

Placental expression of PAPP_A, PAPP_{A2} and PLAC-1 in pregnancies is associated with FGR

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Abstract. Fetal growth restriction (FGR) is a gynecological disorder of varying etiology. In the present study, an expression analysis of pregnancy-associated plasma protein A (PAPP_A), pregnancy-associated plasma protein A2 (PAPP_{A2}) and placenta-specific-1 (PLAC-1) was conducted in pregnancies with FGR and control pregnancies. Placental tissues were collected from pregnancies with FGR (n=16) and control pregnancies (n=16) and the expression of the genes of interest was examined by qPCR. The mean expression levels of PAPP_A and PAPP_{A2} were significantly lower (P<0.001) in placental tissues from FGR pregnancies compared with tissues from healthy subjects, whereas the opposite pattern was observed for PLAC-1 (P<0.001). PAPP_A and PLAC-1 expression in FGR and control subjects correlated with birth weight (P<0.001). The findings suggest a possible pathophysiological link between the development of FGR and the expression of PAPP_A, PAPP_{A2} and PLAC-1.

Introduction

Fetal growth restriction (FGR) is a complex disorder of pregnancy with varying etiology. It is characterized by the failure

of the fetus to achieve its normal growth potential and is associated with perinatal morbidity and mortality, as well as cardiovascular disease in adult life (1). A number of different causes have been attributed to the development of FGR including infections, drug abuse, as well as immunological and anatomical factors. Notably, placental dysfunction is one of the predominant causes of FGR. Despite extensive research into the mechanism underlying the development of FGR, its exact etiology remains elusive. Several hormones that are involved in pregnancy have been investigated with regard to FGR (2). The disease is thought to result from an abnormal placenta and thus identification of the genes involved in abnormal placenta development may enlighten our existing knowledge regarding the pathogenesis of FGR.

Pregnancy-associated plasma protein A (PAPP_A) is a syncytiotrophoblast-derived metalloproteinase that cleaves the complex formed between insulin-like growth factor (IGF) and insulin like growth factor binding protein (IGFBP). Serum levels of PAPP_A have been examined in relation to various pathological disorders such as stillbirth, infant death, preterm birth, and pre-eclampsia, as well as certain chromosomal disorders and anomalies (3). It is generally accepted that plasma levels of PAPP_A are decreased in intrauterine growth restriction (IUGR). Pregnancy-associated plasma protein A2 (PAPP_{A2}) is a protein that shares approximately 40% amino acid homology with PAPP_A and functions in a similar manner as an IGFBP protease. The physiological importance of PAPP_{A2} is not known. However, it is thought that PAPP_{A2} deficiency plays a major role in growth retardation as documented by studies in k/o mice (4). A 25-30% lower body weight and smaller organs were observed for the PAPP_{A2} k/o strain (4). In addition to PAPP_A proteins, placenta-specific-1 (PLAC-1) is a protein that has been examined as a biomarker for genetic and gestational disorders, since its expression is restricted to cells of the trophoblastic lineage and is absent from adult or fetal tissues. PLAC-1 ablation is associated with placentomegaly and IUGR (7). The exact function of this protein remains unknown and PLAC-1 expression has been

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Abbreviations: PAPP_A, pregnancy-associated plasma protein A; PLAC-1 placenta-specific-1; PAPP_{A2}, pregnancy-associated plasma protein A2; FGR, fetal growth restriction; qPCR, quantitative polymerase chain reaction; ISUOG, International Society of Ultrasound in Obstetrics and Gynecology; GA, gestational age

Key words: FGR, PAPP_A, PAPP_{A2}, PLAC-1, pregnancy

further demonstrated in a variety of human cancers, and is likely to have a significant role in modulating proliferation, invasion and survival of cancer cells (6-8).

Although abnormally low PAPP and PAPP2 levels in the first trimester maternal circulation are associated with increased risk of the disease, the expression of the aforementioned markers differs as regards the localization of the proteins in the placenta and/or in the plasma of pregnant women. The aim of the present study was to examine the placental expression levels of PAPP, PAPP2 and PLAC-1 in pregnancies with FGR and healthy pregnancies. Our investigation was further focused on the putative associations of the placental expression of the aforementioned biomarkers with the occurrence of FGR.

