Circulating exosomal and Argonaute-bound microRNAs in preeclampsia

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Abstract

Introduction: microRNAs (miRNAs) play important role in the regulation of placental development, and abnormal miRNA expression is associated with preeclampsia (PE). miRNAs are released from trophoblast cells to maternal blood flow, where they are highly stable, being encapsulated inside extracellular vesicles, like exosomes or bound to Argonaute proteins. In PE, placental dysfunction leads to aberrant extracellular miRNA secretion. hsa-miR-210 is a hypoxia-sensitive miRNA found to be upregulated in PE, however, it is unknown whether it is the cause or the consequence of the disease.
Objective: Our aim was to analyze the expression of several miRNAs, including hsa-miR-210 in placenta, exosome and Ago-bound fractions comparing normal (N) and PE pregnancies. We performed in vitro analyses of extracellular hsa-miR-210 secretion of trophoblast cell cultures (of villous and extravillous origin) under hypoxic condition.
Methods: PE and N placenta samples were collected from C-sections, and blood samples were drawn from each pregnant woman in the third trimester. Htr-8 and Jar cell lines were cultured in exosome-free media and treated with hypoxia-mimetic agents. Exosome and Ago-bound fractions were isolated by membrane affinity spin column method from plasma and cell media. Short RNAs were extracted from exosomes and vesicle-free fractions, and total-RNA was isolated from the placenta samples. The RNA purity and concentration were measured by spectrophotometry. Expression analysis was carried out by qPCR with specific primers to target and reference miRNAs.
Results: The level of hsa-miR-210 was significantly higher in PE placentas, which could cause a minor increase of exosomal and a high elevation of Ago-bound miR-210 in circulation. Hypoxia leads to intracellular hsa-miR-210 upregulation in trophoblast cell lines. In extravillous cell (HTR8) media, only the level of exosomal hsa-miR-210 was increased but no change in Ago-bound hsa-miR-210 level was observed. In contrast, in villous cell (JAR) media, the level of exosomal hsa-miR-210 was increased and enhanced release of Ago-bound hsa-miR-210 was also observed.
Conclusion: Based on our data, we postulate that in PE, exosomal hsa-miR-210 are secreted actively from the trophoblast, and by intercellular communication, it may have a role in disease etiology. In addition, there is a passive release of Ago-bound hsa-miR-210 into the circulation, which may represent by-products of cell-death and is thereby a possible consequence of the disease.

Highlights

- hsa-miR-210 is significantly overexpressed in preeclamptic placenta samples
- hsa-miR-210 profile differs in circulating exosomal and Ago-bound fractions
- Hypoxia induction has a diverse effect on different trophoblast cell lines
- Exosomal hsa-miR-210 is secreted actively from trophoblast cells
- Ago-bound hsa-miR-210 is a possible by-product of trophoblast cell death

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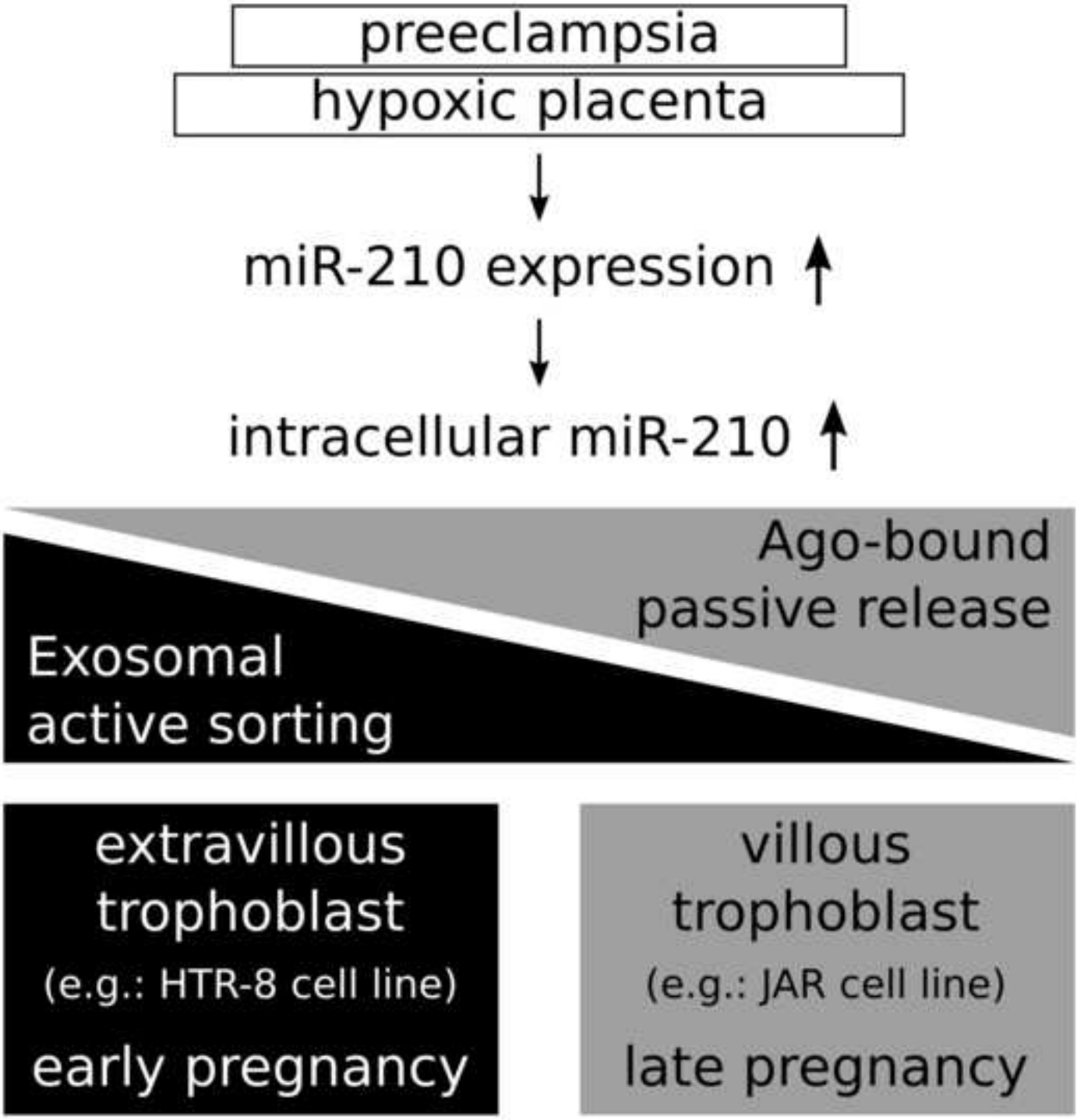

Abbreviations: miRNAs, microRNAs; PE, preeclampsia; N, normal; EOPE, early-onset preeclampsia; LOPE, late-onset preeclampsia; STB, syncytiotrophoblast; EVs, extracellular vesicles; HRE, hypoxia-responsive element; HUVEC, Human Umbilical Vein Endothelial Cell; ISCU, iron-sulfur scaffold homologue

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Keywords: has-miR-210; placenta; HTR-8; JAR

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1. Introduction

1.1. Preeclampsia

Preeclampsia (PE) is of the leading causes of maternal and fetal morbidity and mortality, affecting 3–8% of all pregnancies around the world (Duley, 2009). It is a multisystemic pregnancy-specific disorder, 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 occurrence of other maternal organ failures due to endothelial dysfunction (Tranquilli et al., 2014).

