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Various levels of circulating exosomal total-miRNA and miR-210 hypoxamiR in different forms of pregnancy hypertension

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ABSTRACT

Introduction: Hypertension is a common complication during pregnancy, affecting 10% of pregnant women worldwide. Several microRNA (miRNA) were shown to be involved in hypertensive disorders of pregnancy. In preeclampsia (PE), placental dysfunction causes the enhanced release of extracellular vesicle-derived miRNAs. The hypoxia-sensitive hsa-mir-210 is the most common PE-associated miRNA, but its exosomal profile has not been investigated.

Objectives: Our aims were to measure exosomal total-miRNA concentration and to perform expression analysis of circulating exosomal hsa-miR-210 in women affected by chronic hypertension (CHT) gestational hypertension (GHT) or PE.

Materials and methods: We collected plasma samples from women with CHT, GHT, PE (moderate: mPE and severe: sPE) and from normotensive pregnancies. Exosomal miRNAs were extracted and miRNA concentration was measured. RT-PCR was carried out with hsa-miR-210-3p-specific primers and relative expression was calculated using the comparative Ct method.

Results: The total-miRNA concentration was different in the disease subgroups, and was significantly higher in mPE and sPE compared to the other groups. We found a significant difference in the relative exosomal hsa-miR-210-3p expression between all hypertensive groups compared to the normotensive samples, but significant upregulation was only observed in case of mPE and sPE patients. Both the level of total-miRNA and hsa-miR-210 expression was higher in case of severe PE.

Conclusions: The level of circulating exosomal total-miRNA and hsa-miR-210 was elevated in women with PE, and it was higher in the severe form. We showed that hsa-miR-210 is secreted via exosomes, which may have a role in the pathomechanism of the disease.

1. Introduction

1.1. The different forms of pregnancy hypertension

Hypertension is a common complication during pregnancy, affecting up to 10% of pregnant women worldwide. Considering different types of hypertension in pregnancy, around 1% of pregnancies are affected by chronic hypertension (CHT), 5–6% by gestational or pregnancy-induced hypertension (GHT) and 1–2% by preeclampsia (PE) [1]. CHT is defined as blood pressure (BP) exceeding 140/90 mmHg before pregnancy or prior to 20 weeks of gestation. About 20–25% of women with CHT develops superimposed preeclampsia (SIPE) during pregnancy [2]. In GHT, hypertension develops in the latter part of pregnancy without any other symptoms of PE and followed by BP normalization postpartum. PE is a multisystem disease, characterized by the development of hypertension and proteinuria after 20 weeks of gestation. In the absence of proteinuria, the diagnosis may be established by the presence of other maternal organ dysfunctions including thrombocytopenia, impaired liver functions, renal insufficiency, pulmonary edema and cerebral or visual disturbances previously not experienced [3]. PE is one of the leading causes of maternal and fetal/neonatal morbidity and mortality all around the globe [4]. Stillbirth is twice as common in PE and more than 30% of cases are accompanied by intrauterine growth restriction (IUGR) [5].

Although the pathophysiology of the disease is still elusive, it is believed that PE is a consequence of altered trophoblast invasion and spi-

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ral artery remodeling, which hinders maternal blood flow to the placenta and also leads to high perfusion pressure constituting shear stress to the trophoblast layer [6]. This phenomenon results in injured trophoblast and subsequent release of possibly harmful materials including cell fragments and extracellular vesicles (EVs) into the maternal circulation [7].

1.2. Extracellular vesicles in pregnancy

EVs are lipid bilayer structures that are released from cells into the extracellular environment. They incorporate proteins, lipids, non-coding RNAs, and other regulatory elements. Multiple EV types can be produced by different cells, including red blood cells [8], fibroblasts [9], endothelial cells [10], and trophoblasts [11]. After secretion from cells, EVs may modulate the activity of neighboring cells or travel to distal regions serving a non-hormonal way of intercellular communication. EVs are distinguished by size, function, biogenesis, and morphology into three categories: microvesicles, apoptotic bodies, and exosomes.