Materials and methods

Study group. The study comprised 16 cases of women with FGR and 16 normotensive subjects undergoing healthy pregnancy. Gestational age (GA) was defined by the last menstrual period. GA was corrected at 11-13 weeks of pregnancy in the cases where the gestational dates were uncertain. Medical history and pregnancy characteristics were recorded from maternity computerized records. An ultrasound examination for biometry of the fetus and Doppler studies were conducted in the third trimester of pregnancy. All the protocols were carried out according to the International Society of Ultrasound in Obstetrics and Gynecology (ISUOG) guidelines (9).

During delivery of the fetus samples from the placenta, samples were collected from both FGR and control (CT) groups. The tissue size ranged from 4 to 6 mm³ and tissue specimens were snap frozen in liquid N₂ and further stored at -80°C until processing. The study protocol was approved by the Ethics Committee of the National Kapodistrian University of Athens (Athens, Greece) and written informed consent was obtained from all the participants.

Selection criteria. FGR was defined according to the International Society for the study of Hypertension in Pregnancy (10). Specifically, women after 20 weeks of gestation who presented with a fetus with reduced growth velocity (<10th percentile) were classified as FGR subjects. Women with pre-existing diabetes type I and II, pre-existing hypertension and gestational diabetes mellitus were excluded from the selection criteria. The CT group included women undergoing pregnancies with a normal third trimester ultrasound scan.

RNA extraction and reverse transcription. RNA was extracted with TRIzol as described previously (11). Briefly each tissue was cut to small sections and homogenized with 1 ml TRIzol. The resulting mixture was vortexed with 200 µl chloroform and centrifuged for 15 min at 18,900 g. RNA was extracted from the top layer containing the organic phase and mixed with an equal volume of cold isopropanol. The samples were centrifuged for 10 min at 11,200 g and the resulting RNA pellet was washed once with 70% ethanol and resuspended in 40 µl of diethyl pyrocarbonate-treated water. The cDNA was synthesized using a Takara cDNA synthesis kit (Takara Bio, Inc., Otsu, Japan) following the manufacturer's protocol. The RNA

was incubated with a reaction mixture containing reaction buffer, RNase inhibitor, reverse transcriptase and water at 43°C for 1 h.

Quantitative polymerase chain reaction (qPCR). qPCR was conducted using SYBR at a final reaction volume of 20 µl in a Mx3000 Stratagene PCR amplifier (Agilent Technologies, Inc., Santa Clara, CA, USA). The primers were used at a final concentration of 0.5 µM. The sequences used for amplification of PAPP, PAPP2 and PLAC-1 were as follows: PAPP, forward: GTCATCTTTGCTGGAAGGGAGAA at 56°C (12) and reverse: AGGGCTGTCAACATCAGGATGAC, PAPP2, forward: ACTCACCCAAGAGGGCATAACATGA at 50°C (12) and reverse: GCACTGAGCTGGCAAAGTAGATGT, PLAC-1, forward: ATTGGCTGCAGGGATGAAAG at 50°C (13) and reverse: TGCCTCTGACCATGAACCA.

The quantification was conducted using a standard curve for each primer set. A pool cDNA was prepared that was diluted at: 1:5, 1:25, 1:125 and 1:625. The *GAPDH* and *TOP1* genes were used as housekeeping genes, according to previously published studies (14). The expression levels were presented as normalized values of expression of each gene.

Statistical analysis. Significant differences were determined for the demographic parameters using the Mann-Whitney U test, while statistical comparison of the mean levels of expression of the PAPP, PAPP2 and PLAC-1 genes was conducted using independent variables t-test. Correlation analysis was conducted using the Pearson's correlation test. A P<0.05 was considered to indicate a statistically significant difference.