Although the etiology and pathogenesis of PE are debated, the generally accepted theory denotes placental malperfusion as the main cause of the disease. In the case of early-onset preeclampsia (EOPE, <34 weeks), the placental dysfunction is a consequence of the insufficient extravillous trophoblast invasion and spiral arterial remodeling, therefore the pathophysiological changes take place well before the clinical manifestations. It has been proposed that in late-onset preeclampsia (LOPE, >34 weeks) the placenta exceeds the capacity of the uterus and its vasculature to comply with the increasing demands in term pregnancy, leading to deficient intervillous perfusion and increased hypoxia (Redman, 2017). In both scenarios, maternal blood flow to the placenta is hindered and high perfusion pressure constituting shear stress to the villous trophoblast layer. This phenomenon results in widespread syncytiotrophoblast (STB) damage and subsequent release of toxic materials including cell fragments, antiangiogenic factors, proinflammatory mediators, and extracellular vesicles (EVs) that are capable of activating the maternal endothelium.

1.2. Exosomes

EVs are lipid bilayer structures that are secreted from all kinds of cells into the extracellular space. They comprise proteins, lipids, noncoding RNAs, and other regulatory elements. EVs are distinguished by size, function, biogenesis, and morphology into three categories: microvesicles, apoptotic bodies, and exosomes. Exosomes are the smallest of EVs with a size between 40–120 nm (Kowal et al., 2014). The level and content of placenta-specific exosomes may provide useful information on placental health. In the course of physiologically normal pregnancy, EVs are continuously shed from the STB into the maternal circulation. The release of these particles rises with gestation as the placenta grows in size, and returns to non-pregnant levels in 48 h after delivery (Salomon et al., 2014a).

After secretion from cells, exosomes may have a function in intercellular communication. Over the last decade, several in vitro studies have been carried out investigating the effect of placental vesicles on different target cells (Salomon et al., 2014a, 2014b, 2013a, 2013b; Truong et al., 2017). It was demonstrated that they are capable of modulating the activity of adjacent cells, which may play a role in the progression and maintenance of physiological pregnancy. However, pathological processes can change the number and composition of vesicles, which may lead to the development of various pregnancy complications. In PE, the excessive shear stress resulting from abnormal placentation leads to morphological damage and increased shedding of STB, which may contribute to generalized endothelial dysfunction.

1.3. Placental and cell-free miRNAs

miRNAs are endogenous, 20–22 nucleotides long, non-coding RNA molecules, which play important role in the post-transcriptional regulation of gene expression by either repressing protein translation or inducing mRNA degradation. They are involved in fundamental biological process, such as cell proliferation, differentiation, apoptosis and have

been linked to the pathogenesis of several human diseases (Gebert and MacRae, 2018; Li and Kowdley, 2012).

Several hundred miRNAs are expressed by different layers of the human placenta; however, their function in pregnancy is not fully elucidated. miRNAs take part in the regulation of placental development, and abnormal miRNA expression is associated with pregnancy complications including PE (Fu et al., 2013). miRNAs are released from trophoblast cells to maternal blood flow, where they are highly stable, being encapsulated inside extracellular vesicles (like exosomes) or bound to stabilizing proteins (mainly Argonaute proteins) (Ouyang et al., 2014). Cells seem to selectively release certain non-coding RNAs, thereby affecting the function of surrounding and distant target cells. Exosomal miRNAs can be internalized by recipient cells, where they mediate functional effects by altering host gene expression (Valadi et al., 2007). The miRNA expression patterns of the source cell and released exosomes are usually different, which supports the idea of active exosomal miRNA sorting. However, exosomal miRNAs constitute only a minor fraction of the whole plasma miRNA population, the majority of which are Ago-bound miRNAs (Turchinovich et al., 2011). Non-vesicular miRNAs are believed to be non-specific by-products of general physiological processes and cell death, however, their significance is not known (Schwarzenbach et al., 2014). It has been proposed that vesicle- versus Ago complex-associated miRNAs originate from different cell types and reflect cell type-specific miRNA expression and/or release mechanisms (Arroyo et al., 2011). Ago-specific miRNA profile in blood cells and in the whole plasma differs significantly indicating that most circulating non-vesicular miRNAs are likely to be derived from non-blood cells (Turchinovich and Burwinkel, 2012).

1.4. hsa-miR-210 hypoxamiR

In PE, deficient trophoblast invasion and spiral arterial remodeling lead to prolonged placental hypoxia. hsa-miR-210 is a hypoxia-induced miRNA and considered as one of the hallmarks of hypoxic responses in several cell types, including trophoblast and endothelial cells (Chan and Loscalzo, 2010). HIF-1α is directly recruited to the hypoxia-responsive element (HRE) in the hsa-miR-210 promoter region and triggers miRNA transcription (Huang et al., 2010, 2009).

The direct effect of the elevated hsa-miR-210 level on pregnancy is currently unknown. In our previous study, we have created a miRNA regulated interaction network by the integration of available miRNA and gene expression profiles in preeclampsia, and hsa-miR-210 was the highest degree node in the network (Biró et al., 2017a). It has been associated with preeclampsia before (Sheikh et al., 2016), however, it is questionable whether it is the cause or the consequence of the disease. The mode of miRNA secretion (exosomal or Ago-bound) and the significance of the different forms are not clarified either. In this study, we hypothesized that extracellular hsa-miR-210 profile differs in PE and normal pregnancy which could be the result of abnormal placental miRNA expression and secretion in case of PE.

Our aim was to analyze the expression of hsa-miR-210 in placenta, exosome and Ago-bound fractions comparing PE and N pregnancies. Apart from hsa-miR-210, the expression of hsa-miR-16 and hsa-miR-517c was also measured and used for comparison. hsa-miR-16 is one of the most abundant miRNAs in blood, found to be expressed mainly by red blood cells (Pritchard et al., 2012). hsa-miR-517c is part of the primate-specific C19MC miRNA cluster, which is abundantly expressed in the placenta (Noguer-Dance et al., 2010). We performed an in vitro analysis of extracellular miRNA secretion of different trophoblast cell cultures under normoxic and hypoxic conditions.

