DOI: 10.1111/j.1471-0528.2010.02735.x
www.bjog.org

Reduced placental prolyl hydroxylase 3 mRNA expression in pregnancies affected by fetal growth restriction

V Gourvas,[a] S Sifakis,[b] E Dalpa,[a] N Soulitzis,[a] O Koukoura,[b] DA Spandidos[a]

[a] Laboratory of Clinical Virology, Medical School, University of Crete, Heraklion, Crete, Greece [b] Department of Obstetrics and Gynaecology, University Hospital of Heraklion, Crete, Greece

Correspondence: Prof DA Spandidos, Laboratory of Clinical Virology, Medical School, University of Crete, PO Box 2208, Heraklion 71003, Crete, Greece. Email spandidos@spandidos.gr

Accepted 27 August 2010. Published Online 13 October 2010.

Objective To investigate the role of the hypoxia-inducible factor (HIF) pathway in fetal growth restriction (FGR).

Design A case–control study.

Setting Research laboratory and gynaecology clinic.

Sample Twenty placentas from normal pregnancies and 20 from FGR pregnancies.

Methods RNA extraction, cDNA synthesis, quantitative real-time polymerase chain reaction (qRT-PCR) assay, statistical analysis.

Main outcome measures mRNA expression of HIF-1α, HIF-2α and HIF-β (ARNT), along with prolyl hydroxylase domain 3 (PHD3), which leads to proteasomal degradation of HIF-α subunits.

Results No statistically significant differences in the transcription levels of ARNT and HIF-2α were found between FGR and normal placentas. By contrast, PHD3 and HIF-1α mRNA were downregulated in FGR placentas. PHD3 mRNA expression was associated with gestational age at delivery ($P = 0.008$), birthweight centile ($P = 0.029$) and abnormal umbilical artery (UA) Doppler measurements ($P = 0.034$).

Conclusions As PHD3 regulates the HIF-mediated hypoxic response in FGR, we deduce that fetal adaptation to hypoxia ranges from impaired to adequate, as observed by the gradient of PHD3 downregulation in relation to the severity of FGR.

Keywords ARNT, HIF-1α, HIF-2α, hypoxia, intrauterine growth restriction.

Please cite this paper as: Gourvas V, Sifakis S, Dalpa E, Soulitzis N, Koukoura O, Spandidos D. Reduced placental prolyl hydroxylase 3 mRNA expression in pregnancies affected by fetal growth restriction. BJOG 2010;117:1635–1642.

Introduction

Fetal growth restriction (FGR) is a complex obstetric complication with an incidence of 4–7% of births. It is associated with a between six- and ten-fold increased risk of perinatal morbidity and mortality.[1,2] FGR is not a disease entity with a unique pathophysiology.[3] A variety of factors are involved, including congenital abnormalities, drug abuse, infectious diseases, and immunological and anatomical disorders. However, abnormal placentation (placental development) is observed in most cases.[4]

A successful pregnancy outcome depends on the appropriate development of the fetoplacental vasculature in the villous core, which follows the infiltration of cytotrophoblast in the decidua, and is completed in conjunction with the spiral arteries.[5,6] It is widely accepted that shallow trophoblast invasion can lead to fetal hypoxia and impaired growth.[7] Adequate proliferation and differentiation of villous cytotrophoblast into invasive extravillous trophoblast, a process that is controlled by hypoxia, is critical for successful placentation.[8,9] Thus, the entire repertoire of hypoxia-associated growth factors is remarkably active during placental development.

A prerequisite for many hypoxia-mediated responses are the basic helix-loop-helix (bHLH) Per–ARNT–Sim (PAS) family member transcription factors hypoxia inducible factor-1α (HIF-1α) and HIF-2α.[10,11] After stabilisation, these two molecules heterodimerise into the nucleus with aryl hydrocarbon nuclear translocator protein (ARNT or HIF-β),[12] and then bind to the hypoxia response element (HRE) in responsive genes that play a pivotal role in angiogenesis, glucose transport, glycolysis and erythropoiesis. HIFs are critical in mouse development, as all homozygous knock-out mice manifest embryonic lethality.[13] Degradation

of HIF-α protein is triggered by oxygen, which activates the prolyl hydroxylase domain enzymes (PHDs) that, in turn, hydroxylate proline residues 402 and 564 in HIF-α. These hydroxylated proline residues are required for the recognition and binding of the chaperone von Hippel Lindau (vHL) protein, which is part of a larger complex of proteins that ubiquitinates HIF-α, thereby targeting it for proteosomal degradation.[14,15] As the regulation of HIF activity is mainly mediated by protein stabilisation, vHL, PHD1, 2 and 3 are constitutively expressed in order to achieve complete proteasomal degradation of HIFs (Figure 1).[16]

