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Circulating heat shock protein mRNA profile in gestational hypertension, pre-eclampsia & foetal growth restriction

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***Background & objectives*: Heat shock proteins (Hsp) are ubiquitously distributed phylogenetically conserved molecules that regulate cellular homeostasis and maintain the integrity and function of cellular proteins. Increased levels of Hsp in maternal circulation have been shown to be associated with increased risk of pregnancy related complications. The objective of this study was to explore extracellular *Hsp* mRNA levels in maternal circulation and quantified *Hsp27*, *Hsp60*, *Hsp70*, *Hsp90* and Hsp70 binding protein 1 (*HspBP1*) mRNAs in maternal plasma samples using real-time reverse-transcriptase polymerase chain reaction.**

***Methods*: Pregnancies with gestational hypertension (GH) (n = 33), pre-eclampsia (PE) with or without foetal growth restriction (FGR) (n = 78) and FGR (n = 25) were involved in the study. *Hsp* gene expression was analysed in relation to the severity of the disease with respect to the degree of clinical signs, requirements for the delivery and Doppler ultrasound parameters.**

***Results*: Upregulation of Hsp70 was observed in patients with mild and severe PE ($P = 0.004$ and $P = 0.005$, respectively) and in pregnancies complicated with PE delivering before and after 34 wk of gestation regardless of the degree of clinical signs ($P = 0.015$ and $P = 0.009$, respectively). No difference in the expression of other *Hsp* genes among the studied groups was observed. No association between *Hsp* gene expression and Doppler ultrasonography parameters was found.**

***Interpretation & conclusions*: These data support that maternal circulation can reflect both maternal and foetal pathologic conditions. Hsp70 represents the sole plasmatic marker, and increased *Hsp70* mRNA levels reflect maternal and placental stress response to pregnancy-related complications such as GH and PE, irrespective of the severity of the disease.**

Key words Foetal growth restriction - gestational hypertension - heat shock protein - maternal circulation - pre-eclampsia

Pre-eclampsia (PE) and foetal growth restriction (FGR) is the major cause of maternal and perinatal morbidity and mortality in 2-10 per cent of pregnant women[1,2]. PE is characterized by gestational

hypertension (GH) combined with proteinuria after 20 wk of gestation[3]. FGR is defined as foetal growth less than the 10th percentile for appropriate gestational age. The causes of PE and FGR remain unknown. It is believed that PE results mainly from defective placentation and insufficient maternal spiral artery remodelling that elicit inadequate uteroplacental blood perfusion, ischaemia and finally generalized maternal systemic inflammatory response[4-6]. Elevated amounts of pro-inflammatory cytokines, chemokines and adhesion molecules in the maternal circulation have been shown to play a central role in the excessive systemic inflammatory response and in the generalized endothelial dysfunction characteristics of PE[7]. Moreover, pre-eclamptic patients have been demonstrated to have significantly higher levels of C-reactive protein and particular complement components including terminal complement complex SC5b9[8].

Heat shock proteins (Hsps) are ubiquitously distributed phylogenetically conserved molecules present in the cells of all living organisms. Under physiological conditions, the stress proteins are expressed in low concentrations as the constitutive proteins, regulating cellular homeostasis and maintaining the integrity and function of other cellular proteins[9,10]. Human Hsps are categorized under distinct families based on their functions in the cells, their homologies in the primary structures and their approximate molecular weight, measured in kDa. These families are as follows: a family of small Hsps, Hsp40, Hsp60, Hsp70, Hsp90 and Hsp110[11].

Elevated circulating Hsp70 concentrations reflecting systemic inflammation, oxidative stress and hepatocellular injury were found to be associated with an increased risk of several pregnancy-related complications including transient hypertension of pregnancy, PE, haemolysis, elevated liver enzymes, low platelet syndrome and pre-term delivery[12-17]. Elevation of circulating Hsp70 levels was also observed in pregnant asthmatic women compared to healthy pregnant women[18].