In this article, we will only assay exosomes in relation to pregnancy. Exosomes are the smallest of EVs (40–120 nm) with a density ranging from 1,13 to 1,19 g/mL [12]. They are characterized by endosomal origin with formation through the inward budding of multivesicular bodies and release into extracellular space via exocytosis. Exosomes from different cell types are enriched with endosome-associated proteins (e.g. CD63, CD89, and TSG101) [13]. Placenta-derived exosomes can be detected using the immunoreactive placental alkaline phosphatase protein (PLAP) marker. In the course of physiologically normal pregnancy, exosomes of trophoblast origin can be detected from the 6th week of gestation in the maternal circulation. The number of these vesicles increases until term and returns to non-pregnant levels in 48 h after delivery [14]. The levels of placenta-specific exosomes and their content may serve information of placental health. In PE, placental dysfunction causes the enhanced shedding of trophoblast-derived EVs into the maternal circulation and subsequent release of toxic material including anti-angiogenic factors, pro-inflammatory mediators and microRNAs (miRNAs) [15].

1.3. microRNAs in pregnancy

miRNAs are endogenous, 20–22 nucleotides long, non-coding RNA molecules, which have a special role in fine-tuning gene expression. To date, almost 2000 miRNA sequences have been identified in human [16] and predicted to regulate approximately 30% of all protein-coding genes [17]. miRNAs control gene expression at the post-transcriptional level, by either repressing protein translation of target genes or inducing target mRNA degradation. One particular miRNA is able to control multiple genes, and a single gene can be a target of several miRNAs. miRNAs are involved mainly in all important biological process, such as cell proliferation, differentiation, apoptosis, and metabolism, besides also closely related to the pathogenesis of several human diseases [18].

More than one thousand miRNAs are expressed by different layers of the human placenta, some of which are primate-specific and only present in placental and stem cells [19]. The function of placentally expressed miRNAs is not fully elucidated but is clear that they take part in the regulation of placental development and are essential for normal physiology [20]. Differently expressed miRNAs, both placenta-specific and not placenta-specific, were reported in pregnancy-related hypertensive disorders [21–23].miRNAs are released from trophoblast cells to maternal circulation mainly via the exosomal pathway, but secretion in other forms are described as well, like microvesicles, apoptotic bodies, and protein-bound miRNAs [24]. In either way, extracellular miRNAs have been shown to be stable and protected from RNase degradation, which implicates their use as potential biomarkers [25]. The miRNA profile in exosomes and their donor cells hardly correlate, suggesting specific direct sorting of miRNA [26]. Exosomal miRNAs can be transferred to recipient cells, where they mediate functional effects by altering gene expression [27].

Altered placental development and spiral artery remodeling leads to placental hypoxia, which can change the number and content of secreted EVs. It has been shown that under hypoxic conditions trophoblast cells release harmful agents into the maternal blood flow, which may induce drastic biological changes systemically [28,29].

1.4. Hypoxia-responsive miRNAs and hypertension in pregnancy

miR-210 is one of the so-called “hypoxamiRs”, which are sensitive to the hypoxic environment [30]. It is specifically activated by HIF-1α [31] and considered as one of the hallmarks of hypoxic responses in several cell types including trophoblast and endothelial cells. HIF-1α is directly recruited to the hypoxia-responsive element (HRE) in the miR-210 promoter, which induces miR-210 transcription [32]. Creating a positive feed-back loop, miR-210 stabilizes HIF-1α by targeting an enzyme which decreases HIF-1α stability [33].

The direct consequences of the elevated levels of miR-210 in pregnancy are not clear. Hsa-mir-210-3p is the most common PE-associated miRNA, as it was found to be overexpressed both in the placenta and in the circulation of women with PE [23]. Nevertheless, miR-210 has not been investigated in other forms of pregnancy hypertensions, and the question whether it acts via the exosome-mediated or other pathways has not been addressed either. In this study, our aims were to quantify exosomal total-miRNA concentration and to perform expression analysis of exosomal hsa-miR-210-3p in the circulation of women affected by CHT, GHT, and PE. In the latter group, we also studied whether these factors change with the severity of the disease.

2. Materials and methods

2.1. Participants

Study participants had been recruited during routine prenatal care or following hospital admission between the 24 and 40th weeks of gestation during 2015–2017 at 1st Department of Obstetrics and Gynecology, Semmelweis University, Budapest, Hungary. We collected plasma samples from healthy controls (n = 34) and from pregnant woman affected by different types of hypertension: CHT (n = 16), GHT (n = 14), PE (n = 34, from which 11 were superimposed and 23 were de novo PE). The patient characteristics are summarized in Table 1 in the Results section.