2. Materials and methods

2.1. Study participants

Study participants had been recruited during routine prenatal care or following hospital admission during the third trimester of pregnancy at 1st Department of Obstetrics and Gynecology, Semmelweis University, Budapest, Hungary. In this study 16 plasma and 20 placenta samples were included, 8 vs. 8 and 13 vs. 7 preeclamptic (PE) and normal (N) cases respectively. Inclusion criteria for the PE group were blood pressure greater than 140/90 mmHg with proteinuria>300 mg/24 h or at least 1 g/L (++) on dipstick testing. Pregnancies with ongoing labour, multiple gestations, and congenital malformations were not included. In the normotensive group, exclusion criteria also included the history of pregnancy-related or other forms of hypertension, spontaneous abortion, preterm birth, and intrauterine growth restriction. Placenta samples were collected from C-sections and blood samples were drawn from each pregnant woman in the third trimester.

The study protocol was approved by the Scientific and Research Ethics Committee of the Medical Research Council (ETT TUKEB) [No: 24387-2/2016] and written informed consent was obtained from each patient. The research was conducted in accordance with the Declaration of Helsinki.

2.2. Sample collection and handling

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

Placenta samples were collected according to the protocol described by Pasupathy et al. (Pasupathy et al., 2008). Four areas suitable for sampling were located on the maternal

surface; damaged areas not suitable for further examination (calcification, hematoma, etc.) were excluded. 1-2 mm from the basal membrane was removed and a pea size tissue sample was taken from the placental cotyledons. The samples were washed twice in 1x PBS solution at 4 °C and placed in RNAlater (Thermo Fisher Scientific) stabilizing solution to avoid RNA degradation.

2.3. Cell culture and treatment

We used JAR and HTR-8 placenta-derived cell lines. The former is a choriocarcinoma cell line and has the characteristics of villous trophoblasts (VT), while the latter one is an immortalized extravillous trophoblast (EVT) cell line.

JAR and HTR-8 cell lines were maintained under standard conditions at 37°C and 5% CO_2 in RPMI-1640 (Gibco) or DMEM (Gibco) medium respectively, supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin. 4 x 10^5 cells were seeded per well on six-well plates in media containing 10% exosome-depleted FBS. Cells were treated after 24 hours with hypoxia-mimetic agent (100 μM DFO or 50 μM DFX), or DMSO as control and maintained under standard conditions for another 24 hours. DFO and DFX are well-known iron chelators used for modeling hypoxic conditions in cell cultures.

2.4. Total-RNA and miRNA isolation

Exosomes were isolated from plasma and cell media using the ExoRNEasy kit, which applies membrane affinity spin column method (Qiagen). As compared to ultracentrifugation, this method is faster and yields a cleaner preparation of exosomes (Enderle et al., 2015). Exosomes were bound to the column membrane and protein complexes were partitioned to the flow-through. Short RNAs were extracted from exosomes and vesicle-free fractions.

Total-RNA was isolated from the placenta samples and cultured trophoblast cell lines using the TRIzol ™ lysis reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. The RNA purity and concentration were measured by Nanodrop spectrophotometer (Thermo Fisher Scientific).

2.5. Expression analysis

The expression analysis was performed using the miRCURY LNA™ Universal RT miRNA PCR Assay (Qiagen) according to the manufacturer's instructions. Briefly, RNA samples (5 ng/µl) were reverse-transcribed using the miRCURY LNA™ RT Kit. UniSp6 RNA spike-in template was added to each reaction for cDNA synthesis control. cDNAs of placental and cell culture RNAs were 1:80 diluted, whilst templates from exosomal and Ago-bound miR fractions were 1:10 diluted before subsequent amplification. RT-PCR was carried out using miRCURY SYBR® Green master mix with specific LNA™ PCR primer sets, run on a StepOnePlus™ platform (Thermo Fisher Scientific) using the manufacturer's instructions. The relative expression of the investigated miRNAs was calculated based on the ΔΔCT method and was normalized to hsa-miR-103a internal control miRNA. The miRBase identifiers and seed sequences of investigated and reference miRNAs are listed in Table 1. Melting curve analysis was carried out following each PCR run for evaluating the specificity of the assays.

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. None of the miRNA expression values of placenta and plasma samples followed a normal distribution, thus comparisons between groups were performed using the

nonparametric Mann-Whitney U test and exact probabilities were calculated. The data were represented on box plots, where miRNA levels were shown as median with interquartile ranges. The miRNA expression values of cell culture were logarithmized and one-sided t-test was applied. The data were represented on barplots, where the untransformed miRNA levels were shown as mean with standard error. A p value of <0.05 considered as a statistically significant finding.

3. Results

3.1. Patient characteristics

In the first part of the study, we analyzed the expression of hsa-miR-210 in placenta, exosome and Ago-bound plasma fractions comparing PE and N pregnancies. 16 plasma and 20 placenta samples were included, 8 vs. 8 and 13 vs. 7 PE and N cases respectively. The patient characteristics of the collected plasma and placenta samples are summarized in Table 2 and 3.

3.2. Placental and extracellular hsa-miR-210 expression

The level of placental hsa-miR-210 was significantly upregulated in affected samples comparing to the control group (Figure 1A), which could be related to a minor increase of circulating exosomal (Figure 1B) and significantly elevated Ago-bound hsa-miR-210 (Figure 1C). We found that hsa-miR-210 was overrepresented in the Ago-bound fraction compared to exosomal fraction in the PE but not in the N group.

The level of placental hsa-miR-517c was significantly upregulated in affected samples comparing to the control group (Figure 2A), but there was no significant difference in the level of extracellular hsa-miR-517c between groups (Figure 2B,C). Neither the level of placental hsa-miR-16 (Figure 3A) nor extracellular hsa-miR-16 was altered due to PE (Figure 3B,C).

3.3. In vitro hsa-miR-210 secretion from trophoblast cell-lines

In the second part of the study, we performed an in vitro analysis of extracellular hsa-miR-210 secretion of different trophoblast cell cultures under normoxic and hypoxic conditions.

3.3.1. Effect of hypoxia on JAR cells and secreted cell-free miRNAs

The treatment of villous trophoblast cells with hypoxia-mimetic agent selectively upregulated hsa-miR-210 as compared to the other examined miRNA species (Figure 4A). In the cell media, the level of exosomal hsa-miR-210 was significantly increased and enhanced release of Ago-bound miRNAs was also observed (Figure 4B,C). There was no significant difference in the case of hsa-miR-16, and hsa-miR-517c.

3.3.2. Effect of hypoxia on HTR-8 cells and secreted cell-free miRNAs

The treatment of the extravillous trophoblast cells with hypoxia-mimetic agent strongly upregulated hsa-miR-210 (Figure 5A), and this phenomenon was even more pronounced in secreted exosomal samples: only the level of exosomal hsa-miR-210 was increased (Figure 5B) and a no significant change on Ago-bound hsa-miR-210 level was observed (Figure 5C). There was no significant difference in the case of hsa-miR-16 and hsa-miR-517c is not expressed in this cell line.