Results

Placental levels of PAPP, PAPP2 and PLAC-1. The demographic parameters of the study are shown in Table I. The sample size consisted of 16 pregnant women with FGR and 16 normal pregnancies. The parameters age, height and weight of pregnant women were not significantly different between the control and FGR pregnancies. Significant differences were obtained for GA (P=0.024) and infant weight (P<0.001) with the FGR group exhibiting lower mean values of the two parameters compared to the CT group. This result was expected given the presentation of the disease, which manifested with lower growth characteristics of fetuses compared to normal pregnancies. In addition to the demographic characteristics, the placental mRNA expression levels of *PAPP*, *PAPP2* and *PLAC-1* were determined by qPCR. The mean expression levels of PAPP and PAPP2 were lower in the IUGR group compared to those of the CT group (Fig. 1). The differences were statistically significant as demonstrated by the corresponding P-values (P<0.001) (Table II). In contrast to these observations, PLAC-1 exhibited higher values in the FGR pregnancies compared to those of normal pregnancies (Fig. 1 and Table II), thus presenting an opposite pattern of expression between the two groups compared to PAPP and PAPP2. This difference was highly significant as determined by the independent sample t-test (P<0.001).

Linear regression analysis. Furthermore, correlation analyses revealed positive correlations of PLAC-1 (P<0.05) and PAPP

Table I. Demographic characteristics of mother and infants between IUGR and control group.

| Variables | Groups | No. | Mean | SD | Median | Min | Max | P-value |
|-------------------------|---------|-----|------|------|--------|------|------|---------|
| Age | Control | 16 | 30.1 | 4.1 | 29.0 | 21 | 35 | 0.160 |
| | IUGR | 16 | 33.3 | 7.6 | 33.0 | 22 | 47 | |
| | Total | 32 | 31.7 | 6.2 | 32.0 | 21 | 47 | |
| Height (m) | Control | 16 | 1.6 | 0.1 | 1.6 | 1.54 | 1.76 | 0.717 |
| | IUGR | 16 | 1.6 | 0.1 | 1.6 | 1.50 | 1.74 | |
| | Total | 32 | 1.6 | 0.1 | 1.6 | 1.50 | 1.76 | |
| Weight (kg) | Control | 16 | 71.9 | 8.4 | 68.0 | 60 | 86 | 0.440 |
| | IUGR | 16 | 69.2 | 10.2 | 68.0 | 54 | 95 | |
| | Total | 32 | 70.5 | 9.3 | 68.0 | 54 | 95 | |
| Gestational age (weeks) | Control | 16 | 40.3 | 3.1 | 41.0 | 33 | 44 | 0.024 |
| | IUGR | 16 | 37.8 | 2.7 | 38.0 | 33 | 43 | |
| | Total | 32 | 39.1 | 3.1 | 38.5 | 33 | 44 | |
| Birth weight (kg) | Control | 16 | 3.0 | 0.5 | 3.2 | 1.64 | 3.58 | <0.001 |
| | IUGR | 16 | 2.1 | 0.4 | 2.0 | 0.96 | 2.58 | |
| | Total | 32 | 2.6 | 0.7 | 2.6 | 0.96 | 3.58 | |

IUGR, intrauterine growth restriction; SD, standard deviation.

