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Major differences in the levels of redox status and antioxidant defence markers in the erythrocytes of pre- and full-term neonates with intrauterine growth restriction

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ABSTRACT

Intrauterine growth restriction (IUGR) is a pleiotropic complication of pregnancy. Prematurity and growth abnormalities are common risk factors for perinatal morbidity and mortality. Free radical damage has been recognized as a common pathogenic mechanism of many neonatal diseases. The aim of the present study was to characterize the possible links between the level of maturity, the birthweight and the antioxidant status of neonates born with IUGR. Our data suggest that the stress markers measured on the cord blood of neonates with IUGR and mature, healthy neonates do not necessarily reflect the extent of oxidative stress. However, significant correlations were found between the maturity of the neonates with IUGR and the oxidative damage. The mature IUGRs exhibited $ONOO^-$ accumulation and increased lipid peroxidation more frequently as compared with the pre-term group. The results suggest that the oxidative injury in IUGR may depend on the level of maturity and the birthweight.

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1. Introduction

Pregnancy is a physiological state associated with an enhanced metabolism and an increased demand for oxygen. Premature infants are at particular risk from oxidative stress, as neither the endogenous nor the passively acquired exogenous antioxidant defence system accelerates in maturation until late in the third trimester [1,2]. Intrauterine growth restriction (IUGR) is one of the major complications of pregnancy and accounts for significant neonatal mortality and morbidity [3]. The development of IUGR may be a consequence of various factors, including an abnormal fetomaternal blood circulation, genetic disorders, pregnancy-induced hypertension, pregestational diabetes, a lean umbilical cord, previous intrauterine infections, a poor nutritional state of the mother, frequent cigarette smoking [4] and toxin or drug exposure [5]. However, in the vast majority of the cases the cause remains idiopathic.

IUGR is often complicated by intrauterine hypoxia and may induce the generation of reactive oxygen species (ROS) and foetal oxidative stress, resulting in serious consequences for the foetus, such as low birthweight and prematurity [6]. Neonates with IUGR are more susceptible to ROS-induced oxidative damage because their enzymatic and non-enzymatic antioxidant defence systems and the ability to undergo induction during a hyperoxic challenge are impaired [7].

To eliminate the harmful effects of ROS, cells are equipped with an efficient antioxidant defence system, including enzymes such as superoxide dismutase (SOD), catalase (CAT), hemeoxygenases (HOs), and low-molecular weight antioxidants such as glutathione (GSH) and metallothioneins (MTs) [8,9]. SOD catalyses the reduction of the superoxide anion ($O_2^{\bullet}-$) to hydrogen peroxide (H_2O_2). In a subsequent step, CAT stimulates the degradation of H_2O_2 to molecular oxygen and water [10]. The MTs are small proteins with unusually high cysteine content. This confers their high metal-binding and ROS-reducing properties [11]. The HOs play roles in heme degradation, yielding equimolar quantities of biliverdin, carbon monoxide (CO) with important free radical-scavenging properties and free Fe ions. In mature neonates, a considerable proportion of the plasma total antioxidant capacity originates from bilirubin [12]. HO-2 is a constitutive, and HO-1 an inducible isoform of HO, an antioxidative, anti-inflammatory and cytoprotective enzyme that is induced in response to cellular stress, including oxidative stress [13].

Nitric oxide (NO) may be an important factor for the regulation of blood pressure and oxygen delivery to the foetus [14]. Umbilical cord blood vessels lack innervations, and endothelial cells must

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Table 1
Clinical parameters of the study groups and the maternal age. Data are expressed as means ± SD. The minimum and the maximum values of the parameters are given in parentheses.

	Full-term neonates with normal weight	Full-term neonates with IUGR	Pre-mature neonates with IUGR
Gestational age at delivery (weeks)	39.2 ± 0.77 (38–40 + 2)	38.74 1.31 (37–40+4)	34.01 ± 1.62 (30–36)
Birth weight (g)	3409 ± 455 (3190–4340)	2354 238.8 (2090–2490)	1518 ± 460.5 (980–2120)
The pH of blood samples	7.25 ± 0.11 (7.04–7.42)	7.24 0.088 (7.1–7.36)	7.20 ± 0.142 (7.05–7.3)
1 min APGAR	8.83 ± 1.37 (6–10)	8.36 1.65 (6–10)	7 ± 1.63 (4–9)
Maternal age (years)	29.9 ± 5.74 (22–42)	28.9 6.55 (20–41)	29.3 ± 7.22 (21–42)

