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Study of extracellular placental specific microRNAs in maternal circulation and their utilization in clinical diagnostics of pregnancy-related complications

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ABSTRACT

Objectives: We investigated the possible utilization of extracellular microRNAs as novel non-invasive biomarkers for diagnostics of pregnancy-related complications such as gestational hypertension (GH), preeclampsia (PE) and fetal growth restriction (FGR). First, we identified appropriate pregnancy-associated (placenta specific) microRNAs in maternal circulation in pregnancies with normal course of gestation. Then, we quantified selected extracellular C19MC microRNAs in maternal circulation overtime in normally progressing pregnancies. Subsequently, we compared C19MC microRNA expression profiles in maternal circulation between pregnancies with clinically established pregnancy-related complications (PE, FGR, GH) and gestational-age-matched controls. Finally, monitoring of selected placenta specific C19MC microRNAs in maternal circulation within the first trimester of gestation was performed with the aim to identify extracellular C19MC microRNAs able to differentiate between normal pregnancies and those at risk of subsequent development of pregnancy-related complications.

Methods: The levels and expression profiles of extracellular placental specific microRNAs in circulation of non-pregnant individuals and pregnant women were performed using real-time RT-PCR. The selection of appropriate pregnancy associated microRNAs with the diagnostical potential was based on following criteria: (1) detection rate of 100 % in term placentas, (2) detection rate of $\geq 67\%$ in maternal plasma throughout gestation and (3) detection rate of 0 % in whole peripheral blood and plasma samples of non-pregnant individuals.

Results: We finally identified 7 extracellular C19MC microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) that were simultaneously negative in peripheral blood samples derived from healthy non-pregnant women and were strongly positive in maternal circulation throughout the whole period of gestation. Increased plasmatic levels and gene expression of 5/7 C19MC microRNAs (miR-516-5p, miR-517*, miR-520a*, miR-525, and miR-526a) were observed in women with established preeclampsia, whereas neither plasmatic levels nor gene expression of these C19MC microRNAs differed between control cohort and patients with FGR and GH. First trimester screening approach based on the combination of two placental specific C19MC microRNAs (miR-520h and miR-518b) was able to identify women at risk of subsequent development of GH with a PPV of 82.6% at a specificity of 92.9%. The miR-517-5p biomarker alone had a predictive performance for preeclampsia with a sensitivity of 42.9%, a specificity of 86.2%, a PPV of 52.9%, and a NPV of 80.6%.

Conclusion: Extracellular C19MC microRNA biomarkers were identified that could confirm the diagnosis of preeclampsia or predict later occurrence of GH and preeclampsia. Early diagnosis may afford benefits such as to start early treatment or even to start early prevention of later development of pregnancy-related disorders.

INTRODUCTION

MicroRNAs and their function in gene expression regulation

Previous decades unveiled a number of important cellular roles for diverse subset of non-coding RNA (ncRNA) molecules, among them short RNAs called microRNAs, that are not translated into a protein product, but function as critical structural molecules or regulators of various cellular processes. MicroRNAs as molecules with regulatory function were described, for the first time, in nematode worm *Caenorhabditis elegans* (*C. elegans*). Works by Chalfie et al. (1981) and Ambros (1989) revealed a gene *lin-4* that is important for normal larval development of *C. elegans*, since it is responsible for the progressive repression of the *lin-14* protein levels in first larval stage. Mutant *C. elegans* worms deficient in *lin-4* function had persistently high levels of *lin-14* and displayed developmental timing defects, however, the mechanism for control of *lin-14* expression remained unknown.

Later, in 1993, Lee and colleagues found that *lin-4* does not encode a regulatory protein, but it gives rise to transcripts, which are complementary to sequences in the 3' untranslated region (3'UTR) of the messenger RNA encoding the *lin-14* protein and can regulate *lin-14* mRNA translation via antisense RNA:mRNA interaction. Since research in the field of short regulatory RNAs rapidly progressed, nowadays more than 2588 mature microRNAs were identified.

Human microRNAs are located in all chromosomes except Y chromosome and are non-randomly distributed in the human genome. The genomic distribution of microRNA genes is characterized by the presence of families of several identical or closely related mature microRNAs, encoded within the same genomic cluster. Up to 60% of known human microRNA genes are found in clusters carrying from two to as many as 46 microRNAs. MicroRNAs encoded by genomic cluster frequently contain high sequence homology, particularly within the seed sequence, shared common regulatory regions and are transcribed as polycistronic primary transcripts (Lagos-Quintana et al., 2001; Lau et al. 2001).

Depending on their genomic origin microRNAs can be categorized into four subtypes such as intergenic, intronic, exonic, and others (Ying et al., 2010; Chien et al., 2011). The last three categories can be commonly termed as intragenic microRNAs.