Since most investigators studied Hsp expression at protein level in placental tissues[19-22] and maternal circulation (serum or plasma samples)[12-18], we focused on the examination of Hsp expression at mRNA level. Among differentially expressed proteins, previous studies identified Hsp27 and Hsp70 to be upregulated in placental tissues derived from patients with PE[19-22]. Several researchers pointed to the fact that the expression of various Hsps might vary in different placental zones. Although the protein levels of total Hsp60 and Hsp90 did not differ in placental and decidual tissues between normal and complicated pregnancies[23,24], the expression of Hsp27, Hsp60, Hsp70 and Hsp90 was shown to be higher in the thrombus and lower in the infarction than in control samples[25]. A novel Hsp70 co-chaperone called Hsp70 binding protein 1 (HspBP1) was included in the study since this intracellular protein abundant in tissues, was found to inhibit substantially antiapoptotic function of Hsp70[26].

There is a lack of information on *Hsp27*, *Hsp60*, *Hsp70*, *Hsp90* and *HspBP1* gene expression in patients affected with PE and/or FGR. We therefore, undertook this study to evaluate *Hsp* mRNA levels as also Hsp BP1 level in plasma samples from pregnant women with GH.

Material & Methods

This study was designed in a retrospective consecutive manner within the period ranging from January 2011 to January 2014. Samples of maternal peripheral blood from complicated pregnancies were collected at the Institute for the Care of the Mother and Child (Prague, Czech Republic), mostly during the hospital admission. Samples from pregnancies with the normal course of gestation were collected during regular check-up in the 36th wk of gestation in the Clinic of Obstetrics and Gynecology, University Hospital Motol (Prague, Czech Republic). The study included 78 pregnant women with clinically established PE with or without FGR, 25 pregnancies complicated by FGR, 33 with GH and 39 normal pregnancies. Of the 78 patients with PE, 27 had symptoms of mild PE and 51 women were diagnosed with severe PE. Thirty pre-eclamptic women required the delivery before 34 wk of gestation and 48 women delivered after 34 wk of gestation. PE occurred both in previously normotensive patients (56 cases) and in a form superimposed on previous hypertension (22 cases). Seven growth-retarded foetuses were delivered before 34 wk of gestation and 18 after 34 wk of gestation. Oligohydramnios or anhydramnios was present in nine growth-restricted foetuses. The examination of the flow of blood vessels (Doppler ultrasonography) showed an abnormal pulsatility index (PI) in the umbilical artery (abnormal values above the 95th percentile detected in 12 pre-eclamptic and 14 FGR cases) and/or in the middle cerebral artery (abnormal values below the

5th percentile detected in 9 pre-eclamptic and 5 FGR cases). Cerebroplacental ratio (CPR), expressed as a ratio between middle cerebral artery and umbilical artery PI, was below the 5th percentile in 17 cases (8 pre-eclamptic and 9 FGR cases). Absent or reversed end-diastolic velocity waveforms in the umbilical artery occurred in four cases (2 pre-eclamptic and 2 FGR cases).

Women with normal pregnancies were defined as those without medical, obstetrical or surgical complications at the time of the study and who subsequently delivered full-term, singleton, healthy infants weighing >2500 g after 37 completed wk of gestation. PE was defined as blood pressure >140/90 mmHg in two determinations four hours apart that was associated with proteinuria >300 mg/24 h after 20 wk of gestation[3]. Severe PE was diagnosed by the presence of one or more of the following findings: (*i*) systolic blood pressure >160 mmHg or diastolic blood pressure >110 mmHg, (*ii*) proteinuria >5 g of protein in a 24 h sample, (*iii*) very low urine output (<500 ml in 24 h), (*iv*) signs of respiratory problems (pulmonary oedema or cyanosis), (*v*) impairment of liver function, (*vi*) signs of central nervous system problems (severe headache and visual disturbances), (*vii*) pain in the epigastric area or right upper quadrant, (*viii*) thrombocytopenia, and (*ix*) presence of severe FGR[3]. FGR was diagnosed when the estimated foetal weight, calculated using the Hadlock formula (Astraia Software GmbH), was below the 10th percentile for the evaluated gestational age, adjustments were made for the appropriate population standards of the Czech Republic. The clinical characteristics of the normal and complicated pregnancies are shown in Table I. All patients who participated in this study provided written informed consent. The study was approved by the Ethics Committee of the Third Faculty of Medicine, Charles University in Prague.

Processing of samples and total RNA isolation: Peripheral blood (9 ml) was collected into ethylenediaminetetraacetic acid tubes and centrifuged twice at 1200 g for 10 min at room temperature. Plasma samples were stored at −80°C until further processing. Total RNA was extracted from one ml of maternal plasma using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. To minimize DNA contamination, the eluted RNA was treated with five µl of deoxyribonuclease I (DNase I, Fermentas International, Ontario, Canada) for 30 min at 37°C.