therefore play a major role in the local control of blood flow [15]. NO derived from endothelial nitric oxide synthase (eNOS) is considered the main vasodilator agent in fetoplacental vessels [16]. The simultaneous generation of NO and $O_2^{\bullet-}$ in sufficiently high concentrations in the same compartment favours the production of a toxic reaction product, peroxynitrite anion ($ONOO^-$). $ONOO^-$ and other reactive nitrogen species can affect the cell functions through the oxidation or nitration of various cellular targets [17].

Under stress conditions, genes coding for molecules involved in biological defence and cellular repair are markedly upregulated, and the changes in gene expression can be characteristic, sensitive and measurable endpoints [18]. Members of this antioxidant defence system are useful biomarkers of the oxidant–antioxidant status of neonates with IUGR. The aim of the present study was to characterize the possible links between the level of maturity, the birthweight and the antioxidant status of neonates born with IUGR. We report data on macromolecular damage, the accumulation of powerful oxidants such $ONOO^-$, H_2O_2, the activities of the antioxidant enzymes SOD and CAT, and the expressions of a set of genes coding for members of antioxidant defence system (*sod1*, *sod2*, *cat*, *mt-1*, *mt-2*, *ho-1*, *ho-2* and *enos*) from the aspects of the level of maturity and the birthweight of neonates with IUGR.

2. Materials and methods

2.1. Human subjects

The blood samples were obtained from the Department of Obstetrics and Gynaecology at the University of Szeged, Hungary. The Ethics Committee of the Department of Obstetrics and Gynaecology approved the study protocol (149/2012). 24 mature neonates with normal weight and 28 mature and 28 premature neonates with IUGR of either sex were examined. The neonates were considered premature if they were born before the gestational age of 37 weeks and full-term if the delivery occurred after 37 weeks. Newborns that had a history of difficult delivery and foetal distress, or showed malformations or evidence of genetic disorders were excluded. The nutritional status of the mothers during pregnancy was satisfactory; no case of malnutrition occurred. Smoking mothers and their neonates were also excluded from this study.

Blood was taken from the umbilical artery, before the birth of the placenta. Blood coagulation was inhibited by EDTA. The blood samples were centrifuged at 3000 rpm for 20 min at 4 °C, and the plasma and the buffy coat were removed. The red blood cell (RBC) phase was washed twice with 2 volumes of isotonic saline solution at pH 7.0. The samples were stored at −80 °C until processing (Table 1).

2.2. RNA extraction, reverse transcription and PCR amplification

Approximately, 100 mg of frozen RBC were homogenized in RNA Bee reagent (Tel-Test, Inc.) and total RNAs were prepared according to the procedure suggested by the manufacturer. Total RNA was routinely treated with 100 U RNAse-free DNAseI (Thermo Scientific) to avoid any DNA contamination.

For the quantification of *mt-1* and *mt-2*, *sod1* and *sod2*, *cat*, *enos*, *ho-1* and *ho-2* mRNAs, reverse transcription followed PCR amplifications (RT-PCRs) were performed. First-strand cDNAs were synthesized by using 5 μg total RNAs as templates, 200 pmol of each dNTP (Thermo Scientific), 200 U Maxima H Minus Reverse Transcriptase (Thermo Scientific) and 500 pmol random hexamer primers (Sigma) in a final volume of 20 μL, and incubated for 10 min at 37 °C, followed by 1 h at 52 °C. One microliter reverse transcription product was added to 25 μL DreamTaq Green PCR Master Mix 2x (Thermo Scientific). Amplification was performed in a PTC 200 Peltier Thermal Cycler (MJ Research) using 15 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s for the 18S rRNA, used as internal reference and 30 cycles for *mt-1* and *mt-2*, *sod1* and *sod2*, *cat*, *enos*, *ho-1* and *ho-2* mRNAs, respectively. The amplified products were detected on a 2% agarose gel. The relative levels of mRNAs are expressed as ratios (mRNA/18S rRNA).