In the present study, we investigated the possible involvement of the HIF/PHD pathway in extracted villi of pregnancies complicated by FGR, evaluating HIF-1α, HIF-2α, ARNT and PHD3 placental mRNA expression levels by quantitative real-time polymerase chain reaction (qRT-PCR). We focussed on PHD3, and not on PHD1 or PHD2, because it is strongly upregulated by hypoxia, presumably to rapidly cope with elevated HIF-1α protein abundance upon oxygenation.[17]

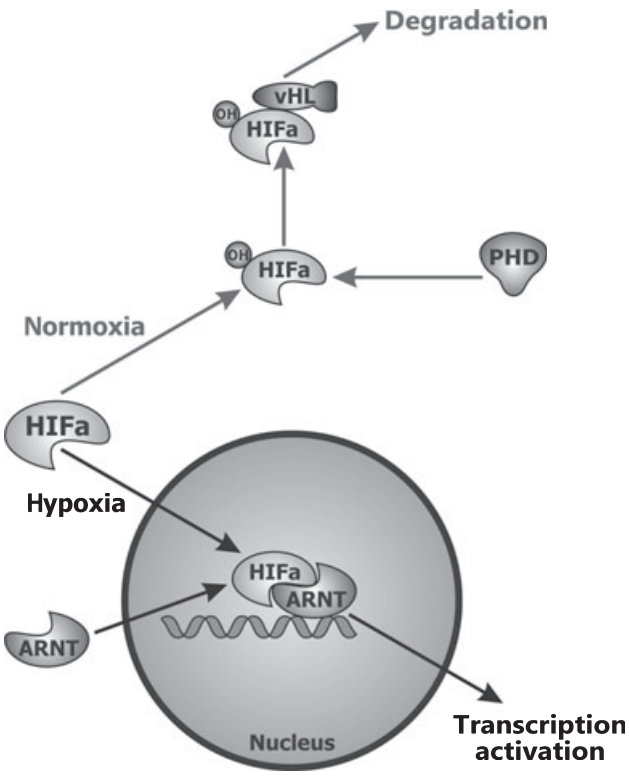

Figure 1. Graphical representation of the HIF pathway during normoxic and hypoxic conditions. In normoxia (grey arrows) PHD hydroxylates HIF-α, which then binds to vHL, leading to its proteosomal degradation. In hypoxia (black arrows) HIF-α enters the nucleus, forms a dimer with ARNT, which then binds to DNA, leading to gene transcription activation.

Methods

Placental collection and processing

Placentas were obtained from women with normal (n = 20) and FGR (n = 20) pregnancies, immediately after delivery. Six basal plate biopsy specimens of the medial part of the maternal–fetal interface were obtained from each placenta, in such a way that each sample contained the decidua basalis and villous placenta, but not the chorionic plate.[18,19] Tissue biopsies were snap-frozen in liquid nitrogen and stored at −80°C. Three of the above six biopsy specimens were randomly chosen for our measurements. The Ethics Committee of the University Hospital of Heraklion approved the study, and written informed consent was obtained from all participants.

Clinical definitions

Clinical details for the study groups are shown in Table 1. FGR was established by fetal weight and size below the tenth centile, along with an asymmetrical pattern of growth

Table 1. Clinical characteristics of the study groups

	FGR pregnancies	Normal pregnancies	*P*
Cases, *n*	20	20	
Maternal age (mean ± SD, years)	28.3 ± 5.6	28.1 ± 5.4	0.91*
BMI (mean ± SD)	23.0 ± 4.0	25.0 ± 6.8	0.28*
Maternal weight gain (mean ± SD, kg)	11 ± 5	15 ± 6	0.056*
Gestational age at delivery (mean ± SD, weeks)	36.6 ± 3.8	38.4 ± 1.7	0.058*
Birthweight (mean ± SD, g)	2022 ± 613	3123 ± 430	**<0.001***
Mode of delivery			
Vaginal (%)	7 (35.0)	11 (55.0)	0.20**
Caesarean section (%)	13 (65.0)	9 (45.0)	
Parity			
Nulliparous (%)	12 (60.0)	9 (45.0)	0.34**
Multiparous (%)	8 (40.0)	11 (55.0)	
Child gender			
Male (%)	9 (45.0)	8 (40.0)	0.75**
Female (%)	11 (55.0)	12 (60.0)	
Smoking			
Yes (%)	11 (55.0)	5 (25.0)	0.053**
No (%)	9 (45.0)	15 (75.0)	
Abnormal UA Doppler			
Yes (%)	8 (40.0)	1 (5.0)	**0.02****
No (%)	12 (60.0)	19 (95.0)	

*Student's *t* test (two-tailed).
**Chi-square or Fisher's exact test (two-tailed).
P-values in bold indicate statistically significant differences between the two study groups.

and abnormal measurements in the second trimester ultrasonography. Based on the above criteria, the fetuses examined in our study were considered to be growth restricted rather than constitutionally small babies. Interestingly, all of them had sizes below the third centile. Pregnant women with pre-eclampsia, pre-existing medical disorders, TORCH [toxoplasmosis, other (congenital syphilis and viruses), rubella, cytomegalovirus and herpes simplex virus] infections and substance abuse were excluded from the study. Additionally, no recognised chromosomal or genetic defects were observed in the neonates, and amniotic fluid index (AFI) values were within the normal range. Finally, pregnancies with a birthweight above the tenth centile, no recognised defect or pathology, and delivery after 37 completed weeks of gestation were classified as normal pregnancies, irrespective of the mode of delivery.