Intergenic microRNAs are transcribed from intergenic regions or gene deserts (Lagos-Quintana et al. 2001; Lau et al. 2001; Saini et al., 2008; Corcoran et al., 2009) between two consecutive protein coding genes and they have their own promoters like the coding genes and the same transcription factors (Lee et al., 2004; Aguda et al., 2008; Pichiorri et al., 2010; Wang et al., 2010).

Intragenic microRNAs can be located within introns or exons (Erdmann et al. 2000; Rodriguez et al., 2004; Saini et al., 2008; Corcoran et al., 2009). Intragenic microRNA precursors located within introns, exons or untranslated regions (UTRs) of protein-coding transcripts are preferentially in the same orientation as the host gene, therefore these microRNAs share common promoters with their host genes and are expressed simultaneously (Beskerville et al., 2005; Rodriguez et al., 2004; Wang et al., 2009; Aravin et al., 2003; Lagos-Quintana et al. 2003; Lai et al. 2003; Lim et al. 2003).

Human microRNA biogenesis is a two-step process, taking place in both, nuclear and cytoplasmic compartments, performed by two RNAIII endonucleases, Drosha and Dicer (Denli et al., 2004; Du and Zamore, 2005; Gregory et al., 2004; Han et al., 2004; Hutvagner et al., 2004; Lee et al., 2002; Lee et al., 2003). The microRNA gene is transcribed to produce primary microRNA (pri-microRNA) transcript containing stem-loop

structured precursor microRNA (pre-microRNA) that is subsequently processed to form microRNA duplex (microRNA:microRNA* duplex; passenger strand is designated with asterisk) which finally delivers mature microRNA (Bartel and Chen, 2004). The single strand mature microRNA is incorporated into the final effector protein complex RISC (RNA induced silencing complex) (Bartel, 2004).

This complex binds microRNA specifically, through Watson-Crick base-pairing, to 3'UTR (untranslated region) of the target mRNA and negatively regulate its expression (Wightman et al., 1993; Lee and Ambros, 1993). A single microRNA guide can regulate several mRNA targets and conversely multiple microRNAs can cooperatively regulate a single mRNA target (Bartel, 2004).

Extracellular microRNAs

For the first time, extracellular microRNAs were described in serum and plasma by Lawrie et al., who detected microRNAs in serum of patients with lymphomas (Lawrie et al., 2008) and Mitchell et al. (2008), who demonstrated the presence of stable microRNAs in the human plasma of healthy subjects and prostate cancer patients. MicroRNAs were subsequently detected in other body fluids and biological samples including saliva (Park et al., 2009), sperm (Li et al., 2012), urine (Hanke et al., 2010) and milk (Pigati et al., 2010).

Further research confirmed the high stability of microRNAs in bodily fluids (Turchinovich et al., 2011; Park et al., 2009; Chen et al., 2008). These observations indicate that microRNAs are protected in the extracellular environment against the effects of RNases and degradation.

Extracellular microRNAs were detected in exosomes (Valadi et al., 2007), microvesicles and apoptotic bodies in peripheral blood samples (Hunter et al., 2008). It was showed later that most extracellular microRNAs are located outside the membrane vesicles in the complex with the Ago2 or NPM1 proteins (Arroyo et al., 2011). A small fraction of extracellular microRNAs was also detected in human plasma in complex with HDL particles (Vickers et al., 2011).

Extracellular microRNAs, which are bound only to protein complexes, are apparently released to extracellular environment passively during cell death (Turchinovich et al., 2012), however active export of microRNA-protein complexes was also described (Wang et al., 2010). MicroRNAs are further secreted into the extracellular environment through microvesicles and apoptotic bodies. It was found that the microRNA spectrum in vesicles not always have correlate with the microRNA profile in the original cells because selective secretion of specific microRNAs is applied (Wang et al., 2010).

microRNAs in normal pregnancy

During pregnancy, development of the highly specialized vital fetal organ – placenta, in maternal body is crucial to support normal growth and development of the fetus (Gude et al., 2004).

Numerous studies have shown that a large number of microRNAs is expressed in trophoblast cells and human placental tissue.

Placenta expresses a large number of ubiquitous as well as specific microRNAs, such as the members of chromosome 19 microRNA cluster (C19MC), chromosome 14 microRNA cluster (C14MC) and miR-371-3 cluster that are almost exclusively or preferentially expressed in the placenta (Morales-Prieto et al., 2012; Donker et al., 2012).

MicroRNAs regulate trophoblast cell proliferation and apoptosis during placental development. While several microRNAs were reported to enhance trophoblast cell proliferation and/or survival (Luo et al., 2012; Pineles et al., 2007; Morales-Prieto et al.,

2011; Segura et al., 2009), other microRNAs act in the opposite way (Li et al., 2013; Dai et al., 2012; Gao et al., 2012; Keniry et al., 2012). Migration and invasion of extravillous trophoblasts (EVTs) to the decidua and myometrium, the critical events during placentation (Chelbi et al., 2008), are influenced by microRNAs, that can exert positive effect (miR-195, miR-376c, and miR-378a-5p)(Fu et al., 2013; Luo et al., 2012) as well as negative effect (miR-210, miR-34a, and miR-29b)(Zhang et al., 2012; Umemura et al., 2013; Pang et al., 2010; Li et al., 2013) on trophoblast cell migration and invasion by modulating the activity of signalling pathways, enzymes, and adhesion molecules.