Analysis of relative Hsp gene expression using real-time RT (reverse transcriptase)-PCR: All PCR reactions were performed using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA) as previously described[27]. Human *Hsp* and *β-actin* primers and probes were designed using Primer Express version 2.0 (Applied Biosystems, USA). The primer/probe sequences and concentrations used for the amplification of the target and reference genes were as follows (5′→3′):

Hsp27, HSPB1: forward primer 5′-TCCCTGGATGTCAACCACTTC-3′ (900 nmol/l); reverse primer 5′-TCTCCACCACGCCATCCT-3′ (900 nmol/l); probe 5′-(FAM) CCCCGGACGAGCTGACGGTC (TAMRA)-3′ (300 nmol/l).

Table I. Clinical characteristics of women with normal and complicated pregnancies

Characteristics	Healthy pregnant women (n=39)	Pre-eclamptic patients (n=78)	Pregnant women with FGR (n=25)	Pregnant women with GH (n=33)
Age (yr)	33 (30-35)	34 (29-37)	32 (28-36)	30 (29-33)
Blood pressure (mmHg)				
Systolic	120 (112-128)	156 (150-163)	127 (115-139)	155 (150-165)
Diastolic	76 (70-80)	100 (90-103)	80 (75-86)	100 (93-100)
Proteinuria (g/24 h)	None	1.23 (0.55-3.4)	None	None
Gestational age at delivery (wk)	40 (39-40)	36 (31-38)	36 (34-38)	39 (37-39)
Mode of delivery (%)				
Vaginal	28 (71.8)	13 (16.7)	9 (36)	19 (57.6)
Caesarean section	11 (28.2)	65 (83.3)	16 (64)	14 (42.4)
Foetal birth weight (g)	3380 (3142-3645)	2450 (1442-3220)	2160 (1925-2610)	3210 (2880-3620)

Data are presented as median (25-75 percentile) for continuous variables and as n (%) for categorical variables. FGR, foetal growth restriction; GH, gestational hypertension

Hsp60, HSPD1: forward primer 5′-GATGTTGATGGAGAAGCTCTAAGTACA-3′ (900 nmol/l); reverse primer 5′-TGCCACAACCTGAAGACCAA-3′ (900 nmol/l); probe 5′-(FAM) TCGTCTTGAATAGGCTAAAG (MGB)-3′ (200 nmol/l).

Hsp70, HSPA1A: forward primer 5′-ACCAAGCAGACGCAGATCTTC-3′(300 nmol/l); reverse primer 5′- GCCCTCGTACACCTGGATCA-3′ (300 nmol/l); probe 5′-(FAM) CCTACTCCGACAACCAACCCGGG (TAMRA)-3′ (200 nmol/l).

Hsp90α, HSP90AA1: forward primer 5′-TGCGGTCACTTAGCCAAGATG-3′ (300 nmol/l); reverse primer 5′-GAAAGGCGAACGTCTCAACCT-3′ (300 nmol/l); probe 5′-(FAM) CCCAGACCCAAGACCAACCGATGG (TAMRA)-3′ (200 nmol/l).

HspBP1, HSPBP1: forward primer 5′-TGGCCGACCTGTGTGAGA-3′ (700 nmol/l); reverse primer 5′-GCAGGTGCATGCCAGACA-3′ (700 nmol/l); probe 5′-(FAM) CATGGACAATGCCG (MGB)-3′ (200 nmol/l).

B-actin, ACTB: forward primer 5′-CCTGGCACCCAGCACAAT-3′ (300 nmol/l for *Hsp27, Hsp60*, *Hsp70*, *Hsp90α* and 200 nmol/l for *HspBP1*); reverse primer 5′-GCCGATCCACACGGAGTACT-3′ (300 nmol/l for *Hsp27*, *Hsp60*, *Hsp70*, *Hsp90α* and 200 nmol/l for *HspBP1*); probe 5′-(VIC) ATCAAGATCATTGCTCCTCCTGAGCGC (TAMRA)-3′ (200 nmol/l for *Hsp27*, *Hsp60*, *Hsp70, Hsp90α* and 100 nmol/l for *HspBP1*).