The umbilical artery (UA) Doppler flow velocity waveform was also measured, and it was considered to be abnormal if the systolic-to-diastolic ratio was >95%, or if the diastolic flow was absent or reversed.[20] However, abnormal UA Doppler measurements alone were not sufficient to categorise a baby as being growth restricted: only in combination with other clinical findings can FGR be diagnosed,[21] as FGR babies can have normal UA Doppler measurements.[22]

RNA extraction and cDNA preparation

A 100-mg sample of each tissue specimen was homogenised in 1 ml of TRIzol® reagent (Invitrogen, Carlsband, CA, USA) using a power homogeniser, followed by the addition of chloroform and centrifugation. Total RNA was precipitated from the supernatant with isopropanol, washed with 75% ethanol and resuspended in 30 μl of diethylpyrocarbonate (DEPC)-treated water. RNA concentration and purity were calculated after measuring the 260-nm absorbance and 260/280-nm absorbance ratio, respectively, on a UV spectrophotometer.

cDNA was synthesised by reverse transcription (RT) with the Thermoscript™ RT kit (Invitrogen), using random hexamers as amplification primers. In detail, 2.5 μg of total RNA, 50 ng of random hexamers and 1 mmol/l deoxynucleotide triphosphates (dNTPs) were heated at 65°C for 5 minutes, in order to remove RNA secondary structures, and placed on ice until the addition of cDNA synthesis mix, which contained 1× cDNA synthesis buffer (50 mmol/l Tris-acetate, pH 8.4, 75 mmol/l potassium acetate and 8 mmol/l magnesium acetate), 5 mmol/l dithiothreitol (DTT), 40 units RNaseOut™ and 15 units Thermoscript™ reverse transcriptase. The final mix (volume 20 μl) was incubated for 10 minutes at 25°C for primer extension, and cDNA synthesis was conducted at 55°C for 50 minutes. The reaction was terminated by heating at 85°C for 5 minutes. In order to remove the RNA template, cDNA was incubated at 37°C for 20 minutes with 2 units of *Escherichia coli* RNaseH, and stored at −20°C until use.

Quantitative real-time polymerase chain reaction assay

The expression of HIF-1α, HIF-2α, PHD3 and ARNT mRNA was measured using a qRT-PCR assay with SYBR® Green I dye. A housekeeping gene, β-actin, was used as an internal control, in order to normalise HIF-1α, HIF-2α, PHD3 and ARNT mRNA expression levels. The mRNA-specific primers, which were designed with Lasergene® (DNASTAR, Madison, WI, USA), and span at least one intron with an average length >800 bp, are listed in Table 2. Their specificity was verified with the BLAST program (www.ncbi.nlm.nih.gov/BLAST). After initial experiments, in order to optimise the concentration and annealing temperature of the primers, 1 μl of cDNA from FGR or control samples was amplified in a PCR reaction containing 2× Brilliant SYBR® Green QPCR Master Mix (Stratagene, La Jolla, CA, USA), 300 nmol/l of each primer and 30 μmol/l Rox passive reference dye, in a final volume of 20 μl. To ensure the accuracy of the quantification measurements, a representative pool of all the samples was diluted in a series of five 2× dilutions, and was run on the same plate, in order to construct a standard curve for the quantification process. After initial denaturation at 95°C for 10 minutes, samples were subjected to 40 cycles of amplification, comprised of denaturation at 95°C for 20 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 30 seconds, followed by a melt-curve analysis (in order to verify the presence of only one PCR product and the absence of dimers), in which the temperature was increased from 60 to 95°C at a linear rate of 0.2°C/second. Data collection was performed during both annealing and extension, with two measurements at each step, and at all times during the melt-curve analysis. PCR