4. Discussion

Exosomes have been shown to be capable of modulating the activity of adjacent cells, which may play a role in the development and maintenance of physiological pregnancy. Over the last decade, several in vitro studies have been carried out investigating the effect of placental vesicles on different target cells. Salamon et al. demonstrated that EVT exosomal signaling may contribute to spiral artery remodeling by enhancing the migration of vascular smooth muscle cells out of the vessel walls. They propose that during a normal placentation, EVT-derived exosomes that contain specific regulatory molecules (e.g. miRNAs) promote loss of vascular cells facilitating remodeling of the spiral uterine arteries (Salomon et al., 2014b). The same group investigated the bioactivity of circulating placental exosomes and they found that treating HUVEC (Human Umbilical Vein Endothelial Cell) culture with these vesicles increases endothelial cell migration. Importantly, the bioactivity of exosomes was the greatest in the first trimester and decreased with the progression of pregnancy. Their results indicate that in a healthy pregnancy, trophoblast-derived exosomes may facilitate the maternal vascular adaptation to pregnancy (Salomon et al., 2013a). They have also shown that in response to changes in oxygen tension, cytotrophoblast cells modify the bioactivity and protein-content of the secreted exosomes, which were able to alter EVT phenotype. Exosomal induction of EVT migration may represent an adaptive response to placental hypoxia in the first trimester (Salomon et al., 2013b).

Pathological processes may change the number and composition of vesicles, which may lead to the development of various pregnancy disorders. In PE, the presence of extensive hypoxia and accumulated proinflammatory cytokines (e.g. TNF-α) may inhibit the beneficial effect of exosomes on vascular cell migration leading to inadequate arterial remodeling. Truong et al. showed that the level of exosomes secreted by EVTs is significantly higher at 1% O_2 than 8% O_2. Exosomes isolated from EVT cells cultured in normoxia contained

miRNAs linked with cell migration, while exosomes isolated from EVT cultured in hypoxia had miRNAs associated with inflammatory response and regulation of cytokine production. They conclude that oxygen tension modifies the effect of EVT-derived exosomes on endothelial cells, thereby hypoxic condition inhibits EVT migration out of the artery and increases the concentration of TNFα at the site of remodeling. Abnormal EVT function may result in compromised placental perfusion and prolonged hypoxia leading to pregnancy complications (Truong et al., 2017).

The placental dysfunction causes the enhanced shedding of trophoblast-derived EVs into the maternal circulation, which may provoke drastic biological changes systemically. There is a growing evidence for STB EV's function in the immunological adaptations to pregnancy. Their role in the activation of the innate immune system has been indicated by that monocytes can bind and internalize STB EVs. As a result, monocytes start producing various cytokines, such as TNF-α, and IL-1β. Based on data from in vitro studies, it can be suggested that STB EVs may support the exaggerated inflammatory state observed in preeclamptic pregnancies (Göhner et al., 2017).

We investigated placental, exosomal and Ago-bound hsa-miR-210 expression profile in PE vs. N groups. The level of hsa-miR-210 was significantly higher in PE placentas, which could be associated with a minor increase of circulating exosomal, and highly elevated Ago-bound hsa-miR-210. In our former study, we found that the concentration of total-miRNA and exosomal hsa-miR-210 was significantly higher in the circulation of women affected by PE (Biró et al., 2017b). The difference in the exosomal expression levels could be due to the different sample numbers and exosome isolation techniques applied it the two studies.

It has been shown that hsa-miR-210 is expressed by several parts of the cardiovascular system (Chan et al., 2012), thus it may be also a potential surface for releasing hsa-miR-210 into the maternal circulation. Similarly to the placenta, cancer cells actively release exosomes

carrying specific regulatory and signaling molecules to communicate with various cells in the tumor microenvironment. Jung et al visualized exosome-mediated transfer of hsa-miR-210 from hypoxic breast cancer cells to neighboring cells using a specific reporter system. They found that exosomal hsa-miR-210 was transferred to cells in the tumor milieu, where it regulated the expression of vascular remodeling related genes to promote angiogenesis. These results demonstrated well that in cancer, particular cellular miRNAs are secreted and transferred to proximal cells via exosomes and through this mechanism, they are able to influence tumor progression (Jung et al., 2017). Hale et al proposed that the Argonaute 2-dependent control of released hsa-miR-210 is also possible and it synchronizes the hypoxic response in different cell-types, preventing redundant activity of delivered hsa-miR-210 in normoxia while potentiating the adaptation of recipient tissues to hypoxic stress. They demonstrated that released hsa-miR-210 can be transferred to recipient cells, where it downregulates the mitochondria-associated iron-sulfur scaffold homologue (ISCU), thereby suppressing mitochondrial metabolism (Hale et al., 2014).

We performed an in vitro analysis of extracellular hsa-miR-210 secretion of different trophoblast cell cultures under normoxic and hypoxic conditions. Hypoxia-induction had a different effect on the villous and extravillous trophoblast cell lines. The intracellular hsa-miR-210 was upregulated in both cases, however, in extravillous cell media, only the level of exosomal hsa-miR-210 was increased and a no change on Ago-bound hsa-miR-210 level was observed. Therefore, in this case, hypoxia resulted in the specific exosomal sorting of hsa-miR-210 – as this cell type has a role in trophoblast invasion, this phenomenon may have a role in placental dysfunction. In villous cell media, the level of exosomal hsa-miR-210 was increased and enhanced release of Ago-bound hsa-miR-210 was also observed. The expression profile was quite similar to that of the placenta and plasma samples – which can be explained by the fact in placenta this cell type is in contact with maternal circulation. Based

on these data, we suggest the use of JAR trophoblast cell-line for modeling mature placenta physiology and function, while the HTR-8 trophoblast cell-line is more appropriate for the investigation of placenta development and pathogenesis of associated diseases.

The hypoxia-induced hsa-miR-210 regulates numerous biological processes both in physiological and pathogenic circumstances. It is overexpressed in most solid tumors, and its levels correlate with a negative clinical outcome. Several genes have been experimentally validated and even more were predicted as targets of hsa-miR-210. These genes play important roles in various cellular processes such as proliferation, apoptosis, differentiation, angiogenesis, and metabolism. Elevated levels of hsa-miR-210 may lead to placental mitochondria dysfunction and oxidative stress by the repression of ISCU (Lee et al., 2011). Supporting its pathogenic role, the targeted mRNA was significantly reduced with concomitant hsa-miR-210 overexpression in preeclamptic placenta samples (Muralimanoharan et al., 2012). Ishibashi et al. showed that hsa-miR-210 regulates 17-beta-hydroxysteroid dehydrogenase (HSD17B1), a steroidogenic enzyme expressed predominantly in the placenta. The plasma HSD17B1 protein levels, as well as the placental expression, was decreased in preeclamptic pregnant women (Ishibashi et al., 2012). Ephrin-A3 (EFNA3) and Homeobox-A9 (HOXA9) are also validated targets of hsa-miR-210, EFNA3 is a key regulator of vascularization and cell migration, while HOXA9 plays an important role in angiogenesis (Fasanaro et al., 2008; Zhang et al., 2012). Anton et al. demonstrated that overexpressing hsa-miR-210 reduces trophoblast invasion and vice versa, hsa-miR-210 inhibition promotes invasion. Moreover, they conclude that it may help to identify at-risk women for monitoring and treatment since elevated first-trimester serum hsa-miR-210 expression was predictive of the disease (Anton et al., 2013). All of the referred mechanisms regulated by hsa-miR-210 are associated with the development of PE, including disturbed trophoblast invasion, and oxidative stress.