In 2009, Luo et al. demonstrated that microRNAs produced by human trophoblast cells can be secreted into maternal plasma or serum through an exosome-mediated pathway (Luo et al., 2009). Further studies confirmed that trophoblast-specific and nonspecific microRNAs are released to the extracellular fluid and are detectable in maternal circulation during pregnancy. For example, Chim et al. (2008) confirmed that 4 miRNAs from tested set with the highest expression in placenta (miR-141, miR-149, miR-299-5p, and miR-135b) were also detected in maternal plasma, with the highest expression rates in late pregnancy (near before the delivery), and with significantly reduced detection rates in postdelivery plasma (Chim et al., 2008). Gilad et al. (2008) reported that serum levels of three placental microRNAs (miR-526a, miR-527, and miR-520d-5p) allowed accurate differentiation between pregnant and non pregnant women (Gilad et al., 2008). Miura et al. (2010) revealed significant increase in plasma concentration of C19MC microRNAs (miR-515-3p, miR-517a, miR-517c, miR-518b, miR-526b) with advancing pregnancy.

Pregnancy-related complications

Hypertensive disorders of pregnancy (HDP) are among the main medical problems encountered during pregnancy (Jim et al., 2010), occurred in about 20.7 million of women in 2013 (GBD 2015 Disease and Injury Incidence and Prevalence Collaborators, 2015). It is estimated that about 10% of pregnancies are complicated by hypertensive disorders worldwide with rates increasing in developing world (WHO Guidelines Approved by the Guidelines Review Committee, 2011; Lo et al., 2013).

Gestational hypertension

- is characterized as new-onset elevation of blood pressure after 20 weeks of gestation, without the presence of proteinuria or other signs of preeclampsia, however approximately 50% of women diagnosed with gestational hypertension between 24 and 35 weeks' gestation ultimately develop preeclampsia (Barton et al., 2001). The failure of blood pressure to normalize postpartum requires changing the diagnosis to chronic hypertension.

Preeclampsia (PE)

- complicates 3-8% of pregnancies worldwide (Kanasaki et al., 2009; Uzan et al., 2011; Sibai, 2005), however geographic, social, economic, and racial differences are thought to be responsible for incidence rates up to 3 times higher in some populations (Sibai 2005). Preeclampsia is one of the leading causes of maternal, as well as perinatal morbidity and mortality, especially in developing countries (Chelbi and Vaiman, 2008; Berg et al., 2009; MacKay et al., 2001), probably accounts for 50,000 - 76,000 maternal and 500,000 infant deaths worldwide every year (Duley, 2005; Ghulmiyyah and Sibai, 2012; Whitworth, 2003).

The sequence of events that leads to the development of PE may be explained by two stages: first (placental) stage, which occurs early in pregnancy, is characterized by defective trophoblastic invasion causing inadequate remodelling of maternal spiral

arteries resulting in a deficient maternal blood supply to the placenta, that causes placental ischemia and hypoxia with local oxidative stress reaction. Following second (peripheral) stage results in a systemic inflammatory response and endothelial dysfunction (Sargent et al., 2006), and leads to the onset of the clinical symptoms of PE (Ness and Roberts, 1996; Sargent et al., 2006).

Fetal growth restriction

Fetal growth restriction (FGR) is a common pregnancy-related complication characterized as failure of a fetus to reach its growth potential, which occurs in 3–10% of all pregnancies, representing one of the leading causes of perinatal morbidity and mortality (Bernstein et al., 2000).

Small for gestational age (SGA) and FGR refers to the fetus who does not achieve the expected in utero growth potential due to multiple factors, involving genetic or environmental factors. SGA and FGR are defined as an estimated fetal weight below 10th percentile.

Dysregulation of microRNA expression in pregnancy-related complications

Recognizing the importance of microRNA expression for the development of pregnancy-related complications is a relatively recent phenomenon, dating to 2007, when the first study on altered placental microRNA expression in PE pregnancies was published (Pineles et al., 2007). Subsequent studies have reported aberrant expression of both, abundant and placenta-specific microRNAs in placental tissues in various pregnancy-related complications such as PE and FGR/IUGR.

Women with preeclampsia exhibit, for example increased placenta expression levels of **miR-210** typically induced by HIF-1 α (Zhang et al., 2012; Pineles et al., 2007; Muralimanoharan et al., 2012; Ishibashi et al., 2012; Enquobahrie et al., 2011; Xu et al., 2014; Zhu et al., 2009). Elevated levels of placental miR-210 may contribute to the pathogenesis of preeclampsia (Anton et al., 2013; Muralimanoharan et al., 2012; Colleoni et al., 2013; Myatt et al., 2014; Kopriva et al., 2013).