Table 2. Primer sequences used for qRT-PCR

Primer pair	Sequence (5′ → 3′)	Amplicon size (bp)
HIF-1α	CGGCGCGAACGACAAGAA GGAAGTGGCAACTGATGAGCAAG	126
HIF-2α	ATGACAGCTGACAAGGAGAAGAA TGGGCCAGCTCATAGAACAC	116
PHD3	CCGGCTGGGCAAATACTAC GTCCACGTGGCGAACATAAC	91
ARNT	GCCATTGCCTCTGGAAACTCT CTGTTCCCTTCTCCATCATCATC	113
β-actin	CGGCATCGTCACCAACTG GGCACACGCAGCTCATTG	70

experiments were conducted on an Mx3000P real-time PCR thermal cycler using software v4.01, build 369, schema 80 (Stratagene). PCR products were electrophoresed on 2% (w/v) agarose gels and stained with ethidium bromide, in order to verify that the corresponding product band (as visualised with the help of a UV transilluminator) had the correct size. Representative bands of all PCR products analysed were extracted from the agarose gels, purified and sequenced, as a final confirmation step that the appropriate gene was amplified at each PCR reaction. After amplification, standard curves were constructed from the samples used in the series of consecutive dilutions. Subsequently, using these standard curves and the cycle threshold (C_t) value of the samples, we calculated the mRNA expression of the genes studied. Samples with no amplification plots or with dissociation curves that exhibited signs of primer–dimer formation or by-products were excluded. To normalise the mRNA expression of each gene, the expression value was divided by the β-actin mRNA value. The normalised values of FGR samples were divided by the average normalised values of normal samples. The result of this division provided the relative expression of an FGR specimen in relation to the control group. This mathematical process is summarised in the following formula:

$$\text{Normalised sample/control} = (1 + E_{\text{gene}})^{-\Delta Ct_{\text{gene}}} / (1 + E_{\beta-\text{actin}})^{-\Delta Ct_{\beta-\text{actin}}}$$

A two-fold increased (i.e. a value ≥2) or decreased (i.e. a value ≤0.5) expression was considered biologically significant (for overexpression or downregulation, respectively). In each PCR reaction two negative controls were included, one with no cDNA template and one with no reverse transcription treatment. All qRT-PCR measurements were conducted in triplicate.

Statistical analysis

The mRNA levels of HIF-1α, HIF-2α, PHD3 and ARNT were first evaluated by the one-sample Kolmogorov–Smirnov goodness-of-fit test, in order to determine whether they followed a normal distribution pattern. Depending on the results, Pearson's or the non-parametric Spearman's rank test was used to examine their relationship pairwise, and their association with continuous variables [maternal age, body mass index (BMI), weight gain, gestational age at delivery and birthweight centile]. Moreover, their association with categorical data (smoking habits, mode of delivery, child gender, parity and UA Doppler measurements) was examined using a Student's *t* test (after an assessment of the equality of variances using Levene's test), or its non-parametric equivalents Mann–Whitney *U*- and Kruskal–Wallis H-tests. Additionally, the chi-square (χ^2) test, replaced by Fisher's exact test when indicated by the analysis, was used to examine the expression status of the four genes with the various clinicopathological parameters after stratification. Statistical analyses were two-sided, and were performed with SPSS 11.5 (SPSS, Chicago, IL, USA). Statistical significance was set at the 95% level ($P < 0.05$).

Power and sample size calculations

Power and sample size calculations were performed with PS POWER AND SAMPLE SIZE CALCULATIONS v3.0.14 (biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize). For sample size calculations, the power (type-II error, or β) was set to 80% and the statistical significance (type-I error, or α) was set to 0.05. For power calculations, the size was set to 20 (the specimen and control groups size in our study), and the type-I error was set to 0.05. For both calculations, the minimum difference in the mean value (0.88) of PHD3 or HIF-1α mRNA expression between FGR samples and controls in the clinical parameters that yielded statistically significant results (gestational age, birthweight centile, UA Doppler and mode of delivery), and the maximum standard deviation (0.92) within the two study groups in each of the above parameters, were used.

Results

The results of our qRT-PCR analysis are shown in Table 3. Compared with controls, mRNA expression levels of HIF-1α and PHD3 exhibited downregulation in 45% (9/20) and 65% (13/20) of FGR samples, respectively. On the contrary, HIF-2α and ARNT transcription levels were similar in the majority of FGR women when compared with controls.

Further analysis revealed statistically significant associations between placental mRNA levels and particular pathological parameters in FGR cases (Figure 2). PHD3 mRNA expression was higher in FGR specimens with a gestational age of <37 weeks (8/20 samples) compared with those with gestational age of >37 weeks (12/20 samples) (2.08 ± 0.92 versus 0.50 ± 0.24, Mann–Whitney *U* test, $P = 0.008$).

Table 3 mRNA expression analysis of HIF-1α, HIF-2α, PHD3 and ARNT in FGR women

Gene	Relative mRNA expression		
	↑ (%)	— (%)	↓ (%)
HIF-1α	5/20 (25.0)	6/20 (30.0)	9/20 (45.0)
HIF-2α	3/20 (15.0)	11/20 (55.0)	6/20 (30.0)
PHD3	3/20 (15.0)	4/20 (20.0)	13/20 (65.0)
ARNT	3/20 (15.0)	11/20 (55.0)	6/20 (30.0)

↑, overexpression; —, normal expression; ↓, reduced expression.