5. Conclusions

We investigated placental, exosomal and Ago-bound hsa-miR-210 expression profile in PE vs N groups. The level of hsa-miR-210 was significantly higher in PE placentas, which could be related to a minor increase of circulating exosomal and highly elevated Ago-bound hsa-miR-210. The hypoxia-induction had a different effect on the villous and extravillous trophoblast cell lines: intracellular hsa-miR-210 was upregulated in both cases, however, in extravillous cell media, only the level of exosomal hsa-miR-210 was increased and a no change on Ago-bound hsa-miR-210 level was observed. Therefore, in this case, hypoxia resulted in the specific exosomal sorting of hsa-miR-210 – as this cell type has a role in trophoblast invasion, this phenomenon may have a role in placental dysfunction. In villous cell media, the level of exosomal hsa-miR-210 was increased and enhanced release of Ago-bound hsa-miR-210 was also observed. The expression profile was quite similar to that of the placenta and plasma samples which can be explained by the fact that in placenta this cell type is in contact with maternal circulation.

Based on our results, we postulate that in PE, exosomal hsa-miR-210 is secreted actively from the trophoblast, which may have a role in intercellular communication and play a role in disease etiology. Moreover, there is also a passive release of Ago-bound hsa-miR-210, which could be the by-product of cell-death and so it is a possible consequence of the disease.

Declarations of interest

None.

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References

Anton, L., Olarerin-George, A.O., Schwartz, N., Srinivas, S., Bastek, J., Hogenesch, J.B., Elovitz, M.A., 2013. miR-210 inhibits trophoblast invasion and is a serum biomarker for preeclampsia. Am. J. Pathol. 183, 1437–45. doi:10.1016/j.ajpath.2013.07.021

Arroyo, J.D., Chevillet, J.R., Kroh, E.M., Ruf, I.K., Pritchard, C.C., Gibson, D.F., Mitchell, P.S., Bennett, C.F., Pogosova-Agadjanyan, E.L., Stirewalt, D.L., Tait, J.F., Tewari, M., 2011. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc. Natl. Acad. Sci. 108, 5003–5008. doi:10.1073/pnas.1019055108

Biró, O., Nagy, B., Rigó, J., 2017a. Identifying miRNA regulatory mechanisms in preeclampsia by systems biology approaches. Hypertens. Pregnancy 36, 90–99. https://doi.org/10.1080/10641955.2016.1239736

Biró, O., Alasztics, B., Molvarec, A., Joó, J., Nagy, B., Rigó, J., 2017b. Various levels of circulating exosomal total-miRNA and miR-210 hypoxamiR in different forms of pregnancy hypertension. Pregnancy Hypertens. 10, 207–212. https://doi.org/10.1016/j.preghy.2017.09.002

Chan, S.Y., Loscalzo, J., 2010. MicroRNA-210: a unique and pleiotropic hypoxamir. Cell Cycle 9, 1072–83. doi:10.4161/cc.9.6.11006

Chan, Y.C., Banerjee, J., Choi, S.Y., Sen, C.K., 2012. miR-210: The Master Hypoxamir. Microcirculation 19, 215–223. doi:10.1111/j.1549-8719.2011.00154.x

Duley, L., 2009. The global impact of pre-eclampsia and eclampsia. Semin. Perinatol. 33, 130–7. doi:10.1053/j.semperi.2009.02.010

Enderle, D., Spiel, A., Coticchia, C.M., Berghoff, E., Mueller, R., Schlumpberger, M., Sprenger-Haussels, M., Shaffer, J.M., Lader, E., Skog, J. and Noerholm, M., 2015. Characterization of RNA from Exosomes and Other Extracellular Vesicles Isolated by a

Novel Spin Column-Based Method. PLoS One 10, e0136133. doi: 10.1371/journal.pone.0136133

Fasanaro, P., D'Alessandra, Y., Di Stefano, V., Melchionna, R., Romani, S., Pompilio, G., Capogrossi, M.C., Martelli, F., 2008. MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. J. Biol. Chem. 283, 15878–83. doi:10.1074/jbc.M800731200

Fu, G., Brkić, J., Hayder, H., Peng, C., 2013. MicroRNAs in Human Placental Development and Pregnancy Complications. Int. J. Mol. Sci. 14, 5519–44. doi:10.3390/ijms14035519

Gebert, L.F.R., MacRae, I.J., 2018. Regulation of microRNA function in animals. Nat. Rev. Mol. Cell Biol. doi:10.1038/s41580-018-0045-7

Göhner, C., Plösch, T., Faas, M.M., 2017. Immune-modulatory effects of syncytiotrophoblast extracellular vesicles in pregnancy and preeclampsia. Placenta 60, S41–S51. doi:10.1016/j.placenta.2017.06.004

Hale, A., Lee, C., Annis, S., Min, P.-K., Pande, R., Creager, M.A., Julian, C.G., Moore, L.G., Mitsialis, S.A., Hwang, S.J., Kourembanas, S., Chan, S.Y., 2014. An Argonaute 2 switch regulates circulating miR-210 to coordinate hypoxic adaptation across cells. Biochim. Biophys. Acta - Mol. Cell Res. 1843, 2528–2542. doi:10.1016/J.BBAMCR.2014.06.012

Huang, X., Ding, L., Bennewith, K.L., Tong, R.T., Welford, S.M., Ang, K.K., Story, M., Le, Q.-T., Giaccia, A.J., 2009. Hypoxia-inducible mir-210 regulates normoxic gene expression involved in tumor initiation. Mol. Cell 35, 856–67. doi:10.1016/j.molcel.2009.09.006

Huang, X., Le, Q.-T., Giaccia, A.J., 2010. MiR-210-micromanager of the hypoxia pathway. Trends Mol. Med. 16, 230–7. doi:10.1016/j.molmed.2010.03.004

Ishibashi, O., Ohkuchi, A., Ali, M.M., Kurashina, R., Luo, S.S., Ishikawa, T., Takizawa, T., Hirashima, C., Takahashi, K., Migita, M., Ishikawa, G., Yoneyama, K., Asakura, H.,

Izumi, A., Matsubara, S., Takeshita, T., 2012. Hydroxysteroid (17-β) dehydrogenase 1 is dysregulated by miR-210 and miR-518c that are aberrantly expressed in preeclamptic placentas: A novel marker for predicting preeclampsia. Hypertension 59, 265–273. doi:10.1161/HYPERTENSIONAHA.111.180232