To perform one-step duplex quantitative RT-PCR assay[27], the *Hsp* gene and endogenous control *β-actin* were amplified in the same tube in a reaction volume of 25 µl consisting of 1× TaqMan One-step RT-PCR Master Mix, TaqMan and/or MGB probes, forward and reverse primers (*Hsp* and *β-actin* primers and probe; Applied Biosystems, USA). Ten nanogram of extracted total RNA from maternal plasma was used for the detection of *Hsp27*, *Hsp60*, *Hsp70*, *Hsp90* and *HspBP1*. Each sample was analysed in duplicate. The thermal profile for one-step real-time RT-PCR included reverse transcription at 48°C for 30 min, denaturation at 95°C for 10 min, followed by 50 cycles of PCR with denaturation at 95°C for 15 sec and annealing/extension at 60°C for one minute. For the analysis of relative changes in gene expression, the comparative C_T method[28] was used to interpret the data. The difference (ΔC_T) between the C_T values of the *Hsp* and the endogenous control was calculated for each sample. RNA isolated from a randomly selected placenta derived from gestation of normal course was chosen as the reference for each comparison. The comparative $\Delta\Delta C_T$ calculation involved finding the difference between each sample's ΔC_T and the reference's ΔC_T. Finally, $\Delta\Delta C_T$ values were transformed to absolute values using the formula $2^{-\Delta\Delta CT}$.

Statistical analysis: Tests of normality of the population distribution of random characteristics (patients' age, systolic and diastolic blood pressure, gestational age at delivery and foetal birth weight) together with the tests of normality of observed data (*Hsp* gene expression in maternal plasma samples) were performed using Shapiro–Wilk test. Half of the clinical characteristics in groups of normal and complicated pregnancies did not show a normal distribution. Further, all experimental data did not follow a normal distribution (Table II). Therefore, *Hsp* mRNA levels were compared between groups by non-parametric tests (Mann–Whitney U-test for the comparison between two groups and Kruskal–Wallis test for the comparison among multiple groups) using Statistica software (version 9.0, StatSoft, Inc., Tulsa, OK, USA). Odds ratio (OR), the likelihood that the outcome occurred in the exposure/intervention group as compared to the control group, was calculated using MedCalc MedCalc Software bvba, Microsoft Partner, Silver Application Development, Ostend, Belgium).

Results

Given that *HspBP1* was not detectable in maternal plasma samples and *Hsp60* showed positive amplification in a limited number of maternal plasma samples, it was decided to exclude them from further analyses. Subsequently, it was determined whether plasma concentrations of *Hsp27*, *Hsp70* and *Hsp90* mRNAs were related to pregnancy complications.

Hsp gene expression in maternal plasma samples in patients with pregnancy-related complications: Overall, significantly increased expression was observed only for *Hsp70* ($P < 0.001$) in women with pregnancy-related complications (GH, PE and/or FGR) compared to pregnancies with normal course of gestation. Kruskal–Wallis analysis revealed *Hsp70* overexpression in the group of patients with PE with

Table II. Shapiro–Wilk test - assumptions of normal distribution

Patients group	Tests for the normality of data									
	Clinical characteristics									
	Maternal age		Systolic blood pressure		Diastolic blood pressure		Gestational age at delivery		Foetal birth weight	
	W	*P*	*W*	*P*	*W*	*P*	*W*	*P*	*W*	*P*
Healthy pregnant women	0.90824	0.00383	0.98340	0.83309	0.95862	0.17108	0.92828	0.01765	0.98826	0.95474
Pre-eclamptic patients	0.96520	0.03145	0.97581	0.24053	0.97639	0.24826	0.91434	0.00008	0.95384	0.01158
Patients with FGR	0.94958	0.24537	0.87791	0.00626	0.94379	0.18097	0.81635	0.00043	0.90262	0.02864
Patients with GH	0.93330	0.04265	0.97521	0.75956	0.94766	0.20433	0.93330	0.04349	0.96289	0.57621

Patients group	*Hsp* gene expression in maternal plasma					
	Hsp27		Hsp70		Hsp90	
	W	*P*	*W*	*P*	*W*	*P*
Healthy pregnant women	0.30623	<0.001	0.55332	<0.001	0.78146	<0.001
Pre-eclamptic patients	0.54569	<0.001	0.46940	<0.001	0.63143	<0.001
Patients with FGR	0.55156	<0.001	0.78182	<0.001	0.79048	<0.001
Patients with GH	0.47074	<0.001	0.35162	<0.001	0.69470	<0.001