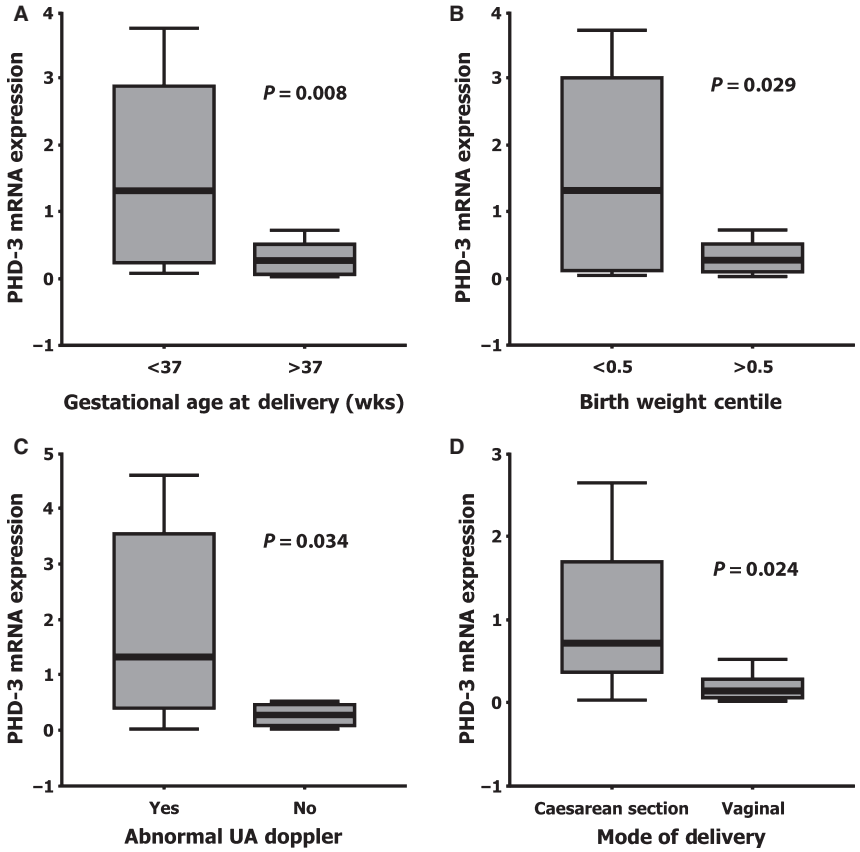

Figure 2. Box and whisker plots depicting statistically significant associations of PHD3 placental mRNA expression with various clinical parameters in FGR-affected pregnancies. (A) PHD3 expression is decreased as gestational duration is elongated [1.32 (2.64) versus 0.28 (0.45), $P = 0.008$]. (B) PHD3 mRNA levels are lower in FGR specimens with birthweights in higher centiles [1.32 (2.90) versus 0.28 (0.42), $P = 0.029$]. (C) PHD3 is downregulated in FGR samples with normal umbilical artery (UA) Doppler measurements [1.32 (3.14) versus 0.27 (0.38), $P = 0.034$]. (D) PHD3 mRNA levels are decreased in FGR samples with vaginal delivery [0.72 (1.33) versus 0.09 (0.22), $P = 0.024$]. All values are presented as medians [interquartile range (IQR) = Q_3–Q_1]. The thick line near the centre of each rectangular box represents the median value, the bottom and top edges of the box indicate the first (Q_1) and third (Q_3) quartiles, and the ends of the whiskers depict the tenth (P_{10}) and 90th (P_{90}) percentiles. Statistical analysis was conducted with a two-tailed Mann–Whitney *U* test.

Moreover, FGR cases with birthweights lower than the 0.5 centile (11/20 samples) had higher PHD3 mRNA levels compared with FGR pregnancies with birthweights higher than the 0.5 centile (9/20 samples) (1.97 ± 0.77 versus 0.30 ± 0.08, Mann–Whitney *U* test, $P = 0.029$). Additionally, FGR pregnancies with abnormal UA Doppler (8/20 samples) exhibited increased PHD3 mRNA expression levels compared with FGR cases with normal UA Doppler (12/20 samples) (2.28 ± 0.92 versus 0.37 ± 0.13, Mann–Whitney *U* test, $P = 0.034$). Finally, HIF-1α mRNA levels were decreased in FGR specimens with birthweights lower than the 0.5 centile (11/20 samples) compared with FGR pregnancies with birthweights higher than the 0.5 centile (9/20 samples) (0.99 ± 0.29 versus 1.87 ± 0.73, Mann–Whitney *U* test, $P = 0.039$).