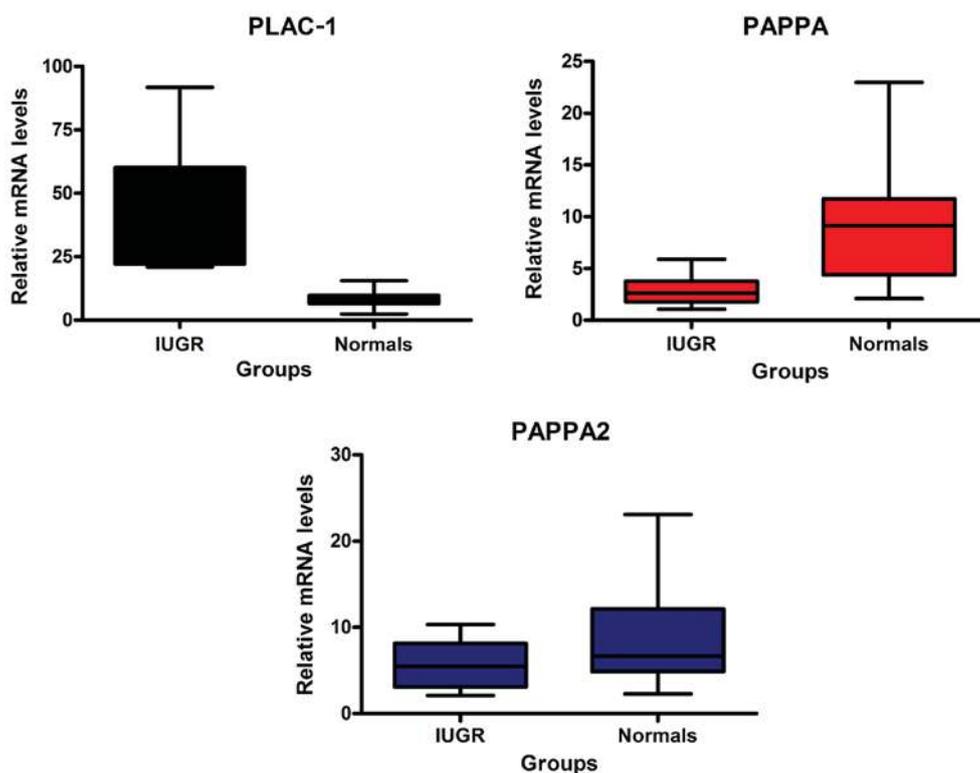


Figure 1. The placental expression levels of the markers PAPP A, PAPP A2 and PLAC-1 in FGR and control pregnancies. The expression levels were evaluated by qPCR analysis and normalized against the housekeeping genes GAPDH and TOP1. The results are expressed as mean \pm SD for FGR patients (n=16) and control pregnancies (n=16). Statistical differences were determined using independent samples t-test and the level of significance was set at 0.05. PAPP A, pregnancy-associated plasma protein A; PAPP A2, pregnancy-associated plasma protein A2; PLAC-1, placenta-specific-1; FGR, fetal growth restriction; SD, standard deviation.

($P < 0.01$) expression levels with birth weight, while with regard to PAPP A2 the results were not statistically significant (Fig. 2 and Table III). No significant correlation was noted

with regard to the expression levels of these genes with the birth weight of the FGR subjects (Table III). However, the correlation of the birth weight of the FGR subjects with the

Table II. Statistical analysis of average and range parameters of the expression levels of *PAPP*, *PAPP2* and *PLAC-1* genes in the FGR and control groups.

| Expression levels | Groups | No. | Mean | SD | Median | Min | Max | P-value |
|-------------------|---------|-----|------|------|--------|------|------|---------|
| <i>PAPP</i> | Control | 16 | 9.4 | 5.9 | 9.1 | 2.1 | 23.0 | <0.001 |
| | FGR | 16 | 2.8 | 1.4 | 2.6 | 1.1 | 5.9 | |
| | Total | 32 | 6.1 | 5.4 | 3.9 | 1.1 | 23.0 | |
| <i>PAPP2</i> | Control | 16 | 8.8 | 5.5 | 6.7 | 2.3 | 23.1 | <0.001 |
| | FGR | 16 | 5.7 | 2.7 | 5.5 | 2.1 | 10.3 | |
| | Total | 32 | 7.2 | 4.5 | 5.9 | 2.1 | 23.1 | |
| <i>PLAC-1</i> | Control | 16 | 8.1 | 3.2 | 8.1 | 2.5 | 15.5 | <0.001 |
| | FGR | 16 | 42.9 | 21.6 | 39.5 | 21.0 | 91.8 | |
| | Total | 32 | 25.5 | 23.3 | 18.3 | 2.5 | 91.8 | |

PAPP, pregnancy-associated plasma protein A; PAPP2, pregnancy-associated plasma protein A2; PLAC-1, placenta-specific-1; FGR, fetal growth restriction; SD, standard deviation.