A link between up-regulation of **miR-155** in preeclamptic placentas (Pineles et al., 2007; Zhang et al., 2010), and the innate immune response has been suggested.

The expression of **miR-141** is elevated in FGR placental tissues, suggesting that this microRNA may play important roles in pathogenesis of the disease by suppressing several target genes such as E2F transcription factor 3 (*E2F3*) and pleomorphic adenoma gene 1 (*PLAG1*).

In FGR placentas, hypoxia significantly increases levels of **miR-424**, which results in reduction of both mRNA and protein levels of mitogen-activated protein kinase 1 (MEK1) and fibroblast growth factor receptor 1 (FGFR1). Since FGFR1 mediates functions of VEGF, it is possible that increased levels of miR-424 contribute to FGR by affecting normal vascularity in placenta (Huang et al., 2013).

AIMS OF THE THESIS

The primary objective of this work was to investigate the possible utilization of recently discovered small non-coding RNA molecules, called microRNAs, as novel non-invasive biomarkers for diagnostics of severe pregnancy-related complications associated with placental insufficiency (preeclampsia and/or fetal growth restriction). Since early and correct diagnosis may afford benefits such as to start early treatment or even prevention of placental-insufficiency related disorders, the discovery of new biomarkers with high sensitivity and specificity remains the desired golden grail in the field of non-invasive prenatal diagnosis (NIPD). To try to achieve this goal, we have performed the following studies:

- Identification of appropriate pregnancy-associated (placenta specific) microRNAs in maternal circulation in pregnancies with normal course of gestation
- Quantification of selected extracellular C₁₉MC microRNAs in maternal circulation overtime in normally progressing pregnancies
- Comparison of C₁₉MC microRNA expression profiles in maternal circulation between pregnancies with clinically established pregnancy-related complications (PE, FGR, GH) and gestational-age-matched controls
- Monitoring of selected placenta specific C₁₉MC microRNAs in maternal circulation within the first trimester of gestation and subsequent identification of extracellular C₁₉MC microRNAs able to differentiate between normal pregnancies and those at risk of subsequent development of pregnancy-related complications

MATERIALS AND METHODS

Materials and methods are entirely described in full-text version of Ph.D. thesis and in related manuscripts.

We can summarize them as follows:

- Collection of peripheral blood and placenta samples
- total RNA was extracted from 1 ml of maternal plasma and placenta samples and highly enriched for small RNAs
- detection of pregnancy-associated microRNAs using two-step quantitative real-time RT-PCR
- both, absolute and relative quantification approaches were used for data evaluation
- receivers operating characteristic (ROC) curves were constructed
- function and functional relationship analysis of predicted targets of extracellular C₁₉MC microRNAs was performed

RESULTS

Identification of appropriate pregnancy-associated (placenta specific) microRNAs in maternal circulation in pregnancies with normal course of gestation

The selection of appropriate pregnancy associated microRNAs with the diagnostical potential was based on following criteria:

- (1) detection rate of 100 % in full-term placentas,
- (2) detection rate of ≥ 67 % in maternal plasma throughout gestation (at least 4 positive wells out of 6 tested wells), and
- (3) detection rate of 0 % in whole peripheral blood and plasma samples of non-pregnant individuals.

From 20 selected microRNAs, all were reliably detectable in the fetal part of the placenta; however, microRNAs with late amplification in the placenta (miR-136 and miR-519a) were undetectable in maternal plasma. On the base of our selection criteria, microRNAs that were detected in blood cells of healthy non-pregnant women (miR-34, miR-224, miR-512-5p, miR-515-5p, miR-518f*, miR-519d, and miR-519e) as well as microRNAs with negative and/or inconsistent results in maternal circulation throughout gestation (miR-34c, miR-136, miR-372, miR-518f*, miR-519e, miR-524-5p, miR-519a, and miR-526b) were excluded from further analysis.

We finally identified seven extracellular C19MC microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) that were simultaneously negative in the whole peripheral blood and plasma samples derived from healthy non-pregnant women (10 negative wells out of 10 tested wells) and were strongly positive in maternal circulation throughout the whole period of gestation.

These seven extracellular C19MC microRNAs (**miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a**) were identified to be pregnancy associated microRNAs with diagnostic potential.

Quantification of selected extracellular C19MC microRNAs in maternal circulation overtime in normally progressing pregnancies

Using both absolute and relative quantification approaches, we explored extracellular C19MC microRNA levels in maternal plasma samples harvested during the first, second and third trimesters from normally progressing pregnancies. Increasing levels of all examined C19MC microRNAs were observed throughout gestation in normal pregnancies (accompanying progression of gestation from the first to the third trimester), which may be linked to the growing mass of the placenta. In accordance with that highest concentrations and expression of circulating placental specific C19MC microRNAs were observed during the third trimester of gestation.