Jung, K.O., Youn, H., Lee, C.-H., Kang, K.W., Chung, J.-K., 2017. Visualization of exosome-mediated miR-210 transfer from hypoxic tumor cells. Oncotarget 8, 9899–9910. doi:10.18632/oncotarget.14247

Kowal, J., Tkach, M., Théry, C., 2014. Biogenesis and secretion of exosomes. Curr. Opin. Cell Biol. 29, 116–125. doi:10.1016/j.ceb.2014.05.004

Lee, D.-C., Romero, R., Kim, J.-S., Tarca, A.L., Montenegro, D., Pineles, B.L., Kim, E., Lee, J., Kim, S.Y., Draghici, S., Mittal, P., Kusanovic, J.P., Chaiworapongsa, T., Hassan, S.S., Kim, C.J., 2011. miR-210 Targets Iron-Sulfur Cluster Scaffold Homologue in Human Trophoblast Cell Lines. Am. J. Pathol. 179, 590–602. doi:10.1016/j.ajpath.2011.04.035

Li, Y., Kowdley, K. V., 2012. MicroRNAs in Common Human Diseases. Genomics. Proteomics Bioinformatics 10, 246–253. doi:10.1016/j.gpb.2012.07.005

Muralimanoharan, S., Maloyan, A., Mele, J., Guo, C., Myatt, L.G., Myatt, L., 2012. MIR-210 modulates mitochondrial respiration in placenta with preeclampsia. Placenta 33, 816–23. doi:10.1016/j.placenta.2012.07.002

Nadkarni, N.A., Rajakumar, A., Mokhashi, N., Burke, S.D., Rana, S., Salahuddin, S., Dang, Q., Thadhani, R., Krishnan, R., Stossel, T.P., Karumanchi, S.A., 2016. Gelsolin is an endogenous inhibitor of syncytiotrophoblast extracellular vesicle shedding in pregnancy. Pregnancy Hypertens. 6, 333–339. doi:10.1016/j.preghy.2016.07.003

Noguer-Dance, M., Abu-Amero, S., Al-Khtib, M., Lefèvre, A., Coullin, P., Moore, G.E., Cavaillé, J., 2010. The primate-specific microRNA gene cluster (C19MC) is imprinted in

the placenta. Hum. Mol. Genet. 19, 3566–3582. doi:10.1093/hmg/ddq272

Ouyang, Y., Mouillet, J.-F.J.-F., Coyne, C.B., Sadovsky, Y., 2014. Review: placenta-specific microRNAs in exosomes - good things come in nano-packages. Placenta 35 Suppl, S69-73. doi:10.1016/j.placenta.2013.11.002

Pasupathy, D., Dacey, A., Cook, E., Charnock-Jones, D.S., White, I.R., Smith, G.C., 2008. Study protocol. A prospective cohort study of unselected primiparous women: the pregnancy outcome prediction study. BMC Pregnancy Childbirth 8, 51. doi:10.1186/1471-2393-8-51

Pritchard, C.C., Kroh, E., Wood, B., Arroyo, J.D., Dougherty, K.J., Miyaji, M.M., Tait, J.F., Tewari, M., 2012. Blood Cell Origin of Circulating MicroRNAs: A Cautionary Note for Cancer Biomarker Studies. doi:10.1158/1940-6207.CAPR-11-0370

Redman, C.W., 2017. Early and late onset preeclampsia: Two sides of the same coin. Pregnancy Hypertens. An Int. J. Women's Cardiovasc. Heal. 7, 58. doi:10.1016/J.PREGHY.2016.10.011

Salomon, C., Kobayashi, M., Ashman, K., Sobrevia, L., Mitchell, M.D., Rice, G.E., 2013a. Hypoxia-Induced Changes in the Bioactivity of Cytotrophoblast-Derived Exosomes. PLoS One 8, e79636. doi:10.1371/journal.pone.0079636

Salomon, C., Ryan, J., Sobrevia, L., Kobayashi, M., Ashman, K., Mitchell, M., Rice, G.E., 2013b. Exosomal Signaling during Hypoxia Mediates Microvascular Endothelial Cell Migration and Vasculogenesis. PLoS One 8, e68451. doi:10.1371/journal.pone.0068451

Salomon, C., Torres, M.J., Kobayashi, M., Scholz-Romero, K., Sobrevia, L., Dobierzewska, A., Illanes, S.E., Mitchell, M.D., Rice, G.E., 2014a. A gestational profile of placental exosomes in maternal plasma and their effects on endothelial cell migration. PLoS One 9, e98667. doi:10.1371/journal.pone.0098667

Salomon, C., Yee, S., Scholz-Romero, K., Kobayashi, M., Vaswani, K., Kvaskoff, D., Illanes,

S.E., Mitchell, M.D., Rice, G.E., 2014b. Extravillous trophoblast cells-derived exosomes promote vascular smooth muscle cell migration. Front. Pharmacol. 5, 175. doi:10.3389/fphar.2014.00175

Schwarzenbach, H., Nishida, N., Calin, G.A., Pantel, K., 2014. Clinical relevance of circulating cell-free microRNAs in cancer. Nat. Rev. Clin. Oncol. 11, 145–156. doi:10.1038/nrclinonc.2014.5

Sheikh, A.M., Small, H.Y., Currie, G., Delles, C., Xiao, H.-S., Chen, C., Bugnicourt, J.-M., 2016. Systematic Review of Micro-RNA Expression in Pre-Eclampsia Identifies a Number of Common Pathways Associated with the Disease. PLoS One 11, e0160808. doi:10.1371/journal.pone.0160808

Tranquilli, A.L., Dekker, G., Magee, L., Roberts, J., Sibai, B.M., Steyn, W., Zeeman, G.G., Brown, M.A., 2014. The classification, diagnosis and management of the hypertensive disorders of pregnancy: A revised statement from the ISSHP. Pregnancy Hypertens. An Int. J. Women's Cardiovasc. Heal. 4, 97–104. doi:10.1016/j.preghy.2014.02.001

Truong, G., Guanzon, D., Kinhal, V., Elfeky, O., Lai, A., Longo, S., Nuzhat, Z., Palma, C., Scholz-Romero, K., Menon, R., Mol, B.W., Rice, G.E., Salomon, C., 2017. Oxygen tension regulates the miRNA profile and bioactivity of exosomes released from extravillous trophoblast cells – Liquid biopsies for monitoring complications of pregnancy. PLoS One 12, e0174514. doi:10.1371/journal.pone.0174514

Turchinovich, A., Burwinkel, B., 2012. Distinct AGO1 and AGO2 associated miRNA profiles in human cells and blood plasma. RNA Biol. 9, 1066–1075. doi:10.4161/rna.21083

Turchinovich, A., Weiz, L., Langheinz, A., Burwinkel, B., 2011. Characterization of extracellular circulating microRNA. Nucleic Acids Res. 39, 7223–33. doi:10.1093/nar/gkr254

Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J.J., Lötvall, J.O., 2007. Exosome-

mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat. Cell Biol. 9, 654–659. doi:10.1038/ncb1596

Xiao, X., Xiao, F., Zhao, M., Tong, M., Wise, M.R., Stone, P.R., Chamley, L.W., Chen, Q., 2017. Treating normal early gestation placentae with preeclamptic sera produces extracellular micro and nano vesicles that activate endothelial cells. J. Reprod. Immunol. 120, 34–41. doi:10.1016/J.JRI.2017.04.004

Zhang, Y., Fei, M., Xue, G., Zhou, Q., Jia, Y., Li, L., Xin, H., Sun, S., 2012. Elevated levels of hypoxia-inducible microRNA-210 in pre-eclampsia: new insights into molecular mechanisms for the disease. J. Cell. Mol. Med. 16, 249–59. doi:10.1111/j.1582-4934.2011.01291.x

Figure Legends

Figure 1. Placental (**A**), exosomal (**B**), and Ago-bound (**C**) hsa-miR-210 expression in N vs. PE group.

miRNA levels are shown as median with interquartile ranges, *: $p < 0.05$, **: $p < 0.01$.

Figure 2. Placental (**A**), exosomal (**B**), and Ago-bound (**C**) hsa-miR-517c expression in N vs. PE group.

miRNA levels are shown as median with interquartile ranges, *: $p < 0.05$.

Figure 3. Placental (**A**), exosomal (**B**), and Ago-bound (**C**) hsa-miR-16 expression in N vs. PE group.

miRNA levels are shown as median with interquartile ranges.

Figure 4. Intracellular (**A**), exosomal (**B**), Ago-bound (**C**) miRNA expression in JAR cells in hypoxia.

Untransformed miRNA levels are shown as mean with standard error, *: $p < 0.05$.

Figure 5. Intracellular (**A**), exosomal (**B**), Ago-bound (**C**) miRNA expression in HTR8 cells in hypoxia.

Untransformed miRNA levels are shown as mean with standard error, *: $p < 0.05$.

Table Legends

Table 1. Investigated and reference miRNAs.

Table 2. Patient characteristics of plasma samples.

Values are given as mean with standard deviation. N: Normal group, PE: Preeclamptic group.

Table 3. Patient characteristics of placenta samples.

Values are given as mean with standard deviation. N: Normal group, PE: Preeclamptic group.

Table 1

miRNA name	mirBase identifier	seed sequence
hsa-miR-210-3p	MIMAT0000267	5’-CUGUGCGUGUGACAGCGGCUGA-3’
hsa-miR-16-5p	MIMAT0000069	5'-UAGCAGCACGUAAAUAUUGGCG-3’
hsa-miR-517c-3p	MIMAT0002866	5'-AUCGUGCAUCCUUUUAGAGUGU-’3
hsa-miR-103a-3p	MIMAT0000101	5'-AGCAGCAUUGUACAGGGCUAUGA-’3

Table 2

	N	**PE**	**p-value**
n	8	8	
gestational age at sampling (weeks)	36,13 ± 3,00	31,00 ± 5,07	<0,05
gestational age at birth (weeks)	37,38 ± 1,85	31,13 ± 5,17	<0,05
birth weight (grams)	3313,75 ± 569,69	1798,29 ± 1254,50	<0,05
maternal age (years)	31,25 ± 5,80	33,43 ± 6,48	>0,05

Table 3

	N	**PE**	**p-value**
n	7	13	
gestational age at birth (weeks)	38,14 ± 0,69	30,39 ± 4,59	<0,05
birth weight (grams)	3446,67 ± 525,23	1529,00 ± 990,75	<0,05
maternal age (years)	30,29 ± 6,16	33,67 ± 5,43	>0,05

Figure 1

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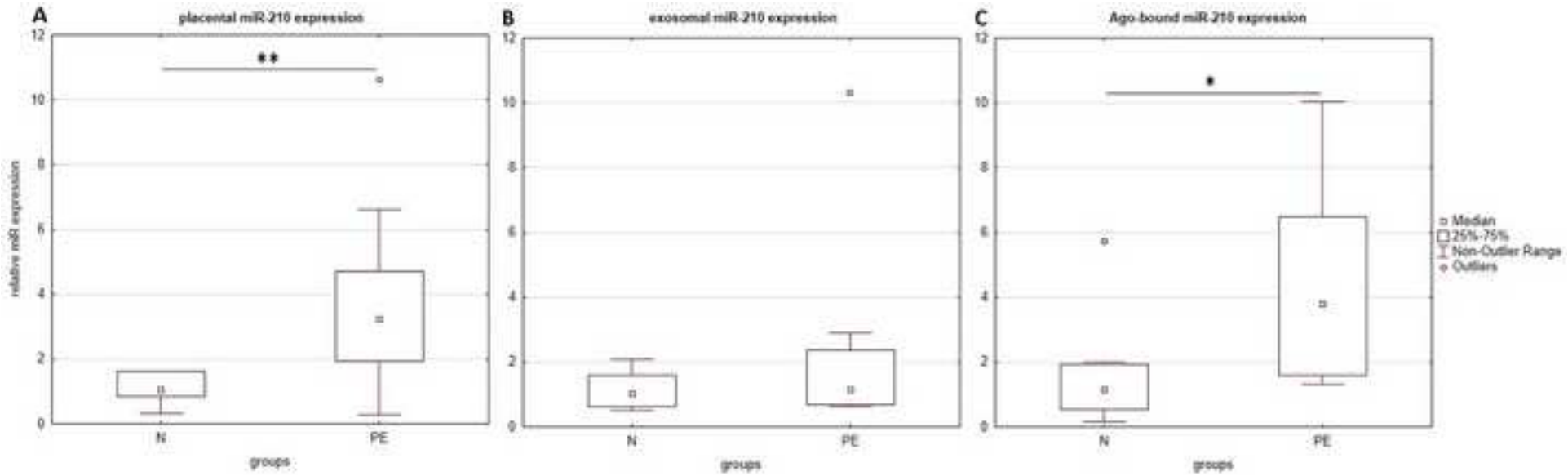

