 weeks. This higher plasma NE concentration occurs during a stage when fetal gonads are developing^{30,31} and are more susceptible to intrauterine factors that might affect its function during postnatal development. In this regard, we have recently described that gestational stress in rats not only increases NE plasma levels during pregnancy but also modifies the female reproductive function in the progeny^{12,32}. Interestingly, previous reports suggest that fetal gonadal development is deranged in girls born from pregnant PCOS mothers¹⁶. Metformin administration to PCOS women regulat-

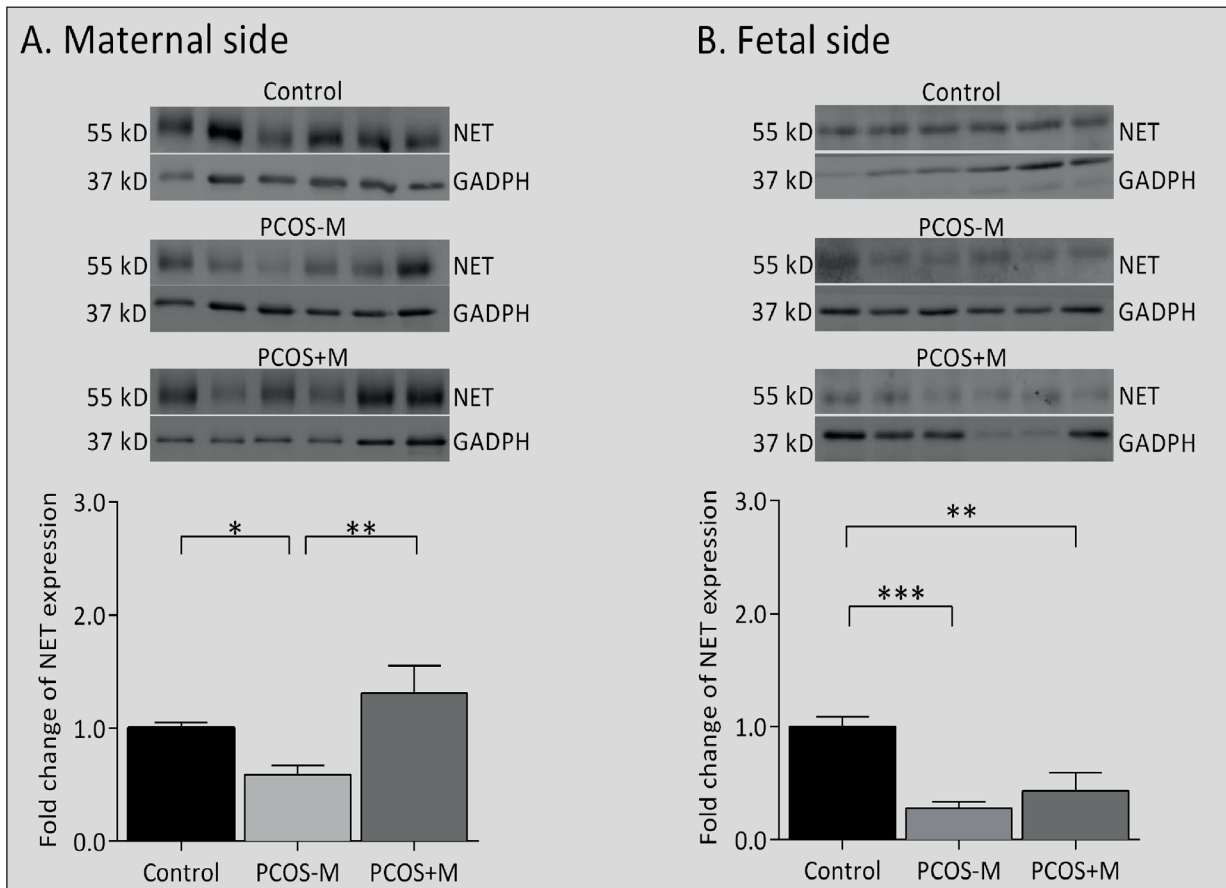


Figure 2. Placental NET expression in maternal side (A) and in fetal side (B) of term placenta from in controls, non-metformin treated PCOS pregnant women (PCOS-M) and metformin treated PCOS pregnant women (PCOS+M). Data are expressed as mean \pm SEM of 8 placentas of each group. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ between the respective bars in the chart. NET: Norepinephrine transporter.

ed the sympathetic nervous system activity in these women. This hypothesis is supported by evidence that metformin administration to hypertensive patients resulted in decreased blood pressure, heart rate and sympathetic nerve activity¹⁷⁻¹⁹. Interestingly, in preeclampsia, metformin reduces endothelial dysfunction and enhances vasodilatation in omental arteries³³. Therefore, our results confirm these suggestions and expand upon previous observations regarding the ability of metformin to regulate the sympathetic nerve activity including pregnant women with PCOS⁵. The mechanisms behind the effect of metformin on the SNA of pregnant PCOS patients are not completely clear. In this regard, metformin has demonstrated to have acute sympathoinhibitory effects on the central nervous system in rats¹⁷. Another mechanism might involve the regulation of androgen secretion, which are well known positive regulators of the biosynthesis of catecholamines at the adrenal medulla³⁴.

Changes in Placental NET Expression in PCOS Patients and the Effect of Metformin Treatment

Placental tissue does not contain nerve fibers to control its activity, but it has numerous monoaminergic transporters such as NET^{11,35}. Catecholamine levels in fetal circulation are very low, although they are important for maintaining cardiovascular homeostasis³⁶. In the sheep fetus, the sympathetic-adrenal axis controls the substantial biosynthesis of catecholamines and the high clearance from fetal circulation³⁶, which is almost exclusively driven by NET^{36,37}. Although there is poor NET expression in the syncytium trophoblasts of the chorionic villi, NET expression is high in trophoblasts of the anchoring villi on the maternal side of the human placenta^{35,37}. A surprising finding of our study was the complete recovery of placental NET expression on the maternal side of the placenta in pregnant PCOS women who were treated with metformin. This may have resulted from a previous metformin-induced decrease in plasma NE levels. Supporting this concept, Na et al³⁸ showed a direct relationship between increased plasma NE levels and decreased placental NET expression in women with preeclampsia. Taking our data together, we suggest that the early increase in maternal NE plasma levels in combination with the decrease in placental NET protein expression could attenuate the ability of NET to clear NE from the fetal circulation and therefore potentially increase fetal

exposure to NE leading to long-term consequences similar to those observed in animal models¹²⁻¹⁴. In this regard, it has been shown that placental NET contributes with nearly 50% of the total fetal NE clearance and an inverse correlation between placental mRNA expression of NET with NE levels in cord blood has been described in pregnancy pathologies^{36,37}.

Conclusions

We are the first to show that placental tissue from patients with PCOS has reduced NE transporter expression, which is associated with high sympathetic nerve activity during pregnancy. The capacity of metformin to increase placental NET expression suggests that this drug would be capable of modulating the sympathetic nervous activity. Although, this study has some limitations such as the small number of patients, the results strongly suggest that the catecholamine's homeostasis is modified in the placenta of PCOS mothers, which could probably affect physiological functions of their progeny during adult life. Larger studies are required to confirm these results.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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