Unfortunately, absolute and relative quantification approaches of C19MC microRNAs in maternal plasma samples derived from normal pregnancies and those with onset of pregnancy-related complications showed no statistically significant difference between the cohorts.

Subsequently, plasma samples derived at various gestational stages from pregnancies that later developed placental insufficiency related complications (1 IUGR, 5

PE, 1 PE and IUGR) revealed significant elevation of extracellular microRNA levels and expression in maternal circulation during early gestation in all cases (between 12th and 16th weeks of gestation). During late gestation (from the 20th week until term) the levels of extracellular microRNAs decreased significantly until they finally reached the corresponding levels of gestational-age-matched normal pregnancies.

The data obtained in this pilot study suggests the potential of extracellular C19MC microRNAs to differentiate, at the beginning of gestation, between patients at risk of later development of placental insufficiency related complications and normal pregnancies.

Comparison of C19MC microRNA expression profiles in maternal circulation between pregnancies with clinically established pregnancy-related complications (PE, FGR, GH) and gestational-age-matched controls

The follow-up study on C19MC microRNA plasmatic levels and gene expression profiles performed on large cohort of patients that can be subdivided into various most specific subgroups showed significant results. Increased plasmatic levels and gene expression of 5 out of 7 C19MC microRNAs (miR-516-5p, miR-517*, miR-520a*, miR-525, and miR-526a) were observed in women with pregnancy-related complications compared to normal pregnancies.

Detailed analysis revealed increased levels and gene expression of miR-516-5p, miR-517*, miR-520a*, miR-525, and miR-526a in the group of patients with established preeclampsia, whereas neither plasmatic levels nor gene expression of these C19MC microRNAs differed between control cohort and patients with FGR and GH. Plasmatic concentrations and/or expression profiles of C19MC microRNAs did not show any association with the severity of the disease with respect to clinical signs and requirements for the delivery. Similarly, association between C19MC microRNA plasmatic levels and/or gene expression and the occurrence of previous hypertension in the cohort of patients with preeclampsia was not determined. While our data may be partially supported by Mouillet et al. (2010), who have recently observed no significant difference in relative placental specific microRNA levels (miR-518b) in FGR plasma samples, they are inconsistent with the study of Yang et al. (2011), who observed up-regulation of extracellular miR-520h in four patients with preeclampsia.

There was no difference in microRNA plasmatic levels and/or gene expression between pregnancies with abnormal and normal blood flow velocity waveforms with the exception of miR-526a, which was up-regulated in the group of patients with abnormal blood flow velocity waveforms in the umbilical artery. Consecutive correlation analysis revealed that the pulsatility index (PI) in the umbilical artery did not correlate with microRNA plasmatic concentrations and/or gene expression. However, a weak negative correlation between the PI in the middle cerebral artery and C19MC microRNA plasmatic concentrations or gene expression (miR-516-5p, miR-517*, miR-520a*, miR-525, and miR-526) was observed. Furthermore, a weak negative correlation between cerebroplacental ratio and C19MC microRNA plasmatic concentrations and gene expression (miR-520a*, and miR-526a) was found.

Overall, results of the study indicated that the up-regulation of miR-516-5p, miR-517*, miR-520a*, miR-525 and miR-526a is a characteristic phenomenon of established preeclampsia.

The function and functional relationship analysis of predicted targets of the five elevated extracellular C19MC microRNAs in patients with established preeclampsia

indicated that a large group of genes was connected to the regulation of the immune system and inflammatory response.

Monitoring of selected placenta specific C19MC microRNAs in maternal circulation within the first trimester of gestation and subsequent identification of extracellular C19MC microRNAs able to differentiate between normal pregnancies and those at risk of subsequent development of pregnancy-related complications

Study performed on the samples collected during the first trimester screening, revealed increased plasmatic levels of miR-516-5p, miR-517*, miR-520h, and miR-518b in those women who subsequently developed gestational hypertension when compared to normal pregnancies. Increased levels (miR-517-5p, miR-518b, and miR-520h) or a trend towards increased plasma levels (miR-520a-5p, and miR-525-5p) of C19MC microRNAs were observed during the first trimester of gestation also in maternal plasma samples derived from women who developed preeclampsia compared to women with normal pregnancies. No difference in plasma levels of C19MC microRNAs between the control group and the group of patients destined to develop IUGR was found.

The predictive accuracy of single first trimester plasmatic microRNA biomarkers was assessed. In patients with gestational hypertension the best positive predictive value (84.6%) and specificity (92.9%) was observed for miR-520h. Although, miR-516-5p had significantly higher AUC than miR-518b, finally miR-518b showed better PPV (73.3%) and specificity (85.7) than miR-516-5p. MiR-516-5p predicted the subsequent occurrence of gestational hypertension with a sensitivity of 80.0%, a specificity of 82.1% and a PPV of 70.6%. In patients, who develop preeclampsia later in pregnancy, miR-517-5p biomarker alone had a predictive performance for preeclampsia with a sensitivity of 42.9%, a specificity of 86.2%, a PPV of 52.9%, and a NPV of 80.6%. There was no additive effect of using the combination of all examined circulating C19MC microRNAs to predict preeclampsia (sensitivity 20.6%, specificity 90.8%, a PPV of 44.8%, and a NPV of 76.0 %). Unfortunately, no extracellular C19MC microRNA predictive biomarkers for later occurrence of IUGR have been identified.