Inclusion criteria for the GHT group were the de novo development of high blood pressure without any of the abnormalities that define PE. CHT was defined as BP exceeding 140/90 mmHg before pregnancy or prior to 20 weeks of gestation. Inclusion criteria for the PE group were BP greater than 140/90 mmHg with proteinuria >300 mg/24 h or at least 1 g/L (++) on dipstick testing. As we wanted to conduct the analysis in the context of severity, PE group was divided to severe (n = 19) and moderate (n = 15) subgroups. Moderate PE was defined as BP greater than 140/90 mmHg, and cases in which BP exceeded 160/110 mmHg or other maternal organ dysfunctions were present including thrombocytopenia, impaired liver functions, renal insufficiency, pulmonary edema and cerebral or visual disturbances, were classified as severe PE [34]. IUGR was determined by ultrasonography when the estimated fetal weight was below the 10th percentile for gestational age [35].

Pregnancies with ongoing labour, multiple gestations, and congenital malformations were not included. In the normotensive group (N), exclusion criteria also included the history of pregnancy-related or

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Table 1
Patient characteristics. Maternal age, gestational age, birthweight, and BMI are expressed as median (min-max). Percentage of sample size in each group was noted at IUGR and nulliparity values.

	N	CHT	GHT	mPE	sPE
sample size	34	16	14	15	19
maternal age	31,30 (23–38)	33 (25–40)	32,36 (22–41)	30,8 (20–41)	30,42 (15–41)
gestational age at sampling	35,58 (30–39)	32,63 (24–40)	35,71 (25–39)	32,47 (24–37)	31 (24–39)
gestational age at birth	38,94 (36,14–40,86)	37,02 (25–40,86)	37,13 (25,86–39,29)	36,63 (29–40)	31,12 (22,42–38,86)
birthweight	3525,81 (2160–4770)	3118 (1100–4130)	2882 (530–4020)	2648,57 (990–3510)	1555,88 (320–3350)
BMI before pregnancy	26,97 (17,92–32,39)	27,61 (18,42–39,12)	29,02 (20,31–39,51)	28,11 (18,3–35,6)	25,23 (18,96–35,6)
BMI at sampling	26,97 (21,30–35,82)	32,24 (21,97–40,96)	31,97 (23,44–41,47)	33,26 (21,72–42,53)	30,11 (21,56–39,79)
IUGR	0 (0%)	4 (25%)	3 (21,43%)	3 (20%)	7 (36,84%)
nulliparity	10 (29,41%)	6 (37,5%)	2 (14,29%)	8 (53,33%)	11 (57,89%)

other forms of hypertension, spontaneous abortion, preterm birth, and IUGR.

The study protocol was approved by the Scientific and Research Ethics Committee of the Medical Research Council (ETT TUKEB) [No: 2379/2014] and written informed consent was obtained from each patient. The research was conducted in accordance with the Declaration of Helsinki.

2.2. Sample handling

Venous blood samples were drawn into 4 mL EDTA tubes and kept at 4 °C until processing. Plasma was separated within few hours by two-step centrifugation (1. 2500*g*, 10 min, 4 °C, 2. 12500*g*, 10 min, 4 °C) and stored in 1.5 ml Eppendorf tubes at −80 °C.

2.3. Exosome and RNA isolation

In the first step, exosomes were isolated from 500 μl plasma using exosome precipitation solution (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and resuspended in 300 μl RNase-free water. miRNAs were extracted from the diluted exosomes with NucleoSpin miRNA Plasma Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and dissolved in 30 μl RNase-free water. A DNase step was included to remove traces of genomic DNA due to co-purified DNA might interfere with qPCR quantification of miRNA.

2.4. Total-miRNA quality and quantity measurement

In order to determine total-miRNA concentration, we applied a miRNA-specific fluorometric assay using a Qubit® 2.0 Fluorometer (Thermo Fischer Scientific Inc., Wilmington, USA). The purity of each sample was checked by Nanodrop spectrophotometer (Thermo Fischer Scientific Inc., Wilmington, USA) based on the A260/A280 ratio.

2.5. Real-time quantitative PCR

The expression analysis was performed using the miRCURY LNA™ Universal RT microRNA PCR protocol for plasma samples (Exiqon A/S, Vedbaek, Denmark) according to the manufacturer's instructions. Briefly, total-miRNA samples were reverse-transcribed using Universal cDNA synthesis kit II. UniSp6 RNA spike-in template was added to each reaction for cDNA synthesis control. cDNA templates were 40× diluted in nuclease-free water before subsequent amplification.