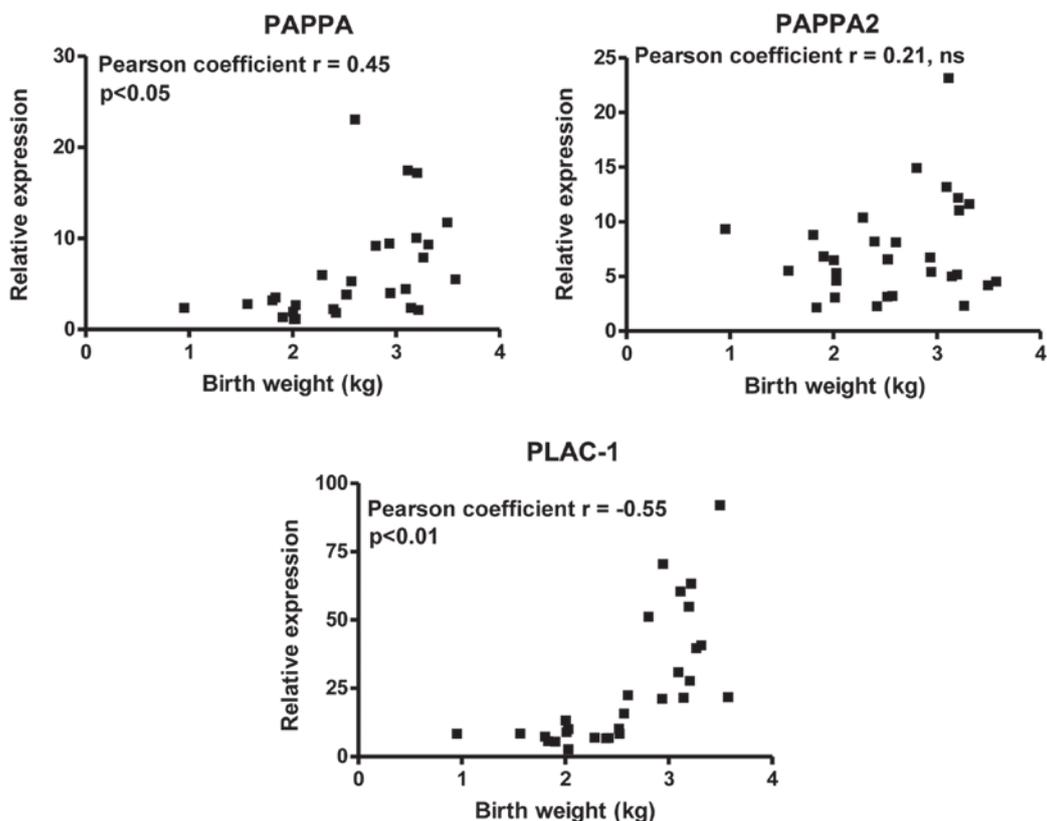


Figure 2. Correlation analysis of PAPP, PAPP2 and PLAC-1 expression with birth weight in FGR and control pregnancies. The results are expressed as correlation scatter plots for FGR patients (n=16) and control pregnancies (n=16) in each plot. The significant differences and the Pearson's coefficient *r* were determined using Pearson's correlation analysis and the level of significance was set at 0.05. PAPP, pregnancy-associated plasma protein A; PAPP2, pregnancy-associated plasma protein A2; PLAC-1, placenta-specific-1; FGR, fetal growth restriction.

expression levels of PAPP2 was stronger compared with that noted in the total population (-0.3358 vs. 0.21, Table III).

Discussion

The present study demonstrated an expression analysis of PAPP, PAPP2 and PLAC-1 in pregnancies with FGR and

control pregnancies. The mean expression levels of PAPP and PAPP2 in placental tissues were significantly lower in FGR pregnancies compared with healthy subjects, whereas the opposite pattern was observed for PLAC-1. The data further demonstrated a correlation of PAPP and PLAC-1 expression in FGR and control subjects with birth weight, suggesting a possible link of these biomarkers with FGR pathology.

Table III. Correlation analysis of PAPP, PAPP2 and PLAC1 expression with birth weight in FGR pregnancies.

| Genes | P-value | r | Significance | 95% CI |
|--------|---------|-------|--------------|------------|
| PAPP | 0.2 | 0.35 | No | -0.21-0.73 |
| PAPP2 | 0.2 | -0.34 | No | -0.73-0.21 |
| PLAC-1 | 0.8 | 0.06 | No | 0.47-0.55 |

The significant differences and the Pearson's coefficient *r* were determined using Pearson's correlation analysis and the level of significance was set at 0.05. PAPP, pregnancy-associated plasma protein A; PAPP2, pregnancy-associated plasma protein A2; PLAC-1, placenta-specific-1; FGR, fetal growth restriction; SD, standard deviation; CI, confidence intervals.