Individual maternal plasma/serum markers have not usually performed well as screening tests for preeclampsia and fetal growth restriction, because the predictive value of each biomarker is low; therefore, combined screening tests to assess the risk of preeclampsia and fetal growth restriction are currently used in practice (Poon et al., 2014; Scazzocchi et al. 2016; Crovetto et al., 2016). In a proposed new approach to prenatal care, screening using a combination of maternal risk factors, mean arterial pressure, uterine artery Doppler, and maternal serum biomarkers (pregnancy-associated plasma protein-A and placental growth factor) can identify up to 95% of cases with early onset of preeclampsia for a false-positive rate of 10% (Poon et al., 2014; Scazzocchi et al., 2016). Another model including maternal characteristics, mean arterial pressure, uterine artery Doppler, placental growth factor, and soluble Fms-like tyrosine kinase-1 achieved an overall detection rate of 71.4% for fetal growth restriction, with a 10% false positive rate (Crovetto et al., 2016).

Our first trimester screening approach for gestational hypertension based on the combination of two placental specific C19MC microRNAs (miR-520h and miR-518b) was able to identify women at risk of subsequent development of GH with a PPV of 82.6% at a specificity of 92.9%. Other C19MC microRNA combinations (miR-516-5p and miR-520h or

miR-516-5p and miR-518b) showed lower PPV than the miR-520h biomarker alone or in combination with miR-518b. However, there was no additive effect of using the combination of all examined circulating C19MC microRNAs to predict preeclampsia (sensitivity 20.6%, specificity 90.8%, a PPV of 44.8%, and a NPV of 76.0%).

Function and functional relationship analysis of predicted targets of up-regulated extracellular C19MC microRNAs indicated an extensive group of pregnancy-related genes (miR-516-5p: 53 out of 349; miR-517*: 21 out of 179; miR-518b: 4 out of 42; miR-520h: 65 out of 509).

Several target genes were previously described as aberrantly expressed in various biological samples derived from patients with clinical symptoms of pregnancy-related complications such as gestational hypertension, preeclampsia (with or without intrauterine growth restriction), HELLP syndrome, fetal growth restriction and/or small for gestational age, gestational diabetes mellitus, spontaneous abortions, miscarriages, recurrent pregnancy loss and ectopic pregnancy. Some of predicted targets such as PAPPA, SP1 (PSG₂, PSG₃, PSG₅, PSG₆, PSG₉, PSG₁₁), LHCGR, FLT1, ANGPT1 have been shown to be potential non-invasive early biomarkers for pregnancy-related complications such as gestational hypertension, preeclampsia, small for gestational age, miscarriage, preterm delivery, stillbirth and aneuploid foetuses.

GENERAL DISCUSSION AND CONCLUSIONS

The goal of our study was to shed, at least partially, some light on complex processes leading to the onset of pregnancy-related complications involving life-threatening conditions for both, the mother and the fetus, with special focus on extracellular placental specific microRNAs, mainly C19MC microRNAs. Special attention was paid to the investigation of appropriate novel biomarkers for non-invasive prenatal diagnosis that could contribute to early and accurate identification of patients at high risk of later development of gestational hypertension, preeclampsia and/or fetal growth restriction.

After discovery of short non-coding RNAs, and mainly microRNAs that significantly affect gene expression, several questions have raised in the field of gynecology and obstetrics. For example, whether placenta specific microRNAs are also released into maternal circulation during normal gestation and if so, could be exploited for non-invasive prenatal diagnosis, at least of chromosomal aneuploidies and pregnancy-related complications. This question was soon partially answered by Chim et al. (2008), who demonstrated that four microRNAs highly expressed in placenta (miR-141, miR-149, miR-299-5p and miR-135b) were abundant in plasma of pregnant women and rapidly cleared from maternal circulation after delivery. Shortly thereafter, Luo et al. observed that placental specific microRNAs are likely constituents of chorionic villous trophoblasts and are released extracellularly into maternal circulation during pregnancy via exosomes (Luo et al., 2009).

In our study we examined, first of all, whether 20 selected microRNAs (miR-518b, miR-34c, miR-372, miR-135b, miR-512-5p, miR-515-5p, miR-516-5p, miR-517*, miR-518f*, miR-519a, miR-519d, miR-519e, miR-520a*, miR-520h, miR-524-5p, miR-525, miR-526a and miR-526b), including C19MC microRNAs, are really highly expressed in placental tissues and should be therefore called "placental specific". As expected all 20 selected microRNAs were detectable in the fetal side of placenta. This was also confirmed later when the expression of C19MC microRNAs has been observed in placental tissue (Donker

et al., 2012; Wang et al., 2012), trophoblast cell lines (Morales-Prieto et al., 2012) and placenta-derived stromal cells (Flor et al., 2012).