RT-PCR was carried out using ExiLENT SYBR® Green master mix with hsa-miR-210-3p specific LNA™ PCR primer set targeting the sequence 5′- CUGUGCGUGUGACAGCGGCUGA-3′. The relative expression was calculated based on the ddCT method and was normalized to hsa-miR-103a-3p internal control miRNA. Melting curve analysis was carried out following each PCR run for evaluating the specificity of the assay.

2.6. Statistics

We used the STATISTICA software package (Statistica, Tulsa, Oklahoma, USA) for statistical analysis. Shapiro-Wilk W test was applied to assess the normality of the dependent variables. Since nor the total-miRNA concentration, neither the hsa-miR-210-3p expression followed a normal distribution, nonparametric tests were used afterward. Comparisons among groups were carried out using Kruskal-Wallis ANOVA integrated with two-tailed Mann-Whitney U test for between group analysis. Spearman Rank Order correlation was calculated to find a possible connection between hsa-miR-210 expression and total-miRNA concentration. miRNA levels were given as median with interquartile ranges, and fold change values (FC) were calculated when the hypertensive and normotensive groups were compared. FC > 2 and FC < 0,5 was regarded as significant over- and underexpression respectively. A p value of <0,05 considered as a statistically significant finding.

3. Results

3.1. Total-miRNA concentration

The median value of total-miRNA concentration in exosome samples varied in the disease subgroups and in the control group as follows: N: 0,26 ng/μl (0,10–0,76 ng/μl); GHT: 0,28 ng/μl (0,16–0,91 ng/μl); CHT: 0,30 ng/μl (0,22–0,38 ng/μl); mPE: 0,83 ng/μl (0,42–1,85 ng/μl); sPE: 1,57 ng/μl (0,25–8,21 ng/μl). The data was represented in box and whisker plot format in Fig. 1. Overexpression

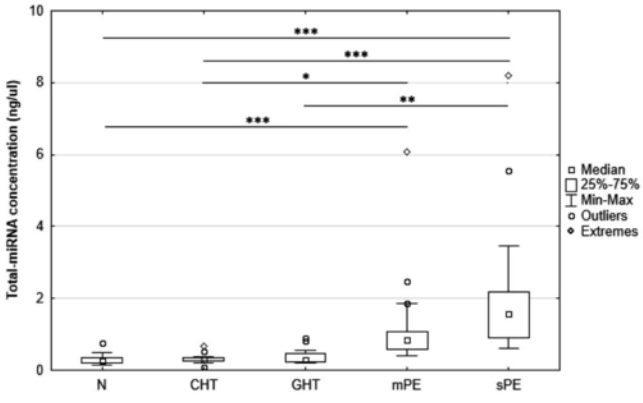

Fig. 1. Comparison of total-miRNA concentration in different groups by box-plot diagram. The groups are arranged in ascending order, N: normotensive, CHT: chronic hypertension, GHT: gestational hypertension, mPE: moderate preeclampsia, sPE: severe preeclampsia. *: p < 0,5, **: p < 0,01, ***: p < 0,001.

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(FC > 2) was only observed in PE groups, the total-miRNA concentration was three-times higher (FC = 3,19, p < 0,001) in mPE and six-times higher (FC = 6,03, p < 0,001) in sPE groups than in the normotensive group. There was significant difference comparing CHT to mPE (p < 0,05) and sPE (p < 0,001), and GHT to sPE group (p < 0,01). De novo and superimposed PE could not be segregated based on the total-miRNA concentration (0,96 (0,25–8,21) vs. 0,57 (0,21–4,00), p > 0,05), and IUGR had no significant impact in the affected groups.

3.2. hsa-miR-210-3p expression

We found significant difference in the relative exosomal hsa-miR-210-3p expression between the hypertension groups and the normotensive group (Fig. 2): N: 0,13 (0,10–0,17); CHT: 0,17 (0,13–0,25); GHT: 0,23 (0,17–0,30); mPE: 0,29 (0,20–0,41); sPE: 0,37 (0,26–0,49). The data was represented in box and whisker plot format in Fig. 2. Overexpression only was observed in mPE (FC = 2,23, p < 0,001) and in sPE groups (FC = 2,85, p < 0,001) but not in CHT (FC = 1,31, p < 0,05) and GHT groups (FC = 1,77, p < 0,01) in comparison to the control group. There was a significant difference comparing CHT and GHT to sPE group (p < 0,05). De novo and superimposed PE could not be separated based on the relative hsa-miR-210 expression (Null,34 (Null,14-Null,73) vs. 0,26 (0,16–0,64), p > 0,05), and IUGR had no significant impact in the hypertensive groups.