Figure 2

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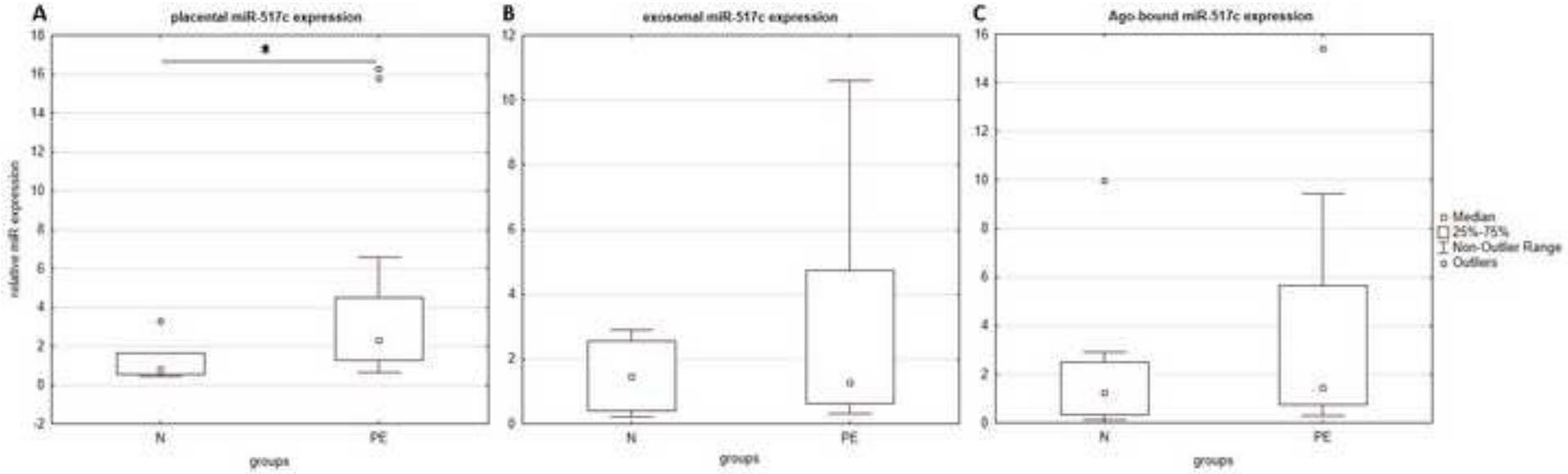

Figure 3

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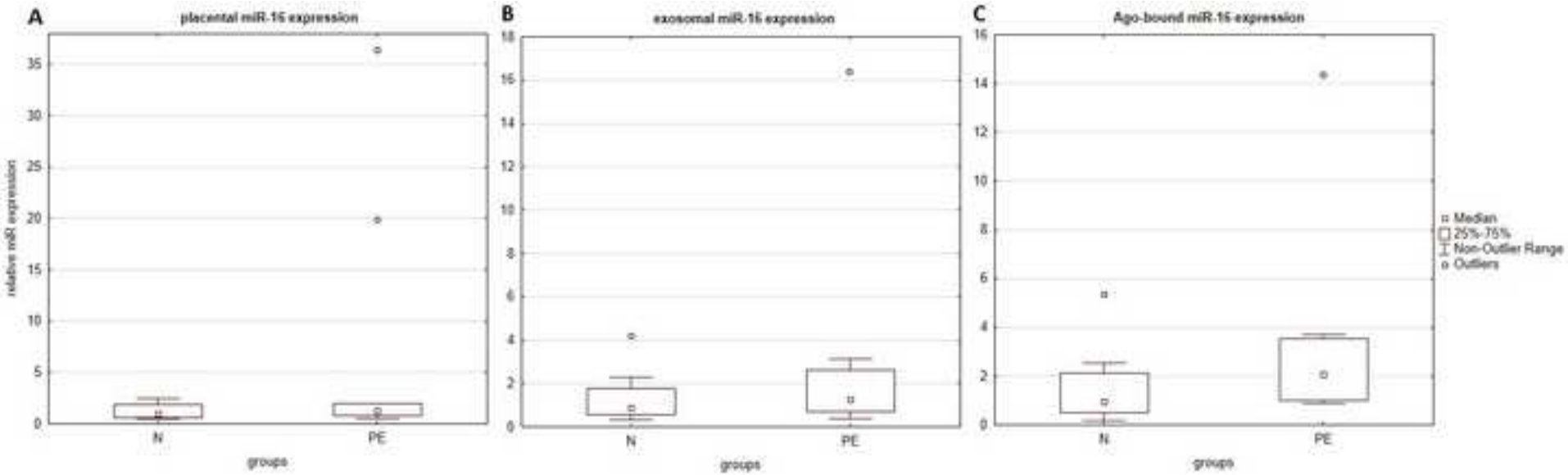

Figure 4

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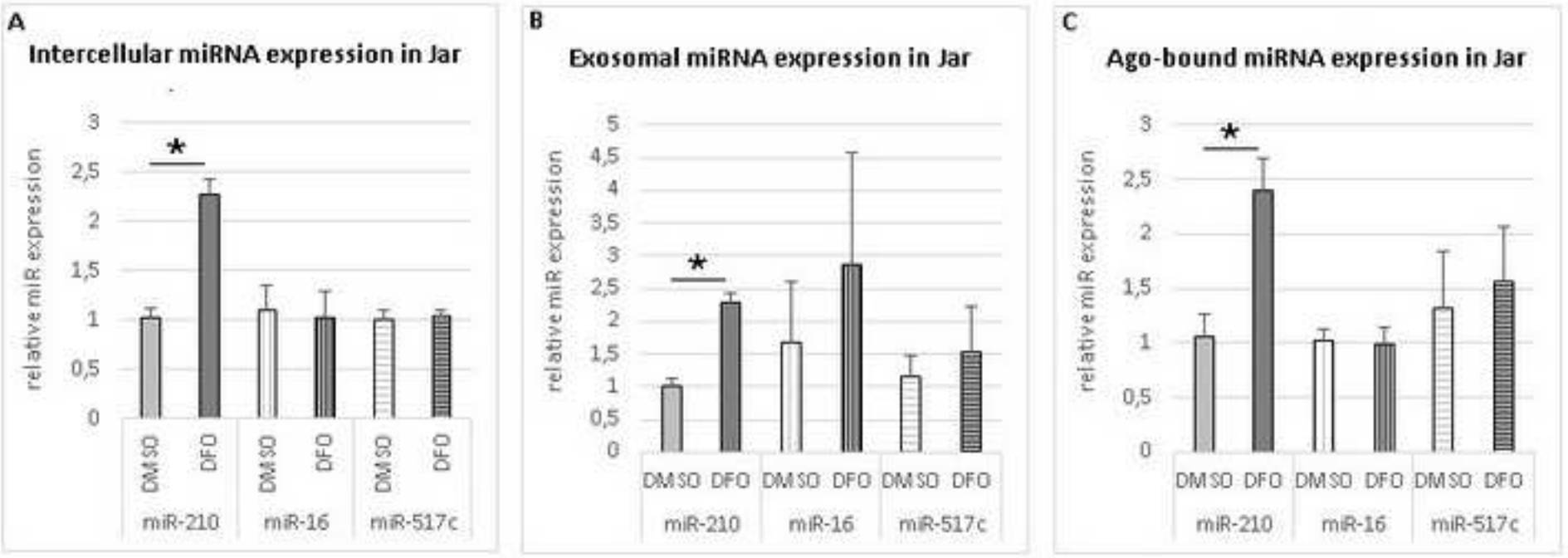

Figure 5

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