Based on our pre-defined criteria for selection we excluded from the consecutive study those microRNAs that were either detected in whole peripheral blood of non-pregnant healthy individuals or were undetectable/inadequately detectable in maternal plasma samples during pregnancy. For example, like Chim et al. (2008), we demonstrated the absence of miR-135b in non-pregnant women and its presence in maternal circulation, but only during late gestation, and therefore we decided to exclude miR-135b from other testing.

Finally, 7 placenta specific C19MC microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525 and miR-526a) that were reliably detected in maternal circulation throughout the whole period of gestation were identified. These C19MC microRNAs also fulfilled our predefined criteria on potential NIPD biomarkers.

Previously published studies showed increasing levels of circulating nucleic acids (DNA and mRNA) with advancing gestation, which reflects the growth of the placenta (Lo et al., 1998; Ng et al., 2003; Sedlackova et al., 2011a, b). Our data obtained from both absolute and relative quantification approaches revealed progressively increasing levels of selected placenta specific C19MC microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) in maternal circulation during normally ongoing pregnancies, which may be linked to the rising mass of the placenta. The highest concentrations and expression levels of circulating C19MC microRNAs were observed during the third trimester of gestation.

Contrarily to our expectancies, as well as to the fact that the onset of preeclampsia and/or IUGR was accompanied by excessive placental trophoblast apoptosis associated with increased extracellular DNA levels in maternal circulation (Farina et al., 2004; Zhong et al., 2007; Tsui et al., 2007; Hromadnikova et al., 2010; Sekizawa et al., 2003), levels of selected placenta specific C19MC microRNAs in maternal plasma showed in our pilot study no statistical difference between normal pregnancies and those with clinically established pregnancy-related complications (preeclampsia and/or IUGR). Our findings may be partially supported by the study of Mouillet et al. who also observed no significant difference in placental specific C19MC microRNA levels (miR-517a and miR-518b) in maternal plasma samples when normal and fetal growth restriction pregnancies were compared (2010).

Within the framework of our pilot study, we surprisingly revealed that levels of examined placental specific C19MC microRNAs exceeded, within 12th to 16th weeks of gestation, in patients who later developed pregnancy-related complications, the explicitly defined cut-offs (mean plus two standard deviations) of the control cohort at the appropriate gestational age. We assume that increased expression of placental C19MC microRNAs might reflect aberrant placentation that leads to inadequate uteroplacental blood perfusion and ischemia followed by an increased apoptosis of placental trophoblasts (Khong et al., 1967).

In consequence to our pilot study, we performed next study on the sufficiently expanded cohort of novel patients with pregnancy-related complications to achieve adequate power of the study. Using both, absolute and relative quantification approaches, an upregulation of circulating C19MC microRNAs (miR-516-5p, miR-517-5p, miR-520a-5p, miR-525 and miR-526a) in patients with clinically established preeclampsia w or w/o fetal growth restriction was demonstrated. Unfortunately, no difference in extracellular C19MC levels was observed between patients with FGR and normal pregnancies.

Furthermore, the dependence between the levels of extracellular C19MC microRNAs in maternal circulation and the pulsatility index in the middle cerebral artery (miR-516-5p, miR-517-5p, miR-520a-5p, miR-525, and miR-526a) and the cerebroplacental ratio (miR-520a-5p, and miR-526a) was observed in patients with preeclampsia and/or FGR. Unfortunately, limited data comparing extracellular C19MC microRNA levels between the groups of normal and complicated pregnancies are available. Our data are inconsistent with Yang et al. (2011), who observed up-regulation of extracellular miR-520h in four patients with preeclampsia. On the other hand, our findings may be supported by Mouillet et al. (2010), who also demonstrated no significant difference in extracellular placental specific microRNA levels, inclusive of miR-518b, in plasma samples between patients with normally progressing pregnancies and those with fetal growth restriction.

Our last two studies were focused on risk assessment for pregnancy-related complications based on maternal plasma concentrations of placental specific C19MC microRNAs in early pregnancy in an unselected population.

Our results demonstrated that up-regulation of circulating C19MC microRNAs (miR-516-5p, miR-517-5p, miR-518b, and miR-520h) is a characteristic phenomenon of early pregnancy destined to develop not only placenta-insufficiency related complications (Hromadnikova et al., 2012), but also gestational hypertension. Similarly, our current study revealed up-regulation of circulating C19MC microRNAs (miR-517-5p, miR-518b, and miR-520h) in early pregnancy in women who later developed preeclampsia. First trimester higher plasmatic levels of miR-520h, miR-518b, miR-516-5p and miR-517-5p certainly appears to be predictive of subsequent gestational hypertension, respectively. Effective screening for the later onset of gestational hypertension and preeclampsia can be achieved during the first-trimester of pregnancy by monitoring of a single extracellular C19MC placental specific microRNA biomarker (miR-517-5p for the prediction of preeclampsia and miR-520h for the prediction of gestational hypertension). Alternatively, the combination of 2 placental specific C19MC microRNA biomarkers (miR-520h and miR-518b) may be used to predict the occurrence of gestational hypertension. Unfortunately, first trimester screening of women had no clinical utility relative to the development of IUGR using extracellular C19MC microRNA biomarkers.