There was no statistically significant association between HIF-1α placental mRNA levels and gestational age at delivery or UA Doppler measurements. Additionally, no statistically significant associations were observed regarding HIF-1α or PHD3 placental mRNA levels and maternal age, BMI, smoking habits, weight gain, gender of the child and parity. Finally, the transcription levels of the other two genes studied (HIF-2α and ARNT), did not exhibit any statistically significant associations with the pathological parameters.

Using Spearman's rank test, we tested the co-expression pattern of the four examined genes in a pairwise manner in the FGR samples (after normalisation). This test examines whether two molecules are upregulated or downregulated together (positive association), or whether when one is overexpressed the other has reduced expression (negative association). The results indicated that HIF-1α is positively associated with PHD3 and ARNT [correlation coefficient (CC) = 0.58, $P = 0.007$; CC = 0.83, $P < 0.001$, respec-

tively], PHD3 is positively associated with ARNT (CC = 0.47, $P = 0.038$), whereas HIF-2α mRNA expression is not associated with HIF-1α, PHD3 and ARNT transcription levels (CC = 0.02, $P = 0.94$; CC = 0.06, $P = 0.82$; and CC = 0.10, $P = 0.66$, respectively).

In FGR-affected pregnancies for which a caesarean section (13/20 samples) was performed, increased PHD3 mRNA levels were observed compared with FGR pregnancies with a vaginal delivery (7/20 samples) (1.64 ± 0.61 versus 0.19 ± 0.07, Mann–Whitney *U* test, $P = 0.024$).

Finally, according to power and sample size calculations, our study had 83.8% power to find the statistically significant associations that were observed. Interestingly, only 18 samples and controls were needed in order for our study to have 80% power.

Discussion

In the present study, we found PHD3 expression in FGR placentas to be downregulated, a finding that differs from the reported PHD3 mRNA levels in pre-eclamptic placentas.[23] It has already been established that increased oxidative stress in the pre-eclamptic placenta is associated with altered proteosomal activity and protein patterns.[24–26] A similar stress, although of different severity, has been reported in FGR placentas.[27] We suggest that reduced PHD3 expression is an impaired hypoxic response of placental cells that occurs in order to activate HIFs. It is also known that vaginal delivery is associated with higher stress to the fetus.[28,29] The impact of this stress on PHD3 expression is additionally revealed by our findings, as among FGR cases that exhibited PHD3 downregulation, those with a vaginal delivery exhibited decreased PHD3 expression compared with those delivered by caesarean section.

This study also demonstrated a less profound downregulation of HIF-1α compared with PHD3, whereas HIF-2α and ARNT mRNA levels showed no significant differences compared with controls. According to the literature, HIF-1α and -2α mRNA expression is not altered in FGR-affected pregnancies,[30] although an increase in protein levels, especially for HIF-2α, has been observed as a result of post-translational modifications.[31] In contrast, in pre-eclampsia, both HIF-1α and HIF-2α are overexpressed.[32–34] There is also evidence to suggest that the impairment of protein degradation rather than increased synthesis causes the inadequate oxygen-dependent reduction of HIF-1α protein in pre-eclamptic placentas.[25]

Based on the above data, we assume that the regulation of HIFs in the placentas of FGR-affected pregnancies is mainly achieved through protein stabilisation (post-translational modification),[17] which could explain why we did not observe large-scale variations in the expression levels of HIFs. Although HIF-1α mRNA appeared to be slightly downregulated, we considered HIF-1α to be more active through minimal proteosomal activity, implying a decrease in HIF-α degradation. HIF-1α downregulation could probably be explained as a result of co-expression with PHD3, because these two molecules share a positive internal feedback mechanism.[35] Regarding ARNT, it is well documented that it is constitutively expressed.[36,37] Consequently, we did not expect to observe alterations in its mRNA expression. From the above we deduce that, although our mRNA data should have been accompanied by experimental work conducted at the protein level, in order to verify and strengthen our observations, our results still indicate that the HIF/PHD pathway is deregulated in the placenta of FGR-affected women.

Notable statistical associations between PHD3 mRNA expression and some of the pathological characteristics of our FGR specimens were also observed. PHD3 expression was inversely related to the gestational age at delivery. Moreover, FGR-affected pregnancies with a birthweight centile higher than 0.5 had decreased PHD3 expression compared with FGR-affected pregnancies with birth weights lower than the 0.5 centile. Additionally, PHD3 mRNA levels were reduced in FGR samples with normal UA Doppler measurements compared with FGR samples with abnormal UA Doppler measurements. A growth-restricted fetus can have a placenta with positive end-diastolic flow, suggesting an adaptive pathway for the placenta in the face of uteroplacental ischemia.[22] Thus, it was observed that FGR-affected pregnancies with a less adverse outcome displayed increased PHD3 downregulation. The severity of FGR appears to be related to differences in the adaptation model to hypoxia, ranging from adequate to impaired or absent adaptation. This approach has already been suggested in FGR cases associated with pre-eclampsia.[38]

It is believed that pre-eclampsia and FGR share common components in their pathogenesis, although some disparate findings have been described.[7,39] Furthermore, it has been suggested that FGR in pre-eclampsia represents the response to a fetal signal stimulated by reduced placental nutrient availability that is inadequate to overcome the subsequent malnutrition stress. Conversely, FGR without pre-eclampsia occurs as a result of the absence of this signal.[38,40] We suggest that this model could be applied to the FGR cases of this study. Based on our results, it can be hypothesised that FGR babies with less severe disease produce a signal stimulated by the reduced placental nutrient and oxygen availability, in contrast to FGR babies with a more adverse outcome that fail to produce this signal.