2.3. Primers

The following primer sets were selected: *sod1*: F: aagatggtgtggccgatgtg and R: ctacagctagcaggataacag; *sod2*: F: caaggctcaggttggggttg and R: gctgggatcattagggtagtatg; *cat*: F: cacagaagatggtaactggg and R: ggcgatgtccatctggaatc; *enos*: F: cactgagcccgtggcagtag and R: ggcaggcagcgccaccgacg; *mt-1*: F: atggaccccaactgctcctg and R: gttcccacatcaggcacagc; *mt-2*: F: atggaccccaactgctcctg and R: cggtcacggtcagggttgtac; *ho-1*: F: gctgctggtggcccacgctt and R: ctctggtccttggtgtcatgg; *ho-2*: F: tggcccacgcatacacccgc and R: ggtctctctggccagtgtgga. For the normalization of *sods*, *cat*, *enos*, *mts* and *hos* mRNAs, the level of carp 18S rRNA was used as internal standard, detected with primer pairs F: gaaacggctaccacatccaagg, and R: ccgctcccaagatccaactacg.

2.4. Densitometry

Images of the ethidium bromide-stained agarose gels were digitized with a GDS 7500 Gel Documentation System and analyzed with the GelBase/GelBlot™ Pro Gel Analysis Software (UVP).

2.5. Enzyme activity measurement

The RBCs were hemolysed by the addition of distilled water at a ratio of 1:9. Except for SOD activity determinations, the aliquots of the hemolysates were used directly. The quantity of protein was determined with Folin reagent, using bovine serum albumin as standard [19]. Biomate 5 Double-Beam UV–vis photometer recording (Thermo Spectronic) was used for SOD measurements and GENESYS 10S UV–vis spectrophotometer (Thermo Scientific) was used for all the other parameters.

SOD (EC 1.15.1.1) activity was determined on the basis of the inhibition of the epinephrine–adrenochrome autoxidation [20]. Spectrophotometric measurement was carried out at 480 nm. The results were expressed in U/mg protein.

CAT (EC 1.11.1.6) activity was determined spectrophotometrically at 240 nm by the method of Beers and Sizer [21] and specific

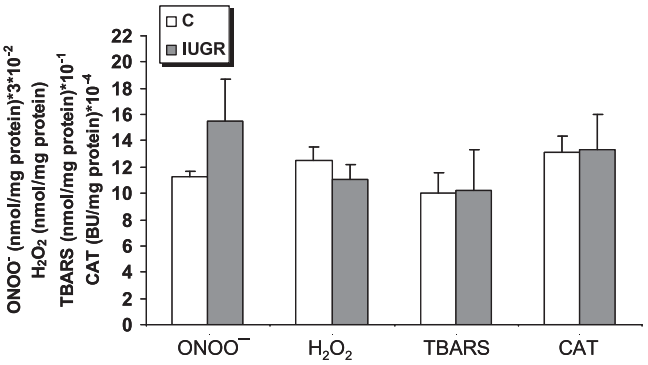

Fig. 1. The levels of $ONOO^-$, H_2O_2, lipid peroxidation (TBARS), and the CAT activity in the cord red blood cells of mature, healthy neonates (C) and neonates with IUGR. Data were expressed as means ± SEM from measurements on 24/56 samples.

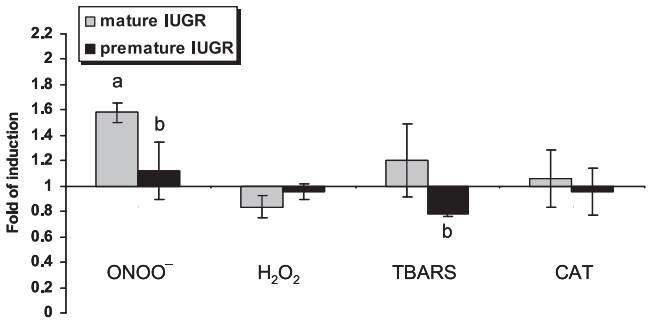

Fig. 3. Fold of change in the levels of $ONOO^-$, H_2O_2, lipid peroxidation (TBARS), and the CAT activity in the cord red blood cells of mature and premature neonates with IUGR. For normalization the levels of mature, healthy neonates were used as reference. Significant difference was accepted at $^{a/b}p \leq 0.05$. (a) A significant difference between the mature, healthy neonates and neonates with IUGR. (b) A significant difference between the mature and premature neonates with IUGR.