Total-miRNA concentration was positively correlated with hsa-miR-210 expression in the hypertensive samples (R = 0,41, p < 0,05) but not in the control samples. This finding was visualized by scatter plot after log-transformation of the data for graphic purposes (Fig. 3).

4. Discussion

In this study, our aims were quantity exosomal total-miRNA and to perform expression analysis of exosomal hsa-miR-210 in the circulation of pregnant women affected by CHT, GHT, mPE and sPE. The median value of total-miRNA concentration was different in the disease subgroups and was significantly higher in sPE and mPE compared to control samples. We found a significant difference in the relative exosomal hsa-miR-210-3p expression between all disease subgroups compared to normotensive group, however, significant upregulation (FC > 2) was only observed in the circulation of PE patients. Total-miRNA concentration was positively correlated with hsa-miR-210 expression in the hypertension groups but no correlation was observed in the normotensive group. It may implicate that miR-210 contribution to the total-miRNA pool was higher in case of hypertension disorders than in normotensive samples.

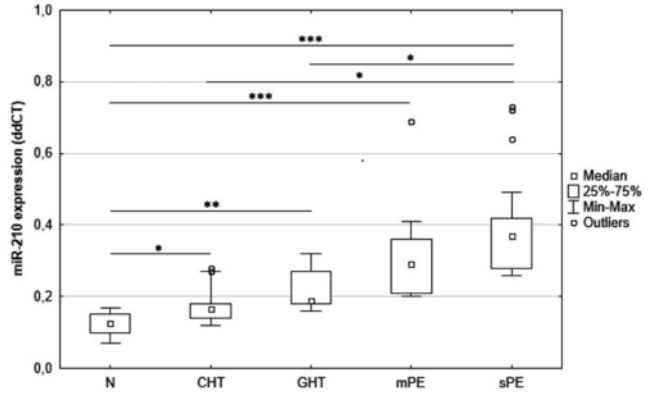

Fig. 2. Comparison of relative exosomal hsa-miR-210-3p expression in different groups by box-plot diagram. The groups are arranged in ascending order, N: normotensive, CHT: chronic hypertension, GHT: gestational hypertension, mPE: moderate preeclampsia, sPE: severe preeclampsia. *: p < 0,5, **: p < 0,01, ***: p < 0,001.

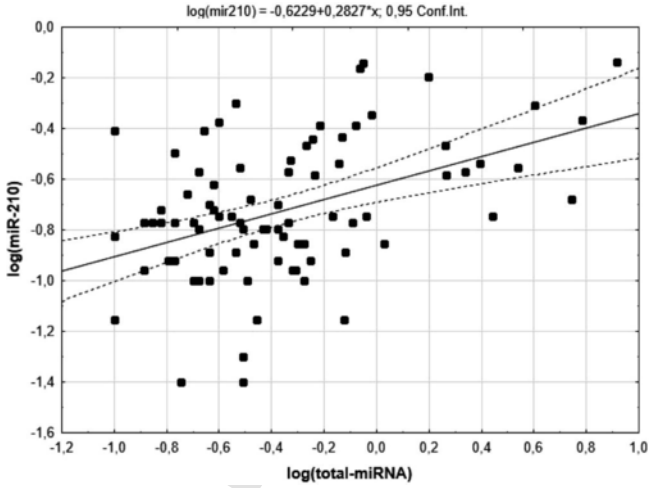

Fig. 3. Correlation between total-miRNA concentration and relative hsa-miR-210 expression in hypertension groups (R = 0,4073, p = 0,002) Log-transformation was performed on the data for graphic purposes.