Conclusion

This pilot study has demonstrated that PHD3 may play an important role in the molecular adaptation that occurs

during oxidative stress in FGR pathophysiology. Further studies are needed in order to verify these results and to investigate whether PHD3 could be used as a prognostic marker of FGR severity.

Disclosure of interests
The authors have no conflicts of interest to declare.

Contribution to authorship
VG, concept and design, data acquisition and interpretation, manuscript drafting and revision; SS, concept and design, data interpretation, manuscript revision; ED, concept and design, data acquisition, manuscript drafting; NS, data analysis, manuscript revision; OK, concept and design; DAS, concept and design, manuscript revision, final version approval.

Details of ethics approval
This study was approved by the University Hospital of Heraklion Ethics Committee (no. 462/01-11-2007).

Funding
This study was financially supported by the Empeirikeion Foundation, Athens, Greece.

Acknowledgements
The authors would like to thank Mr Demetrios Koutroulakis, MD, for his assistance in acquiring the placental tissue samples. ■

References

1 Jarvis S, Glinianaia SV, Torrioli MG, Platt MJ, Miceli M, Jouk PS, *et al.* Cerebral palsy and intrauterine growth in single births: European collaborative study. *Lancet* 2003;362:1106–11.
2 Barker DJ. In utero programming of chronic disease. *Clin Sci (Lond)* 1998;95:115–28.
3 Cetin I, Foidart JM, Miozzo M, Raun T, Jansson T, Tsatsaris V, *et al.* Fetal growth restriction: a workshop report. *Placenta* 2004;25:753–7.
4 Kaufmann P, Black S, Huppertz B. Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia. *Biol Reprod* 2003;69:1–7.
5 Huppertz B, Abe E, Murthi P, Nagamatsu T, Szukiewicz D, Salafia C. Placental angiogenesis, maternal and fetal vessels–a workshop report. *Placenta* 2007;28 (Suppl A):S94–6.
6 Mayhew TM, Charnock-Jones DS, Kaufmann P. Aspects of human fetoplacental vasculogenesis and angiogenesis. III. Changes in complicated pregnancies. *Placenta* 2004;25:127–39.
7 Ness RB, Sibai BM. Shared and disparate components of the pathophysiologies of fetal growth restriction and preeclampsia. *Am J Obstet Gynecol* 2006;195:40–9.
8 James JL, Stone PR, Chamley LW. The regulation of trophoblast differentiation by oxygen in the first trimester of pregnancy. *Hum Reprod Update* 2006;12:137–44.
9 Genbacev O, Zhou Y, Ludlow JW, Fisher SJ. Regulation of human placental development by oxygen tension. *Science* 1997;277:1669–72.
10 Semenza GL. Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol* 1999;15:551–78.
11 Ema M, Taya S, Yokotani N, Sogawa K, Matsuda Y, Fujii-Kuriyama Y. A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development. *Proc Natl Acad Sci USA* 1997;94:4273–8.
12 Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. *Proc Natl Acad Sci USA* 1995;92:5510–4.
13 Maltepe E, Schmidt JV, Baunoch D, Bradfield CA, Simon MC. Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. *Nature* 1997;386:403–7.
14 Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 2001;294:1337–40.
15 Min JH, Yang H, Ivan M, Gertler F, Kaelin WG Jr, Pavletich NP. Structure of an HIF-1alpha -pVHL complex: hydroxyproline recognition in signaling. *Science* 2002;296:1886–9.
16 Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem* 1997;272:22642–7.
17 Schofield CJ, Ratcliffe PJ. Signalling hypoxia by HIF hydroxylases. *Biochem Biophys Res Commun* 2005;338:617–26.
18 Benyo DF, Smarason A, Redman CW, Sims C, Conrad KP. Expression of inflammatory cytokines in placentas from women with preeclampsia. *J Clin Endocrinol Metab* 2001;86:2505–12.
19 Winn VD, Haimov-Kochman R, Paquet AC, Yang YJ, Madhusudhan MS, Gormley M, *et al.* Gene expression profiling of the human maternal-fetal interface reveals dramatic changes between midgestation and term. *Endocrinology* 2007;148:1059–79.
20 Intrauterine growth restriction. clinical management guidelines for obstetrician-gynecologists. American college of obstetricians and gynecologists. *Int J Gynaecol Obstet* 2001;72:85–96.
21 Cnossen JS, Morris RK, ter Riet G, Mol BW, van derPost JA, Coomarasamy A, *et al.* Use of uterine artery Doppler ultrasonography to predict pre-eclampsia and intrauterine growth restriction: a systematic review and bivariable meta-analysis. *CMAJ* 2008;178:701–11.
22 Todros T, Sciarrone A, Piccoli E, Guiot C, Kaufmann P, Kingdom J. Umbilical Doppler waveforms and placental villous angiogenesis in pregnancies complicated by fetal growth restriction. *Obstet Gynecol* 1999;93:499–503.
23 Rajakumar A, Michael HM, Daftary A, Jeyabalan A, Gilmour C, Conrad KP. Proteasomal activity in placentas from women with preeclampsia and intrauterine growth restriction: implications for expression of HIF-alpha proteins. *Placenta* 2008;29:290–9.
24 Wenger RH, Stiehl DP, Camenisch G. Integration of oxygen signaling at the consensus HRE. *Sci STKE* 2005;2005:re12.
25 Rajakumar A, Doty K, Daftary A, Harger G, Conrad KP. Impaired oxygen-dependent reduction of HIF-1alpha and -2alpha proteins in pre-eclamptic placentae. *Placenta* 2003;24:199–208.
26 Hass R, Sohn C. Increased oxidative stress in pre-eclamptic placenta is associated with altered proteasome activity and protein patterns. *Placenta* 2003;24:979–84.
27 Burton G, Yung HW. Placental stress, protein synthesis inhibition, and growth restriction. *Biol Reprod* 2008;78 (Suppl 1):275.
28 Ruth V, Hallman M, Laatikainen T. Corticotropin-releasing hormone and cortisol in cord plasma in relation to gestational age, labor, and fetal distress. *Am J Perinatol* 1993;10:115–8.
29 Bird JA, Spencer JA, Mould T, Symonds ME. Endocrine and metabolic adaptation following caesarean section or vaginal delivery. *Arch Dis Child Fetal Neonatal Ed* 1996;74:F132–4.