CAT activity was expressed in Bergmeyer units (BU)/mg protein (1 BU = decomposition of 1 g H_2O_2/min at 25 °C).

2.6. Lipid peroxidation estimation assay

The level of thiobarbituric acid-reactive substances (TBARS) is regarded as an appropriate indicator of the extent of lipid peroxidation (LPO) [22]. LPO was estimated by a TBARS assay at 532 nm against a blank that contained the thiobarbituric acid (TBA) reagent (0.15 g/mL TCA, 3.75×10^{-3} g/mL TBA and 0.25 M HCl) as described by Serbinova et al. [23].

2.7. Determination of H_2O_2 production

For the assay of H_2O_2, 0.05 mg/mL horseradish peroxidase and 0.1 mg/mL o-dianisidine in sodium phosphate buffer (100 mM, pH 6.5) was used. The H_2O_2 concentration was determined spectrophotometrically at 400 nm and was calculated as nmol/mg protein [24].

2.8. Determination of $ONOO^-$ production

$ONOO^-$ was assayed by diluting samples into 1.0 M NaOH (60:1) and measuring the increase in absorbance at 302 nm. As a control, samples were added to 100 mM potassium phosphate (pH 7.4) (60:1). The decrease in absorbance was measured at neutral pH as $ONOO^-$ decomposes [25]

2.9. Statistical analysis

Statistical differences were calculated with one-way analysis of variance (ANOVA) (MedCalc Statistical Software version 9.4.2.0, Broekstraat, Belgium) with a Student–Newman–Keuls follow-up test. Significant difference was accepted at $^{a,b}p \leq 0.05$, $^{aa}p \leq 0.01$ and $^{aaa}p \leq 0.001$.

3. Results

3.1. Comparative studies of mature, healthy neonates and neonates with IUGR

The levels of oxidant molecules (H_2O_2 and $ONOO^-$) and thiobarbituric acid-reactive substances (TBARS) and the activity of the H_2O_2-degrading CAT did not differ significantly between the IUGR and control groups (Fig. 1).

As concerns the mRNA levels of genes coding for antioxidant molecules, significantly lower amounts were measured for *cat* (~20%), *ho-1* (~40%) and *ho-2* (~50%) in the IUGR group than in the controls. The *mt-2* mRNA level was approximately 1.5-fold higher in the neonates with IUGR than in the control group (Fig. 2). As a consequence of the relatively high individual fluctuations, the averages of the amounts of *mt-1*, *sod*s and *enos* mRNA did not differ significantly (Fig. 2).

3.2. Comparative studies of mature and premature neonates with IUGR

There was no significant difference in the level of H_2O_2 or the activity of CAT between the mature and pre-term neonates with IUGR. However, the mature group exhibited a 1.5-fold higher accumulation of $ONOO^-$, and also a higher TBARS level (Fig. 3). The levels of $ONOO^-$ and TBARS for the pre-term IUGR neonates were was more similar to those for the normal controls.

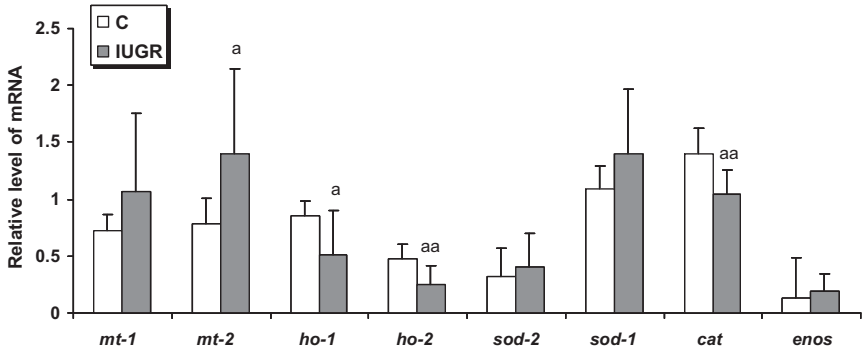

Fig. 2. mRNA expression level of antioxidant genes in the cord red blood cells of mature, healthy neonates (C) and neonates with IUGR. For normalization the level of 18S rRNA was used as an internal standard. Data were expressed as means ± SEM from measurements on 24/28 samples. $^{a}p < 0.05$, $^{aa}p < 0.01.1$.