FGR, foetal growth restriction; GH, gestational hypertension

or without FGR (P = 0.002) and GH (P = 0.003), but not in the group of patients with FGR. Plasma levels of *Hsp70* mRNA between the groups of patients with PE with or without FGR and GH did not differ (Fig. 1). The odds of having *Hsp70* mRNA levels above cut-off (median plus standard deviation of normal pregnancies) was similar between the groups of patients with PE with or without FGR [P < 0.001; OR: 8.31; 95% confidence interval (CI): 2.69-25.62] and GH (P < 0.001; OR: 8.23; 95% CI: 2.38-28.44). The expression of Hsp27 and Hsp90 did not differ between the control group and pregnancies affected with GH, PE with or without FGR and FGR.

Association of Hsp gene expression in maternal plasma samples and severity of disease with respect to clinical signs, requirements for the delivery and Doppler ultrasonography monitoring: Hsp gene expression was analysed in relation to the severity of the disease with respect to the degree of clinical signs (mild and severe PE) and requirements for the delivery (before and after 34 wk of gestation). When compared to normal pregnancies, significant upregulation of *Hsp70* was observed in patients with mild and severe PE (P = 0.004 and P = 0.005, respectively, Fig. 2) and in pregnancies complicated with PE with or without FGR delivering before and after 34 wk of gestation regardless of the degree of clinical signs (P = 0.015 and P = 0.009,

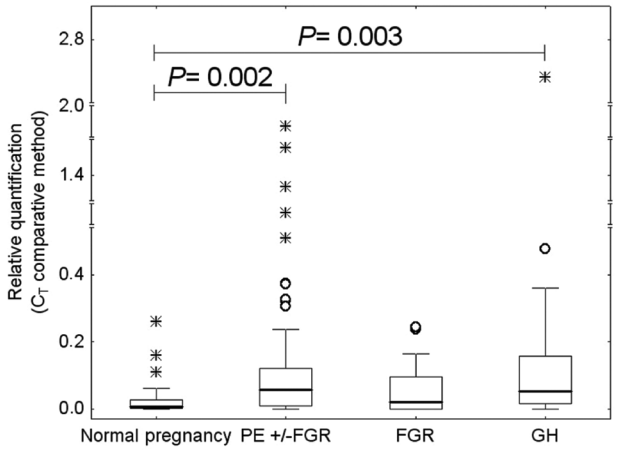

Fig. 1. Heat shock protein 70 (Hsp70) overexpression in maternal plasma samples derived from patients with gestational hypertension (GH) and pre-eclampsia (PE) with or without foetal growth restriction (FGR).

respectively, Fig. 3). However, *Hsp70* mRNA levels did not differ between the patients with mild and severe PE (Fig. 2) and between those who delivered before and after 34 wk of gestation (Fig. 3). The highest estimated odd ratios of having *Hsp70* mRNA levels above cut-off (median plus standard deviation of normal pregnancies) were detected in patients with severe PE (P<0.001; OR: 9.84; 95% CI: 3.05-31.77) and those who delivered

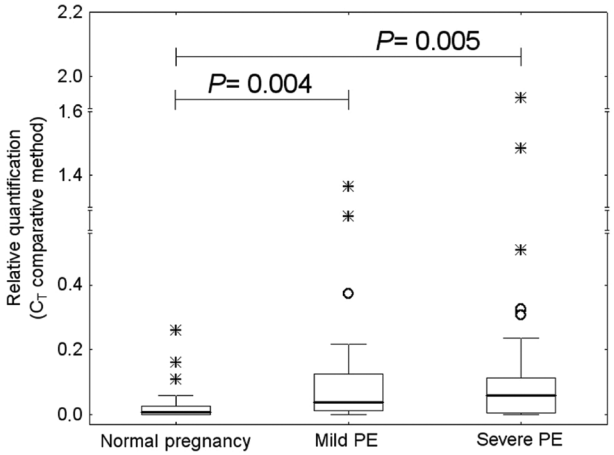

Fig. 2. Increased circulating heat shock protein 70 (*Hsp70*) mRNA levels in patients with mild and severe pre-eclampsia (PE).