Previous studies have shown that PAPP is a biomarker that is decreased in the serum of women who present with FGR in the first trimester (15,16). Giudice *et al* have demonstrated that neonatal weights are associated with serum PAPP levels lower than the 25th centile (17). The exact mechanism that contributes to this outcome remains unclear, although PAPP deficiency has been shown to result in compromised fetal growth and skeletal phenotypes in *k/o* mice (4). However, it is believed that serum PAPP levels cannot be used as a reliable biomarker for FGR pathology, since similar biochemical measurements have been noted for preterm delivery, stillbirth and preeclampsia (18,19). Since PAPP and PAPP2 are expressed at high levels in the placenta we hypothesized that an altered expression of these biomarkers may influence the development of FGR. Although serum levels of PAPP rise and fall during the periods of FGR pregnancy we found that placental levels of PAPP and PAPP2 were significantly lower in FGR compared with control pregnancies, whereas PAPP expression correlated with birth weight. This finding is in agreement with the study of Kodama *et al* where decreased mean levels of placental PAPP mRNA were reported in late onset preeclampsia compared with healthy pregnancy (13). The possible explanation for these observations is attributed to the levels of the substrate IGFBP-5 that are inversely related to PAPP2 levels and regulate cytotrophoblast invasion, a key step in placenta development (20). With regard to PAPP the degradation of the similar protein IGFBP4 leads to the release of IGF-II, which promotes placental development via trophoblast invasion (21). Whether altered expression of these biomarkers is the cause or the effect of FGR remains to be determined.

The present study is in agreement with the report of Kodama *et al*, with the exception of the difference in the pathological state of the subjects examined (FGR vs. pre-eclampsia) (13). In addition, a previous study indicated lower PAPP multiples of median levels in SGA (small for GA) cases compared with control subjects (21). The same effect was noted for PAPP2 following stratification according to maternal hypertension and proteinuria (21). It is believed that the changes in the expression levels of PAPP2 and PAPP are associated with the expression of the substrates IGFBP-5

and IGFBP4 that regulate cytotrophoblast invasion, a key step in placenta development (19,22).

In addition to the PAPP biomarkers, the expression analysis of the novel X-linked gene *PLAC-1* in FGR and normal pregnancies revealed marked differences between the two groups. Disruption of PLAC-1 can cause hyperplasia and FGR, whereas PLAC-1 is also reported to be one of the upregulated genes in the hyperplastic placenta generated by nuclear transfer (23). Although the association of PLAC-1 with the development of FGR during pregnancy has not been examined to date, previous studies demonstrated elevated levels of circulating PLAC-1 mRNA in preeclampsia that were directly related to the disease severity (24-26). In a similar manner, increased levels of PLAC-1 in the placenta of FGR-women were observed in the present study, possibly suggesting a feedback mechanism, in order to overcome the development of the disease. Consistent with this hypothesis is the observation that PLAC-1 participates in the maintenance of pregnancy via the adaptation of the placenta to various physiological and environmental stimuli (27). A key feature of the function of PLAC-1 is the modulation of signaling pathways that regulate the cell membrane response to the extracellular environment, with respect to cell shape, motility and plasticity (27).

In conclusion, the present study demonstrated the selective overexpression of placental PLAC-1 and the reduced expression of PAPP and PAPP2 in pregnancies associated with FGR compared with healthy pregnancies. Future studies with higher number of samples should focus on the clinical value of PLAC-1 as a predictive biomarker for FGR, by addressing the expression of the latter protein in the serum of women throughout the stages of pregnancy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

VPA made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data and was involved in drafting the manuscript or revising it critically for important intellectual content. AP, AV and GIP were involved in drafting the manuscript or revising it critically for important intellectual content. SS and NS made substantial contributions to the acquisition of data and gave final approval of the version to be published. DAS gave final approval of the version to be published.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of the National Kapodistrian University of Athens (Athens, Greece) and written informed consent was obtained from all participants.

Consent for publication

Written informed consent was obtained from all human subjects regarding their participation in the study.

Competing interests

D.A. Spandidos is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article.

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