Other studies evaluated the ability of extracellular microRNA profiles to identify patients at higher risk of later development of pregnancy-related complications *a priori* (during the first trimester of gestation) in unselected population, however majority of studies focused on microRNAs not encoded by C19MC microRNA cluster, therefore we can not discuss our results adequately.

Our study has provided new promising biomarkers for screening approaches for severe pregnancy-related complications. However, other consecutive large scale studies are needed before implementation of extracellular C19MC microRNA biomarkers into routine praxis in the field of gynecology and obstetrics.

LIST OF PUBLICATIONS

1. Publications *in extenso* which are part of the Ph.D. thesis:

Hromadnikova I, **Kotlabova K**, Ivankova K, Krofta L. First trimester screening of circulating C19MC microRNAs and the evaluation of their potential to predict the onset of preeclampsia and IUGR. *PLoS One*. 2017 Feb 9;12(2):e0171756. (**IF₂₀₁₆ = 2.806**)

Hromadnikova I, **Kotlabova K**, Hympanova L, Doucha J, Krofta L. First trimester screening of circulating C19MC microRNAs can predict subsequent onset of gestational hypertension. *PLoS One* 9(12):e113735, 2014. (**IF₂₀₁₄ = 3.234**)

Hromadnikova I, **Kotlabova K**, Ondrackova M, Kestlerova A, Novotna V, Hympanova L, Doucha J, Krofta L. Circulating C19MC microRNAs in preeclampsia, gestational hypertension, and fetal growth restriction. *Mediators Inflamm* 2013:186041, 2013. (**IF₂₀₁₃ = 2.417**)

Hromadnikova I, **Kotlabova K**, Doucha J, Dlouha K, Krofta L. Absolute and relative quantification of placenta-specific microRNAs in maternal circulation with placental insufficiency-related complications. *J Mol Diagn* 14(2):160-7, 2012. (**IF₂₀₁₂ = 3.952**)

Kotlabova K, Doucha J, Hromadnikova I. Placental-specific microRNA in maternal circulation - identification of appropriate pregnancy-associated microRNAs with diagnostic potential. *J Reprod Immunol* 89(2):185-91, 2011. (**IF₂₀₁₁ = 2.966**)

2. Publications *in extenso* without direct connection to Ph.D. thesis:

Hromadnikova I, **Kotlabova K**, Krofta L, Hron F. Follow-up of gestational trophoblastic disease/neoplasia via quantification of circulating nucleic acids of placental origin using C19MC microRNAs, hypermethylated RASSF1A, and SRY sequences. *Tumour Biol.* 2017;39(4):1010428317697548. (**IF₂₀₁₆ = 3.650**)

Hromadnikova I, Dvorakova L, **Kotlabova K**, Kestlerova A, Hympanova L, Novotna V, Doucha J, Krofta L. Circulating heat shock protein mRNA profile in gestational hypertension, pre-eclampsia & foetal growth restriction. *Indian J Med Res* 144(2):229-237, 2016. (**IF₂₀₁₆ = 1.446**)

Hromadnikova I, **Kotlabova K**, Hympanova L, Krofta L. Gestational hypertension, preeclampsia and intrauterine growth restriction induce dysregulation of cardiovascular and cerebrovascular disease associated microRNAs in maternal whole peripheral blood. *Thromb Res.* 2016;137:126-40. (**IF₂₀₁₆ = 2.650**)

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Hromadnikova I, Dvorakova L, **Kotlabova K**, Kestlerova A, Hympanova L, Novotna V, Doucha J, Krofta L. Assessment of placental and maternal stress responses in patients with pregnancy related complications via monitoring of heat shock protein mRNA levels. Mol Biol Rep 42(3):625-37, 2015. (**IF₂₀₁₅ = 1.698**)

Hromadnikova I, **Kotlabova K**, Ondrackova M, Pirkova P, Kestlerova A, Novotna V, Hympanova L, Krofta L. Expression profile of C19MC microRNAs in placental tissue in pregnancy-related complications. DNA Cell Biol 34(6):437-57, 2015. (**IF₂₀₁₅ = 2.574**)

Hromadnikova I, **Kotlabova K**, Hympanova L, Krofta L. Cardiovascular and Cerebrovascular Disease Associated microRNAs Are Dysregulated in Placental Tissues Affected with Gestational Hypertension, Preeclampsia and Intrauterine Growth Restriction. PLoS One. 2015;10(9):e0138383. (**IF₂₀₁₅ = 3.057**)

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