30 Rajakumar A, Jeyabalan A, Markovic N, Ness R, Gilmour C, Conrad KP. Placental HIF-1 alpha, HIF-2 alpha, membrane and soluble VEGF receptor-1 proteins are not increased in normotensive pregnancies complicated by late-onset intrauterine growth restriction. *Am J Physiol Regul Integr Comp Physiol* 2007;293:R766–74.

31 Dai SY, Kanenishi K, Ueno M, Sakamoto H, Hata T. Hypoxia-inducible factor-2alpha is involved in enhanced apoptosis in the placenta from pregnancies with fetal growth restriction. *Pathol Int* 2004;54: 843–9.

32 Rajakumar A, Whitelock KA, Weissfeld LA, Daftary AR, Markovic N, Conrad KP. Selective overexpression of the hypoxia-inducible transcription factor, HIF-2alpha, in placentas from women with preeclampsia. *Biol Reprod* 2001;64:499–506.

33 Jarvenpaa J, Vuoristo JT, Savolainen ER, Ukkola O, Vaskivuo T, Ryynanen M. Altered expression of angiogenesis-related placental genes in pre-eclampsia associated with intrauterine growth restriction. *Gynecol Endocrinol* 2007;23:351–5.

34 Caniggia I, Winter JL. Adriana and Luisa Castellucci award lecture 2001. Hypoxia inducible factor-1: oxygen regulation of trophoblast differentiation in normal and pre-eclamptic pregnancies – a review. *Placenta* 2002;23 (Suppl A):S47–57.

35 Fong GH, Takeda K. Role and regulation of prolyl hydroxylase domain proteins. *Cell Death Differ* 2008;15:635–41.

36 Hirota K, Semenza GL. Regulation of angiogenesis by hypoxia-inducible factor 1. *Crit Rev Oncol Hematol* 2006;59:15–26.

37 Jiang BH, Semenza GL, Bauer C, Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O_2 tension. *Am J Physiol* 1996;271:C1172–80.

38 Roberts JM, Hubel CA. The two stage model of preeclampsia: variations on the theme. *Placenta* 2009;30 (Suppl A):S32–7.

39 Mayhew TM, Wijesekara J, Baker PN, Ong SS. Morphometric evidence that villous development and fetoplacental angiogenesis are compromised by intrauterine growth restriction but not by preeclampsia. *Placenta* 2004;25:829–33.

40 Shibata E, Hubel CA, Powers RW, von Versen-Hoeynck F, Gammill H, Rajakumar A, *et al.* Placental system A amino acid transport is reduced in pregnancies with small for gestational age (SGA) infants but not in preeclampsia with SGA infants. *Placenta* 2008;29:879–82.