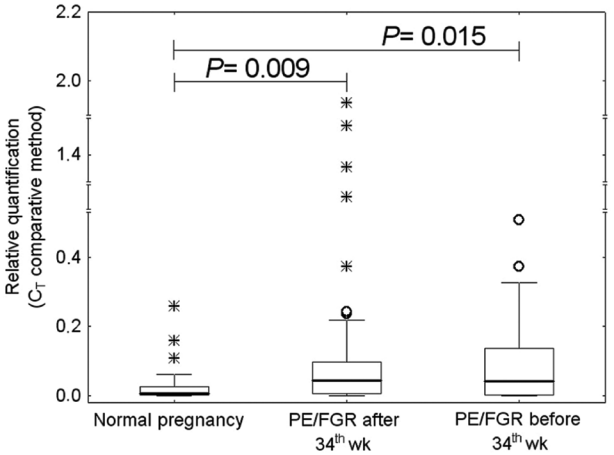

Fig. 3. Increased circulating heat shock protein 70 (*Hsp70*) mRNA levels in pregnancies complicated with pre-eclampsia (PE) with or without foetal growth restriction (FGR) delivering before and after 34 wk of gestation.

before 34 wk of gestation ($P < 0.001$; OR: 11.44; 95% CI: 3.24-40.4) when compared with the group of patients with mild PE ($P = 0.003$; OR: 7.0; 95% CI: 1.94-25.25) and those who delivered after 34 wk of gestation ($P<0.001$; OR: 8.05; 95% CI: 2.47-26.18).

No difference in *Hsp* gene expression between the groups of patients with mild or severe PE and controls was found. Similarly, *Hsp* mRNA levels did not differ between pre-eclamptic pregnancies with requirements for the delivery before and after 34 wk of gestation and controls. In addition, the association between *Hsp* gene expression and the occurrence of previous hypertension in the group of patients with PE was determined. The analyses revealed no difference between the group of PE superposed on chronic hypertension and/or GH and the group of patients with unexpected onset of PE.

The association between *Hsp* gene expression in maternal plasma and Doppler ultrasonography parameters was analysed in the pregnancies complicated with PE and/or FGR. The difference within the group of complicated pregnancies with normal and abnormal values of flow rates was assessed. The analysis showed no effect of the PI in the umbilical artery, the PI in the middle cerebral artery and the CPR on *Hsp* mRNA levels in maternal circulation. Summary of data resulting from statistical analyses is shown in Table III.

Discussion

Circulating nucleic acids such as DNA, mRNA and microRNAs present in maternal plasma or serum samples are increasingly being used as biomarkers for monitoring of pregnancy-related complications. Extracellular nucleic acids present in maternal circulation are predominantly haematopoietic in origin[29]. Placental insufficiency-related pregnancy complications have been shown to be associated with an excessive placental trophoblast apoptosis and shedding of placenta debris[4,5]. The Redman hypothesis proposes that release of a continuous stream of placental debris into the maternal circulation provokes a systemic inflammatory response in all women, which is exaggerated, if the burden of the debris is abnormally high (when the placenta is oxidatively stressed secondary to spiral artery disease) or if the woman responds excessively to the process[4,5]. PE usually appears when the inflammatory responses begin to decompensate. The problem with the placenta is generally considered to be an inadequate uteroplacental circulation leading to placental hypoxia, oxidative stress and, in the most severe cases, infarction. Two abnormalities, affecting the spiral arteries, are known to predispose to PE: the arteries may be either too small because of deficient placentation, or obstructed because of acute atherosis or both[4,5].

We hypothesize that maternal circulation can reflect both maternal and placental pathologic conditions through the mediation of diverse *Hsp* gene expression profiles. Since *Hsp60* and *HspBP1* mRNA are not detectable in maternal plasma samples and *Hsp27* and *Hsp90* mRNA show comparable levels regardless of the course of gestation, Hsp70 represents the sole plasmatic marker. Increased *Hsp70* mRNA levels reflect maternal and placental stress response to