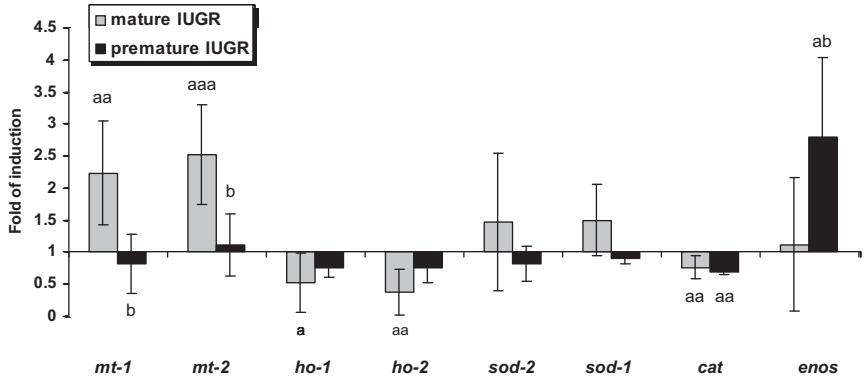

Fig. 4. Fold of changes in the mRNA levels of antioxidant genes in the cord red blood cells of mature and premature neonates with IUGR. Levels of mature, healthy neonates were used as references. Significant difference was accepted at $^{a,b}p \leq 0.05$, $^{aa}p \leq 0.01$, $^{aaa}p \leq 0.001$. (a) A significant difference between the mature, healthy neonates and neonates with IUGR. (b) A significant difference between the mature and premature neonates with IUGR.

Marked differences were also observed between the mRNA levels of selected parameters in the groups of mature and premature IUGR neonates. There was a ~60% difference in the level of the *mt-1* and *mt-2* mRNAs. The mRNA levels of both genes were approximately doubled in the mature IUGR neonates as compared with the pre-term IUGRs and also the normal controls (Fig. 4A). The transcription products of the *ho* genes were considerably lower in both IUGR groups; in the mature IUGRs, the levels of *ho-1* and *ho-2* mRNA were ~50% and 40% of those for the control. In the premature IUGR group, both mRNA levels were lower by 25%, though these changes were not significant. The level of *cat* mRNA was likewise lower in both IUGR groups: a significant ~25% decreases were detected. The transcripts of the *sod* genes in the mature IUGR group were ~1.5-fold those measured in the control and premature IUGR groups. In the premature IUGR group, the *enos* mRNA was ~3-fold that in the mature IUGR group (Fig. 4B). In this case, the mature neonates with or without IUGR exhibited similar mRNA levels.

4. Discussion

Since IUGR is postulated to be a pleiotropic complication of pregnancy, the identification of unit marker molecules or reaction pathways connected with this disorder poses a number of difficulties. Various studies have focused on the antioxidant status of IUGR or premature neonates, but only brief accounts have been given as concerns the comparison of premature and full-term neonates with IUGR from any aspect. The major finding of the present study is the importance of the age-based grouping of neonates with IUGR. Significant differences in the levels of specific stress markers relative to the control group were observed only when the full-term and pre-term IUGR neonates were grouped separately.

A higher degree of oxidative stress was detected in the cord blood of mature IUGR neonates as compared with the control and pre-term IUGR groups; the level of $ONOO^-$ was almost twice as high, indicating increased $O_2^{\bullet-}$ and NO production. The simultaneous generation of NO and $O_2^{\bullet-}$ in relatively high concentrations in the same compartment favours production of the toxic reaction product $ONOO^-$ [17]. Although $ONOO^-$ itself is not a free radical, it is a powerful oxidant, whose toxicity is manifested among others in LPO [17] and which plays a role in the pathophysiology of IUGR [26].

The elevated $ONOO^-$ and $O_2^{\bullet-}$ levels in the cord blood of mature neonates with IUGR were clearly reflected by an increased level of TBARS. NO, however, is a Janus-faced molecule. Since the umbilical cord blood vessels lack innervations, the NO production in the endothelial cells plays a major role in the local control of blood flow and in oxygen delivery to the foetus [15]. In this study, we have demonstrated a significantly higher level of the mRNA of eNOS in the cord blood samples of underdeveloped neonates with IUGR. In these cases, the increase in eNOS expression was not paralleled by $ONOO^-$ accumulation, suggesting an unaltered level of $O_2^{\bullet-}$. This is in accord with the fact that there were no significant differences in the expressions of the *sod* genes, and the activity of SOD was also unaltered (data not shown). Additionally, no significant changes were observed in the TBARS level in the cord blood of the premature neonates with IUGR. An insufficient level of vasodilatation of the umbilical vessels and reduced eNOS activity were earlier reported to be associated with neonates who were small for gestational age [16].