The role of trophoblast-derived EVs in normal and complicated pregnancy could facilitate the improvement of new therapeutic strategies, including neutralizing harmful EVs and boost the release of ones with preferential characteristics. Nadkarni et al. showed that recombinant human plasma gelsolin limits spontaneous EV shedding and subsequent release of toxic material including anti-angiogenic and pro-inflammatory factors [36]. Salomon et al. investigated the bioactivity of circulating placental exosomes and they found that the incubation of HUVEC culture with these vesicles increase endothelial cell migration. Interestingly, the bioactivity of exosomes was the greatest in the first trimester and toned down in the course of pregnancy [14]. These results may indicate that exosomes of trophoblast origin may facilitate the maternal vascular adaptation to pregnancy.

Hsa-miR-210 is consistently overexpressed in the placenta and circulation of women with PE [23]. Moreover, several groups have placental cell lines exposed to hypoxia, including cytotrophoblast, BeWo, and JEG-3 [37–39]. However, hsa-miR-210 expression has not been assessed in EVs, which could shed more light on the mechanism of the disease.

In our previous study, we created an interaction network by integrating miRNA and mRNA expression profiles in normotensive and PE placenta samples. In the group of upregulated miRNAs, we identified miR-210 as a key regulator due to it targeted the most genes in the network [40].

It is possible that EVs and miRNAs have opposing effects depending on different circumstances. For instance, tumor-derived exosomes increase angiogenesis [41], while in PE, circulating exosomes of trophoblast origin may lead to endothelial dysfunction [42]. mir-210 itself is well-studied in cancer and bears tumor suppressor and oncogene functions at the same time [43].

More than sixty genes have been experimentally validated as targets of miR-210 up to now [44], and several others were predicted to be regulated by this miRNA [45]. These genes play important roles in various cell processes such as apoptosis, proliferation, and metabolism.

Luo et al. established that upregulated potassium channel modulator factor 1 (KCMF1), which is a validated target of miR-210 and known to be underexpressed in PE, set back inhibition of trophoblast invasion [46]. Upregulation of miR-210 may lead to placental mitochondria dysfunction and oxidative stress by causing repression of mitochondria-associated iron-sulfur scaffold homologue (ISCU) [47]. Lee et al. demonstrated that ISCU is a direct target of miR-210, exhibiting

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decreased levels in PE [38]. Ishibashi et al. found that miR-210 regulates 17-beta-hydroxysteroid dehydrogenase (HSD17B1), the enzyme which is responsible for the production of estrone into 17β-estradiol. Lower level of circulating HSD17B1 precedes the onset of PE, therefore it is a potential prognostic factor for PE [39]. Ephrin-A3 (EFNA3) and Homeobox-A9 (HOXA9) were then experimentally validated as the functional targets of mir-210 [37]. EFNA3 is important for vascularization and cell migration, especially in the cardiovascular system, and HOXA9 has been shown to be a key regulator of angiogenesis. Anton et al. found that overexpressing miR-210 reduced trophoblast invasion and that elevated concentration in first-trimester serum samples was predictive of the disease. They assumed that miR-210 suppresses trophoblast invasion by targeting the MAPK and ERK signaling pathways. Both pathways are known to participate in the regulation of trophoblast invasion [48]. Kopriva et al. demonstrated that Toll-like receptor 3 (TLR3) activation induces placental miR-210 expression leading to the down-regulation of the STAT6/Interleukin-4 pathway and this may contribute to the development of PE [49].

All of the above-mentioned mechanisms are associated with the main characteristics of PE, such as reduced trophoblast invasion and oxidative stress. Unfortunately, no research had been conducted related to pregnancy, which would have elucidated the regulation of miR-210 in endothelial cells. We hypothesize that circulating exosomal miR-210 originates from the trophoblast layer, however, we cannot exclude other sources for miR-210 in the maternal circulation. It is known that miR-210 is expressed by endothelial cells [50] and endothelial dysfunction is connected to PE. Therefore shedding of exosomal miR-210 from the vasculature of pregnant women may be a source of miR-210 in the circulation.

5. Conclusions

To the best of our knowledge, this was the first study which assessed the exosomal miRNA concentration and expression in pregnancy-related hypertension disorders, including chronic hypertension, gestational hypertension, and preeclampsia. We conclude that the concentration of total-miRNA and exosomal hsa-miR-210 was significantly overexpressed in the circulation of women affected by PE, and it was higher in severe cases. Nevertheless, patient characteristics including BMI, birth weight, and gestational age cannot be excluded as confounding factors in our analysis. Based on our results it can be assumed that in PE, hsa-miR-210 is secreted via the exosomal pathway predominately, and it may contribute to the multisystem nature of the disease.

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