pregnancy-related complications such as GH and PE. Our data supported the finding of other investigators who reported significantly higher protein levels of total Hsp70, constitutive and induced forms of Hsp70 in PE along with oxidative stress in placental tissues[20-22]. Our finding was in compliance with the results of other studies demonstrating elevated circulating Hsp70 concentrations in pregnancy-related complications including transient hypertension of pregnancy and PE[12-17]. Hsp70 represents an antioxidative stress marker. The overexpression of Hsp70 and the heat shock transcription factor (Hsf1) seen in the pre-eclamptic endothelial cells suggests its possible protective role as stress-specific natural adaptive response against the generated stress[30]. As proposed by Molvarec *et al*[15], we hypothesise that haemodynamic stress, oxidative stress (placental or systemic), placental ischaemia, ischaemia of other organs as well as maternal systemic inflammatory response may contribute to the overexpression of Hsp70, resulting in elevated circulating Hsp70 levels. The possible cause of *Hsp70* mRNA overexpression in the maternal circulation of women with PE may be a compilation of several events: the activation of circulating maternal leucocytes, mainly monocytes, as a response to placental hypoxia and excessive amount of placental debris in maternal circulation. Both activated maternal leucocytes and placental debris released into maternal circulation may be a source of high circulating *Hsp70* mRNA levels.

Former studies evaluating the relationship between PE or intrauterine growth restriction and expression of total Hsp60 and Hsp90 revealed that a stress response in placental and decidual tissues, as determined by immunohistochemical and immunofluorescence analyses, was not associated with pregnancy outcome[23,24]. Most likely, there will be very low levels of *Hsp60* mRNA inside placental

Table III. Summary results on association of *Hsp* gene expression and Doppler ultrasonography parameters

Group comparison	*Hsp27*	*Hsp70*	*Hsp90*
	H (3, n=173)=3.280897 P=0.350	H (3, n=174)=16.93786 P<0.001	H (3, n=173)=4.386463 P=0.223
Controls/PE ± FGR (P)	1.0	0.002	0.271
Controls/FGR (P)	1.0	0.996	1.0
Controls/GH (P)	1.0	0.003	1.0
PE/GH (P)	1.0	1.0	1.0
	H (2, n=116)=3.04827 P=0.218	H (2, n=116)=13.68146 P=0.001	H (2, n=116)=4.770630 P=0.092
Controls/mild PE (P)	0.246	0.004	0.114
Controls/severe PE (P)	1.0	0.005	0.335
mild PE/severe PE (P)	0.686	1.0	1.0
	H (2, n=141)=5.827881 P=0.054	H (2, n=141)=10.75257 P=0.005	H (2, n=141)=4.237187 P=0.120
Controls/PE ± FGR delivery before 34 wk (P)	1.0	0.015	0.118
Controls/PE ± FGR delivery after 34 wk (P)	0.183	0.009	0.348
PE ± FGR delivery before versus after 34 wk (P)	0.241	1.0	1.0
Superposed PE/unsuperposed PE (P)	0.842	0.761	0.937
PI in the umbilical artery (P)			
Normal versus abnormal values of the flow rate	0.126	0.872	0.199
PI in the middle cerebral artery (P)			
Normal versus abnormal values of the flow rate	0.465	0.392	0.465
Cerebroplacental ratio (P)			
Normal versus abnormal values of the flow rate	0.145	0.379	0.065

Hsp mRNA levels were compared between groups by non-parametric tests (Mann–Whitney U-test for the comparison between two groups and Kruskal–Wallis test for the comparison among multiple groups). PE, pre-eclamptic pregnancies; GH, pregnancies with gestational hypertension; FGR, foetal growth restriction; Hsp, heat shock protein; PI, pulsatility index

debris released into maternal circulation throughout gestation. Our unpublished data also revealed very low levels of *Hsp60* mRNA in maternal whole peripheral blood samples derived from women with normal and complicated pregnancies. That could be the reason for rarely detectable circulating *Hsp60* mRNA in maternal plasma samples.

We presumed that very low (hardly detectable) *HspBP1* mRNA circulating levels were closely related to the high *Hsp70* mRNA extracellular levels. Hsp70 directly inhibits apoptosis. HspBP1 binds to the ATPase domain of Hsp70 and inhibits its ability to refold denatured proteins[26]. Molar ratio of HspBP1 to Hsp70 in cells is an important determinant of the function of the resulting complex. The excess of HspBP1 enhances the proapoptotic effect in all cells and *vice versa*, the lack of HspBP1 enables Hsp70 to carry out cytoprotective properties through its antiapoptotic function.

In conclusion, our findings support that women with pregnancy-related complications may benefit from the exploration of extracellular *Hsp70* mRNA levels in maternal circulation.

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***Conflicts of Interest*:** None.

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