Evidence has recently been accumulating that CO, best known for its toxicity, can function in a similar manner to NO [27] or may even interact with the NO-producing pathway [28]. CO is formed primarily as a product of heme degradation, which is catalysed by the HOs. The observation of substantial HO activity in the cord tissues supports the possibility of the accumulation of CO in sufficiently high amounts to play a role in fetoplacental blood flow regulation [29]. Our study presents evidence that the expressions of the *ho* genes are markedly low in IUGR neonates (regardless of the level of maturity) versus controls. Though the low expressions of both *ho* genes were characteristic for both IUGR groups, a significant difference was observed only for the full-term neonates with IUGR. Deficiencies in HO-1 have previously been found to be associated with pregnancy disorders, such as recurrent miscarriages, IUGR and pre-eclampsia [30]. Our results support this: the lower expression of *ho* in IUGR neonates might result in a decreased CO level and unsatisfactory fetoplacental blood flow. Moreover our data relating to the selected antioxidant molecules suggest that *ho* could serve as an indicative marker providing a possible link to the IUGR phenotype.

The presence of IUGR in full-term neonates was accompanied by significantly higher levels of expression of both *mt* genes. MT, an important protein which binds bivalent metals, plays a significant role in numerous cellular metabolic processes, such as in maintaining Zn and Cu homeostasis and in Cd and Hg detoxification. Additionally, an increased MT level was demonstrated by Zapata et al. in the erythrocytes of pregnant women [31]. Elevated MT levels have been attributed to the proliferation and differentiation of blood cells in the process of erythropoiesis, the protective role of erythrocytes against the action of free radicals, and increased levels of estrogen and progesterone, hormones which induce the synthesis of MT [32]. A statistically significant increase in the level of MT protein has additionally been revealed in the plasma and RBCs of pregnant women and of their neonates with IUGR [4]. The possible physiological functions of MT in the placenta include

temporary Zn storage and regulation of the Zn flow to the foetus, while restricting toxic metal transfer [33] and protection against the embryotoxic and teratogenic effects of a Zn deficiency [34], which may be caused by cigarette smoke, alcohol, gestational infection and exposure to environmental contaminants, including heavy metals and endocrine disrupters [35].

From an analysis of the parameters relating to the different IUGR groups, we can conclude that full-term neonates with IUGR are at especially high risk. Pre-term neonates, at gestational ages of 33–36 weeks demonstrate less damage in the integrity of the lipid molecules, a significant accumulation of the harmful oxidant $ONOO^-$ cannot be detected, and the expressions of genes coding for antioxidant markers are less affected. The significantly lower LPO in the premature neonates may be correlated with the decreased sensitivity of the RBCs to oxygen radicals, or with the fact that the RBCs of premature newborns contain a smaller quantity of unsaturated fatty acids [36]. This phenotype is not without precedent. The GSH and NADPH levels proved to be lower in those with gestational ages of less than 33 weeks than in pre-term infants with gestational ages of 33–36 weeks, and the levels of these markers were as low in full-term, small-for-age infants as in pre-term infants with gestational ages of less than 33 weeks [7].

5. Conclusions

Our data furnish evidence that there is extreme heterogeneity in ROS production and in the activation of the antioxidant defence system within neonates with IUGR, which is blunted by the use of mean measurements. Intergrouping based on gestational age and birthweight, yielded evidence that the molecular results in the pre-term IUGR group were often similar to those for the mature, appropriate-for-age neonates. However, the background of the IUGR phenotype is pleiotropic, and measurements of various antioxidants provide only a partially adequate picture of the overall condition of the neonates. Data emerging from different approaches to the topic present evidence only of the involvement of additional marker molecules in the development of IUGR disorders. Our study indicates that the levels of expression of the *mt* and *ho* genes are good candidates through which to characterize the IUGR phenotype.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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